

Biological activities of phenolics from leaves of Tunisian *Cydonia oblonga* Miller

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ABSTRACT

We examined the antioxidant and antimicrobial activities of quince (*Cydonia oblonga* Miller) leaf methanol extract of Tunisian variety "Commune". Chromatographic characterization of the leaf extract using HPLC-MS/MS revealed the presence of 9-phenolic acids and flavonoids. Among these polyphenols, 6-were identified, one as caffeoylquinic acid (4-*O*-caffeoylquinic acid), two as quercetin heterosides (quercetin-3-*O*-rutinoside and quercetin-3-*O*-galactoside), and three as kaempferol heterosides (kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glycoside and kaempferol-3-*O*-glucoside). The leaf methanol extract had **DPPH** (2,2-diphenyl-1-picrylhydrazyl) friiee radical-scavenging activity, inhibited the pre-formed radical **ABTS**+ (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) and also inhibited the formation of hydroxyl radical. The antimicrobial potential of quince leaf extract was also tested against 8-pathogenic bacteria by disc diffusion method (namely, *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Enterococcus faecium* (ATCC 19434), *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*).

Key words: Antimicrobial potential, antioxidant activity, bacteria, biological activities, *Cydonia oblonga*, flavonoids, methanol extract, phenolic compounds, quince, quince leaves, yeast.

INTRODUCTION

Interest in edible plants as source of natural bioactive compounds has prompted researchers to investigate their tissue chemical composition and therapeutic potential (28). Quince (*Cydonia oblonga* Miller, Family: Rosaceae) is fruit tree in Asia, central and southern parts of Europe, North Africa, Oceania and the Americas. It is traditionally cultivated in north Tunisia (23). Its fresh or processed fruits are dietary source of health-promoting compounds, due to the presence of biologically active constituents (22), which have antioxidant, antimicrobial, anti-inflammatory, anti-proliferative and anti-ulcerative properties (2,7,14,35,38,39). However, there is also increasing interest in the phenolic content and reactivity of quince non-edible aerial tissues (leaves and seeds) (27,35,39).

Quince leaves are used to prepare decoctions in traditional medicine for their sedative, antipyretic, anti-diarrheic and antitussive properties to treat skin diseases (1,13,15,27). Its vegetative parts and fruits are used in traditional Uyghur medicine (1,15), hence, Uyghur people have greater longevity and lower blood pressure than other central

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Asian populations (1). Therapeutical experiments have shown that quince leaf methanol extract has powerful neuroprotective potential against oxidative damage in rat brain (25), on blood rheology (42), hyperlipidaemia in rats (1), protects human erythrocyte membrane from hemolysis (10) and inhibits the proliferation of human colon cancer cells (Caco-2) (7). All these health-promoting properties are owing to the higher contents of phenolic compounds in Quince tissues. These compounds are present in vascular plant tissues and act as antioxidants to prevent free radical damage to proteins, carbohydrates, lipids and DNA (4,10,14,40). Despite these findings, quince leaves composition has not been studied. Hence, this study aimed to characterize the phenolic composition of quince leaves from the Tunisian variety “Commune” and to test the biological activities of leaf extracts.

MATERIALS AND METHODS

Fresh leaves of quince variety “Commune” (*Cydonia oblonga* Miller) were collected before leaf abscission (November) from 6- orchards in Borj Cédria, Tunisia (36° 44' N, 10° 21' E; altitude: 5-10 m, annual rainfall: 400-450 mm, mean temperature: 19°C). Sampling was done randomly from each tree regardless of leaf size or position. In the laboratory, these leaves were thoroughly rinsed with water and then dried at 40°C for 5 d. Air Dried leaves were ground to fine powder and stored in vacuum-sealed containers at 4°C in dark prior to analysis.

