Antimicrobial activity and chemical composition of *Pistacia chinensis* Bunge leaves

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Abstract

This work was carried out for determining antimicrobial activity of *Pistacia chinensis* leaves methanol extract and identifying the chemical composition of the plant extract. Methanol extract was tested for antimicrobial activity using disc-diffusion assay and the extract was fractionated on silica gel column chromatography for the isolation of the bio-active constituents. The leaves extract of *P. chinensis* showed a significant antimicrobial effect, it strongly inhibited the growth of the test bacteria and yeast studied. Chromatograpic separation of the methanol extract of *P. chinensis* leaves has led to the isolation and characterization of β-sitosterol, luepol, and six flavonoids, quercetin, myricetin, quercetin 3-O-α-rhamnoside, quercetin 3-O-β-glucoside, myricetin 3-O-α-rhamnoside and myricetin 3-O-β-glucuronide using various chromatographic procedures and the interpretation of spectral data in comparison with already existing data reported in the literature. The results presented here may suggest that the leaves extract of *P. chinensis* possess antimicrobial properties, and therefore, can be used as natural preservative ingredients in food and/or pharmaceuticals.

Introduction

The use of herbal medicine for the treatment of diseases remains the main stay of health care system and is gaining increasing popularity in developing countries. Research programs have been going on to assay and improve the medicinal principles found in drugs for use in the development of new pharmacotherapeutic agents in the management and cure of diseases (Sofowara, 1993).

The bacterial organisms including gram positive and gram negative like different species of *Bacillus*, *Staphylococcus, Salmonella* and *Pseudomonas* are the main source to cause severe infections in humans (Nathan, 2004). Resistance to antimicrobials is a significant and growing problem, limiting treatment options, especially for serious gram positive infections, among them *Staphylococcus aureus*, *Streptococcus pneumoniae, Streptococcus pyogenes, Enterococcus faecalis*, and gram negative bacteria such as *Klebsiella pneumonia, Escherichia coli and Pseudomonas aeruginosa*. Plant derived antibacterials are always a source of novel therapeutics. Historically, plants have been placed at top among the sources of novel drugs with antimicrobial activity, as traditional

medicines based on plants and plant extracts have made considerable contributions to human health and well-being.

Pistacia chinensis is a deciduous small tree from Anacardiaceae family. It is widely distributed in China and North America. It is well known as a landscape and shade tree (Widodo, 1998). In Chinese traditional medicine, the oil from its seeds is used for biodiesel production in China and it shows a high resistance to various pests in the United States (Widodo, 1998).

There are few reports about chemical constituents and biological activities from *P. chinensis* plant, two 4-arylcoumarin moieties (neoflavone) dimers were isolated from of *P. chinensis* leaves with estrogen-like activity (Nishimura *et al*., 2000). Also some phenolic compounds such as gallic acid, m-digallic acid, quercetin, 6-O-galloyl arbutin-quercitrin, and quercetin -3-O(6''-galloyl)-β-D-glucosides were isolated from the leaves (Shi and Zuo, 1992) and a new pyrrolidone derivative was isolated from *P. chinensis* tender burgeon, and anthotaxy (Jian *et al*., 2008). *P. chinensis* has the ability to inhibit NO production as anti-inflammatory potential of this plant (Yayeh *et al*., 2012). Aerial parts of the plant

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and its compounds showed an anti-HCV activity (Rashed *et al.*, 2014). The present study aimed to investigate antimicrobial activity of methanol extract of P. chinensis leaves and to identify the constituents responsible for the observed activity.

Materials and Methods

General experimental procedures

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). 1H-NMR and 13C-NMR (Varian Unity Inova). MS (Finnigan MAT SSQ 7000, 70 ev). (Silica gel (0.063-0.200 mm for column chromatography) and Sephadex LH-20 (Pharmacia Fine Chemicals). Thin layer chromatography (TLC) F254 plates. Solvent mixtures, BAW (n-butanol:acetic acid:water 4:1:5 upper phase, 15% acetic acid: water: glacial acetic acid: 85:15). Paper Chromatography (PC) Whatman No.1 (Whatman Led.Maid Stone, Kent, England) sheets for qualitative detection of flavonoids and sugars.

