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XANTHINE OXIDASE INHIBITORY ACTIVITY OF EXTRACTS PREPARED FROM *JUNIPERUS PHOENICEA*

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ABSTRACT: The inhibitory effect of *Juniperus phoenicea* leaves extracts on Xanthine Oxidase activity was evaluated spectro-photometrically at 290 nm. Extracts of *Juniperus phoenicea* showed a potent XO inhibitory activity. In order to isolate the active compounds, an *in vitro* bio-guided fractionation was undertaken by preparative chromatographic techniques. Fractions 2, 3 and 6 of ethyl acetate extract demonstrated substancial XO inhibitory activity (>80% inhibition) at 400 µg/mL. The highest inhibitory effect was obtained for the fraction 3 (87.8%). The major constituents amentoflavone (1) and catechin (2) isolated from the fraction 3 were identified by extensive spectroscopic studies including 1D and 2D NMR experiments. These two compounds have no inhibitory effect on xanthine oxidase, it is deduced that the XOI activity of the fraction 3, was due either to a minor bioactive compound or possible synergisms. However this study showed that the tested plant species is potential source of natural XO inhibitors that can be developed, upon further investigation, for treatment of gout and other XO-related disorders.

INTRODUCTION: A common disease such as inflammation and related disorders are selected for our study and the primary target for treating these disorders includes the enzyme called xanthine oxidase (XO). The over-activity of this enzyme causes a disease called gout [1] and oxidative stress, which is characteristic for many vascular disease states [2] by generating superoxide anions. Gout is one of the most common metabolic disorders that affect humans.

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It is characterized by marked hyperuricemia, leading to the deposition of urrate monohydrate crystals in the joint and kidney, leading to gouty arthritis and uric acid nephrolithiasis.

The deposition of needle-shaped monosodium urate (UMS) crystals in the synovial fluid of the main joints produces acute, extremely painful arthritis with repeated attacks of gout [3]. The treatment of gout involves increasing the excretion of uric acid or reducing its production. Inhibitors of xanthine oxidase (XOI) are very useful [4] because they reduces both vascular oxidative stress and circulating levels of uric acid and they have minor side effects compared to uricosuric and anti-inflammatory agents. Allopurinol, widely used for the treatment of hyperuricemia and gout [5] have

many side effects such as hypersensitivity syndrome, Stevens-Johnson syndrome and renal toxicity [6]. So, finding new XOI would be beneficial not only for treating gout but also for combating various other diseases [7, 8].

Certain active constituents present in crude plant extracts like flavonoids and polyphenolic compounds have been reported to possess XOI [9, 10, 11]. These findings have opened the possibility of isolation of new natural compounds, which can be potent inhibitors of XO, and led to the growing interest in the investigation of medicinal plants.

In this study, the medicinal plant *Juniperus phoenicea* was selected for its XOI activity by its ethnomedical use in the treatment of inflammatory and rheumatic diseases.

Juniperus phoenicea called "Araar" in Arabic, is a tree or shrub up to 8 meters tall. This species extends from the Mediterranean region, occurring in southern Europe, western Asia and North Africa, also in eastern Portugal until in Turkey and Egypt, it extends from the Canary Islands to Saudi Arabia and Jordan, but is more common in the western part of the Mediterranean regions [12, 13].

Previous phytochemical studies of *Juniperus phoenicea* revealed that diterepenes are the main secondary metabolites of the plant [14, 15, 16]. Numerous biflavanoids including amentoflavone, robustaflavone, hinokiflavone, cupressuflavone and mono-O-methyl hinokiflavone were isolated from the leaf extract of *Juniperus phoenicea* [17]. Flavonoids [12], phenylpropanoids [18, 19, 20], and furanones [21, 22] were also isolated from the plant. In the present studies in order to find new natural compounds, which can be potent inhibitors of XO we preceded to an Activity-guided phytochemical investigations of *Juniperus phoenicea*.

MATERIALS AND METHODS:

Chemicals: Analytical grade n-butanol (n-BuOH), ethyl acetate, chloroform and methanol (MeOH) employed for extraction isolation procedures were obtained from Merck, Methanol deuterated (CD3OD) and xanthine powder were purchased from Sigma-Aldrich Co.

General Experimental Procedures: Column Chromatography was performed over Silica gel (63-200) µm, (Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) analysis was performed with precoated silica gel 60 F254 plates (Merck, Darmstadt, Germany), and the spray reagent cerium sulfate (saturated solution in dilute H_2SO_4) and UV (Shimadzu UV-1601, 254 and 366 nm) were used for the spot visualization. Preparative TLC was performed with precoated silica gel 60 F254 aluminium sheets (20 × 20 cm, Merck, Darmstadt, Germany). The absorbance of XO-induced uric acid production from xanthine was measured at 290 nm, using the plate reader FluoSTAR OPTIMA (BMG LABTECH). A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for 1H and 150.858 MHz for 13C, using the WINXNMR software package, was used for NMR experiments in CD3OD.

Plant Material: Aerial parts of *Juniperus phoenicea* were collected in from Tipaza area, located in north Algeria. A voucher specimen has been deposited in the Department of Chemistry, Algiers University.