II. Extraction of phenolics

Prior to methanol extraction, lipids were first extracted from the powdered leaves. For this, 30 g dry powdered leaf material was extracted with 200 mL boiling HPLC-grade n-hexane (Merck, Germany) for 24 h in Soxhlet apparatus. Thereafter leaf material was air-dried and re-extracted with 100% HPLC-grade methanol (200 mL) (Merck, Germany) under the same experimental conditions. The methanol extract was filtered through Whatman No. 1 filter paper, then evaporated to dryness at 40°C in rotary evaporator (Heidolph Laborota 4001, Germany). The residual dry extract was weighed and re-dissolved in methanol at 1 mg/mL concentration prior to analysis.

III. Chromatographic analysis

The phenolic compounds in methanol extract were identified using high-performance liquid chromatography (Agilent 1100, USA) with diode-array detector (HPLC-DAD) coupled to an on-line tandem mass spectrometer (MS/MS) (Waters Micromass Quattro Ultima, USA) fitted with a silica-based reversed phase C18 column (Agilent Superspher 100 RP-18). Mobile phase was ultra-pure water containing 0.1% acetic acid (phase A) and HPLC-grade acetonitrile (phase B) with the elution gradient (%A:%B): 98:2 (0-60 min); 60:40 (60-80 min); 0:100 (80-90 min) and 98:2 (90-110 min). Sample injection volume was 30 µL and oven temperature was constant at 30°C. Data were acquired and analyzed using Masslynx 4.0 software (Waters, USA) after electrospray ionization (ESI).

IV. Total phenols and flavonoids content

The total concentration of phenolic compounds in quince leaves was determined using the Folin-Ciocalteu method. For this, 0.5 mL of the extract solution (1 mg/mL) was mixed with 5 mL of Folin-Ciocalteu reagent (10%) and 4 mL Na_2CO_3 (1 M) at pH 10. The mixture was shaken for 15 min at room temperature in dark and absorbance was determined at 765 nm. Total phenolics contents in the leaf extract were determined with gallic acid (GA) as the reference standard. Results were expressed as mg GA equivalent per g air dry weight (mg GAE/g dw) (21).

Total flavonoids content in the methanol extract was determined by mixing 0.5 mL extract with 1.5 mL methanol, 0.1 mL $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (10%), 0.1 mL $\text{C}_2\text{H}_3\text{NaO}_2$ (1 M) and 2.8 mL of ultra-pure water as per Pourmorad *et al.* (30). The mixture was shaken vigorously and then left to react for 30 min in dark. The absorbance was determined at 430 nm and results were expressed as mg quercetin equivalent per g air DW (mg QE/g dw).

V. Antioxidant activities of leaf extract

The antioxidant properties of leaf methanol extract were measured by colorimetric methods and compared with the reactivity of two synthetic antioxidants: butylated hydroxytoluene (BHT) or ascorbic acid (26). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, was used as standard to compare between the antioxidant activities of leaf extract and BHT by defining their relative Trolox Equivalent Antioxidant Capacity (TEAC) expressed in mmol TEAC/g extract (17).

Antioxidant properties of quince leaves were also evaluated using *5-in-vitro* assays: total antioxidant activity (TAA) (31), ferric reducing ability of plasma (FRAP) (5), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (5), reduction of pre-formed radical monocation 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS^{•+}) (32) and hydroxyl radical inhibition (HRI) (16).

VI. Antimicrobial activity of leaf extract

Antimicrobial activity of quince leaf extract was evaluated using the disc diffusion method using sterilized Mueller-Hinton agar (MHA), (9). In this investigation, 8-pathogenic bacterial strains [*Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Enterococcus faecium* (ATCC 19434), *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Bacillus subtilis*] were used as test microorganisms. These strains were obtained from the culture collection, Pasteur Institute, Tunis and were cultured on a tryptic soy **broth** (TSB) medium. Then, these strains were evenly spread onto MHA medium (20 mL/Petri dish) at $\sim 10^8$ CFU/mL concentration.

The antimicrobial activity of leaf extract dissolved in dimethyl sulfoxide (DMSO) (10 μL /sterile disc) was compared against ampicillin (GRiSP, Poland). For each bacterial strain, one ampicillin- and 3-extract-impregnated discs at 1 mg/mL each were used along with negative controls (containing only DMSO). After 24-h incubation at 37°C, clear zones surrounding the discs were measured in mm.

