BIO-GUIDED ISOLATION AND STRUCTURE ELUCIDATION OF ANTIOXIDANT COMPOUNDS FROM THE LEAVES OF *Ficus sycomorus*

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Summary

This study was performed to investigate the antioxidant activity of the defatted 70% methanol extract of *Ficus sycomorus* leaves using DPPH radical scavenging assay-guided isolation. This extract was fractionated using CHCl₃, EtOAc and n-BuOH. The results revealed that EtOAc and n-BuOH have strong DPPH radical scavenging (SC₅₀ =13.48 and 8.47 µg/ml). Therefore, each EtOAc and n-BuOH was subjected to chromatographic separation and purification. Four compounds were isolated from EtOAc fraction (1-4) and three compounds from n-BuOH (5-7). The structures of the isolated compounds were elucidated using certain spectroscopic methods as quercetin (1) gallic acid (2) quercetin 3-O-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (Rutin) (3), quercetin 3-O-β-D-glucopyranoside (Isoquercitrin) (4), quercetin 3,7-O-α-L-dirhamnose (5), quercetin 3-O-β-D-galactopyranosyl(1→6)-glucopyranoside (6) and β-sitosterol-3-β-D-glucopyranoside (7). Compounds (1-6) were found as major components and principally responsible for the antioxidant activity of *F. sycomorus* by using two in vitro methods, DPPH radical and total antioxidant capacity respectively. This is the first report on the antioxidant activity of the chemical constituents of *F. sycomorus*.

Keywords: DPPH radical; *Ficus sycomorus*; Flavonoid glycosides; Gallic acid; Quercetin.
Introduction

Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions. Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating metals and also by acting as oxygen scavenger. Antioxidants are of interest for the treatment of many kinds of cellular degeneration (1-4). Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis, hypertension, diabetes, hepatic damage and Al-zhheimer disease (5-6). Restriction on the use of the synthetic antioxidants is being imposed because of their hepatotoxicity and carcinogenicity. Thus, the interest in natural antioxidants has been increased considerably (7-8). A number of plant phenols such as flavonoids, coumarins and other natural products such as curcuminioids and terpenes have shown potent antioxidant activity and lower toxicities (8-11).

The genus *Ficus* belongs to the family Moraceae; comprising over 800 species. A number of *Ficus* species are used as food and for treatment of various diseases such as ulcers, vomiting vaginal complains, fever diabetes, inflammation and liver disease (11-13). Flavonoids, coumarins and terpenes and their glycosides have been reported as constituents of this genus (11-15). To the best of our knowledge only a few studies on the chemical constituents have been carried out on *F.sycomorus* but there is no report on antioxidative properties of phenolic compounds from this plant.

In continuation of our screening program for antioxidant agents, it has been found that the aqueous methanol extract of *F.sycomorus* leaves has a considerable free radical scavenging activity on DPPH assay (16). These results suggested that the methanol extract possess identified antioxidant compounds and promoted us to isolate these compounds. In the present study, Bioassay-guided fractionation and isolation of the 70% methanol extract of *F.sycomorus* and its derived ethyl acetate and butanolic fractions was carried out using two *In vitro* methods as free radical scavenging activity with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and phosphomolybdenum method.

Materials and Methods

*General*

Melting points were determined on an electrothermal apparatus and were uncorrected. $^1$H-NMR (300 and 500 MHZ, DMSO-d$_6$) and $^{13}$C-NMR (75 and 125 MHZ, DMSO-d$_6$) spectra were recorded on a varian Mecauy 300 or 500 JEOL GX- Spectrometer. The chemical shifts were referenced to using TMS as internal standard. UV spectra (max) were determined in methanol after addition of different reagent on a UV-601 UV-VIS recording spectrophotometer. ESI-MS was performed on a Micromass Q-TOF Micro-instrument. Silica gel (70-230 mesh, Merck) and Sephadex LH-20 (25-100 µm, Sigma) were used for column chromatography. Thin-layer chromatography and preparative TLC was performed on silica gel GF$_{254}$ precoated plated (Merck). 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$_2$SO$_4$ followed by heating for flavonoids and glycosides whereas aniline phthalate for sugars.
Plant Material

