SOMMAIRE

CURRICULUM VITAE

1- Etat civil

Nom : RANOROMALALA épouse RAKOTO
Prénoms : Danielle Aurore Doll
Sexe : féminin
Nationalité : Malgache
Date et lieu de naissance : 27 Décembre 1958 à Antananarivo
Situation de famille : mariée, mère de 2 enfants
Nom et prénoms du conjoint : RAKOTO Raymond Morris

2- Coordonnées

Adresse personnelle : Ambohimangakely lot 10 B IV
Antananarivo 103 MADAGASCAR
tél. : 00 261 3241 067 77
00 261 3311 584 83 Email : dad.rakoto@yahoo.fr

Adresse professionnelle : Faculté des Sciences BP 906
Antananarivo 101 MADAGASCAR

3- Formation

Formation secondaire et universitaire :

Date	Diplôme obtenu/Stage effectué	Lieu
1976	Baccalauréat série D	Lycée Jules Ferry Antananarivo
1979	DUES en Sciences naturelles	Faculté des Sciences Antananarivo
1981	Licence d'enseignement	Faculté des Sciences Antananarivo
1982	Maîtrise de recherche en Sciences biologiques appliquées	Faculté des Sciences Antananarivo
1984	DEA de 3ème cycle en Biochimie	Faculté des Sciences Antananarivo
1989	Doctorat de 3ème cycle en Biochimie	Faculté des Sciences Antananarivo

Stages et ateliers :

1984-1985 : Stage de pharmacologie au Laboratoire de pharmacologie et toxicologie fondamentales CNRS Toulouse, France

Juillet 1994 : Formation en communication à la Faculté des Sciences, Antananarivo

Septembre 1994 : Stage de formation en criblage de propriétés biologiques à l'Institut malgache de recherches appliquées, Antananarivo

Juillet 1996 : Formation de formateurs en enseignement du français langue étrangère à l'Institut Montpelliérain de langue française, France

Mai-Juin 2000 : Atelier de formation en rédaction d'articles scientifiques organisé par le CIRAD en co-financement avec l'AUF, Antsirabe

Février 2002 : Formation en informatique : logiciels de traitement de texte, tableur, Powerpoint, navigation sur Internet, messagerie,… à la Faculté des Sciences, Antananarivo

Novembre 2002 : Acquisition de techniques d'étude physico-chimique des ignames au Département PERSYST, CIRAD Montpellier, France

Novembre 2006 : Atelier de formation en bioinformatique, organisé par le CIRAD au Cersae.

4- Langues

Langue maternelle : Malgache

Langue	Lu	Parlé	Ecrit	Compris
Français	Très bien	Très bien	Très bien	Très bien
Anglais	Très bien	Bien	Bien	Bien

5- Connaissances en informatique

Maîtrise de Microsoft office, Linux
Connaissances en PAO

6- Responsabilités administratives

Depuis 1996 : Responsable du matériel et équipement des laboratoires du Département de Biochimie fondamentale et appliquée

Depuis 1998 : Intérim en cas d'absence du Chef de département de Biochimie fondamentale et appliquée

Depuis 1998 : Représentant de la Faculté des Sciences ou du département de Biochimie fondamentale et appliquée au cours de rencontres internationales (COI, Cervoi,…)

Depuis 2000 : Responsable administratif et Présidente d'honneur d'Ampon'Ambohitsaina, Chorale de la Faculté des sciences

Depuis février 2010 : Chef du Département de Biochimie fondamentale et appliquée, Faculté des Sciences, Université d'Antananarivo

7- <u>Activités pédagogiques</u>

1982-1985 : Enseignant-chercheur vacataire au Service de Botanique de l'Etablissement d'enseignement supérieur des sciences, Université de Madagascar.

<u>Enseignements dirigés et pratiques de</u> :

- Biochimie structurale en 2ème année de Sciences naturelles
- Biochimie métabolique en 3ème année de Sciences naturelles

1985-1993 : Titularisation en tant qu'enseignant-chercheur **Assistant** au Service de Biologie Végétale et Biochimie, Faculté des sciences, Université d'Antananarivo.

<u>Enseignements dirigés et pratiques de</u> :

- Biochimie structurale en 2ème année de Sciences naturelles
- Biochimie métabolique en 3ème année de Sciences naturelles

Depuis 1993 : Enseignant-chercheur **Maître de conférences** en Biochimie, au Département de Biochimie, Faculté des sciences, Université d'Antananarivo :

- Enseignements théoriques et dirigés de Biochimie structurale en 2ème année de Sciences naturelles
- Enseignements théoriques et dirigés de Biochimie métabolique en 3ème année de Sciences naturelles
- Enseignements théoriques (depuis 2011) dirigés et pratiques de Toxicologie en 4ème année de Sciences naturelles
- Enseignements dirigés et pratiques de Biologie moléculaire en 4ème année de Sciences naturelles

Depuis 2001 : Enseignant-chercheur Maître de conférences vacataire à la Faculté de Médecine, Université d'Antananarivo **:**

- Enseignements théoriques et dirigés de Biochimie structurale et Biochimie métabolique en 1ère année et 2ème année de Médecine
- Enseignements théoriques et dirigés de Biochimie structurale, Biochimie métabolique et Biologie moléculaire en 1ère année et 2ème année de Pharmacie

Depuis 2009 : Enseignant missionnaire à la Faculté des Sciences, Université de Moroni, Comores :

Enseignements théoriques et dirigés de Biologie moléculaire en 3ème année de Sciences de la vie.

8- Expérience en communication

Depuis 1990 : Enseignant-animateur en Didactique et communication en Sciences en langue française (Communication orale, Communication écrite, Communication professionnelle), Faculté des Sciences, Université d'Antananarivo

1995 : Formateur de formateurs en Didactique et communication en Sciences en langue française : Communication orale, Université Nord Madagascar, Antsiranana

1996 : Formateur de formateurs en Didactique et communication en Sciences en langue française : Communication écrite, Université Nord Madagascar, Antsiranana

2006-2010 : Secrétaire de rédaction au quotidien « L'Express de Madagascar », Antananarivo

9- Projets de recherche

Date	Projet	Financement	Lieu
2000	Intervenante dans le contrat-programme « *Laro* »	MNRES	Faculté des Sciences Antananarivo IHSM Toliara
2002	Intervenante dans le projet FSP « *Plantes raticides* »	MADES	Faculté des Sciences Antananarivo
2001-2004	Responsable de volet dans le projet FADES « *Appui à la recherche sur les possibilités de valorisation des ignames à Madagascar* »	Banque mondiale	Faculté des Sciences Antananarivo
2007-2011	Intervenante dans le projet Pôle d'excellence régionale « *Valorisation des ressources de la biodiversité végétale de Madagascar et des Comores pour la sécurité des aliments* »	AUF	Faculté des Sciences Antananarivo
2007-2011	Intervenante dans le projet Qualisann : « *Le cresson à Madagascar* »	AUF	Faculté des Sciences Antananarivo
Depuis 2010	Responsable technique et scientifique du projet « *African food tradition revisited by research : le kitoza à Madagascar* »	Union Européenne	Faculté des Sciences Antananarivo CIRAD Montpellier

10- Participation active au développement de l'unité de recherche « Toxicologie »

J'ai contribué pour une large part à l'extension de l'unité de recherche « Toxicologie », du Laboratoire de Biochimie appliquée aux sciences médicales (Département de Biochimie fondamentale et appliquée) principalement par les actions suivantes :

- Elaboration et mise en œuvre de programmes de recherche : programme *Albizia*, programme « Huile essentielle »

- Mise au point de méthodes de purification et d'analyse : chromatographies sur colonne et sur couche mince,… et d'évaluation des activités biologiques d'extraits : tests sur les végétaux, tests d'activité antimicrobienne, test d'activité antioxydante …
- Développement du partenariat avec diverses institutions (IPM, CIRAD, ASJA, IHSM, CNARP, CNRE, …)
- Recherche de financement pour renforcer les équipements scientifiques, étendre les infrastructures (Laboratoire de Microbiologie et de Mycologie), et prendre les étudiants stagiaires en charge, notamment par le montage de projets.

11- Encadrement d'étudiants

Encadrement et co-encadrement d'étudiants en DEA :

J'ai encadré des étudiants en DEA (au total 31 jusqu'en 2012) sur le thème de recherche « *Etude chimique et biologique des principes toxiques issus de plantes malgaches* ». J'ai également co-encadré des étudiants en DEA qui travaillaient sur le même thème. De la récolte des matériels d'étude jusqu'à la présentation du mémoire, l'encadrement comprend les activités ci-après :

- Enseignement de la recherche bibliographique, dans des documents et en ligne : inventaire des plantes d'intérêt
- Supervision des études sur terrain : élaboration des questionnaires d'enquêtes, détermination des zones d'étude, enquêtes sur terrain, récolte et emballage des échantillons, confection d'herbiers
- Démonstration des techniques d'étude chimique et biologique
- Supervision des travaux de laboratoire : préparation des matériels d'étude, mise au point de techniques d'extraction et des étapes purification de principes actifs, mise au point des techniques d'analyse chimique, établissement des protocoles de tests biologiques, supervision des tests et discussion des résultats
- Correction des écrits des étudiants : rapports de terrain, rapports périodiques et mémoires.

La liste des **étudiants encadrés** ainsi que celle **des étudiants co-encadrés** sont données respectivement en *p. 10 et 13*.

Participation à des jurys de mémoire :

J'ai été sollicitée un grand nombre de fois pour l'examen de mémoires de DEA en Biochimie. La liste de mes participations à des jurys de mémoires, en tant qu'examinateur ou rapporteur (encadreur ou co-encadreur) est présentée à la *p. 4*.

Participation à l'encadrement de doctorants :

J'ai participé à l'encadrement de 07 doctorants du laboratoire de « Biochimie appliquée aux sciences médicales », du Département de Biochimie fondamentale et appliquée : certains d'entre eux étaient des étudiants que j'ai encadrés lors de leurs stages de DEA et qui ont continué sur les mêmes sujets. Ma contribution a essentiellement consisté en la conduite

des missions sur le terrain (enquêtes, collecte de matériels …), l'appui à la conception, l'exécution de certaines expériences (chimie et toxicologie), l'interprétation et la discussion des résultats. A ces titres, je suis co-auteur de publications parues et de publications en soumission.

- RAJEMIARIMOELISOA Clara Fredeline, Isolement, caractérisation chimique et biologique du principe toxique de *Albizia odorata* (Mimosoïdeae, Fabaceae), thèse soutenue le 07/04/2000.
  Co-auteur dans une publication parue

- RANDRIANARIVO Hanitra Ranjana, Isolement, caractérisation chimique et biologique des principes toxiques de *Albizia arenicola* (Mimosoideae, Fabaceae), thèse soutenue le 18/04/2003.
  Co-auteur dans deux publications parues

- RAKOTONDRAZANAKA Lovasoa, Isolement, caractérisation chimique et biologique du principe toxique de *Odosicyos bosseri* (Cucurbitaceae), thèse soutenue le 25/06/2003.
- RAHERINIAINA Christian Edmond, Etudes chimique et toxicologique du principe ichtyotoxique de *Euphorbia laro* (Euphorbiaceae) : Impacts de la pêche au laro, thèse soutenue le 23/03/2004.

- RAKOTOBE Lolona, Etudes chimiques et toxicologiques de deux plantes toxiques malgaches : *Dioscorea antaly* Jum. Et Perr. (Dioscoreaceae) et *Rhodocodon madagascariensis* Baker (Hyacinthaceae), thèse soutenue le 30/10/2009.
  Co-auteur dans une publication parue

- RAVELOMANANA-RAZAFINTSALAMA Vahinalahaja Eliane, Etude chimique et biologique d'une plante médicinale malgache : *Dilobeia thouarsii* (Proteaceae).
  Co-auteur dans deux publications en soumission

- RANDRIAMAMPIANINA Lovarintsoa Judicaël, Etudes chimiques et biologiques des principes toxiques d'*Albizia* (Fabaceae), thèse en cours.
  Co-auteur dans une publication en soumission

12- <u>Responsabilités scientifiques</u>

<u>Responsable du volet « Propriétés autres qu'alimentaires des ignames malgaches » : dans le projet FADES « *Appui à la recherche sur les possibilités de valorisation des ignames à Madagascar* »</u>

Mes attributions en tant que Responsable de volet au sein de ce projet étaient :

- Elaboration du questionnaire d'enquête ethnobotanique et simulation avec les étudiants impliqués dans le volet
- Encadrement sur le terrain des étudiants : enquêtes ethnobotaniques, repérage des matériels d'étude, récolte d'échantillons pour analyse
- Délivrance des consignes pour la rédaction des rapports de mission sur terrain ;

- Correction et finalisation des rapports de mission
- Encadrement des étudiants au laboratoire : mise au point des manipulations, suivi régulier des travaux, analyse des résultats
- Rédaction des rapports trimestriels et finaux du volet
- Contribution à la rédaction des rapports du projet
- Restitution des résultats : intervention (animation, exposé des résultats et recommandations du volet) dans les ateliers sur les sites d'étude, confection de posters, animation des stands au cours des ateliers FADES.

Responsable technique et scientifique du projet « *African food tradition revisited by research (AFTER) : le kitoza à Madagascar* »

En tant que Responsable technique et scientifique, je dois assurer la coordination des activités de l'équipe UT (Université d'Antananarivo) et sa gestion financière, avec la collaboration du Responsable administratif. Mes réalisations et activités sont les suivantes :

- Elaboration des questionnaires d'enquête sur le kitoza, sa production, sa vente et sa consommation ; choix des zones d'étude ; collecte d'échantillons
- Supervision des enquêtes sur terrain, établissement de contacts avec les producteurs et les revendeurs
- Analyse des données d'enquêtes
- Rédaction des rapports d'enquêtes
- Supervision des travaux d'analyse physico-chimique et microbiologique, interprétation des résultats, rédaction des rapports d'analyse
- Participation aux rencontres périodiques du projet : présentation des avancées de l'équipe UT sous forme de communications (Accra, Ghana en mai 2011 et Montpellier en septembre 2011 et novembre 2012), bilan, poursuite des travaux,…
- Organisation de l'atelier « *Value chain analysis* » sur le kitoza à Antananarivo en octobre 2011
- Organisation de l'accueil de chercheurs missionnaires chargés de l'appui dans la réalisation des différentes activités du projet (analyses sensorielles)
- Liaison entre la coordination de Montpellier et l'équipe UT
- Rédaction de « délivrables » : synthèses bibliographiques, protocoles d'échantillonnage des kitoza, rapports périodiques de WP (*Work package*).

12- Travaux de consultance

J'ai été consultante pour une étude d'impact financée par le projet Voarisoa. Sous la direction du bureau d'études Adapt (Antananarivo), les travaux d'évaluation des impacts environnementaux des industries malgaches ont consisté à :

- Déterminer les zones industrielles d'étude
- Descendre sur le terrain pour étudier les installations d'unités industrielles, comprendre leurs processus de transformation et observer leurs systèmes de rejet
- Rédiger des rapports de terrain
- Formuler des recommandations en vue de limiter les impacts négatifs des activités industrielles
- Animer les ateliers de restitution auprès des opérateurs et des autorités locales.

PRODUCTIONS SCIENTIFIQUES

LISTE DES PUBLICATIONS ET COMMUNICATIONS SCIENTIFIQUES

1) Articles dans revues internationales

- **RAKOTO D.A.D.**, RANDRIANARIVO R., EL-YACHOUROUTUI M., ARISOA A.A., RAHARISOA N., RAKOTONDRASOA N., RAONIHARISOA P., JEANNODA V. 2012, Effects of extracts from *Albizia* (Fabaceae) endemic species of Madagascar on vegetable seedling development, *J. Chem. Chem. Eng*. **6**, 313-322.

- **RAKOTO D.A.D.**, RAJEMIARIMOELISOA C., RANDRIANARIVO R., RAMAMONJISON D., RAHERINIAINA C., RAHARISOA N., JEANNODA V. 2011, Antimicrobial activity of some endemic species of *Albizia* (Fabaceae) from Madagascar, *Asian Biotechnology and Development Review* **13**(3), 53-60.

- RAKOTOBE L., MAMBU L., DEVILLE A., DUBOST L., JEANNODA V., **RAKOTO D.**, BODO B. 2010, Clerodane and 19-norclerodane diterpenoids from the tubers of *Dioscorea antaly*, *Phytochemistry* **71**, 1007-1013.

- JEANNODA V., RAKOTONIRINA O., RANDRIANARIVO H., **RAKOTO D.**, WRIGHT P., HLADIK C.-M. 2003, Le principe toxique du bambou consommé par Hapalemur aureus n'est pas neutralisé par la terre ingérée, *Rev. Ecol. (Terre Vie)* **58**, 151-153.

2- Articles en soumission

- Vahinalahaja RAZAFINTSALAMA, Marion GIRARDOT, Ranjàna RANDRIANARIVO, **Danielle RAKOTO**, Samira SARTER, Thomas PETIT, Sylvia RALAMBONIRINA, Alexandre DEVILLE, Philippe GRELLIER, Victor JEANNODA, Lengo MAMBU. Dilobenol A-G, diprenylated dihydroflavonols from the leaves of *Dilobeia thouarsii*. Submitted to the **European Journal of Organic Chemistry.**

- Vahinalahaja RAZAFINTSALAMA, Samira SARTER, Lengo MAMBU, Ranjàna RANDRIANARIVO, Thomas PETIT, Jean-François RAJAONARISON, Christian MERTZ, **Danielle RAKOTO**, Victor JEANNODA. Antimicrobial activities of *Dilobeia thouarsii* Roemer and Schulte, a traditional medicinal plant from Madagascar. Submitted to **South African Journal of Botany.**

- Lovarintsoa RANDRIAMAMPIANINA, Anne OFFROY, Lengo MAMBU, Ranjàna RANDRIANARIVO, **Danielle RAKOTO**, Victor JEANNODA, Simone PUISEUX DAO, Marc EDERY. Marked toxicity of *Albizia bernieri* extracts on embryo-larval development in the medaka fish (*Oryzias latipes).* Submitted to **Toxicon**.

