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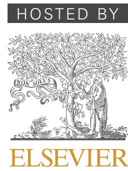

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Original article

Effect of eugenol on cytochrome P450 1A2, 2C9, 2D6, and 3A4 activity in human liver microsomes

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ABSTRACT

This study aimed to provide an understanding of the influence of eugenol on CYP1A2, 2C9, 2D6, and 3A4 in human liver microsomes (HLM). Specific substrate for CYP1A2, 2C9, 2D6, and 3A4 were incubated in HLM with or without eugenol. The formation of their respective metabolites was assessed with HPLC analytical methods. Eugenol at 1, 10 and 100 μM levels inhibited the activity of CYP1A2 and CYP2C9 by 23.38 %, 23.57 %, 39.80 % and 62.82 %, 63.27 %, 67.70 % respectively. While, CYP2D6 and CYP3A4 activity was decreased by 40.70 %, 45.88 %, 62.68 % and 37.41 %, 42.58 % and 67.86 % at 1, 10 and 100 μM eugenol level respectively. The IC_{50} value of eugenol for CYP2D6 and CYP3A4 was calculated as 11.09 ± 3.49 μM and 13.48 ± 3.86 μM respectively. Potential herb-drug interactions was noted when eugenol is administered simultaneously with medications metabolized by these enzymes, most notably CYP2C9, CYP2D6 and CYP3A4.

1. Introduction

Globally, herbal remedies and phytochemicals are increasingly being used to intensify health (Ahad et al., 2020a,2020b). Approximately 80 % of the worldwide population utilizes products made from natural resources (Ekor, 2013; Jagtenberg and Evans, 2003). A survey executed by Barbadia et al. (2007) and Rashrash et al. (2017) found that approximately 20 % of Americans consumed a natural medicine in the past year (Bardia et al., 2007; Rashrash et al., 2017). As of 2017, herbal remedies accounted for more than $8 billion in sales in the United States (Ekor, 2013). The prevalence of herbal remedies amongst chronic disease patients is higher than that of the general population (Alghamdi et al., 2018; Tulunay et al., 2015). A greater understanding of the interactions among herbs and prescription drugs is warranted given the high level of herbal remedy utlization, most prevalently among patients taking prescription medications. There is a substantial contribution of cytochrome P450 (CYP) enzymes in phase I drug metabolism; over 75 % of the medications prescribed to patients are metabolized by CYP enzymes (Zanger and Schwab, 2013; Zhou et al., 2009). Drug-metabolizing enzymes can be induced or inhibited by phytochemicals and herbal extracts; as a consequence, they may impair drug metabolism, leading to therapeutic failure or side effects (Rombola et al., 2020; Wanwimolruk et al., 2014). It has been established that medicinal plants contain a large number of constituents that are able to produce pharmacological effects. There are many plants that contain phenylpropanoids, and typically they are in oxidized form, with an aromatic ring containing a hydroxyl group. The pharmacological activity of phenylpropanoids and their derivatives has been demonstrated in recent studies (de Cassia da Silveira et al., 2014). Eugenol (Fig. 1) is a simple monocyclic terpene found in clove (*Syzygium Aromaticum* (L.) Merr). An essential oil content of 45–90 % can be found in it (Zhang et al., 2013). Food manufacturers use it as a preservative, mainly because of its antioxidant properties, and it is also used as an ingredient for flavoring food and cosmetics (Chatterjee and Bhattacharjee, 2015; Haro-Gonzalez et al., 2021). Additionally, cloves have anti-inflammatory characteristics that may be linked to the anti-inflammatory action of eugenol (Chaieb et al., 2007; Marchese et al., 2017). Numerous medicinal uses have been documented for eugenol: analgesic activities, antipyretic, antibacterial, anesthetic, anticancer, antifungal, and anti-inflammatory (Batiha et al., 2020; Marchese et al., 2017; Mohammadi Nejad et al., 2017; Olea et al., 2019; Vercellini et al., 2023; Zari et al., 2021). Taking into account the bioactive properties of eugenol and its existence in a wide range of foods and plants that have medicinal properties, the purpose of the present study was to probe the influence of eugenol on the metabolic activity of major CYP in human liver microsomes (HLM).