VII. Statistical analysis

All the analytical procedures described in this study were carried out in triplicate and the data was processed using STATISTICA 8.0 software (StatSoft Inc., USA). ANOVA with Student's *t*-test at $P \leq 0.05$ was applied to compare the activities of quince leaf extract with synthetic bioactive compounds. Half maximal 50% inhibitory concentrations (IC_{50}) of quince leaf extracts and BHT were calculated using Origin 6.0 (Microcal Software Inc., USA).

RESULTS AND DISCUSSION

Leaf extract characterization

The cleanup procedure was important to remove lipids from the powdered quince leaves as they contain more than 36 fatty acids and main was palmitic acid (up to 3.5 mg/g dw (11)). The chromatographic profiling using HPLC-MS/MS detected 9-compounds in quince leaf methanol extract (Fig 1). Based on technical data for each peak, these 9-compounds could be divided into two classes: (i). Phenolic acids (C1, C7, C8 and C9) and flavonoids (C2, C3, C4, C5 and C6) Six compounds could be identified corresponding to one phenolic acid (C1: 4-*O*-caffeoylquinic acid) and five flavonoids (namely, C2: quercetin-3-*O*-rutinoside, C3: quercetin-3-*O*-galactoside, C4: kaempferol-3-*O*-rutinoside, C5: kaempferol-3-*O*-glycoside and C6: kaempferol-3-*O*-glucoside). The Quercetin-3-*O*-rutinoside (rutin) is major phenolic compound in Tunisian quince leaves (C2 in Fig. 1). The rutin is present in high concentration (36%) in Tunisian quince fruit peels (14).

The information about the characterization of quince leaf extract is limited (19). Oliveira, (27) using HPLC reported the phenolic profile of Portuguese quince leaves and found 9-compounds, including those identified in this study. The remainder compounds matched exactly with 3-non-identified phenolic acids, (3,5-*O*-dicaffeoylquinic acid, 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid) (Fig 1). 5-*O*-caffeoylquinic acid) was the major compound (36.2%), followed by quercetin-3-*O*-rutinoside (21.1%) and kaempferol-3-*O*-rutinoside (12.5%).

Most research on quince leaf characterization is from Portugal. The 5-*O*-caffeoylquinic acid is major compound in the leaves and its concentration ranged from 10.7 mg/g (7) to 15.7 mg/g (10). The geographical origin and the leaf sampling season influences the phenolics composition in Portugal (27). Costa *et al.* (10) identified only 6-phenolic compounds (3-phenolic acids and 3-flavonoids) in methanol extracts of quince leaves in the same geographical area. The presence of 5-flavonoids in leaf methanol extract (Fig 1), is not surprising, since such compounds act as UV filter, protecting the fragile cell structures (such as chloroplasts) from photo-oxidation damage reactions (7,10).

Quantification of total phenolics and flavonoids in methanol extract revealed that their contents were 52.52 and 4 mg GAE/g dry leaves, respectively. Using comparable analytical procedures, Yilmaz and Seyhan (41) reported that Turkish quince leaf methanol extracts contained 14.9 mg GAE/g. and Oliveira *et al.* (27) reported that total phenolic

content was 10.3 mg/g. In their comparative study, Teleszko and Wojdylo (39) found that the highest concentration of total polyphenols (89.6-175.4 mg/g dw) was in Polish quince leaves. These results suggest that the quince leaves are important source of bioactive constituents.

