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Original Article

Essential Oil Composition, Antioxidant Activity, Phenolic Compounds, Total Phenolic and Flavonoid Contents from Pomace of *Citrus aurantifolia*

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Abstract

The massive pomace of *Citrus aurantofolia* (Christm.) Swingle exhibits a challenging losses exposure difficulty for the processing industries. The resent study was aimed to evaluate the composition and quality of pomace and understand its bioactive compounds to introduce it to different industries such as pharmaceutical, food, medicinal, agricultural etcetera for optimum use. Volatile compounds (VCs) were extracted by water distillation (WD) and Static headspace (SHS) methods. Four different extracts were analyzed for polyphenol compounds identified by HPLC-DAD, total flavonoid content (TFC), total phenolic content (TPC) and antioxidant activity. Limonene (71.7%), β-pinene (8.5%), γ-terpinene (7.3%), myrcene (1.8%) and α-pinene (1.7%), were the main compounds of essential oil (EO) of *C. aurantifolia* pomace by HD extraction. The dominant VCs of *C. aurantifolia* were limonene (49.3%) followed by β-pinene (21.7%), γ-terpinene (9%), α-pinene (6.8%) and sabinene (4.9%), respectively by static headspace analysis. *Trans*-ferulic acid was major polyphenol compound in all studied extracts of *C. aurantifolia* marcs. Methanolic extracts from *C. aurantifolia* pomaces represented higher antioxidant activity, TPC and TFC than other investigated extracts. This studied marc can be introduced to different industries as a source of bioactive and medicinal natural compounds because of its valuable phytochemical characterization.

Keywords: *Citrus aurantifolia*, Phenolic compound, DPPH, Total flavonoid, Total phenol

Introduction

Lemons and limes are cultivated in many countries all over the world. Lemons grow in regions with temperate summers and mild winters, particularly in Mediterranean countries, southern California, and Argentina, whereas limes grow in hot subtropical or tropical regions such as southern Florida, India, Mexico, Egypt, and the West Indies. According to the classification by Swingle [1], lemons and limes belong to two species: *Citrus limon* (L.) Burm. and *Citrus aurantifolia* (Christm.) Swingle (family Rutaceae). *C. aurantifolia* (Persian lime) is a native plant that greatly dispensed in south of Iran. It is the most widespread significant cropped and consumed lime species in Iran. The lime tree has elongated and sometimes round fruits with a thin, yellowish green skin. Its branch is tall and its leaves are matte and elliptical.The color inside the fruit is also slightly greenish and its taste is sour. The content of the seeds are bitter [2]. The most serious commercial fruit crop which is grown in the entire of the world is *Citrus* fruit. It is a major fount of bioactive compounds including pectins, phenolic and flavonoids compounds and ascorbic acid [3]. Three types of flavonoids including flavanones, flavones and flavonols arise in *citrus* fruit [4]. Eriocitrin, naringin, narirutin and hesperidine are the major flavonoids found in *citrus* species [5]. The most palmate remarkable produced and used lime species in Iran is *C. aurantifolia*. Because of its antioxidative activity, this fruit is found as a healthy fruit [6]. In traditional medicine, *C. aurantifolia* is used as a mosquito bite repellent, anthelmintic, antiseptic, for coughs and sore throats, colds, digestive and appetite

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stimulant, arthritis, headache, diuretic, astringent, antiscorbutic, stomach ailments and tonic [7]. Former studies of *C. aurantifolia* have shown terpenoids, coumarins and flavonoids [8,9]. Extraction of solvent is more mostly applied for the extraction of secondary metabolites with distinct polarity, such as phenolic compounds, tannins, flavonoids, terpenoids etc. Various extraction methods have been used for the extraction of phenolic compounds using solvents with different polarities, like for example petroleum ether, ethyl acetate, water and methanol [10]. After pressing of fruits for juice or oil, the solid remains are pomace or marc. It includes the stems, seeds, pulp and skins of the fruit. To our knowledge, this is the first report on the phytochemistry of pomace from *C. aurantifolia* (christm) swingle. All of previous researches were considered the essential oil, volatile compounds and other phytochemistry studies on different parts of fresh or dried of *C. aurantifolia. Citrus* pomaces represent enormous waste from their processing. Pomace, rind, skin and seed of the fruits are the waste materials. They contain well founts of possibly worth bioactive compounds, like oils, enzymes, vitamins, dietary fibers, polyphenols and carotenoids. These phytochemicals may be used in various industries such as the textile and chemical industries, the health industry for pharmaceuticals and medicines and the food industry, for the advancement of enriched or functional foods [11]. During the *Citrus* juice processing, about 40 to 50 percent of the products were retained [12]. It is possible to keep the waste of this crop one hundred thousands of tones were estimated [12]. What remains of fruit juice industry contains all parts of the fruits which are dumped as waste. After pressing of fruits for juice or oil, the solid remains are pomace or marc. It includes the stems, seeds, pulp and skins of the fruit. Unfortunately, these valuable substances, such as fruit pomaces, are unknown in different countries like Iran. Therefore, we decided to investigate volatile compounds (VCs) by water distillation (WD) and static headspace (SHS) methods, total flavonoid content (TFC), total phenolic content (TPC), antioxidant activity and phenolic compounds from *C. aurantifolia* marc for better and optimum applications.

Material and Methods

Plant Materials

Pomaces of *C. aurantifolia* were gathered from a juice factory in Jahrom, Fars province (south of Iran) in October 2018. Pomaces were sliced into small chunks and shaded at room temperature (20-25 °C) for 3 days. Pomaces of *C. aurantifolia* were subjected to WD and SHS to extract essential and volatile oil. In another step, we used 4 extraction methods included, 60 °C water, distilled water, methanol and methanol 80%. Then, we performed four experiments included TPC by Folin-Cicocalteu method, TFC with a colorimetric assay, polyphenol by HPLC analysis and antioxidant capacity via DPPH assay to investigate phytochemistry of *C. aurantifolia* marc and to find the highest values of antioxidant capacity, TPC and TFC in the solvent systems investigated. Each experiment repeated three times.