Plant identification and collection

Leaves of *P. chinensis* were collected from Al-Zohiriya garden, Giza, Egypt in May 2013. The plant was identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereeza Labib consultant of plant taxonomy at the Ministry of Agriculture and director of Orman botanical garden, Giza, Egypt. A voucher specimen is deposited in the herbarium of Al-Zohiriya garden, Giza, Egypt.

Preparation of the extract

Air-dried powder of *P. chinensis* leaves (420 g) was extracted with methanol 80% several times at room temperature until exhaustion by maceration method. The extract was concentrated under reduced pressure to give 26 g of the crude extract.

Isolation of the compounds from P. chinensis *leaves methanol extract*

Twenty five grams of the extract was subjected to silica gel column chromatography eluting with n-hexane, dichloromethane, ethyl acetate and methanol gradually. One hundred and forty fractions of 100 ml conical flask were collected. The fractions that showed similar Paper Chromatography (PC) in Butanol–Acetic acid–Water 4:1:5 (BAW) and 15% acetic acid were combined to give 4 fractions (I, II, III, and IV). Fraction I (2.45 g) was subjected to sub–column of silica gel eluted with n-hexane: dichloromethane (50:50) gave compound 1 and

elution with dichloromethane: n-hexane (80:20) gave compound 2. Fraction II (1.85 g) was subjected to sub–column of silica gel eluted with dichloromethane: ethyl acetate (90:10) yielded compound 3 and elution with ethyl acetate solvent gave compound 4. Compound 5 yielded from elution with ethyl acetate:methanol (90: 10) and compound 6 was obtained from elution with ethyl acetate: methanol (80:20) from fraction III (2.75 g). Compound 7 yielded by elution with ethyl acetate: methanol (75:25) and compound 8 was obtained by elution with ethyl acetate: methanol (60:40) from fraction IV (3.22 g). All the isolated compounds were purified on sephadex LH–20 column using different systems of methanol and distilled water.

General method for acid hydrolysis of flavonoid glycosides

Five milligram of each flavonoid glycoside 5, 6, 7 and 8 in 5 ml 10% HCl was heated for 5 h. The aglycones were extracted with ethyl acetate and identified by co-TLC with authentic standards. The sugars in the aqueous layer were identified by co-paper chromatography (co-PC) with authentic markers on Whatman No. 1 sheets in solvent system (n-BuOH-AcOH-H2O 4:1:5 upper layer).

Antimicrobial tests

The bacterial and yeast strains used are described in Table 1. These microbial strains were isolated from human and food beings and belong to the microbiological laboratory collection of the department of microbiology from Suez Canal University, Egypt. Nutrient agar (for bacterial strains) and Sabouraud dextrose agar media (for yeast strains) were inoculated with this suspension of the respective organism and poured into a sterile petri dish.

The agar diffusion assay was performed according to the modified Kirby-Bauer disc diffusion method (Selim *et al.*, 2013). One ml of each test organism liquid culture was individually suspended in 3 ml of a 0.9% NaCl solution. The essential oil (EO) and methanol extract were dissolved in 10% dimethylsulfoxide (DMSO) to a final concentration of 30 mg/ml as stock solution and sterilized by filtration through 0.45 μm Millipore filters. Antimicrobial tests were then carried out using 100 μl of suspension containing 10^8 cfu/ml of bacteria and 10^6 cfu/ml of yeast spread on nutrient agar and Sabouraud dextrose agar media, respectively. The discs (6 mm in diameter) were impregnated with 100 μg of the essential oil and methanol extract, and then placed onto inoculated agar. Negative controls were prepared using the same solvent employed to dissolve the extract. The