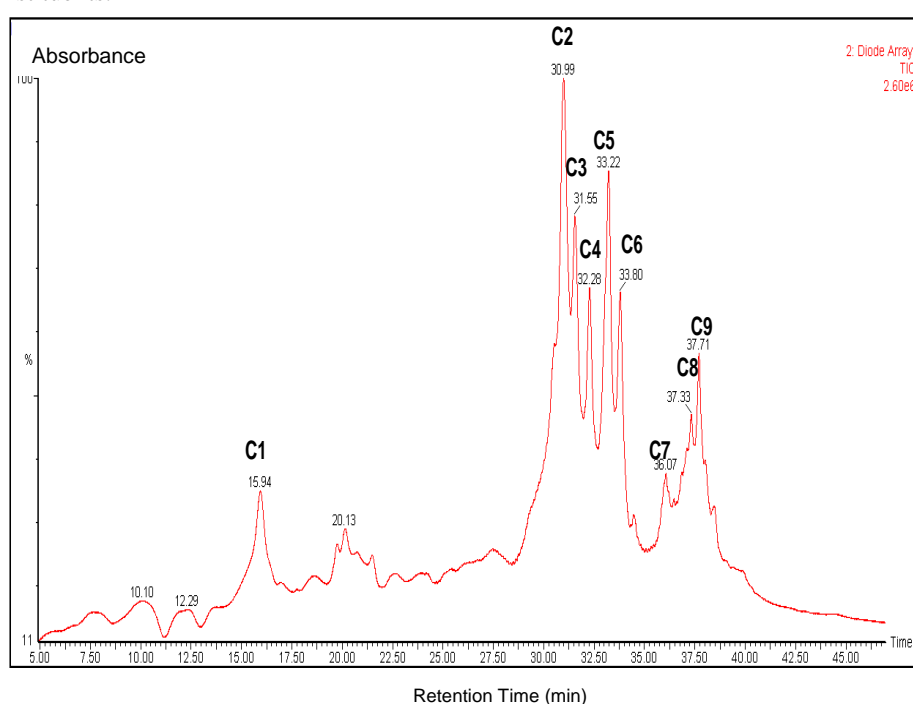


Figure 1. Phenolic profile of quince leaf methanol extract using HPLC-MS/MS. Detector was operated at 250-354 nm. C1: 4-O-caffeoylquinic acid (Phenolic Acid, PA); C2: Quercetin-3-O-rutinoside (Flavonoid, F); C3: Quercetin-3-O-galactoside (F); C4: Kaempferol-3-O-rutinoside (F); C5: Kaempferol-3-O-glucoside (F); C6: Kaempferol-3-O-galactoside (F); C7: n.d. (PA); C8: n.d. (PA); C9: n.d. (PA).

As compared to the composition of quince edible parts (whole fruit, pulp or peel), the total phenolics content in leaves is higher (27,36,39). The Spanish quince peels contain 5.81 mg GAE/g total polyphenols (38), 9-folds lower than in the leaves from the current study. The total phenolic content of the pulp and peel in Tunisian quince fruits was 0.42 and 1.31 mg/g fresh weight, respectively (14). The Indian quince pulp and peel contains still lower contents, 0.67 and 0.97 mg GAE/g fw respectively (24). The quince liquors prepared from whole fruits had about 1.4 times more phenolic compounds than liquors prepared from only pulp (6). This further confirms that the quince leaves contain significantly higher concentration of phenolic compounds than fruits and this is independent of geographical origin and distribution.

Antioxidant activities

Antioxidant assays may be broadly classified as single electron transfer- (SET) and hydrogen atom transfer- (HAT) based assays. SET assays are much easier to measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour upon reduction (29). In this regard, TAA and FRAP assays are based on the reduction of Mo(VI) to Mo(V) and Fe^{3+} to Fe^{2+} , respectively, with subsequent formation of bluish coloured solution under acidic conditions. Thus the antioxidant capacity of quince leaf extract is correlated to colour intensity of the reaction mixture. Comparison to synthetic antioxidants was carried out using comparable concentration range.

(i). **TAA:** TAA (Total antioxidant activity) is used to evaluate the total antioxidant capacity of plant extracts (17). Quince leaf extract showed a concentration-dependent generation of Mo(V), but was consistently lower than ascorbic acid, which is a mild reducing agent and a synthetic antioxidant (Fig. 2).

