

Phytochemical analysis and biological activities of *Scabiosa atropurpurea* L. aerial parts growing on Northern Algeria

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Abstract

The use of natural materials and treatments derived from them is steadily growing in a variety of industries, including medicine, agriculture, food, and cosmetics. UPLC/ESI/MSⁿ was utilized to analyze the aqueous (AqE) and methanol (ME) extracts of *Scabiosa atropurpurea*. The antioxidant activity was tested using hydroxyl radical and hydrogen peroxide scavenging assays. In addition, the *in vitro* anti-protein denaturation and xylene-, croton oil-induced mouse ear and formalin-induced paw edematous assays were applied for anti-inflammatory assessment. Besides, the analgesic activity was evaluated using aspirin as a reference standard. Phytochemical investigation had revealed higher polyphenolic content in ME than AqE, and UPLC-MS-MS analysis of both extracts has resulted in thirty-four secondary metabolites belonging to different chemical classes with major presence of flavonoids and tannins. AqE showed a high antioxidant potential and anti-protein denaturation effect compared to ME. Both extracts revealed a significant and comparable *in vivo* anti-inflammatory activity in xylene, croton oil and formalin induced oedema. ME showed more potent analgesic activity compared to AqE in the acetic acid-induced writhing test. Further investigation into *S. atropurpurea* is warranted for developing antioxidant, anti-inflammatory and analgesic products. Moreover, *in silico* pharmacodynamic profile was performed on the major tentatively identified compounds. It was observed that dicaffeoylquinic acid elicited the most promising inhibitory activity against cyclooxygenase-2 (5F19) with a docking score equal to -10.1 Kcal/mol comparable to the standard drug diclofenac.

1. Introduction

Inflammatory responses are crucial for tissue repair after injury and elimination of pathogens and foreign bodies restoring stability at infection or injury sites. The four hallmarks of inflammatory responses include Dolor (pain), Calor (hyperthermia), Tumor (oedema) and Rubor (erythema), the inflammatory responses are elicited in response to different mediators including instant release of preformed amines such as histamine, intermediate release of lipids such as prostaglandin and leukotrienes followed by slow release of peptides such as interleukins and tumor necrosis factor ¹. While synthetic drugs offer strong anti-inflammatory effects, they carry risks like liver damage, renal failure, and hemorrhage. As a result, some have been banned or are used less frequently for long-term treatment ².

The curative effects of natural products have been recognized since ancient times, attracting both public and scientific interest for treating diseases like cardiovascular disorders, cancer, and inflammation since they are readily available and have less side effects ³. Despite advancements in synthetic chemistry, natural product-derived medicines remain essential in modern drug development ⁴. Herbs have long been a rich source of bioactive components, used in their natural form or as purified substances for disease treatment. Medicinal herbs play a key role in developing potent therapeutic agents. ⁵.

Scabiosa, a famous genus of medicinal plants named after its wide use in the treatment of scabies, is presently categorized under the Caprifoliaceae family ⁶. Genus *Scabiosa* comprises over 80 species primarily found in the Mediterranean region. Of these, around 43 species are found in Europe, while the remaining species are scattered across Asia and Africa ⁷. *Scabiosa atropurpurea* is an annual shrub that has groups of leaves at the bottom and stems with leaves. This species stands out among others by its bluish-lilac blossoms and distinctive fruit morphology. In addition, this plant has traditionally been used to cure many conditions such as influenza, bronchitis, coughs, and acne. Its blooms were also used as a tisane to heal hypoglycemia ⁸. *S. atropurpurea* has distinct biological properties, including hepatoprotective, and anti-diabetic actions ⁹, antibacterial, anti-inflammatory ¹⁰, antifungal and antioxidant activities ¹¹.

In continuation of our search for new natural resources with interesting biological activities, we herein report on the phytochemical investigation as well as the potential antioxidant, anti-inflammatory and analgesic activities of AqE and ME of the entire aerial parts of *S. atropurpurea*, in addition to conducting acute oral toxicity evaluation of both extracts to ensuring the safe use of the extracts as medicinal agents.

2. Results

2.1. Determination of total phenolic and total flavonoid contents

The total phenolic (TPC), flavonoids (TFC), and condensed tannins (CTC) contents in both extracts are presented in Table S1. The findings demonstrated that ME displayed a substantial dose of TPC and TFC with 114.13 ± 0.92 μg gallic acid equivalent /mg dry extract (μg GAE/mg DE) and 100.57 ± 0.93 μg quercetin equivalent/mg dry extract (μg QE/mg DE), respectively. On the other hand, the highest CTC was observed in AqE attaining 41.04 ± 0.64 μg catechin equivalent/mg dry extract (μg CE/ mg DE).

2.2. UPLC/MSⁿ analysis from the aerial parts extracts from *Scabiosa atropurpurea*

Herein, the aerial parts of *S. atropurpurea* were extracted using methanol and water to obtain the ME and AqE, respectively. Both extracts were analyzed using UPLC/MSⁿ analysis in the ESI positive and negative ion modes in order to identify their active metabolites. Thirty-four metabolites were defined tentatively from the two extracts (Tables 1, S2, S3) and their phytochemical classes were presented in Fig. 1. The BPI chromatograms in the ESI positive and negative modes were illustrated in Fig. S1A & 1B and Fig. S2A & 2B. The main identified classes were flavonoids, phenolic acids followed by sesqui- and triterpenoids, saponins, iridoids, tannins, fatty acids and others. The identified components were compared between the two extracts in order to configure the common metabolites and to determine the main components responsible for the biological activities exerted by both extracts.

Table 1. Tentatively identified secondary metabolites from the methanol and aqueous extracts of *Scabiosa atropurpurea* aerial parts (common components only)

No.	Compound name	Chemical Class	Molecular Formula	R _t (min.)	[M-H] ⁻ m/z	[M+H] ⁺ m/z	% Composition		MS/MS fragments	Ref.
							ME.	AqE.		
1	Loganic acid*	Iridoid	C ₁₆ H ₂₄ O ₁₀	0.73	377	381	13.29 (28.33)	5.30 (18.22)	353, 341, 265, 191	12
2	Chlorogenic acid*	Cinnamic acid deriv.	C ₁₆ H ₁₈ O ₉	1.00	353	-	0.79	4.27	339, 294, 265, 239, 191, 179	13
3	Luteolin-hexoside*	Flavonoid	C ₂₇ H ₃₀ O ₁₆	1.19	447	449	0.62	4.39	405, 390, 379, 315, 285, 279, 243	39
4	Apigenin-hexoside*	Flavonoid	C ₂₁ H ₂₀ O ₁₀	9.95	431	433	0.79	4.67	405, 377, 363, 295, 269, 231, 202	23
5	Derivative of syringaldehyde	Miscellaneous	----	11.42	403	449	7.92 (5.03)	5.03	391, 361, 309, 283, 265, 235, 179	16,58
6	Caftaric acid	Phenolic acid	C ₁₃ H ₁₂ O ₉	18.40	311	-	0.61	4.69	262, 257, 197, 191, 157	22,59
7	Hydroxy-octadecatrienoic acid	Fatty acid	C ₁₈ H ₃₀ O ₃	20.24	293	305	9.01 (7.95)	4.70	275, 255, 215, 179, 161	60

Compounds in bold are the major components. R_t; retention time, Ref.; reference(s).

