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Comparative Study of Chemical Profile and Antioxidant Activity of Two *Eugenia* Species Cultivated in Egypt

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ABSTRACT

Objectives: This study aimed to compare the chemical profile and the anti-oxidant activity of the aqueous methanol extracts (AME) of both *Eugenia uniflora* L. (EU) and *Eugenia supraaxillaris* Spring ex Mart. (ES) leaves cultivated in Egypt for the first time. Methods: HPLC/ESI-MS/MS analysis for the AME of both Eugenia species were used to tentatively identify the phenolic compounds. The antioxidant activity was evaluated using DPPH and NO radical scavenging assay. **Results:** HPLC/ESI-MS/MS analysis for the AME led to the tentative identification of fourteen and twelve compounds in *Eugenia uniflora* and *Eugenia supraaxillaris*, respectively. Bioactivity screening showed that the methanolic extract of *E. supraaxillaris* (IC50 = 8.51±0.37, 14.81±0.63 µg/mL) exhibited stronger antioxidant activity than that of *E. uniflora* (IC50 = 13.88±0.59, 32.53±1.75µg/mL) through their ability to inhibit DPPH and NO respectively. Also, we found that *E. supraaxillaris* (IC50 = 8.51±0.37, 14.81±0.63 µg/mL) exerted higher DPPH and NO scavenging activity than that of ascorbic acid (IC50 = 10.21±0.77, 17.95±2.24 µg/mL), respectively, with total anti-oxidant capacity (58.12±2.36 µg/mL) for *E. supraaxillaris* higher than that of *E. uniflora* (49.27±1.89 µg/mL) in comparison to the ascorbic acid as a control (69.75±3.81 µg/mL). **Conclusion:** Our study supports the usage of the AME of both *Eugenia* species, being efficient antioxidants, in the treatment of serious disorders such as aging, cancer, ischemia and rheumatoid arthritis.

***Keywords**: Anti-oxidant, DPPH, Eugenia, HPLC, NO.*

INTRODUCTION

Antioxidants are compounds which delay or inhibit oxidation and so, they prolong the life of the oxidizable matter[1]. Most of diseases are mainly due to oxidative stress resulting from free radicals. The free radicals are species that have very short half-lives, highly reactive and result in damage to many macromolecules like proteins, lipids and DNA[2]. They also affect different enzyme systems causing damage that may lead to serious disorders such as aging, cancer, ischemia and rheumatoid arthritis[3]. Antioxidants lower the oxidative stress in cells so, they are beneficial in the treatment of such human diseases[4].

Natural antioxidants potentiate the strength of the body antioxidants[5]. Polyphenolic compounds constitute the largest group of bioactive secondary metabolites that have antimicrobial, antiviral, anti-allergic, anti-inflammatory, and anti-oxidant[6], as they have a chemical molecule that disable free radicals[7].

The Myrtaceae family is one of the main families of commercial fruit trees in the world, comprising approximately 121 genera[8]. *Eugenia* is ranked as the second genus in the family Myrtaceae, comprising about 1,011 species of aromatic trees and shrubs[9,10]. The most studied species of *Eugenia* is *Eugenia uniflora* L. (known as Pitanga cherry). It is a Brazilian tree known for its cherry, edible sweet fruits, and attractive, aromatic, ornamental leaves[11,12]. The leaves are widely used in folk medicine to treat rheumatism, inflammatory and stomach disorders, hypertension, antioxidants, and fever[13,14,15]. It has been reported as antioxidant, anti-inflammatory and antimicrobial active agents[15-20]. These activities may be due to the presence of different bioactive secondary metabolites, which include flavonoids, leucoanthocyanidins, condensed and hydrolysable tannins, essential oil and steroids and/or triterpenoids[21]. Also, *Eugenia supraaxillaris* Spreng ex Mart., a Brazilian evergreen tree cultivated in subtropical and tropical countries, is a very interesting species. It has shown efficacy in treating inflammation, pain and related oxidative stress diseases[22].

In this study, we comparatively characterized the phenolic content of the leaves of *Eugenia uniflora* L. and *Eugenia supraaxillaris* Spring ex Mart. cultivated in Egypt using HPLC-MS/MS. Also; we evaluated the antioxidant activity of both extracts to demonstrate the correlation between their chemical composition and the proposed bioactivity.

MATERIAL AND METHODS

Plant material

Eugenia uniflora L. and *Eugenia supraaxillaris* Spring ex Mart. leaves were collected in October 2021 from Giza Zoo, Egypt. Dr. Therese Labib, Taxonomist at Mazhar Botanical Garden, Giza, Egypt, kindly authenticated both species. Voucher specimens coded 01 Eun/2021 and 02Esu/2021 were deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

Experimental

Preparation of the plants' extracts

Dried and powdered leaves of *Eugenia uniflora* L. and *Eugenia supraaxillaris* Spring ex Mart. (wt. 30 g each) were exhaustively extracted with 80% aq. MeOH then evaporated under reduced pressure to give 5.75 g and 5.49 g respectively dried methanol extract.

High-performance liquid chromatography-mass spectrometry and tandem MS/MS (HPLC/ESI-MS and MS/MS)

XEVO TQD triple quadruple mass spectrometer, Waters Corporation, Milford, MA01757, U.S.A equipped with electrospray ionization (ESI) operated in the negative ionization mode. Chromatography was carried out on ACQUITY UPLC-BEH C18 1.7 µm – (2.1 × 50 mm) column with a flow rate of 0.2 ml/min. LC/ESI-MS and MS/MS analysis was used for tentative identification of the phenolic compounds present in the methanol extract of the plants under investigation. Qualitative identification of the phenolic constituents was performed by comparison of their mass spectral fragmentation patterns with those reported in the literature data. The experiments were done at the Center for Drug Discovery Research and Development, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

Biological investigation

Evaluation of *In vitro* antioxidant activity

The antioxidant activity of the MeOH extract obtained from the leaves of *Eugenia uniflora* L. and *Eugenia supraaxillaris* Spring ex Mart. were determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University by the DPPH free radical scavenging, NO radical scavenging and TAC assays in triplicates and average values were considered.