Leaves of *Ficus sycomorus* (Family Moraceae) were collected from El-Qualubia Governorate. The plant was kindly identified by Prof.Dr. Wafaa Amer, professor of Plant Taxonomy, Faculty of Science, Cairo University, Giza, Egypt. The fresh leaves of plant was washed with clean water and completely dried in shade place at room temperature and then powdered by electric mill. The leaves powdered of the plant were stored at room temperature in dark places until subjected to the extraction process.

Extraction and Fractionation

The air-dried powdered leaves of *Ficus sycomorus* (1.8 Kg) were extracted with 70% methanol at room temperature for several times. The combined methanolic extract was concentrated under reduced pressure using rotatory evaporator. The dried methanol extract (285 g) was defatted with petroleum ether (60-80 °C). The defatted methanolic extract was dissolved in water and successively extracted with chloroform, ethyl acetate and n-butanol. The obtained fractions were evaporated to dryness and tested for their radical scavenging activity by DPPH assay. The results exhibited that the methanol extract and its derived EtOAc and n-BuOH fractions have significant activity with SC_{50} values of 13.48 and 8.47 µg/ml separately. Accordingly, the EtOAc and n-BuOH extracts were subjected to chromatographic isolation.

Chromatographic Isolation of EtOAc Fraction

The ethyl acetate soluble part from the methanolic extract of *Ficus sycomorus* leaves (20 g) was subjected to open column chromatography (120 x 5 cm) packed with silica gel 60 (70-230 mesh, Merk) adsorbent as stationary phase. Elution was started with CHCl_{3} followed by gradient mixtures of CHCl_{3}: MeOH till reached (100%) methanol at the end. Fractions of 250 ml were collected and combined together according to their PC behavior after screening of their spots over paper chromatography in two common eluents (15%) acetic acid and (n-BuOH:AcOH:H_{2}O; 4:1:5) and over thin layer chromatography (TLC) using different solvent system according to their nature.

Three major fractions I-III were obtained by using different elution systems, CHCl_{3}: MeOH; 90:10, 70:30 and 30:70 respectively. These fractions showed significant scavenging effect with SC_{50} values of 9.68, 11.78 and 10.23 µg/ml. Therefore, these fractions were subjected to rechromatographed isolation and purification.

Fraction I (300 mg) was chromatographed on the Sephadex LH-20 column. Elution was started with 10% aqueous methanol followed by different ratios of aqueous methanol. Elution with 40% methanol led to isolation of compound (1) whereas elution with 85% methanol gave compound (2).
Fraction II (500 mg) was applied to sephadex LH-20 column chromatography. Elution started with 15% aqueous methanol and gradient mixtures of aqueous methanol was used. Elution with 60% methanol gave impure compound (3) which was further purified by preparative TLC on silica gel using solvent system (EtOAc:MeOH:H2O; 25:5:1).

Fraction III was rechromatographed over silica gel column. Elution started with CHCl3 followed by mixtures of CHCl3: MeOH and finally pure methanol. Elution with CHCl3: MeOH; 40:10 give major fraction which was further purified by preparative TLC using CHCl3: MeOH; 40:10 as eluent system to give compound (4).

