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Research article

In vivo anti-inflammatory, analgesic, and hepatoprotective potencies and acute toxicity of chrysanthenone and thymol

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Abstract

Thymol and chrysanthenone are two bioactive compounds primarily found in the essential oils of *Thymus algeriensis* and *Artemisia herba-alba*, respectively. This study aimed to evaluate the anti-inflammatory, analgesic, and hepatoprotective potential of thymol and chrysanthenone, individually and in combination, and assess their acute toxicity. Anti-inflammatory activity was evaluated by measuring the severity of hind paw edema in rats following carrageenan injection, a standard inflammation model. Analgesic activity was assessed using the writhing test, which measures the number of abdominal constrictions. *In silico* evaluations of anti-inflammatory and analgesic activities were performed using the Maestro 12.5 software from the Schrödinger suite. Hepatoprotective effects were tested against carbon tetrachloride (CCl_4)-induced hepatotoxicity in albino rats. Additionally, serum levels of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were measured, and liver tissue from treated animals was histologically examined. The results demonstrated that the oral administration of the thymol/chrysanthenone combination at a dose of 150 mg/kg significantly reduced inflammation, outperforming 1% Diclofenac with maximum edema reductions of 97 ± 3.7% and 88.6 ± 0.81%, respectively. In the writhing test, rats treated with the combination exhibited significantly fewer abdominal contractions (40 ± 0.40) compared to those treated with the serotonin–norepinephrine reuptake inhibitor Tramadol (42.00 ± 2.70), a drug commonly used to manage moderate to severe pain. Molecular docking studies revealed that thymol exhibited strong binding affinities in the active sites of lipoxygenase and cyclooxygenase, with docking scores of –4.805 kcal/mol and –7.821 kcal/mol, respectively. Chrysanthenone also demonstrated favorable binding, with scores of –4.082 kcal/mol and –6.938 kcal/mol at the same targets. The thymol/chrysanthenone combination showed marked hepatoprotective effects, evidenced by the normalization of ASAT and ALAT levels and the prevention of histological liver damage following CCl_4 intoxication. Acute toxicity testing revealed no signs of toxicity, with an LD_{50} greater than 150 mg/kg.

Keywords: Chrysanthenone; thymol; lipoxygenase; cyclooxygenase; hepatoprotective activity; molecular docking

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1. Introduction

Research into novel bioactive molecules with pharmacological effects is ongoing. Numerous epidemiological and experimental studies have suggested that compounds derived from medicinal and aromatic plants represent powerful alternatives to synthetic molecules (Amrati et al., 2023). The frequent and sometimes prolonged use of oral analgesics and anti-inflammatory drugs, despite their therapeutic effectiveness, presents a significant risk of hepatotoxicity. This raises the question of seeking safer alternatives, particularly by exploring medicinal plants' hepatoprotective potential. To what extent can phytotherapy represent an effective and less toxic alternative or complement to conventional treatments.

The pharmacological activities of plant extracts or their essential oils stem primarily from the bioactivity of individual compounds and the synergistic interactions among them. Previous studies have shown that essential oils from *Thymus algeriensis* (family Lamiaceae) and white wormwood (*Artemisia herba-alba*), both individually and in combination, exhibit strong anti-inflammatory, analgesic, and antioxidant properties (Amarti et al., 2010), as well as antifungal, antibacterial, and cytotoxic effects *in vitro*, as demonstrated in our earlier research. These two essential oils

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are primarily characterized by thymol and chrysanthenone as their major constituents, respectively (Bagheri et al., 2016; El Ouahdani et al., 2021).

Chrysanthenone, also known as 2-pinen-7-one, is a monoterpenoid with the chemical formula $C_{10}H_{14}O$. It can potentially be synthesized from its isomer verbenone through chemical rearrangement (Beer et al., 2007), and it is found in several essential oils from medicinal plants such as *Chrysanthemum indicum* L. (Benelli et al., 2016), *Artemisia asiatica*, and *Chrysanthemum boreale* (Braga,2006).

Thymol is a monoterpene phenol, chemically described as 2-isopropyl-5-methylphenol, and appears as a colorless crystalline substance (Bokhtia et al., 2023). It is abundant in several medicinal plants, including *Thymus algeriensis* (Amarti et al., 2010), *Thymus vulgaris* (Bourkhiss et al.,2011), *Nigella sativa* (Chamanara et al., 2019), *Thymus pectinatus* (Chelucci et al., 2014), and *Satureja intermedia* (Da Costa et al., 2023). Thymol is known for various pharmacological activities, including applications in treating respiratory diseases (Dob et al., 2006), inflammatory conditions (El Abdali et al., 2023), and cancer.

The current study aims to investigate the *in vivo* anti-inflammatory, analgesic, and hepatoprotective activities of chrysanthenone and thymol, their acute toxicity, and the potential synergistic effects when administered in combination. This study is the first to demonstrate that combining Thymol and chrysanthenone can induce analgesic, anti-inflammatory, and hepatoprotective effects.

2. Materials and methods

2.1. Chemical Agent

Thymol and chrysanthenone were purchased from Sigma-Aldrich (France). Thymol, a major phenolic compound, was dispersed in ethanol before use (El Ouahdani et al., 2021).

2.2. Anti-Inflammatory Activity: Carrageenan Edema

The anti-inflammatory activity of thymol and chrysanthenone was evaluated using the carrageenan-induced paw edema model, following the method described by Winter *et al* (Eunkung et al., 2013). This model is widely used for assessing the anti-inflammatory potential of test compounds *in vivo*.

Male rats were randomly divided into four groups, with five animals per group. The first two groups received a single oral dose of thymol and chrysanthenone at 150 mg/kg body weight. The third group received a combination of thymol and chrysanthenone in equal proportions, at 150 mg/kg. The fourth group was a positive control group administered 1% diclofenac gel.

All treatments were administered one hour before the induction of inflammation. Paw edema was induced by subplantar injection of 0.1 mL of 1% carrageenan solution (prepared in 0.9% NaCl) into the right hind paw of each rat. The volume of the right hind paw was measured before carrageenan injection and subsequently every hour from the third to the sixth hour post-injection.

