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THE PHARMA RESEARCH

An International Journal of Pharmacy Research

Published on: 15-03-2014 **ISSN: 0975-8216**

EVALUATION OF CYTOTOXICITY, ANTI-HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) AND ANTIBACTERIAL ACTIVITIES OF DIOSPYROS KAKI FRUITS AND PHYTOCONSTITUENTS

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ABSTRACT

This research work deals with the evaluation of in vitro cytotoxicity of the methanol 80% extract of Diospyros kaki fruits in Vero cells, anti-HSV-1 and antibacterial activities against S. aureus, S. epidermidis, E. coli and P. aeruginosa and the determination of its phytochemical content. The methanolic extract from D. kaki fruits showed a high CC50 value of 1900 µg/mL, meaning a low cytotoxicity, and at this non-toxic concentration inhibited partially the development of the HSV-1 induced cytopathic effect using a virus challenge dose of 100xTCID50, while the extract had no effect on bacterial strains. Phytochemical analysis of the methanol extract proves the presence of carbohydrates, tannins, flavonoids, coumarins and triterpenes. Chromatographic separation of the methanol extract resulted in the isolation and identification of gallic, ellagic, quercetin, myricetin, quercetin 3-O-β-glucoside, myricetin 3-O-α-rhamnoside and myricetin 3-O-β-glucuronide. The results indicate that Diospyros kaki fruits methanol extract is a candidate for experiments of biological activity screening that are not antibacterial against the strains evaluated in this study and for anti-HSV-1 evaluation of purified fractions, to be carried on in the future.

Keywords: *Diospyros kaki, fruits, cytotoxicity, anti-HSV-1, antibacterial*

Introduction

Unlike antimicrobial drugs against bacteria and fungi, only a few effective antiviral drugs are available. One of the most important reasons for the lack of success in developing antiviral drugs is due to the nature of the infectious viral agents, which totally depend upon the cell they infect for their multiplication and survival, so that many compounds that may cause the death of viruses also are very likely to injure the host cell that harbour them [1] The sever side

effects and the emergence of drug-resistance mutants during long-term medication with these drugs have often limited their administration to patients [2, 3]. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries (Awadh *et al.,* 2001). Therefore, it is of great interest to carry out a screening of these plants in order to validate their use in folk medicine and to reveal the active principle by isolation and characterization of their constituents (Awadh *et al.,* 2001) and thus, the evaluation of the cytotoxic potential of novel candidates to antimicrobial agents is an important area of research. In searching for natural products as potential antiviral and antimicrobial agents, *Diospyros kaki* is a tree from Ebenaceae family. *D. kaki* is cultivated widely with 90% of production in Korea, China and Japan. *D. kaki* trees are mainly cultivated in the northeast Asian countries and their fruits are classified as sweet and astringent types (George and Redpath, 2008). Due to their nutritional and health benefit functional characteristics, the cultivation and production have been recently increased in Mediterranean countries, such as Spain and Italy (Ancos *et al.,* 2000). *D. kaki* fruits have been used for their medicinal properties, such as their blood pressure-lowering and diuretic effects (George and Redpath, 2008). Recent studies show that *D. kaki* possesses antitumor and multidrug resistance reversal properties (Kawase *et al*., 2003), hypocholesterolemic and antioxidant effects (Gorinstein *et al*., 1998) and antidiabetic effects (Lee *et al*., 2006) and prevents the rise in plasma lipids (Matsumoto *et al*., 2006). These beneficial properties are considered to be related to the various antioxidants, including vitamins, phenolic compounds and carotenoids contained in the fruit. The aim of this study was conducted to investigate the *in vitro* cytotoxicity of the methanol 80% extract of *D. kaki* fruits in Vero cells, anti-HSV-1 and antibacterial activities and also the bioactive constituents.

Material and methods

General experimental procedures

Experimental

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.

Plant identification and collection

Ripe fruits 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 phenol compounds

The fruits (700 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 (38 g) was defatted with n-hexane and the sugars were precipitated by ethyl alcohol absolute and the residue of the extract (26 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 (1.45 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 (1.2 g) 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 (940 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 (1.9 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 further 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 were 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).

