AJAB

Asian J Agric & Biol. xxxx(x).
DOI: 10.35495/ajab.2023.054

Original Article

Antioxidant, anti-inflammatory, anti-arthritic activities and acute toxicity of *Calendula stellata* n-butanol extract from Algeria

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Received:
May 09, 2023

Accepted:
October 02, 2023

Published Online:
October 29, 2023

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Abstract

Calendula stellata (*Asteraceae* family), growing in North-East Algeria was investigated for its biological activities in laboratory animal model studies. The n-butanol extract was prepared from aerial parts using ethanol maceration followed by liquid-liquid extraction then, the total phenolic and flavonoid contents were measured, and the antioxidant activity was evaluated using DPPH, ABTS, CUPRAC, and reducing power assay. Then acute toxicity was tested in mice using the Up and Down test and, the anti-inflammatory anti-arthritic activity was evaluated using formalin induced arthritis (FIA) in Wistar rats. Results indicated that, the extract was rich in phenolic and flavonoid contents (224.097 ±7.31 mg GAE/g and 207.36±10.081mg QE/g, respectively). It possessed considerable antioxidant activity, the extract showed no visible toxicity or mortality signs, and the LD_{50} was > 2000mg/kg body weight. Furthermore, in the FIA, the extract showed significant dose-dependent inhibition in paw edema. It also it decreased the C-reactive protein (CRP) plasmatic, preventing cartilage destruction and liver injury. In conclusion, *C. stellata* n-butanol extract possesses antioxidant and anti-arthritic activities, in addition to protective properties in hepatic tissue.

Keywords: *Calendula stellata*, Antioxidant, Anti-inflammatory, Anti-arthritic, Algeria

How to cite this:

Foughalia A, Zerizer S, Aribi B, Kabouche Z and Bensouici C. Antioxidant, anti-inflammatory, anti-arthritic activities and acute toxicity of *Calendula stellata* n-butanol extract from Algeria. Asian J. Agric. Biol. xxxx(x): 2023053. DOI: https://doi.org/10.35495/ajab.2023.054

Introduction

The Calendula genus (*Asteraceae* family) is primarily distributed in the Mediterranean region. It is well known for the medicinal use, for example, *C. alata* Rech. *f* has been utilized for the treatment of renal disorders, *C. arvensis* Linn has been traditionally used in Italian folk medicine as a disinfectant, antispasmodic, diuretic, as well as for its anti-inflammatory, anticancer, and antipyretic properties

(Arora et al., 2013). Moreover, in North-Eastern Algeria, the aerial parts of *Calendula stellata* have been traditionally employed to treat digestive and hepatic diseases, sore throat, and premenstrual pain (Miara et al., 2021).

Rheumatoid arthritis (RA) is a chronic autoimmune inflammation of joints characterized by cartilage degradation, synovial hyperplasia and joint dysfunction. However, the progression of RA was assessed by the count of tender and inflamed joints, level of C-reactive protein, and pain score (Saleem et al., 2020). Several molecules, including interleukin, tumor necrosis factor (TNF) and prostaglandins (PG), and immune cells such as neutrophils, macrophages, plasmocytes, and adaptive immunity cells are involved in the pathology of RA (Alam et al., 2017; Cecchi et al., 2018). However, free radicals cause oxidative damage that may increase the progression of various disorders in the human body, besides inflammation, such as cancer, arthritis, and ischemic heart disease (Boutennoun et al., 2017; Al-Rifai , 2018). In the case of RA, rheumatoid factor binds IgM, IgG, and IgE when exposed to free radicals and ultimately stimulates the production of more free radicals and then attacks the cartilage matrix (Biswas et al., 2017). However, the antioxidants neutralize free radicals that reduce the biochemical marker in RA patients (Khojah et al., 2016). The contemporary anti-arthritic drugs comprise non-steroidal anti-inflammatory drugs (NSAIDs), immune-suppressants, disease modifying anti-rheumatic drugs (DMARDs) and cytotoxic drugs which may produce various side effects when chronically used (Burmester and Pope, 2017; Phull et al., 2017). These undesired effects stress the need for natural products such as plants and their extracts, which are considered safe, have fewer side effects, and are readily available, making them get option to treat inflammatory disorders such as RA (Gupta et al., 2021).