The percentage inhibition of paw edema was calculated using the following formula:

$$\text{Inhibition} = \frac{((\text{St} - \text{S0})\ \text{Control} - (\text{St} - \text{S0})\ \text{Treated})}{((\text{St} - \text{S0})\ \text{Control})\ \text{x}100} \quad (2)$$

S0 is the circumference before carrageenan injection, and St is the circumference at a given time after carrageenan administration.

2.3. Analgesic Activity: Writhing Test

The analgesic potential of chrysanthenone and thymol was evaluated using the acetic acid-induced abdominal writhing test in mice, a widely accepted model for assessing peripheral analgesic effects of bioactive compounds. This method involves counting the number of abdominal constrictions (writhes) induced by an intraperitoneal injection of acetic acid (0.7%) (Fachini-Queiroz et al., 2016).

Mice were randomly divided into groups of five animals each. The control group received ethanol, while the other groups were orally administered thymol (150 mg/kg), chrysanthenone (150 mg/kg), an equal mixture of the two compounds (total dose of 150 mg/kg), or sodium salicylate at a dose of 150 mg/kg.

One and a half hours (1.5 h) after treatment administration, each mouse received an intraperitoneal injection of 0.7% acetic acid at a 10 mL/kg dose. After a latency period of 5 minutes, the number of writhes was recorded for each animal over a 30-minute observation period.

2.4. Molecular Docking

2.4.1. Ligand Preparation

Chrysanthenone and Thymol molecules were retrieved from the NCBI PubChem database and prepared using the LigPrep subsystem of the Schrödinger suite (version 2018, Schrödinger). The minimization process employed the OPLS3 force field, and all possible ionic states were generated at a target pH of 7.2 ± 2 using Epik. Additionally, for each ligand, plausible stereo isomers and lower-energy ring conformations were generated (Falcone et al., 2005).

2.4.2 Receptor Preparation

Lipoxygenase (PDB: 3V99) (Gavliakova et al., 2013) and cyclooxygenase -2 (PDB: 6COX) (Ghori et al., 2016) were chosen as the target receptors to evaluate anti-inflammatory and analgesic activity, retrieved from the RCSB Protein Data Bank using Maestro v12.5.

The protein preparation wizard of the Schrödinger suite was utilized to process the receptor, involving tasks such as assigning bond orders, adding hydrogen, filling empty side chains and loops with PRIME, and ultimately removing all water from the crystal structures. Following optimizing these crystal structures, a restrained minimization with a root mean square deviation (RMSD) of 0.3 Å was performed using the OPLS3 force field (Hernández-Pérez et al., 2002).

2.4.3. Grid Generation and Molecular Docking

The minimized protein structures were then utilized to generate grids through the "Receptor Grid Generation" panel. A grid was established for each protein with default parameters, including a Van Der Waals scaling factor of 1.00

and a charging cut-off value of 0.25, per the OPLS3 force field. A cubic receptor grid box with dimensions of 20 Å × 20 Å × 20 Å was centered on the selected co-crystallized ligand. The molecular docking assay utilized the Standard Precision (SP) scoring method of Glide, integrated into the Schrödinger suite, Maestro version 12.5 (Jordan et al., 1991).

2.5. Hepatotoxicity effect

The thymol, chrysanthenone, and the combined thymol/chrysanthenone mixture (ratio 1/1) at the dose of 150 mg/kg were dissolved in ethanol before being gavage. At the same time, the mice fasted overnight for 16 hours before intraperitoneal administration of carbon tetrachloride prepared in olive oil (vehicle). Then, the mice were divided into five equal experimental groups (n=6/group). The first group was selected to be untreated and non-intoxicated rats (group 1), the second group received 40% CCl_4/olive oil (1 mg/kg body weight twice a week) intraperitoneally for 8 weeks to induce chronic liver damage. In addition to administration of CCl_4 (group 2), the last three groups (group 3, 4, and 5) were treated simultaneously orally once a week for 8 weeks with thymol, chrysanthenone, and the combined thymol/chrysanthenone at the dose of 150 mg/kg, respectively (Kintzios,2002).

2.6. Acute toxicity

The acute oral toxicity study was conducted according to a protocol adapted from OECD Test Guideline No. 423 (OECD, 2008) (Klein et al., 2010). Mice were randomly divided into three groups, with five animals per group. The first two groups received either thymol (150 mg/kg), chrysanthenone (150 mg/kg), or a combined mixture of both molecules (total dose of 150 mg/kg), administered orally. The control group received ethanol at a dose of 150 mL/kg.

Animals were observed continuously during the first 24 hours and then daily for 14 days to monitor clinical signs of toxicity. Parameters observed included general behavior, changes in body weight, digestion, diarrhea, urination, food intake, sedation, and mortality.

At the end of the 14-day observation period, all animals were euthanized. Blood samples were collected via cardiac puncture, and plasma was separated by centrifugation at 2500 rpm for 15 minutes at 5°C. Biochemical analyses assessed levels of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), creatinine, and urea.

Subsequently, major organs, including the liver, kidneys, and spleen, were excised, weighed to calculate the relative organ weight (ROW), and preserved for histopathological examination.

Euthanasia was performed by intraperitoneal injection of the anesthetic agent: 10% urethanexylan (Manda, 2017).

The liver, kidneys, and spleen were weighed, excised, and examined under a light microscope. The relative weight of each removed organ was calculated using the following formula:

$$\text{Relative organ weight (\%)} = \frac{\text{Organ weight}}{\text{Body weight}} \text{x} 100 \quad (1)$$

2.6.1. Biochemical Parameters

The blood sample tube was centrifuged at 2500 rpm/15 minutes. Then, the plasma was collected to assess the biochemical parameters (ASAT, ALAT, creatinine, and urea). Creatinine levels were measured using the Jaffe method, where this last one produces an orange color quantitatively with picric acid in an alkaline medium (Ku et al., 2013). Urea levels were determined using the enzymatic method (Kyung-Mi et al., 2009), and ASAT and ALAT levels were measured using the IFCC method without pyridoxal-5-phosphate (Lafraxo et al., 2022).