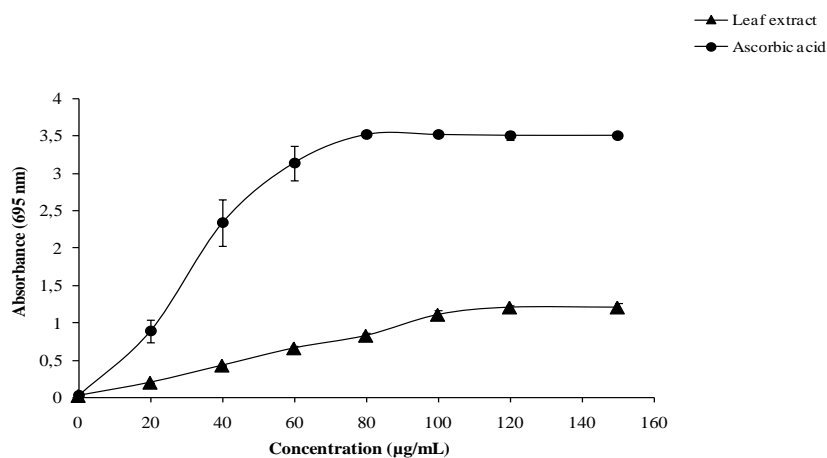


Figure 2. Total antioxidant activity (TAA) of quince leaf extract and ascorbic acid. Values are means of triplicate measurements. At the highest exposure concentration, absorbance means with different lowercase letters are significantly different at $P \leq 0.05$.

In fact, at the highest exposure concentration (150 $\mu\text{g/mL}$), Mo(VI) reduction was 3-folds higher in the presence of ascorbic acid than the methanol extracts. In contrast, Alesiani *et al.* (2) observed that quercetin, rutin and three phenylpropanoid esters isolated from the quince peels had greater TAA than α -tocopherol and ascorbic acid. Similar observations have also been reported for water/methanol extract of medicinal plant *Kalanchoe pinnata* (29).

(ii). **FRAP:** FRAP activity (Ferric reducing ability of plasma) was also measured for leaf extract and results were compared to that of BHT (Butylated hydroxyl toluene). A concentration-dependent increase in ferric reduction was observed for the leaf extracts

but, as with ascorbic acid, BHT exhibited 2-folds higher reducing capacity (Fig. 3). Teleszko and Wojdyło (39) reported that quince leaves have highest FRAP activity of 65.25 mmol TEAC/100 g dw than antioxidant potential of leaves and fruits from 6-plant species (cranberry, apple, chokeberry, Japanese quince, bilberry and blackcurrant). Mir *et al.* (24) reported out that Indian quince peel had the highest FRAP value (1.16 μM), followed by pulp (1.12 μM), seeds (0.98 μM) and mucilage (0.63 μM). This is likely due to the fact that dry quince fruit peels have higher phenolics content and different composition (14,24,27,36). Liquors made from whole fruits also had 3-folds higher FRAP activity than those made with peeled quince (6).

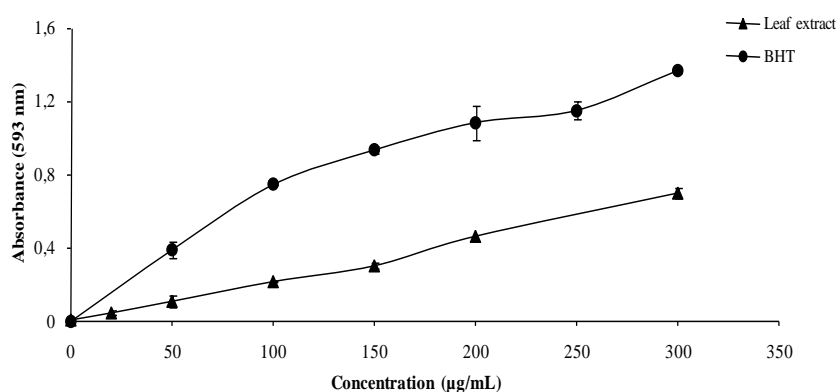


Figure 3. Ferric reducing ability of plasma (FRAP) of quince leaf extract and BHT. Values are means of triplicate measurements. At the highest exposure concentration, absorbance means with different lowercase letters are significantly different at $P \leq 0.05$.

