Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(2): 109-113
Received: 23-01-2016
Accepted: 25-02-2016

Vincent Emile Rasamison
National Center for Applied Pharmaceutical Research, Ivandry, B.P. 702, 101 Antananarivo, Madagascar

Harinantenaina Liva Rakotondraibe
Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA

Vahinalahaja Razafintsalama
National Center for Applied Pharmaceutical Research, Ivandry, B.P. 702, 101 Antananarivo, Madagascar

Stephan Rakotonandrasana
National Center for Applied Pharmaceutical Research, Ivandry, B.P. 702, 101 Antananarivo, Madagascar

Andriamalala Rakotondrafara
National Center for Applied Pharmaceutical Research, Ivandry, B.P. 702, 101 Antananarivo, Madagascar

Michel Alain Ratsimbason
National Center for Applied Pharmaceutical Research, Ivandry, B.P. 702, 101 Antananarivo, Madagascar

Elie Rafidinarivo
High Institute of Technology, Ampasampito, B.P. 8122, 101 Antananarivo, Madagascar

Correspondence
Vincent Emile Rasamison
National Center for Applied Pharmaceutical Research, Ivandry, B.P. 702, 101 Antananarivo, Madagascar

Chemical constituents from stems and leaves of *Diospyros gracilipes* Hiern and the antimicrobial and cytotoxic principles

Vincent Emile Rasamison, Harinantenaina Liva Rakotondraibe, Vahinalahaja Razafintsalama, Stephan Rakotonandrasana, Andriamalala Rakotondrafara, Michel Alain Ratsimbason, Elie Rafidinarivo

Abstract
The ethanolic extract from stems and leaves of *Diospyros gracilipes* Hiern showed moderate antimicrobial activity against *Staphylococcus aureus* (9 mm), *Klebsiella pneumonia* (7 mm) and *Candida albicans* (11 mm) using the disc diffusion assay. Fractionation of the extract led to the isolation of lupenone, ursolic acid, corosolic acid, a mixture of 3α- and 3β- taraxerols, 3β-*E*-coumaroyltaraxerol, pinocembrin, scopoletin, plumbagin and elliptinone as established by spectroscopic techniques. Plumbagin exhibited potent antimicrobial activity against the three test microorganisms with inhibitory zones ranging between 30 and 43 mm at 50 µg/disc, and 21 and 28 mm at 10 µg/disc. Plumbagin displayed significant cytotoxicity (IC_{50}=0.26 µg/mL) against HT-29 colon cancer cell line in the sulforhodamine B assay, whereas elliptinone showed weaker activity (IC_{50}=13.29 µg/mL) compared to plumbagin. The reported anti-progestational activity of plumbagin provides a rationale for the popular use of this plant as abortifacient.

Keywords: *Diospyros gracilipes*; Chemical constituents; Antimicrobial activity; Cytotoxicity; Plumbagin; Elliptinone.

Introduction
Madagascar is classified as a country of biodiversity hot spot. Its flora consists of approximately 12,000 plant species, of which 80% are endemic. The Malagasy forests host a large number of medicinal plants used by local people to treat a variety of illnesses. This biological richness may constitute a reservoir of natural products of great significance as drugs and lead structures.
Diospyros is a large pantropical genus of approximately 500 species in the Ebenaceae family. More than 100 *Diospyros* species are encountered in Madagascar. Most of them, for example *D. gracilipes*, *D. perrieri* and *D. platycalyx*, commonly known as ebonies are reputed to produce a very good quality of wood which is highly demanded and primarily used in making furniture, music instruments or ornamental articles [1]. Many *Diospyros* are used worldwide in indigenous health system of medicine to treat various human diseases as enlisted by Khan and Timi [2]. Some species have been chemically and biologically investigated and reported to contain structurally diverse secondary metabolites including triterpenoids, naphthoquinones and polyphenols endowed with antimicrobial, antioxidant and tyrosinase inhibitory activities [3-5].
In the course of our studies on the bioactive principles from endemic plants that are employed in traditional medicine in Madagascar, we examined the constituents of the stems and leaves of *Diospyros gracilipes* Hiern. It is a tree reaching 15 m in height, found in humid forests in the northern and eastern regions of Madagascar. The barks, leaves and fruits are popularly used to stimulate uterine contractions during childbirth and as abortifacient [1]. There have been no previous reports on the constituents and biological activities of this species. The present work aims at isolating phytochemicals from stems and leaves of *D. gracilipes* and evaluating their antimicrobial and cytotoxic activities.

