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Research Article

INVESTIGATION AND ANTIOXIDANT OF PHENOLIC COMPOUNDS OF THE LEAVES OF *GLEDITSIA TRIACANTHOS* L.

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ABSTRACT

Objective: Nowadays there is an increasing interest to extract and isolate naturally antioxidant compounds especially phenolic compounds that are pharmacologically potent and play an important role as a health-protecting factor. They neutralize the free radicals, which are unstable molecules that are linked with the development of a number of degenerative diseases. This study aims to investigate the antioxidant properties and phenolic compounds from the leaves of *Gleditsia triacanthos* L. (Family Leguminosae).

Methods: The 85% methanol extract of the leaves of *Gleditsia triacanthos* L. and the chloroform and ethyl acetate fractions were estimated for total antioxidant capacity, 1,1-diphenyl picrylhydrazyl (DPPH) free radical scavenging, total phenolic and flavonoid contents using spectrophotometric methods. The ethyl acetate and chloroform fractions were subjected to chromatographic and spectroscopic analysis.

Results: The results showed that the highest content of phenols and flavonoids were found in methanol extract and ethyl acetate fraction whereas the lowest content was recorded in the chloroform fraction and there is correlation between the total phenolic content and the antioxidant activity of these extracts. Also, five compounds have been isolated from the chloroform and the ethyl acetate fractions and their structures have been elucidated as caffeic acid, luteolin, isovitexin, apigenin-7-O-β-D-glucopyranoside and Luteolin-7-O-β-D-glucopyranoside. Evaluation of the five isolated compounds as antioxidant agent against DPPH-radical revealed that the five compounds have antioxidant activity (SC_{50} = 9.53, 11.67, 22.45, 98.44 and 58.91 µg/ml respectively).

Recommendation: This plant extract could be used as a natural antioxidant after comprehensive *in vitro* and *in vivo* biological studies.

Keywords: *Gleditsia triacanthos*; Antioxidant; DPPH; Phenolic compounds

INTRODUCTION

Natural antioxidants have been studied extensively for decades in order to find compounds protecting against a number of diseases related to oxidative stress and free radicals. The plant kingdom offers a wide range of compounds exhibiting antioxidant activities. Polyphenolic compounds including flavonoids, phenolic acids and tannins have been considered as excellent natural antioxidants. They are widely distributed and can be considered as the most abundant plant secondary metabolites [1-3]. Therefore, comprehensive investigation for effective antioxidants from natural sources especially plant kingdom has been undertaken actively [4-5]. Phenolic natural products such as flavonoids are of particular interest because of their antioxidant activity through scavenging oxygen radical. It has been reported that the ability of these compounds for scavenging the free radical, plays an important role in many diseases such as cardiovascular diseases, aging, cancer and inflammatory disorders [6-8]. The stable 1,1-diphenyl picrylhydrazyl (DPPH•) free radical is the most ideal model for detecting the ability of plant extracts and compounds for the free radical- scavenging activity in a relatively short time and it is simpler than the other assay models. This observation has accelerated the search for potential antioxidant principals from plants [8,9]. Previous reports on *Gleditsia* species revealed that they contain many phenolic compounds [9-11]. Therefore, the present work is an attempt for determining the total phenolic and total flavonoids,beside detecting the ability of the 85% methanol extract of the leaves of *G.triacanthos* L. plant for DPPH• scavenging activity as well as isolating and elucidating certain antioxidant compounds from this plant extract.

MATERIALS AND METHODS

Plant materials

Leaves of *Gleditsia triacanthos* L. were collected from El-Orman Garden, Cairo Governorate, Egypt. The plant was kindly identified by Prof. Dr. Wafaa Amer, Professor of Plant Taxonomy, Faculty of Science, Cairo University, Giza, Egypt. The fresh plant leaves were washed with clean water, completely dried in shade place at room temperature and then powdered by electric mill. The dried powders were kept in a dark place until subjected to the extraction process.

General experimental procedures

Melting points were determined on an electrothermal apparatus and were uncorrected. ^{1}H-NMR (500 MHZ, DMSO-d_6) and ^{13}C-NMR (125 MHZ, DMSO-d_6) spectra were recorded on Bruker 500 and JEOL-GX-spectrometer. The chemical shifts were expressed in δ (ppm) with reference TMS and coupling constant (J) in Hertz. UV spectra were determined in methanol after addition of different reagents on spectrophotometer (Milton Roy 601). Silica gel 60 GF_{254} (Fluka) were used for analytical TLC. Sephadex LH-20 (25-100 µm, Sigma) or silica gel (70-230 mesh, Merck) were used for column chromatography. Paper chromatography was carried out on Whatmann No. 1 or No. 3 paper sheets (Whatmann, England). Spots were visualized by absorption of UV radiation and spraying with ethanolic $AlCl_3$ (2 %) or 10 % H_2SO_4 followed by heating for flavonoids and glycosides.

