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A Parasitological Investigation of the Effects Diospyros Kaki Leaves Against Schistosoma Mansoni in Vivo

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In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract
Because of the great need to develop new antischistosomal agents, trials were designed to test the potency of traditional medicinal plants for treating schistosomiasis. That is why the aim of this study was to assess the schistosomicidal effect of the aqueous methanolic extract of Diospyros kaki leaves in-vivo regarding disease progression in a comparative experimental study to praziquantel (PZQ). Phytochemical investigation of Diospyros kaki leaves extract resulted in eleven phenolic and flavonoid compounds. Structures of these compounds were elucidated by UV and 1D⁄2D 1H⁄13C NMR spectroscopy and compared with the literature data. Parasitological parameters (total worm burden, tissue egg load, and oogram pattern) for the infected and treated mice groups had been measured per gram of liver or intestinal tissue. A histopathological examination of the liver granuloma took place as well. Diospyros kaki extract caused a substantial decrease in the number of worms and eggs percentage; the extract also decreased worm and egg burdens moderately. The results demonstrated the leaves extract as a promising antischistosomal activity due to the parasitological and histopathological changes induced, besides it highlights the importance of its polyphenolic constituents.
Keywords: Diospyros kaki; leaves; flavonoids constituents; antischistosomal activity

1. Introduction

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity **[1]**. Schistosomiasis is a parasitic disease that has infected Egyptians since the pharaohs' period. Schistosome eggs embedded in the host liver, which trigger an immune response and cause hepatic granuloma and fibrosis in some patients **[2]**, causes the disease. There are limited options available for the chemotherapeutic treatment of schistosomiasis with the drug of choice still being praziquantel **[3]**. However, after some 30 years of praziquantel usage, a decreased susceptibility to the drug and the emergence of drug-resistant strains of schistosomiases have been observed in several countries **[4-6].** Therefore, search for alternative antischistosomal drugs is an urgent need.

Diospyros kaki belongs to the family of Ebenaceae, The Ebenaceae (Ebony family) are pantropical in distribution and encompass the genera *Diospyros* and *Euclea* with ca. 500 -600 species. Only a few of them extend into temperate zones. Main centers of diversity are in SE-Asia, Madagascar, tropical Africa and South America. The last comprehensive revision of the family dates back to the 19th Century **[7]**. The genus holds more than 2000 species of commercial interest applied to different means such as logging (*Diospyros ebenum*), landscaping (*Diospyros inconstans* and *Diospyros rhombifolia),* tannin extraction (*Diospyros oleifera*) and fruit production (*Diospyros kaki*). The naming of this species reflects its colloquial name of "Food of the Gods" (*Dios*=God, *Pyros*=food) **[8,9].**

The plant showed various biological activities such as antioxidant, anti-diabetes, atherosclerosis, anti-cancer, antifungal potential and multi drug resistance reversal properties **[10-14].** It was reported that the plant contains several phytochemicals like polyphenols, terpenoids, steroids, phenolicacids, carotenoid minerals, and dietary fiber **[15].**

Thus, the present study aims to evaluate the antischistosomal activity of aqueous methanolic extract from *Diospyros kaki* leaves on *schistosoma mansoni* infected mice *in vivo* in a biological mouse model. The study intend to explore the parasitological and histopathological impact of the used natural remedies in different developing stages. Moreover, the extract was subjected to phytochemical investigations using different chromatographic techniques to afford eleven

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compounds (Gallic acid, Catechin, 7-*O*-galloyl-Catechin**,** Quercetin-3-*O*-rutinoside, Myricetin-3-*O*-β-D-glucopyranside, Kaemferol -3-*O*-(3"galloyl) - β-D-glucopyranoside, Quercetin 3-O-β-D-glucopyranoside, Kaemferol -3-*O*- β-D-glucopyranoside**,** Myricetin, Quercetin, Kaemferol).

2. Experimental

2.1. Plant Material

Diospyros kaki (leaves) were collected from EL Monofyia, Egypt during April 2018 (flowering date). The samples were separately air-dried in shed, powdered and kept in tightly sealed round flasks and stored for biological and phytochemical studies.