Cytotoxicity assay

Vero cells (epithelial cells from kidney of *Cercopithecus aethiops*) were cultured in 96-well microplates, and the monolayers were incubated for 72 h at 37°C and 5% CO_2 with DMEM containing 5% fetal bovine serum (FBS) and 1% dimethyl sulfoxide (DMSO), penicillin G (100 IU/ml), enrofloxacin (10 µg/ml) and amphotericin B (1.25 µg/ml) with 2-fold serial dilutions of the extract at different concentrations, ranging from from 3.8 to 1900 µg/ml of *D. kaki* fruits methanol extract. For this, 3 repetitions of 8 wells were used for the evaluation of extract dilution. The *in vitro* toxicity of methanol 80% of *D. kaki* fruits extract which was sterilized by filtration

through PVDF membranes (pore size 0.22 µm), was determined by quantifying the viable cells using 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which is converted into a purple formazan by mitochondrial dehydrogenases (Denizot and Lang, 1986). Fifty percent cytotoxic concentration (CC_{50}) was defined as the extract concentration which could reduce by 50% the number of viable cells, when compared with a control without it, and it was calculated by regression analysis of the dose-response curves.

Antiviral assay

The screening of the antiviral activity of *D. kaki* fruits methanol extract against HSV-1 was carried out on confluent monolayers of Vero cells in 96-well microplates by adding the highest non-toxic concentration determined by the MTT method (1900 µg/mL) during the HSV-1 ($100xTCID_{50}$) adsorption step and after adsorption in the same concentrations within the maintenance medium (DMEM with 5% FBS and 1% DMSO added by antibiotics and antifungal). The viral replication was performed for 72h at 37°C and 5% CO_2 incubation. The inhibition of the HSV-1 replication was related to the absence of any viral induced cytopathic effect at the highest non-toxic concentration of the extract, evaluated by observation of the monolayers under microscope, when compared with a control without it. Thus this test aimed to verify a decrease in the virus titer obtained by the endpoint titration method (Reed and Müench 1938) due to the plant extract.

Antibacterial assay

The antibacterial activity of *D. kaki* fruits methanol extract was evaluated against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) strains by the disk-diffusion method. Briefly, sterilized filter paper disks of 6 mm diameter were impregnated with 10 µL of the control substances (1% DMSO, 0.05% enrofloxacin or 20% chlorhexidine) or with extract solution (1900 µg/mL in water) previously sterilized by filtration on polyvinylidene difluoride membranes with pore diameter of 0.22 µm. The impregnated disks (n=3) containing 19 µg of the extract were positioned on the top of the Petri dishes containing 25 mL of Mueller-Hinton Agar previously seeded with a bacterial suspension adjusted to the 0.5 degree of the McFarland turbidity scale, which corresponds to a concentration of $1.5x10^8$ CFU/mL. The plates were incubated at 35°C for 24 h and after that, the diameter of the zone of inhibition of the bacterial growth around the discs were determined.

Statistical analysis: All biological experiments were statistically expressed as mean ± standard deviation, and analyzed by Student´s *t* test with $P<0.01$. Variables exceeding the upper quantification limit were considered statistically significant.

Results and Discussion

The present investigation evaluated cytotoxicity, antiviral and antibacterial activities of *D. kaki* fruits methanol extract *in vitro,* determined the main phytoconstituents of the extract which are carbohydrates, tannins, flavonoids and triterpenes and also detected the bioactive phytoconstituents of *D. kaki* fruits methanol extract which are gallic, ellagic, quercetin, myricetin, quercetin 3-*O*-*β*-glucoside, myricetin 3-O-α-rhamnoside and myricetin 3-*O*-*β*-glucuronide.

Evaluation of cytotoxicity, antiviral and antibacterial activities

The methanol 80% extract from *Diospyros kaki* fruits showed a lack of cytotoxicity in Vero cells up to 1900 µg/mL, which was the highest tested concentration (Fig. 1), presenting CC_{50} > 1900 µg/mL by the MTT method. This low toxicity value observed here is in agreement with other one already reported for the condensed tannins from *Diospyros kaki* fruits (Ueda et al., 2013), which did not decreased the viability of numerous cell lines such as FL cells, Vero cells, MA104 cells, RAW264.7 cells, LLC-MK2 cells and MDCK cells at 500 µg/mL.

