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Isolation and Antimicrobial and Antioxidant Evaluation of Bio-Active Compounds from *Eriobotrya Japonica* Stems

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ABSTRACT

Purpose: The present study was carried out to evaluate antimicrobial and antioxidant activities from *Eriobotrya japonica* stems as well investigation of its chemical composition.
Methods: Methanol 80% extract of *Eriobotrya japonica* stems was tested for antimicrobial activity against bacterial and fungal strains and for antioxidant activity using oxygen radical absorbance capacity (ORAC) and the trolox equivalent antioxidant capacity (TEAC) assays and also total content of polyphenols with phytochemical analysis of the extract were determined.
Results: The results showed that the extract has a significant antimicrobial activity, it inhibited significantly the growth of *Candida albicans* suggesting that it can be used in the treatment of fungal infections, and it showed no effect on the other bacterial and fungal strains, the extract has a good antioxidant activity, it has shown high values of oxygen radical absorbance capacity and trolox equivalent antioxidant capacity, while it showed a low value of polyphenol content. Phytochemical analysis of the extract showed the presence of carbohydrates, terpenes, tannins and flavonoids, further phytochemical analysis resulted in the isolation and identification of three triterpenic acids, oleanolic, ursolic and corosolic acids and four flavonoids, naringenin, quercetin, kaempferol 3-*O*-*β*-glucoside and quercetin 3-O-α-rhamnoside.
Conclusion: These results may help to discover new chemical classes of natural antimicrobial antioxidant substances.

Introduction

Medicinal plants represent a rich source of antimicrobial agents. A wide range of medicinal plant parts is used for extract as a raw drugs and they possess varied medicinal properties. Natural products are known to play an important role in both drug discovery and chemical biology. Infectious diseases account for approximately one-half of all death in tropical countries, approximately 5 million people in Africa. Bacterial and fungal infection have been a major problem considered for decades that causes spoilage of food products and various diseases in plants and humans, which leads to significant losses in the crop productivity and health problems worldwide. The World Health Organization estimates that plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the worlds population.[1] The effect of plant extracts on microorganism have been studied by a very large number of researchers in different parts of the world.[2-5] *Eriobotrya japonica* Lindl (Rosaceae family) is a well known medicinal plant in Japan and China. In folk medicine, *E. Japonica* leaves have beneficial effects in numerous diseases including asthma, gastroenteric discorders, diabetes mellitus and chronic bronchitis.[6] Previous phytochemical studies on *E. Japonica* proved the presence of various triterpenes, sesquiterpenes, flavonoids, tannins and megastigmane glycosides in the leaves and some of these compounds have been found to possess antiviral, antitumor, hypoglycemic and anti-inflammatory activities.[7-10] The present study were undertaken to evaluate antimicrobial and antioxidant activities from stems of *E. Japonica* as well investigation of its chemical composition.

Materials and Methods

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). ^{1}H-NMR spectra and ^{13}C-NMR spectra: Varian Unity Inova 400 Varian Unity (Graz University, Austria). MS (Finnigan MAT SSQ 7000, 70 ev). Silica gel (0.063-

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0.200 mm for column chromatography) and Sephadex LH-20 (Pharmacia Fine Chemicals). Paper Chromatography (PC) Whatman No.1 (Whatman Led. Maid Stone, Kent, England) sheets**.**

Plant material

Finely ground stems of *Eriobotrya japonica* were collected from the Agricultural Research Centre, Giza, Egypt in May 2011 and the plant was identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC). A voucher specimen is deposited in the herbarium of Agricultural Research Centre, Giza, Egypt.