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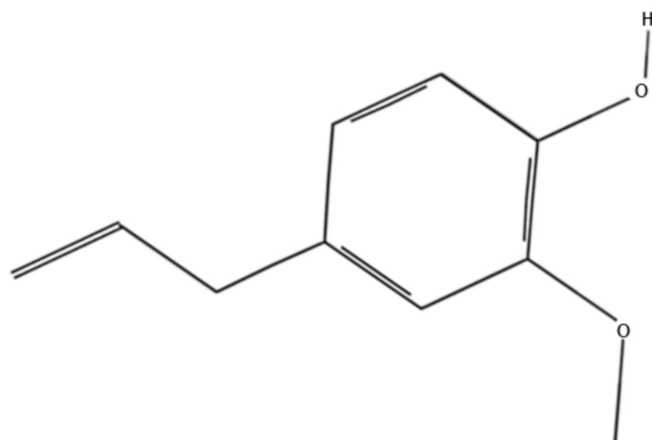

Fig. 1. The chemical structure of eugenol.

2. Materials and methods

2.1. Materials

Eugenol, 6-hydroxytestosterone, testosterone and tolbutamide were supplied by Sigma-Aldrich, MO, USA. Phenacetin, dextrorphan-D-tartrate and dextromethorphan hydrobromide were obtained from "ICN Biomedicals, Inc. Eschwege, Germany". The 4-hydroxytolbutamide was obtained from "Cayman Chemical Company, MI, USA". Corning, MA, USA, provided HLM (UltraPool HLM 150). Acetonitrile of HPLC grade was sourced from "Winlab laboratory and chemicals (Leicestershire, UK)". NADPH was sourced from "Chem-Implex International Inc., Wood Dale, IL, USA". The caffeine and potassium dihydrogen phosphate were provided by "Alfa Aesar, Ward Hill, MA, USA". and "Fisher Scientific in Leicestershire, UK respectively".

2.2. Incubation protocol for CYP1A2 microsomal enzyme

For the CYP1A2 incubation study in HLM, in the first step, 10 μl of phenacetin from a stock of 5 mM stock was transferred to the Eppendorf tubes and then 10 μl from a different eugenol stock (0.5, 2.5, 5, 50, 500, and 5000 μM) was added to the samples. The Eppendorf tubes were and so filled with 50 μl of HLM from stock of 5 mg protein/ml and 0.1 M phosphate buffer pH 7.4. These Eppendorf tubes were gently mixed and then kept in water bath for 5 min at 37 °C that was constantly shaking. Five minutes later, incubation reactions were commenced by the addition of 25 μL of 20 mM NADPH. A 30-minute incubation period was applied to the final mixture. In the final step of the incubation reaction, Eppendorf tubes were filled with 10 μl of 70 percent perchloric acid and the tubes were agitated for 2.0 min to permanently terminate the reaction (Eagling et al., 1998; Kobayashi et al., 1998). Each sample was spiked with a 25 μl solution of caffeine from a 10 μM stock as an internal standard (IS). For metabolite analysis, the samples were centrifuged at 12,000 rpm for 10 min. The resultant supernatant was introduced into HPLC for analysis.

2.2.1. An assessment of acetaminophen in human liver microsomes

In order to measure acetaminophen in HLM, a HPLC analysis was conducted. Metabolite detection was carried out using "Nucleodur C_{18} column (5 μm, 4.6 × 250 mm; Macherey-Nagel, Duren, Germany). A ratio of 15:85 v/v was used of the mobile phase, it contains acetonitrile and phosphate buffer (50 mM) and is pumped at a rate of 1.3 ml/min. A stock solution of acetaminophen, phenacetin, and caffeine was produced in methanol. The detection of phenacetin metabolite (acetaminophen) was achieved at 245 nm (Kobayashi et al., 1998).

2.3. Incubation protocol for CYP2C9 microsomal enzyme

The Eppendorf tubes were loaded with 10 μl of 7.5 mM stock solution of tolbutamide for CYP2C9 incubation. Each sample was then treated with 10 μl of different eugenol stocks (0.5, 2.5, 5.0, 50, 500, and 5000 μm). Following this, 50 μl of HLM from 2.5 mg protein/ml were transferred into Eppendorf tubes containing 0.1 M, phosphate buffer pH 7.4. After evenly mixing, the Eppendorf tubes were positioned in an agitated waterbath for five minutes at 37 °C. Afterwards, each tube was filled with 25 μl of 20 mM NADPH to commence the incubation process at 37 °C for 30 min. The incubation reaction was ended by transferring 250 μl ice-cold methanol to the Eppendorf tubes and agitated the tubes efficiently (Al-Jenoobi, 2010; Korashy et al., 2015). Each tube was spiked with nitrazepam (25 μl) as IS taken from 1.0 μg/ml stock. In order to analyze metabolites, tubes were centrifugated at a speed of 12,000 rpm ten min, the supernatants were collected, and HPLC was used to analyze the samples.