**Chromatographic Isolation of n-BuOH Fraction**

The butanol soluble fraction part of the methanolic extract of *F.sycamorus* leaves (30 g) was chromatographed on silica gel column. Elution was started with CHCl3, different mixtures of CHCl3: MeOH and finally pure methanol. Two major fractions I and II were obtained and showed scavenging activity (SC_{50} =15.25 and 11.27 µg/ml). Thus these fractions were subjected to isolation process. Fraction I was rechromatographed over silica gel column and eluted with mixture of CHCl3: MeOH. Elution with CHCl3: MeOH (7:3) yielded the first subfraction A whereas elution with CHCl3: MeOH (8:2) gave the second subfraction B. The first subfraction A was further separated by preparative TLC using solvent system (EtOAc: MeOH: H2O; 10:1:2) to give compound (5). Also, the second subfraction B was separated by preparative paper chromatography (PC) Whatmann No. 3 using solvent system (HOAc: H2O; 15:85) to give compound (6).

**Compound 1**: yellow powder, m.p. 314-316 °C, Rf 0.50 (CHCl3: MeOH; TLC), UV_{max} nm (MeOH) 255, 297, 370; (NaOMe) 247, 329; (AlCl3) 272, 304, 454; (AlCl3+HCl) 265, 354, 428; (NaOAc) 275, 320, 390; (NaOAc+H3BO3) 261, 304, 388.

**Compound 2**: white powder, m.p. 251-252 °C, Rf 0.56 (15% AcOH; PC); IR (KBr) cm⁻¹; 3369, 3067, 2657, 1706, 1618, 1540, 1245, 1025, 864.

**Compound 3**: yellow powder, m.p. 193-195 °C, Rf 0.31 (CHCl3: MeOH; 4:1TLC); UV_{max} nm (MeOH) 256, 268, 298, 354; (NaOMe) 269, 330, 412; (271, 306, 431; (AlCl3+HCl) 268, 359, 405; (NaOAc) 270, 310, 398; (NaOAc+H3BO3) 260, 290, 372. H-NMR (DMSO-d6) 12.55 (s, 1H, 5-OH), 7.51 (1H, d, J=2.0 Hz, H-2'), 7.49 (1H, dd, J=8.4 and 2.0 Hz, H-6'), 6.81 (1H, d, J=8.4 Hz, H-5'), 6.35 (1H, d, J=2.0 Hz, H-8), 6.16 (1H, d, J=2.0 Hz, H-6), 5.31 (1H, d, J=6.8 Hz, H-1" , Glc), 4.34 (1H, d, J=1.25 Hz, H-1" , Rha) and 0.95 (3H, d, J=6.07 Hz, Rha-6"). C-NMR see Table 1.

**Compound 4**: yellow powder, m.p. 193-195 °C, Rf 0.31 (CHCl3: MeOH; 4:1TLC); UV_{max} nm (MeOH) 256, 297, 357; (NaOMe) 272, 325, 408; (AlCl3) 274, 307, 430; (AlCl3+HCl) 269, 303, 400; (NaOAc) 273, 322, 379; (NaOAc+H3BO3) 261, 395, 377. H-NMR (DMSO-d6) 7.59 (1H, d, J=2.4 Hz, H-2'), 7.56 (1H, d, J=2.1 Hz, H-6), 6.37 (1H, d, J=2.1 Hz, H-8), 6.17 (1H, d, J=2.1 Hz, H-6) and 4.5 (1H, d, J=7.6 Hz, H-1"). C-NMR see Table 1.
Compound 5: m.p. 225-227 °C, Rf 0.52 (EtOAc: MeOH: H2O 10: 1: 2) UV max nm (MeOH) 255, 270\textsuperscript{sh}, 359; (NaOMe) 268, 275, 396; (AlCl\textsubscript{3}) 275, 295\textsuperscript{sh}, 415; (AlCl\textsubscript{3}+HCl) 271, 300\textsuperscript{sh}, 402; (NaOAc) 265, 310\textsuperscript{sh}, 365; (NaOAc+H\textsubscript{3}BO\textsubscript{3}) 261, 320, 370. \textsuperscript{1}H-NMR (DMSO-d\textsubscript{6}) 7.37 (1H, d, J=2.5 Hz, H-2', H-6'), 6.70 (1H, d, J=2.5 Hz, H-6), 6.41(1H, d, J=2.5 Hz, H-8), 6.95 (1H,  d, J=8.5 Hz, H-5'), 5.58 (1H, J=2.1 Hz, H-1''), 1.25 (3H, d, J=6.1 Hz, CH\textsubscript{3}) and 0.95 ( 3H, d, J=6.1 Hz, CH\textsubscript{3}). \textsuperscript{13}C-NMR see Table 1.