Identification of the plant was confirmed by Botany Department, Faculty of Science, Cairo University **[16]**. Voucher specimens (T19) were deposited in the Herbarium of the National Research Centre, Dokki, Cairo, Egypt.

2.2. General methods and drugs

1H (400 MHz) and 13C (100 MHz) – NMR spectra were recorded on a Bruker 400

Spectrometer; the chemical shifts were recorded in DMSO-d6 and are given in ppm values. UV spectra were measured on Shimadzu spectrophotometer model UV-240; CC: was performed using Polyamide 6S and Sephadex LH-20; PC: was carried out on Whatman No.1and 3MM using solvent systems (1) BAW (n- BuOH: HOAc: H2O, 4:1:5); (2) H2O; (3) 15 %AcOH (AcOH: H2O, 15: 85) and visualized under UV light using $AlCl_3$ and NA as spraying reagents; Aniline hydrogen phthalate was used as specific reagent for sugar analysis. Praziquantel (PZQ) was obtained as tablets (Distocide, Egyptian International Pharmaceutical Industries Company, EIPICO) were freshly suspended in 2% Cremphore-EL (Sigma-Aldrich, St Louis, MO, USA) before use.

2.3. Extraction and isolation

1.5 Kg of the air-dried leaves were reduced to a coarse powder and defecated by petroleum ether then successively extracted by maceration with 70% methanol (5x2L) at room temperature till exhaustion. The filtrates were collected, dried under vacuum at 40°C to give 300 gm net weight (w/w). The crude extract was further fractionated by successive extraction by maceration of methylene chloride (2x2L) and then *n*-butanol (5x2L) at room temperature. The filtrates were collected, dried under vacuum at 40 °C to give 50 gm net weight (w/w) with methylene chloride and 70 gm net weight (w/w) with *n*-butanol. Each extract was subjected to TDPC in the solvent system BAW and 15% AcOH whereby both extracts revealed the presence of several polyphenolic spots. Fifty grams of the methylene chloride extract was chromatographed on a Sephadex LH-20 column using *n*-hexane followed by *n*-hexane/methanol mixtures as eluent to give two fractions. Fractions were further separated and purified using Whatman 3MM papers and Sephadex LH-20 column to yield the pure compounds **K_1**, **K_2**, **K_3**.

2.3.1. Investigation of the butanol extract

70 grams of the butanol extract was applied on a Sephadex LH-20 column and eluted by n-butanol-water saturated mixture. The obtained four fractions (I-IV) (500 ml of each fraction) were subjected to paper or column chromatography to obtain the purified eleven compounds.

Compounds **K_4, K_5 & K_6** isolated from Fraction **II** were obtained by applying on Sephadex LH-20 CC using MeOH: water (1:1) for elution at a very low rate.
From fraction **III** Compounds **K_7 & K_8** were further purified on Sephadex LH-20 CC (n-butanol-water saturated for elution) gave one sub fraction with two major spots which were separated by preparative paper chromatography using Butanol-Acetic acid-water (BAW 4:1:5) as eluent to give the two compounds in a purified form.

Compounds **K_9, K_{10} & K_{11}** were isolated from Fraction IV by further purification on sephadex CC LH-20 using MeOH: water (8:2) at a very low rate. Among all the above known compounds two rare compounds will be thoroughly described.