DPPH Radical Scavenging Activity

Freshly prepared (0.004% w/v) methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10°C in the dark. Methanol solutions of the test samples were prepared. A 40 µL aliquot of the methanol solutions were added to 3 ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer at 515 nm. The decrease in absorbance was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula: PI = [{(AC- AT)/ AC} x 100] [23].

Where AC = Absorbance of the control at t = 0 min and AT = absorbance of the sample + DPPH at t = 16 min.

The 50% inhibitory concentration (IC_{50}), the concentration required to 50% DPPH radical scavenging activity was estimated from graphic plots of the dose response curve using Graph pad Prism software (San Diego, CA. USA).

Nitric Oxide Radical (NO) Scavenging Activity

0.2 mL of each sample was mixed with 2 mL of sodium nitroprusside (10 mM/L) and 0.5 mL of saline phosphate buffer. Then the resulting solution was incubated at room temperature for 150 min. The same

volume of the reaction solution without extract but with an ethanol solvent was served as control. After incubation, 1 mL of 33% sulfonic acid in 20% glacial acetic acid was added to 0.5 mL of the prepared combination. After 5 min, 1 mL of N-(1-Naphthyl)-ethylenediamine dihydrochloride (NED) was mixed with the reaction solution. Finally, the composition was kept at dark for 30 min. The absorbance was measured at 546 nm as compared to the blank control solution[24].

Nitric oxide radical scavenging % = [(A control – A sample)/ A control] x 100

Where, A control is the absorbance of the blank control reaction at 546 nm and A sample represents the absorbance of a test reaction at the same wavelength.

Ascorbic acid was used as a reference standard. All the tests were performed in triplicate. Tested material concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition percentage against concentration.

Total Antioxidant Capacity (TAC) Assay

0.2 mL of methanol solution of each sample was mixed with 0.1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated at 95˚C for 90 min in a thermal block. The samples were cooled and their absorbance was read at 695 nm [25]. TAC was measured (as mg gallic acid equivalents).

RESULTS AND DISCUSSION

Tentative characterization of major secondary metabolites

HPLC/ESI-MS/MS analysis for the AME of both *Eugenia uniflora* L. (EU) and *Eugenia supraaxillaris* Spring ex Mart. (ES) leaves cultivated in Egypt led to the tentative identification of fourteen and twelve compounds in *Eugenia uniflora* and *Eugenia supraaxillaris*, respectively **(Table 1 and Figure 1,2)**. They were identified based on comparing retention time, accurate mass, and MS/MS fragmentation pattern with those reported in literature. The major compounds present in both *Eugenia* species were further subjected to MS/MS to establish their structure **(Figure (3-9).**

Compound 1 was eluted at R_t 1.56 min and displayed a molecular ion $[M-H]^-$ at *m/z* 191. The product ion mode for **1** was operated with collision energy (CE) 20 V to produce dehydrated MS^2 fragment ions at *m/z* 173 $[M-H-H_2O]^-$, *m/z* 171 $[M-H_2O-H_2-H]^-$, in addition to *m/z* 93 corresponds to [phenol moiety]$^-$. Other fragments were displayed as MS^3 at *m/z* 127 $[M-H_2O-CO_2.H_2-H]^-$, and MS^4 at *m/z* 109 $[M-2H_2O-CO_2-H_2-H]$ (**Figure 3)**. Hence, **1** was identified as **quinic acid** deduced from its previously reported fragmentation pattern [26, 27].

Compound 2 was recorded at R_t 2.19 min and *m/z* 331. Product ion mode for **2** was operated with collision energy (CE) 25 V to produced fragment ion *m/z* 331 $[M-H]^-$, *m/z* 271 $[M-C_2H_4O_2-H]^-$ after cross-ring fragmentation of glucose (M-H-60), *m/z* 211 $[M-C_4H_8O_4-H]^-$, *m/z* 169 [galloyl group]$^-$ and *m/z* 125. Produced fragment ion *m/z* 169 [galloyl group]$^-$ indicated losses of glucose (162 Da) and the fragment at *m/z* 125, which corresponds to the consecutive losses of 162 amu (hexoside) and 44 amu (CO_2). **Compound** 2 was identified as **monogalloylglucose.** Proposed fragmentation of 3-galloylglucose was shown to represent fragmentation pattern of monogalloylglucose **(Figure 4)**[26].

Compound 3 was recorded at Rt 2.29 min and *m/z* 495. Product ion mode for **3** was operated with collision energy (CE) 30 V to produce fragment ions at *m/z* 343, 325, 191 (quinic acid moiety) and 169 (gallic acid moiety). *m/z* 343, 191 produced due to two successive losses of galloyl moieties ($[M-152-H]^-$ and $[M-304-H]^-$ respectively, as well as a small quantity of the ion at *m/z* 325, corresponding to a loss of gallic acid $[M–170–H]^–$. **Compound 3** was tentatively identified as **digalloyl quinic acid**[28].

Compound 4 was recorded at Rt 2.70 min and *m/z* 647. Product ion mode for **4** was operated with collision energy (CE) 40 V to produced fragment ions at *m/z* 495, 477, 343, 325, 191 (quinic acid moiety) and 169 (gallic acid moiety). *m/z* 495, 343, 191 produced due to three successive losses of galloyl moieties ($[M-152-H]^-$,$[M-304-H]^-$ and $[M-456-H]^-$) respectively, as well as a small quantity of the ion at *m/z* 477, corresponding to a loss of gallic acid $[M–170–H]^–$. *m/z* 325, $[M–152–170–H]^-$ is corresponding to the elimination of galloyl moiety and gallic acid. **Compound 4** was tentatively identified as **trigalloyl quinic acid**[28].