Preparation of Plant Extract

Air dried powder from the stems of *Eriobotrya japonica* (840 g) was extracted with methanol 80% at room temperature several times until exhaustion by maceration method. The extract was concentrated under reduced pressure to give 45 g of crude extract**.** The extract was tested for the presence of bioactive compounds according to following standard tests (Molisch 's test for carbohydrates, Shinoda test for flavonoids, forth test for saponins, Salkowski 's for terpenes and sterols, $FeCl_3$ and Mayer's reagents for detecting of tannins and alkaloids, respectively.[11-13]

Isolation of bio-active compounds from methanol extract of *E. japonica stems:* Methanol extract 42 g was subjected to silica gel column chromatography eluting with n-hexane, dichloromethane, ethyl acetate and methanol gradually. The fractions that showed similar thin layer chromatography (TLC) were collected and according to that four fractions were collected. Fraction 1 (930 mg) eluted with dichloromethane: ethyl acetate (60:40) gave compound 1 (oleanolic acid, 16 mg). Fraction 2 (1.12 g) eluted with dichloromethane: ethyl acetate (50:50) gave compound 2 (ursolic acid, 21 mg) and compound 3 (corosolic acid, 18 mg). Fraction 3 (835 mg) eluted with ethyl acetate: dichloromethane: (70:30) gave compound 4 (naringenin, 12 mg) and further elution with ethyl acetate gave compound 5 (quercetin, 8 mg). Compound 6 (kaempferol 3-*O*-*β*-glucoside, 25 mg) and compound 7 (Quercetin 3-O-α-rhamnoside, 19 mg) were obtained from fraction 4 (840 mg) with further elution of ethyl acetate and methanol. All the compounds were purified on sephadex LH–20 column using methanol and water as eluents.

Acid hydrolysis of flavonoids

Solutions of 5 mg of compounds 6 and 7 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-H_2O 4:1:5 upper layer).

Antimicrobial Assays

The quantitative assay of the antimicrobial activity was performed by broth microdilution method[14,15] in 96–well microplates in order to establish the minimal inhibitory concentration (MIC). The antimicrobial activity was tested against Gram–positive strains (*Staphylococcus aureus, Bacillus subtilis*), Gram–negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*) and fungal strain (*Candida albicans*). The methanol extract of *E. japonica* was tested for its antimicrobial activity using a qualitative screening assay of the antimicrobial properties by the adapted disk diffusion method, Kirby–Bauer method.[16] The quantitative assay of the antimicrobial activity was performed by binary microdilution method,[14] in order to establish the minimal inhibitory concentration (MIC). The antimicrobial activity of the investigated extract was tested against bacterial and fungal strains: Gram positive (*Staphylococcus aureus, Bacillus subtilis*), Gram–negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*) and fungal strains (*Candida albicans*). The microbial strains were identified using a VITEK I automatic system. VITEK cards for the identification and the susceptibility testing (GNS–522) were inoculated and incubated according to the manufacturer's recommendations. In our experiments there were used bacterial suspensions of $1.5x10^8$ UFC/ mL or 0.5 McFarland density obtained from 15–18 h bacterial cultures developed on solid media. The antimicrobial activity was tested on Mueller–Hinton medium recommended for the bacterial strains and Yeast Peptone Glucose (YPG) medium for *Candida albicans*. Solutions of the extract in DMSO (dimethyl sulfoxide) having 2048 µg/ mL concentration were used.

Qualitative screening of the antimicrobial properties of the extract

The antimicrobial activity of the extract was investigated by qualitative screening of the susceptibility spectrum of different microbial strains to the tested extract solubilised in DMSO (1 mg/mL) using adapted variants of the diffusion method. In the 1st variant, 10 µL of the extract solution were equally distributed on the paper filter disks placed on Petri dishes previously seeded "in layer" with the tested bacterial strain inoculums. In the 2nd variant, 10 µL of the tested extract solutions were placed in the agar wells cut in the solid culture medium seeded with the microbial inoculum. In the 3rd variant of the qualitative antimicrobial activity assay, 10 µL of the extract solutions were spotted on Petri dishes seeded with bacterial/yeast inoculum. In all the three variants, the Petri dishes were left at room temperature to ensure the equal diffusion of the compound in the medium or to allow the drop of solution to be adsorbed in the medium and afterwards the dishes were incubated at 37°C for 24 hours. The solvent used was also tested in order to evaluate a potential antimicrobial activity.