Compound 6: yellow amorphous powder, m.p. 185-187 °C, Rf 0.31 (CHCl\textsubscript{3}: MeOH; 2:1; TLC), UV max nm (MeOH) 257, 290\textsuperscript{sh}, 362; (NaOMe) 272, 330\textsuperscript{sh}, 409; (AlCl\textsubscript{3}) 275, 342\textsuperscript{sh}, 438; (AlCl\textsubscript{3}+HCl) 268, 340\textsuperscript{sh}, 405 (NaOAc) 274, 325, 427. \textsuperscript{1}H-NMR (DMSO-d\textsubscript{6}) 12.65 (5-OH), 7.55 ( 1H, d, J=2.5 Hz, H- 2'), 7.52(1H, dd , J=8.5 and 2.5 Hz, H-6'), 6.36 (1H, d, J=2.5 Hz, H-8), 6.17 (1H, d, J=2.5, H-6), 5.33 (1H, d, J=7.2 Hz, H-1'', Glc) and 4.38 (1H, d, J=7.0 Hz, H-1''', Glc). \textsuperscript{13}C-NMR see Table 1.

Compound 7: white powder, m.p. 282-284 °C, Rf 0.40 (CHCl\textsubscript{3}: MeOH; 9: 1). IR (KBr) cm\textsuperscript{-1} 3402, 2935, 2873, 1636, 1460, 1261, 1063, 889, 802, 627. \textsuperscript{1}H-NMR 0.60-0.94, 4.85 (1H, d, J=7.4 Hz, H-1', Glc), 5.28 (1H, d, J=2.1 Hz, 1H, H-6). \textsuperscript{13}C-NMR see Table 1.

Acid hydrolysis

Each isolated glycoside (5 mg each) was reflex 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 with direct comparison with authentic sugars.

DPPH assay

DPPH qualitative antioxidant assay

Primary screening of the ability of column fractions as free radical scavenger was performed with a rapid TLC screening method using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. Briefly, 5 µl of each fraction (1 mg /ml) was carefully loaded on to 20 cm x20 cm TLC layer (Silica gel 60 FG 254; Merck) and allowed to dry then spared with a 0.2% solution of DPPH in methanol and heated for 5 minutes. Active fractions appeared as yellow spots against a purple background. The purple stable free radical 1, 1-diphenyl-2-picrylhydrazyl was reduced to yellow colored diphenyl picrylhydrazine. Ascorbic acid was used as positive control (17). Fractions that showed activity were further subjected to isolation and identification of compounds responsible for the antioxidant activity.

Spectrophotometric assay

DPPH radical scavenging activity

The ability of each extract to scavenge DPPH radicals was measured according to the procedure described by Mensor et al 2001 (18). Briefly; 3 ml of each plant extract at a concentration of 100 µg /ml were mixed with 1 ml of 0.1 mm DPPH in methanol. The mixture was then shaken and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm using a spectrophotometer. Ascorbic acid was used as a reference standard. Control was prepared containing the same solvents and
reagents without any extract and reference ascorbic acid. All experimental were carried out in triplicate.

The scavenging effect (antioxidant activity) of each extract was expressed and SC₅₀ which defined as the concentration of each extract required for 50% scavenging of DPPH radicals compared with that of ascorbic acid which was used as the standard. The lower SC₅₀ value corresponds to a higher scavenging activity (higher antioxidant activity) of plant extract.

