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Comparative UPLC/MS^n study and multimodal assessment of the anti-inflammatory, analgesic, and antioxidant properties of methanol and aqueous extracts of *Scabiosa atropurpurea* L.

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Algerian *Scabiosa atropurpurea* traditionally known for its medicinal uses in treating scabies, respiratory coughs, and internal inflammation. In this study, UPLC/ESI/MS^n profile and total phenolic as well flavonoid contents were determined for aqueous (AqE) and methanol (ME) extracts separately prepared from aerial parts of *S. atropurpurea*. The antioxidant and analgesic activities were evaluated. In addition, acute oral toxicity together with different in vitro and in vivo models *viz*. anti-protein denaturation and xylene, croton oil-induced mouse ear and formalin-induced paw edematous assays were applied for anti-inflammatory assessment. Phytochemical analysis had revealed that both extracts were rich in polyphenolics and flavonoids content. UPLC/MS^n analysis resulted in the tentative identification of thirty-four secondary metabolites belonging to different chemical classes with major presence of flavonoids and tannins. The in silico pharmacodynamic profile was performed on some selected compounds. It was observed that *di*-caffeoylquinic acid elicited the most promising inhibitory activity against cyclooxygenase-2 (5F19) with a docking score equal to -10.1 Kcal/mol. AqE showed a high antioxidant potential (IC_{50} of 6.1 ± 0.34 mg/mL and IC_{50} of 0.13 ± 0.01 mg/mL) for HO. radical and H_2O_2 assays, respectively. It also showed the greatest protein denaturation inhibition (61.97% at 1 mg/ml) compared to ME (33.6% at the same dose). Both extracts showed no signs of toxicity with LD_{50} of exceeds 5 g/kg of body weight for each extract. Both extracts revealed a significant and comparable in vivo anti-inflammatory activity, while ME showed more potent analgesic activity compared to AqE in the acetic acid-induced writhing test. These findings warrant the continuation development of research for drug discovery from *S. atropurpurea* as antioxidant, anti-inflammatory and analgesic effects.

Keywords UPLC/ESI/MS^n, Scabiosa atropurpurea, Molecular docking, Neuropathic pain inhibition, Cyclooxygenase-2 inhibition, Scavenging activity

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 performed 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].

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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* 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].

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[12]. 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[13]. Non-targeted UPLC-MS metabolomics of *Scabiosa tschiliensis* revealed a mixture of flavonoids, triterpenes and coumarin-type metabolites[14]. Targeted LC-MS/HPLC-ESI-MS^n guided work on *Scabiosa sicula* and *S. arenaria* has been used to isolate 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[15].

The simultaneous application of both in silico modeling and in vivo assays is of great importance for precise and reproducible results. Combining both approaches leads to better prediction and confirmation of the molecular mechanisms behind the proposed activity. This combined approach correlates predicted ligand-target binding affinity with observed biological effects, providing stronger evidence for a compound's therapeutic potential or toxicity[16,17].

As an alternative to synthetic inflammation treatment, folklore medicine has used several plants and their extracts as potential anti-inflammatory remedies, with the phenolic compounds being one of their principal components. Phenolic compounds are able to inhibit either the production or the action of pro-inflammatory mediators, resulting in anti-inflammatory capacity[18,19].

The objectives of this study were to search for new sources of antioxidants, anti-inflammatory and analgesic activities using different solvents of extraction to get the best in terms of highly effective phytocontents, low cost and eco-friendly. Depending on the results of phenolic and flavonoids assays, and little documents traced on the bioactivity of the genus *Scabiosa*. The antioxidant, anti-inflammatory and analgesic activities of *S. atropurpurea* aerial parts were carried out on the two different extracts for the first time and most of these results are comparable.

Results

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 (*µ*g GAE/mg DE) and 100.57 ± 0.93 µg quercetin equivalent/mg dry extract (*µ*g 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 (*µ*g CE/ mg DE).

UPLC/MS^n analysis from the aerial parts extracts from *Scabiosa atropurpurea*

Both ME and AqE extracts were analyzed using UPLC/MS^n 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 (Table 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.

