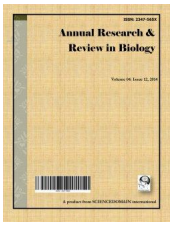

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Anti-inflammatory and Xanthine Oxidase Inhibition Activities of *Santolina chamaecyparissus* Extracts

Meriem Djarmouni[1*], Abderrahmane Baghiani[1], Moufida Adjadj[1] and Lekhmici Arrar[1]

[1]Laboratory of Applied Biochemistry, Faculty of Nature and Life Sciences. Ferhat Abbas University Setif-1, 19000 Setif, Algeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author MD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AB and MA managed the analyses of the study. Author MA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

S. chamaecyparissus aerial parts were extracted with solvent of varying polarity: crude extract (CrE) chloroform extract (CHE), ethyl acetate extract (EAE), and aqueous extract (AE). The content of total phenolics, and flavonoids in all the extracts were determined with spectrophotometric methods. Among all the extracts analyzed, the EAE exhibited a higher phenolic and flavonoids content than other samples: 373.83 ± 0.23 mg gallic acid equivalent and 7.86 ± 61.51 mg quercetin equivalent/g of dried weigh, respectively. CHE and EAE, showed a high inhibition of xanthine oxidase (XO) P>0.05 with IC_{50} (concentration inhibitory of 50% of XO activity): (0.051± 0.0002 mg/ml) and (0.052±0.0003 mg/ml) respectively, followed by CrE (0.091±0.001 mg/ml). The inhibition of xanthine oxidase by CHE and EAE showed a less efficient than allopurinol (IC_{50} = 0.0082 ± 0.0005 mg/ml). The CrE clearly demonstrated anti-inflammatory effects by reduced ear edema induced by PMA with 61.51%.

**Corresponding author: E-mail: magistairebiochimie@gmail.com;*

Our results indicate that EAE possesses potent antioxidant and CE noticed anti-inflammatory properties, and might be valuable natural source that could be applicable to both the medical and food industries.

Keywords: Santolina chamaecyparissus; xanthine oxidase; antiinflammatory; phenolics.

ABBREVIATION

PMA : Phorbol 12-myristate 13-acetate

1. INTRODUCTION

Phenolic compounds are widely distributed in plants and in recent years they have gained much attention, due to their antioxidant activity and free radical-scavenging ability with potential beneficial implications in human health [1].

Abnormal xanthine oxidase (XO) activity and high serum uric acid concentrations are common health problems that occur in many diseases, particularly in gout and metabolic syndromes [2,3].

The potential effects of various natural plant active ingredients on XO regulation and serum uric acid concentrations are being studied in gout-disease models [4,5,6].

Physiological concentrations of free radicals are required to mediate physiological processes such as inflammatory reaction. Indeed, during inflammation, cells of the immune system are recruited to the site of damage. This results in respiratory burst, an overproduction of reactive oxygen species that can propagate inflammation by stimulating release of cytokines and cause oxidative damage to bimolecular constituents in the body which eventually leads to chronic diseases, such as atherosclerosis, cancer, diabetes, aging and other degenerative diseases [7].

Santolina chamaecyparissus L. is a small medicinal herb, cultivated in Europe, Asia and Africa due to the antihelmintic, antiseptic, antispasmodic, bactericidal, fungicidal, digestive and vulnerary properties [8]. The essential oil of this plant is used in perfumery and cosmetics. Phytochemical studies of S. *chamaecyparissus* yielding a number of secondary metabolites such as essential oils [9], flavonoids [10] and coumarins [11].

The aim of our study was to evaluate the potential of *S. chamaecyparissus* aerial part extracts to act as anti-inflammatory and xanthine oxidase inhibition agents.

2. MATERIALS AND METHODS

2.1 Plant Materials

The aerial part of *Santolina chamaecyparissus* (SCE) was harvested from natural resources from Setif, Algeria, during the spring June 2012 in the stage of flowering. The taxonomic identity of the plant was confirmed by the botanist Pr. Laouar houssin (Setif University). The plant were dried under shade and later powdered.

2.2 Animal Materials

Male *Swiss albinos* mice (20 - 30 g) were purchased from Pasteur Institute of Algiers, Algeria and they were group-housed (6-8 mice per cage) with free access to food and water, and kept in a regulated environment at 25C° under 12 h light/12 h dark conditions. They had free access to the standard pellet diet and drinking water during the experiments. All animal experiment and treatment were conducted according to directives of the University ethic committee.