Quantitative assay of the antimicrobial activity

For the quantitative assay of the antimicrobial activity of the extract by the microdilution method[14,17] in liquid medium distributed in 96–well plates, binary serial dilutions of the tested extract solutions were performed. There were obtained concentrations from 1000 µg/mL to 0.97 µg/mL in a 200 µL culture medium final volume, afterwards each well was seeded with a 50 µL microbial suspension of 0.5 MacFarland density. In each test a microbial culture control (a series of wells containing exclusively culture medium with the microbial suspension) and a sterility control (a series of wells containing exclusively culture medium) were performed. The plates were incubated for 24 hours at 37°C.

Antioxidant assays: Extraction: 0.2 g of extract with 10 mL Millipore water boiled was sonic, centrifuged and filtered. Evaluation of antioxidant activity of the extract, using methods Oxygen Radical Absorbance Capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC) and determination of total polyphenols content:

Oxygen Radical Absorbance Capacity (ORAC)

This method determines peroxil radical inhibition capacity, inducing oxidation highlighting the classical radical release; H atom transfer ORAC values were reported as Trolox equivalents, is expressed as micromol TE/DW. The intensity was monitored at 485 nm and 525 nm for 35 min.

Trolox equivalent antioxidant capacity method (TEAC)

This method is based the neutralizing capacity the radical anion $ABTS^+$ [2,2'–azino–bis (3–ethylbenzothiazoline–6–sulphonic acid)] by antioxidants. ABTS is oxidized by radicals peroxil or other oxidants to its radical cation $ABTS^+$, intensely colored (λmax = 734 nm). Antioxidant capacity is expressed compounds tested as potential, to discoloration by direct reaction with it radical $ABTS^+$.

The total content of polyphenols

The blue compounds formed between phenols and Folin–Ciocalteu reagent phenolic compounds are independent of structure, thus developing complex between metal center and phenolic compounds. Absorption was recorded at a wavelength of 765 nm. Total phenol content was expressed as gallic acid equivalents.[18]

Results

The present investigation was focused to evaluate the antimicrobial activity (expressed in µg/ mL) and antioxidant capacity (expressed as Trolox equivalents and total polyphenol content) as can be seen in Figures 1 and 2, as well investigation of the presence of phytochemicals, where phytochemical analysis revealed that carbohydrates, triterpenes, tannins and flavonoids are present in the extract Table 1, estimation of the main bioactive constituents of *Eriobotrya japonica* stems MeOH extract has shown that the bioactive components of *Eriobotrya japonica* stems were three triterpenic acids, Oleanolic, Ursolic and corosolic acids and four flavonoids,, quercetin, naringenin, kaempferol 3-*O*-*β*-glucoside and quercetin 3-O-α-rhamnoside. The chemical structures of the isolated compounds as in Figure 3.