Chemicals

1,1-diphenyl picrylhydrazyl (DPPH)• free radical was purchased from Sigma-Aldrich Co. Also sodium phosphate, ammonium molybdate, Folin-Ciocalteu reagent, ascorbic acid, rutin, gallic acid and sodium carbonate were of the highest analytical grade and were purchased from common sources.

Extraction and isolation procedures

The powdered leaves of *Gleditsia triacanthos* (2.5 Kg) were extracted with 85 % methanol at room temperature and the solvent was removed under reduced pressure to give (200 g) of methanol extract. This extract was defatted with petroleum ether. The defatted residue was dissolved in distilled water and the aqueous filtrate was successively extracted with chloroform followed with ethyl acetate and finally with n-BuOH. The three fractions; chloroform, ethyl acetate and butanol were evaporated until dryness to give 3.5, 8 and 28 g respectively. Preliminary phytochemical screening and the two

dimensional paper chromatography (TDPC) of each of chloroform and ethyl acetate extract using two solvent systems BAW (n-BuOH : AcOH : H_2O ; 4:1:5) and 15 % AcOH (AcOH : H_2O ; 15:85) revealed the presence of many compunds of phenolic and flavonoid components. Therefore these two extracts were submitted to chromatographic isolation.

Determination of total antioxidant capacity

The total antioxidant capacity of each extract was determined according to the phosphomolybdenum method as reported by Prieto *et al*., 1999 [12]. Briefly, 0.3 ml of each extract and ascorbic acid (100 µg/ml) in methanol was mixed in dried tubes with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Tubes containing the reaction mixture were capped and incubated in a boiling water bath at 95 ^oC min. After cooling the samples at room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the sample and it was incubated under the same conditions. All experiments were carried out in triplicate. The antioxidant activity of the extracts was expressed as the number of equivalents of ascorbic acid (AAE).

DPPH radical scavenging activity

The free radical scavenging activity of each extract or pure isolated compound was determined by using a stable (DPPH) according to the procedure described by Mensour *et al.,* 2001 [13]. Briefly, the assay contained 3 ml of each plant extract at a concentration of 1 ml of 0.1 mM DPPH in methanol. The reaction mixture was shaken and left for 30 min at room temperature in the dark. The degree of reduction of absorbance was recorded at 517 nm. Ascorbic acid was used as a reference standard. Control was prepared containing the same solvents and reagents without any extract. All experiments were carried out in triplicate. The scavenging activity (antioxidant activity) of each extract was expressed as SC_{50} which is defined as the concentration of extract required for 50 % scavenging of DPPH radicals compared with that of ascorbic acid which was used as the standard. The lower SC_{50} value corresponds to higher scavenging activity (higher antioxidant activity) of plant extract.

Determination of total phenolic content

The total phenolic content (TPC) of 85% methanol extract of the plant and chloroform and ethyl acetate fractions from it were assayed using Folin-Ciocalteu reagent and expressed as gallic acid equivalents (GAE) [14]. About 100 µl of plant extracts (100 µg/ml) and also 100 µl of gallic acid (100 µg/ml) were mixed with 500 µl of Folin-Ciocalteu reagent and the mixture was kept at room temperature for 5 min then 1.5 ml of 20% aqueous sodium carbonate was added. The mixture was shaken thoroughly and made up to 10 ml using distilled water. After incubation at room temperature for 2 hours, the absorbance of the developed blue color was read at 760 nm against reagent blank using spectrophotometer. All determinations were carried out in triplicate. The total phenolic content of each extract was expressed as mg gallic acid equivalent (GAE) per g extract.

Determination of total flavonoid content

Total flavonoid content (TFC) was determined spectrophotometrically according to the reported procedures by Kumaran and Karunakaran, 2006 [15]. Rutin was used as standard. Briefly, 100 µl of each plant extract was mixed with 100 µl of aluminium trichloride in methanol (20%) and then diluted with methanol to 5 ml. The absorbance of the mixture was read at 415 nm using spectrophotometer after 40 min against the blank (the blank consisted of all reagents and solvents without any extract). All reactions were carried out in triplicate. The total flavonoid in each extract was determined as mg rutin equivalent (RE)/g extract.