3- Communications orales

- RAKOTO D. Purification et étude des propriétés biologiques des principes antibactériens d'une plante médicinale malgache de la famille des Proteaceae, Journée de Pharmacie, 11 Janvier 2010, Académie malgache.

- RAKOTO D.A.D. *In vitro* effects of extracts from five Malagasy species of *Albizia* (Fabaceae) on vegetable seeds germination. The 14th NAPRECA Symposium, 8th-12th August 2011, ICIPE, Kasarani, Nairobi, Kenya.

- RAKOTOBE RANDRIAMOELIARIVONY L., DEVILLE A., DUBOST L., JEANNODA V., **RAKOTO D**., BODO B., MAMBU LENGO A. Etude phytochimique, biologique et toxicologique de *Dioscorea antaly*. Colloque de Toliara, Madagascar, 29-32 Juillet 2009.

4- Communications affichées

- RAKOTO D.A.D. *In vitro* antimicrobial activity of extracts from five Malagasy endemic species of *Albizia* (Fabaceae). The 14th NAPRECA Symposium, 8th-12th August 2011, ICIPE, Kasarani, Nairobi, Kenya. ***Best poster prize***.

JEANNODA V., RATSIMBA A.I., ANDRIAMAMPIANINA H.L., ARNAUD E., **RAKOTO D.A.D**. Quality characterization of *kitoza*, a Malagasy meat product. EFFOST Annual meeting, 20-23 Novembre 2012, Le Corum, Montpellier, France.

5- Communications dans des réunions scientifiques

RAKOTO D.A.D. Kitoza of Madagascar : Survey results, AFTER meeting, 3-6 May 2011, Accra, Ghana.

RAKOTO D.A.D. Kitoza of Madagascar : Process, production, sale and consumption characteristics, AFTER annual meeting, 12-16 September 2011, Montpellier, France.

6- Rapports de projet

JEANNODA V. et coll. (dont **RAKOTO D.A.D.**), Recherche sur les ignames de Madagascar, Rapport du projet FADES « *Appui à la recherche sur les possibilités de valorisation des ignames à Madagascar* », Mai 2005.

ETUDIANTS ENCADRES EN DEA
(31 étudiants au total)

Date	Nom et prénoms	Titre
18/10/1996	RANDRIANARIVO Hanitra Ranjàna	Purification et caractérisation partielles des principes actifs d'*Albizia arenicola (*Mimosoideae, Leguminoseae)
02/04/1999	RAHERINIAINA Christian Edmond	Contribution à l'étude chimique et biologique des principes toxiques d'*Albizia boivini* (Mimosoïdeae, Fabaceae)
21/04/1999	RAHARISOA Noelinirina	Contribution à l'étude chimique et biologique des principes toxiques d'*Albizia bernieri* (Mimosoïdeae, Fabaceae)
03/12/2001	ARISOA Alain Andrianavalona	Etude chimique et biologique d'extraits toxiques de fruits d'*Albizia boivini* (Mimosoïdeae, Fabaceae)
25/04/2003	RAHELINIAINAMANDIMBY Lovasoa	Etude chimique et biologique d'extraits toxiques d'*Olax lanceolata* (Olacaceae)
31/07/2003	RAONIHARISOA Pascaline	Etude chimique et toxicologique des extraits toxiques de graines d'*Albizia tulearensis* (Mimosoideae-Fabaceae)
01/08/2003	RAMIARIMBELOSOA Nancy Hortense	Etude chimique et toxicologique des extraits toxiques du champignon *Boletus sp* (Boletaceae)
27/11/2003	TONIMALALA Miaritiana Hélène	Etude chimique et toxicologique des extraits toxiques du champignon *Cantharellus cf congolensis* de la famille des Cantharellaceae
03/09/2004	RAHANTARINORO Josiane	Etude chimique et toxicologique des extraits toxiques de feuilles de *Ravensara anisata* (Lauraceae)
18/04/2005	RANDRIAMAMONJY Florence Yollande	Purification et caractérisation chimique et toxicologique partielles des principes toxiques des tubercules de *Dioscorea antaly* (Dioscoreaceae)
22/12/2005	ARIJAONA Mino	Purification et caractérisation partielles des principes toxiques de feuilles de *Pittosporum vertivillatum* (Pittosporaceae)

ETUDIANTS ENCADRES EN DEA (suite)

Date	Nom et prénoms	Titre
03/02/2006	ANDRIANTSOANIRINA Landy Valérie	Etude chimique et toxicologique des principes toxiques des feuilles de *Pittosporum ochrosiaefolium* (Pittosporaceae)
04/04/2006	SOIFOINI Toilibou	Etude chimique et toxicologique des extraits de racines de *Rhodocodon madagascariensis* (Liliaceae)
20/08/2007	RAKOTOASIMBOLA Ihasina Harimalala	Purification et caractérisation chimique et toxicologique des feuilles de *Macarisia pyramidata* (Rhizophoraceae)
29/08/2007	ABOUBACAR Moindjie Moissi	Etude chimique et toxicologique des extraits de feuilles d'*Anacardium occidentale* (Anacardiaceae)
28/09/2007	RASOLOFOMANANA Victorien	Purification et caractérisation chimique et toxicologique partielles des principes toxiques des feuilles de *Ficus megapoda* BAKER (Moraceae)
17/10/2008	RASOLOHARIJAONA Franck Yvon	Etudes chimique et toxicologique d'une plante médicinale malgache *Schefflera longipedicellata* (Araliaceae)
15/05/2009	AHMED Abdallah	Etudes chimique et toxicologique des extraits de feuilles d'*Albizia boinensis*(Fabaceae)
03/07/2009	SILMI Abdoullahi	Etudes chimique et toxicologique des extraits de feuilles d'une plante médicinale malgache *Acridocarpus excelsus* (Malpighiaceae)
23/09/2009	DJAZA Djaanfar Peni	Etudes chimique et toxicologique des extraits de feuilles d'une plante médicinale malgache *Macaranga alnifolia* (Euphorbiaceae)
24/09/2009	RAKOTONIAINA Minozanany Mamihery	Caractérisation et purification partielle des propriétés antimicrobiennes des extraits de feuilles de *Macaranga boutonioïdes (*Euphorbiaceae)
20/11/2009	RATOVONIRINA Noël Harijaona	Etudes chimique et toxicologique des fractions lipidique et non lipidique des graines de *Mucunaprurens* (Fabaceae)

ETUDIANTS ENCADRES EN DEA (suite et fin)

Date	Nom et prénoms	Titre
04/10/2010	NOURACHANI Ibrahim	Caractérisation physico-chimique et biologique de l'huile essentielle des écorces de *Cryptocarya crassifolia* (Lauraceae)
07/10/2010	RAHERIMANAMPAMONJY Hanta Lalao Olga	Etudes chimique et toxicologique d'une plante médicinale malgache, *Psorospermum androsaemifolium* (Hypericaceae)
28/10/2011	SEHENOASPIERA Mihaja Nandrianina	Caractérisation physico-chimique et biologique de l'huile essentielle extraite des feuilles d'*Ocotea cymosa* (Lauraceae)
09/12/2011	RANDRIANAIVO Heriniaina Jeannot	Etude des propriétés antibactériennes des huiles essentielles de *Cedrelopsis grevei* Baillon (Rutaceae)
06/04/2012	RATSIMBA Angela Irène	Caractéristiques physico-chimiques et microbiologiques du kitoza de bœuf
24/04/2012	RATSIMIEBO Maholy Pricille	Etudes chimique et toxicologique d'extraits de graines de *Podocarpus madagascariensis* (Podocarpaceae)
30/04/2012	ANDRIAMAMPIANINA Herizo Lalaina	Production, vente et consommation du kitoza dans la province d'Antananarivo, qualité du kitoza de porc
31/05/2012	RASAMOELINA Harintsoa	Etude chimique et toxicologique des extraits d'*Euphorbia primulifolia* var. *primulifolia* (Euphorbiaceae)
02/08/2012	RAKOTOMALALA Andriamirado Tiana	Etude chimique et toxicologique des extraits de feuilles d'*Albizia arenicola* (Fabaceae)

ETUDIANTS CO-ENCADRES EN DEA (43 étudiants au total)

Date	Nom et prénoms	Titre
18/10/1996	RAJEMIARIMOELISOA Clara Fredeline	Contribution à l'étude chimique et biologique des principes actifs d'*Albizia polyphylla* (Mimosoïdeae, Leguminoseae)
21/12/1998	RAMAMONJISON Edouard Delphin	Contribution à l'étude chimique et biologique du principe toxique d'*Albizia sp.* (Mimosoïdeae, Fabaceae)
12/02/1999	RAKOTONDRAZANAKA Lovasoa	Contribution à l'étude chimique et biologique des principes toxiques de *Xerosicyos danguyi* (Cucurbitaceae)
29/06/2000	RAKOTONDRASOA Noelitiana Samuel	Etude chimique et biologique des extraits toxiques de feuilles d'*Albizia polyphylla* (Mimosoïdae, Fabaceae)
27/10/2000	RATIARIVELO Voahangy	Etude chimique et biologique d'extraits toxiques de graines de *Barringtonia butonica* (Lecythidacées)
03/11/2000	RANDRIAMIHARISOA Fidèle	Etude chimique et biologique d'extraits toxiques de feuilles de *Xerosicyos perrieri* (Cucurbitacées)
01/10/2001	HARINJATOVO Hantamalala	Etude chimique et biologique d'extraits toxiques de feuilles de *Pentatropis madagascariensis* (Asclepiadacées)
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05/08/2002	TOSY Ramahafangoza Vaillant	Etude de la fraction lipidique de graines de *Cnestis glabra* (Connaraceae)
11/04/2003	FENORADOSOA Taratra Andrée	Etude chimique et biologique des principes toxiques *d'Asteropeia mc phersonii* (Asteropeiaceae)
13/06/2003	RAKOTOBE Lolona	Etude chimique et toxicologique des extraits toxiques de feuilles de *Deinbollia boinensis* (Sapindaceae)
13/06/2003	RANDRIAMAHAVALISOA Tiana Fanomezantsoa	Etude chimique et toxicologique des extraits toxiques de feuilles d'*Ocotea madagascariensis* (Lauraceae)
24/06/2004	RANDRIANANDRASANA Jaona	Etude chimique et toxicologique des extraits d'écorces de tige d'*Uapaca thouarsii* (Euphorbiaceae)
28/08/2004	RAKOTOARIVELO Nirina Solange Alexandrine	Etude chimique et toxicologique des principes toxiques de graines de *Mimusops commersonii* (SAPOTACEAE)
19/10/2004	ANDRIANJAKANIRINA Lova Mbolaniaina	Etude chimique et toxicologique d'extraits de graine de *Physena madagascariensis* (Physenaceae)

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22/12/2005	RAKOTONDRASOA Andriniaina	Etude chimique et toxicologique des tubercules de *Dioscorea esculenta* (Dioscoreaceae)
21/03/2006	RAZAFINTSALAMA VAHINALAHAJA Eliane	Etude chimique et toxicologique des extraits toxiques de feuilles de *Pittosporum senacia* (Pittosporaceae)
22/03/2006	RANDRIANAMBININTSOA Louis Valentin	Etude chimique et toxicologique d'extraits alcaloïdiques de tubercules de *Dioscorea esculenta* (Dioscoreaceae)
15/06/2006	RANAIVONIARIVO Ieja Tsimiholy	Etude de la fraction lipidique et des principes toxiques de *Cucumis sativus* (Cucurbitaceae)
21/08/2007	MOHAMED Kaniza	Purification et caractérisation partielles des principes toxiques des feuilles de *Pachytrope dimepate* (Moraceae)
05/09/2007	RAKOTO ANJANOROSOA Laingotiana	Etude chimique et toxicologique des extraits toxiques de feuilles de *Cabucala erythrocarpa* (Asclepiadaceae)
13/09/2007	ANDRIAMIARIMANANA Fehivola Mandanirina	Etude chimique et toxicologique des écorces de tige de *Myrica spathulata* (Myricaceae)
21/09/2007	RASOAMAHENINA-ANDRIANAIVORAVELONA Antsa Mirindra	Etude chimique et toxicologique des fruits de *Piper pyrifolium* (Piperaceae)
21/09/2007	RASOLONDRAIBE Onjasoa Nambinina	Etude chimique et toxicologique des extraits toxiques de feuilles de *Xylopia humblotiana* (Annonaceae)
19/09/2008	RANDRIAMAMPIANINA Lovarintsoa Judicaël	Etude chimique et biologique des extraits de feuilles d'une plante médicinale malgache, *Chassalia bojeriana* (Rubiaceae)
17/10/2008	RAZAFINDRATSIMA Haingo	Etudes chimique et toxicologique des extraits de feuilles de *Smilax kraussiana* (Liliaceae) , une plante médicinale
29/10/2008	RABENANDRIANINA Solofomboahangy Mbolatiana	Etudes Chimique et toxicologique des extraits de feuilles de *Dilobeia thouarsii* (Proteaceae), une plante médicinale malgache
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06/06/2009	ABDOU Daniel	Etudes chimique et toxicologique des extraits d'écorces de branches de *Conchopetalum madagascariense* (Sapindaceae), une plante endémique de Madagascar

ETUDIANTS CO-ENCADRES EN DEA (suite et fin)

Date	Nom et prénoms	Titre
19/08/2009	AMDJAD Ali Soudja	Etude chimique et biologique des extraits d'écorces de tige d'*Acridocarpus excelsus*, une Malpighiacee malgache
28/08/2009	ANDRIAMASINORO Sandra Nirina	Caractérisation chimique et biologique des principes antibactériens de l'écorce de tige de *Dilobeia thouarsii* (Proteaceae)
25/09/2009	RASOLOSON Malala Nambinina	Etude chimique et biologique des extraits des écorces de *Dilobeia thouarsii*, (Proteaceae) une plante médicinale malgache
25/09/2009	RAMAHAVORY Hanitriniony Landiharijaona	Analyse des facteurs antinutritionnels et des principes toxiques d'ignames (*Dioscorea*) de la côte est de Madagascar
20/11/2009	RAZAFINDRASOA Marie Annita	Etude des facteurs antinutritionnels et de l'activité biologique d'une igname cultivée (*Dioscorea alata*) des régions de la côte ouest de Madagascar
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28/08/2001	ANDRIANANTENAINA	FaratianaNorosoa	Examinateur
24/09/2001	ANDRIAMAMPIANINA	Rina Hajasoa	Examinateur
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27/02/2003	RAZAFINDRATSIMANDRESY	Richter Mamy Lalaina	Examinateur
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29/11/2003	RAJAONARIVELO	Andriampanarivo Jacob Philémon	Examinateur
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04/10/2010	NOURACHANI	Ibrahim	**Rapporteur**
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18/03/2011	RASOAHANITRALISOA	Lucia Rondroarivelo	Examinateur
08/04/2011	RAKOTONIAINA	Henintsoa Volatiana	Examinateur
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ANNEXES

Copie des publications scientifiques

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Antimicrobial Activity of Some Endemic Species of *Albizia* (FABACEAE) from Madagascar

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Abstract: Plants belonging to the genus *Albizia* (Fabaceae) are traditionally subject of medicinal uses in many countries. Their various properties (larvicidal, antimicrobial, antiparasitic, cytotoxic, effects on nervous system, etc.) were thoroughly investigated. In Madagascar, *Albizia* is represented by 27 species of which 25 are endemic and two were introduced from other countries. Actually, neither chemical nor pharmacological study on the Malagasy species is reported in the literature. We assessed the antimicrobial activity of extracts from five endemic species of *Albizia*. Results showed that the extracts from A_3 and A_5 showed activity against all the tested germs at various degrees. On the other hand, all of the extracts inhibited the growth of *Staphylococcus aureus* and *Candida albicans*. Pure compound from the plant A_2 showed the lowest MIC (6.25 µg/ml) and (MBC) (100 µg/ml) against *Candida albicans*.

Keywords: *Albizia*, seeds, extracts, antimicrobial, minimum inhibitory concentration, minimum bactericidal concentration, Madagascar

Introduction

A large number of people in many developing countries have been relying on traditional medicines, in which plants constitute the principal element, for their health care needs for centuries. Plants belonging to the genus *Albizia* (Fabaceae) are trees found in countries in Africa, Asia and South-America where they are widely used in indigenous pharmacopoeia.[1] *Albizia* species have been the subject of several chemical and pharmacological studies. Thus, many structures (heterosids, alkaloids) were elucidated[2] and various activities such as anthelmintic[3], cytotoxic[4], larvicidal[5] or antimicrobial[6] were found.

In Madagascar, *Albizia* is represented by 27 species of which 25 are endemic and two were introduced from other counties. No previous report on both the chemical constituents and the pharmacological activities of

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these plants could be found in the literature. Since infectious diseases account for the significant proportion of health problems, antimicrobial principles from five Malagasy species of *Albizia* encoded A_1, A_2, A_3, A_4 and A_5, were studied in this work. They were purified and the major secondary metabolites were identified by phytochemical screening. Extracts or pure compounds were tested *in vitro* against two Gram positive bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, three Gram negative bacteria: *Klebsiella pneumoniae, Escherichia coli, Salmonella typhi* and one yeast *Candida albicans*. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) were determined on susceptible germs.

Preparation of Pure Compounds and Extracts

Seeds of plants A_1, A_2, A_3, A_4 and A_5 were used in this study. Fruits were collected in western and southern regions of Madagascar. Seeds were washed, sun-dried and ground into a fine powder, using a microgrinder Culatti. For all the species, the methods of extraction and purification of active principles are shown on figures 1-5. Pure compounds and extracts were subjected to preliminary phytochemical testing for the major chemical groups.[7] The major secondary metabolites identified in extracts are shown in Table 1.