Table 1. Antimicrobial activity of methanol extract of *P. chinensis* leaves

	Origin	Inhibition zone in diameter (mm)
Gram Positive Bacteria		
Bacillus cereus	Food	25
Enterococcus feacalis	Human	25
Serratia marcescens	Human	28
Staphlococcus aureus	Human	-
Gram Negative Bacteria		
Aeromonas hydrophila	Human	18
Acinetobacter baumannii	Human	20
Escherichia coli	Human	18
Klebsiella pneumoniae	Human	22
Brevundimonas vesicularis	Human	16
Pseudomonas aeruginosa	Human	20
Salmonella paratyphi	Food	25
Yeast		
Candida albicans	Human	15
Saccharomyces cerevisiae	Human	9

[a] Inhibition zone in diameter (mm) around the discs impregnated with extract (50 µg/disc).

inoculated plates were incubated at 37°C for 24 h for clinical bacterial strains and 48 h for yeast isolates. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Results and Discussions

The present study was focused on evaluation of the antimicrobial activity of *P. chinensis* leaves methanol extract as can be seen in table 1 as well identification of the main constituents of *P. chinensis* leaves methanol extract which were, β-sitosterol, luepol, and six flavonoids, quercetin, myricetin, quercetin 3-O-α-rhamnoside, quercetin 3-O-β-glucoside, myricetin 3-O-α-rhamnoside, and myricetin 3-O-β-glucuronide. The chemical structures of the isolated compounds are shown in Figure 1.

Structure Elucidation of the isolated compounds

β-sitosterol (1): 17 mg, white needles. ^{1}H-NMR (CDCl$_3$, 400 MHz): δ ppm 5.37 (IH, m, H-6), 3.52 (IH, m, H-3) , 1.09 (3H, s, CH$_3$-19), 0.98 (3H, d, J= 6.5, CH$_3$-21), 0.92 (3H, t, J= 7.4, CH$_3$-29), 0.85 (3H, d, J= 6.7Hz, CH$_3$-26), 0.81 (3H, d, J= 6.7Hz, CH$_3$-27), 0.75 (3H, s, CH$_3$-18). 13C-NMR(CDCl$_3$,100 MHz): δ ppm 140.46 (C-5), 121.52 (C-6), 71.64 (C-3), 57.25 (C-17), 56.48 (C-14), 50.35 (C-9), 46.38 (C-24), 42,82 (C-13, 4), 39.88 (C-12), 37.64 (C-1), 36.75 (C-10), 35.92 (C-20), 34.24 (C-22), 31.78 (C-8, 7), 31.45 (C-2), 29.24 (C-25), 28.46 (C-16), 26.24 (C-23), 24.54 (C-15), 23.48 (C-28), 21.14 (C-11), 19.82 (C-26), 19.58 (C-19), 19.24 (C-27), 18.68 (C-21).

Lupeol (2): 15 mg, white powder. 1H-NMR (CDCl$_3$, 400 MHz): δ ppm 0.75, 0.8, 0.85, 0.96, 0.98, 1.08, 1.75 (each 3H, s), 3.25 (1H, dd, J = 5.6, 10.8 Hz, H-3), 4.58 (1H, s, H-29a), 4.68 (1H, s, H-29b). 13C-NMR(CDCl$_3$, 100MHz): δ ppm 151.46 (C-20), 108.78 (C-29), 78.64 (C-3), 55.82 (C-5), 50.75 (C-9), 48.72 (C-18), 48.42 (C-19), 43.25 (C-17), 43.28 (C-14), 40.82 (C-8), 39.76 (C-22), 38.75 (C-4), 38.58 (C-1), 38.72 (C-13), 37.65 (C- 10), 35.78 (C-16), 34.55 (C-7), 29.42 (C-21), 28.48 (C-23), 27.68 (C-2), 27.65 (C-15), 25.48 (C-12), 21.42 (C-11), 19.45 (C-30), 18.72 (C-6), 18.44 (C-28), 16.56 (C-25), 16.27 (C-26), 15.75 (C-24), 15.28 (C-27).