The institutional ethical committee of care and use of laboratory animals at the Faculty of Sciences, Dhar El Mahraz, Sidi Mohamed Ben Abdallah Fez University, Morocco, reviewed and approved the present study with reference number #04/2024/LBEAS.

2.7 Statistical Analysis

Statistical analyses were performed using Graph Prism software version 8.0.1, for Windows, and one-way analysis of variance was used to examine differences between the means of treatment groups and the control, and the differences were considered significant, $p < 0.05$.

3. Results

3.1. Anti-Inflammatory Activity: Carrageenan Edema

The anti-inflammatory activity of chrysanthenone and thymol was assessed by measuring the percentage reduction in carrageenan-induced right hind paw edema in rats and comparing the results to those of the standard synthetic anti-inflammatory drug, diclofenac (1%) (Figure 1). The results showed that rats treated with the combination of chrysanthenone and thymol exhibited a remarkable anti-inflammatory effect, achieving an inhibition rate of 97.0% ± 3.7. Under the same experimental conditions, the reference drug diclofenac (1%) produced a maximum inhibition of 88.6% ± 0.81.

When administered individually, chrysanthenone and thymol showed inhibition rates of 83.3% ± 0.00 and 79.1% ± 7.29, respectively. These findings indicate that the combined treatment of chrysanthenone and thymol exerts a significantly greater anti-inflammatory effect than either compound alone, and even exceeds the efficacy of diclofenac.

Furthermore, both molecules demonstrated anti-inflammatory activity in a dose-dependent manner, with efficacy observed during all phases of inflammation.

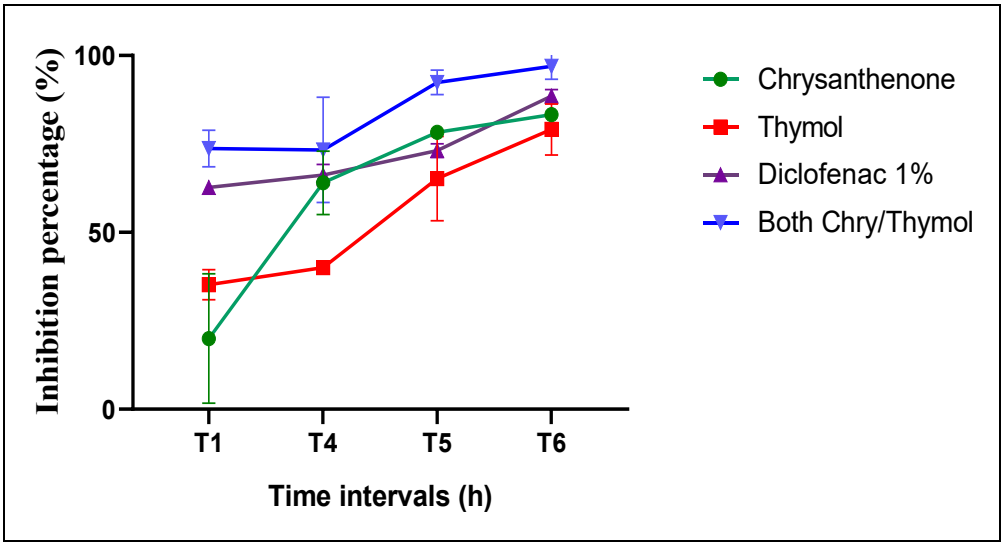

Figure 1. Anti-inflammatory activity of Chrysanthenone, Thymol, and a mixture of the two molecules. Error of the mean (n = 5), ***: $p < 0.001$ compared to negative control and $p < 0.05$ compared to positive control.

3.2. Analgesic Activity: Writhing Test

Analgesic activity was assessed using the acetic acid-induced abdominal writhing test in mice. Abdominal torsions were induced by intraperitoneal injection of a 0.7% acetic acid solution, and the number of writhes was recorded (Figure 2).

Mice treated orally with the combination of chrysanthenone and thymol (150 mg/kg) exhibited a significant reduction in the number of abdominal contortions, with an average of 27.40 ± 0.40**,** compared to the control group, which received the reference analgesic tramadol, showing 42**.**00 ± 2.70 writhes.

When administered individually at the same dose (150 mg/kg), chrysanthenone produced 39**.**60 ± 0.81 writhes, while thymol showed a slightly stronger effect with 32**.**80 ± 0.58 writhes. These results suggest that, although both compounds have peripheral analgesic activity, their combination produces a synergistic effect, resulting in a significantly greater analgesic response than that obtained with either compound alone, and even greater than that of the reference drug.

