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Marianna Rootstock (*Prunus cerasifera* Ehrh. × *Prunus munsoniana* Wight and Hedr.): Phytochemical and Antioxidant Investigation of Different Leaf Extracts

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Abstract

Our study was carried out on leaves of Marianna rootstock (PrunuscerasiferaEhrh. × Prunusmunsoniana Wight and Hedr.) cultivated in Egypt for investigation of antioxidant active metabolites. Leaves were extracted using solvents with increasing polarities (petroleum ether, methylene chloride, ethyl acetate and 80% methanol), in addition to 70% ethanol (the crude extract). Preliminary phytochemical screening revealed presence of sterols and/or triterpenes, carbohydrates and/or glycosides, flavonoids, phenolic acids, saponins and tannins, whereas coumarins and alkaloids were absent. Total phenolic acids and total flavonoids contents were amounted to 46.18 mg gallic acid equivalent (GAE/g) and 44.38 mg catechin equivalent (CE/g), respectively. HPLC analysis of polyphenols revealed 8 compounds identified in crude extract; kaempferol (0.4483 mg/g) was the major flavonoid followed by catechin, quercetin and rutin. Whereas caffeic acid (26.67542 mg/g) was the major phenolic acid followed by chlorogenic acid. Chromatographic investigation of methanol successive extract yielded isolation and spectroscopic identification of 4 flavonoids; kaempferol -3, 7-O-α-L- di-rhamnoside, kaempferol -7-O- α-L-rhamnoside, kaempferol 3, 7-O-β-D-di-glucoside and quercetin-7-O-β-D- glucoside,4'-O-α-L- rhamnoside. In-vitro antioxidant properties of leaves different extractives were evaluated using different assays; DPPH, nitric oxide, metal chelating ability and total antioxidant capacity that showed promising antioxidant efficacy of plant extracts compared with L-ascorbic acid and BHT standards.

Keywords: Marianna rootstock, Prunus, Phytoconstituents, antioxidant activity.

1. Introduction

An excessive number of free radicals are linked to oxidative stress. Radicals from both internal and external sources lead to lipids, proteins, and other vital biological components being damaged in addition to RNA or nucleic acids, leading to the emergence of neurological conditions, such as Parkinson's and Alzheimer's as well as start of cancerous process and developing of metabolic illnesses as diabetes [1]. Free radicals may cause harm to numerous organs and structures, including heart, lungs, kidneys, brain, eyes and joints, causing atherosclerosis, chronic bronchitis, cataracts, heart failure, depression, rheumatism and asthma, additionally oxidative stress might be harmful to fetuses and hasten ageing [2].

In general, various plant species are rich in antioxidant metabolites especially fruits and vegetables, so they are in crucial need for human healthy diet. A high consumption of fruits and vegetables is proven to boost the level of antioxidant activity significantly in human serum [3]. Rosaceae (rose family) is considered as a one of the most economically significant families. It contains a wide

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range of flora. The rose family contains about 100 genera and 3,000 species that can be found almost anywhere especially in the Northern Hemisphere [4& 5]. Prunus (Rosaceae) is a genus of roughly 430 deciduous and evergreen trees and shrubs found primarily in temperate parts of the Northern Hemisphere [6]. Many of prunus species are cultivated for their edible seeds and fruits, especially *Prunusdulcis* Mill. (almonds), *Prunusdomestica* L. (plums), P*runuspersica* L. (peaches), *Prunuscerasus* L. (cherries), and *Prunusamericana* L. (apricots).

Prunus species are rich in flavonoids, which have a wide range of biological functions, in addition to: steroids, terpenes, coumarins, phenolic acids, and carbohydrates that all have been isolated from *Prunus* species [7].

Regarding antioxidant potential of genus *prunus*; previous work reported the antioxidant activity of *P. Avium* L. Stems attributed to high phenolics content**) [8]**. *P. Mume* Siebold and Zucc.flowers extracts were evaluated for antioxidant activity by DPPH, ABTS+, OH free radicals scavenging and ferric-reducing antioxidant power (FRAP) that proved antioxidant properties attributed to chlorogenic acid isomers **[9]**.*P. domestica* L. dried fruits showed powerful antioxidant activity due to many kinds of phenolics present; as caffeoylquinic acid isomers **[10& 11]**. Besides recommended *P. spinosa* L. flowers in traditional herbal medicine for adjunctive treatment of oxidative stress-related diseases **[12],**antioxidant activity of *P. spinosa* L. (blackthorn) fruits extracts was determined by DPPH and photochemiluminescens (PCL) methods that revealed anti-oxidant phenolic chemicals **[13]**. It worth mentioned that leaves of *P. cerasifera* Ehrh.are enriched with biologically active ingredients, notably tannins, flavonoids, and phenolic acids, all of which showed high antioxidant activity **[14]**.

Marianna rootstock (*Prunus cerasifera* Ehrh.×*Prunus munsoniana* Wight and Hedr.) is a member of Family Rosaceae, particularly rich in phenolic compounds and flavonoids (Flavones, flavanones, flavonols, isoflavonoids, dihydroflavonoids, anthocyanins, and proanthocyanidins) **[7]**.

This study aimed to investigate the antioxidant activity of Marianna rootstock leaves crude and successive extracts using different methods compared to standards with isolation and structure characterization of the main polar constituents that may attribute to the activity using different spectroscopic techniques and HPLC.

Material and Experimental

Plant material

Five kilos of fresh leaves of Marianna roostock (*Prunus cerasifera* Ehrh. × *Prunus munsoniana* Wight and Hedr.), family Rosaceae were collected in April and May 2020 from Horticulture Research Institute (HRI), Dokki; Giza, Egypt. The leaves were air dried and ground to yield 1365 g dry powder. Specimen of the plant was authenticated by Dr. Mohamed El Gebaly Senior Botanist, Egypt, and herbarium specimen was kept in museum of pharmacognosy department, faculty of Pharmacy, Cairo University (Voucher no 11.05.2022.I).

Plant extraction

Preparation of crude extract

Air dried powdered of Marianna rootstock leaves (200 g) was macerated on cold in a stoppered container with 70% ethanol (crude extract) and allowed to stand at room temperature with frequent agitation. The mixture was filtered and evaporated under reduced pressure by rotary evaporator at 40°C.The dried residue was weighed (32 g)and kept in a glass vial at -20 °C.

Preparation of successive extracts

One Kg of dried powdered Marianna rootstock leaves were soaked in a percolator and successively extracted with solvents of increasing polarity; petroleum ether, methylene chloride, ethyl acetate and 80% methanol, at room temperature with frequent agitation. After each complete exhaustion with one solvent, the powdered plant was dried and extracted with the next solvent. The collected extracts were evaporated under reduced pressure using rotary evaporator, weighed and kept in glass vials at -20 °C.

Methods

Phytochemical screening of crude and successive extracts profile was followed according to the procedures of Pandey and Tripathi, 2014 **[15].**

Method for Quantitative Determination of Total Flavonoids Content

One g crude extract was mixed with 100 ml methanol and homogenized using the Ultra-Turrax homogenizer. The homogenate was kept at 4 °C for 12 hours and then centrifuged at 10^4 rpm for 20 min. The supernatant was stored at -20 °C till analysis. Total flavonoid content was measured according to method of Zhishen *et al*., 1999 **[16].**Total flavonoids content was recorded as mg catechin equivalent (CE) per g sample using calibration curve of catechin.

Method for Quantitative Determination of Total Phenolics Content

One g of crude extract was mixed with 100 ml methanol and homogenized by the Ultra-Turrax homogenizer. The homogenate was kept at 4 °C for 12 hours and then centrifuged at 10^4 rpm for 20 min. Supernatant was recovered and stored at -20 °C until analysis. Total phenolic content was determined according to Folin-Ciocalteu method **(Zilic*et al.*, 2012) [17].**The total phenolics content was determined by means of a calibration curve prepared with gallic acid, and expressed as mg of gallic acid equivalent (mg GAE) per g sample.

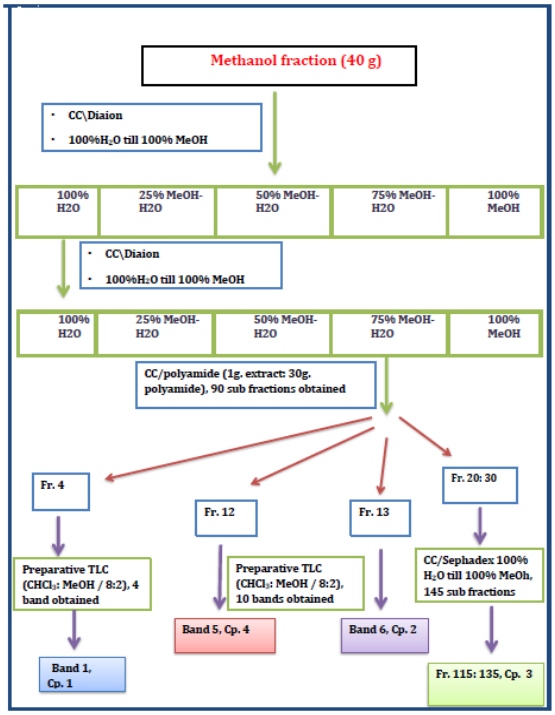
Chromatographic Techniques

Method for HPLC Analysis of Phenolic Compounds

HPLC analysis was performed followed method of Kim *et al*., 2006 **[18].** Mobile phase: acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 ml/ min for about 70 min. The gradient elution was 100% B to 85% B (30 min), then to 50% B (20 min), then to 0% B (5 min) then to 100% B (5 min) with reconditioning period (10 min) after each run. Injection volume was 10 µl, The used detector, diode array detector (DAD), is UV/ visible detector measures at multiple wavelengths. By the aid of DAD, peaks were monitored simultaneously at λ_{maxes} 280, 320 and 360 nm for benzoic acid derivatives, cinnamic acid derivatives and flavonoids, respectively.for derivatives of benzoic&cinnamic acids and 360 nm for flavonoids. Samples were filtered through 0.45 µm Acro disc syringe filter (Gelman Laboratory, MI) prior injection. Peaks were identified depending on retention times and UV spectra compared to the available standards**.**