Standards and Chemicals

Homologous series of C_8-C_{25} n-alkanes, were purchased from Sigma (Sigma–Aldrich, Steineheim, Germany). Folin-Ciocalteu's phenol reagent was provided from Merck (Darmstadt, Germany). Methanol and reference standard of 17 polyphenols (gallic acid (≥99%; Sigma-Aldrich), catechin hydrate (90%; Fluka), caffeic acid (≥98%; Sigma-Aldrich), chlorogenic acid (≥95%; Sigma-Aldrich), quercetin (≥ 95%; Sigma-Aldrich), hesperidin (≥ 80%; Sigma-Aldrich), coumarin (≥99%; Merck), sinapic acid (≥98%; Sigma-Aldrich), trans-ferulic acid (99%; Sigma-Aldrich), vanillin (99%; Sigma-Aldrich), p-coumaric acid (≥98%; Sigma-Aldrich), eugenol (99%; Sigma-Aldrich), hesperetin (≥80%; Sigma-Aldrich), ellagic acid (≥95%; Sigma-Aldrich), rosmarinic acid (≥98% ; Sigma-Aldrich), thymol (≥98.5%; Sigma-Aldrich) and carvacrol (98%; Sigma-Aldrich) were collected for HPLC grade analysis. Deionized water was provided using Millipore Direct-Q UV. All other reagents were of analytical grade.

EO Extraction by Water Distillation (WD)

The EO of pomace of *C. aurantifolia* (60 g) was isolated by WD for 3 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia [13]. The distillated oil was dried over anhydrous sodium sulfate and stored at refrigerator (4 °C), until analysis by GC-FID and GC/MS.

The Static Headspace (SHS) Volatiles Extraction

Based on our previous work [14], the SHS analysis was performed on the Combi-PAL model type (CH-4222 Zwingen, Switzerland). 1 gram of pomace with 1 µL of n-heptadecane including n-hexadecane (as internal standard, 200 ppm) were put in a headspace vial. The incubation temperature was at 85 °C for 15 min, the temperature of the sampling needle was 90 °C, fill speed was 150 µL/s and the injection volume was 1000 µL. Peak area in the GC-MS chromatogram relative to that of the internal standard was used to calculate the response.

Volatile Compounds (VCs) Analysis Procedure

EO extracted by WD method were analysed by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Analytical GC was carried out in a gas chromatograph (Agilent, Model 7890A,G3440A), equipped with a flame ionization

detector (FID), an autosampler (Agilent, Model 7683B), Agilent HP-5 fused silica column (5% phenyl methyl polysiloxane), 30 m × 0.32 mm i.d., film thickness 0.25 µm, and a Agilent ChemStation software system. Oven temperature program was 60–210 °C at the rate of 4 °C/minutes, which was then programmed to 240 °C at the rate of 20 °C/minutes, and finally, held isothermally for 8.5 minutes. Another parameters were as: injector temperature, 250 °C;carrier gas, Nitrogen at 1.0 ml min^{-1}; splitting ratio, 1:50 and detector temperature, 280 °C.The SHS analysis were carried out with an Agilent model 7890-A series gas chromatography and Agilent model 5975-C mass spectrometry coupled with a multipurpose CTC Combi Pal sampler. Separations were performed using HP-5 MS capillary column (phenyl methyl siloxane, 30 m × 0.25 mm i.d × 25 µm). The GC oven temperature was programmed from 60 ºC to 210 ºC at 3 ºC/min, and 210-240 ºC at 20 ºC/min (8.5 min isothermal). Helium was the carrier gas at 1.0 mL/min in constant flow mode. The injector temperature was 280 ºC, and the split ratio was 1:50. Over 50-550 amu with an ionizing voltage of 70 eV, the quadrupole mass spectrometer was scanned. According to the method using n-alkanes (C_8-C_{25}) as standard, the retention indices for the whole ingredients were characterized. The compounds were specified by retention indices (RI, HP-5) comparison with those shown in the literature and by comparison of their mass spectra with the Wiley, Adams and Nist Library data published mass spectra data [15-17].

Preparation of Crude Extract

The definite weight of powdered pomaces extracted using maceration technique by soaking them in methanol, distilled water and methanol 80% (1:10) for about 24 hours and also were immersed in distilled water and placed in a water bath at 60 °C for 1 hr, then filtrating for injecting to HPLC for polyphenolic determination. The crude extracts were concentrated in vacuo at 40 °C by rotary evaporator for antioxidant activity analysis, total phenol and total flavonoid content.

HPLC Analysis

To achieve maximum sensitivity and separation, gradient elution was selected. The elution was done with altering the proportion of solvent A (formic acid 1% in deionized water) to solvent B (methanol (v/v)) as follows: methanol: formic acid 1% (10:90), at zero minute; methanol: formic acid 1% (25:75), at ten minutes; methanol: formic acid 1% (60:40), at twenty minutes and methanol: formic acid 1% (70:30) at thirty minutes, which was retained isocratic up to forty minutes. HPLC analysis was performed on an Agilent 1200 series, provided with a Zorbax Eclipse XDB-C18 column (4.6 (ID) × 150 mm, 5µm (film thickness), RP), and a photodiode array detector (PDA). Elution was monitored at 280 and 320 nm. The column temperature was 30 ºC. The injection volume was 20 µL and it was accomplished automatically by autosampler [18].

DPPH Assay

According to our previous work [18], the antioxidant activity of extracts and the standard antioxidant (gallic acid) were discerned founded upon radical scavenging effect of the stable DPPH (1,1-Diphenyl-2-picrylhydrazyl) free radical. By using a micro-plate reader model biotek ELx808, the DPPH radical inhibition was considered at 515 nm. By Matlab software, the IC_{50} of each sample (concentration in µg/ml required to inhibit DPPH radical formation by 50%) were assessed.

Determination of Total Phenolic

According to the Folin-Cicocalteu method, total phenolic content (TPC) is determined. 500 µl of each extracts were mixed with 2 ml sodium carbonate solution (7.5%) and 2.5 ml Folin- Cicocalteu's (10%) reagent (FCR). For sixty minutes, the mixture was kept at room temperature. Then, by spectrophotometer (Lambda 950, Perkin-Elmer, USA) the absorbance of the samples was read at 765 nm. For the preparation of calibration curve gallic acid solutions (0.0093, 0.0187, 0.0375, 0.075, 0.15 mg L^{-1}) were used (Fig. 1a). For each analysis, the samples were belayed in triplicate. The phenolic content was expressed as mg Ga /g extract [19].

Total Flavonoid Assay

With an aluminium chloride and colorimetric assay, the total flavonoid content (TFC) was determined. The sample contained 1.5 ml of solution of the extract in the concentration of 100 mg/L and 1.5 ml of 2% $AlCl_3$ solution dissolved in methanol. At room temperature, the samples were kept for an hour. Using spectrophotometer at 415 nm (Lambda 950, Perkin-Elmer, USA), the absorbance was measured. For each analysis, the samples were belayed in triplicate and the mean value of the absorbance was afforded. For the standard solution of quercetin (Fig. 1b), the same procedure was repeated. The TFC was expressed in terms of quercetin equivalent (mg of Qu/g of extract) [20].

