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Antioxidant activity of *Malpighia glabra* L., leaves extract.

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Abstract: *Malpighia glabra.* L (Acerola) fruits have wide global medicinal uses due to rich antioxidant contents. Little studies were carried on other parts of plant. *Malpighia glabra.* L leaves (1.5 kg) was subjected to soxhlet exhaustive extraction with methanol and concentrated under vacuum. The dried extract was defatted with petroleum-ether then fractionated with ethyl acetate & n-butanol 3 successive times respectively. The ethyl acetate fraction (9 gm) was subjected to series of silica gel & Sephadex (LH-20) column chromatography. Phytochemical investigation of the Ethyl acetate fraction of *Malpighia glabra*. L leaves in this study resulted in separation of four pure compounds (Caffeic acid, Chlorogenic acid, Quercetin & Kaempferol). The structures of the separated compounds were assigned based on NMR spectral data and mass spectrometer analysis. The methanolic extract showed high values of total phenolics (96.36 mg GAE/g), flavonoids (30.71 mg QE/g) and higher antioxidant activity towards DPPH radical with IC_{50} = 49.8(mg/ml).

Keywords: *Malpighia glabra* L**.** Leaves; DPPH; Caffeic Acid; Chlorogenic Acid; Quercetin; Kaempferol

1. INTRODUCTION

Folk medicine is known to have a great influence on human culture, so that tracing and identifying of active constituents phytochemically and their medicinal effects have taken awide interest overtime [1].

Bioactive compounds present naturally in herbs, foods and dietary supplements that have therapeutic effects and may help in protection from chronic diseases known as "Nutraceuticals" [2].

Malpighiaceae family have about 40-50 species, Malpighia glabra. L genus belongs to this family and known by other terms like Acerola, West Indian cherry, Malpighia punicifolia L, Malpighia emarginata DC and Barbados cherry [3,4] .

Brazil and tropical countries use Malpighia glabra. L fruit juice as one of the highest levels of ascorbic acid, vitamin C and abundance of carotenoids and anthocyanins [5].

Bioactive compounds which separated from Malpighia glabra. L in other studies shows antioxidant, antidiabetic and skin protecting activity.[6]

2. MATERIALS AND METHODS

2.1. Plant material

Malpighia glabra.L leaves were collected from Orman garden, Giza, Egypt. during May/June 2015.

2.2. Extraction and isolation

Air dried powder of Malpighia glabra. L leaves (1.5 kg) was exhaustively extracted with the 99% methanol (3x 10 L) using soxhlet apparatus. The combined extract by methanol was dried and conc. at 40oC under vacuum to give 450 gm of dried extract. The concentrated methanolic extract was dissolved in (500 ml) of distilled water and defatted with petroleum-ether (40 – 60oC). The crude defatted extract (150 gm) was fractionated with ethyl acetate & n-butanol respectively 3 successive times (500 ml). The ethyl acetate (9 gm) fraction was subjected to series of silica gel (Si gel 60, Sigma aldrich) and Sephadex LH-20 (Sigma aldrich) column chromatography to afford four pure compounds.

2.3. Phytochemical screening

The methanolic extract of Malpighia glabra. L leaves was phytochemically studied for their

chemical constituents according to the standard methods) [8-11] .

2.4. Quantification of total phenolic content

Content of phenolics in Malpighia extract were determined Spectrophotometrically 12. (The phenolics concentration was measured from the calibration curve as (mg/ml); then the phenolic content in extracts was expressed as (mg of GAE/g of extract) [12].

2.5. Quantification of total flavonoid content

Contents of total flavonoids were assayed by the aluminum chloride colorimetric method at 510 nm .13

(The total flavonoidal content was expressed in terms of mg quercetin equivalents (QE)/g dry basis) [13].

2.6. Antioxidant assay

Scavenging of (DPPH) was assayed by the modified method [14], (results were expressed as AEAC (ascorbic acid equivalent antioxidant capacity) in grams and the calculation was done according to the followed equation).[14]

AEAC = (µg AA/g) = IC50 (AA)/IC50 (sample) x 1g

3. RESULTS AND DISCUSSION

3.1. Isolation & phytochemical screening

Ethyl acetate fraction was carefully injected to silica gel column chromatography and the elution was done by (CH2Cl2: MeOH) with different concentrations (100:0→60:40) then the collective Fractions was subjected to successive column chromatography on Sephadex LH-20 using methanol to offered four compounds which all subjected to ESI–mass spectroscopy (Ain shams University), and 1H- and 13C-NMR (Cairo university) measurements spectrometer operation at 400 MHz (for 1H) and 100 MHz (for 13C). The phytochemical screening of the 70% alcohol extract of Malpighia glabra. L leaves are presented in Table 1.

