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ANTIINFLMMATORY ACTIVITY OF DIOSPYROS KAKI PEELS AND BIOACTIVE CONSTITUENTS

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ABSTRACT

The aim of this present investigation is to evaluate anti-inflammatory activity of methanol 80% extract of Diospyros kaki peels and to identify the bio-active phytoconstituents present in the plant extract. The extract was tested for anti-inflammatory potential by using nitrite assay and also the extract was fractionated on silica gel for isolation of the bioactive compounds. The results showed that the extract has a significant anti-inflammatory effect, it showed with various concentrations of methanol extract of D. kaki peels remarkably and significantly inhibition of LPS-induced nitrite production. Chromatographic separation of the bio-active extract resulted in the identification of gallic, ellagic, quercetin, myricetin, quercetin 3-O-β-glucoside, myricetin 3-O-α-rhamnoside and myricetin 3-O-β-glucuronide. The results indicated that Diospyros kaki peels methanol extract can act as a potential anti-inflammatory candidate.

Keywords: *Diospyros kaki, peels, antiiflammatory activity, bio-active constituents*

Introduction

Inflammation is a local response of living mammalian tissues to the injury. It is a body defence reaction in order to eliminate or limit the spread of injurious agents. There are various components to an inflammatory reaction that can contribute to the associated symptoms and tissue injury. Oedema formation, leukocyte infiltration and granuloma formation represent such components of inflamemation (1). Inflammation plays an important role in various diseases, such as rheumatoid arthritis, atherosclerosis and asthma, which all show a high prevalence globally. Drugs, which are in use presently for the management of pain and inflammatory conditions, are either narcotics as opioids or non-narcotics as salicylates and corticosteroids. All of these drugs possess

well-known side and toxic effects. Moreover, synthetic drugs are very expensive and on the contrary many medicines of plant origin had been used since long time without any adverse effects. In our search for anti-inflammatory agents from plants, *Diospyros kaki* is a tree from Ebenaceae family. *D. kaki* tree is mainly cultivated in the north-east Asian countries. *D. kaki* has been used for their medicinal properties, such as their blood pressure-lowering and diuretic effects (2). Recent studies show that *D. kaki* possesses antitumor and multidrug resistance reversal properties (3), hypocholesterolemic and antioxidant effects (4) and antidiabetic effects (5) and prevents the rise in plasma lipids (6). These beneficial properties are considered to be related to the various antioxidants, including vitamins, phenolic compounds and carotenoids contained in the fruit. The objective of this study is to evaluate anti-inflammatory activity of *D. kaki* peels and to identify the bioactive constituents responsible for the bio-activity.

Materials and methods

General experimental procedures

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). ^{1}H-NMR and ^{13}C-NMR (Varian Unity Inova). MS (Finnigan MAT SSQ 7000, 70 ev). (Silica gel (0.063-0.200 mm for column chromatography) and Sephadex LH-20 (Pharmacia Fine Chemicals). Thin layer chromatography (TLC) F_{254} plates. Solvent mixtures, BAW (*n*-butanol: acetic acid: water 4:1:5 upper phase, 15% acetic acid: water: glacial acetic acid: 85:15). Paper Chromatography (PC) Whatman No.1 (Whatman Led.Maid Stone, Kent, England) sheets for qualitative detection of flavonoids and sugars were used in this study.

Plant identification and collection

Peels of *Diospyros kaki* were collected from the Agricultural Research Centre, Giza, Egypt in October 2010 and the plant was identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC). A voucher specimen is deposited in the herbarium of Agricultural Research Centre, Giza, Egypt.

Extraction and isolation of the bioactive phenolic compounds

The peels from fruits (420 g) of *D. kaki* were exhaustively extracted with methanol 80% several times at room temperature. The extract was filtered and concentrated on reduced pressure until only H_2O remained. The aqueous extract (29 g) was defatted with n-hexane and the sugars were precipitated by ethyl alcohol absolute and the residue of the extract (18 g) was subjected to silica gel column chromatography using an increasing gradient of ethyl acetate (EtOAc) in methylene chloride (CH_2Cl_2) up to 100%, followed by an increasing gradient of MeOH up to 100%. This gave four fractions A-D. Fraction A (1.25 g) was obtained from CH_2Cl_2: EtOAc (1:1 v/v) and was further subjected to preparative paper