Extraction and Isolation: Air-dried and coarsely powdered (1000g) leaves were macerated at room temperature with MeOH–H_2O (80:20, v/v) for 24 h, and the operation was repeated 3 times. After filtration, the filtrate was concentrated (methanol extract) and dissolved in H_2O (400 ml). The resulting solution was extracted successively with chloroform, EtOAc and n-butanol. The organic phases were concentrated in vacuo at room temperature to obtain the following extracts: Chloroform (1.73g), EtOAc (4.42 g) and n-butanol (22.8 g).

4 g of the EtOAc extract was fractionated over silica gel column using chloroform gradually enriched with ethyl acetate as eluent. Fractions were collected, and combined to 7 major fractions (F1-F7) based on TLC pattern. Fraction 3 was submitted to preparative TLC using as solvent a mixture of MeOH / H_2O (8.5:1.5), to give two major constituents: amentoflavone (1) (5 mg) and catechin (2) (20 mg).

Xanthine Oxidase Assay: The method is based on a modified protocol of Sigma [23]. Spectro-photometric determination of XO activity is based on measuring uric acid production (absorbance)

from xanthine or hypoxanthine substrate at 290 nm for 3 min in a 96-well plate, using the plate reader FluoSTAR OPTIMA. The XO inhibitory effect was determined *via* the decreased production of uric acid. Reagents were 50 mM potassium phosphate buffer, pH 7.5 with 1M KOH, 0.15 mM xanthine solution (pH 7.5), and XO solution (0.2 units/mL). XO, isolated from bovine milk (lyophilized powder). The different extracts (12 mg/mL) were prepared in dimethyl sulfoxide (DMSO). For enzyme activity control, the final reaction mixture was made with 100 µL of xanthine, 150 µL ofbuffer and 50 µL of XO in a 300 µL well. The reaction mixture for inhibition comprised 100 µL of xanthine, 140 µL of buffer, 10 µL of sample and 50 µL of XO. Allopurinol served as positive control. All the experiments were conducted in triplicate. The reaction was initiated by the automatic addition of 0.050 mL of XO solution to a final concentration of 0.006 units/mL.

RESULTS AND DISCUSSION: The XOI activity of extracts was calculated as % xanthine oxidase inhibition activity = 1- (XOIs /XOIo) x 100 %, where XOIs is enzyme activity with test sample and XOIo is enzyme activity without sample. The XOI activity of extracts and fractions were summarized in **Table 1**.

TABLE 1: PERCENTAGE OF DPPH RADICAL SCAVENGING ACTIVITY AND XANTHINE OXIDASE INHIBITORY ACTIVITY

Samples (400 µg/mL)	XOI Activity (% ± SD)
Methanol extract	42.12 ± 3.2
n-butanol extract	70.7 ± 8.4
Ethyl acetate extract	70.2 ± 4.7
F1	58.4 ± 8.2
F2	81.5 ± 2.3
F3	87.8 ± 4.5
F4	75.1 ± 1.3
F5	73.5 ± 1.6
F6	85.6 ± 4.2
F7	70.7 ± 6.5

At 400 µg/mL, all of the investigated extracts displayed significant XO inhibitory activities. Fractions of ethyl acetate extract demonstrated substantial XO inhibitory activity (≥50% inhibition), among them F2, F3 and F6 exhibited a>80% inhibitory effect. The most potent inhibitory effect was obtained for the fraction 3 **Table 1**, hence we chose it to continue the separation of our extract in order to find the compounds responsible for this inhibitory activity (XOI).

The major constituents of this fraction were identified as a bioflavonoid amentoflavone (1) and a flavanol catechin (2) on the basis of spectral evidences and comparison of spectral data with literature value [17, 24]. The presence of compounds 1 and 2 in *Juniperus phoenicea* was already mentioned [17].

Phenolic and flavonoid compounds are reported to possess XOI activities. Flavonoids that selected as potential XO inhibitor should consists either hydroxyl group at C-5 and C-7, or double bond between C-2 and C-3 [25, 26]. According to Nagao et al, lower inhibitory activities among some flavonoids are due to substitution of hydroxyl groups at C-3 and C-7 by glycoside or methyl group [26] and some flavonoids that showed minimal or no XOI are such as catechin, non-planar flavones and isoflavones [26]. In these studies, compounds 1 and 2 do not possess XOI activity, we can deduce that the activity of fraction 3 was due either to a minor bioactive constituent or possible synergisms between polyphenols present in the fraction.

CONCLUSION: These studies revealed the potential of *Juniperus phoenicea* leaves to inhibit xanthine oxidase wich therefore may have a positive impact on the prevention of disease caused by increased activity of XO. It is suggested that further purification such as silica gel vacuum chromatography and HPLC of fractions 2, 3, and 6 shall be carried out to identify a potential chemical entity for clinical use in the prevention and treatment of gout and related inflammatory disorders. Furthermore, *in vivo* XOI activity assay can be done to further prove the antigout property of this medicinal plant.

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CONFLICT OF INTEREST: Nil

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