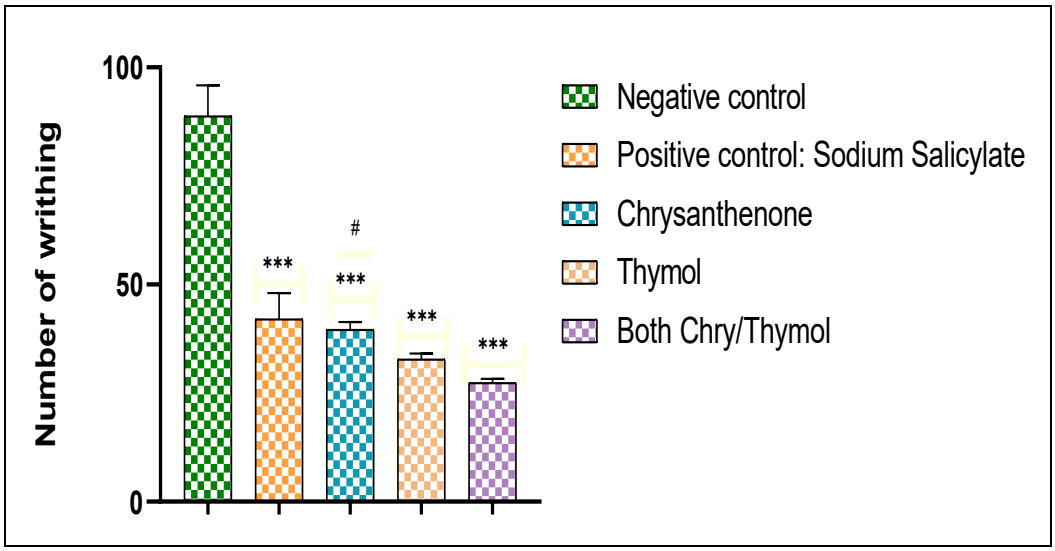

Figure 2. Analgesic activity of the studied molecules. The values are expressed as the mean ± standard error of the mean, ***: $p < 0.001$ compared with negative control; # (n = 5), $p < 0.05$compared with the positive control

3.3. Molecular Docking

During the *in-silico* evaluation of anti-inflammatory activity, thymol exhibited inhibitory activity against lipoxygenase with a glide g score of -4,805 kcal/mol, glide emodel of -32,734 kcal/mol, and glide energy of -23,509 kcal/mol and analgesic activity and inhibitory power against cyclooxygenase with glide gscore of -7,821 kcal/mol, glide emodel of -37,581 kcal/mol, and glide energy of -26,257 kcal/mol (Table 1). 2D and 3D views of the interaction between thymol and the active sites of the receptors showed that this molecule established a single hydrogen bond with residue VAL 671 in the active site of lipoxygenase (Figure 3A and 4A). While in the active cyclooxygenase active site, thymol also established a single hydrogen bond with the SER

530 residue (Figure 3B and 4B). Chrysanthenone had significant inhibitory activity against lipoxygenase with a glide gscore of -4.082kca/mol, a glide emodel of -21.708 kcal/mol, and a glide energy of -15.313 Kcal/mol.

In the active site of cyclooxygenase, this molecule showed inhibitory activity with a glide gscore of -6,938 kcal/mol, a glide emodel of -23,025 kcal/mol, and a glide energy of -17.9 Kcal/mol. In the active sites of lipoxygenase and cyclooxygenase, chrysanthenone did not show any binding (Figure 3C, 4C, 3D, and 4D).

Table 1. Docking results in ligands in two receptors.

	glide gscore	glide emodel	glide energy	glide gscore	glide emodel	glide energy
	Lipoxygenase PDB: 3V99			Cyclooxygenase PDB: 6COX		
Thymol	-4.805	-32.734	-23.509	-7.821	-37.581	-26.257
Chrysanthenone	-4.082	-21.708	-15.313	-6.938	-23.025	-17.9

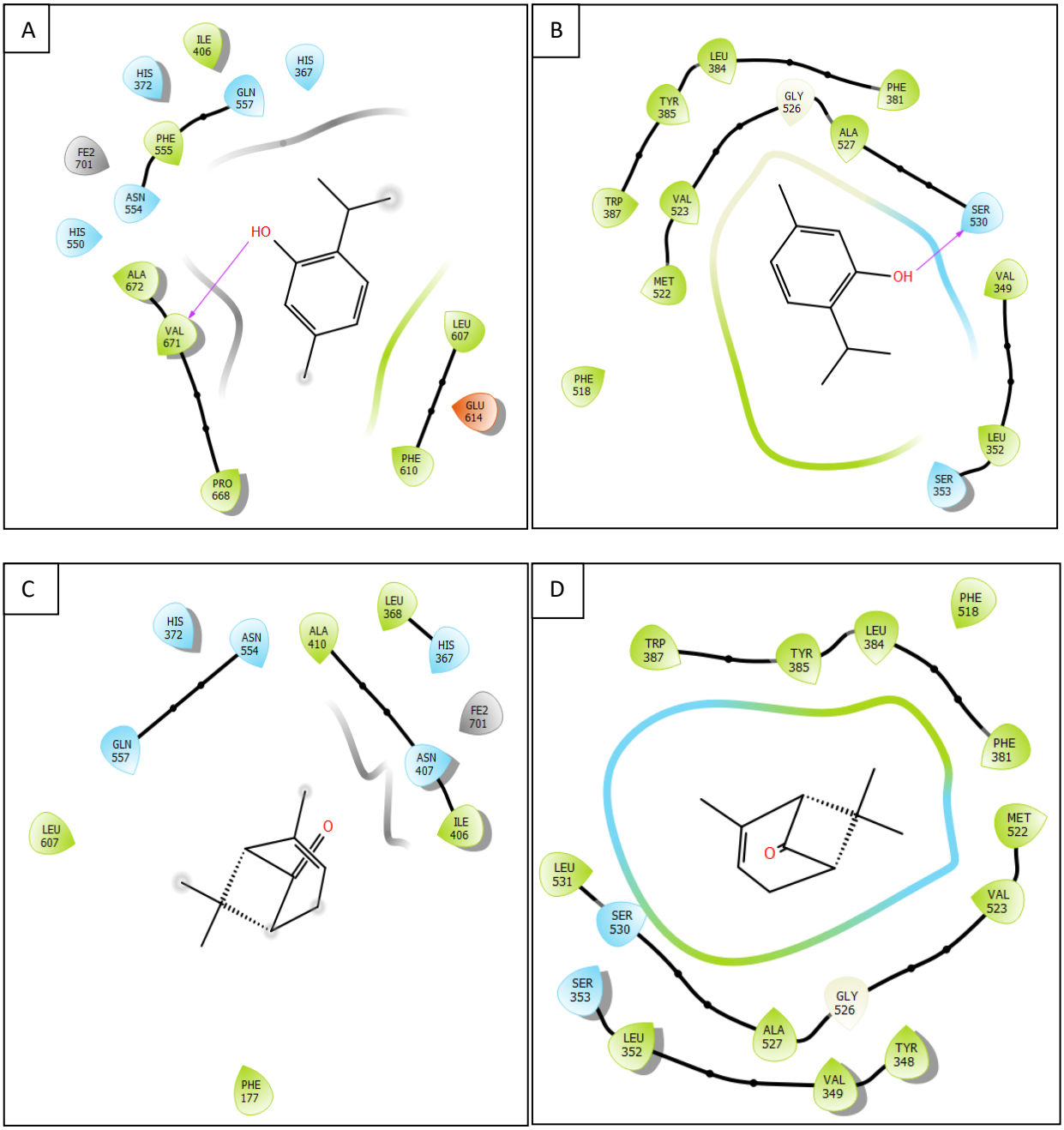

Figure 3. The 2D viewer of ligand interactions with the active site. A and B: Thymol interactions with lipoxygenase and cyclooxygenase active sites; C and D: Chrysanthenone interactions with lipoxygenase and cyclooxygenase active sites.

