

Antibacterial and Phytochemical Study of Iraqi *Salvia officinalis* Leave Extracts

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Abstract

Sage (*Salvia officinalis*), belong to Labiatae family is indigenous to Iraq and other Mediterranean areas but now cultivated world- wide, principally for its use as culinary herb. In the present study preliminary screening for the important phytochemical natural product groups indicated the presence of flavonoid, saponin, hydrolysable and condensed tannin groups. The antibacterial activity of two concentrations 10 mg/ml and 100 mg/ml of chloroform and hydroalcoholic extracts from *Salvia officinalis* leaves was evaluated against four strains of gram negative bacteria (*Escherichia coli*, *Pseudomonas arigenossa*, *Klebsiella pneumonia*, and *Proteus* spp) and two strains of gram positive bacteria (*Staphylococcus aureus*, *Bacillus cerus*) using agar well diffusion method. Chloroform extract 100mg/ml was found active against two types of bacteria, *Staphylococcus aureus* with MIC of 90 mg/ml and *Proteus* spp with MIC of 80 mg/ml. Bioassay guided separation using TLC led to the separation of 6 constituents from the active extract, one of them was identified as thujone.

Key words: *Salvia officinalis*, Antibacterial, TLC, Thujone.

دراسة الفعالية التثبيطية للبكتيريا والمكونات الكيميائية لمستخلصات أوراق الميرمية التي تنمو

في العراق

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الخلاصة

تنتمي الميرمية (*Salvia officinalis*) الى العائلة الشفوية Labiatae والتي تنمو بصورة طبيعية في العراق و مناطق اخرى في الشرق الأوسط و تزرع في مناطق مختلفة من العالم بالدرجة الرئيسية كعشبة للأكل. البحث تمهيدى لنواتج الكيميائية الطبيعية في النبات ادت الى كشف النواتج (flavonoid, saponin, hydrolysable and condensed tannin). و قد أختبرت مستخلصات المذيب العضوي (chloroform) و مذيب الكحولي (75% ethanol) بتركيزين (C1 10mg/ml , C2 100mg/ml) لغرض معرفة الفعالية التثبيطية للبكتيريا مقابل أربعة سلالات من البكتيريا من نوع الصيغة السالبة (*Escherichia coli*, *Pseudomonas arigenossa*, *Klebsiella pneumonia*, *Proteus* spp) ونوعين من الصيغة الموجبة (*Staphylococcus aureus*, *Bacillus cerus*) باستخدام تقنية (agar well diffusion). اظهرت النتائج بان المذيب العضوي 100mg/ml ذو فعالية تثبيطية مقابل سلالتين من البكتيريا، *Staphylococcus aureus* بالحد الأدنى للتركيز المثبط (MIC 90 mg/ml) و *Proteus* spp بالحد الأدنى للتركيز المثبط (MIC 80 mg/ml). لاحقا تم فصل و مطابقة المكونات الرئيسية لمستخلص الفعال لأوراق نبات الميرمية باستخدام التقنية كروموتوغرافيا الصفائح الرقيقة (TLC) و قد تم فصل ستة مكونات رئيسية و تم تشخيص إحداهما بأنها thujone.

الكلمات المفتاحية : TLC ، الميرمية ، الفعالية التثبيطية للبكتيريا

Introduction

Sage (in Kurdish and Arabic, Meramea) derived from (*Salvia officinalis*) belong to Labiatae family, indigenous to Iraq and other Mediterranean areas but now cultivated world- wide, principally for its use as culinary herb. *Salvia* is a perennial herbaceous to shrubby herb growing up to 50cm in height (^{1, 2}). Sage is used as therapeutic agent and in food, beverages, spirits, and cosmetics and in the production of relaxing and curative herbal tea (³). Sage reportedly has antibacterial, fungistatic, virustatic, astringent, secretion-stimulating, and perspiration-inhibiting effects. Phenolic acids (e.g., salvin and salvin monomethyl ether) isolated from sage have antimicrobial

activities, especially against *Staphylococcus aureus* (⁴).The volatile oil of sage consists of about 5% of α - and β - thujone together with cineole, borneol and other constituents (^{2, 3}). Non volatile components of the leaf include diterpenes, phenolic glycosides based on caffeic acid, p- hydroxybenzoic acid and tannins (3-8%) both hydrolysable and condensed tannins (²). Thus, the present study is aimed to carry out preliminary screening for the important phytochemical natural product groups and to investigate and establish the antibacterial activity of Iraqi *Salvia officinalis* leave extracts and finally bioassay guided separation and identification of the main active constituents in the active extract (s).