2.3.1. An assessment of 4-hydroxytolbutamide in human liver microsomes

A Waters C_{18} column ("5 μm, 4.65 mm × 150 mm, Waters, Milford, MA, USA") was used for the determination of 4-hydroxytolbutamide in HLM. In this case, the mobile phase consists of 25 % acetonitrile mixed with 75 % 0.02 M potassium phosphate buffers pH 3.4. The mobile phase was pumped continuously at 1.5 ml/min. Acetonitrile and methanol was used to prepare the stock solutions of tolbutamide and its metabolites. The metabolite was detected at 230 nm (Al-Jenoobi, 2010; Korashy et al., 2015).

2.4. Incubation protocol for CYP2D6 microsomal enzyme

Ten microliters of dextromethorphan solution of 1.25 mM stock was transferred to Eppendorf tubes. After this, 10 μl of the following eugenol stocks were added (0.5, 2.5, 5.0, 50, 500, and 5000 μM). After that, each tube was treated with HLM (final concentration = 0.5 mg protein/ml) in conjunction with phosphate buffer pH 7.4 (0.1 M). The samples were mixed completely before being incubated for 5 min at 37 °C in a water bath. Incubation was executed at 37 °C for 30 min after each tube was filled with 25 μl of 20 mM NADPH and 10 μl of 0.3 M magnesium chloride. After a period of incubation, 10 μl of perchloric acid (70 %) were added to each sample to precipitate the reaction. All samples were vortexed thoroughly. In the final step, the samples were centrifugated for 10 min at a speed of 12,000 rpm. After pipetting out the supernatant from each sample, the clear supernatant was transferred to the HPLC machine to analyze the metabolite (Al-Jenoobi et al., 2010; Al-Jenoobi et al., 2014).

2.4.1. An assessment of dextrorphan-D-tartrate in human liver microsomes

The HPLC method has been applied to examine the content of dextromethorphan metabolite in HLM [30]. The mobile phase was composed of acetonitrile and HPLC-grade water containing 0.1 % trimethylamine and 1.5 % glacial acetic acid. The pH of the mobile phase was kept at 3. The mobile was used at a ratio of 25 % acetonitrile and 75 % HPLC-grade water. The flow rate of 1 ml/min was affirmed for the mobile phase. The samples analyses were performed utilizing a "Nucleodur C_{18} 5 μm, 250 mm × 4.6 mm" HPLC column. Fluorescence detector with 280 nm excitation and 330 nm emission wavelengths was used to detect dextrorphan-D-tartrate (Bendriss et al., 2001).

2.5. Incubation protocol for CYP3A4 microsomal enzyme

Further, the effect of eugenol on CYP3A4 activity was assessed by incubating 10 μl of substrate testosterone (stock of 2.5 mM) filled in a microcentrifuge tube. After this, 10 μl of the following eugenol stocks were added (0.5, 2.5, 5.0, 50, 500, and 5000 μM). In the next step, 50 μl HLM was added from a stock of 2.5 mg protein/ml in each tube. Following a thorough mixing, the samples were kept in a shaker at 37 °C

for five minutes. After 5 min, 25 µl from NADPH solution (20 mM) was added in each samples tube and incubation was accomplished at 37 °C for thirty minutes. In order to stop the incubation reaction after 30 min, in each sample, 250 µl of ice-cold methanol was added and the mixture was thoroughly mixed (Baati et al., 2012; Wang and Yeung, 2011). The IS was used was phenacetin, the stock solution of IS was prepared in methanol (10 µg/ml) and then 10 µl of IS was transferred to each tubes. Samples were centrifuged at 12,000 rpm for 10 min, the supernatant was isolated and filled up in HPLC vials and samples were analyzed for testosterone metabolites by the HPLC analytical method (Borek-Dohalska et al., 2008).

