

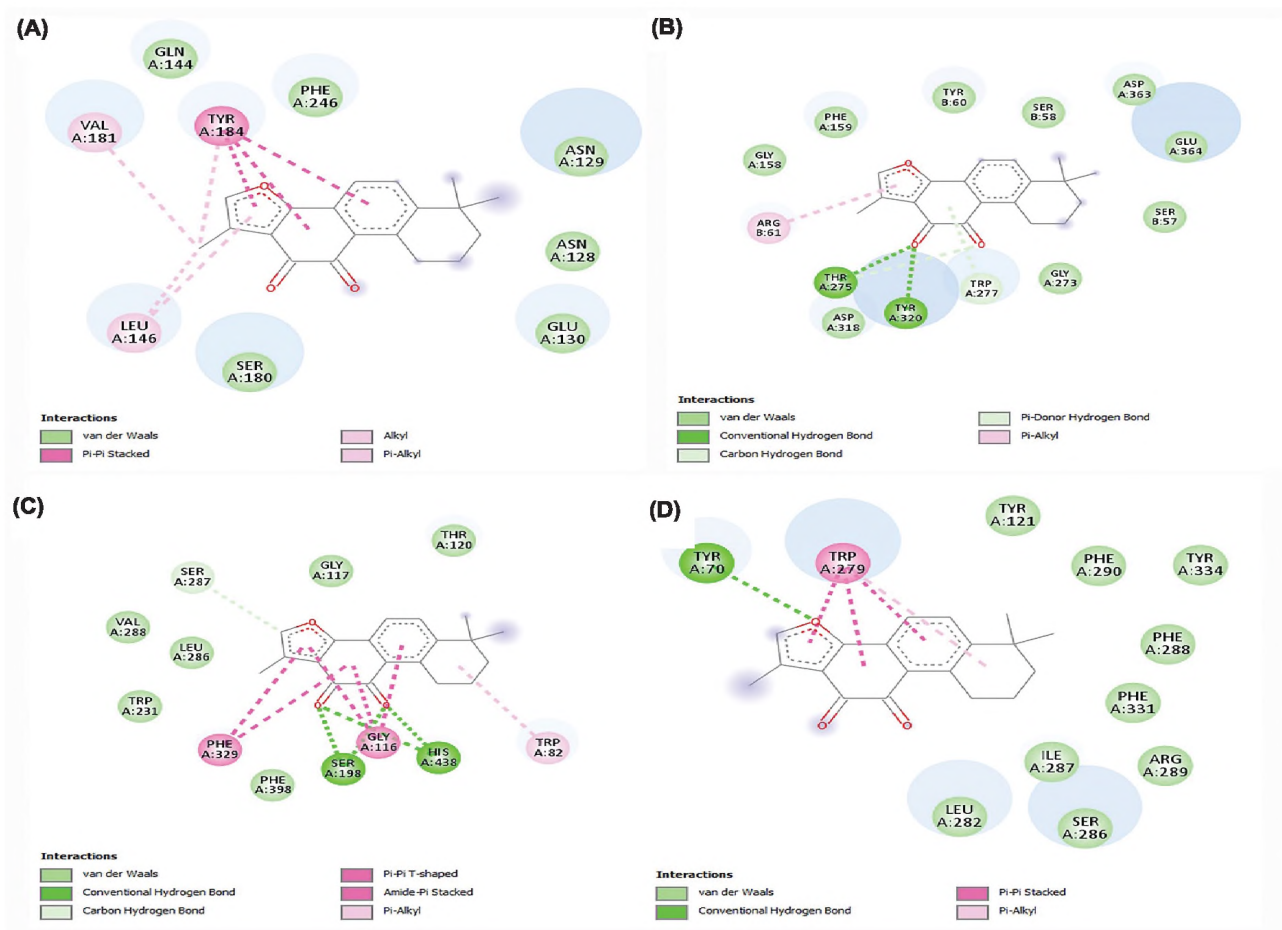


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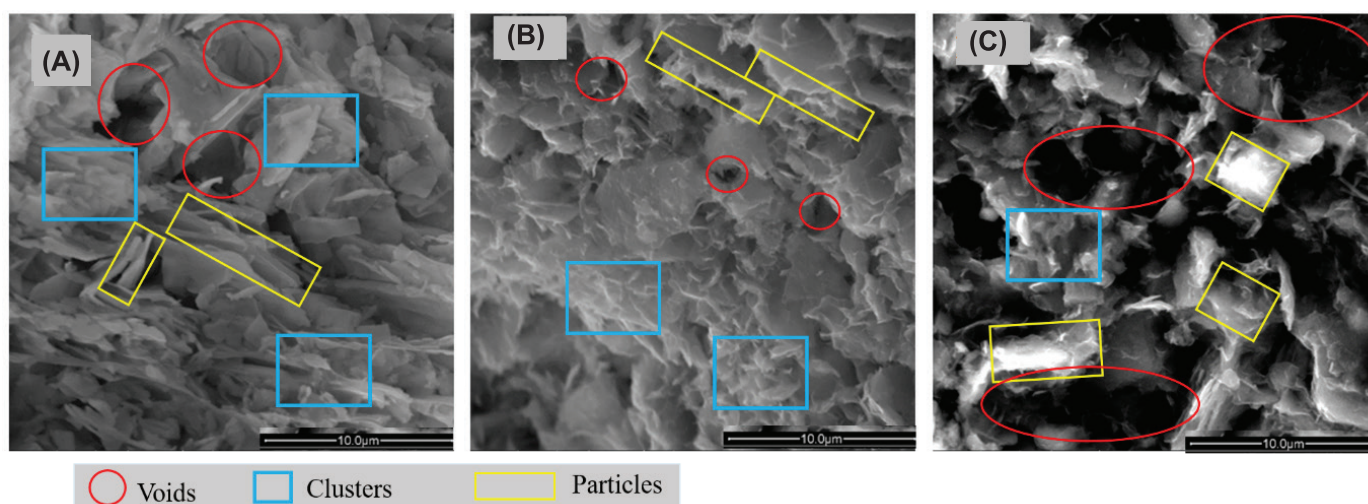
Molecular docking and molecular dynamics simulation revealed potential compounds in *Salvia miltiorrhiza* for inhibiting enzymes and receptors in Alzheimer's disease

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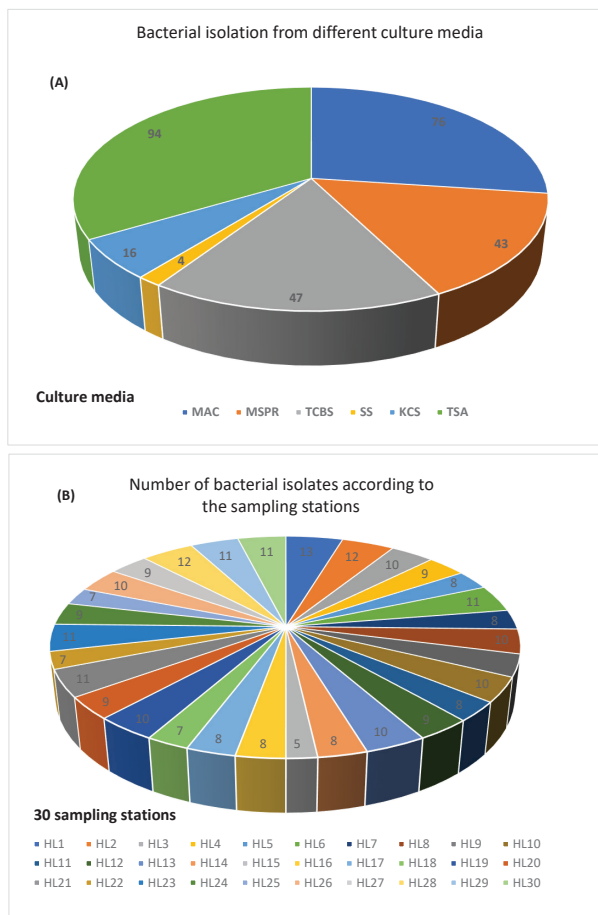
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Physico-chemical analysis in ternary organic mixtures at various temperatures with an acoustical approach

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Abstract:

Thermo-dynamical analyses of organic mixtures are essential across industries. Ultrasonic sound velocity (U) measurements in the ternary organic mixtures of Toluene, Chloroform, and Cyclohexane have been conducted between 303.15, 308.15 and 313.15 K. The experimental/derived data were utilised to compute deviations for their excess values such as adiabatic compressibility (β^E), free length (L_f^E), free volume (V_f^E), impedance (Z^E), internal pressure (π_i^E) and enthalpy (H^E) to investigate and confirm the types of interactions. Using these derived parameters, excess parameters were calculated to further reaffirm the type, strength, magnitude, and potential interactions such as complex formation, dipole-dipole, and dispersive types. Moreover, both ideal and non-ideal behaviours were analysed. The ultrasonic velocity data were validated against some contemporary and well-known models like Namoto's relation (NR), Ideal mixture relation (IMR), Impedance dependence relation (IDR), Collision factor theory (CFT) and Nutch-Kunkies (NK) theorem. From the IMR and IDR are gives well approach to experimental one. And a comparative study of these models was conducted to ascertain the possible types of interactions within the mixtures through their molecular interaction parameter, percentage deviation, standard percentage error deviation.

Keywords: enthalpy, ideality, model, molecular interaction, organic mixture, physical parameter, theory.

Classification numbers: 1.3, 2.1, 2.2

1. Introduction

In recent years, thermo-acoustical investigations of organic, ionic, and electrolyte liquid mixtures have become crucial in numerous industries. Primarily, the intermolecular interactions in liquid mixtures are of significant interest due to their excess properties. Continuing from earlier reports [1-4], the physico-chemical analysis, along with the excess parameters, was computed using standard and experimental data, observing deviations from the ideal mixtures of any solution [5, 6]. Ultrasonic and thermo-dynamical studies in binary liquid mixtures, involving certain alcohols with aniline [7, 8], were undertaken at varying temperatures. The analysis of non-polar liquids, vital in the extraction

processes of coal for compound analysis, and in the membranes of nylon fibre, nylon moulding, resin, paint, varnish, and oils, as well as its use as a plasticiser, is paramount. Such fluid mixtures are utilised for processing and product formation across numerous industries. For instance, organic solvents might be used in the food industry for the gas-sweetening absorption process and as intermediates in the manufacture of other chemicals. To comprehend the interactions among the molecules, particularly in polar and non-polar molecules [9, 10], physico-chemical analyses are indispensable. Moreover, these analyses provide insights into the bonding of associated molecular complexes and other molecular processes.

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The experimental findings were juxtaposed with theoretical models, correlating and validating these against proposed empirical theories [11-13]. The aim of the present investigation, centred on the molecular interactions of adopted systems, necessitates selecting a particular model and applying a concept from the various available ones [14, 15]. If none of the extant models provide a satisfactory prediction of molecular functioning, it might be prudent to propose new models considering the nature of the molecules. Consequently, the theoretical assessment of ultrasonic velocity in the ternary liquid mixtures of organic solutions at different temperatures has been calculated, employing methods such as NR [16], Van Deal-Vangeal's IMR [17], IDR [18], CFT [19], N-K [20]. Furthermore, some supplementary test parameters have also been calculated [21]. In this study, the authors present excess properties at various temperatures for mixtures of Toluene, Chloroform, and Cyclohexane. The types of molecular interaction are then validated against the theoretical model to reaffirm the precision of interaction. The article is structured as follows: Section 2 details experimental procedures; Section 3 covers some excess parameters with pertinent theories/models of ultrasonic velocity; Section 4 presents results and their comprehensive discussion; and Section 5 concludes with the derived outcomes.

2. Experimental

Analytical Grade chemicals were purified using standard procedures [22, 23]. Their purity was estimated at 99.7%. Sound velocities in liquids were measured using an interferometer (2 MHz), with a precision of ± 0.1 m/s. The densities and viscosities of the solutions were determined using a specific gravity bottle (10 ml) and Oswald's viscometer. Temperatures were maintained consistently with a deviation of 0.1 K. Computations were performed in the Excel environment, deriving parameters from the following expressions [1].

3. Theories and models

The excess parameters such as excess compressibility are given as

$$\beta^E = \beta_{\text{exp}} - (x_1\beta_1 + x_2\beta_2 + x_3\beta_3) \quad (\text{I})$$

and the corresponding excess free length is expressed as

$$L_f^E = L_{f_{\text{exp}}} - (x_1L_{f1} + x_2L_{f2} + x_3L_{f3}) \quad (\text{II})$$

The excess free volume is denoted as

$$V_f^E = V_{f_{\text{exp}}} - (x_1V_{f1} + x_2V_{f2} + x_3V_{f3}) \quad (\text{III})$$

and the excess internal pressure is represented by

$$\pi_i^E = \pi_{i_{\text{exp}}} - (x_1\pi_{i1} + x_2\pi_{i2} + x_3\pi_{i3}) \quad (\text{IV})$$

The excess impedance is given by

$$Z^E = Z_{\text{exp}} - (x_1Z_1 + x_2Z_2 + x_3Z_3) \quad (\text{V})$$

and the excess enthalpy takes the following form:

$$H^E = H_{\text{exp}} - (x_1H_1 + x_2H_2 + x_3H_3) \quad (\text{VI})$$

where all notations have their usual meanings.

NR: O. Nomoto (1958) [16] formulated an assumption based on the linear dependence of sound velocity on molar volume in binary mixtures, expressed as

$$U_{NR} = \left(\frac{X_1R_1 + X_2R_2}{X_1V_1 + X_2V_2} \right)^3 \quad (1)$$

From Eq. (1), its expansion to a ternary system is

$$U_{NR} = \left(\frac{X_1R_1 + X_2R_2 + X_3R_3}{X_1V_1 + X_2V_2 + X_3V_3} \right)^3 \quad (2)$$

Molar volume (V) follows the additive rule, e.g.,

$$V = X_1V_1 + X_2V_2 + X_3V_3 \quad (3)$$

Ideal mixture relation (IMR): In this relation, the adiabatic compressibility (β) of the mixture is [17]:

$$\beta = q_1\beta_1 + q_2\beta_2 \quad (4)$$

where q is the volume fraction.

By adopting specific heat, γ , it can be applied to a homogeneous medium as.

$$\beta_{IM} = q_1 \left[\frac{\gamma_1}{\gamma_{IM}} \right] \beta_1 + q_2 \left[\frac{\gamma_2}{\gamma_{IM}} \right] \beta_2 \quad (5)$$

The following relationship is only applicable to ideal systems:

$$\gamma_1 = -\gamma_2 = \gamma_{IM} \quad (6)$$

Using Eq. (4), Eq. (6) can be written as

$$\beta_{IM} = X_1\beta_1 + X_2\beta_2 \quad (7)$$

In binary systems,

$$U_{IMR} = \left(\frac{1}{X_1m_1 + X_2m_2} \right)^{\frac{1}{2}} \left(\frac{X_1}{m_1U_1^2} + \frac{X_2}{m_2U_2^2} \right)^{-\frac{1}{2}} \quad (8)$$

For ternary systems, Eq. (8) can be extended and expressed as

$$U_{IMR} = \left(\frac{1}{X_1m_1 + X_2m_2 + X_3m_3} \right)^{\frac{1}{2}} \left(\frac{X_1}{m_1U_1^2} + \frac{X_2}{m_2U_2^2} + \frac{X_3}{m_3U_3^2} \right)^{-\frac{1}{2}} \quad (9)$$

The degree to which molecules interact (α) is represented by

$$\alpha = \left(\frac{U_{exp}^2}{U_{IMR}^2} \right) - 1 \quad (10)$$

IDR: The impedance or resistance (Z) of liquid mixtures is contingent on their sound velocity and density. This can be computed from the following relation [18]:

$$U_{IDR} = \sum_{i=1}^3 \frac{X_i Z_i}{X_i \rho_i} \quad (11)$$

CFT: This theory's application to ternary systems is given by the following [19]:

$$U_{CFT} = U_a \left(\frac{(X_1S_1 + X_2S_2 + X_3S_3)(X_1B_1 + X_2B_2 + X_3B_3)}{V_m} \right)^3 \quad (12)$$

where V_m is the molar volume; S_i is the collision factor; B_i is the actual volume; X_i is the mole fraction of the component i .

NK theorem: This theorem is formulated from collision factor theory [20], which can be expressed as:

$$U_{NK} = \frac{U_a (\sum X_i S_i) (\sum X_i B_i)^3}{\sum X_i V_i} \quad (13)$$

where all the notations have their usual meaning.

Percentage deviation: The computed value's deviation from the experiment is determined by

$$\left(\frac{\Delta U}{U} \right) \% = \left(\frac{U_{exp} - U_{the}}{U_{exp}} \right) 100 \% \quad (14)$$

Standard percentage error deviation: This error is calculated from the following expression:

$$e = \left(\left(\sum \frac{\Delta U}{U} \right) \% \right)^2 \quad (15)$$

4. Results and discussion

All excess and theoretical velocity values are presented in Table 1. From Fig. 1A, it can be observed that the values of (β^E) show an increasing trend up to 0.06 m, thereafter a decreasing trend is observed with Toluene at all temperatures, with maximum values at 313.15 K. The positive sign of (β^E) is a result of dispersion forces [23-25]. The trend of excess free length mirrors that of (β^E), as shown in Fig. 1B. The dissociation of like molecules and the positive excess contribution indicate weak interactions; the negative sign of β^E strongly suggests increasing solute-solute molecular interactions. This implies the potential formation of complexes between donors and acceptors, causing molecules to come closer and leading to decreased adiabatic compressibility [26]. For these concentrations, the negative values of free length suggest the possibility of close arrangements due to molecular ordering, thus intensifying interactions amongst molecules [27].

From Fig. 1B, the increasing trends of L_f^E values highlight the structural variations among molecules of different sizes [28, 29]. From Fig. 1C, the (V_f^E) values increase and show an upward trend with Toluene across all molalities and temperatures, with peak values at 303.15 K up to 0.06 m, followed by maximum values at 308.15 K. This suggests the strength of the hydrogen bond. A positive V_f^E indicates the formation of complexes and the prevalence of interactions between dissimilar molecules [30, 31]. The values of (π_i^E) are negative and increase with Toluene at all molalities and temperatures. However, the larger values observed at 303.15 K are depicted in Fig. 1D. These suggest weaker interactions between dissimilar molecules [32].

Table 1. The measured and calculated values of ultrasonic velocity (U), their percentage deviation ($\Delta U/U$), standard percentage error (e), and molecular interaction parameter (α) for various molarity (m) of Toluene + Chloroform + Cyclohexane at 303.15, 308.15, and 313.15 K.

(m)	(U) – (m/s)						(ΔU/U) – (m/s)						(a)
	U_{exp}	U_{NR}	U_{IMR}	U_{IDR}	U_{NK}	U_{CFT}	U_{NR}	U_{IMR}	U_{IDR}	U_{NK}	U_{CFT}		
303.15 K													
0.01	1151.2	1178.2	1156.7	1131.3	1168.2	1173.8	-1.2441	-0.2148	0.1246	-1.4321	-3.3765	-0.0316	
0.02	1144.1	1180.3	1158.9	1138.0	1171.2	1195.9	-3.1731	-1.3024	0.5245	-2.3776	-4.5367	-0.0527	
0.03	1136.3	1195.5	1162.2	1142.7	1175.1	1200.9	-5.2377	-2.3063	-0.5897	-3.4419	-5.7130	-0.0700	
0.04	1120.0	1211.6	1165.5	1147.2	1179.2	1222.3	-8.1786	-4.0625	-2.4285	-5.2857	-9.1339	-0.1263	
0.05	1112.6	1228.6	1168.9	1151.7	1183.1	1227.1	-10.486	-5.1169	-3.5701	-6.3938	-10.350	-0.1047	
0.06	1104.7	1246.6	1172.3	1156.0	1187.2	1230.5	-12.917	-6.1865	-4.7101	-7.5362	-11.458	-0.0832	
0.07	1096.2	1265.7	1175.7	1160.3	1191.1	1233.2	-15.484	-7.2719	-5.8667	-8.6770	-12.518	-0.0485	
0.08	1112.4	1285.9	1179.2	1164.5	1195.2	1232.8	-15.638	-6.0431	-4.7212	-7.4820	-10.863	-0.0404	
0.09	1136.9	1307.5	1182.7	1168.6	1199.1	1240.6	-15.097	-4.1102	-2.8697	-5.5545	-9.2077	0.0213	
0.10	1152.1	1330.4	1186.2	1172.7	1203.1	1239.3	-15.486	-2.9687	-1.7968	-4.4357	-7.5781	0.0288	
e	----	0.0379	0.0293	0.0020	0.0349	0.0438							
308.15 K													
0.01	1142.3	1169.2	1139.8	1121.5	1159.2	1189.9	-2.0142	-0.9827	0.6623	-1.3233	-31298	-0.0813	
0.02	1136.3	1170.7	1148.7	1127.3	1161.6	1191.3	-3.0546	-1.1180	0.7659	-2.2535	-4.8680	-0.0763	
0.03	1126.7	1172.9	1151.6	1131.7	1165.1	1198.3	-4.1652	-2.2735	-0.5062	-3.4725	-6.4210	-0.0668	
0.04	1112.3	1175.2	1154.6	1135.9	1168.8	1213.4	-5.6835	-3.8309	-2.1493	-5.1079	-9.1187	-0.1237	
0.05	1104.8	1177.7	1157.6	1140.1	1172.4	1218.3	-6.6757	-4.8551	-3.2699	-6.1957	-10.353	-0.1148	
0.06	1096.1	1180.2	1160.6	1144.1	1175.9	1222.7	-7.6825	-5.8942	-4.3887	-7.2901	-11.560	-0.0931	
0.07	1072.7	1182.8	1163.6	1148.1	1179.6	1222.1	-10.336	-8.5448	-7.0989	-10.037	-14.002	-0.0847	
0.08	1104.5	1185.6	1166.7	1152.0	1183.1	1222.0	-7.3913	-5.6793	-4.3478	-7.1649	-10.688	-0.0500	
0.09	1128.6	1188.5	1169.9	1155.8	1186.7	1232.8	-5.3635	-3.7145	-2.4645	-5.2039	-9.2908	-0.0015	
0.10	1136.3	1191.5	1173.0	1159.6	1190.4	1229.7	-4.8856	-3.2570	-2.0775	-4.7887	-8.2482	0.0060	
e	----	0.0413	0.0330	0.0026	0.0384	0.0484							
313.15 K													
0.01	1141.2	1159.2	1127.3	1109.2	1148.2	1187.2	-0.3165	-0.0021	0.0032	-0.0198	-3.2432	-0.0821	
0.02	1128.0	1160.9	1138.4	1117.3	1151.9	1189.5	-2.9166	-0.9219	0.9486	-2.1188	-5.4521	-0.0731	
0.03	1096.4	1163.3	1141.5	1121.8	1155.7	1190.6	-6.1405	-4.1514	-2.3540	-5.4471	-8.6314	-0.0635	
0.04	1088.2	1165.8	1144.6	1126.3	1159.5	1205.2	-7.1507	-5.2022	-3.5202	-6.5717	-10.772	-0.1209	
0.05	1072.1	1168.3	1147.8	1130.6	1163.3	1216.1	-8.9832	-7.0709	-5.4664	-8.5168	-13.442	-0.1119	
0.06	1056.9	1171.0	1151.0	1134.8	1167.1	1220.1	-10.890	-8.9962	-7.4621	-10.521	-15.540	-0.1031	
0.07	1042.8	1173.8	1154.3	1139.0	1170.9	1221.1	-12.648	-10.777	-9.3090	-12.37	-17.188	-0.0946	
0.08	1088.8	1176.7	1157.5	1143.1	1174.7	1217.6	-8.1525	-6.3878	-5.0643	-7.9688	-11.912	-0.0730	
0.09	1112.2	1179.8	1160.8	1147.1	1178.5	1231.0	-6.0971	-4.3884	-3.1564	-5.9802	-10.701	-0.0245	
0.10	1120.1	1183.2	1164.2	1151.2	1182.3	1229.8	-5.6250	-3.9464	-2.7678	-5.5625	-9.8036	-0.0030	
e	----	0.0429	0.0346	0.0028	0.0402	0.0503							

The excess acoustical impedance (Z^E) generally shows a negative value and an increasing trend up to 0.06 m, after which a decrease is noted with Toluene. The maximum values are observed at 313.15 K (Fig. 1E). Similar patterns have been observed by other researchers [33, 34]. Positive excess values suggest interactions among dissimilar molecules [35]. There exists the potential for additional interactions between molecule constituents, and the formation of charge transfers in complexes is indicated by the positive deviations in acoustic impedance. Thus, deviations in acoustic impedance clearly suggest the presence of more charge transfer complexes.

The value of excess enthalpy shows an increasing trend with concentration and decreases at higher concentrations with negative values (Fig. 1F). These patterns suggest the possibility of molecular attraction during the mixing process, and another potential for molecular attraction due to polarisation between dissimilar molecules. This becomes dominant over the attraction between like molecules, and the effects of temperature lead to variations in these values, especially at low temperatures. Therefore, the deviations diminish at higher temperatures [36].

The experimental and computed ultrasonic velocities, along with their respective parameters for the mixture of toluene, chloroform, and cyclohexane, are presented in Table 1. From Table 1, the observed trend for ultrasonic velocity consistently increases with concentration up to 0.07 m, and a decreasing trend is noted across all temperatures.

From Table 1, IDR closely aligns with the experimental values at all temperatures, while IMR moderately does so. This suggests both a good and exceptional compatibility with the experimental results.

The molecular interactions between the components of a liquid combination may account for the percentage differences between observed and estimated sound velocities. In the context of relevant theories, such interactions have not been fully considered [37-39]. Moreover, they do not comprehensively account for the experimental manifestation of molecular interaction in various ultrasonic processes. The assumptions made in these theories regarding non-polar-non-polar and non-polar-polar interactions between molecules might explain the variation in velocities.

Of the various theories considered, IDR provides an outstanding prediction of sound velocity for all temperatures in this study. With respect to the combination of toluene, cyclohexane, and chloroform, the predictions from the IDR model closely match experimental results

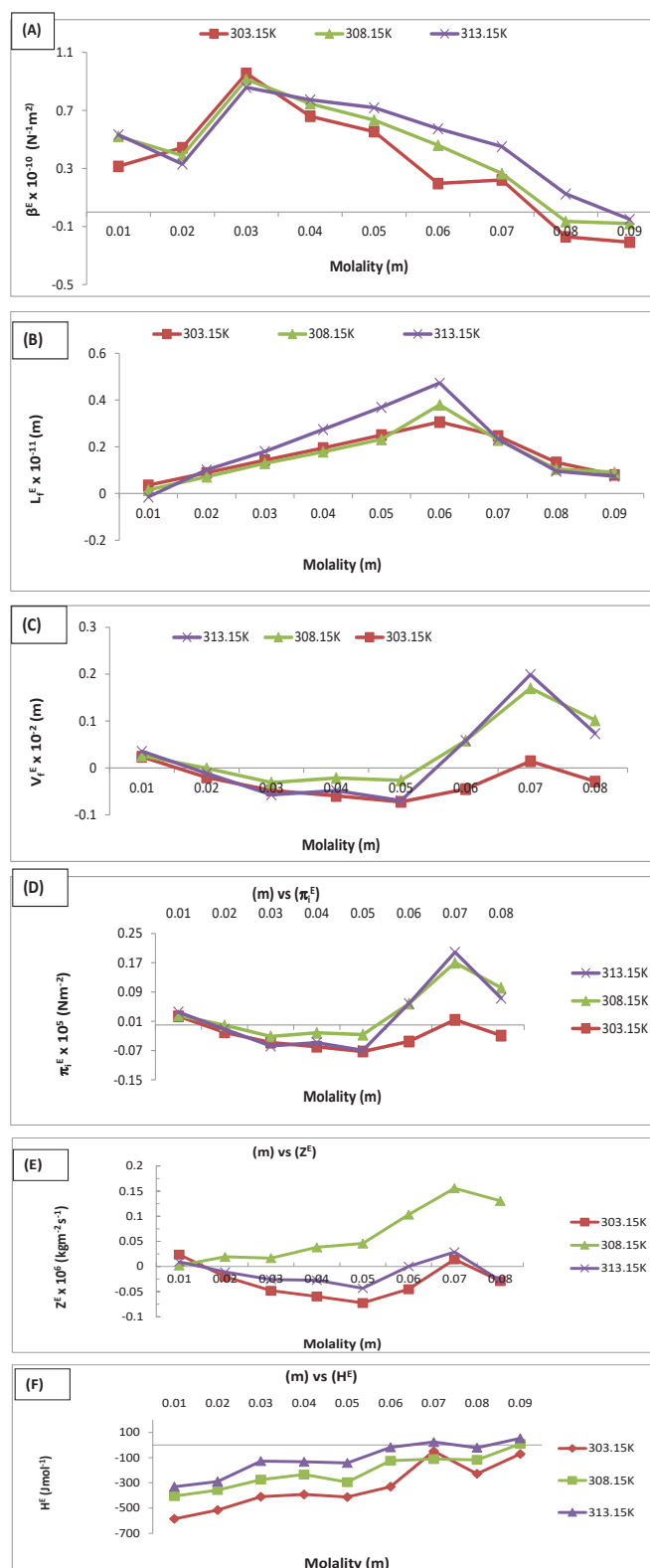


Fig. 1. Excess compressibility (A), excess free length (B), excess free volume (C), excess internal pressure (D), excess impedance (E), and excess enthalpy for various molalities (m) of Toluene + Chloroform + Cyclohexane (F) at various temperatures.

for most molarities in Table 1. Some alignment is also noted for several molarities in IMR. The percentage deviation being close to zero for both IDR and IMR reinforces the reliability of predictions for both in the ternary systems.

Molecular interaction largely depends on the strength of the molecules and is influenced by other factors such as dispersion forces [40]. Furthermore, the values of (α), computed from the IMR values and listed in Table 3, show that α is negative for ternary mixtures across all considered molarities. These negative values suggest the potential formation of intra-molecular hydrogen bonds [2] and further indicate weak dipolar and dispersive interactions. The maximum α value recorded is -0.1850 at 0.07 m, while the minimum is -0.02 and 0.05 at 0.01 and 0.10 m, respectively. This re-emphasises that cyclohexane exhibits much lower interactions compared to the other constituents of the mixture.

The overall molecular interactivity in a liquid mixture is influenced by multiple factors. Given its size, cyclohexane predominantly offers dispersive intra-molecular interactions. This confirms the dispersive interactions between similar molecules and weak dipolar interactions between dissimilar ones [41].

5. Conclusions

1. The in-depth analysis affirms the formation of hydrogen bonds between the molecules.
2. The introduction of toluene results in solute-solvent interactions at lower concentrations, and solvent-solvent interactions at higher ones.
3. Distinct types of interactions among the components are evident.
4. Among the theories considered in this study, IDR aligns exceptionally well with experimental values, while IMR does so to a lesser degree.
5. The mixtures approach ideal behaviour at higher concentrations.

CRedit author statement

S. Nithiyanantham: Methodology, Idea, Editing; V. Vanathi: Data analysis, Drawing figures; S. Mullainathan: Investigation and Writing; S. Mahalakshmi: Support to Editing and Investigation; L. Palaniappan: Validation and Writing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Convective heat transfer by nanofluid jet impingement cooling on automobile radiator with nozzle plate spacing

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Abstract:

Nanofluids are a type of emerging heat transfer fluid with great promise for thermal engineering due to its high heat transfer coefficients. Improving heat transmission remains an ongoing task in engineering fields as diverse as semiconductor technology and high-performance vehicles. This paper investigates and compares the jet impact heat transfer coefficient, thermal conductivity, and viscosity of nanofluids with those of a base fluid in an automobile radiator with nozzle plate spacing. Aluminium oxide (Al_2O_3) and deionised water were selected as the working fluids, and they were used in varying quantities ranging from 0 to 1 volume percent. The nanofluids were prepared using a two-step method with the aid of an ultrasonic homogeniser. The results reveal that the heat transfer coefficient increased by 11.68% at a nanofluid concentration of 1% when nanoparticles were suspended in the base fluid. Furthermore, a 45-49% improvement in the heat transfer coefficient was recorded using a nanoparticle volume concentration of 1% compared to the base fluid.

Keywords: heat transfer coefficient, jet impingement, volume concentration.

Classification numbers: 2.1, 2.3

1. Introduction

Enhancing heat transport is pivotal in heat transfer engineering, with a pressing need to boost the efficiency of thermal devices in various technical fields [1]. Strategies such as increasing the heat transfer area and coefficient have been explored extensively [2]. However, technological advancements in heat transmission have reached their limits. Recent research has shown that incorporating nano-sized nanoparticles into liquids can significantly increase the heat transfer coefficient compared to conventional liquids [3]. This led to the development of “nanofluid”, where particles sized 1 to 100 nm are suspended, markedly improving heat transfer [4]. Subsequent studies have focused on the heat transfer coefficients of various nanofluids.

A. Kumar, et al. (2020) [5] noted an 18% improvement in heat transfer with a 4% volume concentration of copper and copper oxide nanoparticles. A. Banisharif, et al. (2020) [6] found that thermal conductivity of nanofluids increases exponentially with nanoparticle volume concentration. Viscosity, critical for evaluating a material's heating capacity, influences the pressure drop and thus the transport power, making its reduction crucial to increasing heat transmission

[7]. According to experimental research conducted in [8], the viscosity of an Al_2O_3 -DI water nanofluid substantially increases. At a concentration of 5% volume, the viscosity of an Al_2O_3 -DI water nanofluid rises significantly, with a 79% increase at a 5% volume concentration [9].

Another vital aspect of nanofluids is their density, which affects the fluid pressure drop along with viscosity. The nanofluid density does not depend on characteristics such as nanoparticle size, shape, and additives; instead, density primarily depends on the nanoparticle material [10]. The viscosity of nanofluid is shown to rise with increasing nanoparticle concentration since solids have a more significant density than liquids [11]. There is a pressing need for more literature on the topic of nanofluid density.

The specific heat (C_p) of nanofluids is another essential property that determines the nanofluid's thermal ability to conserve energy. Nanofluid C_p is determined by several distinct criteria, including the type, volume concentration of particles, and the base fluids, evaluated at various temperatures [12]. R.S. Vajjha, et al. (2010) [13] were the first to show that particle volume concentration increases in nanofluids containing aluminium oxide/titanium oxide/

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water while the C_p of the nanofluids decreases. J.R. Satti, et al. (2016) [14] researched the specific heat of nanofluids containing aluminium, silicon, and zinc oxide nanoparticles. They also found that the specific heat drops with increasing nanoparticle concentration but rises with nanofluid temperature.

Experiments with alumina-water nanofluids at varying nanoparticle concentrations were previously published [15], which determined particle concentration improved the heat transfer coefficient of the heat transfer of H_2O and 0.4 vol.%. TiO_2 nanoparticles in a parallel flow exchanger under uniform flow conditions [16]. The mass flow rate of the nanofluid and water, as well as the temperature of the nanofluid, were shown to affect the heat transfer coefficient significantly. Nanofluids were shown to have a 16% greater heat transfer coefficient than the primary liquid.

The heat transfer coefficient of aluminium oxide-water nanofluids was studied in a flow with a horizontal heated tube [17]. When combined with water, the aluminium oxide-water nanofluid at 6 vol.% boosted heat transfer at entry and in fully formed regions by 13 and 24%, respectively. Heat transfer properties of TiO_2/Al_2O_3 -water nanofluids were studied in [18, 19] in a heat exchanger with turbulent flow. They found that a 0.5% volume concentration of Al_2O_3 /water nanofluids resulted in a maximum improvement of the total heat transfer coefficient by roughly 27%. At a particle volume concentration of 0.4%, TiO_2 /water nanofluids showed the most significant improvement.

S. Jena, et al. (2020) [20] looked at the metal oxide nanoparticles in oil using scientific methods to improve heat transfer. The results showed that as the nanoparticle volume concentration increases, so does the temperature of the fluid. P.K. Pattnaik, et al. (2021) [21] studied how a micropolar liquid that conducts electricity moves freely over a surface that is being stretched. It shows and discusses how characterising factors affect the flow phenomenon. No matter how much the slip number increases, the data shows an apparent slowing down in the velocity curves due to more drag. S.K. Parida, et al. (2021) [22] investigated the effect of dust particles on nanofluid flow due to the interaction of thermal radiation parameters. The authors discovered that the nanoparticle's volume percentage and radiative energy favour increasing rates of energy transfer near the sheet's surface.

P.K. Pattnaik, et al. (2022a) [23] examined the 2D flow of water-based nanofluids and used a fluid with pores to show how a longitudinal magnetic field works. The nanofluid is made by mixing copper and aluminium oxide nanoparticles with carbon nanotubes in water, the base fluid. The findings

showed that as the particle concentration goes up, the shear rate coefficient does as well, while the heat transfer rate decreases. P. Mathur, et al. (2021) [24] studied second-order velocity slip by moving a nanofluid past a stretched surface. The work describes how several factors that define the flow behave in different situations. The results show that the fluid's velocity slows as the material value increases. P.K. Pattnaik, et al. (2022b) [25] studies how floating forces affect the flow of a conducting nanofluid via hollow walls that change over time. It also looks at the heat absorption factors that affect the nanofluid model. The results show that adding volume fractions makes them move faster near the channel's inner layer and slower near its porous walls.

For their study, P.C. Pattanaik, et al. (2022) [26] examined the Newtonian flow properties of the nanofluid using a parallel path because of thermal stability. As the particle volume concentration goes up, the fluid motion slows down. However, the slowing down of the water-based nanofluid is greater than that of the oil-based nanofluid. B. Mohanty, et al. (2021) [27] put an electrically conductive nanofluid in a curved tube and let it connect to radiated heat energy and a heat sink. The lower alpha potentials show Joule heating also affects the flow processes. The results showed that the Newtonian fluid significantly affects the fluid properties, even if the parameter is visible. S.R. Mishra, et al. (2019) [28] investigated how heat transfer affects a hybrid fluid's flow along an endless flat surface with radiation. The regular electromagnetic field was put in place along the primary flow direction. The results show that the velocity profile got faster when adding the parameter, but the border layer got thinner when the inertia effects were added.

S. Baag, et al. (2018) [29] investigated how heat moves through a thermal boundary layer that flows over a stretched sheet in a steady heat source. The work showed that the force slows down the velocity profile everywhere in the velocity boundary layer. B. Nayak, et al. (2019) [30] studied the flow over a porous rectangular surface with chemical processes rather than heat transmission. The results show that as the number of heavy species increases the reaction time, the quantity of liquid in its boundary region decreases. O.D. Makinde, et al. (2017) [31] examined the gravity forces and changing viscosity affected the mixed flow of a conducting fluid over a porous plate that was heated by convection. It has been found that when an electromagnetic field is present, skin friction decreases, the Reynolds number increases, and the fluid's viscosity decreases.

K.S. Nisar, et al. (2021) [32] investigated the nanofluid flow over an expanded surface that was open, turbulent, and could not be compressed. A steady source of heat and chemical process also improves the heat transfer qualities

of a nanofluid. The results show that raising the fluid concentration lowers the temperature of the fluid when the Nusselt number is high. S. Mishra, et al. (2022) [33] studied the nanofluids using a solid-state reaction process at high rates and pure start ingredients such as tungsten trioxide. The results reveal that the primary crystal structure size is 38 nm, and strains are 0.11%. In their study, S. Mishra, et al. (2021) [34] investigated the radiant flow of a bipolar nanofluid over a flexible film. They looked at the effects of heat flux. The findings show that increasing the radiation and heat production factors improves the temperature distribution.

Automobiles require several vital components, including radiators. The engines are cooled by a heat exchanger system, which is one such system. Like other thermal devices, the engine presents a significant challenge in thermal management. It improves engine efficiency by increasing the heat transfer capacity of the radiator radiators. In the radiator of an automobile, water has often been used instead of other coolants. The performance of automobile radiators can be improved with the recent commercial introduction of 50:50 mixes of ethylene glycol and water [35]. Using nanofluid may enhance the heat transfer efficiency of heat exchangers. However, thermal conductivity, viscosity, and density must first be investigated since the efficiency of the automobile radiator is reliant on these parameters before the heat transfer efficiency can be studied. Only a little amount of research has been done on the viscosity of a nanofluid based on radiator coolant.

The current study examines the heat transfer coefficient, thermal conductivity, and viscosity of nanofluids based on a radiator coolant containing aluminium oxide nanofluids at temperatures varying from 0 to 60°C. Filling the gaps in understanding the fundamental thermophysical characteristics of nanofluids based on radiator coolants is the goal of this study.

2. Preparation of nanofluids

Two-step method for preparation of nanofluids is presented in Fig. 1.

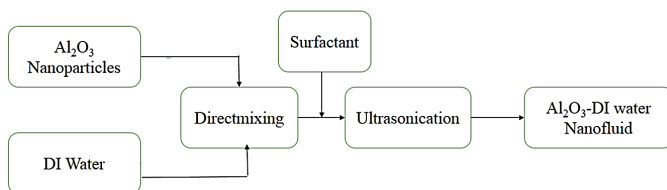


Fig. 1. Two-step method for preparation of nanofluids.

Aluminium oxide nanoparticles were purchased from Sigma-Aldrich Chemicals Ltd. and combined with coolant at a volumetric concentration of 0 to 1% vol.% with a particle size of 20 nm according to the following formula:

$$\text{Volume concentration } (\phi) = \frac{\frac{m_n}{\rho_n}}{\frac{m_n}{\rho_n} + \frac{m_f}{\rho_f}} \quad (1)$$

where m denotes the mass and ρ denotes the concentration of the nanoparticles and coolant; n denotes nano, f denotes fluid. Thus, 100 g of the base fluid was used to make the nanofluid samples, and then the predicted amount of Al_2O_3 was added to the liquid itself. To attain uniform dispersion in the base fluid, the nanoparticle solution was maintained in a sonicator, a mechanical stirrer, and stirred continuously for around 6 hours. The nanoparticles were dissolved in the base fluid, and the surfactant was added to create the nanofluids. According to the formulae for the solid-fluid mixtures, the properties of the nanofluids were assessed. The thermophysical characteristics of Al_2O_3 , the base fluid (DI water), and air at 30°C are listed in Table 1.

Table 1. Properties of the nanofluids.

Fluid	Density (Kg/m ³)	Viscosity (Pas)	Specific heat (kJ/kg K)	Thermal conductivity (W/mK)
Al_2O_3 nanoparticles	3970	22	765	36
DI water	997	10^{-3}	4184	0.6

The density [36], viscosity [37], and thermal conductivity [38] were calculated using the following equations:

$$\rho_{nf} = \phi\rho_p + (1 - \phi)\rho_{bf} \quad (2)$$

$$\mu_{nf} = A\left(\frac{1}{T}\right) - B \quad (3)$$

$$A = 20587\phi^2 + 15857\phi + 1078.3$$

$$B = -107.12\phi^2 + 53.54\phi + 2.8715$$

$$K = \frac{k_p + (n-1)K_f - 2(n-1)\phi(K_f - K_p)}{K_p + 2K_f - (K_f - K_p)}K_f \quad (4)$$

Nomenclature

C_p : specific heat; D : diameter; K : thermal conductivity; m_n : Mass of nanoparticle; N_u : Nusselt number; T : temperature.

Subscripts

Bf: base fluid; f: fluid; h: hydraulic; in: inlet; nf: nanofluid; out: outlet; p: particle.

Greek symbols

ϕ : volume fraction; ρ : density.

2.1. SEM analysis

Scanning electron microscope imaging was used to examine the surface structure of the Al_2O_3 nanoparticles. Images of pure Al_2O_3 nanoparticles are shown as samples in Fig. 2. As seen in the figure, Al_2O_3 nanoparticles are very twisted tubes with a 20 nm diameter.

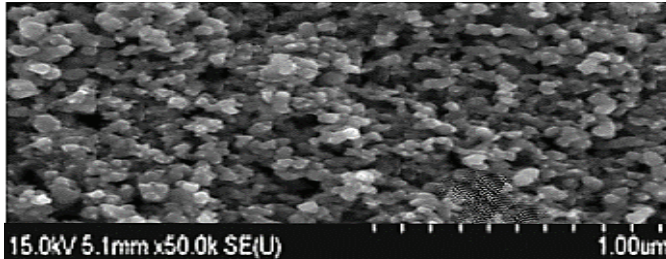


Fig. 2. SEM image of the Al_2O_3 nanoparticles.

2.2. X-Ray diffraction analysis

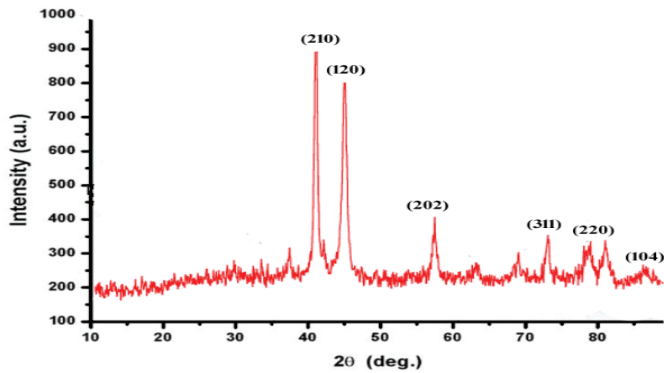


Fig. 3. XRD of the Al_2O_3 nanoparticles.

The size and structure of Al_2O_3 nanofluids are characterised using X-ray diffraction analysis (Fig. 3). Six peaks at 30°C are seen in the XRD pattern shown in Fig. 3, identical to the (210), (120), (202), (311), (220), and (104) orientations. The optimal alteration of Al_2O_3 nanoparticles is demonstrated by a shift in the location of the distinctive peak in the pattern produced by XRD. Further evidence that Al_2O_3 nanoparticles have preserved their initial form after functioning comes from the lack of a dramatic shift in the location of distinctive peaks. This is evidence that the acid-functionalised Al_2O_3 nanoparticles have an ordered flow of structure. Due to the chemical treatment performed throughout the experiment, Al_2O_3 nanoparticles were functionalised with carboxylic groups, increasing their reactivity. These results show that the Al_2O_3 nanoparticles were tightly adhered to the surface due to this functionalisation.

3. Experimental arrangement

The schematic of the experimental setup is presented in Fig. 4.

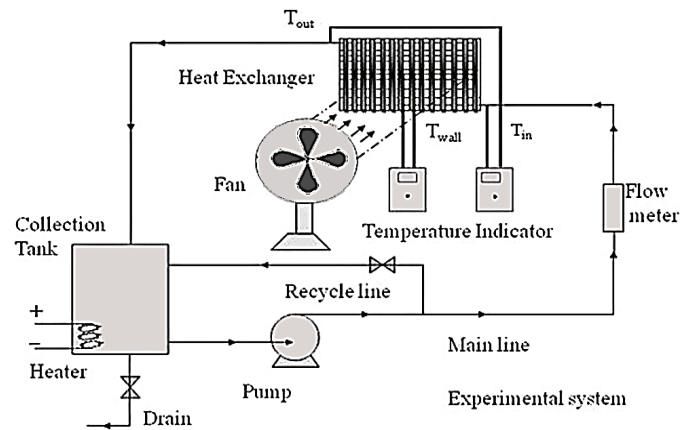


Fig. 4. Schematic of the experimental setup.

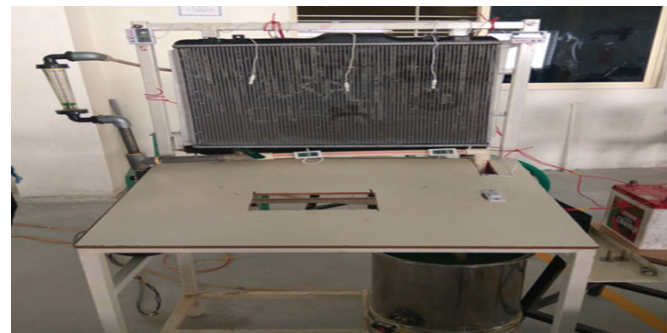


Fig. 5. Image of the experimental setup.

Figure 5 depicts the evaluation of the intended test rig with a conduit attached. The coolant in the container is brought up to the appropriate temperature, and then the pump is activated, allowing the coolant to circulate through the radiator. Finally, the fan is activated to blow air over the radiator, which removes heat from the radiator. Temperatures are recorded at the radiator's intake and output. It is anticipated that the nanofluid will flow with a constant Reynolds number and will be able to tolerate average circulation in the radiator. The coolant circulates via 104 tubes, each 5 mm wide and 0.3 m long. Using a rotameter, it is estimated that the coolant can flow through the radiator at flow rates of 3, 6, 9, and 12 litres per minute. Changing the coolant and air flow rates is necessary to replicate realistic working conditions in an administrative setting. The temperatures of the air entering and exiting the radiator are both measured.

When the desired temperature is reached, the heat exchanger's coolant pump is turned on to circulate high-temperature, durable coolant through the unit. The radiator's coolant entry and exit temperatures are measured using T-type thermocouples. A set of thermocouples measures the air temperature affixed to the front and rear radiator tubes at

various points. Experiments are run with a perforated plate at distances of 10 and 15 cm from the radiator to test the effect of varying L/D ratios. Fig. 6 shows a side view of the test rig, which includes temperature thermocouples for the coolant and air inlets and outlets. Photographs of the nozzle sheet arrangement, which is located between the fan and the radiator, are shown in Fig. 7.



Fig. 6. Side view of the experimental setup.



Fig. 7. Images of nozzle arrangement plate with a diameter of 30 mm.

Estimation of heat transfer coefficient

The formulae below were used to determine the heat flow rate and flow rate of the air passing through the fins to the radiator:

$$Q = hA(T_b - T_w) \quad (5)$$

The heat transfer can be calculated as follows:

$$Q = mC_p\Delta T = mC_p(T_{in} - T_{out}) \quad (6)$$

$$Nu = \frac{h_{exp} d_h}{K} = \frac{mC_p(T_{in} - T_{out})}{A(T_b - T_w)} \quad (7)$$

Nomenclature

C_p : specific heat; D : diameter; K : thermal conductivity; m_n : Mass of nanoparticle; Nu : Nusselt number; T : temperature.

Subscripts

Bf: base fluid; f: fluid; h: hydraulic; in: inlet; nf: nanofluid; out: outlet; p: particle.

Greek symbols

ϕ : volume fraction; ρ : density.

4. Results and discussion

4.1. Effect of heat transfer coefficient

Figure 8 shows the dependence of the total heat transfer coefficient on the nanofluid flow rate for a range of nanofluid volume concentrations. As observed, increasing the nanofluid flow rate significantly raises the nanofluid's total heat transfer coefficient. When comparing the base fluid to the nanofluid, the total heat transfer coefficient increases as the nanoparticle concentration increases. At a nanoparticle concentration of 1 vol.%, the total heat transfer coefficient of Al_2O_3/DI water nanofluids is increased by 12% compared to the base fluid [39]. At a concentration of 0.8 vol.%, the nf improves the total heat transfer coefficient by 9%. The increase in heat transfer coefficient using nf may be described by the heat transfer simulation, the reduction in thermal boundary layer thickness, and an increase in thermal conductivity. Furthermore, the significant reason for this improvement in thermal conductivity of nf in the radiator pipe flow [40]. This represents the inconsistent particle concentration in the tubes.

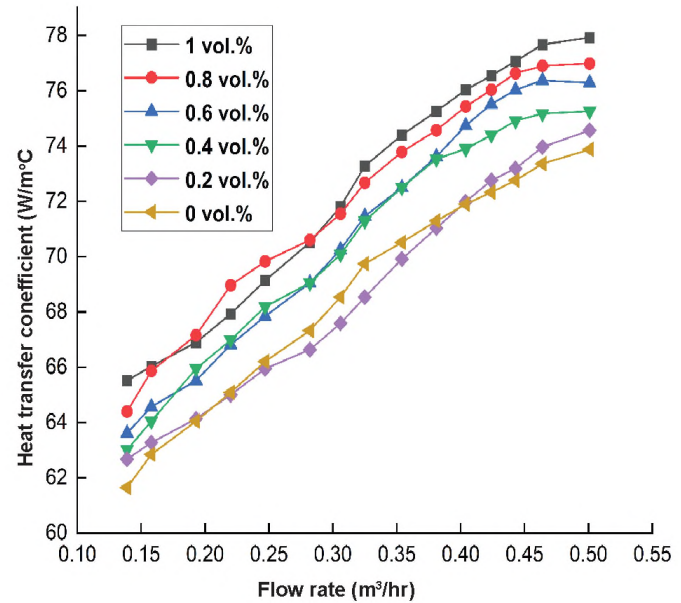


Fig. 8. Variation in nanofluid flow rate as a function of concentration and heat transfer coefficient.

4.2. Effect of thermal conductivity

Figure 9 shows the temperature-dependent thermal conductivity of aluminium oxide-radiator coolant nanofluid at varying volume concentrations. The illustration demonstrates that the nanofluid's thermal conductivity increases as both concentration and temperature rise. The thermal conductivity of both the base fluid and the nanofluid

increases with temperature, but the nanofluids exhibit better thermal conductivity than the base fluid at all concentrations due to the motion of particles during heating. As the fluid's temperature rises, the particles move more rapidly against one another, increasing the fluid's thermal conductivity.

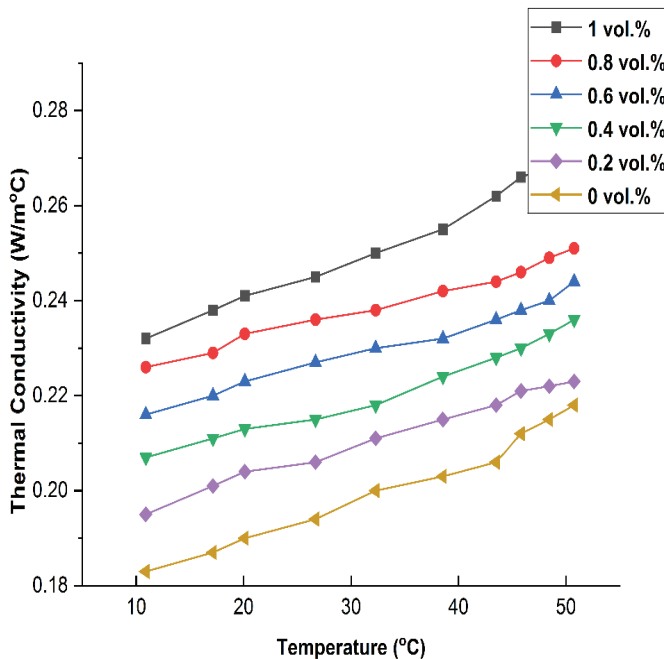


Fig. 9. Changes in nanofluid thermal conductivity with temperature.

The thermal conductivity of the base fluid was 0.250 W/mK at 10°C, whereas that of the nanofluid was 0.270 W/mK with a concentration of 1 vol.%, an increase of 8%. This indicates that the nanofluid's thermal conductivity improves as its concentration rises. At 50°C, the thermal conductivity of the base fluid and the nanofluid at 1 vol.% were 0.259 and 0.280 W/mK, respectively [41]. At a concentration of 1 vol.%, the thermal conductivity of the nanofluid is 8.30% higher than that of the base fluid at the same temperature. In every instance, the nanofluid was shown to have superior thermal conductivity than the base fluid. The thermal conductivity of nf has been observed to increase almost linearly. However, the lines on the graph do not match up perfectly. The nanofluid's thermal conductivity may be affected by factors other than particle concentration and temperature, such as particle size and shape.

Radiator coolant-based nanofluids experimental data on their thermal conductivity are shown in Fig. 10. This data compares the thermal conductivity values of Al_2O_3 -radiator cooled nanofluid at 27°C with several empirical models at

various volume concentrations ranging from 0 to 1% [42, 43]. Both experiments and established models show that as concentration increases, so does thermal conductivity. However, when using an Al_2O_3 -based radiator coolant, the thermal conductivity model of R.L. Hamilton, et al. (1962) [43] is 1.92% higher than that of N. Kumar, et al. (2018) [42]. The experimentally determined thermal conductivities for a 1 vol.% concentration of Al_2O_3 -based nanofluid were 4.26 and 2.20% higher than the estimated thermal conductivities using the N. Kumar, et al. (2018) [42] and R.L. Hamilton, et al. (1962) [43].

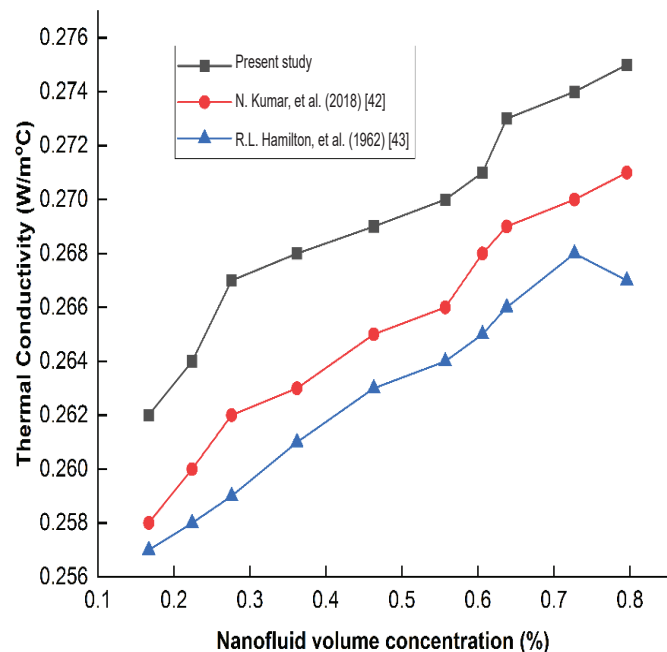


Fig. 10. Comparison of the experimental data with models.

4.3. Effect of viscosity

From 10 to 50°C, and at 0 to 1 vol.% nanoparticle concentration, the viscosities of Al_2O_3 -DI water-based nanofluid were measured. Fig. 11 demonstrates that as the temperature increases, nanofluid viscosity decreases rapidly. The nanofluid has a higher viscosity at all volume concentrations than the base fluid. Moreover, the viscosity of the nanofluid is shown to rise with the concentration in the base fluid and to decrease with an increase in temperature. The viscosity of the nanofluid falls by 61.05% when raised from 10 to 50°C, whereas the viscosity of the base fluid decreases by 72.11% over the same temperature range. In general, liquids tend to become less dense as temperatures rise [44].

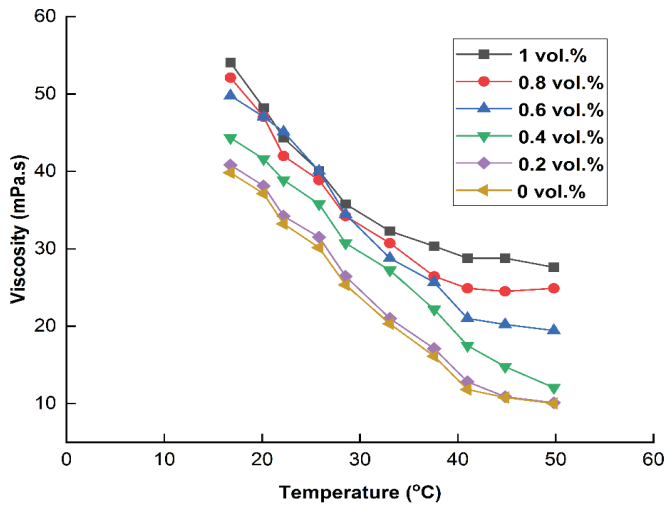


Fig. 11. Al_2O_3 nanofluid vs. temperature for different volume concentrations.

The viscosities of the Al_2O_3 -DI water nanofluids at 30°C were calculated and compared to experimental values from the literature. M. Kole, et al. (2010) [45] results on the viscosity of Al_2O_3 /DI water were chosen for comparison and are presented in Fig. 12. At 0.2 vol.%, the viscosity of Al_2O_3 -DI water nanofluid is the same as that of M. Kole, et al. (2010) [45]. However, the observed viscosity of this study appeared to be greater than that of M. Kole, et al. (2010) [45]. While the nanoparticles and base fluid were the same in both situations, their sources were unique. As a result, variations in the purity and concentrations of the two sources are possible, resulting in this minor discrepancy in viscosity values. Such differences were also observed in other researchers' experimental data for the same nanofluids.

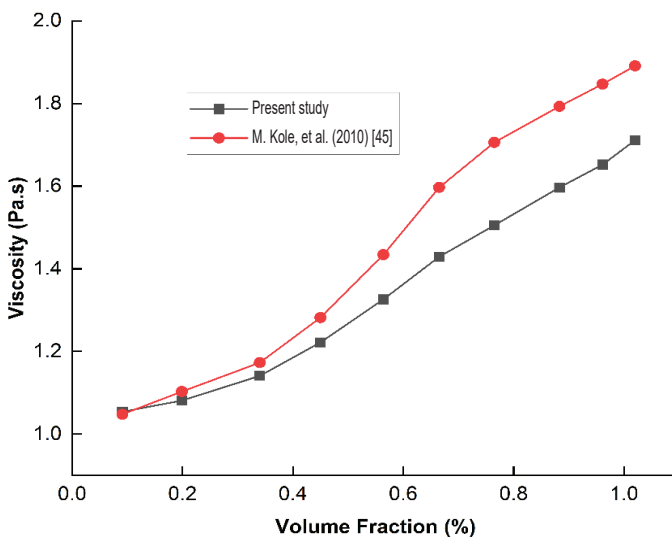


Fig. 12. Model comparison with experimental values [45].

4.4. Comparison of base fluid

To ensure the consistency and precision of the experimental apparatus, preliminary tests were conducted using pure water before proceeding with systematic investigations on the use of nanofluids in the radiator. Fig. 13 presents the experimental findings obtained with a consistent input temperature of 49°C . As anticipated, there is a positive correlation between the Nusselt and Reynolds numbers. Additionally, a comparison was conducted between the experimental results and two established empirical correlations, one proposed [46].

$$Nu = 0.0236Re^{0.82}Pr^{0.3} \quad (8)$$

Figure 13 demonstrates notable concurrence between the Dittus equation and the experimental data within the Reynolds number range employed in this investigation [47]. The findings indicate that the correlation proposed [48] did not align with the current experimental data regarding water flow in flat tubes. The results concerning various water temperatures at the radiator intake, particularly 37, 44, and 49°C , indicate that the Dittus relation exhibits an absolute average inaccuracy of 7%, while the V. Gnielinski's (2009) [48] correlation demonstrates a value of 30% for the exact measurement.

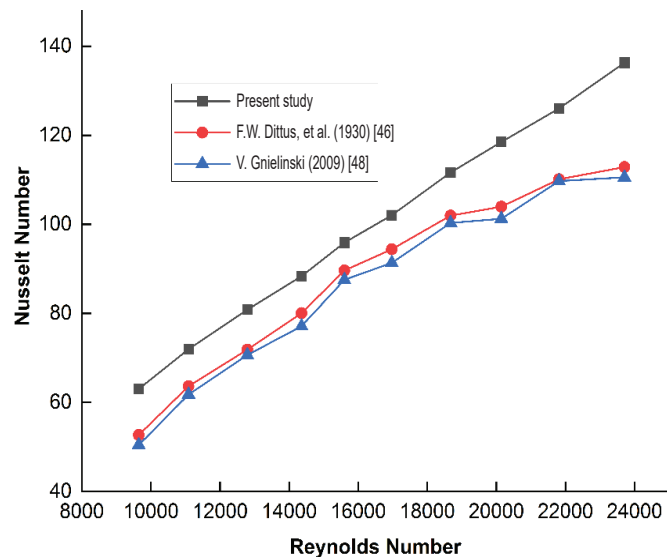


Fig. 13. Experimental results for DI water in comparison with the existing correlations.

5. Conclusions

1. With a nanoparticle concentration of 1 vol.%, the total heat transfer coefficient of Al_2O_3 /DI water nanofluids increased by 12% compared to the base fluid.

2. The thermal conductivity of the base fluid was 0.250 W/mK at 10°C, whereas that of the nanofluid was 0.270 W/mK with a concentration of 1 vol.%, an increase of 8%.

3. The nanofluid's viscosity fell by 61.05% when the temperature was raised from 10 to 50°C, whereas the viscosity of the base fluid decreased by 72.11% over the same temperature range.

4. The density of Al₂O₃-DI water nanofluid and radiator coolant decreased with increasing temperature, which is connected to the rise in fluid volume caused by increasing temperatures.

Strengths and limitations: It is easy to change the focus on an area and quickly eliminate a lot of heat. Crossflow comprises compressed air to a group of jets that hit a small area. The liquids and air may absorb and transfer heat, but not as effectively as required.

CRedit author statement

M. Peeraiah: Concept, Design, and Data collection; K. Nagamalleswara Rao: Analysis and Interpretation of results; B. Balakrishna: Draft manuscript preparation.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Development and validation of quantitative procedure of clotrimazole and eugenol in gel product using high-performance liquid chromatography

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Abstract:

Vulvovaginitis is a prevalent gynaecological ailment worldwide, often attributed to bacteria, *Candida* fungi, or *Trichomonas*. The synergistic action of antifungal agents and essential oils can enhance the efficacy against pathogenic microorganisms. In this study, a simultaneous quantitative method for determining clotrimazole and eugenol in a laboratory-compounded gel product was developed using high-performance liquid chromatography with a photodiode array detector (HPLC-PDA). The HPLC-PDA system utilised in this study was the Shimadzu LC-20AD, with a HiQ Sil RP C18 HS column (250 x 4.6 mm, 5 µm), and a mobile phase consisting of methanol-water in an 80:20 (v/v) ratio, employing isocratic elution at a flow rate of 1.0 ml/min. The injection volume was set at 20 µl, and detection was performed at a wavelength of 229.0 nm. The analytical procedure was validated in accordance with ASEAN guidelines for the validation of analytical procedures, successfully meeting the criteria for specificity, accuracy, system suitability, repeatability, intermediate precision, and linearity within the concentration range of 2.25 to 36.00 ppm for eugenol and 12.00 to 200.00 ppm for clotrimazole. Subsequently, this validated procedure was applied to quantify the clotrimazole and eugenol content in bulk materials and three different lots of gel products. The results for clotrimazole and eugenol content in both the raw materials and the gel product lots fell within an acceptable range, with deviations of less than ±10% compared to the labelled content.

Keywords: antibacterial activity, antifungal activity, clotrimazole, high-performance liquid chromatography, *Ocimum gratissimum* essential oil.

Classification numbers: 2.2, 3.3

1. Introduction

Vaginitis is a medical condition encompassing various conditions characterised by infection or inflammation of the vagina, with vulvovaginitis being the inflammation affecting both the vagina and vulva. In a healthy vaginal environment, *Lactobacillus* bacteria play a vital role by metabolising glucose into lactic acid, thereby maintaining a normal vaginal pH level ranging from 3.5 to 4.6. The acidic pH fosters the proliferation of *Lactobacillus* by providing advantageous conditions for their growth. *Lactobacillus* also produces hydrogen peroxide, which effectively combats bacteria and hampers the growth of pathogenic microorganisms. The presence of *Lactobacillus* in vaginal discharge typically amounts to approximately 10⁶ colony-forming units per millilitre (CFU/ml) [1]. Pathogenic bacteria can reduce the concentration of *Lactobacillus* to below 10³ CFU/ml. When vaginal pH levels rise, it creates a conducive environment for the proliferation of pathogenic bacteria, ultimately leading to vulvovaginitis [2].

Clotrimazole is a broad-spectrum antifungal compound, first discovered in the late 1960s, belonging to the azole group. This group is the largest class of antifungals used in clinical treatments, comprising two primary subgroups: imidazole and triazole, with clotrimazole falling under the imidazole subgroup [3]. Clotrimazole operates by inhibiting the methylation of 14- α -lanosterol through cytochrome P450, a critical step in the biosynthesis of ergosterol in fungi. This results in the replacement of ergosterol with 14- α -methyl sterol, an atypical sterol, causing disruptions in the flexibility and permeability of the fungal cell membrane. Clotrimazole not only impedes enzyme activity related to cell membrane synthesis but also enhances the leakage of cell contents through the membrane. Clotrimazole is considered an inhibitor at lower concentrations and a fungicidal agent at higher concentrations [4].