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Materials and Methods

Plant material

Salvia officinalis leaves were collected during September 2009 in Erbil and authenticated by the Department of Pharmacognosy, College of Pharmacy-Hawler Medical University.

Phytochemical screening

Fifty g of dried powdered *Salvia officinalis* leaves were extracted using 75% ethanol for the phytochemical investigation using ultra sonic bath for 1hr at 40 °C⁽⁵⁾. Preliminary qualitative tests were carried out on the plant extracts to detect the most important natural product groups⁽⁶⁾.

Alkaloid test: Hydroalcoholic extract of sage leaves (75% ethanol) was treated with dilute sodium hydroxide (5% NaOH) solution; extraction is then carried out with chloroform. The concentrated organic liquid is then shaken with 5% HCl and allowed to separate. The aqueous extract was used for detection of alkaloidal compounds⁽²⁾. Few drops of Dragendorff reagent were added to 1ml of extract. The appearance of a reddish-brown to orange precipitate indicated the presence of alkaloid^(2, 7, and 8).

Flavonoid test: 2 ml of hydroalcoholic extract of sage leaves were tested for the presence of flavonoid glycoside by the addition of few drops of NaOH solution. The formation of intense yellow color which turn to colorless on addition of few drops of dilute acid solution indicated the presence of flavonoids⁽⁹⁾.

Anthraquinone test: The tested sage extract was boiled with 1ml of dilute HCl in a test tube over a pre-heated water bath for 5 minute. The contents were cooled and extracted with chloroform, the chloroform layer was separated and ammonia solution was added. The appearance of a rose-pink color in ammonia layer is indicated the presence of anthraquinone glycoside⁽⁹⁾.

Cardioactive glycoside test: The dried sage extract was dissolved in chloroform, and then evaporated to dryness. Dissolve the residue in (0.4ml) glacial acetic acid with few drops of FeCl₃, concentrated sulphuric acid (H₂SO₄) was added along the side of the test tube to settle at the bottom. The reddish brown color changing to bluish green color appears at the junction of the two reagents within 2-5 min. spreading slowly into the acetic acid layer indicated the presence of cardioactive glycoside⁽⁹⁾.

Saponin test: A volume of 2ml of hydroalcoholic sage extract were shaken with 1ml of water. The formation of semi-

permanent foam (15 min) indicated the presence of saponin natural products⁽⁶⁾.

Tannin test: A few drops of ferric chloride reagent 1% were added to 1ml of sage extract. The appearance of blue color indicated the presence of hydrolysable tannins and appearance of green color indicated the presence of condensed tannins^(2, 6).

Antibacterial evaluation

Extraction: Plant material was collected, dried in air for seven days, and all plants were powdered with mechanical grinder. 50gm of dried powdered plant material was extracted separately with 1000 ml of chloroform using ultra sonic bath for one hour at 40 °C⁽⁵⁾. The extracts were obtained by filtration through Buckner funnel, and evaporated to dryness by rotary evaporator yielding chloroform extract (5.4 g). The residual plant materials were dried then re-extracted using 75% ethanol using ultra sonic bath for one hour at 40 °C⁽⁵⁾. The obtained extracted from filtration by Buckner funnel evaporated to dryness by rotary evaporator yielding ethanol extract (9.2 g).

Plant extract preparation: The plant extracts were evaluated separately at two different concentrations 10mg and 100mg. the chloroform extracts and ethanol extracts were dissolved separately in 1ml of 20% tween 80 and 10% dimethyl sulfoxide (DMSO) respectively.

Tested microorganism: Bacteria which were used in the process of investigation are obtained from biology department, Science College, Salahaddin University. Bacterial strains include two gram positive bacteria (*Staphylococcus aureus*, *Bacillus cerus*) and four gram negative bacteria (*Escherichia coli*, *Pseudomonas arigenossa*, *Klebsilla spp* and *Proteus spp*). The bacterial samples are frozen at -4 °C in cooled incubator, later reactivated before it's used.

