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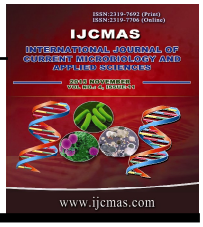

Original Research Article

HPLC Analysis of Quercetin and Antimicrobial Activity of Comparative Methanol Extracts of *Shinus molle* L.

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Keywords

Schinus molle, Quercetin, Supercritical fluid extraction, RP-HPLC analysis, Antimicrobial activity.

A B S T R A C T

Schinus molle L., also known as pepper tree, had been reported to have antimicrobial, antifungal, anti-inflammatory, and antispasmodic properties. This work studied two comparative techniques, for quercetin content and antimicrobial activity, maceration extraction (ME) and supercritical fluid extraction (SFE) to obtain two methanolic crude extracts from the aerial parts of *Schinus molle*. Quercetin and quercetin 7-*O*-glucoside were isolated in a pure form by CC, identified by TLC and ^{1}HNMR. RP-HPLC was employed to quantify quercetin in both extracts. The *in vitro* antimicrobial activity of both extracts and that of the isolated two compounds were evaluated against gram-positive (*Bacillus subtilits*, *Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria, yeast (*Candida albicans*) and filamentous fungi (*Aspergillus niger*) using an overlay method. Quercetin percentage was found to be 4.48% for ME and 5.90% for SFE. Quercetin showed the strongest antifungal and antibacterial activities. While SFE showed stronger activities than ME. This study displayed the antimicrobial properties of interest related to quercetin content (polyphenols), and route of extraction of *Schinus molle* L. The use of natural compounds as antimicrobial agents can provide synergistic benefits with their natural biological efficacy. We concluded that aerial parts of *Schinus molle* L. could afford an interesting natural raw material for the production of health- benefit products as food preservatives solving problems of large incidence in poor tropical countries.

Introduction

Shinus molle L. (also known as California pepper or pepper tree) is a native plant from South America, Anarcardiaceae family (Munoz, 2000). It has been distributed widely and naturalized in many tropical, Mediterranean and subtropical countries (National Parks Service, 2002; Taylor, 2005). The fruits and leaves of this genus have been used in traditional medicine as antibacterial, antifungal, and for treating

tuberculosis, bronchitis and cough (Molina *et al.,* 2007). All parts of the plants have been used medicinally by indigenous people as a digestive, stimulant, diuretic, astringent, antidepressant and for tropical use as wound healer and skin antiseptic (Martinez and Barboza, 2010).

It presents high oil contents with a pleasant spicy scent (Marongiu *et al.,* 2004). Total of 22 volatile compounds were detected and identified, characterized mainly by the presence of sabinene, α- and β- pinene, α-cadinol and high concentrations of myrcene (Gomes *et al.,* 2013). Previous phytochemical studies of *Shinus molle* have resulted in the isolation of mono-sesqui and triterpenes, flavonoids, gallotannins, fatty acids (H□nsel *et al.,* 1994) and antioxidant flavonol glycosides (Marzouk *et al.,* 2006).

Pharmacological studies carried out with extracts from *Shinus molle* show that this plant exerts antitumoral (Ruffa *et al.,* 2002), anti-inflammatory activity (Yueqin *et al.,* 2003) and antidepressant-like effect (Machado *et al.,* 2008). Extracts of pepper tree were shown to be the most effective of a number aromatic and medicinal plant species in suppressing several important pathogenic bacteria (Martinez *et al.,* 1996). The plant extracts also demonstrated repellent and insecticidal properties (Wimalaratne *et al.,* 1996).

Different route of extraction show similar qualitative chemical profiles, but containing different amounts of the main bioactive compounds. The main task of the present study was to; firstly, investigate the amount of quercetin and its glycoside, which is the main flavonol isolated from the methanol crude extract of the aerial parts of *Schinus molle* L., in two comparative extracts, obtained by maceration and supercritical Co_2 extraction technique. In addition, this study sought to evaluate the influence of the route of extraction on the antimicrobial activity investigated.

Material and Methods

Plant material

Aerial parts (leaves and fruits) of *Shinus molle* var. areira L. were collected in April from trees in the botanical gardens of Giza. A voucher specimen was identified and deposited in herbarium of the National Research Center. The plant material was air-dried at room temperature for 1 week.