Materials and Methods
Plant material
The stems and leaves of *D. gracilipes* were collected in October 2011 in the Integrale Natural Reserve of Tsaratanana, Commune of Mangindrano in the North of Madagascar. The species was identified by one of us (S.R.) at the Botany and Ethnobotany Department of the National

Center for Applied Pharmaceutical Research, Antananarivo, Madagascar, where a voucher specimen (ST1486) is also deposited.

Extraction and isolation

The stems and leaves of *D. gracilipes* (350 g) were powdered and extracted with EtOH at room temperature for 48 hours, resulting in the crude ethanolic extract (11.3 g). A portion (8.5 g) was suspended in 90% aqueous MeOH (200 mL) and extracted with *n*-hexane (3 x 200 mL). The aqueous MeOH layer was then diluted to 60% aqueous MeOH by addition of water before partitioning with $CHCl_3$ (3 x 200 mL). Evaporation of the solvents *in vacuo* provided dried *n*-hexane-soluble (1.7 g), $CHCl_3$-soluble (2.1 g) and MeOH-soluble (4.3 g) fractions.

The hexane-soluble fraction was first chromatographed over silica gel column with different solvents of increasing polarity (*n*-hexane/CH_2Cl_2 followed by CH_2Cl_2/MeOH) to yield six fractions (A1-A6). Fractions A1 (429.7 mg) was further fractionated over silica gel column (*n*-hexane/EtOAc, 100:0→0:100) to yield five sub-fractions (A11-A15). Sub-fractions A12 and A14 were crystallized from MeOH to give a mixture of compounds 4 and 5 (3.6 mg), and compound 6 (2.3 mg), respectively. Fraction A4 was subjected to RP-18 silica gel column (MeOH/H_2O, 4:1) as eluent to afford compound 2 (8.3 mg). Purification of fraction A5 (178.6 mg) on Sephadex LH-20 ($CHCl_3$/MeOH, 7:3) furnished compound 8 (1.5 mg).

The $CHCl_3$-soluble fraction was loaded on a silica gel column with gradient elution of CH_2Cl_2/MeOH to give eight fractions (B1-B8). Further separation of fraction B1 (67 mg) by silica gel column chromatography with different mixtures of EtOAc in *n*-hexane gave six subfractions (B11-B16). Sub-fraction B13 was washed with EtOAc to leave compound 10 (0.4 mg). Fraction B6 (129.1 mg) was further separated on Sephadex LH-20 ($CHCl_3$/MeOH, 7:3) and RP-18 silica gel (MeOH/H_2O, 7:3 and 3:2) to afford compound 3 (6.6 mg).

Sub-fractions A11 and B11 from the above column chromatography were pooled because they showed almost identical TLC patterns. The combined fraction (total weight 140.7 mg) was purified by silica gel column (*n*-hexane/EtOAc, 15:1) and RP-18 silica gel column to give compound 1 (1.5 mg) and compound 9 (2.3 mg). Similarly, the combined sub-fractions A13 and B14 (total weight 89.8 mg) were subjected to a silica gel column (*n*-hexane/EtOAc, 7:1) and RP-18 silica gel column to furnish compound 7 (1.4 mg).

Purity of compounds was checked on normal phase silica gel TLC or RP-18 silica gel TLC. TLC plates were developed with suitable eluents. Spots were first visualized under UV light at 254 and 366 nm and then by using the vanillin sulphuric spray reagent.

Structural elucidation

The chemical background of the genus and the Rf value, fluorescence and color on TLC of the studied compound gave a first idea of the chemical class to which it belongs. Structures were elucidated by means of spectroscopic techniques (^{1}H NMR, ^{1}H-^{1}H COSY, MS). Spectra were carefully interpreted and data obtained were compared with those published in the literature.