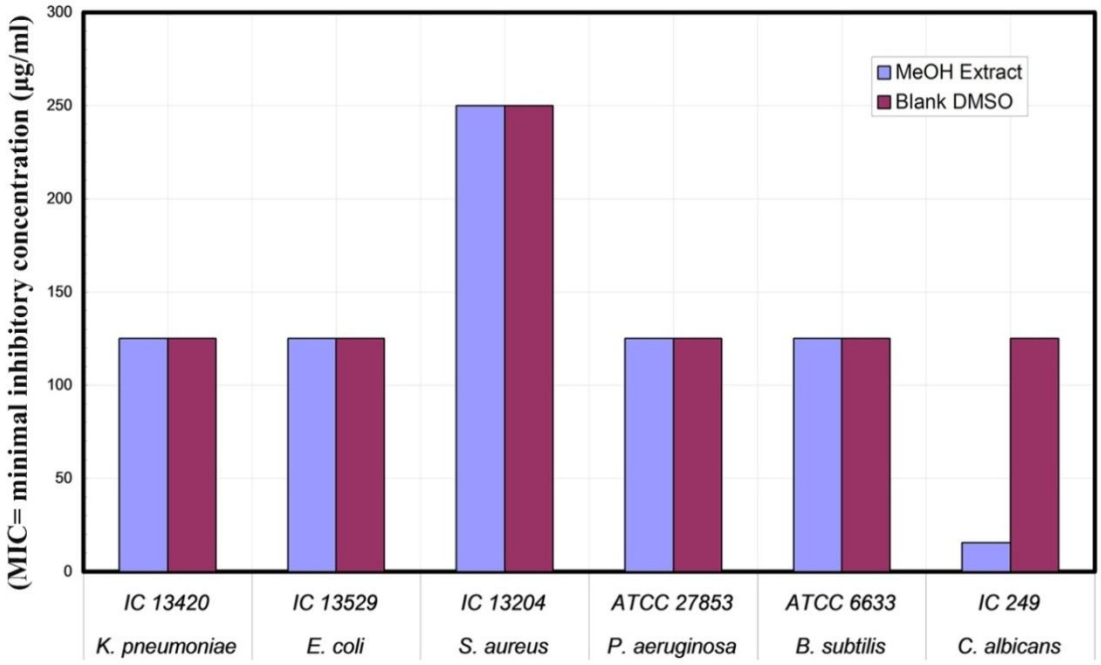

Figure 1. Antimicrobial activity of *Eriobotrya japonica* methanol extract

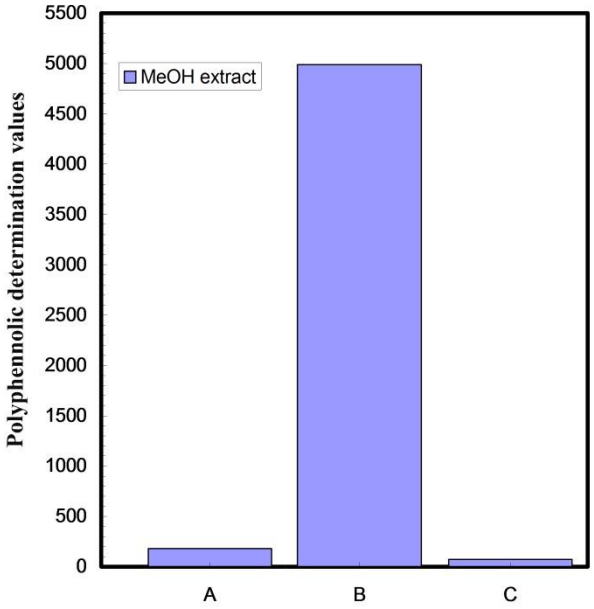

Figure 2. Antioxidant capacity of *Eriobotrya japonica* methanol extract was expressed as Trolox equivalents**. (A):** Total polyphenol content (gallic acid mg/g DW), (**B**): Oxygen radical absorbance capacity (ORAC) assay values, (**C**): Trolox equivalent antioxidant capacity (TEAC) assay values.

Table 1. Phytochemical Analysis of the *Eriobotrya japonica* methanol extract

Constituents	Methanol extract
Triterpenes and /or Sterols	+
Carbohydrates and/or glycosides	+
Flavonoids	+
Coumarins	-
Alkaloids and/or nitrogenous compounds	-
Tannins	+
Saponins	-
(+) presence of constituents, (-) absence of constituents	

Structure Elucidation of the isolated compounds

Oleanolic acid (1): White amorphous powder. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.23 (IH, t, J=3.4, H-12), 3.17 (1H, dd, J=10, 4.2 Hz, H-3), 2.74 (1H, dd, J=12.5, 4 Hz, H-18), 0.95 (3H, s, Me-23), 0.76 (3H, s, Me-24), 0.85 (3H, s, Me-25), 0.77 (3H, s, Me-26), 1.23 (3H, s, Me-27), 0.89 (3H, s, Me-29), 0.95 (3H, s, Me-30). (+) ESI-MS: m/z 455 $[M-H]^+$.

Ursolic acid (2): White powder. ^{1}H-NMR ($CDCl_3$, 400 MHz):δ 5.26 (IH, t, J=3.5, H-12), 3.17 (1H, dd, J=10, 4.2 Hz, H-3), 2.15 (1H, d, J=11.5 Hz, H-18), 1.92 (1H, dd, J=12.8, 4.2 Hz, H_b-22), 1.12 (1H, m, H_a-22), 1. 22 (3H, s, Me-23), 0.94 (3H, s, Me-24), 0.75 (3H, s, Me-25), 1.04 (3H, s, Me-26), 1.12 (3H, s, Me-27), 0.92 (3H, d, J= 6.4 Hz, Me-29), 0.89 (3H, d, *J*=5.8Hz, Me-30). (+) ESI-MS: m/z 455 $[M-H]^+$.