Furthermore, clotrimazole exhibits pharmacological effects such as inhibiting the activity of the Ca²⁺-ATPase

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channel, disrupting intracellular calcium storage, blocking calcium-dependent potassium channels, and voltage-dependent calcium channels. *In vitro* studies have shown that clotrimazole inhibits the growth of certain normal and cancer cell types. It is also reported to have neuroprotective effects, reducing the toxicity of metal cations to cells. Additionally, clotrimazole demonstrates activity against the malaria parasite *in vitro* due to its inhibitory action on the hemoperoxidase enzyme [4, 5].

Numerous studies have focused on quantifying clotrimazole in various sample types, including biological fluids. For instance, N. Tamilselvi, et al. (2016) [6] developed and validated a novel quantitative procedure for clotrimazole in human plasma using a reversed-phase C18 column and a mobile phase of 0.5% TEA in water-acetonitrile (20:80, v/v). M. Locatelli, et al. (2017) [7] established a robust method for the simultaneous determination of 12 azole compounds in body fluids (plasma and urine). This method employed HPLC-PDA combined with fabric phase sorptive extraction, a Luna C18 column, and a gradient elution mode. C. Campestre, et al. (2017) [8] analysed 12 azole drugs in biological samples using HPLC-PDA with micro-extraction by packed sorbent. The analytical procedure employed a C18 packing column and a gradient mode involving a mixture of phosphate buffer (pH 2.5) and acetonitrile.

Ocimum gratissimum essential oil is extracted from the aerial parts of *Ocimum gratissimum*, a member of the Lamiaceae family. Eugenol constitutes the predominant component, accounting for 45-75% of *Ocimum gratissimum* essential oil, followed by terpinolene and germacrene D. This essential oil effectively inhibits pathogenic bacterial strains, including *Staphylococcus aureus* and *Enterobacteriaceae* such as *Escherichia coli*, *Salmonella typhimurium*, *Salmonella typhi*, *Shigella flexneri*, *Salmonella enteritis*, and *Proteus mirabilis*, with a minimum inhibitory concentration (MIC) ranging from 3 to 12 mg/ml and a minimum bactericidal concentration (MBC) twice that of the MIC [9]. *Ocimum gratissimum* essential oil also demonstrates the ability to inhibit various pathogenic fungi, including *Botryosphaeria rhodia*, *Rhizoctonia* sp., *Alternaria* sp., *Cryptococcus neoformans*, and numerous skin fungi [10].

While clotrimazole is an effective antifungal agent, its activity against bacteria is limited, making it suitable primarily for treating vulvovaginitis resulting from fungal infections. In recent years, researchers have explored the combination of antifungal drugs and essential oils to broaden the spectrum of targeted microorganisms. Some

studies have indicated that the coadministration of essential oils with antifungal drugs can significantly enhance their inhibitory activity against various microorganisms. However, limited research has been conducted on the combination of clotrimazole and essential oils. In a previous study, the combination of clotrimazole and *Ocimum gratissimum* essential oil exhibited potent activity against nine bacterial and fungal strains [11]. Building upon this finding, we formulated a laboratory-produced gel product. In this study, we have developed and validated a procedure for quantifying the content of clotrimazole and eugenol, the primary component of *Ocimum gratissimum* essential oil, in this gel product.

2. Materials and methods

2.1. Materials

Clotrimazole was supplied by the Medical Biomaterial and Pharmaceutical Joint Stock Company, Ho Chi Minh City, Vietnam, with a purity of 99%. *Ocimum gratissimum* essential oil, sourced from McWay Beauty Care company (Saroma Brand) in Ho Chi Minh City, Vietnam. Reference standards of clotrimazole were obtained from the Institute of Drug Quality Control Ho Chi Minh City, and eugenol from Sigma-Aldrich, with purities of 99.38 and 99.00%, respectively.

Gel product formulation: Clotrimazole 0.50 g; *Ocimum gratissimum* essential oil 0.15 g; ingredients (propylene glycol, hydroxypropyl methylcellulose, polyethylene glycol 4000, benzyl alcohol, stabiliser) until the total is 100.00 g.

2.2. Methods

Surveying quantitative procedure conditions: Initially, we investigated the composition and proportions of the mobile phase. Standard samples containing clotrimazole and eugenol references, as well as test samples with comparable concentrations, were prepared. The experiments were conducted under the suggested chromatographic conditions, which included:

- Column: HiQ Sil RP C18 HS (250 x 4.6 mm, 5 µm) or Supelco RP C18 (250 x 4.6 mm, 5 µm).
- Detection wavelength: 229.0 nm.
- Flow rate: 1.0 ml/min.
- Injection volume: 20.0 µl.
- Mobile phase: 1. Acetonitrile-water 90:10 (v/v) or 2. Methanol-water 80:20 (v/v) or 3. Methanol-water 90:10 (v/v).

Based on the chromatographic parameters for clotrimazole and eugenol peaks, we selected the most suitable mobile phase and column type. Subsequently, we explored two sample treatment methods:

Method 1: Weighed 1.0 g of gel, diluted it with methanol in a 10.0 ml volumetric flask, then further diluted this solution ten-fold with the same solvent. Afterwards, the solution was filtered through a 0.45 µm membrane filter to obtain the test sample.

Method 2: Weighed 1.0 g of gel, diluted it with dichloromethane, and completely evaporated the solvent. The resulting residue was then diluted with methanol in a 10.0 ml volumetric flask, followed by a ten-fold dilution with the same solvent. The solution was then filtered through a 0.45 µm membrane filter to obtain the test sample.

We conducted experiments using the chosen conditions with mobile phases 2 and 3. The most suitable sample treatment method was selected based on the chromatogram parameters. Lastly, we investigated the number of extractions with dichloromethane. The test sample was treated with Method 2, subjected to repeated extractions with dichloromethane, and chromatography was carried out until no clotrimazole and eugenol peaks were detected on the chromatograms.

Validation of the simultaneous quantitative procedure of clotrimazole and eugenol: The quantitative procedure was validated following the ASEAN guidelines for the validation of analytical procedures, which includes assessments for specificity, linearity and range, accuracy, and precision [12].

Determination of clotrimazole and eugenol contents in bulk materials and products: Standard samples of clotrimazole and eugenol were prepared with concentrations of 50.0 and 9.0 ppm, respectively. The clotrimazole sample was prepared by weighing 1.0 g of clotrimazole standard, dissolving it in methanol, and diluting it with the same solvent to obtain a 50.0 ppm solution. The *Ocimum gratissimum* essential oil sample was prepared by weighing 1.0 g of the oil, dissolving it in methanol, and diluting it with the same solvent to obtain a 9.0 ppm solution.

Both test samples and standard samples underwent the chromatography procedure, and the content of clotrimazole and eugenol in the materials was calculated. For the gel products, test samples and standard samples were prepared and subjected to the chromatography process according to the specified procedures. The contents of clotrimazole and eugenol in 100 g of the gel product were calculated using the following equation:

$$X = \frac{S_T}{S_S} \times \frac{a}{1000} \times C\% \times \frac{K_T}{K_S} \times \frac{100}{m}$$

where, X : the weight of eugenol (clotrimazole) in 100 g of gel product (g); S_T : the peak area of eugenol (clotrimazole) in the test sample; S_S : the peak area of eugenol (clotrimazole) in the standard sample; a : the weight of eugenol (clotrimazole) standard (g); $C\%$: the purity of eugenol (clotrimazole) standard; K_T , K_S : the dilution level of the test sample and standard sample; m : the weight of the product (g).

3. Results and discussion

3.1. Surveying quantitative procedure conditions

Until this point, there has been no prior research dedicated to the development of a simultaneous quantitative procedure for eugenol and clotrimazole in a product. This lack of research can be attributed to the disparate structural and physicochemical characteristics of these compounds. Eugenol is typically quantified using gas chromatography, whereas clotrimazole quantification methods often rely on HPLC. Considering this, and after consulting relevant literature, it was established that both eugenol and clotrimazole could be quantified individually using HPLC, which is a widely adopted and suitable technique for laboratory use. Consequently, we proceeded to develop a simultaneous quantitative procedure for eugenol and clotrimazole utilising HPLC-PDA.

Figure 1 illustrates that the Supelco column had the capability to separate the eugenol and clotrimazole peaks, but the associated parameters did not meet the desired criteria. Conversely, the HiQ Sil column effectively separated the eugenol and clotrimazole peaks while yielding superior parameters. The use of an acetonitrile-water solvent led to an asymmetrical peak and prolonged chromatographic duration. Hence, we opted for the HiQ Sil column in conjunction with a methanol-water mobile phase for the chromatographic procedure.

Most references in the field employed a reversed-phase C18 column for the quantification of eugenol and clotrimazole. We investigated two types of columns, Supelco and HiQ Sil, both of which employed octadecyl carbon chain bonded silica as the stationary phase. However, they differed in terms of stationary phase particles and silanisation content [13, 14]. The HiQ Sil column employed high-purity silica gel particles and a high carbon loading content, resulting in sharper and more symmetrical peaks. Our quantitative findings revealed that the eugenol and clotrimazole peaks obtained using the Supelco column exhibited tailing, whereas those achieved with the HiQ Sil column displayed

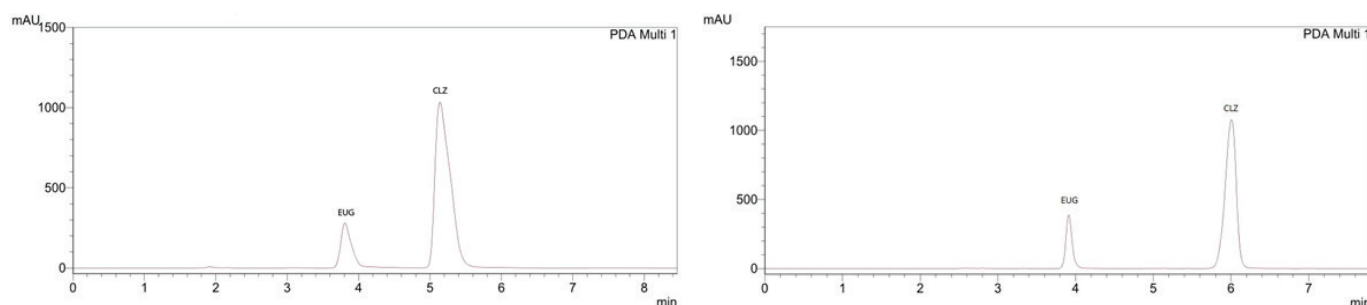


Fig. 1. Chromatograms of eugenol and clotrimazole with Supelco column (left) and HiQ Sil column (right).

satisfactory asymmetry factors. Consequently, we selected the HiQ Sil column for the simultaneous quantification of eugenol and clotrimazole.

Upon reviewing prior research, we ascertained that standard mobile phases such as acetonitrile-water and methanol-water were typically used, with buffer solutions designed specifically for clotrimazole quantification. S.S. Santos, et al. (2012) [15] employed methanol-water (90:10, v/v) with isocratic elution at a flow rate of 1.0 ml/min to determine clotrimazole content. S.J. Liu, et al. (2010) [16] utilised methanol-water (65:35, v/v) in an isocratic elution mode at a flow rate of 1.0 ml/min for eugenol quantification. Although acetonitrile was also tested as a mobile phase on both columns, the resulting peaks did not exhibit satisfactory asymmetry factors and necessitated extended analysis times. As a result, acetonitrile was excluded from the quantification process. Following experimentation with various methanol-

water ratios, we concluded that the ratio of 80:20 (v/v) was optimal for the simultaneous quantification of eugenol and clotrimazole.

Results of the test sample treatment method survey are depicted in Fig. 2. Method 1 was straightforward and easily implemented, but the ensuing chromatograms displayed multiple impurity peaks that remained unresolved from the primary analytical peaks. Conversely, Method 2, although more intricate and comprising multiple stages, effectively eliminated a substantial portion of impurities and separated the primary peaks. The peak obtained using methanol-water 80:20 (v/v) achieved the desired level of purity and met all other requisite parameters, while the peak obtained using methanol-water 90:10 (v/v) exhibited unsatisfactory purity.

In terms of the number of extraction times, the chromatogram obtained after the 4th extraction did not

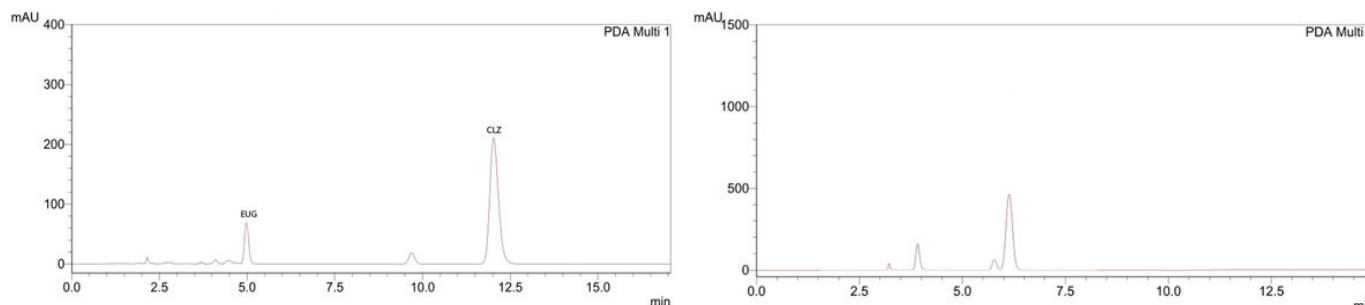


Fig. 2. Chromatograms of test samples treated by Method 2 using methanol-water 80:20 (v/v) (left) and methanol-water 90:10 (v/v) (right).

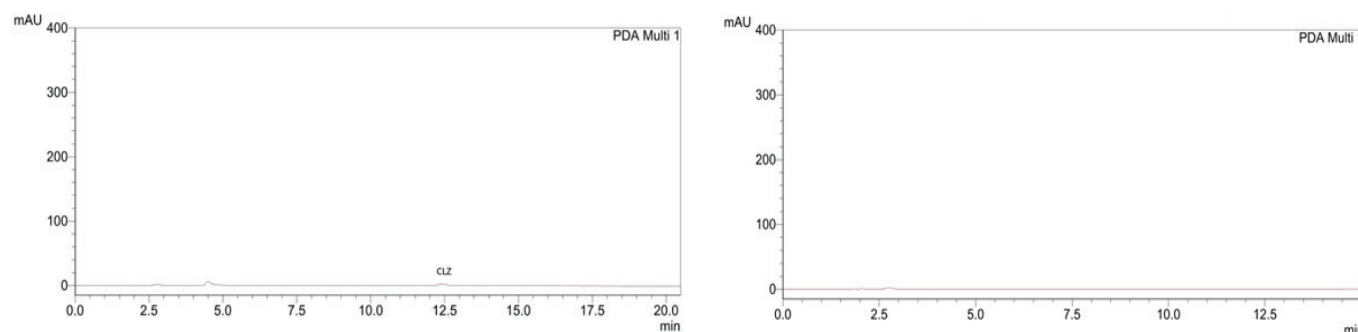


Fig. 3. Chromatograms of eugenol and clotrimazole of the 3rd (left) and 4th (right) extraction.

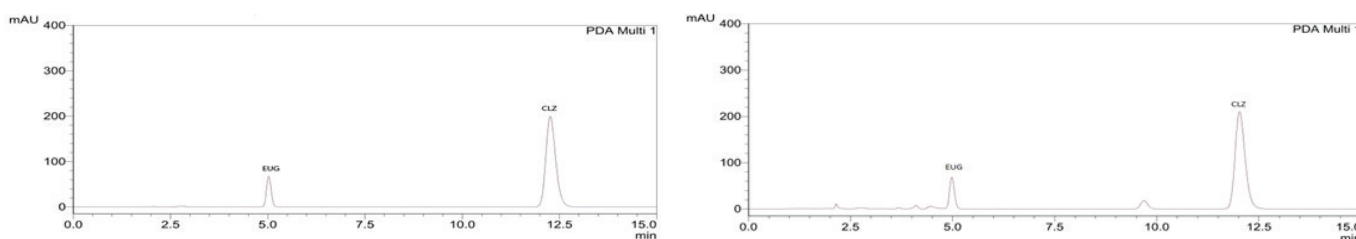


Fig. 4. Chromatograms of eugenol and clotrimazole in the standard sample (left) and test sample (right) under the optimal conditions.

exhibit any eugenol and clotrimazole peaks (Fig. 3). This implies that conducting 3 extraction cycles was adequate to fully extract eugenol and clotrimazole from the test sample.

The effectiveness of the simultaneous quantitative procedure for eugenol and clotrimazole relied on the treatment method employed to eliminate impurity peaks from the chromatograms, which can impact the quantification results. To extract eugenol and clotrimazole from the gel product and eliminate impurity peaks, we utilised dichloromethane as a solvent, followed by dissolving the extracted components in methanol. This process successfully removed numerous impurity peaks, completely separated the primary peaks, and yielded favourable chromatographic parameters. Incomplete extraction of the active components would have introduced quantification errors. Consequently, we also investigated the number of extraction cycles with

dichloromethane, ultimately determining that a three-step extraction process was optimal for sample treatment.

The optimal chromatography conditions were chosen:

- Instrument: HPLC-PDA Shimadzu LC-20AD System.
- Column: HiQ Sil RP C18 HS (250 x 4.6 mm, 5 μ m).
- Mobile phase: Methanol-water 80:20 (v/v).
- Elution mode: Isocratic.
- Flow rate: 1.0 ml/min.
- Injection volume: 20.0 μ l.
- Detection wavelength: 229.0 nm.

With these selected chromatography conditions, the chromatograms exhibited well-resolved eugenol and clotrimazole peaks with satisfactory peak parameters (Fig. 4). The retention time of eugenol and clotrimazole in the test sample matched that in the standard sample.

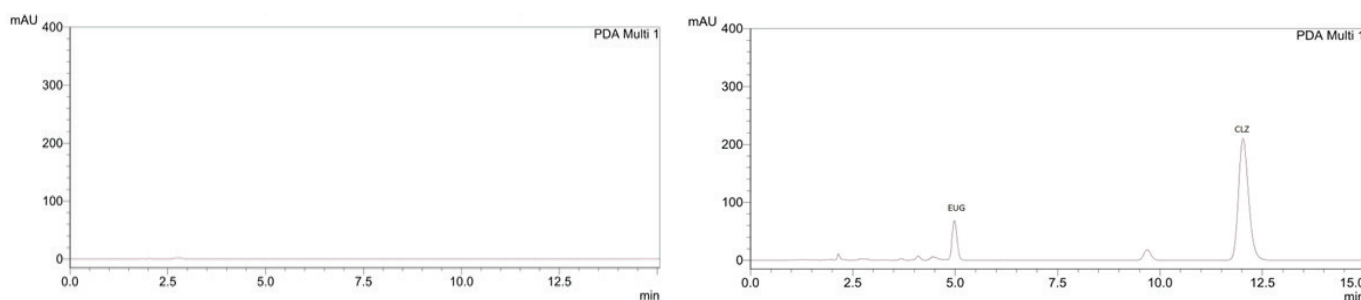


Fig. 5. Chromatograms of the blank sample (left) and test sample (right).

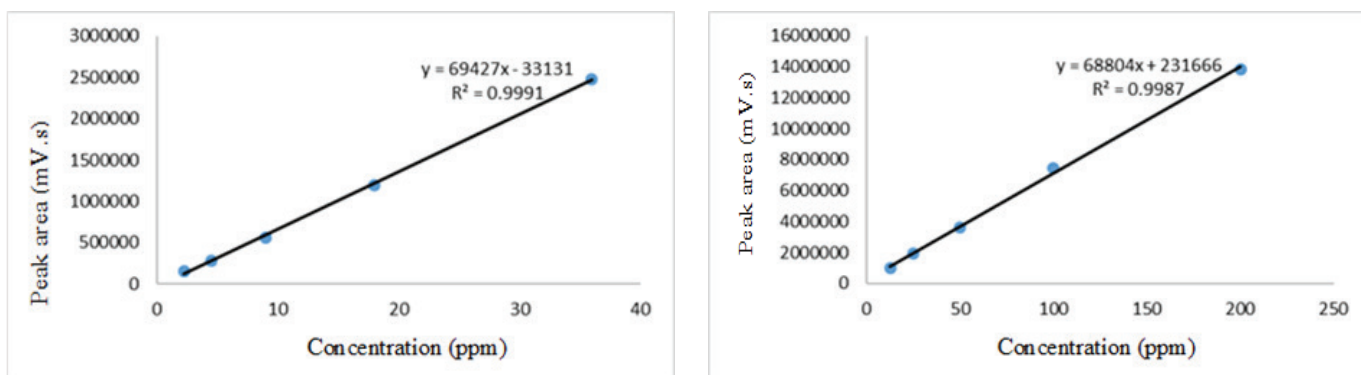


Fig. 6. Regression equations of eugenol (left) and clotrimazole (right) peaks.

3.2. Validating the quantitative procedure

Specificity: Fig. 5 confirms that the chromatograms of the blank sample did not display any peaks with the same retention time as the eugenol and clotrimazole standards. In the chromatogram of the test sample, eugenol and clotrimazole peaks with similar retention times to those of the standards were observed. In the chromatograms of the standard samples, the eugenol and clotrimazole peaks were entirely separated from impurity peaks. The eugenol and clotrimazole peaks in the chromatograms of both the test and standard samples exhibited good purity (peak impurity of approximately 100%). The spectral overlay coefficient of the eugenol and clotrimazole peaks in the chromatograms of the test and standard samples was approximately 1.0.

Linearity and range: Fig. 6 illustrates the linearity of the quantitative procedure for eugenol and clotrimazole. The suitability of the regression equation and the significance of the coefficients "a" and "b" were verified using the T-test, F-test, and ANOVA analysis with Microsoft Excel software. The regression equation for eugenol was $\hat{y}=69,427x$ ($R^2=0.9991$), and for clotrimazole, it was $\hat{y}=68,804x$ ($R^2=0.9987$). The linear range for eugenol and clotrimazole was 2.25-36.00 ppm and 12.50-200.00 ppm, respectively, with R^2 values exceeding 0.99.

Accuracy: Table 1 illustrates the accuracy of the quantitative procedure for eugenol and clotrimazole.

The quantitative procedure exhibited a recovery rate of 95.0-105.0% for eugenol and 97.0-103.0% for clotrimazole, with $RSD\% \leq 2.0\%$ (Table 1).

Table 1. The accuracy of the eugenol and clotrimazole quantitative procedure.

Concentration	Eugenol			Clotrimazole		
	Amount of added standard	Amount of found standard	Recovery rate (%)	Amount of added standard	Amount of found standard	Recovery rate (%)
80%	7.20	6.83	94.84	40.00	40.61	101.53
	7.20	6.83	94.87	40.00	39.76	99.40
	7.20	6.98	96.93	40.00	41.07	102.67
	Average		95.55	Average		101.20
	RSD%		1.25	RSD%		1.64
100%	9.00	8.70	96.65	50.00	50.24	100.48
	9.00	8.77	97.48	50.00	51.56	103.12
	9.00	8.52	94.66	50.00	49.91	99.83
	Average		96.26	Average		101.14
	RSD%		1.51	RSD%		1.73
120%	10.80	10.47	96.98	60.00	60.29	100.49
	10.80	10.41	96.43	60.00	60.11	100.18
	10.80	10.54	97.61	60.00	60.74	101.24
	Average		97.01	Average		100.64
	RSD%		0.61	RSD%		0.54

Table 2. The system suitability of the eugenol and clotrimazole quantitative procedure.

No.	Eugenol				Clotrimazole			
	RT	Peak area	Peak height	Asymmetry	RT	Peak area	Peak height	Asymmetry
1	5.026	537,646	59,206	1.17	12.355	3,435,165	188,728	1.18
2	5.028	513,952	56,550	1.17	12.329	3,266,724	179,641	1.18
3	5.033	521,251	57,303	1.17	12.330	3,319,747	182,651	1.18
4	5.031	525,383	57,758	1.16	12.347	3,325,773	183,014	1.24
5	5.034	525,366	57,847	1.17	12.353	3,345,920	183,582	1.18
6	5.029	532,316	58,980	1.17	12.344	3,362,791	180,214	1.19
Average	5.030	525,986	57,941		12.343	3,342,687	182,972	
SD	0.003	8,294.70	1,006.38		0.011	55,766.49	3,234.52	
RSD%	0.06	1.58	1.74		0.09	1.67	1.77	

Precision: The system suitability: The RSD% values for the retention time and peak area of eugenol and clotrimazole were $\leq 2.0\%$. All other parameters of the two active ingredients met the standard criteria for liquid chromatography methods (Table 2).

Repeatability: The RSD% values for the eugenol and clotrimazole quantitative procedure in test samples were $\leq 2.0\%$. Other parameters of the two active components met the standard criteria for the liquid chromatography method (Table 3).

Table 3. The repeatability of the eugenol and clotrimazole quantitative procedure.

Active ingredient content (%)		
	Eugenol	Clotrimazole
1	0.088	0.496
2	0.088	0.501
3	0.089	0.507
4	0.089	0.510
5	0.089	0.511
6	0.088	0.496
Average	0.088	0.503
SD	0.0004	0.0068
RSD%	0.49	1.36

Intermediate precision: In test samples, the RSD% value for the eugenol and clotrimazole quantitative procedure was $\leq 2.0\%$, and the difference in active ingredient content between the two analysts was $\leq 2.0\%$ (eugenol 1.67% and clotrimazole 1.54%) (Table 4). In summary, the method was validated according to ASEAN guidelines for the validation of analytical procedures, encompassing specificity, linearity and range, accuracy, system suitability, repeatability, and intermediate precision.

Table 4. The intermediate precision of the eugenol and clotrimazole quantitative procedure.

	Eugenol		Clotrimazole	
	Analyst 1	Analyst 2	Analyst 1	Analyst 2
1	0.088	0.090	0.496	0.515
2	0.088	0.089	0.501	0.492
3	0.089	0.092	0.507	0.517
4	0.089	0.091	0.510	0.503
5	0.089	0.088	0.511	0.502
6	0.088	0.091	0.496	0.505
Average	0.088	0.090	0.503	0.506
SD	0.0004	0.0015	0.0068	0.0092
RSD%	0.49	1.70	1.36	1.81

The procedure was developed to quantify eugenol and clotrimazole in a combination gel product containing 0.5% clotrimazole and 0.15% *Ocimum gratissimum* essential oil (equivalent to 0.09% eugenol). The peaks of eugenol and clotrimazole met all specified parameters. The procedure was validated according to the ASEAN Guidelines for the validation of analytical procedures, covering specificity, linearity and range, accuracy, system suitability, repeatability, and intermediate precision. Validation results for all criteria were excellent, with linear ranges of 2.25-36.00 ppm for eugenol and 12.50-200.00 ppm for clotrimazole.

3.3. Determination of the eugenol and clotrimazole content in material and gel products

The content of clotrimazole and eugenol in the material and three lots of gel products has been documented in Tables 5 and 6.

Table 5. Content of eugenol and clotrimazole in the material.

Bulk sample	Eugenol content (%)	Clotrimazole content (%)
<i>Ocimum gratissimum</i> essential oil	60.71	
Clotrimazole		98.46

Table 6. Content of eugenol and clotrimazole in three lots of gel product.

Gel sample	Content of active ingredients in 100 g of gel product	
	Eugenol (g)	Clotrimazole (g)
Lot 1	0.087	0.483
Lot 2	0.092	0.498
Lot 3	0.084	0.527

The procedure was employed to ascertain the eugenol and clotrimazole content in the bulk and three lots of gel products, which were compounded in the laboratory. The clotrimazole content in the bulk and eugenol in the *Ocimum Gratissimum* essential oil was found to be consistent with the specifications provided in the bulk certificate of analysis. These materials were deemed pure and of high quality, suitable for use in the formulation of pharmaceutical products. In accordance with the Vietnamese Pharmacopoeia 5th Edition, the allowable variation for active ingredient content in topical products was within $\pm 10\%$ of the label contents [17]. Analysis of the eugenol and clotrimazole contents in the three lots of gel products revealed that they fell within the permissible limits.

4. Conclusions

This study has successfully developed and validated a simultaneous quantitative procedure for determining the content of clotrimazole and eugenol. The method was effectively applied to assess the content of these components in the raw materials and in three batches of the final product. The procedure exhibited robust performance in terms of specificity, linearity, accuracy, system suitability, repeatability, and intermediate precision. The results obtained from the analysis of the bulk materials and three batches of the gel product demonstrated that the eugenol and clotrimazole content adhered to the standards set by the Vietnamese Pharmacopoeia 5th Edition.

CRedit author statement

Mong To Tam Tran: Methodology, Investigation, Writing; Thi Thanh Vy Dinh: Formal analysis, Validation; Hoai Hieu Vo: Validation; Thi Hoang Yen Pham: Writing; Thi Loan Le: Editing; Dinh Nga Nguyen: Conceptualisation, Supervision.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Chemical structure of steroidal saponin isolated from the leaves of *Dracaena draco* L.

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Abstract:

Saponins are naturally occurring compounds ubiquitously present in various organisms, predominantly in plants. They are noted for forming colloidal solutions in water, which foam on shaking and precipitate cholesterol. Several *in vitro* and *in vivo* tests on mixtures and individual saponins have revealed a broad spectrum of biological and pharmacological activities, including cancer-related activity, antiphlogistic and antiallergic, immunomodulating, antihepatotoxic, antiviral, hypoglycemic, antifungal and molluscicidal activities. The *Dracaena* genus is renowned for an abundance of steroidal saponins utilized in treating digestive and hematological disorders. Previous studies on this genus revealed the presence of steroidal saponins, some of which showed cytotoxic activities against cultured tumor cells and interesting biological activities. This study procured steroidal saponin from the foliage of *D. draco* L. using various chromatographic techniques and 1D and 2D nuclear magnetic resonance (NMR) spectroscopic analysis. The structure was deduced as 26-*O*- β -D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1 β ,3 β ,22 α ,26-tetrol 1-*O*-{*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl}; a compound previously isolated from the underground parts of *Ruscus aculeatus* in Japan. Future evaluation of the saponin's biological activity is imperative to enhance chemotaxonomic data concerning the chemical composition and biological functionality of *D. draco* L. and, by extension, the *Dracaena* genus.

Keywords: Asparagaceae, *Dracaena draco* L., nuclear magnetic resonance, ornamental plant, steroidal saponin.

Classification numbers: 2.2, 3.3

1. Introduction

The *Dracaena* genus, part of the Asparagaceae family, comprises approximately 110 species predominantly native to tropical and subtropical zones [1]. Species from this genus are employed in treating digestive ailments. Traditional Chinese medicine has documented the substitution of the red resin from *D. cochinchinensis* since the 1970s to augment blood circulation and treat inflammation, diarrhea, diabetes, and haemorrhage [2]. Chemical studies on *Dracaena* species have disclosed the presence of saponins with antituberculosis, anti-inflammatory, antimicrobial, and cytotoxic properties [3-6].

D. draco L. is indigenous to the Canary Islands but is also observed in Vietnam. Its trunk and leaves exhibit a dark red hue, known as 'dragon's blood', and are employed in traditional medicine. Previous studies have examined the

isolation and structural elucidation of steroidal saponins with biological efficacy from *D. draco* L. [7]. To facilitate comparison between specimens found in Japan and Vietnam, an exhaustive phytochemical analysis of *D. draco* L.'s leaves was undertaken, culminating in the isolation of a steroidal saponin via chromatographic and spectroscopic techniques. The paper delineates the isolation and structural elucidation of this saponin.

2. Materials and methods

2.1. Plant material

The foliage of *D. draco* L. was gathered in Thai Nguyen Province, Vietnam, during July 2019 and was authenticated by Dr. Hung Duc Nguyen, a co-author. The voucher specimen is conserved at the Faculty of Biology, Thai Nguyen University of Education (Fig. 1).

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Fig. 1. The species *D. draco* L.

2.2. General experimental procedure

Nuclear magnetic resonance (NMR) spectra were acquired utilising an Inova NMR spectrometer (Agilent Technologies®, USA) at frequencies of 600 MHz for ^1H and 2D NMR (HSQC, HMBC, ROESY) and 150 MHz for ^{13}C NMR. Chemical shifts (δ) are reported in parts per million (ppm), with samples dissolved in pyridine- d_5 . Electrospray Ionisation Mass Spectrometry (ESI-MS) readings were conducted on a microTOF II mass spectrometer (Bruker®, Germany). Extractions utilised an Elmasonic S10H ultrasound cleaner (Elma, Switzerland). Isolations employed a Medium-Pressure Liquid Chromatography (MPLC) system with a silica gel 60 (40–63 μm , Merck®, Germany) stationary phase. Reversed-phase silica gel RP-18 (75–200 μm , SiliCycle, Canada) and Sephadex LH20 (Sigma Aldrich, France) were utilised for Vacuum Liquid Chromatography (VLC) and Column Chromatography (CC), respectively. Thin-layer chromatography (TLC) was conducted on pre-coated silica gel plates 60F254 (Merck, Germany). The saponins were detected with a spray reagent of vanillin-sulphuric acid, comprising 1% vanillin in an $\text{EtOH}:\text{H}_2\text{SO}_4$ solution (50:1, v/v).

2.3. Extraction and isolation of saponin

Dried, powdered leaves (130.6 g) underwent successive extractions via an ultrasound-assisted method with an $\text{EtOH}:\text{H}_2\text{O}$ (75:35, 800 ml each) solvent system at 30 W and 50°C for 35 minutes. Post evaporation under reduced pressure at 55°C using a Büchi Rotavapor R-210, 6.8 g of ethanolic-aqueous extract was procured and then dissolved in 30 ml H_2O , subsequently subjected to VLC using silica gel RP-18 and 500 ml of solvents sequentially: 100% H_2O , 50% EtOH -50% H_2O , and 100% EtOH . This process yielded three fractions, including the saponin-rich fraction DR.2 (418.1 mg). Fraction DR.2 was further separated on an MPLC with silica gel 60 using a CHCl_3 - MeOH - H_2O solvent

system at varying ratios (80-20-2, 70-30-7, 60-32-7, v/v/v) to produce five subfractions (DR.2.1-DR.2.5). Subfraction DR.2.2 (35.6 mg) underwent further MPLC to yield three subfractions, DR.2.2.1-DR.2.2.3. The subfraction DR.2.2.2 (6.3 mg) was purified using CC on Sephadex LH20 with MeOH , resulting in compound 1 (DSC02) (2.9 mg) (Fig. 2).

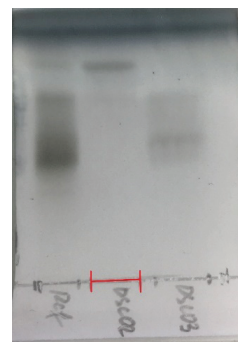


Fig. 2. TLC of compound 1 (in red).

Table 1. Spectroscopic data of compound 1 ($\text{C}_{45}\text{D}_5\text{N}$, δ in ppm, J in Hz).

Carbon	δ_c	δ_H	Carbon	δ_c	δ_H
1	83.7	3.71 dd (11.3, 2.9)	23	37.3	2.20, 2.25
2	37.3	2.34, 2.68	24	28.3	2.66, 2.70
3	67.7	3.79	25	146.6	-
4	43.1	2.52, 2.72	26	72.1	4.34 br d (12.3), 4.59 br d (12.3)
5	139.0	-	27	110.9	5.03 br s, 5.35 br s
6	124.4	5.54 d (5.1)	OCH_3	47.2	3.30 s
7	31.5	1.81, 1.84	Ara-1	100.7	4.67 d (7.0)
8	33.1	1.55	2	74.7	4.52
9	50.5	1.45	3	75.2	4.17
10	42.6	-	4	69.9	4.26
11	23.5	1.60, 2.94	5	66.9	3.72, 4.25
12	40.2	1.35, 1.66	Rha-1	101.6	6.29 br s
13	40.4	-	2	72.3	4.75 br s
14	56.7	1.15	3	72.2	4.64 dd (9.4, 3.1)
15	32.2	1.42, 2.04	4	73.9	4.38 dd (9.4, 9.3)
16	81.5	4.86 q (7.0)	5	69.4	4.88 dq (9.3, 5.8)
17	63.2	1.93	6	18.9	1.72 d (5.8)
18	16.7	0.94 s	Glc-1	103.6	4.90 d (7.6)
19	14.6	1.41 s	2	74.9	4.08
20	40.2	2.28 t (7.0)	3	78.2	4.29
21	16.5	1.26 d (7.0)	4	71.5	4.20
22	112.1	-	5	78.1	3.97
			6	62.6	4.35, 4.56

Compound 1: amorphous powder. ^1H -NMR (pyridine- d_5 , 600 MHz) and ^{13}C -NMR (pyridine- d_5 , 150 MHz), see Table 1. ESI-MS (positive mode) m/z 923.4602 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_{45}\text{H}_{72}\text{NaO}_{18}$, 923.4616).

3. Results and discussion

Compound **1** was obtained as a white amorphous powder and formed a soapy lather when shaken with water. The ESI-MS peak of compound **1** at m/z 923.4602 gave evidence for the molecular formula of $C_{45}H_{72}O_{18}$. The 1H NMR spectrum of compound **1** showed distinct signals for one olefinic proton at δ_H 5.54 (d, $J=5.1$ Hz, aglycone H-6), two exomethylene protons at δ_H 5.03 and 5.35 (br s, aglycone H-27), two tertiary methyl groups at δ_H 0.94 (s, aglycone 18-Me) and 1.41 (s, aglycone 19-Me), one secondary methyl group at δ_H 1.26 (d, $J=7.0$ Hz, aglycone 21-Me), and a methoxyl group at δ_H 3.30 (s) (Fig. 3). The ^{13}C NMR spectrum displayed a total of 27 carbons for the aglycone, excluding one for the methoxyl carbon at δ_C 47.2 (Fig. 4). Accordingly, the structural feature of the aglycone moiety was inferred to be a 22-methoxyl furostanol derivative, which is in agreement with literature [8]. The configurations of the H-1, H-3, H-6, H-9, H-16 and H-21 positions in the aglycone were determined to be in the α -axial orientation via observations

from the ROESY spectrum at δ_H/δ_H 3.71 (dd, $J=11.3, 2.9$ Hz, aglycone H-1)/3.79 (aglycone H-3), δ_H/δ_H 3.79 (aglycone H-3)/5.54 (d, $J=5.1$ Hz, aglycone H-6), and δ_H/δ_H 1.26 (d, $J=7.0$ Hz, aglycone 21-Me)/ 4.86 (q, $J=7.0$ Hz, aglycone H-16). On the other hand, the configurations of the H-8, H-14, H-18, H-19, H-16 and H-21 positions in the aglycone were inferred to be of β -axial orientation by observations of the ROESY spectrum at δ_H/δ_H 1.55 (aglycone H-8)/0.94 (s, aglycone 18-Me), δ_H/δ_H 1.55 (aglycone H-8)/1.41 (s, aglycone 19-Me), δ_H/δ_H 1.55 (aglycone H-8)/1.15 (aglycone H-14), δ_H/δ_H 1.15 (aglycone H-14)/0.94 (s, aglycone 18-Me), δ_H/δ_H 1.15 (aglycone H-14)/1.41 (s, aglycone 19-Me), and δ_H/δ_H 0.94 (s, aglycone 18-Me)/1.41 (s, aglycone 19-Me) (Fig. 5). Furthermore, the cross peak observed in the ROESY spectrum between δ_H 4.86 (q, $J=7.0$ Hz, aglycone H-16), and δ_H 3.30 (s, aglycone methoxyl proton) confirmed the C-22 α configuration. Thus, the structure of the aglycone in compound **1** was confirmed to be 22-*O*-methyلفurosta-5,25(27)-diene-1 β ,3 β ,22 α ,26-tetrol.

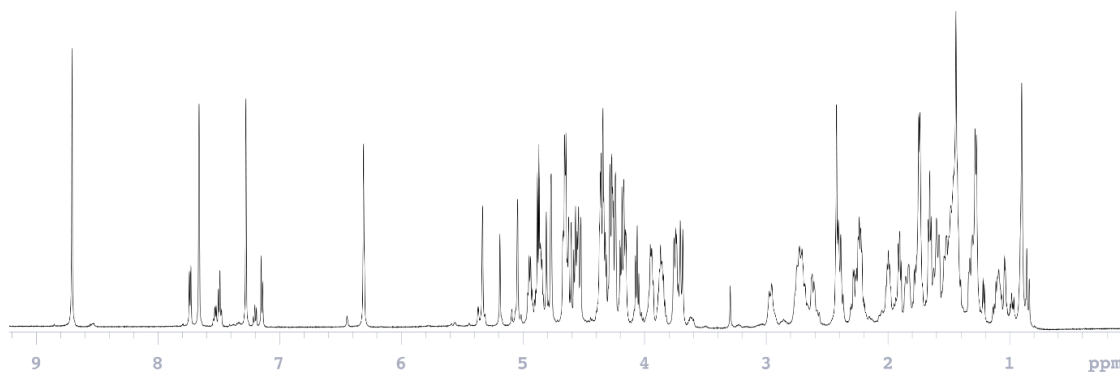


Fig. 3. 1H NMR spectrum of compound **1**.

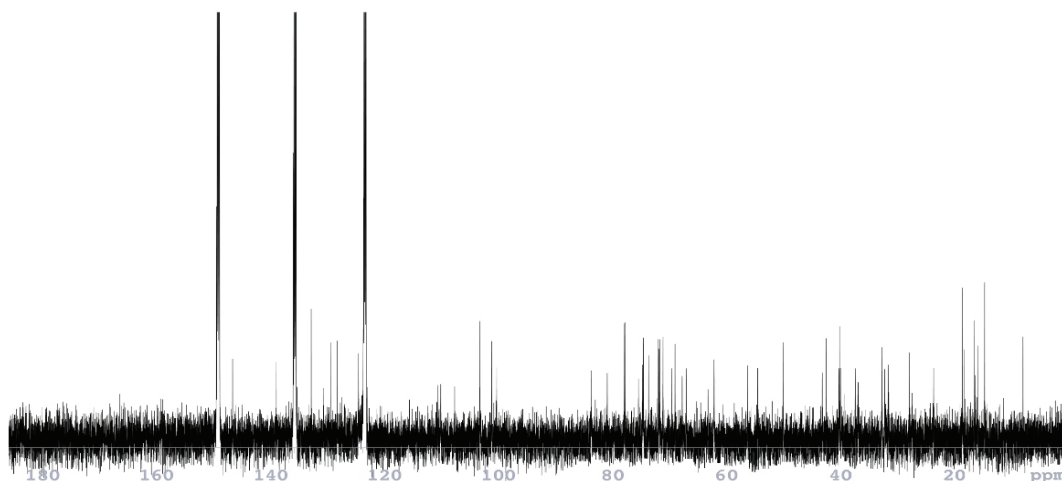


Fig. 4. ^{13}C NMR spectrum of compound **1**.

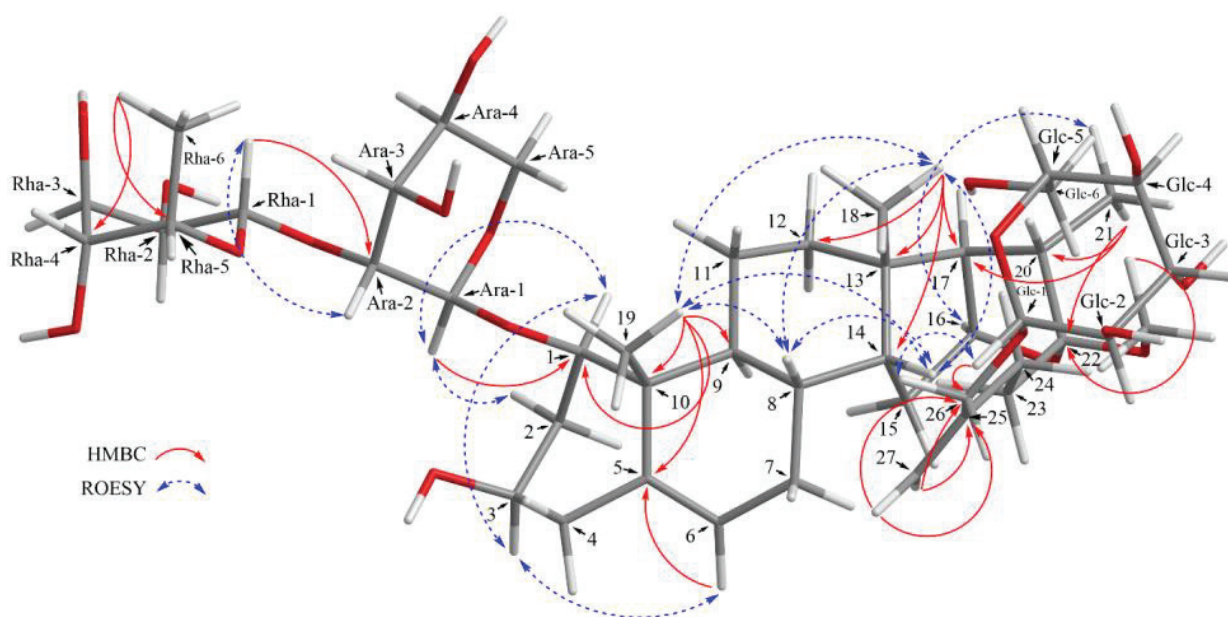


Fig. 5. Three-dimensional structure of compound 1 (HMBC correlations in red, ROESY correlations in blue).

The ^1H NMR spectrum in the sugar region of compound 1 exhibited the presence of three anomeric protons at δ_{H} 4.67 (d, $J=7.0$ Hz), 4.90 (d, $J=7.6$ Hz), and 6.29 (br s), which were further revealed to be one arabinose (Ara), one glucose (Glc), and one rhamnose (Rha) unit, respectively. These were delineated through systematic ^1H - ^{13}C HMBC and ^1H - ^1H ROESY spectra mapping from the anomeric proton signals. Acid hydrolysis and comparison with antecedent research data [9] elucidated their absolute configurations as α -L for Rha and Ara, and β -D for Glc [9]. The Ara moiety's connection to the C-1 position of the aglycone was confirmed through the HMBC correlation between δ_{H} 4.67 (d, $J=7.0$ Hz, Ara H-1) and δ_{C} 83.7 (aglycone C-1), and a ROESY correlation with δ_{H} 3.71 (dd, $J=11.3, 2.9$ Hz, aglycone H-1). The sugar sequence extending from the C-1 position of the aglycone was conclusively identified as

α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl. This was validated by the HMBC correlation from δ_{H} 6.29 (br s, Rha H-1) to δ_{C} 74.7 (Ara C-2), and the ROESY correlation between δ_{H} 6.29 (br s, Rha H-1) and δ_{H} 4.52 (Ara H-2). Furthermore, the linkage of the Glc unit to the C-26 position of the aglycone was established via the HMBC correlation from δ_{H} 4.90 (d, $J=7.6$ Hz, Glc H-1) to δ_{C} 72.1 (aglycone C-26), complemented by the ROESY correlation with δ_{H} 4.59 (br d, $J=12.3$ Hz, aglycone H-26a). Consequently, the complete molecular architecture of compound 1 was resolved as 26-O- β -D-glucopyranosyl-22-O-methylfurosta-5,25(27)-diene-1 β ,3 β ,22 α ,26-tetrol 1-O-{O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl} (Fig. 6), analogous to the compound isolated from the subterranean segments of *Ruscus aculeatus*, a member of the Asparagaceae family, sourced from Japan [10].

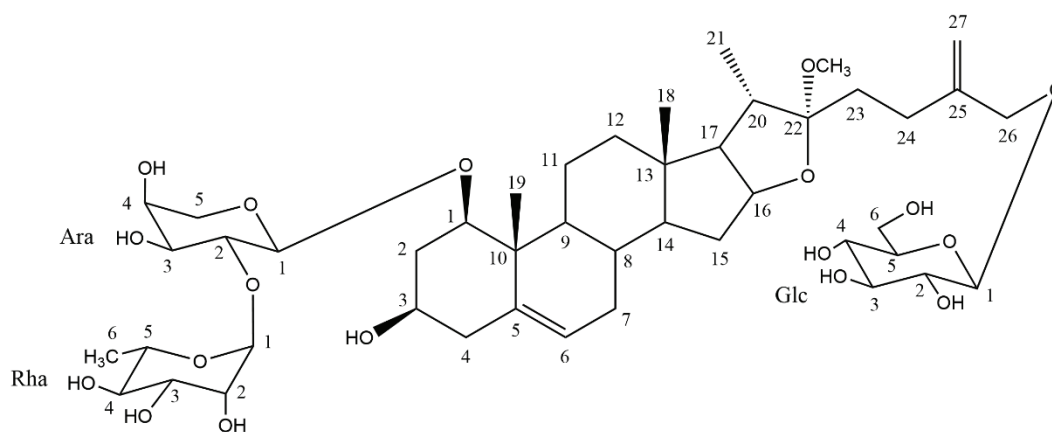


Fig. 6. Structure of compound 1.

Saponins are recognized for their hemolytic, antimicrobial, piscicidal, molluscicidal, insecticidal, antifeedant, antifertility, cytotoxic, and antitumor properties. Notably, three steroidal saponins including namonin A, namonin B, and (23*S*,24*S*)-spirosta-5,25(27)-dien-1 β -3 β -23,24-tetrol 1-*O*-{[2,3,4-tri-*O*-acetyl- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)]-[β -D-xylopyranosyl-(1 $\rightarrow$ 3)]- α -L-arabinopyranoside} 24-*O*- β -D-fucopyranoside isolated from the roots and rhizomes of *D. angustifolia* showed significant antiproliferative activity against HT-1080 fibrosarcoma cells with IC₅₀ values of 0.2, 0.3, and 0.6 μ M, respectively, compared to doxorubicin with IC₅₀ values of 0.2 μ M [11]. The biological activity of saponins correlates with their chemical structure. For example, mannioside A and other compounds such as pennogenin, floribundasaponin A, and spiroconazole A, isolated from the stem bark of *D. mannii* collected in Cameroon, were evaluated for anti-inflammatory effects. Results indicated that after one-hour post-carrageenan injection, the maximal inhibitory activity of 80.57, 71.22, 66.19, and 30.21% was achieved for mannioside A, floribundasaponin A, spiroconazole A, and pennogenin, respectively. It was inferred that the attachment of a Glc unit to the aglycone markedly enhances the anti-inflammatory efficacy of these compounds. Additionally, the inclusion of a Rha unit to the C-3 position of the Glc unit in mannioside A resulted in marginally greater activity compared to floribundasaponin A. Interestingly, two Rha units connected at the C-2 and C-3 positions of Glc in spiroconazole A slightly diminished the anti-inflammatory effect [12]. Further studies on the cytotoxic activity of saponins from the *Dracaena* genus were conducted by Y. Luo, et al. (2014) [13] on *D. cambodiana*'s dragon's blood collected in China, which led to the isolation of thirteen steroidal saponins. Among these, diosgenin-3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 3)]- β -D-glucopyranoside, pennogenin-3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[α -L-rhamnopyranosyl-(1 $\rightarrow$ 3)]- β -D-glucopyranoside, and spirost-5,25(27)-dien-1 β ,3 β -diol-1-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[β -D-xylopyranosyl-(1 $\rightarrow$ 3)]- α -L-arabinopyranoside exhibited significant inhibitory activities against various cancer cell lines with IC₅₀ values indicating potent efficacy, whilst other compounds were inactive (IC₅₀ > 10 μ M), on par with paclitaxel [13]. This overview suggests that the *Dracaena* genus is a promising source of saponins with remarkable biological activities, particularly in cytotoxicity against

cancer cell lines. Consequently, future testing of the biological activity of saponin 26-*O*- β -D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1 β ,3 β ,22 α ,26-tetrol 1-*O*-{*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl} is warranted to augment chemotaxonomy data on the chemical composition and biological activity of the *D. draco* L. species and the *Dracaena* genus at large.

4. Conclusions

In this study, a steroidal saponin was extracted from the foliage of *D. draco* L. using some chromatographic and spectroscopic methods. The structure of this compound was determined to be 26-*O*- β -D-glucopyranosyl-22-*O*-methylfurosta-5,25(27)-diene-1 β ,3 β ,22 α ,26-tetrol 1-*O*-{*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl}, which is similar to the saponin isolated previously from the underground portions of *Ruscus aculeatus* collected in Japan.

CRedit author statement

Hung Duc Nguyen: Investigation, Methodology, Writing original draft, Editing, Data analysis; Tan Quang Tu: Investigation and Writing, Visualisation; Mau Hoang Chu: Writing - Original editing, Conceptualisation, Supervision, Methodology, Idea.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Stochastic modelling of material variability in structural dynamics: A threefold comparison of Monte Carlo, polynomial chaos, and random sampling techniques

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Abstract:

This article investigates the influence of random elastic modulus on beam eigenfrequencies using multiple simulation techniques: Monte Carlo simulations (employing Cholesky decomposition (MCS-CD) and Kosambi-Karhunen-Loève expansion (MCS-KKL)), Polynomial Chaos expansion (PCE), and a proposed Random Sampling method (RSM). Anomalies in Monte Carlo simulations, where normally distributed elastic modulus led to negative values and imaginary eigenfrequencies, were effectively addressed by adopting a log-normal distribution. Comparative analyses focused on covariance variation of the first three eigenfrequencies with correlation length and standard deviation of the random field, highlighting nuanced differences between normal and log-normal distributions. PCE exhibited distinct responses, showcasing variations in covariance with different distributions. The study culminates in eigenfrequency estimation using the proposed RSM, wherein the beam is discretised into n elements with randomly assigned elastic moduli. The mean and variance of eigenfrequencies are compared with existing methods, which represent an alternative method for achieving similar outcomes. These comparative studies provide a comprehensive understanding of how different statistical treatments and simulation methods impact the reliability and accuracy of eigenfrequency predictions in beams with random elastic properties, thus contributing valuable insights for structural analysis and design under uncertainty.

Keywords: Kosambi-Karhunen-Loève expansion, Monte Carlo, polynomial chaos, probabilistic method, stochastic, vibration.

Classification number: 2.3

1. Introduction

Structural dynamics and vibration analysis are crucial aspects of engineering design, particularly when dealing with complex systems such as beams subjected to varying loads and uncertainties. In this context, the elastic modulus of materials plays a pivotal role in determining the response characteristics of structures. While traditional approaches often assume deterministic material properties, overlooking the intrinsic variability observed in real-world scenarios, Young's modulus has been reported to display randomness rather than consistency in various investigations [1-5].

In this article, the author investigates the impact of random elastic modulus on beam eigenfrequencies. The author analyses the consequences of using various simulation methods to better understand these effects. The investigation encompasses MCS with MCS-CD, MCS-KKL, PCE, and a proposed RSM. The primary focus is to unravel the nuances

associated with these methods and determine their efficacy in capturing the dynamic behaviour of beams subjected to uncertain elastic modulus distributions.

During simulations, it was observed that some elastic moduli were found to be negative in MCS with MCS-CD, leading to imaginary eigenfrequencies, particularly under certain conditions of normal distribution. The study unfolds by scrutinising this anomaly and reveals that transitioning to a log-normal distribution mitigates the issue. Comparative analyses of covariance structures between normal and log-normal distributions provide insights into the impact of distribution choice on the structural response.

Building upon this foundation, the article introduces MCS-KKL, showcasing similar negative modulus occurrences. The investigation culminates in an examination of eigenfrequency estimation using PCE and a proposed RSM. The latter involves discretising the beam into distinct

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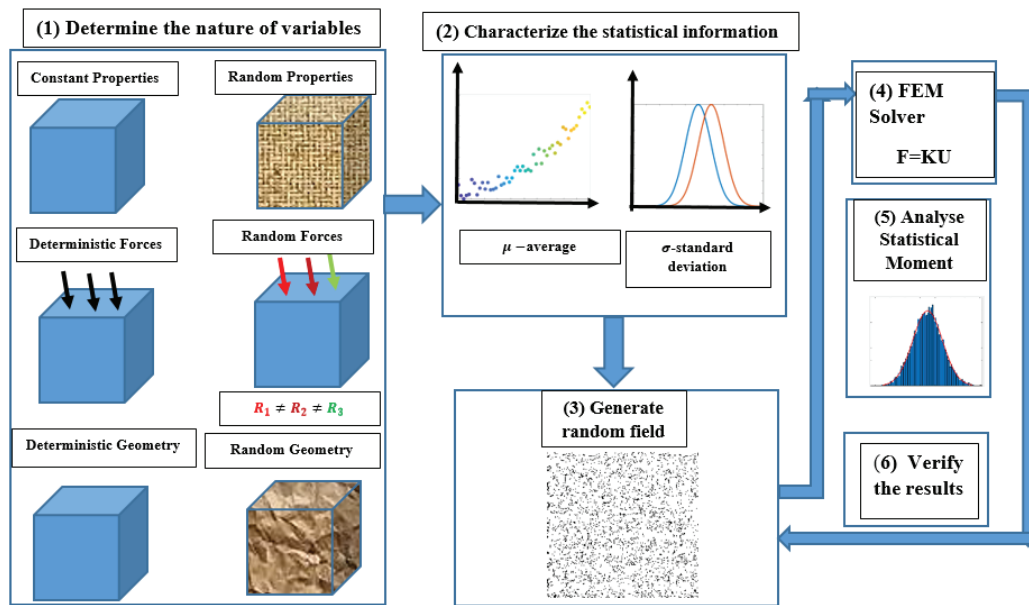


Fig. 1. Schematic of random material and stochastic finite element method.

elements with randomly assigned elastic moduli, exploring a vast range of permutations to obtain a comprehensive understanding of the system's dynamic behaviour.

This research contributes not only to the fundamental understanding of how random elastic modulus influences beam eigenfrequency but also provides a comparative evaluation of simulation techniques. The insights gained herein are expected to advance the field of structural dynamics, guiding engineers and researchers towards more robust and accurate modelling approaches for systems subjected to material property uncertainties.

Figure 1 illustrates the adopted approach for structural analysis, incorporating randomness in material properties, loading, and geometry. The statistical properties of the structural response are inherently linked to the stochastic nature of the input parameters. Material properties, such as the elastic modulus, may exhibit spatial distribution and correlation across the structures. This necessitates a more comprehensive understanding of the probabilistic aspects of these parameters for robust structural design.

In literature, there are generally three methods for analysing how systems respond to random input variables. Firstly, perturbation methods [6-8] use Taylor series expansions, often truncated due to computational challenges. Secondly, polynomial chaos expansion [9, 10] relies on Hermite multivariate polynomials, evolving into Generalised Polynomial Chaos [11, 12]. Lastly, MCS directly computes responses for a large set of random inputs, estimating statistical indicators [13].

PCE efficiently represents system responses with random inputs. For instance, the elastic modulus can be represented by a random process, often exhibiting correlation between different points. The KKL decomposition is similar to Fourier expansion, expressing the random process as an infinite sum of orthogonal functions. This method can be truncated after a few terms [14-17]. The eigenvalues and eigenfunctions are obtained by solving Fredholm integral equations, with available closed-form solutions for some covariance functions [18]. Numerical methods, such as Galerkin and Nystrom methods, can be employed to solve these integral equations [16, 19].

The orthogonal polynomials obtained through KKL expansion form a basis for PCE, offering a concise representation of the stochastic system response. It represents a random variable using combinations of orthogonal polynomials. If system input parameters are random, the response is represented by a polynomial chaos expansion, minimizing errors through Galerkin projection.

Various researchers have proposed diverse methods to find eigenfrequencies, solving stochastic eigenvalue problems. Ghanem and Ghosh's approach provides a comprehensive probabilistic description of eigenvalues and eigenvectors using the Newton-Raphson algorithm [20]. Pascual and Adhikari introduced hybrid methods, like reduced spectral power and inverse power [21]. S.B. Mulani, et al. (2006) [22] presented a non-statistical eigenvalue extraction algorithm, demonstrating accuracy through generalised polynomial chaos. C.V. Verhoosel, et al. (2006)

[23] proposed a spectral expansion algorithm for random non-symmetric matrices, demonstrating efficient and robust performance. E. Sarrouy, et al. (2012) [24] utilised polynomial chaos expansion for complex eigenvalues and eigenvectors in damped structures.

P.C. Nguyen, et al. (2022) [4] used MCS, utilising finite element analysis and an iterative algorithm, to explore the natural frequencies of a beam on an elastic foundation with uncertain material characterised by a homogeneous Gaussian random field of elastic modulus, generated through the spectral representation method. The stochastic analysis of the FGM beam is conducted through MCS, in which elastic modulus and mass density are considered as one-dimensional homogeneous stochastic processes [25].

Cholesky decomposition factorises the covariance matrix into a product of an upper triangular matrix and its transpose 'L', where 'L' is lower triangular matrix. The lower triangular matrix L when applied to an uncorrelated vector u gives a sample vector Lu having covariance properties as that of the modelled system.

2. Materials and methods

2.1. Monte Carlo simulation by Cholesky decomposition

In the present study, the vibration of a cantilever beam of length 'L' having variable elastic modulus 'E' along its length is considered. The elastic modulus of the beam is treated as a random property. It is assumed that the elastic modulus changes stochastically (with Gaussian distribution) and is a function of the location from one end of the beam. Therefore, one can represent the random variation of elastic modulus as [26]:

$$E(x) = E_0 [1 + \alpha(x)] \quad (1)$$

where E_0 is mean value of the elastic modulus; $\alpha(x)$ is the level of randomness; and x indicates the position from the root of the beam. The expected mean of random variation is zero, i.e., $E[\alpha(x)] = 0$. Here, the symbol E represents the average value. Furthermore, the autocorrelation is defined as:

$$R_{\alpha\alpha}(\xi) = \mu[\alpha(x)\alpha(x+\xi)] \quad (2)$$

where ξ is the distance between two points x and $x+\xi$. This can extend to a 3-dimensional case using vector representation.

Considering this stochastic field as homogeneous and isotropic [27], the autocorrelation function of the spatial variation is a function of the distance $|\xi|$ only and is characterised by the variance σ_α^2 . Thus, the autocorrelation function can be represented as:

$$R_{\alpha\alpha}(\xi) = \sigma_\alpha^2 \exp\left(-\frac{|\xi|}{d}\right) \quad (3)$$

where d is a correlation length that governs the rate at which the autocorrelation function decays. The standard deviation σ_α of $\alpha(x)$ represents the coefficient of variation (COV) of the elastic property of $E(x)$. If the beam is divided into n elements (and assuming that each element has one specific value of elastic modulus), it will have n random values of homogeneous stochastic field $\alpha_i = \alpha(x_i)$; $i=1, 2, \dots, n$ with mean zero, but are correlated. The correlation of elastic modulus between these n elements can be defined by a covariance matrix of size $n \times n$. The covariance matrix, $\text{cov}(\alpha\alpha)$, is defined as:

$$\text{cov}(\alpha\alpha) = E[(\alpha - E[\alpha])(\alpha - E[\alpha])^T] \quad (4)$$

$$= R_{\alpha\alpha} - E[\alpha] E[\alpha]^T \quad (5)$$

where $E[\alpha]$ represents expectation.

Since, $E[\alpha(x)] = 0$, the correlation matrix $R_{\alpha\alpha}$ can be defined as $R_{\alpha\alpha} = E[\alpha\alpha^T]$.

Therefore,

$$\text{cov}(\alpha_i \alpha_j) = R_{\alpha\alpha}(\xi_{ij}) = L L^T \quad (6)$$

Now we can state

$$\alpha = [L]Z \quad (7)$$

where vector $\{Z\} = \{Z_1, Z_2, \dots, Z_N\}$ is an independent Gaussian stochastic vector with mean of zero and standard deviation of one, and $[L]$ is a lower triangular matrix obtained from the Cholesky decomposition of the covariance matrix as in:

$$\begin{aligned} E[\alpha\alpha^T] &= E[LZ(LZ)^T] \\ &= E[L(ZZ^T)L^T] = L E[ZZ^T] L^T \end{aligned} \quad (8)$$

Now, $E[ZZ^T] = 1$, therefore:

$$E[\alpha\alpha^T] = L L^T = \text{cov}(\alpha_i \alpha_j) \quad (9)$$

Figure 2 illustrates the variation of elastic modulus (E) with length for σ values of 0.3, 0.4, and 0.5, considering 50 realisations. It is evident that for $\sigma=0.4$ and $\sigma=0.5$, some elastic modulus values become negative, which is physically unrealistic and contradicts practical expectations. Additionally, with an increase in σ , the number of negative elastic modulus values rises. The literature recognises the challenge of encountering negative values post Cholesky decomposition of the random vector. It emphasises careful exclusion during simulations, particularly when significant variance exists in the random process, to ensure robust and reliable results [28].

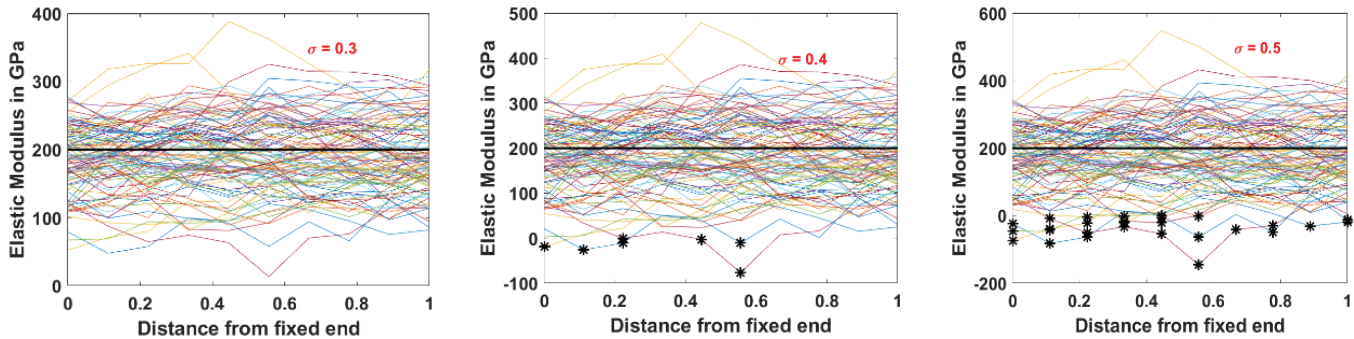


Fig. 2. Variation of elastic modulus with length (elastic modulus normally distributed).

It is crucial to acknowledge that the elastic modulus may exhibit negativity at any σ value due to our utilisation of a Gaussian normal distribution. To address this issue, we propose adopting a log-normal distribution for elastic modulus samples. This shift ensures the generated values remain strictly positive, aligning with physical expectations and resolving the observed problem.

To overcome the challenges encountered, we have integrated the log-normal transformation through the Cholesky decomposition method. Specifically, a vector of independent standard normal random variables, Z , is generated and transformed into correlated variables, α , using the lower triangular matrix L obtained from Cholesky decomposition as given in Eq. (7).

The transformation is expressed as:

$$\log\left(\frac{E}{E_0}\right) = \alpha \quad (10)$$

Here, $\alpha(x)$ represents the zero-mean random process capturing the random variation in elastic modulus, and the

logarithmic transformation guarantees the strictly positive nature of the elastic modulus [29].

The impact of this log-normal transformation is depicted in Fig. 3, illustrating the variation of elastic modulus with length for different σ values (0.3, 0.4, and 0.5) based on 50 realisations. The results demonstrate that the adjustment effectively eliminates negative values of elastic modulus, ensuring that our model adheres more closely to physical expectations. As σ increases, the log-normal transformation prevents the occurrence of physically unrealistic negative values, providing more accurate and meaningful insights into the behaviour of elastic modulus in structures subjected to random mechanical fields.

