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Synthesis, characterization, anticancer, antibacterial and antioxidant activities of novel thymol ester compound

Inas Bsharat [a,*], Lubna Abdalla [b], Ashraf Sawafta [b], Ibrahim M. Abu-Reidah [c,d,**], Mohammed A. Al-Nuri [a,e,*]

[a] Department of Chemistry, Science College, An-Najah National University, P.O. Box 7, Nablus, Palestine
[b] Biology and Biotechnology Department, An-Najah National University, P.O. Box 7, Nablus, Palestine
[c] CREAIT, Memorial University of Newfoundland, St. John's, NL A1C 5S7, Canada
[d] Faculty of Sciences, Arab-American University, P.O Box 240 Jenin, 13 Zababdeh Jenin, Palestine
[e] Faculty of Pharmacy, Arab-American University, P.O Box 240 Jenin, 13 Zababdeh Jenin, Palestine

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ABSTRACT

Background: Thymol and its derivatives have been proven in earlier studies to exhibit a broad range of biological activities, particularly given that many of them have therapeutic uses.
Objectives: This study focused on preparing a new ester of thymol, and the new compound was studied as antibacterial, anticancer and as antioxidant agent.
Methodology: Thymol was condensed with indole-3-carboxylic acid in DCM solvent at room temperature (25 °C) without catalyst to form the new thymol ester compound **E2** (Fig. 1), and then the new compound was identified through FT-IR and NMR analysis, and the biological properties were tested and studied.
Results: The results showed that the novel **E2** ester molecule was variably effective against all tested cancer and bacterial cells in different amounts.
Conclusion: According to this study, thymol ester **E2** preserved normal muscle cells more effectively than thymol. In contrast to thymol, the **E2** derivative had superior efficacy against the Gram-positive bacteria under investigation (*S. aureus* and *S. epidermis*) in the antibacterial trial. Additionally, its inhibition zone was larger than Gentamicin against *S. epidermis*. Additionally, the **E2** ester molecule exceeded thymol in an antioxidant assay for scavenging capacity at low concentrations.

1. Introduction

Esters are one of the most significant organic chemical components found in food [1], plasticizers [2,3], cosmetics production [4,5], production of fertilizer [6], and in the medical field [7,8].

Numerous investigations have demonstrated the wide range of biological activities exhibited by monoterpenoids and the compounds derived from them [9–15]. With the chemical formula $C_{10}H_{14}O$, a monoterpenoid phenol derivative of p-Cymene that occurs naturally is called thymol (2-isopropyl-5-methylphenol). At 51.34 %, thymol has the greatest concentration of any thyme essential oil [16]. Thymol possesses antioxidant, antiseptic, antibacterial, and antifungal effects [17–19]. A prior study found that after increasing thymol concentrations for 24 h, the cell viability of gastric cancer cells reduced from 89.56 % to 50.75 % [20]. Lung cancer can benefit from thymol treatment [21]. Thymol may be effective against a variety of malignancies because it causes mitochondrial malfunction and apoptosis [20]. Molecules having an acetohydrazone linkage in thymol hydrazone derivatives have anticancer and antioxidant effects [22] and thymol esters exhibit a variety of biochemical properties [23–29]. *In vitro*, thymol and thymol octanoate both demonstrated biological activity against C. parvum while exhibiting minimal damage towards HCT-8 cells. When evaluating the impact on parasite growth, thymol had a similar effect to thymol octanoate against C. parvum invasion [30]. Additionally, the ROS produced by acetic acid thymol ester is significantly lower at ~0.08 μg/mL compared to thymol at ~60 μg/mL and it exhibits cytotoxic and genomic effects [31].

By forming hydrogen bonds with biological molecules, heteroatoms

* Corresponding authors.
** Corresponding author at: CREAIT, Memorial University of Newfoundland, St. John's, NL A1C 5S7, Canada.
E-mail addresses: i.bsharat@najah.edu (I. Bsharat), iabureidah@gmail.com, iabureidah@mun.ca (I.M. Abu-Reidah), Maalnuri@najah.edu (M.A. Al-Nuri).

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such as nitrogen in the indole ring can increase the compound's binding affinity and selectivity. Furthermore, heteroatoms change the way a molecule's electrons are distributed, which affects how reactive it is and how it interacts with biological targets. This can enhance the biological qualities of the phenolic group of thymol (an oxygen atom attached to a benzene ring). Thus, this work focused on the condensation reaction between thymol and indole-3-carboxylic acid compound in dichloromethane at room temperature (25 °C) to prepare a new ester. Utilizing FT-IR, ^{1}H, and ^{13}C NMR spectroscopy, the new thymol ester compound's chemical structure was identified. A novel ester molecule was also tested for its potential as an antioxidant, antimicrobial, and anticancer activity (Fig. 1).

2. Materials and methods

2.1. Material and instrumentation

We bought thymol, indole-3-carboxylic acid, triethylamine, and oxalyl chloride from Sigma-Aldrich Chemical Company. We purchased hexane, dichloromethane, ethyl acetate, methanol, dimethyl sulfoxide, and dimethylformamide from Chemical Science Company. We did not perform any further purification while using all of the chemicals and solvents. A Bruker 500 MHz-Avance III was utilized by the University of Jordan's Chemistry Department to identify ^{1}H and ^{13}C NMR Nuclear Magnetic Resonance Spectra. An-Najah National University's Chemistry Department identified infrared spectroscopy (FT-IR) using a Fourier Transform Infrared Spectrophotometer (Nicolet 1s5-Id3). Sigma-Aldrich Chemical Company provided the Merck Kiesel gel 60 F254 pre-coated silica gel plates utilized in the TLC investigation. A UV lamp was used for visualization.