Using this highest non-toxic concentration (1900 µg/mL) as a upper limit to evaluate its anti-HSV-1 activity potential, it was found that the extract did not inhibit entirely the development of the HSV-1 induced cytopathic effect (rounded cells), but the monolayers infected with virus and incubated with extract had the extension of this cytopathic effect decreased, as observed in Fig. 2. This effect could not be quantitatively measured by the method employed here to evaluate the antiviral activity. It was reported an anti-HSV-1 activity for the extract obtained from green persimmon fruits (Ueda et al., 2013), which was attributed to its high percentage of tannins. In a similar way, this tannin-rich extract suppressed significantly the virus titer (from $TCID_{50}$ ~ 6.5 to a $TCID_{50}$ ~ 1.5) at 5000 µg/mL but did not inhibit entirely the HSV-1 replication. Taking into account that acyclovir had an EC_{50}=20 $\pm$ 1 µg/mL, the methanol extract from *D. kaki* fruits had a much lower antiviral activity.

Table 1. Phytochemical Analysis of *Diospyros kaki* fruits 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 constituents, (-) absence of constituents

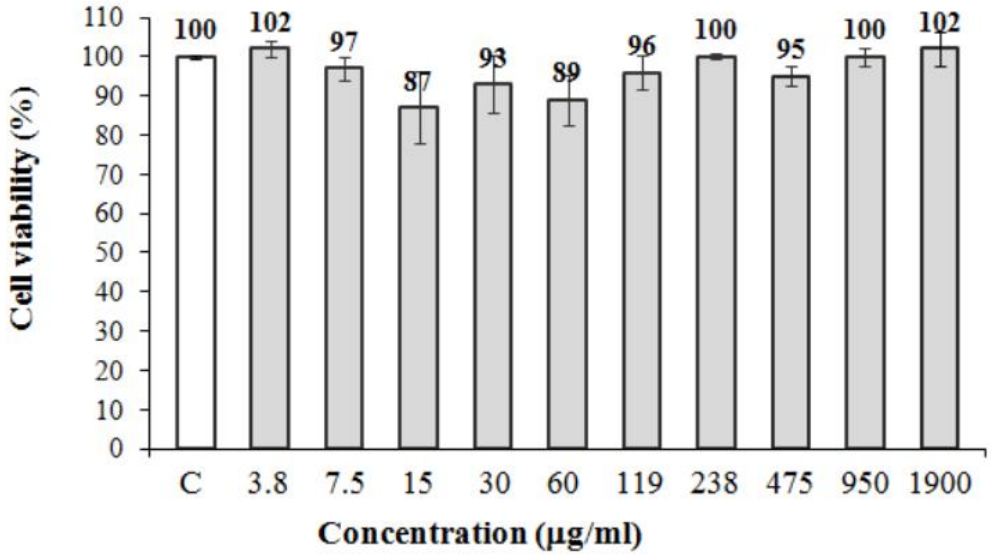


Figure 1. Evaluation of cytotoxicity of *D. kaki* fruits methanol extract in Vero cells after 72h of incubation at 37°C and 5% CO_2 by the MTT assay. Control: without extract treatment. *Bars* represent means, with *vertical lines* indicating standard deviations, $n = 3$, $^*P < 0.01$.

Figure 2. Screening of the anti-HSV-1 activity of *D. kaki* fruits methanol extract in Vero cells after 72h of incubation at 37°C and 5% CO_2 in 96-well microplates. A: negative control, DMEM 5% FBS; B: positive control, 100x$TCID_{50}$ of HSV-1; C: 100x$TCID_{50}$ of HSV-1 + methanol 80% extract of *D. kaki* fruits at 1900 µg/mL. Magnification of 200x.

The methanol 80% extract of *D. kaki* fruits at 1900 µg/mL did not inhibit the growth of *S. aureus*, *S. epidermidis*, *P. aeruginosa and E. coli* bacteria, despite the inhibitory activities of the 20% clorexidine and 0.05% enrofloxacin controls, both of which promoted a development of a clear zone of growth inhibition by the disk-diffusion method (Figure 3). Working with a catechol isolated from the methanol extract from the roots of *D. kaki*, and after that with catechol derivatives, Jeong *et al.* (2009) reported antibacterial activities against *Bifidobacterium breve*, *B. longum*, *Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli*, and *Lactobacillus casei*. The lack of activity observed here for the methanol extract of the fruits against *E. coli* could be probably related to differences in the chemical composition of the extracts obtained from different parts of the plant.

Phytochemical analysis

Phytochemical analysis of methanol extract of *D. kaki* fruits revealed that it contained carbohydrates, tannins, flavonoids and triterpenes (Table 1). Chromatographic separation and purification of methanol extract of *D. kaki* fruits 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 4). Their structures were elucidated on the basis of UV, ^{1}H-NMR, ^{13}C-NMR and MS analyses.