Chemicals and Reagents

Column chromatography (CC) was performed using silica gel and sephadex LH-20 (Pharmacia, Merck, Darmstadt, Germany). Thin layer chromatography was carried out on pre-coated silica gel F_{254} plates (Merck) developed with methylene chloride: methanol (8:2) and ethyl acetate: formic acid: acetic acid: H_2O (30:1.5:1.5:7). Whatmann no.3 was used for paper chromatography (PC), developed with *n*-butanol: acetic acid: H_2O (BAW) 4:1:5, and 15% acetic acid (AC). Spots were detected using Neu's reagent (1% 2-aminoethyl diphenylborinate in methanol, Aldrich). NMR was recorded on a Delta 2 spectrometer, operating at 500 MHz in DMSO.

Extraction and Isolation

The plant material was grounded to moderately fine powder and extracted (750 g) four times by maceration in aqueous methanol (80%), at room temperature. The methanol extract (ME) was filtered and concentrated in vacuum to obtain a crude extract (80 g). Part of this extract (70 g) was dissolved in H_2O and extracted successively

with *n*-hexane (hexane fraction, 14 g), ethyl acetate (ethyl acetate fraction, 12 g) and *n*-butanol (butanol fraction, 9 g). A portion of the ethyl acetate fraction (7 g) was applied to silica gel column (400 g) which was eluted with methylene chloride containing increasing amounts of methanol (up to 100%) to give 20 combined fractions. Fractions 10-12 (0.9 g) and 16-18 (1.4 g) was submitted to sephadex LH- 20 column, which was eluted with aqueous methanol (95%) to yield **1**(quercetin, 65 mg) and **2** (quercetin 7-*O*-glucoside, 50 mg). These compounds were identified by comparison of its spectral data (^{1}H-NMR) with reported values in the literature (Marzouk *et al.*, 2006), and direct comparison (TLC, HPLC) with authentic sample of quercetin.

Supercritical Extraction

A sample of dried plant material was used for extraction in a supercritical extraction (SE) pilot plant (Cassel *et al.,* 2010). The solvent used was 99.9% CO_2 with a flow rate of $1.38x10^{-4}$ kg s^{-1} through the extraction vessel. Optimal supercritical fluid extraction (SFE)conditions were identified as 46–50 °C temperature, 160–200 kg cm^{-2} pressure and 6–7% ethanol as modifier for maximum extract yield (Barroso *et al.*,2011).

HPLC Analysis

HPLC- grade acetonitrile, methanol, tetrahydrofuran, dimethyl sulphoxide (DMSO) and acetic acid were obtained from Fisher, England. All other chemicals, reagents and solvents used, of analytical grade, double distilled water was used. Quercetin (98%, Sigma, Aldrich) stock solution was prepared and used as an external standard.

The analyses were performed using a high-performance liquid chromatographic system (PERKIN Elmer, Germany) consisting of a solvent delivery pump (series 200 mic IC), auto sampler with a 200-Ul loop (series 200) and an integrator (Model UV Jasco 975). Separation was performed on analytical column a YMC ODS pack (250x4.6 mm I.D., 5 µm particle size) (YMC CO. Ltds, Japan) proceeded by a guard column (20X4.6 mm I.D.) dry packed with pellicular ODS material (37-53 µum) (Whatman, Kent, UK).

The mobile phase consisted of tetrahydrofuran/water/aceitc acid (21:77:2, v/v/v) and was filtered through a 0.45-µm pore size nylon filter (Alltech, Deerfield, IL, USA) and degassed by ultrasonic treatment before use. The HPLC system was operated isocratically at flow rate of 0.85 ml /min at ± 34^{o}C with injection volume 10 µl, and the detector was set at 280 nm. The integrator attenuation was 8 and the chart speed was 0.1 cm/min. Analysis was performed after triplicate extractions of each sample, ME and SFE. Each extract was injected in triplicate (n=3). Quercetin in the samples was identified by comparison of its retention time (t_R) with the standard quercetin (Phani *et al.,* 2010).

Antimicrobial activity

The ability to inhibit the growth of Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi was observed using an overlay method (Williams *et al,*. 1983).

The common pathogenic and food spoilage microorganisms were selected for their relevance in bakery products and other food. The antibacterial, anticandidial and antifungal activity of the endophytic extracts were tested against two Gram positive bacteria (*Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* NRRL-B-4219), one

Gram negative bacteria (*Escherichia coli* ATCC 25922), and yeast (*Candida albicans* ATCC 10231) and filamentous fungi (*Aspergillus niger*).