2.2. Synthesis of thymol ester (E2)

Ten milliliters of dry dichloromethane were mixed with one equivalent of indole-3-carboxylic acid (0.525 g, 3 mmol) and two drops of dry dimethylformamide in a round-bottom flask kept in an ice water bath under nitrogen atmosphere. After adding two equal parts of oxalyl chloride (1 mL, 12 mmol) to the mixture, it was stirred for three hours at 25 °C without catalyst. Subsequently, the solvent was removed under vacuum. A stirred residual solution in 10 mL of dry dichloromethane was combined with a solution of thymol (1 equivalent) (0.55 g, 3.7 mmol) and triethylamine (1 equivalent) (0.5 mL, 4.5 mmol) under nitrogen atmosphere. The mixture was then left to stir for 24 h at room temperature (25 °C). Saturated sodium bicarbonate was used to quench the reaction with dichloromethane (1:1) three times. After using $MgSO_4$ drying agent to dry the mixed organic layer, after filtering the sample, the solvent was vacuum-evaporated. The product was purified using flash chromatography and an eluant system consisting of hexane:ethyl acetate (70:30). To produce the **E2** pure compound, crystals were formed from the end product by crystallization using solvents dichloromethane and hexane (4.5:0.5).

2.3. Characterization

2-isopropyl-5-methylphenyl 1H-indole-3-carboxylate (E2)

Purified by flash chromatography using a hexane: ethyl acetate (70:30) as an eluant. Light pink colored crystal; 77.8 % yield; Melting point 183–184 °C; FT-IR functional groups: 3273.66 cm^{-1} $_{N\text{-}Hindole}$, 2961.49 $cm^{-1}_{C\text{-}H\ aromatic}$, 2869.82 $cm^{-1}_{C\text{-}H\ aliphatic}$, 1690.16 $cm^{-1}_{C=O\ ester}$, 1619.9 $cm^{-1}_{C=C\ thymol}$, 1581 $cm^{-1}_{C=C\ indole}$. ^{1}H NMR (500 MHz, DMSO, ppm): 12.14 (1H, s, broad), 8.31 (1H, d, J = 7.60 Hz), 8.01 (1H, d, J = 7.60 Hz), 7.54 (1H, d, J = 7.60 Hz), 7.23–7.26 (3H, m), 7.06 (1H, d, J = 7.90 Hz), 6.95 (1H, s), 3.03 (1H, sept, J = 7.00 Hz), 2.29 (3H, s), 1.14 (6H, d, J = 7.00 Hz). ^{13}C NMR: (ppm), 136.39 (1C, -C=O), 148.34 (1C, -C-O-), 137.62 (1C, -NH-C=C- indole), 137.04 (1C, -O-C=C-CH$(CH_3)_2$), 136.49 (1C, -C=C-CH_3), 126.26 (1C, -NH-C=C- indole), 105.90 (1C, -C=C-C=O- indole), 133.36 (1C, -NH-C=C-C=O- indole), 128.55 (1C, CH aryl thymol), 127.20 (1C, CH aryl thymol), 123.60 (1C, CH aryl thymol), 122.22 (1C, CH aryl indole), 120.60 (1C, CH aryl indole), 118.10 (1C, CH aryl indole), 112.50 (1C, CH aryl indole), 26.90 (1C, -CH$(CH_3)_2$), 23.02 (2C, 2CH_3), 21.82 (1C, CH_3).

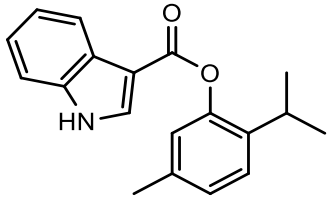


Fig. 1. 2-isopropyl-5-methylphenyl 1H-indole-3-carboxylate (E2).

2.4. Biological assays

2.4.1. Anticancer activity

Normal muscle cells (L6, ATCC number:CRL-1458, human, from the skeletal muscle), human breast cancer cells MCF7 (ATCC number:HTB-22), human prostate cancer cells (PC3, ATCC number:CRL-1435, human, from the human prostate), human liver cancer cells (HepG2, ATCC number:HB-8065, human, from the epithelial cells of the liver), and human cervix cancer cells (ATCC number:CCL-2, from a cervical carcinoma) were all grown in culture in RPMI medium supplemented with 10 % fetal calf serum. All cell lines were grown at temperature of 37 °C in a humidified atmosphere of 95 % air and 5 % CO2. All of the chemicals were bought from Biological Company, however SIGMA Company sold the amphotericin B and MTT reagent. Five cell lines in total were bought from ATTC. On a 96-well plate, cells were seeded at a density of (2 × 104) cells per well with a confluence of (40–50 %). Several concentrations of the synthetic thymol ester compound (500, 250, 125, 62.5, and 31.25 μg/mL) were then added to the cells, and they were then incubated for one day. The MTT test was performed to assess the cells' viability, and a microplate reader from Labtech (UK) was used to detect absorbance at 570 nm.

2.4.2. Antibacterial activity assay

Five bacterial isolates were used for antibacterial screening, which was sourced from the American Type Culture Collection (ATCC). They are Staphylococcus aureus (ATCC 6538P), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 25,922), *Klebsiella pneumoniae* (ATCC 13,883), and *Staphylococcus epidermis* (ATCC 12,228). Using the disk diffusion method, all compounds' antibacterial activity against the prior strains of bacteria was ascertained [32]. The antibacterial activity we used is the broth microdilution method for determination of MIC and MBC values for E2. This method is considered the standard parameter to determine the susceptibility of microorganisms to antimicrobial agents, mainly compounds *in vitro* [33]. The entire sterile Mueller-Hinton agar surface was streaked with the adjusted bacteria (1.5 × 108 CFU/mL) on nutrient agar plates. Next, the previously inoculated agar plates were topped with 6 mm disks containing 0.2 mg/mL of each substance under research. After an 18 h incubation period at 37 °C, the inhibition zone diameter (IZD) of each plate was measured to the nearest millimeter in order to assess the presence of bacterial growth inhibition. A positive control of the antibiotic gentamicin and a negative control of 10 % DMSO were used. The micro-broth dilution method was used to measure each compound's minimum inhibitory concentration (MIC) [34]. Samples were serially diluted (500, 250, 125, 62.5, 31.25, 15.625, 7.81, 3.91, 1.95, and 0.98 μg/mL) in Mueller-Hinton broth medium and then inoculated with 1 μL of 5*107 CFU/mL of each bacteria. The final two identical wells served as negative controls and were not vaccinated. Following inoculation, the micro-plates were incubated at 37 °C for 18 h. The lowest chemical concentration (MIC) that prevented the tested bacterium from growing was determined. The minimal bactericidal

concentration (MBC) was assessed for additional antibacterial analysis. After streaking the contents of the MIC wells onto an agar plate devoid of any antibacterial agents, the plate was incubated for eighteen hours at 37 °C. The lowest chemical concentration at which bacterial growth was not observed was referred to as MBC. The MTT assay is a widely accepted, non-radioactive, colorimetric-based assay that measures the activity of mitochondria as a means of assessing numbers of living cells. Also, the MTT assay is well-suited to large-scale screening [35].