In order to generate a Monte Carlo simulation, we need to determine the structural dynamic characteristics of the beam. The determination of structural dynamic characteristics of the beam using deterministic FEM is described as follows:

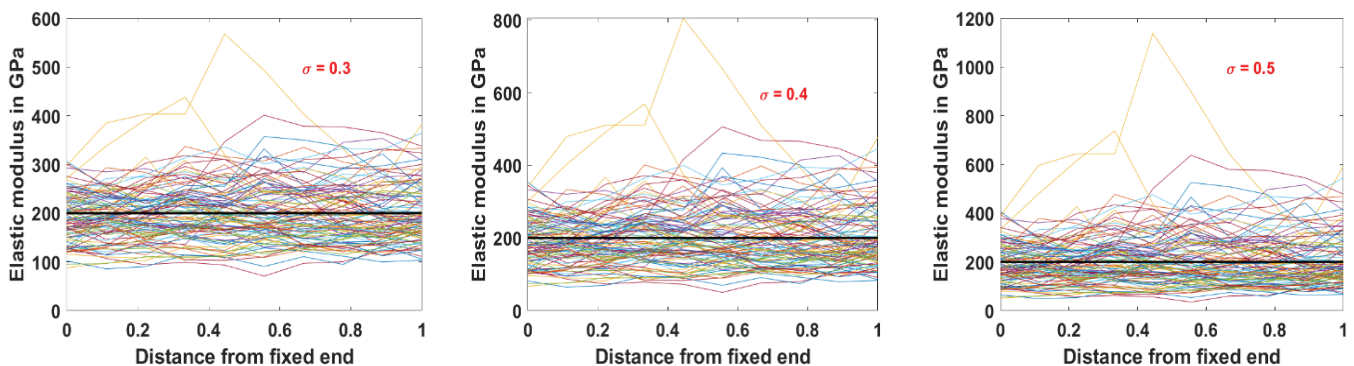


Fig. 3. Variation of elastic modulus with length (elastic modulus log normally distributed).

The Euler-Bernoulli equation for beam bending is

$$\rho \frac{\partial^2 v}{\partial t^2} + \frac{\partial^2}{\partial x^2} \left(EI \frac{\partial^2 v}{\partial x^2} \right) = q(x, t) \quad (11)$$

where $v(x, t)$ is the transverse displacement of the beam; ρ is mass density per volume; EI is the beam rigidity; $q(x, t)$ is the externally applied pressure loading; t is time; and x is spatial distance along the beam axis. The average weighted residual of Eq. (11) is:

$$\int_0^L \left\{ \rho \frac{\partial^2 v}{\partial t^2} + \frac{\partial^2}{\partial x^2} \left(EI \frac{\partial^2 v}{\partial x^2} \right) - q(x, t) \right\} w \, dx = 0 \quad (12)$$

where L is the length of the beam; and w is test function. Galerkin's method is considered, where the test function is equal to the trial function.

The weak formulation is obtained from integrations by parts twice for the second term of the equation. In addition, discretisation of the beam into a number of finite elements gives

$$\sum_1^n \left[\int \rho \frac{\partial^2 v}{\partial t^2} w \, dx + \int EI \frac{\partial^2 v}{\partial x^2} \frac{\partial^2 w}{\partial x^2} \, dx - \int q w \, dx \right] + \left[V w - M \frac{\partial w}{\partial x} \right]_0^L = 0 \quad (13)$$

The boundary conditions for the cantilever beam are as follows:

Displacement and slope are zero at $x=0$ i.e. $v(0)=w(0)=0$; $\theta = \frac{\partial v}{\partial x} \Big|_{(L=0)} = \frac{\partial w}{\partial x} \Big|_{(L=0)} = 0$ and Shear Force (V) and Moment (M) is zero at $x=L$.

Applying the boundary conditions, the last term of Eq. (13) becomes zero. Also, for free vibration, $q(x, t)=0$. Therefore, Eq. (13) becomes:

$$\sum_1^n \left[\int \rho \frac{\partial^2 v}{\partial t^2} w \, dx + \int EI \frac{\partial^2 v}{\partial x^2} \frac{\partial^2 w}{\partial x^2} \, dx \right] = 0 \quad (14)$$

There are four nodal variables for the beam elements, therefore, assuming a cubic polynomial function for $v(x)$:

$$v(x) = c_0 + c_1 x + c_2 x^2 + c_3 x^3 \quad (15)$$

Since an Euler-Bernoulli beam has been assumed, the slope is computed from Eq. (15) as $\theta = dv/dx$ where:

$$\theta(x) = c_1 + 2c_2 x + 3c_3 x^2 \quad (16)$$

Hermitian shape functions are applied:

$$v(x) = H_1(x)v_1 + H_2(x)\theta_1 + H_3(x)v_2 + H_4(x)\theta_2$$

where

$$H_1(x) = 1 - \frac{3x^2}{l^2} + \frac{2x^3}{l^3}; H_2(x) = x - \frac{2x^2}{l} + \frac{2x^3}{l^2}; H_3(x) = \frac{3x^2}{l^2} - \frac{2x^3}{l^3}; H_4(x) = -\frac{x^2}{l} + \frac{x^3}{l^2}$$

Applying Hermitian shape functions and Galerkin's method to the second term of Eq. (14) we obtain the stiffness matrix of the beam element. Specifically,

$$[K]^e = \int_0^L [B]^T EI [B] \, dx \quad (17)$$

where $[B] = \{H_1'', H_2'', H_3'', H_4''\}$ and the corresponding element nodal degrees of freedom is $d^e = \{v_1, \theta_1, v_2, \theta_2\}^T$, and v_i and θ_i are deflection and slope nodal variables, respectively. The double prime denotes the second derivative of the function.

For dynamic analyses of beams, the transverse deflection is a function of x and t . The deflection is interpolated within a beam element as given below:

$$v(x) = H_1(x)v_1(t) + H_2(x)\theta_1(t) + H_3(x)v_2(t) + H_4(x)\theta_2(t)$$

The shape functions are used to interpolate the deflection in terms of the spatial domain and the nodal variables are functions of time. The first term in Eq. (14) becomes:

$$\int_0^L \rho A [H]^T [H] \{\ddot{v}\} \, dx \quad (18)$$

where $[H] = [H_1 \ H_2 \ H_3 \ H_4]$ and the superscript dots denote time derivatives. From Eq. (18), the element mass matrix becomes:

$$[M]^e = \int_0^L \rho A [H]^T [H] \{\ddot{v}\} \, dx$$

The elemental stiffness and mass matrix are assembled to obtain global stiffness (K) and global mass matrix ($\mathcal{M}$). Thus, the equation of free vibration of beam becomes the Eigen value problem of the equation:

$$KX - \omega^2 \mathcal{M}X = 0 \quad (19)$$

where ω is eigenfrequency. Making the substitution $\omega^2 = \lambda$, the eigenfrequencies are obtained by finding the eigenvalue of the following equation:

$$(K - \lambda \mathcal{M})X = 0 \quad (20)$$

For MCS, the variations of elastic modulus (E) are generated using Eqs. (6), (7), and (1), which are substituted in Eq. (17). For each value of E , a corresponding eigenvalue and eigenvector can be obtained using Eq. (20). This procedure is repeated for a large number of random data sets of elastic moduli to produce an ensemble of the structural dynamic response characteristics. These values are used to compute the mean and standard deviation of the structural dynamic characteristics represented by the first three eigenfrequencies.

2.2. Kosambi - Karhunen - Loève expansion

In this method the cantilever beam of length ' L ' having variable elastic modulus ' E ' along its length is considered. The random field of elastic moduli has infinite-dimensional probability space (Ω, Θ, P) , where Ω is set of elementary

events, Θ a sigma algebra generated on Ω , and P is a probability measure on (Ω, Θ) . However, after the spatial discretisation, we have a finite dimensional representation of the random elastic field, which can be represented as a random vector:

$$x = [x_1, x_2, \dots, x_M]^T \quad (21)$$

where M can be regarded as the number of elements in a discretised model. So, for each x_i , $i=1, 2, \dots, M$ is a random variable that represents the random elastic property in each element. The beam is discretised into M number of elements so that x_i is an M dimensional vector.

Now, assume we are given N number of realisations of the random elastic field $[x_1, x_2, \dots, x_N]$, where each realisation is represented as an M -dimensional column vector. The random vector x_i obtained after the discretisation are highly correlated. In order to obtain uncorrelated elastic moduli, we consider a nonlinear mapping φ that relates the input space R^M to another space F . We will refer to F as the feature space. These realisations x_i are mapped onto the feature space F to $\varphi(x_i)$, denoting the mean of the φ -mapped data by:

$$\bar{\varphi} = \frac{1}{N} \sum_{i=1}^N \varphi(x_i) \quad (22)$$

and defining the centred map as:

$$\tilde{\varphi} = \varphi(x) - \bar{\varphi} \quad (23)$$

The covariance matrix C in the feature space can be obtained as:

$$C = \frac{1}{N} \sum_{i=1}^N \tilde{\varphi}(x_i) \tilde{\varphi}(x_i)^T \quad (24)$$

The dimension of the covariance matrix is $N_F * N_F$ where N_F is the dimension of the feature space. The KKL expansion involves solving the eigenvalue problem $CV = \lambda V$. As explained [30], N_F could be extremely large. As a result, it will be impossible to compute the C matrix and solve the eigenvalue problem directly. Thus, a kernel eigenvalue problem, which uses only dot products of vectors in the feature space, is formulated to solve the original eigenvalue problem indirectly [30]. The dot product in the feature space is defined as:

$$[\tilde{\varphi}(y_i) \cdot \tilde{\varphi}(y_j)] = \tilde{\varphi}(y_i) \tilde{\varphi}(y_j)^T \quad (25)$$

We now have to find eigenvalues $\lambda \geq 0$ and eigenvectors $\in F$ such that $CV = \lambda V$ [31], which implies all solutions of V with $\lambda \neq 0$ lie in the span of $\varphi(x_1), \varphi(x_2), \dots, \varphi(x_N)$. For us, this has two useful consequences. First, we may instead consider the set of equations:

$$(\tilde{\varphi}(x_i) x C V) = \lambda (\tilde{\varphi}(x_i) x V), \forall i=1, 2, \dots, N \quad (26)$$

Second, there exist coefficients $\beta_j \forall j=1, 2, \dots, N$ such that we can expand the solution V as:

$$V = \sum_{j=1}^N \beta_j \tilde{\varphi}(x_j) \quad (27)$$

Now, combining Eq. (26) and Eq. (27), we obtain:

$$\begin{aligned} \frac{1}{N} \sum_{j=1}^N \beta_j \sum_{k=1}^N (\tilde{\varphi}(x_i) \cdot \tilde{\varphi}(x_k)) (\tilde{\varphi}(x_k) \cdot \tilde{\varphi}(x_j)) \\ = \lambda \sum_{j=1}^N \alpha_j \tilde{\varphi}(x_i) \cdot \tilde{\varphi}(x_j) \end{aligned} \quad (28)$$

for $i = 1, 2, \dots, N$

Defining the $N \times N$ kernel matrix R , which is the dot product of vectors in the feature space $\mathcal{F}$ $R_{ij} = \varphi(x_i) \cdot \varphi(x_j)$ and the corresponding centred kernel matrix:

$$\tilde{R}: \tilde{R}_{ij} = \tilde{\varphi}(x_i) \cdot \tilde{\varphi}(x_j) \quad (29)$$

We obtain $\tilde{R}^2 \beta = N \lambda_i \tilde{R} \beta \forall i=1, 2, \dots, N$

where $\beta = [\beta_1, \beta_2, \dots, \beta_N]$.

This is equivalent to the following kernel eigenvalue problem:

$$\tilde{R} \beta = N \lambda_i \beta \quad (30)$$

We rewrite above equation in the following matrix form:

$$\tilde{R} U = \Lambda U \quad (31)$$

where $\Lambda = \text{diag}(\lambda_1, \lambda_2, \dots, \lambda_N)$ is the diagonal matrix of the corresponding eigenvalues, $U = [\beta_1, \beta_2, \dots, \beta_N]$ is the matrix containing the eigenvectors of the Kernel matrix $\tilde{R}$, and each column consists of the i^{th} eigenvector $\beta_i = [\beta_1, \beta_2, \dots, \beta_N]$.

Therefore, through Eq. (27), the i^{th} eigenvector of the covariance matrix C in the feature space can be shown to be [30, 32]:

$$\tilde{V}_i = \sum_{j=1}^N \tilde{\beta}_{ij} \tilde{\varphi}(x_j) \quad (32)$$

where $\tilde{\beta}_{ij} = \frac{\beta_{ij}}{\sqrt{\lambda_i}}$. It is easy to verify that Eq. (32) satisfies the orthonormal condition:

$$\begin{aligned} \tilde{V}_i \cdot \tilde{V}_i &= 1 \\ \tilde{V}_i &= \sum_{j=1}^N \beta_{ij} \sqrt{\lambda_i} \tilde{\varphi}(x_j) \end{aligned} \quad (33)$$

Let x be a realisation of the random field with a mapping $\varphi(x)$ in $\mathcal{F}$. The random function $\varphi(x)$ can be decomposed in the following way:

$$\varphi(x) = \sum_{i=1}^N z_i \tilde{V}_i + \bar{\varphi} \quad (34)$$

where z_i is the projection coefficient onto the i^{th} eigenvector $\tilde{V}_i$:

$$z_i = \tilde{V}_i \cdot \tilde{\varphi}(x) = \sum_{j=1}^N \tilde{\beta}_{ij} \tilde{\varphi}(x_j) \cdot \tilde{\varphi}(x_j) \quad (35)$$

Normalising the coefficient z_i as $\varepsilon_i = \frac{z_i}{\lambda_i^{1/2}}$ where ε_i are zero mean, mutually uncorrelated random variables with unit variance [32], we can represent $\varphi(x)$ as:

$$\varphi(x) = \sum_{i=1}^N \varepsilon_i \lambda_i^{1/2} \tilde{V}_i + \bar{\varphi} \quad (36)$$

From Eq. (29), it can be seen that in order to compute the kernel matrix, only the dot products of vectors in the feature space F are required, while the explicit calculation of the map $\varphi(x)$ does not need to be known. As shown [33], the dot product can be computed through the use of the kernel function. Not all arbitrary functions except the Mercer kernels can be used as a kernel function [30]. The kernel function $R(x_i, x_j)$ calculates the dot product in space F directly from the vectors of the input space $\mathbb{R}^M$. Mercer proved that any positive definite function $R(x_i, x_j)$ with $x_i, x_j \in \mathbb{R}^D$ defines an inner product of another vector space V . $R(x_i, x_j)$ is called the covariance or kernel function [34]. In this article, we have used exponential covariance function $R_{\alpha}(\xi) = \sigma_{\alpha}^2 \exp(-\frac{|\xi|}{d})$. Kernels depend on a parameter d called the correlation length, describing the correlation between two points of the field. The limit $d \rightarrow \infty$ generates a fully correlated random field, whereas $d \rightarrow 0$ produces a random field without any spatial correlation [35].

Since R is real and symmetric, it has an Eigen decomposition of the form:

$$R(s, t) = \sum_i \lambda_i V_i(s) V_i(t) \quad (37)$$

where λ_i are eigenvalues and V_i are eigenvectors of R .

The eigenvectors are obtained by solving the homogenous Fredholm integral equation of the second kind [36] as:

$$\int_a^b R(x, y) V_k(x) dx = \lambda_k V_k \quad (38)$$

Writing random functions as $\varphi(x, \theta) = E(x, \theta)$, $\bar{\varphi} = E_0$, and $\sum_{i=1}^N \varepsilon_i \lambda_i^{1/2} \tilde{V}_i = E_0 * \alpha(x, \theta)$, we have:

$$E(x, \theta) = E_0 [1 + \alpha(x, \theta)] \quad (39)$$

where $E[E(x, \theta)] = E_0$ is the mean value of the random elastic field and $\alpha(x, \theta)$ is a zero mean random process.

Figure 4 shows the Mercer Eigen functions. Fig. 5 shows the realisations of the random function α having zero mean.

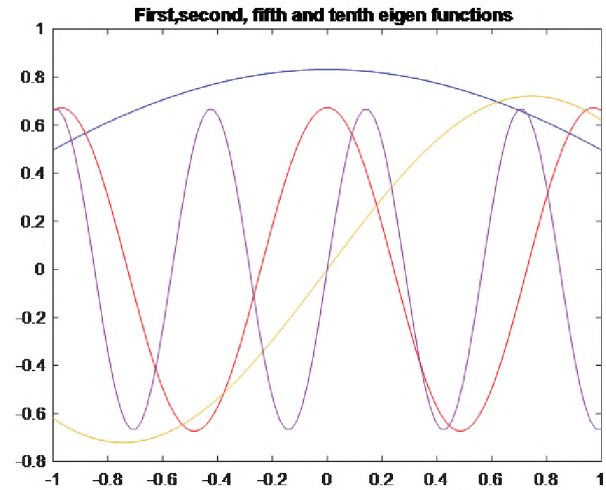


Fig. 4. Representation of eigenvalues.

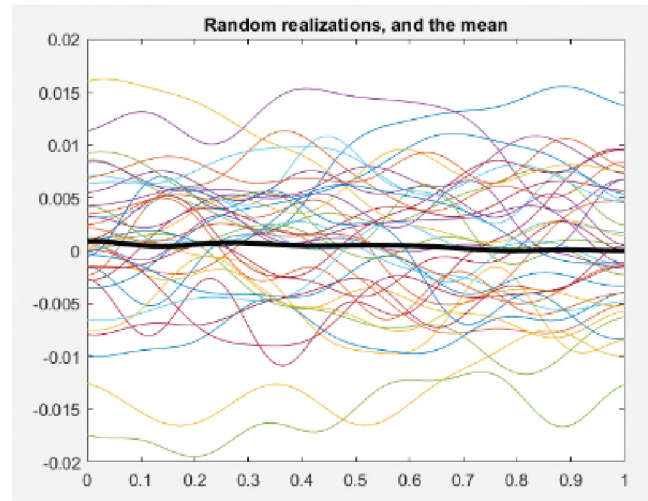


Fig. 5. Realisations of random function alpha.

2.3. Truncation of Kosambi-Karhunen-Loève expansion

In order to obtain a random process, an infinite sum of linear combination of eigenfunction is not required. The series can be truncated after a finite number of terms, the specific value of which depends on the desired level of accuracy. The proof for truncation is explained below.

Random coefficients ξ_m of the KKL expansion are uncorrelated having zero mean and unit variance. The variance of α is simply the sum of the variances of the individual components of the sum:

$$\text{var}[E(x, \theta)] = \text{var}[E_0 * \alpha] = \sum_{k=1}^N \lambda_k V_k^2 \text{var}[\varepsilon_k] \quad (40)$$

Since $\text{var}[\xi_k] = 1$,

$$E_0^2 \text{var}[\alpha] = \sum_{k=1}^N \lambda_k V_k^2 \quad (41)$$

Using the orthonormality of V_k , we obtain the total variance of the process:

$$E_0^2 \text{var}[\alpha] = \sum_{k=1}^N \lambda_k \quad (42)$$

The total variance of the T-truncated approximation is given by the sum of the first T eigenvalues denoted as $\sum_{k=1}^T \lambda_k$. Consequently, the T-truncated expansion explains a proportion of the variance equal to $\frac{\sum_{k=1}^T \lambda_k}{\sum_{k=1}^N \lambda_k}$ of the variance.

For instance, if we seek an approximation that accounts for 95% of the variance, we must determine a value of T such that

$$\frac{\sum_{k=1}^T \lambda_k}{\sum_{k=1}^N \lambda_k} \geq 0.95 \quad (43)$$

The truncation order 'T' obtained is 5 for 95% accuracy and 3 for 90% accuracy. Note that the statement "The truncation order 'T' obtained is 5 for 95% accuracy and 3 for 90% accuracy" indicates the level of approximation achieved through truncating the MCS-KKL. In essence, it means that by considering the first 5 terms in the expansion, we can capture 95% of the total variance of the process, while for 90% accuracy, only the first 3 terms are necessary. This is based on the fact that Eq. (42), the total variance of the process, can be represented as the sum of the eigenvalues associated with each term in the expansion. By truncating at a certain order, we essentially prioritise the dominant modes that contribute most to the variance of the process.

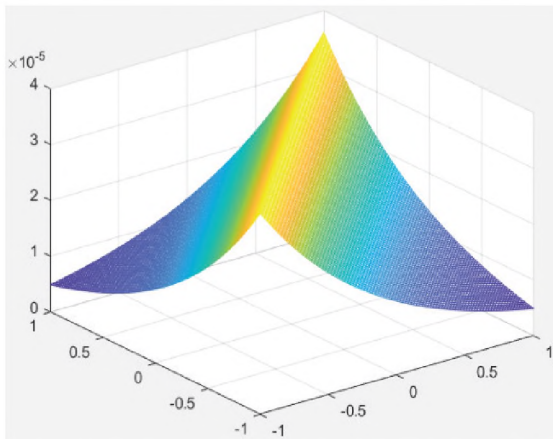


Fig. 6. Bending stiffness covariance $R = \sigma^2 \exp(-\frac{|\xi|}{d})$.

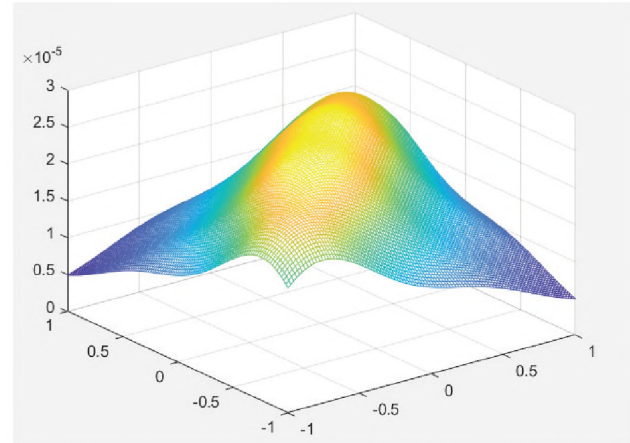


Fig. 7. Bending stiffness covariance for truncated Kosambi-Karhunen-Loève expansion of order T=5.

Figure 6 shows the bending stiffness covariance $R = \sigma^2 \exp(-\frac{|\xi|}{d})$ and Fig. 7 shows the bending stiffness covariance for a truncated KKL expansion of order T=5. Fig. 8A shows the realisation of alpha (zero mean random process) after a truncation order T=5. Fig. 8B, shows the bending stiffness in GPa after a truncation of the KKL expansion of order T=5.

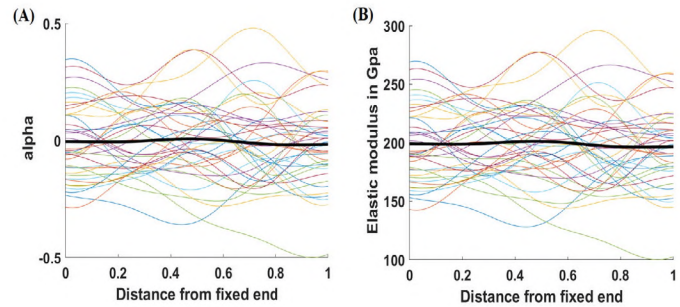


Fig. 8. Variation of elastic modulus with length for Kosambi-Karhunen-Loève expansion of truncation order 5.

The bending stiffness of the beam $K(x, \theta)$ is linearly dependent on a random process $E(x, \theta)$, i.e.,

$K(x, \theta) = K(E(x, \theta))$. Now $K(x, \theta)$ can be written as:

$$K(x, \theta) = \mathcal{K}(E(x, \theta)) \approx K_0 + \sum_{j=1}^T K_j \xi_j(\theta) \quad (44)$$

where K_0 is the stiffness matrix of the mean system. The deterministic matrices K_j are given by the operator:

$$K_j = \begin{cases} \mathcal{K}(E_0) & j = 0 \\ \mathcal{K}(\sqrt{\lambda_j} V_j(x)) & j = 1 \dots T \end{cases} \quad (45)$$

The bending stiffness for a given realisation can thus be calculated by Eq. (44).

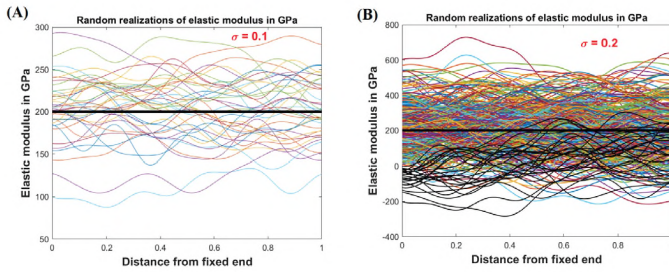


Fig. 9. Variation of alpha and elastic modulus with length for normal distribution.

Figure 9A shows the realisation of elastic modulus with length for $\sigma=0.1$, while Fig. 9B displays the realisation of elastic modulus with length for $\sigma=0.2$. The black curve in Fig. 9B represents instances where the elastic modulus values are negative, which is physically impossible. To address this issue, the elastic modulus is considered as lognormal, and it is depicted in Figs. 10A and 10B for $\sigma=0.1$ and $\sigma=0.2$, respectively.

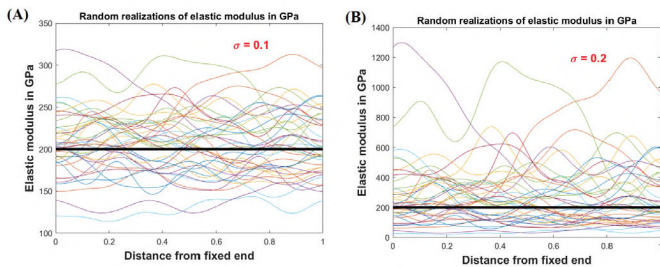


Fig. 10. Variation of alpha and elastic modulus with length for lognormal distribution.

In order to calculate MCS for KKL, the process is described as follows:

- (i) Assemble the system matrices K_j .
- (ii) Generate the sets of independent standard Gaussian variables $\{\xi_1(\theta_i), \dots, \xi_T(\theta_i)\}$ with $i=1, \dots, n_{MCS}$.
- (iii) Assemble the stiffness matrix $K(\theta_i)$, for every realisation i .

- (iv) The eigenfrequency is obtained by finding the eigenvalue of the equation

$$KX = \omega^2 MX = (K - \lambda M)X = 0 \quad (46)$$

- (v) Solve the deterministic system of equations $K(\theta_i) X = \lambda M X$.

The mean and variance of MCS obtained after KKL decomposition converges nearly after 1500 samples as shown in Fig. 11. For calculation purposes, the number of MCS is taken as 2000.

2.4. Polynomial chaos expansion

In the polynomial chaos method, the solution $u(\theta)$ of differential equations are projected on a vector space spanned by polynomials $\psi_i(\xi)$. In this article, we discuss the Galerkin-based intrusive spectral projection method. In this approach, a random variable is represented as spectral representation in terms of a set of polynomial basis functions:

$$u(\theta) = \sum_{i \in \mathbb{N}} u_i \psi_i(\xi) \quad (47)$$

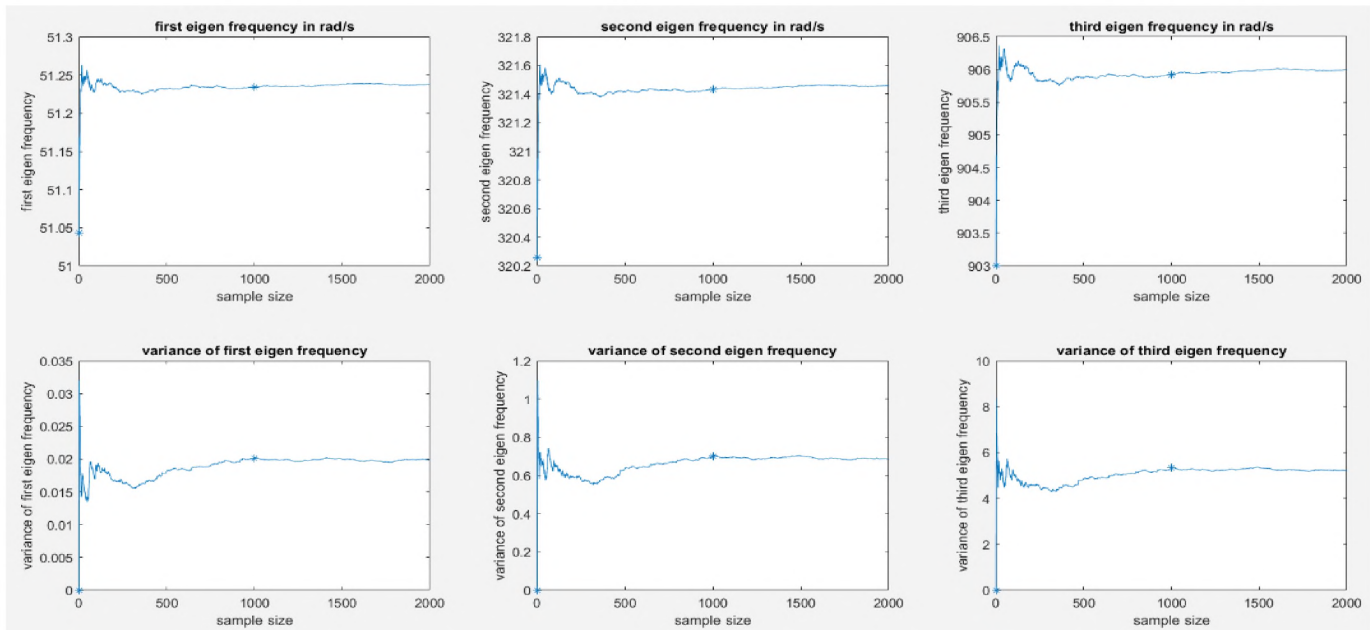


Fig. 11. Mean and variance of the first three eigenfrequencies of Monte Carlo simulation obtained after Kosambi-Karhunen-Loève expansion decomposition having truncation order 'T'=5.

where $u(\theta)$ is a random variable and $\psi_i(\xi)$ is a polynomial function of another random variable ξ .

The polynomials are chosen to be orthogonal with respect to the joint probability of these random variables:

$$\langle \psi_i \psi_j \rangle = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} \psi_k(\xi) \psi_k(\xi) \rho(\xi) d\xi = \delta_{ij} \langle \psi_i^2 \rangle \quad (48)$$

where u_i are deterministic coefficients, referred to as PC coefficients.

Wiener in his original article (1938) considered the spectral representation of random variables by Hermite polynomial with germs as Gaussian random variables. The PCE was later generalised by D. Xiu, et al. (2002) [12] known as generalised Polynomial chaos or gPC. Table 1 mentions the polynomial type and its corresponding random variable or germ.

Table 1. Generalised polynomial chaos (adapted from [12]).

Uncertain parameter type	Optimal polynomial type
Gaussian	Hermite
Gamma	Laguerre
Beta	Jacobi
Uniform	Legendre

In this article, Gauss-Hermite PCE type has been considered, and the basis functions are Hermite polynomials as a function of Gaussian random variables ξ .

In order to obtain the deterministic coefficient u_i , the random variable u is projected on to the space spanned by Hermite polynomials as its basis. The number of degrees of freedom of the random variables determines the dimension of the germ ξ . However, if the random variable has infinite degrees of freedom, then in that case the number of terms (T) after which the KKL expansion is truncated determines the dimensionality of ξ . Given the space dimension T (number of random variables) and the order of the polynomial chaos p , a basis dimension P containing the number of PC used in the expansion can be computed as [18, 37]:

$$P + 1 = \frac{(T + p)!}{p! T!}$$

where p depends on the information contained about the random variable that has been represented. Table 2 shows the number of terms of P for a given truncation T and order of polynomial p . If the truncation order T is 3 and the order of polynomial is 1, $P=5$. If the truncation order T is 5 and the order of the polynomial is 2, then $P=20$.

Table 2. Hermite polynomial chaos in 1D and 2D (adapted from R.G. Ghanem, et al. (2012) [10]).

Order p	1D		2D	
	Index k	PC ($\psi_k(\xi_1)$)	Index k	PC ($\psi_k(\xi_1, \xi_2)$)
0	0	1	0	1
1	1	ξ_1	1	ξ_1
			2	ξ_2
2	2	$\xi_1^2 - 1$	3	$\xi_1^2 - 1$
			4	$\xi_1 \xi_2$
			5	$\xi_2^2 - 1$
3	3	$\xi_1^3 - 3\xi_1$	6	$\xi_1^3 - 3\xi_1$
			7	$\xi_1^2 \xi_2 - \xi_2$
			8	$\xi_1 \xi_2^2 - \xi_1$
			9	$\xi_2^3 - 3\xi_2$
4	4	$\xi_1^4 - 6\xi_1^2 + 3$	10	$\xi_1^4 - 6\xi_1^2 + 3$
			11	$\xi_1^3 \xi_2 - 3\xi_1 \xi_2$
			12	$\xi_1^2 \xi_2^2 - \xi_1^2 - \xi_2^2 + 1$
			13	$\xi_1 \xi_2^3 - 3\xi_1 \xi_2$
			14	$\xi_2^4 - 6\xi_2^2 + 3$
5	5	$\xi_1^5 - 10\xi_1^3 + 15\xi_1$	15	$\xi_1^5 - 10\xi_1^3 + 15\xi_1$
			16	$\xi_1^4 \xi_2 - 6\xi_1^2 \xi_2 + 3\xi_2$
			17	$\xi_1^3 \xi_2^2 - \xi_1^3 - 3\xi_1 \xi_2^2 + 3\xi_1$
			18	$\xi_1^2 \xi_2^3 - \xi_1^2 - 3\xi_1 \xi_2^2 + 3\xi_1$
			19	$\xi_1 \xi_2^4 - 6\xi_1 \xi_2^2 + 3\xi_1$
			20	$\xi_2^5 - 10\xi_2^3 + 15\xi_2$

The equation of motion of a free vibration of a beam can be written as:

$$[M]\{\ddot{u}\} + [K]\{u\} = 0$$

Now, uncertainty in the modulus of elasticity will result in uncertainty in the stiffness matrix (K) such that:

$$K_i(\xi) = K_0 + \sum_{i=1}^M K_i \xi_i(\theta) \quad (49)$$

Since uncertainty in K will induce uncertainty in u , a polynomial chaos expansion for u is specified:

$$u = \sum_{k=0}^{\infty} u_k \psi_k(\xi) \quad (50)$$

where u_k is a deterministic unknown coefficient.

The eigenvalue equation of the vibration of a beam can be written as:

$$(K - \lambda M)u = 0 \quad (51)$$

Substituting the value of u and K in Eq. (51), we obtain:

$$\left\{ \left(K_0 + \sum_{i=1}^T K_i \xi_i \right) - M\lambda \right\} \left\{ \sum_{j=1}^P u_{n_j} \langle \psi_j \rangle \right\} = 0 \quad (52)$$

Multiplying both sides of the equation with ψ_k , and taking the expectation with respect to the basis germ ξ , we obtain:

$$\sum_{i=0}^T \sum_{j=1}^P \sum_{k=1}^P K_i u_{n_j} \langle \xi_i \psi_j \psi_k \rangle - M \sum_{j=1}^P \sum_{k=1}^P u_{n_j} \lambda \langle \psi_j \psi_k \rangle \quad (53)$$

Taking $u(n_j)$ common, we obtain:

$$\left(\sum_{i=0}^T \sum_{j=1}^P \sum_{k=1}^P K_i \langle \xi_i \psi_j \psi_k \rangle - M \left(\sum_{j=1}^P \sum_{k=1}^P \lambda \langle \psi_j \psi_k \rangle \right) \right) u_{n_j} = 0 \quad (54)$$

Substituting $\langle \xi_i \psi_j \psi_k \rangle = e_{ijk}$, we obtain:

$$\left(\sum_{i=0}^T \sum_{j=1}^P \sum_{k=1}^P K_i e_{ijk} - M \left(\sum_{j=1}^P \sum_{k=1}^P \lambda \langle \psi_j \psi_k \rangle \right) \right) u_{n_j} = 0 \quad (55)$$

where

$$e_{ijk} = E[\xi_i \psi_j \psi_k] = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} \xi_i \psi_j(\xi) \psi_k(\xi) \rho(\xi) d\xi \quad (56)$$

The assembly of the stiffness matrix K is given in Refs. [38] and [39] as:

$$K = \sum_{i=0}^T K_i \otimes e_{ijk} \quad (57)$$

where $\otimes$ denotes the Kronecker product. Finally, K can be represented as:

$$\begin{bmatrix} \sum_{i=0}^T K_i e_{i11} & \dots & \sum_{i=0}^T K_i e_{i1P} \\ \vdots & \ddots & \vdots \\ \sum_{i=0}^T K_i e_{jP1} & \dots & \sum_{i=0}^T K_i e_{iPP} \end{bmatrix} \quad (58)$$

The assembly of mass matrix M is given in Ref. [39] as:

$$M = M \otimes e_{0jk} \quad (59)$$

and

$$\lambda^T = [\lambda_1 \dots \lambda_P]^T \quad (60)$$

where M is the mass matrix and λ is the eigen value.

The mean (m_U) and variance of the PC expansion can be given by Eq. (61) and Eq. (62), respectively, as:

$$m_U = \int \sum_{p=0}^P \lambda_p \psi_p(\xi) \rho(\xi) d\xi = \lambda_1 \quad (61)$$

$$\text{Variance} = \int \left[\sum_{p=1}^P \lambda_p \psi_p(\xi) - \lambda_1 \right]^2 \rho(\xi) d\xi \quad (62)$$

2.5. Random sampling method

In the RSM for a given mean and standard deviation of the elastic modulus, one can randomly choose a vector of size n assuming normal distribution. Using these n values of elastic modulus, for all possible combinations, the structural dynamic characteristics of the beam is evaluated. For example, if the beam is divided into two elements, then each element can have one value of elastic modulus. Considering E_1 and E_2 as two possible values of elastic modulus, the possible combinations are $[E_1 E_1, E_1 E_2, E_2 E_1, E_2 E_2]$. Similarly, for N elements with N possible values of elastic modulus, the total number of combinations will be N^N . It may be noted that the total number of possible cases increases rapidly with number of elements of the beam. For ten elements, the number of possible combinations is 10^{10} .

Figure 12 illustrates the convergence plots of the first three eigenfrequencies as a function of the number of elements. In Figs. 12A and 12B, it is evident that the first two natural frequencies converge satisfactorily with five elements. However, Fig. 12C reveals that achieving convergence for the third frequency necessitates at least six elements.

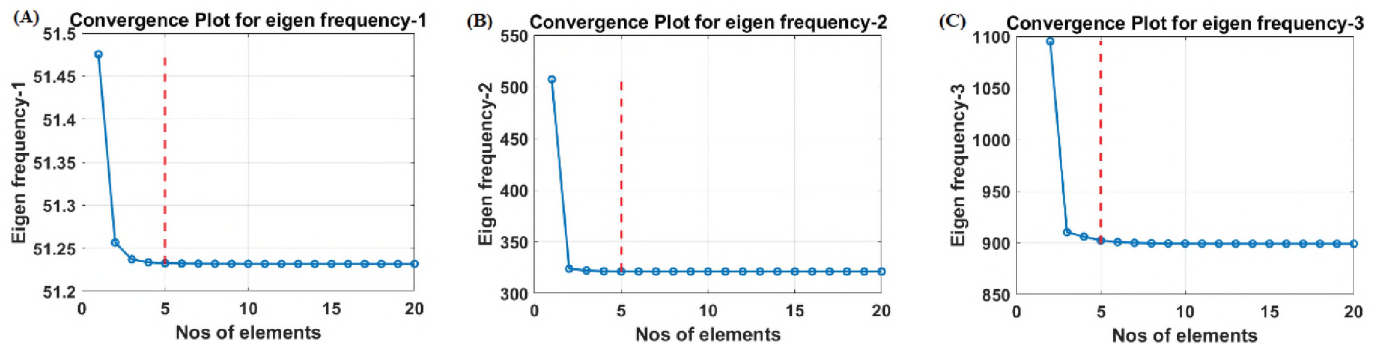


Fig. 12. Convergence plot of eigenfrequencies with number of finite elements.

The computational cost, particularly for the RSM, exhibits an exponential increase with the number of elements, escalating from 3125 to 46656 evaluations. This steep rise poses a significant challenge for practical implementation, highlighting the need for a balanced approach.

To strike a compromise between accuracy and computational cost, this paper adopts five elements for RSM and six elements for MCS-CD, MCS-KKL, and PCE methods. This decision is made to optimise computational efficiency while ensuring sufficient accuracy for reliable analysis.

By implementing this approach, the study aims to achieve a practical balance, enabling effective analysis of natural frequencies in uniform beams without imposing prohibitive computational burdens.

3. Results and discussion

In this study, a cantilever beam of length 1 m, width 12 mm, and depth 10 mm is taken as the sample problem. The first three natural frequencies and mode shapes are treated as the response quantities to study the effect of randomness of the elastic modulus. The density of material is 7850 kg/m³. The mean value of modulus of elasticity (E) is taken as 200 GPa. The cantilever beam is discretised into finite elements, with each element having two nodes. The nodal degrees of freedom represent the nodal displacement and rotation. Cubic Hermite polynomials are used as interpolating functions between the two nodes.

In this study, the beam has been discretised into five elements for RSM and six for the other two methods. For MCS, a sample size of 2000 sets is generated. It may be noted that the mean and variance converge after a sample size of 1500 sets.

Table 3. Mean of eigenfrequencies.

Eigenfrequency in rad/sec	Random sampling	KKL M=5	PC M=5 p=1	PC M=5 p=2	Cholesky
First eigenfrequency	5.12E+01	5.12E+01	5.12E+01	5.12E+01	5.12E+01
Second eigenfrequency	3.21E+02	3.21E+02	3.21E+02	3.21E+02	3.21E+02
Third eigenfrequency	9.01E+02	9.01E+02	9.00E+02	9.00E+02	9.01E+02

Table 4. Variance of eigenfrequencies.

Variance of eigenfrequencies	Random sampling	KKL M=5	PC M=5 p=1	PC M=5 p=2	Cholesky
First eigenfrequency	3.64E-03	2.58E-03	4.33E-04	1.06E-04	2.09E-02
Second eigenfrequency	9.47E-02	1.01E-01	1.70E-02	4.17E-03	7.38E-01
Third eigenfrequency	6.17E-01	7.97E-01	1.34E-01	3.28E-02	5.66E+00

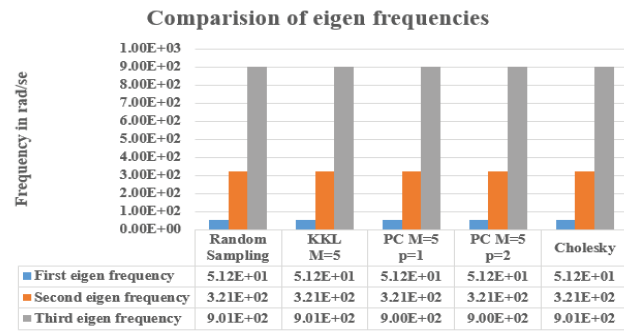


Fig. 13. Comparison of mean of eigenfrequency by Random Sampling method, Kosambi-Karhunen-Loève expansion, Polynomial Chaos expansion, and Monte Carlo simulations with Cholesky decomposition.

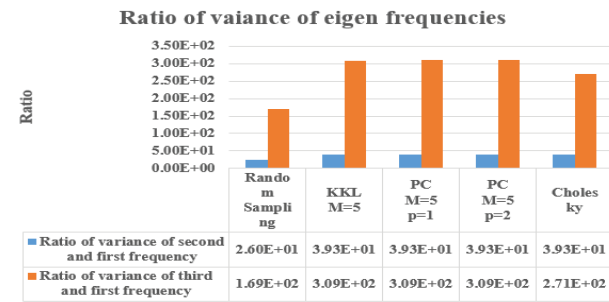


Fig. 14. Comparison of ratio of variance of third and second eigenfrequency with first eigenfrequency.

Figure 13 illustrates that all four methods yield nearly identical eigenfrequencies. However, there is a notable disparity in the variance among these methods. Additionally, within each method, there is a sharp increase in variance across different eigenfrequencies (Table 3). Fig. 14 highlights this observation, showing that the ratio of the variance of the third to the first eigenfrequency is approximately 100. The elevated variance of the third eigenfrequency holds significant implications, particularly considering its crucial role in the vibration characteristics of rotor blades.

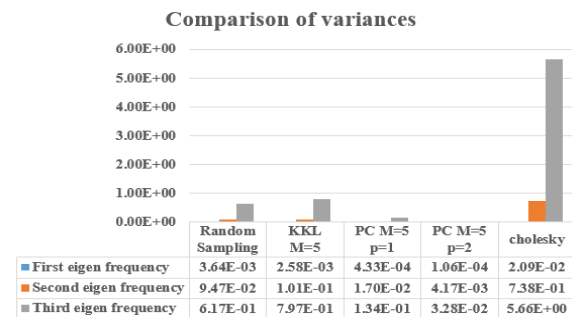


Fig. 15. Comparison of variance of eigenfrequency by Random Sampling method, Kosambi-Karhunen-Loève expansion, Polynomial Chaos expansion, and Monte Carlo simulations with Cholesky decomposition (Table 4).

From Fig. 15, it can be observed that PCE has least variance whereas (MCS-CD) has highest variance. The observed difference in variances between polynomial chaos and MCS-CD can be attributed to the inherent characteristics of each method in handling uncertainty.

Polynomial chaos expansion systematically represents the uncertain input variables (in this case, random elastic modulus) as a series of orthogonal polynomials, allowing for efficient and accurate estimation of the response statistics. This method excels in capturing the variability in the system with a reduced number of simulations, leading to a lower variance in the results. Essentially, polynomial chaos provides a more focused and structured approach to handle uncertainty.

On the other hand, MCS-CD rely on random sampling and, in certain scenarios, may encounter challenges, such as the occurrence of outliers. These anomalies can lead to significant variations in the results, resulting in higher variances. Cholesky decomposition, particularly when applied to normal distributions with specific covariance conditions, might introduce instabilities that contribute to the increased variance observed in the eigenfrequency estimates.

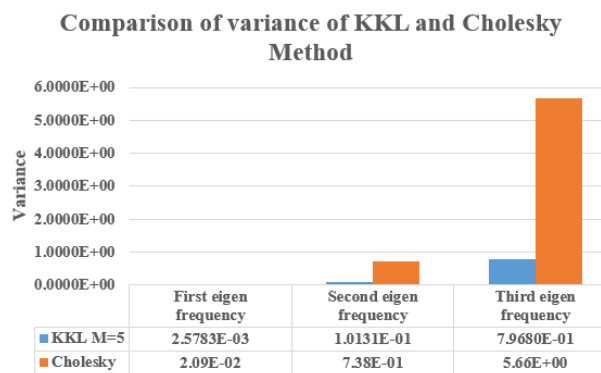


Fig. 16. Comparison of variance of eigenfrequency by Monte Carlo simulations with Cholesky decomposition and Monte Carlo simulations with Kosambi-Karhunen-Loève expansion.

Similarly, from Fig. 16, it can be observed that MCS-CD has higher variance compared to MCS-KKL. The observed difference in variances between MCS using the KKL expansion method and MCS-CD can be attributed to the specific characteristics and advantages of the KKL expansion approach in handling uncertainty.

KKL expansion is a technique that efficiently represents a random field in terms of uncorrelated modes. These modes capture the major variations in the random field, allowing

for a more effective dimensionality reduction. When applied to MCS, the KKL expansion method effectively captures the dominant modes of variability in the elastic modulus field, leading to a more focused and reduced set of samples needed for estimation.

On the other hand, Cholesky decomposition involves transforming a covariance matrix to obtain uncorrelated samples. In certain situations, particularly when the covariance structure is intricate or when dealing with specific distributions, Cholesky decomposition may lead to challenges, including potential numerical instabilities. These challenges can contribute to a broader spread of samples and, consequently, a higher variance in the results compared to the KKL expansion method.

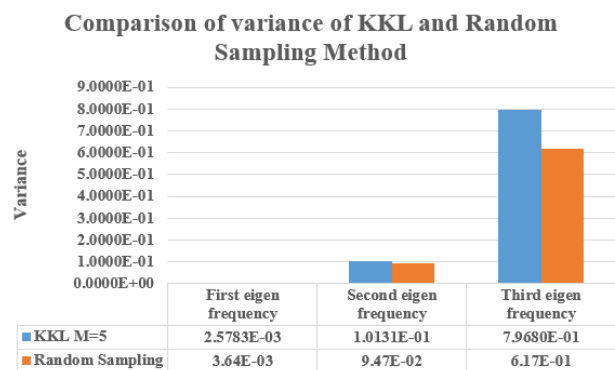


Fig. 17. Comparison of variance of eigenfrequency by Monte Carlo simulations with Kosambi-Karhunen-Loève expansion and Random Sampling method.

From Fig. 17, it can be observed that RSM and MCS-KKL have nearly identical variance. The similarity in variance between the proposed RSM and KKL expansion can be explained by their shared focus on efficiently representing and exploring the dominant modes of variability in the system. Both methods aim to capture the key patterns in the random field, allowing for a more focused analysis and reducing the overall variability in the results.

Similarly, the reason that RSM has lower variance as compared to MCS-CD is that the permutation-based nature of RSM introduces a level of variability, particularly when exploring a wide range of possible combinations. In contrast, MCS with Cholesky can face challenges related to numerical instability and negative modulus values, potentially leading to a broader spread of samples and higher variance.

Comparing the variance of RSM with PCE, it can be observed that RSM has higher variance. This can be attributed to the fact that Polynomial chaos relies on structured representations using orthogonal polynomials,

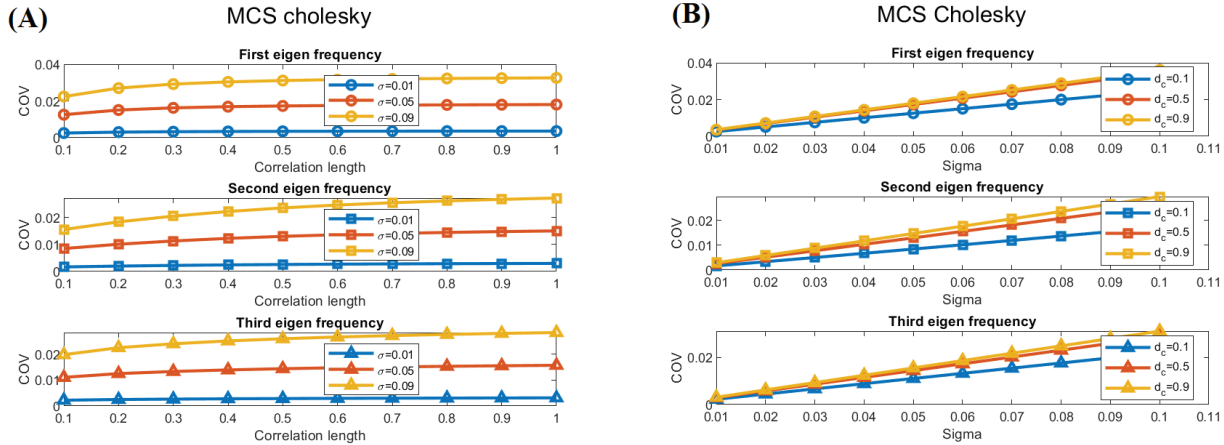


Fig. 18. COV of eigenfrequencies with correlation length and sigma for normal distribution.

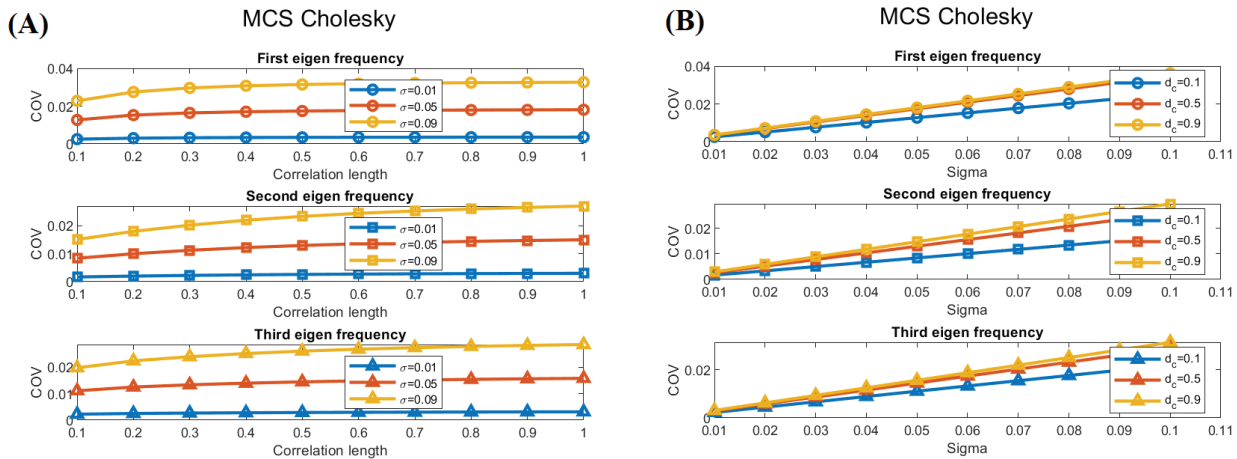


Fig. 19. COV of eigenfrequencies with correlation length and sigma for lognormal distribution.

contributing to a more controlled and focused analysis, resulting in lower variances. RSM, while comprehensive in exploration, may introduce more variability due to its unstructured permutation-based nature.

Figure 18A displays the variation of the COV of eigenfrequencies with correlation length for different values of sigma, while Fig. 18B depicts the variation of COV with sigma for different values of correlation length. These analyses were conducted using MCS-CD for both normally distributed elastic modulus. Similarly, Fig. 19A displays the variation of the COV of eigenfrequencies with correlation length for different values of sigma, while Fig. 19B depicts the variation of COV with sigma for different values of correlation length for log normally distributed elastic modulus for MCS-CD. It can be observed that both normal and lognormal distribution of elastic modulus give similar results. This can be reasoned because the transformation from a normal distribution to a lognormal distribution

involves an exponential function. It is possible that the transformation of the random variable by the exponential function cancels out terms of the covariance of the response. Also, the statistical properties of the normal and lognormal distributions are different, but when we compute the covariance of the response, the specific transformation might lead to similar results. The correlation structure and variability may be preserved even after the distribution change.

Figure 20A displays the variation of the COV of eigenfrequencies with correlation length for different values of sigma, while Fig. 20B depicts the variation of COV with sigma for different values of correlation length. These analyses were conducted using MCS-KKL for both normally-distributed elastic moduli. Similarly, Fig. 21A displays the variation of the COV of eigenfrequencies with correlation length for different values of sigma, while Fig. 21B depicts the variation of COV with sigma for different

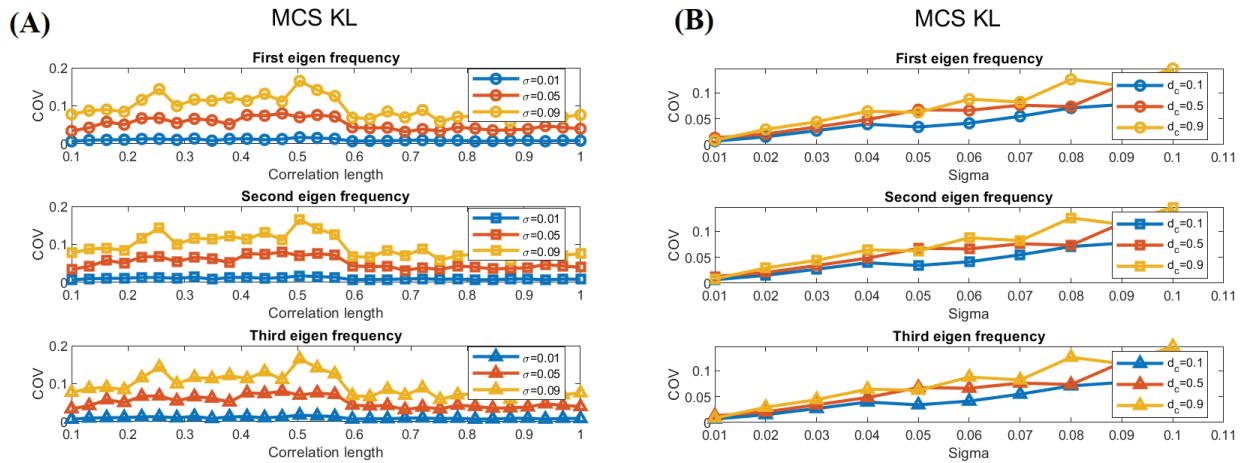


Fig. 20. COV of eigenfrequencies with correlation length and sigma for normal distribution.

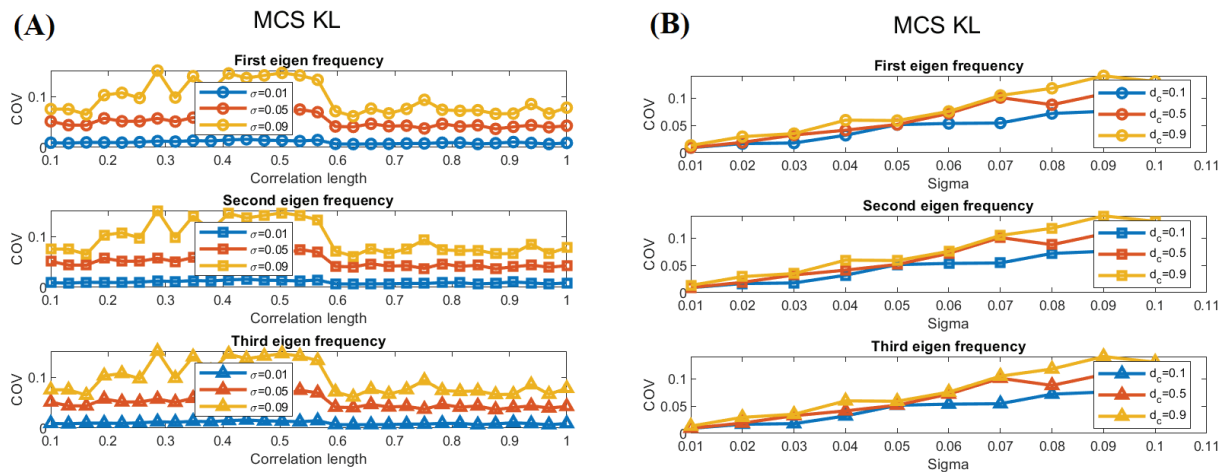


Fig. 21. COV of eigenfrequencies with correlation length and sigma for lognormal distribution.

values of correlation length for lognormally distributed elastic moduli for MCS-KKL. In this case, normal and lognormal distributions do not have any significant effect on the COV of the response. This can be attributed to the fact that MCS methods such as Cholesky decomposition and KKL expansion may be less sensitive to the choice of distribution for certain types of problems. The algorithms used in these methods could handle the transformation without significantly impacting the covariance of the response. The correlation length and the correlation structure of the random elastic modulus may have a more dominant effect on the covariance of the response than the specific choice between a normal and lognormal distribution.

Figure 22A displays the variation of the COV of Eigenfrequencies with correlation length for different values of sigma, while Fig. 22B depicts the variation of COV with sigma for different values of correlation length. These analyses were conducted using PCE for normally

distributed elastic modulus. Similarly, Fig. 23A displays the variation of the COV of eigenfrequencies with correlation length for different values of sigma, while Fig. 23B depicts the variation of COV with sigma for different values of correlation length for log normally distributed elastic modulus for PCE. In this case, the COV of response varies significantly for normal and lognormal distribution. This can happen because PCE relies on orthogonal polynomials as the basis functions. These polynomials are specifically designed to work well with certain types of distributions. If the distribution changes from normal to lognormal, the orthogonality properties of the polynomial basis may be affected, leading to different behaviours in the PCE. Also, PCE involves the expansion of the response in terms of orthogonal polynomials of the random variables. The transformation of the random variable from a normal to a lognormal distribution involves different mathematical operations (exponential function), and this transformation can affect the representation of the response in the PCE.

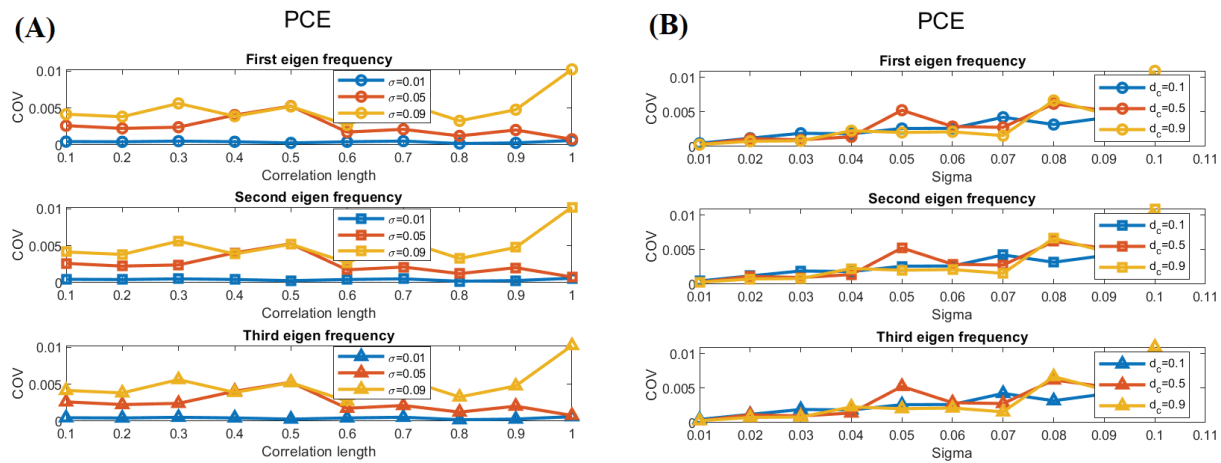


Fig. 22. COV of eigenfrequencies with correlation length and sigma for normal distribution.

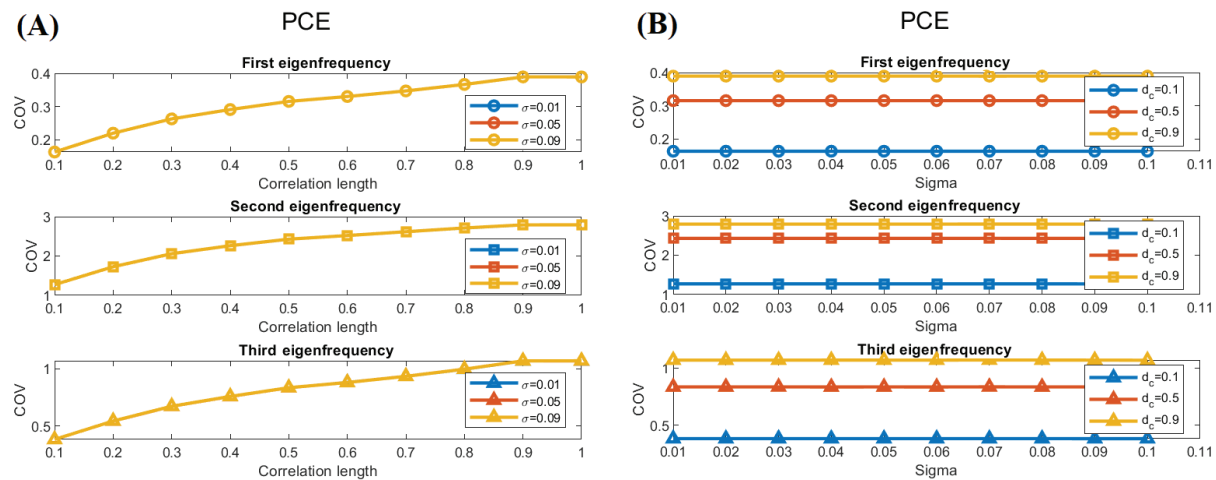


Fig. 23. COV of eigenfrequencies with correlation length and sigma for lognormal distribution.

Table 5. Computational cost.

Method	Number of simulations	Computational time (in seconds)
MCS-CD	2000	19.95792
MCS-KKL	2000	1.312695
PCE (T=5, p=1)	5	0.017394
PCE (T=5, p=2)	20	0.03018
RSM (nos. of elements=5)	3125	31.63806

Table 5 provides a comparison of the computational cost for different RSMs including MCS-CD, MCS-KKL, PCE with different polynomial orders, and RSM.

The computational cost for RSM is relatively higher compared to MCS-CD, MCS-KKL and PCE. This can be attributed to the nature of RSM, which involves evaluating the structural dynamic characteristics of the beam for all possible combinations of elastic modulus values.

Despite requiring a larger number of simulations compared to other methods, RSM provides a comprehensive analysis by considering all possible combinations of elastic modulus values, this exhaustive approach ensures a thorough exploration of the structural behaviour under varying material properties.

MCS-KKL demonstrates lower computational cost compared to MCS with MCS-CD, suggesting that MCS-KKL may be a more efficient RSM for the given analysis. KKL offers optimised sampling strategies that lead to faster convergence and reduced computational cost. It leverages the eigenstructure of the covariance matrix to efficiently sample from the underlying distribution, resulting in fewer iterations required to achieve accurate results compared to traditional methods like Cholesky decomposition.

It may be noted that RSM may require more computational resources due to the exponential increase in possible combinations with the number of elements, it offers a comprehensive analysis of the structural behaviour, which can be valuable for understanding the effects of material variability on the system response.

Figure 24 depicts the mode shapes of MCS-CD, MCS-KKL, RSM, and PCE. Upon examination of Figs. 24A and 24B, it becomes apparent that the spread of mode shapes in MCS-CD is larger compared to the proposed RSM and MCS-KKL. Furthermore, the spread of mode shapes in MCS-KKL and RSM appears to be nearly identical. This difference can be attributed to variations in their respective variances. The observed differences in the spread of mode shapes among MCS-CD, MCS-KKL, RSM, and PCE models bear practical significance in structural design and analysis. Understanding the variations in mode shapes can provide valuable insights into the dynamic behaviour of the system under consideration. For instance, a larger

spread of mode shapes, as seen in MCS-CD, may indicate increased uncertainty or variability in structural response, necessitating robust design strategies. Conversely, the similarity in mode shapes between MCS-KKL and RSM suggests a comparable predictive capability between these methods, offering potential efficiency gains in computational modelling and analysis tasks.

4. Conclusions

In this study, we have thoroughly investigated the influence of random elastic modulus on beam eigenfrequencies using various simulation techniques, including MCS-CD and MCS-KKL, PCE, and a proposed RSM. We successfully addressed anomalies encountered in MCS by adopting a log-normal distribution for the elastic modulus, effectively mitigating negative values and imaginary eigenfrequencies. Through comparative analyses focusing on covariance variation of the first three eigenfrequencies with correlation length and standard

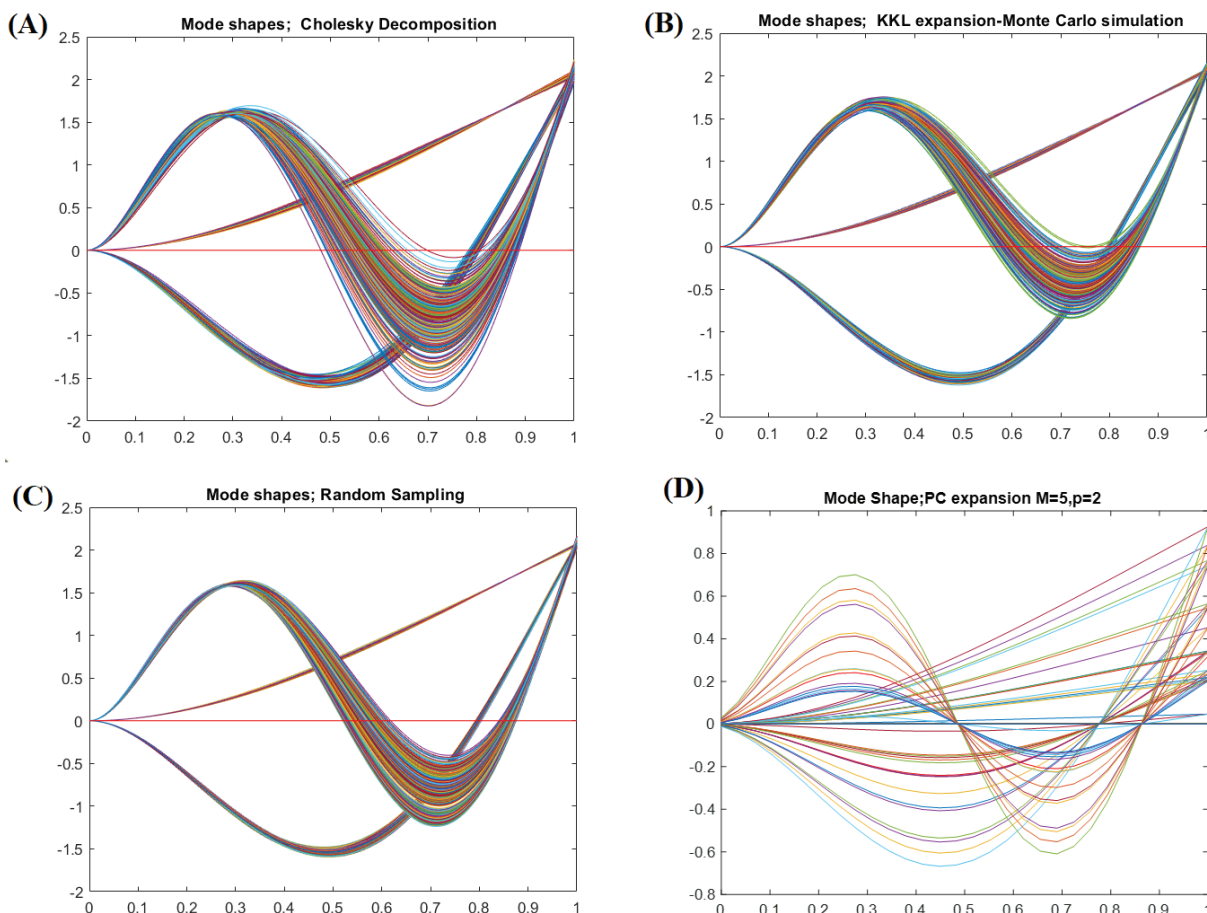


Fig. 24. Mode shapes for Monte Carlo simulations with Cholesky decomposition, Monte Carlo simulations with Kosambi-Karhunen-Loève expansion, Random Sampling method and Polynomial Chaos expansion.

deviation of the random field, we highlighted nuanced differences between normal and log-normal distributions. Additionally, PCE revealed distinct responses, showcasing variations in covariance with different distributions.