Antimicrobial assay

The microorganisms used in this study consisted of the Gram-positive bacteria *Staphylococcus aureus* (ATCC 11632), the Gram-negative bacteria *Klebsiella pneumoniae* (from the stock culture of the Pharmacology Department of the National Center for Applied Pharmaceutical Research, Antananarivo, Madagascar) and the yeast *Candida albicans* (ATCC 10231). Antimicrobial activity and susceptibility-screening tests were performed using the disc diffusion method. Each microorganism was suspended in Muller-Hinton broth (Difco, Detroit, MI) for the bacteria and Sabouraud broth (Difco, Detroit, MI) for the yeast. They were diluted with peptone water to provide cell counts of the inoculum at 10^6 CFU/ml. Bacterial strains were then inoculated on Mueller–Hinton agar plates and the yeast in Sabouraud agar, each in triplicate. Sterilized filter paper discs of 6 mm diameter (Biomérieux, Marcy l'Etoile, France) were saturated with 10 µL of the ethanolic extract (100 µg/disc) and the compounds (10 and 50 µg/disc). Soaked discs were then placed on the plates and incubated for 24 h, after which the diameter of the inhibitory zone was measured (mm). Negative controls consisted of the solvents used to dissolve the samples. Each assay was done in triplicate. The results were expressed as the mean value of the inhibition zones. Tetracyclin and miconazole (Bio-Rad, Marnes-la-Coquette, France) were used as positive controls.

Cytotoxicity assay

Cytotoxic assay against HT-29 colon cancer cell line was performed at the College of Pharmacy, The Ohio State University using the sulforhodamine B assay as previously described [6].

Results

Isolated compounds

The hexane and chloroform fractions of the ethanolic extract from stems and leaves of *D. gracilipes* were subjected to column chromatography over silica gel, Sephadex LH-20 and RP-18 silica gel. The fractionation procedure led to the isolation and the identification of the following compounds: lupenone (compound 1), ursolic acid (compound 2), corosolic acid (compound 3), a mixture of 3α- and 3β-taraxerols (compounds 4 and 5, respectively), 3β-*E*-coumaroyltaraxerol (compound 6), pinocembrin (compound 7), scopoletin (compound 8), plumbagin (compound 9) and elliptinone (compound 10) (Fig. 1). ^{1}H NMR spectral data of isolated compounds are in good agreement with those published in the literature [7-15]. ^{1}H-^{1}H COSY and MS spectra helped to unambiguously establish the structure of elliptinone (compound 10).

This is the first report on the chemistry of *D. gracilipes*, and to our knowledge, the first report of corosolic acid and 3β-*E*-coumaroyltaraxerol from the genus *Diospyros*.

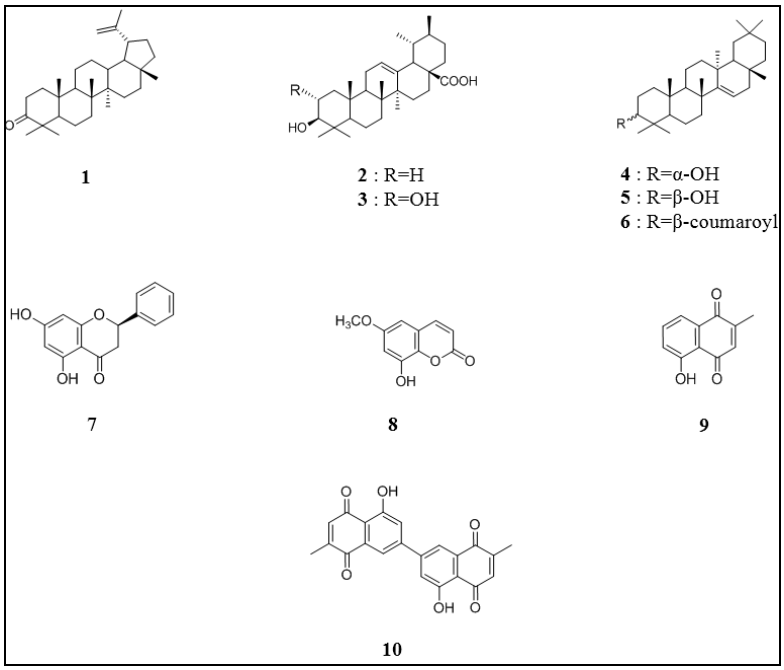

Fig 1: Structures of compounds 1-10 isolated from *D. gracilipes* stems and leaves