2.3.2. Characterization of some rare isolated natural compounds:

2.3.2.1. Myricetin-3-O-β-D-glucopyranside, (K_5).

K_5 was isolated as a yellow amorphous powder, gave a brown color under UV light with Rf values (x100):47(BAW), 19 (15% AcOH), 05(H_2O); UV Spectral Data λ_{max}(nm): MeOH: 258, 365, (a) + NaOMe: 266,395, (a) + NaOAc (b): 266, 340, (b) +H_3BO_3: 257, 305sh, 395, (a) +$AlCl_3$ (c):269, 310sh, 405, (c) +HCl: 270, 360sh,400; ^{1}H NMR (DMSO-d_6) spectral data: δ (ppm)7.28 (s, H-2' and H-6'), 6.44 (d, *J* =1.8 Hz, H-8), 6.25 (d, *J* = 1.8 Hz, H-6), 5.40 (d, *J* = 7.77 Hz, H-1"), 3.36-3.73 (m, rest of glucose protons) ; ^{13}C NMR spectral data (DMSO-d_6) spectral data: δ (ppm) at 156.47 (C-2), 133.99 (C-3), 177.64 (C-4), 161.44 (C-5), 98.91 (C-6),164.36 (C-7), 93.62 (C-8), 156.36 (C-9),104.14 (C-10), 120.23 (C-1'), 108.80 (C2'), 145.58 (C-3'), 136.89 (C-4'), 145.58 (C-5'), 108.80 (C-6'); 3-O-β-D- glucopyranoside moiety: δ at 102.30 (C-1"), 71.45 (C-2"), 73.50 (C-3"), 68.25 (C-4"), 76.11 (C-5") and 60.64 (C-6").

2.3.2.2. Kampferol-3-O-β-D-(3''-galloyl) glucopyranoside, (K_6)

K_6 was isolated as a light yellow amorphous powder, gave a brown color under UV light with Rf values (x100): 34 (BAW), 30 (15% AcOH); MeOH (a): 266,356, (a)+ NaOMe: 275 324sh, 395, (a)+ NaOAc (b): 274, 383, (b) +H_3BO_3: 267, 355, (a)+$AlCl_3$) (c): 276, 399, (c)+ HCl: 274, 396; ^{1}H NMR (DMSO-d6) spectral data: δ (ppm) 8.029 (d,*J*=8.4Hz, H-2'and H-6'), 7.035 (s,galloyl H-2 and H-6), 6.922 (d,*J*=8.4 Hz, H-3'and H-5'), 6.411 (d,*J*=1.8Hz, H-8), 6.18

(d,*J*=1.8Hz,H-6), 5.82 (d,*J*=8.4Hz,H-1"), 4.964 ppm (*J*=8.8 Hz, H-3"), 3.17-3.78 (m, rest of glucoseprotons; ^{13}C NMR spectral data (DMSO-d_6) spectral data: δ (ppm) 156.81 (C-2), 132.87 (C-3), 177.46 (C-4), 160.60 (C-5), 98.60(C-6), 115.67 (C-7), 94.21 (C-8), 156.46 (C-9), 104.18 (C-10), 121.11 (C-1'), 131.28 (C-2' & 6'), 115.67 (C-3'&5'), 165.81 (C-4'). 3- O-β-D-glucopyranoside moiety: δ (ppm) at 99.37 (C-1"), 74.70 (C-2"), 78.18 (C-3"), 70.62(C-4"), 78.18(C-5"), 61.05(C-6"). galloylmoiety: δ (ppm) at 115.67 (C-1), 145.99 (C-3 &5), 132.87 (C-4), 109.41(C-2&6), 165.65 (C=O).

2.3.3. Animals

Thirty Male Swiss albino mice (CD-1) were obtained from SBSC of TBRI, Giza, Egypt, weighing 18–20 g each, were housed under environmentally controlled room temperature of 20–22 °C, a 12 h light/dark cycle and 50-60% humidity with access to food and water ad libitum throughout the acclimatization and experimental cycles. Mice were infected with S. mansoni cercariae (provided by SBSC) using body immersion **[17]** through exposure to 80 ± 10 cercariae/mouse. The entire experimental animal was conducted in accordance with the Laboratory Animals Guide and approved by the Institutional Review Board of TBRI. The cercarial suspension (0.1 ml) will be gently mixed, stained with picric acid solution and counted.

2.3.4. Experimental design

In 2 percent of Cremophore-EL (SigmaAldrich, St Louis, MO, USA) *Diospyros kaki* extract and PZQ were freshly suspended. Infected mice were divided into 3 groups, each composed of 10 mice at the beginning of the experiment. Plant extract will be orally administered for 5 consecutive days at the 7th week post infection. Group 1: Control group for untreated infected mice (IC). Group 2: Infected mice were treated with PZQ in a dose of 200 mg/kg. Group 3: Infected mice were treated with (DK) in a dose of 200 mg/kg.