All samples were dissolved in dimethyl sulfoxide (DMSO, RFCL Limited, New Delhi, India) at 10 mg/mL concentration. Antibiotic discs of streptomycin (S) (10 μg), oxytetracycline (T) (30 μg) and tetracyciline (TE) (30 μg) were used as positive control for bacteria, Neomycin (N) (30 μg), was used for fungi. The bacteria were slanted on nutrient agar (Merck, Darmstadt, Germany), Yeast was slanted and mentioned on Sabaroud's agar medium (Lab M., Bury, Lancashire, UK) and the fungi was slanted and mentioned on the potato dextrose agar medium (Lab M Limited, Bury, Lancashire, UK). Mueller-Hinton agar (Lab M., Bury, Lancashire, UK) following the manufacturer's instructions for the assay.

The antibacterial screening was determined by the well diffusion agar method described by Jorgensen and Turnidge (2007) with some modification.

The organisms were streaked in radial patterns on the agar plates. Plates were incubated under aerobic conditions at 37°C and 28°C for 24 h and 48 h for bacteria and fungi, respectively. In order to obtain comparable results, all prepared solutions were treated under the same conditions under the same incubated plates.

All tests were performed for triplicates. After incubation, plates were examined, confluent bacterial growth was observed. Evidence of antimicrobial activities, represented by a zone of inhibition of microorganism's growth around the holes and diameters of clear zones, was measured in mm (Cruickshank *et al.*, 1975).

Results and Discussion

In the present investigation, quercetin and quercetin 7-*O*-glucoside are isolated from the ethyl acetate fraction of aqueous methanol extract, using silica gel column and further purification on a sephadex column or PPC. Quercetin (1) is isolated as a yellowish white amorphous powder, identified by TLC with authentic sample. ^{1}H-NMR spectrum (500 MHz, DMSO) δ: 8.14 (2 H, d, J=8.8 Hz, H-2', H-6'), 6.80 (1H, d, J=8.8 Hz, H-5'), 6.64 and 6.79 (2H, d, J=2.5 Hz, H-6, H-8). While ^{1}H-NMR spectrum of quercetin 7-*O*-glucoside (2) shows the aglycone protons, besides 3.36 for the glucosyl protons and 5.39 for glucose anomeric H-1" (Mizuo *et al.*, 1992).

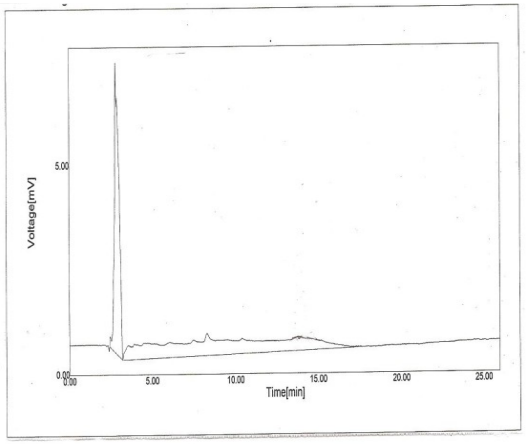
Quercetin is quantified at 280 nm in both extracts, ME and SFE, of *Shinusmolle* using peak area by comparison to a calibration curve derived from standard quercetin. Comparing the HPLC chromatograms from ME and SEF, the main difference is in peak eluted at 2.78 min. The peaks in this study show marked increase in peak area in case of SFE, on comparison with standard quercetin, other peaks in both chromatograms indicate the presence of other chemical constituents. From calibration curve results, the amount of quercetin is calculated as 5.90% of SEF sample, while that of ME is 4.48% (Fig. 1).

The results in table 1, (Fig. 2), show that the extracts and isolated compounds have a moderate antibacterial activity against most of the tested pathogens. ME, SFE and the two compounds show a moderate inhibitory effect against gram-positive bacteria, *Bacillus subtilits*, except quercetin, which shows a strong inhibition effect in comparing with the antibacterial standard antibiotics used. The inhibitory zone diameters range from 10–20 mm.

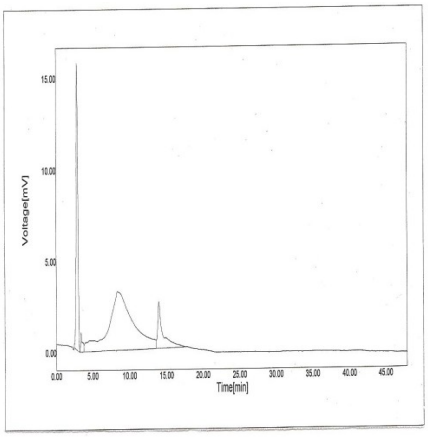
Table.1 Antimicrobial activity measured by inhibition zone diameter (mm) of extracts and isolated compounds at 10 mg/mL using agar diffusion method