Column Chromatography (CC)

Forty grams of 80% methanol extract of Marianna rootstock leaves were subjected to column chromatography in which the stationary phase was ion exchange resin, Diaion (in ratio 5:1) and eluted with gradient elution using solvent system of decreasing polarity starting with 100% water then 25% methanol: water, 50%, 75% and ending with 100% methanol fractions. Each fraction of approximately 2-3 L were collected, concentrated under vacuum and investigated by paper chromatography (Whatman1 MM) using solvent systems: 15% acetic acid and *n*-butanol- acetic acid-water (BAW, 4:1:5), chromatograms were visualized under UV light before and after exposure to ammonia vapor and spraying with 1% ethanolic $AlCl_3$ solution. Each fraction was subjected to the suitable chromatographic technique. The most promising fractions was75% methanol sub-fraction so it was selected for further fractionation and compounds isolation using (Polyamide, Sephadix & PTLC) as illustrated in schematic diagram, scheme (1) supplementary.



Scheme 1supplementary: Chromatographic fractionation of the methanol extractive of Marianna rootstock leaves

Antioxidant Activity Characters

Scavenging properties of plant leaves crude and successive extracts

Free radical scavenging effect

Free radical scavenging property of Marianna rootstock (*Prunus cerasifera* Ehrh.× *Prunus munsoniana* Wight and Hedr.) crude and successive extracts (petroleum ether, dichloromethane, ethyl acetate, and methanol) was measured at five consecutive concentrations (50, 100, 250, 500 and 1000µg/ ml) by 1, 1-diphenyl-2-picryl-hydrazil (DPPH•) using the method of Yamaguchi et al., 1998 [19], % scavenging efficacy was recorded as:

DPPH• scavenging effect

(%) =100 − [(A0-A1)/A0) ×100]

Where A0 (absorbance of control), A1 (absorbance of the tested sample)

Nitric oxide (NO) radical scavenging effect

NO· radical scavenging effect of different extracts was determined by using a sodium nitroprusside (SNP) generating NO· system. NO· was generated from SNP in aqueous solution at physiological pH

reacts with oxygen to produce nitrite ions which were measured by the Greiss reagent as stated by Marcocciet al., 1994 [20].

Total antioxidant capacity (cation radical scavenging capability)

Total antioxidant capacity was measured in triplicates according to the method described by Miller and Rice-Evans, 1997) [21]. Absorbance at 734 nm was measured to represent the total antioxidant capacity and calculated as:

Total antioxidant activity (%) = [1- (A sample/A control)] x 100.

Superoxide anion scavenging effect

Measurement of superoxide anion scavenging ability of crude and successive extracts as well as standards; L. Ascorbic acid and BHT, was based on the method described by Liu et al., 1997 [22]. Ascorbic acid and BHT were used as positive controls. Decrease in absorbance of the reaction mixture indicated increased superoxide anion scavenging capability. Superoxide anion generation percentage that was inhibited was performed using the following formula:

Inhibition % = [(A0-A1)/A0] X 100

Where A0 (absorbance of control), A1 (absorbance of tested samples or standards)

Scavenging of hydrogen peroxide

The ability of crude and successive extracts to scavenge hydrogen peroxide as compared to standards was determined according to the method of Ruchet al., 1989 [23]. The percentage of hydrogen peroxide scavenging was calculated using the following equation:

H_2O_2 (%) = ((A0-A1)- A0) X 100

A0 (absorbance of control), A1 (absorbance of tested materials and standards)

Oxidative stress promoters controlling characters

Reduction power

Reduction capability of plant crude, successive extracts and standards was determined according to method of Oyaizu, 1986 **[24].** L. Ascorbic acid and BHT were used as controls. Increase in absorbance of mixture indicates potential reducing power.

Metal chelating ability

The chelation of ferrous ions ability by the plant crude, successive extracts and standards at five concentrations in triplicates was estimated by the method of Singh and Rajini 2004 **[25].**

% inhibition of ferrozine-Fe^{2+}complex was recorded by the formula: Inhibition (%) = $[(A_0-A_1)/ A_0] \times 100$,

Where A_0 (absorbance of control), A_1 (absorbance of sample and standards), triplicates were recorded. Control contains $FeCl_2$ and ferrozine.

Lipid Peroxidation by linoleic assay

Inhibition of lipid peroxidation of plant extract, successive extracts and standards was determined according to the method of Gülçin*et al*., 2004 **[26]** with some modifications. Inhibition of lipid peroxides production was recorded according to equation: Inhibition (%) = $[(A_0 - A_1) / A_0] \times 100$,

Where A_0 (absorbance of the control reaction), A_1 (absorbance of extracts or standards)

Data analysis

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were recorded as mean of triplicates ± SD. One-way (ANOVA) was applied, $P<0.05$. Multiple comparisons between averages of different samples were recorded by one-way ANOVA followed by post hoc testing by Duncan's t-test.

Results

Preliminary phytochemical screening and % yield of different extracts.

Preliminary phytochemical screening of *Marianna rootstock* leaves revealed the presence of sterols and/or triterpenes, carbohydrates and/or glycosides, flavonoids, phenolic compounds, saponins and tannins, whereas coumarins and alkaloids were absent, Table 1.

Table 1: Percentage yields and results of phytochemical examination

Extract	% yield	Constituents
Ethanol 70%	16	S⁄tr, flav, tan, carb⁄gly
Pet. ether	3.6	S⁄tr
Dichloromethane	2	S⁄tr, flav
Ethyl acetate	1.67	Flav, tan, carb⁄gly
Methanol	8	Flav, carb⁄gly

Carb⁄gly: carbohydrates⁄or glycosides; flav: flavonoids; s⁄tr: sterols and⁄ortriterpenes; tan: tannins

Colorimetric determination of total Flavonoids content

Calibration curve of catechin standard (equivalent to 2.5- 25 µg) was carried out. The results were recorded from the average of three determinations to reveal that, total flavonoids content of Marianna rootstock leaves crude extract calculated as catechin per gram sample was 44.38 mg.

Colorimetric determination of total phenolics content

Calibration curve of gallic acid standard (equivalent to 5-50 µg) was carried. The results were recorded as the average of three determinations. The total phenolics content of Marianna rootstock leaves calculated as gallic acid per gram sample was found to be 46.18 mg GAE/g crude extract.

HPLC Analysis of Phenolic compounds

HPLC method is established as the most convenient method which enables qualitative and quantitative estimation of compounds. The present study was performed to estimate the flavonoids and phenolic acids in 70% ethanolic extract of Marianna rootstock leaves comparing the retention times (R_t) with those of available authentic. Results were expressed as mg/g extract for each identified compound from the total area; results are shown in Table 2 and Figures 1& 2. From Table 2, it was revealed that there are 8 compounds identified out of 18 standards analyzed in crude extract. Kaempferol (0.4483mg/g) was the major flavonoid followed by catechin, quercetin and rutin. Whereas caffeic acid (26.67542 mg/g) was the major phenolic acid followed by chlorogenic acid (0.60174 mg/g), while syringic and vanillic acids were present in lower amounts. Caffeic acid was reported before from *P. Domestica* L. dried fruits **[10]** and fruits of *P. cerasifera*Ehrh**[27].**While Chlorogenic acid was isolated from *P. Domestica* L., *P. dulcis*Mill, *P. Mume* Siebold and Zucc and *P. Spinosa* L **[28& 29]**.Vanillic acid was isolated from *Prunusamygdalus* Mill.**[30],***Prunusceraus* L.**[31],***Prunusspinosa* L.dried fruits**[10]** and from fruits of *Prunus cerasifera* Ehrh along with caffeic and other phenolic acids **[27].** While from ethyl acetate extract of *Prunus cerasifera* Ehrh leaves, phenolic acids were identified including; caffeic acid, chlorogenic acid with its isomers and others **[14].**

Table 2: Results of HPLC analysis of flavonoids and phenolic acids of 70% ethanolic extract of Marianna rootstock leaves

	Flavonoids and Phenolic acids	R_t	Conc.(mg/g extract)
1	Gallic acid	3.69	ND
2	Protocatechuic acid	7.66	ND
3	Gentisic acid	12.36	ND
4	*p*-hydroxybenzoic acid	12.46	ND
5	Catechin	16.18	0.4389
6	Chlorogenic acid	17.78	0.6017
7	Caffeic acid	18.57	26.6754
8	Syringic acid	21.21	0.2464
9	Vanillic acid	23.16	0.01712
10	Ferulic acid	33.12	ND
11	Sinapic acid	35.05	ND
12	Rutin	38.36	0.2338
13	*p*-Coumaric acid	38.4	ND
14	Apigenin-7-glucoside	47.7	ND
15	Cinnamic acid	54.1	ND
16	Quercetin	55.6	0.3296
17	Kaempferol	58.61	0.44839
18	Chyrsin	61.26	ND
Total			**28.9913**