Recently, the extraction of medicinal plants, identifying and quantifying active ingredients, then assess their biological activities *in vivo* and /or *in vitro* was the most exciting objective of research in biology and organic chemistry (Lekouaghet et al., 2020). Although there is extensive ethnobotany use of *C. stellata*, there is no scientific data of anti-inflammatory and anti-arthritic effects *in vivo* in literature. Therefore, this work aims were to investigate the antioxidant and the *in vivo* anti-inflammatory and anti-arthritic properties of *C. stellata* n-butanol extract (CSB).

Material and Methods

Chemical reagents

All solvents and chemicals stated in the text were analytical grades obtained from Sigma Aldrich (Sigma, St Louis, MO, USA).

Plant material

Aerial parts of *C. stellata. (Asteraceae),* were collected from Guelma, North-East Algeria, during the flowering season from March to April 2018, The authentication of the plant material was conducted by Mr. Kamel Kabouche, and a type specimen (LOST.Cs.3.18) was stored in the Laboratory of Therapeutic Substances at the University frères Mentouri-Constantine1, Algeria.

Animals

Adult male *Mus musculus* mice (25 – 30g, eight weeks old) and Adult male Wistar rats (170 – 220g, eight weeks old), were obtained from the Central Pharmacy Institute of Constantine, Algeria. They were maintained under standard laboratory conditions of temperature and humidity (12h/12h, light/dark) and free access to feed and water.

Ethics statement

The animal exploration was performed following the procedure of the code number of the research project (F00920140076) obtained from the Ministry of Scientific Research, Algeria. The ethical principles and experiments were executed strictly with the OECD ethical principles and guidelines for monitoring and supervising animal experiments (OECD Test No. 420, 2002).

Preparation of the n-butanol extract

The extraction process was achieved according to the method of Lemoui et al. (2018). The aerial parts of *C. stellata* were air dried and powdered, and then 500 g of powder was macerated with EthOH-H_2O (8:2, v/v) for 48H three times. The filtrate was evaporated to dryness at 40 °C under vacuum. Then, 100 g of concentrated material was put in 1 L of distilled water and extracted successively with different solvents depending on the polarity (300 mL × 3 times) to obtain petroleum ether, chloroform, ethyl acetate and n-butanol extracts, respectively (6.45 g, 2.371g, 2.06g and, 20.54g respectively). The resulting n-butanol

extract (CSB extract) was then used in all experiments.

Total phenolic content (TPC)

The *CSB* extract's TPC was carried out using the Folin-Ciocalteu method (Müller et al., 2010) and Gallic acid monohydrate (25 - 200 µg/ml) was used as standard. The results were expressed as mg of Gallic acid equivalents per g of dry extract weight (mg GAE/g DW).

Total flavonoid content (TFC)

The *CSB* extract's TFC was evaluated according to the method described by Topçu et al. (2007), and Quercetin (25 - 200 µg/ml) was used as a standard. The results were expressed as mg of Quercetin equivalents per g of dry extract weight (mgQE/g DW).

Antioxidant activity

The methanol was used as a blank, and Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and Ascorbic acid were used as reference standards.

DPPH (1,1-Diphényl-2-Picryl-Hydrazyl) scavenging assay

The DPPH assay was done according to the method of Blois (Lakhal et al., 2011). The principle is measures the extract's ability to inhibit the stable 1,1-Diphényl-2-Picryl-Hydrazyl free radical. The absorbance was then measured at 515 nm. The percentage of inhibition (I%) free radical DPPH was calculated using the following formula (Khalfallah et al., 2017):

$$I\% = [(A_{control} - A_{extract}) / A_{control}] \times 100$$

Where, $A_{control}$ and $A_{extract}$ are the absorbance of control and sample extracts respectively.

DPPH scavenging activity is represented by IC50 value, which is the concentration of extract needed to scavenge 50% of free radicals in the solution.

ABTS scavenging assay

The ABTS test was performed according to the method of Re et al. (1999). The absorbance was measured at 734 nm. The percentage of inhibition was calculated using the following formula:

ABTS scavenging effect (%)=[($A_{control} - A_{extract}$) / $A_{control}$] x100

Where, $A_{control}$ and $A_{extract}$ are the absorbance of control and sample extracts respectively.

Cupric-reducing antioxidant capacity (CUPRAC) assay

The *CUPRAC* assay was carried out using the method of Apak et al. (2004). The absorbance was measured at 450 nm. The results were calculated as $A_{0.5}$ (µg/mL). Which indicates the concentration of extract corresponding to the absorption at 0.50 nm.