Method of antibacterial evaluation: The antibacterial activity of the two types of plant extracts were tested against six strains of bacteria using agar- well diffusion method^(10, 11). From the frozen bacteria inoculation was done into nutrient agar media, and incubated at 37°C for 24 hr. The grown bacteria were suspended in a normal saline solution (0.85% sodium chloride w/v) to a turbidity of 0.5 Mc Farland standards (108 cfu/ml). The prepared bacterial suspension was used to inoculate into Muller-Hinton agar plate with a sterile non-toxic cotton swab on a wooden applicator. Four wells were done by a sterile cork borer of 5mm in diameter in each plate. 100 µl of dilution of plant extracts in 10% DMSO for ethanol extract and 20% tween 80 for

chloroform extract to give final concentration of 1mg and 10mg of each plant extract was added in each well, 10% DMSO and 20% tween 80 were used as negative control and streptomycin antibiotic used as positive control in concentration of (100mg/ml) added in a well in each plate. Plates are incubated at 37°C for 24 hr.

Determination of minimum inhibitory concentration (MIC): MIC values for biologically active extracts against *Staphylococcus aureus* and *Proteus spp.* were determined by agar well diffusion method^(10,11).

Separation and Identification of the main active constituents

Thin layer chromatography (TLC) technique was applied for the separation and identification of active constituents using silica gel GF 254 nm, layer thickness 0.2 mm; 20x20 cm aluminium cards (fluka, Switzerland)^(7,12).

Different solvent systems were tried as a mobile phase [Toluen: Ethyl acetate (93:7), Toluen: Ethyl acetate: Formic acid (5:4:1), Ethyl acetate: Formic acid: glacial acetic acid: water (10:1.1:1.1:2.6), Benzene: Pyridine: Formic acid (7.2:1.8:1)]^(13, 14). Fifty mg of dried chloroform extract was dissolved in 1 ml chloroform and 1µL of thujone reference substance (Chromadex, USA) was dissolved in 1ml toluene, 10 µL of both solutions were applied to TLC plate and developed in different mobile phase systems. The plate was dried at room temperature. The TLC plate was sprayed with anisaldehyde - sulphuric acid reagent after the development process. The plate was heated to 100–110°C for 5–10 min. and visualized under visible light⁽¹³⁾. A number of constituents were separated, and their R_f values were calculated, the procedure was triplicated and mean values were taken, table (1).

Table 1: R_f value and spot color of separated constituent (S3)

Separated constituent	R _f value				Spot color
	Mobile phase (1)	Mobile phase (2)	Mobile phase (3)	Mobile phase(4)	
S ₃	0.5	0.31	0.13	0.43	Violet
Standard Thujone	0.52	0.34	0.15	0.44	Violet

Mobile phase (1): Toluen: Ethyl acetate (93:7)

Mobile phase (2): Toluen: Ethyl acetate: Formic acid (5:4:1)

Mobile phase (3): Ethyl acetate: Formic acid: glacial acetic acid: water (10:1.1:1.1:2.6)

Mobile phase (4): Benzene: Pyridine: Formic acid (7.2:1.8:1)

Results

Sage leaves chloroform extract shows activity against two strain of bacteria *Staphylococcus aureus* and *Protues spp.* at 100mg/ml concentration with inhibition zone diameter (20±0.265 and 10±0.2) respectively with corresponding MIC value 90 mg/ml against *Staphylococcus aures* and 80 mg/ml against *Proteus spp.*, table (2). the phytochemical screening tests showed positive results for flavonoid, saponin hydrolysable and condensed tannin, while the other natural product groups including alkaloid, anthraquinone, and cardioactive were found absent. The results of separation by TLC revealed the presence of a number of constituents in chloroform extract, one of the constituent (S₃) was found to be with identical color and R_f value with that of thujone reference substance, figure (1).