Certain metabolites were present in common in the ME and AqE but in different compositions and were listed in Table 1. The ME showed the unique presence of twenty-two components (Table S2). 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, Iridoids such as loganic acid showed a deprotonated peak at *m/z* 377(381) with fragments at *m/z* 353, 341, 265, 191[20]. This iridoid appeared as a major peak in the ME (13.29%) compared to the AqE (5.30%).

Cinnamic acid derivatives

Chlorogenic acid 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* 191[12]. It showed a % composition of 4.27% in the AqE and only traces in the ME. 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

No.	Compound name	Chemical Class	Molecular Formula	R_t (min.)	$[M-H]^-$ m/z	$[M+H]^+$ m/z	% Composition ME.	AqE.	MS/MS fragments	Ref.
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	[20]
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	[12]
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	[24]
5	Derivative of syringaldehyde	Miscellaneous	–	11.42	403	449	**7.92 (5.03)**	**5.03**	391, 361, 309, 283, 265, 235, 179	[25,60]
6	Caftaric acid	Phenolic acid	$C_{13}H_{12}O_9$	18.40	311	-	0.61	**4.69**	262, 257, 197, 191, 157	[23,61]
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	[62]

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

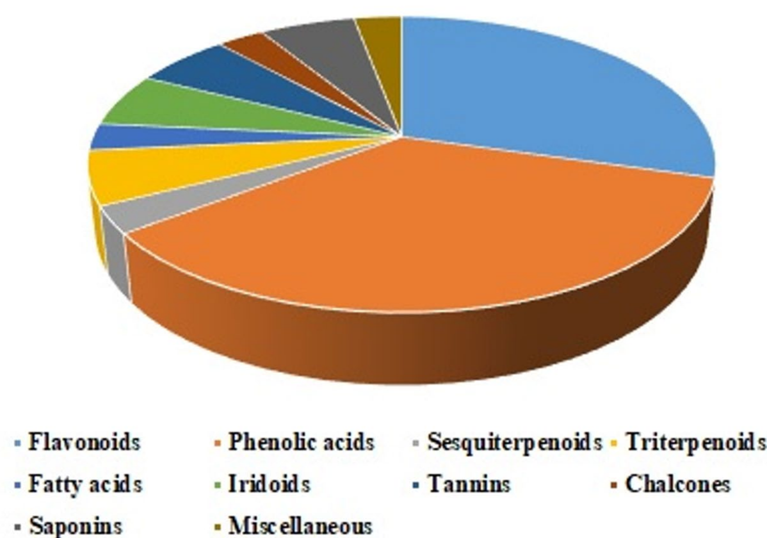

Fig. 1. Pie chart showing the tentatively identified metabolites from *Scabiosa atropurpurea* aerial parts extracts.

acid (*m/z* 293) was present in the two extracts with more proportion towards the ME. 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 compound[21]. Four cinnamic acid peaks were also detected from the AqE, at *m/z* 315, a protocatechuic acid-hexoside (previously identified from genus *Scabiosa*) was defined[20]. Two abundant peaks were traced at *m/z* 265 for feruloyl-caffeoyl-quinic acid derivative and its isomer[22,23]. Moreover, a deprotonated molecular ion peak appeared at *m/z* 325 for *p*-coumaric acid-hexoside (previously identified from genus *Scabiosa*)[24].

Flavonoids

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. A deprotonated molecular ion peak appeared at *m/z* 601 (4.48%) and was assigned to myricetin-(galloyl)-pentoside[25–27].

Miscellaneous

On the other hand, eustomoside had its deprotonated peak at *m/z* 389(401) with fragments at *m/z* 353, 233, 225, 179[12].

Terpenoids

Moreover, a ursolic acid derivative[25,28] 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 reports[29], it is also worthy noted that ursolic acid itself is reported for genus *Scabiosa*[30]. Moreover, one sesquiterpenoid peak appeared at *m/z* 295 $[M-H]^-$ and *m/z* 298 $[M + 2 H]^+$ which was assigned to *alpha*-artemisic acid[16].