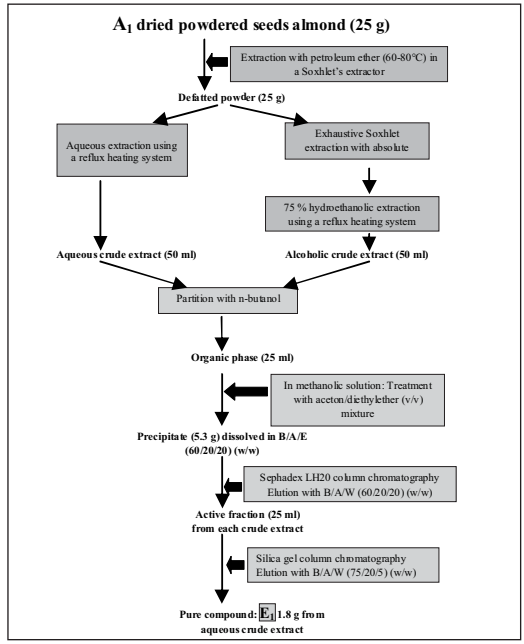
Except E_1 which didn't contain triterpenes, all extracts showed the presence of unsaturated sterols, triterpenes and deoxysugars, indicating glycosidic nature of active principles. The presence of saponins, in addition with positive foam test and hemolytic effect (not shown) mean that antimicrobial compounds may be saponins. Saponins and other glycosides were isolated and identified from other species of *Albizia*.[8]

Table 1: Phytochemical Screening of Extracts from 5 Malagasy Species of *Albizia* (A_1 to A_5)

Phytochemical compounds	Extracts					
	E_1	E_{21}	E_{22}	E_3	E_4	E_5
Alkaloids	–	–	–	–	–	–
Flavonoids	–	–	–	–	–	–
Anthocyanins	–	–	–	–	–	–
Phenols	–	–	–	–	–	–
Quinons	–	–	–	–	–	–
Unsaturated sterols	+	+	+	+	+	+
Triterpenes	–	+	+	+	+	+
Deoxysugars	+	+	+	+	+	+
Saponins	+	+	+	+	+	+

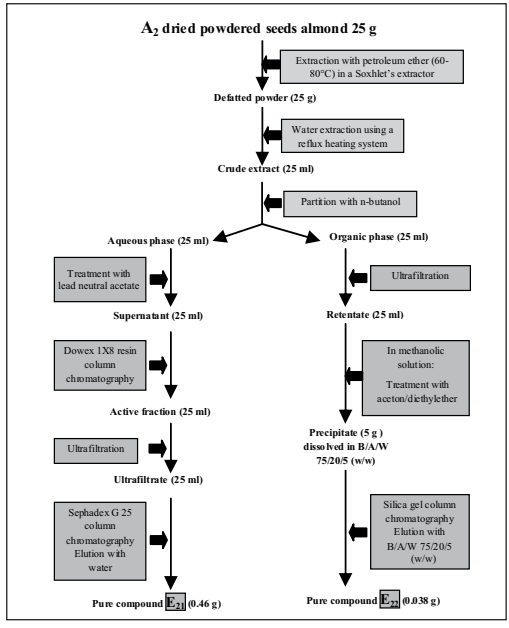
Source: Authors' compilation.
- : negative test + : positive test
E_1, E_3, E_4, E_5 : purified extracts from plants A_1, A_3, A_4 and A_5 respectively
E_1, E_{21}, E_{22} : pure compounds from plant A_1, A_2 respectively

Figure 1: Extraction and Purification of Active Principle from A_1 Seeds



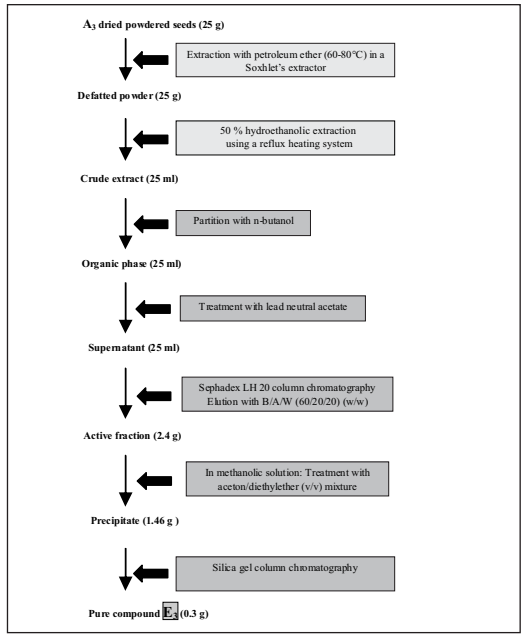
Source: Authors' compilation.

Figure 2: Extraction and Purification of Active Principle from A_2 Seeds



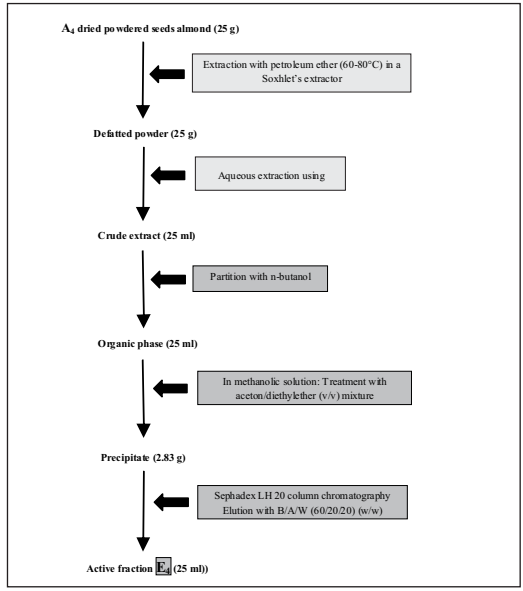
Source: Authors' compilation.

Figure 3: Extraction and Purification of Active Principle from A_3 Seeds



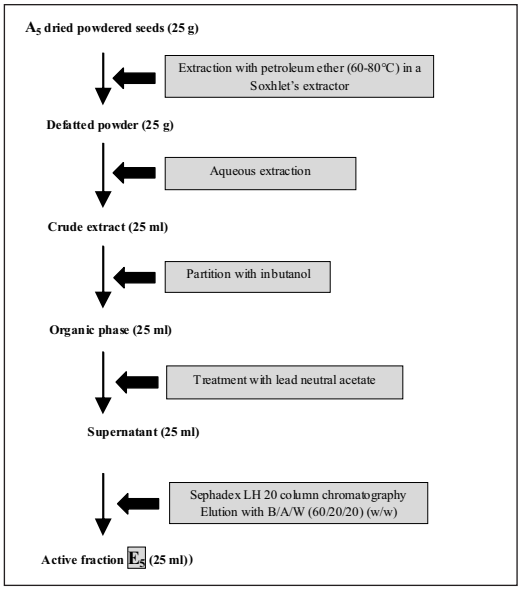
Source: Authors' compilation.

Figure 4: Extraction and Purification of Active Principle from A_4 Seeds



Source: Authors' compilation.

Figure 5: Extraction and Purification of Active Principle from A_5 Seeds



Source: Authors' compilation.

Assays on Micro-organisms

The pathogenic micro-organisms consisted of two Gram positive bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, three Gram negative bacteria : *Klebsiella pneumoniae, Escherichia coli, Salmonella typhi* and one yeast *Candida albicans*. They were isolated and identified from heterogeneous cultures available in *Institut Pasteur de Madagascar*. The antimicrobial tests were carried out by disc diffusion method in Mueller Hinton agar.[9] The average inhibition zone (mm) is shown in Table 2.

Table 2: *In vitro* Antimicrobial Activity of Extracts from Five Malagasy Species of *Albizia*

Microorganisms	Extracts (µg/disc)						
	E_1 (25)	E_{21} (31,25)	E_{22} (31,25)	E_3 (400)	E_4 (400)	E_5 (500)	Reference antibiotics
Gram positive bacteria							
Staphylococcus aureus	8	12	10	11	9.5	11	Vancomycin (30 µg) 23 mm
Bacillus subtilis	–	ND	ND	13	11	16	Furan (25 µg) 33 mm

Table 2 continued....

Table 2 continued....

Gram negative bacteria							
Klebsiella pneumoniae	–	10	10	9	7	10	Furan (25 µg) 32 mm
Escherichia coli	9	–	–	11	9	13	Furan (25 µg) 34 mm
Salmonella typhi	–	8	–	9	7	11	Furan (25 µg) 30 mm
Yeast							
Candida albicans	14	20	12	10	8	13	Amphotericin B (100µg) 12 mm

Source: Authors' compilation. – : No activity ND : Not determined

According to these results, the extracts E_3 and E_5, respectively from A_3 and A_5, showed activity against all the tested germs. *Bacillus subtilis* seemed to be the most susceptible bacterium (13 mm inhibition zone for E_3 and 16 mm for E_5) to these extracts. On the other hand, all of the extracts inhibited the growth of *Staphylococcus aureus* and *Candida albicans* at the tested concentrations. E_{21} (pure compound) exhibited the strongest activity against the fungus (20 mm). In a general manner, Gram positive germs, including *Candida albicans,* were more susceptible than Gram negative ones. Similar results were obtained with some other species of *Albizia*.[10]

MIC was determined for each extract on the most susceptible germ by broth dilution method.[11] Each medium showing no visible growth is subcultured on Mueller Hinton agar plates. After 24 hours at 37°C, MBC was the corresponding concentration required to kill 99.9 per cent of the cells.[12] MIC and MBC determined are given in Table 3.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Extracts from Five Malagasy Species of *Albizia*

Extracts	Sensitive germs	MIC (µg/ml)	MBC (µg/ml)
E_1	*Staphylococcus aureus*	320	2500
E_{22}	*Candida albicans*	6.25	100
E_{22}	*Klebsiella pneumoniae*	50	800
E_3	*Escherichia coli*	2500	10 000
E_4	*Staphylococcus aureus*	625	10 000
E_4	*Escherichia coli*	1250	20 000
E_5	*Escherichia coli*	12 500	12 500

Source: Authors' compilation.

Pure compound E_{22} from the plant A_2, showed the lowest MIC (6.25 µg/ml) and MBC (100 µg/ml) against *Candida albicans*. With MIC values

corresponding to 100 µg/ml and 12.5 µg/ml respectively, *Albizia myriophylla* and *Albizia gummifera*[13] showed lower activity than A_2 against this germ.

Conclusion

Infectious diseases account for a significant proportion of health problems in Madagascar. Hence five species of *Albizia* plants native to Madagascar were tested for their antimicrobial properties. The tests confirmed the presence of triterpenes, unsaturated salts and deoxysugars in these plants. Out of the five species, two reported activity against all the five tested germs.

Endnotes

1. Githiori *et al.* (2003); Geyid *et al.* (2005); Zheng *et al.* (2006); Murugan *et al.* (2007); Rukayadi (2008).
2. Zou *et al.* (2006); Rukunga *et al.* (2007).
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4. Zou *et al.* (2006).
5. Murugan *et al.* (2007).
6. Agyare *et al.* (2005); Geyid *et al.,* (2005); Sudharameshwari *et al.* (2007).
7. Farnsworth (1966); Marini-Bettolo et al. (1981).
8. Pal *et al.* (1995); Debella *et al*. (2000); Zou *et al*. (2006).
9. Rios *et al.* (1988).
10. Mbosso *et al.* (2010); Rukayadi (2008); Sudharameshwari *et al*. (2007).
11. Duval and Soussy (1990); Ferron (1994).
12. *ibid.*
13. Mbosso *et al.* (2010); Rukayadi (2008).

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Effects of Extracts from *Albizia* (Fabaceae) Endemic Species of Madagascar on Vegetable Seedling Development

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Abstract: The effects of extracts from five *Albizia* (Fabaceae) endemic species from Madagascar (*A. arenicola*, *A. boivini*¸ *A. bernieri*, *A. polyphylla* and *A. tulearensis*) were tested on vegetable seedling development. Crude extracts were obtained through cold or hot extraction methods on dried powdered seeds, seed teguments, leaves or empty pods. They were thereafter purified using techniques based on physicochemical properties of active substances. Assays were carried out on seedling growth of Monocotyledons and Dicotyledons representatives. Results showed that all extracts exerted significant dose dependent inhibition on epicotyl and hypocotyl growth. However, some extracts exhibited a slight stimulation effect at low doses. Moreover, *A. arenicola* crude extract (E_{23}) slightly inhibited axillary bud growth, while *A. tulearensis* crude extract (E_{71}) showed a stimulation effect. According to preliminary phytochemical screening results, these effects might be due to saponins or alkaloids.

Key words: *Albizia*, extracts, saponins, alkaloids, inhibition, seedling development, Madagascar.

1. Introduction

Trees belonging to the genus *Albizia* (Fabaceae) grow in tropical areas such as Africa, Asia, Central and South-America where they are widely used in traditional medicine [1-5]. Chemical and pharmacological investigations on number of these plants led to the isolation of novel structures with various properties, indicating the efficiency of the healers' herb preparations. Thus, ethanolic extract from *A. lebbeck* exhibited anticonvulsive activity [6]. The structure of cytotoxic triterpenoidal saponins from *A. julibrissin* was established [3]. Sedative activity of flavonol glycosides from this species was reported [1]. Antiplasmodial spermin alkaloids were isolated from *A. gummifera* [7]. Water extracts from *A. anthelmintica* were demonstrated to be highly anthelmintic several years ago [8-9]. Antimicrobial activity of many species such as *A. ferruginea*, *A. gummifera*, *A. lebbeck* was reported [10-12]. It is also of importance to note that some *Albizia* species are toxic [10, 13]. However, no investigation on *Albizia* effects on plants has been reported yet. The increasing use of chemical herbicides which always threatens scientists and users who search for new weedkillers with low toxicity and without side effects is encouraged. In addition, high application of herbicides leads to resistance of many weeds [14-15].

The purpose of this study was to assess the effects of malagasy *Albizia* species extracts on plants development. In Madagascar, *Albizia* genus is represented by twenty-four endemic and three

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introduced species [16]. Up to now, neither chemical constituents nor pharmacological activities were reported except our recent publication on antimicrobial activity of five of these species [17]. Effects of A. arenicola, A. boivini, A. bernieri, A. polyphylla and A. tulearensis extracts on Monocotyledons and Dicotyledons seedling development were evaluated. Vegetables were used as plant tests since calibrated seeds were available.

2. Materials and Methods

2.1 Plant Materials

2.1.1 Collection and Processing of Plants

Albizia materials were collected in western (Mahajanga) and southern (Toliary, Morondava) regions of Madagascar. Parts used for each plant are presented in Table 1. Seeds (for A_2, A_5, A_7) were washed and all plant materials (seeds, pods and leaves) were sun-dried. Dried seeds (for A_5, A_7), empty pods (for A_4) and leaves (for A_6) were ground into a fine powder, using a microgrinder Culatti. For A_2, teguments were separated from almond by several grinding/sieving cycles. Seed teguments were washed to remove almond residues, then sun-dried and powdered.

2.1.2 Tests of Seed

Calibrated vegetable seeds used for tests came from the collection of *National Research Center for Farming* (Fofifa, Antananarivo) seed bank (Table 2). For each seedling test, experiments were performed on one Monocotyledons representative and one Dicotyledons representative.

2.2 Preparation of Extracts

2.2.1 Crude Extracts

Prior to extraction, powdered dried seeds were defatted by extraction with petroleum ether (60-80 °C) (A_5) or with hexane at 45 °C for 18 h (A_7) in a Soxhlet's extractor. Using the same procedure, powdered dried leaves (A_6) were depigmented with acetone. Plant materials were then submitted to cold or hot extraction using distilled water or organic solvents.

2.2.2 Purified Extracts

Crude extracts were purified using methods based on solubility, molecular weight or electric charge of active principles. Extraction and purification protocol is summarized for each plant on Figs. 1-5.

2.3 Phytochemical Screening

Extracts were subjected to preliminary phytochemical testing for the major chemical groups [18-19].

2.4 Assays on Plants

2.4.1 Assays on Seedling Growth

The effects of crude (E_{23}, E_{41}, E_{71}) or purified (E_5, E_6) extracts were studied on epicotyl and hypocotyl growth, using an adapted method for determining weed response to herbicide [20-21]. Different batches of ten vegetable seeds were soaked for 48 h at 30 °C in darkness, and then transferred onto Petri dishes layered with cotton. Batches were germinated and regularly watered with a range of concentrations of the tested extracts. One of them was watered with distilled water

Table 1 Used plant parts.

	Collected materials				
Species	*A. arenicola* (A_2)	*A. boivini* (A_4)	*A. bernieri* (A_5)	*A. polyphylla* (A_6)	*A. tulearensis* (A_7)
Plant parts	Seed teguments	Empty pods	Seeds	Leaves	Seeds

Table 2 Extracts and vegetable seeds used.

Plants	*A. arenicola* (A_2)	*A. boivini* (A_4)	*A. bernieri* (A_5)	*A. polyphylla* (A_6)	*A. tulearensis* (A_7)
Extracts	CE = E_{23} PE = E_{24}	CE = E_{41}	PE = A_5	PE = E_6	CE = E_{71} PE = E_{72}
Seeds	Rice, Bean	Rice, Bean	Rice, Bean	Maize, Pea	Rice, White tissam

CE = Crude extract; PE = Purified extract.

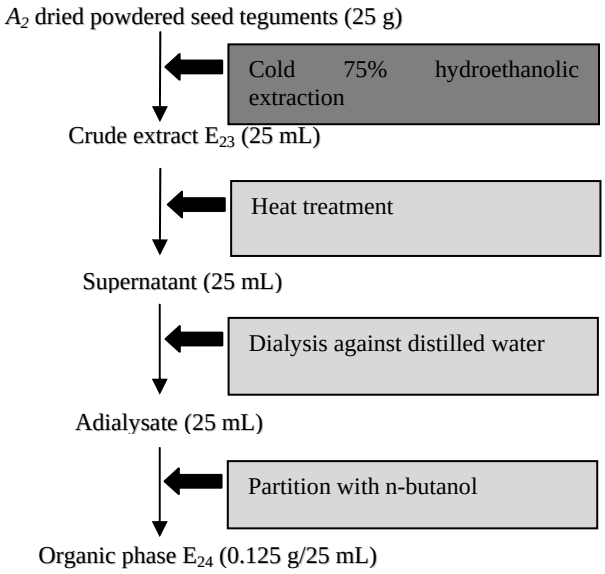

Fig. 1 Extraction and purification of active principles from *A. arenicola* (A_2) teguments.