2.4.3. Antioxidant assay

A 96-well microplate was used for the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical test [36]. To put it briefly, 100 µL of a 0.01 % methanolic DPPH solution was mixed with 100 µL of different chemical concentrations in methanol (ranging from 500 to 0.5 µg/mL). Using a microplate reader (Labtech, UK), the absorbance of the plate was measured at 540 nm after it had been incubated for 30 min at room temperature in the dark. As a standard, ascorbic acid was utilized at varying doses (500–0.5 µg/mL). The DPPH radical scavenging activity (%) was calculated as follows:

DPPH scavenging activity (%) = [(*A control* − *A sample*) / *A control*] × 100Where the absorbance of control was [DPPH + Methanol without sample] and the absorbance of sample was [DPPH + Sample (compound/standard)].

Using Microsoft Excel, non-linear regression was used to determine the concentration that would inhibit 50 % of DPPH radical scavenging activity (IC_{50}). The % inhibition vs. concentration was plotted to form the dose-response curve. The antioxidant assay in this study is the DPPH assay, which is a rapid, simple, inexpensive, and widely used method to assess antioxidant properties for natural products in several solvent systems [37].

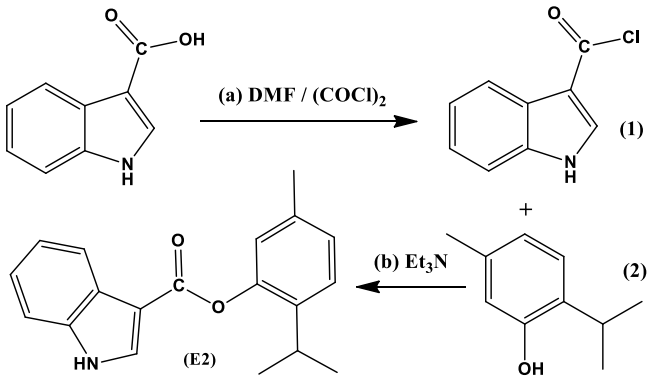
3. Result and discussion

3.1. Synthesis

A condensation reaction between one equivalent of 1H-indole-3-carbonyl chloride (**1**), one equivalent of thymol (**2**), and one equivalent of triethylamine (**b**) produced a good yield of 2-isopropyl-5-methylphenyl 1H-indole-3-carboxylate **E2** (77.8 %) (the thymol ester compound) Scheme 1. The synthesized thymol ester compound's chemical structure was detected by ^{1}H NMR and FT-IR analysis. All of the different spectral data clearly confirmed the synthesis of the thymol ester **E2**.

3.2. FT-IR spectral analysis

The broad peaks of OH absorption for (hydroxy group of thymol at 3231.99 cm^{-1}) and for carboxylic acid compound (hydroxy group of indole-3-carboxylic acid at 2359.75–3305.78 cm^{-1}) were disappeared as well as the appearance of a new C=O peak for ester compound at 1960.16 cm^{-1} were used to interpret the FT-IR analysis results. Before an esterification reaction, the FT-IR spectra of thymol and indole-3-carboxylic acid were analyzed, and the FT-IR spectra of the ester product following an esterification reaction were compared. The bond of C-$H_{aromatic}$ observed at 2961.49 cm^{-1}, C-$H_{aliphatic}$ at 2869.82 cm^{-1}, C=O_{ester} at 1690.16 cm^{-1}, C=C_{aryl} at 1619.9 cm^{-1}, C=C_{hetero} at 1581.03 cm^{-1} and N-H_{indole} at 3273.66 cm^{-1}were appeared in new thymol ester as shown in Fig. 2.This proves that the successful formation of the thymol ester compound **E2** was accomplished.



Scheme 1. Synthesis of thymol ester (**E2**).

3.3. ^{1}H and ^{13}C NMR spectral analysis

As described in the experimental section, an actual ^{1}H NMR analysis of the thymol ester compound was carried out in DMSO. Fig. 3**a** exhibits the usual 1H-NMR spectra of **E2**. Fig. 3**a** displays each of the **E2** compound's anticipated signals as follows:12.14 (1H, s, N-H indole), 8.31 (1H, d, J= 3 Hz, Ar-H indole), 8.01 (1H, d, J= 7.6 Hz, CH-NH indole), 7.54 (1H, m, J= 7.66- 7.44 Hz, Ar-H indole), 7.23 (3H, dt, J= 15.1, 7.4 Hz, Ar-H thymol and indole), 7.06 (1H, d, J= 7.9, Ar-H thymol), 6.95 (1H, s, Ar-H thymol), 3.03 (1H, hept, J= 7 Hz, C-H thymol), 2.29 (3H, s, CH_3 thymol), 1.14 (6H, d, J= 6.9, $2CH_3$ thymol). Both the signal for the proton of the COOH group in indole-3-carboxylic acid and the signal for the proton of the OH group in the thymol molecule at 5.24 ppm and 11 ppm, respectively, were disappeared. This proves that the successful formation of the thymol ester compound **E2** was accomplished. The primary proton signals for thymol derivatives compounds in ^{1}H NMR analysis in earlier investigations were (1.21–1.27 ppm) for 6H of $2CH_3$ groups, (2.31–2.39 ppm) for 3H of Ar-CH_3 group, and (3.05–3.11 ppm) for the single proton in CH group. These findings agree with those of our study [38,39].

