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PHYTOCHEMICAL AND BIOLOGICAL STUDY OF *DIOSPYROS KAKI* L. GROWING IN EGYPT

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ABSTRACT

The leaves of *Diospyros kaki* L. is a traditional medicine used in Japan for the treatment of hypertension, angina and internal hemorrhage. The present study aims to investigate the antitumor and antimicrobial activities of the ethanolic extract (EE), unsaponifiable matter (USM), ethyl acetate extrac (EtOAc) of this plant. Moreover, seven compounds were isolated from the leaves and their activities were evaluated. The anticancer activities were studied using MCF-7 breast cancer cell line and HeLa-cervical carcinoma cell line. The antimicrobial activity was studied by *in vitro* diffusion agar method. The EE, USM and EtOAc extract showed anticancer activities against MCF-7 breast cancer cell line (IC_{50}=50µg/mL, 25.4µg/mL and 19.8 µg/mL respectively) and HeLa-cervical carcinoma cell line (IC_{50}=46.2µg/mL, 7.8 µg/mL and 16.7µg/mL respectively). Among the isolated compounds, lupeol (**1**) was the most active showing anticancer activity against MCF-7 breast carcinoma cell line and HeLa-cervical carcinoma cell line (IC_{50} = 20.7µg/mL and 23.7µg/mL respectively). The EtOAc extract showed strong antimicrobial activity against all tested microorganisms with zones of inhibition ranging from 13.6 to 24.6 mm at a concentration of 5mg/mL. Isolated compounds showed different activities against the tested microorganisms, with kaempferol **(5)** being the most active.

Keywords: *Diospyros kaki L.,E benaceae, anticancer, antimicrobial.*

1. INTRODUCTION

Diospyros kaki L. is a deciduous tree belonging to family Ebenaceae. The plant is commonly named as Japanese persimmon.[1] The leaves of the plant are used for the traditional treatment of hypertension, angina and internal hemorrhage[2], also powdered leaf supplement was reported to have a hypolipidemic effect.[3]Five triterpenoid isolated from *D. kaki* leaves are reported to have antioxidant and antigenotoxic activities.[4] Kaempferol isolated from *D. kaki* leaves had antimicrobial activity against some bacteria[5] and the water soluble proanthocyanidins have antihypertensive effect.[6]The methanolic extract of persimmon calyx has cytotoxic effect against human cancer cells (HT-29, HeLa and PANC-1cells).[7] Reviewing the available literature, no reports on the antitumor activity of *D.kaki* L. leaves against MCF-7 breast cancer cell line and HeLa cervical carcinoma cell line, were traced. Therefore, the present work is performed to study the antitumor effect of the leaves of *D. kaki* L. growing in Egypt and its phytochemical and antimicrobial activity of the leaf extracts and isolated compounds.

2. MATERIALS AND METHODS

2.1. General experimental procedures

Precoated silica gel F_{254} TLC plates (Sigma Aldrich Chemicals-Germany), normal phase silica gel 60 (Sigma-Aldrich chemicals Germany), Sephadex LH_{20} (Uppsala, Sweden) were used. All the solvents are of analytical grade. Jeol mass spectrophotometer (70 eV), Electro thermal 9100, for determination of melting points (U.K) and NMR Bruker Biospin 400 MHz were used and the NMR spectra were recorded in DMSO-*d6*, $CDCl_3$ and MeOD.

2.2. Plant material

The leaves of *D.kaki* L. were collected from Modereyet El-Tahrir Farm (El Beheira governorate, Egypt), June 2010. A voucher specimen no. (BUPD29) is deposited in Pharmacognosy Department, Beni Suef University. The leaves were dried in shade, powdered and stored in an air-tight container for phytochemical and biological study.

2.3. Preparation of the extracts

The air-dried powdered leaves (100 g) were exhaustively extracted with 80% ethanol and the solvent was evaporated under reduced pressure. The residue obtained was kept for biological study. Powdered leaves (1.5kg) were subjected to successive fractionation using solvents of increasing polarities [petroleum ether, chloroform, ethyl acetate, and butanol (which is kept for futher assay)]. The successive extractives were evaporated under reduced pressure to give

30, 57, 40.3 and 245.8 g respectively. The petroleum ether extract of the leaves (30 g) was saponified by reflux with 300 mL of 10% alcoholic KOH at room temperature. The unsaponifiable matter (USM) was extracted with ether and saved for further analysis.