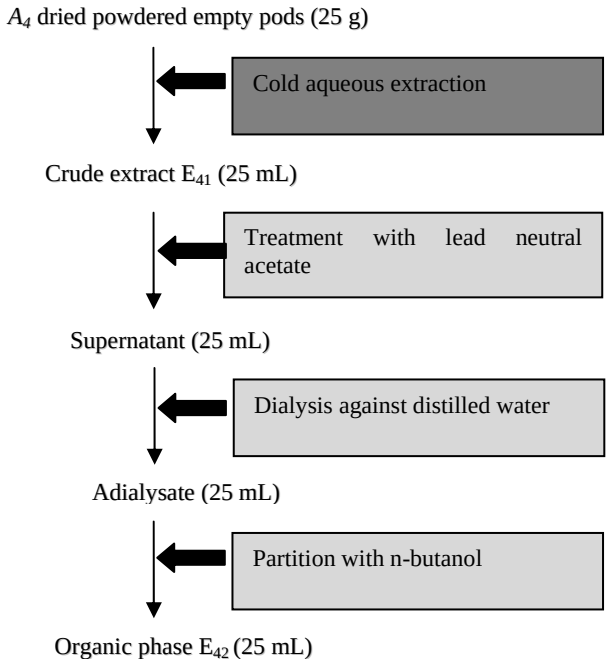

Fig. 2 Extraction and purification of active principles from *A. boivini* (A_4) empty pods.

as control. Epicotyl and hypocotyl lengths were measured every 2 days during 14 days.

2.4.2 Assays on Axillary Bud Growth

Assays were realized on 15-day-old pea seedlings previously sectioned above the second axillary bud. Effects of extracts were compared with those of the plant growth regulators gibberellins and auxin. Tested

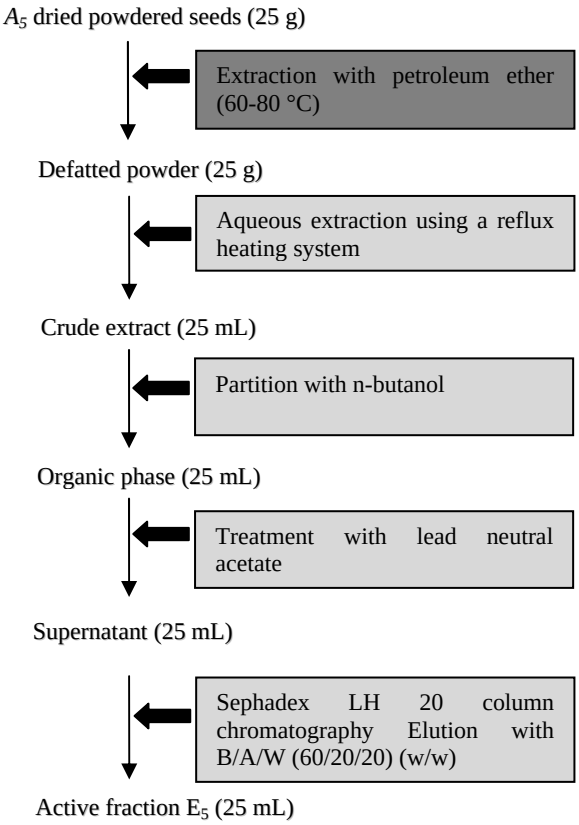

Fig. 3 Extraction and purification of active principles from *A. bernieri* (A_5) seeds.

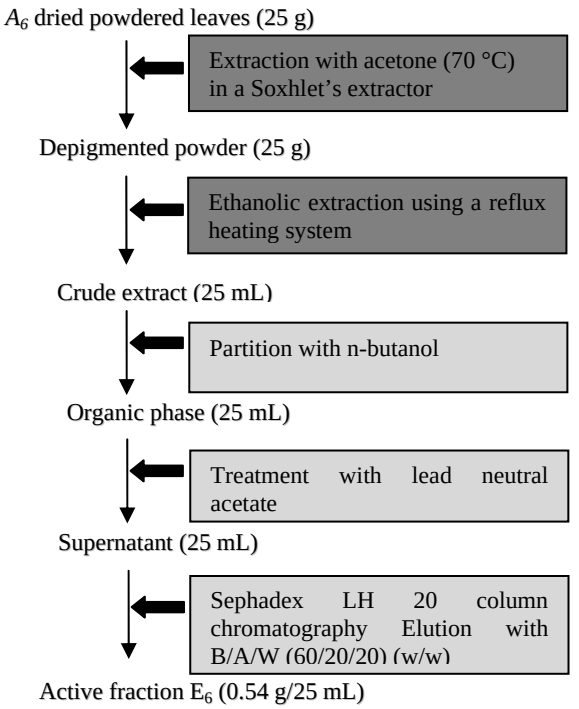

Fig. 4 Extraction and purification of active principles from *A. polyphylla* (A_6) leaves.

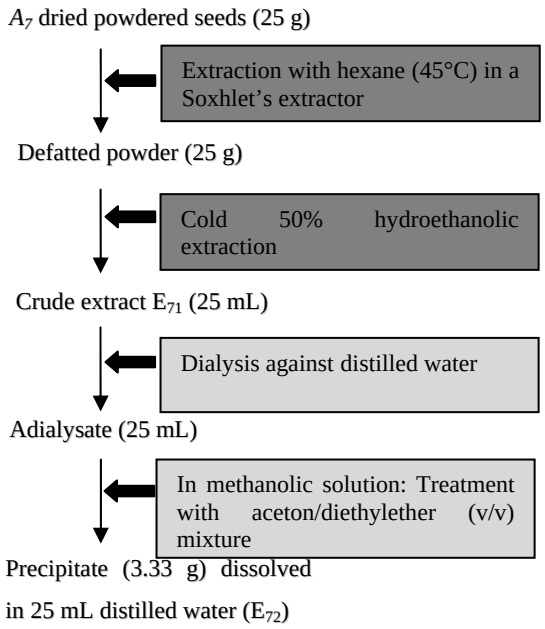

Fig. 5 Extraction and purification of active principle from *A. tulearensis* (A_7) seeds.

solutions (1µL) were mixed with lanolin and deposited on the top of the sectioned part. Five groups of 5 plants each were studied: group 1, received gibberellin (positive control); group 2, auxin (negative control); group 3, crude extract; group 4, purified extract and group 5, 1µL distilled water (control). Axillary bud growth was measured every 2 days during 14 days.

2.5 Statistical Analysis

One-way analysis of variance (ANOVA) which was followed by Newman Keuls comparison test with Statitcf® software was used for statistical analysis. Statistical estimates were made at confidence interval of 95%.

3. Results

The used extracts for each *Albizia* species and the vegetable seeds tested in assays are shown in Table 2. Vegetable seeds (Monocotyledons and Dicotyledons) were chosen among those which did not germinate during preliminary tests with extracts at 1 mg/mL.

3.1 Effects on Seedling Growth

All the extracts showed statistically significant and dose dependent ($p < 0.05$) epicotyls and hypocotyls growth inhibition at the tested concentrations (Figs. 6-15). Response from the seed-tests occurred at different concentrations when assays were carried out on the same vegetable. Thus, when the rice (Monocotyledon) was tested in the presence of extracts from several plants such as *A. arenicola* (A_2), *A. boivini* (A_4), *A. bernieri*, (A_5), *A. tulearensis* (A_7), the used concentrations were quite different. The same results were observed for bean (Dicotyledon) on *A. arenicola* (A_2), *A. boivini* (A_4), *A. bernieri* (A_5) extracts which were used (Figs. 6-11, 14).

In some cases (in the presence of E_5 and E_6, Figs. 10-13), epicotyls appeared to be more susceptible than hypocotyls. However, a slight stimulation effect was exhibited by some extracts at low concentrations, such as 0.23 mg/mL for E_{23} (Fig. 6 and 7), 0.035 mg/mL for E_{41} (Fig. 9) or 0.117 mg/mL and 0.234 mg/mL for E_6 (Fig. 13). E_5 at 1.31 mg/mL was the most active extract as demonstrated by the total inhibition of the growth which noted for epicotyls of rice and bean seedlings (Figs. 6 and 7).

3.2 Effects on Axillary Bud Growth

Effects of crude and purified extracts from A_2 (extracts E_{23} and E_{24} from seed teguments of *A. arenicola*) and from A_7 (extracts E_{71} and E_{72} from seeds of *A. tulearensis*) which were available at sufficient amounts were assessed.

Crude extract E_{23} slightly inhibited pea axillary bud growth. This effect was lower than auxin at the same dose. On the contrary, purified extract E_{24} exhibited no effect (Fig. 16). Active principles were probably removed by purification.

Extracts E_{71} and E_{72} stimulated axillary bud growth but in less extent than gibberellin. Even at 9 times and 4.7 times higher doses respectively, than gibberellin, stimulation effect remained weaker than positive control (Table 3).

3.3 Phytochemical Screening

The inhibition activity of the extracts from the various

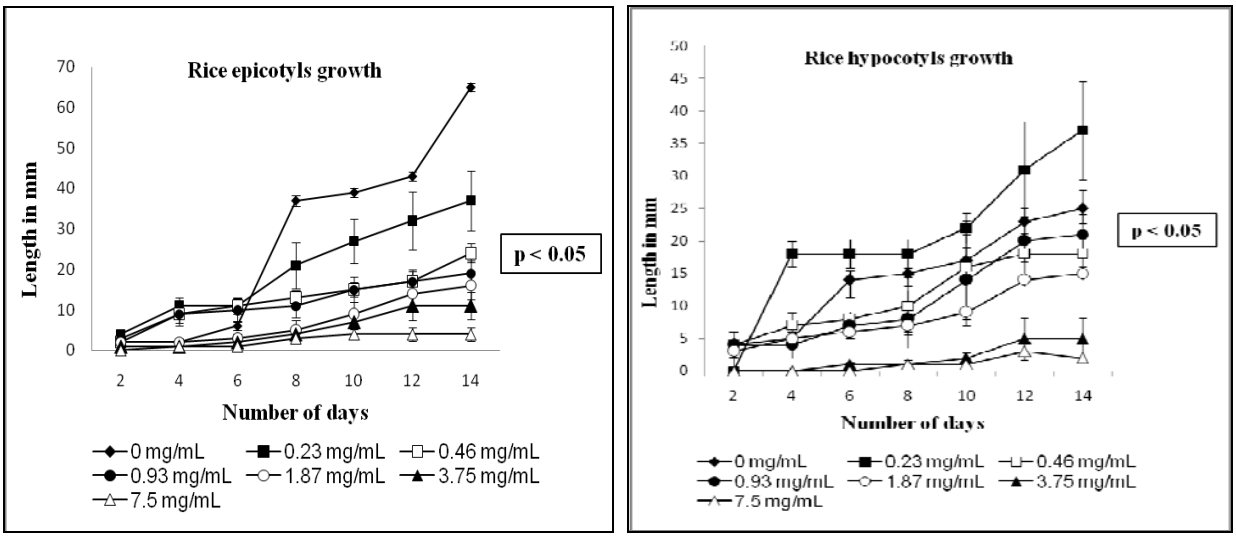

Fig. 6 Rice seedling growth in the presence of crude extract E_{23} from *A. arenicola.*

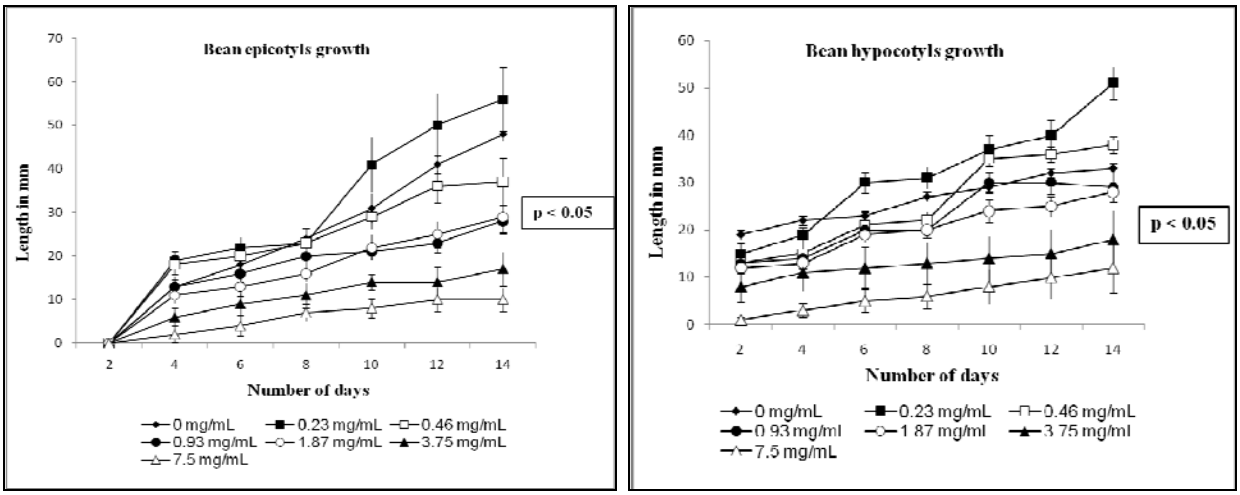

Fig. 7 Bean seedling growth in the presence of crude extract E_{23} from *A. arenicola.*

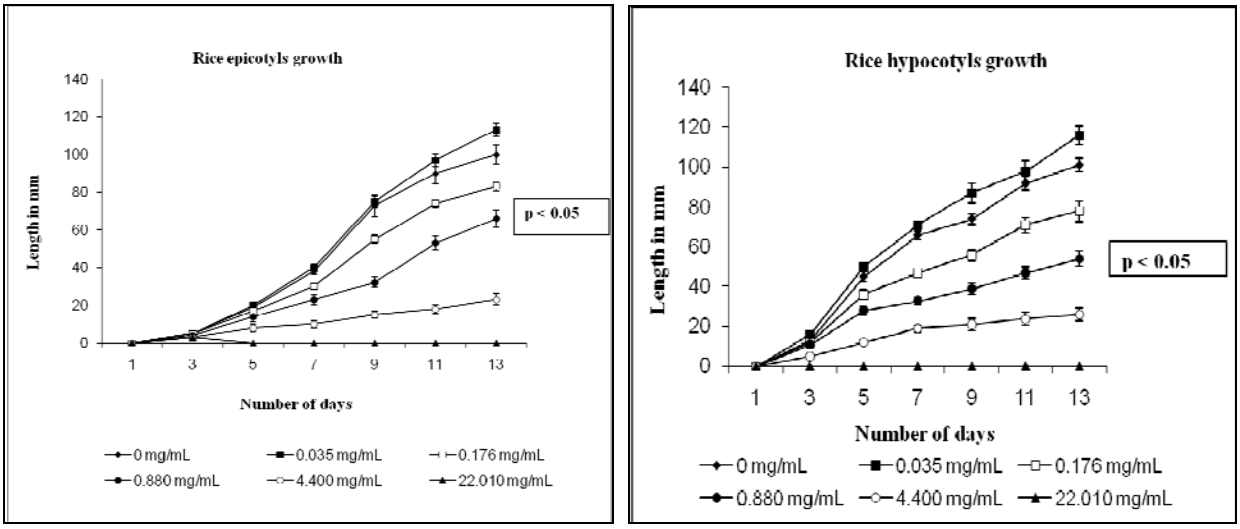

Fig. 8 Rice seedling growth in the presence of crude extract E_{41} from *A. boivini.*

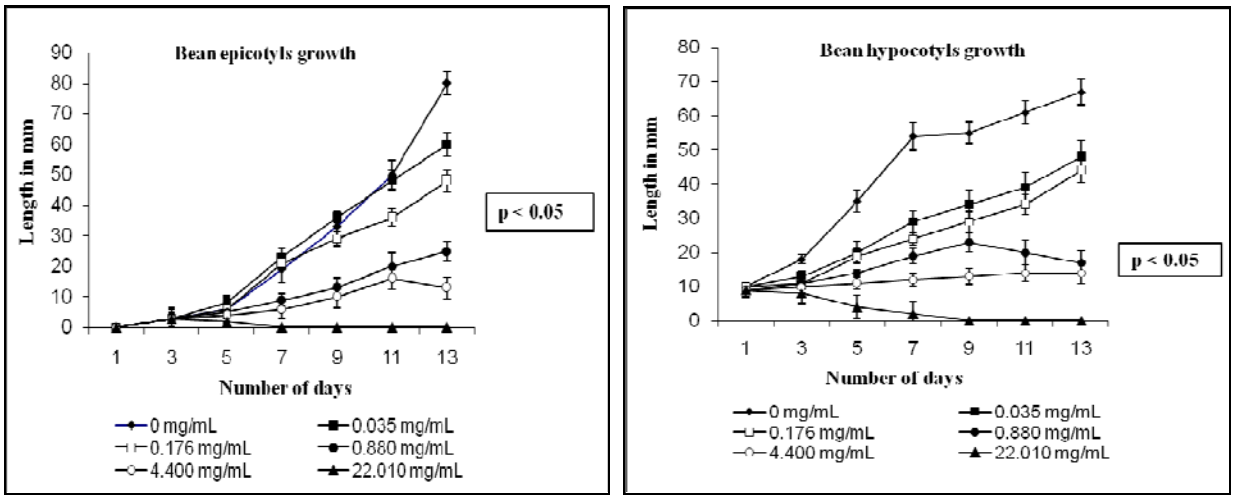

Fig. 9 Bean seedling growth in the presence of crude extract E_{41} from *A. boivini.*