Statistical Analyses

Data were represented as the means ± standard error of the mean of four autonomous experiments accomplished in triplicate. The data was statistically analyzed using one-way ANOVA by the program SPSS (23.0). By Duncan's multiple range test, significant differences among means from a triplicate analysis at (P<0.01, 0.05) were assessed.

Results and Discussion

Identification of the VCs

The oil isolated from the pomaces of *C. aurantifolia* was pale yellow colored liquid with the oil yield of 1.63%. Quantitation of EO was obtained by GC-FID and quantification with GC/MS. Using an Agilent Chemstation Integrator, the percentage of relative amounts was calculated from peak areas. Data gained from quantitative and qualitative assessment of the EO and VCs from pomace of *C. aurantifolia* are displayed in Table 1. 39 compounds, representing 98.9% of the oil, were identified (Table 1). The major constituents of the oil were limonene (71.7%), β-pinene (8.5%), γ-terpinene (7.3%), myrcene (1.8%) and α-pinene (1.7 %), respectively. 99.1% of the volatile of *C. aurantifolia* were thirty seven compounds which were recognized in the pomace samples by SHS. The main VCs of *C. aurantifolia* were limonene (49.3%) followed by β-pinene (21.7%), γ-terpinene (9.0%), α-pinene (6.8%) and sabinene (4.9%), respectively. In Mexico, abundant VCs in the EO of *C. aurantifolia* were limonene, linalool, sabinene and bergamol [21]. Numerous oxygenated monoterpenes, mono and sesquiterpene hydrocarbons have been found in *C. aurantifolia* peel [22]. Limonene, β-ocimene, and caryophyllene were collected at higher concentrations in the young shoots of *C. aurantifolia* by solid phase microextraction and analyzed in a gas chromatograph coupled to a mass selective detector [23]. In the lemon juice of the region of Kalamata, the most plentiful volatile compound was limonene followed by γ-terpinene, sabinene and neryl acetate [24]. In Spanish lemon fibres, the compounds such as α-terpineol, terpinen- 4-ol, linalool, terpinolene, γ-terpinene, limonene, p-cymene, α-pinene and nonanal have been recently recognized [25]. In southern Florida, distilled oil of fruit peels of *C. aurantifolia* was studied with limonene (32.6%), α-terpineol (12.5%), and β-pinene (6.3%) as the major compounds [26]. 50 compounds was found in the fruit peel EO of Iranian *C. aurantifolia*, the most plentiful of them were limonene (53.53%), α-terpineol (9.41%), and γ-terpinene (6.26%) [27]. We studied the VCs by SHS method and HD. Our results showed similar compounds in comparison with other studies. Similar to previous reports limonene was the main constituent in studied pomace. Our results showed that monoterpene hydrocarbons were the major constituents in studied pomace. The compoundes in EO were separated into 3 classes: monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons. The major constituents were monoterpene hydrocarbons (92.8%), oxygenated monoterpenes (3.4%) and sesquiterpene hydrocarbons (2.7%), respectively in the oils of *C. aurantifolia* pomace. 98.9% of constituents by SHS were contained monoterpene hydrocarbons but sesquiterpene hydrocarbons and oxygenated monoterpenes such as *(E)*-caryophyllene and terpinen-4-ol, respectively, were known in this marc. Limonene is the main odor constituent of *citrus* (Rutaceae). This compound is utilized in drug and food manufacturing, i.e. as a fragrance material in hand cleaners, bath products, after shave lotions, perfumery, etc. and as a flavoring agent to conceal the bitter taste of alkaloids utilized in some medicines. Limonene has a piney, turpentine-like odor and can be used, as a botanical insecticide. In natural and alternative medicine, limonene is applied because of its ability to becalm heartburn and gastro esophageal reflux disease [28].

Phenolic Composition

To determine the quantitative content of polyphenol of *C. aurantifolia* pomace calibration curves were provided in the concentration range from 1 to 500 mg L^{-1}. Equations of calibration curves and their correlation coefficients are presented in Table 2. The R^2 quantities were in the span from 0.992 to 0.999 which confirmed the linearity of the method. Results are also presented in Fig. 2. The variance analysis illustrated significant differences in polyphenol compounds among the different extracts in *C. aurantifolia* (P<0.01; Table 2, 3). The content of seventeen studied polyphenols are displayed in table 2. Our results showed that 11 polyplenol constituents from 17 considered ones, were detected in *C. aurantifolia* pomace. Among them, sinapic acid, caffeic acid, thymol, coumarin, eugenol and carvacrol were not detected in *C. aurantifolia* pomace extracts. *Trans*-ferulic acid was major polyphenol compound in all studied extracts. *Trans*-ferulic acid was varied from 2.30 (60 °C water extract) to 2.86 mg/g (water extract) in *C. aurantifolia*. Hesperidin as another main phenolic compound, was ranged from 0.33 mg/g (water extract) to 2.12 mg/g (80% methanolic extract). The wide amount of ellagic acid (1.83 mg/g) was determined in methanolic extract and the lowest content (0.21 mg/g) was in 60 °C water extract.
Quercetin was differed from 80% methanolic extract (0.79 mg/g) to 60 °C water extract (0.03 mg/g). As our results showed, we found the variation of polyphenol compounds in investigated extracts of *C. aurantifolia* marc. The vast content of rosmarinic acid (0.33 mg/g) and hesperetin (0.32 mg/g) were determined in 80% methanolic extract (Fig. 2). The highest amount of gallic acid was found in methanolic extract (0.20 mg/g). Catechin (0.60 mg/g), chlorogenic acid (0.61 mg/g), p-coumaric acid (0.16 mg/g) and vanillin (0.12 mg/g) have great content in water extract. Ellagic acid and gallic acid were prevailing phenolic compounds in methanolic extract. The hesperidin and naringin were found in maximum quantity in peel of *C. aurantifolia* fruit during growth and maturation [29]. An organic compound, ferulic acid which is a hydroxycinnamic acid, is a plentiful phenolic phytochemical detected in plant cell

walls. It is covalently linked as side chains to molecules like arabinoxylans. Hydroxycinnamic acids are compounds containing a cinnamic acid where the benzene ring is hydroxylated. *Trans*-ferulic acid is an extremely weak basic (essentially neutral) compound (based on its pKa).