Corosolic acid (3): White amorphous powder. ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 4.07 (1H, ddd, *J* = 3.8, 9.4, 11.0 Hz, H-2), 3.41 (1H, d, *J* = 9.5 Hz, H-3), 5.42 (1H, br s, H-12), 2.61 (1H, d, *J* = 10.9 Hz, H-18), 1.36 (3H, s, H-23), 1.12 (3H, s, H-24), 1.09 (3H, s, H-25), 1.04 (3H, s, H-26), 1.21 (3H, s, H-27), 1.03 (3H, d, *J* =6 Hz, H-29), 0.96 (3H, d, *J* = 6.0 Hz, H-30). (^{13}C-NMR, pyridine-d_5) δ 48.58 (C-1), 69.14 (C-2), 84.35 (C-3), 40.39 (C-4), 56.46 (C-5), 19.35 (C-6), 34.9 (C-7), 41.2 (C-8), 49.5 (C-9), 39.2 (C-10), 23.7 (C-11), 126.28 (C-12), 139.85 (C-13), 43.8 (C-14), 29.5 (C-15), 25.9 (C-16), 48.65 (C-17), 54.28 (C-18), 40.22 (C-19), 40.45 (C-20), 31.58 (C-21), 38.4 (C-22), 29.7 (C-23), 19.1 (C-24), 18.6 (C-25), 17.95 (C-26), 25.2 (C-27), 181.2 (C-28), 18.24 (C-29), 21.95 (C-30). EI-MS: *m/z* 472.

Naringenin (4): Pale yellow needles, ^{1}HNMR (CD_3OD, 500 MHz,) δ 7.25 (2H, d, *J* = 8.2 Hz, H-2', 6'), 6.89 (2H, d, *J* = 8.2 Hz, H-3', 5'), 5.82 (1H, d, *J* = 2.4 Hz, H-8), 5.8 (1H, d, *J* = 2.4 Hz, H-6), 5.28 (1H, dd, *J* = 13 3.5 Hz, H-2), 3.12 (1H, dd, *J* = 17.3, 13.1 Hz, H-3a), 2.72 (1H, dd, *J* = 17.3, 3.5 Hz, H-3b). EI-MS: m/z :272.

Quercetin (5): Yellow powder. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.74 (1H, d, J = 8, 2 Hz, H-2'), 7.55 (1H, d, J = 2 Hz, H-6'), 6.92 (1H, d, J = 8 Hz, H-5'), 6.42 (1H, d, *J* = 1.2 Hz, H-8), 6.15 (1H, d, *J* = 1.2 Hz, H-6). (+) ESI-MS: m/z 303 $[M+H]^+$.

Kaempferol 3-*O*-*β*-glucoside (6): Yellow powder. UV λmax (MeOH): 266, 344; (NaOMe): 274, 327sh, 401; ($AlCl_3$): 274, 302, 349, 396; ($AlCl_3$/HCl): 274, 347, 394; (NaOAc): 274, 307, 391; (NaOAc/H_3BO_3): 267, 352. 1H–NMR (DMSO–d_6, 500 MHz): δ 8.1 (2H, d, J = 8.5 Hz, H–2',6'), 6.92 (2H, d, J=8.5 Hz, H–3',5'), 6.54 (1H, d, J = 2 Hz, H–8), 6.22 (1H, d, J = 2 Hz, H–6), 5.35 (1H,d, J=7.5, H–1``), 4–3.1 (5H,m, other sugar protons).

Quercetin 3-O-α-rhamnoside (7): Yellow crystals. ^{1}H–NMR (DMSO–d_6, 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, br s, H–1") 0.78 (3H, d, *J*=6Hz). ^{13}C–NMR (DMSO–d_6,100 MHz): 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").