Quercetin (3): 9 mg, yellow powder. UV λmax (MeOH): 255, 267, 371; (NaOMe): 270, 320, 420; (AlCl$_3$): 270, 455; (AlCl$_3$/HCl): 264, 303sh, 315sh, 428; (NaOAc): 257, 274, 318, 383; (NaOAc/
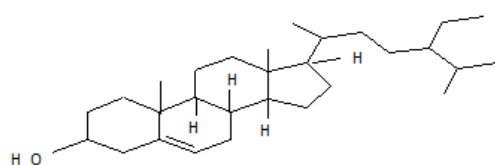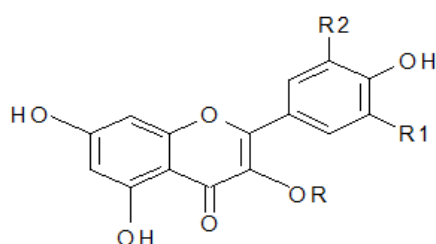
1. *β*-sitosterol
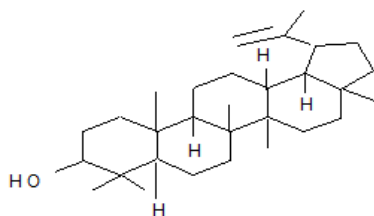
2: Lupeol

3: Quercetin (R=R1=H, R2=OH)
4: Myricetin (R=H, R1=R2=OH)
5: Quercetin 3-O-α-rhamnopyranoside (R=rhambose, R1=H, R2=OH)
6: Quercetin 3-O-β -glucoside (R=glucose, R1=H, R2=OH)
7: Myricetin 3-O-α-rhamnopyranoside (R=rhamnose, R1=R2 =OH)
8: Myricetin 3-O-β-glucronoide (R=glucuronic acid, R1=R2 =OH)

Figure 1. Structures of chemical compounds isolated from *P. chinensis* leaves

H3BO3): 259, 387. EI–MS: m/z 302.

Myricetin (4): 10 mg, yellow powder. UV λmax (MeOH): 254, 272sh, 374; (NaOMe): 262sh, 285sh, 322sh, 423(Dec.); (AlCl$_3$): 271, 316sh, 450; (AlCl$_3$/HCl): 266, 275sh, 308sh, 360sh, 428; (NaOAc): 269, 335(Dec.); (NaOAc/H3BO3): 258, 304sh, 392. EI-MS: m/z 318.

Quercetin 3-O-α-rhamnoside (5): 16 mg, yellow crystals. 1H–NMR (DMSO–d6, 400 MHz) δ ppm 7.26 (2H, m, H–2`, 6`), 6.83 (1H, d, J=9 Hz, H–5'), 6.49 (1H,d, J=2.5 Hz, H–8),6.14 (1H, d, J=2.5Hz, H–6), 5.25 (1H, s, H–1'') 0.78 (3H, d, J=6Hz). 13C–NMR (100 MHz, DMSO–d6): δ ppm 177.42 (C–4), 167.45 (C–7), 161.40 (C–5), 157.01 (C–2), 157 (C–9), 149.19 (C–4`), 145.57 (C–3`), 134.12 (C–3), 131.97 (C–6`), 121.40 (C–1`), 115.71 (C–2`), 115.40 (C–5`), 103.10 (C–10), 101.97 (C–1``), 99.98 (C–6), 94.47 (C–8), 71.47 (C–4''), 70.94, 70.85, 70.62 (C–2``, C–5``, C–3''), 17.78 (C6'').

Quercetin 3-O-β-glucoside (6): 14 mg, yellow crystals. 1H-NMR (DMSO-d6, 400 MHz): δ ppm 7.78 (1H, dd, J=2, 8.5 Hz, H-6'), 7.54 (1H, d, J=2

Hz, H-2'), 6.82 (1H, d, J=8.5 Hz, H-5'), d 6.42 (1H, d, J= 2 Hz, H-8), 6.24 (1 H, d, J= 2 Hz, H-6), 5.5 (1H, d, J=7.5 Hz, H-1''). (-) ESI-MS: m/z 463 [M-H]⁻.