2.4. Anticancer activity

MCF-7and HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). They were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50µg/mL Gentamycin. They were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and subcultured two to three times a week.The anticancer activity was evaluated according to the reported protocol.[8]

2.5. Antimicrobial screening

Selected strains of fungi: *Trichophyton mentagrophytes* (RCMB 0925), *Aspergillus fumigates* (RCMB 02564), *Candida albicans* (RCMB 05035), *Geotricum candidum* (RCMB 05096), *Penicillium marneffei* (RCMB 01267), and *Syncephalastrum racemosum* (RCMB 05922); Gram positive bacteria: *Staphylococcus aureus* (RCMB 010027), *Staphylococcus epidermidis* (RCMB 010024), *Streptococcus pyogenes* (RCMB 010015), *Bacillus subtillis* (RCMB 010063) and *Enterococcus faecalis* (RCMB 010068); Gram negative bacteria**:** *Neisseria gonorrhoeae* (RCMB 010076), *Proteous vulgaris* (RCMB 010085), *Klebsiella pneumoniae* (RCMB 0010093), *Shigella flexneri* (RCMB 0100542), *Pseudomonas aeruginosa* (RCMB 010043) were used. The tested organisms were subcultured on Saboroud dextrose agar (Oxoid laboratories, UK) for fungi[9] and nutrient agar medium (Oxoid laboratories, UK) for bacteria.[10] Amphotericin B used as a standard for fungi, Penicillin G for Gram positive bacteria and Streptomycin for Gram negative bacteria. The antimicrobial activity was determined using the agar well diffusion test.[11]Minimum inhibitory concentration (MIC) was determined for the isolated compounds.[12]

2.6. Isolation and Purification of various compounds

USM (12 g) were subjected to vacuum liquid chromatography (VLC) using 200 g silica and EtOAc/*n*-hexane gradient in 5% increment for elution. Fraction 6 (5%EtOAc/*n*-hexane) was recrystallized from *n*-hexane to yield compound **(1)**. Fraction 9 (15% EtOAc/*n*-hexane) (200 mg) was rechromatographed on silica gel column using petroleum ether-EtOAc in 1% increment till 20 %. Collected subfractions (27-30) yielded compound **(2)**. Subfractions (47 and 48) yielded compound **(3)**.

EtOAc extract (25 g) was subjected to silica VLC column. Elution was performed with MeOH/CH_2Cl_2 (0%-100%). Collected fractions 15-20 (5%MeOH/ CH_2Cl_2) were subjected to Sephadex- LH_{20} column to yield compounds **(4)** and **(5)**. Fractions 40 and 41 (20%MeOH/ CH_2Cl_2) were rechromatographed on a Sephadex-LH_{20} column to give compound **(6)** and another subfraction that was rechromatographed on silica gel using MeOH/CH_2Cl_2 to yield compound **(7).**

3. RESULTS AND DISCUSSION

3.1. Identification of the isolated compounds

The identification of all isolated compounds fig. (1) was achieved using different spectroscopic techniques (^{1}H-^{13}C- NMR, and MS) and by comparison with the previously reported spectral data.

Compound **(1):** 1.2 g, white powder, m.p 215℃, soluble in CH_2Cl_2, gives a positive Liebermann–Burchard test. MS data displayed a molecular ion peak at m/z 426 [M^+]; compatible with the molecular formula $C_{30}H_{50}O$.^{1}H and^{13}C NMR data were in good agreement with those reported for Lupeol.[13]This compound was previously isolated from *kaki* root[14]and for the first time from the leaves.

Compound (**2**): 120 mg, colorless needle crystals, m.p 135-136°C, freely soluble in *n*-hexane, gives a positive Liebermann–Burchard test. MS spectrum displayed a molecular ion peak at m/z 414 suggesting the molecular formula of $C_{29}H_{50}O$. Compound (**2**) was identified as *β*-sitosterol by co-TLC with authentic sample using different mobile phases, in addition to examination of ^{1}H-NMR data which were in good agreement with those of *β*-sitosterol.[15] This compound was previously isolated from *kaki* calyx[16] and isolated from the leaves for the first time.