2.5.1. An assessment of 6-β-hydroxytestosterone in human liver microsomes

The estimation of testosterone metabolite in HLM was conducted by HPLC analytical method. The testosterone and its metabolite stock solution were produced in methanol. In this method, a "C_{18} column, 5 µm, 4.6 × 250 mm" was employed for testosterone metabolite estimation. The mobile phase (flow rate 0.5 ml/min) contained methanol and water in a 70:30 ratio. Formation of testosterone metabolite was measured using UV detector at 242 nm (Pan et al., 2012).

2.6. Statistical analysis

"GraphPad Prism 6 (GraphPad, Software Inc., San Diego, CA, USA)" computed the "half-maximal inhibitory concentration" (IC_{50}) values. "An ANOVA followed by Dunnett's test was conducted to compare the groups from control"; "$P < 0.05$ considered significant".

3. Results

In this investigation, pooled HLM was used to assess the eugenol's effect on CYP1A2 enzyme activity. Phenacetin at various concentrations was incubated with eugenol. A CYP1A2-mediated reaction was performed in HLM to assess eugenol's effects on acetaminophen formation. According to our findings, eugenol substantially inhibits the CYP1A2 enzyme in a dose-related fashion. At 0.01, 0.05 and 0.10 µM levels non-significant CYP1A2 inhibition was observed which was 1.65 %, 5.32 % and 9.95 % respectively.

Eugenol suppressed significantly ($P < 0.05$) CYP1A2 expression by 23.38 ± 4.72 % at 1 µM. Comparable inhibition of CYP1A2 activity about 23.57 ± 5.68 % was noted at 10 µM (Fig. 2). The maximum statistically significant ($P < 0.05$) inhibition of CYP1A2 39.80 ± 3.43 % was noted at the highest level of eugenol (100 µM) (Fig. 2), however, the inhibition did not surpass the 50 % level for IC_{50}. Hence, the IC_{50} was unable to be calculated for CYP1A2 because the enzyme was weakly inhibited.

The impact of eugenol on the metabolic activity of CYP2C9 was tested by detecting 4-hydroxytolbutamide production by CYP2C9 from tolbutamide in HLM incubated with six (0.01, 0.05, 0.1, 1, 10 and 100 µM) different concentrations of eugenol. Statistically significant inhibition of CYP2C9 by 57.33 ± 4.27 %, 59.43 ± 2.18 % and 62.27 ± 0.79 % was observed with eugenol at 0.01, 0.05 and 0.1 µM (Fig. 3). Further, 1, 10 and 100 µM eugenol levels significantly inhibited the activity of CYP2C9 by 62.82 ± 0.89 %, 63.27 ± 2.25 % and 67.70 ± 0.96 % (Fig. 3). In this case, the IC_{50} could not be accurately determined because the CYP2C9 enzyme was inhibited more than 50 % even at lowest level of eugenol. Hence, the predicted IC_{50} value of eugenol was interpreted as < 0.01 µM. Based on the IC_{50} value, which was low; it appears that the inhibition was strong.

A CYP2D6 enzyme incubation activity using HLM was accomplished to inquire the potential influence of eugenol on this enzyme. In this experiment, the assay assesses the production of dextromethorphan metabolite in HLM when eugenol at six different concentrations (0.01, 0.05, 0.1, 1, 10, and 100 µM) was incubated with dextromethorphan.

A percentage of control activity was calculated based on the results. Significant ($P < 0.05$) decreased in dextrorphan-D-tartrate formation by eugenol (Fig. 4A) was noted. A statistically significant ($P < 0.05$) inhibition of CYP2D6 activity was observed with eugenol at 0.01 µM, 0.05 µM and 0.1 µM (Fig. 4A) and the inhibition was found to be 23.45 ± 2.73 %, 23.62 ± 2.70 and 28.36 ± 1.56 % respectively. While at 1 µM and 10 µM eugenol level, the activity of CYP2D6 was decreased by 40.70 ± 3.94 % and 45.88 ± 3.71 %. Eugenol inhibited the enzyme at 100 µM with a maximum efficiency of 62.68 ± 0.26 % (Fig. 4A). The IC_{50} value of eugenol was calculated as 11.09 ± 3.49 µM (Fig. 4B). A potent inhibitory effect of eugenol on CYP2D6 was demonstrated.