Reducing power assay (RP)

The RP assay was carried out using the method of Oyaizu (1986). The absorbance was measured at 700 nm. The results were calculated as $A_{0.5}$ (µg/mL).

Acute oral toxicity (Up and Down test)

The acute oral toxicity study was performed under the "Up and Down" method using the 2000 mg/kg body weight as the dose limit (Bruce, 1985). Eighteen adult male mice were divided into two groups; CSB extract group and control group. Before treatment began, the animals were fasted overnight, with free access to water. In the CSB extract group, one animal was treated with CSB extract orally at a dose of 2000mg/kg for the first time, and it was observed for any clinical signs or mortality during the first hour, then every hour for 3 hours then, periodically until 48 h. If the animal survived, the eight additional animals were given the same dose (2000 mg/kg). In parallel, the control group was treated with distilled water. The animals were observed periodically during the first 24, then once a day for 14 days and the number of mice that died over the experiment period was recorded.

Formalin induced arthritis

Arthritis was induced via the injection of 0.1 ml of formalin (5%) into the right hind paws of rats in each group (except normal control) on the first and third days, 30 min after the administration of vehicle or drugs (Saleem et al., 2020). To perform this test, thirty adult male rats were divided into six groups (N, F, S, EI, EII, EIII): Group (N) received vehicle 0.9% NaCl. Group (F) formalin injected and received vehicle 0.9% NaCl. Group (S) formalin injected and treated with Diclofenac sodium (anti-inflammatory standard drug) (10 mg/kg). Group (EI, EII, and EIII) formalin injected and treated with CSB extract at different concentrations (25 mg/kg, 50 mg/kg, and 200 mg/kg) respectively. All groups were treated by a daily gavage (p.o) for 10 days. The anti-inflammatory anti-arthritic effects were evaluated by following the edema

evolution in injected paw on the 1st, 2nd, 4th, 8th, and 10th day of study by using digital Vernier Caliper. The Mean differences in paw thickness after the injection (mm) compared to initial paw thickness were calculated on respective days using the following equation:

$$\Delta E = Ex - E0$$

Where, ΔE**:** the mean paw edema since day 0 to day x;

E0: the initial paw thickness (mm) on day "0" (before formalin injection);

Ex**:** the injected paw thickness (mm) on day "x" (after formalin injection).

These mean differences were used to calculate the inhibition percentage "%Inh" of arthritis with respect to the formalin group, which was calculated using the following equation:

$$\%\text{Inh} = 100[1 - \frac{\Delta Et}{\Delta Ec}]$$

Where, ΔEt: The mean paw edema since day 0 to day x of the treated rat;

ΔEc: The mean paw edema from day 0 to day x of formalin injected rat.

At the end of the experiment after blood drawing, each rat was sacrificed and the hind paw (right and left) were cut off and weighed to calculate the weight of paw edema.

Blood investigation

At the last day of the experiment, blood samples were collected from the vena cava in heparinized tubes. The hs-C-reactive protein (CRP) assay was conducted on the separated plasma using an immunoturbidimetric method. The analysis was conducted at the AL AMINE laboratory in Constantine, utilizing a Cobas Integra 400 plus analyzer (Roche).

Histological sections

All rats were dissected to cut off the liver and right hind paws; the organs underwent a rapid saline solution (0.9%) rinse followed by fixation in formalin 10%. Afterwards, the tissues were embedded in paraffin, sectioned at a thickness of 5µm, and stained using the hematoxylin-eosin staining technique (H&E).

Statistical analysis

The data were analyzed using one way ANOVA and Tukey's multiple comparison tests (SPSS version 20). The values of, ***P<0.001, **P< 0.01 and *P< 0.05 were considered to indicate the significance levels.

Results

The percentage yield of all extracts was calculated, *C. stellata* ethanolic extract percentage yield was 27.452% for aerial parts powder, and petroleum ether, chloroform, ethyl acetate, and n-butanol extracts percentages yield were 6.456%, 2.371%, 2.064 % and 20.544 % of ethanolic extract respectively.

Total phenolic and flavonoid compounds contents

The results found that this CSB extract contains a relatively high concentration of polyphenols (224.097 ±7.31 mg GAE/g DW) and a high concentration of flavonoids (207.36±10.081 mg QE/g DW) (Table1).