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 syrinaldehyde were present in higher percentages in the AqE.

Biological study

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 of 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.

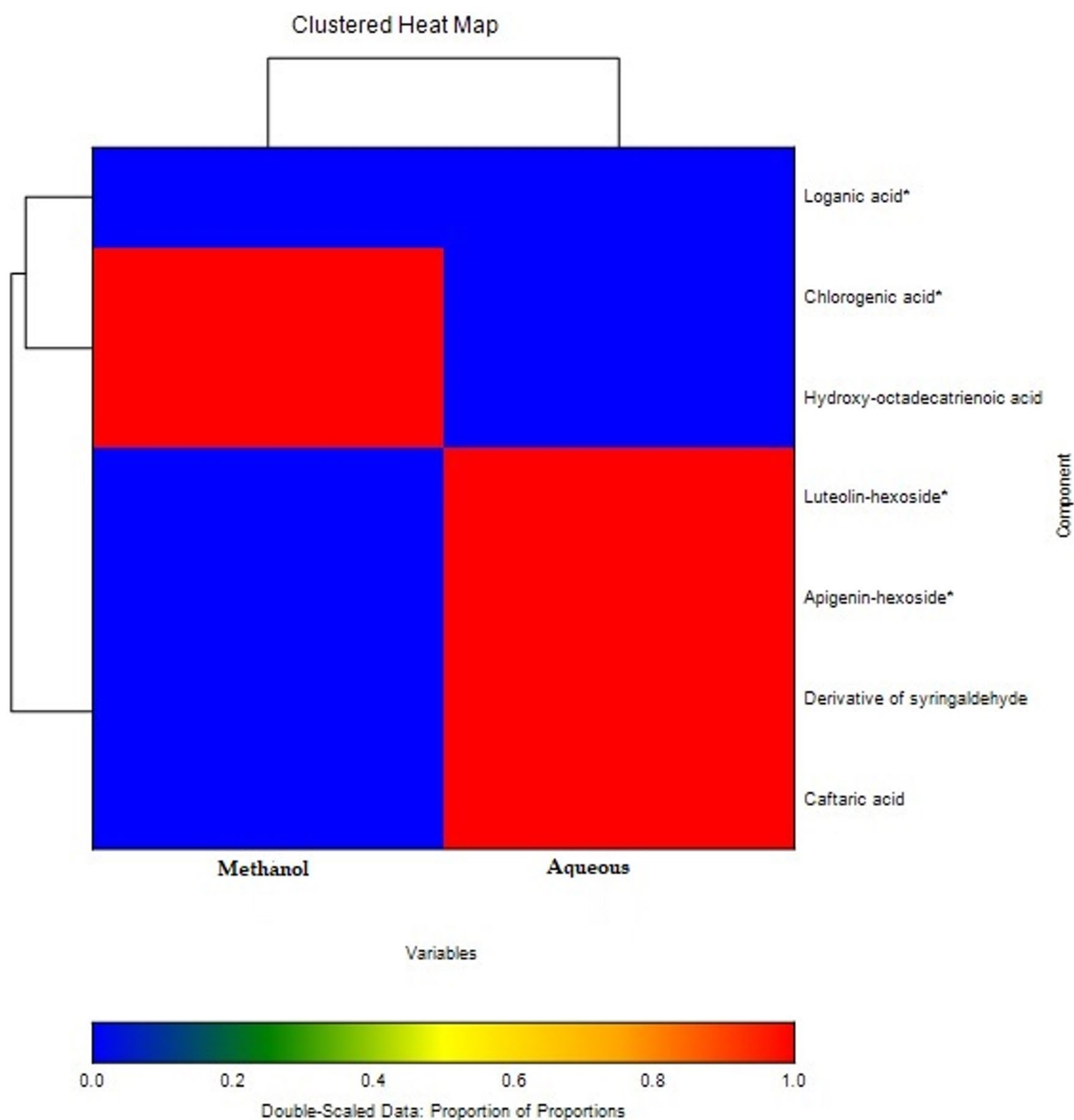

Fig. 2. Clustered heat map for the common metabolites identified from the methanol and aqueous extracts of *Scabiosa atropurpurea*.