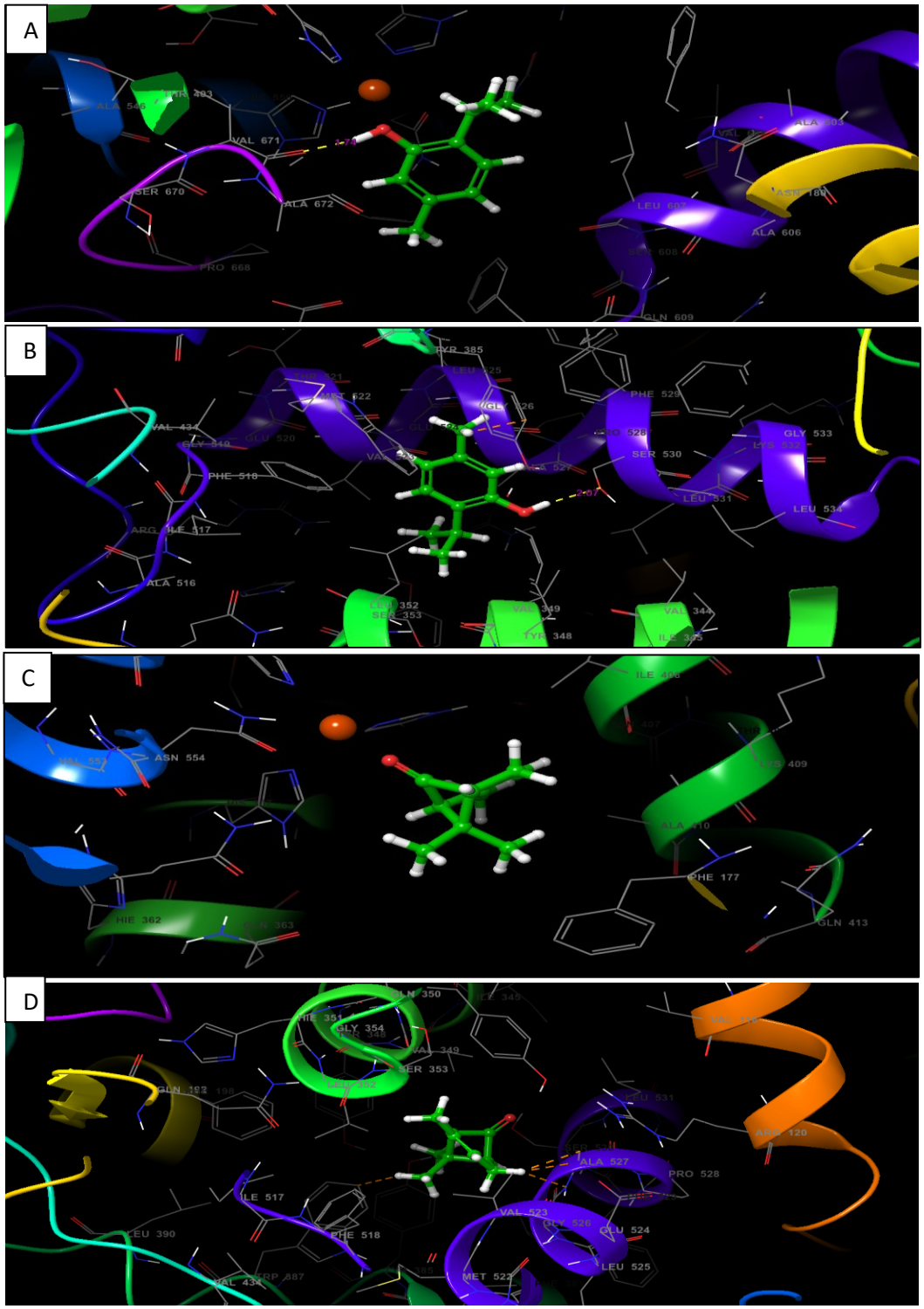

Figure 4. The 3D viewer of ligand interactions with the active site. A and B: Thymol interactions with lipoxygenase and cyclooxygenase active sites; C and D: Chrysanthenone interactions with lipoxygenase and cyclooxygenase active sites.

3.4. Hepatotoxicity

3.4.1. Liver enzymes in blood serum

The hepatoprotective potential of a compound is typically evaluated by its ability to preserve liver tissue integrity, maintain hepatic function, and mitigate damage induced by hepatotoxic agents. In this study, the levels of hepatic enzymes ASAT (aspartate aminotransferase) and ALAT (alanine aminotransferase) in the blood were measured to assess the hepatoprotective effects of thymol, chrysanthenone, and their combination.

A significant increase in ASAT and ALAT levels was observed in rats exposed to carbon tetrachloride **(CCl_4)**, indicating severe hepatocellular damage (Group 2: ASAT = 872 ± 58.89; ALAT = 73 ± 2.31), compared to normal control rats (Group 1). In contrast, pre-treatment with thymol at 150 mg/kg (Group 3) markedly reduced liver enzyme levels (ASAT = 389 ± 25.83; ALAT = 51.86 ± 2.93), demonstrating

a partial protective effect. Similarly, pre-treatment with chrysanthenone at the same dose showed an even more pronounced decrease (ASAT = 236 ± 19.48; ALAT = 44.40 ± 1.47), though enzyme levels remained elevated compared to the untreated control group.