Compound 5 was recorded at R_t 2.76 min and *m/z* 169. Product ion mode for **5** was operated with collision energy (CE) 20 V to produced fragment ion *m/z* 169 $[M-H]^-$, *m/z* 125 $[M-CO_2-H]^-$, *m/z* 107 $[M-CO_2-H_2O-H]^-$ and *m/z* 79 $[M-CO_2-H_2O-CO-H]^-$. The fragmentation pattern of compound (3) agrees with gallic acid fragmentation (**Fig. 5**). **Compound 5** was identified as **gallic acid**[26].

Compound 6 was recorded at R_t 3.05 min and *m/z* 291. Product ion mode for **6** was operated with collision energy (CE) 20 V to give an intense product ion at *m/z* 247 $[M-H-44]^–$ indicating the loss of CO_2, as reported for brevifolin carboxylic acid. **Compound 6** was tentatively identified as **brevifolin carboxylic acid**[29].

Compound 7 was recorded at R_t 3.44 min and *m/z* 183. Product ion mode for **7** was operated with collision energy (CE) 20 V to produce fragment ion *m/z* 183 $[M-H]^-$, *m/z* 124 $[M-H-CH_3-CO_2]^-$, *m/z* 168 $[M-CH_3-H]^-$. **Compound 7** was identified as **methyl gallate**[30].

Table 1. Tentatively identified metabolites from EU and ES extracts by HPLC/ESI-MS and MS/MS.

No.	R_t	$[M-H]^-$	MF	MS/MS	Metabolite name	E. u.	E. s.	References
1	1.56	191	$C_7H_{12}O_6$	191 (100), 173, 171, 127, 111, 109, 93, 87, 85, 59	Quinic acid	+	+	24, 25
2	2.19	331	$C_{13}H_{16}O_{10}$	331, 271, 211, 169 (100), 125	Monogalloyl glucose	+	-	24
3	2.29	495	$C_{21}H_{20}O_{14}$	343, 325, 191, 169 (100)	Digalloyl quinic acid	-	+	26
4	2.70	647	$C_{28}H_{24}O_{18}$	495, 477, 343, 325, 191, 169 (100)	Trigalloyl quinic acid	-	+	26
5	2.76	169	$C_7H_6O_5$	169, 125 (100), 107, 79	Gallic acid	+	-	24
6	3.05	291	$C_{13}H_8O_8$	247 (100)	Brevifolin carboxylic acid	-	+	27
7	5.92	433	$C_{20}H_{18}O_{11}$	301, 300 (100), 271, 255, 151	Quercetin-3-*O*-pentoside	-	+	28
8	6.99	451	$C_{21}H_8O_{12}$	451, 313, 169 (100)	Trigalloyl acid lactonized	+	-	29
9	7.37	783	$C_{34}H_{24}O_{22}$	783, 301 (100), 275	Di-HHDP-glucose (Pedunculagin)	+	-	30
10	7.79	183	$C_8H_8O_5$	183, 168, 124 (100)	Methyl gallate	+	+	31
11	8.40	469	$C_{21}H_{10}O_{13}$	469, 301, 300 (100), 299, 271, 229, 151	Valoneic acid dilactone	+	-	32, 33
12	8.65	467	$C_{20}H_{20}O_{13}$	423 (100), 315, 169	Di-*O*-galloyl-rhamnose	+	-	28
13	8.65	479	$C_{21}H_{20}O_{13}$	479, 317, 316 (100), 271	Myricetin-3-*O*-galactoside	+	-	34, 35
14	9.07	463	$C_{21}H_{20}O_{12}$	316 (100), 271, 179, 136	Myricetin-3-*O*-rhamnoside (Myricitrin)	+	-	35
15	9.69	447	$C_{21}H_{20}O_{11}$	447, 301, 300 (100), 273, 179, 151	Quercetin-3-*O*- rhamnoside (Quercitrin)	+	+	36, 37
16	9.82	483	$C_{20}H_{20}O_{14}$	483, 331, 313, 271, 211, 169 (100), 151, 125	Digalloyl glucose	+	-	24
17	10.26	615	$C_{28}H_{24}O_{16}$	615, 463, 317 (100)	Myricetin galloyl rhamnoside	+	+	35
18	13.97	255	$C_{15}H_{12}O_4$	255 (100), 213, 171, 151	Pinocembrin	-	+	38
19	15.91	223	$C_{11}H_{12}O_5$	223, 208 (100), 180, 179, 164	Sinapic acid	-	+	39
20	16.77	283	$C_{16}H_{12}O_5$	283, 268 (100), 240	Calycosin	-	+	40
21	19.69	329	$C_{17}H_{14}O_7$	329, 314 (100), 299, 271	Trihydroxy dimethoxyflavone (Quercetin dimethyl ether)	+	+	38

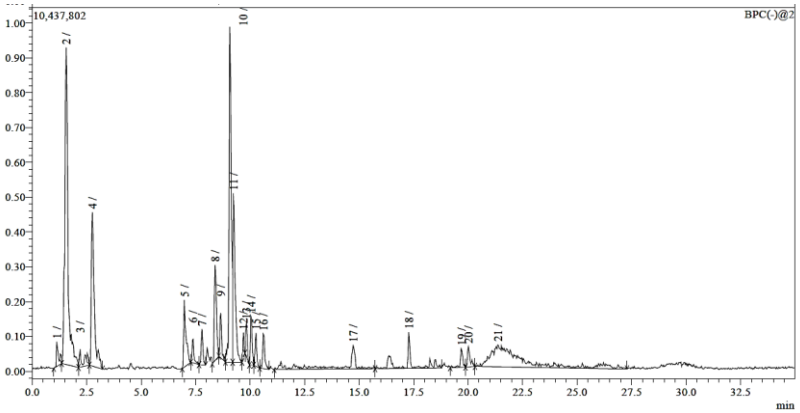

Figure 1. Negative ESI mass chromatogram of *Eugenia uniflora* leaves methanol extract