Compound **(3):** 71 mg, white powder, m.p. 230-231°C, freely soluble in CH_2Cl_2; gives positive Liebermann–Burchard test.^{1}H-and^{13}C-NMR data were in good agreement with those of uvaol,[17] this compound was previously isolated from *kaki* leaves **(**2).

Compound **(4):** 43 mg, white powder, m.p. 184-186° C, freely soluble in CH_2Cl_2; it gives positive Liebermann–Burchard test. It showed molecular ion Peak at m/z 426. Examination of ^{1}H and^{13}C-NMR data revealed α-amyr in structure.[18] This compound was previously isolated from *kaki* leaves.[4]

Compound **(5):** 21 mg, yellow powder, m.p. 271-274°C, soluble in MeOH. ^{1}HNMR (δppm, 400MHz, DMSO-*d6*) showed four doublet signals at δ 6.17(*d*,1H,H-6, *J*=2.0 Hz),6.42(1H,*d*,H-8, *J*=2.0 Hz), 6.91(2H,*d*,H-3′,H-5′, J=9 Hz), 8.02(2H,*d*,H-6′,H-8′,*J*=9 Hz). From the abovementioned data and by comparison with an authentic sample and published data[(5)]. The structure of compound **(5)** was identified as 3,5,7,4′-tetrahydroxy flavones (Kaempferol).

Compound **(6):** 20 mg yellow powder, m.p. 212-215°C, soluble in MeOH. ^{1}HNMR (δppm,400MHz,DMSO-d_6) showed signals at δ 5.98 (1H, *d*, H6), 6.17 (1H,*d*,H-8), 6.84 (2H,*d*,H-3′,H-5′), 8.02 (2H,*d*,H-6′,H-8′), 5.4 (1H,d,H-1″), 3.28 (1H,*m*,H2″), 3.21(1H,*m*,H3″), 3.19 (1H,*m*,H4″), 3.09 (1H,*m*,H5″). Accordingly compound **(6**) was identified as astragalin.[19] This compound was previously isolated from *kaki* leaves. [20]

Compound **(7):** 26 mg, white powder, m.p. 298°C, freely soluble in MeOH, positive with Liebermann–Burchard test. ^{1}H- and ^{13}C-NMR datawere in good agreement with those of barbinervic acid.[21] This compound was previously isolated from *kaki* leaves.[22]

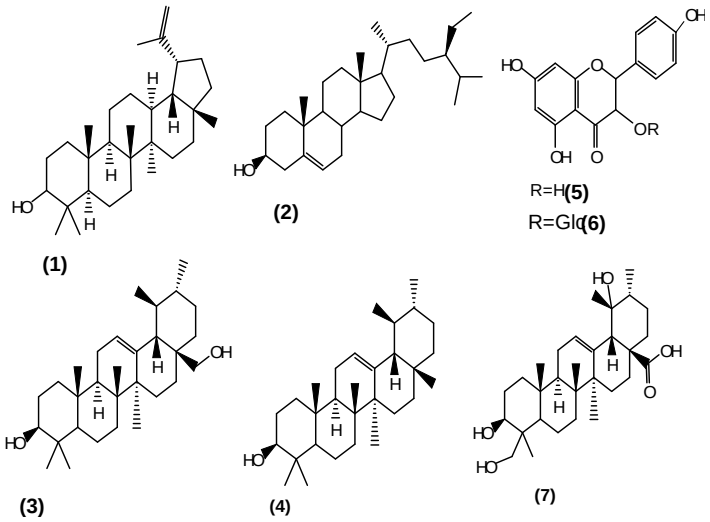

Figure (1) Isolated compounds from the leaves of *Diospyros kaki* L.

3.2. Anticancer activity

The EE, USM, EtOAc of the leaves were evaluated for their anticancer activities against MCF-7 and HeLa cell lines. Results are presented in table (1) revealed that EE has moderate anticancer activities against MCF-7 and HeLa cell lines with IC_{50}>50 and 46.2µg/mL respectively. The USM showed IC_{50} of 25.4 and 7.8 µg /mL respectively, while EtOAc extract IC_{50} is 25.4 and 16.7 µg/mL. Based on these data USM and EtOAc extracts were subjected to chromatographic study and the isolated compounds were further studied

biologically; Lupeol **(1)** showed significant anticancer activity against MCF-7 and HeLa with IC_{50} 20.7 and 23.4 µg/mL respectively. Results of IC_{50} of Uvaol **(3)**, *α*-amyrin **(4)** and Kampferol **(5)** against MCF-7 were 46.2, 47.1 and 43.4 µg/mL respectively. No activity was observed with those compounds against HeLa cells. Also no anticancer activity was detected for *β*-sitosterol (**2**) and baribenervic acid **(7)** against both cancer cell lines.