Table-1. Total phenolic (TPC), Total flavonoid contents (TFC), and Antioxidant activity of CSB extract.

Simple	CSB extract	BHA	BHT	Ascorbic acid
TPC (mg GAE/g)	224.097 ±7.31	-	-	-
TFC (mg QE/g)	207.36±10.081	-	-	-
DPPH-IC_{50} (µg/ml)	18.08±0.64	6.14±0.41	12.99±0.41	-
ABTS-IC_{50} (µg/ml)	65.86±1.72	1.81±0.10	1.29±0.30	-
CUPRAC-$A0_{50}$ (µg/ml)	37.97± 0.51	3.64±0.19	9.62±0.87	-
RP-$A0_{50}$ (µg/ml)	16.6± 1.65	-	-	6.77±1.15

Results are shown as mean ± SD (n=3).

Antioxidant activity

The CSB extract exhibited significant antioxidant activity, as demonstrated by the results from Table 1. It showed high scavenging potential against DPPH and ABTS radicals, with IC50 values of 18.08±0.64 µg/ml and 65.86±1.72 µg/ml, respectively. Additionally, the extract was evaluated for its ability to reduce metallic ions using the CUPRAC and RP assays and results revealed that the extract possessed effective reducing capabilities, with A0.50 values of 37.97±0.51 µg/ml in CUPRAC assay and 16.6±1.65 µg/ml in RP assay, indicating its potential as a powerful antioxidant.

Acute oral toxicity (Up and Down test)

On the Up and Down acute toxicity test, the first mouse survived, and no signs of toxicity or mortality were noted at a dose of 2000mg/kg of CSB extract

during the first 48h. For that, the same dose was given orally to the other mice. After observation for 14 days, no mice death occurred and all of them survived until the experiment's end. Moreover, no significant changes were observed in the weights of mice.

Formalin induced arthritis

The efficacy of the CSB extract in treating formalin-induced arthritis was assessed by measuring changes in paw edema, which is a crucial indicator of inflammation severity and serves as a measure of the effectiveness of anti-arthritis drugs. As shown in figure 1(A), throughout the experiment, the paw size of the untreated group (N) remained consistently stable. However, on the first day after 1 h of formalin injection, the extract demonstrated a dose-dependent protective effect against edema accumulation compared to the formalin group (F) and Diclofenac treated group (S). After 24h following the second formalin injection (day 4), there was a modest increase in paw edema size for all groups injected with formalin, except the group treated with the high extract dose (200mg/kg), Interestingly, this particular group exhibited a significant decrease in paw edema size as compared to formalin group (F) and Diclofenac treated group (S). On day 8, it shows a decrease in the paw edema size for all treated groups, and this decrease continued to the last day of experience (day 10). The decrease in paw edema size was highly significant and more important in groups treated with the extract (EI, EII, and EIII) at different doses (25, 50, and 200mg/kg) as compared to the formalin group (F) and Diclofenac treated group (S). The changes in paw edema weight at the end of the experiment confirmed these results. (Figure 1(B)), which shows a highly significant dose-dependent decrease in all treated groups as compared to group (F).

The inhibition percentage of arthritis "% Inh" is shown in Table 2. A significant increase of about 55%, 69%, and 77 % was attained at the last day of experiment in groups treated with CSB extract (EI, EII, and EIII) at different doses 25, 50, and 200 mg/kg, respectively. These percentages are highly significant and more important than what was attained in Diclofenac treated group (28%).

The C-reactive protein (CRP) concentration in rats' plasma at the end of the experiment, reported an increase in the formalin group (F) compared to the untreated group (N). Although, in the groups treated with CSB extract at different doses (25, 50, and 200 mg/kg) or Diclofenac (10 mg/kg) for 10 days, the CRP concentration was decreased but did not achieve the CRP value of the untreated group. An important decrease was reported in the highest dose treatment (200 mg/kg) (EIII) with a value of 0.04±0. 043 mg/L (Table 2).

The histopathological evaluation of the paw joint revealed normal joint histology in the untreated group (N). However, in formalin group (F) and Diclofenac treated group showed histological change; pannus formation (PF) in the hyaline cartilage, inflammatory cells infiltration (ICI) into synovium accompanied with cartilage destruction (CD), and synovial hyperplasia (SH). In contrast, treatment with CSB extract at different doses ameliorated the degree of cartilage destruction, increased chondrocyte proliferation (CP) and regeneration, and decreased the inflammatory cells infiltration. The CSB extract in a dose of 200mg/kg exhibited the mostly protective effect compared to other doses (Figure 2).