% Composition is on g of compound per g of dry extract.

* For compounds previously identified from genus *Scabiosa*.

Certain metabolites were present in common in the ME and AqE but in different compositions and were listed in Table 1. Moreover, each extract showed a unique chemical profile where certain components were only present in the ME (Table S2) while other different compounds were only detected for the AqE (Table S3).

As presented in Table 1, loganic acid showed a deprotonated peak at m/z 377(381) with fragments at m/z 353, 341, 265, 191¹². This iridoid appeared as a major peak in the ME (13.29%) compared to the AqE (5.30%). Chlorogenic acid, a cinnamic acid derivative, showed a deprotonated peak at m/z 353 with fragments at m/z 339, 294, 265, 239, 191, 179 where the characteristic quinic acid peak was seen at m/z 191¹³. It showed a % composition of 4.27% in the AqE and only traces in the ME. Similarly, two flavonoid hexosides were also detected in higher % in the AqE (4.39% and 4.67%, respectively) which showed their deprotonated molecular ion peaks at m/z 447(449) and 431(433) in ESI negative and positive modes, respectively. In addition to that, a derivative of syringaldehyde appeared equally in the two extracts. Besides, Caftaric acid with peak at m/z 311 appeared mainly in the AqE while hydroxy-octadecatrienoic acid (m/z 293) was present in the two extracts with more proportion towards the ME.

The ME showed the unique presence of twenty-two components (Table S2). Caffeoyl quinic acid dimer appeared at m/z 707(732) with two main fragments at m/z 353 for the caffeoyl quinic acid and at m/z 191 for quinic acid which were defining for this compound¹⁴. On the other hand, eustomoside had its deprotonated peak at m/z 389(401) with fragments at m/z 353, 233, 225, 179¹³. Moreover, a ursolic acid derivative^{15,16} appeared at m/z 547 with the characteristic fragmentation patterns at m/z 447, 417, 395, 300, 291, 253, 241, 149 where these fragments were reported to be characteristic for ursolic acid according to literature reports¹⁷, it is also worthy noted that ursolic acid itself is reported for genus *Scabiosa*¹⁸. Moreover, one sesquiterpenoid peak appeared at m/z 295 $[M-H]^-$ and m/z 298 $[M + 2H]^+$ which was assigned to *alpha*-artemisiic acid¹⁶.

In addition to that, five main metabolites were detected only in the AqE (Table S3). A deprotonated molecular ion peak appeared at m/z 601 (4.48%) and was assigned to myricetin-(galloyl)-pentoside^{16,19,20}. Four cinnamic acid peaks were also detected from the AqE, at m/z 315, a protocatechuic acid-hexoside (previously identified from genus *Scabiosa*) was defined¹². Two abundant peaks were traced at m/z 265 for feruloyl-caffeoyl-quinic acid derivative and its isomer^{21,22}. Moreover, a deprotonated molecular ion peak appeared at m/z 325 for *p*-coumaric acid-hexoside (previously identified from genus *Scabiosa*)²³.

2.3. Multivariate data analysis using clustered heat map

A clustered heat map was constructed with Euclidean distance and the unweighted pair group method (Fig. 2). The common components present in both the AqE and ME were utilized for the clustered heat map. As shown in Fig. 2, the % composition for each component was presented as color gradient where components with high percentage were in red while those in blue are with low percentage. Due to difference in polarity between the two solvents certain components were seen in common with variable percentage composition. Chlorogenic acid and hydroxyl-octadecatrienoic acid were the abundant components in the ME while luteolin-hexoside, apigenin-hexoside, caftaric acid and a derivative of syringaldehyde were present in higher percentages in the AqE.

2.4. Biological study

2.4.1. Hydroxyl radical scavenging effect

The effect of hydroxyl radical scavenging ability of *S. atropurpurea* was measured as an IC_{50} value and the findings are shown in Fig.S3. Regardless the effect of BHT (0.22 ± 0.00 mg/mL) as a reference; AqE recorded the highest scavenging effect with an IC_{50} of 6.1 ± 0.34 mg/mL followed by ME with an IC_{50} of 9.64 ± 0.74 mg/mL.

2.4.2. Hydrogen peroxide scavenging effect

The Hydrogen peroxide scavenging ability of *S. atropurpurea* was determined as an IC_{50} value and the results are presented in Fig.S4. Both extracts exhibited a significant scavenging effect against hydrogen peroxide species. ME showed a significant effect with an IC_{50} of 0.15 ± 0.00 mg/mL, while AqE had the highest activity with an IC_{50} of 0.13 ± 0.01 mg/mL.

2.4.3. *In vitro* anti-inflammatory activity

The *in vitro* anti-inflammatory capability of *S. atropurpurea* was examined by employing protein denaturation inhibition procedure. Findings are reported in Table S4. Both extracts revealed a dose-dependent activity on reducing protein denaturation. AqE showed the greatest inhibiting ability across all tested doses (0.125, 0.25 and 0.5 mg/mL) attaining 61.97% at 1 mg/ml, while ME demonstrated a lower value of 33.6% at the same dose.

2.4.4. Evaluation of Acute oral toxicity

The consumption of AqE and ME from *S. atropurpurea* showed no lethality among the experimented mice over the 14-day experimental period. Furthermore, there were no behavioral changes or evident signs of acute poisoning. Consequently, the LD₅₀ of both extracts exceeds 5g/kg of body weight.

2.4.5. *In vivo* evaluation of the anti-inflammatory activity

2.4.5.1. Xylene-induced oedema

The results of xylene-induced ear oedema in mice are illustrated in Fig. 3. Both extracts demonstrated a significant and dose-dependent anti-edematous effect at the given concentrations (50, 150, 300 mg/kg). ME and AqE exhibited equivalent potential to indomethacin; the reference medicine ($p > 0.05$), with oedema reduction at 300 mg/kg achieving 81.48% and 77.37%, respectively, whereas indomethacin's impact at a dose of 50mg/kg was 82.51%.

2.4.5.2. Croton oil-induced oedemas

The oral anti-inflammatory efficacy was evaluated utilizing the croton oil-induced ear oedema Approach in mice, with results depicted in Fig. 4. Both extracts reduced the oedema response in a dose-dependent way. At doses of 150 and 300 mg/kg, the impact was statistically comparable to that of indomethacin (NSAID) ($p > 0.05$). AqE and ME decreased swelling by 58.97% and 61.03% at a dosage of 300 mg/kg, respectively. In contrast, indomethacin produced a 63.08% reduction in oedema at a dosage of 50 mg/kg.