Sample	Bacteria			Fungi	
	G+ve		G-ve	unicellular	filamentous
	B. subtilits	***S. aureus***	***E. coli***	***C. albicans***	***A. niger***
ME (10 mg/ml)	10	10	10	12	8
SFE (10 mg/ml)	15	15	12	20	15
Quercetin (10 mg/ ml)	20	20	20	00	15
Quercetin 7-*O*-glucoside(10 mg/ml)	13	15	11	00	13
S* =10µg	14	00	00	12	00
TE* =30µg	18	00	00	23	00
N* =30 µg	00	00	00	00	16
T* =30µg	30	00	28	00	00

Figure.1 **a**= HPLC chromatogram of quercetin, **b**=HPLC chromatogram of ME, **c**=HPLC chromatogram of SFE.



a



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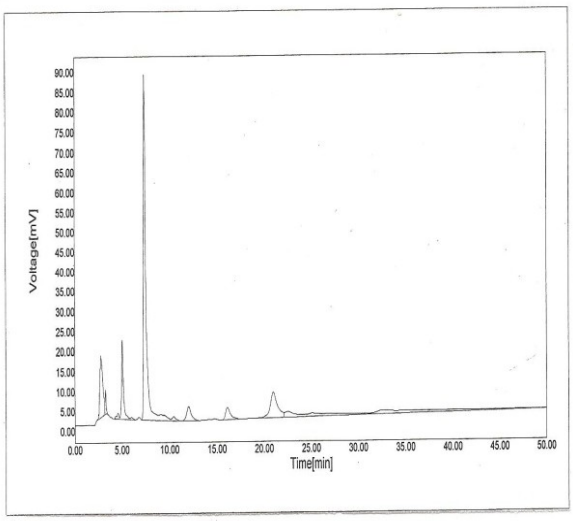
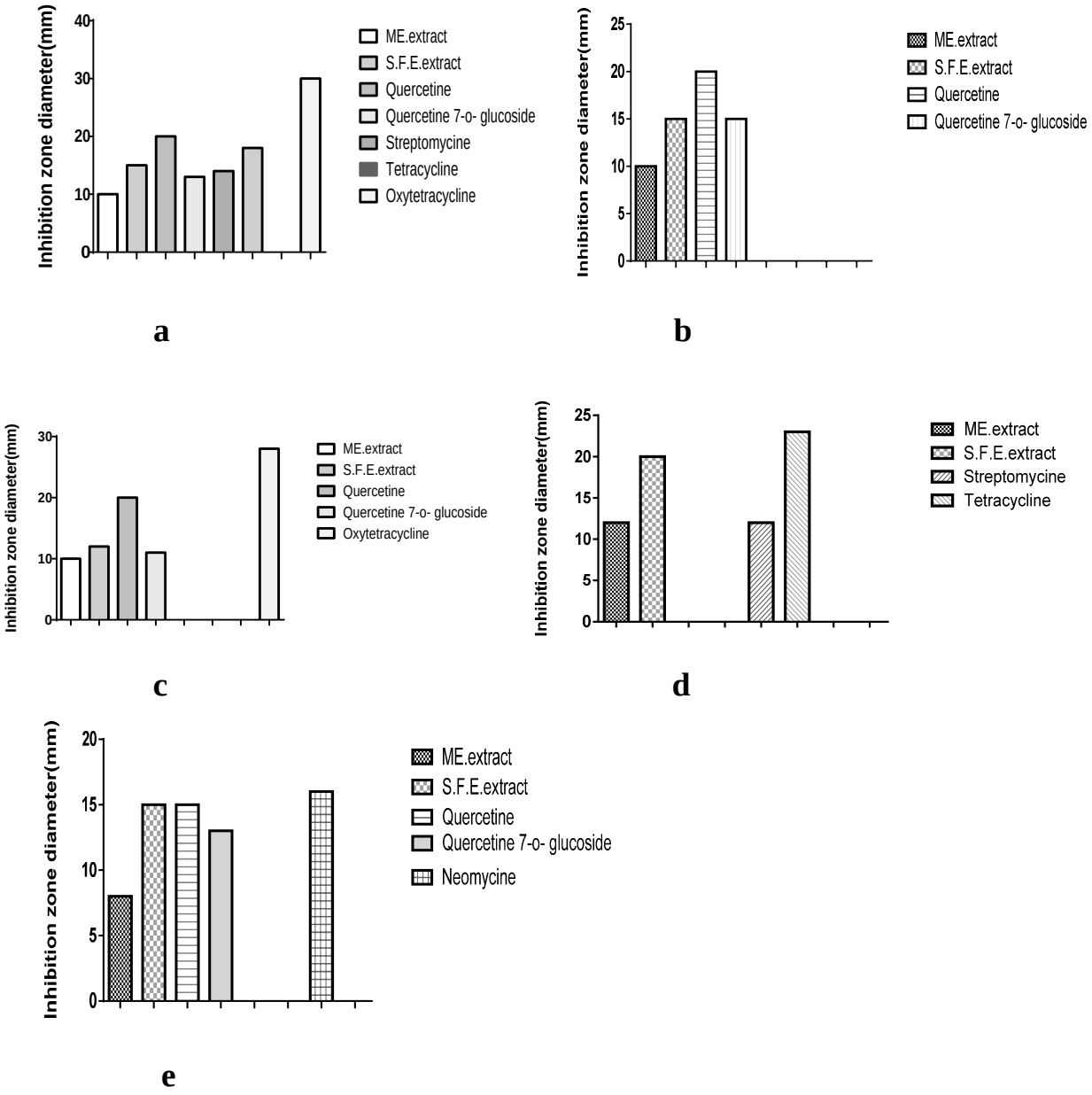