**Determination of total antioxidant capacity**

The antioxidant activity of each extract was determined according to phosphomolybdenum method Prieto et al 1999 (19) using ascorbic acid as standard. In this method, 0.3 ml of each extract (100 µg /ml) in methanol was combined in dried vials with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials containing the reaction mixture were capped and incubated in a thermal block at 95 °C min. After the samples had cooled 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).

**Statistical analysis**

All experimental were run in triplicate, and statistical analysis were performed using SPSS software. Analysis of variance was performed by ANOVA procedures. Results were given as means ± standard deviation. Significance level was defined as P < 0.05.

**Results And Discussion**

DPPH radical scavenging assay of the defatted 70% methanol extract of the leaves of *F.sycamorus* showed that this extract possess a stronger antioxidants activity (SC₅₀=13.55 µg/ml). Therefore, the defatted methanol extract was subjected to fractionation using different organic solvents including CHCl₃, EtOAc and n-BuOH. It was found that EtOAc and n-BuOH gave high scavenging activity (SC₅₀=13.48 and 8.47 µg/ml), therefore, the two extracts were isolated and the structure of the isolated compounds was elucidated by using certain spectroscopic methods as UV, NMR (¹H-NMR and ¹³C-NMR) and MS as well as by comparing with authentic samples and reported data.

**Identification of compounds 1-7**

**Compound 1** gave bright yellow with NH₃ vapor, yellow with AlCl₃ (20, 21). Its UV spectrum in methanol and with different reagents was in agreement with that quercetin reported in the literature (22, 23). The UV spectrum in methanol showed two major absorption bands at 370 nm and 255 nm which confirmed the flavonol structure. Degradation with MeONa and hypochromic shifts with AlCl₃/HCl and NaOAc/H₃BO₃ supported the presence of 3, 3', 4'-tri-hydroxy system. Bathochromic shifts with NaOAc were related to 7-hydroxy and the bathochromic shift with AlCl₃/HCl to 5-hydroxy. Also, the compound was identified as quercetin by comparison with authentic sample (CO-TLC, m.p. and Rf) (24).
Compound 2 gave a positive test with FeCl₃ and effervescence with NaHCO₃ solution indicating the presence of phenolic and carboxylic groups respectively. The presence of peaks at 3369 and 1706 cm⁻¹ in the IR spectrum (KBr) νₘₐₓ cm⁻¹ indicated the presence of hydroxyl and carbonyl groups respectively (25,26). UV spectral data λₘₐₓ (nm) in methanol was 218 and 275 nm (27-28). The compound was identified as gallic acid (3, 4, 5-trihydroxybenzoic acid) through the comparison of its chromatographic behavior, mixed melting point and superimposable IR spectrum with authentic sample of gallic acid (27-28).

Compound 3 showed faint yellow color in visible light and dark purple color under UV light changing deep yellow with ammonia vapor and orange with Naturstoff reagent and green color with FeCl₃ (21). The UV spectrum of the compound showed two major absorption bands in methanol at 354 nm and 254 nm which indicated the presence of flavonol structure. Bathochromic shift with MeONa supported the presence of 4'-hydroxy and with NaOAc indicated the presence of 7-hydroxy functions. The AlCl₃ and AlCl₃/HCl spectrum of the compound showed 5-hydroxy and orthodihydroxy in B-ring. This fact indicated that the 3-hydroxy was absent or substituted (29-31). The ¹H-NMR spectrum clearly established the presence of chelated hydroxyl group with the occurrence of a sharp singlet at δ 12.55 for 5-OH. In addition, the ¹H-NMR spectrum showed the presence of five protons which appeared as doublets at δ 7.51 (1H, d, J= 2.0 Hz), 7.49 (1H, dd, J= 8.4 and 2.0 Hz), 6.81 (1H, d, J= 8.4 Hz), 6.35 (1H, d, J= 2.0 Hz) and 6.16 (1H, d, J= 2.0 Hz) assigned to H-2', H-6', H-5', H-8 and H-6 respectively. H-1'' for glucosyl moiety was observed as doublet at δ 5.31 (1H, d, J= 6.85 Hz). The one singlet peak at δ 4.34 revealed the presence of one anomic proton H-1''' for rhamnosyl moiety in which the methyl group of rhamnosyl appeared at δ 0.95 (3H, d). This was confirmed the presence of twenty-seven carbon signals in the ¹³C-NMR spectrum of the compound as shown in (Table 1), which consisting ten quaternary carbons (C-4, C-5, C-7, C-9, C-2, C-10, C-4', C-3', C-3 and C-1'), fifteen methine carbons (C-6', C-5', C-2', C-6, C-8, C-1'', C-1'', C-3'', C-5'', C-2'', C-4'', C-4'', C-3''', C-2''' and C-5''''), one methyl carbon at C-6'' and one methylene carbon at C-6'' (23,31,32). Acid hydrolysis of the compound gave quercetin as aglycone and the sugar component was identified as glucose and rhamnose by comparing with authentic samples on PC. On the basis of the above data compound (3) was identified as quercetin-3-O-α-L-rhamnopyranosyl (1→6) β-D-glucopyranoside (Rutin).