It exists in all living species, ranging from bacteria to humans and is found, on average, in the highest concentration in corns. *Trans*-ferulic acid has also been detected, but not quantified in, several different foods, such as burdocks, ginkgo nuts, persian limes, kiwis, and chickpea. This could make *trans*-ferulic acid a potential biomarker for the consumption of these foods [30]. This compound, like many natural phenols, is an antioxidant in vitro in the sense that it is reactive toward free radicals such as reactive oxygen species. If added to a topical preparation of ascorbic acid and vitamin E, ferulic acid may reduce oxidative stress and formation of thymine dimers in skin [31]. A flavanon glycoside detected in *citrus* fruits is hesperidin. Hesperetin is its aglycone form "Hesperidium" which is produced by *citrus* trees is the origin word of hesperidin. Hesperidin exhibits multiple biological properties: antioxidant, anti-inflammatory, anti-hypercholesterolemic, anti-hypertensive, anti-carcinogenic, antimicrobial, and anti-allergenic [32]. Hesperidin is the main flavonoid in lemons and sweet oranges [32]. Hesperetin is a cholesterol lowering flavanoid found in a number of *citrus* juices. It appears to reduce cholesteryl ester mass [33].

Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid and as hydrolyzable tannin; catechin is a flavan-3-ol, a kind of antioxidant and natural phenol; chlorogenic acid refers to a related polyphenol family of esters; quercetin is a plant flavonol from the flavonoid group of polyphenols; a hydroxy derivative of cinnamic acid is a hydroxycinnamic called p-coumaric acid; vanillin is a phenolic aldehyde; ellagic acid is a natural phenol antioxidant and as hydrolyzable tannin; hesperetin is the 4'-methoxy derivative of eriodictyol, a flavanone and rosmarinic acid is a phenolic compound and ester of caffeic acid [34-38]. Phenolic compounds in *C. aurantifolia* pomace in our study included 2 hydroxybenzoic acids (gallic and ellagic acids; and as hydrolyzable tannins), 4 hydroxycinnamic acids (*trans*-ferulic, rosmarinic, p-coumaric and chlorogenic acids), one flavanon glycoside (hesperidin), one trihydroxyflavanone (hesperitin), one phenolic aldehyde (vanillin), one flavonol (quercetin) and one flavan-3-ol (catechin).

IC_{50} was used to represent the radical-scavenging activity on DPPH. This quantity was the concentration in µg/ml involved to exclude DPPH radical formation by 50%. The highest activity was found in 100% methanolic extract (2045.95 µg ml^{1}) (Table 4, Fig. 3a).

The antioxidant potential of fractions was found to be moderate ($P<0.01$). 60 °C water extract showed the lowest antioxidant capacity (2664.44 µg ml^{-1}) (Table 4, Fig. 3a). In *C. aurantifolia*, the highest antioxidant ingredients yield was in methanol with 16.67 g/100 g for peel and 40 g/100 g for pulp [39]. The juices of ripen and unripen of *C. aurantifolia* have potent antioxidant activity [40]. In fact, compare to methanol, water due to its high polarity is not an efficient solvent for the phenols extraction. According to some studies, pure water is not an efficient solvent to extract polyphenols because these compounds are more soluble in solvents less polar than water [41, 42].

Although we considered the waste of the studied samples, they showed high to moderate antioxidant activity rather than new and real samples of *Citrus* species. It means that the pomaces could be rich potent for antioxidant capacity. In these extracts, the phenolic compounds (including phenolic hydroxyls) are majorly detected and might be imputable to the perceived great antiradical traits of them [43, 44].

Antioxidant Capacit

Total Phenolic Content (TPC)

Phenols on reaction with an oxidizing agent phosphomolybdate in FCR under alkaline conditions, lead to the formation of a molybdenum blue colored complex, the intensity of which can be measured at 765 nm colorimetrically. Milligram GA/g dry weight (dw) extract is used to express the TPC values. The results are represented in table 4. Methanolic extract of *C. aurantifolia* has the highest amount of TPC (130.85 mgGa/g) (Table 4, Fig. 3b). The results indicated that there was no meaningful variation found in the TPC among the extracts got from *C. aurantifolia* (Table 4). The TPC were ranged from 94.95 to 130.85 mgGA/g in *C. aurantifolia* samples. Also, our reported values of TPC in *C. aurantifolia* was much greater as compared with the other investigate from Nakhal region of Oman (96.55 µg/mg extract) [45]. The TPC of *C. aurantifolia* extracts from India varied from 5.0 to 45.0 mgGA/g and 2.5 to 22.5 mgGA/g of peel and pulp segments, respectively [39].

The results revealed that the most efficient solvent for extraction of TPC for *C. aurantifolia* pomace is pure methanol, followed by 80% methanol, 60 °C water and distilled water. Pure and 60 °C water extracts showed the lowest TPC. Our study shown that the highest amounts of phenolic compounds in methanolic extracts were *trans*-ferulic acid, ellagic acid, hesperidin and quercetin, respectively, in which could be responsible for the highest TPCs in this studied marc. TPC plays a probable role in preventing different diseases related to oxidative stress such as cardiovascular, cancer and neurodegenerative diseases. Total Flavonoid Content (TFC)

Table 1 Volatile compounds of pomace of *Citrus aurantifolia* (Christm.) Swingle by WD and SHS methods