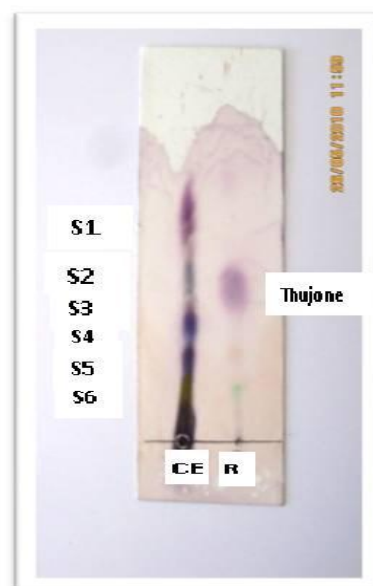


Figure 1: TLC chromatogram of Sage leaves, mobile phase [Toluen: Ethyl acetate (93:7)]; chloroform extract (CE), thujone reference substance (R).

Table 2: Antibacterial activity for Sage leaves chloroform extract, C1 (10mg/ml) and C2 (100mg/ml).

No.	Microorganism	Inhibition zone diameter (mm), MIC value (mg/ml)			
		Chloroform extracts		Ethanol extracts	
		C1	C2	C1	C2
1-	<i>Staphylococcus aureus</i>	---	20±0.265, (90)*	---	---
2-	<i>Pseudomonas aeruginosa</i>	---	---	---	---
3-	<i>Escherichia coli</i>	---	---	---	---
4-	<i>Klebsila pneumoniae</i>	---	---	---	---
5-	<i>Bacillus cerus</i>	---	---	---	---
6-	<i>Proteus spp.</i>	---	10±0.2, (80)*	---	---

*All readings were repeated three times, the mean of triplicates ± SD values were taken.

Discussion

Sample preparation is the crucial first step in the analysis of herbs, because it is necessary to extract the desired chemical components from the herbal materials for further separation and characterization. Thus, the development of modern sample preparation techniques with significant advantages over conventional methods analysis of medicinal plants is likely to play an important role in the overall effort of ensuring and providing high quality herbal products to consumers worldwide ⁽¹⁵⁾. The plant expresses antibacterial activity against two strains of bacteria. From the results, the chloroform extract was active while hydroalcoholic extract was inactive. The activity of the plant as showed in the literature review may be due to phenolic acids (example, salvin and salvin monomethyl ether) previously isolated from sage, especially against *Staphylococcus aureus* ⁽⁴⁾. The obtained results were in agreement with the finding of Zhi-He and Hiroyuki, (1996) ⁽¹⁶⁾ who were found that sage leaves extract at 0.2% concentration was gave strong activity against *Staphylococcus aureus*, in the same time Rogério *et al*, (2004) ⁽¹⁷⁾ found that the essential oils of *Salvia officinalis* was inhibited 83.3% of *Proteus spp.* while Gislene *et al*, (2000) ⁽¹⁸⁾ were found that the collected sage in Brazil was inactive against *Staphylococcus aureus* and *Proteus spp.* Literature reviews on *Salvia officinalis* revealed that there was few phytochemical studies conducted on the Iraqi species of sage, and due to the antibacterial activity of its chloroform extract and from phytochemical screening which was found to contain four groups of phytochemical natural product groups, it was tried to separate and identify the main active constituents in the active extract by using TLC. The proposed TLC method was regarded as suitable for quality control of

herbal products containing *Salvia officinalis* and for its extracts. The method is simple, sensitive, and specific, and both the standards and the samples can be analyzed simultaneously, without the requirement for sophisticated equipment ⁽¹³⁾. Separation of the main active constituents in chloroform extract by the TLC revealed the presence of six constituents figure (1). One of the constituents (S₃) had the same R_f value and color as thujone the reference compound. Chemotaxonomic markers are sometimes used as indicators of botanical identity; thujone is the marker for *Salvia officinalis* ⁽¹⁹⁾. Miladinović and Miladinović (2000) ⁽²⁰⁾ found that the main component of the *Salvia officinalis* essential oils was thujone (24.88%) and camphor (16.3%). The carriers of antimicrobial activity of the sage oil were found to be thujone and camphor ^(19, 21). Further study using advanced spectroscopical methods and gas-liquid chromatography is recommended for more confirmation of the identity of (S₃) constituents and to identify the other separated constituents in the chloroform extract.

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