Our study culminated in eigenfrequency estimation using the proposed RSM, where the beam was discretised into n elements with randomly assigned elastic moduli. Comparative analysis with existing methods showed that the RSM yields nearly identical results for small numbers of finite elements, with the variance of the proposed method closely approximating that of the KKL method. However, it is essential to note that the proposed method becomes computationally expensive as the number of elements increases. For instance, when the number of elements increases from $n=6$ to $n=7$, the computational complexity rises significantly from $6^6=46,656$ to $7^7=823,543$. Therefore, while the RSM offers promising results, its scalability may pose challenges in computational resources for larger structural models.

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COMPETING INTERESTS

The author declares that there is no conflict of interest regarding the publication of this article.

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Comprehensive analysis of cement-stabilised dredged marine soil: Evaluating physical, microstructural, and mechanical characteristics

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Abstract:

This study evaluates the index, microstructural, and mechanical behaviour of cement-stabilised soft soil. The marine soil dredged from Hangzhou is taken for the study. Different percentages of ordinary Portland cement (OPC) by dry mass were added to the soils during reconstitution, i.e., 5, 10, 15, and 20%. The reconstituted samples were analysed in the laboratory to determine basic properties such as moisture content, void ratio, density, specific gravity, consistency limits, and particle size distribution. The microstructures of the reconstituted samples were also evaluated through scanning electron microscopy. Results inferred a significant impact on the physical properties of the soil. An increase in cement content increases the consistency limits. Additionally, the specific gravity, plasticity index, and density of the treated soil initially decreased but increased upon further addition of cement content. The microstructure of the soil transformed from a dispersed to a flocculated structure, with larger and denser particles. The change in microstructure was also evident in the particle size distribution, with an increase in cement content leading to larger size particles. Finally, the unconfined compression test conducted on the reconstituted soil indicates a substantial enhancement in strength with higher cement content and prolonged curing periods.

Keywords: cement, mechanical properties, microstructure, self-pozzolanic, soft soils, soil stabilisation.

Classification number: 2.3

1. Introduction

Soil is the product of various physical and chemical phenomena of nature, varying from place to place and over time due to factors influencing its formation. A significant factor in soil formation is water, whether flowing, stagnant, frozen, or sub-surface. Consequently, weak soils are often prevalent along riverbanks and coastlines. Nevertheless, construction on such flatlands with weak soil has been commonplace since ancient times. Many major cities worldwide have been established on riverbanks, lacustrine deposits, or coastal areas. Consequently, weak soil with poor mechanical properties poses a challenge for engineers in constructing infrastructure in these urban areas due to excessive deformation and failure in the foundation and substructure. To address these challenges, various methods of ground improvement have been employed to either compact or chemically modify soft soil to enhance its index and engineering characteristics. Common chemical stabilisers used for this purpose include cement, lime, fly ash, silica fumes, rice husk ash, eggshells, among others [1-5]. Following technological advancements, chemical stabilisation using cement has become the predominant

ground improvement technique. However, many studies have pointed out sustainability issues using cement as the chemical stabiliser [6-7]. This method is preferred over other chemical stabilisation techniques such as lime and fly ash. Lime treatment may lead to an increase in site pH, while many fly ash products contain heavy metals. Moreover, for soft soil with high compressibility and low permeability, cement treatment is favoured over traditional techniques like dynamic compaction, which can cause detrimental vibrations in urban areas.

Numerous studies have been conducted to quantify the strength of cement-treated clays and have reported significant strength improvements compared to untreated clays [8-12]. Similarly, research has explored the mechanical behaviour of cement-treated clays, resulting in various constitutive models. However, limited attention has been given to the physical changes and behaviour of cement-treated clays [9, 13-16]. Therefore, this research aims to experimentally investigate soft soil to determine changes in physical, microstructural, and mechanical behaviours resulting from chemical reactions between soil and cement.

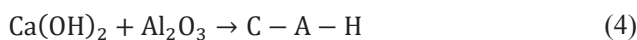
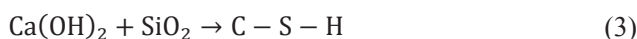
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The improvement in soil properties is primarily attributed to chemical reactions with cement. Portland cement, comprising tricalcium silicates (C_3S), dicalcium silicates (C_2S), tricalcium aluminate (C_3A), and tetracalcium aluminoferrite (C_4AF), is the most commonly used cement in soil improvement. Major reactions between soil and cement include dehydration, ion exchange, and pozzolanic reaction [17, 18]. The primary hydration reactions and pozzolanic reactions processes are outlined below [18, 19]:

Primary cementitious product



Secondary cementitious product



The hydrated lime releases calcium ions (Ca^{2+}), causing a rise in pH, which is crucial for pozzolanic reaction. These calcium ions then react with the soil silica and soil alumina to form cement hydrates; hydrated calcium silicates (C-S-H) and hydrated calcium aluminates (C-A-H), respectively [20]. This reaction formed soil-cement structures, which play a vital role in strength development. The term “structure” was coined by R.J. Mitchell (1970) [21] and defined by J.B. Burland (1990) [22] as the amalgamation of bonding and fabric. The fabric component deals with the arrangement of soil particles, while bonding defines the forces between soil fabric. Under load, the degradation of cementation mainly occurs due to the loss of bonding structures [23, 24] while the fabric component remains stable and unaffected by loading. The process of soil-cement hardening was first proposed by S. Saitoh, et al. (1985) [25], as shown in schematic diagrams (Fig. 1), which illustrates the change in the soil-cement structures during the hardening process. Specifically, Fig. 1A shows that initially the clusters of clay particles are surrounded by a cement slurry. Then, the primary hydration reaction occurs in the cement slurry so that hardened cement is formed around the cluster of clay particles (Fig. 1B).

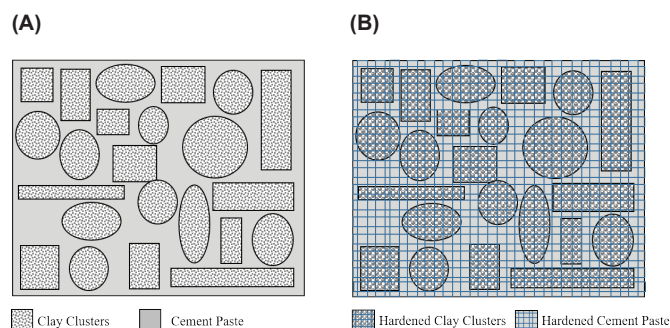


Fig. 1. Schematic illustrations of improved soil: immediately after mixing (A), after hardening (B).

The microstructure of treated soils is primarily influenced by the type of admixture used for stabilisation. For example, the absence of primary hydration reaction in lime-treated soil may result in a distinct microstructure compared to cement-treated soil. Several studies have investigated microstructure using techniques such as X-Ray Diffraction (XRD) and scanning electron microscope (SEM) [26–30]. Research on Singapore clay noted that untreated clay exhibits an open microstructure, while cemented clay shows a flocculated microstructure where clay-cement clusters are separated by large intercluster voids [28]. Similarly, a study on very expansive Handan clay treated with 12% cement content observed structural changes from a dispersed to a flocculated and finally agglomerated structure over the long term [31]. The effect of cement on microstructure is significant, with higher cement content leading to a more flocculated nature of soil fabrics [17].

The major factors controlling physical changes in cement-stabilised soil are the amount of cement and the curing period [18, 26, 30]. However, few studies have focused on evaluating the physical properties of cemented soil. One comprehensive laboratory investigation on cement-treated Bangkok clay reported negligible changes in liquid limit (LL) due to treatment [11]. However, plastic limit (PL) gradually increased with the amount of cement and curing period, leading to a significant decrease in the plasticity index (PI) of treated soil. Similarly, the specific gravity of treated soil decreased with increasing cement content and curing period. The predominant parameter for the decrease in specific gravity (G_s) and PL is the curing period, allowing time for reactions between cement and clay constituents. Another study on treated soil cured without confinement showed a higher LL than cured under pressure, attributed to entrapped water within soil particles that did not affect particle interaction [29]. This increase in LL is attributed to entrapped water within the soil particles, which does not affect the interaction between particles. Moreover, the study on Bangkok clay revealed that the unit weight of the treated specimen increases with the increase in the cement and curing period.

2. Materials and methods

2.1. Materials

Soil: Dredged soil collected from the coastal region of Hangzhou, China, underwent examination for its physical characteristics and chemical composition, as detailed in Table 1. This marine soil has an LL of 52.33%, a PI of

16.29%, and 4.41% organic matter. According to the Unified Soil Classification System (USCS), it falls into the category of high-plasticity silt. The primary chemical components of this soil consist of SiO_2 and Al_2O_3 . Additionally, a gradation analysis revealed that over 99% of the soil is fine-grained, with clay-sized particles ($<2\ \mu\text{m}$) constituting only 16%, as depicted in Fig. 2.

Table 1. Physical properties and chemical composition of soils.

Physical Properties		Chemical composition	
Liquid limit (LL)	52.33%	SiO_2	58.69%
Plastic limit (PL)	36.04%	Al_2O_3	19.63%
Plasticity index (PI)	16.29%	Fe_2O_3	8.24%
Specific gravity (G_s)	2.76	Na_2O	4.42%
Clay (%), size range $<2\ \mu\text{m}$	15.84%	MgO	3.69%
Silt (%), size range $2-75\ \mu\text{m}$	84.14%	K_2O	2.74%
Sand (%), size range $75-200\ \mu\text{m}$	0.02%	CaO	2.39%
Organic content	4.41%	MnO	0.15%
Soil classification	MH		
Depth of sampling	-		
Location	Hangzhou, China		

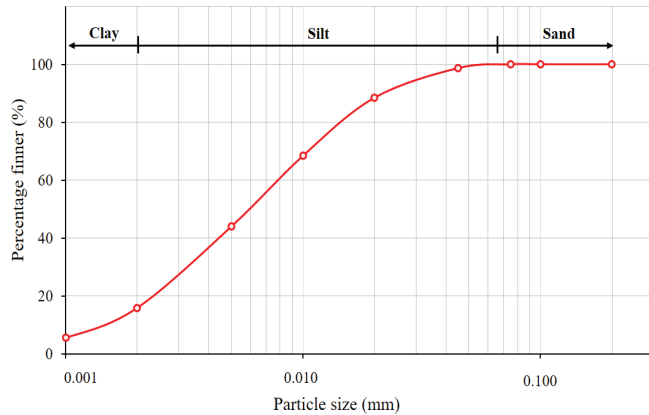


Fig. 2. Particle size distribution of dredged marine soil.

Cement: Regular Portland cement was employed for chemical stabilisation in this study. The composition of ordinary Portland cement (OPC) was determined using X-ray fluorescence spectrometry (XRF) and comprises: CaO 57.25%, SiO_2 23.54%, Al_2O_3 6.6%, Fe_2O_3 3.25%, SO_3 2.74%, Na_2O 0.15%, and K_2O 0.71%, along with a loss on ignition of 3.13%. Reconstituted samples with varying cement content were prepared to assess the effect of cement.

2.2. Sample preparation

The soil was dried at room temperature, and after seven days, negligible moisture loss was observed, thus considered as dry soil. The air-dried soil was pulverised

using a wooden hammer, sieved through a $425\ \mu\text{m}$ [No 40] sieve, and stored in an airtight plastic bag to prevent further moisture exchange. Before sample preparation, distilled water was de-aired using a vacuum pump with a capacity of 100 kPa.

The soil was then mixed with predetermined amounts of cement and water. The water content used for reconstitution was taken as 100% of dry soil mass [32, 33]. The water content is selected based on the natural water content of soil found in different places like, Singapore, Hong Kong, Bangkok, and Japan, which varies from 80 to 120% and the commonly used water content in deep cement mixing and grouting also varies from 90 to 110% [34]. The samples were prepared following methods from the literature [5, 30, 35]. As presented in the Fig. 3, the soil-cement-water mixture was mixed for 10 minutes, considering the significance of mixing time [36]. The mixture was then poured into a mold with a diameter of 39.1 mm and a height of 80 mm. These samples were cured under free drainage conditions at a constant temperature of 25°C for different periods of up to 56 days. Cement-treated soil samples were prepared as presented in Table 2 to evaluate the effect of cement on the physical, microstructural, and mechanical behaviour of reconstituted soil through various laboratory investigations (Figs. 3I-3IV).

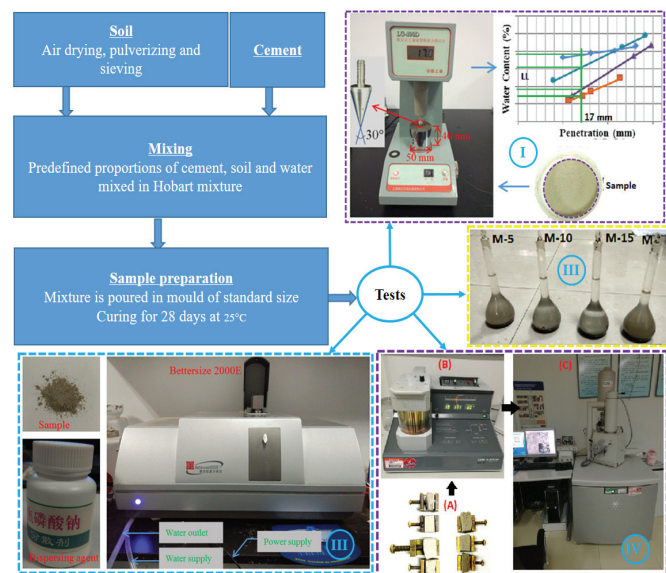


Fig. 3. Sample preparation flow chart and laboratory equipment. (I) Specific gravity, **(II)** Liquid limit and plastic limit, **(III)** Particle size distribution analysis using Bettersize 2000E, and **(IV)** with **(A)** SEM samples, **(B)** BAL-TEC/SCD 050 sputter coater and **(C)** FEI Quanta 200 ESEM/VPSEM scanning electron microscope.

Table 2. Characterisation of cement-treated samples.

Soil	Cement content (%)	Water content (%)	Curing days	Curing temperature	Sample code
Marine Soil	5	100	28	25°C	M-5
	10	100	28	25°C	M-10
	15	100	28	25°C	M-15
	20	100	7, 14, 28, 56	25°C	M-20

3. Results and discussion

The changes in physical properties due to cement were thoroughly studied, considering various physical quantities. Furthermore, microstructural behaviour was examined through SEM, and mechanical behaviour was assessed using uniaxial compressive strength (UCS). Dredged marine soil reconstituted with 5, 10, 15, and 20% cement content was utilised to evaluate behaviours. The results from the laboratory investigation are discussed as follows.

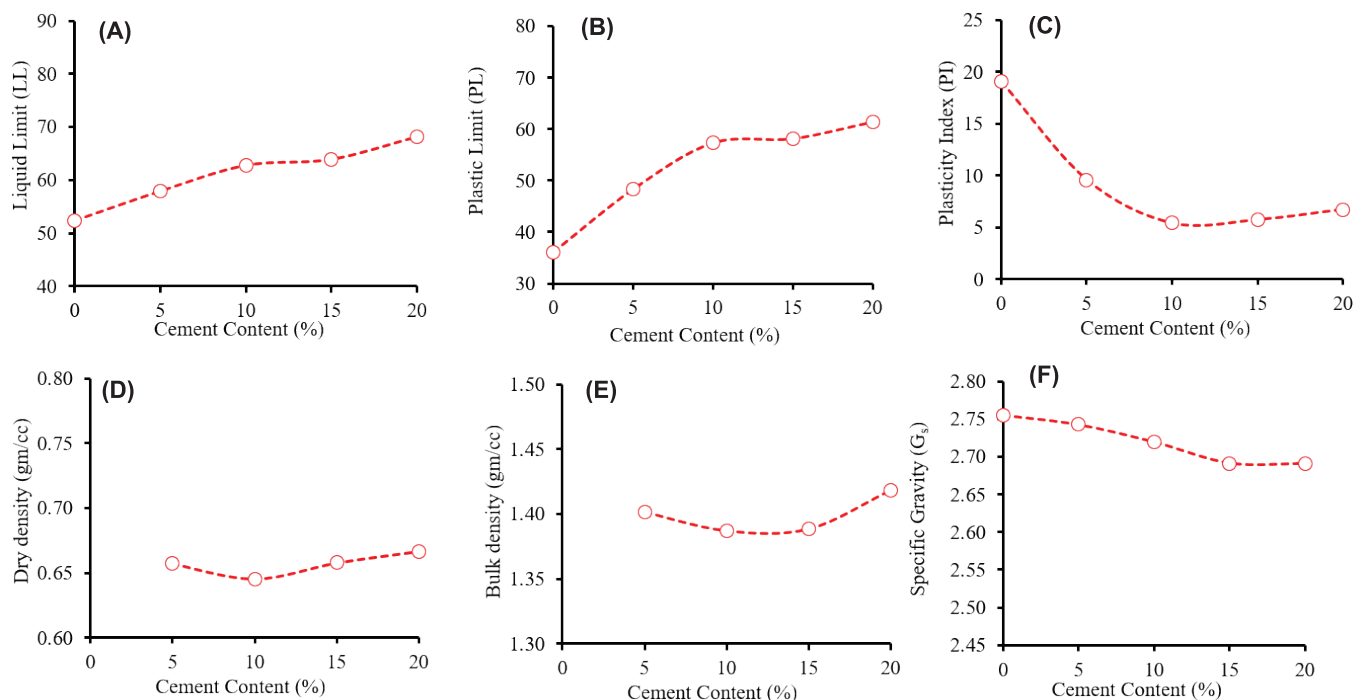
3.1. Effect of cement on the specific gravity of stabilised soils

The variation in specific gravity (G_s) of the cement-treated soil is presented in Fig. 4F. For dredged marine soil, the G_s value decreased from 2.76 to 2.69 when the cement content increased to 20% in the untreated soil. The rate of decrease is small at lower cement content but increases with the increase in cement content (up to 15%) and remains constant afterwards. A study on cement-treated Bangkok clay concluded that an increase in cement

content significantly reduced the specific gravity [11]. The decrease in the rate of reduction of G_s at higher cement content resembles the behaviour observed in this study. This reduction may be attributed to the agglomeration of fine particles forming clusters with entrapped air [37]. Therefore, it can be inferred that an initial increase in cement content significantly reduces the G_s of treated clay, but after a certain cement content, G_s increases with further cement content.

3.2. Effect of cement on index properties of stabilised soils

The variations in the LL of the treated samples are presented in Fig. 4A. The result shows that the increase in cement content significantly increases the LL. Similar observations have been noted in lime-treated Louisville clay [38] and cement-treated Singapore clay at lower cement content [17]. In Singapore clay, the LL slightly increased for cement content higher than 10%. This increase in LL may be due to the entrapped water in the cluster formed by aggregation and cementation of particles. On the other hand, H. Brandl (1981) [37] reported a divergent trend depending on the plasticity of untreated soil. He concluded that with more plastic soils, the LL decreases with an increase in cement content because of the formation of the clay clusters. Moreover, the trend is the opposite for low plastic soils, the presence of entrapped water in soil clusters plays the dominant role.

**Fig. 4. Variation of physical properties of cement-treated soils.**

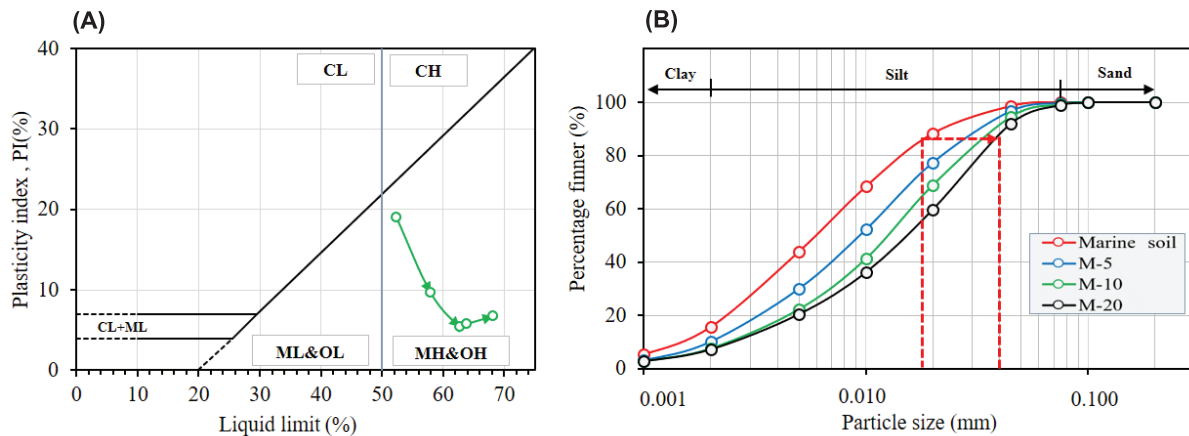


Fig. 5. Phase transformation of the cement-treated soils: plasticity chart (A), particle size distribution (B).

From Fig. 4B, it is evident that the PL of the cement-treated soils was higher than untreated soils. PL increases gradually until the 10% cement content, with an increment of 21.32%. However, further increases in cement content do not significantly affect the PL. A similar trend for PL was reported in cement-treated Bangkok clay [8]. The results show that both the LL and PL of the cement-treated soils increase with the increase in cement content. However, the increment rate of PL is higher than LL for lower cement content, resulting in a sudden decrease in PI (Fig. 4C). In contrast, PI for all ranges of cement content used in this study was found to be lower than untreated soil, although PI increases beyond a certain cement content (i.e. 10%).

The summary of Atterberg's limits is plotted on the plasticity chart based on the USCS (Fig. 5A). The chart infers that the soil lies in the high plasticity silt or organic soil category, with or without cement modification. The soil exhibits more silty behaviour after treatment with 15% cement content. The soil regains plasticity characteristics upon further increase in cement content but remains on the lower side compared to untreated soil.

3.3. Effect of cement on sample density of stabilised soils

The density for each specimen was calculated and plotted in Figs. 4D and 4E. The results show that the minimum density was found at 10% cement content, and the treated soil is denser on both sides of the cement content. A similar trend is observed in the dry density, with a minimum density at 10% cement content. According to a study treating soft, lean clay with cement up to 10%, the density rises with cement content [39]. The difference in the trend is primarily due to the presence of organic matter in the soil used in this study, unlike the inorganic soil used in previous research. However, the variation in density is found to be small and insignificant.

3.4. Effect of cement on the particle size distribution of the stabilised soils

The results of particle size distributions of treated and untreated dredged marine soil are presented in Fig. 5. Fig. 5B shows that the particle size distribution curve significantly shifts to the right side (coarser side) for the cement-treated marine soil. When the soil is treated with 5% cement content, the effective size (D_{10}) increases to 1.967 from 1.454 μm . Furthermore, D_{10} increases to 2.45 μm after increasing the cement content to 10%. This trend is even more pronounced for the mean particle size (D_{50}) of cemented soil, which increases from 5.914 to 15.56 μm when treated with 20% cement content. This increase in particle size is the result of aggregation of soil particles. A similar trend has been observed in cement-treated Singapore marine clay [17]. The increased pH value causes dissolved bivalent calcium ions (Ca^{2+}) to replace the monovalent ions (Na^+ , K^+) of the minerals. The presence of Ca^{2+} on the surface of clay particles brings them together, resulting in increased particle size [40, 41].

3.5. Microstructural behaviour of cement stabilised soil using scanning electron microscope

The SEM analysis of the soil reveals how the soil structure has transformed from a dispersed to a flocculated fabric after being treated with cement, as shown in Fig. 6. The examination of untreated soil Fig. 6A indicates the presence of fine, flaky particles with many small-sized voids distributed throughout the section. Additionally, the particles formed small clusters in a more or less homogeneous manner. However, the soil treated with 10% cement content (Fig. 6B) exhibited a transformed structure characterized by open configurations and a few needle-like microstructures, along with some indications of reticulation. Reticulation was prevalent when cement content increased from 10 to 20%. The size of the particle significantly increases with

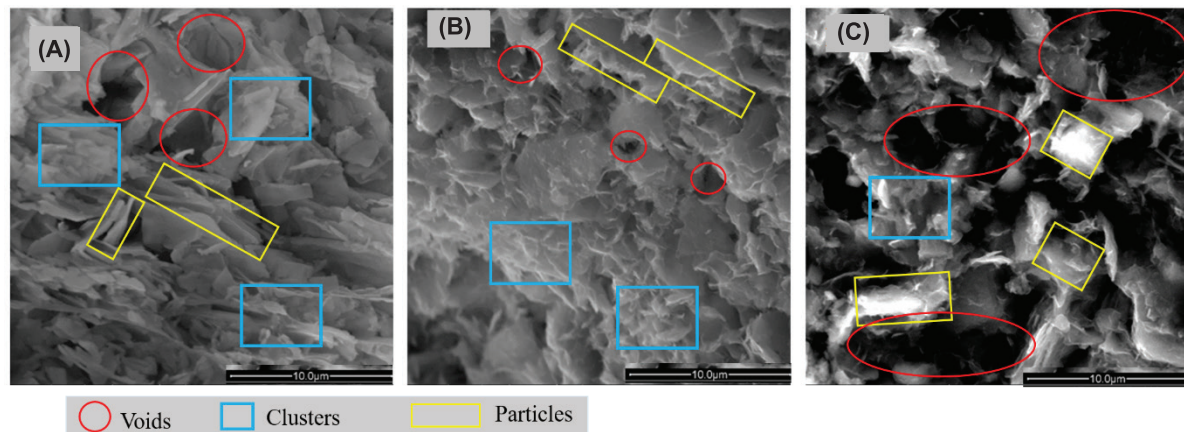


Fig. 6. SEM image of cement-treated soils. (A) Untreated, (B) M-10, (C) M-20.

dense structure, increased pore size, and decreased number of pores. Furthermore, some precipitated crystals were found individually or as clusters creating the cementation bonds between the soil particles. These aggregated and precipitated crystals create an open-type structure in both soils. These observations have been corroborated in similar studies, wherein the degree of reticulation increases as the amount of cement increases in dredged materials from flood spill channels, Singapore marine clay, and Ballina clay [42-44]. This aggregation and cementation of the soil structures significantly improved the compressive strength [35, 45, 46], yield strength, and compression characteristics of the soil [11, 47]. Furthermore, the microstructure of solidified soils has been defined by five different parameters: particle circularity, particle direction fractal dimension, particle size fractal dimension, pore area ratio, and particle equivalent diameter, and found that these parameters are well-correlated with the strength of soils [48].

3.6. Effect of cement content and curing time on the strength of the stabilised soil

The findings from compression tests conducted on marine soil [11] were further extended with different curing periods along with the strength development pattern corresponding to various cement contents, which are illustrated in Fig. 7A. The outcomes reveal that the sample treated with 5% cement content exhibits lower strength. Notably, a distinctive peak in the stress-strain curve is observed when the cement content is increased to 10%, resulting in an approximately fourfold increase in strength ($q_u = 86.94$ kPa). Likewise, as the cement content further doubles to 20%, the UCS values are increased by more than 3 folds (290.4 kPa). These results signify the enhancement in the strength of marine soil with increasing cement content and curing period, aligning with the findings of previous researchers [49-51].

Moreover, as depicted in Fig. 7A, the increase in cement content leads to a reduction in failure strain (ϵ_f). Specifically,

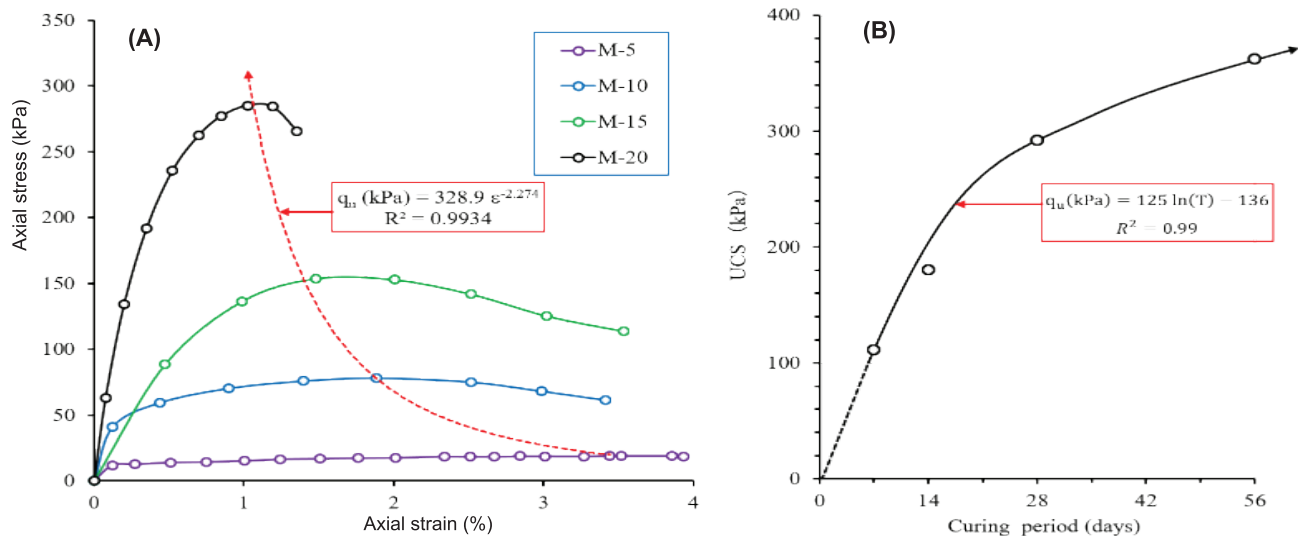


Fig. 7. Variation of unconfined compressive strength with cement content (A), curing period (B).

ϵ_f decreases to 1.65, 1.45, and 1.08% at cement contents of 10, 15, and 20%, respectively, compared to the higher value (3.84%) observed at 5% cement content. This trend indicates that an increase in cement content results in higher brittleness in the treated soil.

Additionally, the soil treated with a cement content of 20% was cured for different durations (7, 14, 28, and 56 days) to assess the impact of the curing period on soil strength. Fig. 7B illustrates a substantial increase in soil strength with prolonged curing periods. The mobilised peak strength of the soil was recorded as 111.31 kPa after 7 days of curing, which increases to 180.56 kPa at 14 days and further increases to 362.09 kPa after 56 days of curing. The improved strength of stabilised soil is attributed to the increased particle size, change in the soil microstructure and cementation between the particles. The trend observed in the study is aligned with the studies involving different cement-treated clays [52, 53].

4. Conclusions

Dredged marine soil was reconstituted with different cement contents. The reconstituted samples were used for laboratory tests and results were analysed to determine the physical, microstructural, and mechanical behaviours. The findings of this study are summarised below:

- The cementation significantly affects the physical behaviour of the treated soils. The specific gravity of dredged marine soil decreases from 2.76 to 2.69 when modified with 20% cement content.

- Both the LL and PL of treated soil increased with cement content. However, the rate of increase in the PL was higher, causing a decrease in the PI. However, PI levels recover after a certain amount of cement content.

- The effect of the cement content on cementation and aggregation of the clay particles resulted in larger particle sizes. The effective size (D_{10}) increased to 2.45 μm from 1.454 μm with a cement content of 10%. A similar trend is found for the mean particle size (D_{50}) of cemented soil, which increases from 5.914 to 15.56 μm when treated with 20% cement content.

- Analysis of SEM images supports the results obtained from the gradation analysis. The treated soil had a more flocculated microstructure, with larger particle size and more sizeable voids than the untreated clay. The changes in the microstructure are crucial for enhancing the mechanical behaviour of modified soil.

- The changes observed in the stabilised soil, including improvements in its physical properties, increased particle size, and changes in microstructure, led to a substantial enhancement in strength. This increase in strength was particularly noteworthy, with a nearly seventeen-fold improvement observed when comparing soil treated with 5% cement content and cured for 28 days to soil treated with 20% cement content and cured for 56 days.

CRedit author statement

Bhim Kumar Dahal: Methodology, Data collection, Data analysis, Writing, Editing; Jun Jie Zheng: Conceptualisation, Data analysis, Reviewing, Editing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Determination of critical properties of orange peel for a polymer reinforcement material

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Abstract:

Accurately determining the critical properties of orange peels ensures composite engineers can successfully monitor and control in-service composite failures. However, the current approaches to property determination are deficient, lack critical analysis, and are not wide-ranging. Thus, they fail to accurately establish the important properties of orange peels. To overcome this challenge, this article establishes the compressive strength, heat absorption, and electrical conductivity of an orange peel polymer to understand the behaviour of the material in lightweight composite applications. Three experimental sets were used to determine the three mentioned properties. The results obtained showed that the average heat energy absorbed by the samples of collected orange peels in 28 poly bags was 79.32 J. The highest and lowest heat absorbed by the samples was found to be 162.03 and 11.08 J, respectively. The average electrical conductivity was measured at 0.025σ while the average resistance, length, and surface area of 20 orange peel samples were found to be 27.24Ω , 2.92 cm, and 7.02 cm^2 , respectively. The resistance of the orange peels had the greatest influence on their electrical conductivity. Lastly, the compressive shear strengths of the 0.300, 0.425, and 0.600-mm orange peel particulates were found to be 41.86, 14.82, and 9.58 kN/m², respectively. With this information, composite design engineers using orange peel reinforcements could improve their design accuracies and extend in-service composite lives.

Keywords: composites, electrical conductivity, heat energy absorption, orange peels, resistance.

Classification numbers: 2.3, 5.1, 5.3

1. Introduction

The composite material development industry is governed by the imperative to adhere to carbon neutrality and the peaking policies of governments [1, 2]. Therefore, the development of new green materials, particularly orange peels, which are plentiful and are free at the source, is crucial [3]. In the past, the behaviour of orange peels was evaluated mechanically [3-5]. However, it was recognised that in-service orange peel-based composites absorb infrared radiation and warm up as the waves strike them. The resulting heat stress triggers dehydration, influencing the lifespan of the composites. Moreover, it has been observed that orange peel-based composites can fail in ductility. Additionally, extremes in soil electrical conductivity can impede crop growth. Therefore, considering the importance of heat absorption, electrical conductivity, and compressive strength, incorporating these into a set of mathematical measures is vital for the advancement of property evaluation. As material property assessment progresses, confining evaluations to mechanical, rheological, and chemical

properties are increasingly insufficient. Consequently, a method that encompasses heat absorption, electrical conductivity, and compressive strength is necessary to ensure consistent design standards and reliable data for reinforcement materials, such as orange peel.

Despite extensive research on the mechanical properties of orange peels, their potential for heat absorption has been overlooked [3-5]. The literature has also neglected the relevance of compressive strength and the opportunity to enhance the electrical conductivity of orange peel-based composites in design [3-5]. Therefore, despite the abundant knowledge, the heat absorption properties that elucidate the heat transfer rates within orange particles remain underexplored [6-9]. Moreover, the overlooked compressive strength in the literature has implications for structural integrity in designs, and the neglect of electrical conductivity overlooks the durability of structures based on orange peel composites.

However, electrical conductivity measurements, compressive strength, and heat absorption of materials have

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been substantial investigation topics in materials science and engineering. Pioneering work on electrical conductivity is attributed to Benjamin Franklin, while Stephen Timoshenko significantly contributed to our understanding of compressive strength. Newton is largely credited for his work on heat transfer in materials.

Recent studies, such as S.M. Jubihulla, et al. (2022) [10], have determined various properties of the *Albizia Julibrissin* plant stem, revealing high cellulose content and low lignin levels. R. Kumar, et al. (2022) [11] presented chemical composition values for *Acacia nilotica* L. fibre, focusing on cellulose, ash content, and lignin. Additionally, R. Vijay, et al. (2022) [12] evaluated the properties and tensile strength of *Vachellia farnesiana* fibre material. J.S.N. Raju, et al. (2022) [13] assessed the thermal, XRD, physical, morphological, and chemical properties of *Symphirema involucratum* stem fibre.

From the literature, there has been a noticeable increase in property evaluation research for green materials, many of which are utilised for polymer reinforcement in composite development. There are also some specific papers tackling the niche aspect of polymer property assessment. O.A. Ajibade, et al. (2016) [14] applied the grey relational analysis for the optimisation of tapped density for particular orange peels. They focused on characterising optimisation information on masses and volumes of the two-grade peels (i.e. 0.425- and 0.600-mm peel particulates) in concept type such that black represents conditions where no information exists and white where perfect information could be obtained. The paper strongly emphasised experimental runs and their conversion of optimal parametric settings. This emphasis is also shown in a comparative attempt of the Taguchi method with the grey relational analysis. However, the optimisation data presented in the results section of the article is limited to only one property, which is tapped density, hence does not represent the major aspects of the much-desired material properties of orange peel particulates. It does not contain information about compressive strength, electrical conductivity, and heat energy absorption. Although tapped density measures the heat effects on particulates after the mobility of the particulates, thus is expensive and not sustainable in a production process. In summary, the paper offers a superior understanding of volumetric and mass changes for orange particulates, but it lacks evaluation of the rise in temperature of the particulate over the experimental period. It has no means of evaluating the applied load and the gauge's cross-sectional area in a compressive strength evaluation. Again, the orange peel particulate's ability to conduct electricity was not previously determined.

A highly relevant article is also credited to O.A. Ajibade, et al. (2015) [15]. They used the Taguchi method to evaluate

the optimisation potentials of coconut shell particulates. In this area and on the aspect considered in this work, the papers offer the most comprehensive range of optimisation methods on free swell for coconut material. However, the heat aspects, information on compressive strength, and details on electrical resistivity are absent. The work only refers to the orthogonal array of L_{27} , which is the foundation for determining the optimal settings for the Taguchi-simplex methods. The study contains substantially relevant information on the properties of green waste for interested stakeholders. Notwithstanding, their study also ignores the ideas of temperature evaluation, applied load, gauge cross-sectional area, and the ability to conduct electricity, which is useful for the determination of compressive strength, electrical conductivity, and heat energy absorption. Overall, to evaluate the most desirable properties of orange peel particulates, the information stated above, which is different in O.A. Ajibade, et al. (2016) [14] and O.A. Ajibade, et al. (2015) [15], is essential. It is thus desirable to evaluate the properties of the compressive strength, electrical conductivity, and heat energy absorption for orange peel particulates.

Furthermore, additional reports on orange peels are as follows: A. Victor, et al. (2013) [16] focused on reinforcing high-density polyethylene with orange peels. P. Kumar (2012) [17] and S. Ojha, et al. (2012) [18] both investigated the mechanical properties of an orange peel-based reinforced polymer composite. Preliminary experimental results revealed the vast potential that orange peel particulates have in providing the properties desired in the sporting equipment manufacturing industry. Unfortunately, there exists no significant accessible document that provides information on green reinforcement materials, particularly orange-peel particulates. In addition, investigations into orange peel particulates that gave attention to particulate size considerations and their effects on fabricated material have not been reported in detail. Hence, it is the objective of this work to address the issue of orange peel particulate properties using a systematic approach.

The literature indicates that the utilisation of reinforcement materials for composites has largely been confined to metals and non-metals. In general, these investigations focus more on aluminium alloys than many other materials. However, applications of orange peel particulates in both natural, dried form and carbonised (burnt) form employing various compressive strengths in the formation process and under various viscosity measures in room temperature applications have not yet been documented. By considering the eco-friendliness and cost benefits of using green reinforcement materials for sporting equipment and related facilities, a proposal is undertaken to utilise orange

peel particulates as a composite reinforcement material to achieve an economic production of equipment and facilities for the sporting industry. The demonstration of orange peel particulate-based composite through experimental means provides an attractive alternative in composite formulation, fabrication, and development and is a worthwhile endeavour for sporting equipment manufacturers and competitors. The responsibility of exploring the development of new materials and their transfer to industries is expected to be the major benefit of this investigation.

Yet, much research has omitted critical concerns such as heat absorption, electrical conductivity, and compressive strength for orange peel reinforcement materials in composite fabrication. The absence of heat transfer data in the literature restricts the understanding of heat flow duration under sun exposure. Information essential for controlling the rate of heat flow within the material is also lacking. The absence of electrical conductivity data hinders insights into electrostatic industrial coating applications on orange peel composite structures, including implications of conductivity on overspray losses and particle adherence. Furthermore, knowledge of compressive strength is crucial for understanding the load-bearing capacity of orange peels until failure.

Therefore, this study introduces electrical conductivity, heat absorption, and compressive strength measurements for orange peel samples as materials for composite development. The aim is to fundamentally understand the property dimensions of orange peel particulates in terms of electrical, heat, and compressive stress from experimental perspectives. The experimental setup is described, results discussed, and conclusions drawn. The study's principal contributions are:

1. A multi-property evaluation system for orange peels is constructed. This system utilises infrared data to generate compressive strength, electrical conductivity, and heat absorption data for sun-dried orange peel particulates.
2. The experimental approaches are comprehensive. Through theoretical and practical analysis, the efficacy and potential applications of sun-dried orange peel data are explored.

2. Materials and methods

2.1. Materials

Orange peels were collected in polyethylene bags, chosen for their malleability under heat and pressure. Given the irregular shapes and bulk collection, polyethylene bags were suitable for storing and labelling the orange peels. The collected masses were air and sun-dried for approximately two weeks, ensuring adequate drying for grinding and sieving

into different grades for compressive testing in the civil engineering laboratory. The experiments employed sieves and a compressive testing machine. A multimeter was used for electrical conductivity and resistivity measurements, while the temperature variations of the orange peels during hot sun-drying were recorded to calculate the heat energy absorbed.

2.2. Procedure for the compressive strength tests

Dried orange peels were milled to fine particles and sieved to different sizes. Each category was treated collectively. Given that orange peel composite reinforcement could be exposed to water in outdoor applications, understanding the compressive strength of the wet samples is essential. A graded set of orange peels mixed with water formed a paste used to fill a cylindrical mould. Known loads were applied to the paste to determine compression extents, with readings taken on compressive testing equipment at the University of Lagos, Nigeria.

2.3. Procedure for the electrical conductivity tests

Orange peel samples were collected from fruit vendors, placed in polyethylene bags, and labelled by category and weight. They were spread out for daily electrical resistivity readings using a multimeter. A Vernier calliper measured sample dimensions, and an equation relating electrical resistivity and temperature was used to determine the relevant electrical conductivity and resistivity.

2.4. Procedure for heat absorption energy tests

Wet orange peels of various shapes such as spherical and needle-like were collected from fruit vendors and subjected to open-sun drying. Open-sun drying is a process that triggers the evaporation of water from the orange peels by the sun's heat. The circulation of air aids the fast evaporation of the water contained in the orange peel waste. The literature suggests that temperatures can reach approximately 98°F during this process. Based on the rise in temperature of the product as the sun's heat falls on it, it is argued that a change in temperature from an initial stage when the orange peels are laid out in the open air when no sun emerges to a final stage when the heat from the sun is the highest, is represented as ΔT where it is $T_f - T_i$, T_f is the final temperature experienced by the orange peels while T_i is the initial temperature experienced by the orange peels when the sun is not yet active, Eq. (1). This is known as the heat-absorbed energy of the orange peels.

$$\Delta H = C_p m \Delta T \quad (1)$$

The mass of the set of orange peels is represented as m , the specific heat capacity of orange peels, obtained from literature sources, is defined as C_p .

3. Results and discussion

The specified objectives and methods for determining the heat absorption, compressive strength, and electrical conductivity properties of orange peels were previously outlined. Experimental investigations on the collected orange peel samples were conducted and the findings are graphically presented to elucidate the correlations between several parameters and their resultant properties: heat absorbed versus mass of orange peels for heat absorption; electrical conductivity and resistance of orange peels for electrical conductivity; electrical conductivity against surface area and length of orange peels for electrical conductivity; and stress versus strain for compressive strength behaviour.

The electrical conductivity and compressive strength property measurements were divided into two categories (i.e., relationship concerning the surface area and length of orange peels) for electrical conductivity and three categories (i.e., relationship regarding 0.300, 0.425, and 0.600-mm particulates) for compressive strength. To determine the heat-absorbed energy, parameters such as the mass and specific heat capacity of the orange peels were used. During the analysis of the heat absorbed by the orange peels, the specific heat capacity was obtained from W. Zhou, et al. (2008) [19] as $3.77 \text{ J kg}^{-1} \text{ K}^{-1}$. This value was used to compute the heat absorbed by the orange peels examined in this work. The details of the analysis and discussion of results are henceforth given.

3.1. Heat absorbed by orange peels

The relationship between the mass of the orange peels and heat absorbed is presented in Fig. 1.

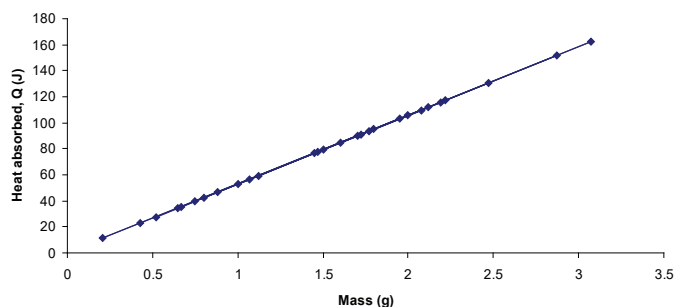


Fig. 1. Relationship between heat absorbed and mass of orange peels.

The mass is taken to be the net loss between the initial and final weights of the orange peels, measured in grams. This figure represents the mass of orange peels that have absorbed heat during the drying process. The calculation

for heat absorption involves the product of the mass, the specific heat capacity, and the change in temperature. For this investigation, orange peels were gathered into 28 separate polyethylene bag samples. The relationship between the heat absorbed and the mass is depicted by an upward-sloping curve. It was noted that the amount of heat absorbed, measured in Joules, rose with an increase in sample mass. The minimum energy uptake was observed in sample 6, with a heat absorption of 11.08 J at a mass of 0.21 kg. Samples 2, 3, 5, 6, 13, 17, 18, 23, 24, 27, and 28 displayed similar heat absorption values, ranging from 11.08 to 56.47 J, correlating with their comparable masses. Conversely, samples 1, 4, 7, 8, 9, 10, 12, 15, 16, 19, 20, 25, and 26 showed a heat absorption range of 76.53 to 117.17 J, which is attributed to their greater masses. Samples 14, 28, and 11 exhibited the highest heat absorption at 130.37, 151.48, and 162.03 J, respectively, which is consistent with their larger mass, lying between 2.47 and 3.07 kg. These results clearly demonstrate that the mass of the samples is a primary determinant of the heat energy absorbed. The experiments were conducted under consistent atmospheric conditions of humidity and temperature, assuming a constant specific heat capacity. The findings underscore the significance of mass in the heat energy absorption by the orange peel samples.

This knowledge is particularly relevant in the context of composite material production, where orange peels serve as reinforcement. It becomes crucial when orange peels are integrated into composites for use in thermal storage applications such as heat sinks, storage tanks for volatile substances, and insulation. In such cases, the ratio of orange peel volume/weight to the composite matrix must be optimised to ensure the composite can absorb or dissipate the requisite amount of heat for the application.

3.2. Electrical conductivity of orange peels

The electrical conductance of orange peels depends on three basic quantities, namely, resistance, surface area, and length. The individual relationship of these three quantities will be discussed.

Relationship between electrical conductivity and resistance of orange peels: The resistance of a material is inversely proportional to its electrical conductivity. In this study, the correlation between the resistance and electrical conductivity of selected orange peel samples is depicted by a curve in Fig. 2.

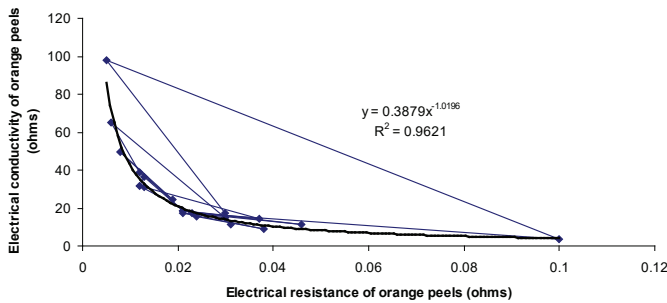


Fig. 2. Relationship between electrical conductivity and resistance of orange peels.

The curve shows a steep decline in conductivity from 98.1 to 38.6 S/m, corresponding to an increase in resistance from 0.005 to 0.012 Ω . This indicates that a higher resistance significantly diminishes electrical conductivity. The high resistance observed in the orange peels may be attributed to their moisture content and fibrous composition. The other samples exhibit closely grouped resistance values, resulting in the scattered data points seen in Fig. 3. Consequently, samples with lower resistance demonstrate higher conductivity. The sample with the utmost conductivity recorded a resistance of 3.6 Ω , possibly due to enhanced drying, reducing the moisture and oil content in the peels.

Relationship between electrical conductivity and surface area of orange peels: Unlike the rapidly descending curve observed between resistance and conductivity, the relationship between the surface area and electrical conductivity is characterised by a series of linearly aligned points in Fig. 3.

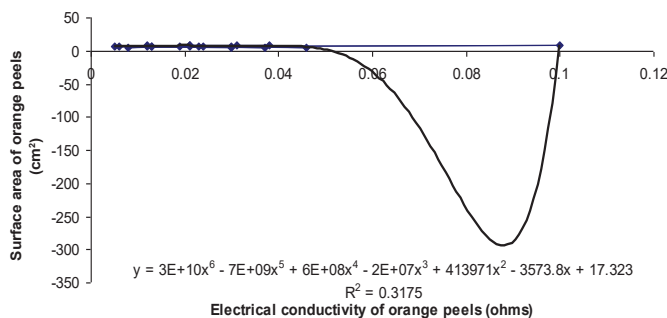


Fig. 3. Relationship between electrical conductivity and surface area of orange peels.

The data from the monitored orange peel samples do not present a consistent pattern regarding the impact of surface area on conductivity. Additionally, the surface area values did not significantly affect electrical conductivity in the same manner as resistance. Thus, certain samples with larger surface areas exhibited lower conductivity, while others with similar dimensions showed higher conductivity. The influence of surface area on conductivity reflects the potential cross-sectional region available for conductance.

Relationship between electrical conductivity and length of orange peels: The length of the individual orange peel samples impacts their conductivity. Specifically, the length denotes the electrode gap which facilitates the travel of electric current through the caps of the orange peels. This correlation is depicted in Fig. 4.

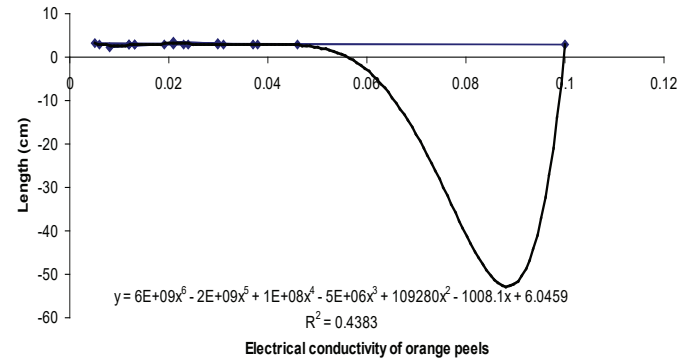


Fig. 4. Relationship between electrical conductivity and length of orange peels.

A longer peel tends to exhibit higher conductance and current flow. However, length is not the sole determinant of conductivity. This is evident as different samples measuring 2.8 cm in length displayed varying conductivities of 0.012, 0.03, 0.031, 0.037, 0.038, and 0.046 S/m, respectively. Therefore, the conductivity of orange peels is the result of a complex interaction of various factors.

3.3. Compression test results

The compression test generated three distinct stress-strain curves for the 0.300, 0.425, and 0.600 mm particulate sizes, as illustrated in Figs. 5, 6, and 7, respectively. Each curve represents unique compressive strength behaviour.

Compression strength behaviour of 0.300-mm orange peel particulates: The compressive strength behaviour for the 0.300-mm particulates is outlined in Fig. 5. The curve appears to be segmented into three distinct phases, reflecting different stress-strain responses. Initially, the material exhibited no strain until the compressive stress reached 8.68 kN/m², causing deformation. The strain then increased to 0.52 as the compressive stress rose from 8.68 to 13.01 kN/m², indicating elastic behaviour. At this juncture, had the stress been removed, the material would likely revert to its original shape without lasting damage. The 0.300 mm sample reached its proportional limit at an applied stress of 13.01 kN/m², which is evident as a straight line on the curve, confirming adherence to Hooke's Law; where stress is directly proportional to strain. Subsequent stress application, from 13.01 to 15.13 kN/m², resulted in an increased strain of 0.78.

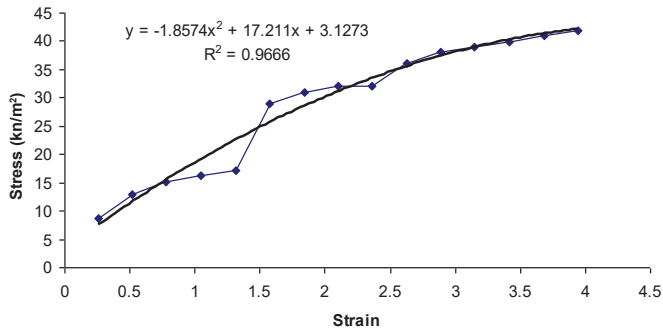


Fig. 5. Relationship between stress and strain for 0.300-mm orange peel particulates.

At this stage, the material is considered to have reached its yield point, with the stress of 15.13 kN/m² acting as the elastic limit or yield strength for the 0.300 mm sample. This marks the transition from elastic to plastic behaviour, with noticeable and significant deformation. The sample's yield strength indicates the elastic and plastic regions. As stress increased further to 16.16 kN/m², the strain escalated to 1.05, indicating plastic deformation. In the plastic region, the material would not revert to its original shape if stress were relieved. The stress continued to rise, correlating with increased strain, until reaching a failure point at 41.86 kN/m², where the strain was 3.94. At this stress level, the material began to bulge and shear, indicating the compressive shear strength of the 0.300 mm orange peel particulates was at 41.86 kN/m².

Compression strength behaviour of 0.425-mm orange peel particulates: Fig. 6 illustrates the stress-strain curve for the 0.425 mm sample. The initial strain of 0.26 was recorded when the compressive strength reached 1.09 kN/m².

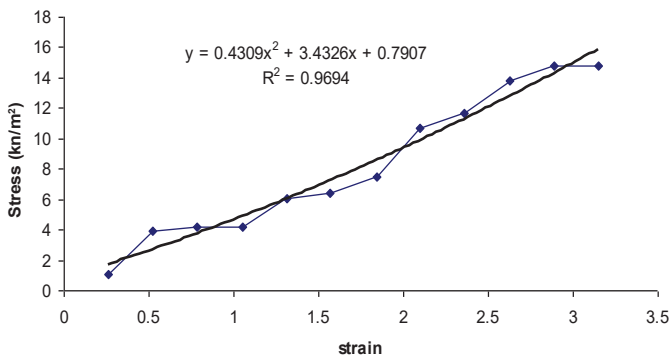


Fig. 6. Relationship between stress and strain for 0.425-mm orange peel particulates.

The stress and strain increase progressively until the material reaches its proportional limit at 4.75 kN/m², where the stress is directly proportional to the strain, signifying compliance with Hooke's law. This is represented by the linear portion of the curve. Within this elastic region, if the

stress were removed, the material would revert to its initial state. As the stress increases further, the material reaches its yield point and yield strength at 5.17 kN/m². This transition signifies the end of elastic behaviour and the onset of plastic deformation, characterised by a sharp increase in strain with each increment of stress. At this stage, any removal of stress would not restore the material to its original shape due to plastic deformation. The material ultimately fails at an applied stress of 14.82 kN/m², which corresponds to a strain of 2.89. The compressive strength at which the material begins to bulge, and shear is identified as 14.82 kN/m².

Compression strength behaviour of 0.600-mm orange peel particulates: For the 0.600-mm sample, the stress-strain curve presented in Fig. 7 demonstrates its compressive strength behaviour. An initial strain of 0.26 corresponds with an applied stress of 1.09 kN/m², indicating the material is within its elastic range and would return to its original state if stress was removed.

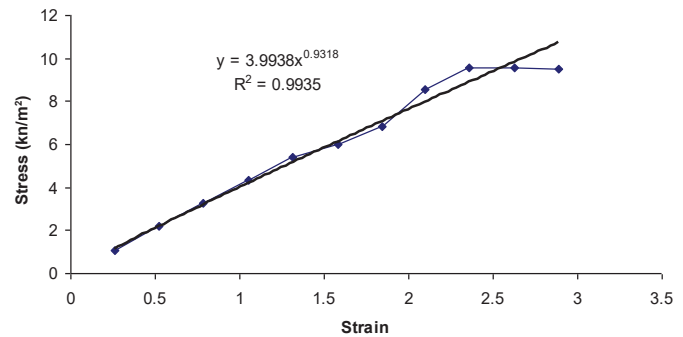


Fig. 7. Relationship between stress and strain for 0.600-mm orange peel particulates.

The material exhibits a proportional limit at a stress of 4.31 kN/m², resulting in a strain of 1.05. This limit is denoted by the straight section of the curve, maintaining a proportional relationship between stress and strain in accordance with Hooke's law. The material's elastic behaviour persists until the stress reaches 5.00 kN/m² with a corresponding strain of 1.20, marking the end of the elastic region and the start of plastic deformation. At this yield point, with a stress of 5.00 kN/m² (the yield strength or elastic limit), the material undergoes permanent deformation. Failure occurs through bulging or shearing at a stress of 9.58 kN/m² and a strain of 2.36. The compressive strength of the 0.600 mm sample is established at 9.58 kN/m².

The varying values of proportional limit, yield strength, and compressive strength across the samples highlight specific characteristics. For instance, the 0.300 mm sample required more stress, 8.86 kN/m², to produce an initial strain of 0.26 compared to the 1.09 kN/m² necessary for both the 0.425 and 0.600 mm samples. Similarly, the yield

strength for the 0.300 mm sample is the highest at 15.13 kN/m², while the 0.425 and 0.600-mm samples exhibit lower yield strengths of 5.17 and 5.00 kN/m², respectively. The highest compressive strength recorded was for the 0.300 mm sample at 41.86 kN/m².

4. Conclusions

This study presents the outcomes of three sets of experiments that examine the properties of orange peel and its particulates when used as reinforcement materials in the fabrication of green composites. One of the major challenges addressed by this research is the substantiation of the foundational assumptions in the determination of heat absorption, electrical conductivity, and compressive strength properties, along with methodological considerations. The findings provide design engineers with empirical values to develop composites that exhibit superior in-service performance and extended lifespans [20]. The salient conclusions drawn are as follows:

- The heat energy absorption of the collected samples is primarily dependent on their individual masses.
- The minimum energy absorption was observed in sample 6, with 11.08 J, whereas the maximum was 162.03 J in sample 11.
- The electrical conductivity of orange peel particulates is significantly influenced by their electrical resistance.
- Moisture content within the fibrous structure of orange peels may increase resistance and hinder electrical conductivity.
- No consistent pattern was detected in how surface area affects electrical conductivity, indicating that surface area did not markedly influence conductivity in the studied orange peels.
- The length of orange peel samples was not as determinative of conductivity as other factors; thus, samples of equal length exhibited varying electrical conductivities.
- In essence, the electrical conductivity of orange peel samples is largely dictated by the complex interplay of multiple factors.
- The compressive shear strength for the 0.300 mm orange peel particulate samples is established at 41.86 kN/m².
- The compressive shear strength for the 0.425 mm particulates is determined to be 14.82 kN/m².
- The compressive shear strength for the 0.600 mm particulates is recorded at 9.58 kN/m².

- The varying compressive shear strengths across different samples can be attributed to the strength of cohesive forces between the grains. It was noted that these cohesive forces, and hence compressive strength, decreased as the particulate size increased from 0.300 to 0.600 mm.

- The greater the cohesive forces within the samples, the higher the compressive stress they can endure, which explains the robust compressive strength, exhibited by the 0.300 mm OPP samples.

CRediT author statement

Oluwaseyi Ayodele Ajibade: Methodology, Formal analysis, Original draft preparation, Visualisation; Johnson Olumuyiwa Agunsoye: Conceptualisation, Data curation, Investigation; Sunday Ayoola Oke: Investigation, Visualisation, Formal analysis.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Chemical constituents, antioxidant and antifungal activities of the essential oil from Vietnamese *Ocimum tenuiflorum* L. leaves

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Abstract:

In this study, the authors aim to determine the chemical compositions of the essential oil from *Ocimum tenuiflorum* leaves and to evaluate its antioxidant and antifungal activities. The essential oil used in this research, extracted by hydrodistillation, was chemically profiled using gas chromatography/mass spectrometry (GC/MS) analysis. Methyl eugenol (46.07%), caryophyllene (15.65%), eugenol (11.83%), β -elemene (4.33%), β -copaene (4.18%), caryophyllene oxide (3.18%), and α -copaene (1.95%) were identified as major components of the essential oil. The results indicated that the essential oil of *O. tenuiflorum* leaves possessed relatively high 2,2-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities with IC_{50} values of 53.5 and 55.1 μ g/ml, respectively, compared with 67.2 and 63.2 μ g/ml, respectively, for the positive control. The reducing powers of *O. tenuiflorum* essential oil and butylated hydroxytoluene (BHT) at a concentration of 200 μ g/ml were 0.95 and 0.73, respectively. Additionally, the essential oil of *O. tenuiflorum* at a concentration of 200 μ g/ml strongly inhibited the mycelial growth of *Rhizoctonia solani*.

Keywords: eugenol, *Ocimum sanctum*, *Rhizoctonia solani*, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid), 2,2-diphenyl-2-picrylhydrazyl.

Classification number: 3.1

1. Introduction

Ocimum tenuiflorum (synonym: *Ocimum sanctum*) is a fragrant plant of the Lamiaceae family native to tropical and subtropical regions in Southern Asia. It is used in the cosmetics and food industries and as a traditional medicine to treat various conditions, including influenza, coughs, and headaches. It is readily differentiated from other *Ocimum* species by its internally glabrous calyces and spreading pedicels. Notable components in the essential oil of *O. tenuiflorum* composition include eugenol and methyl eugenol [1, 2]. It thrives in the plains, midlands, and low mountains, growing slowly at altitudes above 1.000 m, above sea level. The height of the plant varies from 0.5 to 1.5 m, and it has the ability to regenerate shoots. Inflorescences are often situated in the interstitial leaves or branches of the plant. The leaves, arranged in two successive pairs perpendicular to each other, are simple, petioled, and purple with an ovate blade up to 5 cm long and a slightly toothed margin. The purplish flowers are placed in close whorls on elongated racemes [2].

The biologically active components of the essential oil of *O. tenuiflorum*, which possess antimicrobial, anti-stress, anti-inflammatory, immunomodulatory, antipyretic, antiasthmatic, hypoglycaemic, hypotensive, and analgesic effects, have made the plant one of nature's "mother medicines" [1, 3]. Its application is known to retain skin moisture, reduce skin roughness and scaliness, prevent wrinkles, and make the skin smooth. It may also play a major role in treating inflammatory diseases [4]. Many studies related to the chemical composition and biological activity of extracts from *Ocimum* species, especially their antibacterial and antioxidant properties, have been conducted. The health benefits of *Ocimum* species could invariably be attributed to their abundant plant-derived phytochemicals [4-9].

To the best of our knowledge, only a few reports regarding the antifungal activity of the essential oil from Vietnamese *O. tenuiflorum* leaves have been published. However, a study by T.T.Q. Cung, et al. (2018) [10] shows

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the *O. sanctum* essential oil inhibited three important pathogenic fungi, namely *Rhizoctonia solani* and *Fusarium oxysporum*, with the inhibition zone ranging from 20 to 30 mm. Furthermore, the T.T.T. Vo, et al. (2019) [11] results in Binh Dinh province showed that *O. sanctum* essential oils have strong inhibitory activity against the growth of *Lactobacillus fermentum* and *Staphylococcus aureus*. It also inhibits the development of *Bacillus subtilis* and *Pseudomonasa eruginosa*. The essential oil of *O. sanctum* grown in Hanoi shows strong activity against three fungal strains of human skin pathogen (*Malassezia furfur* VNF01, *M. furfur* ATCC14521 and *M. globosa* VNG02) with the minimum concentration inhibiting the growth of fungal strains of 2.5 µl/ml. In addition, the cell number of the tested yeast strains was eliminated by 74.7-80.7% after 20 min of treatment at this concentration [12].

The purpose of the present research is to determine the chemical constituents of *O. tenuiflorum* leaves grown in Hanoi via GC/MS analysis and to elucidate their antioxidant and antifungal activities against plant pathogenic fungi (*F. oxysporum*, *F. solani*, *Phytophthora capsici*, *R. solani*).

2. Materials and methods

2.1. Materials and chemicals

Fresh leaves of *O. tenuiflorum* (1 kg, 30-35% moisture) were collected from the National Institute of Medicinal Materials (Hanoi, Vietnam) in October 2022. They were then hydrodistilled separately for 4 h, with the process performed in triplicate. The obtained essential oil was separated, dehydrated, and stored at 4°C until analysis.

Pathogenic fungi (*Fusarium oxysporum* KACC 40032, *Fusarium solani* KACC 40484, *Phytophthora capsici* KACC 40473, *Rhizoctonia solani* KACC 40111) were provided by the Korean Agricultural Culture Collection (KACC). DPPH, potassium persulphate, potassium ferricyanide, and dimethyl sulfoxide were purchased from Sigma-Aldrich. ABTS and BHT were purchased from Merck. All solvents were of analytical grade.

2.2. Gas chromatography/mass spectrometry (GC/MS) analysis

The chemical composition of the essential oil of *O. tenuiflorum* was analysed by GC/MS. A GC/MS (QP2020) system from Shimadzu Company (Japan) with a capillary column SH-Rxi-5Sil MS (30 m x 0.32 mm x 0.25 µm, non-polar phase: 100% dimethyl polysiloxane) was used. The essential oil of *O. tenuiflorum* was diluted in n-hexane and mixed using a vortex mixer. For the MS analysis, the

electron ionisation source was set at 200°C and the interface source was set at 250°C to isolate and fragment the ions. The GC/MS (QP2020) scan mode was used with an MS range from 50 to 900 m/z. Peaks were observed in the total ion chromatogram. Interpretation of the mass spectrum of GC/MS was done using the NIST mass spectral search program. The mass spectrum was compared with the spectrum of the components stored in the NIST library. The name, chemical structure, and molecular weight of the major components were determined [13, 14].