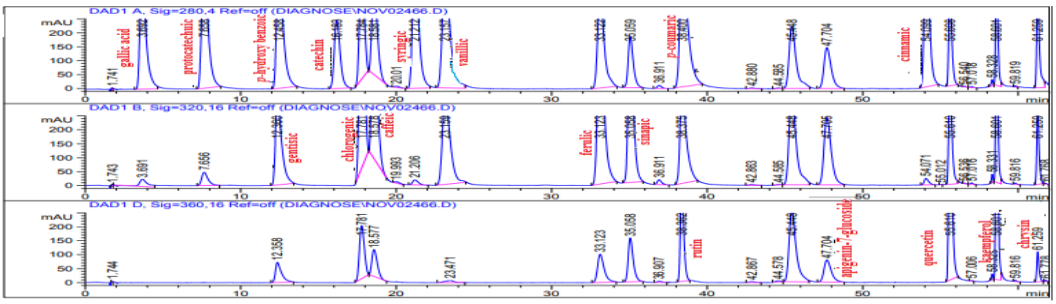


Figure 1: Chromatogram of authentic phenolic acids and flavonoids used as standards by HPLC\DAD

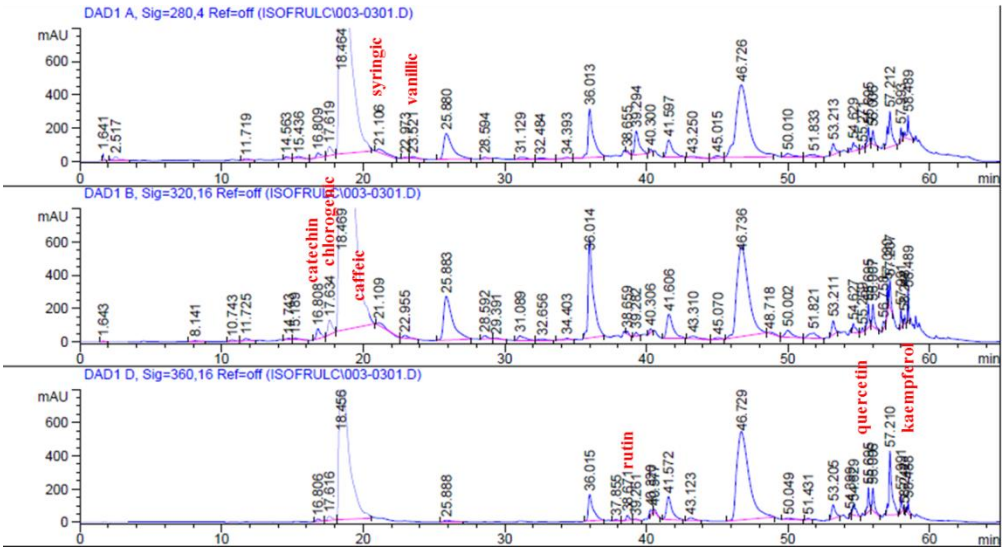


Figure 2: Chromatogram of phenolic acids and flavonoids detected in 70 % ethanolic extract by HPLC\DAD at 3 wavelengths; A: at 280 nm, B: at 320 nm and C: at 360 nm

Isolation and identification of the main constituents of Marianna rootstock leaves methanol extract

Compound (1):kaempferol 3, 7-*O*-*α*-L-dirhamnoside (Kaempferitrin)

Chromatographic data: yellow residue (17 mg), appeared as a dark purple spot on PC under UV light changed to yellow upon exposure to ammonia vapor and $AlCl_3$ spraying. R_f= 0.48 and 0.59 on paper chromatography (1MM) in solvent systems BAW (4:1:5) and acetic acid (15%), respectively. From chromatographic data, the compound could be identified as a flavonoid glycoside.

UV spectral data: UV spectral data indicated a flavonol type structure with substituted 7-OH group, 4`-OH free and absence of *O*-dihydroxyl groups at ring-B **[32]**.

^{1}H-NMR δ ppm (400 MHz, DMSO):6.47 (1H-6,d, *J* = 4 Hz), 6.79 (1H-8, d, *J* = 4 Hz), 6.93 (2H-3`, 5`, d, *J* = 8 Hz), 7.80 (2H-2`, 6`, d, *J* = 8Hz), 0.81 (3H-6″, d, *J* = 4 Hz), 1.14 (3H-6‴, d, *J* = 4 Hz), 5.56 (1H-1″, s), 5.31 (1H-1‴, s).

^{13}C-NMR δ ppm (100 MHz, DMSO)

156.52 (C-2), 135 (C-3), 178.4 (C-4), 161.39 (C-5), 98.8 (C-6), 162.16 (C-7), 95.07 (C-8), 158.26 (C-9), 106.2 (C-10), 120.8 (C-1'), 131.18 (C2', C-6'), 115.89 (C3', C-5'), 160.6 (C-4'), 99.93(C-1″), 102.3(C-1‴), 17.9 (C-6″), 18.3(C-6‴).

By comparing UV, ^{1}HNMR and ^{13}CNMR data with the previously published data **[33],** compound 1 was identified as kaempferol 3, 7-*O*-*α*-L-dirhamnoside (Kaempferitrin), this compound is first time to be isolated from the plant.

Compound (2): kaempferol 3,7-*O*-*β*-D-di-glucoside

Chromatographic data: Yellow residue (15 mg), appeared as a dark purple spot on paper chromatography(1MM) under UV light changed to yellow upon exposure to ammonia vapor and $AlCl_3$ spraying. R_f= 0.45 and 0.53 on PC in solvent systems BAW (4:1:5) and acetic acid (15%) respectively. Chromatographic data suggested flavonoidal glycoside.

UV spectral data: UV spectral data indicated a flavonol type with substituted 7-OH group, 4`-OH free and absence of *O*-dihydroxyl groups at ring-B **[32]**.

^{1}H-NMR δ ppm (400 MHz, DMSO): 6.2 (1H-6, d, *J*=2), 6.4 (1H-8, d, *J*=2), 6.8 (2H-3`,5`, d, *J* = 8.19 Hz), 8 (2H-2`,6`, d, *J* = 8.19 Hz), 5.4(1H-1‴, d, *J*=6.2), 5.2 (1H-1″, d, *J*=6.2).

^{13}C-NMR δ ppm (100 MHz, DMSO):156.5 (C-2), 134.78 (C-3), 175.4 (C-4), 162.2 (C-5), 97.65 (C-6), 162.63 (C-7), 92.37 (C-8), 158.5 (C-9), 104.00(C-10), 120.8 (C-1'),130.61 (C2', C-6'), 112.4 (C3', C-5'), 160.6 (C-4`), 97.6(C-1″), 102.87(C-1‴).

From UV, ^{1}H-NMR and ^{13}C-NMR spectral data, compound 2 was identified as kaempferol 3, 7-*O*-*β*-D-diglucoside, first isolated from the plant.

Compound (3): kaempferol 7-*O*-*α*-L-rhamnoside

Yellow residue (20mg), appeared as a dark purple spot on PC under UV light changed to yellow upon exposure to ammonia vapor and $AlCl_3$ spraying. R_f= 0.47 and 0.66 on paper chromatography (1MM) in solvent systems BAW (4:1:5) and acetic acid (15%) respectively, suggesting flavonoid glycoside

UV spectral data: UV data indicated a flavonol type with substituted 7-OH and absence of *O*-dihydroxyl groups at ring-B **[32]**.

^{1}H-NMR δ ppm (400 MHz, DMSO): 6.43 (1H-6, d, *J* = 1.5Hz), 6.83 (1H-8, d, *J* = 1.5Hz), 6.95 (2H-3`-5`, d, *J* = 8 Hz), 8.09 (2H-2`-6`, d, *J* = 8 Hz), 5.49 (1H-", s), 1.14 (3H-6", d, *J* = 4 Hz).

^{13}C-NMR δ ppm (100 MHz, DMSO)

148 (C-2) , 136.49 (C-3), 176.5 (C-4), 160.8(C-5), 97.7(C-6), 161.8(C-7), 94.8(C-8), 156.2 (C-9), 105.1 (C-10), 121.9(C-1'), 130.1(C-2', C-6'), 115.9 (C-3', C-5'), 159.9 (C-4`), 18.4(C-6′′).

^{1}H-NMR of compound 3 showed two *meta* coupled protons resonating at 6.43(*J* = 1.5 Hz) and δ 6.83 (*J* = 1.5Hz) attributed to *meta*-split of H-6 and H-8. Two signals resonating at δ 6.95 (*J* = 8 Hz) and 8.09 (*J* = 8Hz) attributed to 3`-5` and 2`-6` respectively. A singlet at 5.49 ppm attributed to anomeric proton with a peak at δ 94.8 in ^{13}C-NMR spectrum for anomeric carbon, and a doublet at 1.14 ppm assigned to protons of methyl group resonating its carbon at 18.4 ppm in ^{13}C-NMR spectrum confirming rhamnose sugar moiety. From UV, ^{1}HNMR and ^{13}CNMR compound 3 was identified as kaempferol 7-*O*-*α*-L-rhamnoside, first isolated from the plant.