2.3.5. Assessment of parasitological criteria of cure

All mice were sacrificed and perfused fourteen days after treatment, and the number of worms recovered (worm burden) was quantified and sexed **[18, 19]**. We calculated the number of eggs per gram of liver or intestinal tissue **[20]**. The percentage of egg developmental stages (oogram pattern) has been studied **[21]**, identifying and counting eggs at different maturity stages (from I to IV). Mature eggs and dead eggs (granular, dark, and semi-transparent) were also counted in three intestinal fragments and the mean number was calculated for each stage.

2.3.5. Histological examination

For light microscopy inspection, liver specimens were fixed in 10% formalin, processed to paraffin blocks, sectioned (4 µm thick) and coated with hematoxylin/eosin (H&E). The presence of hepatic granulomas and associated histological changes were examined for the liver sections. Only transverse sections (250 µm apart) of H&E stained granulomas showing a central, viable egg, were considered for measurements. Granuloma diameter (30/mouse), composition and egg viability were investigated.

2.3.6. Statistical analysis

The percentage reduction of worm/egg burden in each treated group was calculated according to the owing equation: % reduction = [(No. of worms/eggs in control group) - (No. of worms/eggs in treated group)] / (No. of worms/eggs in control group) × 100. Results were expressed as mean ± SEM. A two-tailed Student's t-test was used to detect the significance of difference between the means of different groups. Results were considered significant when the P value is <0.05.

3. Results & Discussion

The phytochemical investigation of the aqueous methanolic extract of *Diospyros kaki* leaves extract afforded eleven compounds; (**K_1**), gallic acid, (**K_2**), catechin, (**K_3**), 7-O-galloyl catechin, (**K_4**), quercetin-3-O-rutinoside, (**K_5**), myricetin-3-O-β-D-glucopyranside, (**K_6**), kaemferol-3-O-(3"galloyl)-β-D-glucopyranoside, (**K_7**), quercetin-3-O-β-D-glucopyranoside, (**K_8**), kaemferol-3-O-β-D-glucopyranoside, (**K_9**), myricetin, (**K_{10}**), quercetin and (**K_{11}**), kaemferol.

The use of extract of *Diospyros kaki* leaves showed a clear effect as an antidote to intestinal schistosomiasis and revealed significant reduction in both the hepatic and intestinal tissue egg loads upon administration of extracts in comparison with infected untreated control. The results of *Diospyros kaki* leaves extract was significant as anti-schistosomiasis whereby it led to a decrease in the burden of worms by (58.9% worm reduction). The mean total worms and couples in the Liver, Porto-mesenteric were 1.20 ± 0.37 and 0.0 ± 0.0, 0.0 ± 0.0 respectively (PZQ group). In the extract group, the mean total worms and couples in the Liver, porto-mesenteric were 13.00 ± 1.94 and 0.80 ± 0.58, 4.60 ± 0.8 respectively. Comparing these data with the infected untreated control group (31.6 ± 1.43 mean total worms and 4.80 ± 0.37, 9.00 ± 0.44 mean total couples in the Liver and porto-mesenteric. The difference was statistically significant at $p < 0.01$ (Table2) Reduction in the mean total tissue egg load was still evident in mice given *D. Kaki* extract (5.653±0.733and 3.570±0.558).The difference was statistically significant from infected untreated control mice at $p < 0.01$ (Table 2). All stages of ova development were found in control infected untreated mice. However, mice given praziquantel at 200 mg/kg to *S. mansoni*-infected mice for five consecutive days, revealed disappearance of the immature stages of ova development with rise in the dead ova in the oogram. The difference was statistically significant from infected untreated control mice at $p < 0.001$ (Tables 1 and 2). Significant

decrease in the percentage of total immature eggs for plant extract of *KaKi* (P<0.01; 11.0%) and a significant increase in the percentage of mature (P<0.01; 74.0%) and dead eggs (P<0.01; 15.0%) compared with the infected untreated control. These results also showed decrease in the egg load in liver and intestinal tissues by 93.89% and 96.5%, respectively and an increase in the percentage of dead eggs. The decrease in the total stages of immature eggs also led to the worms not mating. These results are in consistence with **Pellegrino *et al***. [22] who stated that if viable ova of any of immature stages are disappearance after specific chemotherapy in experimental schistosomiasis, the drug is considered active.