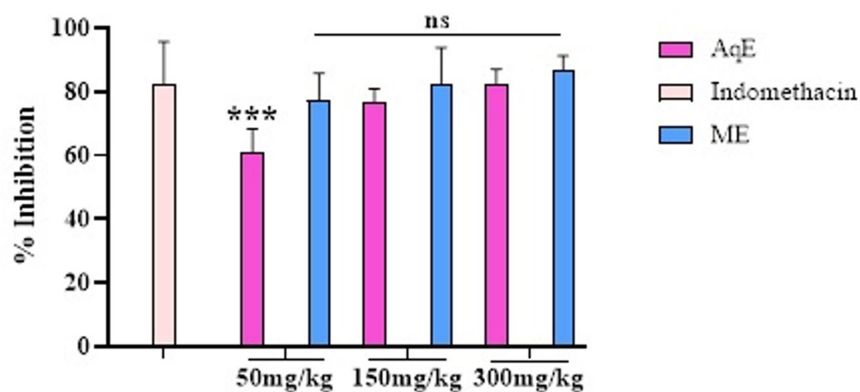

Fig. 3. Inhibition percent of xylene-induced ear edema of *S. atropurpurea*, at different concentration. Values are represented as mean ± SEM ($n = 6$). ns: no significant difference, ***$P < 0.001$. AqE: aqueous extract, ME: methanol extract.

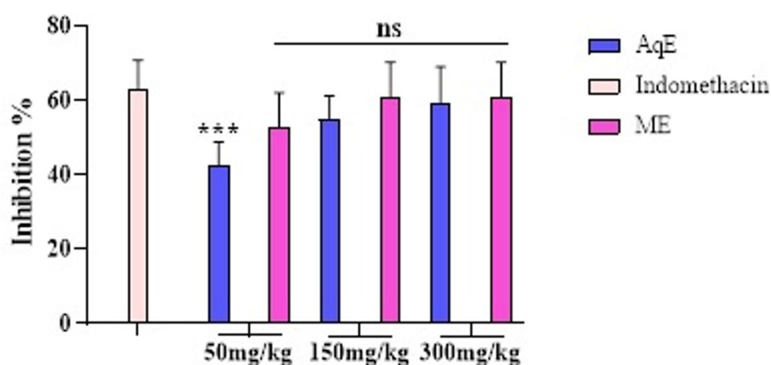

Fig. 4. Inhibition percent of croton oil-induced ear edema of *S. atropurpurea*, at different concentrations. Values are shown as mean ± SEM ($n = 6$). ns: no significant difference, ***$P < 0.001$. AqE: aqueous extract, ME: methanol extract.

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.

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.

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 LD_{50} of both extracts exceeds 5 g/kg of body weight.

In vivo evaluation of the anti-inflammatory activity

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 50 mg/kg was 82.51%.

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 edema reduction was significant compared 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.

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 h 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.

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).

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[31]. 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[32]. 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.