Table 1: Anticancer activity of the extracts and the isolated compounds of *Diospyros kaki* leaves against MCF-7 breast cancer cell line and HeLa- cervical carcinoma cell line.

Sample	IC_{50} (µg/mL)	
	MCF-7	HeLa
Total ethanol extract	>50	46.2
Unsaponifiable matter	25.4	7.8
Ethyl acetate extract	19.8	16.7
Lupeol (1)	20.7	23.7
***β*-sitosterol (2)**	>50	>50
Uvaol (3)	46.2	>50
***α*- amyrin (4)**	47.1	>50
Kaempferol (5)	43.4	>50
Barbinervic acid (7)	>50	>50

3.3. Antimicrobial activity

Zones of inhibition produced by EE, USM and EtOAc extract of the leaves of *D. kaki* L ranged from11.7-19.4 mm. EtOAc extract was active against all tested microorganisms. Therefore, the minimum inhibitory concentrations (MIC) of the isolated compounds from EtOAc extract were determined, Table (2). Kaempferol **(5)** was the most active compound with inhibitory activity against six types of dermatophytes (*T. mentagrophytes***,** *A. fumigatus, C. albicans, G. candidum, P. marneffi* and *S. racemosum),* and MIC (3.9, 15.63, 31.25, 0.98, 3.9 and 31.25 µg/mL respectively), giving good opportunity for using leaf extract in dermatological preparations. Kaempferol **(5)** also showed a significant activity against Gram positive bacteria (*Staph. aureus, Staph. epidermidis, S. pyogenes, B. subtillis and E. faecalis*) with MIC (0.98, 0.24, 15.63, 0.12 and 62.5µg/mL respectively) and Gram negative bacteria (*N. gonorrhoeae, P. vulgaris, K. pneumoniae, S. flexneri and P. aeruginosa)* with MIC (125,0.98, 0.12,1.95 and 62.5µg/mL respectively).

Table 2 Minimum inhibitory concentration of isolated compounds from ethyl acetate extract of *Diospyros kaki* L. leaves.

Tested Microorganisms	MIC (µg/mL)			Standard
	Barbinervic acid	Kaempferol	Astragalin	
Fungi:				*Amphotericin B*
Trichophyton mentagrophytes	NA	3.9	NA	3.9
Aspergillus fumigates	NA	15.63	NA	0.98
Candida albicans	NA	31.25	NA	0.06
Geotricum candidum	NA	0.98	NA	0.12
Penicillium marneffei	NA	3.9	NA	0.98
Syncephalastrum racemosum	NA	31.25	NA	0.007
Gram Positive Bacteria:				*Ampicillin*
Staphylococcus aureus	500	0.98	125	0.015
Staphylococcus epidermidis	125	0.24	125	0.12
Streptococcus pyogenes	500	15.63	500	0.06
Bacillus subtilis	62.5	0.12	31.25	0.007
Enterococcus faecalis	NA	62.5	NA	0.98
Gram negative bacteria:				*Gentamycin*
Neisseria gonorrhoeae	NA	125	125	3.9
Proteus vulgaris	NA	0.98	125	0.49
Klebsiella pneumoniae	NA	0.12	500	0.06
Shigella flexneri	NA	1.95	31.25	0.12
Pseudomonas aeruginosa	NA	62.5	NA	31.25

NA: No Activity

On the other hand, barbinervic acid **(7)** and astragalin **(6)** showed weak antibacterial activity against some Gram positive bacteria, but no activity against tested fungi and Gram negative bacteria (Table 2).

The significantly higher growth inhibitory activity observed for EtOAc extract against all tested microbial strains could be due to the presence of Kaempferol.

CONCLUSION

The present study reports the anticancer and antimicrobial activity of the leaves. The EtOAc extract and USM of D. *kaki* L. leaves showed interesting anticancer activity against MCF-7 and HeLa cell lines. The significant antimicrobial activity of the EtOAc extract, gives a good opportunity for using the leaf extract in dermatological preparations and in treatment of microbial infection.

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