(iii). **DPPH:** DPPH (2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging) activity is used as screening method to test the anti-radical potential of a variety of compounds (34). Hence, it is most used antioxidant assay for different quince tissues. Both the quince leaf extract and BHT presented a concentration-dependent antiradical activity up to 120 $\mu\text{g/mL}$ (Fig. 4). This was reflected by comparable IC_{50} values for DPPH activity at $P \leq 0.05$ (38.4 and 36.5 $\mu\text{g/mL}$, respectively). Costa (10) investigated the DPPH free radical-scavenging capacity in 12-healthy quince leaf samples collected from various seasons and regions of Portugal and reported that IC_{50} values ranged between 14.5 and 27.6 $\mu\text{g/mL}$ with mean of 21.6 $\mu\text{g/mL}$. This is higher than the antiradical activity observed in Tunisian quince leaves but lower than green tea (12.7 $\mu\text{g/mL}$) and ascorbic acid (8.1 $\mu\text{g/mL}$) (10). On the other hand, methanol extracts of quince edible pulp and peel showed IC_{50} values of 600 and 800 $\mu\text{g/mL}$ for DPPH activity, respectively, while seed extracts had much lower antioxidant potential (12.2 mg/mL) (22). This is, nonetheless, far lower than he reported antiradical activity of leaf extracts. We also noted comparable TEAC values for DPPH activity of leaf extracts and BHT: 1.25 and 1.20 mmol TEAC/g dw, respectively. Yilmaz and Seyhan (41),

found even higher DPPH activity for methanol extract of Turkish quince leaves (5.65 mmol TEAC/g dw). In their study on Tunisian quince fresh fruits, Fattouch *et al.* (14) found higher TEAC values for peel than for pulp: 4.27 against 3.33 g TEAC/100 g fw, respectively. Liquors made from whole fruits from different cultivars had higher DPPH activity (up to 16 mmol TEAC/100 mL) than those made from pulp (6). Silva *et al.* (35,36) suggested that hydroxycinnamic acid derivatives (phenylpropanoids) are likely to be the principle components involved in the DPPH free radical-scavenging activity. The antioxidant effects of caffeoylquinic acids (C1, C7, C8 and C9) can be explained by the presence of a catechol group, which confers stability to phenoxyl radicals by participating in electron delocalization. Additionally, the conjugated double bond in the side chain of catechol group is likely to further stabilize the putative phenoxyl radical and thus, enhance the antiradical activity (10,35,36).

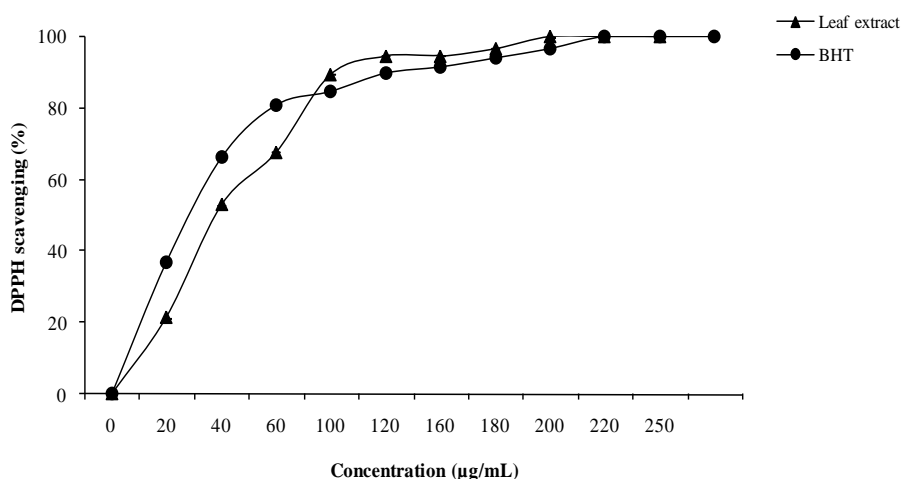


Figure 4. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of quince leaf extract and BHT.