Table 1: Phytochemical screening results of the 70% methanolic extract of ***Malpighia glabra*. L** leaves*

1-	Crystalline sublimate	- ve
2-	Volatile oil	+ ve
3-	Carbohydrates and/or glycosides	+ ve
4-	Oxidase enzyme	- ve
5-	Flavonoids:	++ ve
	- Aglycones	++ ve
	- Glycoside	+ ve
6-	Tannins	++ ve
7-	Sterols and/or triterpenes	++ ve
8-	Saponins	– ve
9-	Cardiac glycosides	– ve
10-	Alkaloids	– ve
11-	Anthraquinone derivatives	– ve

*– ve = Absent + ve = Present ++ve = Strongly present

Screening of Malpighia glabra.L leaves phytochemical substances revealed the existence of volatile oil, carbohydrates and/or glycosides, sterols, tanins and/or triterpenes and flavonoids, and absence of crystalline sublimate, cardiac glycosides, saponins, alkaloids , oxidase enzyme and Anthraquinone derivatives .

3.2. Isolated compounds

Chromatographic investigation of Malpighia glabra.L leaves extract yielded four compounds, compound 1, Caffeic acid(figure.1), compound 2, Chlorogenic acid(figue. 2), compound 3, Quercetin (figure.3), & compound 4, Kaempferol (figure.4)

Compound 1: It is appeared as white powder with amorphous character. ESI-MS of the compound

was measured on (TQD triple quadruple instrument (Waters Corporation, Milford, instrument), and showed [M-H] + peak at m/z 179.1, which is compatible with the molecular formula C9H8O4. The 1H NMR spectrum of the compound exhibited signals which is characteristic for δ 7.57 (1H, d, J = 16 Hz, H-7), 7.06(1H, d, J = 2.0 z, H- 2), 6.96(1H, dd, J = 8.0, 2.0 Hz, H-6), 6.94(1H, d, J = 8.0 Hz, H-5), 6.26 (1H, d, J = 16 Hz, H-8).

The ^{13}C-NMR spectral data of the compound showed signals at δ 169.80(C-9), 148.03 (C-4), 145.76 (C-3), 145.5C-7), 126.43 (C-1), 121.57 (C-6), 115.15 (C-5), 114.11(C-2), 113.77 (C-8). From these data, compound 1 was identified as caffeic acid, which is confirmed by the direct comparison with published data [15].

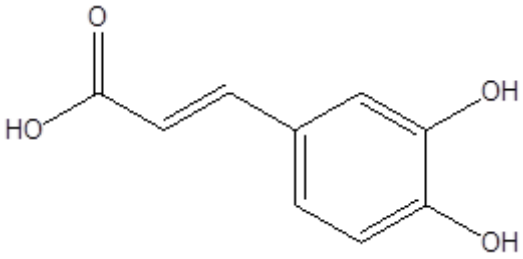

Figure 1: Chemical structure of Caffeic acid [15]

Compound 2: It was isolated as white powder with amorphous shape. ESI-MS of the compound revealed [M-H] $^{+}$peak at m/z 353.4, which is compatible with the molecular formula $C_{16}H_{18}O_9$.

The ^{1}H NMR spectrum of the compound exhibited signals which characteristic for δ 7.44 (1H, d, J = 16 Hz, H-7'), 7.04 (1H, d, J = 2.0 Hz, H- 2'), 6.99 (1H, dd, J = 8.0, 2.0 Hz, H-6'), 6.78 (1H, d, J = 8.0 Hz, H-5'), 6.17 (1H, d, J = 16 Hz, H-8'), 5.08 (1H, ddd, J = 6.8, 6.8, 4.4 Hz, H-5), 3.95 (1H, ddd, J = 7.2, 3.6, 3.6 Hz, H-3), 3.58(1H, dd, J = 6.8, 2.4 Hz, H-4), 2.04 (1H, m, H-2ax), 2.01 (1H, m, H-6ax), 1.96 (1H, dd, J = 13.2, 4.0 Hz, H-6eq), 1.82 (1H, dd, J = 13.2, 7.6 Hz, H- 2eq)

The ^{13}C-NMR spectrum of the compound revealed signals at δ 175.00 (C-7), 165.83 (C-9'), 148.40 (C-4'), 145.63 (C-3'), 145.03 (C-7'), 125.70 (C-1'), 121.46 (C- 6'),115.85 (C-5'), 114.84 (C-2'), 114.40 (C-8'), 73.59 (C-1), 70.95 (C-5), 70.51 (C- 4), 68.20 (C-3), 37.28 (C-2), 36.39 (C-6).