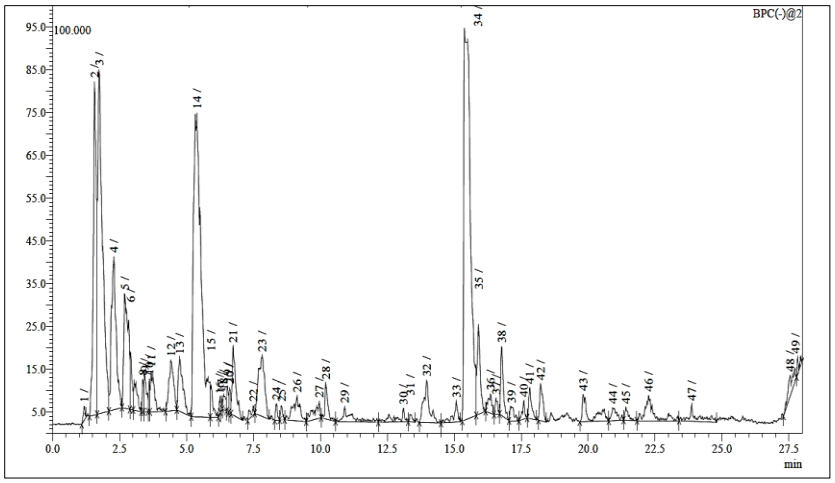

Figure 2. Negative ESI mass chromatogram of *Eugenia supraaxillaris* leaves methanol extract

Compound 8 was recorded at R_t 6.99 min and *m/z* 451. Product ion mode for **8** was operated with collision energy (CE) 30 V to produce fragment ion *m/z* 451 $[M-H]^-$, 313$[M-H-138]^-$ and 169 that were attributed to galloyl and lactonized galloyl losses. *m/z* 124 $[M-H-CH_3-CO_2]^-$, *m/z* 168 $[M-CH_3-H]^-$. **Compound 8** was tentatively identified as **lactonized trigalloyl acid**[31].

Compound 9 was recorded at R_t 7.37 min and *m/z* 783. Product ion mode for (5) was operated with collision energy (CE) 40 V to produce fragment ion *m/z* 783 $[M-H]^-$, *m/z* 301 (M-482, loss of HHDP glucose) represents the HHDP moiety (loss of HHDP glucose via cleavage of ester link and spontaneous lactonization of HHDP moiety in form of ellagic acid *m/z* 301), *m/z* 275 produced by loss of a CO group from the HHDP moiety. **Compound 9** was identified as **di-HHDP-glucose (pedunculagin)**[32].

Compound 10 was recorded at R_t 7.79 min and *m/z* 183. Product ion mode for **10** was operated with collision energy (CE) 20V to produce fragment ion *m/z* 183 $[M-H]^-$, *m/z* 124 $[M-H-CH_3-CO_2]^-$, *m/z* 168 $[M-CH_3-H]^-$. **Compound 10** was identified as **methyl gallate**[33].

Compound 11 was found at R_t 8.40 min and *m/z* 469. Product ion mode for **11** was operated with collision energy (CE) 30 V to produce fragment *m/z* 469 $[M-H]^-$ and three main fragments at *m/z* 301 (-168 amu), 300 (-169 amu) and 299 (-170 amu) consistent with the structure of valoneic acid dilactone (**Figure 6).** *m/z* 301 represents ellagic acid fragment. Ellagic acid peaks at *m/z* 300, 271 were also found. Hence, **compound 11** was finally identified as **valoneic acid dilactone**[34, 35].

Compound 12 was found at R_t 8.65 min and *m/z* 467. Product ion mode for **12** was operated with collision energy (CE) 30 V to produce fragment *m/z* 467 $[M-H]^-$ and three main fragments at *m/z* 315 (-152 amu) corresponding to loss of galloyl group, 169 (-152 amu - 146 amu) corresponding to loss of galloyl group plus deoxyhexose group. Therefore, this compound was tentatively identified as **di-*O*-galloyl-rhamnose**[30].

Compound 13 was found at R_t 8.65 min and *m/z* 479. Product ion mode for **13** was operated with collision energy (CE) 30 V to produce fragment *m/z* 479 $[M-H]^-$, *m/z* 317 $[M-H-162Da]^-$, main fragment *m/z* 316 $[M-2H-162amu]^-$ which indicates the loss of a galactosyl moiety from the precursor ion at *m/z* 479, *m/z* 271 $[317-CO-H_2O]^-$. Hence, **compound 13** was assigned as **myricitin-3-*O*-galactoside** [36,37].

Compound 14 was found at R_t 9.07 min and *m/z* 463. Product ion mode for **14** was operated with collision energy (CE) 45 V to produce fragment *m/z* 316 $[M-2H-146]^-$ by the loss of a rhamnoside moiety (146 amu). The fragment at *m/z* 316 corresponded to myricetin. Other fragments at *m/z* 271 $[317-CO-H_2O]^-$ **(Figure 7)**, *m/z* 179 and *m/z* 136 were in agreement to the presence of **compound 14**. **Compound 14** was identified as **myricetin-3-*O*-rhamnoside** due to its fragmentation pattern[37].

Insert Figure 7

Compound 15 was found at R_t 9.69 min and *m/z* 447. Product ion mode for **15** was operated with collision energy (CE) 30 V to produce fragment *m/z* 447 $[M-H]^-$, *m/z* 301 $[M-H-146]^-$ by the loss of a rhamnoside moiety (146 amu), *m/z* 300 $[M-2H-146]^-$. The fragment at *m/z* 301 corresponded to quercetin. Quercetin-derivatives can be easily identified by the presence of the typical fragment ions in the mass spectra at *m/z* 301, 273, 179 and 151 derived from the fragmentation of the quercetin aglycone[39] **(Figure 8)**. Therefore **compound 15** was identified as **quercetin-3-*O*- rhamnoside** due to its fragmentation pattern [38, 39].