c

Figure.2 Sensitivity of bacterial isolates No. **a,b,c** preliminary identified as *B. subtilis* (NRRL-B-4219), *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922) respectively, and fungal isolates No. **d,e** identified as *C. albicans* (ATCC 10231), and *A. niger* to ME, SFE extracts and two single compounds.



Also quercetin gives a strong inhibitory effect (20 mm) against *Escherichia coli*, as example of gram-negative bacteria while the others show a moderate inhibition effect. The antifungal activities presented, show that, in case of unicellular fungi, the SFE extract shows a strong antifungal effect against *Candida albicans*, while the ME is characterized by moderate effect. In case of filamentous fungi, all compounds and extracts have a moderate antifungal effect against *Aspergillus niger* in comparison to the antifungal standard antibiotic that is used in this study.

Quercetin is a flavonol, one of the most abundant natural flavonoid, being extensively found in many plants, including *Shinus* species. It is of great interest because of its pharmacological function. Reversed-phase HPLC has been applied for the identification of flavonoid derivatives, as well as, quantitative analysis of their variation in various plants extracts (Harborne, *et al.*, 1985; Nikolova, *et al.*, 2004). Unlike other processes, SFE is a technique, widely used in separation processes of natural products, time saving, where the solvent- CO_2- is non-toxic, odorless, tasteless, inert and inexpensive, leaving no residue behind. Moreover, the pressure increase at constant temperature promotes a solvent density increase with low viscosity, low surface tension, high diffusivity, which allows the solvent to extract compounds from the solid matrix (Taylor, 1996). Finally, evaluation reported in this work, promotes SFE to be useful for the scale-up of the extraction process and/or during industrial operation.

Earlier studies have shown that *Shinus* species are indicated for the treatment of stomatitis (Xavier, 1995) and are used externally as antiseptic and healing agents (Sim□es, 1989). It has been proved that the hydro alcoholic extract of the bark of these species presented anti-inflammatory healing activities, allied with antihistaminic effect, besides the antimicrobial action in relation to *Staphylococcus aureus* (Matos, 1988), moreover, it has been reported that the topical use of ethanolic and hexanic extracts from leaves of *Schinus molle* var. areira L. would be safe, either as a therapeutic agent or as an inert repellent (Bras *et al.*,2011), confirming the results presently obtained. Notice that, all of the samples have a considerable strong antifungal and antibacterial activity. Concerning the higher antimicrobial activity of quercetin which is a free flavonoid aglycone, compared to its glucoside, this may support the hypothesis, that phenolic hydroxyl groups are able to form hydrogen bonds with active site of target enzymes, on the other hand, there is negative impact of high glycosidation on the activity of flavonoids. In addition, the synergistic effect of different flavonoidal and phenolic constituents within the extracts lead to some activity of them compared to pure compounds. This may be due to a synergistic effect of several constituents present in the medicinal plant. This is consistent with the finding of Kaatz (2005) who conclude that, synergy is the key, and a complete plant extract rather than a single compound, that it will come to be generally accepted, that all medicinal plants contain multi-drug resistant inhibitors.

As the inherent activity of the extracts may be related to their chemical composition and the proportions of the main components, our finding reveal the presence of antimicrobial metabolites in *Schinus molle*, that could justify it to be potential source of natural antimicrobial agents.

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