Compound (4): quercetin -7-*O*-*β*-D-glucoside -4',-*O*-*α*-L-rhamnoside

Chromatographic data: yellow precipitate (16 mg), appeared as a dark purple spot on PC under UV light slightly changed upon exposure to ammonia vapor and $AlCl_3$ spraying. R_f= 0.45 and 0.57 on PC in solvent systems BAW (4:1:5) and acetic acid (15%) respectively.

UV spectral data: MeOH; 267, 350, NaOMe; 267, 377, 388, NaOAc; 267.5, 356; NaAc\ H_3BO_3 ; 267.5, 355, $AlCl_3$; 267, 346, 397; $AlCl_3$\ HCl; 267, 350, 398

^{1}H-NMR δ ppm (400 MHz, DMSO): 6.04 (1H, d, *J*= 2 Hz), 6.2 (1H, d, *J*= 2 Hz), 6.8 (1H, d, *J*= 9Hz), 7.9 (1H, m), 8.08 (1H, d, *J* = 2 Hz), 5.2 (1H, d, *J* = 6.3 Hz), 5.5 (1H, s), 1 (3H, d, *J* = 6 Hz).

Chromatographic data suggested that the compound belongs to flavonoid glycoside indicated a flavonol type with substituted 7-OH confirmed from no NaOAc bathochromic shift relative to methanol maximum and substituted 4`-OH confirmed from NaOMe absorption maximum with no increase in intensity **[32].**

The ^{1}H-NMR of compound 4 showed two protons resonating at δ 6.03 and δ 6.2 attributed to H-6 and H-8. Three signals were observed at δ 6.8 (*J* = 9 Hz), 7.9 (*m*) and 8.08 (*J* = 2Hz) attributed to 5`, 6` and 2` respectively. A singlet at 5.5 ppm was attributed to the anomeric proton of a rhamnose moiety confirmed from a doublet at 1 ppm (3H, d, *J* = 6Hz) attributed to protons of the rhamnose methyl group. Another anomeric doublet signal at 5.2 (*J* = 6Hz) were attributed to proton of a glucose moiety, confirmed by acid hydrolysis and co-chromatography with authentic sugar using Aniline phthalate reagent. The position of glucose moiety was assigned to 7-OH as its anomeric proton is observed upfield at 5.2 ppm **[32]**. Regarding UV and ^{1}HNMR spectral data, compound 4 was suggested to be quercetin -7-*O*-*β*-D-glucoside -4',-*O*-*α*-L-rhamnoside, first isolated from the plant. Structures of the isolated compounds are illustrated in Figure 3.

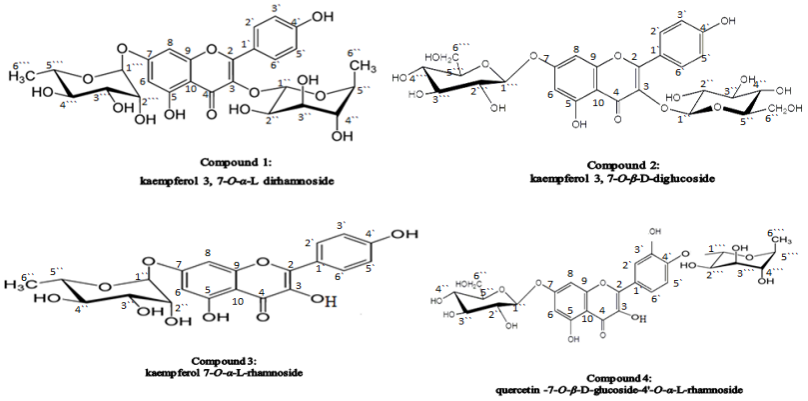


Figure 3: Structures of the isolated flavonol glycosides

Biological study

Antioxidant characters

Scavenging properties of Marianna rootstock crude and successive extracts:

DPPH Free radical scavenging effect

1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a stable (in powder form) free radical with deep violet color which turns to yellow when scavenged in reaction media. The DPPH assay uses this character to assess free radical scavenging ability of materials. Data in Figure 4 showed that the crude, successive extracts and standards scavenged DPPH radicals in concentration-dependent manner. The crude extract exhibited a potent scavenging effect, which was magnified with increasing the concentration from 50µg/ ml (52.71±1.41%) to 1000µg/ ml (97.51± 1.00%); this effect manner was similar to that of other extracts. Insignificant difference was observed among crude extract, pet.ether, dichloromethane, ethyl acetate, 80% methanolic extracts and Ascorbic acid at the concentration 1000µg/ml. (Table 3). In parallel, insignificant difference was recorded among crude, dichloromethane, and ethyl acetate extract which was significant as compared to Ascorbic acid and BHT at the concentration 500µg/ ml ($P<0.05$). (Table 3), whereas reference materials showed the same efficacy of methanoilc extract at 100 and 500µg/ ml. Regarding IC_{50} values, The less IC_{50} value was recorded for plant crude extract as free radical scavenger (45.11µg/ ml), whereas the greatest value was obtained (182.14 µg/ml) for ethyl acetate extract, compared with L. Ascorbic acid (47.68 µg/ml) and BHT (43.02µg/ml) (Table 3). There was insignificant difference among crude extract, Ascorbic acid and BHT (45.11, 47.68 and 43.02 µg/ml, respectively).

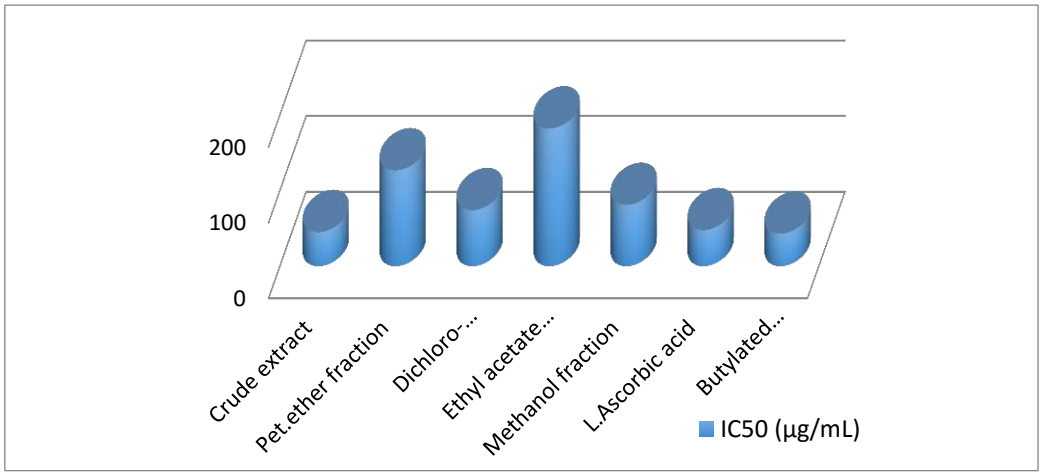


Figure 4: $IC_{50}$of DPPH radical scavenging efficacy of Marianna rootstock crude extract, successive extracts and standards

Table 3: DPPH radical scavenging efficacy of crude, successive extracts and standards at different concentrations

Tested material. / Concn.	Crude extract	Pet. Ether extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	L. Ascorbic acid	Butylated hydroxyl-toluene
50µg/ml	24.868±1.63$^{*\#}$	52.71±1.41^{a}	41.48±1.34$^{*\#v}$	31.66±1.68$^{*\#}$	39.21±1.27$^{*\#}_{v}$	50.12±1.36^{a}	52.33±1.40^{a}
100µg/ ml	40.36±1$^{*\#d}$	71.22±1.36$^{*\#}$	73.25±1.75$^{*\#}$	41.09±1.45$^{*\#d}$	59.52±1.68$^{\#y}$	62.51±1.41^{y}	64.11±1.53^{y}
250µg/ ml	73.91±2.34$^{r\#}$	88.69±1.96$^{*\#}$	80.22±2.37^{h}	64.29±2.02$^{*\#}$	83.46±2^{*p}	77.82±1.73^{r}	78.16±1.89^{ph}
500µg/ ml	83.46±1.72$^{*\#}$	93.39±1.32$^{*\#i}$	99.76±1.81$^{*\#}$	95.25±1.59$^{*\#l}$	89.99±1.55^{k}	89.13±1.49^{k}	87.18±1.26^{k}
1000µg/ ml	100±1.01$^{u*\#}$	97.51±1.00^{u}	100±0.94$^{*\#u}$	99.78±1^{u}	96.16±0.97^{un}	95.22±0.98^{n}	94.33±1.10^{n}
IC_{50} (µg/ ml)	**45.11**	**126.71**	**74.16**	**182.14**	**81.03**	**47.68**	**43.02**

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3 replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test. Occurring of * means significant difference as compared to ascorbic acid whereas # means significant difference as compared to BHT. Groups have the same letter means insignificant difference between them

Nitric oxide scavenging potential role of Marianna rootstock crude and successive extracts

The plant crude extract and successive extracts inhibited the NO• liberation from SNP through their effect as nitric oxide radical scavenger, the effect was concentration-dependent. NO• radical scavenging efficacy was promising in the crude alcoholic extract (93.33±2 at 1000µg/ml) as compared to standards at the same concentration (91.14 and 91.45%, respectively). However, petroleum ether and ethyl acetate extracts presented moderate NO• radical scavenging ability, Figure 5. All of tested materials (crude extract, pet.ether, dichloromethane, ethyl acetate and methanolic extracts) presented gradual NO scavenging efficacy increased with concentration increment. Insignificant difference was obtained at 1000µg/ml among the tested materials and reference compounds (93.33, 95.72, 95.72, 95.50 and 92.04, 91.14 and 91.45 % for crude, pet.ether, dichloromethane, ethyl acetate, methanolic successive extracts, Ascorbic acid and BHT, respectively). In addition, crude extract produced the highest scavenging ability at all concentration as compared to the other tested extract comparing to reference material, Table 4. Considering IC_{50} values, crude extract showed a value of 47.64µg/ ml which indicates its valuable efficacy as scavenger. In contrast, ethyl acetate showed the less efficacy (IC_{50} value, 145.33µg/ ml) in comparison with Ascorbic acid (54.67µg/ml) and BHT (58.34µg/ ml). Additionally, mild scavenging efficacy was recorded for dichloromethane and methanolic extracts (67.11 and 66.12 µg/ ml, respectively), Table 4.