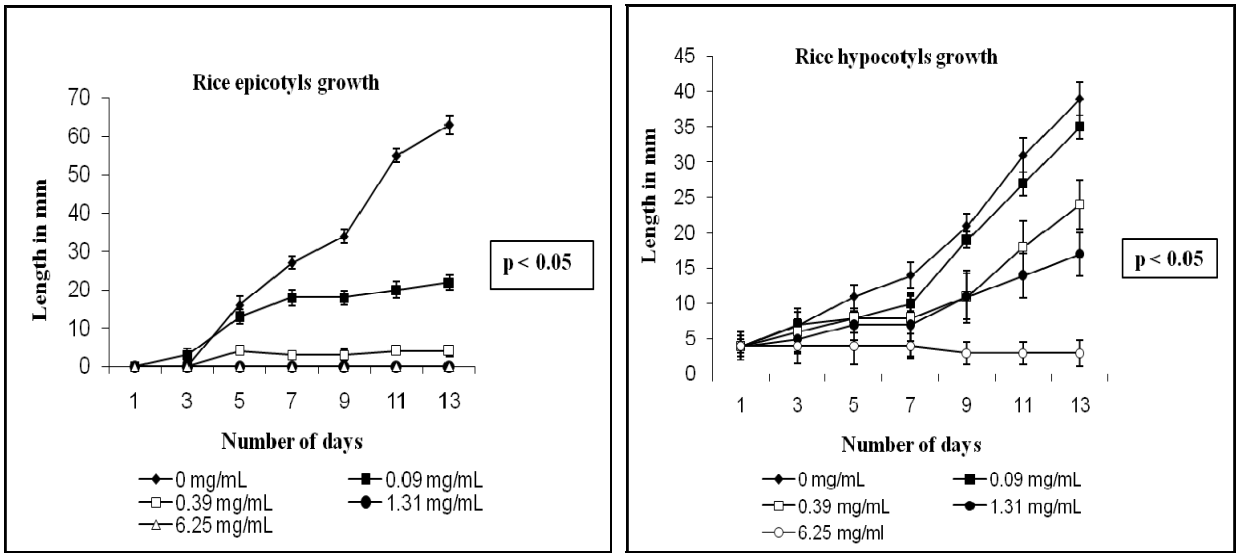

Fig. 10 Rice seedling growth in the presence of purified extract E_5 from *A. bernieri.*

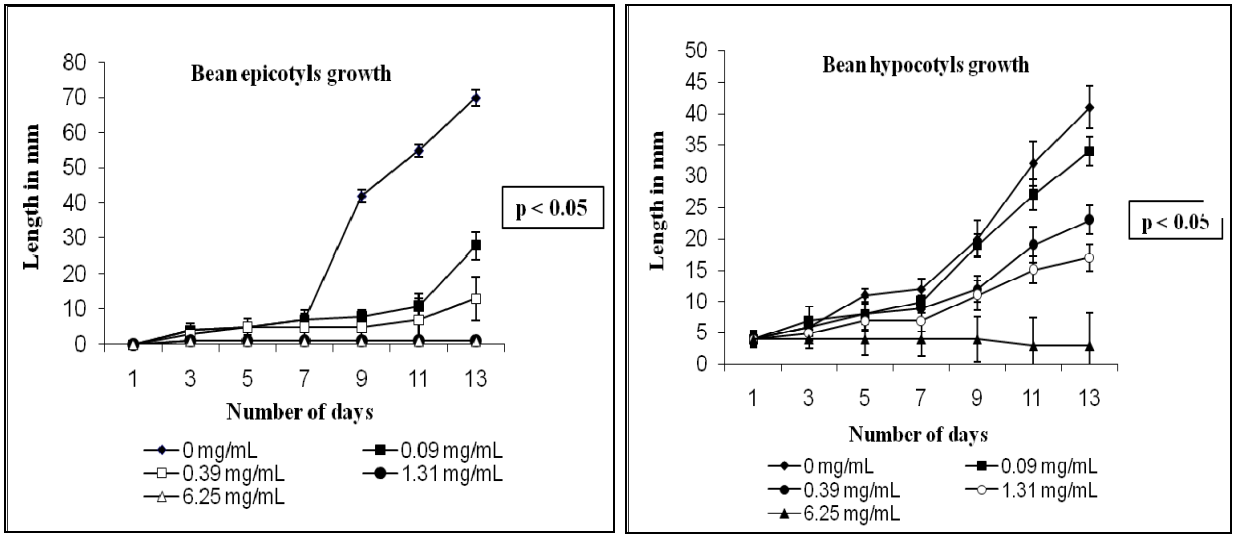

Fig. 11 Bean seedling growth in the presence of purified extract E_5 from *A. bernieri.*

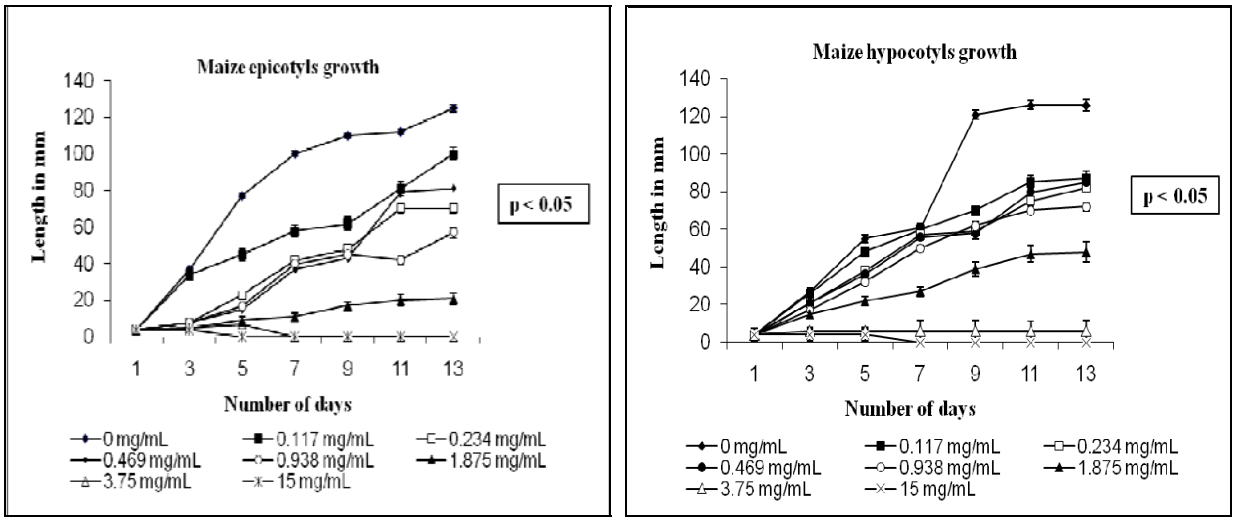

Fig. 12 Maize seedling growth in the presence of purified extract E_6 from *A. polyphylla.*

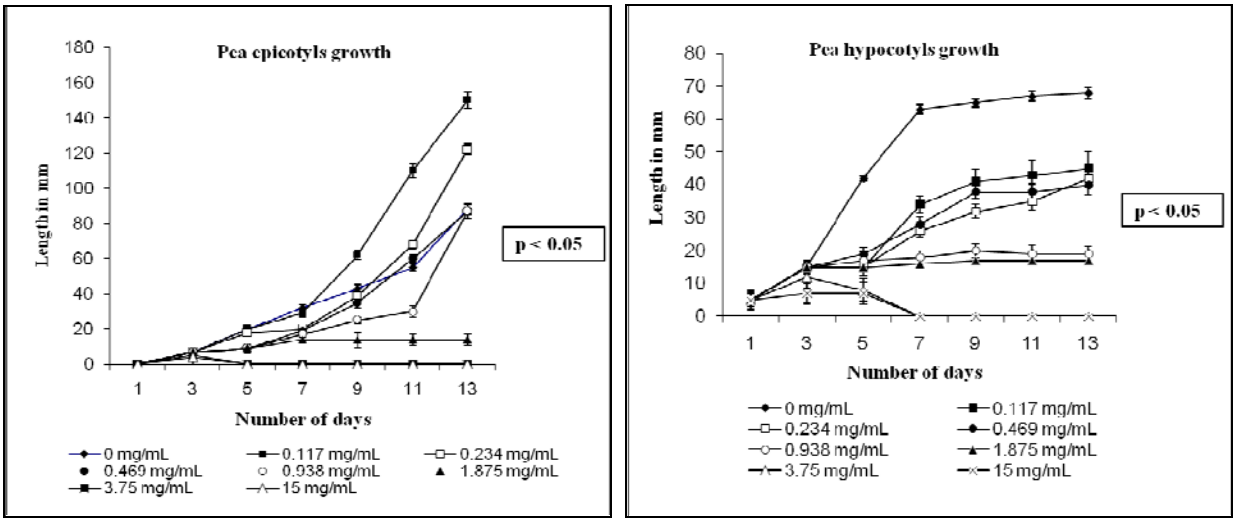

Fig. 13 Pea seedling growth in the presence of purified extract E_6 from *A. polyphylla.*

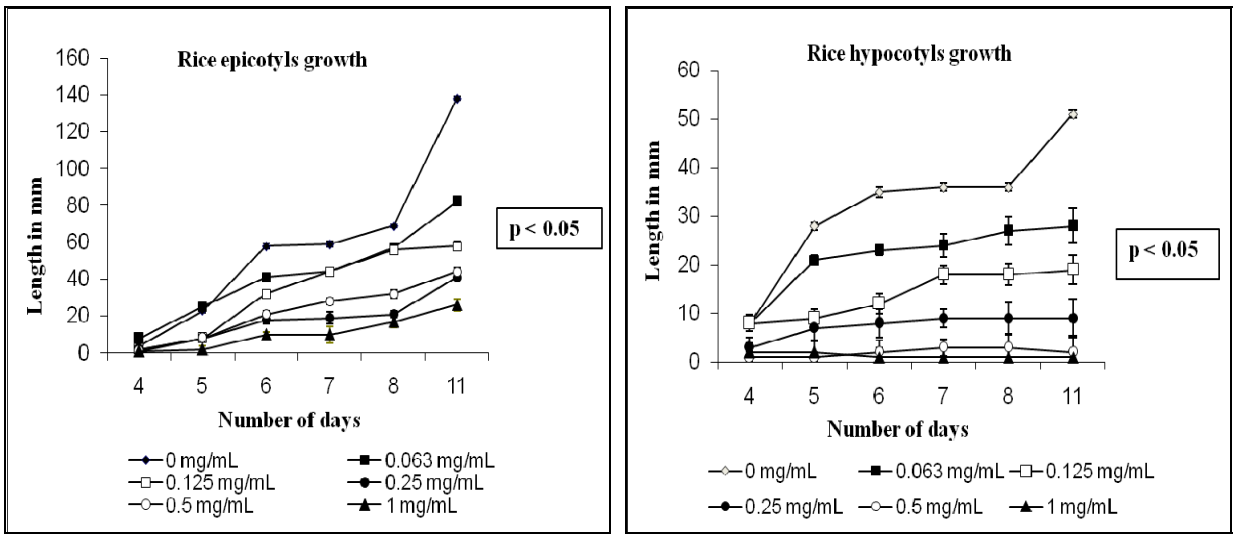

Fig. 14 Rice seedling growth in the presence of crude extract E_{71} from *A. tulearensis*.

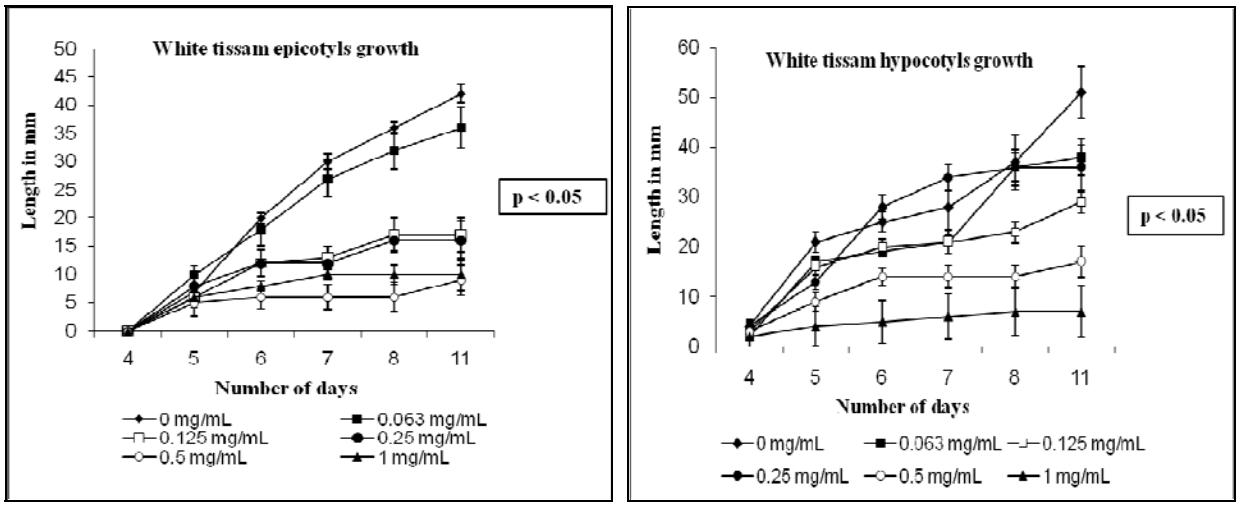

Fig. 15 White tissam seedling growth in the presence of crude extract E_{71} from *A. tulearensis.*

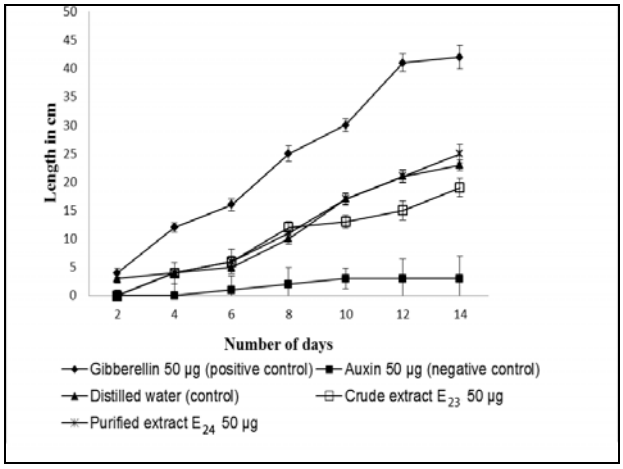

Fig. 16 Pea axillary bud growth in the presence of A_2 extracts.

Table 3 Effects of A_7 extracts on pea axillary bud growth after 8 days.

	Control	Gibberellin (28 µg)	Auxin (28 µg)	E_{71} (255 µg)	E_{72} (133 µg)
Axillary bud length (mm)	43	180	28	127	141

parts of the investigated plants could be due to saponins (A_4, A_5, A_7) or alkaloids (A_2, A_6) which were identified by phytochemical screening as shown in Table 4. Extract legends were shown in Table 2

4. Discussion

Extracts from the five *Albizia* species were demonstrated to disturb seedling development at the used levels in this study. It is of interest to note that this

Table 4 Phytochemical screening of extracts from species A_2, A_4, A_5, A_6 and A_7.

Phytochemical compounds	Extracts						
	E_{23}	E_{24}	E_{41}	E_5	E_6	E_{71}	E_{72}
Alkaloids	+	+	−	−	+	−	−
Flavonoids	−	−	−	−	+	−	−
Anthocyanins	+	−	−	−	−	−	−
Phenols	−	−	−	−	−	−	−
Quinons	−	−	−	−	−	−	−
Unsaturated sterols	+	+	−	+	−	+	+
Triterpenes	+	+	+	+	−	+	+
Deoxysugars	−	−	+	+	−	+	+
Saponins	−	−	−	+	−	+	+

activity is specific for each tested plant since the used vegetables which were the most susceptible during preliminary germination tests were different. On the other hand, the activity of *Albizia* species was exhibited at different concentrations on the same plant-test, indicating various resistance levels of the vegetables. In addition, some *Albizia* extracts showed stimulation effect at low doses. Seedling assay in Petri dishes is a rapid and cheap method for detecting herbicide performance [20]. According to the results here reported, these *Albizia* species or some of them could probably contain principles both specifically active against weed and non toxic on the cultivated plant(s). More accurate studies consisting in assessment of seed weed susceptibility to *Albizia* extracts should provide reliable results for this aspect.

The seedling growth inhibition may be caused by action on different targets such as photosynthesis, nutrients absorption, respiration, metabolism routes. Assays on axillary bud growth were carried out in order to obtain more information on action mechanism of active substances. Thus, extracts from *A. arenicola* (A_2) and *A. tulearensis* (A_7) developed opposite effects against pea axillary bud, confirming the specific character of the activity of the involved principles. In all cases, these extracts appeared to act as auxin and gibberellin on axillary bud elongation. These first results do not yet enable us to precise the action mechanism of these extracts. Besides, future works should include plant-plant interactions aspects.

Saponins and alkaloids were found in *A. boivini* (A_4), *A. bernieri*, (A_5), *A. tulearensis* (A_7) extracts and *A. arenicola* (A_2), *A. polyphylla* (A_6) extracts. This is in accordance with literature data which indicate saponosidic [22-23] and alkaloidic [7, 24] nature of active molecules from *Albizia*. Further chemical studies are required to elucidate number and structure of the involved molecules.

5. Conclusion

In conclusion, this is the first report on the effects of *Albizia* species against other plants. The use of these *Albizia* extracts as herbicides is conceivable. However, toxicological studies must be done to elucidate their mechanism of action and assess their possible noxious effects on health and environment. Their toxicity on microorganisms is already demonstrated [17] and toxicological studies on mammals, insects and other animals are currently undertaken in the laboratory.