Relative amounts (%)[c]							
No	Compounds	RI[a]	RI[b]	WD	RSD[d] (%)	SHS	RSD (%)
1	n-Nonane	901	900	-	-	t [e]	-
2	Tricyclene	923	926	-	-	t	-
3	α-Thujene	927	930	0.3	2.6	1.5	3.6
4	α-Pinene	934	935	1.7	2.3	6.8	2.8
5	Camphene	952	950	t	-	0.3	4.7
6	Thuja-2,4(10)-diene	958	960	-	-	t	-
7	Sabinene	974	972	0.5	4.3	4.9	7.3
8	β-Pinene	976	977	8.5	1.5	21.7	3.5
9	6-methyl-5-Hepten-2-one	987	986	-	-	t	-
10	Myrcene	992	991	1.8	2.6	3.2	5.6
11	n-Decane	1000	999	-	-	t	-
12	α-Phellandrene	1002	1004	t	-	0.1	3.6
13	p-Mentha-1(7),8-diene	1004	1008	-	-	t	-
14	α-Terpinene	1017	1014	0.3	1.1	0.6	2.1
15	p-Cymene	1025	1024	t	-	0.2	3.4
16	Limonene	1029	1031	71.7	1.2	49.3	1.5
17	*(Z)*-β-Ocimene	1037	1036	t	-	0.2	5.1
18	*(E)*-β-Ocimene	1047	1050	0.2	2.4	0.6	3.4
19	γ-Terpinene	1059	1060	7.3	1.7	9.0	4.7
20	*Trans*-Linalool oxide	1073	1071	t	-	-	-
21	Terpinolene	1089	1090	0.5	3.0	0.5	3.1
22	Linalool	1099	1100	0.2	1.2	t	-
23	*endo*-Fenchol	1116	1115	t	-	-	-
24	Citronellal	1153	1150	t	-	t	-
25	Borneol	1166	1165	t	-	-	-
26	Terpinen-4-ol	1177	1179	0.6	1.5	0.1	1.2
27	α-Terpineol	1190	1189	1.1	2.4	t	-
28	n-Dodecane	1199	1200	-	-	t	-
29	n-Decanal	1206	1202	-	-	t	-
30	Citronellol	1229	1226	-	-	t	-
31	Nerol	1230	1229	0.2	2.8	-	-
32	Neral	1241	1239	0.1	3.6	t	-
33	Geraniol	1256	1253	0.2	1.4	-	-
34	Geranial	1269	1268	0.2	1.3	t	-
35	Thymol	1291	1290	t	-	-	-
36	Carvacrol	1299	1298	t	-	-	-
37	n-Tridecane	1301	1300	-	-	t	-
38	δ-Elemene	1337	1338	0.1	1.2	t	-
39	Citronellyl acetate	1353	1352	t	-	-	-
40	Neryl acetate	1365	1362	0.6	2.3	t	-
41	Geranyl acetate	1385	1384	0.2	1.5	t	-
42	β-Elemene	1392	1390	t	-	t	-
43	*(E)*-Caryophyllene	1418	1417	0.6	2.1	0.1	2.4
44	*Trans*-α-Bergamotene	1435	1436	0.6	2.6	t	-
45	α-Humulene	1453	1455	0.1	1.1	-	-
46	*(E)*-β-Farnesene	1459	1457	t	-	-	-
47	Valencene	1494	1498	0.2	2.3	-	-
48	β-Bisabolene	1506	1505	1.1	2.5	t	-
49	*(Z)*-α-Bisabolene	1508	1509	t	-	-	-
Total	-	-	-	98.9	-	99.1	-

[a]Linear retention indices were calculated using a homologous series C_8-C_{25} n-alkanes; [b]Retention indices taken from literature;[c]Relative area percent (peak area relative to total peak area); %; [d]percentage relative standard deviation [e]trace<0.1

Table 2 Plolyphenol compounds from pomace of *Citrus aurantifolia* (Christm.) Swingle

Polyphenol	Linear regression equation[a]	Correlation coefficient(R^2)	60 °C water	Distilled water	Methanol	Methanol 80%
Gallic acid	Y=40.507x-33.427	0.999	0.04±0.004 [b]d	0.07±0.004 c [c]	0.20±0.0001 a	0.17±0.0002 b
Chlorogenic acid	Y=24.112x-8.6696	0.999	0.41±0.0001 b	0.61±0.0008 a	0.12±0.0002 d	0.24±0.0004 c
Sinapic acid	Y=12.843x-82.917	0.999	nd [d]	nd	nd	nd
Coumarin	Y=55.203x+186.22	0.999	nd	nd	nd	nd
Hesperetin	Y=30.574x-141.76	0.999	0.06±0.00001 c	0.06±0.00004 d	0.12±0.0001 b	0.32±0.0004 a
Carvacrol	Y=10.675x-12.921	0.998	nd	nd	nd	nd
Caffeic acid	Y=15.247x+152.14	0.997	nd	nd	nd	nd
Vanillin	Y=42.74x+59.464	0.999	0.08±0.0003 c	0.12±0.0003 a	0.09±0.00003 b	0.08±0.0002 d
Trans-Ferulic acid	Y=30.718x-214.48	0.998	2.30±0.005 d	2.86±0.0006 a	2.63±0.0005 c	2.71±0.002 b
Quercetin	Y=14.927x+72.349	0.998	0.03±0.00001 d	0.11±0.0002 c	0.28±0.0002 b	0.79±0.0002 a
p-Coumaric acid	Y=82.241x+287.72	0.999	0.09±0.002 c	0.16±0.002 a	0.04±0.00002 d	0.11±0.0005 b
Rosmarinic acid	Y=24.232x-101	0.990	0.08±0.00002 c	0.12±0.0002 b	0.07±0.0001 d	0.33±0.0005 a
Hesperidin	Y=16.849x+40.817	0.997	1.20±0.0002 b	0.33±0.004 d	0.70±0.0001 c	2.12±0.0002 a
Ellagic acid	Y=17.803x-185.06	0.992	0.21±0.0005 d	0.62±0.0002 c	1.83±0.0002 a	0.63±0.0002 b
Eugenol	Y=11.32x-147.17	0.994	nd	nd	nd	nd
Thymol	Y=8.7065x-68.159	0.998	nd	nd	nd	nd
Catechin	Y=9.2191x-72.022	0.997	0.29±0.0002 b	0.60±0.002(A)	0.24±0.0003(D)	0.25±0.0001 c

[a]Y: Area; X: Concentration; [b]Calculated mean amount of the polyphenol (mg/g) based on the weight of the ground dry pomaces in three replicates ± SD; [c]Duncan's mean separation; [d]nd: not detected

Table 3 Compare of 4 different extracts of *Citrus aurantifolia* (Christm.) Swingle for extraction of polyphenol compounds using one-way ANOVA

Polyphenol	MS	CV (%)	Polyphenol	MS	CV (%)
Gallic acid	0.02 **	2.34 **	Quercetin	0.36 **	0.06 **
Chlorogenic acid	0.14 **	0.13 **	p-Coumaric acid	0.008 **	1.43 **
Sinapic acid	-	-	Rosmarinic acid	0.05 **	0.18 **
Coumarin	-	-	Hesperidin	1.81**	0.18 **
Hesperetin	0.05 **	0.15 **	Ellagic acid	1.46 **	0.04 **
Carvacrol	-	-	Eugenol	-	-
Caffeic acid	-	-	Thymol	-	-
Vanillin	0.001 **	0.25 **	Catechin	0.09 **	0.29 **
Trans-Ferulic acid	0.17 **	0.10 **	-	-	-

**: significantly different at 1% level

Table 4 Compare of 4 different extracts of *Citrus aurantifolia* (Christm.) Swingle for antioxidante activity, total phenolic and flavonoid contents using one-way ANOVA