Notably, rats pre-treated with the combined thymol/chrysanthenone mixture at 150 mg/kg (Group 4) exhibited ASAT and ALAT levels of 201 ± 12.95 and 42.94 ± 1.73, respectively, values that were not significantly different from those of the normal control group (Group 1), and well within physiological norms (Table 2).

These results clearly indicate that all pre-treated groups experienced a significant reduction in hepatic enzyme levels compared to the CCl_4-intoxicated group, with the combined treatment showing the most potent hepatoprotective effect.

The normalization of ASAT and ALAT levels in the combined treatment group highlights the ability of the thymol/chrysanthenone mixture to stabilize hepatocyte membranes, thereby preventing the leakage of intracellular enzymes into the bloodstream. This suggests a synergistic effect between the two compounds, offering enhanced protection against CCl_4-induced liver toxicity.

Table 2. Effect of pretreatment with different thymol, chrysanthenone, and their mixture (dose 150 mL/ kg bw/ day) on serum levels of liver enzymes (ASAT/ALAT) of rats with CCl4-induced hepatotoxicity.

Biochemical parameters	Groups of rats				
	Negative control Group 1	Group 2 CCl_4 alone	Group 3 Thymol/CCl_4	Group 4 Chry/CCl_4	Group 5 Mix/CCl_4
ASAT (UI/I)	196±40.72	872±58.89	389±25.83	236±19.48	201±12.95
ALAT (UI/I)	43.13±1.041	73±2.31	51.86±2.93	44.40±1.47	43.94±1.73

Values are presented as mean ±standard error of mean (n=6)

3.4.2. Macroscopic and histopathological alterations

The results of the histological sections of the liver were in the same direction, since decreases in serum liver enzyme levels were greater with the combined mixture (thymol/chrysanthenone) than with the separated thymol and chrysanthenone.

Microscopic observation of liver sections of normal rats (group 1) revealed a normal lobular architecture marked by the presence of centrilobular veins surrounded radially by normal hepatocytes separated by sinusoids lined with endothelial cells (Figure 5A). However, histological sections of the liver of rats intoxicated by CCl_4 (Figure 5B) showed severe centrilobular and fat histopathological changes, indicating hypertrophy, bloated degeneration, microvacuolar steatosis, cavitation, and lymphocyte infiltration with the presence of Kupffer cells as well as cysts of different sizes reflecting major liver damage. After preventive treatment with thymol at a dose of 150 mg/kg in rats intoxicated by CCl_4, histological sections emphasize that there is regression of chemo-induced liver damage, so hepatic parenchyma remains almost normal with signs of lympho-plasmocytic inflammatory infiltrates without any signs of necrosis, steatosis, or degeneration (Figure 5C). Rats treated preventively with chrysanthenone at 150 mg/kg; there is no less pronounced evidence of liver distress. Near normal liver structure was observed with minimal signs of toxicity, showing significant congestion persisting around the centrilobular veins, such that hepatocytes were near normal. No steatosis, necrosis, or degeneration was observed (Figure 5D). When rats were exposed simultaneously to thymol and chrysanthenone, histological examination of the liver of rats treated in a preventive way shows a recognizable hepatic parenchyma with disappearance of all signs of toxicity that appeared after intoxication by CCl_4 (Figure 5E).

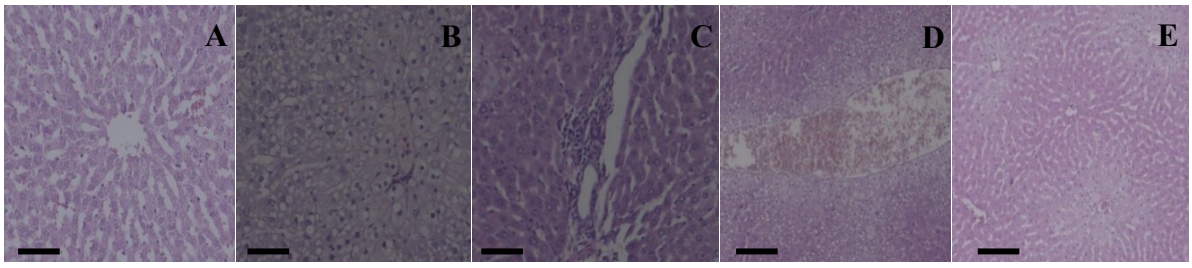

Figure 5. Microscopic photomicrographs of liver sections stained by Hematoxylin and Eosin. Magnification: x40 (A): Hepatic section of the normal parenchyma of untreated rats (group 1); (B): Hepatic section of the parenchyma of rats intoxicated by CCl_4 (group 2) with bloated degeneration, micro vacuolar steatosis and cavitation; (C): Hepatic section of rats intoxicated with CCl_4 receiving thymol (group 3); (D): hepatic section of rats intoxicated with CCl_4 and treated with chrysanthenone(group 4); (E): hepatic section of parenchyma of rats intoxicated with CCl_4 treated with combined thymol/chrysanthenone mixture. Scale bar: 10 mm.

3.5. Acute toxicity

The toxicity of thymol was determined by the use of methods described by the Organization for Economic Cooperation and Development (OECD) guideline 407. Single oral administration of chrysanthenone alone and the combined mixture caused no significant change in general behavior (signs of toxicity). Rats died, while the thymol-treated group showed some changes in general behavior, such as diarrhea and anorexia (Table 3).