Myricetin 3-O-α-rhamnoside (7): 22 mg, yellow amorphous powder. UV λmax (MeOH): 260, 296sh, 352; (NaOMe): 273, 321, 392; (AlCl$_3$): 272, 312, 420; (AlCl$_3$/HCl): 270, 310, 404; (NaOAc): 270, 317, 364; (NaOAc/H$_3$BO$_3$): 260, 303, 376. 1H-NMR (400 MHz, DMSO-d6): δ ppm 6.89 (2H, s, H-2`, 6`), 6.24 (1H, d, J = 2.5 Hz, H-6), 6.37 (1H, d, J = 2.5 Hz, H-8), 5.2 (1H, s, H-1``), 3.9-3.2 (m, remaining sugar protons), 0.82 (CH3-rhamnosyl, d, J = 6 Hz, H-6``).

Myricetin 3-O-β-glucuronide (8): 19 mg, yellow amorphous powder. UV λmax (MeOH): 262, 298sh, 349; (NaOMe): 272, 324, 392; (AlCl3): 272, 312, 428; (AlCl$_3$/HCl): 270, 310, 404; (NaOAc): 270, 318, 366; (NaOAc/H$_3$BO$_3$): 260, 300, 374. 1H-NMR (CD3OD, 400 MHz): δ ppm 7.42 (2H, s, H-2', 6'), 6.45 (1H, d, J = 1.2 Hz, H-8), 6.22 (1H, d, J = 1.2 Hz, H-6), 5.47 (1H, d, J = 7.5 Hz, H-1''). 13C-NMR (CD3OD, 100 MHz)): δ 177.52 (C-4), 174.25 (C-6''), 165.84 (C-7), 162.68 (C-5), 158.46 (C-9), 148.28 (C-2), 146.92 (C-3',5'), 137.55 (C-3), 137.15 (C-4'), 123.32 (C-1'), 108.86 (C-2',6'), 104.74 (C-10), 104.18 (C-1''), 99.52 (C-8), 94.64 (C-6), 78.26 (C-3''), 78.42 (C-5''), 75.66 (C-2''), 73.48 (C-4'').

Chromatographic separation of *P. chinensis* leaves methanol extract resulted in the isolation and identification of compound 1 which gave dark spot under short UV light on thin layer chromatography (TLC) that changed to violet colour on spraying with vanillin sulphuric and heating in an oven at 110°C for 5 min. NMR spectral data has shown signals very close to β-sitosterol (Pateh *et al.*, 2009). Compound 2 afforded a dark spot under short UV light and changed to pink to violet upon spraying with vanillin-sulphuric acid and heating in an oven at 110°C for 5 min. NMR spectral data showed signals very similar to lupeol (Abdullahi *et al.*, 2013). Compounds 3, and 4 gave a yellow spot and appeared as fluorescence yellow colour after spraying with AlCl$_3$ and its spectral data are very similar to quercetin, and myricetin (Manguro *et al.*, 2005). Compound 5 yielded deep purple spot under UV light and changed to yellow when subjected to ammonia and AlCl$_3$ and complete acid hydrolysis gave quercetin as an aglycone and rhamnose as sugar moiety and its spectral data was very similar to that of quercetin 3-O-α-rhamnoside (Lawrence *et al.*, 2005). Compound 6 (quercetin 3-O-β-glucoside) is obtained as a deep purple spot and and gave yellow colour when exposed to ammonia vapour and a bright yellow colour when spraying with AlCl$_3$.

Complete acid hydrolysis of the compound gave quercetin as an aglycone and glucose as sugar moiety.

Spectral data is very close to spectra of quercetin 3-O-β-glucoside (Song *et al*. 2007). Compounds 7, and 8 are obtained as deep purple spot and both gave yellow colour when exposed to ammonia vapour and gave a bright yellow colour when spraying with AlCl$_3$. Complete acid hydrolysis of the both compounds gave myricetin as an aglycone, rhamnose and glucuronic acid as sugar moieties, respectively. Spectral data of both compounds are very close to spectra of myricetin 3-O-α-rhamnopyranoside, and myricetin 3-O-α-glucronoide (Manguro *et al*., 2005).