	MS	CV (%)	60 °C water	Distilled water	Methanol	Methanol 80%
Antioxidant activity by DPPH (µg/ml)	382686.49 **	1.49	2664.44±50.44 d a	2567.86±43.77 c	2045.95±13.77 a	2130.34±15.48 b
Total phenolic content (TPC) (mg/gr extract)	959.43 ns	3.20	96.94±3.52 c	94.95±2.93 c	130.85±3.22 a	121.50±4.39 b
Total flavonoid content (TFC) (mg QU/gr extract)	23.55 **	6.77	11.08±0.82 b	10.59±1.04 b	16.06±0.89 a	15.21±0.82 a

[a]Duncan's mean separation; **: significantly different at 1% level; ns: not significantly different

Phenolics and flavonoids compounds are highly effective free radical scavengers and antioxidants. Table 4 illustrates the TFCs in studied extract samples. Our results were correlated with antioxidant and the TPC reports. It was resulted that the highest amounts of the TFC were available in 100% methanol followed by 80% methanol from *C. aurantifolia* pomace extracts, respectively (P< 0.01; Table4, Fig. 3c). Pure and 60 °C water extracts showed the lowest TFC. In this study, the TFC were limited from 10.59 to 16.06 mg QU/gr extract in *C. aurantifolia* samples, respectively.

All the extracts analyzed in this research showed high levels of phenol contents. One of the greatest groups of polyphenols is flavonoids. They are thought to illustrate advantageous health effects for their chelating and antioxidant attributes, and are the main participant to the plants antioxidant valence. They function either by breaking lipid peroxidation chain reactions, by scavenging free radicals, or by blocking the generation of hypervalent metal forms [46]. In *Citrus* fruit, flavonols, flavones and flavanones are 3 kinds of flavonoids [4]. In *citrus* species, the major flavonoids are eriocitrin, naringin, narirutin and hesperidine [5]. 41.38-64.2 µg of QU/mg of dry extract was the TFC in *C.aurantifolia* leaves grown in two regions of Oman [45]. Among investigated phenolic constituents, hesperidin, quercetin, hesperetin and catechin were classified as flavonoid compounds. So, we considered that TFC of *C. aurantifolia* pomace might be resulted from catechin, hesperidin, quercetin and hesperetin as flavonoid compounds. *C. aurantifolia* pomaces were rich of flavonoids. Various investigations [47-49] found that phenolic compounds in herbs and spices considerably brought about their antioxidant attributes.

The Correlation between TPC, Antioxidant Activity, Phenolic Compound, and TFC

Figure 3 display the correlation between DPPH assay, TPC, TFC and phenolic constituents of studied extracts. Our results demonstrated highly positive correlation coefficient between DPPH assay, TPC and TFC of *C. aurantifolia* extracts (P<0.01; R^2=0.92, 0.89; Fig. 4a,b). Also, Pearson's correlation coefficients (R^2) was 0.98 between TPC and TFC in this plant (P<0.01; Fig. 4c). A positive correlation between antioxidant capacity, TPC and TFC with gallic acid content as hydrolyzable tannin was observed (P<0.01, R^2=0.98; P<0.01, R^2=0.92; P<0.01, R^2=0.86, respectively; Fig. 4d, e, f). According to figures 4g-i, there was no correlation between TFC with hesperetin, quercetin and catechin of *C. aurantifolia* extracts (P<0.05). Several studies illustrate a linear correlation of flavonoid quantity and total phenolic with antioxidant content [50]. By comparing the correlations, it can be proposed that flavonoid and phenolic groups are extremely accountable the antioxidant activity of the *C. aurantifolia* extracts. The findings were in accord with the former studies [51, 52], which indicated phenolic compounds controlled total antioxidant content of *citrus* fruits.

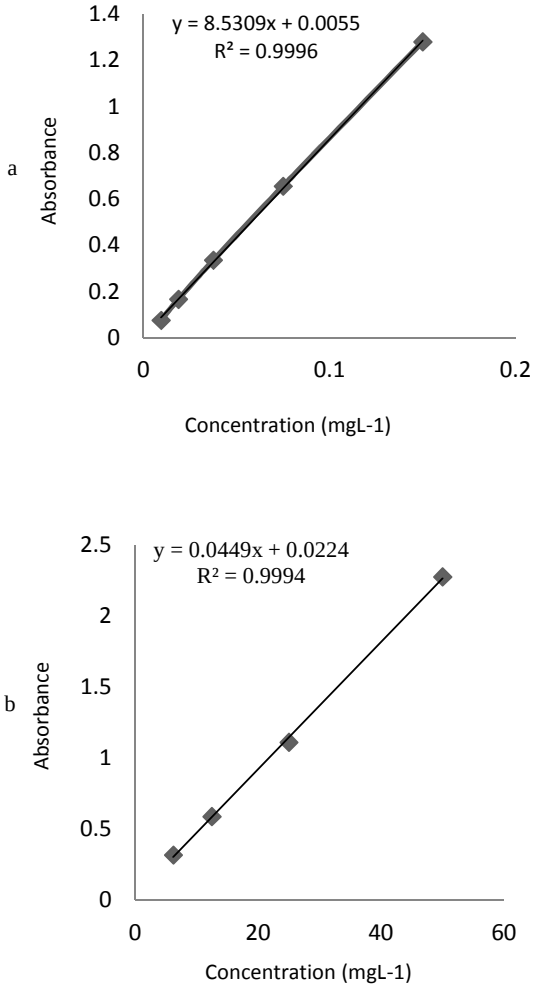
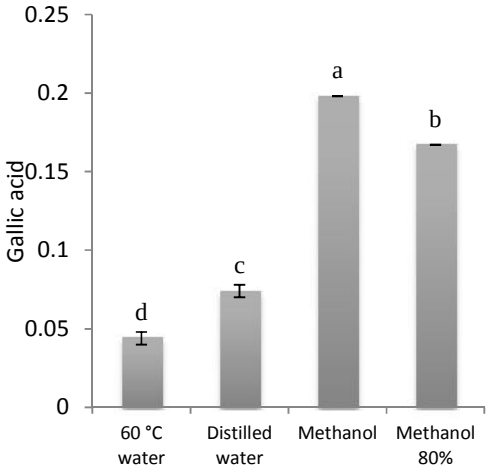


Fig. 1 The calibration curves of gallic acid solutions for determination of total phenolic (a) and quercetin solutions for total flavonoid assay (b).