Further, an experiment was conducted to probe the impact of eugenol on CYP3A4 metabolic activity. In this study, the HLM was treated with testosterone, and the metabolite of testosterone produced by CYP3A4 was quantified by HPLC analytical method. The outcomes expressed that eugenol suppressed the activity of the CYP3A4 enzyme in a dose-related pattern (Fig. 5). Non-significant decrease in the activity of CYP3A4 was noted at 0.01, and 0.05 µM eugenol levels and the CYP3A4 activity was reduced by 0.87 % and 2.15 % ($P > 0.05$, Fig. 5A). While at

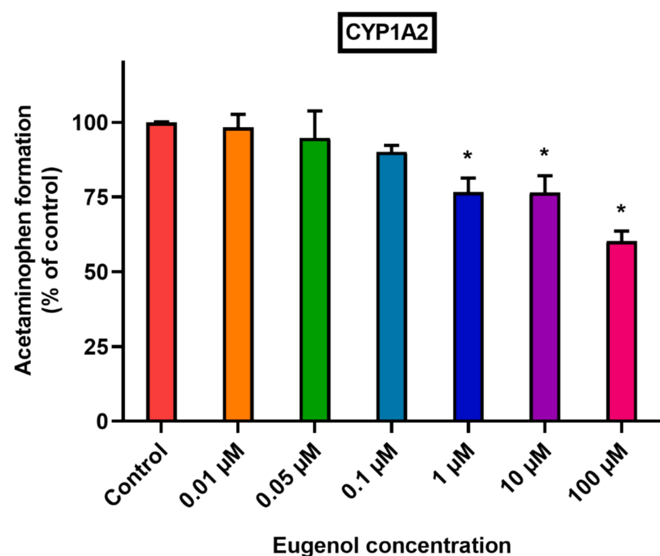

Fig. 2. Influences of various concentration of eugenol on the metabolite activity of CYP1A2 in HLM (Mean ± SEM). *$p < 0.05$ in comparison to control.

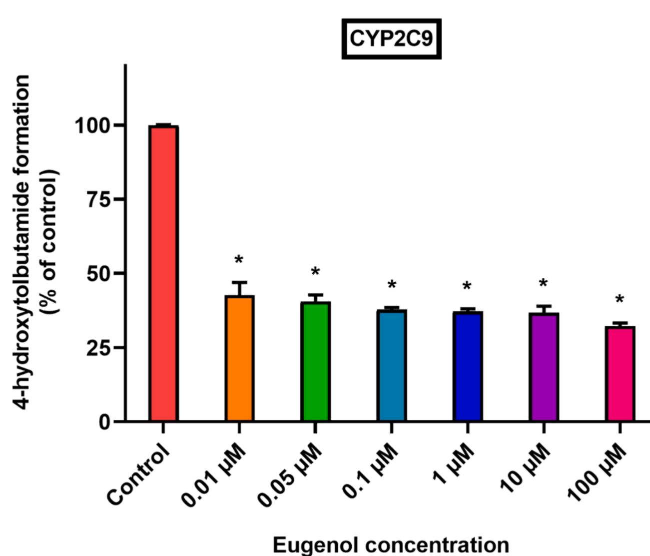

Fig. 3. Influences of various concentration of eugenol on the metabolite activity of CYP2C9 in HLM (Mean ± SEM). *$p < 0.05$ in comparison to control.