chromatography using BAW (*n*-butanol:acetic acid:water 4:1:5 upper phase) as eluent, a violet band and shine band under short Ultraviolet (UV) light were detected and each band was cutted off and was washed with methanol to give compounds 1 and 2. Fraction B (900 mg) was eluted with EtOAc: CH_2Cl_2 (80:20) to give compound 3 which was purified through Sephadex LH-20 column using absolute ethyl alcohol as eluent. Fraction C (840 mg) was eluted with EtOAc 100% to give compound 4 which was purified through Sephadex LH-20 column using water: methanol (80:20 v/v). Fraction D (1.35 g) was eluted with EtOAc:MeOH (70:30 v/v) to give compound 5 and further elution with methanol yielded compounds 6 and 7 which also were subjected further column chromatography using Sephadex LH-20 using 50% MeOH as eluent. Compound 8 resulted from elution with EtOAc:MeOH (50:50 v/v) and it was futher purified on Sephadex LH-20 column using 50% MeOH as eluent.

General method for acid hydrolysis of flavonoid glycosides

5 mg of each flavonoid glycoside 5, 6 and 7 in 5 ml 10% HCl was heated for 5h. The aglycones were extracted with EtOAc and identified by co-TLC with authentic standards. The sugars in the aqueous layer was identified by co-paper chromatography (co-PC) with authentic markers on Whatman No. 1 sheets in solvent system (*n*-BuOH-AcOH-H_2O 4:1:5 upper layer).

Nitrite assay

The RAW 264.7 cells are treated methanol extract of *D. kaki* peels at various concentrations and LPS (*Escherichia coli* 0127: E8) (100 ng/mL) or LPS only for 24 h. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction. After centrifugation at 1000 *g* for 20 min, 100 µL of each supernatant medium was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was measured with an enzyme-linked immunosorbent assay plate reader. Methanol extract of *D. kaki* peels was dissolved in dimethylsulfoxide (DMSO) and control cells were treated with 0.05 % DMSO as vehicle.

Results and Discussion

To investigate the anti-inflammatory effects of methanol extract of *D. kaki* peels, the level of nitrite in the culture media of RAW 264.7 cells was examined. As shown in **Table 1**, the level of nitrite was increased in LPS-treated group (13.0 ± 0.1 □M). However, cells co-treatment with various concentrations of methanol extract of *D. kaki* peels remarkably and significantly inhibited LPS-induced nitrite production. The present investigation evaluated anti-inflammatory potential of *D. kaki* peels methanol extract (Table 1), determined the main phytoconstituents of the extract which are carbohydrates, tannins,

flavonoids and triterpenes and also detected the bioactive phytoconstituents of *D. kaki* peels methanol extract which are gallic, ellagic, quercetin, myricetin, quercetin 3-*O*-*β*-glucoside, myricetin 3-O-α-rhamnoside and myricetin 3-*O*-*β*-glucuronide (Fig. 1). Among these bioactive compounds, gallic acid has been reported to suppress LPS-induced NO production through down-regulation of inducible nitric oxide synthase (7). Ellagic acid isolated from strawberry also inhibited LPS-induced iNOS protein expression in macrophage as well as pro-inflammatory cytokines production in UVB- irradiated mouse skin (8, 9). Furthermore, a number of studies suggest quercetin as a potent anti-inflammatory agent against LPS-stimulated NO production in different cell types (10, 11, 12). Thus, we suggested the potential of methanol extract of *D. kaki* peels on suppression of NO production was contribute to those bioactive compounds. In addition, this inhibitory effect may work in combination of those bioactive compounds that synergy to down-regulate NO production. However, further experiments are needed to further elucidation.

Table 1. Anti-inflammatory activity of *D. kaki* peels methanol extract

Concentration	Methanol extract of *D. kaki* peels
Control	1.4 ± 0.1
LPS (100 ng/mL)	13.0 ± 0.1
20 µg/mL+LPS	9.8 ± 0.1[a]
40 µg/mL+LPS	8.4 ± 1.6[a]
60 µg/mL+LPS	6.4 ± 0.6[a]
80 µg/mL+LPS	3.1 ± 0.6[b]
100 µg/mL+LPS	3.6 ± 0.2[b]

The cells were treated with 100 ng/mL LPS only or with different concentrations of *D. kaki* peels methanol extract. After incubation for 24 h, 100 µL of culture media were collected for nitrite assay. The values are expressed as means ± SE of triplicate tests. [a]$P < 0.05$ and [b]$P < 0.001$ indicate statistically significant differences from the LPS-treated group.

Phytochemical analysis

Phytochemical analysis of methanol extract of *D. kaki* peels revealed that it contained carbohydrates, tannins, flavonoids and triterpenes (Table 2). Chromatographic separation and purification of methanol extract of *D. kaki* peels allowed the identification of gallic, ellagic, quercetin, myricetin, quercetin 3-*O*-*β*-glucoside, myricetin 3-O-α-rhamnoside and myricetin 3-*O*-*β*-glucuronide (Figure 1). Their structures were elucidated on the basis of UV, ^{1}H-NMR, ^{13}C-NMR and MS analyses.