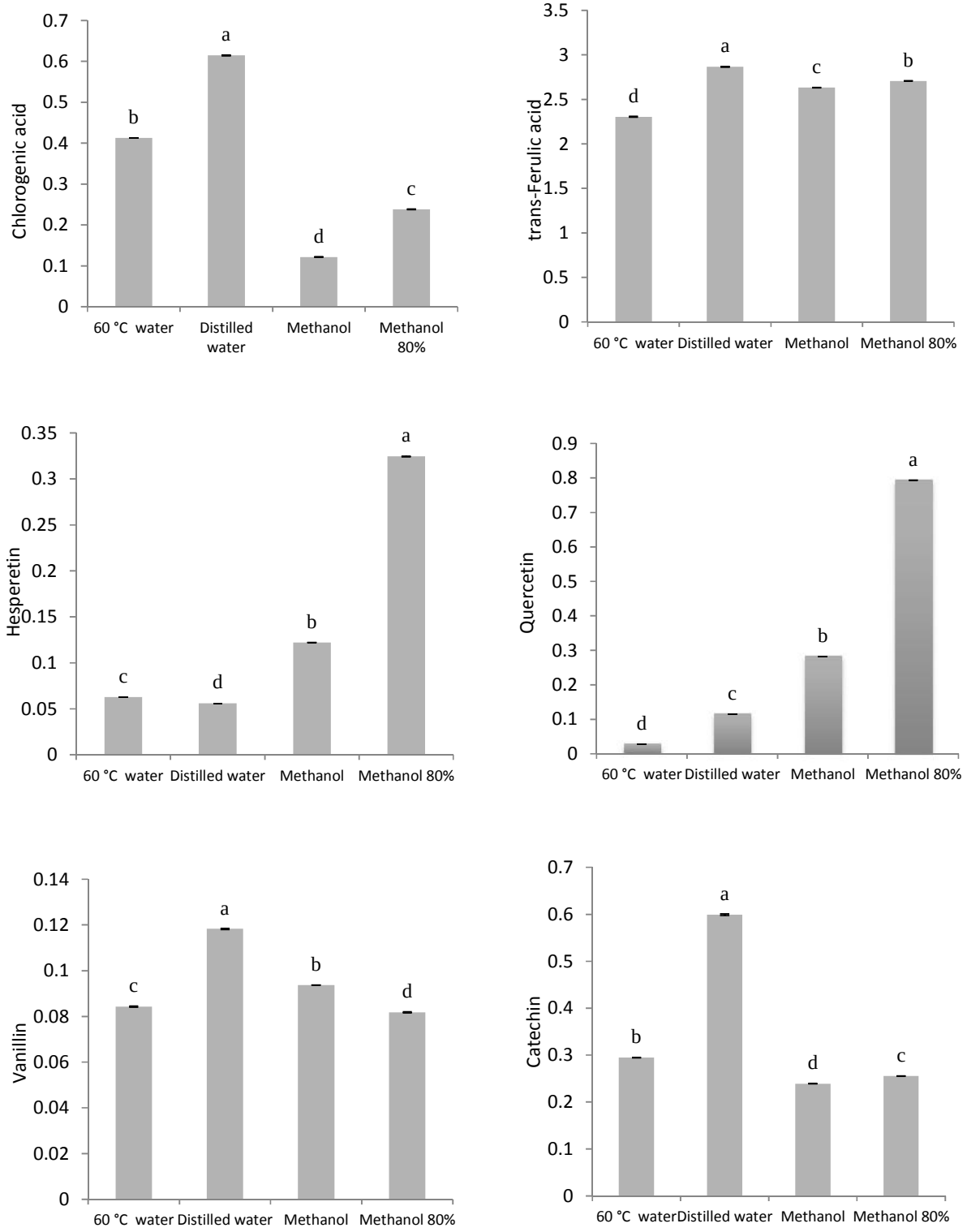


Fig. 2 Comparison of the phenolic compounds of *Citrus aurantiifolia* (Christm.) Swingle in different extracts