Compound 4 showed a faint yellow visible light and dark purple under UV light, changing to yellow with ammonia and orange with Naturstoff reagent indicating that the compound is 3-O-substituted flavonol (21, 33). UV spectral analysis of the compound in MeOH and with the usual shift reagents 1) appeared that the absorption band in methanol at 357 nm (band I) is comparable to 3-hydroxy substituted flavonol. The presence of a free 4'-OH group was confirmed by the bathochromic shift of band I (51 nm) with NaOMe. The 5-OH group was confirmed by the bathochromic shift of band I (43 nm) with AlCl₃/HCl. The presence of 7-OH group was indicated by the bathochromic shift 17 nm with NaOAc (band II). The bathochromic shift (20 nm) band I with NaOAc/H₃BO₃ suggested the presence of O-dihydroxy groups in B-ring (22, 34). ¹H-NMR of the compound in aglycone region exhibited an ABX system at δ 7.59 (1H, d, J= 2.4 Hz, H-2'), 7.56 (1H, dd, J= 8.5 and 2.4 Hz, H-6') and 6.84 (1H, d,

J= 8.5 Hz, H-5') due to 3', 4'-disubstituted of ring B and a typical meta-coupled pattern for H-6 and H-8 protons at (δ 6.17 and 6.37, d, J=2.1 Hz respectively). The doublets at δ 5.45 (1H", d, J= 7.6 Hz) was assigned to anomeric proton of hexoses and suggested a glycosidic β-linkage (30, 35-37). The 13C-NMR spectrum of the compound (Table 1) showed the presence of 15 aromatic carbon signals in aglycone region including ten quaternary carbons at 156.17, 133.33, 177.40, 161.22, 164.60, 156.38, 103.85, 121.16, 144.87 and 148.56 ppm assigned to C-2, C-3, C-4, C-5, C-7, C-9, C-10, C-1', C-3' and C-4' respectively. The five methine carbons occurred at δ 98.82, 93.63, 116.22, 120.90 and 121.61 assigned to C-6, C-8, C-2', C-5' and C-6' respectively (31, 36, 37). Acid hydrolysis of the compound gave quercetin as aglycone and glucose as sugar which were identified by Co-chromatography with authentic sample. On the basis of these data, compound (4) was identified as quercetin-3-O-β-D-glucopyranoside (isoquercitrin).