Chromatographic separation of chloroform and ethyl acetate extracts

The chloroform extract (3 g) was subjected to column chromatography (3 x 90 Cm) packed with silica gel (70-230 mesh, Fluka). The elution was started with petroleum ether followed by a gradient mixture of chloroform/methanol till pure methanol. Factions (150 ml) were collected, concentrated and examined by PC using two solvent systems (n-BuOH: AcOH : H_2O; 4:1:5) and 15 % AcOH. Fractions eluted with $CHCl_3$-MeOH; 70:30 give a major compound with few minor compounds. This fraction was purified on Sephadex LH- 20 column (2 x 70 Cm) using methanol as eluent to yield compound 1. The fractions eluted with $CHCl_3$: MeOH; 90:10 contained mixture of two major compounds. These fractions were subjected to preparative paper chromatography (PPC, 3 MM and 15 % Acetic acid as eluent system) to give compounds 2 and 3.

The ethyl acetate fraction (7 g) was submitted to silica gel column (3 x 90 Cm). Elution was started with pure chloroform followed by a gradient mixture of chloroform/methanol till pure methanol. Fractions (150 ml) were collected, concentrated and examined by TLC using solvent system (n-BuOH:AcOH:H_2O; 7:3:1) and PC using solvent system 15 % AcOH. The two major fractions eluted with $CHCl_3$:MeOH; 95:5 and 80:20 were separately purified on Sephadex LH-20 column using 80 % MeOH and pure methanol to give compounds 4 and 5.

Compound 1 was obtained as a yellow-brown powder with melting point 137-139 ^oC and R_f = 0.40 ($CHCl_3$: MeOH; 9:1) on silica gel TLC and 0.18 (15 % AcOH on PC). IRν max: 3368, 2788, 1685, 1618, 1595, 1520, 1127 and 715 Cm^{-1}. UV λ_{max} (nm): (MeOH) 245, 285sh, 352, (NaOMe) 254, 310, 350, ($AlCl_3$) 270, 290, 387, ($AlCl_3$+ HCl) 275, 290, 380, (NaOAc) 272, 318, 369, (NaOAc+H_3BO_3) 252, 267, 347. ^{1}H-NMR: δ 7.52 (1H, d, *J*=15.01 Hz, H-7), 7.11 (1H, *s*, H-2),7.02 (1H, *J* = 8.02 Hz, H-6), 6.87 (1H, d, *J* = 8.02 Hz, H-5), 6.30 (1 H, d, *J* = 15.01 Hz, H-8). ^{13}C-NMR: δ 125.45 (C-1), 114.85 (C-2), 145.25 (C-3), 148.34 (C-4), 115.76 (C-5), 121.36 (C-6), 141.45 (C-7), 127.49 (C-8), 174.50 (C-9).

Compound 2 was obtained as a yellow powder, mp 325-327^oC, R_f = 0.78 ($CHCl_3$:MeOH: H_2O; 8: 4:1; TLC) and 0.08 (15 % AcOH ; PC). UV λ_{max} (nm): (MeOH) 257, 289, 354; (NaOMe) 267, 327sh, 405, ($AlCl_3$) 274, 308 sh, 390, ($AlCl_3$ + HCl) 268, 350, 385, (NaOAc) 275, 319, 389, (NaOAc+H_3BO_3) 270, 279, 376. ^{1}H-NMR: δ 12.31 (1H, *s*, 5-OH), 7.53 (1H, d, *J* = 2.1 Hz, H-2/), 7.48 (1H, dd, *J*= 2.1 Hz, *J*= 8.2 Hz, H-6/), 6.97 (1H, d, *J* = 8.1 Hz, H-5/), 6.78 (1H, d, *J* =2.1 Hz, H-8), 6.72 (1H, *s*, H-3), 6.42 (1H, d, *J*= 2.1 Hz, H-6). ^{13}C-NMR: δ 182.60 (C-4), 165.40 (C-7), 165.10 (C-2), 163.18 (C-5), 156.23 (C-9), 149.50 (C-4/), 145.77 (C-3/), 121.10 (C-1/), 118.10 (C-6/), 116.21(C-5/), 114.10 (C-2/), 105.39 (C-10), 104.25 (C-3), 99.42 (C-6), 94.32 (C-8).