Values are means of triplicate measurements. Half maximal 50% inhibitory concentrations (IC₅₀) of quince leaf extracts and BHT were calculated using Origin 6.0 software.

The inhibition of the pre-formed radical mono-cation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) is illustrated in Fig 5. As for DPPH activity (Fig. 4), quince leaf extracts and BHT reduced ABTS^{•+} in a dose-dependent manner until 100% inhibition was seen at 500 µg/mL. However, BHT effect was slightly stronger within this concentration range, yielding an IC₅₀ value of 128.2 µg/mL than leaf extract (161.4 µg/mL). In addition, there was no significant difference in their TEAC (0.73 and 0.87 mmol/g dw, respectively). Slightly higher values were reported by Teleszko and Wojdyło (39) for Polish quince leaves (1.16 mmol TEAC/g dw), which are considered the most active among all plant species studied. In their investigation on plant species of the genus *Chaenomelese*, a close relative of quince, Wu *et al* (12) reported strong ABTS^{•+}

activity in fruits, with values ranging from 0.19 to 0.31 mmol TEAC/g fw. As reported for DPPH activity, the presence of peels in the quince fruit liquors perhaps conferred a higher ABTS^{•+} activity (up to 3.35 mmol TEAC/100 mL) than liquors made from pulp alone (6).

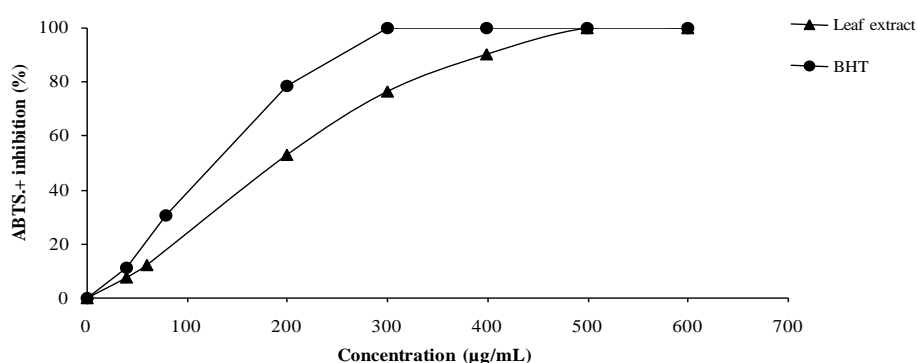


Figure 5. ABTS^{•+} (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) reducing activity of quince leaf extract and BHT. Values are means of triplicate measurements. Half maximal 50% inhibitory concentrations (IC₅₀) of quince leaf extracts and BHT were calculated using Origin 6.0 software

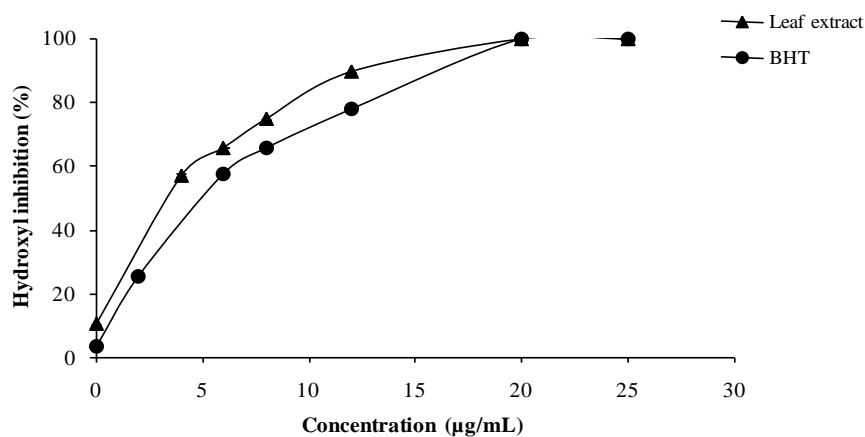


Figure 6. Hydroxyl radical inhibition (HRI) activity of quince leaf extract and BHT. Values are means of triplicate measurements. Half maximal 50% inhibitory concentrations (IC₅₀) of quince leaf extracts and BHT were calculated using Origin 6.0 software.