Structure elucidation of the isolated compounds

Gallic acid (1): 14 mg, white amorphous powder. UV λmax (MeOH): 270. ^{1}H-NMR

(DMSO-d_6, 400 MHz): δ 7.1 (2H, s, H-2,6). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 166.9(-COOH), 145.4 (C-3, 5), 137.8 (C-4), 121.4 (C-1), 109.6 (C-2, 6).

Ellagic acid (2): 16 mg, white amorphous powder. ^{1}H-NMR (DMSO-d_6,400 MHz): δ 7.44 (2H, s, H-4,9). 13C-NMR (DMSO-d_6, 100 MHz): d 158.8 (5,10-CO),147.8 (C 3,8), 139.3 (C-2,7), 136.1 (C-1a,6a), 112 (C-4b,9b), 110.2 (C-4,9), 107.3 (4a,9a).

Quercetin (3): 12 mg, yellow powder. UV λmax (MeOH): 255, 267, 371; (NaOMe): 270, 320, 420; ($AlCl_3$): 270, 455; ($AlCl_3$/HCl): 264, 303sh, 315sh, 428; (NaOAc): 257, 274, 318, 383; (NaOAc/H_3BO_3): 259, 387. EI-MS: m/z 302.

Myricetin (4): 12 mg, yellow powder. UV λmax (MeOH): 254, 272sh, 374; (NaOMe): 262sh, 285sh, 322sh, 423(Dec.); ($AlCl_3$): 271, 316sh, 450; ($AlCl_3$/HCl): 266, 275sh, 308sh, 360sh, 428; (NaOAc): 269, 335(Dec.); (NaOAc/H_3BO_3): 258, 304sh, 392. EI-MS: m/z 318.

Quercetin 3-*O*-*β*-glucoside (5): 10 mg, yellow crystals. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.78 (1H, dd, *J*=2, 8.5 Hz, H-6'), 7.54 (1H, d, *J*=2 Hz, H-2'), 6.82 (1H, d, *J*=8.5 Hz, H-5'), d 6.42 (1H, d, *J*= 2 Hz, H-8), 6.24 (1 H,d, *J*= 2 Hz, H-6), 5.5 (1H, d, *J*=7.5 Hz, H-1''). (-) ESI-MS: m/z 463 [M-H]$^-$.

Myricetin 3-O-α-rhamnopyranoside (6): 23 mg, yellow amorphous powder. UV λmax (MeOH): 260, 296sh, 352; (NaOMe): 273, 321, 392; (AlCl3): 272, 312, 420; (AlCl3/HCl): 270, 310, 404; (NaOAc): 270, 317, 364; (NaOAc/H_3BO_3): 260, 303, 376. ^{1}H-NMR (400 MHz, DMSO-d_6): δ ppm 6.89 (2H,s, H-2`/6`), δ 6.2 (1H, d, *J* = 2.5 Hz, H-6), δ 6.37 (1H, d, *J* = 2.5 Hz, H-8), 5.2 (1H, s, H-1``), 3.9-3.2 (m, remaining sugar protons), 0.8 ($CH_3$-rhamnosyl, d, *J* = 6 Hz, H-6``).

Myricetin 3-*O*-*β*-glucronoide (7): 24 mg, yellow amorphous powder. UV λmax (MeOH): 262, 298sh, 349; (NaOMe): 272, 324, 392; ($AlCl_3$): 272, 312, 428; ($AlCl_3$/HCl): 270, 310, 404; (NaOAc): 270, 318, 366; (NaOAc/H_3BO_3): 260, 300, 374. ^{1}H-NMR (MeOD, 400 MHz): δ 7.42 (2H, s, H-2',6'), 6.45 (1H, d, *J* = 1.2 Hz, H-8), 6.22 (1H, d, J = 1.2 Hz, H-6), 5.47 (1H, d, *J* = 7.5 Hz, H-1''). ^{13}C-NMR (MeOD, 100 MHz)): δ 177.5 (C-4), 174 (C-6''), 165.8 (C-7), 162.6 (C-5), 158.4 (C-9), 148.2 (C-2), 146.9 (C-3',5'), 137.5 (C-3), 137.1 (C-4'), 123.3 (C-1'), 108.8 (C-2',6'), 104.7 (C-10), 104 (C-1''), 99.5 (C-8), 94.6 (C-6), 78.2 (C-3''), 78 (C-5''), 75.6 (C-2''), 73.4 (C-4'').