Compound 3 was obtained as a yellow powder, m.p 230-232 ^oC, R_f = 0.35 (EtOAc:MeOH : H_2O; 10:2:1; TLC) and 0.19 (15 % AcOH, PC). UV λ_{max} (nm) (MeOH) 270, 338 (NaMe) 275, 330, 397; ($AlCl_3$) 278, 305 381 ($AlCl_3$ +HCl) 279, 303, 344, 381 (NaOAc) 279, 305, 351, 387, (NaOAc+H_3BO_3) 273, 345. ^{1}H-NMR: δ 7.89 (2H, d, *J*= 8.5 Hz, H-2/, H-6/), 6.90 (2H, d, *J* =8.7 Hz, H-3/, H-5/), 6.54 (1H, *s*, H-3) 6.49 (1H, *s*, H-8), 4.80 (1H, d, *J* = 10.20 Hz, H-1//). ^{13}C-NMR: δ 182.60 (C-4), 163.50 (C-2), 163.45 (C-7), 161.30 ((C-4/), 161.04 (C-5), 158.79 (C-9), 128.20 (C-2/,C-6/), 121.20 (C-1/), 116.20 (C-3/,C-5/), 108.90 (C-6), 102.87 (C-10), 102.70 (C-3), 94.30 (C-8), 81.60 (C-5//), 78.86 (C-3//), 75.30 (C-1//), 71.50 (C-2//), 70.62 (C-4//), 61.80 (C-6//).

Compound 4 was obtained as a yellow powder, mp 216-218 ^oC, R_f =0.54 (EtAC : MeOH: H_2O; 1: 2: 1;TLC) and 0.24 (PC; 15 % AcOH). UV λ_{max} (nm) (MeOH): 267, 329, (NaOMe) 274, 319, 398, ($AlCl_3$) 276, 296, 381, ($AlCl_3$+HCl) 274, 339, 385, (NaOAc) 272, 355, 385, (NaOAc+H_3BO_3) 276, 357. ^{1}H-NMR: δ 12.52 (1H, *s*, C_5-OH), 7.69 (2H, d, *J* = 8.5 Hz, H-2/, H-6/), 6.85 (2H, d, *J*=8.5 Hz, H-3/, H-5/), 6.79 (1H, *J*= 2.0 Hz, H-6), 6.73 (1H, d, *J* = 2.0 Hz, H-8), 6.52 (1H, *s*, H-3), 4.78 (1H, d, *J* = 7.8 Hz, H-1//). ^{13}C-NMR: δ 181.50 (C-4), 164.16 (C-2), 163.85 (C-5), 161.80 (C-4/), 160,15 (C-9), 159.74 (C-7), 128.14 (C-2/,C-6/), 123.41 (C-1/), 116.20 (C-3/, C-5/) 109.19 (C-10), 106.26 (C-3), 104.45 (C-6), 99.35 (C-8), 103.26 (C-1//), 77.89 (C-5//), 76.32 (C-3//), 74.34 (C-2//), 70.87 (C-4//), 61.60 (C-6//).

Compound 5 was obtained as a yellow powder, mp 254-256 ^oC, R_f = 0.35 ($CHCl_3$: MeOH: H_2O ; 6:2:1 ;TLC) and 0.21 (15 % AcOH, PC). UV λ_{max} (nm) (MeOH): 254, 286 sh, 352, (NaOMe) 264, 318, 398, ($AlCl_3$) 269, 325, 410 ($AlCl_3$ + HCl) 260, 358, 390 (NaOAc) 265, 318 sh, 380,

(NaOAc + H_3BO_3) 254, 368, 426. ^{1}H-NMR: δ 12.41 (1H, *s*, br, 5-OH), 7.42 (1H, dd, *J*=8.1, 2.1 Hz, H-6/), 7.40 (1H, d, *J*= 2.1 Hz, H-2/), 6.86 (1H, d, *J* = 8.1 Hz, H-5/), 6.75 (1H, d, *J*= 2.1 Hz, H-8), 6.70 (1H, *s*, H-3), 6.42 (1H, d, *J* = 2.1 Hz, H-6), 5.04 (1H, d, *J*= 7.5 Hz, H-1//). ^{13}C-NMR δ 182.10 (C-4), 164.30 (C-2), 163.20 (C-7), 162.89 (C-5), 156.70 (C-9), 149.80 (C-4/). 146.10 (C-3/), 120.20, (C-1/), 118.30 (C-6/), 112.5 (C-2/) 104.90 (C-10), 102.98 (C-3), 99.75 (C-1//), 94.20 (C-8), 77.10 (C-5//), 76.40 (C-3//), 73.50 (C-2//), 69.50 (C-4//), 61.40 (C-6 //).

Acid hydrolysis

Five mg of compounds 4 and 5 were refluxed with 10% HCl in aqueous methanol for two hours. The reaction solution was concentrated and extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness. The obtained aglycones were identified by direct comparison with an authentic sample whereas the aqueous layer was neutralized, filtered and concentrated and the sugar moieties were identified by direct comparison with authentic sugars.