Hydroxyl radical ([•]OH) is the most reactive species among the reactive oxygen species and can induce severe damage to adjacent biomolecules such as proteins, DNA and lipids (20). Quince leaf extracts showed significantly higher hydroxyl radical inhibition (HRI) activity than BHT as reflected by their corresponding IC₅₀ values (2.07 and 5.48 µg/mL, respectively) (Fig. 6). We also noted low but similar TEAC as compared to the

reactivity of Trolox (0.2 and 0.17 mmol TEAC/g dw for leaf extracts and BHT, respectively). This HRI activity is more significant than that reported for fruit ethanol extract of medicinal plant *Cassia fistula* ($IC_{50} = 609 \mu\text{g/mL}$) (33), confirming the rationale for the historical therapeutic use of quince leaves (1). Indian quince fruits showed the following HRI activities for different tissues: peel (34.2%) > pulp (28.5%) > seeds (24.6%) > mucilage (14.2%); and this was highly correlated with tissue phenolic content (24).

Antibacterial activity

The clear inhibition zones surrounding discs impregnated with leaf extract were observed (Table 1) with only 3-pathogenic bacteria viz., *Enterococcus faecium* (ATCC 19434), *Bacillus subtilis* and *Streptococcus agalactiae* (group B *Streptococcus*). However, the antimicrobial activity was lower than ampicillin added at the same concentrations as leaf extract (1 mg/mL in DMSO). Very few have studied the antimicrobial activity of quince leaf extracts and the results are mixed. For instance, Cerempei *et al.* (8) did not notice any inhibitory effects of quince leaf extract on *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922), while Silva and Oliveira (37) observed significant effect on *S. agalactiae* (22-26 mm) but no inhibition of *S. aureus* and *E. coli*. Quince leaf ethanol extract at 200 mg/mL concentration or higher are inhibitory to fungus *Aspergillus niger* (3). In contrast, Tunisian quince pulp and peel extracts showed significant antimicrobial activity against *S. aureus* ATCC6538 and *Pseudomonas aeruginosa* but weak activity against *E. coli* (ATCC 8739), *Salmonella sp.*, *A. niger* and the yeast *Candida albicans* (14).

Table 1. Effects of quince leaf extract and ampicillin on microbial growth

| Bacteria | Halo diameter (mm) | |
|--|----------------------|------------------------|
| | Ampicillin (1 mg/mL) | Leaf extract (1 mg/mL) |
| <i>Salmonella typhimurium</i> ATCC 14028 | 27 | - |
| <i>Escherichia coli</i> ATCC 6538 | 22 | - |
| <i>Enterococcus faecalis</i> ATCC 29212 | 27 | - |
| <i>Enterococcus faecium</i> ATCC 19434 | 45 b* | 11 a |
| <i>Staphylococcus epidermidis</i> | 22 | - |
| <i>Staphylococcus aureus</i> | 29 | - |
| <i>Streptococcus agalactiae</i> | 30 b* | 11 a |
| <i>Bacillus subtilis</i> | 44 b* | 10 a |

*Mean halo diameters followed by different lowercase letters (a or b) are significantly different at $P \leq 0.05$. - :No inhibition

In general, the Gram positive bacteria appear to be more sensitive to bactericidal polyphenols than Gram negative species (14). Karar *et al.* (18) proposed that this difference is due to repulsion between the phenolics and the surfaces of Gram negative bacteria, which contain lipopolysaccharide.

CONCLUSIONS

Antioxidant and antimicrobial properties of quince leaves extract as determined in this study, showed that the quince leaves may serve as rich and economic alternative

source for health promoting compounds. Disparities between the antioxidant and antimicrobial activities reported in literature are due to variability in quince leaf composition, owing to the cultivars, growing region and sampling date. The leaves of Tunisian variety “Commune” have a unique composition distinct from the leaves of other regions.

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