Vol. 12/2 (2012) 129-134

JOURNAL OF NATURAL REMEDIES

Antiprotozoal and anthelmintic naphthoquinones from three unexplored species of *Diospyros*

M.J Amar Dev[1], N.Rajarajeshwari[1]*, S.Ganapaty[2], B. Parixit[2], R. Brun[3]

1.V.V.Puram Institute of Pharmaceutical Sciences, KIMS campus, Bangalore- 590 070, India
2. University. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam- 530 003, India
3. Swiss Tropical Institutes 57, PO Box, CH-4002 Basel, Swirtzerland

Abstract

The genus *Diospyros* is a rich source for napthols and naphthoquinones. During the study, we have chemically examined the roots of three unexplored species of *Diospyros, D. oocarpa, D. nigresence* and *D. candolleana* for their chemical profile and biological activities. The plant materials on conventional extractions with organic solvents and sequential chromatography yielded seven naphthoquinone derivatves, habibone [1], 8'-hydroxyisodiospyrin [2], diospyrin [3], 4-hydroxy-5methoxy-2-naphthaldehyde [4], 5-hydroxy-4-methoxy-2-naphthaldehyde [5], 4-hydroxy-3,5-dimethoxy-2-naphthaldehyde [6], and 2-methyl anthraquinone [7] These isolates were characterized by spectral data (2D NMR, ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY). In addition, these compounds were evaluated for their potential to inhibit parasitic protozoa belonging to the genera *Trypanosoma, Leishmania* and *Plasmodium* using *in vitro* antiprotozoal assay and anthelmintic activity against adult earth worms, *Pheritima posthuma.* The study showed that the isolates enjoy significant anthelmintic and antiprotozoal activity and supports its use in folk medicine.

***Keywords*:** anthelmintic, antiprotozoal, naphthoquinones, *D. oocarpa, D. nigresence,* and *D. candolleana*

1. Introduction

The genus *Diospyros*, a member of Ebenaceae family is represented by 450 species and nearly 90 species are distributed in India. These are mostly trees and rarely shrubs in which fewof thespecies are endemic. Phytochemical investigation of more than 130 species led to the isolation of variety of compounds, the majority of which are naphthoquinones[1]. This genus is so interesting that almost all plants were found to possess potential biological properties and used for distinctive purposes in folkloric medicine as anthelmintic, antihypertensive, antidiarrhoeal, antitubercular, anti-inflammatory and antiprotozoal [2,3].

In continuation of our investigations [4,6] on Indian *Diospyros* species, we have studied three unexplored species, hitherto unreported, for their chemical profile and folklore claimed biological activities to give scientific validation. In this study,

* Corresponding author
Email: rajuvips@gmail.com

we have focused mainly on the search for metabolites with antiprotozoal and anthelmintic activity.

2. Materials and Methods

2.1 Collection and preparation of plants

The root material of *D.oocarpa* were collected at Amboli ghat and *D. nigriscence* from Tillari ghat of Maharastra State . The roots of *D. condolleana* were collected at Ram ghat, Goa, India. These were identified by Prof. Jadav, Taxanomist, Dept of Botany, Shivaji University, Kolhapur, Maharashtra, India and voucher specimens (SGDO-1, SGDN-2 and SGDC-3) have been deposited at the herbarium, College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India.

2.2 Extraction and Isolation

The plant materials were dried in shade at ambient temperature and finely powered in Willy mill and exhaustively extracted with chloroform. The extracts so obtained, were then concentrated under *vacuum* to get the corresponding residues. These, on column chromatography over silica gel (Acme 100-200 mesh) and elution with solvents of increasing polarity afforded seven compounds.

The root material of *D. oocarpa gave* habibone, 8'-hydroxyisodiospyrin, diospyrin and *D. nigresence* yielded diospyrin, 4-hydroxy-5-methoxy-2-naphthaldehyde, 5-hydroxy-4-methoxy-2-naphthaldehyde and the root parts of *D. candolleana* delivered 5-hydroxy-4-methoxy-2-naphthaldehyde, 4-hydroxy-3,5-dimethoxy-2-naphthaldehyde,2-methyl anthraquinone and diospyrin.