2.4.5.3. Formalin-induced paw edematous

Extracts of *S. atropurpurea* were evaluated for their efficacy in inhibiting formalin-induced paw oedema in mice. The findings are displayed in Table S5. Extracts at doses of 25, 50, and 100 mg/kg exhibited a significant and dose-related reduction for 3 hours post-inflammation induction using a 1% formalin solution in the right paw of mice, compared to diclofenac at 25 mg/kg. The peak reduction of inflammation by ME and AqE occurred during the third hour, achieving 61.11% and 57.91%, respectively, at a dose of 100 mg/kg. Nonetheless, the reference medication inhibited paw swelling by 70.25% after three hours.

2.4.6. Evaluation of the Analgesic activity of different extracts of *S. atropurpurea*

The analgesic properties of *S. atropurpurea* extracts was evaluated using the acetic acid-induced writhing in mice. The effect increased progressively with higher doses, showing a dose-dependent effect when compared to the standard drug. ME demonstrated the strongest analgesic activity at all administered doses (50, 150, and 300 mg/kg). At a dose of 300 mg/kg, both AqE and ME significantly ($p > 0.05$) decreased writhing, with inhibition rates of 65.32% and 67.63%, respectively, closely matching the 72.83% reduction observed with aspirin at 100 mg/kg (Fig. S5).

2.5. *In silico* molecular docking study

The major compounds identified using UPLC-ESI-MS/MS were tested *in silico* against cyclooxygenase-2 (5F19). Previous reports showed that diclofenac possessed anti-inflammatory activity by inhibiting cyclooxygenase-2 enzyme, therefore, it was used as standard ²⁴. The results revealed that it exhibited a docking score equal to -7.9 Kcal/mol. Furthermore, all the tested compounds showed agreeable binding affinities with negative binding scores. In addition, it was observed that the compound namely, di-caffeoylquinic acid elicited the highest inhibitory activity with binding score = -10.1 kcal/mol. Moreover, it was observed that the compounds namely, apigenin-hexoside, luteolin hexoside, chlorogenic acid, caffeoyl quinic acid dimer, *p*-coumaric acid-hexoside, caffeic acid-hexouronide, caftaric acid, ursolic acid, protocatechuic acid-hexoside, eustomoside exhibited binding affinity higher than the standard drug diclofenac with binding scores equal to -9.9, -9.7, -9.5, -9.4, -9.2, -8.9, -8.6, -8.5, -8.2, -8.1 kcal/mol,

respectively. The observed inhibitory activity was attributed to the hydrophobic interactions and hydrogen bonds ²⁵. In addition, π -stacking, π -cation interactions and salt bridge formation were observed. The binding scores and the interactions are listed in Table S6 and illustrated in Figs. 5, 6 and 7.

3. Discussion

Plant extracts are recognized as a valuable natural reserve comprising bioactive secondary metabolites, which exhibit a variety of chemical structures and extensively utilized in the food, cosmetic and pharmaceutical sectors ^{26,27}. Numerous investigations on *Scabiosa* species have definitively shown the existence of many beneficial compounds.

The results of the phytochemical analysis showed a high tenor of polyphenols, flavonoids and condensed tannins, especially when compared to other *S. atropurpurea* species such as *S. maritima* different parts ²⁸, *S. atropurpurea* hydro-ethanol extract of aerial part ⁹, *S. atropurpurea* leaves methanol extract ²⁹ and *S. maritima* aqueous extract of the whole plant ³⁰.

These variations in phytochemical content can be ascribed to factors such as plant part, solvent employed, period of collection, extraction procedure and geographical influences ²⁸.

Moreover, the UPLC/MS identification indicated the existence of 34 metabolites pertaining to phenolic acids, flavonoids, saponins, iridoids and other classes. Many studies on *Scabiosa* genus, especially *S. atropurpurea* demonstrated the existence of a variety of bioactive compounds most of them are in accordance with our results. The UPLC/MS analysis of different species from genus *Scabiosa* showed a phytochemical profile with abundant phenolic acids, flavonoids and (in some species) iridoids and other terpenoids. For example, LC-ESI-FT-MS and NMR analysis of *Scabiosa atropurpurea* lead to the tentative identification of twenty-eight compounds principally iridoids, several flavonoids and a handful of phenolic acids ¹³. LC-MS/MS profiling of *Scabiosa columbaria* leaf and flower methanol extracts identified chlorogenic acid as a major phenolic and also detected kaempferol, quercetin, rutin, protocatechuic, caffeic and *p*-coumaric acids ³¹. Non-targeted UPLC-MS metabolomics of *Scabiosa tschiliensis* revealed a mixture of flavonoids, triterpenes and coumarin-type metabolites with seasonal and treatment-dependent shifts in their relative abundances ³². Targeted LC-MS/HPLC-ESI-MSⁿ guided work on *Scabiosa sicula* and *S. arenaria* has been used to isolate and structurally elucidate several phenolics and related glycosides (flavonoid glycosides and simple phenolic acids) that explain much of their antioxidant activity ⁹. More recent LC-MS surveys of other taxa (e.g., *S. rotata* and multi-taxa comparative studies) continue to highlight chlorogenic and other caffeic-derived acids plus rutin/quercetin-type flavonoids as recurring, bioactive markers across the genus ³³.

The difference in solvent polarity between the ME and AqE lead to the presence of certain common components while each extract showed a unique chemical composition discriminated from the other. The most abundant metabolites in the AqE were cinnamic acid derivatives *viz.* protocatechuic acid-hexoside, feruloyl-caffeoyl-quinic acid derivative and its isomer, *p*-coumaric acid-hexoside in addition to one flavonoid named as myricetin-(galloyl)-pentoside. On the contrast, the ME showed less polar components like caffeoyl quinic acid dimer, eustomoside, a ursolic acid derivative and *alpha*-artemismic acid. This difference in the phytochemical composition of the two extracts was also visually illustrated using a clustered heat map which was applied for the components commonly present in the two extracts where a variation in their % composition was obvious. Therefore, flavonoid glycosides and phenolic acids *viz.* luteolin-hexoside, apigenin-hexoside, caftaric acid and a derivative of syraldehyde due to their relatively higher solubility were present in higher percentages in the AqE while iridoids and fatty acids like loganic acid and hydroxyl-octadecatrienoic acid were the abundant components in the ME.