According to Fig. 3**b**, the^{13}C-NMR spectra of **E2** indicated three different types of carbons: three CH_3 group carbons, nine CH group carbons, and seven C group carbons. These carbons were shown in the experimental part.

3.4. Anticancer activity

We utilized the Hela cancer cell line, the MCF7 breast cancer cell line, the PC3 prostate cancer cell line, and the HepG2 liver cancer cell line to assess the in *vitro* anticancer activity of the thymol ester molecule **E2**. The outcomes demonstrated the cytotoxicity of the generated thymol ester **E2** at different concentrations to the four cancer cell lines. Throughout the tested concentration range, the viability of Hela cancer cells, MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells were determined to be (38.6–42.6 %), (28–33 %), (28–30.3 %), and (30.8–33.2 %), respectively. The thymol ester molecule **E2** concentration rose in proportion to the decrease in cell proliferation (Figs. 4 and 8).

Through a cytotoxic experiment, ester compound **E2** reduced the evaluated viability of Hela cancer cells from 38.6 % at conc. 500 µg/mL to conc. 42.6 % at 31.25 µg/mL (Fig. 4). Ester moleculeE2decreased the viability of the tested Hela cancer cells in a cytostatic experiment from 56.7 % at conc. 500 µg/mL to 62 % at conc. 31.25 µg/mL (see Fig. 5). When it comes to cytotoxic and cytostatic assays on Hela cancer cells at all tested concentrations, the prepared thymol ester compound E2 is more effective than thymol because the evaluated Hela cancer cells' viability decreased when exposed to thymol; these cells went from 58 % at conc. 500 µg/mL to 63 % at conc. 31.25 µg/mL and from 69.7 % at conc. 500 µg/mL to 73.5 % at conc. 31.25 µg/mL, respectively (Figs. 4 and 5).

In addition to being tested on Hela cancer cells, the novel thymol ester **E2** and thymol compound were also investigated on L6 normal muscle cells, a type of live cell. In a cytotoxic assay, thymol ester **E2** reduced the evaluated L6 normal cells' viability from 50 % at conc. 500 µg/mL to 55.3 % at conc. 31.25 µg/mL (see Fig. 6). Thymol ester

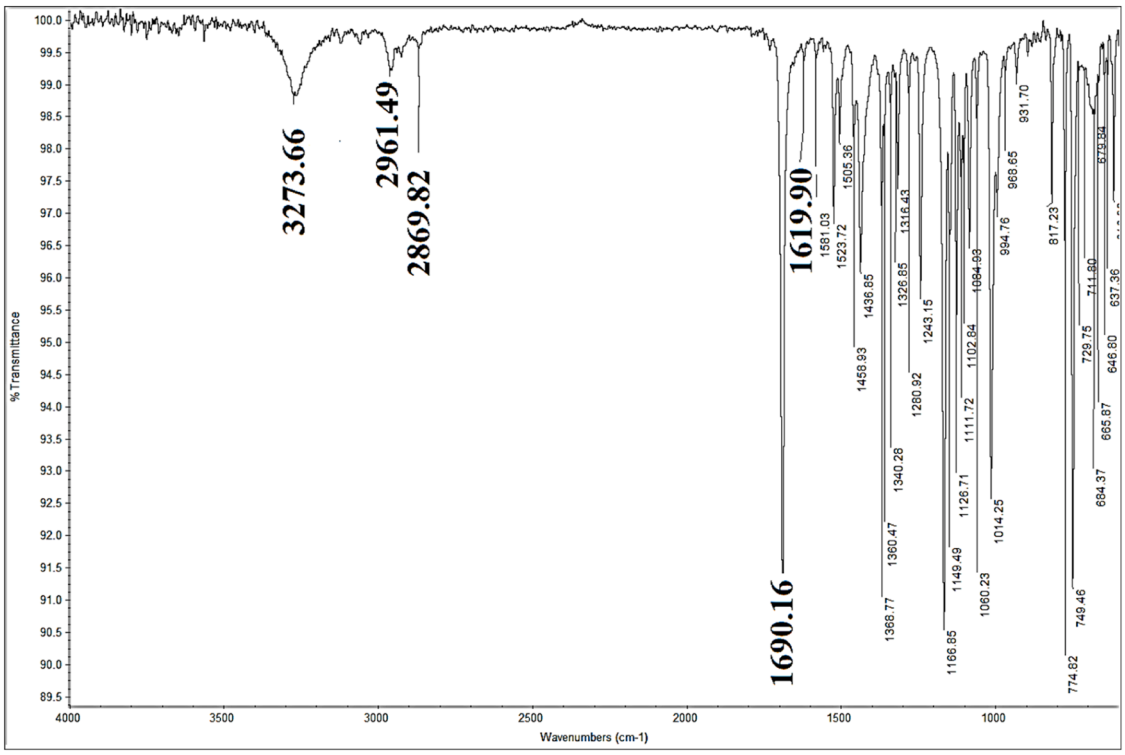


Fig. 2. IR spectra of thymol ester **E2**.

E2decreased the evaluated L6 normal cells' viability in a cytostatic assay from 54.4 % at conc. 500 μg/mL to 60 % at conc. 31.25 μg/mL (Fig. 7). In the cytotoxic and cytostatic tests on L6 normal cells at all tested concentrations, thymol compound is marginally more effective than thymol ester compound **E2**. This is because thymol decreased the viability of the tested L6 normal cells, which went from 61.05 % at conc. 500 μg/mL to 66.5 % at conc. 31.25 μg/mL and from 62.5 % at conc. 500 μg/mL to 64.2 % at conc. 31.25 μg/mL, respectively (Figs. 6 and 7). This does not diminish the efficacy of thymol ester **E2**, particularly when applied at low quantities (less than 31.25 μg/mL).