Insert Figure 8

Compound 16 was recorded at R*t* 9.82 min and *m/z* 483. Product ion mode for **16** was operated with collision energy (CE) 30 V to produce fragment ion *m/z*

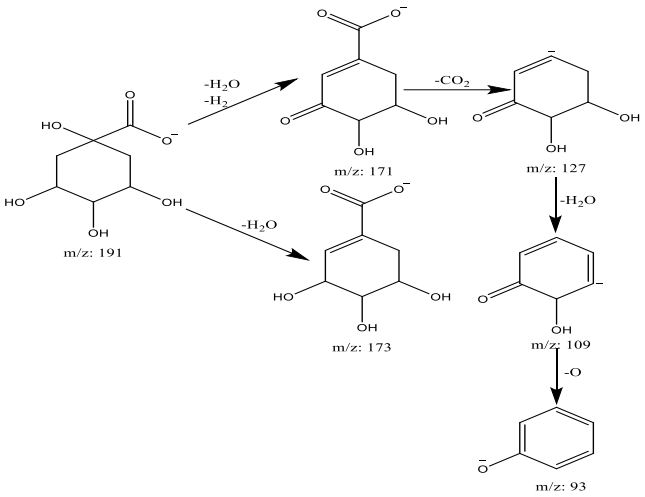

Figure 3. Proposed fragmentation scheme of quinic acid

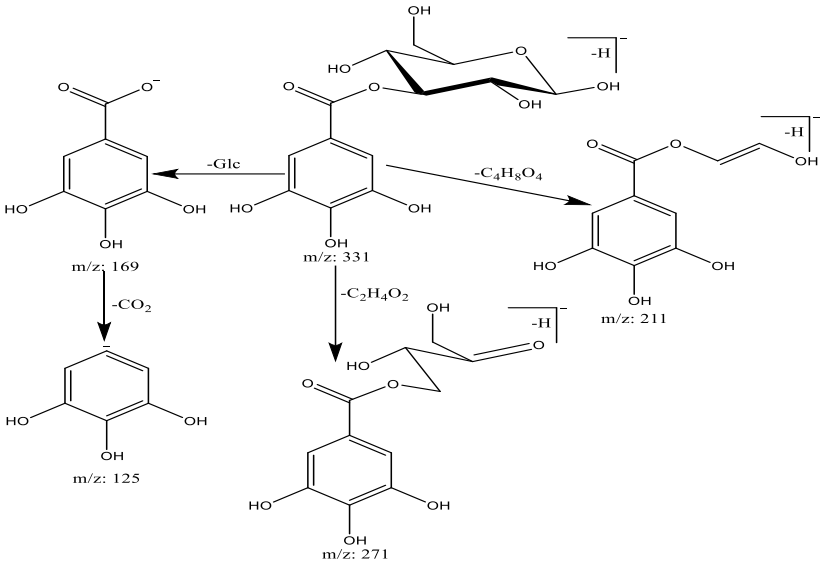

Figure 4. Proposed fragmentation of 3-*O*-galloylglucose as representing monogalloylglucose