From the data mentioned above, compound 2 was identified as chlorogenic acid, which confirmed by direct comparison with published data [16]

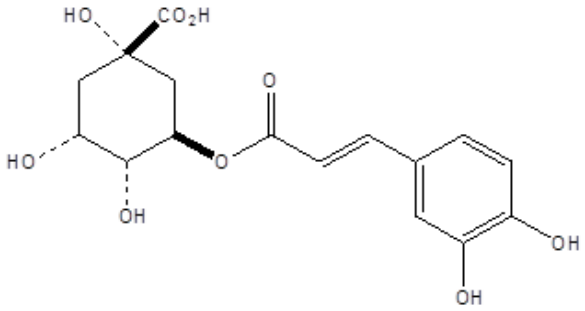

Figure 2: Chemical structure of Chlorgenic acid.[16]

Compound 3: The compound was isolated as a yellow powder with amorphous characters. The ESI-MS of the compound showed a peak at m/z 301.0[M-H] + indicating that the molecular formula is $C_{15}H_{10}O$. The ^{1}H-NMR spectrum of the compound showed a clear flavonol type pattern of aromatic proton signals; two meta coupled protons each at δ 6.19 ppm (d, J= 2.0, H-6) and δ 6.41 ppm (d, J= 2.0, H-8), doublet of one proton at δ 6.90 ppm (d, J=8.4 Hz, H-5`) and a meta coupled doublet of one proton at δ 7.68 ppm (d, J= 2.0, H-2`) and H-6' appear as doublet of doublet at 7.55 ppm (dd, J= 8.4, 2.0, H-6`). This data indicated that the compound is a 3, 5, 7, 3, 4` –penta oxygenated flavone derivative, which is in a good agreement with Quercetin. The ^{13}C-NMR (100 MHz, DMSO-*d6*) spectrum of the compound supporting the structure assignment made above, revealing the presence of 15 carbon signals, including 8 oxygenated at [δ 145.11 (C-2), 160.78 (C-5), 163.97 (C-7), 156.21 (C-9), 146.86 (C-3`), 135.78 (C-3), δ 175.89 (C-4) and 147.75 (C-4`)]. 7 non-oxygenated at [δ 98.26 (C-6), 93.42 (C-8), 103.07 (C-10), 122.04 (C-1`), 115.14 (C-2`), 115.67 (C-5`) and 120.05 (C-6`)]17,18 . Compound 3 is Quercetin according to the literature data. 17,18

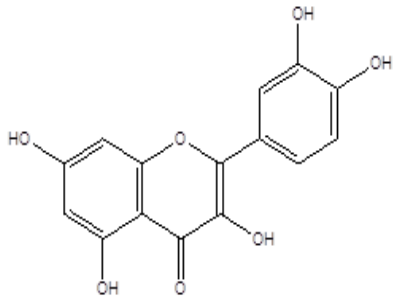

Figure 3: Chemical structure of Quercetin [17, 18]

Compound 4: Compound 4 was isolated as fine yellow needles. The ESI-MS spectrum of compound 4, showed a molecular ion peak at m/z 286 [M]$^{+}$, corresponded to the molecular formula $C_{15}H_{10}O_6$.

Peaks due to RDA fragmentation at m/z 153 corresponding to ring-A, respectively, indicating a kaempferol nucleus

The ^{1}H-NMR spectrum of compound 4 revealed a clear Flavonol-type pattern of aromatic proton signals; two meta-coupled protons each at δ 6.19 ppm (d, j=2 H-6) and δ 6.44ppm (d, j =2 H-8) and an (AA`BB`) system, characteristic to a 1,4-disubstituted aromatic B ring at δ 8.06 and 6.94 (each 2H, each d, J= 8.8 Hz, H-2`, 6` and H-3`, 5`, respectively) .

The ^{13}C–NMR spectrum of compound 4 showed 15 carbon signals at 147.24 (C- 2), 136.12(C- 3), 176.36(C- 4), 161.17(C- 5), 98.71(C- 6), 164.52(C-7), 93.96(C- 8), 156.65(C- 9), 103.45(C- 10), 122.14(C- 1'), 129.96(C- 2'/6'), 115.91(C- 3'5'/), 159.66(C- 4') identical with those reported for kaempferol nucleus. The p-substitution of B ring was confirming by the same chemical shifts for the equivalent C–3`/C–5` and C–2`/C–6`. The ^{1}H-NMR & ^{13}C-NMR spectral data were similar to with those reported for kaempferol [17,18]. Compound 4 is kaempferol according to the literature data. [17,18].