Methods

Antiprotozoal assay

2.3 In vitro assay for Trypanosoma brucei rhodesiense

T. brucei rhodesiense STIB 900 strain and the standard drug melarsoprol (Arsobal) were used for the assay. Minimum Essential Medium (50μL) supplemented with 2-mercaptoethanol and 15% heat-activated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123μg/mL and then added to the wells. Then 10^4 bloodstream forms of *Trypanosoma b. rhodesiense* STIB 900 in 50μL were added to each well and the plate incubated at 37°C under a 5% CO_2 atmosphere for 72hours. 10μL of resazurin [7,8] solution (12.5mg resazurin dissolved in 100mL distilled water) was then added to each well and incubation continued for a further 2-4 hours. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536nm and emission wavelength of 588nm [9] (Raz *et al.*, 1997). Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic program Softmax Pro (Molecular Devices) which calculated IC_{50} values.

2.4 In vitro assay for Trypanosoma cruzi

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100μL RPMI 1640 medium with 10% FBS and 2mM L-glutamine. After 24 hours the medium was removed and replaced by 100ìL per well containing 5000 trypomostigote forms of *T. cruzi* (Tulhahuen strain C-2, C-4 containing the β-galactosidase (Lac Z) gene [8] (Weniger et al., 2006).. Forty-eight hours later the medium was removed from the wells and replaced by 100μL fresh medium with or without a serial drug dilution. Seven 3-fold dilutions were used covering a range from 90μg/mL to 0.123μg/mL. Each drug was tested in duplicate. After 96 hours of incubation the plates were inspected under an inverted microscope to assure growth

of the controls and sterility. Then the substrate CPRG/Nonidet (50µL) was added to all wells. A colour reaction developed within 2-6 hours and could be read photometrically at 540nm. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) and IC_{50} values calculated. Benznidazole was the standard drug used.

2.4 In vitro assay for Leishmania donovani (axenic amastigote assay)

50µL of culture medium, a 1:1 mixture of SM medium [10] and SDM-79 medium [11] at pH 5.4 supplemented with 10% heat-inactivated FBS, was added to each well of a 96-well microtiter plated (Costar, USA). Serial drug dilutions in duplicates were prepared covering a range from 30 to 0.041µg/mL. Then 10^5 axenically grown *Leishmania donavani* amastigotes (strain MHOM-ET/67/L82) in 50µL medium were added to each well and the plate incubated at 37ºC under a 5% CO_2 atmosphere for 72 hours. 10µL of resazurin solution [8] (12.5mg resazurin dissolved in 100mL distilled water) were added to each well and incubation continued for a further 2-4 hours. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536nm and emission wavelength of 588nm [9]. Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) and IC_{50} values calculated. Miltefosin (Zentaris GmbH, Germany) was used as a positive reference.

2.5 In vitro assay for Plasmodium falciparum

Antiplasmodial activity was determined using the KI strain of *P. falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [^{3}H]-hypoxanthine incorporation assay was used [12]. Briefly, infected human red blood cells in RPMI 1640 medium with 5% Albumax II were exposed to serial drug dilutions in micro titer plates. After 48hours of incubation at 37ºC in a reduced oxygen atmosphere, 0.5µCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated at 37ºC in a reduced oxygen atmosphere, 0.5µCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated for a further 24hours before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Beta plate TM liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves IC_{50} values were calculated. The IC_{50} values are the means of four values of two independent assays carried out in duplicate.

Anthelmintic activity

The anthelmintic activity was evaluated on adult earth worms [13,14] *Pheritima posthuma* owing to its anatomical and physiological resemblance with the round worm parasites of human beings [15,16,17]. Worms were collected from the water logged areas of forte lake, Belgaum, Karnataka, India. The method of Dash *et al.* was followed for the study [18.19]. The test samples of all the isolates were prepared at the concentration of 5 and 10 µg/ ml using chloroform. Six worms of approximately equal size were placed in 9 cm Petri dish containing above concentration of isolates, time for paralysis were notted and death of worms was recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50° C) [20].

3. Results and Discussion

The isolates of *Diospyros* (1-7) were studied by antiprotozoal activity in *in-vitro* methods on *Trypanosoma brucei rhodensiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*. In addition, the cytotoxicity of the test compounds was performed using rat skeletal myoblasts (L-6 cells), in order to establish if the *in vitro* activity of the metabolite was due to its general cytotoxic activity or if it possesses a selective activity against the *Leishmania* parasite.