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

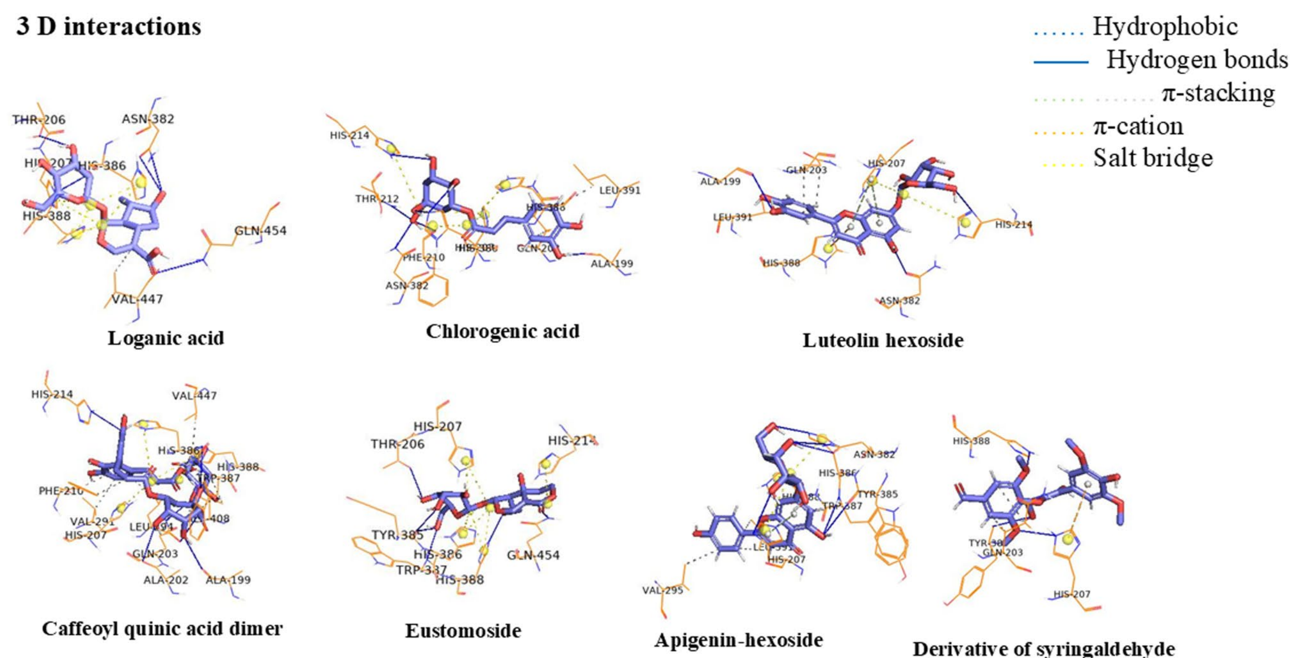

Fig. 5. Three-dimensional interactions of compounds namely, loganic acid, chlorogenic acid, luteolin hexoside, caffeoyl quinic acid, eustomoside, apigenin-hexoside and derivative of syringaldehyde with cyclooxygenase-2 (5F19) active sites.