Statistical analysis

All experiments were run in triplicate and statistical analyses were performed using SPSS software. The results were given as means ± standard deviation.

RESULTS AND DISCUSSION

Determination of total antioxidant capacity

Each of the total antioxidant capacity of the defatted 85 % methanolic extract of *G. triacanthos* as well as chloroform and ethyl acetate fractions derived from the methanolic extract was measured spectrophotometrically by phosphomolybdenum method. This method is based on the reduction of Mo(IV) to Mo(V) by the tested agents and the subsequent formation of green phosphate /Mo (V) compounds with a maximum absorption at 695 nm. A high absorbance value of the sample indicates its strong antioxidant capacity. Results in table 1 exhibited that the defatted 85% methanol extract of *G. triacantho*s and the chloroform and ethyl acetate fractions derived from it, have antioxidant activity with the two used methods. The total antioxidant capacity were 288.51±2.19, 174.51±2.92 and 418.53±3.78 (mg equivalent to ascorbic acid / g extract) respectively. The results exhibited that, the ethyl acetate extract is the most active whereas the chloroform extract is the lowest one. These results are in full agreement with previous studies on other plant species [3, 12].

Scavenging activity (DPPH) assay

The measurement of the scavenging of DPPH radical allows one to determine exclusively the intrinsic ability of substance to donate hydrogen atom or electrons to this reactive species in a homogenous system. The method is based on the reduction of methanolic DPPH solution because the presence of antioxidant substances having hydrogen-donating groups such as phenols and flavonoid compounds due to the formation of non radical DPPH-H form [13].

The SC_{50} values for DPPH assay of the samples have been given in table 1. The methanol, $CHCl_3$ and ethyl acetate extracts of *G. triacanthos* have proved to be active antioxidants (SC_{50} = 47.10 ±0.39, 98.53 ± 0.82 and 15.42± 0.53 µg/ml respectively). The mechanism of the reaction between antioxidant compounds and DPPH depends on the structural conformation of these compounds. It has been reported that the free radical-scavenging activity of flavonoids is dependant on the presence of free OH groups especially 3-OH [18,19]. In the present study, the antioxidant activity of the methanol, chloroform and ethyl acetate extracts may be attributed to the collective antioxidant effects of the phenolic compounds and these results are in full agreement with previous studies on many plant species [3,6,7].

Table 1: Yield, total antioxidant capacity and free radical scavenging activity (DPPH) of the defatted 85% methanolic extract of *G. triacanthos* as well as chloroform and ethyl acetate fractions derived from it.

Extract	Yield %	Total antioxidant capacity [mg equivalent to ascorbic acid /g extract][a]	DPPH free radical scavenging activity SC_{50} [µg/ml][b]
85% MeOH	8	288.51 ± 2.19	47.10 ± 0.39
$CHCl_3$ fraction	1.50	174.51 ± 2.92	98.53 ± 0.82
EtOAC fraction	3.5	418.53 ± 3.78	15.42 ± 0.53
Ascorbic acid		-	7.82 ± 0.3

Values of SC_{50} and total antioxidant capacity are expressed as mean of triplicate determinations ± standard deviation.

[a]Antioxidant capacity monitored by the phosphomolybdenum method expressed by mg equivalent to ascorbic acid /g extract.

[b]SC_{50}, Concentration in µg /ml required scavenging the DPPH radical (100 µg /ml) by 50%.

Determination of total phenolic contents

Phenolic compounds are considered as a major group of antioxidant molecules that contribute to the antioxidant activities of plants because of their ability to scavenge free radicals by virtue of the presence of hydroxyl groups [3,14]. In this study, total phenolic content of defatted methanol extract of *G. triacanthos* and the chloroform and the ethyl acetate fractions derived from it was determined by using Folin-Ciocalteau assay. Total phenolic content in this method is expressed in terms of gallic acid equivalent and the values obtained for the concentration of total phenols are expressed as mg of gallic acid (GAE)/g of extract. The results in table 2 showed that the total phenolic contents in the three examined extracts are 240.32 ± 1.56, 160 ± 1.65 and 275.42 ± 1.53 GAE/g respectively. The highest concentration of phenols was measured in ethyl acetate fraction 275.42 ± 1.53 GAE/g followed by the defatted methanolic extract 240.32 ± 1.56 GAE/g whereas the chloroform had the lowest content (160 ± 1.65 GAE/g). These results are in full agreement with the previous studies [3,14-16] which reported that the percent of the phenolic compounds from the plants depends on the nature of the solvent used in the extraction process of the plant. Also, there are relationship between the phenolic content of the examined extracts and their abilities as antioxidant agents.