In a cytotoxic assay conducted on MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells, thymol ester **E2** decreased the viability of the cells from 28 % at conc. 500 μg/mL to 33 % at conc. 31.25 μg/mL, 28 % at conc. 500 μg/mL to 30.3 % at conc. 31.25 μg/mL, and 30.8 % at conc. 500 μg/mL to 33.2 % at conc. 31.25 μg/mL, respectively, (Fig. 8). In Fig. 9, thymol ester **E2** decreased the viability of the studied MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells in a cytostatic assay. The cells went from 55.4 % at conc. 500 μg/mL to 57.5 % at conc. 31.25μg/mL, 57.46 % at conc. 500 μg/mL to 60.8 % at conc. 31.25 μg/mL, and 60.57 % at conc. 500 μg/mL to 63.44 % at conc. 31.25 μg/mL, respectively. The aforementioned findings align with earlier research demonstrating the significant potential of several chemicals generated from indole as anti-cancer medicines [40–42].

The outcomes indicated that Hela cancer cells, MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells are all susceptible to cytotoxic and cytostatic experiments of the thymol ester compound **E2**. In the cytotoxic experiment, the values of the IC_{50} of thymol and thymol ester **E2** are 13.9 μg/mL and 14.5 μg/mL for Hela carcinoma cells, respectively. The cytostatic experiment yielded the following IC_{50} values for thymol and thymol ester **E2** on Hela cancer cells: thymol 0.18 μg/mL and thymol ester **E2** 0.0937 μg/mL, as indicated in Table 1. For MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells, the synthesized thymol ester **E2** had IC_{50} values of 17.21 μg/mL, 19.98 μg/mL, and 17.13 μg/mL in the cytotoxic experiment, respectively. In the cytostatic experiment, the IC_{50} values of the synthesized thymol ester **E2**on MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells were 7.08 μg/mL, 10.26 μg/mL, and 13.04 μg/mL, respectively, as shown in Table 1. For L6 normal cells, IC_{50} values in a cytotoxic experiment were 9.2 μg/mL and 8.066 μg/mL, respectively, for thymol and thymol ester **E2** that was developed from it. Furthermore, thymol and thymol ester **E2** IC_{50} values for L6 normal cells in a cytostatic experiment were 5.37 μg/mL and 7.26 μg/mL, respectively.

3.5. Antibacterial activity

In this work, the agar disk diffusion method of thymol ester **E2** and thymol demonstrated antibacterial efficacy against the bacterial isolates that were examined. In comparison to thymol, Fig. 10 shows that the thymol ester **E2** had the strongest antibacterial activity against the tested Gram-positive bacteria (*S. aureus* and *S. epidermis*), producing inhibition zones measuring 12 mm and 15 mm against each isolate, respectively. Despite this, the thymol ester **E2** inhibition zone (15 mm) against S. epidermis was greater than the gentamicin inhibition zone (7 mm) in Fig. 10.

By figuring out the minimum inhibitory concentration (MIC) of each molecule against the five bacterial isolates, the antibacterial activity of the thymol ester **E2** and thymol under investigation was quantitatively evaluated. In this experiment, Fig. 11 demonstrated unequivocally that the thymol ester **E2** outperformed thymol in terms of effectiveness against Gram positive bacteria. Therefore, **E2**'s MIC value against *S. aureus* was found to be 15.625 μg/mL, whereas that of *S. epidermis* was found to be 62.5 μg/mL.

For every concentration in this study that was shown to have inhibitory effects, the minimal bactericidal concentrations (MBC) were found. The thymol eater **E2** was the most effective, as demonstrated by Fig. 11, killing *S. epidermis* at the lowest dose of 31.25 μg/mL among the screened compounds.

Through a number of methods, such as breakdown of cell membranes, decrease of biofilms, inhibition of membrane-bound ATPases, inhibition of efflux pumps, and inhibition of motility, thymol