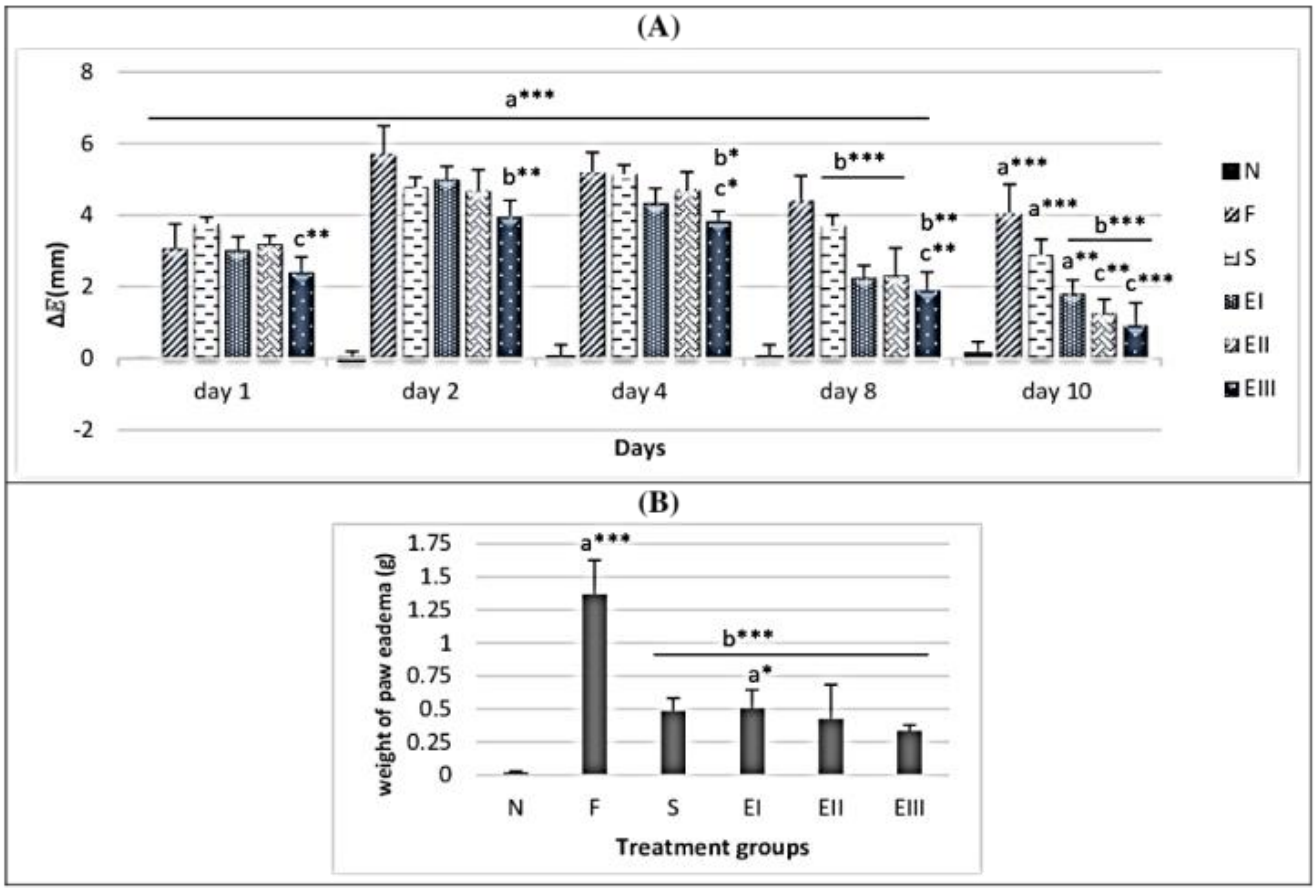

Figure-1. Effect of CSB extract on paw edema diameter changes ΔE (mm) (A) and on weight of paw edema (B) in FIA test.