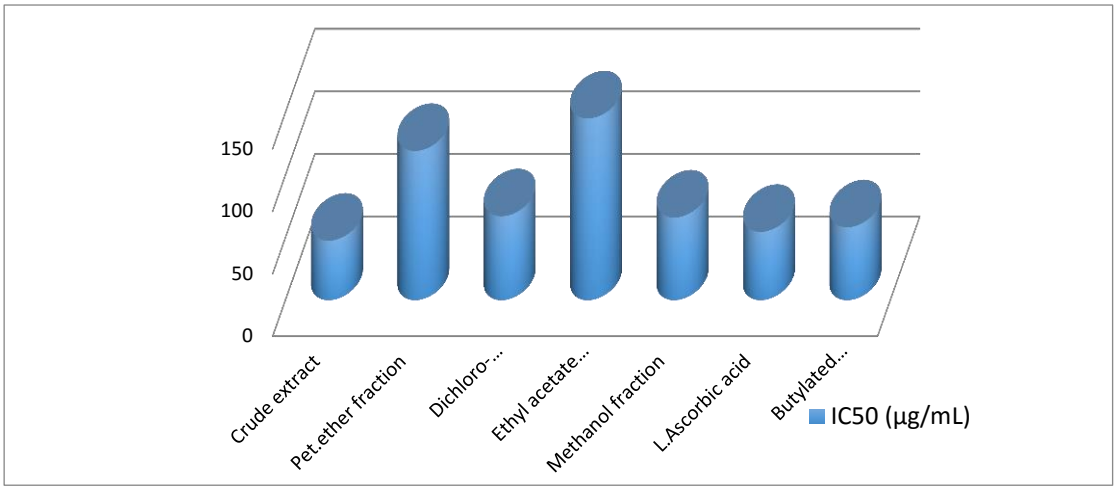


Figure 5: IC_{50} of Potential effect of Marianna rootstock crude and successive extracts as nitric oxide accumulation inhibitor comparing to standards

Table 4: Potential effect of Marianna rootstock crude and successive extracts as nitric oxide accumulation inhibitor comparing to standards

Tested material / Concn.	Crude extract	Pet. Ether extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	L. Ascorbic acid	Butylated hydroxyl-toluene
50µg/ml	24.38±1.51$^{*\#}$	51.67±1.43^{a}	40.63±1.39$^{*\#b}$	31.03±1.41	38.44±1.54$^{*\#b}$	49.13±1.46^{a}	47.65±1.44^{a}
100µg/ml	41±1.32$^{*\#f}$	72.35±1.28$^{*\#e}$	74.42±1.25$^{*\#e}$	41.75±1.31$^{*\#f}$	59.52±1.33^{c}	65.51±1.29^{d}	62.51±1.30^{cd}
250µg/ml	73.66±1.22^{g}	88.39±1.20^{h}	79.95±1.19^{i}	64.08±1.21$^{*\#}$	83.18±1.24^{h}	77.56±1.20^{gi}	75.47±1.19^{gi}
500µg/ml	80.17±1.01$^{*\#}$	59.71±0.97$^{*\#}$	95.83±1$^{*\#j}$	91.49±1.02$^{*\#j}$	85.45±0.99^{k}	85.62±1.01^{k}	86.22±1^{k}
1000µg/ml	95.72±1.97^{l}	93.33±2^{l}	95.72±1.89^{l}	95.50±1.99^{l}	92.04±2.01^{l}	91.14±1.97^{l}	91.45±1.95^{l}
IC_{50} (µg/ml)	**47.64**	**119.34**	**67.11**	**145.33**	**66.12**	**54.67**	**58.34**

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test. Occurring of * means significant difference as compared to ascorbic acid whereas # means significant difference as compared to BHT. Groups have the same letter means insignificant difference between them

Superoxide radical scavenging character of crude and successive extracts

Crude extract of leaves andsucessiveextracts inhibited generation of O_2^- radical in PMS NADH-NBT system as compared with two standard compounds, Figure 6. All of tested materials (crude extract, pet.ether, dichloromethane, ethyl acetate and methanolicextracts) presented gradual O_2^- scavenging efficacy increased with concentration increment. Crude extract showed activity ranged from 52.34±1.25to 100±2.11at 50–1000 µg/ml, compared to Ascorbic acid (49.18±1.21to 96.45±2.18) and BHT (53.31±1.08to 100±2.24) at the same concentrations. This efficacy was similar to other extracts at 1000 µg/ml. The IC_{50} of plant extracts for O_2^- radical scavenging ranged between 46.26 µg/ml for leaves alcoholicextract and 135.67 µg/ml for petroleum ether extract, Table 5. IC_{50} of crude extract is close to these of l. Ascorbic acid and BHT (55 and 48.53 µg/ml, respectively).

Insignificant difference was obtained at 1000µg/ml among the tested materials and reference compounds (100, 100, 100, 100, 97.40, 96.45 and 100 % for crude, pet.ether, dichloromethane, ethyl acetate, methanolic extracts, Ascorbic acid and BHT, respectively). In addition, crude extract produced the highest scavenging ability at all concentration as compared to the other tested extracts in comparing to reference material, Table 5.

Considering IC_{50} values, crude extract recorded the least value (46.26µg/ ml) that means its valuable efficacy as scavenger. In contrast, pet.ether showed the less mean value efficacy (IC_{50} value, 135.67µg/ ml) in comparison with Ascorbic acid (55µg/ml) and BHT (48.53µg/ ml). Additionally, mild scavenging efficacy was recorded for dichloromethane and methanolic extracts (65.54 and 64.82 µg/ ml, respectively), Table 5.

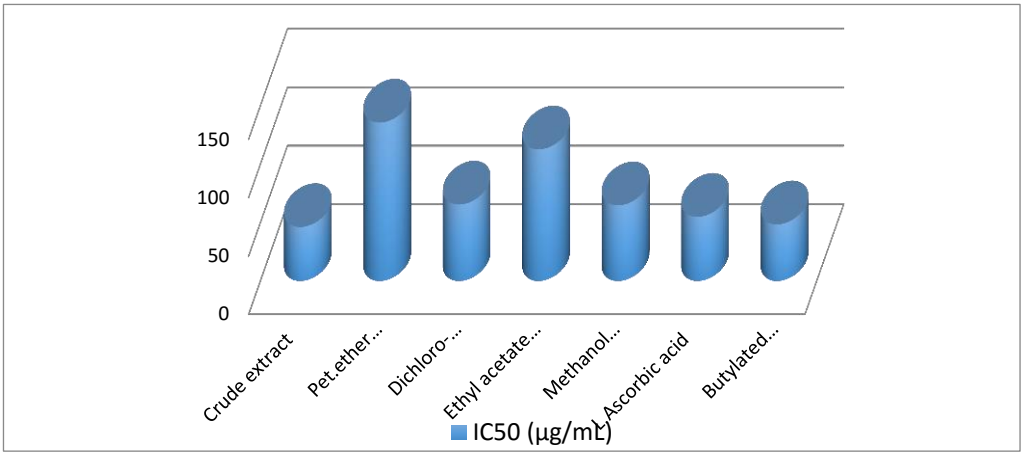


Figure 6: IC_{50} of Superoxide radical scavenging property of Marianna rootstock crude and successive extracts in comparing with standards

Table 5. Superoxide radical scavenging property of Marianna rootstock crude and successive extracts in comparing with standards

Tested material / Concn.	Crude extract	Pet.Ether extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	L. Ascorbic acid	Butylated hydroxy-toluene
50µg/ml	31.95±1.31$^{*\#}$	52.34±1.25^{a}	40.69±1.19$^{*\#b}$	31.07±1.20$^{*\#}$	38.47±1.16$^{*\#b}$	49.18±1.21^{a}	53.31±1.08^{a}
100µg/ml	42.43±1.46$^{*\#}$ ^{c}	73.89±1.39$^{*\#d}$	77.01±1.40$^{*\#d}$	43.20±1.35$^{*\#c}$	62.57±1.29^{e}	65.72±1.42^{e}	66.92±1.33^{e}
250µg/ml	75.48±2.23$^{\#f}$	82.70±2^{g}	81.93±2.01^{g}	65.66±1.97$^{*\#}$	75.24±1.98$^{\#h}$	79.48±2.13^{fhg}	80.23±2^{g}
500µg/ml	82.67±1.62$^{\#i}$	87.61±1.53^{j}	93.59±1.55$^{*\#}$	59.36±1.62$^{*\#}$	84.43±1.60$^{\#i}$	83.62±1.57$^{\#i}$	89.47±1.54^{j}
1000µg/ml	100±2.30^{k}	100±2.11^{k}	100±2.22^{k}	100±2^{k}	97.40±2.31^{k}	96.45±2.18^{k}	100±2.24^{k}
IC_{50} (µg/ml)	**46.26**	**135.67**	**65.54**	**112.71**	**64.82**	**55**	**48.53**

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3 replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test. Occurring of * means significant difference as compared to ascorbic acid whereas # means significant difference as compared to BHT. Groups have the same letter means insignificant difference between them.