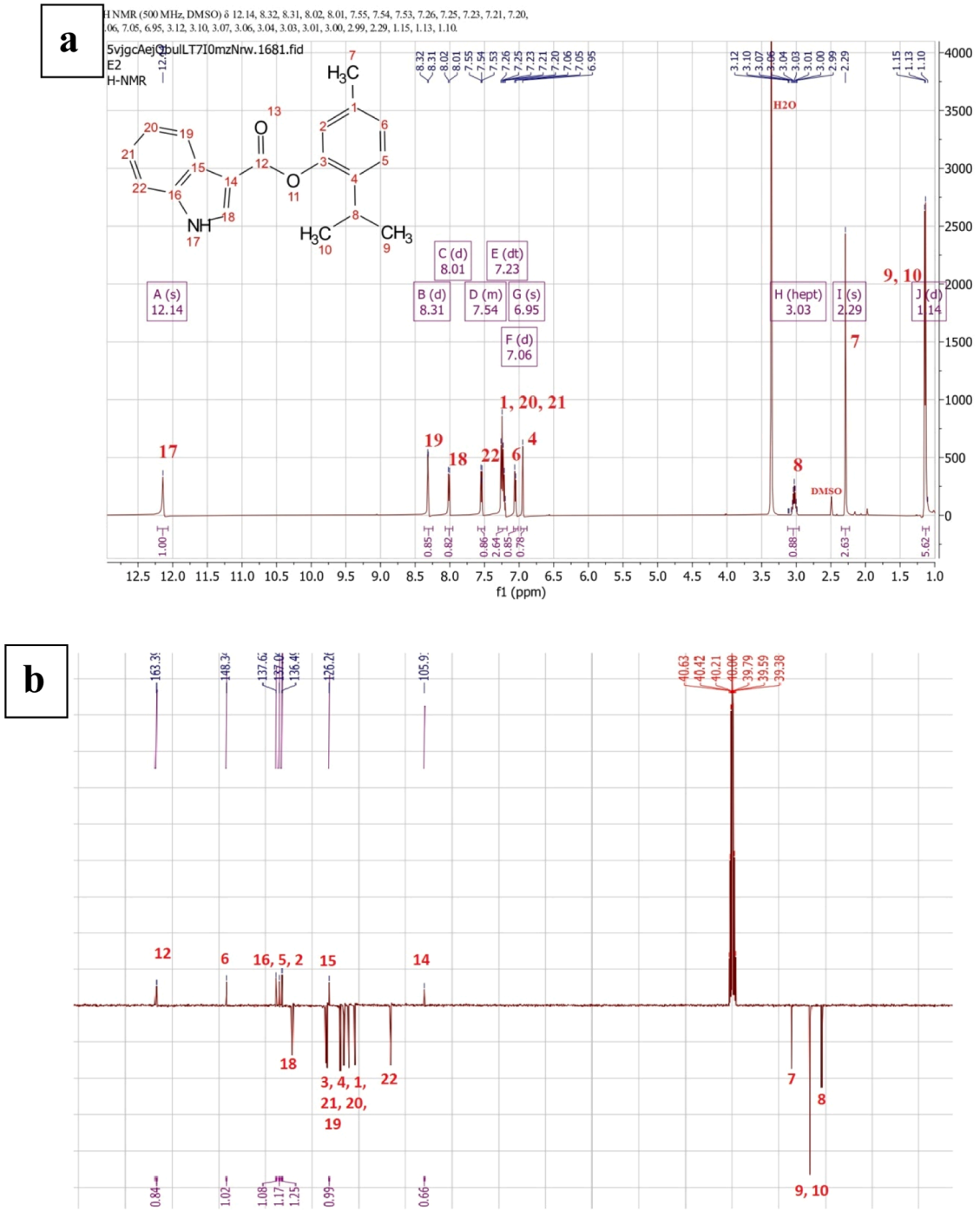


Fig. 3. (a) ^{1}H NMR spectra of thymol ester **E2(b)**^{13}C NMR in DMSO at room temperature.

demonstrates its antibacterial and bactericidal effects [43]. Additionally, thymol has antibacterial activity against a variety of species, including microbes that have lodged themselves in biofilms. Despite having antibacterial properties, thymol is not often used as an antibacterial agent. This is because, in addition to being poisonous to animal cells, thymol needs a high dose in order to have bactericidal effects. Therefore, more research is required to use nontoxic doses of thymol to achieve optimal antibacterial action [44]. This work examined the antibacterial activity of a recently synthesized thymol ester derivative with an indole functional group **E2** against a variety of bacterial species. Compound **E2** demonstrated noteworthy antibacterial activity against Gram positive bacteria when tested against the investigated bacterial species. Gram-positive bacteria are typically more susceptible to the effects of thymol and its derivatives because they are terpenes [45]. Terpenes' antibacterial mechanism of action is often attributed to their lipophilic nature. Additionally, monoterpenes alter the topology of the membrane's proteins, increase fluidity and permeability, and disrupt the respiration chain in order to affect membrane structure [45]. A prior investigation on thymol and its ester derivatives, considering the **E2** chemical structure, indicated that the greatest improved action of these esters showed to be against Gram positive bacteria [24]. In fact, additional research concentrated on thymol derivatives that are coupled to various five-membered heterocyclic moieties through blocked phenolic hydroxyl groups. These compounds have both insecticidal and antibacterial properties [46]. Additionally, the Nagle group produced thymol with pyridine moieties, which demonstrated superior antifungal

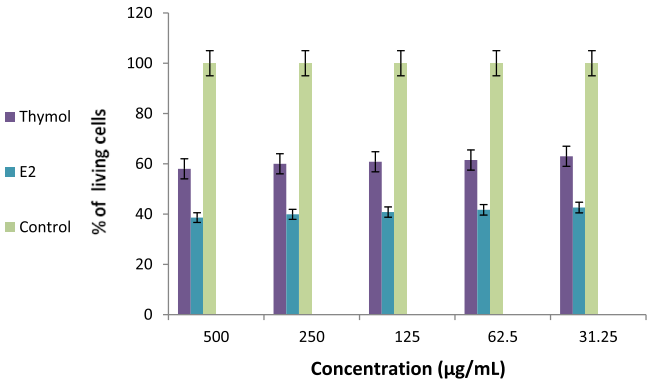


Fig. 4. Thymol and thymol ester compound (**E2**) in *vitro* effect at different doses (500, 250, 125, 62.5, and 31.25 µg/mL) on Hela cancer cells in a cytotoxic experiment.

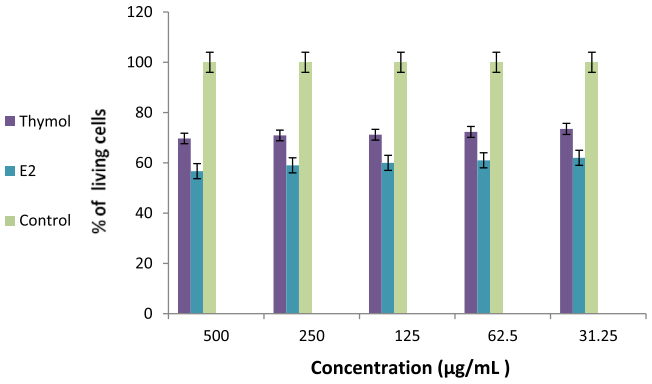


Fig. 5. Thymol and thymol ester compound (**E2**) in *vitro* effect at different doses (500, 250, 125, 62.5, and 31.25µg/mL) on Hela cancer cells in a cytostatic experiment.

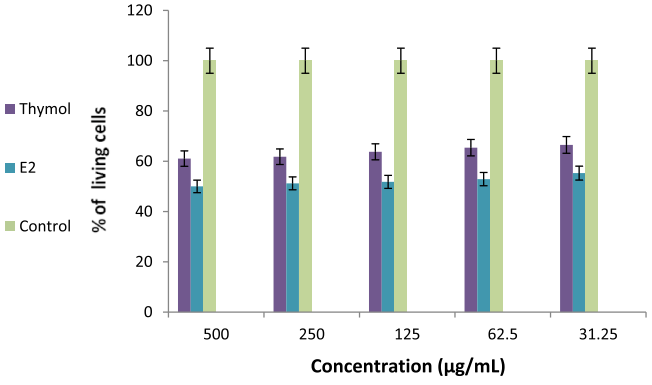


Fig. 6. Thymol and thymol ester compound (**E2**) in *vitro* effect at different doses (500, 250, 125, 62.5, and 31.25 µg/mL) on L6 normal cells in a cytotoxic experiment.