Antimicrobial activity

As shown in Table 1, the ethanolic extract from stems and leaves of *D. gracilipes* was found to be active against the two bacteria *S. aureus* and *K. pneumonia* and the yeast *C. albicans* with inhibition zones of 9±1.2, 7±2 and 11±1.5 mm, respectively, at the concentration of 100 µg/disc in the disk diffusion assay. Compounds 2-5 and 9 which were obtained in sufficient amounts were submitted to antimicrobial assay against the three targeted microorganisms. The observed inhibition zones of plumbagin at 50 µg/disc against *S. aureus* (43±2.9 mm), *K. pneumonia* (30±2 mm), and *C. albicans* (37±8.8 mm) were greater than those produced by 30 µg/disc of tetracycline against *S. aureus* and *K. pneumonia* (32±2 and 15±2 mm, respectively) and 50 µg/disc of miconazole against *C. albicans* (25±2 mm). When the compound was tested at 10 µg/disc, the activity against *S. aureus* was reduced by half (21±1.2 mm). However, at this concentration, it was still more active than 30 µg/disc of tetracycline against *K. pneumonia* (28±2 mm) and as active as 50 µg/disc of miconazole against *C. albicans* (23±8.9 mm).

Table 1: Antimicrobial activity of the ethanolic extract and plumbagin

Microorganisms	Ethanolic Extract (µg/dis)	Plumbagin (µg/disc)		Tetracycline (µg/disc)	Miconazole (µg/disc)
	100	50	10	30	50
	Zone of inhibition (mm)[a,b]				
S. aureus	9±1.2	43±2.9	21±1.2	32±2	NT
K. pneumonia	7±2	30±2	28±2	15±2	NT
C. albicans	11±1.5	37±8.8	23±8.9	NT	25±2

[a] Values are expressed as ±S.D (Standard Deviation)
[b] Including the diameter of the paper disk (6 mm)
NT: not tested

Cytotoxic activity

All the isolated compounds were evaluated for their antiproliferative activity against HT-29 colon cancer cell line. Plumbagin displayed strong cytotoxicity (IC_{50}=0.26 µg/mL), whereas elliptinone showed weaker activity (IC_{50}=13.29 µg/mL) compared to plumbagin. Compounds 1-8 were inactive in this assay.

Discussion

Plants still hold a conspicuous place in natural product research due to their relative high diversity, good availability and abundant uses in traditional medicine. A considerable number of plants have been screened for biologically active constituents in multiple therapeutic areas. In the present study, chemical investigations of *D. gracilipes* were initiated after the ethanolic extract from stems and leaves inhibited the growth of *S. aureus*, *K. pneumonia* and *C. albicans* during a primary screen for antimicrobial compounds from medicinal plants endemic to Madagascar. Ten compounds were characterized and corresponded to six triterpenoids, one coumarin, one flavonoid and two naphtoquinones. The occurrence of 1,4-naphtoquinones represented herein by plumbagin and elliptinone reinforces the view that this class of natural products is widely distributed in *Diospyros* species and may be used as good biochemical markers in this genus [16].

The significant antimicrobial activity of plumbagin against the three targeted pathogenic microorganisms lends strength to previous results that this compound is effective against a number of both Gram-positive and Gram-negative bacteria, and yeasts [14, 17]. The isolation of plumbagin is consistent with the antimicrobial activity detected for *D. gracilipes*. Furthermore, literature search revealed that this compound is a strong anti-progestational substance by exhibiting 100% abortifacient activity in rats at the dose of 2 mg/kg, p.o. [18].

Therefore, it can be assumed that the claimed abortifacient property of *D. gracilipes* may be attributed to the presence of plumbagin.