Discussion

Antimicrobial activity of E. japonica stems methanol extract

For the antimicrobial qualitative methods, i.e. paper filter disks impregnated with the tested extract solution and disposal of the respective solutions in agar wells, the reading of the results was performed by measuring the microbial growth inhibition zones around the filter disks impregnated with the testing extract and around the wells, respectively. The most efficient qualitative method proved to be the direct spotting of the tested solutions on the seeded medium, the results being very well correlated with the results of the (minimal

inhibitory concentration) MIC quantitative assay. For the quantitative methods of the antimicrobial activity of the tested extract by the microdilution method in liquid medium, the MIC was read by wells observation: in the first wells containing high concentrations of extract, the culture growth was not visible, the microbial cells being killed or inhibited by the tested extract. At lower concentrations of the tested extract, the microbial culture becomes visible. The lowest concentration which inhibited the visible microbial growth was considered the MIC (µg/mL) value for the extract. In the next wells, including the standard culture growth control wells, the medium become muddy as a result of the microbial growth. In the sterility control wells series, the medium had to remain clear. From the last well without any visible microbial growth and from the first one that presented microbial growth, Gram stained smears were performed for the results confirmation. In Figure 1, there are the results of the quantitative assay of the antimicrobial activity of the *E. japonica* stems methanol extract. Our results have shown that the extract was highly active against *C. albicans*, suggesting its possible use in the treatment of fungal infections, but it *was* not active on other bacterial strains.