2.3 Extraction and Fractionation

The extractions were carried out using various polar and non polar solvents, according to the method of Markham [12]. One hundred grams of powdered aerial part were macerated 24 h with 85% aqueous-methanol. Then, the macerates were extracted twice using 50% with the same solvent for 4 h with continuous stirring, then the extracts were filtered and evaporated on rotary evaporator (BÜCHI) to give crude extract (CrE).

CrE was successively extracted with hexane, chloroform and ethyl acetate extraction. Each organic layer was evaporated to dryness under reduced pressure to yield Hexane (HxE), CHE, EAE and Aqueus (AqE) fractions, respectively. All of these fractions were stored at -20C° prior to use.

2.4 Determination of Total Phenolic Compounds and Flavonoid Content in Extract

Total phenolic content was determined by the Folin–Ciocalteau method [13]. 200 µl of SCE was mixed with 1 ml of of 1:10 diluted Folin–Ciocalteau reagent for 4 min and 800µl of 75 g/l Na_2CO_3 were then added. The absorbance was measured at 765 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents.

Total flavonoids content was estimated according to a literature procedure [14]. To 1 ml of each sample, 1 ml of 2% $AlCl_3$ methanol solution was added. After 10mn at room temperature, the absorbance was measured at 430 nm. Total flavonoid content was calculated as quercetin from a calibration curve.

2.5 Xanthine Oxidase Activity Assay

Xanthine oxidase activity was evaluated by the spectrophotometric measurement of the formation of uric acid by xanthine at 295 nm. An aliquot of a 100 µM solution of xanthine in 50 mM phosphate buffer (pH 7.4), containing 0.1 mM EDTA with (1227 ηmole/min/mg of XO). Different concentrations of tested fractions were added to samples before the enzyme had been added and their effect on the generation of uric acid was used to calculate regression lines and IC_{50} values [15]. Allopurinol was used as a positive standard.

2.6 Superoxide Anion Generation by Xanthine-/Xanthine Oxidase Assay

Anti-radical activity was determined spectrophotometrically according to Robak and Gryglewski [16], by monitoring the effect of SCE extracts on superoxide anion radicals produced by xanthine/xanthine oxidase system. The reaction mixture contained xanthine (100 µM), horse heart cytochrome c (25 µM), in air-saturated sodium phosphate buffer (50 mM, pH 7.4), supplemented with 0.1mM EDTA and various concentrations of SCE extracts. The reactions were started by addition of XO. Within 1 min, reduced cytochrome c was spectrometrically determined at 550 nm against enzyme-free mixture.

2.7 Anti-inflammatory Activity

The PMA-induced mice ear oedema test has been used as an experimental model for screening the anti-inflammatory activity. According to a modified method of Garrido et al. [17]. We used three groups of 6-8 mice each (control, Diclophenac and CrE groups), 4µg per ear of PMA, in 20 µl of DMSO, was applied to both surfaces of the right ear of each mouse. The left ear received the vehicle (DMSO, 20 µl). Crude extract was administered topically (100 mg/kg) 1 h before PMA application. The reference group was treated with diclofenac (10 mg/kg). Six hours after PMA application, mice were killed by cervical dislocation and a 6mm diameter disc from each ear was removed with a metal punch and weighed. Ear edema was calculated by subtracting the weight of the left ear (vehicle) from the right ear (treatment), and was expressed as edema weight.

2.8 Statistical Analysis

All experiments were done in triplicate and results were reported as mean ± SD. Data were analyzed by one way ANOVA. Statistically significant effects were further analyzed and means were compared using Tukey test.

3. RESULTS AND DISCUSSION

3.1 Determination of Total Polyphenols and Flavonoids Content

Phenolic antioxidants are products of secondary metabolism in plants and their antioxidant activity is mainly due to their redox properties and chemical structure, which might play an important role in chelating transition metals and scavenging free radicals. Consequently, the antioxidant activities of plant extracts are often explained by their total phenolic and flavonoid contents. Also, the sterical structures of antioxidants or free radicals are known to play a more important role in their abilities to scavenge different types of free radicals [18].

The yields of Crude, chloroform, and ethyl acetate extracts of SCE were 9.71%, 1.487% and 1.352%, respectively. The total phenolic and flavonoid contents of the SCE extracts as determined by Folin–Ciocalteu and $AlCl_3$ method (Table 1). Among the three extracts, EAE was containing highest (373.83 ± 0.23 mg gallic acid/g, and 61.51±7.86 mg quercetin/g) amount of phenolic and flavonoid compounds respectively followed by CrE and CHE.