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Clerodane and 19-norclerodane diterpenoids from the tubers of *Dioscorea antaly*

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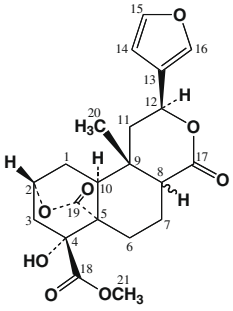
ABSTRACT

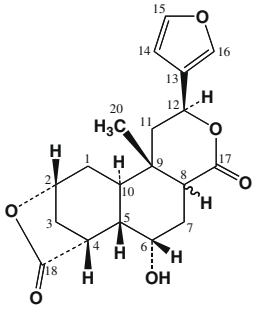
Two clerodane diterpenoids, antadiosbulbins A and B and two 19-norclerodane diterpenes, 8-epidiosbulbins E and G along with the known diosbulbin E as well as nine known phenolics including five phenanthrenes and stilbenes and four flavonoids were isolated from the ethyl acetate soluble part of the methanolic extract of the tubers of *Dioscorea antaly*, a yam endemic to Madagascar. Structures were determined by analysis of the spectral data, mainly 2D-NMR and mass spectrometry.

1. Introduction

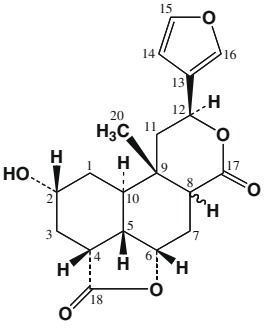
Dioscorea antaly Jum. and H. Perrier (Dioscoreaceae) is a liana endemic to Madagascar and found in the West and North-West regions. In times of scarcity, its tubers are used as food after prerequisite detoxification to remove bitters and toxic principles. As a part of our research program devoted to chemical knowledge and possible commercial use of Madagascar yams, we have chemically investigated this species. The present article describes the isolation from the EtOAc soluble part of the methanol extract of the tubers, and structure determination of the four new diterpenoids, two clerodanes, antadiosbulbins A (**1**) and B (**2**), and two furanoid 19-norclerodanes, 8-epidiosbulbin E (**3**) and 8-epidiosbulbin G (**4**) together with the complete ^{1}H and ^{13}C NMR assignments for the known diterpenoid diosbulbin E (**5**) (Ida et al., 1978a). In addition, nine known phenolics were isolated, which were five stilbene or phenanthrene derivatives, i.e. 3,7-dihydroxy-2,4-dimethoxyphenanthrene (Leong et al., 1997), (*E*)-piceatannol (Brinker and Seigler, 1991), 3,4,3′,5′-tetrahydroxy-dihydrostilbene or dihydropiceatannol (Mannila et al., 1993; Matsuda et al., 2001), the latter being obtained for the first time from a natural source, cassigarol D (Baba et al., 1992), scirpusin B (Nakajima et al., 1978), and four flavonoids, catechin (Davies et al., 1996), 3-*O*-[*β*-D-glucose-(6 → 1)-*α*-L-rhamnose]-kaempferol (Kartnig and Bucar-Stachel, 1991; Markham et al., 1978), kaempferol and quercetin.



Antadiosbulbin A (**1**): 8α-H
Antadiosbulbin B (**2**): 8β-H



8-Epidiosbulbin E (**3**): 8β-H
Diosbulbin E (**5**): 8α-H



8-Epidiosbulbin G (**4**): 8β-H
Diosbulbin G (**7**): 8α-H

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Table 1
NMR data for antadiosbulbins A (**1**) and B (**2**) (400 MHz for ^{1}H).

Position	Antadiosbulbin A (**1**) in CD_3OD				Antadiosbulbin A (**1**) in $CDCl_3$				Antadiosbulbin B (**2**) in CD_3OD				Antadiosbulbin B (**2**) in $CDCl_3$			
	δ_C	δ_H	*Mult.*	*J* (Hz)	δ_C	δ_H	*Mult.*	*J* (Hz)	δ_C	δ_H	*Mult.*	*J* (Hz)	δ_C	δ_H	*Mult.*	*J* (Hz)
1a	28.1	2.32	*dddd*	13.4; 11.2; 5.2; 2.1	27.1	2.31	*m*		28.0	2.34	*dddd*	13.5; 11.2; 5.2; 2.2	27.1	2.27	*m*	
b		1.63	*dd*	13.4; 8.2		1.57	*dd*	13.3; 8.8		1.62	*dd*	13.5; 8.3		1.57	*m*	
2	74.3	4.88	*dd*	5.2; 5.2	72.1	4.90	*dd*	5.2; 5.2	74.1	4.92	dd	5.2; 5.2	71.8	4.90	*dd*	5.2; 5.2
3a	40.2	2.77	*d*	14.8	38.6	2.70	*ddd*	14.9; 1.1; 1.1	39.8	2.76	*d*	15.0	38.2	2.50	*dd*	15.0; 0.7
b		2.13	*ddd*	14.8; 5.2; 2.1		2.26	*m*			2.16	*ddd*	15.0; 5.2; 2.2		2.31	*m*	
4	76.5	–			75.9	–			77.0	–			76.1	–		
5	53.4	–			52.2	–			53.3	–			51.8	–		
6eq	24.1	2.62	*ddd*	15.1; 3.9; 3.0	23.1	2.34	*m*		21.6	2.40	*dddd*	14.8; 3.5; 3.5; 0.8	20.5	2.10	*m*	
ax		2.06	*ddd*	15.1; 14.5; 5.0		2.14	*m*			1.90	*ddd*	14.8; 14.2; 4.0		2.10	*m*	
7eq	19.7	1.91	*dddd*	14.8; 5.0; 3.0; 3.0	18.3	2.04	*dddd*	14.2; 4.8; 3.2; 3.2	19.9	2.30	*dddd*	14.3; 4.0; 3.5; 3.3	19.0	2.39	*dddd*	14.0; 3.8; 3.8; 3.8
ax		1.56	*dddd*	14.8; 14.5; 12.4; 3.9		1.44	*dddd*	14.2; 14.2; 12.0; 3.2		1.75	*dddd*	14.3; 14.2; 4.5; 3.5		1.55	*m*	
8	48.0	2.78	*dd*	12.4; 3.0	46.7	2.48	*dd*	12.0; 3.2	48.1	2.53	*dd*	3.5; 3.3	47.3	2.36	*m*	
9	37.2	–			36.0	–			36.3	–			35.0	–		
10	47.8	2.20	*dd*	11.2; 8.2	46.2	2.09	*m*		38.8	2.08	*dd*	11.2; 8.3	37.1	2.11	*m*	
11eq	44.0	2.07	*dd*	14.5; 6.3	43.6	1.94	*dd*	14.1; 6.4	42.2	2.09	*dd*	14.8; 3.8	41.7	2.03	*dd*	14.8; 3.5
ax		1.86	*dd*	14.5; 12.0		1.83	*dd*	14.1; 11.0		1.86	*dd*	14.8; 12.5		1.75	*dd*	14.8; 12.3
12	71.6	5.50	*dd*	12.0; 6.3	69.9	5.33	*dd*	11.0; 6.4	72.3	5.48	*dd*	12.5; 3.8	70.2	5.27	*dd*	12.3; 3.5
13	125.9	–			124.0	–			126.5	–			124.6	–		
14	109.7	6.48	*dd*	1.8; 0.8	108.4	6.37	*dd*	1.9; 0.9	109.6	6.53	*dd*	1.9; 0.9	108.3	6.38	*dd*	1.9; 0.9
15	145.1	7.48	*dd*	1.8; 1.6	143.8	7.40	*dd*	1.9; 1.7	144.9	7.48	*dd*	1.9; 1.7	143.7	7.39	*dd*	1.9; 1.7
16	141.6	7.56	*ddd*	1.6; 0.9; 0.8	139.6	7.42	*ddd*	1.7; 0.9; 0.9	141.5	7.59	*ddd*	1.7; 0.9; 0.8	139.8	7.43	*ddd*	1.7; 0.9
17	176.7	–			172.9	–			174.5	–			171.0	–		
18	174.0	–			173.9	–			174.0	–			173.6	–		
19	177.2	–			172.1	–			177.0	–			172.3			
20	20.6	0.90	*s*		20.2	0.83	*s*		24.0	0.98	*s*		24.1	0.98	*s*	
OMe	53.3	3.80	*s*		53.5	3.83	*s*		53.4	3.82	*s*		53.4	3.85	*s*	
4-OH	–	–			–	3.45	*s*		–	–			–	3.44	*s*	

2. Results and discussion

The dried and ground tubers of *D. antaly* were defatted with cyclohexane and then extracted with MeOH at room temperature. After concentration to dryness, the MeOH extract was partitioned between EtOAc and water. The EtOAc soluble part on fractionation by a combination of column and MPL chromatographies on silica gel, C-18 reverse phase and Sephadex LH-20, led to the isolation of the four new compounds antadiosbulbins A (**1**, 3 mg) and B (**2**, 3 mg), 8-epidiosbulbins E (**3**, 534 mg) and G (**4**, 26 mg), together with the known diosbulbin E (**5**, 16 mg) and the nine phenolic substances.

Compound **1** was an optically active $[\alpha]_D^{20}$ −45° (*c* 0.7, $CHCl_3$) colourless amorphous solid. Its HR-ESI-MS showed the protonated molecular ion $[M+H]^+$ at *m/z* 405.1519 (calc. for $C_{21}H_{25}O_8$: 405.1548) a molecular formula indicative of ten degrees of unsaturation. The IR spectrum of **1** displayed absorption bands at 1740 (–COO–), 3447 (OH), and 3150 and 1506 cm^{-1} suggesting the presence of a furan ring. The ^{13}C *J*-modulated NMR spectrum (CD_3OD) of **1** exhibited the resonances of 21 carbons consisting of one methyl, one methoxyl at δ_C 53.3, five methylenes, seven methines including three sp^2 carbons at δ_C 109.7, 141.6 and 145.1, seven quaternary carbons including three carbonyls at δ_C 174.0, 176.7 and 177.2 and one ethylenic carbon at 125.9 (Table 1). Taking into account the ten degrees of unsaturation, compound **1** should include five rings. The ^1H NMR spectrum (CD_3OD) displayed typical signals of a methyl singlet at δ_H 0.90, the methoxyl of an ester at δ_H 3.80, three ethylenic protons at δ_H 7.56, 7.48 and 6.48 and two sp^3 oxymethine protons at δ_H 4.88 and 5.50 (Table 1).

Detailed analysis of the ^1H–^1H COSY and HSQC spectra established the presence of the four sub-structures: **a** (>CH–CH_2–CH(O)–CH_2–), **b** (–CH_2–CH_2–CH<), **c** (–CH_2–CH(O)–) and **d** (a monosubstituted furan ring) which are marked with bold bonds in Fig. 1, and further assembled from the cross-peaks observed in the HMBC spectrum. The C=O signal at δ_C 174.0 (C-18) was assigned as a methyl ester at C-18 because of the correlations with protons at δ_H 2.77 (H-3eq) and 4.88 (H-2; $^4J_{H-C}$ *W* coupling) and the methoxyl at 3.80 (CH_3-21) pointing C(O)-4 at δ_C 76.5. Similarly the C=O at δ_C 177.2 was assigned as C-19 due to the correlations with protons at δ_H 2.62 and 2.06 (CH_2-6) and 2.20 (CH-10), and the correlation with the oxymethine proton at 4.88 (H-2) indicated the lactone ring closure to C-2. The second lactone C=O at δ_C 176.7 was assigned to C-17 because it correlated with protons at δ_H 1.91, 1.56 (CH_2-7) and 2.78 (H-8), while its correlation with the proton at δ_H 5.50 (H-12) indicated the second lactone ring closure to C-12. The protons of the methyl at δ_H 0.90 (C-20) were strongly correlated with carbons at δ_C 48.0 (C-8), 37.2 (C-9), 47.8 (C-10) and 44.0 (C-11) indicating this methyl to be linked to C-9, hence sub-structures **a**–**c** could be assembled as shown in Fig. 1. The furan group was linked to C-12, because the quaternary carbon of this ring at δ_C 125.9 (C-13) correlated with the protons at δ_H 5.50 (H-12) and 1.86 (H-11ax). Other HMBC data confirmed this assemblage of sub-structures (**a**–**d**) to form the proposed planar structure for **1** (Fig. 1). This structure was confirmed by analysis of the 1D- and 2D-NMR spectra of **1** in solution in $CDCl_3$ and especially the linkage of the free hydroxyl group at δ_H 3.45 to C-4 (Table 1). The large coupling constant of H-12 with H-11ax (^3J = 11.0 Hz), indicated they were in a *trans* relationship on the half-chair C-ring. A *trans*-diaxial disposition was observed for H-8, which showed a large coupling (^3J = 12.4 Hz) with H-7ax and a gauche coupling (^3J = 3.0 Hz) with H-7eq. The large coupling constant of H-10 (^3J = 11.2 Hz) with one of the CH_2-1 protons indicated it was axial on the B-ring. Methine H-2 gave gauche couplings with only one proton of each of its vicinal methylenes at C-1 (H-1a, at lower fields) and C-3 (H-3b, at higher fields) and in addition a *W* coupling (2.1 Hz) was observed between these two protons (Table 1). These

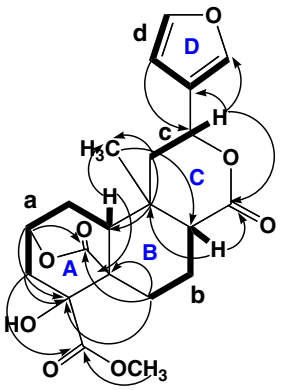

Fig. 1. Sub-structures (**a**–**d**), key COSY (bold bonds) and HMBC (arrows) correlations for antadiosbulbin A (**1**).

data, together with the NOEs observed in both solvents ($CDCl_3$ and CD_3OD) between α-face protons (H-8 with H-7eq, H-10 and H-12; H-10 with H-6ax), or the β-face protons (CH_3-20 with H-1b, H-3a, H-7ax, H-11eq and H-14) whereas H-6eq was correlated with the hydroxyl group at C-4, and the OCH_3-21 with H-3a, H-7ax and CH_3-20, led to the relative configuration proposed in Fig. 2. Hence the structure (relative configuration) of **1** was established as methyl 15,16-epoxy-4-hydroxyclero-13(16),14-diene-17,12;19,2-diolide-18-carboxylate and the name antadiosbulbin A was proposed for this novel furano-clerodane with two δ-lactone rings bridging carbonyl C-19 to C-2 and carbonyl C-17 to C-12.

Antadiosbulbin B (**2**) was an optically active $[\alpha]_D^{20}$ −28° (*c* 0.4, $CHCl_3$) colourless amorphous solid. It depicted the same molecular formula $C_{21}H_{24}O_8$ as antadiobulbin A (**1**) and had similar IR and NMR (in CD_3OD as well as in $CDCl_3$) spectra, suggesting they were isomers (Table 1). Analysis of the COSY spectrum of **2** led to the same sub-structures **a**–**d** as **1** (Fig. 1). When compared with those of antadiosbulbin A, the chemical shifts of carbons C-6, C-9, C-10, C-11 and C-17 of **2** (in $CDCl_3$) were shifted upfield, with $\Delta\delta_C$ = −2.6, −1.0, −9.1, −1.9 and −1.9 ppm, whereas those of C-7, C-8 and C-20 were shifted downfield with $\Delta\delta_C$ +0.6, +1.6 and +3.9, respectively (Table 1), with the upfield shift of C-10 being notable. The second main distinction between the two compounds were the coupling constants of H-8 (CD_3OD), one large and one small for **1** (^3J = 12.4 and 3.0 Hz) and two small for **2** (^3J = 3.5 and 3.3 Hz) due to its β-equatorial disposition with respect to the B-ring in **2**, instead of α-axial as in **1**. This equatorial disposition of H-8 on the β-face was confirmed from the NOESY spectrum where this proton did not give cross-peak with H-12, but gave a strong one with the methyl CH_3-20 which was also correlated with H-1b, H-3a, H-7ax (Fig. 3). The other NOE data indicated for the various chiral centers the same relative configurations as for antadiosbulbin A. With a *cis*-fused A(cyclohexane)/B-ring and a *cis*-fused B/C-ring the structure of **2** was established as methyl 15,16-epoxy-4-hydroxyclero-13(16),14-diene-17,12;19,2-diolide-18-carboxylate and the name antadiosbulbin B was proposed for this new compound **2**, which is thus the 8-epimer of antadiosbulbin A.