In this study, the antioxidant activity of *S. atropurpurea* was assessed using the hydroxyl radical scavenging assay. Indeed, this test is a widely utilized technique to assess the antioxidant ability of bioactive compounds found in plants ⁶². The antioxidant efficacy of an extract is directly proportional to its capacity to neutralize hydroxyl radicals ⁶³. Additionally, the hydrogen peroxide scavenging effect of *S. atropurpurea* was also tested. Actually, hydrogen peroxide is not very reactive oxygen species, but it can be harmful to cells due to its potential to generate hydroxyl radicals in cells and this may be the origin of many of its toxic effects. Consequently, the elimination of hydrogen peroxide is crucial for antioxidant protection in cellular or dietary systems. Polyphenols have demonstrated efficacy in safeguarding biological systems from damage triggered by hydroxyl radicals. The

elimination of hydroxyl radicals and hydrogen peroxide may result from the existence of hydrogen-donating phenolic and flavonoids substances in our extracts. These polyphenols are known to have the capacity of scavenging reactive species of oxygen, such as hydrogen peroxide and hydroxyl radicals, so aiding in the prevention or reduction of illnesses associated with oxidative harm. Therefore, polyphenols are extensively known as potent antioxidants ^{64,65}.

Although the ME contained more total phenolics, its antioxidant activity was lower than that of the AqE. This difference may be due to lower levels of specific phenolic compounds that play more important role in antioxidant defense. It's well known that the effectiveness of an extract depends not just on the total amount of phenolics, but on the specific types present. This highlights how complex plant extracts can be. Their antioxidant potential often results from the combined effect of many compounds working together. Isolating a single compound that can match the activity of the whole extract is rarely possible. To fully understand an extract's antioxidant strength, each compound's ability to neutralize free radicals needs to be examined individually ⁶⁶.

Protein denaturation is widely used as an *in vitro* method for evaluating the anti-inflammatory effects of plant extracts ⁶⁷. This process plays a crucial role in the development of inflammation and arthritic conditions, as it is closely associated with various inflammatory mediators and

contributes significantly to inflammatory responses. The denaturation of proteins is essential to the inflammatory process, encompassing a sequence of molecular processes ⁶⁸. Furthermore, numerous inflammatory diseases, including rheumatoid arthritis and serum sickness, are typically linked to the denaturation of proteins' tissue. This protein anti-denaturation effect can be attributed to the existence of phytochemicals like phenols and flavonoids and could represent one of the processes implicated in the anti-arthritic activity of the medicinal plant ⁶⁹. Furthermore, the plants' extracts have been discovered for their ability to inhibit denaturation of proteins to a certain level ⁷⁰.

The security of phytopharmaceuticals, comprising extracts, isolated fractions and medicinal plants, is well-documented and remains an important issue ⁷¹. Medicinal herbs are supposed to have less toxicity owing to their extended application in humans. Studies on acute toxicity serve as an advanced approach for evaluating the safety profile of certain herbs or medicines using single and multiple concentration assessments ⁷².

Herein, the oral administration of AqE and ME from *S. atropurpurea* did not cause mortality in the experimental mice within the fourteen-day trial period. Thus, the LD₅₀ of both extracts surpasses 5 g/kg of body weight. These outcomes agree with those presented by ⁶¹ on *S. atropurpurea* var. *maritima* aqueous extract of the whole plant; which indicated the safety of the species at dose of 4 g/kg.

The xylene and croton oil-induced mouse ear edematous assays are frequently employed to estimate the anti-inflammatory effectiveness of novel pharmaceuticals ⁷³. Topical application of xylene causes acute epidermal ear inflammation, characterized by the release of pro-inflammatory mediators, cellular infiltration, vascular dilatation and the development of oedema ⁶⁷. Croton oil encompasses a mixture of phorbol-12, 13-diester and 12-o-tetradecanoyl phorbol 13-acetate (TPA), which stimulate protein kinase C and multiple inflammatory mediators, resulting in irritative and cutaneous symptoms close to psoriasis ⁷⁴. Topical application of croton oil leads to cutaneous inflammation by initiating an enzymatic cascade involving phospholipase A₂ (PLA₂), which subsequently facilitates the secretion of platelet-activating agent, arachidonic acid (AA), and prostaglandins. This process results in oedema, heightened vascular permeability, leukocyte infiltration, eicosanoid synthesizing *via* cyclooxygenase (COX) and lipoxygenase (5-LOX), along with the release of serotonin and histamine ³⁴⁵. This research evaluates the anti-inflammatory impact of AqE and ME from *S. atropurpurea*, which significantly reduced ear edematous caused by xylene and croton oil. The anti-edematous property is attributed to the plant's polyphenols, which inhibit inflammatory markers and signaling pathways. Polyphenols reduce the expression of inflammatory genes while also modulating oxidative stress levels and interfering with NF- κ B (nuclear factor κ B) DNA binding. Additionally, polyphenols and saponins regulate the MAPK (mitogen-activated protein kinase) and arachidonic acid pathways, further contributing to their anti-inflammatory effects ^{76,77}. Arifin et al (2015) reported

that quercetin inhibits COX-2 and 5-LOX, key enzymes in eicosanoid synthesis, while resveratrol blocks the release of pro-inflammatory mediators and modulates immune responses³⁵. Furthermore, flavonoids and tannins scavenge free radicals, thereby inhibiting pro-inflammatory enzymes⁷⁰.

Additionally, the acute anti-inflammatory response of *S. atropurpurea* was estimated by applying the formalin-induced paw oedema, noted for its biphasic response. Neurogenic pain occurs in the first stage (0–1 h), followed by an inflammatory response marked by the release of mediators such as cytokines (TNF- α , IL-1 β , IL-6), bradykinin, histamine, serotonin, prostaglandins and nitric oxide (NO) in the following stage (2–3 h)⁶⁷.

Our data indicate that AqE and ME displayed a potential anti-inflammatory impact in alleviating paw oedema generated by formalin. This action is ascribed to the plant's bioactive components, such as quercetin, which can modify NF- κ B signaling and inhibit COX-2⁷⁹. The impact of extracts on oedema may be attributed to the suppression of pro-inflammatory substance synthesis and the prevention of inflammatory mediator release, which are the anti-inflammatory processes of polyphenols, flavonoids, and saponins.

Generally, Polyphenols possess anti-inflammatory properties, making them interesting and promising choices for treating and preventing inflammatory illnesses. The anti-inflammatory effectiveness of polyphenols can be varied according to several elements, including their quantity, delivery path, chemical structure and individual differences³⁶.

The results of the *in silico* study showed that *di*-caffeoylquinic acid exhibited the highest inhibitory against cyclooxygenase-2 enzyme. Previous reports revealed that the compound is a natural alternative to non-steroidal anti-inflammatory drugs as it elicited anti-inflammatory activity by inhibiting the activation of the nuclear factor- κ B and MAPK pathways³⁷. In addition, it was previously reported that phenolic compounds exert their action in a similar way as NSAIDs. Moreover, they could inhibit other pro-inflammatory mediators along with COX by reducing their activity or gene expression. Furthermore, some phenolic compounds can up/downregulate transcriptional factors, like nuclear factor- κ B (NF- κ B) or Nrf-2, in inflammatory and antioxidant pathways³⁸. Thus, the results of the *in silico* study substantiate the anti-inflammatory role of the phenolic compounds.