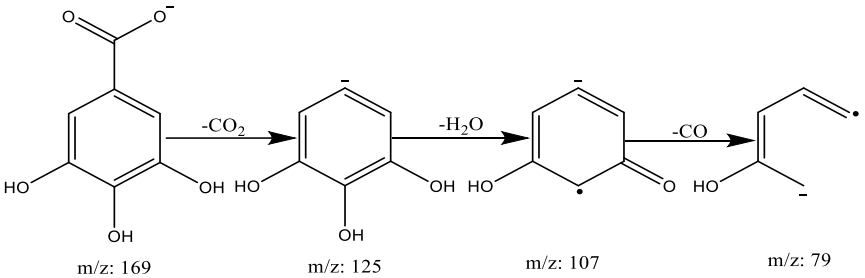

Figure 5. Proposed fragmentation of gallic acid

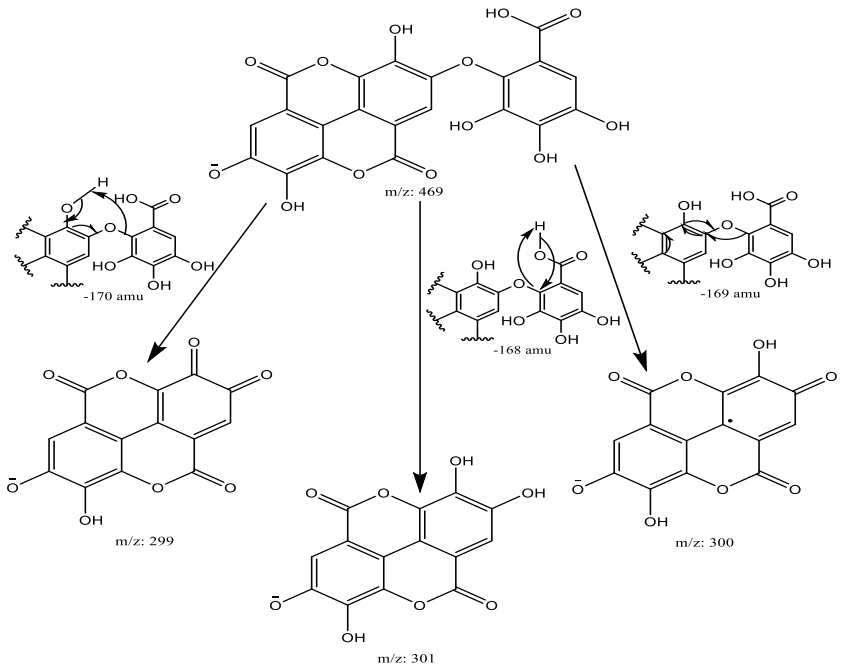

Figure 6. Proposed fragmentation of valoneic acid dilactone

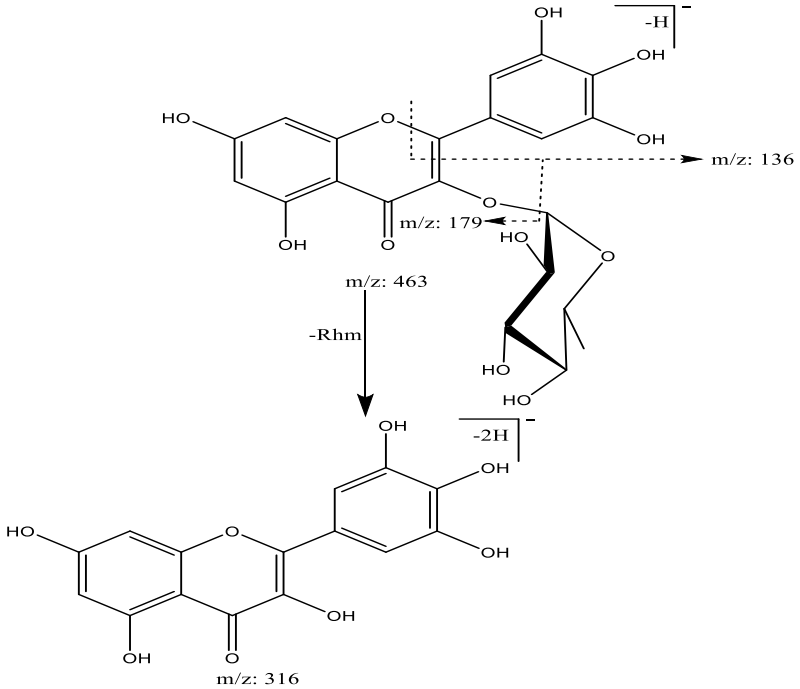

Figure 7. Proposed fragmentation of myricetin-3-*O*-rhamnoside

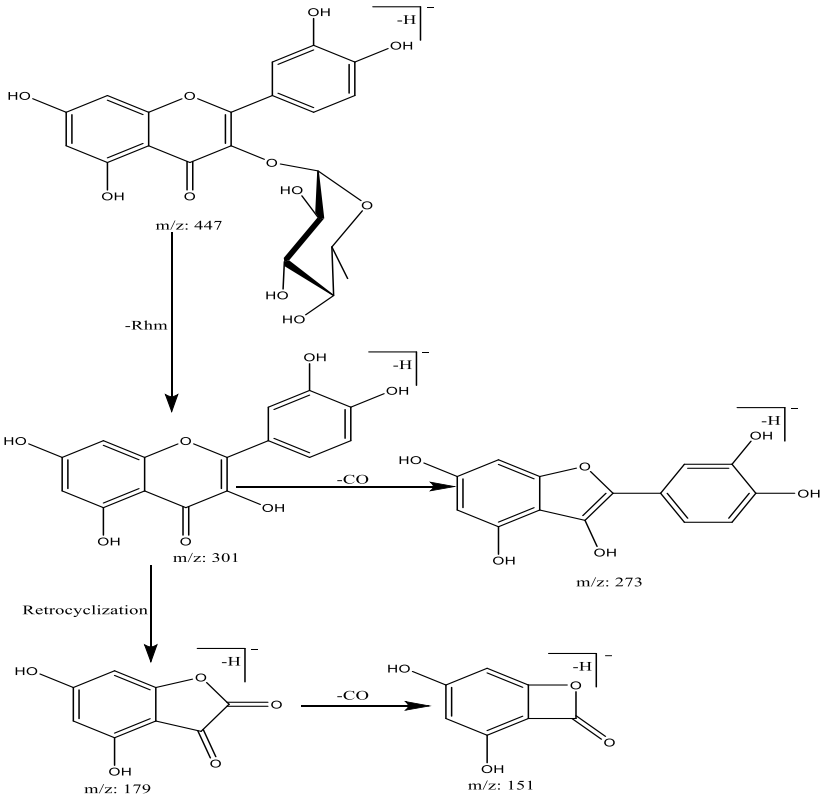

Figure 8. Proposed fragmentation of Quercetin -3-*O*-rhamnoside

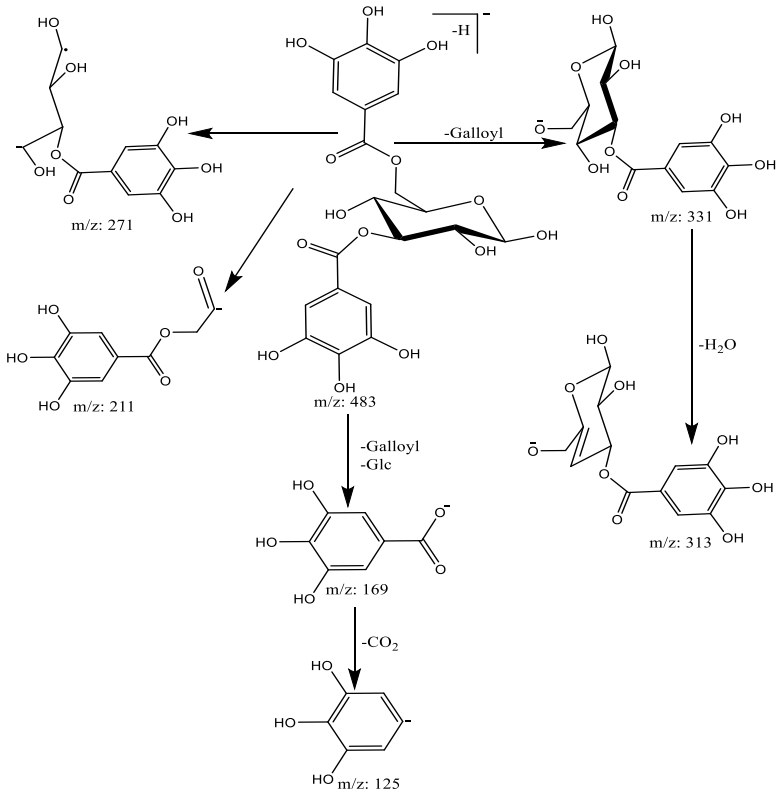

Figure 9. Proposed fragmentation of digalloyl glucose

483 $[M-H]^-$, *m/z* 331 $[M-H-152]^-$ after sequential removal of the galloyl group (152 amu), *m/z* 313 $[M-H-170]^-$ after sequential removal of the gallic acid (170 amu), *m/z* 271$[M-H-212]^-$ was observed as cross-ring fragment ions of a glucose molecule, and ion of the gallic acid moiety, *m/z* 211 $[M-H-272]^-$, *m/z* 151 [galloyl group-$H]^-$, *m/z* 169 $[M-H-152-162]^-$ is a deprotonated ion of the gallic acid moiety produced after sequential removal of the galloyl group (152 amu) and the hexose moiety (162 amu), *m/z* 125 [gallic acid-CO_2-$H]^-$. **Compound 16** was identified as **digalloyl glucose**[26]. The proposed fragmentation of digalloyl glucose is shown in **Figure 9**.