Cancer remains a major health problem all over the world by causing millions of death and new cases each year. Efforts to find new anticancer agents intensify especially from natural resources [19]. In this connection, all the compounds isolated in this work were evaluated for their cytotoxicity in vitro against HT-29 colon cancer cell line. The results indicate that plumbagin was very active in this assay, but its dimerization to elliptinone considerably reduced the cytotoxicity. Interestingly, elliptinone previously reported from *D. wallichii* was found to be cytotoxic about five times stronger than plumbagin against the MCF-7 human breast carcinoma cell [15]. Plumbagin constantly displays appreciable cytoinhibitory activity in different experimental models [14, 15, 20]. The selectivity of elliptinone cytotoxicity could be linked to steric effects inherent to its high molecular weight compared to that of plumbagin. Further studies are needed to verify this hypothesis.

D. gracilipes is a prominent source of medicinally active phyto-elements as inferred from the reported bioactivities of the other isolated compounds. They have been shown to exhibit cytotoxic, anti-inflammatory, antioxidant and anti-diabetic activities [21-24]. These findings add value to this medicinal plant and pave the way for more analyses to explore its pharmaceutical potentials.

Conclusion

The present study gives the first information about the chemistry and pharmacology of *D. gracilipes*. The current pharmacological data of plumbagin and elliptinone together with those of other researchers could be a basis for further investigations for the development of new drugs of natural origin to fight against infectious diseases and cancer. The reported anti-progestational activity of plumbagin provides a rationale for the popular use of this plant as abortifacient.

Conflict of interests

Declared none

Acknowledgement

We are grateful to the National Center for Applied Pharmaceutical Research, Antananarivo, Madagascar for financial support during the plant collection and the supply of chemicals critical to this study. We also thank The Ohio State University, College of Pharmacy NMR facility at The Ohio State University for facilitating the acquisition of the NMR and Mass spectra.

References

1. Lemmens RHMJ, Louppe D. Plant Resources of Tropical Africa 7(2). Timbers 2. Fondation PROTA, Wageningen, Pays-Bas, 2012, 341-343.
2. Khan MR, Timi D. Constituents of the root and stem barks leaves and fruits of Diospyros hallierii. Fitoterapia 1999; 70(3):320-321.
3. Prachayasittikul S, Saraban P, Cherdtrakulkiat R, Ruchirawat S, Prachayasittikul V. New bioactive triterpenoids and antimalarial activity of Diospyros rubra Lec. EXCLI J, 2010; 9:1-10.
4. Cesari I, Hoerle M, Simoes-Pires C, Grisoli P, Queiroz EF, Dacarro C *et al.* Anti-inflammatory, antimicrobial and antioxidant activities of Diospyros bipindensis (Gurke) extracts and its main constituents. Journal of Ethnopharmacology. 2013; 146(1):264-270.
5. Xue Y-L, Miyakawa T, Hayashi Y, Okamoto K, Hu F, Mitani N, *et al.* Isolation and Tyrosinase Inhibitory Effects of Polyphenols from the Leaves of Persimmon, Diospyros kaki. Journal of Agriculture and Food Chemistry. 2011; 59(11):6011-6017.
6. Addo EM, Chai HB, Hymete A, Yeshak MY, Slebodnick C, Kingston DGI *et al*. Antiproliferative constituents of the roots of Ethiopian Podocarpus falcatus and Structure revision of 2α-Hydroxynagilactone F and Nagilactone I. Journal of Natural Products. 2015; 78(4):827-835.
7. Prakash CVS, Prakash I. Isolation and Structural Characterization of Lupane triterpenes from Polypodium Vulgare. Research Journal of Pharmaceutical Sciences. 2012; 1(1):23-27.
8. Kriwacki RW, Pitner TP. Current aspects of practical two-dimensional nuclear magnetic resonance spectroscopy: applications to structure elucidation. Pharmaceutical Research, 1989; 6(7):531-554.
9. Zong W, Zhao G. Corosolic acid isolation from the leaves of Eriobotrta japonica showing the effects on carbohydrate metabolism and differentiation of 3T3-L1 adipocytes. Asian Pacific Journal of Clinical Nutrition. 2007; 16(Suppl 1):346-352.
10. Corbett RE, Gumming SD, Whitehead EV. Lichens and fungi. Part X. 14α-Taraxerane. Journal of the Chemical Society, Perkin Transactions 1, 1972, 2827-2829.
11. McLean S, Reynolds WF, Yang J-P, Jacobs H, Jean-Pierre LL. Total assignment of the ^{1}H and ^{13}C chemical shifts for a mixture of *cis*- and *trans*-p-hydroxycinnamoyl esters of taraxerol with the aid of high-resolution, ^{13}C-Detected, ^{13}C-^{1}H shift correlation spectra. Magnetic Resonance in Chemistry, 1994; 32(7):422-428.
12. Laskar RA, Sk I, Roy N, Begum NA. Antioxidant activity of Indian propolis and its chemical constituents. Food Chemistry, 2010; 122(1):233-237.
13. Fatiany PR, Fiatoa B, Raoelson G, Randrianirina AO, Randriantsoa A, Andrianjara C *et al.* Vasodilator effects of Cymbopogon pruinosus (Poaceae) from Madagascar on isolated rat thoracic aorta and structural elucidation of its two bioactive compounds. Journal of Pharmacognosy and Phytochemistry, 2016; 5(1):46-55.
14. Gu J-Q, Graf TN, Lee D, Chai H-B, Mi Q, Kardono LBS, *et al.* Cytotoxic and Antimicrobial Constituents of the Bark of Diospyros maritima Collected in two Geographical Locations in Indonesia. Journal of Natural Products. 2004; 67(7):1156-1161.
15. Salae A-W, Karalai C, Ponglimanont C, Kanjana-Opas A, Yuenyongsawad S. Naphthalene derivatives from Diospyros wallichii. Canadian Journal of Chemistry, 2010; 88(9):922-927.
16. Fatiany PR, Robijaona B, Randrianarivo E, Raharisololalao A, Marie-Therese M, Ngbolua K. Isolation and structural elucidation of cytotoxic compounds from the root bark of Diospyros quercina (Baill.) endemic to Madagascar. Asian Pacific Journal of Tropical Biomedicine. 2014; 4(3):169-175.
17. Mabona U, Viljoen A, Shikanga E, Marston A, Vuuren SV. Antimicrobial activity of southern African medicinal plants with dermatological relevance: From an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound. Journal of Ethnopharmacology. 2013; 148(1):45-55.