Compound **3** was a colourless amorphous solid, $[\alpha]_D^{20}$ −16° (*c* 0.3, $CHCl_3$), with the molecular formula $C_{19}H_{22}O_6$ based on the protonated molecular ion $[M+H]^+$ at *m*/*z* 347.1485 (calc. 347.1493 for $C_{19}H_{23}O_6$) indicative of nine degrees of unsaturation. Detailed analyses of the 2D-NMR spectra allowed full assignment of protons and carbons and indicated that **3** was a furanoid 19-norclerodane derivative (Table 2). The spin systems observed on the ^1H–^1H COSY spectrum (Fig. 4) were connected from the HMBC spectrum to form the norclerodane nucleus and determine the functionalities location. From the NOE data and ^1H–^1H vicinal coupling constants, proton H-2 was β-equatorial due to its gauche *J* values ($J_{H2-H1eq}$ = 4.9 and $J_{H2-H3eq}$ = 5.5 Hz) and H-4 was also β-equatorial because of its small coupling constants (*J* = 5.1 and 1.1 Hz) with H-3eq and H-5ax on the A-ring which is now a chair (Fig. 5). The coupling of H-5 with H-10 (*J* = 12.5 Hz) and the lack of NOE interaction between them indicated they were in a *trans*-diaxial disposition and hence the junction between the A- and B-rings was *trans*. Proton H-12 was α-axial because of its large coupling constant (*J* = 12.3 Hz) with β-axial H-11. The small coupling constants of H-6 and H-8 with the two protons at CH_2-7 indicated that they were both β-equatorial. Finally NOEs were observed between β-face protons (H-8 with CH_3-20, H-7ax and H-11ax; CH_3-20 with H-5 and H-1ax; H-1ax with H-3ax; H-6 with H-4 and H-5), and between α-face protons H-10 and H-12 (Fig. 5). The structure was confirmed by analysis of the 2D-NMR spectra in C_5D_5N (Table 2). Hence compound **3** was 15,16-epoxy-6α-hydroxy-19-nor-clero-13(16),14-diene-17,12;18,2-diolide, the epimer at C-8 of diosbulbin E (**5**), a substance previously isolated from *Dioscorea bulbifera* L. (*forma spontanea* Makino et Nemoto) (Ida et al., 1978a), and thus named 8-epidiosbulbin E. Acetylation of **3** by acetic anhydride in pyridine afforded in good yield the mono-acetate **6**, which had spectral data identical with those of 8-epidiosbulbin E acetate previously

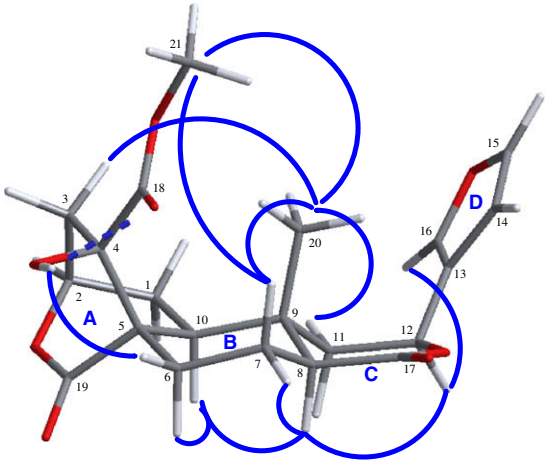

Fig. 2. Selected NOESY correlations (blue lines) and proposed 3D-structure for antadiosbulbin A (**1**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

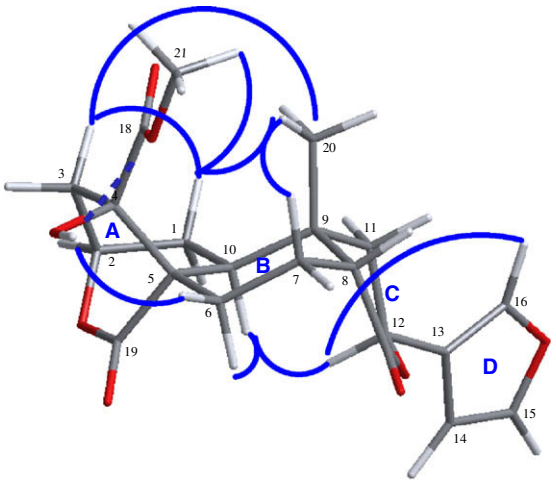

Fig. 3. Selected NOESY correlations (blue lines) and proposed 3D-structure for antadiosbulbin B (**2**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
NMR data for 8-epidiosbulbin E (**3**) and diosbulbin E (**5**) (400 MHz for ^{1}H).

Position	8-Epidiosbulbin E (**3**) in $CDCl_3$				8-Epidiosbulbin E (**3**) in C_5D_5N				Diosbulbin E (**5**) in $CDCl_3$			
	δ_C	δ_H	*Mult.*	*J* (Hz)	δ_C	δ_H	*Mult.*	*J* (Hz)	δ_C	δ_H	*Mult.*	*J* (Hz)
1eq	28.2	2.10	*dddd*	13.3; 6.0; 4.9; 1.7	28.4	1.97	*dddd*	12.3; 6.0; 5.0; 1.5	28.5	2.12	*brd*	13.2
ax		1.45	*ddd*	13.3; 12.2; 0.9		1.31	*ddd*	12.3, 12.1; 1.2		1.42	*ddd*	13.2; 12.5; 0.7
2	78.0	4.85	*dd*	5.5; 4.9	77.6	4.73	*dd*	5.5; 5.0	78.4	4.92	*brdd*	5.3; 5.0
3eq	38.3	2.52	*dddd*	11.6; 5.5; 5.1; 1.7	38.6	2.32	*dddd*	11.5; 5.5; 5.5; 1.5	38.6	2.53	*dddd*	11.7; 5.5; 5.3; 1.9
ax		1.77	*d*	11.6		1.62	*d*	11.5		1.78	*brd*	11.7
4	43.3	2.65	*brdd*	5.1; 1.1	43.6	2.63	*dd*	5.5; 1.8	43.6	2.68	*brdd*	5.4; 1.5
5	42.7	1.80	*ddd*	12.5; 2.0; 1.1	43.4	1.75	*ddd*	12.1; 1.8; 1.5	42.9	1.72	*ddd*	12.4; 2.0; 1.5
6	69.4	4.14	*ddd*	2.5; 2.2; 2.0	68.8	4.19	*brdddd*	3.4; 2.2; 1.5; 1.5	69.4	4.23	*ddd*	2.6; 2.6; 2.0
7eq	29.7	2.75	*ddd*	14.7; 3.2; 2.2	31.2	2.99	*ddd*	14.2; 3.4; 2.0	28.9	2.14	*brdd*	14.6; 2.6
ax		1.88	*ddd*	14.7; 6.3; 2.5		1.91	*ddd*	14.2; 6.5; 2.2		1.75	*ddd*	14.6; 12.2; 2.6
8	45.5	2.25	*dd*	6.3; 2.1	45.9	2.41	*dd*	6.5; 2.0	41.0	3.20	*dd*	12.2; 3.6
9	34.6	–			34.8	–			35.9	–		
10	29.7	2.71	*ddd*	12.5; 12.2; 6.0	30.1	3.09	*ddd*	12.1; 12.1; 6.0	39.0	2.39	*ddd*	12.5; 12.4; 5.5
11eq	39.6	2.08	*dd*	15.4; 3.1	39.5	2.04	*dd*	14.6; 3.1	42.3	2.13	*dd*	15.0; 6.1
ax		1.74	*dd*	15.4; 12.3		1.80	*dd*	14.6; 12.6		1.77	*dd*	15.0; 11.2
12	69.4	5.49	*dd*	12.3; 3.1	69.5	5.56	*dd*	12.6; 3.1	70.0	5.36	*dd*	11.2; 6.1
13	125.0	–			126.4	–			124.2	–		
14	108.5	6.40	*dd*	1.8; 0.9	109.4	6.53	*dd*	1.9; 0.8	108.5	6.39	*dd*	1.7; 1.0
15	143.5	7.37	*dd*	1.8; 1.7	143.9	7.56	*brs*		143.7	7.40	*dd*	1.7; 1.7
16	139.7	7.45	*ddd*	1.7; 0.9; 0.5	140.4	7.59	*brs*		139.6	7.44	*dd*	1.7; 1.0
17	171.8	–			171.8	–			174.2	–		
18	179.2	–			178.3	–			180.0	–		
20	22.0	1.10	*s*		21.9	0.98	*s*		18.3	0.98	*s*	
OH	–	2.30	*brs*		–	5.69	*s*		–	2.35	*brs*	

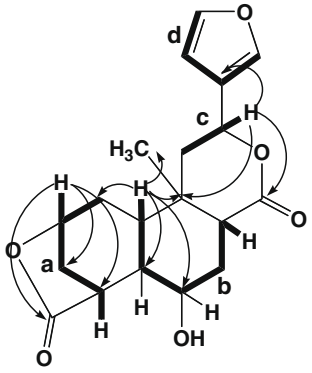

Fig. 4. Sub-structures (**a**–**d**) and key COSY (bold bonds) and HMBC (arrows) correlations for 8-epidiosbulbin E (**3**).

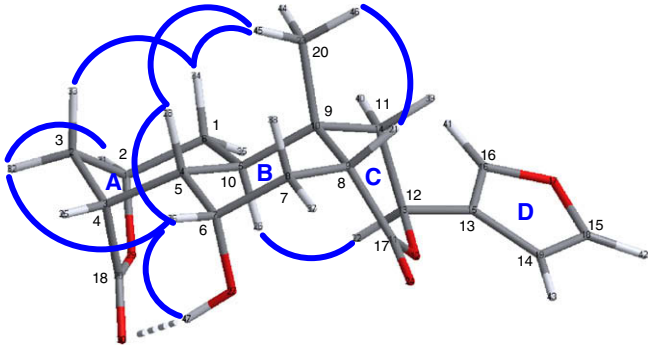

Fig. 5. Selected NOESY correlations (blue lines) and proposed 3D-structure for 8-epidiosbulbin E (**3**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

isolated from *D. bulbifera* L. var. *sativa* (Murray et al., 1984; Shriram et al., 2008). Diosbulbin E (**5**) has been also isolated in the present work and its absolute configuration was established earlier by circular dichroism (Ida et al., 1978a). However, no detailed NMR data for **5** has so far appeared in the literature and we now report its complete ^{1}H and ^{13}C NMR assignments (Table 2). Compared with 8-epidiosbulbin E (**3**), C-7, C-8 and C-20 of **5** (in $CDCl_3$) were shifted upfield by −0.8, −4.5 and −3.7 ppm, whereas C-9, C-10, C-11 and C-17 were shifted downfield by +1.3, +9.3, +2.7 and +2.4 ppm (Table 2). Again, a change from H-8β to H-8α results in a shift of about δ_C +10 ppm for C-10. The NMR data for 6-*O*-coumaroyl derivative of diosbulbin E recently isolated from *D. bulbifera*, are in agreement with the above observation (Wang et al., 2009). Thus **3** is the 8β-epimer of diosbulbin E (**5**).

Compound **4**, another optically active colourless amorphous solid, $[\alpha]_D^{20}$ −20° (*c* 0.3, $CHCl_3$) with molecular formula $C_{19}H_{22}O_6$ exhibited the protonated molecular ion $[M+H]^+$ at *m*/*z*: 347.1403 (calc. 347.1493 for $C_{19}H_{23}O_6$). All the 2D-NMR data indicated that **4** had the same planar structure as diosbulbin G (**7**) (Ida et al., 1978b). The junction of the A- and B-chair rings was *trans* due to the *trans*-diaxial disposition of H-5 and H-10 ($^3J_{H\text{-}5/H\text{-}10}$ = 12.2 Hz). NOEs were depicted between equatorial H-2 and its geminal-hydroxyl and its vicinal protons H-1eq, H-1ax, H-3eq and H-3ax (Fig. 6). NOEs were also observed between β-face protons (H-6 with H-4eq, H-5ax and H-7ax; H-4eq with H-5ax and H-3ax; CH_3-20 with H-1ax, H-5, H-8) and between α-face protons (H-12 with H-10). Thus compound **4** differed from diosbulbin G in the configuration of C-8, H-8 being β in **4** instead of α. The full ^{13}C NMR assignment of diosbulbin G (**7**) in Pyr-d_5, has been published by Ternai and co-workers (Lentini et al., 1986). It is interesting that the chemical shift of C-10 in **4** where H-8 is β, is more than 10 ppm downfield ($\Delta\delta_C$ +13.5) from its shift in diosbulbin G where it is α. The β-orientation of H-8, which implies the α-orientation of the C-17 carbonyl group in 8-epidiosbulbin G (**4**), induces steric constraints which are responsible for this shift, is also true for antadiosbulbin B and 8-epidiosbulbin E. Compound **4** may by thought of as an isomer of 8-epidiosbulbin E (**3**) from which it differs in the direction of cyclization of the C-18 carboxyl which forms a γ-lactone involving C-6 instead of C-2.

Dioscorea species are known to biosynthesize diterpenoids of the clerodane or of the 19-norclerodane types which are responsible of the bitter taste, in addition to alkaloids such as dioscorine and steroid sapogenins responsible for their toxicity (Sautour

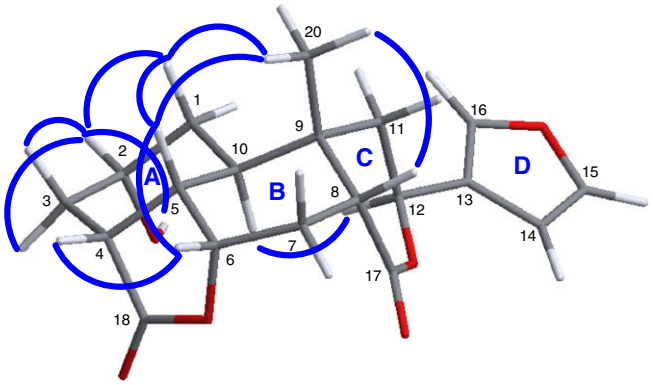

Fig. 6. Selected NOESY correlations (blue lines) and proposed 3D-structure for 8-epidiosbulbin G (**4**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2007). The bitter taste of some Nepalese species has been assigned to bitter components identified as furanoid norditerpenes, especially to diosbulbin B (Bhandari and Kawabata, 2005). Only diterpenoids were isolated from *D. antaly* in the course of our work and no saponins were detected, which suggests that *D. antaly* and *D. bulbifera* are close taxonomically and distinct from other *Dioscorea* species. Only one saponin, a pennogenin glycoside, has been isolated from *D. bulbifera* var. *sativa* (Teponno et al., 2006), all other phytochemical investigations on *D. bulbifera* have not produced any steroid sapogenins (Wang et al., 2009). The water-soluble extract of the tubers of *D. antaly* was slightly toxic when medaka fishes were incubated in a medium containing this extract, with LD_{50} around 0.86 mg/ml (Rakotobe et al., 2010).

In summary, two C-8 epimers antadiosbulbins A and B, as well as two new 19-norclerodanes, 8-epidiosbulbins E and G have thus been isolated from the tubers of *D. antaly* in addition to the known diosbulbin E. A general scheme for the biosynthesis of *Dioscorea* diterpenes from geranyl–geranyl diphosphate might lead to a trihydroxylated tri-carboxylic acid intermediate, in which the asymmetric center at C-8, α to an acid function might undergo racemization (Fig. 7). Cyclization could then yield antadiosbulbins A and B. After decarboxylation, the resulting 19-norclerodane might further be cyclized to yield either diosbubin E and 8-epidiosbulbin E by forming of a lactone ring between the carboxylic function at C-18 and the alcohol at C-2 or diosbulbin G and its epimer at C-8 by cyclization with the alcohol group at C-6.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin Elmer model 341 polarimeter at 20 °C and the $[\alpha]_D$ values are given in deg cm^2 g^{-1}. IR spectra were taken on a Shimadzu FTIR-8400S spectrophotometer. Mass spectra data were recorded using on an API Q-STAR Pulsar I of Applied Biosystems. ^{13}C NMR spectra were recorded on an AC 300 BRUKER spectrometer operating at 75.47 MHz (for ^{13}C). ^{1}H and 2D-NMR spectra were on an Avance-400 BRUKER spectrometer operating at 400.13, equipped with ^{1}H-broad-band reverse gradient probe head. The ^{1}H and ^{13}C NMR chemical shifts are given in ppm relative to TMS, with coupling constants (*J*) reported in Hz. For the HMBC experiments, the delay (1/2*J*) was 70 ms and for the NOESY experiments the mixing time was 150 ms. TLC was carried out on precoated Si gel 60 F_{254} plates (Merck). Spots were detected under UV (254 and 366 nm) before spraying with phosphomolybdic acid solution in EtOH or Liebermann–Burchard reagent or vanillin–sulfuric solution followed by heating the plate at 110 °C. Column chromatography was performed on 200–400 mesh silica gel 60 (Merck). Preparative medium-pressure liquid chromatography (MPLC) was performed with a pump K-120 (Knauer) and Flashsmart cartridges (Si and C-18 gels 20–40 μm, AIT, France).

3.2. Plant material

Tubers of *D. antaly* Jum. and H. Perrier were collected in May 2004 in the Menabe region near Morondava (Beroboka) located 600 km South-West Antananarivo (Madagascar). The plant was identified by Prof. V.H. Jeannoda and voucher specimens (MT 066 to MT 069) were deposited at the Herbarium of the Department of Botany, University of Antananarivo.

3.3. Extraction and isolation

Tubers were washed, cut into small pieces, air-dried and milled. The powdered plant material (546 g) was extracted successively with cyclohexane (4 × 250 ml) and MeOH (4 × 250 ml) at rt and concentrated to dryness under reduced pressure. The crude methanolic extract (15.7 g) was partitioned between EtOAc (200 ml) and water (300 ml) to yield EtOAc (6.2 g) and aqueous (9 g) extracts after evaporation of solvents. The EtOAc-soluble extract (6.2 g) was further chromatographed over silica gel column (300 g) eluted with a cyclohexane/EtOAc gradient of increasing polarity, followed by EtOAc–MeOH gradient to afford 27 fractions (500 ml each). Fractions 11, 12 and 13 (290 mg) which showed similar profiles on TLC were grouped together and purified on Sephadex LH-20 eluted with MeOH/CH_2Cl_2:90/10 to yield 3,7-dihydroxy-2,4-dimethoxyphenanthrene (17 mg).

Purification of fraction 16 (560 mg) by CC on Sephadex LH-20 eluted with MeOH and then by MPLC yielded dihydroxypiceatannol (25 mg), piceatannol (20 mg), compound **1** (7 mg) and a mixture of compound **1** and **2** (161 mg). This mixture was further separated by isochratic MPLC using CH_2Cl_2/EtOAc:98/2 at flow rate of 1 ml/min and furnished further 25 mg of compound **2**. Fraction 17 (1.18 g) was subjected to repeated column chromatography on Sephadex LH-20 eluted with MeOH to furnish 23 sub-fractions (90 ml each) from which sub-fractions 17-7 and 17-23 contained pure catechin (150 mg) and cassigarol D (162 mg), respectively. Flash separation of sub-fraction 17-3 on MPLC with gradient elution CH_2Cl_2/MeOH:99/1 afforded diosbulbin E (**5**, 16 mg), antabiosbulbins A (**1**, 3 mg) and B (**2**, 3 mg). Purification of sub-fraction 17-16 (15 mg) on a C-18 reverse phase flash column (3 g) on MPLC using ACN/H_2O + 0.1% TFA (20:80) yielded scircupsin B (1 mg).

Recrystallisation of fraction 19 (1473 mg) in MeOH yielded 8-epidiosbulbin E (**3**, 534 mg) and that of fraction 20 (559 mg) yielded 8-epidiosbulbin G (**4**, 26 mg). By successive repurification on Sephadex LH-20 followed by MPLC of fraction 21 afforded (3-*O*-[β-D-glucose-(6 → 1)-α-L-rhamnose]-kaempferol) (32 mg). Kaempferol (12 mg) and quercetin (6 mg) were obtained from fraction 22 (313 mg) by chromatography on Sephadex LH-20 eluted with MeOH.