2.3. Antioxidant activity determination

The DPPH free radical scavenging ability was conducted according to the spectrophotometric method described in G. Miliauskas, et al. (2004) [15] with slight modifications [16]. Briefly, various concentrations of essential oil (0.1 ml) were mixed with 0.9 ml of 0.2 mM DPPH dissolved in methanol. The samples were then vortexed and incubated at 25±1°C for 15 min in the dark. The absorbance was measured at 517 nm using a BioTek MicroFill Dispenser (Agilent, Santa Clara, CA, USA). The ability to scavenge DPPH radicals was calculated as

$$\text{DPPH radical-scavenging activity (\%)} = [(C - T)/C] \times 100 \quad (1)$$

where, C and T are the absorbances of the DPPH radicals in methanol and essential oil, respectively. Meanwhile, BHT was used for comparison. The assays were performed twice, with three replicates for each treatment.

The ABTS free radical scavenging ability was conducted according to the method of R. Re, et al. (1999) [17] with some modifications [16]. For the ABTS assay, various concentrations of essential oil (0.2 ml) were mixed with 1.8 ml of ABTS solution consisting of a mixture of ABTS (7.4 mM) and potassium persulphate (2.6 mM). The reaction mixture was incubated at 25±1°C for 2 h in the dark. The sample was absorbed at 734 nm using a BioTek MicroFill dispenser. The ABTS radical-scavenging activity was calculated as

$$\text{ABTS radical-scavenging activity (\%)} = [(C - T)/C] \times 100 \quad (2)$$

where, C and T are the absorbances of the ABTS radicals in methanol and essential oil, respectively. BHT was used as a positive control. The experiments were performed twice, with three replicates for each treatment.

The reducing power of *O. tenuiflorum* was determined according to the method of M. Oyaizu (1986) [18] as described by T.H. Luong, et al. (2022) [19]. Various concentrations of the essential oil were mixed with 2.5 ml of 0.2 M phosphate buffer and 2.5 ml of 1% (w/v) potassium

ferricyanide, followed by incubation at 50°C for 20 min. Thereafter, 2.5 ml of 10% (w/v) trichloroacetic acid was added to the sample, followed by centrifugation at 3000×g for 20 min. Finally, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride, and the absorbance was measured at 700 nm using a BioTek MicroFill dispenser. Increased absorbance by the sample indicates an increase in reducing power. BHT, known for its antioxidant properties, was also used for comparison.

The concentration of the *O. tenuiflorum* essential oil that was used to evaluate the antioxidant activity ranged from 12.5, 25, 50, 100 to 200 µg/ml.

2.4. Antifungal activity determination

The agar diffusion technique was used to determine the antifungal activity of the essential oil on various plant pathogens [20]. Various concentrations of the essential oil from stock solutions were added to 20 ml of sterilised potato dextrose agar in Petri dishes (90×15 mm) to provide final concentrations of 0, 12.5, 25, 50, 100, or 200 µg/ml. Test fungi (*R. solani*, *F. oxysporum*, *F. solani*, and *P. capsici*) were inoculated using 6 mm-diameter mycelial plugs and fungal growth was recorded after 3-9 days of incubation at 25°C until full fungal growth had occurred in the control plates. The percentage of growth inhibition was calculated from mean values as

$$\text{Inhibition (\%)} = [(C - T)/C - 6] \times 100 \quad (3)$$

where, C and T are the colony diameters in the methanol control and the essential oil-treated sample, respectively. The assay was performed twice, with three replicates for each treatment.

The minimum inhibitory concentration (MIC) that inhibited any visible fungal growth was analysed by using the twofold serial dilution method [21]. The essential oil of *O. tenuiflorum* was dissolved in ethanol to obtain a stock solution of 4000 µg/ml, then further diluted with the same solvent to obtain twofold serial dilutions of 400, 200, 100, 50, 25, and 12.5 µg/ml. These were added to Potato Dextrose Broth (PDB) in the wells of a 24-well Microtest™ tissue culture plate to obtain the desired essential oil concentration. A 6 mm-diameter plug cut from the actively growing region of a 1-week-old colony of *R. solani* was added to the culture. A control culture treated with methanol only was also prepared. The cultures were incubated at 25°C for 72 h. Four replicates for each were run simultaneously. The MIC value was defined as the lowest essential oil concentration from *O. tenuiflorum* without any visible fungal growth of *R. solani*.

2.5. Statistical analysis

Treatment effects were determined by analysis of variance conducted according to the general linear model of the Statistical Analysis System 9.4 [22]. Means were separated with Tukey's honestly significant difference (HSD) test, with a significance of $p \leq 0.05$. IC_{50} values were calculated based on linear regression of DPPH and ABTS radical scavenging activity at different concentrations of essential oil. The experiments were performed twice with three replicates for each treatment ($n=6$). All data are presented as mean values $\pm$ standard deviation, based on the number of observations.

3. Results and discussion

3.1. The composition of the essential oil from *Ocimum tenuiflorum* leaves

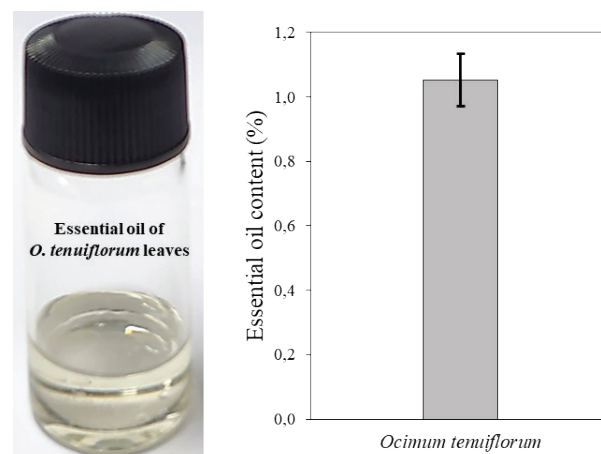


Fig. 1. Extracted essential oil of *Ocimum tenuiflorum* leaves (Hanoi, Vietnam).

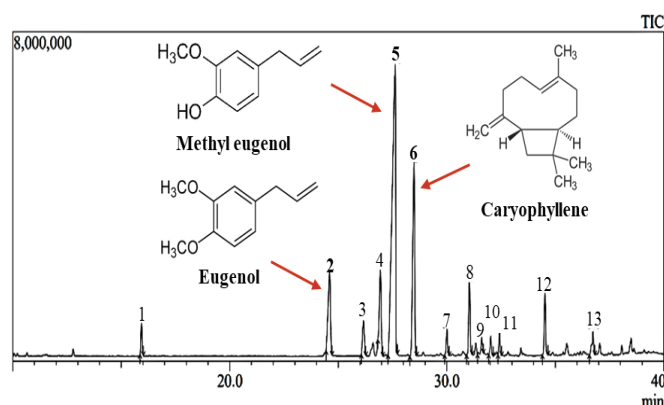


Fig. 2. A GC-MS profile showing the compounds in the essential oil obtained from the leaves of *Ocimum tenuiflorum*.

Table 1. Chemical composition of the essential oil from the leaves of *Ocimum tenuiflorum* identified via GC-MS.

Peak#	Compound	RT (min)	Retention Index		Content (%)
			RI_{Cal}	RI_{Db}	
1	Endo-borneol	15.931	1165±2	1165	1.92
2	Eugenol	24.596	1358±3	1356	11.83
3	α -Copaene	26.163	1376±3	1377	1.95
4	β -Elemene	26.937	1384±5	1384	4.33
5	Methyl eugenol	27.625	1410±4	1412	46.07
6	Caryophyllene	28.491	1426±2	1426	15.65
7	Humulene	30.009	1452±3	1455	1.63
8	β -Copaene	31.045	1483±6	1482	4.18
9	α -Selinene	31.613	1508±2	1508	1.02
10	Guaia-1(10),11-dien	32.027	1511±1	1510	1.14
11	β -Cadinene	32.431	1522±5	1524	1.32
12	Caryophyllene oxide	34.527	1583±1	1583	3.18
13	Neointermedeol	36.740	1632±2	1633	1.84
Monoterpene hydrocarbon (peak Nos.)					0
Oxygenated monoterpenes (peak Nos. 1, 2, 5)					58.82
Sesquiterpene hydrocarbon (peak Nos. 3, 4, 6-9, 11)					30.08
Oxygenated sesquiterpenes (peak Nos. 10, 12, 13)					6.16
Total identified					96.06

RT: retention time (min); RI_{Cal} : retention index calculated using a standard non-polar GC column and library data from NIST: 17 (Software NIST MS Search v2.3); RI_{Db} (Column Rxi-1 MS); retention index from the database.

The obtained essential oil from *O. tenuiflorum* leaves is transparent, with a recovery yield of $1.05 \pm 0.081\%$ (Fig. 1). A total of 13 compounds were detected and identified by cross-referencing their mass spectra and retention indexes with those in GC/MS libraries (NIST) (Fig. 2, Table 1). As reported in Table 1, the essential oil contained three oxygenated monoterpenes (1, 2, and 5), seven sesquiterpene hydrocarbons (3, 4, 6-9, and 11), and three oxygenated sesquiterpenes (10, 12, and 13), accounting for 58.82, 30.08, and 6.16% of the total, respectively. The oxygenated monoterpene profile of the essential oil was characterised

by the presence of methyl eugenol (46.07%), caryophyllene (15.65%), and eugenol (11.83%); the sesquiterpene hydrocarbon profile featured β -elemene (4.33%) and β -copaene (4.18%); and the sesquiterpene profile comprised caryophyllene oxide (3.18%).

Comparatively, the essential oil of *O. tenuiflorum* contains methyl eugenol, β -caryophyllene [2], methyl eugenol, (E)-caryophyllene, eugenol, and, β -elemene [23, 24]. According to İ. Gülçin (2011) [25], eugenol has powerful antioxidant and radical-scavenging abilities and is largely responsible for the therapeutic potential of the essential oil of *O. sanctum* [4]. The essential oil of *O. sanctum* identified 19 main substances, accounting for 99.88% of the total substances in the essential oil sample grown in Hanoi, with eugenol being the most predominant accounting for 42.4% [12]. Additionally, T.T.T. Vo, et al. (2019) [11] showed that the essential oil of *O. sanctum* contains 71.21% eugenol when grown in Binh Dinh province. Also, the eugenol content in *O. sanctum* essential oil grown in Iran was 15.7-37.5% [26]. Meanwhile, in this study, the main component in essential oils of *O. tenuiflorum* was methyl eugenol.

3.2. Antioxidant activity of the essential oil from *Ocimum tenuiflorum* leaves

Table 2. Radical-scavenging activity of the essential oil obtained from *Ocimum tenuiflorum*.

Concentration ($\mu\text{g/ml}$)	DPPH		ABTS		Reducing power	
	OT	BHT	OT	BHT	OT	BHT
12.5	27.5±2.0 ^e	24.5±2.5 ^c	32.2±1.9 ^f	28.3±2.2 ^f	0.08±0.01 ^d	0.05±0.01 ^d
25	48.5±2.9 ^d	44.2±3.2 ^d	45.5±1.8 ^e	42.2±3.2 ^e	0.19±0.02 ^d	0.13±0.04 ^d
50	76.0±0.8 ^{bc}	69.6±3.3 ^c	69.1±0.9 ^d	65.6±3.3 ^d	0.40±0.03 ^c	0.31±0.02 ^{cd}
100	83.3±2.4 ^b	76.6±3.6 ^{bc}	81.4±1.2 ^c	77.9±3.6 ^c	0.82±0.03 ^b	0.60±0.02 ^c
200	89.9±1.3 ^a	79.9±4.1 ^b	94.2±0.8 ^a	89.2±4.1 ^b	0.95±0.02 ^a	0.73±0.03 ^b
IC ₅₀ ($\mu\text{g/ml}$)	53.5±2.1	67.2±1.8	55.1±1.3	63.2±2.7	-	-

The results are expressed as the mean $\pm$ SD from triplicate measurements. Different letters in the column indicate a significant difference at each observation time based on a Tukey's HSD test with significance $p \leq 0.05$; OT: *Ocimum tenuiflorum*.

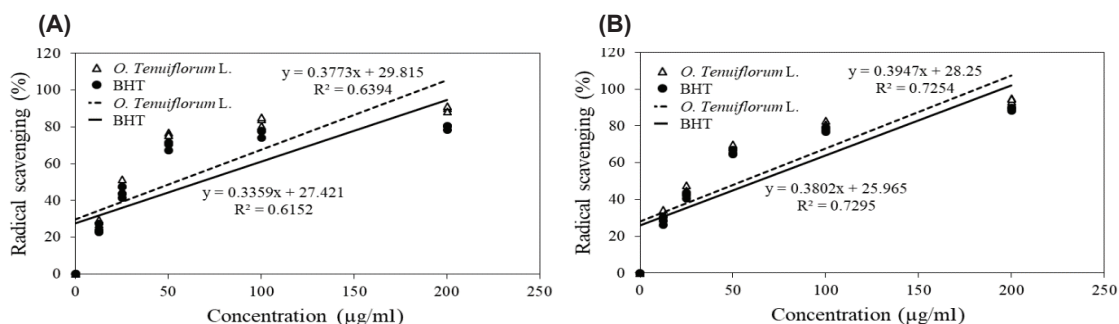


Fig. 3. Percentage radical scavenging vs. concentration of *Ocimum tenuiflorum* essential oil along with the linear regression equations used to calculate the values for 2,2-diphenyl-2-picrylhydrazyl (A) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (B). Different letters above the standard error bars indicate a significant difference at each observation time based on Tukey's HSD test, with a significance of $p \leq 0.05$.

According to the studies conducted by R. Saravanan, et al. (2022) [27] and N.K.D.N. Silva, et al. (2016) [28], methyl eugenol is one of the derivatives of eugenol. Methyl eugenol is a phenylpropanoid chemical that is found in the essential oil of *O. sanctum* and has been reported to have antioxidant activity [28, 29]. It has also been reported that methyl eugenol has antioxidant activity [29, 30]. Eugenol is a similar compound to methyl eugenol and has demonstrated relevant biological potential with well-known antimicrobial and antioxidant action. However, eugenol has a dual effect on oxidative stress, which can act as an antioxidant or prooxidant agent [30]. Antioxidants play an important role in protecting the body against damage from free radicals by stopping the formation of new free radical species, converting existing ones to less toxic molecules that can be easily eliminated, and preventing radical chain reactions [31]. Various *Ocimum* species and their extracts or essential oils have shown antioxidant activity [32]. In our study, 0, 12.5, 25, 50, 100, and 200 µg/ml of the *O. tenuiflorum* essential oil provided DPPH radical-scavenging activity of 0.0, 27.5, 48.5, 76.0, 83.3, and 89.9%, and BHT radical-scavenging activity of 0.0, 24.5, 44.2, 69.6, 76.6, and 79.9%, respectively (Table 2). Additionally, the IC₅₀ values for the DPPH radical-scavenging activity of the *O. tenuiflorum* essential oil and BHT were 53.5 and 67.2 µg/ml, respectively, which were significantly different (Table 2; Figs. 3A, 3B). In the ABTS radical-scavenging assay with 200 µg/ml of the essential oil, the anti-radical activity of *O. tenuiflorum* was 94.2%, while that of BHT was 89.2% (Table 2). The IC₅₀ values of the ABTS radical-scavenging activity by the *O. tenuiflorum* essential oil was 55.1 µg/ml, which was significantly different from that of BHT (63.2, Table 2). The reducing power values (ΔOD) of 200 µg/ml of *O. tenuiflorum* essential oil and BHT were 0.95 and 0.73, respectively (Table 2).

The study conducted by İ. Gülçin (2011) [25] suggested that eugenol inhibited 96.7% of lipid peroxidation in a linoleic acid emulsion at a concentration of 15 µg/ml. At the same concentration, butylated hydroxyanisole, butylatedhydroxytoluene, α-tocopherol, and Trolox displayed 95.4, 99.7, 84.6, and 95.6% inhibition of peroxidation, respectively. Furthermore, E. Nagababu, et al. (2010) [33] demonstrated that eugenol significantly inhibits the rise in glutamic-oxaloacetic transaminase activity and cell necrosis without protecting the endoplasmic reticulum damage as assessed by its failure to prevent a decrease in glucose-6-phosphatase activity. The protective action of eugenol has been found to be due to interception of secondary radicals derived from endoplasmic reticulum lipids rather than interfering with primary radicals of CCl₄.

F.L. Hakkim, et al. (2008) [32] demonstrated that all *Ocimum* species extracts were capable of scavenging DPPH free radicals at a dose of 1 µg/ml. However, none of the *Ocimum* species extracts were as effective as the positive control BHA (85.2%) in scavenging DPPH· radicals. The cultivar also had a significant impact on both FRAP (ferric reducing antioxidant power) and DPPH antioxidant capacities. Study of A. Chaudhary, et al. (2020)

[29] conducted that *O. sanctum* extracted by butanol (OsB) was rich in both total polyphenolic content (TPC) (212.26±6.3 mg GAE/g extract) and total flavonoid contents (TFC) (54.51±3.5 mg QE/g extract). Similarly, *O. sanctum* extracted by ethylacetate (OsE) also had significantly high TPC (202.71±5.5 mg GAE/g extract). The EC₅₀ values based on DPPH (3.91±0.3 µg/ml), ABTS (1.6±0.1 µg/ml), and phosphomolybdate (2.31±0.1 µg/ml) for OsB, hydroxyl (5.3±0.4 µg/ml), superoxide (7.32±0.9 µg/ml) radicals for *O. sanctum* extracted by methanol (OsM), and DPPH (8.61±0.6 µg/ml), phosphomolybdate (2.43±0.1 µg/ml), and ABTS (5.3±0.4 µg/ml) for OsE, were lower than that of ascorbic acid, indicating potential antioxidant properties [29]. The essential oils of *Ocimum* sp. also exhibited antioxidant activities through the DPPH radical scavenging assay, with an EC₅₀ value of 9.43 ng/ml [34]. In this study, the essential oil of *O. tenuiflorum* leaves could reduce the pink-coloured free radical to yellow-coloured diphenyl picrylhydrazine, confirming its DPPH radical scavenging activity and indicating that the essential oil possessed relatively high antioxidant activity.

3.3. The inhibitory effect of *Ocimum tenuiflorum* essential oil on the growth of fungal mycelia

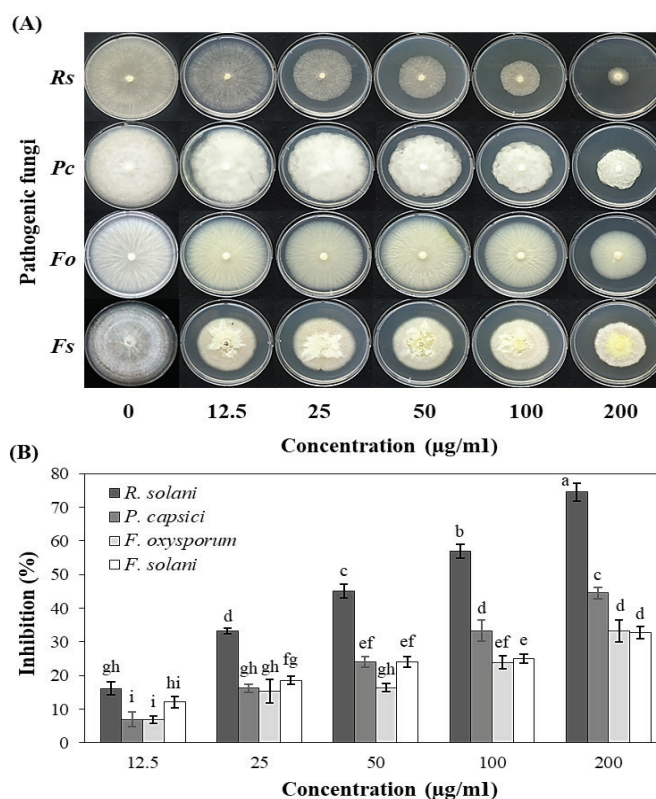


Fig. 4. The antifungal activity of essential oil of *Ocimum tenuiflorum* against four pathogenic fungi: (A) photos and (B) a bar chart of the mycelial growth inhibition. The error bars represent the standard error of the mean. The calculated mean values are from three replicates. Mean values with the same letter are not significantly different at $p \leq 0.05$ when compared using the least significant difference. Rs: *R. solani*; Pc: *P. capsici*; Fo: *F. oxysporum*; Fs: *F. solani*.

In the present study, the essential oil from *O. tenuiflorum* leaves displayed potent antifungal activity and inhibited the mycelial growth of *R. solani* in a dose-dependent manner, with a significant increase as the essential oil concentration was increased from 0 to 200 µg/ml (Fig. 4). Meanwhile, it showed weak-to-medium strong mycelial growth inhibition of *P. capsici*, *F. oxysporum*, and *F. solani*. In summary, 200 µg/ml of the essential oil from *O. tenuiflorum* markedly inhibited the mycelial growth of *R. solani*, *P. capsici*, *F. oxysporum*, and *F. solani* by 74.6±2.6%, 44.5±1.6%, 33.3±3.2%, and 32.7±1.8%, respectively (Fig. 4). Similar to our study, T.T.Q. Cung, et al. (2018) [10] suggested that essential oil of *O. sanctum* inhibited growth of *R. solani* and *F. oxysporum*. In particular, the effect of eugenol of different concentrations on the growth of mycelium was tested on a PDA medium against *R. solani*. The EC₅₀ and EC₉₀ values for eugenol were 52.77 and 166.37 µg/ml, respectively [35]. The fungicidal activity of eugenol (IC₅₀ = 58.9 and 52.9 µg/ml, respectively) against *R. solani* and *F. oxysporum* was also evaluated [36]. Also, no mycelial growth at concentrations of 200, 400 and 800 µg/ml was observed. According to the definition of MIC, 200 µg/ml was the MIC value for eugenol on *R. solani* [35]. Moreover, after treatment with eugenol, the mycelia lost water and shrank, accompanied by surface depression, roughness and unevenness.

Destruction of the mycelia was more serious at a eugenol concentration of 166.37 µg/ml than at 52.77 µg/ml. Meanwhile, mycelial morphology of *R. solani* showed that the mycelia in the control were regular, plump and uniform [35]. X. Zhou, et al.'s study on the antifungal activity of eugenol against *F. oxysporum* showed that eugenol treatment at half MIC decreased the spore germination of *F. oxysporum* by 90.22%, whereas the spore germination of *F. oxysporum* was almost completely inhibited at the MIC of eugenol, and there was no significant difference between 0.5 g/l of eugenol and 8 g/l of chlorothalonil. Especially, the mycelial morphology of *F. oxysporum* was wrinkled and rough after 3 day of eugenol treatment. Also, the structural integrity of the cells was damaged to varying degrees [37]. The antibacterial activity analysis demonstrated that β-caryophyllene presented MIC values equivalent to 32 µg/ml against *Staphylococcus aureus* [38].

4. Conclusions

In this work, we investigated the chemical constituents, antioxidant and antifungal activity of the essential oils of *O. tenuiflorum*. The major compounds of *O. tenuiflorum* were methyl eugenol, caryophyllene, eugenol, β-elemene, β-copaene, and caryophyllene oxide, with the methyl eugenol being the most predominant compound, accounting for a very high share of 46.07%. The essential oil of *O. tenuiflorum* demonstrated a great potential as an alternative to synthetic fungicides for the control of plant pathogens. These results suggest that the essential oil of *O. tenuiflorum* could be a promising natural antioxidant and fungicide agents. However, the antioxidative activities of eugenol, methyl eugenol and

other major compounds from the essential oil of *O. tenuiflorum* identified in this study should be subjected to more thorough evaluation in future in-depth studies.

CRedit author statement

Thi-Hoan Luong: Conceptualisation, Methodology, Data analysis, Software, Data curation, Investigation, Original draft preparation, Writing; Huu-Khanh-Tan Tran: Methodology and Data analysis; Woo-Jin Jung: Methodology, Data analysis, and Editing; Van-Viet Nguyen: Methodology and Data analysis; Dang-Minh-Chanh Nguyen: Conceptualisation, Methodology, Data analysis, Writing - Reviewing and Editing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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A novel mutation in the transthyretin gene in amyloidosis: A cluster case report in Vietnam

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Abstract:

Transthyretin amyloidosis (ATTR) is a slowly progressive condition characterised by the abnormal accumulation of a protein called amyloid in the body's organs and tissues. There are three main subtypes of amyloidosis (AL), including primary or AL, secondary or AA AL, and hereditary or familial AL. Hereditary AL, which is less common, is caused by an autosomal-dominant mutation, most frequently in the transthyretin (TTR) gene, and has a more favourable prognosis. We report the case of a 51-year-old Vietnamese male who presented with severe dizziness, fainting episodes, low BMI, slow heart rate, and a blood pressure of 60/40 mmHg. The patient had a history of digestive disorders, weakness, and pain in the legs even after disc spine surgery. All symptoms onset occurred at 43 years old. A pathogenic mutation in the TTR gene c.209G>T was confirmed in the patient and 7 out of 15 individuals in his extended family. This is the first case reported in Vietnam diagnosing a new variant of TTR causing typical AL. Based on this study, we conclude that the TTR variant c.209G>T is a pathogenic gene. This study also emphasises the need for increased knowledge regarding AL associated with this pathogenic variant. However, further extensive research is required to provide a comprehensive understanding of the pathogenesis of TTR in the future.

Keywords: amyloid, digestive disorders, hereditary, slow heart rate.

Classification number: 3.2

1. Introduction

AL is a heterogeneous disease, acquired or hereditary in nature. Its common cause lies in the accumulation of toxic insoluble beta-sheet fibrillar protein aggregates within various organs, resulting in impaired organ function. The process of amyloid formation originates from a wide array of misfolded proteins converging towards a similar fibril structure. This accumulation of amyloid induces cellular stress and death, altering tissue architecture, and leading to organ dysfunction and eventual mortality [1]. Organs frequently affected by AL encompass the heart, kidneys, liver, spleen, nervous system, and digestive tract, presenting with diverse clinical symptoms such as cardiomyopathy, hepatomegaly, proteinuria, macroglossia, autonomic dysfunction, ecchymoses, neuropathy, renal failure, hypertension, and corneal and vitreous abnormalities. The disease can manifest as localized or systemic, with certain types of AL showing comorbidity with other diseases [2].

AL is classified into numerous subtypes based on the precursor protein involved, including AL with monoclonal immunoglobulin light chain, ATTRm with mutant TTR,

and ATTRwt with wild-type TTR. Among hereditary AL, TTR is the most prevalent subtype, with over 100 pathogenic TTR mutations reported to date. Mutations in the TTR gene are accountable for hereditary AL, with the TTR gene located on 18q.12.1. TTR is a 55kDa tetrameric transport protein comprising four identical subunits of 127 amino acids. It is secreted from the liver into the blood and cerebrospinal fluid to facilitate the transportation of thyroid hormone thyroxine and retinol to the liver. Various mutations in the TTR gene have been identified in patients with familial amyloid polyneuropathy (FAP) or familial cardiac AL, which typically manifests with adult onset, follows an autosomal dominant inheritance pattern, and is primarily characterised by progressive and irreversible peripheral nerve damage.

Among the various types of AL that can culminate in life-threatening organ failure, amyloid cardiomyopathy stands out, characterised by the infiltration of amyloid into the heart muscle and other organs, leading to impaired cardiac and organ function [3, 4]. In the context of ATTR, amyloid fibril

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deposition in the heart, stemming from the conversion of TTR homotetramers, precipitates heart failure, predominantly presenting as cardiomyopathy in the elderly. Extracellular deposition of amyloid fibres initiates intracellular stress responses, culminating in heightened inflammation, apoptosis, and organ dysfunction. Recent studies propose that cardiac amyloid derived from TTR may significantly contribute to the pathogenesis of heart failure with preserved ejection fraction. The incidence of this rare disease is estimated to be approximately 5-8 cases per 1 million people in the United States and the United Kingdom [5].

In this study, we diagnose and examine a family presenting with complex and diverse symptoms of TTR, particularly involving the cardiovascular system, commonly known as amyloid cardiomyopathy.

2. Case presentation

2.1. Chief complaints

A 51-year-old Vietnamese male presented to Hanoi Medical University with severe dizziness and fainting episodes persisting for one week.

2.2. History of past illness

In 2015, at 43 years old, the patient began experiencing digestive disorders, particularly loose stools postprandially, which progressively worsened over time, leading to significant debilitation and a weight loss of 20 kg. In 2018, at 46 years old, the patient underwent disc surgery in the L4-L5 region of the spine, after which his leg weakness and pain were exacerbated, especially during exertion. Approximately two years ago, in 2021, at 49 years old, the patient started experiencing increasing fatigue, shortness of breath, weakness, dizziness, and episodes of fainting upon sitting up. Additionally, the patient observed spontaneous bruising of the left eyelid. Despite seeking medical care at multiple hospitals in Vietnam and Singapore and undergoing comprehensive gastrointestinal endoscopy, cranial magnetic resonance imaging, and various tests, no definitive diagnosis was made, and treatments failed to alleviate symptoms.

2.3. Physical examination upon admission

Upon admission, the patient was 1.58 m tall, weighed 37 kg, resulting in a BMI of 14.8 kg/m². His heart rate was only 12-15 bpm, and his blood pressure measured 60/40 mmHg.

2.4. Imaging examinations

Electrocardiogram on admission revealed a 3rd-degree AV block with a very low ventricular rate of approximately 12-15 bpm (Fig. 1).

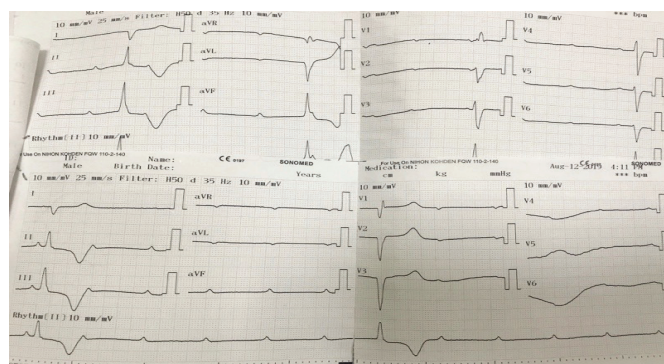


Fig. 1. Electrocardiogram in the emergency department. Third-degree heart block with an extremely slow heart rate of 12 bpm.

Echocardiography depicted a significantly thickened and brightly echogenic myocardium, with preserved left ventricular systolic function (EF 58%). However, severe diastolic dysfunction was noted, along with speckle pattern changes in total longitudinal strain (GLS) at the apex (Fig. 2).

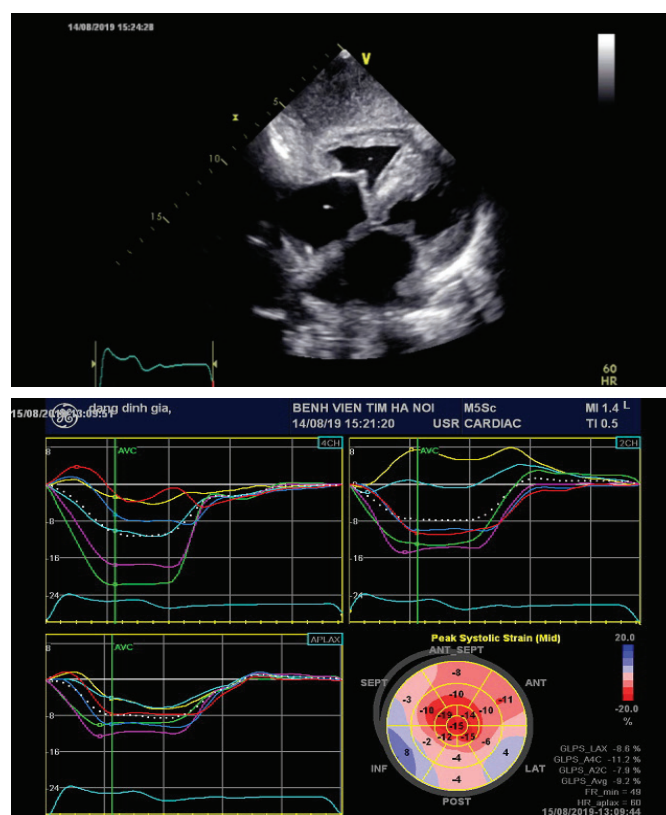


Fig. 2. Echocardiography.

Cardiac magnetic resonance displayed diffuse late gadolinium enhancement in two ventricles and two atriums, significant wall thickness exceeding 18 mm, minimal pericardial effusion, and preserved left ventricular function (EF≥60%) (Fig. 3).

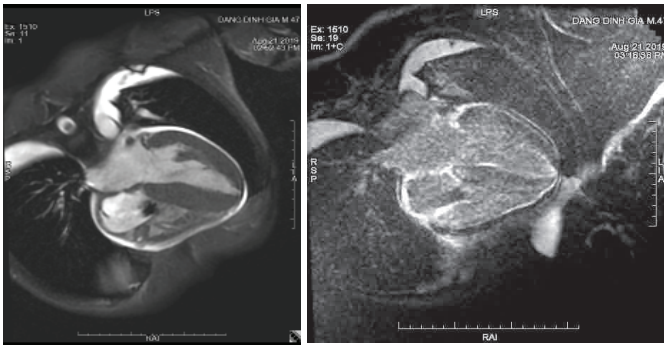


Fig. 3. Diffuse late gadolinium enhancement on cardiac magnetic resonance.

Coronary angiography revealed no signs of damage. The 99m Technitium-Pyrophosphate imaging strongly indicated the presence of TTR AL, with a semi-quantitative imaging score of 3 and a cardiac/contralateral ratio (H/CL ratio) of 2 (Fig. 4).

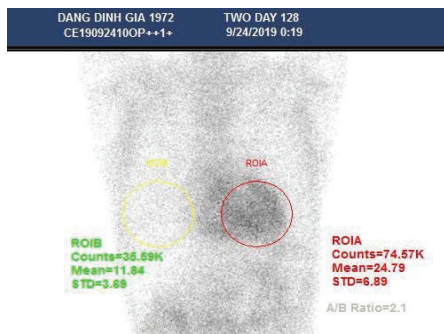


Fig. 4. The 99m Tc-PYP uptake by the heart using the contralateral heart-to-lung ratio (H/CL) =2.1.

2.5. Laboratory examinations

Blood tests revealed renal dysfunction (eGFR 34 ml/min), myocardial trauma (high-sensitive Troponin T: 252 pg/ml), and heart failure (NT-proBNP: 13259 pg/ml).

Serum immunofixation tests yielded normal results. Abdominal fat and tongue biopsies did not exhibit Congo red staining, indicating a negative result for AL.

2.6. Sequencing of TTR gene

Peripheral blood was collected from the patient and sent to the Green Cross laboratory in Korea for sequencing of the TTR gene. The Diagnostic Exome Sequencing method was utilised, scanning 5870 genes, revealing a heterozygous mutation for c.209G>T (p.Ser70Ile) on the TTR gene. Sanger sequencing was subsequently performed on samples harbouring the mutation. The primer sequences are shown in Table 1. The results of the samples are shown in Fig. 5.

Table 1. Summary of clinical and genetic findings in patients with TTR variant c.209G>T.

Clinical symptoms	1 st Cousin (III:4)	1 st older sister (III:5)	2 nd Cousin (III:6)	3 rd Cousin (III:7)	2 nd older sister (III:10)	Granddaughter (IV:8)	Grandson (IV:9)	Patient (III:15)
Sex	M	M	F	M	F	F	M	M
Year old	1970	1976	1969	1972	1981	1999	2004	1972
Fainting	+	-	+	-	-	-	-	+
Low blood pressure	+	-	+	-	-	-	-	+
Heart failure	+	-	+	-	-	-	-	+
Chronic kidney disease	-	-	+	-	-	-	-	+
Heart arrhythmia	+	-	+	-	-	-	-	+
Cardiovascular symptoms: tricuspid regurgitation, myocardial hypertrophy...	+	+/-	+	+	-	-	-	+
Age of onset of digestive disorders	47	+/-	48	50	-	-	-	42
Digestive symptoms: colon, stomach, liver...	+++	+/-	+++	+	+/-	-	-	+++

(-): none; (+): yes; (+++): yes in severe degree.

2.7. Medical management

A permanent chamber pacemaker compatible with MRI was implanted, and low-dose vasopressors were administered to stabilise blood pressure. Digestive enzyme supplements and multivitamins were prescribed to alleviate gastrointestinal

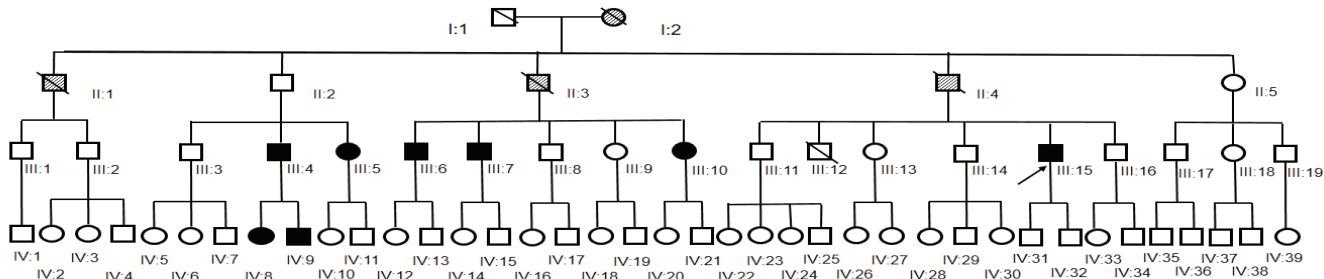


Fig. 5. Pedigree of an extended family with four generations. The proband was indicated by black arrow. Male individuals were represented by squares, while female individuals were represented by circles. Individuals with mutation were denoted by black color; individuals without mutation were denoted by white color; individuals who had died were denoted with the cross; individuals who had the similar clinical symptoms were denoted by grey color.

disturbances. Although fainting episodes improved post-pacemaker implantation, the patient continued to experience low blood pressure (around 80/60 mm Hg) and dizziness upon position changes, with persistent loose stools following meals. Given the laboratory and imaging findings, the patient was diagnosed with ATTR-type AL.

Since treatment for this disease had not been reported in Vietnam, the patient's records were forwarded to colleagues in the United States for further evaluation. Upon reviewing the tests and imaging conducted in Vietnam, the American colleagues confirmed the diagnosis of ATTR AL without the need for additional testing. The patient was fortunate to be enrolled in a clinical trial providing specific treatment free of charge. The treatment involved a short double-stranded interfering RNA targeting the liver, which led to a notable improvement in symptoms shortly after initiation.

3. Final diagnosis

ATTR Amyloidosis.

4. Other cases in the patient's family

The pedigree of the extended family spanning four generations is depicted in Fig. 6. Following receipt of the sequencing results, samples were collected from an additional 15 members of the patient's extended family, including five cousins (III:4, III:5, III:6, III:7, III:10) and some of their offspring (IV:5, IV:6, IV:7, IV:8, IV:9, IV:10, IV:11, IV:12, IV:13, IV:14, IV:15), for sequencing tests. Clinical data were obtained for all members of the extended family. The patient's paternal grandmother (I:2), uncles (II:1, II:3), and father (II:4) all exhibited similar symptoms, unfortunately resulting in their demise.

The sequencing test results revealed that five other individuals, who were cousins of the patient (III:4, III:5, III:6, III:7, III:10), exhibited identical results. Among them, one family from the patient's uncle (II:2) had two out of three children (III:3, III:4) carrying the disease gene. Furthermore, one child with the disease gene from this family (III:4) had a son and a daughter (IV:8, IV:9), both of whom carried the disease gene and manifested symptoms (Table 1).

5. Discussion

ATTR arises from abnormal fibres derived from TTR, a protein primarily synthesised by the liver, which aggregate and deposit in tissues and organs. The clinical manifestations of this disease are diverse, contingent upon the organs affected. In the patient's history, the onset of gastrointestinal and radicular symptoms preceding cardiovascular symptoms suggests a singular association with AL. However, definitive testing confirming the disease was not performed at that time. All clinical features observed in this patient are consistent with sensory-autonomic symptoms.

Upon the patient's admission to the hospital, cardiovascular symptoms were most pronounced. In his extended family, digestive and cardiovascular symptoms were more prevalent and readily identifiable than other manifestations. Cardiomyopathy, particularly ATTR-associated cardiomyopathy (ATTR-CM), is a common presentation of ATTR AL and is linked to a notably diminished life expectancy of 2 to 6 years post-diagnosis. Patients with ATTR-CM endure debilitating physical symptoms akin to heart failure, such as exercise intolerance and fatigue, culminating in reduced activity capacity, compromised quality of life, and eventual mortality. ATTR-CM can manifest as either the wild-type TTR subset (ATTRwt) or be inherited from multiple genetic variants of TTR (mutant transgenic AL ATTRm; also recognised as hereditary ATTR) [6].

Conforming to the consensus recommendations for the suspicion and diagnosis of cardiac AL published in Circulation Heart Failure in September 2019, screening for monoclonal protein AL is imperative in symptomatic patients exhibiting suggestive electrocardiography, echocardiography, cardiac MRI, and AL biomarkers. This involves conducting three tests: serum kappa/lambda free light chain ratio (abnormal if the ratio falls below 0.26 or exceeds 1.65), serum immunofixation (abnormal if monoclonal protein is detected), and urine immunofixation (abnormal if monoclonal protein is detected). These tests typically yield normal results for patients with ATTR AL [2].

Our patient underwent treatment for various digestive and neurological ailments across multiple medical facilities, yet no definitive diagnosis was reached. It was only upon the onset of cardiovascular symptoms, with echocardiogram findings indicative of thickened and brightened heart walls, that suspicion of this disease arose. However, our understanding of the disease at that juncture was limited. Subsequent testing confirmed the diagnosis, based on myocardial perfusion with 99m pyrophosphate, indicating TTR AL. An abnormal genetic test further confirmed the hereditary pattern of the patient's ATTR.

The genetic mutation detected in our patient is seldom documented in the literature. The most prevalent variant worldwide is Val122Ile (or pV142I), occurring in 3-4% of black Americans with an indeterminate gene penetrance [7]. The cardinal clinical presentation of this TTR Val122Ile variant is cardiomyopathy, with an estimated 10% of Black Americans over 60 years old with heart failure harbouring this variant. The Val30Met variant is the most prevalent in manifesting neuropathy [8]. Our patient's genetic mutation is heterozygous c.209G>T (p.Ser70Ile), previously reported in select Asian cases. For example, ATTR Ser50Ile was reported in two Japanese patients by Nishi and Saeki. Briefly, TTR gene PCR products were denatured in the presence of formamide and were electrophoresed in undenatured polyacrylamide gels to detect electrophoretic changes due to sequence variation. An abnormal DNA fragment was visualised by silver staining in exon 3 PCR products from the patient. Subsequent

sequencing analysis revealed a T-to-A transition and resulted in a substitution of Ser by Ile at codon 50 of the TTR gene. Saeki found the exon 3 variant at the 50th codon, and the AGT encoding for Ser was changed to the ATT encoding for Ile. The mechanism through which variant TTR molecules are deposited remains incompletely understood, although mutations at phylogenetically conserved sites of the TTR molecule may play a pivotal role in amyloid formation [9, 10].

In a study detailing the distinctive phenotypic and genetic features of hereditary ATTR within a multiracial South - East Asian cohort in Singapore, the variant ATTR-S50I (p.Ser70Ile) was identified in patients exhibiting somatic neuropathy diseases, including carpal tunnel syndrome, autonomic dysfunction, and cardiac dysfunction. These cases were characterised by early onset and a positive parental history [11].

Data on the occurrence of hypertrophic cardiomyopathy (HCM) in individuals with hereditary ATTR AL have been collected by the Hypertrophic Cardiomyopathy Genetics Oversight Committee. This data indicates that some individuals with hereditary ATTR AL exhibit characteristics of HCM and/or restrictive cardiomyopathy (RCM). The prevalence of HCM and/or RCM in hereditary ATTR AL is high, but the exact percentage has not been accurately determined due to the high prevalence of HCM and RCM (approximately 25%) in cardiac AL associated with aging. This condition is caused by the accumulation of misfolded TTR in the heart over time. Certain variants, such as TTR-V30M, TTR-T60A, and TTR-V122L, are commonly associated with cardiac AL.

The first autopsy case report by N. Sakashita, et al. (2000) [10] described the clinical pathological findings for two cases of FAP with the single amino acid mutation ATTR Ser50Ile. The report demonstrated systemic amyloid deposition in various organs and tissues of the autopsy cases. Initially, signs and symptoms in FAP (ATTR Ser50Ile) did not differ from those of typical FAP (ATTR Val30Met). However, cardiac symptoms, particularly congestive heart failure, became prominent in the early clinical stage. Consistent with the present cases, previous reports have highlighted severe heart failure and fatal arrhythmias as the most serious issues in this type of mutation. The transplantation of pacemakers may improve prognosis. This present examination revealed significant amyloid deposition in the cardiovascular system, akin to that described in previous autopsy reports of the TTR-associated FAPs of Ser50Arg and Tyr114Cys [12-14]. The total amount of amyloid in the current autopsy case was immeasurable compared to the 20 FAP cases (ATTR Val30Met) previously reported [13-15].

The ATTR Ser50Ile mutation has been documented in several studies conducted in Japan [12-16]. DNA sequence analysis by K. Sadamatsu, et al. (1997) [12] revealed the presence of the 50TTR Ile mutation in one patient. This individual presented clinical features of hereditary sensory and autonomic neuropathy, alongside autonomic dysfunction and

amyloid cardiomyopathy. The 58-year-old Japanese woman experienced weakness, numbness, and chest pain during exertion, alongside sensory disturbances in her lower limbs over four years. A notable amyloid deposition was detected in her colon. The onset age for these four patients ranged from the fourth to fifth decade of life. Hence, FAP associated with the TTR Ile 50 mutation is likely to manifest in middle age, predominantly affecting peripheral nerves and the heart [12].

Due to financial constraints, the patient's family could only conduct testing on 16 individuals, revealing that 50% of them carried the disease-causing gene. Among those carrying the gene, two-thirds exhibited cardiovascular and gastrointestinal symptoms. Moreover, a case within the patient's extended family was identified where the father carried the disease-causing gene, and both the son and daughter were also affected. This, alongside relevant clinical evidence, supports the attribution of disease-causing potential to the TTR c.209G>T (p.Ser70Ile) variant, the first variant identified in Vietnam for diagnosing hereditary ATTR disease. These findings contribute to genetic counselling and prevention strategies for individuals carrying the disease-causing gene, aiming to mitigate the risk of future disease occurrence.

Despite study limitations, such as a small sample size, it still underscored the disease-causing impact of the variant from a genetic standpoint. Furthermore, the inability to analyse the patient's parents and siblings to explore potential associated variants, lack of biopsy to confirm the diagnosis, and challenges in investigating the patient's neurological symptoms complicated the assessment of the relationship between neuro symptoms and the disease. Moreover, diagnosing hereditary ATTR disease within a clinical setting poses challenges. Although efforts were made to analyse relevant reports on the TTR c.209G>T (p.Ser70Ile) variant, currently, no documented reports on this variant exist in the literature worldwide or in Vietnam. Thus, establishing the role of this variant in the disease's pathogenesis remains challenging. With the advancements in molecular genetic testing, new technologies in genetic sequencing could facilitate the identification of multigene panels, including the TTR gene and other genes with numerous variants [17-20]. If this multigene panel could be implemented in diagnostics, genotype-phenotype correlations and disease causation could be elucidated more clearly.

Currently, there have been no reports published regarding the penetrance of the TTR gene. However, based on the findings from a cluster of cases and some related reports, we can cautiously suggest that hereditary ATTR disease may exhibit incomplete penetrance, depending on the age of the affected individuals and the varying levels of expression, which often occur later in life. Therefore, identifying individuals carrying the disease-causing gene is of significant importance in preventing disease progression and plays a crucial role in counselling, screening, prenatal diagnosis, and premarital assessment.

6. Conclusions

Hereditary TTR AL is a rare disease with diverse clinical manifestations, but its nonspecific nature makes diagnosis a significant challenge for clinicians. We report a cluster of hereditary TTR AL cases in Vietnam with a different gene mutation than those previously reported worldwide. This study emphasises the importance of genetic testing in disease diagnosis, as hereditary TTR AL presents with late-onset and multi-organ involvement. Diagnosis often requires a multidisciplinary approach, and treatment remains a complex issue due to the high cost of targeted therapies. Further in-depth research should be conducted to improve diagnostic capabilities and gain a better understanding of the underlying disease mechanisms, aiming to reduce treatment costs for patients when the disease is in advanced stages.

CRedit author statement

Ngoc Lan Thi Nguyen: Reviewing, Methodology, Data analysis, Data collection, Writing, Editing; Thu Anh Nguyen: Data collection; Quy Van Vu: Data analysis; Huyen Thi Vu: Writing, Editing; Phuong Lan Thi Dam: Writing, Editing; Duc Tuan Nguyen: Data analysis.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Epidemiological, clinical characteristics and the association between influenza vaccination, oseltamivir treatment, and complications in children with seasonal influenza

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Abstract:

Influenza, a historical and contemporary public health challenge, is an acute respiratory infection caused mainly by influenza A or B viruses. This descriptive case series describes 194 children treated for seasonal influenza at the Department of Pediatrics, 103 Military Hospital, Vietnam Military Medical University, from May 2022 to May 2023. Influenza primarily occurred during winter and early spring, peaking in December and January, 57.2% were aged 12-60 months, boys. Vaccination coverage was limited (36.3%), especially post-COVID-19. Complications were seen in 75.3% of the cases, including febrile seizures (42.3%), bronchopneumonia (40.2%), respiratory failure (8.2%), acute myocarditis, influenza-associated encephalopathy, and septic shock. The study reveals bacterial coinfections, affected 20% of the cases, mainly by *Streptococcus pneumoniae* and *S. aureus*, and atypical bacteria *Mycoplasma pneumoniae*. Non-vaccinated children faced significantly higher risks of complications, oseltamivir within 48 hours reducing the risks of complications. Influenza predominantly affects 12-60 month-olds, males, and peaks annually in winter. Severe conditions stem from bacterial coinfections, emphasizing the importance of vaccination and prompt antiviral treatment to mitigate complications. The study highlights a critical need for increased vaccination, especially in the post-COVID-19 era, to manage influenza in children effectively.

Keywords: children, complications, influenza, vaccination.

Classification number: 3.2

1. Introduction

Influenza, a perennial public health challenge, has historically wielded substantial socio-economic repercussions, leaving an indelible mark on global mortality and morbidity rates. This viral infection, caused predominantly by influenza viruses type A or B (occasionally type C), contributes significantly to the landscape of acute infectious respiratory diseases, which collectively impose a noteworthy burden on public health worldwide [1]. The transmission of the influenza virus occurs via aerosol droplets during coughing, sneezing, or close contact with an infected individual, potentially leading to upper or lower respiratory tract infections and complications affecting various extra-pulmonary organs, such as acute encephalopathy, and myocarditis [2, 3].

Yearly global outbreaks, typically prevalent during the winter season in temperate climate regions, affect an estimated 32 million people, resulting in approximately

5 million hospitalizations due to influenza-induced acute respiratory tract infections (ARI) in adults [1]. Alarming, children under 5 years old bear a significant brunt, with approximately 110 million reported cases of influenza virus infection in 2018. In developing countries, where vaccination rates are constrained, these young children frequently experience acute respiratory tract inflammation, leading to elevated rates of hospital admissions and mortality [4]. In Vietnam, the under-5 age group exhibits the highest incidence rate of seasonal influenza, with a mortality rate of 0.3% associated with ARI or severe acute respiratory infections (SARI) [5].

The burgeoning incidence of ARI hospitalizations poses an escalating burden on both the healthcare system and economic society. Recognizing influenza vaccination as a crucial strategy for safeguarding children and the broader community, particularly in the wake of co-circulating pathogens like severe acute respiratory syndrome-

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coronavirus 2 (SARS-CoV-2), the American Academy of Pediatrics (AAP) and World Health Organization (WHO) recommend annual influenza vaccine injections for all children without medical contraindications, starting at 6 months of age. This targeted vaccination not only bolsters individual immunity but also collectively alleviates the overall burden of respiratory illnesses [1, 6, 7].

However, the emergence of the COVID-19 pandemic has ushered in a new era, marked by social distancing measures, reduced children's exposure to infections, and a notable decline in vaccination rates. This unprecedented shift has not only impacted the trajectory of respiratory virus spread but has also set the stage for a potential resurgence of seasonal influenza [8, 9]. Acknowledging the intricate interplay of these factors, we embarked on a comprehensive study titled "Epidemiological, clinical characteristics and the association between influenza vaccination, oseltamivir treatment and complications in children with seasonal influenza". This study aims to unravel the multifaceted implications of influenza in the post-COVID-19 landscape, offering critical insights for informed public health interventions and preparedness strategies.

2. Objects and method

2.1. Objects

Inclusion criteria: A total of 194 patients aged 1 month to 16 years exhibiting suspected seasonal influenza symptoms, such as high fever, cough, runny nose, sore throat, post-orbital pain, and muscle aches, were included. Patients tested positive for influenza antigen via rapid test or real-time RT-PCR from throat swab specimens, adhering to Ministry of Health guidelines, Vietnam. The study was conducted between December 2022 to May 2023 and took place at the Department of Pediatrics, 103 Military Hospital, Vietnam Military Medical University. Data collection encompassed epidemiological factors, clinical symptoms, selected indicators, chest X-rays, and treatment outcomes.

Exclusion criteria: Patients with underlying conditions (congenital heart disease, immunodeficiency, lung disorders, chromosomal abnormalities) or those with family members declining participation were excluded.

2.2. Methods

A descriptive case series study utilized a convenience sampling method.

2.3. Data processing

Statistical software: SPSS 26 and Microsoft Excel in a Medical Context facilitated data analysis. Results were presented as percentages, with continuous variables expressed as mean and standard deviation. P-values <0.05 were considered statistically significant. Univariate and

multivariate regression models analyzed the relationship between complications, influenza vaccination, and oseltamivir treatment.

2.4. Research ethics

Researcher presentations ensured understanding of study content and objectives among children's families, emphasizing voluntary participation and the right to withdraw. Confidentiality of information was guaranteed for research purposes only, ensuring no negative impact on participating patients.

3. Results

3.1. Epidemiological characteristics

The prevalence of influenza was higher in male children than female children, accounting for 56.2%. The average age was 36.73 ± 3.13 months, with the age group from 12 to 60 months representing 57.2%. Influenza A and B detection rates using rapid antigen testing and real-time RT-PCR with nasopharyngeal samples were 85.6 and 9.8%, respectively (Table 1).

Table 1. Epidemiological characteristics of the objects.

Epidemiological characteristics		Number of pediatric patients (n=194)	Percentage (%)
Sex	Male	109	56.2
	Female	85	43.8
Age	<12 months old	26	13.4
	Between 12-60 months old	111	57.2
	>5 years old	57	29.4
	X $\pm$ SD	36.73 $\pm$ 3.13	
	Min	1-month-old	
	Max	15 years old	
Classification	Influenza A	166	85.6
	Influenza B	19	9.8
	Influenza A and B	9	4.6

Influenza usually occurs between winter and early spring. From April to October, the percentage of people suffering from influenza remains low, approximately under 5% (Fig. 1). The infection increased dramatically to 18.5% in November and reached its peak of 27% in December and January.

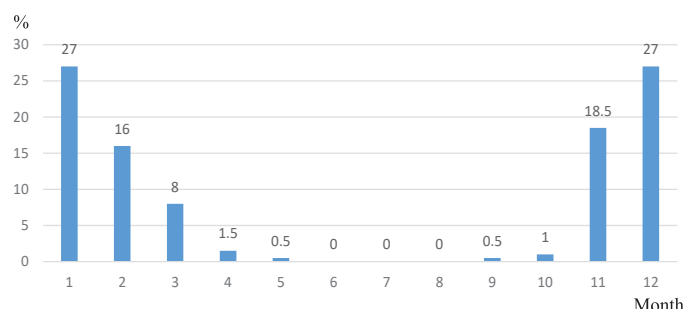


Fig. 1. Monthwise distribution of influenza.

The percentage of vaccinated children accounted for 36%, which is just a little more than half when compared to 64% of children who are either unvaccinated or have an unknown status (Fig. 2).

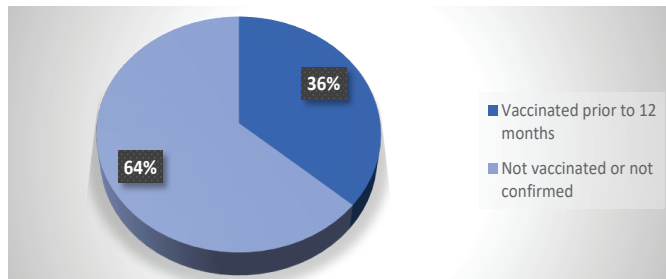


Fig. 2. The proportion of children received the influenza vaccination.

3.2. Clinical and laboratory manifestations

The average hospitalization duration was 3.78 ± 2.13 days. All research patients presented with fever, with an average temperature of $38.79 \pm 0.67^\circ\text{C}$ upon admission (Table 2). The average duration of fever was 4 ± 1.43 days, with the longest recorded case lasting 9 days. The prevalence of sore throat, runny nose, and cough symptoms was 72.7 and 96.9%, respectively, while shortness of breath accounted for 4.1%. Muscle pain was reported in 37.6% of children, generalised rash in 6.2%, swollen and painful lymph nodes in the neck region in 4.1%, and gastrointestinal symptoms in 10.3%.

Table 2. Clinical characteristics of the objects.

Clinical symptoms		Number of pediatric patients (n=194)	Percentage (%)
Hospitalization duration (days)	$\bar{x} \pm \text{SD}$	3.78±2.13	
	Min	1	
	Max	11	
Fever duration (days)	$\bar{x} \pm \text{SD}$	4±1.43	
	Min	1	
	Max	9	
Temperature upon admission ($^\circ\text{C}$)	$\bar{x} \pm \text{SD}$	38.79±0.67	
	Min	38	
	Max	41	
Fever		194	100
Cough		188	96.9
Sore throat		141	72.7
Runny nose		141	72.7
Muscle pain		73	37.6
Shortness of breath		8	4.1
Swollen and painful lymph nodes in the neck region		8	4.1
Generalised rash		12	6.2
Gastrointestinal symptoms		20	10.3

The average number of WBC was 7.59 ± 2.22 ($\times 10^3/\text{mm}^3$), with the highest recorded value being 16.6 ($\times 10^3/\text{mm}^3$) (Table 3). The average Hb and HCT levels were 118.83 ± 14.14 g/l and $38.88 \pm 3.29\%$, respectively. The average PLT count was 218 ± 111 ($\times 10^3/\text{mm}^3$), and the CRP level was 8.25 ± 3.22 mg/l, with one case showing an elevated CRP level of 50 mg/l.

Table 3. Laboratory findings of the study population.

Indicator	$\bar{x} \pm \text{SD}$	Min	Max
WBC ($\times 10^3/\text{mm}^3$)	7.59±2.22	3.14	16.6
Hb (g/l)	118.83±14.14	89	156
HCT (l/l)	38.88±3.29	30.9	46
PLT ($\times 10^3/\text{mm}^3$)	218±111	100	551
CRP (mg/l)	8.25±3.22	0.12	50

WBC: white blood cell count; PTL: platelet count; Hb: hemoglobin; HCT: hematocrit, CRP: C-reactive protein.

Bronchial wall thickening, consolidation, and interstitial infiltration frequently feature in children diagnosed with influenza. Among them, bronchial wall thickening was the most common occurrence, observed in approximately 32.98% of patients (Fig. 3). In contrast, the pleural effusion was rarely seen, with a percentage of around 2.06%.

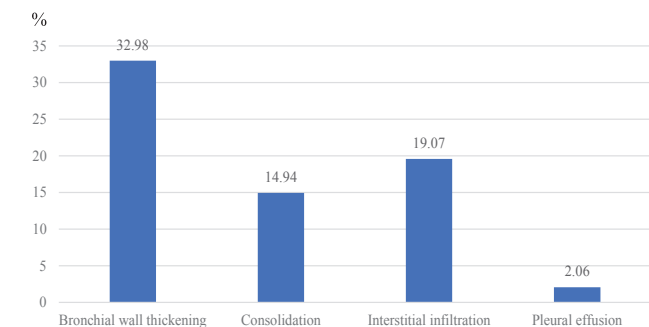


Fig. 3. Chest radiography findings in pediatric patients diagnosed with influenza viruses type A and B infection.

Gram-positive bacteria *S. pneumoniae* and *S. aureus* are observed in 30% of patients (58/194) and 23.71% (46/194), respectively (Fig. 4). While the rate of coinfection with atypical bacteria *Mycoplasma pneumoniae* was 19.07% (37/194).

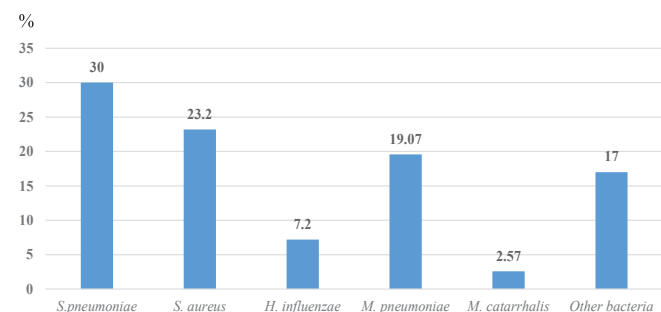


Fig. 4. Incidence of influenza and bacterial coinfections.

3.3. Association between influenza vaccination and Oseltamivir treatment with selected complications in patients with influenza

The number of cases with complications accounted for 146/194 (75.3%), with 82/194 (42.3%) patients experiencing febrile seizures, 78/194 (40.2%) cases of bronchopneumonia, 16/194 (8.2%) cases of respiratory failure, and 7/194 (3.6%) cases of acute otitis media (Table 4). In some cases, influenza-associated with acute myocarditis and encephalopathy 1/194 (0.52%) and septic shock 3/194 (1.55%). The incidence of acute diarrhea was 6/194 (3.1%), with no cases of influenza-associated neurological complications.

Table 4. Selected complications of the study population.

Influenza-related complications	Number of pediatric patients (n=194)	Percentage (%)
Febrile seizures	82	42.3
Bronchopneumonia	78	40.2
Acute otitis media	7	3.6
Diarrhea	6	3.1
Respiratory failure	16	8.2
Acute myocarditis	1	0.52
Septic shock	3	1.55
Influenza-associated encephalopathy	1	0.52
Presence of complications	146	75.3

The risk of experiencing febrile seizures, and bronchopneumonia, in the non-vaccinated group was significantly higher, with an odds ratio of 4.44 (95% CI: 2.4-8.18), 2.81 (95% CI: 1.55-5.1), respectively, compared to the group that received influenza vaccination (Table 5).

Table 5. Association between influenza vaccination and complications.

Complications	Vaccinated n (%)	Not vaccinated n (%)	p-value OR (95%CI)
Febrile seizures	25(30.5%)	57(69.5%)	<0.05 4.44 (2.4-8.18)
Bronchopneumonia	28(35.9%)	50(64.1%)	<0.05 2.81 (1.55-5.1)
Acute otitis media	4(57.1%)	3(42.9%)	>0.05
Diarrhea	2(33.3%)	4(66.7%)	>0.05

P value less than 0.05 indicates a statistical difference; OR: odds ratio CI: confidence interval.

The group without complications initiated Oseltamivir treatment was at an average of 1.22±0.56 days from admission (Table 6).

Table 6. Association between influenza complications and Oseltamivir treatment.

Use Oseltamivir	Complications		Without complications		p-value
	n	%	n	%	
Before 48 hours	108	71.5%	43	28.5%	<0.05
After 48 hours or untreated	38	88.4%	5	11.6%	
The number of days to starting Oseltamivir after disease onset	2.45±0.34		1.22±0.56		<0.05

4. Discussion

4.1. Epidemiological characteristics of the study population

Our study population had a mean age of 36.73±3.13 months, with ages ranging from 12 to 60 months accounting for 57.2% and males being more affected than females (56.2 and 43.8%, respectively). Most patients were infected with influenza virus type A (166/194, 85.6%). The vaccination rate for influenza immunisation within the past 12 months was 36.3%, while 63.4% of patients were either not vaccinated or had unclear vaccination status. Our study also yielded similar results to the study conducted by S.E. Coffin, et al. (2007) [10], on 745 pediatric patients over a 4-year period, which found a higher proportion of male cases (59% versus 41%), with the age group of 6 months to 5 years accounting for 52% of the cases. Furthermore, the prevalence of influenza virus type A was higher than that of type B (79% versus 21%). Similar findings were reported in the study by Y. Shi, et al. (2021) [11], which included a sample size of 36,047 children, where males accounted for 55% of the cases, and the mean age was 36±4.14 months. According to the study conducted by H.V. Pham, et al. (2021) [12] on 200 children, males accounted for 55.5% of the cases, with a mean age of 27 months. The prevalence of influenza type A was 93.5%, and the vaccination rate for influenza was 26.5% within the 12 months before illness. The number of flu patients tends to peak during the winter and spring, with the highest incidence in December and January. This phenomenon can be attributed to the lipoprotein structure of the flu virus, rendering the immune system weaker and more susceptible to infection in the absence of sunlight radiation. Consequently, the prevalence of flu is lower in warmer temperatures [13]. The vaccination rate for seasonal flu in the study population is 36.3%, significantly lower than the vaccination rates in the Regions of the Americas and Europe, which stand at 89%. This difference may be explained by variations in vaccination policies, economic capabilities, and vaccine distribution among high and upper-middle-income countries, and several low and middle-income countries (LMICs) [7]. Three regions Africa, the Middle East, and Southeast Asia-comprise 50% of the global population but receive only 6% of distributed seasonal influenza vaccine doses, leading to limited vaccine accessibility and hindering efforts to improve flu vaccination rates [14].

4.2. Clinical and laboratory characteristics of the objects

The average hospital stay for the patients was 3.78±2.13 days. The typical symptoms of influenza observed were fever (100%), with an average duration of 4±1.43 days, and an average temperature of 38.79±0.67 degrees Celsius upon admission. Other common symptoms included cough (96.9%), sore throat and runny nose (72.7%), dyspnea (4.1%), myalgia (37.6%), swollen lymph nodes under the jaw (4.1%), generalized rash (6.2%), and gastrointestinal symptoms (10.3%). These findings are consistent with the study conducted by H.V. Pham, et al.

(2021) [12], which reported fever in 100% of cases, cough in 85.5%, runny nose in 62.5%, and sore throat in 58.5%. Additionally, H. Silvennoinen, et al. (2009) [15] conducted a study involving 353 patients over 2 years and found that fever was present in 95% of cases, cough in 77%, runny nose in 78%, muscle aches in 7%, and gastrointestinal symptoms in 9%. These results are also consistent with our study.

The clinical laboratory values showed that the average white blood cell and platelet counts were 7.59 ± 2.22 ($\times 10^3/\text{mm}^3$) and 218 ± 111 ($\times 10^3/\text{mm}^3$), respectively. The highest recorded white blood cell count was 16.6 ($\times 10^3/\text{mm}^3$), while the lowest platelet count was 100 ($\times 10^3/\text{mm}^3$). Hemoglobin levels exhibited a downward trend, with an average of 118.83 ± 14.14 g/l; the lowest recorded level was 89 g/l. The C-reactive protein level was also measured to be 8.25 ± 3.22 mg/l, with the highest recorded level reaching 50 mg/l. Our results are consistent with Y. Shi, et al.'s (2021) [11] study on 36,047 children. The authors of that study found no significant differences in the levels of platelets and white blood cells. However, they observed decreased haemoglobin levels and increased C-reactive protein levels among children with severe influenza infection.