Table 3. Results of the observation of the rats every day for 2 weeks after oral administration of thymol

Clinical signs of toxicity/lot	Group 1 Negative control	Group 2 Thymol (150mg/kg)	Group 3 Chrysanthenone (150 mg/kg)	Group 4 Both Thymol/Chry (150mg/kg)
Drowsiness	-	-	-	-
Anorexia	-	+	-	-
Diarrhea	-	+	-	-
Breathing difficulties	-	-	-	-
Sensitivity to pain and noise	-	-	-	-
Abdominal pain (contortion)	-	-	-	-
Convulsion	-	-	-	-
Tremor	-	-	-	-
Coma	-	-	-	-
Mortality	-	-	-	-

All rats remained alive after 14 days of observation. Body masses in the control group and both chrysanthenone and mixture groups remained almost stable throughout the study period, with a non-significant difference in body weight variation between treated rats and the control group for 14 days, while thymol-treated rats showed a very significant decrease in body weight compared to the control group (Table 4). In agreement with Medicines Agency reports European on the plant (EMA/HMPC/166517/2015) the mixture at a dose of 150mg/kg and chrysanthenone at a dose of 150mg/kg are considered non-toxic and safe, and according to the Globally Harmonized System of Classification and Labeling of Chemicals the LD_{50} of the mixture is greater than 150 mg/kg. After 14 days of treatment, the rats are euthanized, and their organs, including liver, kidneys, and spleen, are removed to measure their relative masses. The relative organ masses of the rats treated with chrysanthenone alone and the mixture (Thymol/chrysanthenone) at 150 mg/kg showed no significant difference from the relative organ masses of rats in the control group. However, the relative organ masses of rats treated with thymol alone at 150mg/kg were highly significant relative to the control group (Table 4).

Results of the biochemical study confirm conclusions based on relative organ masses and showed no significant increase in concentrations of liver enzymes ASAT, ALAT, and Creatinine, in the chrysanthenone-only and mixture groups in rats (Thymol/chrysanthenone) at a dose of 150 mg/kg. In contrast, the levels of these parameters are significantly elevated compared to the control group (Table 5).

Table 4. Effect of oral administration of thymol/chrysanthenone on relative masses of organs removed from rats after 14 days of treatment.

Organs	Doses mg/kg			
	Negative control	Thymol (150mg/kg)	Chrysanthenone (150 mg/kg)	Both Thymol/Chry (150mg/kg)
Liver	1,507 ± 0.094	1.604±0.096	1.511±0.007	1.505±0.008
Kidney	0,450± 0.005	0.503±0.08	0.462±0.003	0.453±0.002
Spleen	0,154 ± 0.006	0.159±0.009	0.145±0.004	0.156±0.001

Values are presented as mean ±standard error of mean (n=5)

Table 5. Effect of oral administration of thymol/chrysanthenone on biochemical parameters in rats after 14 days of treatment.

Parameters	Negative control	Thymol (150 mg/kg)	Chrysanthenone (150 mg/kg)	Both Thymol/Chry (150 mg/kg)
"Urea (g/l)"	0,39 ± 0,02	0.42±0.05	0.37±0.07	0.38±0.02
"Creatinine (mg/l)"	3,67 ± 0,33	3.99±0.42	3.71±0.24	3.63±0.23
"ASAT (UI/l)"	196 ± 2,72	203±2.05	194±2.09	193±1.86
"ALAT (UI/l)"	26,67 ± 2,19	28.04±2.41	25.94±1.64	23.17±2.06

Values are presented as mean ±standard error of mean (n=5)

4. Discussion

Bioactive molecules have several pharmacological effects and can be extracted from essential oils (Lisin et al., 1997; Li et al., 2018). Essential oils of *Thymus algeriensis* and *Artemisia herba-alba* are characterized by the presence of thymol and chrysanthenone as the major compounds, respectively. Several studies demonstrated the efficacy of thymol vis-à-vis several acute and chronic pathologies like ROS (Marsik et al., 2005; Manuela et al., 2020), dyslipidemia (Mastelic et al., 2008), pathologies related to inflammation (Marzouk et al., 2015; El Ouahdani et al., 2021), cardiovascular diseases (Meister et al., 1999), respiratory (Mendes et al., 2010), and an anti-cancer effect (Nagoor et al., 2015) and cytotoxic (Nagoor et al., 2010).

Non-steroidal anti-inflammatory drugs (NSAIDs) that can reduce or suppress inflammation symptoms exert their effect by inhibiting cyclo-oxygenase 2, a constitutive protein that plays an important role in tissue integrity (OECD, 2002). However, inhibition of inflammation by NSAIDs can cause multiple adverse effects (Raza et al., 2023), such as gastric ulcers, renal dysfunction, drowsiness, and nausea. All these adverse effects have resulted in researchers seeking new bioactive molecules that can reduce inflammation without causing the adverse effects caused by NSAIDs. Acute inflammation induced in mice by the injection of carrageenan is a standard and practical model, widely used for the evaluation of anti-inflammatory properties of different agents (Reitman et al., 1957). The combination of thymol/chrysanthenone at a dose of 150 mg/kg shows an anti-inflammatory effect superior to that of Diclofenac, a reference anti-inflammatory. Thymol, isolated from the essential oil of the leaves of Lippia gracilis, administered at a dose of 200mg/kg, has an inhibitory power of the edema induced by the injection of carrageenan, similar to that of acetylsalicylic acid (Salehi et al., 2018). At a 7.5 mg/kg dose, thymol induces inhibition of peroxidation, glycation, dyslipidemia, and chemically induced inflammation (Suresh et al., 2018). The percentage of inhibition of edema caused by the carrageenan injection of the thymol/chrysanthenone combination was greater than that of thymol and chrysanthenone dosed separately, demonstrating the synergistic effect between these two molecules.

The Writhing Test was employed to evaluate the analgesic potential of thymol and chrysanthenone. In that assay, intraperitoneal acetic acid injection stimulates the activation of peripheral pain mechanisms through the production and release of different chemical mediators such as histamine, serotonin, bradykinin, and prostaglandins PGE 2 and PGEα (Tang et al., 2011). Thymol and its carvacrol isomer suppress expressions of TNF-α and IL-6 *in vitro* and *in vivo,* two inflammation biomarkers (Tilaoui et al., 2011). However, results of another study show that thymol exerts its anti-inflammatory effect by inhibiting pro-inflammatory cytokines such as IL-1β and TNF-α (Wang et al., 2009) and via α2 adrenergic receptors in nerve cells (Winter,1962). The thymol/chrysanthenone mixture has a greater analgesic effect than salicylic acid, a reference analgesic, and the two molecules tested separately.