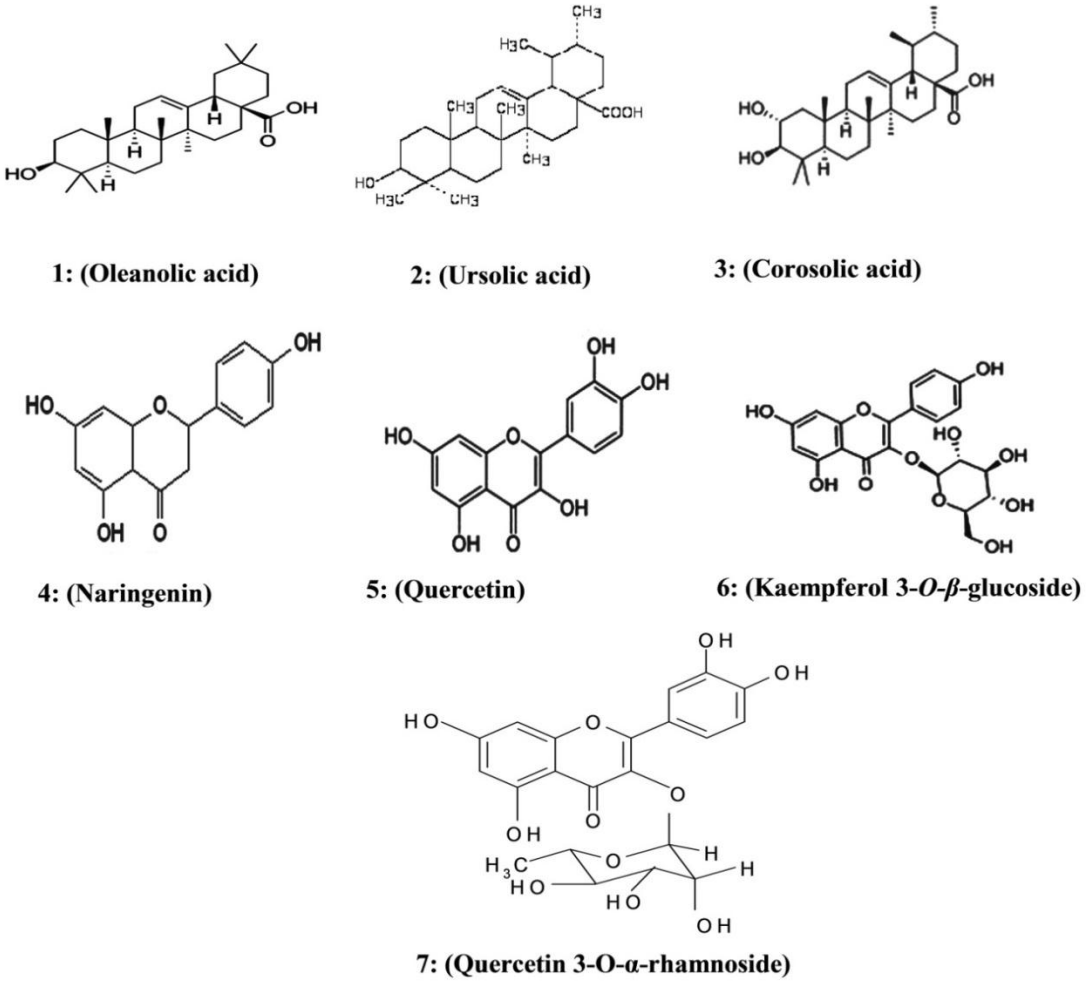

Figure 3. Compounds isolated from *Eriobotrya japonica* stems methanol extract

Antioxidant activity E. japonica stems methanol extract *and Total polyphenol content*

Antioxidant activity was evaluated by Oxygen Radical Absorbance Capacity (ORAC) and Trolox equivalent antioxidant capacity method (TEAC) assays. In (TEAC) assay, *E. japonica* methanol extract showed high TEAC value 179±6.6 mM TE/g, and also in (ORAC) assay, it showed a high ORAC value (4988 mM TE/g) (Figure 2), while it showed a low total polyphenol content 74.43 ±1.60 mg/g (Figure 2) which was expressed as gallic acid equivalents and these results suggest the antioxidant activity of *E. japonica* extract and the activity can be proved by phytochemical analysis of the extract which has shown the presence of triterpenes, flavonoids, tannins and carbohydrates (Table 1).

Identification of the isolated compounds

Chromatographic separation of *Eriobotrya japonica* stems Methanol extract resulted in the isolation and identification of compound 1 (Oleanolic acid), compound 2 (ursolic acid) and compound 3 (corosolic

acid) were monitored by TLC, and the spot of each compound was detected by heating the plates at 110°C after spraying with p-anisaldehyde–sulfuric acid, also spectral data were in agreement with published data.[19] Compound 4 (naringenin) is obtained as a deep purple spot on TLC and it gave yellow colour with AlCl3 reagent, the molecular formula, $C_{15}H_{12}O_5$, was obtained of the [M+] ion at m/z 272 in EI-MS is in agreement with naringenin chemical structure and the spectral data showed basically agreement with the reported literature of naringenin.[20] Compound 5 (quercetin) gave as yellow spot on paper chromatography (PC) under UV light and its spectral data very similar to that of Lawrence et al., 2005.[21] Compound 6 (kaempferol 3-O-β-glucoside) and 6 (quercetin 3-O-α-rhamnoside) were obtained as deep purple spots under UV light and each gave yellow colour with $AlCl_3$ reagent, Complete acid hydrolysis of compounds 6 and 7 revealed the presence of kaempferol as an aglycone and glucose as the sugar moiety for compound 6 and the presence of quercetin as an aglycone and rhamnose as the sugar moiety for compound 7. UV spectral data and NMR signals for both compounds 6 and 7 are very similar to that of compound 6 (kaempferol 3-O-β-glucoside) and compound 7 (quercetin 3-O-α-rhamnoside.[21] Antimicrobial and antioxidant activity of *Eriobotrya japonica* stems methanol extract is due to the presence of flavonoids which have shown a significant antimicrobial activity,[22,23] as well for tannins which have shown a significant antimicrobial and antioxidant activities,[24] also some isolated compounds from the extract has shown a good antioxidant activity as ursolic acid which proved a significant antioxidant activity, it has a significant DPPH radical scavenging activity at various degrees,[25] and kaempferol 3-*O*-*β*-glucoside has shown a significant antimicrobial and antioxidant activity.[26]

Conclusion

Based on our results and discussion, it can be concluded that *E. japonica* stems methanol extract possess a significant antimicrobial and antioxidant activity due to the presence of bioactive compounds as flavonoids and triterpenes and also for the isolated and identified compounds from the extract. These results also suggest that the extract could serve as potential source of bioactive compounds. Further research is needed in which the extract could possibly be exploited for pharmaceutical use.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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