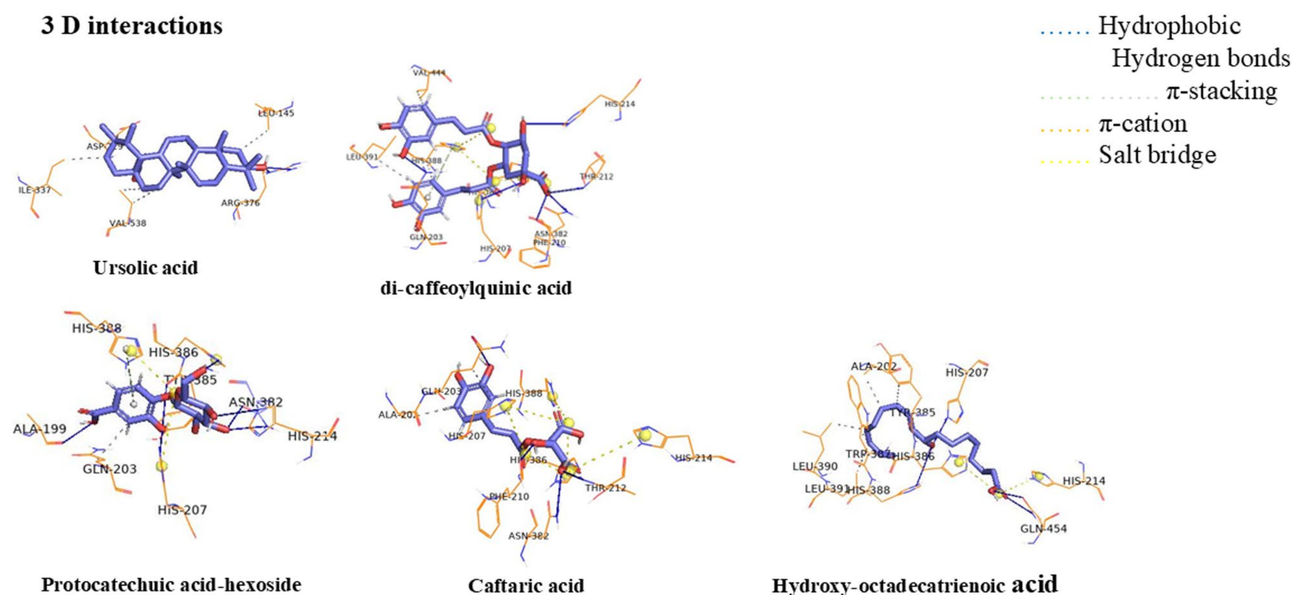

Fig. 6. Three-dimensional interactions of compounds namely, ursolic acid, *di*-caffeoyl quinic acid, protocatechuic acid, caftaric acid, and hydroxy-octadecatrienoic acid with cyclooxygenase-2 (5F19) active sites.

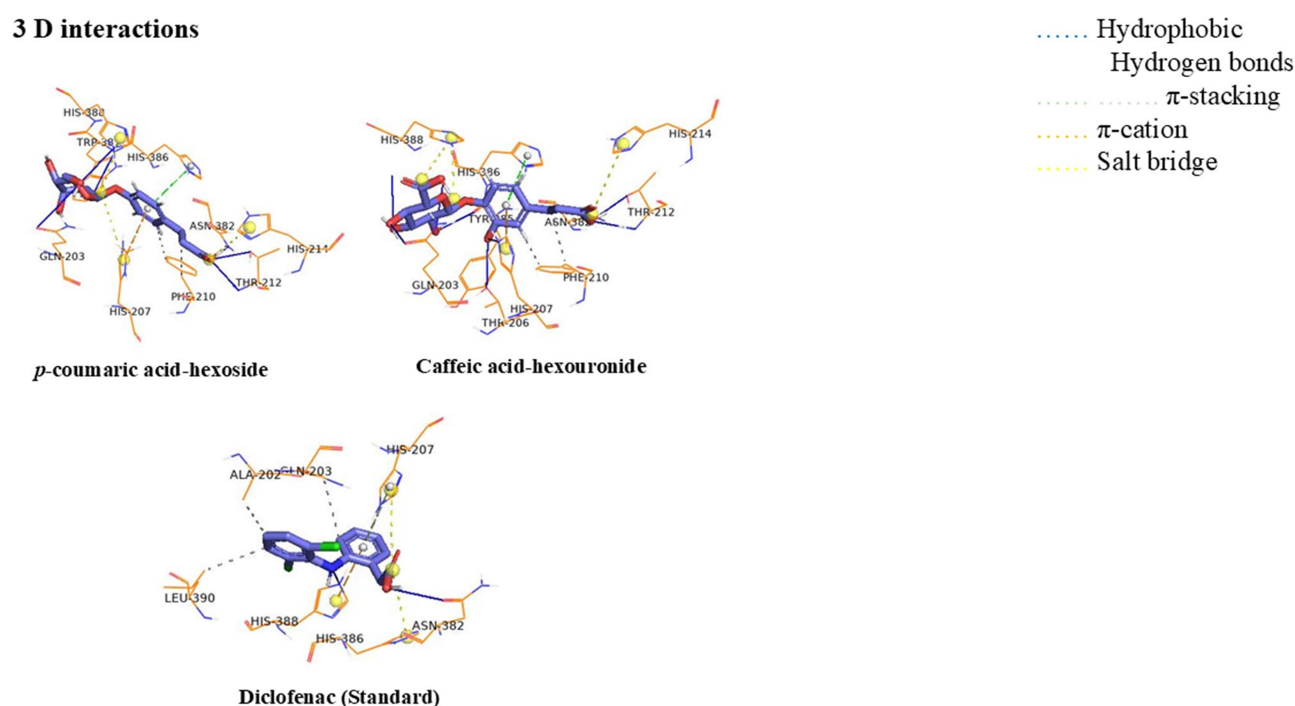

Fig. 7. Three-dimensional interactions of compounds namely, *p*-coumaric acid-hexoside, caffeic acid-hexouronide and diclofenac with cyclooxygenase-2 (5F19) active sites.