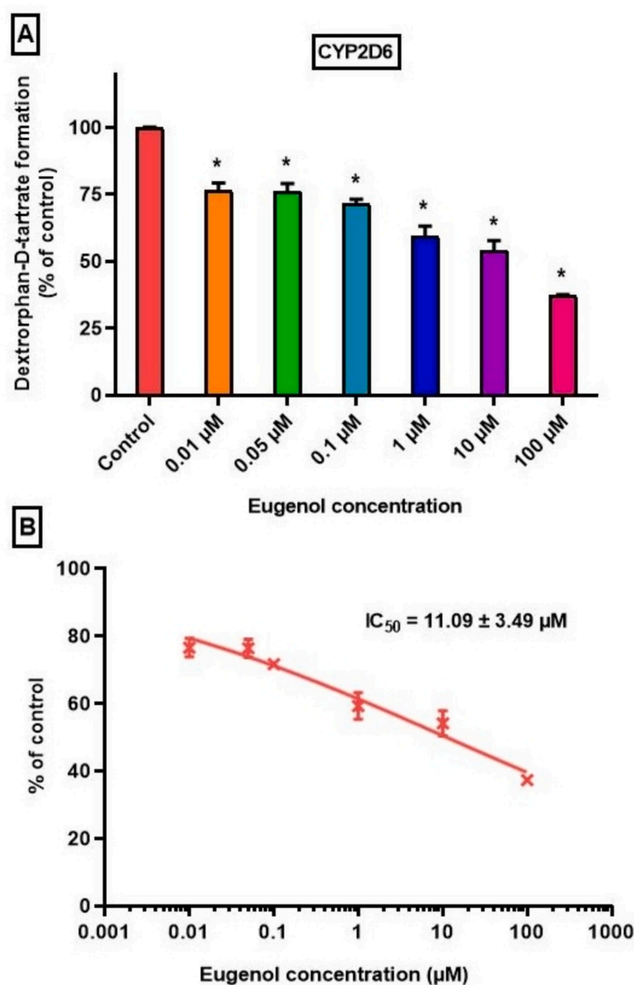

Fig. 4. (A) Influences of various concentration of eugenol on the metabolite activity of CYP2D6 in HLM. (B) Showing the IC_{50} curve and values of eugenol for CYP2D6 (Mean ± SEM). *p < 0.05 in comparison to control.

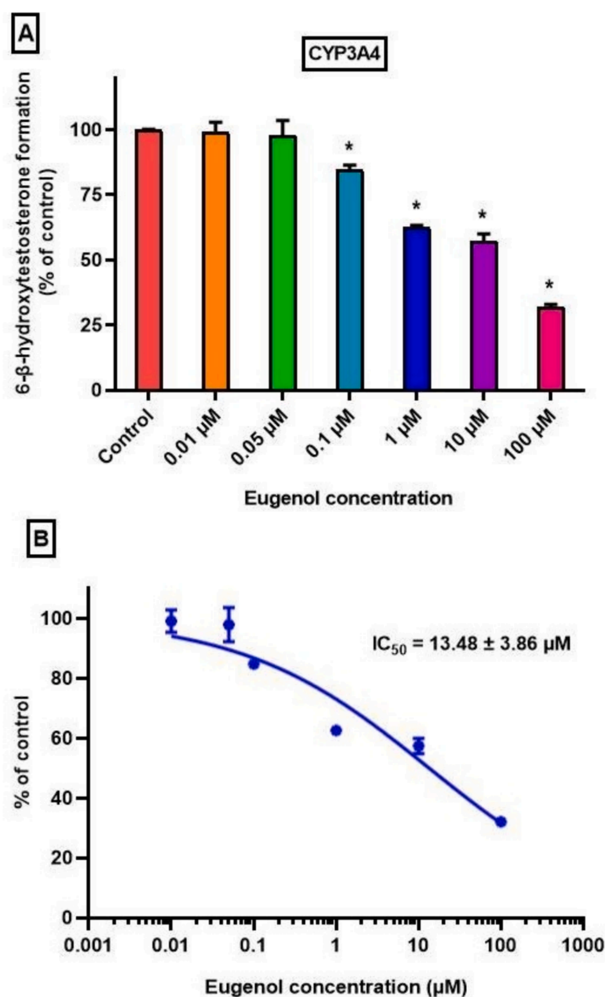

Fig. 5. (A) Influences of various concentration of eugenol on the metabolite activity of CYP3A4 in HLM. (B) Showing the IC_{50} curve and values of eugenol for CYP3A4 (Mean ± SEM). *p < 0.05 in comparison to control.

0.1 µM eugenol level, significant ($P < 0.05$) metabolic activity of CYP3A4 was inhibited by 15.28 ± 1.71 %. Similarly, at a moderate eugenol concentration (1 µM and 10 µM) a significant ($P < 0.05$) inhibition of 37.41 % and 42.58 % was observed (Fig. 5A). Its maximum inhibition of 67.86 ± 0.92 %, $P < 0.05$ was recorded at the maximum value of eugenol 100 µM (Fig. 5A). The IC_{50} value of eugenol was calculated as 13.48 ± 3.86 µM (Fig. 5B).