The study recorded the kind of damage to the lung on chest X-rays, such as bronchial wall thickening, consolidation, interstitial infiltration, and pleural effusion. The bronchial wall thickening is the most found, accounting for around 32.98% (64/194). However, pleural effusion is rare, with 3.1% (6/194). P. Rohani, et al. (2016) [16] conducted chest x-rays on patients diagnosed with influenza et al pneumonia and admitted to the ICU in the first 24 hours. The significant radiographic findings were ground-glass opacities (89%), consolidation (89%), reticular opacities (33%), peri broncho vascular in 61% patients, and multifocal patchy airspace opacities (56%). Chest radiographic changes in influenza pneumonia range from mild interstitial infiltration to poorly defined, patchy areas of consolidation, to extensive airspace disease due to hemorrhagic pulmonary edema. Pleural effusion is rare and usually represents bacterial coinfections.

In our study, gram-positive bacteria *S. pneumoniae* and *S. aureus* were observed in 30% of patients (58/194) and 23.71% (46/194), respectively. The rate of coinfection with atypical bacteria *Mycoplasma pneumoniae* was 19% (37/194), gram-negative bacteria *Haemophilus influenzae* and *Moraxella catarrhalis* were observed in 7.2% (14/194) and 2.5% (12/194), respectively. M. Qiao, et al. (2023) [17] conducted a systematic review of 39,762 influenza cases, 5578 of which had bacterial co-infection. The prevalence of influenza bacterial co-infection was 20.3%. Compared with influenza single-infection, bacterial co-infection increased the risk of death by 2.55-fold intensive care unit admission by 1.87-fold, and requirement for mechanical ventilation by 1.78-fold, approximately 23.8% of influenza deaths were attributable to bacterial co-infection. A systematic review and meta-analysis performed by E.Y Klein, et al. (2016) [18],

including 3215 participants, indicated that coinfecting bacteria were *S. pneumoniae* and *S. aureus*, accounting for 35% and 28% of infections, respectively. Q.Y. Wang, et al. (2023) [19] retrospectively analyzed data for 64 pediatric patients with severe influenza virus-associated pneumonia, susceptible to bacterial infection, with gram-negative bacteria, especially *Haemophilus influenzae* and *Moraxella catarrhalis*, being the most common pathogens. In a systematic review and meta-analysis J.A. Herrero, et al. (2023) [20] studied 48,259 patients hospitalized with influenza of any age. Coinfecting bacteria were diagnosed in 11.2%. *S. pneumoniae* (30.7%) and *S. aureus* (30.4%) were the most frequent microorganisms, followed by *Haemophilus influenzae* (7.1%) and *Pseudomonas aeruginosa* (5.9%). Coinfecting bacteria increased death risk by 3.36-fold (95% CI: 2.56-4.41) compared with Influenza virus infection alone. The mechanisms by which bacteria act synergistically with influenza viruses include increased binding and invasion of bacteria, increased viral replication, and modification of the host inflammatory response. As the viral neuraminidase cleaves sialic acid to release new viral particles from host cells, damage to the epithelial layer of the airways occurs, exposing binding sites necessary for adherence to *S. pneumoniae* [21].

4.3. Relationship between influenza vaccination, Oseltamivir treatment, and certain complications in patients with influenza

The number of children with complications accounted for 146 out of 194 (75.3%) in our study. Among them, the most common complications were febrile seizures (42.3%) and bronchopneumonia (40.2%). Other less common complications included acute otitis media (3.6%), respiratory failure (4.1%), and acute diarrhoea (3.1%). Additionally, cases of acute myocarditis, influenza-associated encephalopathy, and septic shock were found, comprising 0.51% and 1.54%, respectively. A study by H.V. Pham, et al. (2021) [12] on 200 patients from 2020 to 2021 observed that febrile seizures and bronchopneumonia accounted for the highest proportions at 45% and 41%, respectively. However, respiratory failure and acute otitis media cases were higher, accounting for 25.5% and 11.5%, respectively. The complications in our patient group were lower compared to Y. Shi, et al.'s (2021) [11] study on 36,047 patients over 5 years from 2014 to 2019, which reported 118 severe influenza cases. The rates of complications such as bronchopneumonia, respiratory failure, and heart failure were 83.1, 84.7, and 12.7% of children experienced influenza-associated encephalopathy, 6.8% developed septic shock, and 15.3% experienced acute kidney injury. The differences observed may be attributed to our study's smaller sample size, geographical variations, and differences in immunization programs between countries. Influenza vaccination is an important strategy for protecting children and the broader community, as well as reducing the overall burden of respiratory illnesses when other viruses are cocirculating. The AAP recommends annual influenza vaccination of all children without medical contraindications starting at 6 months of age, children are at risk for hospitalization and death from influenza [22].

In our study, febrile seizures, bronchopneumonia, and respiratory failure were more prevalent in the group of children who were not vaccinated, with corresponding odds ratios of 4.44 (2.81 and 2.25), respectively. However, the two groups had no significant difference regarding complications such as diarrhea, acute otitis media, acute myocarditis, influenza-associated encephalopathy, and septic shock. A study by B. Flannery, et al. (2017) [23] conducted over 4 years from 2010 to 2014 on 358 deceased children aged 6 months to 17 years associated with seasonal influenza found that only 26% of children received influenza vaccination, which reduced the risk of death in the high-risk group of children with influenza. A study by J.M. Ferdinands, et al. (2014) [24] conducted in 21 pediatric intensive care units (PICUs) over 2 years from 2010 to 2012 found that full influenza vaccination reduced the risk of severe influenza complications by 75%. Oseltamivir has been shown to benefit adults with influenza, but its safety and efficacy are not as well demonstrated in children. Oseltamivir is an antiviral neuraminidase inhibitor with potent and selective competitive inhibition of the influenza virus neuraminidase, an enzyme necessary for viral replication. Oseltamivir interferes with the release of progeny influenza virus from infected host cells and thus halts the spread of infection to new host cells. Oral oseltamivir treatment reduces the duration by about 0.5 to 3 days and the severity of acute influenza and may decrease the incidence of secondary complications. In 2012, the United States Food and Drug Administration (FDA) approved oseltamivir for the treatment of influenza within 2 days of illness onset in children aged ≥ 14 days [25-27]. In our study, the group without complications initiated oseltamivir treatment at an average of 1.22 ± 0.56 days from admission hospital. The group treated with Oseltamivir within 48 hours had a reduced risk of complications, compared to the group treated with Oseltamivir after 48 hours or left untreated. A study by R.J. Whitley, et al. (2001) [28] on 2 groups of patients treated with oseltamivir and a placebo group, randomized, double-blind, placebo-controlled study, it was found that the oseltamivir treatment group reduced fever duration by 36 hours (26%, $p < 0.01$), symptoms of cough 32 hours (45%, $p < 0.01$), the rate of complications and severity in the oseltamivir treatment group decreased by 40% and 29% compared with no treatment group. J. Qin, et al. (2022) [29] conducted an observational real-world study at Shanghai Children's Medical Center in 2018 showing that Oseltamivir treatment does not significantly shorten the duration of fever, nor does it significantly relieve influenza-like symptoms in children with infection of influenza. R.E. Malosh, et al. (2017) [27] conducted a systematic review to identify RCTs of oseltamivir therapy in children and showed that treatment with oseltamivir significantly reduced the duration of illness in those with influenza and lowered the risk of developing otitis media. However, children treated with oseltamivir had a higher frequency of emesis compared with those receiving

placebo. P.S. Walsh, et al. (2022) [30] conducted a cohort study, a multicenter propensity score-weighted retrospective analysis of data for 55 799 children hospitalized with influenza between 2007 and 2020 revealed that early (hospital day 0 or 1) oseltamivir use was associated with shorter hospital stay and lower odds of 7-day readmission, transfer to the PICU, and in-hospital mortality or use of ECMO (extracorporeal membrane oxygenation). The AAP recommends antiviral treatment should be initiated as soon as possible and children with suspected or confirmed influenza who are hospitalized, have severe or progressive disease, or have underlying conditions that increase their risk of complications of influenza should be used, regardless of the duration of illness [22].

5. Conclusions

Influenza is predominantly observed in children aged 12 to 60 months, it happens more frequently in boys than in girls. The seasonal influenza vaccination rate was low, and the influenza patients easily cooperated with other bacteria like Gram-positive and atypical bacteria. Patients with influenza commonly present with fever, respiratory tract inflammation, gastrointestinal symptoms, varying degrees of mucocutaneous manifestations, and mostly normal laboratory parameters. Common complications included seizures, bronchiolitis, respiratory failure, and diarrhea. In contrast, the ratio of acute myocarditis and cases associated with nervous system problems dramatically decreased. Administration of seasonal influenza vaccine within 12 months and treatment with Oseltamivir within 48 hours of symptom onset in vaccinated children would likely reduce the duration of symptoms and the incidence of complications.

CRedit author statement

Manh Cuong Nguyen: Methodology, Formal analysis, Original draft preparation, Visualisation; Thi Thuy Hang Le: Supervision, Validation, Writing - Reviewing and Editing; Thanh Nam Do: Conceptualisation, Data curation, Investigation; Thai Son Pham: Supervision, Validation, Writing - Reviewing and Editing; Hoang Long Trinh: Investigation, Visualisation, Formal analysis; Thi Bich Thuy Phung: Supervision, Validation, Writing - Reviewing and Editing; Yen Thi Hoang: Investigation, Visualisation, Formal analysis; Quang Khai Tran: Methodology, Formal analysis, Original draft preparation, Visualisation.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Molecular docking and molecular dynamics simulation revealed potential compounds in *Salvia miltiorrhiza* for inhibiting enzymes and receptors in Alzheimer's disease

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Abstract:

Alzheimer's disease is an age-related neurodegenerative disorder that leads to cognitive and functional decline. Potential target proteins for managing Alzheimer's disease include Acetylcholinesterase (AChE), Butyrylcholinesterase (BuChE), Beta-secretase cleavage enzyme (BACE1) and *N*-methyl-D-aspartate (NMDA) receptors. In this study, we conducted *in silico* evaluations of the inhibitory effects of *Salvia miltiorrhiza*'s compounds on AChE, BuChE, BACE1, and NMDA receptors. The Lipinski's rule of five was employed to compare phytochemicals with drug-like and non-drug-like properties. Based on previous publications, we compiled a list of 30 compounds from *S. miltiorrhiza* with potential for improving Alzheimer's disease. Among these 30 compounds, six natural compounds exhibited potential inhibition of all four target proteins. Five of these six compounds, namely Tanshinone I, Tanshinone IIA, Isotanshinone I, Dihydroisotanshinone I, and Dihydroisotanshinone II, possessed drug-like properties, good absorption potential, and the ability to cross the blood-brain barrier. In conclusion, these five compounds show the highest potential as future drug candidates for the treatment of Alzheimer's disease.

Keywords: Alzheimer, *in silico*, molecular docking, molecular dynamics, *Salvia miltiorrhiza*.

Classification numbers: 3.3, 3.5

1. Introduction

Alzheimer's disease is an age-related neurodegenerative disorder resulting in cognitive and functional decline. Research has identified three stages of Alzheimer's disease: preclinical Alzheimer's disease, mild cognitive impairment (MCI) due to Alzheimer's disease, and dementia due to Alzheimer's disease. In the first stage, individuals exhibit no symptoms such as memory loss, but they already display early signs of the disease indicated by biomarkers. In the subsequent two stages, clinical symptoms of Alzheimer's disease become more evident. Alongside biomarker evidence, such as increased levels of beta-amyloid, patients with MCI due to Alzheimer's disease experience more pronounced cognitive decline than individuals of the same age. The final stage, dementia due to Alzheimer's disease, is characterized by numerous symptoms related to memory, cognition, and behavior, significantly impairing an individual's daily functioning [1]. Vietnam, one of the world's fastest-aging countries, is witnessing a rising number of people with Alzheimer's disease and related dementias [2].

Therapeutically, Alzheimer's disease management focuses on two primary strategies: symptomatic and mechanism-based therapeutic approaches targeting β -amyloid and tau pathologies.

Firstly, in the strategy for symptomatic improvements, the inhibition of brain cholinesterase activity and the regulation of glutamate activity have been considered the two main treatment avenues for Alzheimer's disease [3].

Inhibition of brain cholinesterases was believed to raise acetylcholine levels since acetylcholine, the neurotransmitter, is rapidly degraded by the hydrolytic activity of cholinesterases following its release into the synaptic cleft [3]. Increasing acetylcholine levels is thought to reduce symptoms of Alzheimer's disease. In the human brain, the most prominent enzyme responsible for acetylcholine hydrolysis is AChE. AChE plays a vital role in catalysing the breakdown of acetylcholine into choline and acetic acid, a reaction necessary for cholinergic neurons to return to their resting state after activation [4]. Another study suggests that BuChE may also hydrolyse acetylcholine in the brain, contributing to cholinergic transmission [5]. Therefore, inhibiting these two enzymes is considered a therapeutic approach to enhancing cognitive and neural cell function.

The other therapy to improve Alzheimer's disease symptoms involves regulating glutamate activity. Glutamate excitotoxicity, mediated through excessive activation of NMDA receptors, has been linked to neuronal death observed in Alzheimer's disease and other neurodegenerative conditions [3]. This results in neuronal cell death and a decline in cognitive function. Consequently, NMDA receptor antagonists are believed to alleviate Alzheimer's disease symptoms by preventing glutamate-mediated neurotoxicity.

Second are mechanism-based therapeutic approaches targeting β -amyloid and tau pathologies. Alzheimer's disease is characterized by the presence of extracellular amyloid β

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(A β) plaques and intracellular deposition of neurofibrillary tangles (NFTs) [3, 6]. A β is an aggregated form of amyloid β peptide, while NFTs consist of hyperphosphorylated tau proteins. BACE1 plays a crucial role in producing amyloid- β proteins by cleaving proteins from amyloid precursor proteins, leading to neurotoxicity and neuronal death [7, 8]. Therefore, inhibiting BACE1 to prevent A β production holds potential as a therapy for Alzheimer's disease. In this study, we selected AChE, BuChE, NMDA receptor, and BACE1 as target proteins.

Molecular docking is a widely used computational structure-based drug design (SBDD) method that has been employed since the early 1980s [9]. Molecular docking aims to predict the most favourable position and configuration for a substrate molecule to bind to a protein. This method simplifies the identification of compounds with the best pharmacological effects without the need for experiments. Additionally, this *in silico* method saves considerable time, cost, and effort [10].

Salvia miltiorrhiza Bunge belongs to the *Salvia* Linn. genus, the largest in the Lamiaceae family. *S. miltiorrhiza*, native to China and Japan, is primarily produced in China, with smaller production areas in countries like Japan and Vietnam. In Vietnam, *S. miltiorrhiza* thrives in the northern regions. *Salvia miltiorrhiza* Bunge is a well-known traditional herb with a history of use in preventing and treating various ailments, including neurological diseases, cancer, inflammation, and cardiovascular diseases [11].

In this study, we employed *in silico* techniques to identify natural compounds from *Salvia miltiorrhiza* Bunge that could potentially be effective in treating Alzheimer's disease and other neurodegenerative disorders.

2. Materials and methods

2.1. Retrieval and preparation of protein structure

Initially, the three-dimensional (3D) structures of the NMDA receptor (PDB ID: 1PBQ), enzyme β -secretase 1 (PDB ID: 4X7I), enzyme BuChE (PDB ID: 4BDS), and enzyme AChE (PDB ID: 1EVE) were obtained from the Protein Data Bank RCSB. Subsequently, all water molecules and co-crystallised ligands were removed from the protein structures using Discovery Studio Visualizer v21.1.0.20298 software. MGL Autodock Tools 1.5.6 software was utilised to regenerate the active site after supplying missing hydrogen atoms with Autodock Vina. Finally, these proteins were saved in pdbqt format in preparation for the docking program.

2.2. Ligands preparation

Based on prior publications, we gathered 30 compounds from *S. miltiorrhiza* with potential to improve Alzheimer's disease (Table 1). Next, the 3D structures were obtained from PubChem in SDF format and then converted into PDB format using Chimera 1.17.3 software. Subsequently, these structures were optimised using Avogadro 1.2.0 software with conjugate gradients and converted to PDBQT format using Autodock Tools software.

Table 1. Structures of the 30 compounds from *S. miltiorrhiza*.

No	Name	Molecular formula	No	Name	Molecular formula
1	Tanshinone I	C ₁₈ H ₁₂ O ₃	16	4-Methylenemiltirone	C ₁₈ H ₁₈ O ₂
2	Tanshinol A	C ₁₈ H ₁₂ O ₄	17	Caffeic acid	C ₉ H ₈ O ₄
3	Przewaquinone B	C ₁₈ H ₁₂ O ₄	18	Isotanshinone I	C ₁₈ H ₁₂ O ₃
4	Tanshinone IIA	C ₁₉ H ₁₈ O ₃	19	Dihydroisotanshinone I	C ₁₈ H ₁₄ O ₃
5	Hydroxytanshinone IIA	C ₁₉ H ₁₈ O ₄	20	Dihydroisotanshinone II	C ₁₈ H ₁₄ O ₃
6	Tanshinone IIB	C ₁₉ H ₁₈ O ₄	21	Danshexinkun A	C ₁₈ H ₁₆ O ₄
7	Danshensu	C ₉ H ₁₀ O ₅	22	Danshenxinkun B	C ₁₈ H ₁₆ O ₃
8	Methyl tanshinonate	C ₂₀ H ₁₈ O ₅	23	Danshenxinkun C	C ₁₆ H ₁₂ O ₃
9	Tanshinolaldehyde	C ₁₉ H ₁₈ O ₄	24	Isoferulic acid	C ₁₀ H ₁₀ O ₄
10	Tanshindiol A	C ₁₈ H ₁₆ O ₅	25	Salvianolic acid F	C ₁₇ H ₁₄ O ₆
11	Rosmarinic acid	C ₁₈ H ₁₆ O ₈	26	Salvianolic acid G	C ₁₈ H ₁₂ O ₇
12	Przewaquinone A	C ₁₉ H ₁₈ O ₄	27	Sugiol	C ₂₀ H ₂₈ O ₂
13	Tanshindiol C	C ₁₈ H ₁₆ O ₅	28	Sibiriquinone A	C ₁₉ H ₂₀ O ₂
14	Methyl dihydronortanshinonate	C ₂₀ H ₂₀ O ₅	29	Trijunanone B	C ₁₈ H ₁₆ O ₃
15	Miltirone	C ₁₉ H ₂₂ O ₂	30	Neo-przewaquinone A	C ₃₆ H ₂₈ O ₆

2.3. Molecular docking study

The selected phytochemicals were docked into the active sites of proteins using Autodock Vina software. The scoring function of Autodock Vina was employed to calculate the energy of the ligand-protein interactions. Molecular interactions between proteins and the selected compounds with higher binding affinity were visualised using Discovery Studio Visualizer v21.1.0.20298.

2.4. Validation of docking protocol

After separating the co-crystallised ligands from the proteins using Discovery Studio Visualizer v21.1.0.20298 software, the molecules in PDB format were saved and converted to PDBQT format. Subsequently, a re-docking process was carried out where the co-crystallised ligands were re-docked into the active sites of targets prior to screening. The procedure was considered successful if the root mean square deviation (RMSD) value was not higher than 1.5 Å [9].

2.5. Lipinski's rule of five

Lipinski's rule of five is a technique used to compare drug-like molecules and those that are not. It is employed to screen molecules with potential pharmacological profiles similar to drugs. In this study, an online tool was used to evaluate Lipinski's rule of five. The chemical structures were retrieved from the PubChem database and set at pH 7.0.

2.6. Prediction of ADMET by computational analysis

ADMET profiling was employed to assess the physicochemical efficiency of inhibiting the target proteins. Only compounds that satisfy Lipinski's rule of five were evaluated by ADMET profiling. ADMET profile predicts five parameters: absorption, distribution, metabolism, excretion, and toxicity, which are crucial for determining a drug's potential success. Standard ranges for these criteria were

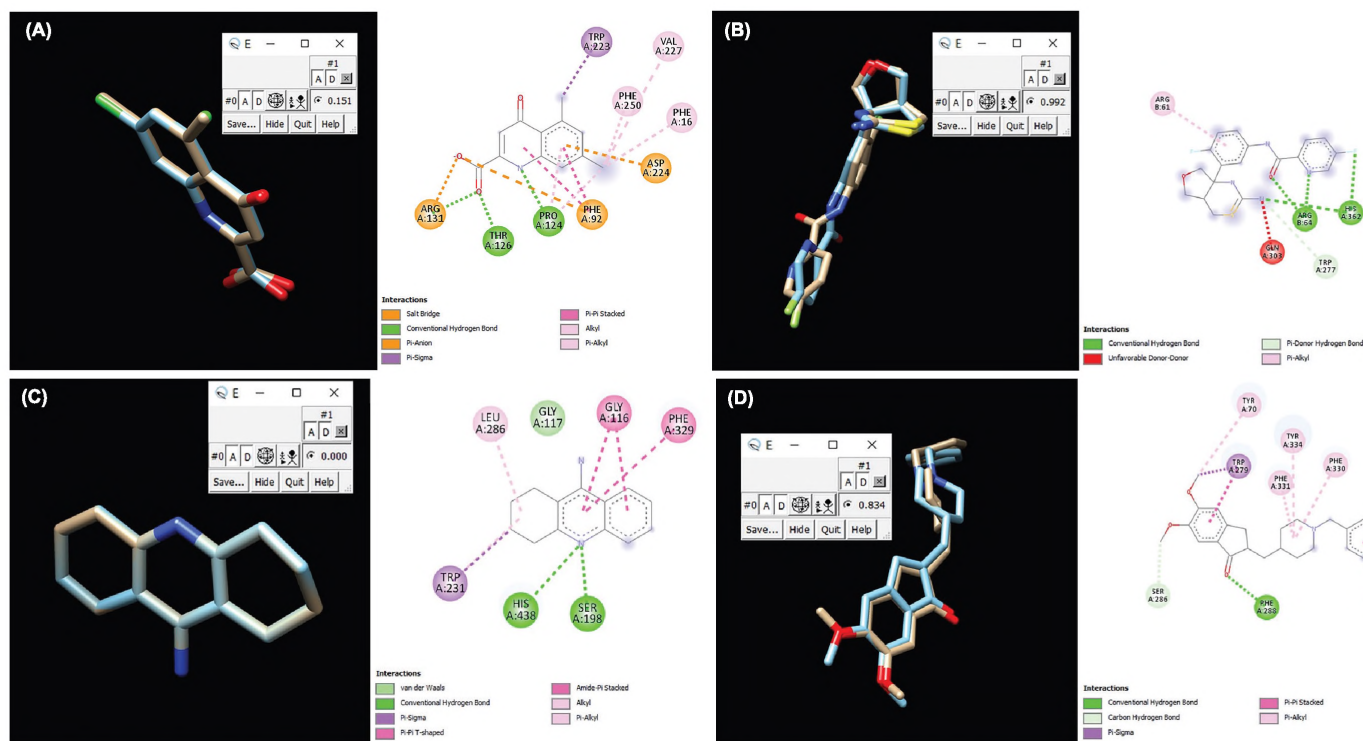


Fig. 1. Results of re-docking and interactions of the co-crystallised ligands with proteins. (A) Re-docking and interactions of the co-crystallised ligand 5,7-dichloro-4-hydroxyquinoline-2-carboxylic acid with NMDA. **(B)** Re-docking and interactions of the co-crystallised ligand LY2886721 with BACE1. **(C)** Re-docking and interactions of the co-crystallised ligand Tacrine with BuChE. **(D)** Re-docking and interactions of the co-crystallised ligand Donepezil with AChE.

tested for compliance, and ADMET profiling was predicted using the pkCSM tool. Canonical SMILES molecular structures of collected phytochemicals were retrieved from PubChem.

2.7. Molecular dynamics simulation of the most potent compounds

Molecular dynamics was employed to assess the physical movement and interactions of all atoms in the protein-ligand complexes, including their interactions with the surroundings. MOE version 2015.10 was used for molecular dynamics (MD) simulations of the complexes with the least binding energy pose. The Nosé-Poincaré-Andersen (NPA) equations of motion were applied in MOE dynamics simulation. The system was optimised for equilibrium over 600 ps, followed by a production run at 310 K for 500 ps. The RMSD was determined using the formula:

$$\text{RMSD} = \sqrt{\frac{1}{N} \sum_{i=1}^N d_i^2}$$

where N is the total number of atoms in the complex and d_i is the distance between atom i at two separate times.

3. Results

3.1. The result of a validation

Before proceeding with screening, it is crucial to validate the docking protocol. To achieve this, the co-crystallised ligand was extracted from the protein and then re-docked into the active site of the target. The RMSD was calculated, and the structural similarity

was assessed using Chimera 1.17.3 software. The superposition of the co-crystallised ligand structures before and after docking with NMDA, BACE1, BuChE, and AChE resulted in RMSD values of 0.151 Å, 0.992 Å, 0 Å, and 0.834 Å, respectively. All RMSD values were found to be less than 1.5 Å, indicating the reliability of the molecular docking outcomes. The results of the re-dock and interactions between the co-crystallised ligands and proteins are presented in Fig. 1.

As depicted in Fig. 1, the co-crystallised ligand 5,7-dichloro-4-hydroxyquinoline-2-carboxylic acid displayed interactions with TRP 223, PHE 250, VAL 227, PHE 16, ASP 224, PHE 92, PRO 124, THR 126, and ARG 131 of NMDA. For BACE1, the co-crystallised ligand LY2886721 interacted with ARG 61, HIS 362, ARG 64, and GLN 303 of the target protein. Tacrine, on the other hand, interacted with LEU 286, GLY 116, PHE 329, SER 198, HIS 438, and TRP 231, which are amino acids in BuChE. Lastly, in the case of AChE, Donepezil interacted with TYR 70, TRP 279, PHE 331, TYR 334, PHE 330, TRP 84, PHE 288, and SER 286.

3.2. Molecular docking of compounds with the target proteins

Following the preparation of ligands, we conducted docking experiments involving 30 phytochemicals and four target proteins: NMDA, BuChE, AChE, and BACE1. To evaluate the potential of these compounds to inhibit these four target proteins, we compared the docking scores of the ligands with those of four reference drugs: memantine, LY2886721, rivastigmine, and donepezil, respectively. The results are presented in Table 2.

Table 2. Docking results of 6 compounds potential and reference compounds with target proteins.

No	Name	Binding energy with proteins (kcal/mol)			
		<i>IPBQ-NMDA</i>	<i>4X7I-BACE1</i>	<i>4BDS-BuChE</i>	<i>IEVE-AChE</i>
1	Tanshinone I	-9.4	-10.5	-9.8	-11.3
2	Tanshinone IIA	-9.4	-10.3	-10.1	-11.2
3	4-Methylenemiltirone	-8.8	-9.7	-9.1	-11.1
4	Isotanshinone I	-9.9	-10.6	-9.9	-11.1
5	Dihydroisotanshinone I	-10.3	-10.6	-10.3	-11.9
6	Dihydroisotanshinone II	-10.4	-10.4	-10.3	-11.1
+	Memantine	-6.9			
+	LY2886721		-9.0		
+	Rivastigmine			-6.9	
+	Donepezil				-10.9

Memantine, an NMDA receptor antagonist, was FDA-approved in 2013 for managing Alzheimer’s disease [12]. Rivastigmine, another drug, is known as a dual BuChE and AChE inhibitor, FDA-approved in 2000 for treating mild to moderate Alzheimer’s dementia [13, 14]. Donepezil, an AChE inhibitor, has been used to manage behavioural and cognitive symptoms of Alzheimer’s

disease and related dementias since its initial FDA approval in 1996. In 2014, its extended-release form was approved in combination with memantine for treating patients in moderate and severe stages of Alzheimer’s dementia (Donepezil FDA label). In 2015, a study showed that LY2886721 has the potential to be a BACE1 inhibitor. Experiments on mice, dogs, and humans indicated that LY288672 causes robust central Aβ pharmacodynamic responses [15]. Considering this evidence, these four reference drugs were selected as positive control molecules.

Table 2 presents the docking results of six compounds that simultaneously exhibited lower free energies of binding than all four positive control compounds.

Among the 30 compounds, only six compounds, including Tanshinone I, Tanshinone IIA, 4-methylenemiltirone, Isotanshinone I, Dihydroisotanshinone I, and Dihydroisotanshinone II that potentially inhibited all four target proteins.

Therefore, we have chosen these six compounds for further evaluation of their drug-like properties due to their significant potential to inhibit targets in Alzheimer’s treatment.

Table 3 and Figs. 2-7 present the active site residues of the target protein interaction with six potential magnetic compounds.

Table 3. Active site residues of target proteins with compounds from *S. miltiorrhiza*.

No	Compounds	Active site residues atom range			
		<i>IPBQ-NMDA</i>	<i>4X7I-BACE1</i>	<i>4BDS-BuChE</i>	<i>IEVE-AChE</i>
1	Tanshinone I	PHE 250, ARG 131, PHE 92	Asp 62, TYR 60, ARG 61, TRP 277	LEU 286, PHE 329, SER 198, HIS 438, GLY 116	PHE 330, TYR 334
2	Tanshinone IIA	VAL 181, TYR 184, LEU 146	ARG 61, THR 275, TYR 320	SER 287, PHE 329, SER 198, GLY 116, HIS 438, TRP 82	TYR 70, TRP 279
3	4-Methylenemiltirone	ARG 131, SER 180, PHE 92	TYR 71	SER 198, HIS 438, PHE 329, PHE 398, GLY 117, LEU 286, TRP 231, TRP 82	PHE 330, TRP 84, TYR334
4	Isotanshinone I	TYR 184, PHE 246, LEU 146,	TYR 71, ASP 32	GLY 116, HIS 438, PHE 329, SER 198, VAL 288, LEU 286, TRP 231	TYR 334, TRP 279
5	Dihydroisotanshinone I	TRP 223, SER 180,	VAL 361	TRP 82, HIS 438, SER 198, GLY 117, TRP 231, LEU 286, PHE 329, TYR 332, TRP 82	TYR 334, PHE 330, TRP 279
6	Dihydroisotanshinone II	TRP 223, PHE 92, PHE 250	TYR 71, TRP 76	TYR 128, TRP 82, HIS 438	PHE 330, TYR 334, TYR 121

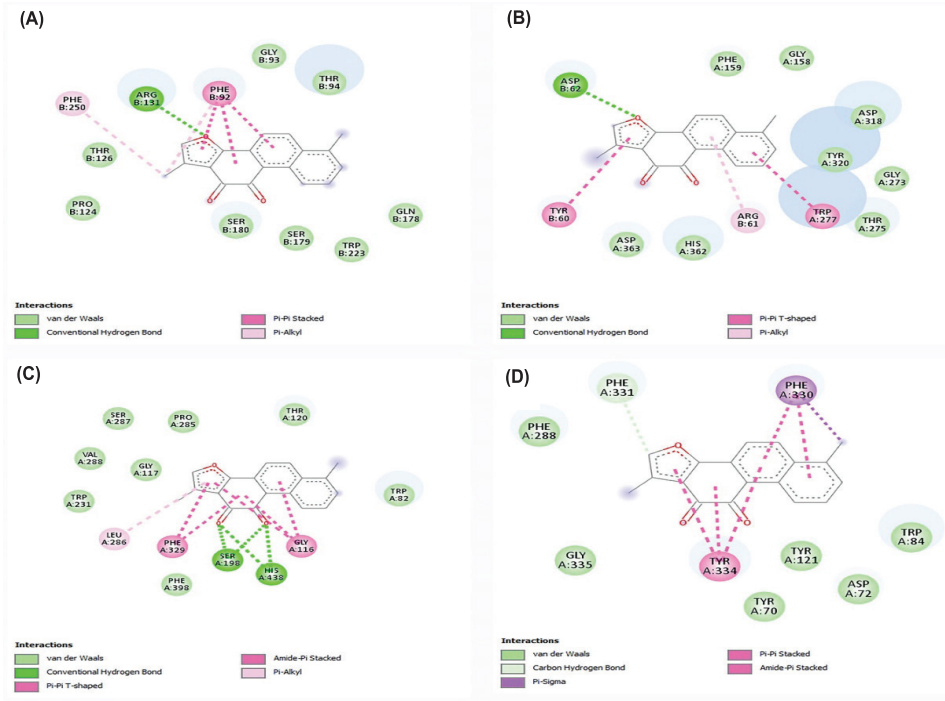


Fig. 2. Binding interactions of Tanshinone I with NMDA (A), BACE1 (B), BuChE (C), and AChE (D).

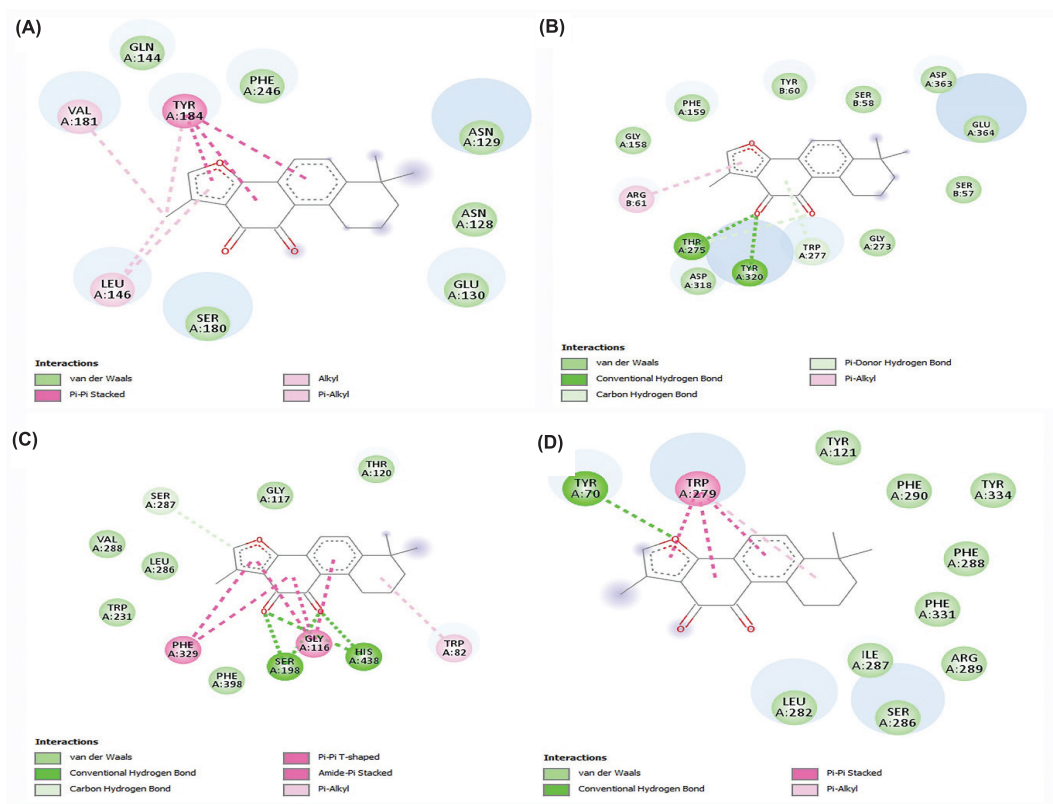


Fig. 3. Binding interactions of Tanshinone IIA with NMDA (A), BACE1 (B), BuChE (C), and AChE (D).

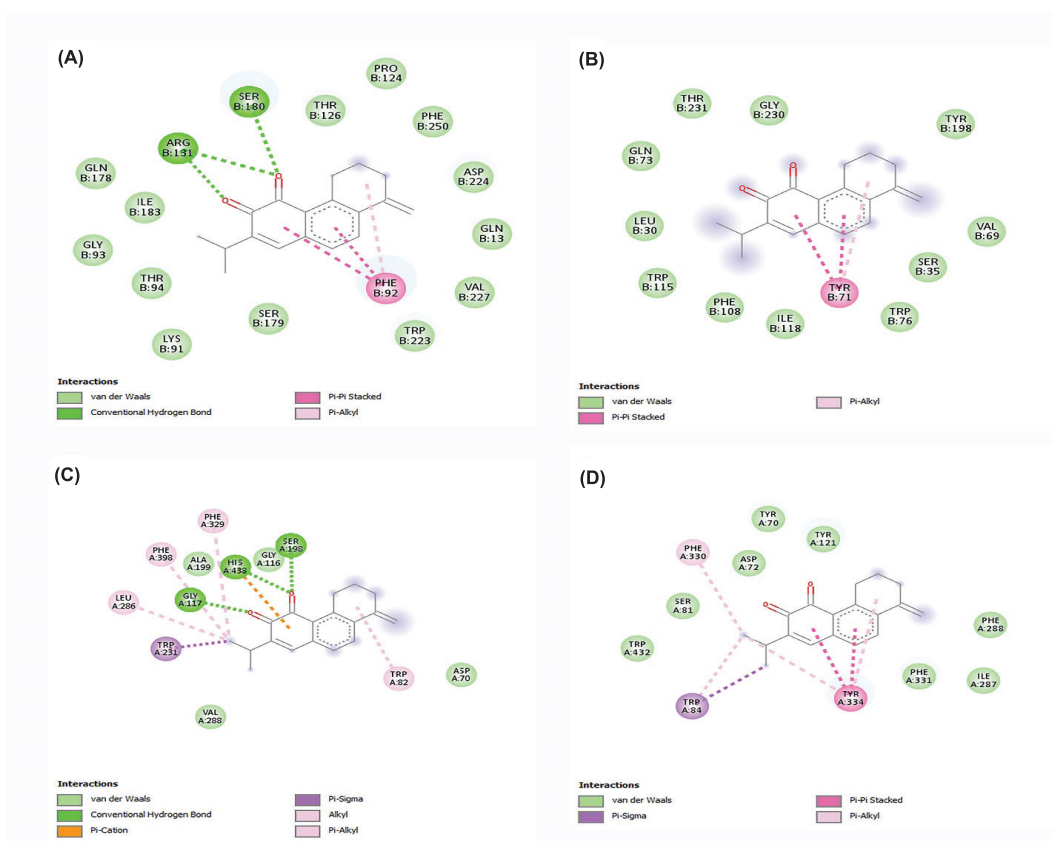


Fig. 4. Binding interactions of 4-Methylenemiltirone with NMDA (A), BACE1 (B), BuChE (C), and AChE (D).

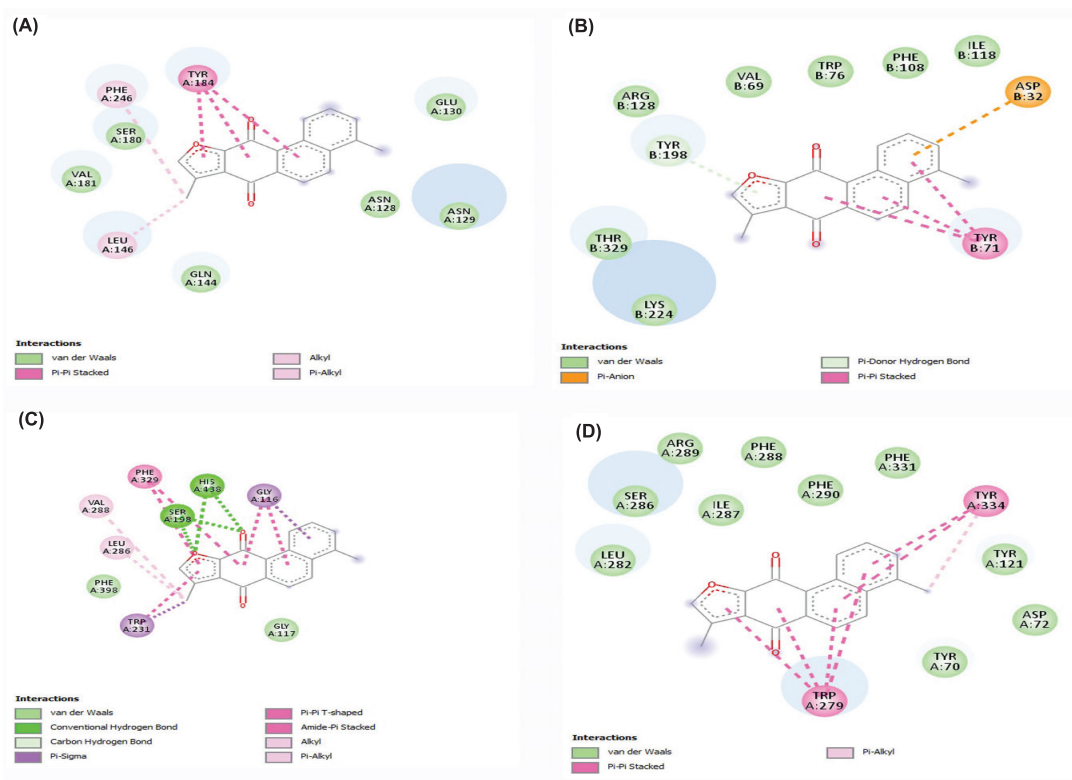


Fig. 5. Binding interactions of Isotanshinone I with NMDA (A), BACE1 (B), BuChE (C), and AChE (D).

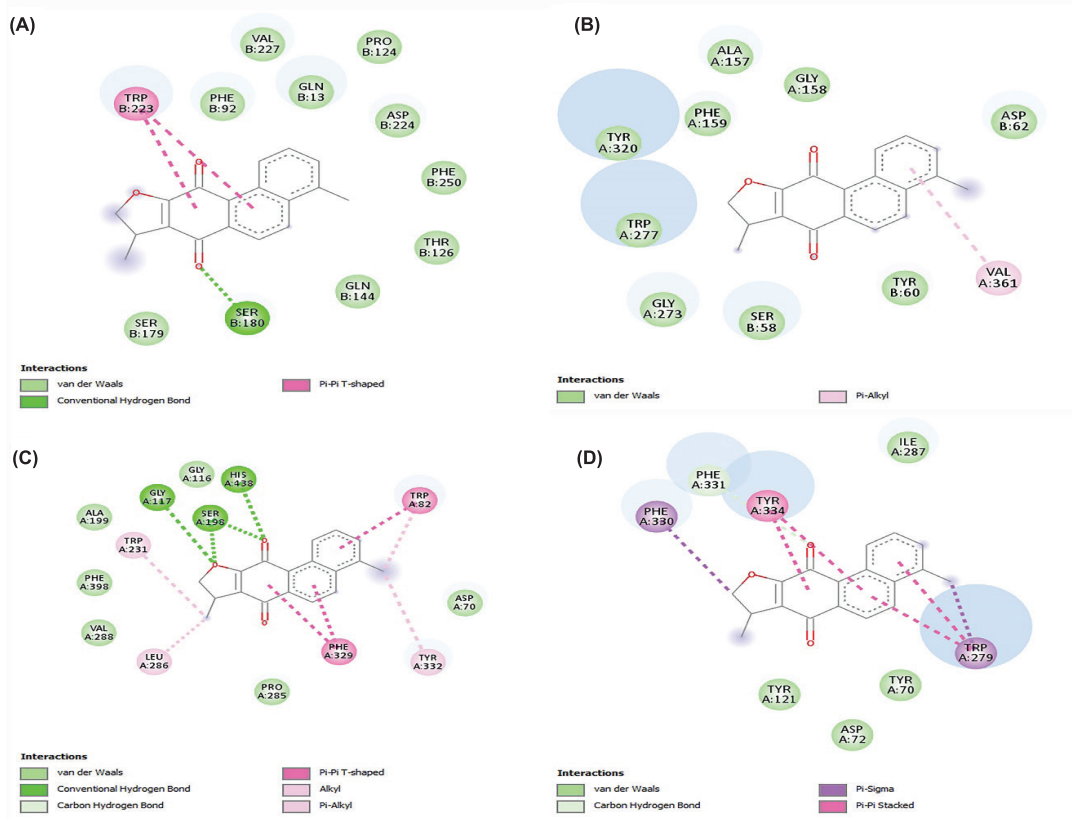


Fig. 6. Binding interactions of Dihydroisotanshinone I with NMDA (A), BACE1 (B), BuChE (C), and AChE (D).

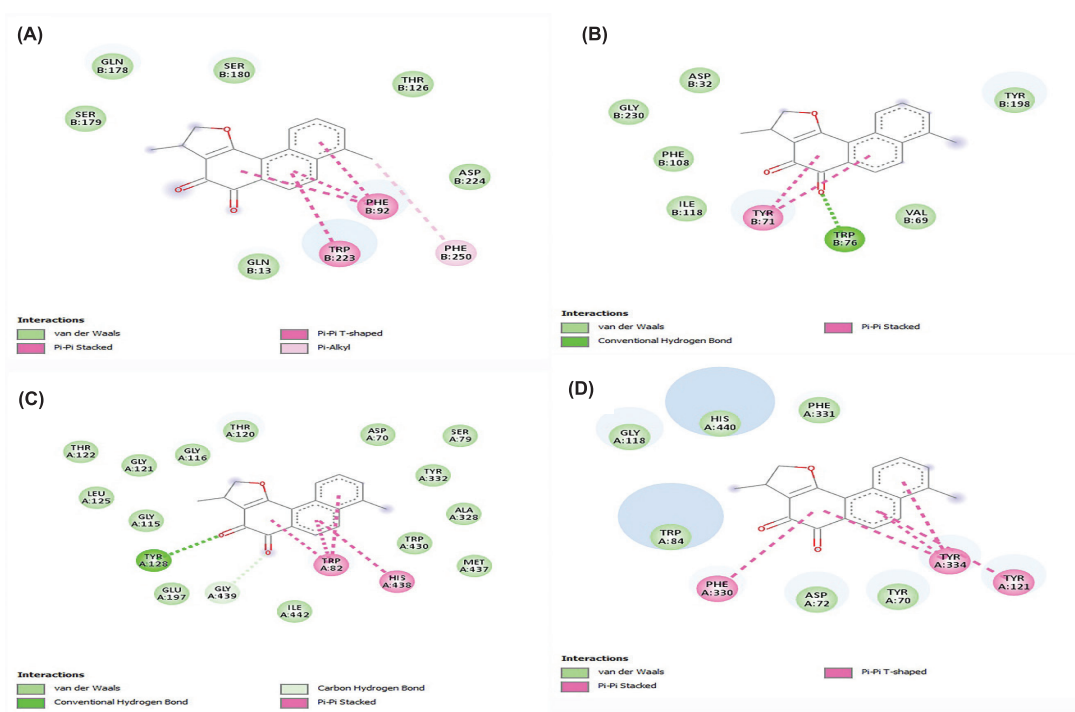


Fig. 7. Binding interactions of Dihydroisotanshinone II with NMDA (A), BACE1 (B), BuChE (C), and AChE (D).

3.3. Lipinski's rule of five

According to Lipinski's rule of five, compounds that satisfy at least two of the following criteria will have the potential to become oral drugs, including: molecular mass (MW) less than 500 Da; high lipophilicity (expressed as LogP less than 5); fewer than 5 hydrogen

bond donors (HBD); fewer than 10 hydrogen bond acceptors (HBA1); molar refractivity (MR) between 40 and 130.

The results indicate that all six compounds meet the criteria outlined in Lipinski's rule of five (Table 4).

3.4. Prediction of absorption, distribution, metabolism, excretion, and toxicity (ADMET) profile

In silico ADMET profiling was employed to assess the physiochemical efficiency of the six compounds as inhibitors of the target proteins. ADMET profiling predicts five key parameters: absorption, distribution, metabolism, excretion, and toxicity. All five parameters are crucial in determining the likelihood of a compound's success as a drug. Table 5 is the ADMET evaluation results of six potential compounds.

Table 4. Results of Lipinski's rule of five.

No	Compounds	Molecular weight (Da)	HBD	HBA	Log P	Molar refractivity (MR)	Drug likeness
1	Tanshinone I	276.0	0	3	4.1	79.8	Yes
2	Tanshinone IIA	294.0	0	3	4.2	83.5	Yes
3	4-Methylenemiltirone	266.0	0	3	3.8	80.6	Yes
4	Isotanshinone I	276.0	0	3	3.8	78.9	Yes
5	Dihydroisotanshinone I	278.0	0	3	3.4	79.5	Yes
6	Dihydroisotanshinone II	278.0	0	3	3.3	80.1	Yes

Table 5. ADMET assessment results.

Properties	Tanshinone I	Tanshinone IIA	4-Methylenemiltirone	Isotanshinone I	Dihydroisotanshinone I	Dihydroisotanshinone II
Absorption						
Water solubility (log mol/l)	-4.443	-4.494	-4.986	-4.567	-3.731	-3.883
Caco2 permeability (log Papp in 10 ⁻⁶ cm/s)	1.401	1.419	1.774	1.353	1.355	1.366
Intestinal absorption (human) (%)	98.909	96.253	96.635	99.51	99.561	98.628
Distribution						
VDss (human) (log l/kg)	0.561	0.325	0.853	0.629	-0.013	-0.02
BBB permeability (log BB)	0.447	0.302	0.285	0.419	0.56	0.377
Metabolism						
CYP2D6 substrate	No	No	No	No	No	No
CYP3A4 substrate	Yes	Yes	Yes	Yes	Yes	Yes
CYP2D6 inhibitor	No	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	Yes	No	No
Excretion						
Total clearance (log ml/min/kg)	0.209	0.821	0.03	0.15	0.103	0.138
Toxicity						
AMES toxicity	Yes	No	No	Yes	Yes	No
Hepatotoxicity	No	No	Yes	Yes	No	No
Skin sensitisation	No	No	No	No	No	No

In the context of absorption progress, Table 5 shows that the water solubility of all six compounds is notably poor, with concentrations ranging from $10^{-4.9}$ to $10^{-3.7}$ mol/l. CaCo2 membrane permeability (log Papp in 10^{-6} cm/s) with a value higher than 0.9 is indicative of good permeability. Therefore, all six compounds, including Tanshinone I, Tanshinone IIA, 4-methylenemiltirone, Isotanshinone I, Dihydroisotanshinone I, and Dihydroisotanshinone II, are likely to exhibit significant permeability through the CaCo2 cell membrane, with log Papp values of 1.401, 1.419, 1.774, 1.353, 1.355, and 1.366 (in 10^{-6} cm/s), respectively. Additionally, these compounds are predicted to be completely absorbed through the human gut, with absorption rates exceeding 96%.

In terms of distribution, a logBB value greater than 0.3 indicates the likelihood of a compound being absorbed across the blood-brain barrier. The ability to cross the blood-brain barrier is crucial for certain pharmacological effects, such as inhibiting the AChE enzyme. With the exception of 4-methylenemiltirone, the other five compounds have logBB values exceeding 0.3. Tanshinone I and Tanshinone IIA have logBB values of 0.447 and 0.302, while Isotanshinone I, Dihydroisotanshinone I, and Dihydroisotanshinone II have logBB values of 0.419, 0.56, and 0.377, respectively.

Regarding metabolism, cytochrome P450 isozymes play a significant role in drug metabolism in the liver. All six compounds are CYP3A4 substrates, while only Isotanshinone I is a CYP3A4 inhibitor, indicating that they are metabolised by CYP3A4.

The toxicity profile reveals that Tanshinone I, Isotanshinone I, and Dihydroisotanshinone I exhibit AMES toxicity, which assesses the mutagenic potential of chemical compounds. Furthermore, both 4-methylenemiltirone and Isotanshinone I are predicted to cause liver toxicity. Therefore, only Tanshinone IIA and Dihydroisotanshinone II do not exhibit any form of toxicity, including AMES toxicity, hepatotoxicity, and skin sensitisation.

3.5. Molecular dynamics

The ADMET profile results indicate that five compounds, namely Tanshinone I, Tanshinone IIA, Isotanshinone I, Dihydroisotanshinone I, and Dihydroisotanshinone II, are likely to cross the blood-brain barrier, suggesting their potential for managing Alzheimer's disease.

To assess the stability of the docking poses of these five promising compounds, molecular dynamics simulations of the protein-ligand complexes were performed using the docking data as the starting configuration. The molecular dynamics results for all complexes, as depicted in Figs. 8-12, demonstrate that the stable model with free energy reaches equilibrium after approximately 100 ps.

The resulting RMSD values for all complexes are small and generally remain steady. These findings indicate that after 600 ps of molecular dynamics simulation, the positions of the complex's atoms show minimal differences. However, it is essential to further investigate the long-term stability of the protein-ligand complexes.

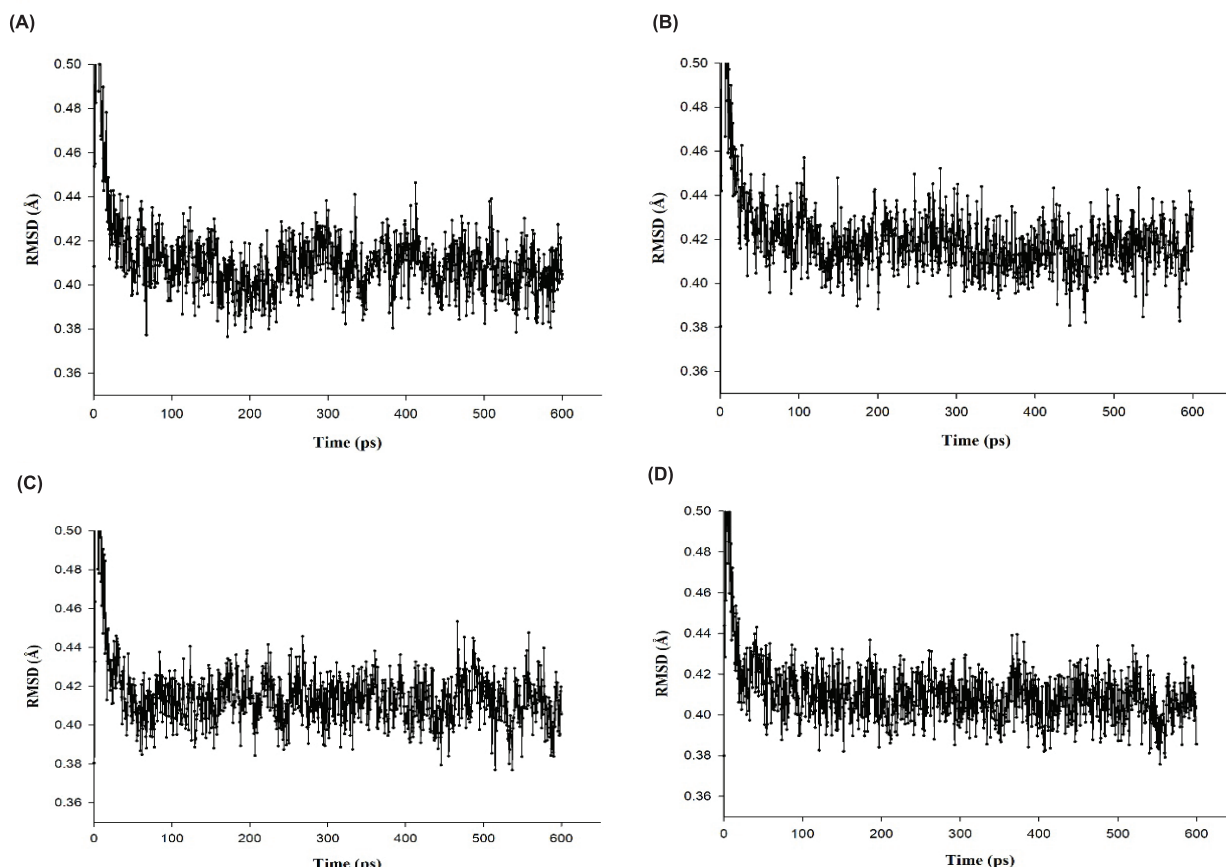


Fig. 8. RMSD of NMDA - Tanshinone I (A), BACE1 - Tanshinone I (B), BuChE - Tanshinone I (C), and AChE - Tanshinone I (D) complexes during 600 ps of molecular dynamics simulation.

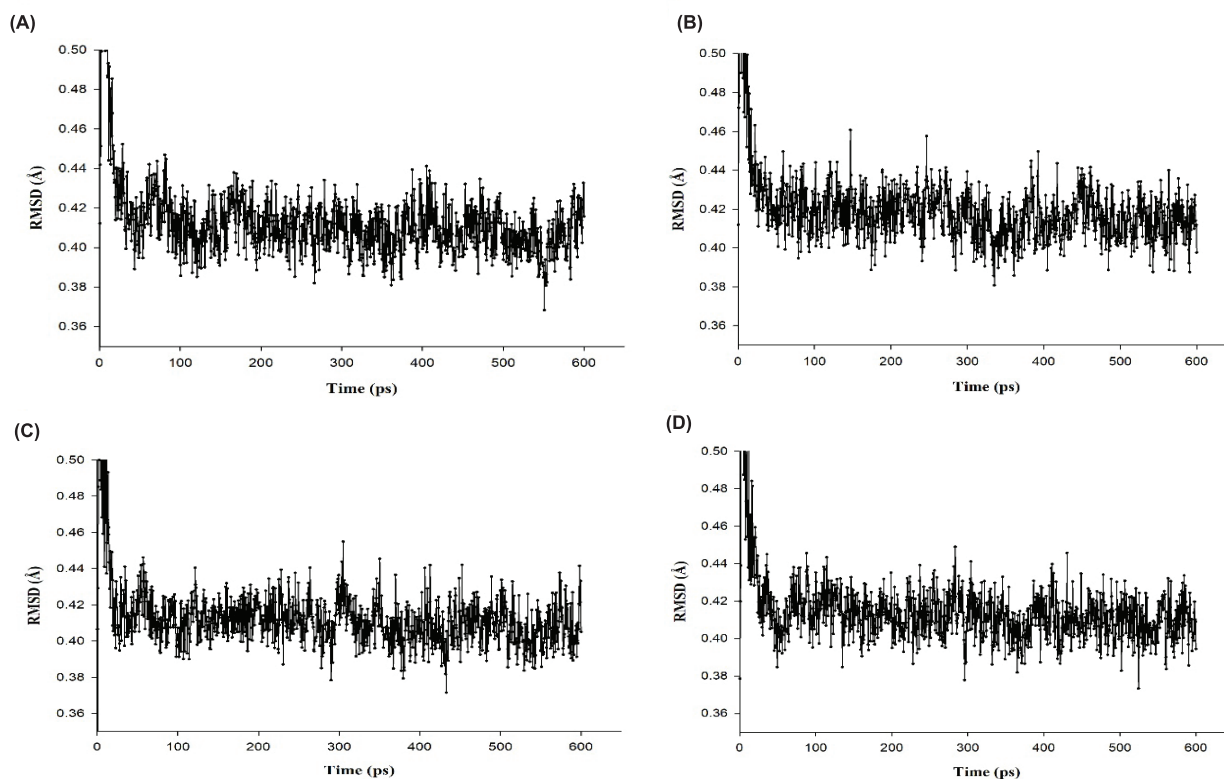


Fig. 9. RMSD of NMDA - Tanshinone IIA (A), BACE1 - Tanshinone IIA (B), BuChE - Tanshinone IIA (C), and AChE - Tanshinone IIA (D) complexes during 600 ps of molecular dynamics simulation.

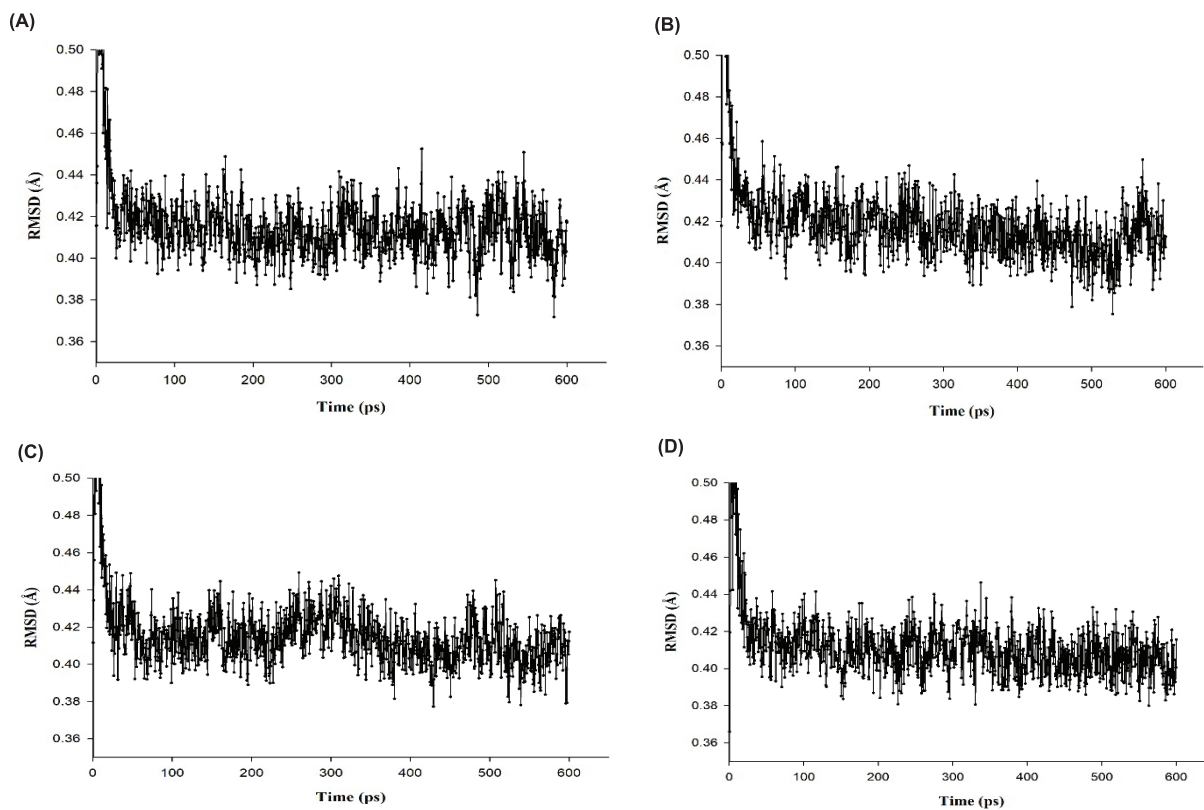


Fig. 10. RMSD of NMDA - Isotanshinone I (A), BACE1 - Isotanshinone I (B), BuChE - Isotanshinone I (C), and AChE - Isotanshinone I (D) complexes during 600 ps of molecular dynamics simulation.

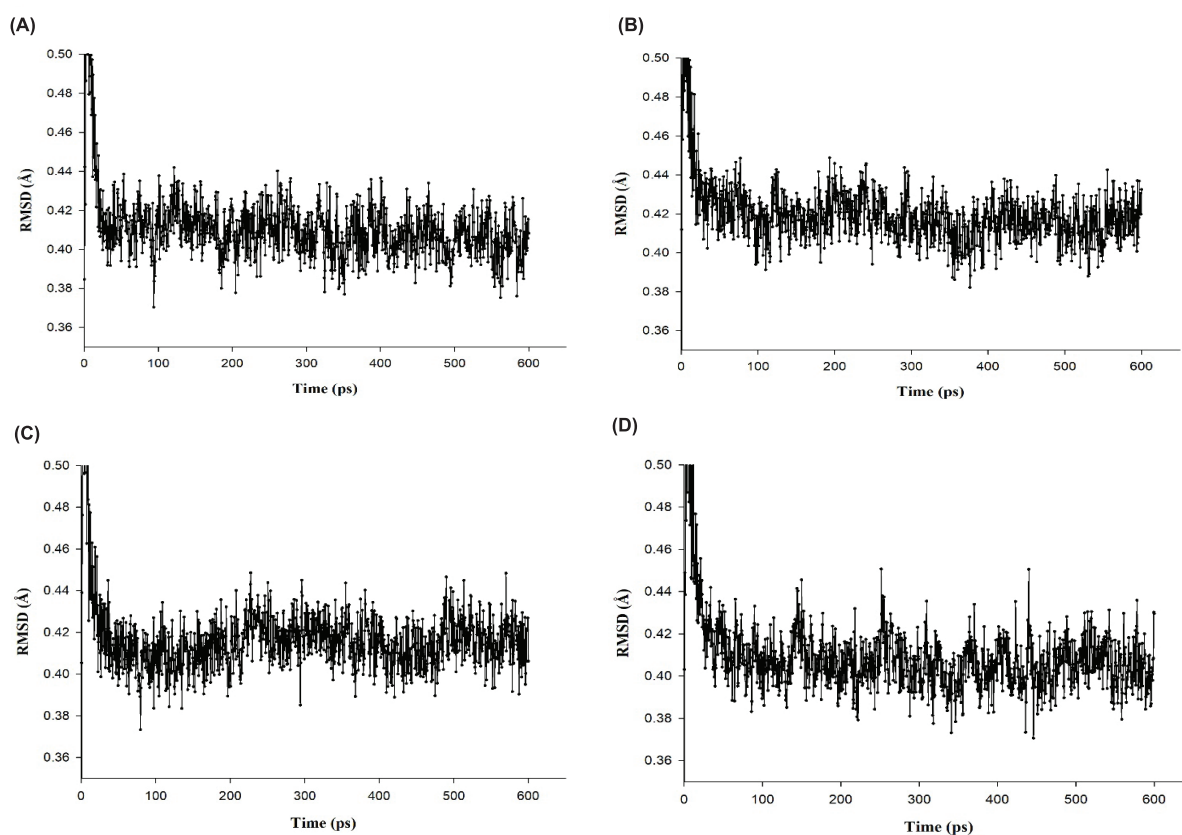


Fig. 11. RMSD of NMDA - Dihydroisotanshinone I (A), BACE1 - Dihydroisotanshinone I (B), BuChE - Dihydroisotanshinone I (C), and AChE - Dihydroisotanshinone I (D) complexes during 600 ps of molecular dynamics simulation.

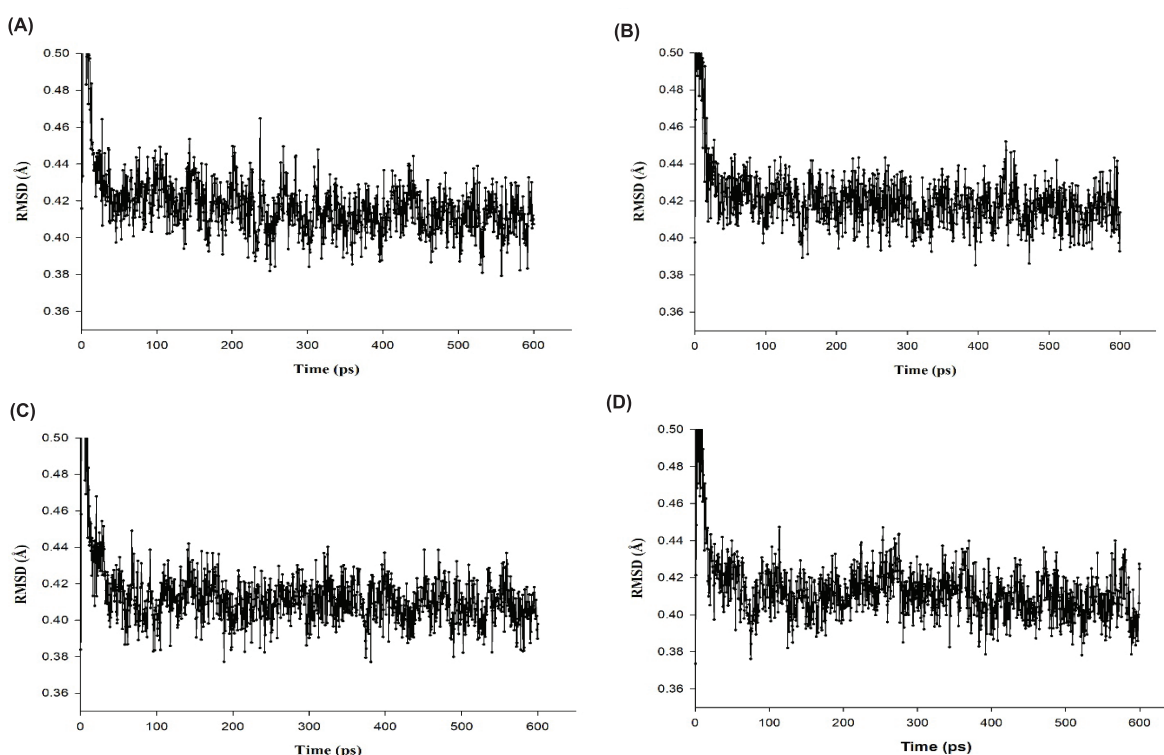


Fig. 12. RMSD of NMDA - Dihydroisotanshinone II (A), BACE1 - Dihydroisotanshinone II (B), BuChE - Dihydroisotanshinone II (C), and AChE - Dihydroisotanshinone II (D) complexes during 600 ps of molecular dynamics simulation.