sectors[33,34]. *S. atropurpurea* is native to Algeria with many folk medical uses related to Scabies, skin conditions, inflammation, respiratory conditions like fever and cough.

The study results showed the strong linkage between the phytochemical profiles detected and its impact on the observed biological results. The in silico study proposed that the main identified components were of potential effect against COX-2 which was ascertained through the in vivo results which suggested a significant inhibition for the assayed inflammatory markers. Moreover, the antioxidant and analgesic potential of the two extracts were strongly linked to the abundant presence of phenolic acids and flavonoids as demonstrated by their quantitative contents in both extracts side by side to the tentative profiling through UPLC/MS^n.

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[35], *S. atropurpurea* hydro-ethanol extract of aerial part[9], *S. atropurpurea* leaves methanol extract[36] and *S. maritima* aqueous extract of the whole plant[37].

Moreover, the UPLC/MS^n 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.

The variations in phytochemical content can be ascribed to factors such as plant part, solvent employed, period of collection, extraction procedure and geographical influences[35]. Herein, 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. 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.

In this study, the antioxidant activity of *S. atropurpurea* was assessed using the hydroxyl radical scavenging assay. Additionally, the hydrogen peroxide scavenging effect of *S. atropurpurea* was also tested. 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. Therefore, polyphenols are extensively known as potent antioxidants[45,54].

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 synergistic effect of the whole extract components[55].

In our current study, the analgesic effectiveness was determined by acetic acid-induced writhing[38–40]. The analgesic effect of plant extracts such as AqE and ME in our study may be clearly linked to their richness with polyphenolic compounds such as phenolic acids, flavonoids and cinnamic acid derivatives which help in downregulating the pain signaling process and alleviating pain sensation eventually.

Protein denaturation is widely used as an in vitro method for evaluating the anti-inflammatory effects of plant extracts[56]. This process plays a crucial role in the development of inflammation.

and arthritic conditions[57]. 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[58]. Furthermore, the plants' extracts have been discovered for their ability to inhibit denaturation of proteins to a certain level[59].

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[37].

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 regarded as safe. These outcomes agree with those presented by[61] on *S. artropurperea 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. Topical application of croton oil leads to cutaneous inflammation by initiating an enzymatic cascade involving phospholipase A_2 (PLA2). 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[41]. In our study, AqE and ME extracts 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. 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 responses[42]. Furthermore, flavonoids and tannins scavenge free radicals, thereby inhibiting pro-inflammatory enzymes.

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-1β, IL-6), bradykinin, histamine, serotonin, prostaglandins and nitric oxide (NO) in the following stage (2–3 h)[56].

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-κB and MAPK pathways[43]. 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-kB (NF-κB) or Nrf-2, in inflammatory and antioxidant pathways[18]. Thus, the results of the in silico study substantiate the anti-inflammatory role of the phenolic compounds.

The aforementioned study results may be extrapolated in the future using fractions from the studied extracts or their isolated compounds. Our future work may also involve detailed mechanistic observations highlighting the key enzymes and receptors standing behind the observed analgesic, antioxidant and anti-inflammatory

findings. A formulation for the most active extract/fraction or if available pure components would be beneficial in promoting the bioavailability of the identified compounds thus enhancing their effectiveness.

Methods

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. This study was conducted and reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org) for reporting animal research". The experiments were authorized under the approval No. 88– 08/1988 by the 'Algerian Association of Sciences in Animal Experimentation' associated with veterinary medical activities and animal health protection (N° JORA: 004/1988). The number of animals used was the minimum number necessary to show consistent effects of the drug treatments. At the end of the experiments, the animals were anesthetized with 60 mg/kg ketamine plus 7.5 mg/kg xylazine, and euthanized by anesthetic depth. All animal handling and care throughout the experiment was as per the National Institute of Health Guidelines for the Care and Use of Laboratory Animals[44].