Compound 17 was recorded at R_t 10.05 min and *m/z* 615. Product ion mode for **17** was operated with collision energy (CE) 40 V to produce fragment ions at *m/z* 615 $[M - H]^-$, *m/z* 463 $[M -152–H]^-$ which correspond to the loss of galloyl unit and *m/z* 317 $[M – 152– 146 – H]^-$ which correspond to the sequential loss galloyl and rhamnoside moieties. **Compound 17** was tentatively identified as **myricetin galloyl rhamnoside**[37].

Compound 18 was recorded at Rt 13.97 min and *m/z* 255. Product ion mode for **18** was operated with collision energy (CE) 20 V to produce fragment ions at *m/z* 255 $[M – H]^-$, 213, 171 and 151. According to the literature it was tentatively identified as **pinocembrin**[40].

Compound 19 was recorded at Rt 15.91 min and *m/z* 223. Product ion mode for **19** was operated with collision energy (CE) 20 V to produce fragment ions at *m/z* 223 $[M – H]^-$, 208, 180, 179 and 164. According to the literature it was tentatively identified as **sinapic acid**[41].

Compound 20 was recorded at Rt 16.77 min and *m/z* 283. Product ion mode for **20** was operated with collision energy (CE) 20 V to give significant $[M-H]^-$ ions in the negative ion mode at *m/z* 283. The MS/MS spectrum showed *m/z* 268 $[M-H-CH_3]^-$ as the base peak, suggesting the presence of a methoxyl group and *m/z* 240 $[M-H-CH_3-CO]^-$, resulting from a loss of CO. The compound was therefore identified as **calycosin**[42].

Compound 21 was found at R_t 19.69 min and *m/z* 329. Product ion mode for **21** was operated with collision energy (CE) 25 V to produce fragment *m/z* 329 $[M-H]^-$, *m/z* 314 $[M-H-CH_3]^-$, *m/z* 299 $[M-H-2CH_3]^-$, *m/z* 271 $[M-H-2CH_3-CO]^-$. **Compound 21** was identified as **quercetin dimethyl ether** due to its fragmentation pattern[40].

In vitro antioxidant activity

Different natural sources were reported as antioxidants due to the correlation between radical scavenging activity and their phenolic content[43]. Several *in vitro* complementary assays are used to evaluate the antioxidant activity of natural compounds as the scavenging ability of DPPH free radical and inactivation of NO radicals[44]. We noticed that methanolic extract of *E. supraaxillaris* (IC_{50} = 8.51±0.37, 14.81±0.63 µg/mL) exhibited stronger antioxidant activity than that of *E. uniflora* (IC_{50} = 13.88±0.59, 32.53±1.75µg/mL) through their ability to inhibit DPPH and NO respectively. Also, we found that *E. supraaxillaris* (IC_{50} = 8.51±0.37, 14.81±0.63 µg/mL) exerted higher DPPH and NO scavenging activity than that of ascorbic acid (IC_{50} = 10.21±0.77, 17.95±2.24 µg/mL), respectively. Moreover, the methanolic extract of *E. supraaxillaris* showed a total anti-oxidant capacity (58.12±2.36 µg/mL) higher than that of *E. uniflora* (49.27±1.89 µg/mL) in comparison to the ascorbic acid as a control (69.75±3.81 µg/mL). **(Tables 2-5, Figures 10- 13)**

Table 2. Dose-response effect of the antioxidant DPPH scavenging activity of methanol extracts of both species compared to the reference standard drug (ascorbic acid).

Sample	% DPPH Scavenging Activity									
	2	3.9	7.8	15.6	31.25	62.5	125	250	500	1000
***E. uniflora* MeOH (µg/mL)**	18.26	27.43	39.18	53.06	71.78	79.64	82.42	90.85	92.71	93.89
***E. supraaxillaris* MeOH (µg/mL)**	30.41	36.27	47.80	72.19	80.46	87.33	90.68	92.76	94.02	95.63
Ascorbic acid (µg/mL)	22.19	31.28	43.01	65.05	79.35	86.42	90.89	94.25	96.34	98.65

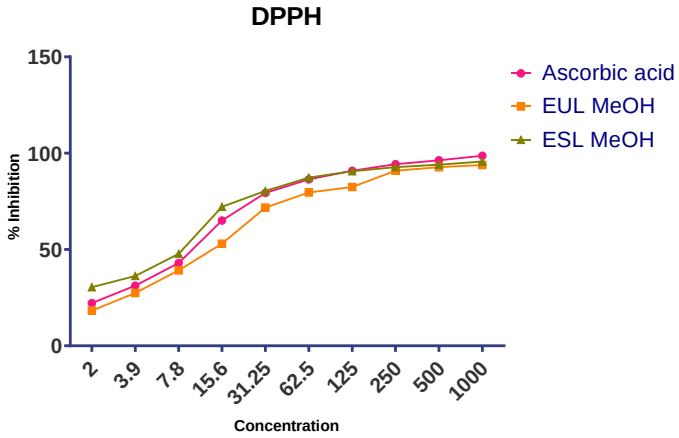

Figure 10. Dose-response effect of tested leaves methanol extracts of *Eugenia uniflora* (EUL) and *Eugenia supraaxillaris* (ESL) on the oxidative marker, DPPH. Each value is expressed as the mean of three independent experiments (n=3) compared to the reference standard drug ascorbic acid.