When making a comparison between the yields of extracts, it was found that the polar solvents gave better extraction yields, which is true in the

study of Chigayo et al. [19] Since biologically active compounds occur naturally in very small concentrations, the choice of an extraction method and the corresponding suitable solvent is an important step in the drug discovery process.

Addition of water to methanol proved that the extraction efficiency can be increased significantly as the extraction yields obtained with 80% methanol (52.9% yield) and 60% methanol (46.9% yield) were much higher than the yield obtained from the use of pure methanol (36.6% yield) [19].

3.2 Xanthine Oxidase Inhibitory and $O_2^{\bullet-}$ Scavenging Activity

The enzyme XOD plays a crucial role in the production of uric acid, catalyzing the oxidation of hypoxanthine and xanthine. During the reoxidation of XOD, molecular oxygen acts as an electron acceptor, producing superoxide radical and hydrogen peroxide. Both, inhibition of XOD and the scavenging effect on the superoxide anion, were measured.

From our results, CHE and EAE, showed a high inhibition of XO P>0.05 with concentration inhibitory of 50% of XO activity (IC_{50}) of (0.051± 0.0002 mg/ml) and (0.052±0.0003 mg/ml) respectively, followed by CrE (0.091±0.001 mg/ml). The inhibition of XO by CHE and EAE showed a less efficient than allopurinol (IC_{50} = 0.0082 ± 0.0005 mg/ml), as a specific inhibitor for XOR, by 6.2-folds and 6.3-folds, respectively (Fig. 1).

The effect of SCE were studied for their ability to scavenge $O_2^{\bullet-}$ generated by the xanthine/xanthine oxidase system. The most potent scavengers of superoxide anion radical were observed for EAE with concentration inhibitory of 50% of $O_2^{\bullet-}$ scavenging activity (IC_{50}) IC_{50} of (0.057± 6.65 10^{5-} mg/ml) followed by CHE with IC_{50} of (0.074±0.001 mg/ml), which was approximately 1.3-folds higher than that of EAE, however CrE showed the lowest effect in compared with previous extracts ($p \leq 0.001$) (Fig. 2).

Table 1. Yields, total polyphenols and flavonoids content of *S. chamaecyparissus* extracts

Extracts	Yeild (%)	Total phenols content µg GAE/mg of extract	Flavonoids content µg QE/mg extract
CrE	9.71 ± 0.20	196.08±0.35	16.94±1.96
HXE	9.71 ± 0.20		
CHE	1.487±0.22	143.08±0.47	8.71±0.98
EAE	1.352±0.34	373.83±0.23	61.51±7.86

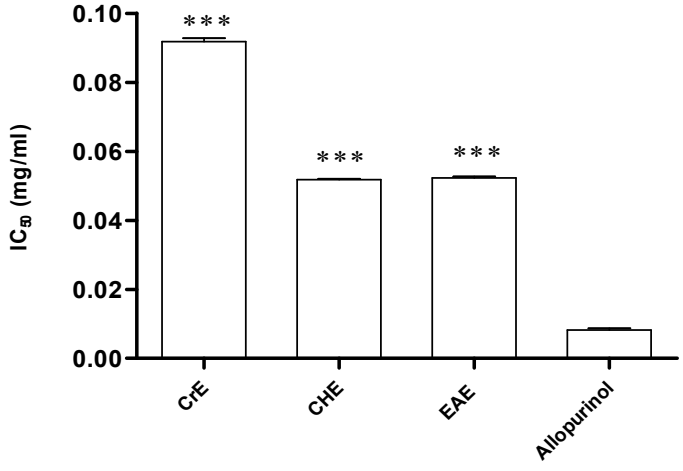

Fig. 1. IC_{50}, Inhibitory concentration of SCE for 50% of XO activity. Comparison was realized against allopurinol