In vitro biological activity tests (Table1) against parasitic protozoa were performed on seven isolates. Habibone (1) showed considerable activity against *Trypanosoma brucei rhodensiense* (strain STIB 900, stage trypomastigotes) with IC_{50} 0.25 and 0.34 μg/ml and with *T. cruzi* (strain Tulahuen C4, stage trypomastigotes) 1.71 and 4.05 μg/ml. Diospyrin (3) exhibited moderate activity against both *Trypanosoma brucei rhodensiense* and *T. scruzi* of IC_{50} 0.41 and 15.3 μg/ml. The two isomeric naphthaldehydes namely, 4-hydroxy-5methoxy-2-naphthaldehyde (4) and 5-hydroxy-4-methoxy-2-naphthaldehyde (5) showed low activity against *Leishmania donovani* (strain MHOM-ET-67, stage amastigotes) with IC_{50} of 26.7, 17.9 μg/ml, while the 4-hydroxy-3,5-dimethoxy-2-naphthaldehyde (6) was comparatively more active against *T. brucei*, *T. cruzi* and *L. donovani* with IC_{50} of 4.6, 2.85 and 9 μg/ml respectively.

Habibone (1) and 8'-Hydroxyisodiosprin (2) exhibited good activity against *Plasmodium falciparum* (strain K1 and NF54, stages IEF) with IC_{50} of 2.20, 3.06, 3.48 μg/ml respectively. Cytotoxicity studies towards rat skeletal myoblasts L-6 cells showed Habibone (1), 8'-Hydroxyisodiospyrin (2) and diospyrin (3) with IC_{50} of 1.93, 1.94, 3.63 and 2.39 μg/ml respectively. 2-methyl anthraquinone (7) exhibited low response to all parasitic protozoa.

The data for standard control drugs Melarsoprol, Benznidazole, Miltefosine, Chloroquine/ Artemisinin and Podophyllotoxin were included for comparison purposes. Of the three dimeric naphthoquinones, habibone (1) and diospyrin (3) were equally potent but possessed low *in vitro* activity that too, only against *T. cruzi*.

With regard to the anthelmintic activity (Table-2) of the compounds, 8'-hydroxyisodiospyrin (2), exhibited highly significant activity and isolates 4-hydroxy-5-methoxy-2-naphthaldehyde (4) and 4-Hydroxy-3,5-dimethoxy-2-naphthaldehyde (6) showed significant activity when compared with the standard at the dose level of 5 and 10 μg/ml. Infact, compound 2 showed better activity than the standard. This study suggests that these isolates of unexplored species of *diospyros* showed good activity. The experimental evidence obtained in laboratory model could provide rationale for the use of these isolates as antiprotozoal and anthelmintic by rural folk.

4. Acknowledgement

One of the authors (R.N) is grateful to ICMR New Delhi, for financial assistance ICMR Major research project. Grant No.21/12/17/09/HSR Dated:17/03/2011

Table 1: *In-Vitro* Antiprotozoal activity of the isolated compounds from three *Diospyros* species (DOR, DNR, DCR) (1-7)

Compounds (μg/mL)	IC_{50} values (μg/mL)			Cytotoxicity MIC	
	T.b. rhod	***T. cruzi***	***L. don. axen***	***P. falc*** K1	(L-6 Cells)
Standards	0.001	0.183	0.23	0.0916	0.006
1.	0.341	4.05	2.5	3.0683	1.945
2.	5.055	28.6	6.5	3.4878	3.631
3.	0.4195	15.3	3.3	2.194	2.393
4.	18.3	>30	26.7	>5	63.392
5.	10.8	>30	17.9	>5	63.834
6.	4.6	2.85	9	>5	20.603
7.	12.8	9.7	>30	>5	28.283

Standards used**:** For *T.b. rhod* (Melarsoprol), *T. cruzi* (Benznidazole), *L. don*. (Miltefosine), *P.falc*. (Chloroquine / Artemisinin) and L-6 cells (Podophyllotoxin)

Table 2: Effect of isolates of *D. oocarpa*, D. *nigrisence D. candolleana* species on *Pheritima posthuma*