18. Dhar SK, Rao PG. Hormonal profile of plumbagin. Fitoterapia, 1995; LXVI(5):442-446.
19. Liu Y, Cheng E, Rakotondraibe LH, Brodie PJ, Applequist W, Randrianaivo R *et al.* Antiproliferative compounds from Ocotea macrocarpa from the Madagascar dry forest. Tetrahedron Letters 2015; 56(23):3630-3632.
20. Yan C-H, Li F, Ma Y-C. Plumbagin shows anticancer activity in human osteosarcoma (MG-63) cells via the inhibition of S-Phase checkpoints and down-regulation of c-*myc.* International Journal of Clinical and Experimental Medicine, 2015; 8(8):14432-14439.
21. Ahn KS, Hahm MS, Park EJ, Lee HK, Kim IH. Corosolic acid isolated from the fruit of Crataegus pinnatifida var. psilosa is a protein kinase C inhibitor as well as a cytotoxic agent. Planta Medica, 1998; 64(5):468-470.
22. Yao X, Li G, Bai Q, Xu H, Lü C. Taraxerol inhibits LPS-induced inflammatory responses through suppression of TAK1 and Akt activation. International Immuno pharmacology, 2013; 15(2):316-324.
23. Tanjung M, Tjahjandarie TS, Sentosa MH. Antioxidant and cytotoxic agent from the rhizomes of Kaempferia pandurata. Asian Pacific Journal of Tropical Disease. 2013; 3(5):401-404.
24. Xu F, Wu H, Wang X, Yang Y, Wang Y, Qian H *et al*. RP-HPLC characterization of lupenone and β-sitosterol in Rhizoma musae and evaluation of the anti-diabetic activity of lupenone in diabetic Sprague-Dawley rats. Molecules, 2014; 19(9):14114-14127.