In this study, the analgesic effectiveness was determined by acetic acid-induced writhing, a model for abdominal inflammatory pain triggered by the secretion of endogenous mediators that stimulate nociceptors^{39,40}. Intraperitoneal injection of acetic acid induces the secretion of proinflammatory cytokines, such as TNF- α , IL-1 β and IL-8 from peritoneal mastocytes and macrophages. Consequently, an acute inflammatory response transpires in the peritoneal of mice, resulting in characteristic writhing behavior. Compounds with anti-inflammatory characteristics can diminish the incidence of writhing with time⁴¹.

Bioactive molecules are known for their analgesic characteristics by regulating pain responses and alleviating aches related to inflammation in particular phenolic molecules, which have garnered considerable attention for their influence on inflammatory signaling processes⁴². Moreover, bioactive substances demonstrate potential effectiveness in both the prevention and treatment of neuropathic pain. Saponins exhibit anti-nociceptive properties in numerous animal experiments by suppressing the secretion of IL-6, IL-1 β , COX-2, and iNOS, hence decreasing neuronal harm and facilitating peripheral nerve regeneration and functional recovery⁸³.

4. Methods

4.1. Animals

Female albino mice ranging around 25 and 30 g were tested. The mice bought from the 'Institute Pasteur of Algeria'. Prior to the start of the research, the mice were placed in caged and maintained under norms over one week. Mice received water and food (ad libitum) and kept in compliance with the guidelines specified in the Animals By-Laws N° 425–2008. The experiments were authorized under the approval No. 88– 08/1988 by the 'Algerian Association of Sciences in Animal Experimentation'.

4.2. Plant collection

S. atropurpurea was obtained from the Chaabia district of Ouricia, Setif, located in northern Algeria, in the flowering and fruiting season (May to July) 2021. The species was authenticated by Pr. Laouer Hocine, a botanist at the Laboratory of Valorization of Natural Biological Resources, University Ferhat Abbas Setif 1, Algeria, and established using a voucher specimen labelled (020/DBEV/UFA/22). The entire aerial specimen was carefully washed, dried in a shaded place at ambient temperature, pulverized using an electric grinder, and thereafter conserved for further studies.

4.3. Preparation of plant extracts

AqE was prepared by decocting 100g of dried aerial parts in one liter of filtered water for 20 minutes at 100°C. Subsequently, the resultant solution was filtered by muslin cloths and then subjected to centrifugation at 3000 rpm for 15 minutes. The resultant solution was evaporated at 40°C and entirely desiccated.⁴³

ME was prepared by defatting the plant material through maceration of 4 kg of the aerial component in 15 liters of petroleum ether. Subsequently, the plant was dried and macerated with 15 liters of 100% methanol for 7 days at room temperature, followed by evaporation under vacuum at 45°C using a rotary evaporator⁴⁴.

The obtained dried extracts (AqE and ME) were weighed and thereafter kept in an opaque vials at 4°C.

4.4. Estimation of total phenolic and total flavonoid contents

A polyphenol analysis was carried out using the Folin–Ciocalteu process⁴⁵. 0.1 mL of each extract was mixed with 0.5 mL of Folin–Ciocalteu reagent, followed by the addition of 0.4 mL of 7.5% sodium carbonate solution. After 2 hours of incubation, the absorbance was measured at 765 nm. TPC was expressed as µg GAE/mg DE.

For the measuring of the total flavonoid content; aluminum chloride reagent test was employed⁴³. After mixing 0.5 mL of samples with 0.5 mL of 2% ACR, the produced mix was left for 10 minutes. Spectrophotometry was performed at 430 nm, and the data were presented as µg QE/mg DE.

A modifying vanillin method was employed to assess the concentration of condensed tannins⁴⁵. 375 µL of vanillin reagent (4%) was mixed with 250 µL of tested samples. Following that, 187.5 µL of hydrochloric acid was added. After twenty minutes, a spectrophotometric analysis was carried out at 550 nm. Results expressed as µg CE/mg DE.

4.5. UPLC/MSⁿ analysis

The UPLC/ESI/MS analysis was performed for the *S. atropurpurea* extracts using XEVO TQD triple quadrupole instrument, Waters Corporation, Milford, MA01757 U.S.A, with ESI-MS positive and negative ion acquisition modes using ACQUITY UPLC - BEH C18 column (1.7 µm – 2.1 × 50 mm) with Flow rate of 0.2 mL/min applying a gradient of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid; selected precursor ions were subjected to further analysis applying suitable fragmentation criteria to obtain MS2 data^{19,22,46–48}.

4.6. Multivariate data analysis using clustered heat map

A clustered heat map was built using NCSS. 12[®] software with Euclidean distance and the unweighted pair group method^{47,49,50}.

4.7. Evaluation of antioxidant activity

4.7.1. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was estimated according to the method described by²⁰. 150 µL of the extracts/ BHT at various concentrations was mixed with 300 µL of a stock solution containing 9 mM FeSO₄ and 0.3% hydrogen peroxide (H₂O₂), then incubated at 32°C for 15 minutes. Subsequently, 75 µL of salicylic acid (20 mM) was added, and the reaction mixture was incubated again at 32°C for 15 minutes. Absorbance was measured at 562 nm. Results were expressed as IC₅₀ values, indicating the concentration required to inhibit 50% of the hydroxyl radicals in the solution.

4.7.2. Hydrogen Peroxide Scavenging activity

The hydrogen peroxide scavenging capacity was assessed according to a modified method ²¹. 300 µL of extracts/ ascorbic acid at various concentrations was mixed with 300 µL of hydrogen peroxide solution prepared in phosphate buffer (pH 7.4, 40 mM). The reaction mixture was kept for 10 min and the absorbance of hydrogen peroxide was measured at 230 nm.

4.8. *In vitro* anti-inflammatory activity

The evaluation of the anti-inflammatory effect of the extracts was using the bovine serum albumin denaturation procedure described by ²². 500 µL of 0.2% BSA suspension (in Tris-HCl buffer; 20 mM, pH 6.4) was mixed with 500 µL of different doses of each sample or diclofenac (as a reference). The reaction solution was kept for 15 min at 37°C, subsequent by 10 min at 70°C. Protein denaturation was detected at 660 nm and the inhibition percent was obtained using the formula:

$$I\% = 100 \times (A_c - A_t) / A_c$$

Where A_c and A_t represent the absorbances of the negative control and the tested extracts.