4. Discussion

Alzheimer's disease, with its complex multifactorial pathophysiology, poses a significant challenge in terms of management. Therefore, it is imperative to explore multiple therapeutic strategies for Alzheimer's disease. Potential targets for Alzheimer's disease treatment include AChE, BuChE, BACE1, and NMDA receptors (NMDARs). This study focuses on compounds derived from *S. miltiorrhiza* that demonstrate the potential to inhibit most, if not all, of these target proteins.

Salvia miltiorrhiza Bunge, commonly known as danshen or red sage, holds a prominent place in traditional herbal medicine. Several studies have indicated that *S. miltiorrhiza* possesses neuroprotective properties relevant to Alzheimer's disease. Aqueous extracts of *S. miltiorrhiza* have demonstrated protection against neurotoxicity induced by Aβ₂₅₋₃₅ in SH-SY5Y cells by reducing oxidative stress and mitigating the mitochondria-dependent apoptotic pathway [16]. Additionally, these extracts have been shown to block NMDA-evoked currents in whole-cell patch-clamp experiments [17].

In our research, we conducted docking simulations of 30 compounds from the medicinal herb *S. miltiorrhiza* with four proteins: AChE, BuChE, BACE1, and NMDAR to evaluate their potential to simultaneously inhibit all these proteins. Following the docking process, six compounds were identified that exhibited the ability to inhibit all four target proteins. Pharmacokinetic and toxicological predictions (ADMET) revealed that five of these six compounds possessed favourable pharmacokinetic properties, could cross the blood-brain barrier, were well-absorbed in the intestine, and did not induce skin irritation. These five compounds were Tanshinone I, Tanshinone IIA, 4-Methylenemiltirone, Isotanshinone I, Dihydroisotanshinone I, and Dihydroisotanshinone II. Furthermore, molecular dynamics simulations indicated that all five of these compounds maintained stable docking poses with the four target proteins. However, some potential risks, such as AMES toxicity, hepatotoxicity, and skin sensitisation, should be considered. Therefore, further *in vivo* or *in vitro* studies are necessary to validate and test the accuracy of these predictions.

Tanshinone I displayed strong interactions with all four target proteins, as evidenced by its negative binding energies with NMDA, BACE1, BuChE, and AChE, measuring -9.4, -10.5, -9.8, and -11.3 kcal/mol, respectively. In addition to the previously mentioned important amino acids of NMDA, this compound also interacted with PHE 250, ARG 131, and PHE 92, binds to BuChE through key amino acids, including PHE 329, SER 198, and GLY 116. Tanshinone I met all the requirements of Lipinski's rule of five. Moreover, it demonstrated the potential to cross the blood-brain barrier (Table 5). However, it exhibited AMES toxicity, as indicated by the ADMET profile.

Tanshinone IIA exhibited the potential to inhibit NMDA, BACE1, BuChE, and AChE due to its free binding energies with these proteins, which were -9.4, -10.3, -10.1, and -11.2 kcal/mol, respectively. In contrast to Tanshinone I, Tanshinone IIA did not exhibit any form of toxicity. Previous research indicated that Tanshinone IIA, at a dose of 1 µg/g, played a neuroprotective role, potentially related to decreased [Ca²⁺] aggregation, alterations in

NMDA receptor expression, and inhibition of calcium transportation [18-20]. It was reported that both Tanshinone I and Tanshinone IIA improved memory deficits induced by scopolamine (1 mg/kg, i.p.) in a mouse model (the passive avoidance test) by enhancing cholinergic signalling [21].

Isotanshinone I potentially inhibited all four target proteins, including NMDA, BACE1, BuChE, and AChE, due to its lower binding energy compared to reference drugs (-9.9, -10.6, -9.9, and -11.1 kcal/mol, respectively). However, according to ADMET profile results, it exhibited both AMES toxicity and hepatotoxicity. Isotanshinone I also interacted with some important amino acids previously identified in other studies, such as its interaction with ASP 32 of BACE1 and various important amino acids of BuChE, including GLY 16, PHE 329, and SER 198.

Dihydroisotanshinone I demonstrated strong interactions with all four target proteins, as evidenced by its negative binding energies with NMDA, BACE1, BuChE, and AChE, measuring -10.3, -10.6, -10.3, and -11.9 kcal/mol, respectively. Similar to Tanshinone I, Dihydroisotanshinone I exhibited AMES toxicity but did not show hepatotoxicity or skin sensitisation. Accumulated studies have primarily focused on the interaction between Dihydroisotanshinone I and AChE, a well-established anti-Alzheimer's disease target. Dihydroisotanshinone I is a high-affinity inhibitor of human AChE (K_i=0.6-0.8 µM). In a non-cell-based enzymatic assay, it inhibited AChE activities with an IC₅₀ of 1.0 and 25 µM. Another study demonstrated that Dihydroisotanshinone I inhibited brain AChE and BuChE with IC₅₀ values of 0.89 and 5.51 µM, respectively. *In vivo*, Dihydroisotanshinone I improved learning and memory impairments in mice induced by scopolamine, partially mediated by AChE inhibition [22].

Dihydroisotanshinone II potentially inhibited NMDA, BACE1, BuChE, and AChE, as indicated by its binding energies with these proteins, which were -10.4, -10.4, -10.3, and -11.1 kcal/mol, respectively. Dihydroisotanshinone II also interacted with PHE 250 of NMDA, an important amino acid mentioned earlier. Similar to Tanshinone IIA, Dihydroisotanshinone II did not exhibit any form of toxicity. It appears that there have been limited studies conducted on the potential of dihydroisotanshinone II in Alzheimer's disease treatment. Therefore, based on the results of this study, dihydroisotanshinone II could be a promising bioactive compound that warrants further investigation for the treatment of Alzheimer's disease.

It is noteworthy that the interactions between these five compounds and the active site residues significantly contributed to their binding energy with target proteins. Firstly, these compounds also interacted with important amino acids similar to those identified in previous studies. Secondly, hydrogen bonds and π -stacking interactions were the most prominent interactions observed in ligand-protein complexes in this study. Hydrogen bonds, as reported in previous studies, are the second most frequent type of interaction in various ligand-protein complexes [23]. They are known to significantly enhance the binding affinity between ligands and proteins. In this study, compounds formed hydrogen bond interactions with certain amino acids, such as Tanshinone I with ARG 131 of NMDA or Tanshinone I with ASP 62 of BACE1, and

SER 198. In addition to hydrogen bonds, π -stacking interactions were also observed in many ligand-protein complexes in this study. π -Stacking interactions also referred to as aromatic interactions, play a crucial role in ligand-protein recognition and drug design. They rank as the third most frequent interactions in ligand-protein complexes [23]. Evidence suggests that the formation of π -stacking interactions enhances the binding affinity of inhibitors for their targets. In our study, compounds exhibited π -stacking interactions with certain amino acids, such as Tanshinone IIA with TYR 184. π - π stacking interactions can be categorised into three types: edge-to-face stacked (T-shaped), offset stacked, and face-to-surface stacked [24]. T-shaped π - π stacking interactions were observed in various ligand-protein complexes in this study. For example, Tanshinone I formed T-shaped π - π stacking interactions with TYR 60 and TRP 277. This interaction was also observed in the Dihydroisotanshinone II-NMDA complex. Besides hydrogen bonds and π - π stacking interactions, compounds in this study also exhibited various other interactions with target proteins, such as amide- π stacked interaction, van der Waals, or pi-alkyl interaction. All of these interactions contributed to the binding energy of ligand-protein complexes. However, it should be noted that these interactions are not the sole determinants of binding affinity.

5. Conclusions

In this study, we found five natural compounds in *S. miltiorrhiza* Bunge with significant potential for Alzheimer's disease inhibition, utilising molecular docking and molecular dynamics simulations. All five compounds, namely Tanshinone I, Tanshinone IIA, Isotanshinone I, Dihydroisotanshinone I, and Dihydroisotanshinone II, exhibit drug-like properties, good absorption capabilities, and the potential to penetrate the blood-brain barrier.

CRedit author statement

Tung Bui Thanh: Conceptualisation, Design, Supervision, Critical Reviews; Trang Vu Dai: Design, Resources, Literature Search, Writing; Huong Le Thi: Resources, Literature Search, Writing.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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The occurrence of potential pathogenic antibiotic-resistant bacteria in the marine environment in Ha Long Bay, Vietnam

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Abstract:

This study aimed to investigate the occurrence of potential pathogenic bacteria from surface water samples collected at 30 stations along Ha Long Bay, Vietnam. Among 116 bacterial isolates identified, the composition analysis indicated 24 distinct bacterial genera, with *Vibrio* (29.3%), *Pseudomonas* (23.3%), *Staphylococcus* (8.6%), and *Bacillus* (6.0%) being the most dominant. The taxonomic analysis revealed 61/116 (52.6%) isolates as potential pathogens (risk group 2) belonging to 21 bacterial species. Potential pathogens were detected in 28/30 (93.3%) sampling stations, with the most common pathogens including *Vibrio alginolyticus* (n=25), *Vibrio parahaemolyticus* (n=5), *Enterobacter cloacae* (n=3), *Pseudomonas mendocina* (n=3), *Aeromonas hydrophila* (n=2), *Enterobacter asburiae* (n=2), *Klebsiella oxytoca* (n=2), *Bacillus cereus* (n=2), and *Staphylococcus haemolyticus* (n=2). The selected pathogens were highly resistant to Amoxicillin (100%), Piperacillin (100%), Amoxicillin + Clavulanate (85.7%), Ticarcillin (71.4%), Cefoxitin (54.4%), Kanamycin (50%), and Fosfomycin (50%), and less resistant to Trimethoprim + sulfamethoxazole, Fusidic Acid, Tetracycline, Erythromycin, Pristinamycin, Dalacin, and Rifampicin (<25%), and susceptible to the remaining antibiotics. Notably, 12/23 (52.2%) isolates were resistant to at least three antibiotics and were multidrug-resistant bacteria. Our findings underline that the occurrence of pathogenic antibiotic-resistant bacteria in the marine environment of Ha Long Bay presents a potential threat to human health. Further genomic surveillance studies are necessary to manage and control the emergence and spread of pathogenic-resistant bacteria in this area.

Keywords: antibiotic resistance, Ha Long Bay, marine environment, multidrug resistance, pathogenic bacteria.

Classification numbers: 3.5, 5.3

1. Introduction

Infectious diseases (IDs) and emerging infectious diseases (EIDs) affecting humans and wildlife are a significant and growing threat to health, the economy, resources, and biodiversity on a global scale [1, 2]. Each year, infectious diseases kill millions of people worldwide, resulting in an economic cost of \$ billions in the United States alone [3]. For instance, it is estimated about 7.15 million waterborne illnesses occurs in the United States annually resulting in 6,630 deaths and with the indirect healthcare costs of US \$3.33 billion [4]. IDs and EIDs can also greatly influence ecosystem and wildlife health, contributing to population declines and sometimes extinction [5, 6]. Bacteria represent the pathogens more often (54.3%) involved in EID events in the last decades [7, 8]. IDs and EIDs are inherently difficult to study for various reasons, including the fact that their etiologic agents are poorly characterized. Therefore, identifying high-risk pathogens ranks among the greatest challenges facing modern science; critical to this effort is the need to predict geographic locations where disease outbreaks are likely to occur, identify the reservoir hosts from which pathogens will emerge, and predict host species at greatest risk of pathogen-mediated declines.

Waterborne infections are strongly associated with high morbidity and mortality globally. Previous studies [7, 8] have reported the occurrence of waterborne pathogens and antibiotic resistance in surface water, including coliforms, *Pseudomonas* sp., *Vibrio* sp., *Salmonella* sp., *Campylobacter* sp., *Acinetobacter* sp., *Aeromonas* sp., and new and re-emerging pathogens worldwide [9-14]. Unfortunately, most research on IDs and antimicrobial resistance has focused on inland systems with comparatively little effort directed towards marine habitats. However, marine environments can function as transmission foci for pathogens or antimicrobial resistance because seawater carries a large quantity of microorganisms, some harmful to humans, marine species, and ecosystems. Additionally, the aquatic life histories of many vectors and intermediate hosts can affect species interactions and trigger disease emergencies [9-12]. There is also evidence of microbial and antimicrobial resistance dispersal mechanisms between marine and terrestrial biomes. The direct transmission of human or marine livestock pathogens from marine habitats is therefore not negligible, but the major risk to public health and aquaculture production is assumed to be the development of a reservoir of resistance genes transferable to human or marine livestock pathogens and the emergence of pathogens with newly acquired antibiotic resistance. This risk increases in developing regions

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where the treatment of hospital and household sewage, important sources of pathogens, antibiotic-resistant genes, and antibiotics in the coastal environment, is limited (often non-existent) and where intensive aquaculture has developed. The risk of infection linked to seawater is therefore a major world health challenge [9-12].

Ha Long Bay is a spectacular scenic spot nominated as one of the seven world heritage sites by UNESCO in 1994. Located in Quang Ninh province in north-eastern Vietnam, Ha Long Bay covers approximately 65,650 ha with 1,133 islands and islets, featuring several creeks and arches, predominantly composed of limestone (<https://whc.unesco.org/en/list/672/>). However, the water environment in Ha Long Bay is polluted with oil, coliforms, heavy metals, chlorinated pesticides, organic matter, and plastics due to the rise of industrial, agricultural, domestic waste, and tourism activity. Intensive marine aquaculture is also one of the most important activities carried out in this area. In 2023, the total aquaculture area of Quang Ninh province reached 42,292 hectares with total aquaculture products reaching 175,324.6 tons. The seafood production value was 6,943 billion VND, accounting for nearly 50% of the value of the province's agriculture and rural sectors. All these activities could make Ha Long Bay a reservoir for the emergence and spread of potential pathogens in the marine environment to the human population. Unfortunately, little is known about its effects on microbial biodiversity, particularly the emergence and transmission of potential pathogens. In this context, this study aimed to investigate the occurrence of potential pathogenic antibiotic-resistant bacteria in marine water along Ha Long Bay, which are fundamental elements to reveal the water quality and risk to human health.

2. Materials and methods

2.1. Sample collection

Following the mission of the Centre for Marine Environmental Monitoring and Analysis, the Vietnam People's Navy, the quality of seawater in Ha Long Bay, Quang Ninh province, is assessed to evaluate potential risks to human health. This study was responsible for investigating potential pathogenic bacteria circulating in Ha Long Bay. The selected 30 sampling stations covered the entire coastal area within Ha Long Bay. The seawater samples were taken from 30 different stations (HL1-HL30) with geographical coordinates between 20°50'00.1" N - 20°56'05.7" N latitudes and 106°59'52.4" E - 107°12'25.3" E longitudes. At each sampling station, surface water was collected in autoclavable 500-ml polypropylene bottles with screw caps and kept cold at 4°C for microbial analysis.

2.2. Bacterial isolation

In this study, six culture media were selected for the isolation of potential pathogenic bacteria, including Tryptic Soy Agar (TSA, Sigma-Aldrich, Germany) for the isolation and cultivation of total cultivable bacteria, MacConkey Agar (MAC, Oxoid, UK) for the isolation and selection of Enterobacteriaceae species, Mannitol

Salt Phenol Red Agar (MSPR, Sigma-Aldrich, Germany) for the isolation and selection of Staphylococcus, Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS, Merck, Germany) for the isolation and selection of *Vibrio* species, Salmonella-Shigella Agar (SS, Sigma-Aldrich, Germany) for the isolation and selection of Salmonella and Shigella species, and Klebsiella ChromoSelect Agar (KCS, Sigma-Aldrich, Germany) for *Klebsiella* species. For each sample, 1 ml of seawater was spread on a petri plate followed by incubation at 35°C for 18-24 hours. Bacterial colonies were primarily differentiated by morphological characteristics and then purified by sub-culturing using the streaking plate method. All purified isolates were maintained in TSB with 50% glycerol solution and stored at -80°C.

2.3. Bacterial identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

The bacterial isolates were rapidly identified using the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) Biotyper system (Bruker, Germany). Briefly, a full loop of pure colonies was suspended in 300 µl of 70% ethanol, and the cells were harvested by centrifugation at 12,857 rcf (relative centrifugal force) for 5 minutes. Cell pellets were incubated with a mix containing 50 µl of 70% formic acid and 50 µl of acetonitrile for 5 minutes. The cell suspension was centrifuged at 12,857 rcf for 5 minutes, and then 1 µl of the clear supernatant was transferred onto a 96-spot MALDI target plate and air-dried at room temperature. Each spot was overlaid with 1 µl of MALDI matrix and dehydrated for 15 minutes, followed by processing on the MALDI-TOF Biotyper system. The protein spectra obtained were analysed by BioTyper 3.0 system software and compared against the CDC database for bacterial identification. A log (score) of MALDI Biotypes above 1.8 indicates a valid identification, while a score under 1.7 suggests poor performance and represents uncertain identification.

2.4. Bacterial identification by 16S rDNA gene sequencing

For isolates that were not identified using the MALDI-TOF MS system, they were further identified by 16S rDNA gene sequencing. A 500-bp DNA fragment of the 16S rDNA gene was amplified with the primers 16S-8F (Forward): 5'-GCTGGATAGGTTAAGGGCGG-3' and 16S-518R (Reverse): 5'-ATTACCGCGGCTGCTGG-3'. The thermal cycling parameters included a 1-minute denaturation at 95°C followed by 35 cycles of 15 seconds at 95°C, 15 seconds at 55°C, and 10 seconds at 72°C. PCR amplicons were examined by agarose gel electrophoresis. After that, the PCR amplicons were purified and sequenced at the First Base Company (Singapore). The obtained DNA sequences were processed using BioEdit software and then searched on the GenBank, National Center for Biotechnology Information (NCBI) database using the nucleotide BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for bacterial identification based on sequence comparison.

2.5. Antibiotic susceptibility testing

The antibiotic susceptibility was determined for selected potential pathogenic bacteria on Mueller Hinton Agar (MHA, Sigma, Germany) using the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), M100 version: performance standards for antimicrobial susceptibility (CLSI 2020). Specifically, *Aeromonas* species were tested for 6 antibiotics including fusidic acid (10 µg), Cefepime (30 µg), Ceftazidime (10 µg), Ciprofloxacin (5 µg), Trimethoprim + sulfamethoxazole (25 µg), and Levofloxacin (5 µg). *Vibrio* species were examined for 7 antibiotics including Cefotaxime (5 µg), Ciprofloxacin (5 µg), Trimethoprim + sulfamethoxazole (25 µg), Erythromycin (15 µg), Meropenem (10 µg), Piperacillin + Tazobactam (36 µg), and Tetracycline (30 µg). *Enterobacter* and *Klebsiella* (Enterobacteria family) were tested for 16 antibiotics including nalidixic acid (30 µg), amikacin (30 µg), amoxicillin + clavulanic acid (30 µg), amoxicillin (20 µg), Cefotaxime (5 µg), Cefoxitin (30 µg), Ceftazidime (10 µg), Ciprofloxacin (5 µg), Trimethoprim + sulfamethoxazole (25 µg), Ertapenem (10 µg), Gentamicin (10 µg), Imipenem (10 µg), Levofloxacin (5 µg), Piperacillin (30 µg), Ticarcillin (75 µg), and Tobramycin (10 µg). *Bacillus cereus* and *Staphylococcus haemolyticus* were tested for 14 antibiotics including Gentamicin (10 µg), Trimethoprim + Sulfamethoxazole (25 µg), Tobramycin (10 µg), Tetracycline (30 µg), Ofloxacin (5 µg), Fosfomycin (15 µg), Erythromycin (15 µg), Kanamycin (30 µg), Rifampicin (5 µg), fusidic acid (10 µg), Pristinamycin (15 µg), Cefoxitin (30 µg), Dalacin (10 µg), and Teicoplanin (30 µg). The sizes of the inhibition zones were interpreted to determine whether the microorganism was susceptible, intermediately resistant, or resistant to each antibiotic according to the CLSI guidelines. Multiple antibiotic-resistant phenotypes were reported for isolates that showed resistance to at least 3 antibiotics. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as quality controls for all tests.

3. Results

3.1. Bacterial isolation and purification

A total of 280 bacterial colonies were isolated from the six culture media according to their morphological characteristics (Fig. 1A). Most were isolated from TSA (n=94, 33.6%), followed by MAC (n=76, 27.1%), TCBS (n=47, 16.8%), and MRSP (n=43, 15.4%). Only a few bacterial colonies were recovered from KCS and SS media (5.7% and 1.4%, respectively). Overall, 14 out of 30 sampling spots yielded ≥10 bacterial colonies (Fig. 1B). Specifically, the number of bacterial isolates was highest in stations HL1 (n=13), HL2 (n=12), HL28 (n=12), HL6, HL21, HL23, HL29, and HL30 (each station, n=11), while fewer bacteria were isolated in stations HL15 (n=5), HL18, HL22, and HL25 (each station, n=7). The low number of bacteria isolated suggests that the culture media may not be suitable for the growth and survival of marine bacteria.

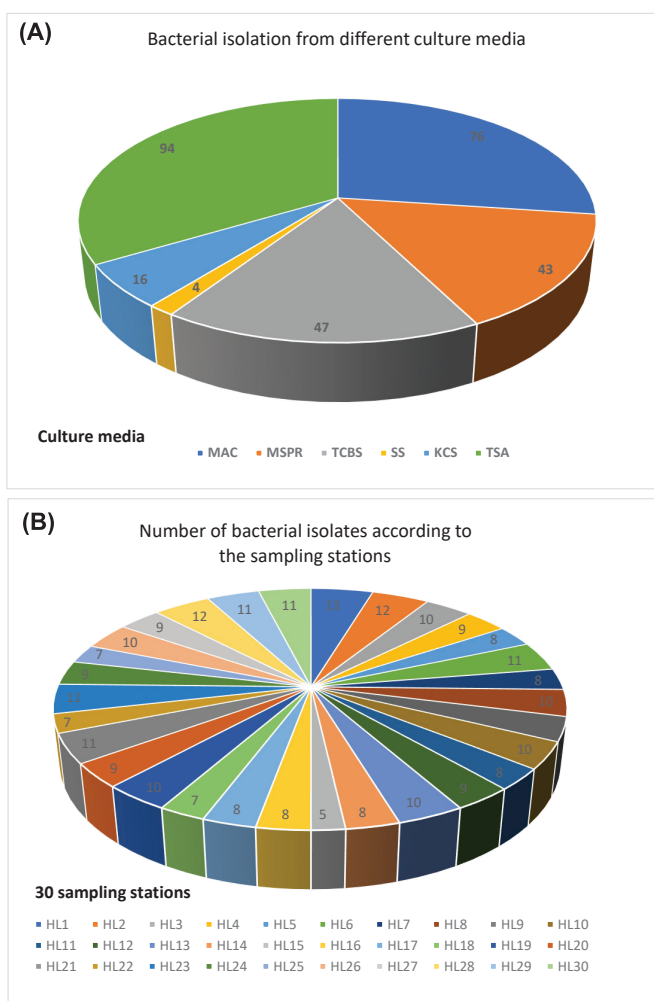


Fig. 1. Distribution of bacterial isolates according to the selected culture media (A) and sampling stations (B).

3.2. Identification of potential pathogenic bacteria

Among the 280 bacterial isolates, 85 could not be regrown; thus, 195 were subjected to taxonomic identification by MALDI-TOF MS. Since these bacterial isolates originated from the marine environment, the media used and culture conditions might not be suitable for their growth and survival. After removing duplicate isolates within each sampling spot, 116 distinct isolates were available for taxonomic analysis. The bacterial identification results showed high diversity in the marine water environment in Ha Long Bay. These isolates belonged to 24 different bacterial genera, with *Vibrio* being the most dominant (n=34, 29.3%), followed by *Pseudomonas* (n=27, 23.3%), *Staphylococcus* (n=10, 8.6%), and *Bacillus* (n=7, 6.0%). The remaining genera were less common, accounting for less than 3% of total isolates. *Vibrio* was widely distributed in Ha Long Bay, found in 26 out of 30 (86.7%) sampling stations, followed by *Pseudomonas* (19/30, 63.3%), *Staphylococcus* (8/30, 26.7%), and *Bacillus* (7/30, 23.3%).

The 116 isolates were further identified as 54 distinct bacterial species. The most common bacterial species were

Vibrio alginolyticus (n=25, 21.5%), followed by *Pseudomonas stutzeri* (n=10, 8.6%), *Pseudomonas* sp. (n=6, 5.2%), and *Vibrio parahaemolyticus* (n=5, 4.3%). Notably, 21/54 (38.9%) identified bacterial species were recognised as potential human pathogenic bacteria according to the classification of the risk group by ZKBS Germany (<https://www.zkbs-online.de/ZKBS/DE/Datenbanken/Organismen>) (Fig. 2). *Vibrio alginolyticus* was found in 25/30 (83.3%) sampling stations, and other potential pathogens were sporadically distributed in Ha Long Bay. Overall, potential pathogens were detected in 28/30 (93.3%) sampling stations, except for stations HL22 and HL26 (Fig. 2).

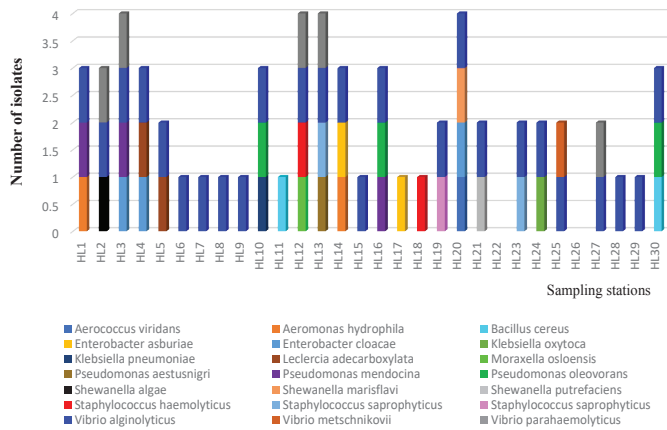


Fig. 2. Distribution of potential pathogenic bacteria according to the 30 sampling stations.

3.3. Antibiotic susceptibility testing

Among the gram-negative pathogenic bacteria isolated, *A. hydrophila* (n=2), *Enterobacter asburiae* (n=2), *Enterobacter cloacae* (n=3), *Klebsiella oxytoca* (n=1), *Klebsiella pneumoniae* (n=1), *Vibrio alginolyticus* (n=5), and *Vibrio parahaemolyticus* (n=5) were selected for evaluating susceptibility to selected antibiotics. *A. hydrophila* HL1 was sensitive to five antibiotics and was only intermediately resistant to ceftazidime. *A. hydrophila* HL14 was sensitive to three antibiotics but was resistant to Levofloxacin and intermediately resistant to Ciprofloxacin and Trimethoprim + Sulfamethoxazole. All seven enteric bacterial strains (*Enterobacter asburiae*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*) were resistant to 3-5 antibiotics. Specifically, they were resistant to Amoxicillin and Piperacillin (7/7, 100%), followed by Amoxicillin + Clavulanate (6/7, 85.7%), Cefoxitin and Ticarcillin (5/7, 71.4%). Nevertheless, all these strains were sensitive to antibiotics belonging to aminoglycosides, quinolones, and carbapenems. Finally, all 10 *Vibrio* isolates were sensitive to the seven antibiotics tested.

Regarding potential pathogenic gram-positive bacteria, *Bacillus cereus* (n=2) and *Staphylococcus haemolyticus* (n=2) were selected for phenotypic susceptibility testing towards 14 antibiotics. Specifically, *B. cereus* HL11 and *B. cereus* HL30 were intermediately resistant/resistant to nine antibiotics, belonging to at least five antibiotic groups. *S. haemolyticus* HL12 and *S.*

haemolyticus HL18 were also intermediately resistant/resistant to five and four antibiotics, respectively, belonging to four antibiotic groups.

Table 1. Antibiotic-resistant profiles of the selected bacterial isolates.

No.	Bacterial species	Isolates sensitive to all antibiotics	Isolates intermediately resistant or resistant to ≤ 2 antibiotics	Isolates intermediately resistant or resistant to multiple antibiotics (n ≥ 3)	Total tested isolates
1	<i>Aeromonas hydrophila</i>	0	1	1	2
2	<i>Enterobacter asburiae</i>	0	0	2	2
3	<i>Enterobacter cloacae</i>	0	0	3	3
4	<i>Klebsiella oxytoca</i>	0	0	1	1
5	<i>Klebsiella pneumoniae</i>	0	0	1	1
6	<i>Vibrio alginolyticus</i>	5	0	0	5
7	<i>Vibrio parahaemolyticus</i>	5	0	0	5
8	<i>Bacillus cereus</i>	0	0	2	2
9	<i>Staphylococcus haemolyticus</i>	0	0	2	2
Total		10	1	12	23

The antibiotic-resistant profiles showed that 12/23 (52.2%) bacterial isolates tested were multidrug-resistant phenotypes (Table 1). These multidrug-resistant bacteria included *A. hydrophila*, *E. asburiae*, *E. cloacae*, *K. oxytoca*, *K. pneumoniae*, *B. cereus*, and *S. haemolyticus*. Resistance to at least 18 out of 29 antibiotics tested was detected (Fig. 3). The selected pathogens were completely resistant to Amoxicillin and Piperacillin (100%). A high proportion of bacterial isolates were resistant to Amoxicillin + Clavulanate (85.7%), Ticarcillin (71.4%), Cefoxitin (54.4%), Kanamycin (50%), and Fosfomycin (50%). Resistance to Trimethoprim + sulfamethoxazole, Fusidic Acid, Tetracycline, Erythromycin, Pristinamycin, Dalacin, and Rifampicin was also detected at a low proportion (<25%). These bacterial isolates were still susceptible to 10 antibiotics including Amikacin, Tobramycin, Gentamicin, Amikacin + Tobramycin, Ciprofloxacin, Levofloxacin, Trimethoprim + Sulfamethoxazole, Nalidixic acid, Ofloxacin, and Ertapenem.

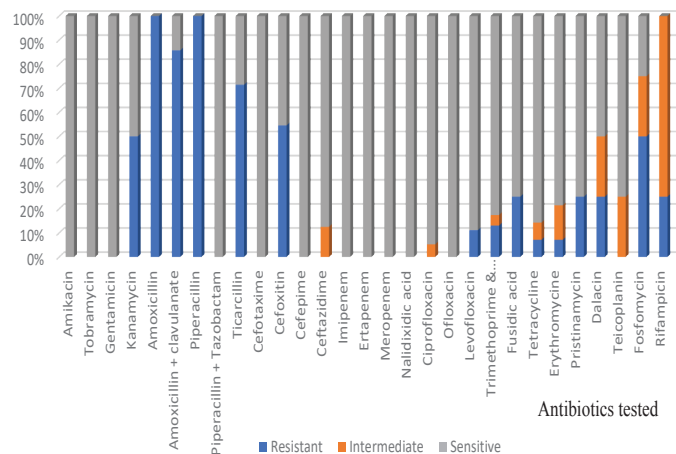


Fig. 3. Proportion of antibiotic-resistant bacteria in the marine water in Ha Long Bay.

4. Discussion

Pathogenic bacteria in marine environments can present a major threat to human health, with bathing and consumption of seafood being the major transmission routes to humans [12, 13]. The primary sources of microbial contamination in marine environments and transmission mechanisms are the faecal-oral route, involving sewage, runoff, river discharge, groundwater seepage, and sewage sludge disposal [14, 15]. The survival and growth of potential pathogenic bacteria in marine environments are greatly influenced by human activities and environmental factors, mainly triggered by man-made eutrophication. Unfortunately, the health threat posed by pathogenic bacteria in marine environments is not yet well understood [16-18]. Therefore, the present study investigated the distribution and antibiotic resistance of potential pathogenic bacteria in the marine environment of Ha Long Bay, Vietnam.

Among the 116 identified isolates, 21 bacterial species were potential pathogens to humans and animals. Among them, *Vibrio* (29.3%), *Pseudomonas* (23.3%), *Staphylococcus* (8.6%), and *Bacillus* (6.0%) were the most dominant. It is worth noting that these pathogens were found in 93.3% of the studied areas. In nature, *Vibrio* and *Aeromonas* species typically exist at low abundance in marine environments but represent a reservoir from which epidemics can arise [19, 20]. These pathogens are often found in marine environments with high-intensity aquaculture activities and are considered indicators of water quality in aquaculture. Particularly, *Vibrio* strains and their associated infections are on the rise globally due to increasing sea surface temperatures, representing an emergent threat to human and animal health, and causing significant economic losses in the aquaculture industry worldwide [20, 21]. In the present study, *Vibrio* species were found in 27/30 sampling stations in Ha Long Bay. The absence of *Vibrio* in the remaining 3 stations could be explained by the loss of *Vibrio* strains during the isolation and culture steps. Among marine *Vibrio* species, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* can cause serious seafood poisoning, wound and ear infections, with fatal diseases including necrotising soft-tissue infections, bacteraemia, septic shock, and multiple organ failures. These two pathogens are considered the major causative agents for seafood poisoning outbreaks in Asia [22-24]. Additionally, *Aeromonas hydrophila*, a well-known opportunistic aquatic pathogen, is now recognised as an emerging foodborne pathogen capable of causing human gastroenteritis, with its main reservoir being the aquatic environment. Foodborne outbreaks associated with *Aeromonas hydrophila* have been reported worldwide [25, 26]. This pathogen can be transmitted via the faecal-oral route through direct consumption of contaminated water or food. Notably, this pathogen can survive at very low temperatures, meaning frozen seafood contaminated with *Aeromonas hydrophila* still poses a potential risk of food poisoning. Furthermore, *Bacillus cereus*, a common food-poisoning pathogen, along with other human pathogenic species of Enterobacteriaceae (*Enterobacter asburiae*, *Enterobacter cloacae*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae*), were

also detected in Ha Long Bay. Our findings emphasise the possible harm to human health that eating raw seafood poses, as it can lead to foodborne infections and microbiological food poisoning.

Recently, antibiotic-resistant bacteria have become a severe issue that endangers people's lives globally and places a heavy financial strain on the health system [27]. In this study, resistance to amoxicillin and piperacillin was the most commonly detected (100%), followed by amoxicillin + clavulanate (85.7%), ticarcillin (71.4%), cefoxitin (54.4%), kanamycin (50%), and fosfomycin (50-85.7%). Resistance to trimethoprim + sulfamethoxazole, fusidic acid, tetracycline, erythromycin, pristinamycin, dalacin, and rifampicin was less commonly detected (<25%). It has been reported that beta-lactams, tetracycline, erythromycin, and quinolones are commonly used in fisheries for the prevention and treatment of bacterial infections [28]. Therefore, the presence of *Aeromonas*, *Enterobacter*, *Klebsiella*, *Bacillus*, and *Staphylococcus* isolates resistant to at least one of these antibiotics is consistent with previous studies [11, 29, 30]. For *Vibrio* species, previous studies have reported that the prevalence of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolates resistant to antibiotics is often relatively low. Nevertheless, the high prevalence of antibiotic-resistant genes in marine environments [30, 31], combined with the high incidence of bacterial infections in aquaculture and the overuse of antibiotics and horizontal gene transfer mechanisms, would promote the selection and transmission of antibiotic-resistant bacteria in this area. Finally, we found that 52.3% of the selected bacterial isolates were multidrug-resistant phenotypes. These pathogens exhibited resistance to 3 to 9 antibiotics. The presence of multi-resistant bacteria in these samples highlights the potential risks to the health of marine organisms and humans. Additionally, this coastal area also receives discharges from urban canals and rivers, meaning human pathogens and AMR on land can be transported to the sea. Therefore, it is necessary to conduct monitoring and genomic surveillance studies to rapidly respond to the emergence of new pathogens in this important marine environment.

5. Conclusions

The wide distribution of potential human bacterial pathogens and the occurrence of multidrug-resistant strains in the surface water environment of Ha Long Bay pose a threat to the environment and community health. Thus, further studies should focus on monitoring the distribution, prevalence, and transmission pathways of antibiotic-resistant genes in this area to provide insight into the risk of the emergence and spread of multidrug-resistant bacteria. This knowledge can contribute to developing appropriate management and policy measures to reduce the human health risks associated with bathing and consuming seafood in Quang Ninh province.

CRedit author statement

Nguyen Quang Huy: Methodology, Experiment, Data analysis, Writing, Editing; Pham Quynh Trang: Experiment, Data analysis; Nguyen Thi Loi: Sample Collection, Data analysis, Writing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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Comparing the efficacy of online vs. offline methods in modulating emotional responses through mental stress tasks

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Abstract:

Emotion regulation and stress represent two complex, intertwined processes. Yet, there is a paucity of studies examining the effect of stress on emotional regulation among Vietnamese students, specifically the influence of online and offline experimental conditions. This study investigates these dynamics by subjective assessment of the emotional responses of the subjects participating in this study. The research was structured in three primary phases: preliminary, online (conducted via the Internet), and offline (conducted in person) experiments. The preliminary phase (N=16) aimed to validate the appropriateness of the International Affective Picture System (IAPS) picture library and emotional stimulation videos from previous studies. Participants in the online (N=30) and offline (N=45) experiments were divided into control and stress groups, distinguished by the difficulty and urgency of the mental arithmetic task (MAT). Participants were then asked to view photos and videos and to rate their subjective feelings using the Self-assessment Manikin (SAM). The online experimental session revealed that subjects under acute stress exhibited heightened sensitivity, as indicated by increased valence scores in the positive and neutral groups, and decreased scores for negative imagery (Positive: MD=0.292, $p=0.0113$; Neutral: MD=0.245, $p=0.001$; Negative: MD=-0.435, $p<0.0001$). An increase in arousal scores in the stress group was also observed for positive ($p<0.0001$) and neutral sets ($p<0.0001$). However, these findings were not replicated in the offline experiment session. In conclusion, the IAPS pictures have proven suitable for emotional studies among Vietnamese students.

Keywords: emotional response, offline experiment, online experiment, stress, Vietnamese student.

Classification number: 3.6

1. Introduction

Emotion regulation, the process by which individuals modulate their emotional experiences and expressions through various automatic and controlled physiological, behavioural, or cognitive processes, is critical for adaptive function and overlaps with stress-coping mechanisms throughout life [1]. Particularly, stress can negatively influence an individual's emotional state, often leading to an increase in rumination [2-4], which in turn increases the possibility of enhanced negative emotions [4]. Persistent negative emotions can result in mental disorders, thereby decreasing the quality of life. However, acute stress can also bring about some positive emotions [5, 6], underlying the importance of understanding the relationship between stress and emotional regulation. Additionally, the COVID-19 pandemic has underscored the importance of conducting experiments online, particularly in light of social distancing measures [7]. Indeed, social distancing during the pandemic can challenge cognitive performance via multiple elements [8], suggesting that participants' performance could vary between online and offline experimental settings, particularly considering the changes brought about during and after periods of social distancing. This study aims to clarify any potential differences between online and offline experimental environments.

University students represent one of the most stress-prone demographics within the Vietnamese population, with many factors contributing to this, including academic stress [9], familial

expectations, and peer pressure. Moreover, a high prevalence of mental disorders, such as stress, anxiety, and depression, has been reported among Vietnamese university students [10]. Stress can have detrimental effects on a student's lifestyle, such as causing reductions in cognitive performance [11], which often culminates in poor academic performance [12]. Stress also impacts the three components of emotions: arousal (ranging from a state of calm to excitement or agitation post-stimulus) [13-15], valence (indicating a spectrum from extremely negative to highly positive, or the pleasantness of the stimulus) [15, 16], and dominance (the capacity to control one's emotions following a stimulus) [16]. Notably, stress is responsible for an increase in emotional distress, leading to an increase in arousal [13-15] and a decrease in valence [15, 16]. Despite these findings, no research to date has specifically focused on these three components of emotion (valence, arousal, and dominance) among Vietnamese university students following exposure to stressors. Furthermore, there is a lack of suitable picture and video libraries targeting this demographic. As such, there is a pressing need to assess the emotional responses of Vietnamese university students after encountering acute stress. Such understanding would provide valuable insights into how stress can affect emotional responses, especially within the Vietnamese student population.

Consequently, this study will comprise three experimental stages: preliminary, online, and offline. For the preliminary phase, a set of images from the International Affective Picture System (IAPS) libraries and various categories of videos will be used to determine

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their suitability for evaluating the emotional responses of Vietnamese university students. This evaluation will be critical not only for this project, but also future research related to the emotional responses of Vietnamese students. Subsequently, the selected library of pictures and videos will be used in both the offline and online phases to evaluate the emotional response of Vietnamese students, focusing on changes in valence, arousal, and dominance after experiencing acute stress induced by Arithmetic Tasks.

2. Subject and methodology

2.1. Study design

This study comprised three main phases: the preliminary, online, and offline phases (Fig. 1). In the preliminary phase, volunteers completed experiments and a subsequent SAM survey to select appropriate pictures for the emotional trigger task. These images and videos were then used to develop the platform for the main experiment (both offline and online phases), which was divided into three parts: 1) Stress induction or control protocol via the MAT; 2) Emotional triggers involving ninety pictures or five videos; 3) A mid-experiment Stroop Effect Task to assess the attention of the participants. The study's second phase, conducted remotely (online) during the COVID-19 quarantine via Zoom and AnyDesk, spanned two months. The final phase, carried out in person (offline) at the International University, Vietnam National University, lasted six months. At the start of both the online and offline phases of the main experiment, participants were asked to fill out a pre-screened questionnaire to record their current physical and mental states. The video sessions were scheduled two to three weeks after the picture sessions. The platform was displayed on the computer screen via PsychoPy software, with participants responding to the task by typing on the keyboard.

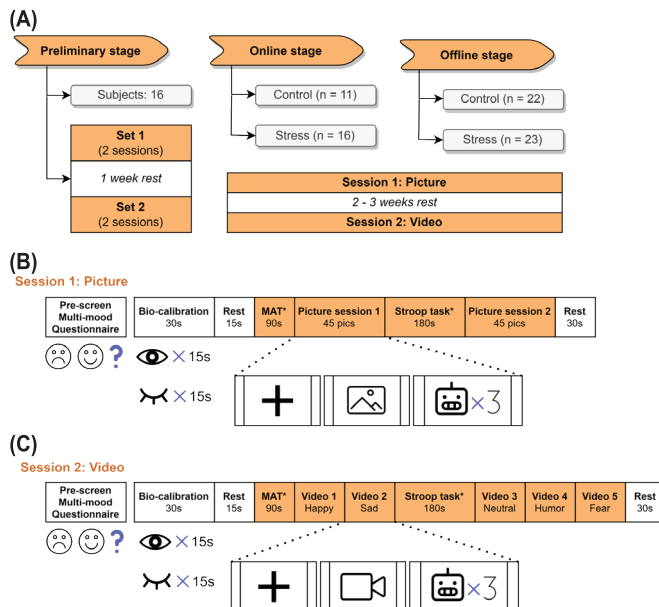


Fig. 1. The three-phase experimental design: preliminary, online, and offline (A); platform design of the first experiment with 90 pictures inducing positive, neutral, or negative emotions (B); the design of the second experiment with five videos inducing pleasure, sadness, neutral, amusement, and fear (C).

2.2. Participants

Ninety-five subjects were recruited to participate in this study, including sixteen subjects for the preliminary phase, thirty subjects for the online phase, and forty-nine subjects for the offline phase. All participants were college students of ages between 18 and 25 years old. The demographic information of the subjects is summarized in Table 1. Before their participation, subjects were screened to ensure consistency in physical and mental health in the same manner as in similar studies [17, 18]. All participants signed an informed consent form before participating in the experiment. The study protocol was approved by the School of Biomedical Engineering, International University, Vietnam National University - Ho Chi Minh City.

Table 1. Demographic information of participants in the three phases.

Phase	Prelim (N=16)	Online (N=30)	Offline (N=45)
Group	N/A	Control Stress	Control Stress
N	16	11 19	22 23
Gender (M/F)	6/10	1/10 4/15	4/18 9/14
Age (Mean±SD)	21.45±1.95	21.09±1.04 21.32±1.42	20.33±0.92 20.48±1.6

M: male; F: female; SD: standard deviation; N/A: not applicable.

2.3. Picture and video selection

This study selected images and videos used as emotional stimuli based on previous studies on emotional elicitation and responses [19-21]. The pictures for this study were sourced from the IAPS database (<http://csea.php.ufl.edu/media/iapsmessage.html>), a renowned resource offering approximately 900 images for the evaluation of human emotions [22]. The emotional rating of pictures within IAPS is characterized by three values: arousal, dominance, and valence. We selected a total of 40 pictures from IAPS, incorporating three categories: negative, neutral, and positive pictures. This selection was based on the valence value, the exclusion of alarming keywords, and arousal scores (i.e., neutral images have the lowest arousal scores, while negative and positive images have the highest arousal scores). These pictures were subsequently divided into two sets for the preliminary assessment.

For the selection of videos, we relied on the emotional clips documented by Y. Deng, et al. (2017) [23] and divided them into two sets. Each set included five types of emotional triggers: Pleasure, Sadness, Neutral, Amusement, and Fear. A detailed description of the two sets of videos is summarized in Table 2.

Table 2. Description of selected videos for the preliminary phase.

Emotion	Duration (seconds)	Description
Set 1		
Pleasure	88	A girl is giving a speech at the graduation ceremony.
Sadness	180	The daughter's regret at the father's sacrifice
Neutral	97	Drive on a mountain road
Amusement	350	A poor man and a rich man sleep together
Fear	103	Ghosts hang up people in their house
Set 2		
Pleasure	39	A happy conversation between father and son
Sadness	274	Thoughts of an abandoned dog
Neutral	97	Drive on a mountain road
Amusement	190	Teachers' funny competition
Fear	120	An aggressive blind woman in the hospital

2.4. Platform design

The platform of this study was developed and executed by PsychoPy Software version 2021.2.3, running on Python 3.8. The experiment comprised two sessions: one involving pictures (Fig. 1B) and the other, videos (Fig. 1C). At the start of each session, a 30-second bio-calibration was performed (including 15 seconds with eyes open and 15 seconds with eyes closed), followed by a 30-second rest period. Subsequently, the MAT was displayed for 90 seconds, featuring a high difficulty level as an acute stress inducer for the test group, and a lower difficulty level for the control group.

After the stress-inducing step, subjects were exposed to emotional photographs or videos. For the picture session, subjects were given a three-second rest period before each photo was displayed for five seconds. Subsequently, participants were required to respond to the Manikin survey, assessing valence, arousal, and dominance, within 15 seconds (five seconds per question). This procedure was repeated 90 times (i.e., 90 trials), with the photographs displayed randomly. After the presentation of 45 pictures, the Stroop Task was conducted for 180 seconds before continuing the picture session. In the video session, the procedure mirrored the picture session, including a four-second rest period, emotional video stimulation, and a 15-second Manikin assessment. The videos were shown in the following order: Pleasure, Sadness, Neutral, Amusement, and Fear, with the Stroop Task presented after the Sadness video. Finally, at the conclusion of the experiment, subjects were given a 30-second rest period.

2.5. Emotions and mood self-evaluated scales

2.5.1. Momentary mood state measures

The momentary mood state measures (MMSM) were conducted to analyse participants' moods before each experiment session. This 15-item measure utilized a 5-point Likert-type rating scale to evaluate 7 aspects of a positive state: happy, cheerful, proud, relaxed, cooperative, caring, and friendly, and 8 aspects of a negative state: angry, nervous, lonely, frustrated, strained, worried, irritated, and stressed [24]. The measures were translated into the participants' native language (i.e., Vietnamese) and revised by expert psychologists.

2.5.2. Self-assessment Manikin

The SAM is a non-verbal measure of the three directions of emotional response including valence, arousal, and dominance [25, 26]. In this study, we used a 5-point scale to measure the score of each direction in response to the three categories of IAPS images and videos. The SAM scale is shown in Fig. 2.

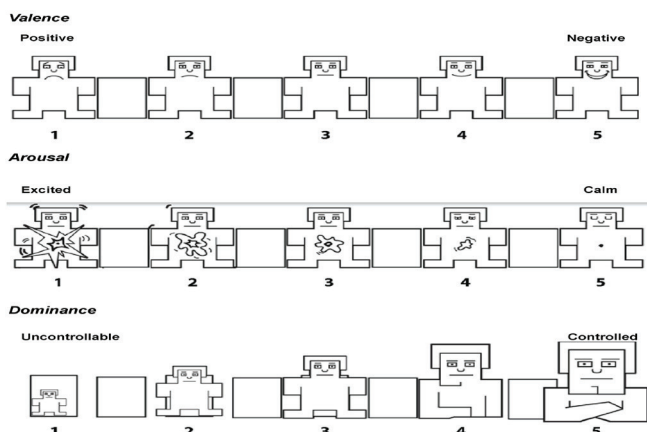


Fig. 2. The self-assessment Manikin scale measuring valence, arousal, and dominance [27].

2.6. Mental arithmetic task

The MAT was used as a stress-inducing task [18, 28, 29]. Participants were tasked with solving a set of arithmetic problems. For the control group, basic calculations involving the addition and subtraction of three digits ranging from one to ten were provided, with no time limit set for each calculation. Conversely, the stress group was assigned more complicated calculations, including the addition, subtraction, and multiplication of three integers less than 100. Participants in this group were allotted three seconds to perform each calculation, with a total time limit of 90 seconds for the entire MAT section. Subsequently, we used a 7-point Likert-type rating scale to survey their perception of the task's difficulty and stress levels.

2.7. Data analysis

The responses to the pre-screen questionnaire were queried on a Google Answer Spreadsheet. The picture and video session results were extracted from a .csv file exported by PsychoPy, including the MAT and Manikin responses. Any mistyped values were considered as missing values. Subjects with more than 30% of values missing were excluded from the study.

2.8. Statistical analysis

All data in this study were illustrated as mean $\pm$ standard error of the mean (SEM) and analysed by the one-way analysis of variance (ANOVA) test and *t*-test. Statistical analysis was performed using a 0.05 level of significance and conducted by GraphPad Prism software, version 8.3.1.

3. Results

3.1. Identification of picture and video library to induce emotional response

We analysed the self-evaluated Manikin scale distribution from the preliminary phase to identify suitable pictures and videos that could elicit emotional responses in Vietnamese students. Figs. 3A, 3D illustrate that the videos in Set 2, which induced positive emotion, showed a score distribution entirely on a positive scale. This result affirms the suitability of these videos in Set 2 for inducing emotional responses of pleasure and amusement subsequent experiments. Regarding sadness-triggered videos, the distribution of Set 1 focused more on the positive valence than Set 2 (Fig. 3B). This suggests that the footage in Set 1 stimulated more than one emotion. Therefore, the sadness video in Set 2 was chosen for the subsequent experiments. Two videos designed to trigger fear exhibited no difference on both valence and arousal scales (Fig. 3E). Overall, the videos in Set 2 proved to be a better fit for the purpose of the experiment and were implemented in subsequent experiments. The pictures from both sets yielded valence and arousal scores that aligned with their designated emotions, namely positive, neutral, and negative, with no discernible differences (Figs. 3F-3H). After removing outlier pictures whose valence scores did not correspond with the expected emotional activation, 45 images for each emotion type were randomly selected from both sets for further experiments.

3.2. Evaluation of the stress-inducing effect using mental arithmetic task

Independent *t*-tests were initially conducted on the participants' feedback regarding difficulty and stress levels to determine the stress-inducing effects of MAT under both online and offline experimental

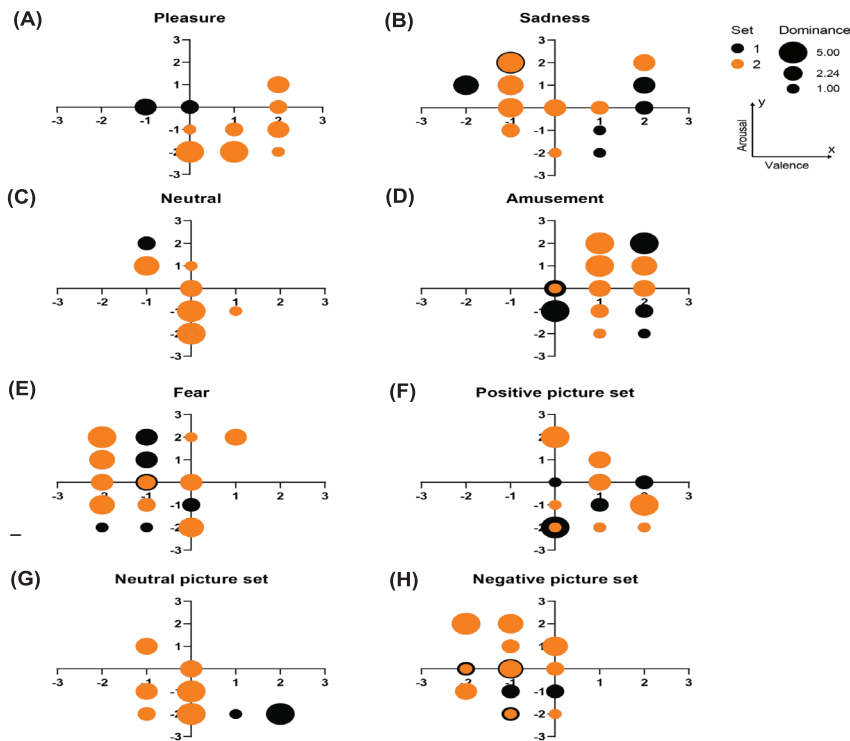


Fig. 3. The self-evaluated Manikin scale distribution of pictures and videos from the preliminary phase. Videos were targeted to induced specific emotional responses: (A) Pleasure, (B) Sadness, (C) Neutral, (D) Amusement, and (E) Fear. Pictures were targeted to induce the following specific emotional responses: (F) Positive, (G) Neutral, and (H) Negative (Set 1: N=16; Set 2: N=16). Each circle represents the mean value. The size of the circle indicates the mean value of the dominance scale.

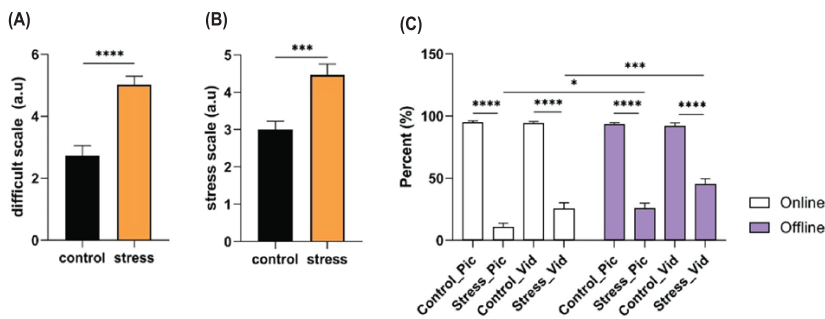


Fig. 4. The participants' self-evaluated difficulty and stress levels when performing mental arithmetic tasks in the control and stress groups. (A) Difficulty; (B) Stress level; (C) The results from the mental arithmetic task of the control and stress groups. Control online: N=11, stress online: N=16, control offline: N=22, stress offline: N=23.

conditions. When comparing the two groups, the stress group reported significantly higher levels of difficulty ($MD=2.291 \pm 0.4193$, $p<0.0001$, Fig. 4A) and stress ($MD=1.465 \pm 0.3679$, $p=0.0002$, Fig. 4B). This result is consistent with the subjects' performance presented in Fig. 4C, where the control group achieved significantly higher accuracy in all evaluated sessions ($p<0.0001$). Particularly, in the first online experimental session, the control group surpassed the stress group by $83.74 \pm 6.380\%$. This gap slightly decreased in the second session to $68.97 \pm 5.926\%$. A similar trend was observed in the offline experiments, wherein the first session, the control group surpassed the stress group by $67.31 \pm 4.324\%$, and in the

second session, the accuracy gap was reduced to $46.61 \pm 4.512\%$. Notably, significant differences were found within the stress cohorts between the two experimental conditions. Regarding arithmetic tasks for the stress groups, the offline cohort yielded significantly higher accuracy in both experimental trials (first trial: $MD=15.06 \pm 4.980$, $p=0.0119$; second trial: $MD=19.80 \pm 4.972$, $p=0.0004$). In contrast, the control group showed no difference between the two experimental conditions. The difference in accuracy exhibited by the stress group between two experimental conditions, online and offline, is hypothesized to be influenced by the COVID-19 quarantine and will be further elucidated in the following section.

3.3. The difference between offline and online platforms in emotional response

Differences in emotional response between two experimental groups, control and stress, were evaluated through three parameters: valence, arousal, and dominance. In the first experimental session of the online cohort, an elevated sensitivity was evident in subjects' responses to all picture sets: the valence differed significantly in all sets, with the stress group deviating in emotional tendencies (Positive: $MD=0.292$, $p=0.0113$; Neutral: $MD=0.245$, $p=0.001$; Negative: $MD=-0.435$, $p<0.0001$). The stress-induced group also experienced more intense emotions, reflected in higher arousal scores, particularly noticeable in positive ($p<0.0001$, Fig. 5A) and neutral sets ($p<0.0001$, Fig. 5B). This trend persisted in the second session using video stimulation, as the stress group sustained their valence deviation more notably than the control group (Supplementary Fig. 1). However, this trend did not apply to sadness ($MD = 0.11$) as the stress group showed a slightly higher valence score compared to the control (Supplementary Fig. 1B). Nevertheless, the arousal scores were consistently higher in the stress group across all emotions (significantly higher in positive and neutral, $MD=0.32-0.52$, respectively, $p<0.0001$), suggesting they were experiencing more intense emotions (Fig. 5).

In the first experimental session of the offline platform, no differences were observed between the two groups (Fig. 6). Overall, both groups expressed emotional valence in alignment with the intended mood as they responded with high valence scores to positive pictures (Fig. 6A) and low valence scores to negative pictures (Fig. 6C). On the other hand, the stress group experienced more intense emotions, as evidenced by their arousal score, especially in response to positive pictures ($MD=0.169$; $p=0.0341$, Fig. 6A). In the second session, which involved video stimulation, there was no significant difference detected between the two experimental groups, similar to the online cohort (Supplementary Fig. 2).

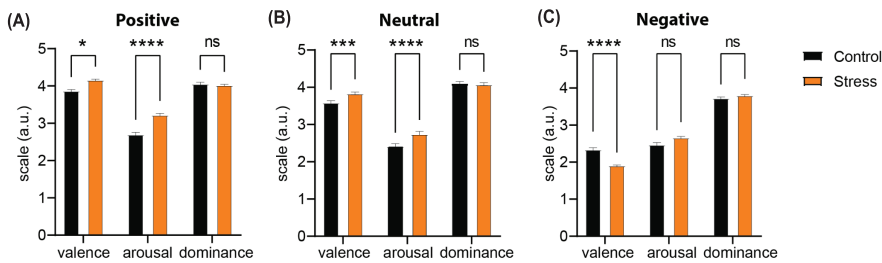


Fig. 5. Comparing emotional response levels of the two experimental groups in the online session to pictures of specific emotional triggers through the Manikin emotional self-rating scale. (A) Positive (control: n=487, stress: n=828); (B) Neutral (control: n=244, stress: n=428); (C) Negative (control: n=245, stress: n=418) (online control: N=9, online stress: N=15).

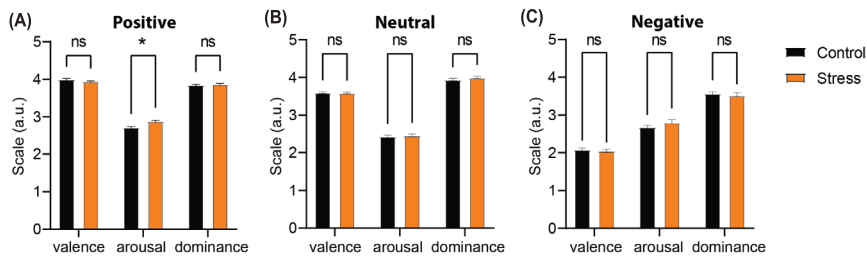


Fig. 6. Comparing the emotional response levels of the two experimental groups in the offline session to pictures of specific emotional triggers through the Manikin emotional self-rating scale. (A) Positive (control: n=720, stress: n=671); (B) Neutral (control: n=720, stress: n=673); (C) Negative (control: n=1433, stress: n=1305) (online control: N=25, online stress: N=24).

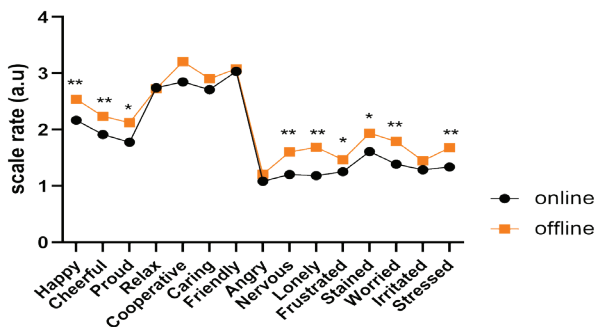


Fig. 7. Mean of momentary mood states measure scale in the online and offline experiments.

Subsequently, multiple comparisons were conducted on participants' mood evaluations to highlight any potential differences between two experimental cohorts, namely online and offline. As illustrated in Fig. 7, it can be deduced that the offline cohort experienced more intense emotions. This observation is based on the overall trend where offline subjects provided higher intensity ratings for 13 out of 15 evaluated moods. Notably, 9 out of these 15 moods demonstrated significant differences between the two cohorts, including happiness ($p=0.00352$), cheerfulness ($p=0.00611$), pride ($p=0.0126$), nervousness ($p=0.000480$), loneliness ($p=0.000185$), frustration ($p=0.0263$), strain ($p=0.0134$), worry ($p=0.00145$), and stress ($p=0.00484$). Particularly, the most distinctive moods were loneliness ($MD=0.501$) and nervousness ($MD=0.400$). These results implied a heightened sensitivity in the offline cohort's emotions prior to intervention compared to the online cohort, which may be related to the respective cohort's living conditions. This point will be elaborated in the next section.

4. Discussion

Numerous prior studies have explored the suitability of the IAPS library in emotional responses, cognition, and regulation across various socio-cultural contexts [21, 30, 31]. However, given socio-cultural differences, the impact of the IAPS library on Vietnamese students has not been established, nor has any effort been made to build a library of images and videos to support studies of emotional responses in Vietnamese individuals. Our preliminary results suggest that our designated IAPS library evokes emotions in Vietnamese students in a manner similar to trends observed in previous studies [21, 22, 30]. Both IAPS libraries (Sets 1 and 2) effectively induced emotions as indicated by their labels: high scores on the valence scale for positively induced emotions (>0), low scores for negatively induced emotions (<0), and scores ranging from -1 to 1 for neutral picture sets [30]. Similarly, the results for each emotional-inducing video also displayed valence scores consistent with each type of emotion.

The differences in self-rating stress and difficulty levels, as well as arithmetic accuracy, between the two experimental groups (Fig. 4) were similar to those found in previous studies [18, 32, 33]. These results demonstrate that the designated MAT is capable of inducing acute stress in the stress group. MAT performance is commonly used to assess the level of working memory involved in cognitive processing [34-36]. Interestingly, the accuracy achieved by the online stress group was found to be significantly lower than that of the offline stress group (Fig. 4C). This difference implies the effect of environmental factors on subjects' MAT performance in the two experimental conditions: online or offline. Previous studies have shown a negative association between anxiety and working memory task performance [37]. In our study, the two experimental stages were conducted during different periods of the COVID-19 pandemic - the online phase took place at the end of the COVID-19 quarantine period, and the offline phase occurred during the resurgence of SARS-CoV-2 infections [38]. We hypothesize that the pandemic outbreak during the offline phase may have caused anxiety among the stress group, which could explain the higher performance in MAT among the offline stress cohort.

Previous studies have demonstrated the effects of acute stress on emotional sensitivity and recognition in experimental subjects [19, 39]. The online experimental results showed that MAT heightened awareness of positive and negative emotions in the stress groups by increasing the valence score in the positive and neutral emotion-triggered picture sets and decreasing it in the negative emotion-triggered set (Fig. 5). Sensitivity was also enhanced, as evidenced by an increase in arousal scores regardless of emotion types. However, this trend was not as significant in the offline experiments: Only the positive picture set recorded a substantial increase in the stress group's arousal score (Fig. 6A), while two other picture sets only saw a slight increase (Figs. 6B, 6C). On the other hand, a higher negative valence score and lower scores in neutral and positive valence scores were found in the offline stress

group compared to the online group. Regarding the control group, there was no difference between the two experimental conditions. This result suggests that the online cohort was more sensitive in their emotional responses. Previous studies have clarified the positive correlation between social stressors and negative emotional responsiveness and perception [6, 40]. Therefore, environmental stressors during the online session are hypothesized to be the primary cause of different emotional responses between the two experimental conditions. It is noteworthy that the online experiment was conducted during the easing of social lockdown, and the offline experiment was conducted during the “new normal” - after the COVID-19 lockdown had been lifted. So, were environmental factors involved in subjects’ emotional resilience in two experimental conditions?

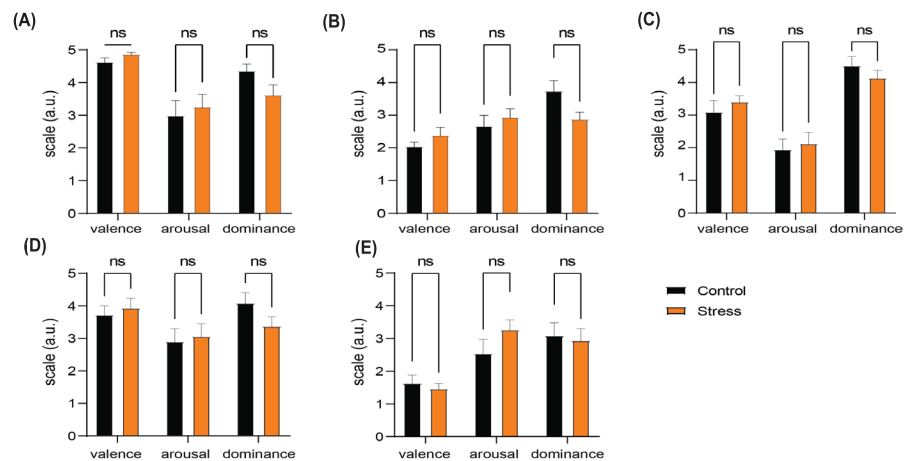
The impact of the COVID-19 pandemic can be inferred from the analysis of Mean of Momentary Mood States Measure (MMSM) results (Fig. 7). The offline cohort exhibited higher intensity ratings for 13 out of 15 surveyed moods. This trend indicated an increase in mood instability in young adults [41]. Studies investigating the effects of stress posed by the COVID-19 pandemic on mood swings after lockdown have been documented in other countries [42, 43]. However, the Vietnamese population reported lower stress level than other countries in the region and developed countries [44]. Students under social lockdown reported that being with their families during the pandemic partly reduced their core stressors, such as tuition fees, living alone, and transportation problems [45]. In contrast, at the time of the offline experiment, students had returned to study on campus, and problems such as job loss, financial crises of sponsors, and living away from home affected students. Furthermore, between March and May, there was a sudden increase in SARS-CoV-2 infections [38]. These unique circumstances might explain the difference in mental health between the online and offline cohorts. However, there have been limited studies investigating the influence of post-lockdown social factors on students’ mental health. Further work needs to be conducted to clarify the relationship between these two factors.