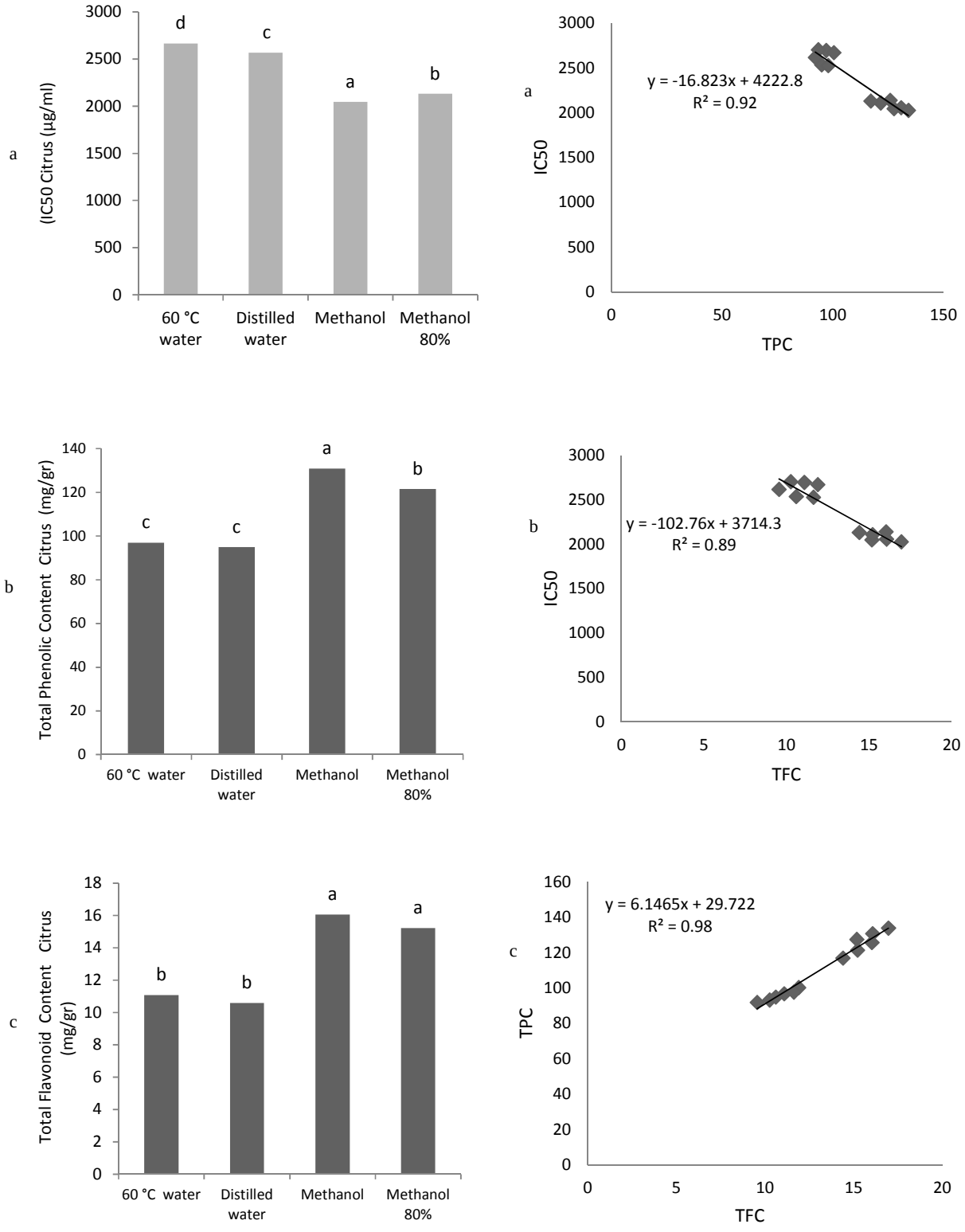


Fig. 3 Antioxidante activity (a), Total ph e nolic (b) and flavonoid (c) contents of Citrus aurantiifolia (Christm.) Swingle in four different extracts.

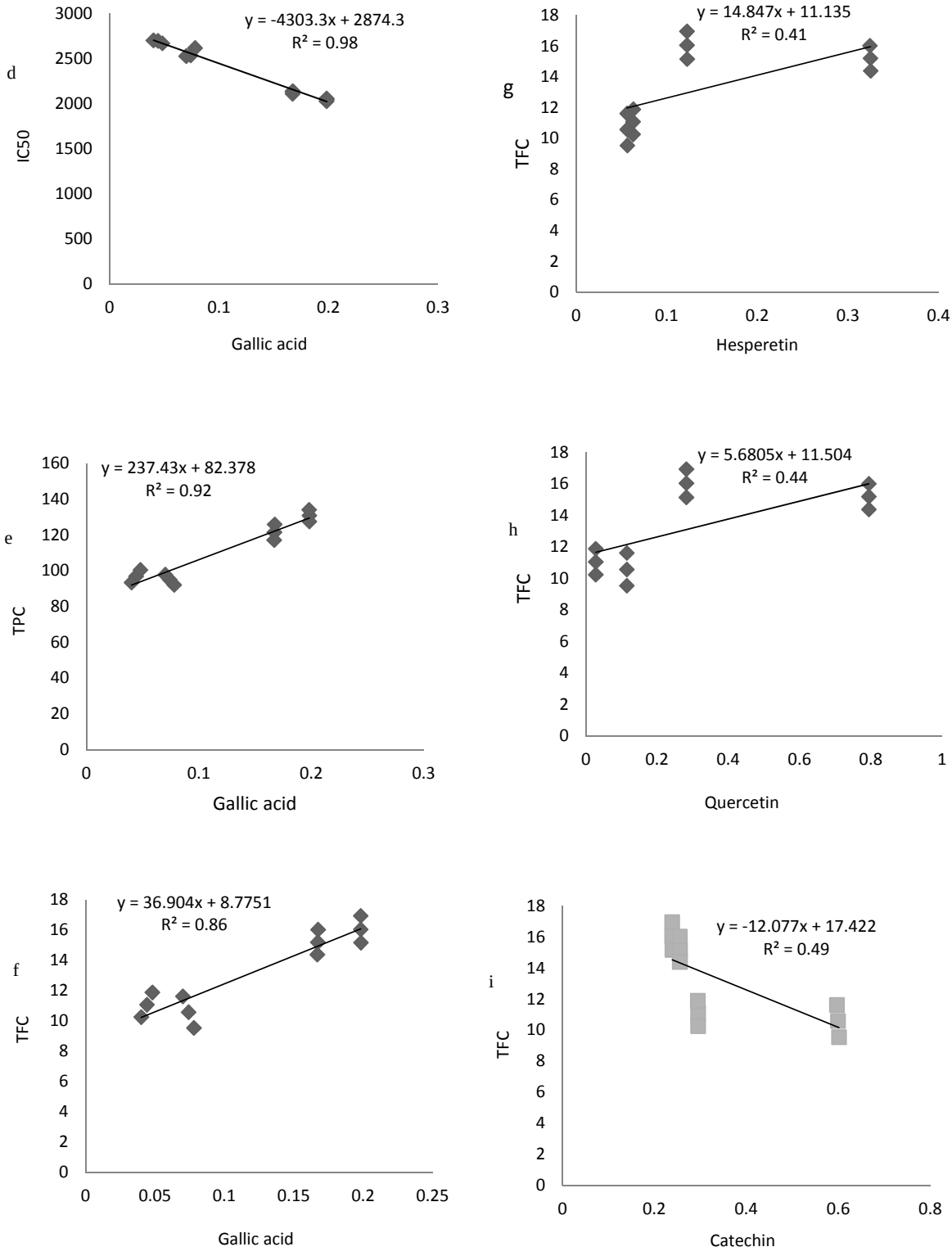


Fig. 4 The correlation between antioxidant activity (DPPH free radical-scavenging activity), phenolic compound, total phenolic and flavonoid contents of *Citrus aurantiifolia* (Christm.) Swingle extract

Conclusions

In the current trial, the antioxidant activity illustrates that pomace of this studied medicinal plant with greater flavonoid and phenolic quantities might be a considerable fount of natural products. The redox attributes of compounds causes the radical scavenging activities, which may act in decomposing peroxides, quenching singlet and triplet oxygen or neutralizing free radicals. Moreover, a large group of plant polyphenols is flavonoid with radical scavenging activity and intense antioxidant capacity. According to the findings of this study, the marc of *C. aurantifolia* is natural source of phenolic compounds. We concluded that amid the solvent systems investigated, the extracts received from 100% methanol, had the highest values of antioxidant capacity, TPC and TFC as compared with the other extracts. The results for antioxidant assay, TPC and TFC were correlated with each other in *C. aurantifolia* pomaces. Also, TFC in *C. aurantifolia* extracts could be correlated to another flavonoids (flavonols and flavones such as eriocitrin, naringin, narirutin, kaempferol) in which we have not studied in this research. Whereas desirable utilization of agricultural waste such as pomaces of *Citrus* will reduce costs and environmental hazards in which results from their disposal and remaining in the environment, our studies are continuing to investigate and introduce marcs of *C. aurantifolia* as strong natural resources and we are going to use these marcs for other goals.

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