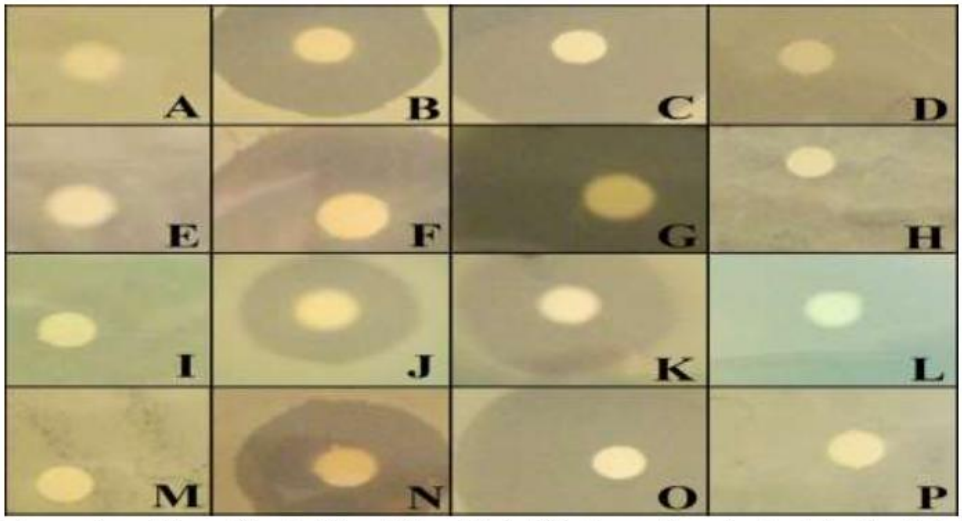
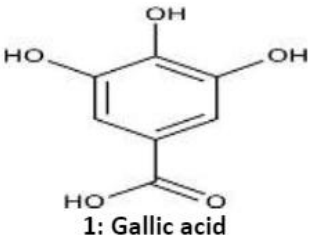
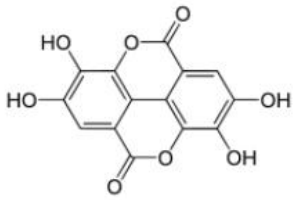


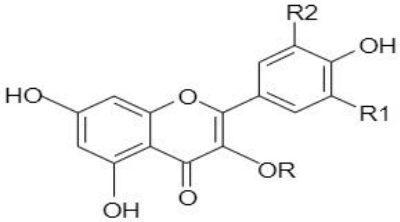
Figure 3. Screening of the antibacterial activity of *D. kaki* fruits methanol extract by the disk-diffusion method after 24h of incubation at 37°C. A: *S. aureus* and water; B: *S. aureus* and 20% chlorhexidine; C: *S. aureus* and 0.05% enrofloxacin; D: *S. aureus* and extract at 1900 µg/mL; E: *S. epidermidis* and water; F: *S. epidermidis* and 20% chlorhexidine; G: *S. epidermidis* and 0.05% enrofloxacin; H: *S. epidermidis* and extract at 1900 µg/mL; I: *P. aeruginosa* and water; J: *P. aeruginosa* and 20% chlorhexidine; K: *P. aeruginosa* and 0.05% enrofloxacin; L: *P. aeruginosa* and extract at 1900 µg/mL; M: *E. coli* and water; N: *E. coli* and 20% chlorhexidine; O: *E. coli* and 0.05% enrofloxacin; P: *E. coli* and extract at 1900 µg/mL.



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 4. Chemical structures of compounds isolated from *D. kaki* fruits methanol extract

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 (5): 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* fruits 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 (Gohar *et al.*, 2003). 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 agrrement with the published literature (Naira and Karvekar,

2010). 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 Manguro *et al.*, 2005. 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*. (2007). 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 Manguro *et al.*, 2005.

Conclusion

The methanol extract of the fruits of *D. kaki* presented a low cytotoxicity and a weak anti-HSV-1 activity at the highest non-toxic concentration observed in Vero cells and did not show antibacterial activities against *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli*. This indicates that purified fractions of this extract should be considered as candidates to re-evaluate the anti-HSV-1 activity in the future. The main phytoconstituents isolated from the extract are gallic, ellagic, quercetin, myricetin, quercetin 3-*O*-*β*-glucoside, myricetin 3-O-α-rhamnoside and myricetin 3-*O*-*β*-glucuronide.

Conflict of interest

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

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