5. Conclusions

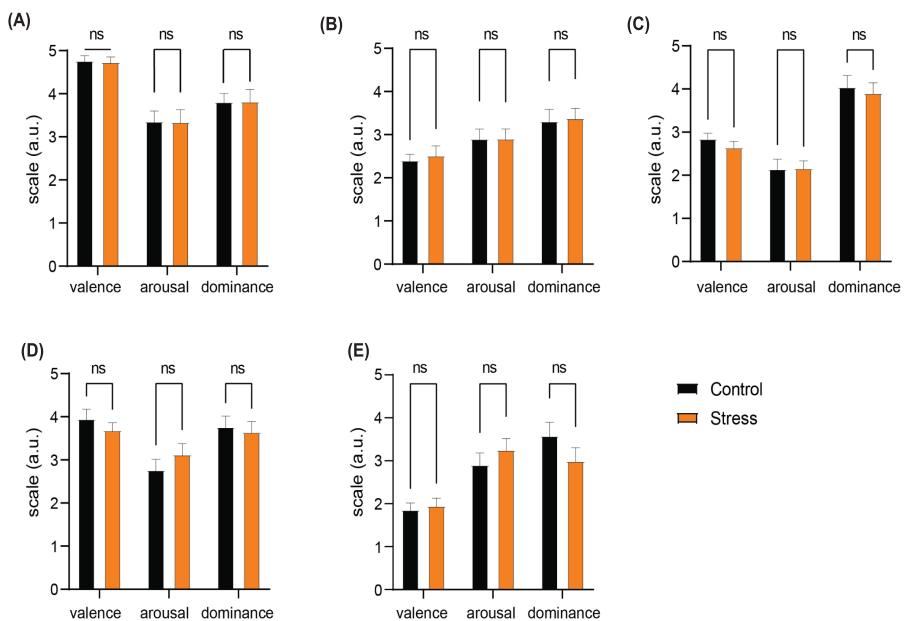
To date, no picture or video library has been specifically designed to trigger emotional responses in Vietnamese individuals. The impact of acute stress on emotional responses in Vietnamese students also remains unexplored. This pioneering study demonstrates that the IAPS picture and video libraries, adapted from foreign studies, effectively trigger emotions in Vietnamese students consistent with their designated labels. Consequently, a selection of IAPS pictures and videos was implemented in subsequent experiments to evaluate the effects of acute stress on emotions in this demographic.

The online experiments revealed that subjects under acute stress were more sensitive across three emotional categories via increased valence scores in the positive and neutral groups and decreased scores for negative pictures. This emotional sensitivity was also reflected in arousal scores of each emotion. However, a similar significance was not observed in the offline experiment. Through the MMSM results, increases in both positive and negative states were noted among subjects in the offline experiment. These findings suggest the potential influence of social factors, such as COVID-19 - considering the timing of the experiments - on participants’ mental health. In one interpretation, these results underscore the enduring impact of the COVID-19 crisis on the mental health of Vietnamese students.

SUPPLEMENTARY



Supplementary Fig. 1. Comparison of the emotional response levels of the two experimental groups in the online session to videos of specific emotional triggers through the Manikin emotional self-rating scale. (A) Pleasure; (B) Sadness; (C) Neutral; (D) Amusement; (E) Fear. Online control: N=11, online stress: N=19.



Supplementary Fig. 2. Comparison of emotional response levels of the two experimental groups in the Offline session to videos of specific emotional triggers through the Manikin emotional self-rating scale. (A) Pleasure; (B) Sadness; (C) Neutral; (D) Amusement; (E) Fear. Offline control: N=22, offline stress: N=23.

CRedit author statement

Luan Van Tran: Conceptualisation, Project administration, Software, Investigation, Methodology, Data analysis, Writing, Editing; Anh Phuc Hoang Le: Methodology, Investigation, Data analysis, Writing, Editing, Reviewing; Thao Thi Mai Le: Methodology, Writing, Editing; My Thi Tra Nguyen: Investigation, Data analysis, Writing, Reviewing; Quang Lam Nguyen: Investigation, Writing, Reviewing, Editing; Huong Thi Thanh Ha: Supervision, Resources, Conceptualisation, Reviewing, Funding acquisition.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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GPR investigation of the cause of persistent failure of a university road

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Abstract:

We present the results of a multi-frequency ground-penetrating radar (GPR) survey over a university road in south-western Nigeria that has experienced persistent failures. The goal was to evaluate causes of failure to aid remediation efforts. Data was collected concurrently at 250, 500, and 1000 MHz using a UTSI electronic trivue system. Data were processed and interpreted using Reflex-Win. Visual observations of major features apparent on the road surface including failed sections were noted. Imaged features included possible bases of the surface course, base course and sub-base, subsurface projections of an outcropping and buried basement rocks, three deeply cut and back-filled sections that lie beneath sections of the road that have experienced persistent failure, and the ringing effects and rebar-associated reflection hyperbolae of two reinforced concrete drains. Indications of preferential wetting of the shallow subsurface beneath much of the failed sections suggest that failure is due to excessive moisture content in these sections. These failed sections generally correspond to regions of deep cut and backfill, and regions cut and filled for the construction of reinforced concrete drains. Apparently, the backfills at these locations have failed to prevent water influx into the subsurface and more effective remediation measures need to be deployed.

Keywords: ground-penetrating radar, moisture content, road failure, shallow basement.

Classification number: 4.3

1. Introduction

GPR is a nondestructive testing (NDT) technology device that has found applications in various fields of civil engineering, agricultural studies, archaeology, and environmental studies around the world. Electromagnetic waves of various frequency band ranges are radiated into the ground by the transmitters and the reflections are recorded by receivers. The acquired signals enable the determination of the location and disposition of the subsurface anomalies [1]. GPR applications in geotechnical investigation and engineering projects include the evaluation of the ground structure around tunnels and linings [2, 3], foundation assessment and identification of foundation strata [4, 5], location and identification of underground utilities [6, 7], inspection of road pavements [8, 9], railway subgrade and ballast characterization [10, 11], bridge deck condition assessments [12, 13], and more.

In the assessment of pavement layers for road monitoring and remediation, GPR has been applied in the detection of construction changes [14], the location of voids and sinkholes [15, 16], the identification of wet patches (indicating poor support), locating reinforcing bars [1] and mapping excess sub-base moisture [17, 18].

Many highways and roads use a bituminous binding material to make a solid, bound upper-pavement layer. Usually, the upper-pavement layer will itself consist of several individual layers of slightly varying materials. The essence is to achieve a section that provides good load distribution, prevents water ingress, and ensures a smooth ride for vehicles [14]. The basic sections of the pavement include a sub-base, a base course and a surface course (Fig. 1). The sub-base is the layer of aggregate material laid

on the subgrade (*in situ* soil). The materials used may be either unbound granular, or cement-bound. It is often the main load-bearing layer of the pavement [19]. The base course is a layer usually of loosely-bound aggregates placed on the sub-base that acts to distribute traffic loads to the underlying sub-base and subgrade layers, facilitate drainage, as well as provide support to the wearing surface (surface course) to prevent tensile fatigue or cracking [20]. The surface course is the wearing course or friction course. In rigid pavements, the road consists of a concrete slab made of Portland cement while in flexible pavements, it is an asphalt concrete, made up of a construction aggregate and a bituminous binder [21]. Since these components (surface bituminous course, base course, sub-base and subgrade [soil]) usually have differing dielectric constants [14], this leads to varying levels of reflection of energy emanating due to variations in the materials within the pavement structure and the subgrade beneath.

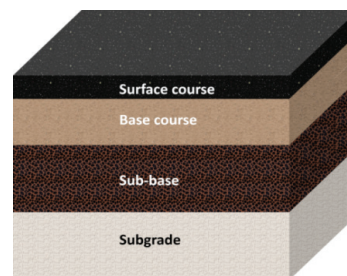


Fig. 1. Basic flexible pavement structure.

W. Xue, et al. (2023) [22] developed a back-propagated neural network algorithm for automatic detection of underground targets beneath urban roads from GPR data. The procedure involved clutter

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suppression via robust principal component analysis, target domain statistical analyses, neural network recognition of target's horizontal domain and a fusion and delete (FAD) algorithm to optimize the definition of the horizontal target domains. In experimental trials, the algorithm allowed the detection of targets at a higher accuracy rate and in less time compared to two traditional segmentation recognition methods.

J.Y. Rhee, et al. (2021) [1] addressed the applications and the limitations of the GPR in the investigation and monitoring of the subsurface beneath Korean highways with a view to improving the efficient application of GPR technology. They study the depth of investigation and detection performance of anomalous objects of two different multichannel GPR systems on the Korean Expressway Corporation's nondestructive testing test-bed for subsurface detection. The results showed that the combination of the plan view by depth and cross- and longitudinal sections of the GPR data achieved from multichannel 3D GPR made analysis easier and more accurate.

S. Colagrande, et al. (2011) [23] conducted GPR investigations to study degraded road pavements built in a cut-and-fill section. They assess road integrity via quantitative analysis of power curves on GPR data collected at 1600 and 600 MHz. Comparison between the absorption coefficients of damaged and undamaged road sections allowed the determination of the cause of road degradation. Degradation was linked to fatigue and thermal shrinkage of road pavement material when the absorption angles were similar but were added to differential compaction of the subgrade if absorption angles were significantly dissimilar.

M.S. Abdullah, et al. (2022) [24] conducted a GPR survey at a municipal pumping station to estimate the type of soil causing differential settlement of the ground beneath the station. The study involved two surveys, involving a 160 and 450 MHz antenna, run once before and once after the injection of cement slurry into the subsurface for ground improvement. The results showed soil creep in some sections of the soil beneath the facility's foundation. Further, they effectively determined the thickness of the raft foundation beneath the structure.

J. Ling, et al. (2022) [25] developed a road subgrade monitoring method based on time-lapse full-coverage 3D GPR surveying. The focus was solving key problems related to time and spatial position mismatches in experimental data. The study used the time zero consistency correction, 3D data combination, and spatial position matching methods, which greatly improved the 3D imaging quality of underground spaces. Time-lapse attribute analysis was further used to obtain detailed characteristics and an overall pattern of the dynamic subgrade change.

T. Thitimakorn, et al. (2016) [26] used the GPR technique to investigate the potential presence of subsurface voids under the road surface in the Bangkok metropolitan area. The GPR data were collected with a 400 MHz antenna mounted on a survey cart. Several void-like anomalies were detected from the GPR data and later drilled to confirm the existence of the voids. Some GPR anomalies were found not to be voids but came from areas of past road maintenance or manholes with a hidden cover (asphalt concrete overlay).

These studies demonstrate the versatility of the GPR technique in road monitoring and maintenance. The goal of the current study was to investigate the sources of multiple recurrent failure indices on a major university road. Although the road surface had been remediated on multiple occasions, such remediations have not achieved the desired result and have not arrested the failure.

2. Ground-penetrating radar principle

The GPR technique involves transmitting high-frequency electromagnetic pulses (radar) from a transmitting antenna (or an array of antennas) into the subsurface and recording the time taken for reflected energy to return to a receiver antenna. The passage of radar waves through a material is dependent on the material type, condition, water content and pore fluid content. These properties impact the dielectric constant of the material, which in turn determines the speed of the radar pulse through the material. If two materials have sufficiently contrasting dielectric properties, some radar energy is reflected to the surface from the material boundary (Fig. 2).

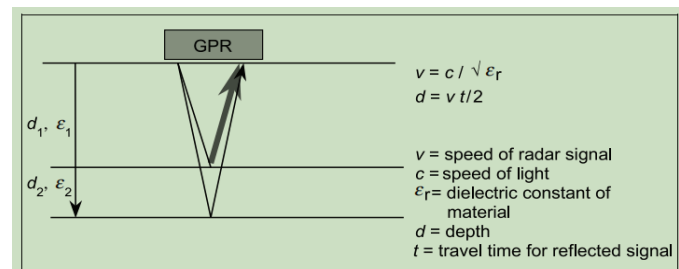


Fig. 2. Schematic of GPR technique indicating the calculation of radar pulse speed and depth [14].

GPR systems use a wide range of frequencies, usually, 100 to 2000 MHz. Higher frequencies (≥ 400 MHz) provide higher resolution in the shallow sections of the material but a much-limited penetration depth. This encourages their use for detailed near-surface surveys such as pavement monitoring and mapping reinforced concrete rebar conditions. Conversely, lower frequencies (≤ 400 MHz) provide greater depths of investigation but lower resolution capacity. They therefore find much use in geological mapping and deep archaeological studies.

3. Site of investigation

Adekunle Ajasin University is located in Akungba-Akoko in the north-eastern region of Ondo State, south-western Nigeria (Fig. 3A). Akungba-Akoko is located between $05^{\circ}43'E$ and $05^{\circ}47'E$, and $07^{\circ}27'N$ and $07^{\circ}31'N$. The township bounds consist of a gently sloping central low-lying region surrounded in a perimeter-like pattern by high-rising granitic hills to the north, west, east, and southeast. Topographic relief is generally beyond 345 m above sea level. Surrounding perimeter hills could rise to 420 m above sea level. The local lithology suite includes rocks of the migmatite-gneiss complex of the basement rocks characteristic of the southwestern regions of Nigeria [27]. The suite of rocks consists of biotite-rich grey gneiss, granite gneiss and lesser amounts of charnockitic rock. The area is drained by seasonal streams that start from the high reliefs in the northeastern region of the area. Regolith development

in the area is generally thin. This is appreciated from visual reconnaissance evaluation of the area; basement rocks frequently outcrop in several parts of the area. The rocks of the Akungba-Akoko area have undergone at least two episodes of tectonic deformation characteristic of the Precambrian basement complex rock of south-western Nigeria. This has engendered the development of a series of fractures and complex minor folds. Drainage courses are generally controlled by persistent major fracture systems. The survey site is a section of a major road spanning 370 m in the western campus of Adekunle Ajasin University (Fig. 3A). This university road has multiple failed sections that have been the focus of repeated and perennial remediation efforts (Fig. 3B).

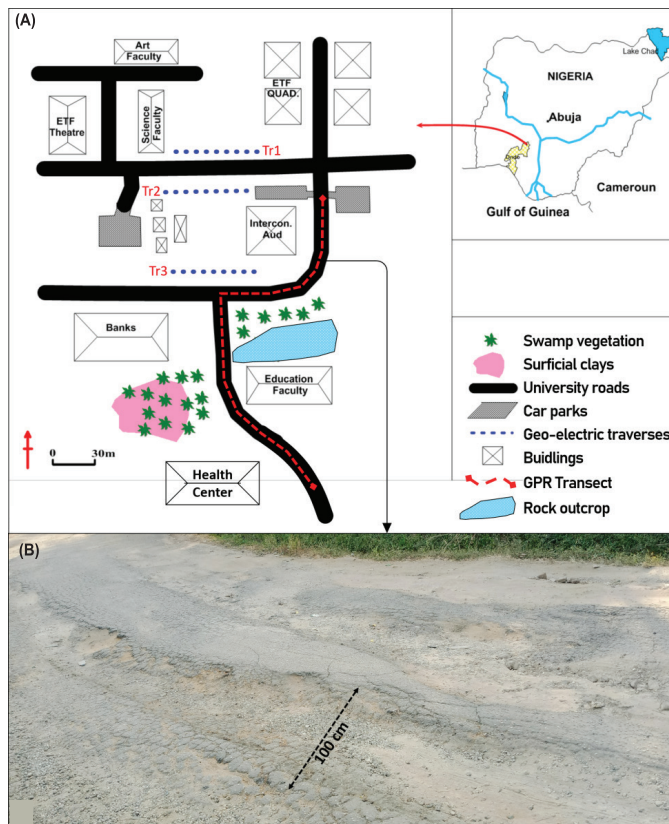


Fig. 3. Survey location. (A) GPR transect, red dashed line, traverses northwards, and takes a right turn eastwards before continuing northwards (Modified after [28]); (B) Failed section.

4. Methodology

A GPR transect was surveyed in this study. The transect starts from the proximate front of the University Health Centre, travels northwards and then takes an eastward turn around the south-eastern flanks of the Intercontinental Bank Auditorium before terminating north a few meters before a four-way junction (Fig. 3). The transect length was 370 m. GPR data was collected using a triple-frequency UTSI Electronic Trivue system with bandwidths of 125-500, 200-800, and 500-2000 MHz centred on 250, 500 and 1000 MHz, respectively. The antennas are co-centred. Record times were 80, 60, and 40 ns for the 250, 500, and 1000 MHz records, respectively. Triggering was via an encoder wheel system which

also records the length of the transects. Data was collected in the month of October (2023) towards the end of the rainy season. Visual observations of key features along the road were made and documented. These included failed road portions, speed bumps, reinforced concrete drains, and outcropping basement rocks (Table 1). Data processing was accomplished in Reflex-Win version 10.1 and followed a sequence including (Table 2, Fig. 4): (1) Dewow, (2) Static correction, (3) Gain, and (4) Background removal. The outputs were 2D GPR radar-grams plotted in time (ns) versus lateral position. The final sections are presented in grayscale as this provides the best opportunity to identify features.

Table 1. Features noted along the GPR transect.

No.	Feature	Position (m)
1	Start of transect	0
2	Failed section 1 - (concrete mend)	7-20 (7-20)
3	Speed bump 1	12
4	Failed section 2	48-59
5	Reinforced concrete drain	63-65
6	Failed section 3	108-162
7	Outcropping basement rock	145-161
8	The first turn starts (right)	208
9	Failed section 4	210-212
10	Reinforced concrete drain	210-211
11	Failed section 5	232-242
12	The second turn starts (left)	268
13	Failed section 6 (concrete mend)	273-342 (311-330)
14	Speed bump 1	340
15	End of transect	370

Table 2. GPR processing sequence.

No.	Processing sequence ID	Parameter		
		250 MHz	500 MHz	1000 MHz
1	Dewow	64 ns	32 ns	16 ns
2	Static correction	Variable	Variable	Variable
3	Gain (exponential)	0.5	1	2
4	Background removal	Automatic	Automatic	Automatic

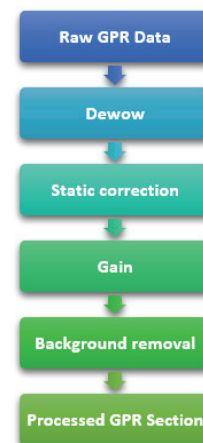


Fig. 4. Workflow sequence used for processing the GPR data.

5. Results

Only the 500 and 1000 MHz sections are presented in this study (Figs. 5-8). These sufficiently highlight the various features identified in the study area and enable ample interpretation. The 250 MHz sections were of very low resolutions for most features and as the area is generally of thin overburden thickness, no significant features were imaged at the greater depths it provided. Generally, the 1000 MHz section provided higher resolutions and more detailed imaging of the very shallow subsurface (0-8 ns) and allowed a better interpretation of features in the very shallow subsurface compared to the 500 MHz section. Conversely, the 500 MHz allowed better imaging and interpretation of major features at depth and allowed the identification of further extensions of features not completely imaged on the 1000 MHz section.

Persistent reflections are identified at 0.5, 1.5, and 4 ns, respectively on the 1000 MHz section. They however appear lumped together and indistinct in the 500 MHz section. These reflections are generally continuous all through the transect except at failed portions of the road, specifically at 7-20, 48-49, 108-162, 232-242, and 273-342 m. At these locations, the reflections become discontinuous and have much lower reflection amplitudes and lower reflection cycles. They may also present as undulating reflections, or as hyperbola-interrupted reflections. These undulations are particularly apparent at 90-110, and 130-145 m at average depths of 2-4 ns two-way time (tw). Speed bump locations present with concave upwards reflections at 10-11 and 341-342 m with ringing effects that persist with depth (Figs. 5 and 8). The known concrete drains present strong elevated platform-like amplitude reflections at 62-65 and 209-211 m (Figs. 5 and 7). On either side of the drain at 62-65 m, reflections show a sag at 4 ns that extends to about 12 ns twt. The drain reflections also show a ringing effect continuous at depth. In the 500 MHz sections, multiple tightly packed and overlapping hyperbolae are visible. Slight variations occur between the noted locations of the speed bumps and concrete drain (Table 1) and their reflected locations on the GPR transect.

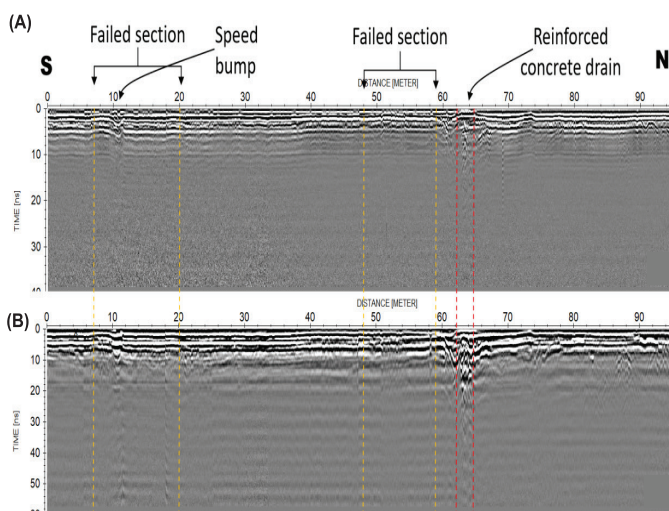


Fig. 5. 0-90 m GPR composite image of processed (A) 1000 MHz, (B) 500 MHz section. Failed road sections, a speed bump and a reinforced concrete drain are indicated at 7-20, 48-59, 11, and 62-53 m, respectively along the transect.

At 132-155 m, strong amplitude reflections occur in the first 4 ns twt (Fig. 6). Beneath this region, steeply dipping strong amplitude reflections coincide with the lateral position of a rock mass outcropping to the east of the Transect. The reflections are continuous, strongly undulating and the southwards dipping arm extends beyond 40 ns twt in the 500 MHz section. The northward arm appears to dip at greater angles but is only imaged to less than 30 ns twt.

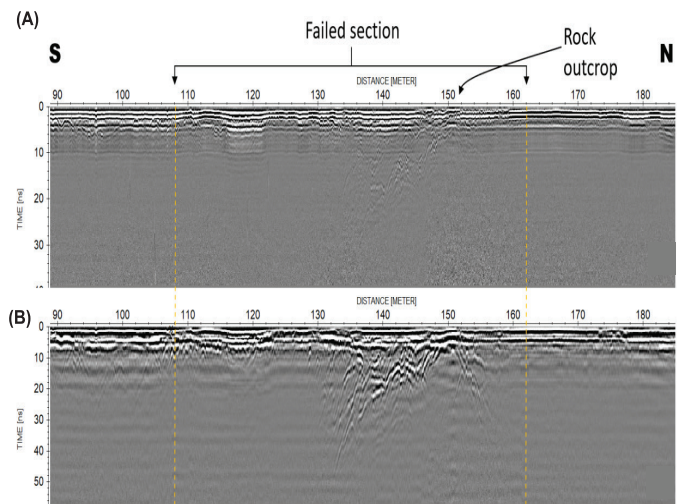


Fig. 6. 90-180 m GPR composite image of processed of (A) 1000 MHz, (B) 500 MHz section. The failed road section spans 108-162 m. Lateral amplitude loss is noted at 90-116 m, and 122-136 m in the shallow (<8 ns) section of the 1000 MHz image. Strong amplitude dipping reflections occur beneath the location of the outcropping rock noted at 145-161 m and extend to over 45 ns.

Significant lateral loss of reflection amplitudes within the upper 8 ns of the 1000 MHz section occurs at 72-82, 90-116, 122-136, 213-217, 232-260, and 278-311 m (Figs. 5-8). At these locations, the reflection amplitude is much dimmer than in adjacent locations. In the 500 MHz, these locations may display little amplitude loss. They, however, frequently present as more discontinuous reflections relative to adjacent locations. At 215-228 m, a second dipping strong amplitude reflection is well imaged on the 500 MHz section (Fig. 7). This reflection is not imaged on the 1000 MHz section. At its upper end, it borders the location of the reinforced concrete drain at 209-211 in a zone characterised by chaotic reflections. This zone presents with significant amplitude loss in the 1000 MHz section.

Three regions of relatively chaotic low amplitude reflections are identified at depths beyond the 10 ns mark on the 500 MHz sections (Figs. 7 and 8). These regions occur at 225-250, 275-305, and 317-343 m. These regions extend up to 25, 37, and 26 ns, respectively. The bottom of the 225-250 region is a smooth basin-like structure but the bottoms of the 275-305 and 317-343 m regions present as undulating bases. Within these basin-like sections, the reflection character differs considerably and is more chaotic relative to adjacent sections. At 311-330 m, a concrete overlay has been applied to the failed road (Fig. 8). In shallow (<5 ns) sections beneath the concrete overlay, strong amplitude and continuous reflection akin to the shallow continuous reflection earlier noted at 0.5, 1.5, and 4 ns are observed. However, in-between the reflections, the reflection character is chaotic.

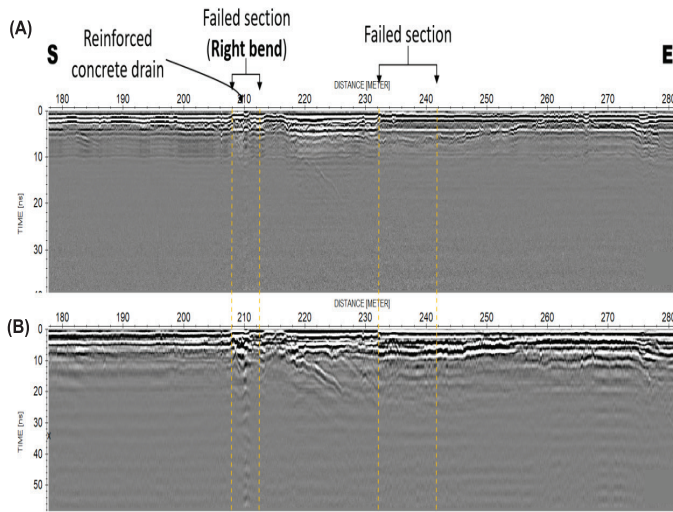


Fig. 7. 180-280 m GPR composite image of processed of (A) 1000 MHz, (B) 500 MHz section. Failed road sections span 208-212 m and 232-242 m. Lateral amplitude loss is noted at 213-217 and 232-260 m in the shallow (<8 ns) section of the 1000 MHz image. A second strongly dipping set of high-amplitude reflections is imaged at 215-228 m on the 500 MHz image. At 225-250 m, significantly disturbed reflections are observed beyond 10 ns.

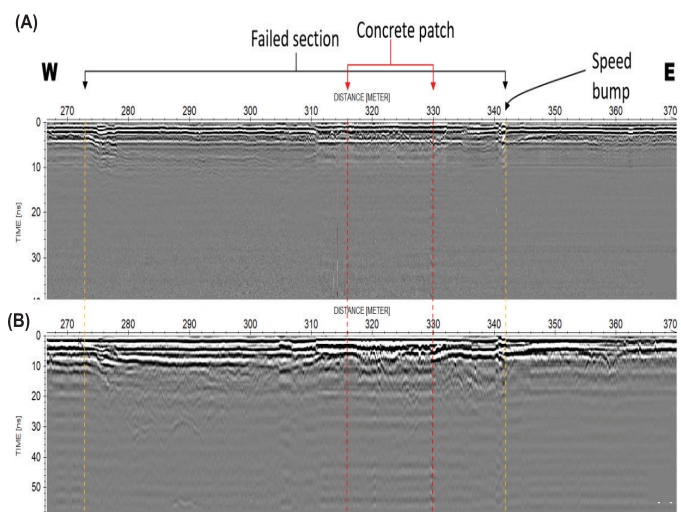


Fig. 8. 270-370 m GPR composite image of processed of (A) 1000 MHz, (B) 500 MHz section. The failed road section spans 273-342 m. A concrete patch has been applied to 316-330 m. At 275-305 and 317-343 m, significantly disturbed reflections which form bowl-shaped morphologies extend to depths beyond 25 ns.

6. Discussion

Figure 9 represents a cartoon of our interpretation of the GPR transect along the road. Previously, a geo-electric traverse was

surveyed close to a section of the transect in this study. Details of the survey and interpretation can be found in [28]. The locations of the geo-electric traverses are indicated in Fig. 3.

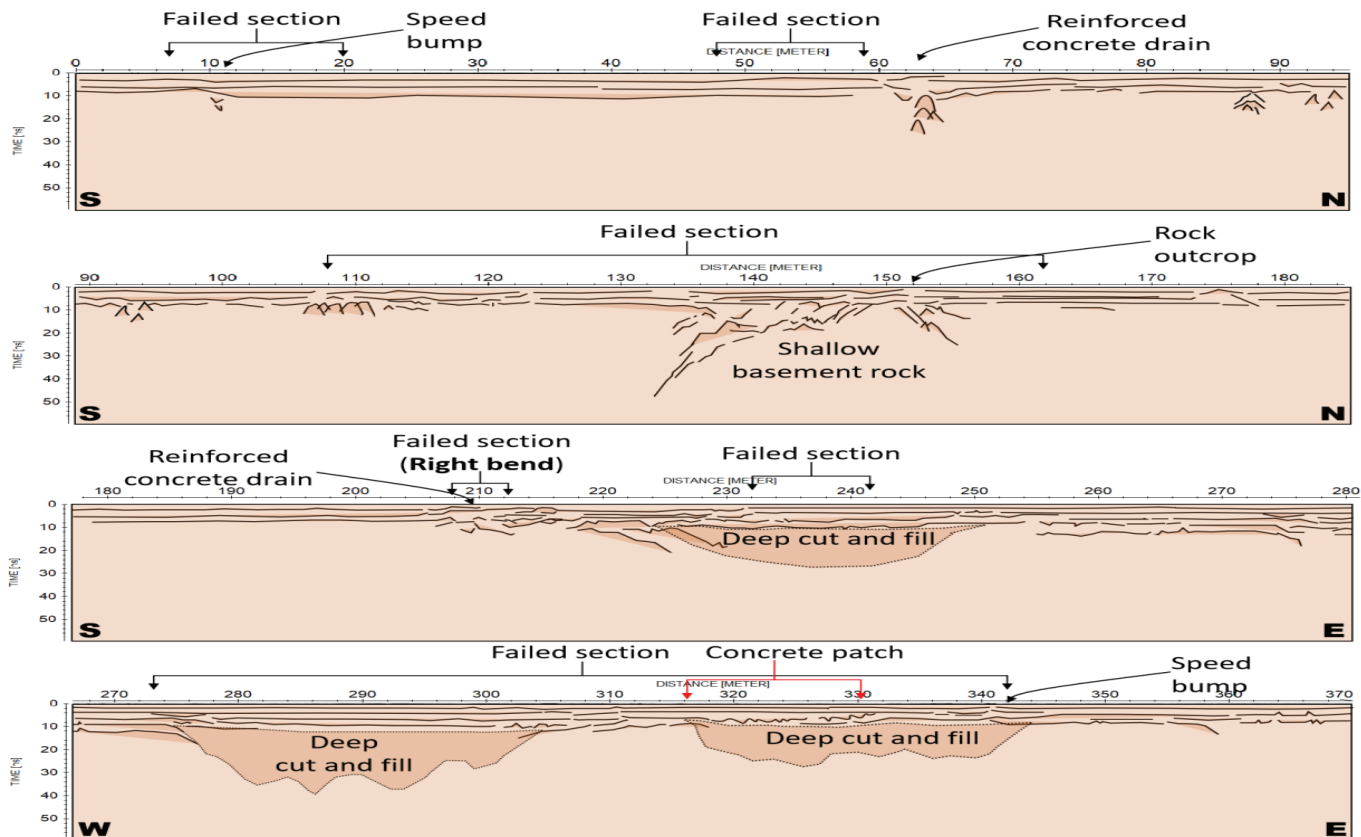


Fig. 9. Interpretation cartoon of the subsurface along the road transect. Road failure is associated with regions with very shallow-to-outcropping bedrock at 215-228 and 145-161 m, respectively, and deeply back-filled sections at 275-305 and 317-343 m.

Based on a transmission velocity of 0.1 m/ns that has been recommended for subsurface materials beneath tarmac [29], the continuous reflections identified at 0.5, 1.5, and 4 ns twt, on the 1000 MHz section correspond to depths 0.025, 0.075, and 0.2 m, respectively. These reflections may correspond to the base of the surface course, the base of the base course, and the base of the sub-base along the road, respectively. We do not have independent confirmation of these interpretations but these values represent typical values of surface course, base course and sub-base course in the area. These reflections become way more chaotic toward the second half of the transect (>210 m). The presence of ringing at depth beneath the reinforced concrete drains is due to the air gap between the drains' upper slab and its floor [29]. Tightly packed hyperbolae at the drain (62-65 m) likely represent reflections from individual rebars in the upper slab reinforcement of the drain. We interpret the reflection sags observed on either side of the drain as evidence of previous excavation and backfill during the construction of the drain. Differential compaction between the original subgrade and fill material may have preserved the line of cut and given rise to the apparent sag.

We further interpret the lateral amplitude losses along reflections at 72-82, 90-116, 122-136, 213-217, 232-260, and 278-311 m as related to elevated water content at these locations [14, 29, 30]. The presence of water in the near-surface slows the GPR wave and broadens it in the time direction. We interpret the three chaotic regions at 225-250, 275-305, and 317-343 m to represent areas of considerably thicker overburden relative to the rest of the transect. These regions likely represent regions of deeply excavated and back-filled overburden. They align considerably well with failed sections of the road at 210-212, 232-242, and 273-342 m.

We contend that failure along the road is primarily due to the presence of excess amounts of water in the subsurface at the failed sections. The outcropping rock mass at 132-155 m likely sheds much precipitation runoff during the rains into the shallow subsurface at the failed section at 108-162 m. The second dipping high amplitude reflection at 215-228 m likely represents the presence of a second near-surface rock mass at this location. Only one arm (east dipping) of the rock mass is indicated as the other arm (likely west dipping) lies west of the first turn (and is beyond the coverage) of the GPR

transect. It is worth noting that [28] imaged a neck-like weathering-resistant shallow basement over which the university road passes at this junction (Fig. 10).

Outcrops of this rock can be seen much further west, north of the Banks' location. The chaotic regions imaged at depth (extending >10 ns) lie in regions coincident with a clayey aquitard imaged in [28] (Fig. 10). This clayey aquitard extends to depths in excess of 10 m in the eastern bend of the current transect. The bowl-shaped chaotic regions appear to lie within a region that is a lateral equivalent to this clayey aquitard and may represent deep excavations and backfill of the clayey aquitard along the road. The chaotic internal reflections here likely represent coarser rock fill intended to stabilise the subsurface beneath this section of the road. Apparently, the excavation and fill have not been sufficiently effective to prevent water from saturating the near-surface materials along the sections of the road. This is where the road fails extensively, and even though a section of it has been remediated with a concrete overlay, the road still experiences much damage. The basement arm imaged at 215-228 possibly also sheds much runoff eastwards into this cut and fill section encouraging its wetness and attendant instability.

7. Conclusions

We have presented the results of a GPR survey aimed at determining the causes of multiple failures of a road that have defied repeated remediation courses. The major factors causing the failures, in our opinion, are related to deeply excavated and back-filled regions and regions cut and filled for the construction of reinforced concrete drainage. Though the deeply excavated regions were meant to remediate a section initially filled with a clayey aquitard, they have failed to prevent the ingress of groundwater which saturates the shallow subsurface at these locations and leads to much instability at the surface. A further contribution to the saturation of the shallow subsurface at failed locations likely comes from their proximity to shallow basement rocks that serve to shed precipitation runoff toward these sections. The result of the survey allowed a re-construction of the shallow subsurface along the road and the identification of the major causes of recurrent failure. It is hoped this would help in the design of effective remediation measures to prevent future failure.

CRediT author statement

Muslim Babatunde Aminu: Conceptualisation and Methodology, Writing - Original draft preparation; Femi Ologunaye: Methodology and Editing.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

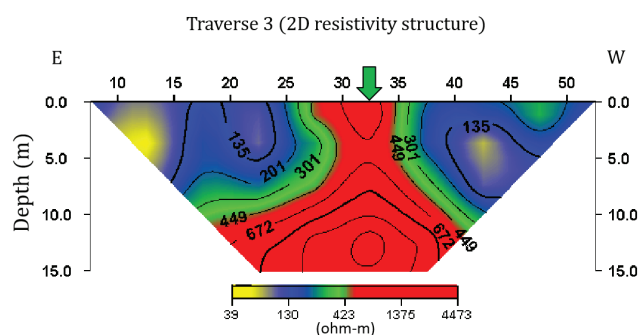


Fig. 10. 2D subsurface resistivity structure along the southernmost geoelectric traverse (Tr 3) which runs adjacent to part of the GPR transect [28] (see Fig. 3 for spatial relationship with the GPR transect).

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Research on households' willingness to pay for the options of price calculation of household solid waste management services in Hanoi

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Abstract:

This study used the contingent valuation method (CVM) to investigate and analyse the current status of household solid waste management and evaluate people's consensus and willingness to pay (WTP) for household solid waste collection, transportation, and treatment services among urban, rural, and mountainous areas in Hanoi. It also utilised regression analysis to assess factors affecting people's WTP. The study surveyed a sample size of 405 households. To ensure a basis for comparison among urban, rural, and mountainous areas, the study distributed and conducted an equal number of 135 surveys in each area through random sampling. The research results indicate that the average household solid waste in Hanoi is highest in urban areas at 1.27 kg/household/day, followed by rural areas at 1.21 kg/household/day, and mountainous areas at 1.067 kg/household/day. However, most household solid waste has not been classified at the source. Regarding WTP for household solid waste collection, transportation, and treatment services, the results show that 85% of urban households agreed to pay higher than the proposed price, with an average WTP of 1,136 VND/kg. In rural households, 47% agreed to pay 778.96 VND/kg, and 11% of mountainous households agreed to pay an average of 673.04 VND/kg. These insights can guide tailored waste management pricing strategies across diverse urban and rural settings.

Keywords: contingent valuation method, Hanoi, household solid waste, solid waste management, willingness to pay.

Classification number: 5.3

1. Introduction

Solid waste management is a major environmental problem in Vietnam [1]. According to a World Bank report, the amount of household solid waste generated by Vietnamese people is estimated to be 1.14 kg/person/day, higher than the average of other middle-income countries at 0.79 kg [2].

Globally, many countries have practical policies for managing, collecting, and treating solid waste. They have introduced various measures and regulations to encourage compliance, increase recycling rates by classifying waste at the source, and apply modern waste treatment and recycling technologies. Intentional violations are severely punished. To reduce the financial burden on residents, many governments subsidise waste collection companies.

Amornchai Chalcharoenwattana and Chanathip Pharino, in a report on urban waste management in Thumbon Phang Khon, Sakon Nakorn Province, Thailand, describe a voluntary waste collection service operated by a government agency, collecting municipal fees of 1.95 USD (1 USD = 30.73 Thai Baht) per household per year for daily waste collection. Additionally, the landfill charges 16.27 USD/ton for municipal waste from operating agencies and 32.54 USD/ton from private units. The research shows that community-based urban waste management reduces greenhouse gas emissions and landfill costs by about 7.41 USD/ton of urban waste, potentially reducing costs to 6.55 USD/ton according to estimated scenarios.

This study provides important lessons and demonstrates that urban waste reduction through community activities can be scaled up and implemented in approximately 50% of communities in Thailand and Southeast Asia, where populations still reside in rural or peri-urban areas [3].

M.D.M. Samsudina's 2013 publication on municipal solid waste management in Malaysia describes the country's situation, estimating that Malaysia generated about 5,475 million tons of municipal solid waste in 2001, equivalent to 0.81-1.7 kg/person/day [4]. The most common method is landfilling, primarily using public landfills due to its low cost, posing significant challenges for solid waste management with relatively poor management levels and a lack of organisation. The average solid waste management fee in Malaysia is only about 0.06 RM (~311.09 VND) per kg of waste, equivalent to about 1.2 USD/ton (~27,600 VND/ton). Compared to other developing countries, this fee is much lower, such as Argentina and Brazil, where fees range from 5-18 USD/ton (~115,000-414,000 VND/ton); Chile (5-17 USD/ton ~115,000-391,000 VND/ton); Mexico (4-17 USD/ton~92,000-391,000 VND/ton); South Africa (12 USD/ton~276,000 VND/ton); Hong Kong (10 USD/ton~230,000 VND/ton); and equivalent to countries in the region such as Indonesia (1.3 USD/ton~30,000 VND/ton) and China (2.5 USD/ton~57,500 VND/ton). Given these low fees, Malaysia's solid waste management charges need careful consideration to ensure the necessary resources for solid waste management can be secured and sustained.

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Currently, the Vietnam's Environmental Protection Law 2020 has introduced many new provisions related to household solid waste management. A fundamental change is the shift in regulations on payment for household solid waste collection, transportation, and treatment services from state subsidies to self-paying entities. Clause 1, Article 79, stipulates that the price of household waste management services should be calculated based on the mass or volume of classified waste, including recyclable and reusable waste, and hazardous waste. It requires classification of household solid waste at the source into three categories: recyclable solid waste, food waste, and other common household solid waste [5]. The Vietnam Ministry of Natural Resources and Environment will provide guidance on pricing methods for household solid waste management services, establish economic and technical norms for the collection, transportation, and treatment of municipal solid waste, and offer technical guidance on household solid waste classification. Regulations on collecting fees for household solid waste management will be implemented from 2025 [6, 7]. For these regulations to be effectively implemented, public understanding and cooperation with the Government are essential.

Hanoi, as a metropolitan area and the centre of economic, political, cultural, technological, and educational activities in Vietnam, faces increasing domestic waste due to rapid economic development, high living standards, and high consumption. This growth places significant pressure on the collection, transportation, and treatment of waste. On average, Hanoi generates about 6,500 tons of household solid waste daily. Most urban solid waste has not been classified at the source, as classification is not mandatory. The collection rate of household solid waste in inner-city areas is about 95%, while it is 66% in suburban areas [2]. However, different types of waste are often mixed and transported to treatment sites, where 89% of household solid waste is treated by landfilling and 11% by incineration [2]. The management of household solid waste, from classification and collection to transportation and treatment, remains outdated, posing significant environmental pollution risks and public health concerns [1].

Research in Vietnam has primarily focused on the theoretical basis and general models of household solid waste management, along with the current situation related to municipal solid waste. Few studies have examined factors influencing the implementation of solid waste analysis, collection, and transportation, or the WTP among urban, rural, and mountainous populations for household solid waste services. This study aims to fill this research gap by analysing and evaluating the differences in WTP among communities in Hanoi.

To assess the readiness to implement the new regulations of the Vietnam Environmental Protection Law 2020 regarding household solid waste management pricing based on classified mass or volume, and to understand how socio-economic status influences WTP across different urban, rural, and mountainous communities within Hanoi, this study was conducted. The findings are crucial for stakeholders, including policymakers, urban planners, and the community, to develop more effective and equitable waste management policies that can improve compliance rates and sustainability.

2. Methodology

2.1. Data sources and collection

2.1.1. Secondary data sources and collection methods

The collected data include socio-economic conditions, census reports, documents and reports from the Hanoi Department of Natural Resources and Environment, District Department of Natural Resources and Environment, current legal documents related to household solid waste management, and reports from the Environment and Urban Environment Limited Company (URENCO) with data on household solid waste management and treatment planning in Hanoi [8]. Published papers in journals related to the research subject, including studies on WTP using the CVM random assessment method [9-13], and published papers on people's WTP and pricing plans for solid waste management services in Vietnam [14-17], were also reviewed.

2.1.2. Primary data sources and collection methods

Primary data were collected through a questionnaire survey to gather information about the current status of household solid waste management. The study conducted online and face-to-face interviews with household heads or income earners in households in Hanoi.

2.2. Method for forecasting the amount of household solid waste generated by 2030

To forecast the amount of household solid waste generated in Hanoi by 2030, the study is based on the improved Euler model. The improved Euler formula (Eq. 1) is a mathematical formula used to forecast population, expressed as follows [14]:

$$N_{i+1}^* = N_i + r \times N_i \times \Delta t \quad (1)$$

where, N_{i+1}^* : population after one year (people); N_i : initial population (people); r : population growth rate (%/year); Δt : time (year).

From the results of the projected population over the years calculated, the average amount of waste generated in the following years is calculated according to the formula (Eq. 2) [14]:

$$X_{i+1} = X_i + n \times X_i \quad (2)$$

where X_{i+1} : the average amount of waste generated in year $i+1$; X_i : the average amount of waste generated in year i ; n : rate of increase in discharge rate (%).

2.3. Contingent valuation method

To estimate people's WTP to implement a plan to calculate the price of household solid waste management services based on the mass or volume of classified waste, the study followed the steps of the CVM. CVM essentially provides values (in terms of WT) or willingness to accept compensation - WTA) of the concerned people for an environmental good as stated by them during a survey. WTP and WTA are used because many of the project impacts to be included in the economic analysis will be unmarketable, such as biodiversity preservation, or incompletely marketed, such as water supply and sanitation benefits.

The steps to conduct specific research are as follows:

2.3.1. Set up investigations and interviews

Purpose: To collect information about the socio-economic characteristics of the interviewee's household, activities generating household solid waste, waste composition, and the WTP for implementing the plan to calculate the price of household solid waste management services based on the mass or volume of classified waste in Hanoi.

Structure of the survey form: i) Set up questions to collect information related to the current status of waste classification and management in household activities; ii) Develop a scenario and set up questions to collect information about people's WTP to implement a plan to calculate the price of household solid waste management services based on the mass or volume of classified waste in Hanoi; iii) Collect general information such as full name, year of birth, gender, address, phone number, ID card, education level, occupation, average income, number of people in the household, and area of the house.

2.3.2. Conduct interviews with a specified number of samples

The survey sample size is calculated according to the following formula [18]:

$$n = \frac{N}{1 + Ne^2} \quad (3)$$

where, n : is the sample size; N : is the total number of households in the study area; e : is the standard error (this study chooses $e=0.05$).

According to the 2020 statistical yearbook, the total number of households in Hanoi was 2,224,107. From this value, the following sample size is calculated as:

$$n = \frac{2,224,107}{1 + 2,224,107 \times 0.05^2} \approx 400$$

Thus, the study was conducted with a sample size of 405 questionnaires. To form a basis for comparison between the research areas (urban, rural, and mountainous), the study distributed and conducted 135 surveys in each of the three areas using random sampling.

Before the official investigation, the research team conducted a pilot survey of 20 questionnaires to adjust the script and questionnaire accordingly. In the pilot survey, prices were given in the form of open-ended questions, and respondents indicated the price they were willing to pay. The results from the trial survey obtained three price levels: 650, 900, and 1,500 VND/kg. This formed the basis for including prices in the official questionnaire according to the double-bounded random survey model.

In the official survey, the research team used valuation questions in the form of an auction and conducted the survey according to the double-bounded random survey model. The iterative randomised survey model is depicted in Fig. 1 [19].

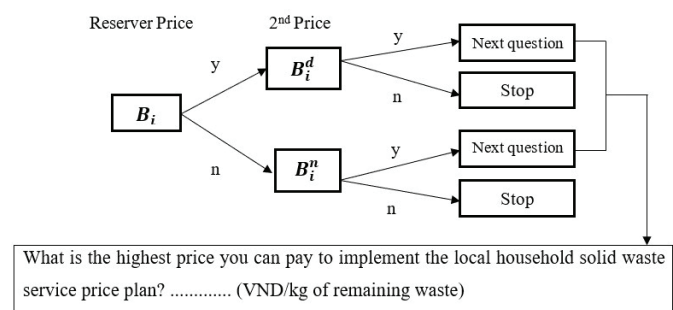


Fig. 1. The double-bounded model.

According to the double-bounded survey model, the interviewee will be asked, "Do you agree with the starting WTP price?" In this study, the starting price is taken from the average WTP price of several people in the trial survey form. If people answer "yes," it means they can pay a higher price, and they will continue to be asked their WTP a higher price until they find the highest WTP. If the person answers "no," it means they will only pay the lower price, and they will be asked the lower price, then asked about their highest WTP. This is the basis for choosing the appropriate price in the iterative double-bounded survey model.

2.4. Data processing methods

Microsoft Excel was used to perform calculations with encrypted data from the questionnaire after the interviews were completed.

- Interview results were analysed using the descriptive statistics tool in Excel to conduct statistics and calculate the average WTP.

- The total WTP was calculated in the following manner:

TWTP of all households = Average WTP × Total population of Hanoi × % of people WTP

The WTP level varies due to a wide dependence on many factors such as income, education level, age, gender, and household average monthly water volume use. Thus, the WTP function has the following form:

$$WTP_i = \beta_0 + \beta_1 (\text{Income}) + \beta_2 (\text{Education}) + \beta_3 (\text{Gender}) + \beta_4 (\text{Age}) + \beta_5 (\text{Members}) + \beta_6 (D) + \beta_7 (K) \quad [14]$$

where WTP: willingness to pay (VND); β_0 : intercept; $\beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6, \beta_7$: coefficients.

The coefficients are explained as follows:

Income: The study employs a correlation model using pseudo-variables for the income variable coded as follows:

- Equal to 1 if less than 5 million VND/month.
- Equal to 2 for 5-10 million VND/month.
- Equal to 3 for 10-15 million VND/month.
- Equal to 4 for 15-20 million VND/month.
- Equal to 5 for 20-25 million VND/month.
- Equal to 6 for 25-30 million VND/month.
- Equal to 7 for 30-35 million VND/month.
- Equal to 8 for over 35 million VND/month.

Education: The study uses dummy variables for the education level variable coded as follows:

- Degree 0: no degree.
- Degree 12: high school.
- Degree 16: university/college/intermediate.
- Degree 18: post-graduate.

Gender: The study uses dummy variables for gender coded as follows:

- Equal to 1 for male.
- Equal to 0 for female.

Age: The study uses dummy variables for age coded as follows:

- Equal to 1 for under 18 years old.
- Equal to 2 for 18-24 years old.
- Equal to 3 for 24-55 years old.
- Equal to 4 for over 55 years old.

Members: Number of people in household.

Jobs (D): The study uses dummy variables for jobs coded as follows:

- Equal to 1 for civil servants.
- Equal to 2 for free business.
- Equal to 3 for students.
- Equal to 4 for workers.
- Equal to 5 for housewives/retirees.
- Equal to 6 for other professions.

Volume of household solid waste (K): Volume of household solid waste in kg/day/household.

3. Results and discussion

3.1. Assessing the current status of household solid waste generation and collection in Hanoi

According to the results from a survey of 405 households, the average amount of daily household solid waste is 1.182 kg/household/day. The amount of household solid waste varies across different areas, with urban areas generating the highest rate at 1.27 kg/household/day, followed by rural areas at 1.21 kg/household/day, and mountainous areas at 1.067 kg/household/day. This discrepancy can be attributed to the higher population density and demand for goods and services in urban areas compared to rural and mountainous areas. Additionally, the predominant housing type in urban areas, such as apartment buildings with dense populations, makes recycling household solid waste more challenging than in the other two areas, resulting in a higher per capita waste generation in urban areas.

Comparing the results of the household solid waste volume generation coefficient in urban, rural, and mountainous areas of Hanoi residents (Fig. 2) with data from the 2019 State of the Environment Report shows a smaller coefficient. Specifically, the average generated volume in urban areas is approximately 3 kg/household/day, and in rural and mountainous areas, it is approximately 2.5 kg/household/day. The discrepancies in the survey results may be due to a limited number of sample forms, short survey duration, and infrequent survey repetitions, which could affect accuracy.

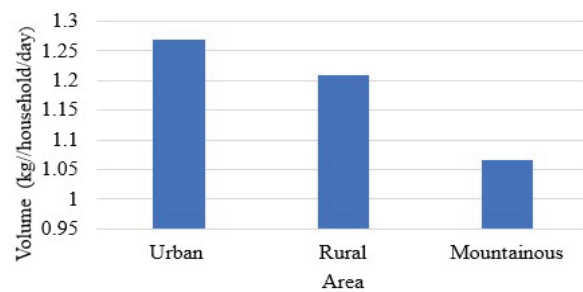


Fig. 2. Household solid waste volume for each study area.

The survey results from 405 households registered for household solid waste collection services revealed that 81.23% of respondents believe the frequency of household solid waste collection is reasonable, while 18.77% consider it unreasonable. Additionally, 83.46% of respondents think the timing of household solid waste collection is reasonable, whereas 16.54% do not. The assessment of collection frequency (81.23%) from the questionnaire is almost similar to the collection results in Hanoi's report on the current state of the environment from 2016-2020 (93%). The discrepancy in the assessment results, particularly in rural and mountainous areas, may be due to the equal distribution of the questionnaire across these three areas.

According to the survey results, 59% of respondents recognise the importance of separating household solid waste before treatment. Most questions on household solid waste pertained to two categories: organic waste and inorganic waste (36% of total questions). However, a significant portion (41%) indicated no classification, with 90% citing the inconvenience of garbage separation as the primary reason. Other reasons include time constraints and the mixing of domestic waste with other types of waste. These findings suggest that respondents in Hanoi are not fully aware of the benefits and drawbacks of not segregating waste at the source. In-depth interviews with URENCO staff revealed that while URENCO complies with standard engineering, shipping, and handling procedures, and possesses modern and sophisticated machinery, equipment, and technology for cleaning and maintenance, domestic solid waste management and public understanding of URENCO's operations vary between urban, rural, and mountainous areas. Urban residents have a better understanding of business operations and benefits from domestic solid waste management than those in rural and mountainous areas. Based on evaluation data, the study proposes measures for effective communication and management of domestic solid waste in Hanoi. Currently, the collection of household solid waste on small roads is not yet mechanised; collection vehicles are often rusted, causing overflow and leakage of wastewater, which creates an unhygienic environment during collection and transportation.

3.2. Forecasting the amount of household solid waste generated in Hanoi by 2030

Based on the population and annual population growth rate of the locality, the study calculated the current amount of household waste and estimated the volume generated by 2030. The forecast

population is calculated according to Eq. 1. According to the Hanoi Socio-Economic Development Plan for the period up to 2025 and the vision to 2030, the forecast population growth rate from 2025 to 2030 is 2.2%.

According to the Hanoi City Socio-Economic Development Plan for the period up to 2025 and vision to 2030, the forecast population growth rate from 2025 to 2030 is 2.2%.

According to statistics from the General Department of Environment (2014), the rate of solid waste generation in the Red River Delta region is 10% per year. Based on the 2019 environmental status report, the average amount of waste generated per person in Hanoi is 0.81 kg/person/day. The household solid waste collection efficiency is 93%. The results of calculating the amount of household solid waste generated over the years are presented in Table 1.

Table 1. Household solid waste generated in Hanoi by 2030.

Year	Population growth rate (%)	Population (thousands of people)	Average amount of household solid waste generated (kg/person/day)	Average amount of household solid waste generated (tons/day)	Volume of household solid waste collected and treated (tons/day)
2020	2.2	8,230.2	0.89	7,333.1	6,819.76
2021	2.2	8,411.2	0.98	8,243.8	7,666.78
2022	2.2	8,596.3	1.08	9,267.7	8,618.99
2023	2.2	8,785.4	1.19	10,418.8	9,689.47
2024	2.2	8,978.7	1.30	11,712.8	10,892.90
2025	2.2	9,176.2	1.43	13,167.5	12,245.80
2026	2.2	9,378.1	1.58	14,802.9	13,766.73
2027	2.2	9,584.4	1.74	16,641.5	15,476.56
2028	2.2	9,795.3	1.91	18,708.3	17,398.74
2029	2.2	10,010.8	2.10	21,031.9	19,559.67
2030	2.2	10,231.0	2.31	23,644.1	21,988.98

Source: Compiled by the authors.

It is forecasted that the amount of household solid waste generated will be 13,167.5 tons/day by 2025 and 23,644.1 tons/day by 2030. The increasing volume of household solid waste necessitates improvements in the current system of collection, transportation, and treatment to meet future requirements.

3.3. Evaluating willingness to pay for implementing a solution and calculating prices for household solid waste collection, transportation, and treatment services in Hanoi

The results in Table 2 show that, of the 405 surveyed households, there are 218 male heads (54%) and 187 female heads (46%). Thus, the gender disparity among the respondents is not significant.

The majority of interviewees in Hanoi belong to the 18-24 age group, indicating that most respondents are of appropriate school age and possess knowledge of environmental protection, thus providing reliable answers. Additionally, the diversity in age groups allows for an equitable assessment of payment levels.

Table 2. Characteristics of the interviewees.

Characteristics of the subject	Rate
Gender	Male (54%), Female (46%)
Ages	Under 18 age (2.0%), from 18-24 age (55.1%), from 24-55 age (38.5%), over 55 age (4.4%)
Educations	No degree (1%), secondary (15%), university/college/intermediate (68%), Postgraduate (16%).
Jobs	Students (52%), officer/civil servant (13%), freelance business (24%), worker (6%), housewife/retired (3%), other (2%)
Members	One person (4.2%), two people (18.8%), three people (12.8%), four people (35.8%), five people (14.6%), six people (6.9%), seven people (5.9%), eight people (1%)
Income	Under 5 million VND/month (19%), 5-10 million VND/month (19%), 10-15 million VND/month (21%), 15-20 million VND/month (11%), 20-25 million VND/month (4%), 25-30 million VND/month (4%), 30-35 million VND/month (7%), >35 million VND/month (15%)

Source: Compiled by the authors.

The average number of people per household is 3.87. Households with four members constitute the highest rate (35.8%), while those with eight members constitute the lowest rate (1%). Most households have 3-5 members, affecting the volume of household solid waste generation.

Regarding education level, 15% of the households have secondary education, 68% have university/college/intermediate degrees, and 16% have postgraduate degrees. Thus, the education level of the household heads is predominantly at the university/college/intermediate level or higher. The high education level is likely a key factor influencing people's actions and perceptions of the environment, as well as their WTP.

In terms of occupation, the industry is relatively diverse. Students represent the largest proportion (52%), followed by self-employed individuals (24%), and civil servants (13%). The proportion of people working in non-state occupations (workers, traders, other professions) is about three times higher than those in the public sector. The high percentage of students indicates a strength in making informed and reasonable choices due to their knowledge and learning.

Income distribution among the respondents shows that 21% have incomes in the range of 10-15 million VND/month, primarily state employees. Incomes below 5 million and from 5 to 10 million VND/month account for 19% each (students, workers, and other professions). Incomes over 35 million VND/month, mainly from business and trading, account for 15%. Thus, the interviewees' income is generally average or higher, with a higher percentage of middle-income households or above. This higher income correlates with a greater WTP for improved sanitation services, reflecting the household's conditions.

Table 3. Descriptive statistics of WTP for domestic solid waste collection, transportation, and treatment services based on the volume or waste classification in Hanoi.

Target	Unit	WTP _{urban area average}	WTP _{rural area average}	WTP _{mountainous areas average}
Mean	VND/kg	1,136	778.96	673.04
Standard error		30.19	12.40	10.70
Median	VND	930	650	650
Mode	VND	1,500	650	650
Minimum	VND	650	500	300
Maximum	VND	1,500	1000	1,500
Sum	VND	153,360	105,160	90,860
Count		135	135	135

The results in Table 3 show the average WTP of different households in each area:

Households in urban areas have an average WTP of 1,136 VND/kg. The study shows that 85% of households are willing to pay a price higher than the proposed price of 930 VND/kg, while 15% are not.

Households in rural areas have an average WTP of 778.96 VND/kg. The study shows that 47% of households are willing to pay a price higher than the proposed price of 930 VND/kg, while 53% are not.

Households in mountainous areas have an average WTP of 673.04 VND/kg. The study shows that 11% of households are willing to pay a price higher than the proposed price of 930 VND/kg, while 89% are not.

3.4. Analysing factors affecting and comparing willingness to pay in the three research areas

Regression analysis was conducted using Microsoft Excel to identify factors affecting WTP, with independent variables including age, gender, demographics, occupation, education level, income, and the amount of household solid waste generated. The results of the regression model are shown in Table 4.

The regression equation is given for each area below.

For households in urban areas:

$$WTP_{\text{urban areas}} = 237.5\text{Constant} + 122.64\text{Income} + 6.66\text{Education} + 44.67\text{Gender} - 20.22\text{Age} - 13.33\text{Members} + 33.76\text{D} + 66.99\text{K}$$

The analysis results show that the multiple correlation coefficient (Multiple R) is approximately 0.769, indicating that the selected linear regression model is very suitable. R-Square=0.591 means that the independent variables in the model (income, education, gender, age, demographics, jobs, and the amount of domestic solid waste) explain about 59.5% of the volatility of Y (WTP). About 40.9% is due to random factors and other factors not included in the model (online survey factor).

Table 4. Regression results of willingness to pay dependent variable of surveyed households.

Targets	WTP _{urban areas}		WTP _{rural areas}		WTP _{mountainous areas}	
	Coefficients	p-value	Coefficients	p-value	Coefficients	p-value
Constant	237.50	0.169	611.39	4.00694E-07	560.01	1.19001E-07
Income	122.64	2.3598E-21	58.61	1.6264E-08	57.26	1.21422E-10
Education	6.66	0.325	5.17	0.151	4.75	0.231
Gender	44.67	0.274	-10.27	0.614	-3.80	0.836
Age	-20.22	0.554	25.55	0.171	5.03	0.814
Members	-13.33	0.371	8.15	0.349	-4.60	0.521
D (occupation)	33.76	0.061	1.76	0.864	-8.25	0.560
K (volume of domestic solid waste)	66.99	0.041	27.26	0.031	23.80	0.034

For households in rural areas:

$$WTP_{\text{rural areas}} = 611.39\text{Constant} + 58.61\text{Income} + 5.17\text{Education} - 10.27\text{Gender} + 25.55\text{Age} + 8.15\text{Members} + 1.76\text{D} + 27.26\text{K}$$

The analysis results showed that the multiple correlation coefficient (Multiple R) is approximately 0.639, indicating that the selected linear regression model is very suitable. R-Square=0.508 means that the independent variables in the model (income, education, gender, age, demographics, jobs, and the amount of domestic solid waste) explain about 50.8% of the volatility of Y (WTP). About 49.2% is due to random factors and other factors not included in the model (online survey factor).

For households in mountainous areas:

$$WTP_{\text{mountainous areas}} = 560.01\text{Constant} + 57.26\text{Income} + 4.75\text{Education} - 3.80\text{Gender} + 5.03\text{Age} - 4.6\text{Members} - 8.25\text{D} + 23.8\text{K}$$

The analysis results showed that the multiple correlation coefficient (Multiple R) is approximately 0.614, indicating that the selected linear regression model is very suitable. R-Square=0.477 means that the independent variables in the model (income, education, gender, age, demographics, jobs, and the amount of domestic solid waste) explain about 47.7% of the volatility of Y (WTP). About 52.3% is due to random factors and other factors not included in the model (online survey factor).

Observation of the model showed that the variables of income and the volume of domestic solid waste are directly proportional to the WTP, while the remaining variables have varying relationships with WTP depending on the area.

Firstly, the higher the income, the higher the WTP. When other variables are constant, if the income in urban areas increases by 1 unit, the WTP increases by 122.64 VND. Calculation results: p-value for urban areas (2.3598E-21)<0.05 shows that the income variable has a close relationship with the WTP variable.

When other variables are constant, if the income in rural areas increases by 1 unit, the WTP increases by 58.61 VND. Calculation results: P-value for rural areas ($1.6264E-08$) <0.05 shows that the income variable has a close relationship with the WTP variable.

When other variables are constant, if the income in rural areas increases by 1 unit, the WTP increases by 58.61 VND. Calculation results: p-value for rural areas ($1.6264E-08$) <0.05 shows that income variable has a close relationship with WTP variable.

When other variables are constant, if the income in mountainous areas increases by 1 unit, the WTP increases by 57.26 VND. Calculation results: p-value for mountainous areas ($1.21422E-10$) <0.05 shows that the income variable has a close relationship with the WTP variable.

Secondly, the greater the amount of household solid waste, the greater the WTP and has important implications in paying waste fees. When other variables are constant, if the amount of household solid waste in urban areas increases by 1 unit, the WTP increases by 66.99 VND. P-value for urban areas (0.041) <0.05 shows that the amount of household solid waste variable has a close relationship with the WTP variable.

When other variables are constant, if the amount of household solid waste in rural areas increases by 1 unit, the WTP increases by 27.26 VND. P-value for rural areas (0.031) <0.05 shows that the amount of household solid waste variable has a close relationship with the WTP variable.

When other variables are constant, if the amount of household solid waste in mountainous areas increases by 1 unit, the WTP increases by 23.80 VND. P-value for mountainous areas (0.034) <0.05 shows that the amount of household solid waste variable has a close relationship with the WTP variable.

Research results show that among the major factors directly affecting the WTP variable for domestic solid waste, the two factors “income” and “the amount of solid waste generated” have the greatest influence. This will be the basis for research to develop appropriate solutions. For example, the city government of Hanoi can implement solutions to improve environmental knowledge and promote the behaviour of classifying domestic waste at the source to help reduce the amount of waste discharged into the environment.

Although there have been many efforts to collect and investigate data on the status of household solid waste, as well as the WTP for household solid waste collection, transportation, and treatment services in Hanoi, the CVM method has several limitations related to the technique of assessing WTP. The process of collecting information still faces many difficulties because people are not familiar with the investigation method and may not understand the questions correctly when hypothetical situations are presented.

3.5. Proposing policy recommendations in household solid waste management in Hanoi

Based on model research results, it is evident that the variables of income and volume of household solid waste generated are directly proportional and closely correlated with the WTP variable. Thus, these findings form the basis for making recommendations. To improve the effectiveness of solid waste management policies in line with the 2020 Environmental Protection Law in Hanoi, the following recommendations are proposed:

Firstly, management agencies need to assess the characteristics of each region in household solid waste management, considering the impact factors. Policymakers should consider costs and financial support, particularly for infrastructure required for classifying household solid waste at source. It is crucial for managers to understand the aspirations and financial capacities of people in each region, allowing for adjustments in policies and flexible service pricing plans tailored to each area. When implementing differential pricing, the management agency should consider the variability in payment capacity across urban, rural, and mountainous areas as revealed by WTP analyses.

Secondly, policies should be established to support low-income households in participating in the solid waste management process. Support measures may include funding for collection, transportation, and waste treatment, as well as providing equipment and tools for waste classification and collection. This helps balance the cost burden and enables low-income households to actively engage in sustainable solid waste management.

Thirdly, public cooperation is essential for the success of waste management policies. Comprehensive public awareness and education campaigns should be conducted to inform residents about the importance of waste management and the specifics of new policies. Local media, schools, and community centres should be utilised for outreach.

Although there have been significant efforts to collect and investigate data on the status of domestic solid waste and the WTP for household solid waste collection, transportation, and treatment services in Hanoi, the CVM method has limitations related to assessing WTP. The information collection process faces challenges as people are not familiar with the investigation method and may not understand the questions correctly when hypothetical situations are presented. The study focuses on certain areas of Hanoi, and due to economic and time constraints, the experimental scale is limited. It is recommended that future studies be conducted on a larger scale and with a larger sample size.

4. Conclusions

The study used the CVM to investigate, compile statistics, and analyse the current status of household solid waste management. It also estimated people’s consensus and differences in WTP for household solid waste collection, transportation, and treatment services among urban, rural, and mountainous populations in Hanoi.

Research results showed that the average household solid waste in urban areas is the highest, at 1.27 kg/household/day, followed by rural areas at 1.21 kg/household/day, and mountainous areas at 1.067 kg/household/day. However, most household solid waste has not been classified at source. Eighty-five percent of urban households agreed to pay higher than the proposed price, with an average WTP of 1,136 VND/kg; forty-seven percent of rural households agreed to pay 778.96 VND/kg, and eleven percent of mountainous households agreed to pay an average of 673.04 VND/kg. Among the major factors that directly affect the WTP for domestic solid waste, two factors - income and the amount of solid waste generated - have the greatest influence.

The research results are crucial and can influence policymaking, affect public behaviour, and potentially lead to improvements in environmental, economic, and social sustainability in Hanoi. The study has proposed several well-founded and appropriate solutions for the Hanoi area, as well as specific implementing entities, to increase financial resources, raise community awareness, and improve the quality of household solid waste management services for local residents. These provide a practical basis for developing effective household solid waste management solutions suitable to local conditions.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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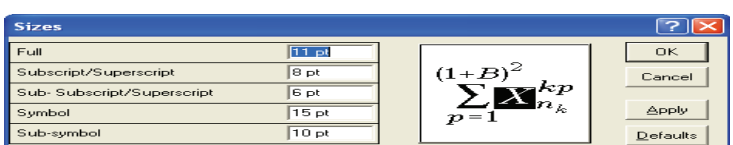


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