Plant collection

S. atropurpurea was obtained from the Chaabia district of Ouricia, Setif, according to national guidelines, 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.

Preparation of plant extracts

AqE was prepared by decocting 100 g of dried aerial parts in one liter of filtered water for 20 min at 100 °C. The decoction was filtered, evaporated under vacuum then lyophilized[45].

ME was prepared by defatting the plant material through maceration of 4 kg of the aerial parts in 15 L of petroleum ether. Subsequently, the defatted plant was macerated in absolute methanol then filtered, evaporated under vacuum then lyophilized[46]. The obtained dried extracts (AqE and ME) were weighed and thereafter kept in an opaque vials at 4 °C.

Estimation of total phenolic and total flavonoid contents

A polyphenol analysis was carried out using the Folin–Ciocalteu process[45].

For the measuring of the total flavonoid content; aluminum chloride reagent test was employed[45].

A modifying vanillin method was employed to assess the concentration of condensed tannins[47]. 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.

UPLC/MS^n analysis

The UPLC/ESI/MS analysis was performed for the *S. atropurpurea* extracts using XEVO TQD triple quadruple 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 actonitrile containing 0.1% formic acid; selected precursor ions were subjected to further analysis applying suitable fragmentation criteria to obtain MS^2 data[23,26,48–50].

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[49,51,52]

Evaluation of antioxidant activity

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was estimated according to the method described by[63]. 150 µL of the extracts/ BHT at various concentrations was mixed with 300 μL of a stock solution containing 9 mM $FeSO_4$ and 0.3% hydrogen peroxide (H_2O_2), then incubated at 32 °C for 15 min. Subsequently, 75 µL of salicylic acid (20 mM) was added, and the reaction mixture was incubated again at 32 °C for 15 min. Absorbance was measured at 562 nm. Results were expressed as IC_{50} values, indicating the concentration required to inhibit 50% of the hydroxyl radicals in the solution.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging capacity was assessed according to a modified method[64]. 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.

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[65]. 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.

In vivo evaluation of acute oral toxicity

The acute oral toxicity of AqE and ME was evaluated in mice according the OECD criteria[66]. 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 h. Subsequently, the monitoring of any harmful indicators was conducted daily for a duration of fourteen days.

In vivo anti-inflammatory activity

Xylene-induced oedema

The oral anti-edematous activity was examined following the protocol outlined in[53]. 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.

Croton oil-induced oedema

The anti-inflammatory capacity was tested adopting the croton oil-induced oedema technique as reported by[54]. 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.

Formalin-induced paw oedema

Inflammation is triggered by the administration of formalin at the plantar arch of the right paw of mice[49,55,56]. 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 min 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.

Analgesic activity

The analgesic activity of extracts was assessed by inducing abdominal contractions in mice with acetic acid[50,57,58]. 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 min after acetic acid injection and lasting for 30 min. The inhibition percent of writhing was measured utilizing the following equation:

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

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.

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 $p < 0.05$.

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[59]. 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[31]. 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.

Conclusions

This study concluded that *Scabiosa atropurpurea* L. aerial parts represent a rich source of secondary metabolites mainly polyphenols, flavonoids and condensed tannins. These metabolites are recognized for their biological characteristics as well as effective therapeutic components. To the best of our knowledge, this study is the first study comparing two extracts from genus *Scabiosa* on basis of their phytochemical content and biological effectiveness. 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. Molecular docking further emphasized the observed biological activity. The acute oral toxicity study revealed the relative safety of these plant extracts thus their identified phytocnstituents could be applied as a green source for semi- or de novo drug leads synthesis.

Data availability

All data are available upon request from the first author.

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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.

Declarations

Competing interests
The authors declare no competing interests.

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).

Additional information
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