4.9. *In vivo* evaluation of acute oral toxicity

The acute oral toxicity of AqE and ME was evaluated in mice according the OECD criteria ²³. Mice were assigned to groups of five individuals each. Following a 12-hour fasting period, mice received a single dosage of each of the two extracts (2 and 5 g/kg), while the control group received just distilled water. Mice were checked for symptoms of poisoning during the initial hour post-treatment and then at regular intervals over the next 24 hours. Subsequently, the monitoring of any harmful indicators was conducted daily for a duration of fourteen days.

4.10. *In vivo* anti-inflammatory activity

4.10.1. Xylene-induced oedema

The oral anti-edematous activity was examined following the protocol outlined in ⁵¹. Mice were assigned to groups of six individuals. Following one hour of oral administration of various doses of extracts (50, 150, 300 mg/kg), indomethacin (50 mg/kg), and distilled water (negative control), oedema was locally caused in the ears of mice using 30 µL of xylene. A digital caliper was employed for measuring the thickness of the ears both prior to and two hours following the induction of oedema. The inhibition percent of oedema was measured as follow:

$$I\% = 100 * (\Delta n - \Delta) / \Delta n$$

Where Δn and Δ indicates the variance in ear oedema thickness between the negative and treated groups.

4.10.2. Croton oil-induced oedema

The anti-inflammatory capacity was tested adopting the croton oil-induced oedema technique as reported by ⁵². Mice were assigned to groups of six. Each group received indomethacin orally at a dosage of 50 mg/kg, distilled water as a negative control, and different doses of AqE and ME (50, 150, 300 mg/kg). After a period of one hour, swelling was induced topically in the ears of mice by the application of a solution containing croton oil (80 µg/15 µL). After six hours, ear thickness was measured with a digital caliper. The percentage of inflammatory reduction was calculated using the previously mentioned formula.

4.10.3. Formalin-induced paw oedema

Inflammation is triggered by the administration of formalin at the plantar arch of the right paw of mice ^{47,53,54}. Mice were assigned to groups of five and were administered intraperitoneally with varying dosages of extracts (25, 50, and 100 mg/kg), while the positive and negative controls were given diclofenac (25 mg/kg) and 0.9% NaCl (10 mL/kg), respectively. Oedema was caused in the right hind paw of mouse with a subplantar injection of 0.025 mL of 1% formaldehyde 30 minutes after extracts injection. The oedema of the paw swelling was measured using a caliper over a three-hour duration post-injection. The effectiveness of items in inhibiting paw oedema was measured as a percent of inhibition employing the following equation:

$$I\% = 100 * (\Delta_T - \Delta) / \Delta_T$$

Where Δ_T and Δ : The variance in mice paw edema thickness between the negative and treated groups.

4.11. Analgesic activity

The analgesic activity of extracts was assessed by inducing abdominal contractions in mice with acetic acid ^{48,55,56}. Mice were grouped into groups of five. The negative control was administered pure water, whereas the positive control was supplied with aspirin at a dose of 100 mg/kg. Test groups received extracts at concentrations of 50, 150, and 300 mg/kg, respectively. Writhing was triggered intraperitoneally by 0.7% acetic acid (10 mL/kg body weight) one hour after treatment of all groups. The writhes number was documented for each group, beginning 5 minutes after acetic acid injection and lasting for 30 minutes. The inhibition percent of writhing was measured utilizing the following equation:

$$\text{Inhibition (\%)} = 100 * (\text{Cn} - \text{Ct}) / \text{Cn}$$

where Cn and Ct are the mean of constriction' count in mice in the negative control and the treated groups with different concentrations of extracts or aspirin.

4.12. Statistical analysis

Application of one-way ANOVA test, along with Dunnett and Tukey tests for multiple comparison. The data were presented in triplicate, with the mean value and the standard error (SD)/ standard error of the mean (SEM). The IC_{50} determined from graphic plots of the dose response curve for each concentration. The analysis was carried out utilizing GraphPad Prism-8. Variation considered significant at $p < 0.05$.

4.13. *In silico* molecular docking study

The major identified compounds by UPLC-MS-MS analysis were subjected to molecular docking study using the AutoDock Vina platform to assess their anti-inflammatory activity ⁵⁷. The crystal structures of the target cyclooxygenase-2 with the PDB ID (5F19) were retrieved from the Protein Data Bank (www.rcsb.org). Diclofenac was used as standard drug owing to its anti-inflammatory activity ²⁴. The structures of the compounds were drawn using ChemSketch. In addition, the binding interactions between the ligands and the target were generated using the Protein-Ligand Interaction Profiler web tool (<http://plip-tool.biotec.tu-dresden.de>). Furthermore, the 3D interactions were visualized using PyMOL. Validation of the docking was carried out by docking the co-crystallized ligand in the receptor, then, calculation of RMSD by comparing the co-crystallized pose and the docked pose.

5. Conclusions

This study concluded that, *Scabiosa atropurpurea* L. aerial parts represent a rich source of secondary metabolites. UPLC/MSⁿ analysis was utilized for the analysis of the ME and AqE from this plant where thirty-four phytoconstituents were tentatively identified with the abundant presence of polyphenols, flavonoids and condensed tannins. These metabolites are recognized for their biological characteristics as well as effective therapeutic components. Both extracts exhibited considerable antioxidant, anti-inflammatory and analgesic activities which may be attributed to the synergistic biological activity exhibited by the diverse phyto-metabolites in each of them. The acute oral toxicity study revealed the relative safety of these plant extracts which allows for further use as potential source for the discovery of new drug leads.

Declarations

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Author contributions

ANBS: Conceptualization, supervision and revised the manuscript draft, **EAE, HSZ:** data analysis and wrote the original manuscript draft, **HSZ, AK, NC, LA:** performed the biological assays and revised the manuscript draft, **SS:** performed the molecular docking study and revised the manuscript draft, **AME:** performed the UPLC-MS-MS analysis and revised the manuscript draft. All authors revised and approved the final version of the manuscript.

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Data availability

All data are available upon request from the first author

Ethics approval and consent to participate

S. atropurpurea was collected from the Chaabia district of Ouricia, Setif, located in Northern Algeria, according to national guidelines. The species was authenticated by Pr. Laouer Hocine, a botanist at the Laboratory of Valorization of Natural Biological Resources, University Ferhat Abbas Setif 1, Algeria, and established using a voucher specimen labelled (020/DBEV/UFA/22)

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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Figures

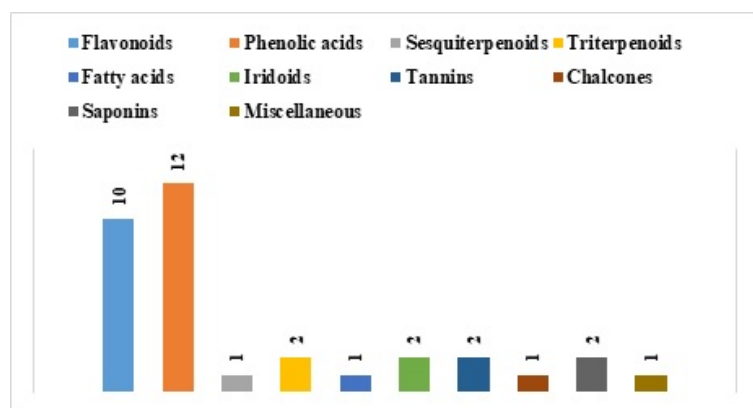


Figure 1

Bar chart showing the tentatively identified metabolites from *Scabiosa atropurpurea* aerial parts extracts.