Determination of total flavonoid contents

In this study, the total flavonoid content of the extracts was evaluated by aluminum colorimetric assay. Rutin was used as a standard and the total flavonoid contents was expressed as rutin equivalents (RE) in milligram per gram dry extract. The results in table 2 showed that ethyl acetate has high flavonoid content (39.21 ± 0.22) followed by the methanolic extract (23.53 ± 0.18) and the chloroform is the lowest one (15.34 ± 0.52 ;mg rutin equivalent/g extract).

Results in tables 1,2 revealed that there is a positive correlation between the total antioxidant and DPPH radical-scavenging activities of the defatted methanolic extract of G. triacanthos and the two fractions ; chloroform and ethyl acetate which were derived from this extract and their phenolic and flavonoid contents. These results are in full agreement with several studies which proved that there is a linear correlation between the total phenolic content and total antioxidant capacity and DPPH scavenging activities of many plants [14,16].

Table 2: Total phenolic content (TPC) and total flavonoid content (TF) of the defatted 85% methanolic extract of *G. triacanthos* as well as chloroform and ethyl acetate fractions derived from it.

Extract	TPC (mg gallic acid equivalent/g extract)[a]	TF (mg rutin equivalent/g extract)[b]
85% methanol	240.32 ± 1.56	23.53 ± 0.18
$CHCl_3$ fraction	160 ± 1.65	15.34 ± 0.52
EtOAC fraction	275.42 ± 1.53	39.21 ± 0.22

Values of total phenolics and flavonoids are expressed as mean of triplicate determinations ± standard deviation

[a] Total phenolics expressed by mg equivalent to gallic acid /g extract.

[b] Total flavonoids expressed by mg equivalent to rutin /g extract.

Identification and the antioxidant of the isolated compounds

Each of the chloroform and ethyl acetate fraction was submitted to chromatographic separation and the structure of the isolated compounds (Fig. 1) was elucidated using certain spectroscopic analysis as follows:-

The IR spectrum of compound 1 showed absorption band at 3368 cm^{-1} for the hydroxyl groups, 1685 cm^{-1} for C=O of carboxylic group [17,18]. The ^{1}H-NMR spectrum of compound 1 displayed two ortho-coupled doublet for (*J*=8.02 Hz) each for 1H at δ 7.02 and 6.87 for H-6 and H-5 and one singlet for H-2 at δ 7.11(this indicated that the compound has trisubstituted aromatic ring). This was confirmed by presence of the carbons which are carrying the three hydrogen in ^{13}C-NMR at δ 121.36 (C-6), 115.76 (C-5) and 114.85 (C-2) respectively. Also, the ^{1}H-NMR spectrum showed two doublets (*J* = 15.01) each for 1H at δ 7.52 (H-7) and 6.30 (H-8) indicating the presence of trans-disubstituted ethylene part in compound 1 [17, 19]. This was confirmed by presence of two carbon signals for C-7 and C-8 at δ 141.45 and 127.49 respectively. The carbon signal at δ 174.50 proved the presence of carboxylic group which was confirmed by IR spectrum. Also, the upfield chemical shifts of one of the ethylenic carbon C-8 exhibited that the carboxylic group is located at C-8 [18,19]. From this data and by comparison with authentic sample, compound 1 was identified as caffeic acid.

The UV spectrum of compound 2 in methanol showed two characteristic bands at 354 and 257 which indicated that compound 2 belongs to the group of flavonoids. Bathochromic shift of band I from 354 to 390 (16 nm) with $AlCl_3$ indicated the presence of OH group in position 5. Also after adding NaOAc,band II was shifted from 257 to 275 (18 nm) which indicated the presence of OH-group at C-7. Adding of NaOAc+H_3BO_3, band I was shifted from 354 to 357 (21 nm) and indicated the presence of OH-group in position 3/ and 4/ [19,20]. The ^{1}HNMR spectrum of compound 2 showed a downfield signal at δ 12.31 indicating the presence of OH group at C-5 position. It also showed two meta-coupled doublets (*J*= 2.1 Hz) at δ 6.78 and 6.42 each integrating for one proton and they were assigned to H-8 and H-6 respectively of ring A of 5,7-dihydroxy flavonoids. The only singlet at 6.72 integrating for one proton that is attributed to H-3 of the flavonoid compounds. The presence of ABX system at δ 7.53, 7.48 and 6.97 is characteristic of 1,2,4-trisubstituted phenyl unit [19-20]. The ^{13}C-NMR data of compound 2 showed the presence of a ketone carbonyl at δ 182.60, two olefinic carbons at δ 165.10 and 104.25 and four hydroxyl carbons at δ 165.40, 163.18, 149.50 and 145.77. By comparing this data with the literature [19,20], it appeared that compound 2 is luteolin.