4. Discussion

A growing trend of natural medicines is being observed in the current era, and the demand for herbal products is increasing year by year (Falzon and Balabanova, 2017; Sen and Samanta, 2015). The use of herbal medicines in combination with many recommended remedies is highly popular and is frequently used by patients (Arora et al., 2022). Drug interactions can often happen when co-administered drugs or foreign chemicals influence a drug's metabolism, which could result in adverse effects. There has been evidence that ancient medicines modulate CYP enzymes, leading to the belief that conventional treatments are having negative outcomes (Korobkova, 2015). This contradicts the generally accepted view of herbal medicine in nations where it is widely practiced. Moreover, experimental models and human studies have found that natural medicines and supplements differentially modulate CYP stimulation (Harris et al., 2003; Zhou et al., 2003).

Drug interactions are generally severe when a product, food, drug, or natural product interferes with the metabolic clearance of one active. In addition, drug-metabolizing proteins may be inhibited, and this can ultimately result in elevations in plasma levels of concurrently administered drugs. Consequently, prolonging their influence on the body, and increasing the risk of drug-induced toxicity and harmful side effects (Angela and Kuerzel, 2013; Arora et al., 2022; Harris et al., 2003; Parvez and Rishi, 2019; Zhang et al., 2022; Zhou et al., 2003).

Essential oils extracted from cloves contain eugenol as their primary component. In spite of eugenol's increasing medicinal use, there are few studies investigating its interactions with CYP enzymes (Gardner et al., 1997; Iwano et al., 2014).

In this study, a dose-related inhibition of the CYP1A2 enzyme was detected with eugenol. Non-significant CYP1A2 inhibitions were noted at the 0.01, 0.05 and 0.10 µM dose levels. Eugenol inhibited CYP1A2 expression by 23 % and 39.80 % at 1 µM, and 100 µM concentrations. CYP1A2 inhibition was significantly inhibited at these two concentrations. It appears that eugenol's inhibitory effect on CYP2C9 activity was strong based on the IC50 value. It was found that eugenol at 1, 10 and 100 µM significantly suppressed CYP2C9 by 62 %, 63 %, and 67 % respectively. Eugenol has a strong inhibitory consequence on CYP2D6, and the IC50 value is 11.09 ± 3.49 µM. Eugenol reduced CYP2D activity by 40 % and 45 % at 1 µM and 10 µM respectively. CYP2D6 activity was maximum inhibited by 62 % at 100 µM. CYP3A4 enzyme activity was reduced by eugenol dose-dependently. Eugenol's IC50 value was

estimated as 13.48 ± 3.86 μM. CYP3A4 enzyme activity decreased by 37 % and 42 % at 1 μM and 10 μM respectively. Eugenol 100 μM inhibited CYP3A4 activity by 67 %. Results indicate that drug interactions are likely to appear when herbs containing eugenol are concurrently consumed with medications that are metabolized via investigated CYPs. As a result of inhibition or stimulation of these CYP enzymes, drugs' plasma levels might increase or decrease, changing drug pharmacological effects, and enhancing the probability of toxic drug reactions. Hence, eugenol's suppressive action on four important CYP enzymes indicates the possible contribution that some herbs and substances contained in herbs could lead to adverse pharmacological reactions. It is imperative for the efficient use and assurance of medication safety, notably for low-therapeutic-index medicines.

5. Conclusion

The present research indicates that eugenol, which is the principal chemical constituent of clove, profoundly affects the metabolic performance of four enzymes those participating in drug metabolism in HLM–CYP1A2, CYP2D6, CYP2C9, and CYP3A4. Eugenol has the greatest impact on the CYP2C9 enzyme compared to the other enzymes in this interaction study. This indicates the potential for substantial drug-herb interactions, especially for drugs metabolized by this enzyme. It appears that eugenol could modulate these enzymes' metabolic activities in the human body. Moreover, the present study showed that the inhibitory effect was dose-dependent. Thus, it could be possible that the level of inhibition could be modified by adjusting the concentration of eugenol. In particular, it is recommended to exercise caution when a herb containing eugenol is used in conjunction with prescribed medications predominantly broken down by CYP1A2, CYP2D6, CYP2C9, and CYP3A4 enzymes, in particular narrow-spectrum drugs.

Ethical Statement.

"This article does not contain any studies with human participants or animals performed by any of the authors".

CRediT authorship contribution statement

Naif Fahad M. Alharbi: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Abdul Ahad:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Yousef A. Bin Jardan:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Fahad I. Al-Jenoobi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, 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".

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