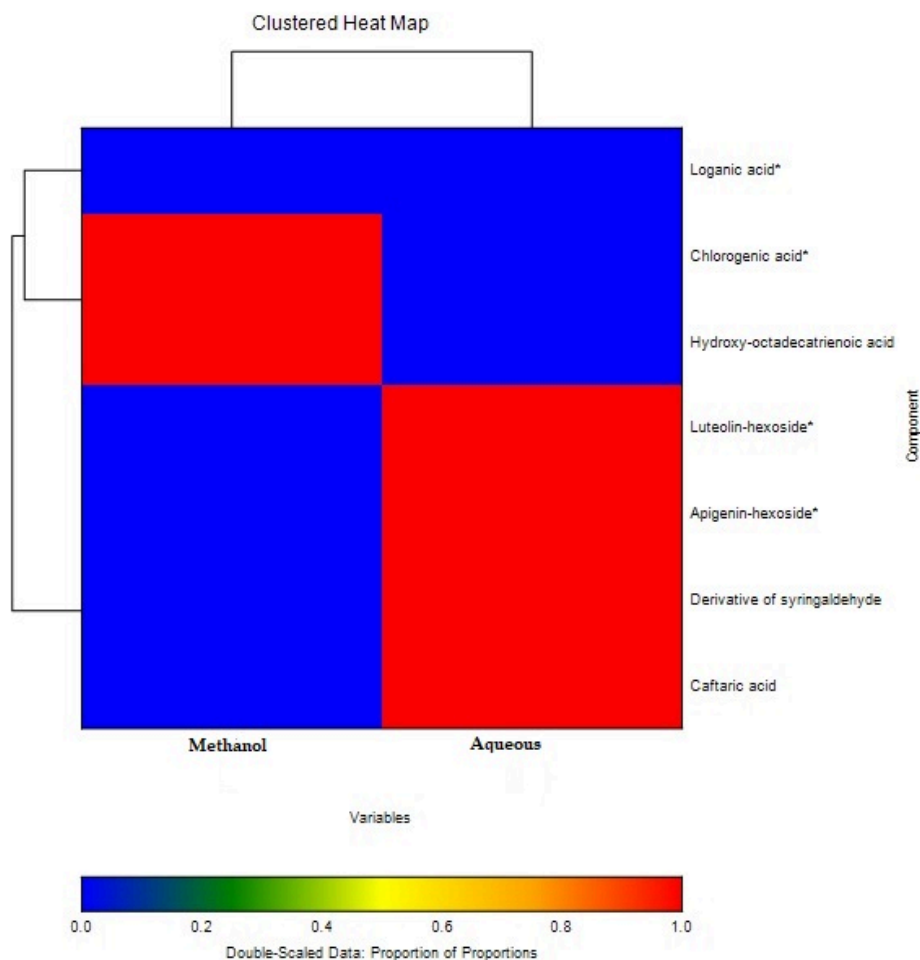


Figure 2

Clustered heat map for the common metabolites identified from the methanol and aqueous extracts of *Scabiosa atropurpurea*.

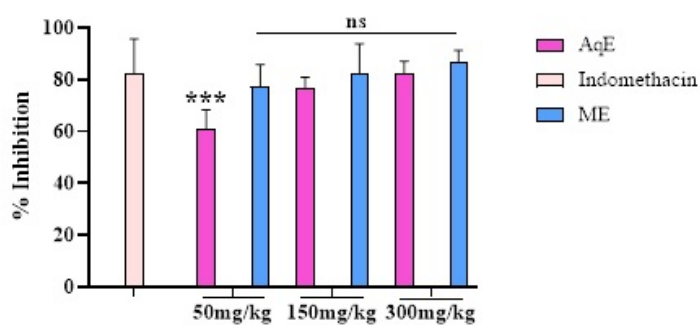


Figure 3

Inhibition percent of xylene-induced ear edema of *S. atropurpurea*, at different concentration. Values are represented as mean $\pm$ SEM (n = 6). ns: no significant difference, ***P<0.001. AqE: aqueous extract, ME: methanol extract.

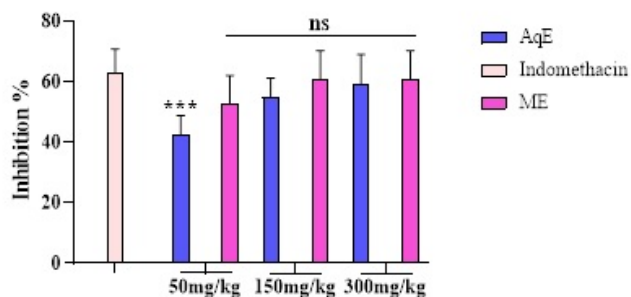


Figure 4

Inhibition percent of croton oil-induced ear edema of *S. atropurpurea*, at different concentrations. Values are shown as mean $\pm$ SEM (n = 6). ns: no significant difference, ***P<0.001. AqE: aqueous extract, ME: methanol extract.