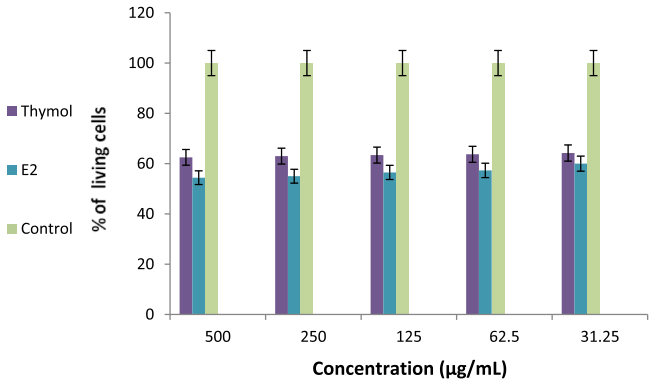


Fig. 7. Thymol and thymol ester compound (**E2**) in *vitro* effect at different doses (500, 250, 125, 62.5, and 31.25 µg/mL) on L6 normal cells in a cytostatic experiment.

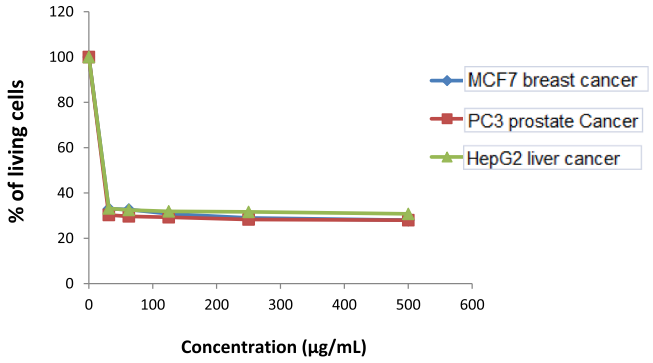


Fig. 8. Thymol ester compound (**E2**) in *vitro* effect at different doses (500, 250, 125, 62.5, and 31.25 µg/mL) on MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells in a cytotoxic experiment.

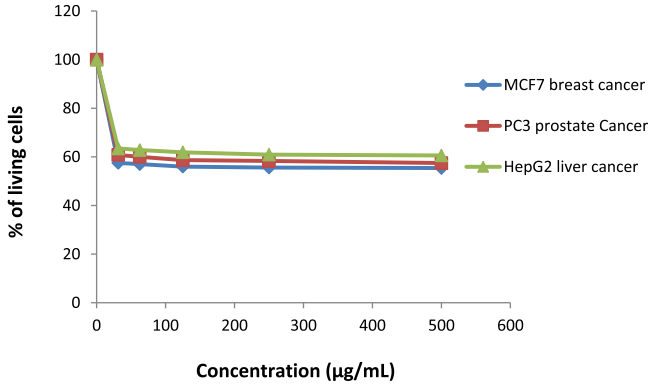


Fig. 9. Thymol ester compound (**E2**) in *vitro* effect at different doses (500, 250, 125, 62.5, and 31.25 µg/mL) on MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells in a cytostatic experiment.

and antibacterial action compared to thymol [47]. Furthermore, it is commonly recognized that the greatest class of antifungal medicines consists of imidazoles and triazoles [48]. Like **E2**, all these compounds have an N-containing structure, which could be the reason for their antibacterial activity. Furthermore, since **E2** contains indole, research has indicated that bioactivity is dependent on both the side chains and the indole ring [49]. Indole derivatives are therefore useful and appropriate for more research and development [50]. **E2** derivative under study could be considered a good bacteriostatic agent when compared to other thymol derivatives reported in several literatures. In comparison to the eight thymol derivatives in the previous study [51], **E2** MIC value against *S. aureus* (62.5 µg/mL) was better than **2, 7** and **8** derivatives and similar to **6** derivatives. Also, the same effect of **E2** and all screened derivatives except 5 in the same study against *E. coli*. Moreover, **E2** derivative was exhibited the same effect as derivative **1** against *P. aeruginosa* (MIC 125 µg/mL) [52]. Another noticeable effect of **E2** antibacterial efficacy was shown when compared with thymol derivatives prepared in the previous study [53], as **E2** was better than these thymol derivatives in its action against *E. coli, K. pnaemonae, P. aeruginosa* and *S. aureus*. In addition to that, **E2** inhibition zone

Table 1
IC50 values for the new thymol ester **E2** and thymol in terms of their cytotoxic and cytostatic effects on Hela cancer cells, L6 normal cells, MCF7 breast cancer cells, PC3 prostate cancer cells, and HepG2 liver cancer cells.

	Thymol	E2
Hela cytotoxic	13.90	14.50
Hela cytostatic	0.18	0.094
L6 cytotoxic	9.27	8.066
L6 cytostatic	5.37	7.26
MCF7 breast cytotoxic	-	17.21
MCF7 breast cytostatic	-	7.08
PC3 prostate cytotoxic	-	19.98
PC3 prostate cytostatic	-	10.26
HepG2 liver cytotoxic	-	17.13
HepG2 liver cytostatic	-	13.04

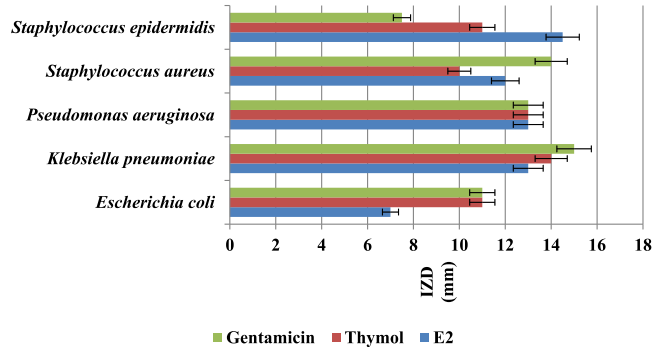


Fig. 10. Antibacterial activity of thymol and **E2** derivative against *E. coli, K. pneumoniae, P. aeruginosa, S. aureus* and *S. epidermis* using disk diffusion method; (IZD) Inhibition zone diameter (mm).