Cation radical scavenging property of crude and successive extracts (ABTS)

The ABTS/H_2O_2 discoloration method is thought to represent the total antioxidant capacity of plant extracts. As shown in Figure 7, Pet.ether, dichloro methane, and ethyl acetate extracts showed remarkable antioxidant capacity as compared to crude extract and standards at 1000µg/ml. The antioxidant capacity was significantly enhanced by increasing extract concentration in a concentration dependent response for all extracts. The less efficacy was recorded for methanol extract at all concentrations. Insignificant difference was observed among pet.ether, dichloro methane, ethyl acetate extracts and standards at the concentration 1000 µg/ml, Table 6. In contrast, significant difference was recorded among crude extract and methanol extract as compared to ascorbic acid and BHT at 500µg/ ml and 1000µg/ ml. Regarding IC_{50} values, The IC_{50} value was recorded for plant crude extract as free radical scavenger (87.13µg/ ml), whereas the greatest value was 492.37µg/ ml for methanol extract, compared to Ascorbic acid (51.05µg/ ml) and BHT (77.68µg/ ml), Table

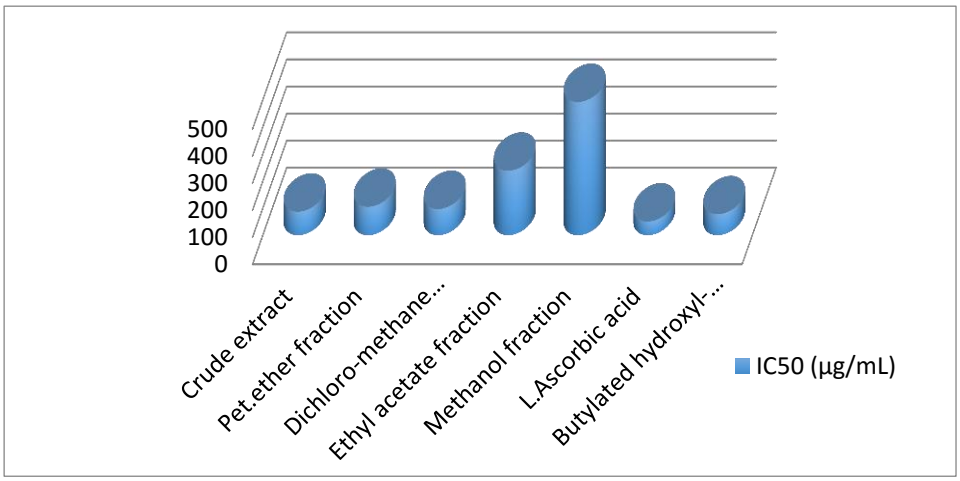


Figure 7: IC_{50}Cation radical scavenging efficacy of Marianna rootstock crude extract, successive extracts and standards

Table 6: Cation radical scavenging efficacy of Marianna rootstock crude, successive extracts and standards at different concentrations

Tested material / Concn.	Crude extract	Pet. Ether extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	L. Ascorbic acid	Butylated hydroxyl-toluene
50µg/ml	44.18±1.03*a	44.71±1.11*^{a}	29.22±1.23*#	23.76±1.17*#	11.35±1.25*#	48.27±1.10^{f}	45.73±1.23^{af}
100µg/ml	52.42±1.20*#z	49.62±1.25*#	50.42±1.43*#z	39.94±1.20*#	20.48±1.47*#	65.43±1.46^{c}	61.11±1.33^{c}
250µg/ml	60.12±1.24*#	71.21±1.22*#	66.36±1.16*#	53.34±1.29*#	32.83±1.31*#	80.37±1.35	81.28±1.40
500µg/ml	63.63±1.10*#	86.92±1.30^{q}	82.17±1.17*#	71.92±1.31*#	49.02±1.22*#	91.76±1.37^{q}	92.16±1.51^{q}
1000µg/ml	76.52±1.43*#	90±1.45*#x	91.67±1.31*#x	93.62±1.79*#x	54.66±1.26*#	100±1.40^{n}	100±1.22^{n}
IC50 (µg/ml)	**87.13**	**105.24**	**97.28**	**238.37**	**492.37**	**51.05**	**77.68**

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3 replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test. Occurring of * means significant difference as compared to ascorbic acid whereas # means significant difference as compared to BHT. Groups have the same letter means insignificant difference between them.

Oxidative stress promoters controlling characters

Reduction power

The ability of tested extracts to play as reductant in the reaction medium was increased dependently in response to concentration. Dichloromethane and ethyl acetate successive extracts were found to have remarkable reducing power as compared to standard materials (L-ascorbic acid and BHT) at all concentrations (50, 100, 250,500 and 1000µg /mL) and the effect was concentration-dependent. Reducing power of extracts and standards at high concentration followed the order; Ascorbic> Ethyl acetate extract > Dichloromethane extract> Crude extract >pet.ether extract > BHT > methanol extract (data in Table 7). All of tested materials (crude extract, pet.ether, dichloromethane, ethyl acetate and methanolic extracts) presented gradual efficacy increase with concentration increment. Insignificant difference was obtained at 1000µg/ml among crude extract, pet.ether, dichloromethane, ethyl acetate successive extracts and reference compounds (0.733, 0.62, 0.78, 0.78, 0.79and 0.62 % for crude, pet.ether, dichloromethane, ethyl acetate extracts, ascorbic acid and BHT, respectively), Table 7.

Table 7: Reduction capability of Marianna rootstock crude, successive extracts and standards at different concentrations

Tested material / Concn.	Crude extract	Pet.ether extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	L.Ascorbic acid	Butylated hydroxyl-toluene
50µg/ml	0.342±0.16*e	0.345±0.12e	0.404±0.1*#s	0.404±0.1*#s	0.381±0.05#f	0.385±0.1#f	0.29±0.06*
100µg/ml	0.409±0.28*#	0.446±0.16*#	0.621±0.15*#s	0.624±0.12*#s	0.385±0.08*p	0.564±0.13#	0.378±0.10*p
250µg/ml	0.559±0.54*#	0.447±0.12*#	0.650±0.17q	0.643±0.17*#q	0.391±0.11*#	0.631±0.11#q	0.51±0.07*
500µg/ml	0.656±0.18*#	0.502±0.14*#	0.675±0.05*#d	0.673±0.14*#d	0.396±0.10*#	0.74±0.09#	0.573±0.10*
1000µg/ml	0.733±0.3*#	0.62±0.21*#x	0.781±0.06#t	0.781±0.10#t	0.453±0.12*#	0.79±0.12#t	0.62±0.13*x

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3 replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test.
Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3 replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test.

Chelation of ferrous ion by crude extract and successive extracts

The purpose of ferrous ion chelating test is determining the capacity of the plant crude extract and successive to bind to the ferrous ion catalyzing oxidation as compared to standards (ascorbic acid and BHT). The ferrous ion chelating effect of crude extract, successive and standards at different concentrations, 50, 100,250, 500, and 1000 µg/ml, is presented in Figure 8. The data showed that all of tested materials (crude extract, pet.ether, dichloromethane, ethyl acetate and methanol extracts) presented gradual increase in chelating efficacy with concentration increment.

Insignificant difference was obtained at 1000µg/ml among the tested materials and reference compounds (100, 98.84, 98.84, 100, 100 and100 % for crude, dichloromethane, ethyl acetate, methanol, ascorbic acid and BHT, respectively), Table 8, Figure 8.

In addition, crude extract produced the highest scavenging ability at all concentration as compared to the other tested extract in comparing to reference material. Considering IC_{50} values, crude extract recorded less value (46.11µg/ ml) regarding its valuable efficacy as chelator, as well as, methanol fraction (51 µg/ ml). In contrast, pet.ether showed the lowest efficacy (IC_{50} value, 149.26µg/ ml) in comparison with Ascorbic acid (72.16µg/ml) and BHT (77.13µg/ ml) (Table 8). Additionally, mild scavenging efficacy was recorded for dichloromethane and ethyl acetate extracts (74.36 and 75.34 µg/ ml, respectively), Table 8.