3 D interactions

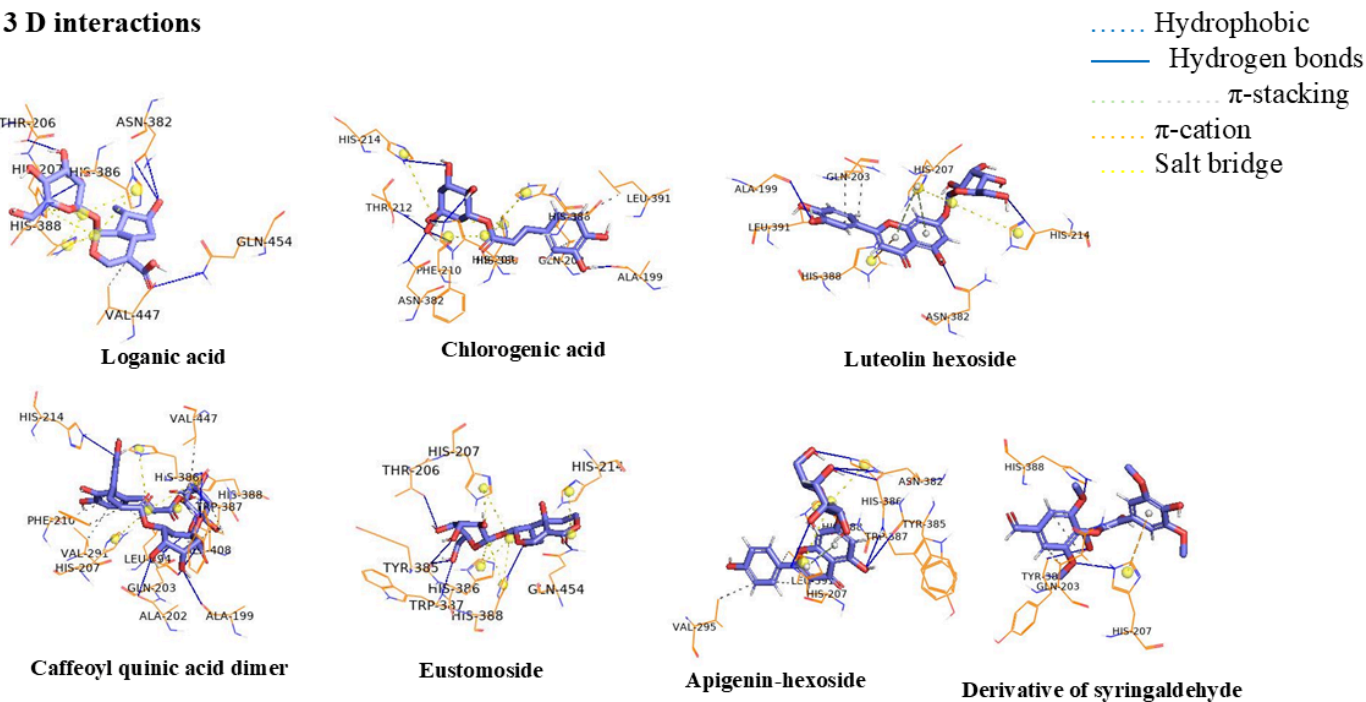


Figure 5

Three-dimensional interactions of compounds namely, loganic acid, chlorogenic acid, luteolin hexoside, caffeoyl quinic acid, eustomoside, apigenin-hexoside and derivative of syringaldehyde with cyclooxygenase-2 (5F19) active sites.

3 D interactions

- Hydrophobic
- Hydrogen bonds
- π -stacking
- π -cation
- Salt bridge

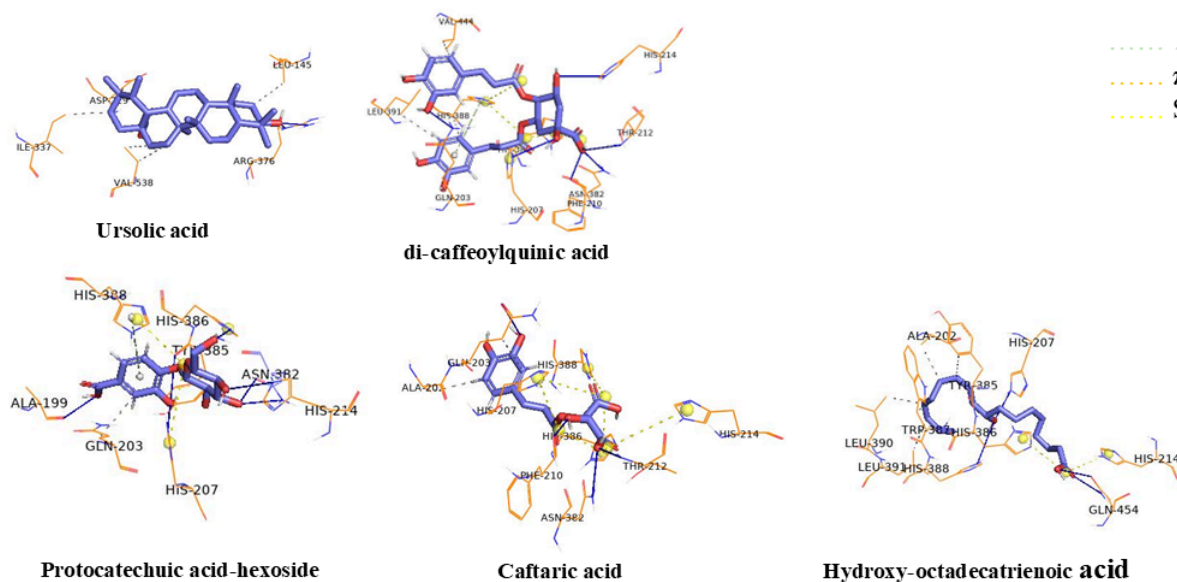


Figure 6

Three-dimensional interactions of compounds namely, ursolic acid, di-caffeoyl quinic acid, protocatechuic acid, caftaric acid, and hydroxy-octadecatrienoic acid with cyclooxygenase-2 (5F19) active sites.

3 D interactions

- Hydrophobic
- Hydrogen bonds
- π -stacking
- π -cation
- Salt bridge

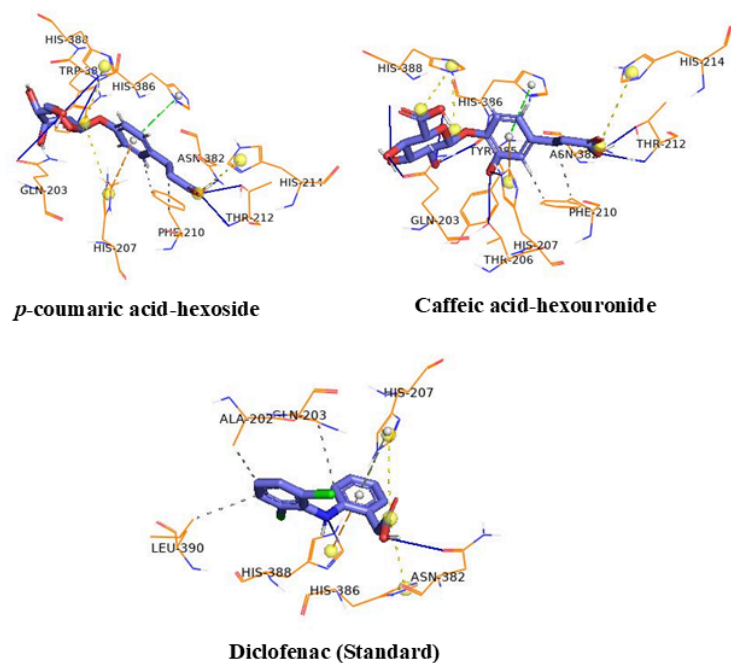


Figure 7

Three-dimensional interactions of compounds namely, *p*-coumaric acid-hexoside, caffeic acid-hexouronide and diclofenac with cyclooxygenase-2 (5F19) active sites.

Supplementary Files

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