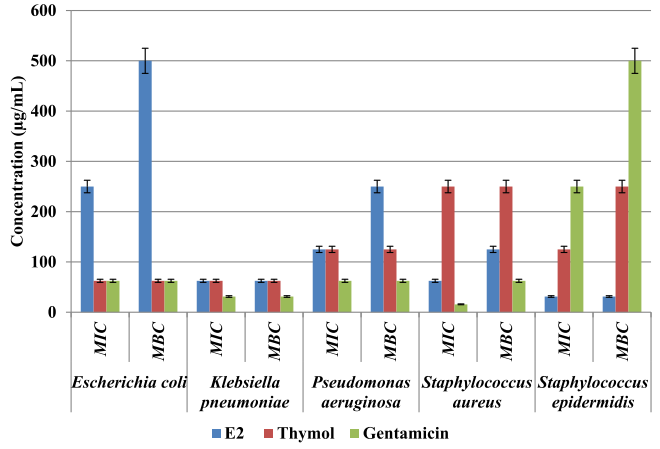


Fig. 11. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in µg/mL by using micro-broth dilution method for thymol and **E2** derivative against *E. coli, K. pneumoniae, P. aeruginosa, S. aureus* and *S. epidermis*.

measurement against *S. aureus* (12 mm) and *K. pnaemonae* (13 mm) revealed its valuable effect when compared to various thymol derivatives [54].

Studies on the mechanism of thymol's antibacterial effect show that it increases the surface curvature through its incorporation into the lipid layer of the cell membrane. The molecule's hydrophilic part interacts with the membrane's polar part, while the aliphatic side chains and hydrophobic benzene ring sink into the biological membrane's core. These interactions lead to changes in membrane structure through lipid layer destabilization, decrease membrane elasticity, and increase its fluidity, which in turn result in increased permeability to ions and affected internal membrane proteins [55]. The cytotoxicity of thymol against cancer cell lines may be through induction of apoptosis via caspase-dependent and caspase-independent pathways [56]. Moreover, the molecular docking studies revealed that indole-3-carboxylic acid conjugates exhibited encouraging binding interaction networks and binding affinity with DNA gyrase that led to their antibacterial activity [57]. The presence of an indole moiety in **E2** which is a significant basic subunit for an enormous figure of natural products and pharmaceuticals. Hence, tryprostatins A, tryprostatins B, and vincristine are all utilized as antibiotics used for chemotherapy [58]. In literature, indole-based compounds have been reported to inhibit bacterial growth by targeting cell division and FtsZ [59].

3.6. Antioxidant activity

The antioxidant assay that is frequently used in labs to determine if natural compounds can scavenge free radicals is the DPPH free radical scavenging assay. In comparison to ascorbic acid, the results of this experiment demonstrated that the thymol ester **E2** exhibited a modest level of antioxidant capacity. Other than that, Fig. 12 showed that this derivative's low concentrations (16–0.5 µg/mL) had more scavenging power than thymol.

Thymol's phenolic structure and redox characteristics play a critical role in the neutralization and adsorption of free radicals and breaking down peroxides. Its antioxidant activity is responsible for these characteristics [60]. Furthermore, it was looked at whether thymol derivatives with various structures could scavenge free radicals. These derivatives' capacity to function as antioxidants results from the intramolecular hydrogen bond formation [61].

4. Conclusion

By condensing thymol and indole-3-carboxylic acid in a dichloromethane solvent at room temperature (25 °C), the new thymol ester **E2** was synthesized. The process was tracked using TLC and FT-IR, and the final product was identified using FT-IR, ^{1}H NMR and ^{13}C NMR analysis. The biological features of the new chemical were tested on several types of cancer and bacterial cells. In all cytotoxic and cytostatic tests, the ester compound **E2** shown greater efficacy against PC3 prostate cancer cells, MCF7 breast cancer cells and HepG2 liver cancer cells. Also, thymol ester **E2** was better at preserving normal muscle cells than thymol. When compared to thymol in the antibacterial experiment, the **E2** derivative exhibited the best activity against the studied Gram-positive bacteria (*S. aureus* and *S. epidermis*), and its inhibition zone was bigger than that of gentamicin against *S. epidermis*. Furthermore, in an antioxidant assay, **E2** ester molecule outperformed thymol in terms of scavenging power at low doses. Based on the effectiveness of the new thymol ester compound **E2** on all cancer cells and bacteria cells studied,

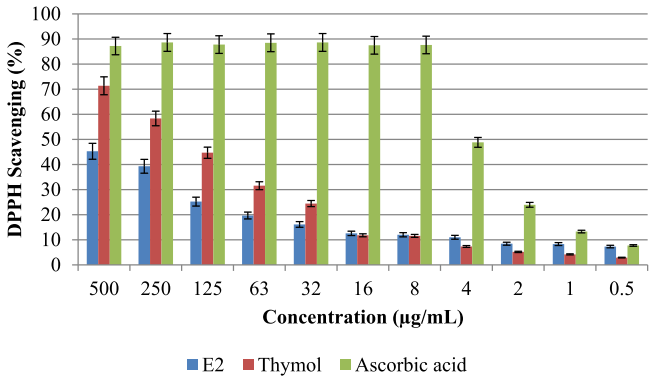


Fig. 12. Percentage of DPPH inhibition activity of thymol, **E2** derivative and ascorbic acid.

it will become possible in the future to study precisely the mechanism of the biological effect of this crystalline compound on cells. Further *in vivo* studies concerning the effect of the examined thymol derivative for its antibacterial, anticancer, and antioxidant properties are recommended. Further experimentation is necessary to precisely understand the mechanism of action of this thymol derivative in the screened assays. Also, more pharmacologically elaborated experiments are required to examine the required dose of this derivative in parallel to its cytotoxicity evaluation.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

CRediT authorship contribution statement

Inas Bsharat: Writing – review & editing, Writing – original draft, Software, Formal analysis, Data curation. **Lubna Abdalla:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ashraf Sawafta:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation. **Ibrahim M. Abu-Reidah:** Writing – review & editing, Validation, Investigation. **Mohammed A. Al-Nuri:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2025.141771.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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