Identification of the active compounds of *D. kaki* peels methanol extract

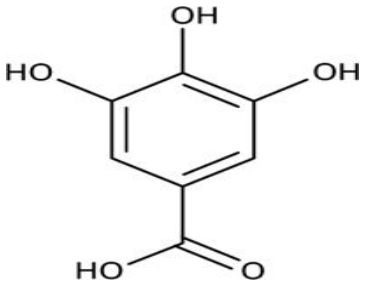
Compound 1 (gallic acid) gave a violet spot under short UV light and gave a specific dark green colour with $FeCl_3$, NMR data are with in accordance with the published literature (13). Compound 2 (ellagic acid) yielded a shine spot under short UV light and it gave a bluish grren colour with $FeCl_3$ and this indicates that it is a phenolic compound, NMR data was in agreement with the published literature (14). Compound 3 (quercetin) and compound 4

(myricetin) gave yellow green colour and when exposed to ammonia vapour and gave a bright yellow colour when spraying with $AlCl_3$ and their spectral data were identical to that of Lawrence et al. (15). Compound 5 (quercetin 3-*O*-*β*-glucoside) is obtained as deep purple spot and the compound gave yellow colour when exposed to ammonia vapour and gave a bright yellow colour when spraying with $AlCl_3$. Complete acid hydrolysis of the compound gave quercetin as an aglycone and glucose as sugar moiety. Spectral data of this compound is very close to spectra of Song *et al*. (16). Compound 6 (myricetin 3-O-α-rhamnopyranoside) and compound 7 (myricetin 3-*O*-*β*-glucronoide) are obtained as deep purple spot and both gave yellow colour when exposed to ammonia vapour and gave a bright yellow colour when spraying with $AlCl_3$. Complete acid hydrolysis of the both compounds gave myricetin as an aglycone, rhamnose and glucuronic acid as sugar moieties, respectively. Spectral data of both compounds are very close to spectra of Lawrence et al. (15). The significant anti-inflammatory activity of the extract of *D. kaki* peels is proved from the phytoconstituents present in the extract, The flavonoids from plant extracts have been found to possess antioxidants and anti-inflammatory properties in various studies (17, 18). The presence of terpenoids have shown anti-inflammatory properties (19). Strong presence of tannins in the plant extract may explain its potent bioactivities as tannins are known to possess potent antioxidants (20), and anti-inflammatory properties (21). Quercetin 3-*O*-*β*-glucoside was also identified in an anti-inflammatory fraction of *Solanum melonga* leaves (22).

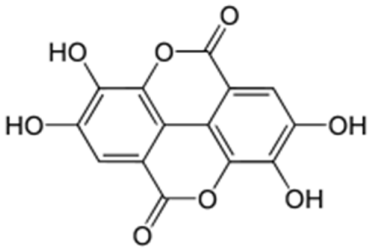
Table 2. Phytochemical Analysis of *Diospyros kaki* peels methanol 80% extract

Constituents	Methanol 80% extract
Triterpenes and /or Sterols	+
Carbohydrates and/or glycosides	+
Flavonoids	+
Coumarins	-
Alkaloids and/or nitrogenous compounds	-
Tannins	+
Saponins	-

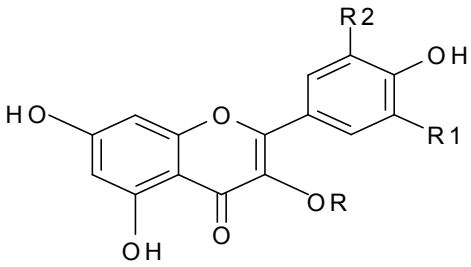
(+) presence of the constituents, (-) absence of the constituents



1: Gallic acid



2: Ellagic acid



3: Querectin (R=R1=H, R2=OH)
4: Myriectin (R=H, R1=R2=OH)
5: Quercetin 3-O-β-glucoside (R=glucose, R1=H, R2=OH)
6: Myricetin 3-O-α-rhamnopyranoside (R=rhamnose, R1=R2 =OH)
7: Myricetin 3-*O*-β-glucronoide (R=glucuronic acid, R1=R2 =OH)

Figure 1. Chemical structures of the compounds isolated from *D. kaki* peels methanol extract

Conclusion: It can be concluded from this study that methanol extract of *D. kaki* peels possess significant anti-inflammatory activity. This supports the traditional use of this plant in various painful inflammatory diseases. The Information of the present study may help for discovery of a potential source of chemically interesting and biologically important drug candidates.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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