Moreover, histopathological examination of liver sections from different examined groups (Figure 1) showed a significant reduction in the number of egg granulomas in the PZQ treated group. However, no significant reduction in granulomas count were detected between the control and the treated plant extract. For the granuloma diameter; the largest mean granuloma diameter recorded in the untreated control infected mice was (418.3±64.52 µm), while the granuloma size in the PZQ (287.18±55.14 µm) and plant extract (275.88±62.54 µm) compared to the control group gave significant reduction (table 3). In addition, most of egg granulomas of different groups were fibrocellular with mild increase in the number of cellular granulomas in the control group compared to the other treated groups. Considering the measurement of liver fibrosis in tissue sections, it was found that the PZQ treated group showed significant reduction in fibrosis (measured as area/LPF) compared to the control group. The extract treated group showed less significant reduction in fibrosis compared to the control group.

Thus, the results confirmed that the extract *Diospyros kaki* leaves was active and promising as anti-schistosomiasis, this may be attributed due to the chemical content of polyphenolic compounds.

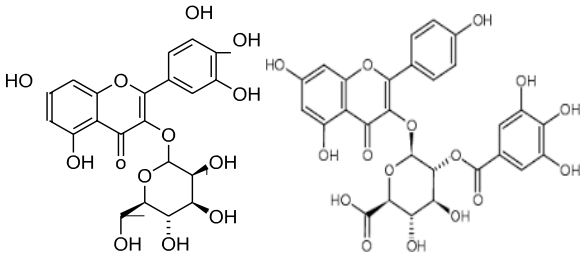

Fig. 1: Structure of the rare natural isolated compounds of *Diospyors kaki*

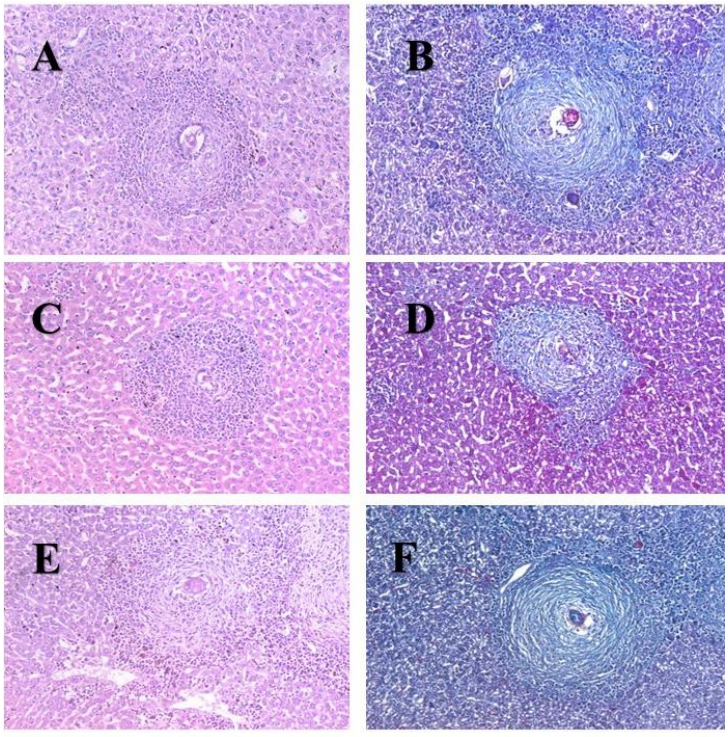

Fig. 2: Section in mouse liver of the different groups tested in case of *Diospyors kaki*