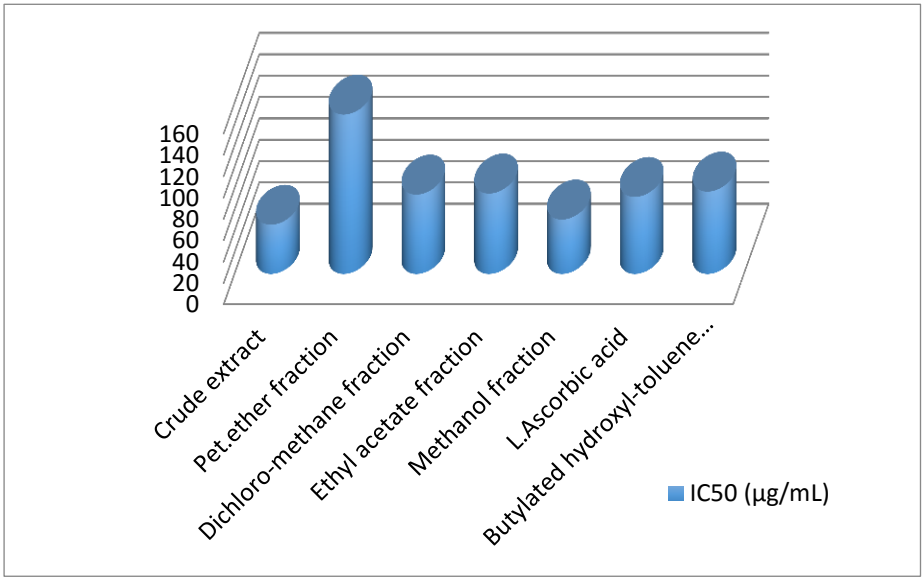


Figure 8: IC_{50} of Chelation of ferrous ions efficacy of Marianna rootstock crude, successive extracts and standards

Table 8: Chelation of ferrous ions efficacy of Marianna rootstock crude, successive extracts and standards at different concentrations

Tested material / Concn.	Crude extract	Pet. Ether extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	L. ascorbic acid	Butylated hydroxyl toluene
50µg/ml	37.37±1.32$^{*\#}$	52.32±1.01$^{*\#}$	44.05±1.02^{a}	44.05±1.45^{a}	49.47±1.42	42.33±1.41^{a}	44.11±1.27^{a}
100µg/ml	45.74±2.01$^{*\#}$	68.42±1.84$^{*\#m}$	69.37±2.13$^{*\#m}$	69.37±2.00$^{*\#m}$	72.53±2.53$^{*\#}$	63.17±2.22^{u}	58.18±2.11^{u}
250µg/ml	71.74±1.53$^{*\#}_{k}$	73.68±1.73$^{*\#k}$	77.05±1.31^{r}	77.05±1.33^{r}	83.36±1.63^{r}	81.09±1.02^{r}	84±1.12^{r}
500µg/ml	86.89±1.54$^{*\#}_{h}$	87.58±1.61$^{*\#h}$	89.42±1.64$^{*\#h}$	89.42±1.62$^{*\#h}$	100±1.81^{y}	98±1.25^{y}	100±1.36^{y}
1000µg/ml	92.74±1.00$^{*\#}$	100±0.98^{t}	98.84±0.99^{t}	98.84±1.13^{t}	100±1.00^{t}	100±0.91^{t}	100±1.14^{t}
IC_{50} (µg/ml)	**46.11**	**149.26**	**74.36**	**75.34**	**51**	**72.16**	**77.13**

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3 replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test. Occurring of * means significant difference as compared to ascorbic acid whereas # means significant difference as compared to BHT. Groups have the same letter means insignificant difference between them

Hydrogen peroxide scavenging efficacy

As shown in Figure 9, the presented data show that all of tested materials (crude extract, pet.ether, dichloromethane, ethyl acetate and methanolic successives) presented gradual hydrogen peroxide scavenging efficacy increased with concentration increment. Crude alcoholic extract represented the highest H_2O_2 scavenging activity, higher than that represented by other successives and similar to that of standards. Insignificant difference was obtained at 1000µg/ml between crude extract and pet.ether successive extract compared to reference compounds (92.16, 100, 100 and 100 % for crude, pet.ether, ascorbic acid and BHT, respectively). In addition, crude extract produced the highest scavenging ability at all concentration as compared to the other tested extract in comparing to Butylated hydroxyltoluene as reference material, Table 9. The crude extract and pet.ether extract reached the nearest values to standards at 500 and 1000 µg/ml (85.67% and 92.16% for crude extract and 83.83 and 100% for pet.etherextract) compared with two standard compounds; L. ascorbic acid (89.52 %and 100%) and BHT (86.29%and 100%) at the same concentrations. Considering IC_{50} values, crude extract recorded less value (51.22µg/ ml) regarding its valuable efficacy as scavenger. In contrast, pet.ether showed the lowest efficacy (IC_{50} value, 164.32µg/ ml) in comparison with Ascorbic acid (47.56 µg/ml) and BHT (50µg/ ml) (Table 9). Additionally, mild scavenging efficacy was recorded for dichloromethane and ethyl acetate extracts (86.17 and 91.28 µg/ ml, respectively), Table 9.

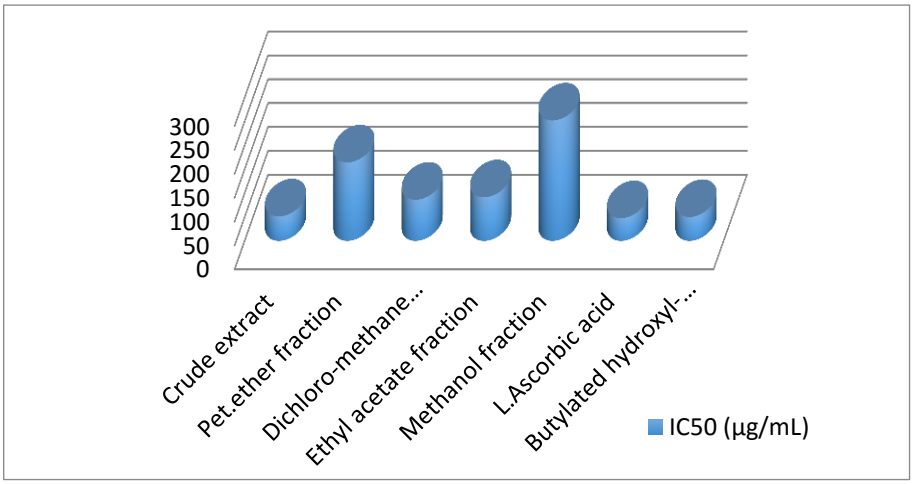


Figure 9: IC_{50} of Hydrogen peroxide scavenging property of Marianna rootstock crude and successive extracts in comparing with standards

Table 9: Hydrogen peroxide scavenging property of Marianna rootstock crude, successive extracts and standards at different concentrations

Tested material / Concn.	Crude extract	Pet. ether extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	L. Ascorbic acid	Butylated hydroxyl-toluene
50µg/ml	25.03±1.30$^{*\#}$	49.26±1.24^{a}	41.74±1.28$^{*\#b}$	29.59±1.31$^{*\#}$	39.46±1.26$^{*\#b}$	50.44±1.30^{a}	48.91±1.25^{a}
100µg/ml	44.93±2.15$^{*\#cd}$	68.44±2^{f}	53.27±1.97$^{*\#e}$	42.49±2.06$^{*\#c}$	48.03±2.11$^{*\#de}$	69.61±2.07^{f}	66.48±2.22^{f}
250µg/ml	75.34±2^{g}	79.56±2.06^{g}	62.81±2.18$^{*\#h}$	64.49±1.98$^{*\#h}$	50.96±2.23$^{*\#}$	79.33±2.08^{g}	78.41±2.11^{g}
500µg/ml	83.83±1.78^{*i}	85.67±1.64^{*i}	70.11±1.59$^{*\#}$	74.27±1.72$^{*\#}$	53.24±1.68$^{*\#}$	89.52±1.57^{j}	86.29±1.63^{ij}
1000µg/ml	100±1.97^{k}	92.16±2$^{*\#}$	81.43±1.97$^{*\#m}$	81.39±2.07$^{*\#m}$	60.48±2.01$^{*\#}$	100±1.89^{k}	100±2.12^{k}
IC_{50} (µg/ml)	**51.22**	**164.32**	**86.17**	**91.28**	**252**	**47.56**	**50**

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3 replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test. Occurring of * means significant difference as compared to ascorbic acid whereas # means significant difference as compared to BHT. Groups have the same letter means insignificant difference between them.

Inhibition of lipid peroxide production by crude and successive extracts

Lipid peroxidation was significantly inhibited by all plant extracts at all tested concentrations, Figure 10. All of tested materials (crude extract, pet.ether, dichloromethane, ethyl acetate and methanol successive) revealed gradual increase in lipid peroxide inhibition with concentration increment. The maximum inhibition percentage was presented with highest extract concentration. Plant extracts inhibition percentages at highest concentration arranged in the following order; Ethyl acetate extract>Dichloro-methane extract>pet.etherextract> Crude extract> Methanol extract (Table, 10). Insignificant difference was obtained at 1000µg/ml among the tested materials and reference compounds (93.62, 91.67, 90, 100 and 100 % for ethyl acetate dichloromethane, pet.ether successive, ascorbic acid and BHT, respectively), In parallel, significant difference was recorded between crude extract and, methanol successive extract as compared to ascorbic acid and BHT at the concentration 1000 µg/ ml (Table 10). Considering IC_{50} values, crude extract recorded less value (100µg/ ml) regarding its valuable efficacy as lipid peroxide inhibitor. In contrast, methanol extract showed the lowest efficacy (IC_{50} value, 255µg/ ml) in comparison with Ascorbic acid (51.02 µg/ml) and BHT (73.41µg/ ml) (Table 10). Additionally, mild scavenging efficacy was recorded for dichloromethane and ethyl acetate successive (101.07 and 101.34 µg/ ml, respectively), Table 10.