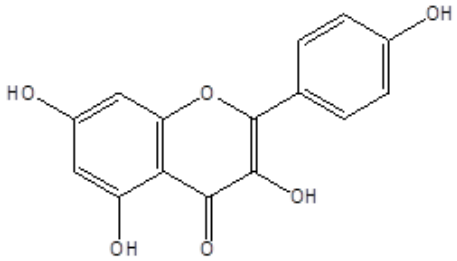

Figure 4: Chemical structure of Kaempferol [17,18]

The total phenolics of ***Malpighia glabra***.L leaves extract was determined spectrophometrically at 765 nm which is found to be 96.36 mg GAE/g of extract compared to gallic acid as in graph (figure.5)

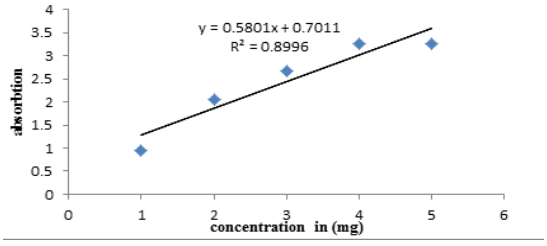

Figure 5: Standard curve for Gallic acid [12]

3.4. Quantification of total flavonoid contents:

The results that observed in the graph (fig.6) had declared that *Malpighia glabra*. L rich in flavonoids (total flavonoid content: 30.71 mg QE/g extract).

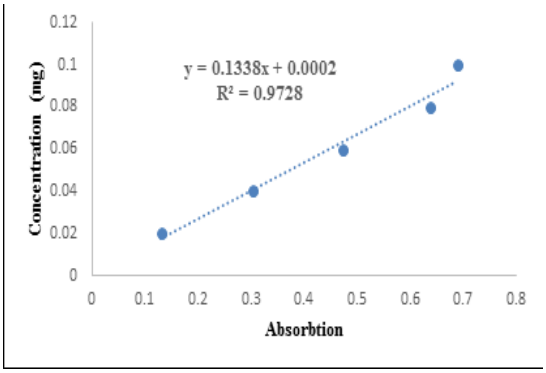

Figure 6: Standard curve for quercetin [13]

Phenolic acids, flavonoids content and free radical scavenging agents [19,20] have a positive relation to each other. Concentration of phenolic compounds and the antioxidant effect have a significant linear correlation, many studies had ensured the correlation between the phenolics and free radical scavenging activity.

3.5. Antioxidant assay

Plant extract which contains high levels of phenolics shows great antioxidant activity and resulted in free radical scavenging activity [21]

Complementing this result, DPPH test of Malpighia glabra. L leaves extract shows significant decrement of DPPH radicals which is nearly similar to the considerable antioxidant agent "ascorbic acid", and lead to DNA protection specially in fruits that contains antioxidant compounds[22], carotenoids and poly phenols [23-25] but consistency of vit C in unripe fruits is higher than ripe fruits.[26]

The DPPH assay result of ***Malpighia glabra. L*** using ascorbic acid as positive control shows that (IC_{50} = 5.159 µg/ml) validate the assay, while the amount of the extract which required to capture the DPPH radicals is (IC_{50} = 4.98 µg/ml) acerola leaves **(Table 2)**.

Table 2: Antioxidant assay of **Malpighia glabra. L** extract

sample	Inhibition per concentration		IC_{50} (mg/ml)	IC_{50} (µg/ml)	AEAC(µg/ml)
	100mg/ml	gm/ml			
ascorbic acid	100.4	1000.4	51.59	5.16	1.000
extract	96.9	969	49.8	4.98	0.965

4. CONCLUSIONS

Separation of four pure compounds from Ethyl acetate fraction of ***Malpighia glabra***.L leaves, that compounds are Caffeic acid, Chlorogenic acid, Quercetin & Kaempferol. Moreover, **Malpighia *glabra*. L** extract shows high values of total phenolic and total flavonoid contents reflecting high free radicals scavenging activity.

Conflict of interest: All authors declared no conflict of interest.

Author contribution: AMF performed the extraction, participated in chromatographic separation of the pure compounds and wrote the paper. AIM and MAE revised the paper and conceived the phytochemical and biological study. WAE shared in the biological study. MSA participated in the structure elucidation of the isolated compounds, wrote and conceived the study.

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