The UV absorption of compound 3 in methanol and the shifts observed after the addition of shift reagents were consistent with the presence of a 5,7,4/-trihydroxyflavone structure in this compound [21,22]. In the ^{1}H-NMR spectrum of compound 3, two doublets were observed at 7.89 and 6.90 due to H-2/,H-6/ and H-3/,H-5/ of the para-substituted B-ring of the compound 3 respectively.The singlet at 6.49 was assigned to H-8 proton of the A-ring due to the long-range correlations observed from H-8 to C-10 (102.87), C-7 (163.45) and C-9 (158.79). A singlet proton resonance at 6.54 which correlated to the carbon resonance at 102.70 in the HMQC spectrum was assigned to H-3 of the aglycone. HMBC correlations observed from H-3 to C-2 (163.50), C-10 (102.87) and C-1/ (121.20) supported this assumption [21,22]. The chemical shift value of the anomeric carbon atom at 75.30 and the coupling constituent value of H-1/ (*J*= 10.20 Hz) indicated that the linkage of glucose was through a C-bond. A prominent long-range correlation between the anomeric proton (4.80) of the glucose unit and C-6 (108.90) of the aglycone exhibited the attachment of the sugar moiety at the C-6 position [21,22]. Thus, compound 3 was elucidated as isovitexin.

The UV spectrum of compound 4 recorded before and after the addition of shift reagents pointed out the sugar moiety attached to OH group at C-7 apigenin [23]. Also in the ^{1}H-NMR spectrum of this compound,the aromatic proton signals of two m-coupled doublets at δ 6.97 and 6.73 (each, *J* = 2.0 Hz) showed HMQC correlations to the carbon resonances at δ 104.45 and 99.35 respectively, were attributed to the H-6 and H-8 of the A-ring. Two vicinally coupled doublets at 7.69 and 6.85 (each, 2H, *J* = 8.5 Hz) showed long-range couplings with the ^{13}C-NMR signal at δ 161.80 (C-4/) and therefore, were assignated to (H-2/, H-6/) and (H-3/, H-5/) respectively of the B-ring. Additionally a singlet at 6.52 was ascribed to H-3 [19,21,22]. The assignment of H-3 was confirmed by its hetero-nuclear long-range correlations to C-2 at 164.16 and C-1/ at 123.41. Additional resonances arising from a β -D-glucopyranoside unit at δ 4.78 in ^{1}H-NMR and signal at δ 103.26 in the ^{13}C-NMR spectrum. Upon acid hydrolysis, this compound liberated apigenin as aglycone and glucose as sugar moiety. Each of them was identified by direct comparison with authentic samples. According to the above data, compound 4 was identified as apigenin 7-O- β -D-glucopyranoside.

The ^{1}H-NMR spectrum of compound 5 showed a downfield signal at 12.41 indicating the presence of OH group at C-5 position. It also showed two meta-coupled doublets (*J*= 2.1 Hz) at δ 6.75 and 6.42 each integrating for one proton and they were assigned to H-8 and H-6 respectively of ring A of 5,7-dihydroxy flavonoids. The only singlet at δ 6.70 integrating for one proton which is attributed to H -3 of the flavonoid compounds. The presence of ABX system at 7.42, 7.40 and 6.86 characteristic of 1,2,4-trisubstituted phenyl unit. The ^{13}C-NMR data of compound 5 showed the presence of a ketone carbonyl at 182.10, two olefinic carbons at δ 164.30 and 102.98 and four hydroxyl carbons at 162.0, 162.89, 149.80 and 146.10. The ^{1}H and ^{13}C-NMR spectra of compound 5 were almost identical to those of compound 2. However, additional resonances arising from the sugar moiety which showed that the anomeric proton of the sugar part at δ 5.04 with coupling constant (*J*=7.5 Hz) and chemical shifts of the sugar carbons at δ 99.79, 77.10, 76.40, 73.50, 69.50 and 61.40. Acid hydrolysis of the compound gave luteolin as aglycone and the sugar component was identified as glucose by comparison with authentic samples on PC. As compound 5 has the typical spectral data (UV and NMR spectra) of luteolin -7-O-glucoside isolated earlier from *Vitex agnus casts* and *Vernonia cinerea* [24], thus compound 5 proved to be luteolin-7-O-β-D-glucopyranoside.