Antimicrobial activity of P. chinensis *leaves methanol extract*

The *in vitro* antimicrobial potential of *P. chinensis* leaves methanol extract against a panel of microorganisms is shown in Table 1. The extract showed a significant antimicrobial activity against all tested bacteria, including gram positive, and gram negative ones with diameter zones of inhibition 9 to 28 mm. In the comparison of microbial sensitivity to the extract, Serratia marcescens seems to be more sensitive than other infectious pathogens such as *E. coli*. No remarkable activity was observed against *S. aureus*. The antimicrobial activity of *P. chinensis* leaves methanol extract was more pronounced against gram-positive than gram-negative bacteria. Generally, the higher resistance among gram-negative bacteria could be described to the presence of their outer phospholipidic membrane, almost impermeable to lipophilic compounds (Burt, 2004). The absence of this barrier in gram-positive bacteria allows the direct contact of the hydrophobic constituents with the phospholipids bilayer of the cell membrane, where they bring about their effect, causing either an increase of ion permeability and leakage of vital intracellular constituents, or impairment of the bacteria enzyme. The antimicrobial activity of *P. chinensis* leaves methanol extract could, in part, be associated with their major constituents such as triterpenes and flavonoids. These components have been reported to display antimicrobial effects (Scortichini and Rossi, 1991; Cushnie and Lamb, 2005). The antimicrobial compounds from plant source have increasing attention in recent years. The antimicrobial activity of *P. chinensis* leaves may be due the presence of phenolics, flavonoids and terpenoids. The antimicrobial activity of the plant extract is majorly attributed to the presence of phenolic compounds. The isolated compounds from the methanol extract of *P. chinensis* leaves prove the observed antimicrobial activity. according to the literature, β-sitosterol has antimicrobial activity via an agar disk diffusion method, the antimicrobial activity ranged from 10 mm to 14 mm for *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Klebsiella pneumoniae*. In comparison with standard antibacterial compound gentamicin (20 µg/ml), it was found that β-sitosterol (20 µg/ml) has antimicrobial activity almost equivalent to the standard (Amit *et al.*, 2012). The triterpene compound (lupeol), protocatechuic acid, and trans-cinnamic acid isolated from *Solanum spirale* Roxb. Leaves showed antibacterial activity against *E. coli* with the minimal inhibitory concentration (MIC) of 250 µg/mL (Sukanya *et al*., 2012). Myricetin inhibits *Escherichia coli* DnaB helicase according to a mechanism dominated by noncompetitive behavior, myricetin inhibits *E. coli* DnaB helicase with an inhibitory concentration at 50% maximal (IC$_{50}$) of 11.3± 1.6 Mm (Mark *et al*., 2007). The ethanol extract of *Mentha longifolia* has antimicrobial activity against some clinical bacteria. The phytochemical results showed that the ethanol extract contained mainly five flavonoids identified as luteolin-7-O-glycoside, luteolin-7, 3'-O-diglycoside, apigenin, quercetin-3-O-glycoside and kaempferol-3-O-glycoside. Among these molecules, quercetin-3-O-glucoside had the highest antibacterial activity, but the synergism between apigenin, quercetin-3-O-glycoside, and kaempferol-3-O-glycoside affored the best minimal inhibitory concentration for each tested species. The substitution type of flavonoids had and impact on the antibacterial activity (Souâd *et al*., 2009).

Conclusion

This present study provided scientific evidence that *P. chinensis* leaves methanol extract has a significant antimicrobial activity and this activity may be due to the presence of the constituents isolated from the extract. As the industries tend to reduce the use of chemical preservatives in their products, *P. chinensis* leaves methanol extract with potentially active antimicrobial properties might be considered as a natural source for the maintenance or extension of the shelf life of the products. These results indicate the possibility of exploitation of *P. chinensis* leaves extract as effective inhibitor of microorganisms. This is the first report on the antimicrobial activities of *P. chinensis* extract in the literature. The isolated compounds are expected to be useful for the study of antimicrobial agents in the future.

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