****: $p \leq 0.001$*

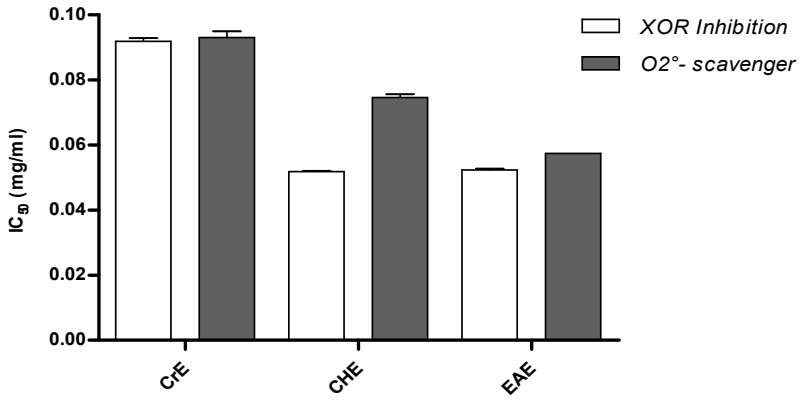

Fig. 2. Evaluation of *SCE* as inhibitors of xanthine oxidase and as scavengers of superoxide produced by the action of XO enzyme

Each value is represented as mean ± SD (n = 3)

SCE have an inhibitory effect on XO activity, indicating that their scavenging effects on superoxide anion is due to dual effect of the extracts on XO activity and superoxide anion scavenging. This activity could be related to the presence of flavonoids in SCE and their structural differences. The insaturation in the C ring and the free hydroxyl group at C-7 enhanced the activity [20]. XO converts xanthine to uric by transferring an oxygen atom to xanthine from the enzyme's molybdenum center. Flavonoids work by competitively binding at the binding site of xanthine [21].

To our knowledge, there have been no studies or reports to date regarding inhibition of XO activity by SCE.

3.3 Anti-inflammatory Activity

Ear oedema was induced by the application of PMA to the mouse ears. As shown in Fig. 3, the application of PMA for 6 h significantly increased the weight of ear edema. Diclofenac, a common clinical non-steroidal anti-inflammatory drug, was used as positive control for the inhibitory effect on the ear edema. The pre-treatment of 10 mg/kg of Diclofenac could effectively reduce ear edema after 6 h PMA stimulation ($p < 0.01$). Similarly, pre-administration of CrE (100 mg/kg) markedly inhibited the PMA-induced ear edema with 61.51% inhibition ($p < 0.001$).

Topical application of PMA offers a skin inflammation model appropriate for evaluating

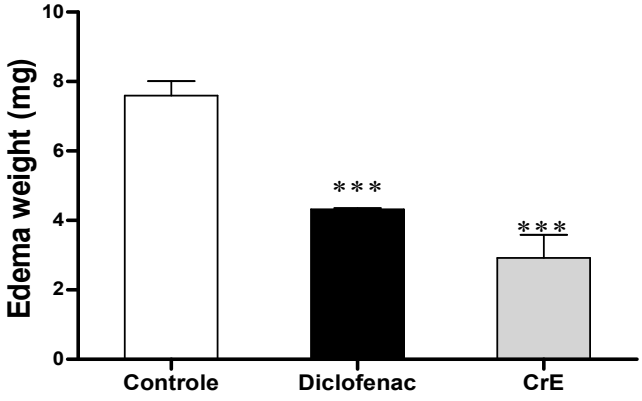

Fig. 3. Inhibition of PMA induced ear edema by CrE and Diclofenac applied topically before PMA application

Each point represents the mean ± SEM (n = 7-8). P < 0.01, P < 0.001 vs. control group

anti-inflammatory agents. PMA induces inflammation by activating PLA2, which subsequently activates the release and metabolism of arachidonic acid. The COX-2 inhibitors are very effective in suppressing PMA induced ear oedema, indicating the role of prostaglandins. Topical application of CrE significantly inhibited PMA induced ear inflammation suggesting that the extract can act as a topical anti-inflammatory agent and that prostaglandins are implicated in its antiinflammatory activity. It is appealing in perspective of antiinflammatory agents to explore the probable mechanism underlying their potentially beneficial activity to ascertain their mode of action [22].

Also, polyphenols and flavonoids such as caffeic acid, quercetin, luteolin, have been recognized as potent inhibitors of cyclo-oxygenase in other studies. These reports, together the results of Khouya et al. [7] suggest a possible relationship between the protective effects of aqueous extracts in an acute inflammatory animal model and the rich content of polyphenol in these extracts.

4. CONCLUSION

The antioxidant and anti-inflammatory properties of these plants could justify its importance in the fight against oxidative stress diseases and its corollaries. The presented study could explain the effectiveness of plants in traditional medicine in Algeria.

In biological activities, the plant extracts presented some potential antioxidant and anti-inflammatory effects. The near future is to isolate the bioactive compounds form these plants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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