Values are shown as mean ± SD (n=5). Statistical significant as compared to normal group (a), as compared to formalin group (b), and as compared to Diclofenac group (c); *P<0.05, **P<0.01, ***P<0.001. N: untreated group (0.5 ml /rat of 0.9% NaCl); F: formalin injected (0.5 ml /rat of 0.9% NaCl); S: Formalin injected and Diclofenac treated (10mg/kg); Group EI, EII and EIII: formalin injected, received CSB extract (25, 50, and 200 mg/kg, respectively).

Table-2. Effect of CSB extract on percentage inhibition of arthritis (% Inh) and plasma C-reactive protein (CRP) levels in FIA.

Groups	% Inh					CRP
	Day 1	Day 2	Day 4	Day 8	Day 10	
N	-	-	-	-	-	0.033±0.04
F	0	0	0	0	0	0.136±0.073
S	22.561±5,839**	16.389±4.97	0.969±4.68**	16.252±6.68*	28.914±10.76**	0.123±0.095
EI	2.275±12.6^{**c}	12.368±6.37	17.089±8.24^{**c}	48.915±7.59^{*c}	55.489±9.36^{**c}	0.11±0.088
EII	-2.990±8.37**	17.764±10.04	9.370±9.36**	47.965±17.64^{*c}	69.079±9.68^{**b}	0.14±0.112
EIII	22.431±14.55$^{**}_{b}$	30.728±7.91	26.382±5.25^{**b}	57.233±11.7^{*c}	77.400±15.7^{**b}	0.04±0.043

Results are shown as mean ± SD (n=5). Statistical significant between groups is shown as *P<0.05, **P<0.01, ***P<0.001 and statistical significant as compared to Diclofenac treated group is shown as P^{a}<0.05, P^{b}<0.01, P^{c}<0.001. N: untreated group (0.5 ml /rat of 0.9% NaCl); F: formalin injected (0.5 ml /rat of 0.9% NaCl); S: Formalin injected and Diclofenac treated (10mg/kg); Group EI, EII and EIII: formalin injected, received CSB extract (25, 50, and 200 mg/kg, respectively).

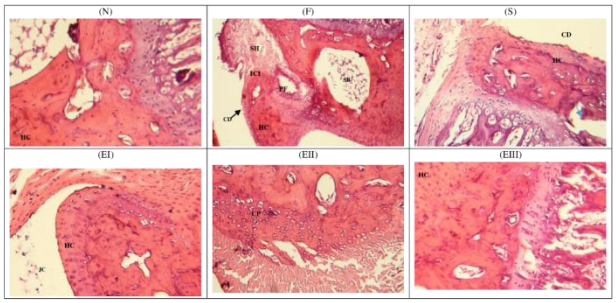

Figure-2. Histological section of rats knee joints. Tissues were stained with H&E staining (×100).
PF: pannus formation, CD: cartilage destruction, SH: synovial hyperplasia, CP: chondrocyte proliferation, ICI: inflammatory cells infiltration, IHC: intact hyaline cartilage, JC: joint cavity. N: untreated group (0.5 ml /rat of 0.9% NaCl); F: formalin injected (0.5 ml /rat of 0.9% NaCl); S: Formalin injected and Diclofenac treated (10mg/kg); Group EI, EII and EIII: formalin injected, received CSB extract (25, 50, and 200 mg/kg, respectively).

Histopathological evaluation of the livers (Figure 4) revealed that treatment with CSB extract at various doses (EI, EII, and EIII) did not cause any changes in the liver tissue when compared to the untreated group (N). In contrast, in formalin group (F) and Diclofenac treated group (S) the histopathological evaluation showed an immune cells infiltration (ICI) and hepatocytes with pyknotic nuclei (Figure 3).

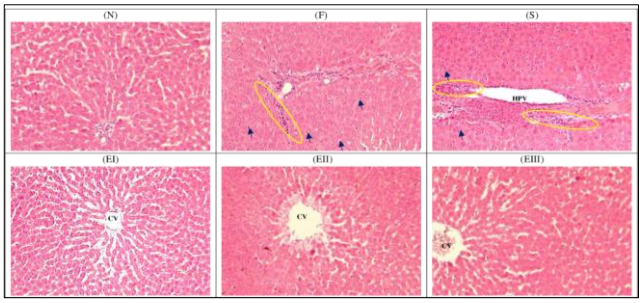

Figure-3. Histological section of liver. Tissues were stained with H&E staining (×100).
Yalow circles indicate immune cells infiltration (ICI), the blue arrow indicate hepatocyte with pyknotic nuclei, HPV: hepatic portal vein, CV: central vein. N: untreated group (0.5 ml /rat of 0.9% NaCl); F: formalin injected (0.5 ml /rat of 0.9% NaCl); S: Formalin injected and Diclofenac treated (10mg/kg); Group EI, EII and EIII: formalin injected, received CSB extract (25, 50, and 200 mg/kg, respectively).