Evaluation of the five isolated compounds as antioxidant agents against DPPH-radical revealed that the five compounds have antioxidant activity (SC_{50} = 9.53, 11.67, 22.45, 98.44 and 58.91 µg/ml respectively).

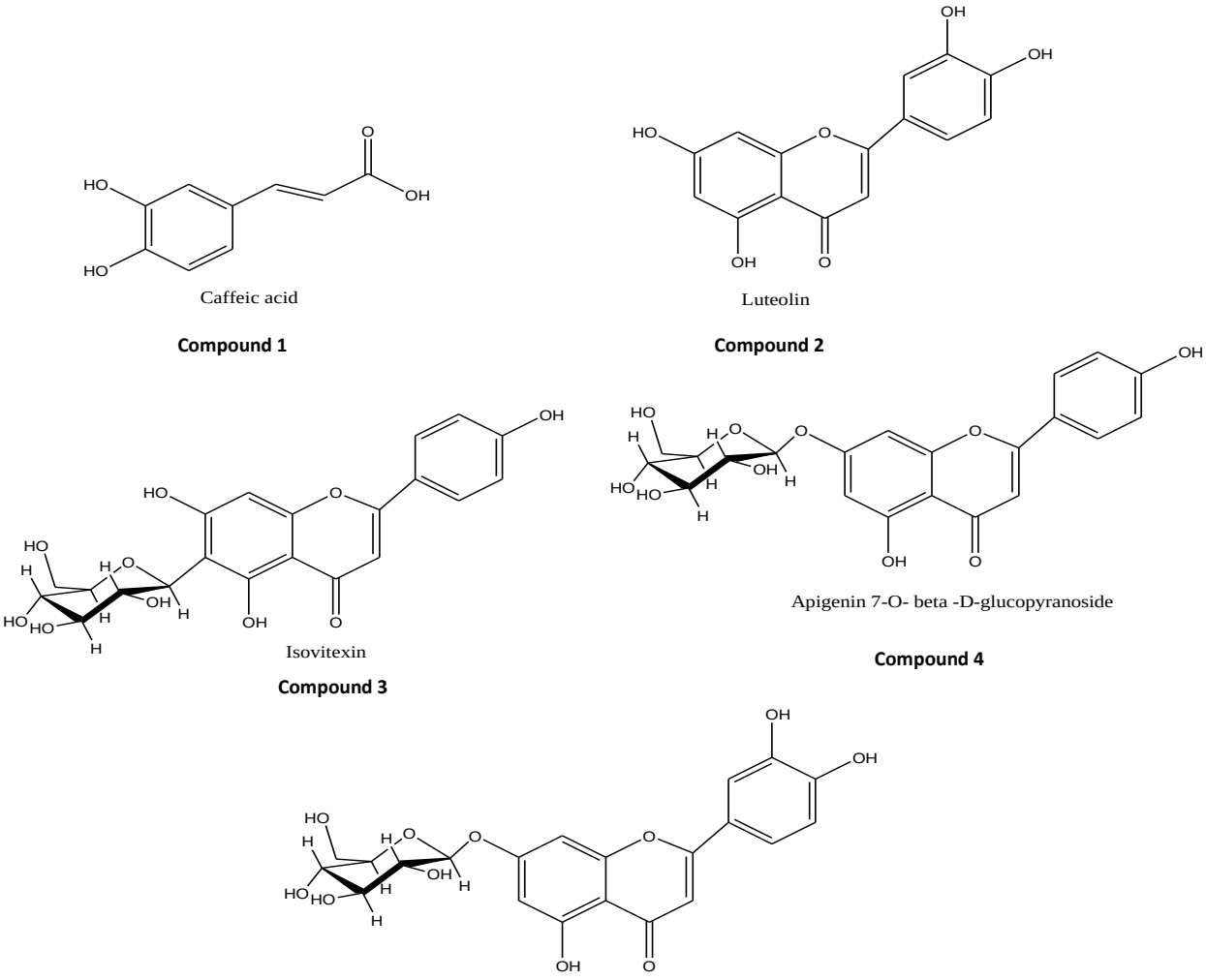

Fig. 1: Chemical structure of compounds 1-5 isolated from *G. triacanthos* leaves.

CONCLUSION

The present study indicated that the methanolic extract of *G. triacanthos* has high amount of phenolic and flavonoid contents. The antioxidant activity of the plant is correlated with the total phenolic and flavonoid contents. The methanolic extract of the plant can be recommended as a natural source of antioxidants.

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