SL.No.	Groups		Concentration μg/ ml	Time of Paralysis (P) and Death (D) of *Pheritima posthuma* in min. (Mean± SEM)	
				P	D
1.	Piperazine citrate		10 mg/ml	1.6 ± 0.06	41 ±0.006
2.	Chloroform		1 ml	> 90	> 90
3.	Habibone	DNR 2	5 μg/ ml	2.62±0.38	51±0.06
			10 μg/ ml	3.23±0.28	45±0.02
4.	8'Hydroxy	DNR 4	5 μg/ ml	1.53±0.44	33 ± 0.17
	isodiospyrin		10 μg/ ml	1.29±0.34	31±0.27
5.	Diospyrin	DOR 8	5 μg/ ml	2.28±0.52	55 ±0.91
			10 μg/ ml	2.00±0.17	49 ±0.11
6.	4-Hydroxy-5-methoxy-2-naphthaldehyde	DOR 3	5 μg/ ml	2.11±0.30	43±0.38
			10 μg/ ml	1.01±0.00	42±0.30
7.	5-Hydroxy-4-methoxy-2-naphthaldehyde	DOR 2	5 μg/ ml	4.62±0.19	58±0.29
			10 μg/ ml	3.71±0.88	49±0.79
8.	4-Hydroxy-3,5-dimethoxy -2-naphthaldehyde	DOR 4	5 μg/ ml	1.90±0.36	41±0.30
			10 μg/ ml	1.20±0.12	40.±0.56
9.	2-Methyl anthraquinone**.**	DCR 2	5 μg/ ml	6. 91±0.19	58± 0.65
			10 μg/ ml	5.71± 0.30	53± 0.54

References

1. Mallavadhani UV, Panda AK, Rao YR, (1998) *Phytochemistry*. 49(4): 901-951.

2. Adzu B, Amos S, Dzarma S, Muazzam I, Gamaniel KS. (2003) *J.Ethnopharmacol*. 82: 191-195.

3. Bouquet A, Debray M (1974): *Plantes Medicinales de la cote D'lvoire*. ORSTOM, Paris; 80-82.

4. Ganapaty S, Steve Thomas P, Karagianis G, Waterman PG, Brun R. (2006): *Phytochemistry*. 67: 1950-1956.

5. Ganapaty S, Thomas PS, Fotso S, Laatsch H. (2004) *Phytochemistry* 65: 1265-1271.

6. Ganapaty S, Thomas PS, Karagianis Peter G W. (2006) *Nat. Prod. Res* 20: 783-787.

7. Orhan I, Sener B, Atici T, Brun R, Perrozo R, Tasdemir D. (2006) *Phytomedicine*. 13: 388-393.

8. Weniger B, Vonthron-Se Necheau C, Kaiser M, Brun R, Anton R. (2006) *Phytomedicine*. 13: 176-180.

9. Raz B, Iten M, Grether-Buhler Y, Kaminsky R, Brun R. (1997) *Acta. Trop*. 68: 139-147.

10. Cunningham I. (1977) *J. Prtozool*. 24: 325-329.

11. Brun R, Jenni L, Tanner M, Schonenberger M, Chell KF. (1979 *Acta. Trop*. 36:289-292.

12. Matile H, Pink JRL. (1990): *Plasmodium falsiparum malarial parasite cultures and Immunological Methods*, Academic press: San Diego, 221-234.

13. Mali RG, Mahajan SG, Mehta AA.(2007) *PHCOG MAG*. 3: 73-76.

14. Shivkar YM, Kumar VL, (2003) *Pharm. Biol* 41(4): 263-265.

15. Thorn GW, Adams RD, Braun Wald KJ (1997): Isselbacher and Peter Sclorf RG. *Harrisons Principles of Internal Medicine*. MC Graw Hill Co: Newyork: 216.

16. Vigar Z (1984): *Atlas of Medical Parasitology*. PG publishing House, Singapore: 216.

17. Chaterjee KD (1967): *Parasitology and Helminthology*. Guha Ray Sree Saraswathy press: Culcutta; pp 168-169.

18. Dash GK, Suresh P, Sahu SK,Kar DM , Ganapaty S, Panda B. (2002) *J. Nat. Rem.* 2 : 182-185.

19. Dash GK, Mishra B, Panda A, Patro P, Ganapaty S. (2003) *Ind. J. Nat. Prod* 28: 19-24.

20. Mali RG, Hindwale JC, Sonawane RS, Patil RN, Hatapakki BC. (2004) *Ind. J Nat. Prod* 20(4): 110-13.