Discussion

The CSB extract of aerial parts had a higher concentration of phenolic and flavonoids compounds (224.097±7.31mgGAE/g and 207.36±10.081mg QE/g respectively) compared to n-butanol extract of *C. tripterocarpa* Rupr aerial parts (52.62±0.04 mg GAE/g and 89.29±0.05 mg QE/g, respectively) (Al-Rifai, 2018) and, compared to aqueous extract (47.89±2.441 mg GAE/g and 74.93±1.5 mg QE/g, respectively) and, hexanol extract (50.26±0.18 mg GAE/g and 174±58.21 mg QE/g, respectively) of *C. arvensis* flowers (Abudunia et al., 2017).
On the other hand, the CSB extract was found to be a high scavenging agent against DPPH and ABTS. Moreover, it showed remarkable reducing properties. These results may be explained by the high content of polyphenols and flavonoids, which exhibit a strong linear correlation with antioxidant activity (Shi et al., 2021). Several studies demonstrated the antioxidant activities of genus *Calendula* extracts; *Calendula officinalis* (Chroho et al., 2021), *Calendula suffruticosa* (Ismahene et al., 2018), *Calendula arvensis* (Abudunia et al., 2017).
Acute oral toxicity demonstrates that the minimum lethal dose of CSB extract by oral administration in mice is more than 2000mg/k; similar toxicological concentration were achieved for dichloromethane extract of *Stachys circinata* by Slimani et al. (2020).
Formalin arthritis is a chronic animal model that is highly suitable for testing NSAIDs in terms of their anti-inflammatory and anti-arthritic properties (Thite et al., 2014). Formalin injection induced biphasic arthritis, initially producing pain, which has been attributed to histamine and serotonin; this was followed by prostaglandin release and mediated by bradykinin, and leukotrienes, which were produced by tissue macrophages. These factors are thought to be responsible for various effects, including the formation of inflammatory exudates, increased infiltration of neutrophils and lymphocyte CD4+, and the production of TNF-, IL-1, IL-6, and INF which are considerate as pro-inflammatory mediators. Additionally, this process was accompanied by fibrosis and tissue destruction (Gutiérrez-Rebolledo et al., 2018; Saleem et al., 2020). In the present study, we showed that n-butanol extract of CSB inhibited the joint's inflammatory response to formalin. This inhibition was exhibited by a significant decrease of paw edema size and weight as compared to the formalin group (F) and Diclofenac treated group(S).

These results, confirmed by "% Inh" of arthritis, suggest that components of the extract may be targeting the biphasic processes of formalin arthritis. The key mediators for inflammation are the eicosanoids (prostaglandin (PGE2, PGD2, PGI2) and thromboxane A2 (TXA2), which are produced by polyunsaturated arachidonic acid through the enzymatic activity of COX 1 and COX 2. PGD2 is a potent chemotactic for eosinophils and migration of lymphocytes. Moreover, the combination of PGD2 and PGI2 significantly increases edema formation and leukocyte infiltration by facilitating increased blood flow to the inflamed area (Kaithwas et al., 2012).

The CRP plasmatic concentration is a clinical parameter of RA disease activity. Furthermore, earlier researchers have documented a direct relationship between levels of CRP and disease activity (progressive joint damage and functional status of joints) in RA patients (Shen et al., 2015; Shrivastava et al., 2015). As stated earlier, CRP plasma levels were reduced also by CSB extract treatment compared to those in the formalin group (F). Thereby, its inhibition activity of CRP synthesis may be associated with the inhibition of the pro-inflammatory cytokines, most notably IL-6 which is reported to be the major stimulator of CRP gene expression in hepatocytes (Sproston and Ashworth, 2018). Our findings align with the study conducted by Kehili et al. (2016), demonstrating a reduction in edema size and CRP levels in mice injected with formalin and subsequently treated with Algerian *Phoenix dactylifera* fruit.