(A) Control group showing an egg granuloma with central intact ova showing fibrocellular egg granuloma with central ova and peripheral condensation of neutrophils and concentric layers of fibrous tissue (H&E stain, X200). **(B)** (MT stain, X200). **(C)**: PZQ treated group showing a cellular egg granuloma consists mostly of neutrophils and mononuclear cells (H&E stain, X200). **(D)**: PZQ treated group showing a fibrocellular egg granuloma (MT stain, X200). **(E)**: *Diospyros kaki* treated group showing an egg granulomas with central intact ova and peripheral mono and polymorphnuclear inflammatory cells (H&E stain, X200). **(F)**: *Diospyros kaki* treated group showing an intact ova surrounded by fibrocellular tissue reaction (green) (MT stain, X200).

Table (1): Effect of PZQ and Diospyros kaki leaves extract (200 mg/kg/day for 5 days) on worm load and sex in S. mansoni-infected groups

Groups	Worm load and Sex							
	Liver			Portomesenteric			Total worms	Percent worm reduction%
	Total males	Total females	Total Couples	Total Males	Total Females	Total couples		
Infected control	1.40±0.24	1.20±0.20	4.80±0.37	1.00±0.44	0.40±0.24	9.00±0.44	31.60±1.43	_
PZQ	1.20±0.37	0.00±0.00	0.00±0.00***	0.00±0.00	0.00±0.00	0.00±0.00***	1.20±0.37***	96.20%
Diospyros kaki leaves	1.40±0.67	0.80±0.37	0.80±0.58**	0.00±0.00	0.00±0.00	4.60±0.81**	13.00±1.94**	58.90%

PZQ and *Diospyros kaki* leaves extract were administered orally 7 weeks post *S. mansoni* infection in doses of 200 mg/kg/day for 5 days. Results are presented as mean ± SEM.
** Significant difference from infected control at P<0.01.
*** Significant difference from infected control at P<0.001.

Table (2): Effect of PZQ and *Diospyros kaki* leaves extract (200 mg/kg/day for 5 days) on tissue egg load and percentage egg developmental stages in *S.mansoni*-infected mice.

Groups	Tissue egg load		% of egg developmental stages		
	Hepatic count x10^3	Intestinal count x10^3	% immature	% mature	% dead
Infected control	14551.41±1659.2	20818.29±2024.6	58.8±3.92	34.20±3.62	7.00±0.54
PZQ	5.653±0.733**	3.570±0.558**	0.00±0.00***	4.00±2.44**	96.0±2.44**
Diospyros kaki	7.9038±1.12**	6.3026±1.10**	11.00±5.09**	74.00±2.44**	15.00±3.16**

PZQ and *Diospyros kaki* leaves extract were administered orally 7 weeks post *S. mansoni* infection in doses of 200 mg/kg/day for 5 days. Results are presented as mean ± SEM.
** Significant difference from infected control at P<0.01.
*** Significant difference from infected control at P<0.001.

Table (3): Hepatic granuloma size in *S. mansoni* infected mice treated with PZQ and *Diospyros kaki* leave extract (200 mg/kg/day for 5 days) versus untreated control animals.

Group	**Granuloma Egg**			
	Number (N/5 HPF)	**Type**	**Diameter (µm)**	**Fibrosis (area µm^2/LPF)**
Infected control	29	Fibrocellular 90%/Cellular 10%	418.3±64.52	167.83±12.2
PZQ	13	Fibrocellular 95%/Cellular 5%	287.18±55.14**	130.47±52.55**
Diospyros Kaki	20	Fibrocellular 90%/Cellular 10%	275.88±62.54**	160.55±9.92

**: Significant difference with the control group (p<0.01)
*: Significant difference with the control group (p<0.05)

4. Conclusion

The results confirmed that the extracts of *Diospyros kaki* leaves were active and promising as significantly active anti-schistosomiasis. This may be attributed due to the chemical content of polyphenolic compounds.

5. Conflicts of interest

There are no conflicts to declare.

6. References

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