Table 3. Dose-response effect of the antioxidant NO scavenging activity of methanol extracts of both species compared to the reference standard drug

Sample	% NO Scavenging Activity									
	2	3.9	7.8	15.6	31.25	62.5	125	250	500	1000
***E. uniflora* MeOH (µg/mL)**	10.72	19.83	26.47	41.38	58.40	67.29	75.16	83.48	88.26	91.73
***E. supraaxillaris* MeOH (µg/mL)**	9.85	16.88	32.76	51.92	63.84	74.15	82.73	86.29	90.54	92.31
Ascorbic acid (µg/mL)	22.59	30.08	37.87	47.67	62.89	73.52	79.39	84.79	89.47	93.82

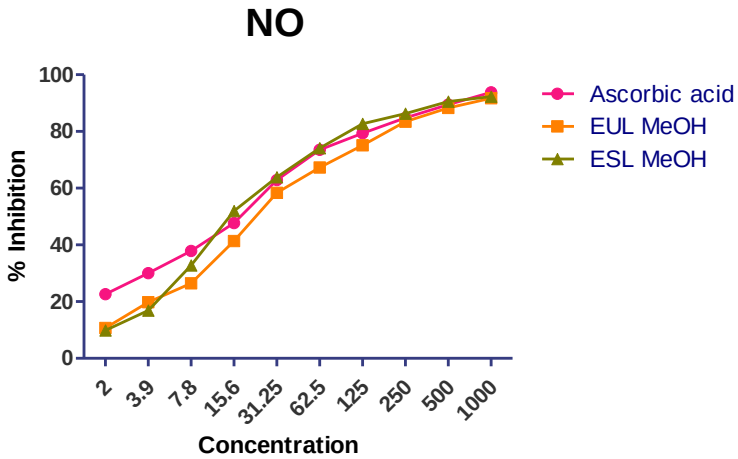

Figure 11. Dose-response effect of tested leaves methanol extracts of *Eugenia uniflora* (EUL) and *Eugenia supraaxillaris* (ESL) on the oxidative marker, NO. Each value is expressed as the mean of three independent experiments (n=3) compared to the reference standard drug ascorbic acid.

Table 4. Antioxidant effect (represented by IC_{50} ±SD) of *E. uniflora* L. *and E. supraaxillaris* Spring ex Mart. leaves methanol extracts in DPPH and NO scavenging assays.

Tested Sample	IC_{50} ±SD	
	DPPH	NO
***E. uniflora* MeOH (µg/mL)**	13.88±0.59	32.53±1.75
***E. supraaxillaris* MeOH (µg/mL)**	8.51±0.37	14.81±0.63
Ascorbic acid (µg/mL)	10.21±0.77	17.95±2.24

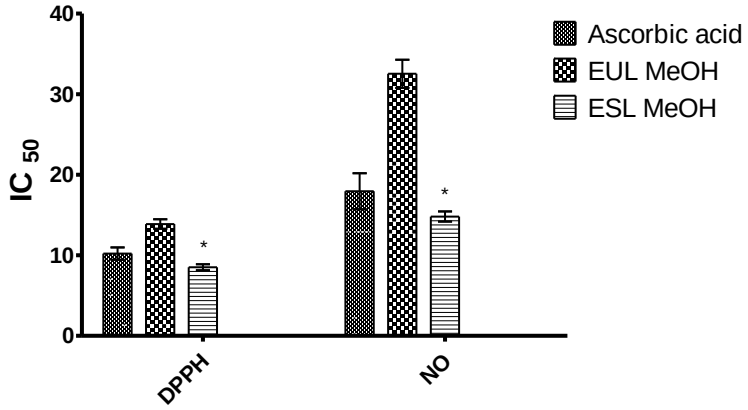

Figure12. IC_{50} ±SD of the leaves methanol extracts of *Eugenia uniflora* (EUL) and *Eugenia supraaxillaris* (ESL) on the oxidative markers, DPPH and NO. Each value is expressed as the mean of three.

Table 5. Antioxidant effect of the methanol extracts of *E. uniflora* L. *and E. supraaxillaris* Spring ex Mart. leaves using total antioxidant capacity method (TAC) expressed as mg Gallic acid Equivalent per gram sample. Each value is expressed as the mean of three independent experiments (n=3) and compared to a reference standard drug ascorbic acid.

Tested Sample	TAC (mg GAE/g)
***E. uniflora* MeOH (µg/mL)**	49.27±1.89
***E. supraaxillaris* MeOH (µg/mL)**	58.12±2.36
Ascorbic acid (µg/mL)	69.75±3.81

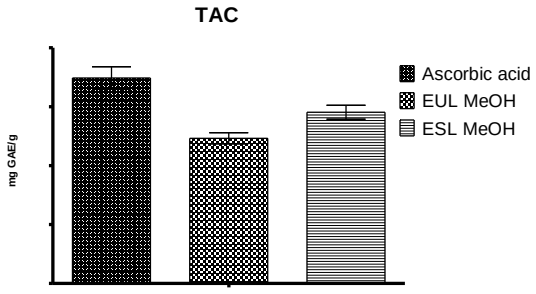

Figure 13. Total antioxidant capacity (TAC) of the leaves methanol extracts of *Eugenia uniflora* (EUL) and *Eugenia supraaxillaris* (ESL) expressed as mg Gallic acid Equivalent per gram. Each value is expressed as the mean of three independent experiments (n=3) and compared to a reference standard drug ascorbic acid.

CONCLUSION

This study provides information on comparative phytochemical constituents and antioxidant activity of the methanolic extracts of the leaves of *Eugenia uniflora* L. and *Eugenia supraaxillaris* Spreng ex Mart. They are rich source of phytoconstituents as polyphenolics that correlated to more significant anti-oxidant properties. This is due to their redox properties, (the ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers, and to some extent, could also be due to their metal chelating properties).

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Conflict of interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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