Thymol is a natural, monoterpene phenol compound derived from plants, particularly from the herb, commonly known as thyme (*Thymus vulgaris*). Thymol has been used for centuries for its various medicinal properties, including its antimicrobial, antifungal (Xin et al., 2012), antioxidant (Yadav et al., 2009), anti-inflammatory (Yu et al., 2016), and analgesic properties (El Ouahdani et al., 2023). It is commonly used as an ingredient in pharmaceuticals, cosmetics, and food products, as well as in aromatherapy and herbal medicine (Tilaoui et al., 2011). Thymol is known for inhibiting the growth of bacteria, fungi, and other microorganisms, making it a valuable compound in medicine and health care.

Chrysanthenone is a bicyclic ketone compound with a molecular formula of $C_{10}H_{14}O$. It is found naturally in certain plant species, particularly in the genus Chrysanthemum, from which it derives its name. Chrysanthenone has been identified as the predominant constituent of the essential oil of *Thymus algeriensis.* This plant has garnered attention due to the notable anti-inflammatory and analgesic activities that might be due to chrysanthenone (El Ouahdani et al., 2021). Based on the results obtained from the *in-silico* evaluation of anti-inflammatory and analgesic activities, it is evident that both thymol and chrysanthenone exhibit inhibitory activity against key enzymes involved in the inflammatory and analgesic process, specifically lipoxygenase and cyclooxygenase.

Thymol and chrysanthenone demonstrated significant inhibitory activity against lipoxygenase and cyclooxygenase, as their favorable glide g-scores indicated. This suggests a strong binding affinity to the active sites of lipoxygenase and cyclooxygenase, potentially inhibiting their enzymatic activity.

The hepatoprotective effect of the combined mixture can be attributed to the phytochemical compounds' thymol and chrysanthenone. The carvacrol, thymol isomer, has a hepatoprotective impact in preventing liver steatosis induced by a high-fat diet via SIRT1-AMPK signaling pathway activation (El Ouahdani et al., 2021). Pre-treatment with thymol induces a significant decrease in serum enzyme levels (ASAT, ALAT, and alkaline phosphatase) in rats with paracetamol-induced hepatotoxicity. The transformation of carbon tetrachloride, CCl4, into trichloromethyl reactive radical is the key pathogenicity factor of CCl_4. Thus, pre-treatment with an inhibitor of CCl_4 metabolism, an antioxidant agent, a lipid peroxidation inhibitor, or an anti-inflammatory can reduce the hepatotoxicity and protect liver homeostasis.

Thymol and chrysanthenone showed a significant antiradical effect in vitro, superior to those of synthetic antioxidants, and a crucial anti-inflammatory effect in vivo, exceeding the reference anti-inflammatory. Nevertheless, it has been suggested that thymol can inhibit lipid peroxidation and non-

enzymatic oxidation (El Ouahdani et al., 2021). Consequently, the hepatoprotective mechanism of thymol might be due to its antioxidant and anti-inflammatory effects. This illustrates the hepatoprotective effect of the combined mixture (thymol/chrysanthenone) at a dose of 150 mg/kg, either by its oxidizing effect, which is more important compared to the effects of thymol and chrysanthenone studied separately, that is, essentially the synergy between these two molecules, a synergy that was proven in other pharmacological tests.

The results of tests with thymol and chrysanthenone separately at a dose of 150 mg/kg show that the latter cannot fully repair hepatic tissue damage induced by CCl_4, but could limit it. However, the results of the preventive test by the combined mixture at a dose of 150 mg/kg were spectacular; all signs of intoxication induced by CCl_4 disappeared.

Carbon tetrachloride (CCl_4) is a hepatotoxin with a dose-dependent action. Its toxicity is mainly due to an increased production of oxygenated reactive species that induce oxidative stress and peroxidation of membrane lipids, leading to the destruction of hepatocyte membranes and consequently the leakage of hepatic enzymes in the blood circulation (Suresh et al., 2018). Elevated serum levels of liver enzymes, as well as the various tissue lesions that appeared during the study of hepatic histological sections, will provide reliable indicators of liver dysfunction caused by CCl_4 intoxication. In addition, our two compounds were hepatoprotective; this protective effect was manifested by the decrease in serum levels of hepatic enzymes (ASAT/ALAT) and the reduction of signs of liver pain. Repeated administration of thymol and chrysanthenone separately at 150 mg/kg protects the liver from damage caused by CCl_4. Alternatively, the mixture combined with a dose of 150 mg/kg demonstrated a hepatoprotective effect, where it acted effectively against the intoxication induced by CCl_4 and maintained the tissue integrity and the functioning of the liver enzymes. Histology of the liver of rats treated with the mixture shows a normal liver and a total disappearance of signs of liver intoxication (Yadav et al., 2009). In total, the preventive treatment with the combined mixture (thymol/chrysanthenone) seems to have the best hepatoprotective effect against intoxication with CCl_4. The toxicity study of thymol and chrysanthenone at a dose of 150 mg/kg shows that the oral administration of the combination of these two molecules did not adversely affect the health of rats and did not result in any signs of toxicity, encouraging us to study other activities and, why not, move on to clinical studies.

5. Conclusion

The very significant results obtained in this pharmacotoxicological study show that the combination of thymol/chrysanthenone at a dose of 150 mg/kg has interesting therapeutic potential against inflammation and pain in relation to Diclofenac and salicylic acid reference drugs, respectively. A spectacular hepatoprotective effect of the combined (thymol/chrysanthenone) mixture at a dose of 150 mg/kg. However, this combination showed no signs of toxicity. These interesting results open the door to clinical research and development of a new anti-inflammatory, analgesic, and hepatoprotective drug alternative to reference drugs with several adverse effects.

Supplementary Materials: Not applicable

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