The anti-arthritic activity observed with CSB extract may be attributed to their phenolic richness and interesting antioxidant activities showed in this study. These results suggest that the extract's components target the biphasic processes of arthritis. Previously, Lehbili et al. (2017) reported that five previously undescribed molecules were identified in the ethanolic extract of *C. stellata*. (bidesmosidic triterpenoid saponins named calendustellatosoide A-E), and the majority of them have at their structures the oleanolic acid (calenduloside B, calenduloside D, silphioside B, arvensoside B, osteosaponin-I, acanthopanaxoside E, Chikusetsusaponin, zingibroside R1, udosaponin B, calenduloside G, calendulaglycoside A, calendulaglycoside C). Oleanolic acid can be found in two forms: as a free acid or as an aglycone precursor for triterpenoid saponins. In triterpenoid saponins, it can bind with one or more sugar chains (Pollier and Goossens, 2012). Research has demonstrated the anti-inflammatory effects of oleanolic acid through its ability to inhibit certain enzymes involved in pro-inflammatory mediation (COX2, iNOS), that may inhibit the production of NO and PGE2 (Jin et al., 2021). Moreover, Oleanolic acid demonstrated the ability to reduce pro-inflammatory responses induced by LPS. So that by suppression the expression of nuclear factor-κB (NF-κB) and TNF-α, as well as by upregulating the expression of anti-inflammatory cytokines (Ayeleso et al., 2017).

Histological study showed that formalin injection caused synovial hyperplasia combined by hyaline cartilage destruction thus confirms that formalin injection induced arthritis. Our findings are according to the study conducted by Farrukh et al. (2022), reporting that; *Sarcococca saligna* methanolic extract reduces the histopathological manifestation of arthritis in rats (cartilage destruction, infiltration of immune cells, synovial hyperplasia, pannus formation, infiltration of lymphocytes and erosion of bone) and has accompanied with a significant decrease of serum levels of HSP-70, IL-6, TNF-α, as well as a decrease in the mRNA expression of IL-1β, TNF-α, NF-κB and COX-2 compared to the disease group. The cartilage damage is the key histological manifestation of chronic inflammatory joints like osteoarthritis (OA) and RA. In RA, fibroblast like synoviocytes (FLS) are activated by the inflamed and hyperplastic synovial membrane, particularly by pro-inflammatory factors and immune cells accumulate at the inflamed site, attack the cartilage and contribute to its progressive destruction (Pap and Korb-Pap, 2015). In RA, FLS exhibits up-regulation and activation of NF-κB which enhances the production of pro-inflammatory cytokines and matrix metalloproteinases. Furthermore, it promotes their proliferation and inhibits apoptosis, ultimately leading to disease progression and sustaining chronic inflammation (Nejatbakhsh et al., 2020). This study suggests that the CSB extract prevents accumulation of immune cells at the synovium and/or inhibits the activation of FLS and cartilage destruction in contrast to the anti-inflammatory drug Diclofenac. In the liver histological section, hepatic injury in the group treated with Diclofenac was observed, that was the potent side effect of NSAIDs, because the metabolism of Diclofenac is carried out in hepatocytes, the mechanism of hepatic injury caused by Diclofenac are not completely known, however, an immune and inflammatory pathway was mediated (Schmeltzer et al., 2016). In contrast, CSB extract did not induce any injury or toxicity to hepatic tissue, which reinforces

the results of the acute toxicity test. Therefore, it could be suggested that this extract is safe and these preventive effects may be due to its phenolic compounds and its antioxidant properties.

Conclusion

The finding of this study indicated that the n-butanol extract of the medicinal plant *C. stellata* is rich in phenolic compounds and exhibits excellent antioxidant activity. Moreover, this extract demonstrates significant anti-inflammatory, anti-arthritic activities in rats by reducing the inflammatory manifestations (edema and CRP) and preventing the cartilage destruction. It was further concluded that it does not produce any adverse effects on the liver as well.

Disclaimer: None.
Conflict of Interest: None.
Source of Funding: None.

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Contribution of Authors

Foughalia A: Conceived idea, developed research methodology, collected and analysed data and wrote the manuscript

Zerizer S: Supervised the study, developed the main ideas for discussion and revised and edited the manuscript

Aribi B: Helped in the protocol development for the study

Kabouche Z & Bensouici C: Provided materials for the extraction of plants and *in vitro* study