Compound 5 appeared as a violet fluorescent on PC under UV light and changed to yellow with ammonia indicating the presence of flavone with free hydroxyl at C-4', C-3', and C-5 (20). UV spectra of the compound in MeOH gave two major spectral peaks at 255 nm (Band II) and 359 nm (Band I) and shifts obtained with diagnostic reagents; NaOMe, 268, 271, 396; AlCl₃, 275, 415; AlCl₃+HCl, 271, 300 [8], 402; NaOAc, 265, 310, 365 and NaOAc+H₃BO₃, 261, 320, 370 suggested it is a 3,7-disubstituted quercetin glycoside with free hydroxyl groups at 3', 5 and 4'-positions (38,39). The 1H-NMR spectrum of the compound showed two meta coupled doublets each with J= 2.5 Hz at δ 6.70 and 6.41 ppm for the A-ring H-6 and H-8-protons respectively. An ABX system for three protons at δ 6.95 (1H, d, J= 8.5 Hz), δ 7.37 (1H, d, J= 8.5 Hz) and δ 7.57 (1H, d, J= 8.5 Hz) assignable to H-5', H-6' and H-2' respectively for B-ring. Two anomic protons at δ 5.58 (1H, d, J= 2.1 Hz) and δ 5.35 (1H, d, J= 2.1 Hz) were attributed to rhamnosyl moiety (α-configuration) directly linked to the aromatic rings at the 7 and 3 positions respectively. The two secondary methyl groups of sugar moieties appeared as doublets at 0.95 (3H, d, J= 6.1 Hz, CH₃) and 1.25 (3H, d, J= 6.1 Hz, CH₃) (38-40). In the 13C-NMR spectrum (Table 1) of the compound 27 carbon signals were shown. Fifteen carbon signals were assigned to quercetin moiety and 12 carbon signals were assigned to the two rhamnoside units. Complete acid hydrolysis of the compound yielded quercetin as aglycone and rhamnose as sugar, the aglycone was identified by comparison with authentic sample. Also sugar moiety was detected by comparison with authentic sugar (solvent system; CHCl₃: Me₂CO: MeOH: H₂O, 3:3:2:1 respectively). Therefore, compound (5) was identified as quercetin 3,7-O-a-L-dirhamnoside.

Compound 6 was obtained as yellow amorphous powder. Its UV absorption in MeOH were consistent with the presence of 3, 5, 7, 3', 4'-pentahydroxyflavone structure. Also, the presence of free 5, 7, 3' and 4'-hydroxy groups during its UV survey indicated that glycosidation linkage must be attached at 3-hydroxyl group in the aglycone (34, 41, 42). Quercetin, glucose and galactose were liberated by acid hydrolysis of the compound. It's 1H-NMR and 13C-NMR spectra (Table 1) showed the presence of aromatic system and sugar moieties. The 1H-NMR of the compound revealed 3 aromatic protons at δ_H 7.55 (d, J= 2.5 Hz), 7.52 (dd, J= 8.5 and 2.5 Hz) and 6.84 (d, J= 8.5 Hz) which were observed as an ABX suggesting the presence of an O-disubstituted B-ring (Liu et al., 2008; Han et al., 2004). Moreover, meta coupled signals in the aromatic region at δ_H 6.17 (d, J= 2.5 Hz, H-6) and 6.36 (1H, d, J=2.5 Hz,
H-8) were consistent with 5, 7-dihydroxy substituted A-ring of flavonoid. Two anomeric protons appeared in $^1$H-NMR spectrum at $\delta$ 5.33 and 4.38. The configuration of the anomeric protons and the sugar moieties was proposed to be $\beta$-type on the basis of the coupling constant (7.70 and 7.20 Hz). Sugar was found to be $\beta$-glucopyranoside according to $^1$H-NMR data and by comparison with the literature (43, 44). In the $^{13}$C-NMR spectrum of the compound 27 carbon signals were shown. Fifteen carbon signals were assigned to quercetin moiety 12 carbon signals were assigned to glucose and galactose units. The carbon signal of C-6 was observed at $\delta$ 67.0 ppm owing to the glycosidation affect which is usually observed at 61.5 ppm in galactose (37, 43). The ESI-MS (m/z) of the compound exhibited a pseudomolecular ion peak [M+Na]$^+$ at 649.57 and [M+H]$^+$ at 626.57 respectively. Other prominent ions at 464.41 [M+H-162]$^+$ and 301.14 [M+H-324]$^+$ m/z indicated the successive elimination of 2 hexoses units respectively. Therefore, compound (6) was identified as quercetin-3-$O$-$\beta$-D-galactopyranosyl (1→6) glucopyranoside.