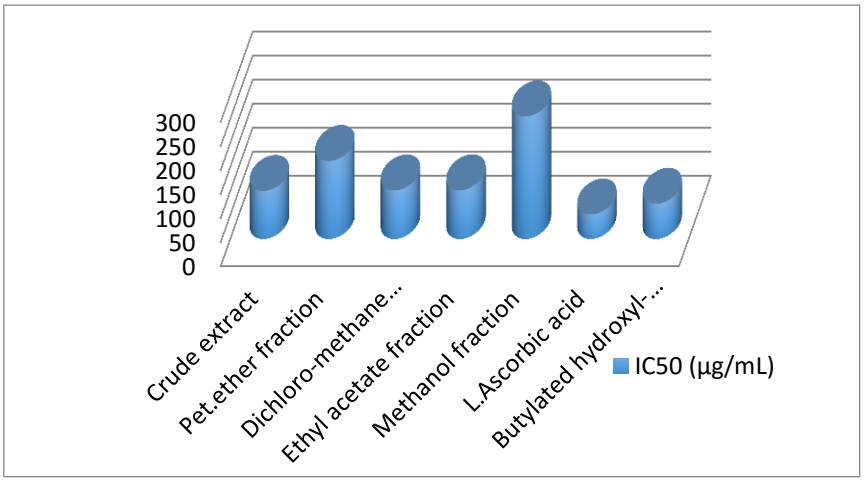


Figure 10: IC_{50} of Efficacy of Marianna rootstock crude and successive extracts as lipid peroxide formation inhibitor as compared to standard materials

Table 10: Efficacy of Marianna rootstock crude and successive extracts as lipid peroxide formation inhibitor in comparison to standard materials

Tested material / Concn.	Crude extract	Pet. Ether extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	L.Ascorbic acid	Butylated hydroxyl-toluene
50µg/ml	44.95±1.63^{a}	45.49±1.46^{a}	10.43±1.20$^{*\#}$	24.17±1.17$^{*\#}$	11.55±1.21$^{*\#}$	49.11±1.31^{a}	46.32±1.34^{a}
100µg/ml	50.67±1.41$^{*\#c}$	47.96±1.32$^{*\#c}$	49.76±1.26$^{*\#c}$	38.60±1.20$^{*\#}$	19.79±1.13$^{*\#}$	63.24±1.20^{b}	60.31±1.39^{b}
250µg/ml	56.11±1$^{*\#e}$	66.46±0.97$^{*\#f}$	61.39±1.01$^{*\#ef}$	49.78±0.99$^{*\#}$	30.64±1.04$^{*\#}$	75.01±1.01^{d}	75.19±1.01^{d}
500µg/ml	59.89±1$^{*\#}$	81.81±0.95^{g}	76.15±1.12$^{*\#}$	67.69±1.02$^{*\#}$	46.14±1$^{*\#}$	86.37±1^{g}	85.41±1^{g}
1000µg/ml	76.52±1.72$^{*\#}$	90±1.53$^{*\#h}$	91.67±1.47$^{*\#h}$	93.62±1.51$^{*\#h}$	54.66±1.40$^{*\#}$	100±1.22^{i}	100±1.46^{i}
IC_{50} (µg/ ml)	**100**	**162.34**	**101.07**	**101.34**	**255**	**51.02**	**73.41**

Data were served into the Statistical Package for Social Sciences (SPSS ver. 25). Data were presented as mean ± standard deviation. A one-way analysis of variance (ANOVA) was applied for comparisons between groups (n= 3 replicates), *P<0.05*. Multiple comparisons between averages of different groups were performed by one-way ANOVA followed with post hoc testing by Duncan's t-test. Occurring of * means significant difference as compared to ascorbic acid whereas # means significant difference as compared to BHT. Groups have the same letter means insignificant difference between them.

Discussion

The most common natural antioxidant metabolites are flavonoids and phenolic acids. There is no extensive reported data on Marianna rootstock (*Prunus cerasifera* Ehrh. × *Prunus munsoniana* Wight and Hedrick) from the phytochemical and biological aspects therefore, the current study focused on the antioxidant property of this plant with phytoconstituents investigation of polar metabolites that may attribute to this activity.

Reactive oxygen species (ROS), such as hydroxyl radical, superoxide anion and hydrogen peroxide are natural metabolic byproducts of oxygen **[34].** Oxidative stress resulted from disturbance in protective system leading to imbalanced ROS formation and elimination. There are many risk factors that may increase ROS levels in our body causing oxidative stress, for example, radiations, pollution, stress and UV radiation that troubling our defense mechanism and damaging DNA, leading to various diseases *vs*., cancer, arthritis, aging, Alzheimer's, *etc*., **[35].** Therefore natural antioxidants that act against imbalanced ROS are in crucial needs to protect our body from such oxidative stress. Natural antioxidants are generally considered safe for the human body. They are chemicals that halt the catalytic effects of free radicals, various illnesses, and chain processes to delay the onset of early ageing **[36].**

Antioxidants work by different mechanisms that act on initiation and/or propagation of the oxidation process *vs*., scavenging free radicals, chelating metals or by exert reducing potential **[37& 38]** and all these mechanisms are tested and profiled here in our study (DPPH Free radical scavenging effect, nitric oxide scavenging potential, Superoxide radical scavenging character, cation radical scavenging property, reduction power, chelation of ferrous ion, hydrogen peroxide scavenging efficacy and inhibition of lipid peroxide production) that proved the plant different extracts antioxidant potential in an approach to find its medicinal benefits**.**

Estimation of total phenolics and total flavonoids in Marianna rootstock total ethanolic extract revealed good contents recording 46.18 mg GAE/g and 44.38 mg CE/g calculated as gallic acid and catechin equivalent, respectively. To profile the flavonoidal and phenolic acids contents, HPLC as a sensitive and rapid analytical technique developed 8 metabolites first identified from this plant. Regarding Phenolic acids including in our study, hydroxybenzoic acids (vanillic and syringic) and hydroxycinnamic acids (caffeic and chlorogenic) were detected, while flavonoids detected including: flavonols (quercetin&kaempferol) and flavan (catechin) structures. According to the promising activity of polar extract as antioxidant and the good yield of estimated phenolics in our study, consequently to give deep insight, successive methanol extract, the most polar fraction, was subjected for more investigation by chromatographic fractionation leads to isolation of 4 compounds identified for the first time from the plant.The isolated compounds revealed flavonol structure with glycosidic linkage and were identified as "kaempferol -3, 7-*O*-*α*-L- dirhamnoside, kaempferol -7-*O*- *α*-L- rhamnoside, kaempferol 3, 7-*O*-*β*-D- diglucoside and quercetin-7-*O*-*β*-D-glucoside, 4'-*O*-*α*-L- rhamnoside".

Regarding phenolic acids structure activity relationship (SAR), antioxidant potential is affected by the numbers of hydroxyl groups in the molecule, along with the effect of steric hindrance and the electron-withdrawing properties of the carboxylic acid functional group that exerts negative action on proton donating ability of hydroxy benzoates and cinnamates**[38].**

Focusing on phenolic acids recorded in our HPLC profile, vanillic acid is used mainly in food as flavoring agent, it showed potential bioactivities *vs*., antioxidant, neuroprotective, anti-inflammatory and immune-stimulant action **[39].** Vanillic acid and its derivatives were *in-vivo* assayed to prove its powerful antioxidant property compared with Trolox "a tocopherol water soluble analogue" that showed greater free radicals scavenging activity than the standard attributed to the lipophilic nature **[40].**

Caffeic acid was reported for its antioxidant potential that tested by various assays compared with Trolox as a reference **[41]**, besides its action as carcinogenesis inhibitor and prevention of cardiovascular disorders **[42].**

For flavonoids SAR, specifically the presence and location of hydroxyl (-OH) substitutions and the catechol-type groups are linked to the antioxidant properties, for example: the *ortho*-dihydroxy or catechol group at ring-B, the 2, 3-double bond conjugated to 4-oxo (=O) alongwith hydroxyl substitution at positions 3 and 5 are responsible for the structural characteristics of a powerful antioxidant capacity **[36].**

Flavonoids were reported for various actions that could inhibit enzymes like cyclooxygenases and protein kinases where they are part of cell proliferation and apoptosis processes **[43].** Rutin, a diglycosidicflavonol, reported for improvement of harmful effects caused by radiations **[44].** Quercetin showed protective action for cells from oxidative

stress that interacts with aryl hydrocarbon receptor **[45].**

Kaempferol a dietary flavonoid, presents in numerous fruits and vegetables found to possess antioxidant potential beside other diverse actions; cardioprotective, neuroprotective, anti-inflammatory, antidiabetic, antimicrobial, and anti-cancer activities **[46].** Otherwise, it was reported that combination between quercetin and kaempferol revealed synergistic effect rather than single action **[47].**

Finally, it was concluded that antioxidant activity of Marianna rootstock crude and successive extracts that determined using different assays (DPPH Free radical scavenging effect, nitric oxide scavenging potential, Superoxide radical scavenging character, cation radical scavenging property, reduction power, chelation of ferrous ion, hydrogen peroxide scavenging efficacy and inhibition of lipid peroxide production) when compared to reference materials may be attributed to the diverse phenolics identified here for the first time from the plant.

Conclusion

According to this early investigation, the antioxidant activity of crude and successive extracts of Marianna rootstock could be attributable to the existence of active phenolic compounds. This research representsa solid foundation for additional research into mechanism of antioxidant action of active metabolites isolated from Marianna rootstock. As a result, this plant could be exploited to develop new lead structures for antioxidant natural agents.

Ethics Approval Number:

17086 (Medical Research Ethics Committee, National Research Centre, Egypt**)**

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Conflict of interest

There is no conflict of interest.

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