**Compound 7** gave positive results with Liebermann-Burchard and Molisch's tests. Its IR spectrum (KBr) $\nu_{max}$ cm$^{-1}$ showed absorption bands at 3402.78 (-OH), 2935.13 (-CH$_2$), 2873.42 (-CH), 1636.94 (-C=C-) and 1068.37 (C-O-C) cm$^{-1}$ (45, 46). Its IR, NMR and mass spectral data obtained were comparable to the published previously of $\beta$-Sitosterol-3-$O$-$\beta$-glucopyranoside (46-48). In the $^1$H-NMR of this compound six methyl groups appeared at rang 0.60-0.94 ppm, an olefinic proton at $\delta$ 3.28 and anomeric proton at $\delta$ 4.85 ppm. This data indicated that the compound (7) is sterol with a double bond at C-5 and C-6. This was confirmed by presence of two carbon signals at 140.96 and 121.73 for C-5 and C-6. Also, a signal of anomeric sugar proton appeared in $^1$H-NMR at 4.86 ppm as doublet with $J=7.8$ Hz which pointed out the $\beta$-configuration of glycosidic bond (47, 48). This was confirmed by appearing of anomeric sugar carbon at $\delta$ 101.36 in its $^{13}$C-NMR (Table 1) (47, 48). ESI-Ms of compound (7) showed a prominent peak of [M+Na]$^+$ at m/z 576.41 and [2M+Na]$^+$ at m/z 1175.86. Other fragment of [M+Na-Glc]$^+$ appeared at m/z 413.26. Thus compound (7) was identified as $\beta$-Sitosterol-3-$O$-$\beta$-D-glucopyranoside.

**Conclusion**

This is the first report on the phytochemical and antioxidant studies the chemical constituents of *F.sycomorus* leaves. The results revealed that quercetin, gallic acid, rutin, isoquercitrin, quercetin 3, 7-$O$-$\alpha$-L-dirhamnoside are the major constituents of the plant and represent the antioxidant ingredients with DPPH radical and Phosphomolybdenum methods ($SC_{50} =$3.97, 4.80, 7.79, 9.25, 10.01, and 6.95 $\mu$g/ml) and 77.2, 690.34, 463.69, 321.58, 266.63 and 502.20 mg AAE/g compound). These results are in full agreement with the previous studies (1-5) which revealed that the free radical scavenging property of phenolic natural products is mainly owing to their ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers. Also in some cases the activity of these compounds are due to their metal chelation potential. Thus, the presence of these free radical scavengers in *F.sycomorus* might be relevant in relation to this plants various biological properties and medicinal uses.
Table 1

$^{13}$C-NMR [(DMSO-$d_6$), $\delta$ values (ppm)] spectral data for the isolated compounds (3-7).

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$^a$ $^{13}$C-NMR spectra were measured in (125 MHz) instrument.

$^b$ $^{13}$C-NMR spectra were measured in (75 MHz) instrument.
Table 2
Antioxidant activities of compounds (1-6) isolated from *F. sycomorus* using DPPH free radical scavenging activity and total antioxidant capacity methods.

<table>
<thead>
<tr>
<th>Compound</th>
<th>DPPH ( SC_{50} ) [µg /ml]</th>
<th>Total antioxidant capacity (mg AAE /g compound.)</th>
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<tr>
<td>Ascorbic acid</td>
<td>7.90 ± 2.10</td>
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Results are (means ± S.D.), \((n = 3)\).

* Significant
Fig. 1. Structure of the isolated compounds (1-7) from *F. sycomorus* leaves.
References