3.3.1. Antadiosbulbin A (*1*)

Colourless amorphous solid; $[\alpha]_D^{20}$ −45° (*c* 0.7, $CHCl_3$); HR-ESI-MS positive mode, *m/z*: 405.1519 $[M+H]^+$, $C_{21}H_{25}O_8$ (calc.: 405.1548); IR ($CHCl_3$) ν_{max} (cm^{-1}): 3447, 3150, 2953, 2928, 1740, 1506, 1448, 1368, 1265, 1157, 1115, 1082; ^{1}H and ^{13}C NMR data, see Table 1.

3.3.2. Antadiosbulbin B (*2*)

Colourless amorphous solid; $[\alpha]_D^{20}$ −28° (*c* 0.4, $CHCl_3$); HR-ESI-MS positive mode, *m/z*: 405.1527 $[M+H]^+$, $C_{21}H_{25}O_8$ (calc.: 405.1548); IR ($CHCl_3$) ν_{max} (cm^{-1}): 3447, 3150, 2953, 1750, 1734, 1505, 1464, 1437, 1204, 1113; ^{1}H and ^{13}C NMR data, see Table 1.

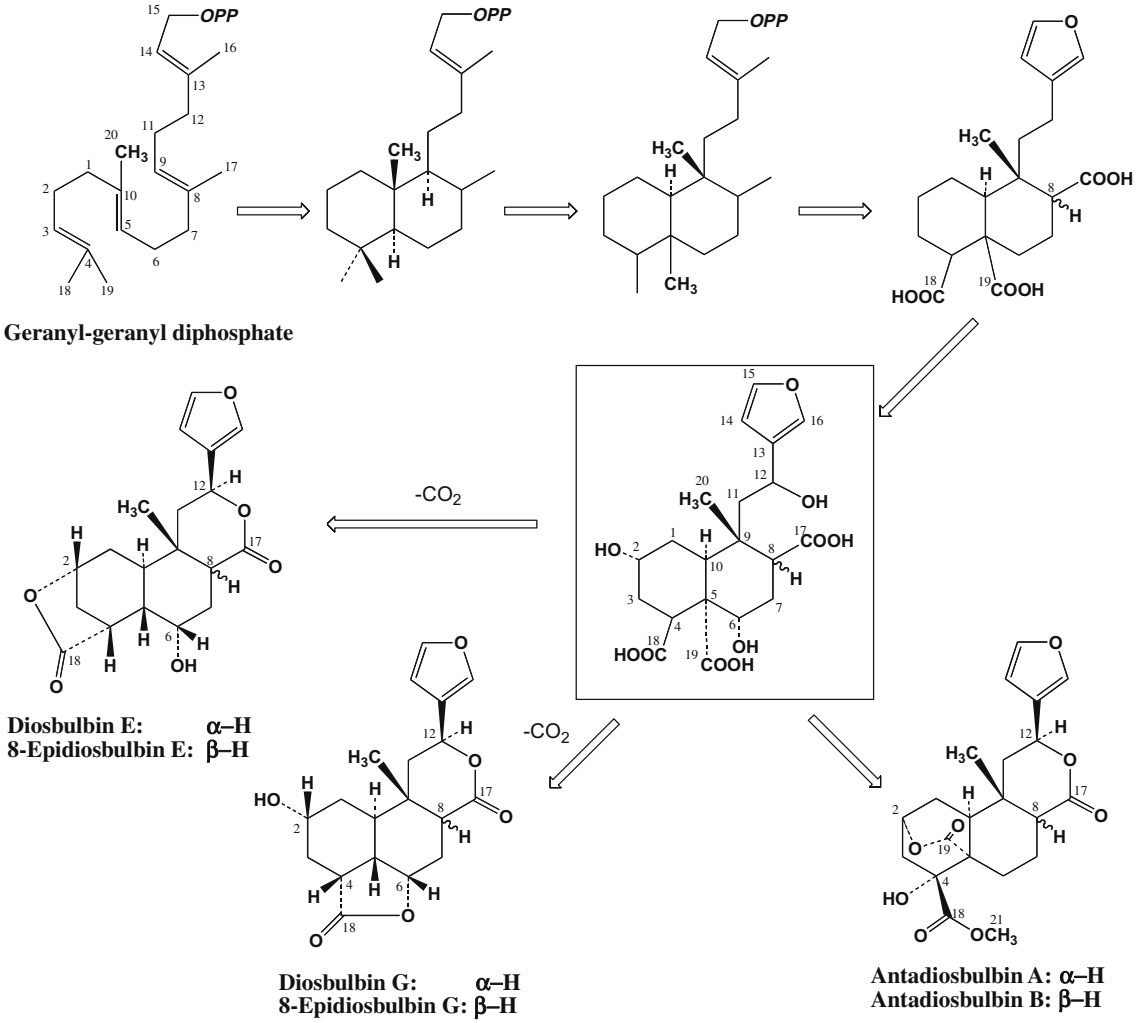

Fig. 7. Plausible pathway for the biosynthesis of the *Dioscorea* diterpenoids.

3.3.3. 8-Epidiosbulbin E (**3**)

Colourless amorphous solid; $[\alpha]_D^{20}$ −16° (*c* 0.3, $CHCl_3$); HR-ESI-MS positive mode, *m*/*z*: 347.1485 $[M+H]^+$, $C_{19}H_{23}O_6$ (calc.: 347.1493); IR ($CHCl_3$) ν_{max} (cm^{-1}): 3636, 3150, 2926, 1744, 1725, 1505, 1204, 1109; ^1H and ^{13}C NMR data, see Table 2.

3.3.4. 8-Epidiosbulbin G (**4**)

Colourless amorphous solid; $[\alpha]_D^{20}$ −20° (*c* 0.3, $CHCl_3$); HR-ESI-MS positive mode, *m*/*z*: 347.1497 $[M+H]^+$, $C_{19}H_{23}O_6$ (calc.: 347.1493); ^1H and ^{13}C NMR data see Table 3.

3.3.5. Diosbulbin E (**5**)

Colourless amorphous solid; HR-ESI-MS positive mode, *m*/*z*: 347.1496 $[M+H]^+$, $C_{19}H_{23}O_6$ (calc.: 347.1493); IR ($CHCl_3$) ν_{max} (cm^{-1}): 3484, 3150, 2930, 1769, 1718, 1503, 1298, 1209, 1147, 1076, 1024; ^1H and ^{13}C NMR data, see Table 2.

3.3.6. 8-Epidiosbulbin E acetate (**6**)

Compound **3** (5 mg) was added to a mixture of 1 ml of pyridine and 1 ml of Ac_2O at room temperature and stirred for 24 h. To accelerate the acetylation, 7 mg of dimethylaminopyridine (DMAP) were added to the reaction mixture and stirring was maintained for 24 h; then 10 ml of aqueous HCl (5%) were added to the reaction mixture. After extraction with CH_2Cl_2 and washing with

Table 3
^{13}C and ^1H NMR data for 8-epidiosbulbin G (**4**) in Pyr-d_5 (400.13 MHz for ^1H).

Position	8-Epidiosbulbin G (**4**)			
	δ_C	δ_H	*Mult.*	*J* (Hz)
1eq	30.6	1.88	*dm*	12.7
ax		1.16	*ddd*	12.7; 12.2; 2.2
2	64.5	4.35	*m*	
3eq	30.3	2.54	*dm*	14.8
ax		1.79	*ddd*	14.8; 7.8; 3.7
4	40.5	2.85	*ddd*	7.8; 6.2; 1.5
5	38.5	2.22	*ddd*	12.2; 6.2; 4.9
6	76.2	4.48	*ddd*	5.9; 4.9; 4.0
7eq	25.8	3.00	*ddd*	15.3; 5.9; 4.0
ax		2.18	*ddd*	15.3; 5.9; 5.9
8	46.3	2.51	*dd*	5.9; 5.9
9	33.5	–		
10	28.7	2.65	*ddd*	12.2; 12.2; 2.2
11eq	40.4	2.23	*dd*	14.6; 2.8
ax		1.83	*dd*	14.6; 12.5
12	70.2	5.82	*dd*	12.5; 2.8
13	126.1	–		
14	109.3	6.51	*dd*	2.0; 1.0
15	144.0	7.54	*dd*	2.0; 1.8
16	140.4	7.59	*brs*	
17	172.1	–		
18	178.2	–		
20	21.6	0.97	*s*	
2-OH	–	5.10	*s*	

10 ml H_2O the organic layers were dried over Na_2SO_4 and concentrated under vacuum. Purification on silica gel column chromatography (cyclohexane/EtOAc:95/5) afforded 3 mg of 8-epidiosbulbin E acetate (**6**).

Compound **6**: colourless amorphous solid; ESI-MS positive mode, *m*/*z*: 389.1607 $[M+H]^+$, $C_{21}H_{25}O_7$ (calc.: 389.1599); ^{13}C NMR ($CDCl_3$): 28.2 (C-1), 76.4 (C-2), 38.6 (C-3), 42.0 (C-4), 41.2 (C-5), 69.0 (C-6), 26.8 (C-7), 45.6 (C-8), 34.5 (C-9), 31.6 (C-10), 39.7 (C-11), 69.6 (C-12), 124.9 (C-13), 108.6 (C-14), 143.9 (C-15), 139.9 (C-16), 170.8 (C-17), 175.7 (C-18), 21.9 (C-20), 170.7 (C-21), 20.9 (C-22). ^1H NMR ($CDCl_3$): 2.10 (1H, *brddd* 13.3, 6.0, 5.0, H-1a), 1.46 (1H, *ddd* 13.3, 12.0, 1.1, H-1b); 4.85 (1H, *dd* 5.5, 4.7, H-2); 2.51 (1H, *dddd* 11.4, 5.7, 5.5, 1.7, H-3a); 1.77 (1H, *d* 11.5, H-3b); 2.57 (1H, *dd* 5.8, 1.6, H-4); 1.92 (1H, *ddd* 12.3, 2.0, 2.0, H-5); 5.15 (1H, *brddd* 2.8, 2.7, 2.7, H-6); 2.83 (1H, *ddd* 15.1, 3.1, 2.1, H-7a); 1.87 (1H, *ddd* 15.1, 6.2, 2.6, H-7b); 2.31 (1H, *dd* 6.2, 2.1, H-8); 2.60 (1H, *ddd* 12.3, 12.0, 4.0, H-10); 2.11 (1H, *dd* 14.7, 3.3, H-11a); 1.78 (1H, *dd* 14.7, 12.6, H-11b); 5.51 (1H, *dd* 12.6, 3.3, H-12); 6.41 (1H, *brdd* 1.9, 0.8, H-14); 7.40 (1H, *brdd* 1.9, 1.6, H-15); 7.48 (1H, *ddd* 1.6, 0.8, 0.8, H-16); 1.16 (1H, *s*, H-20); 1.99 (1H, *s*, H-22).

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LE PRINCIPE TOXIQUE DU BAMBOU CONSOMMÉ PAR *HAPALEMUR AUREUS* N'EST PAS NEUTRALISÉ PAR LA TERRE INGÉRÉE

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SUMMARY

The toxicity of the bamboo shoots of *Cephalostachium viguieri*, eaten by *Hapalemur aureus*, has been tested before and after mixing the crushed plant material with the soil which is also consumed by the animal. Although in most instances, geophagy allows primates to detoxify plant secondary compounds such as tannins or glycoalkaloids, in the present case, the adsorption by the soil was not efficient. Other physiological or behavioral responses are still to be found to explain how the Golden Hapalemur copes with the bamboo toxicity.

INTRODUCTION

L'Hapalémur doré, *Hapalemur aureus*, une espèce dont la découverte est relativement récente (Meier *et al.*, 1987 ; Wright *et al.*, 1987) se nourrit essentiellement des repousses d'un bambou dont la toxicité est bien connue (Glander *et al.*, 1987). Ce prosimien vit en sympatrie avec deux autres espèces du genre *Hapalemur* (*H. griseus* et *H. simus*), dans le Parc National de Ranomafana, sur le versant oriental de Madagascar (Wright, 1997, 1999). Il semble surprenant de trouver dans un même milieu trois espèces d'un même genre et se nourrissant des pousses et des feuillages des bambous, dont les différences de poids corporel ne sont pas particulièrement importantes : 700 g à 1 kg pour *H. griseus*, 1 500 g pour *H. aureus*, et 2 500 g pour *H. simus* (Glander *et al.*, 1992 ; Tan, 1999). La différenciation des niches écologiques porte en fait sur les espèces de bambou consommées, l'accès aux repousses

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des grands bambous très coriaces étant la spécialité de la plus grosse espèce (*H. simus*), tandis que les repousses des petites formes sont davantage consommées par l'Hapalémur gris (*H. griseus*). Pour l'Hapalémur doré, il pourrait sembler que la niche écologique de consommateur de bambou, déjà occupée par deux espèces de poids corporel voisin, entraîne une compétition excessive (Wright & Randrianamanantena, 1989 ; Tan, 1999 ; Wright & Andriamihaja, 2002).

En fait, parmi les bambous du Parc National de Ranomafana, il existe une espèce toxique, *Cephalostachium viguieri* (le bambou géant), qui n'est jamais consommée, ni par l'espèce la plus petite (*H. griseus*), ni par l'espèce la plus grande (*H. simus*), alors que le régime alimentaire de l'Hapalémur doré (*H. aureus*) est centré sur cette espèce, ce qui exclut toute compétition entre les espèces du genre *Hapalemur*. Parmi les produits toxiques du *Cephalostachium viguieri*, Glander *et al.* (1989) ont noté la présence de composés cyanogéniques et l'analyse de la teneur en cyanures du sang de l'Hapalémur doré a révélé un taux anormalement élevé de ces produits. Cependant, d'autres familles chimiques de composés toxiques (glycosides non cyanogéniques) ont été mises en évidence au laboratoire de Toxicologie du Département de Biochimie Fondamentale et Appliquée de la Faculté des Sciences d'Antananarivo. Cette consommation d'une plante très toxique pose le problème des adaptations physiologiques ou comportementales de l'animal.

OBSERVATIONS

Une réponse à cette question aurait pu être fournie par le comportement de géophagie de l'Hapalémur doré. En effet, outre les observations de plusieurs étudiants travaillant sous la direction de P. Wright (Tan 1999, 2000), l'un des guides de Ranomafana, Diamondra, avait observé l'Hapalémur doré descendre au sol pour consommer de la terre, après avoir mangé les pousses du bambou toxique. On connaît bien les effets d'adsorption des substances argileuses sur les tannins et les glycoalcaloïdes. Hladik & Guéguen (1974) en avaient proposé l'hypothèse pour expliquer le comportement de géophagie des primates et Johns (1990, 1999) a pu vérifier expérimentalement (*in vitro*) l'efficacité de ce phénomène d'adsorption.

Les échantillons de la plante consommée, ainsi que du sol prélevé à l'emplacement où l'Hapalémur doré en avait ingéré, ont été rapidement transportés à notre laboratoire, afin d'effectuer les tests de toxicologie sur des souris.

Les extraits, obtenus après broyage de pousses de bambou, selon qu'ils furent effectués en présence ou en l'absence de la terre, donnèrent, après centrifugation, une solution parfaitement limpide dans le premier cas et une solution opalescente lorsque la terre n'était pas jointe au broyat. Mais les deux extraits sont létaux pour les souris (albinos mâles de 25 g) : les animaux ayant reçu, par voie intra-péritonéale, 0,2 ml (12 ml/kg de poids) d'extrait préalablement stérilisé par filtration sur membrane Millipore 0,22 µm sont morts au bout d'une minute, après des crises convulsives. Par gavage intra-gastrique, les effets furent moins rapides, mais également létaux.

CONCLUSION

Ce résultat démontre la présence, dans les pousses du bambou, d'un ou de plusieurs composé(s) toxique(s) non adsorbable(s) par les argiles de la terre ingérée. Il

faut donc chercher d'autres explications, d'ordre physiologique ou comportemental, à la résistance de l'Hapalémur doré à la toxicité de *Cephalostachium viguieri*, qui a permis à ce prosimien de subsister en sympatrie avec d'autres espèces consommatrices de bambous.

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Dilobenol A-G, Diprenylated dihydroflavonols from the leaves of *Dilobeia thouarsii*

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Keywords: Natural products / Prenylated dihydroflavonols / Structural elucidation / Antimicrobial activity / Cytotoxicity

The study of the EtOAc extract of the leaves of *Dilobeia thouarsii* led to the isolation and identification of seven new diprenylated dihydroxyflavonols named dilobenol A-G (1-7). Their structures were elucidated by spectroscopic analysis including UV, IR, 1D and 2D NMR and MS as well as by chemical hydrolysis.

The isolated compounds were assessed for their antibacterial, antiplasmodial and cytotoxic activities. They exhibited moderate growth inhibitory activities against *Staphylococcus aureus, Vibrio* spp., *Bacillus* spp, and *Plasmodium falciparum* without significant toxicity against mammalian cell line L-6.

Introduction

Dilobeia thouarsii Roem & Schult (Proteaceae) syn *D. madagascariensis* Chancerel or *D. boiviniana* Baill. is an endemic tree to Madagascar growing in forest and is characterized by male and female feet bearing slightly different leaves.[1] Its wood is particularly appreciated for its resistance to pathogens such as fungi or insects and is used in house construction and carpentry. The leaves are used in traditional medicine for the treatment of infected wounds and as antihelmintic, diuretic and tonic. It is also used to prevent the risks of abortion.[2] A literature survey revealed that no phytochemical or pharmacological studies have been reported from the genus *Dilobeia* which is constituted by two endemics species to Madagascar: *D. thouarsii* and *D. tenuinervis*. The family Proteaceae with 75 genera is represented by 1500 species and found in Southern hemisphere, particularly in Australia, New Caledonia, Madagascar and South Africa. These species are less common in South America. [3]
Early phytochemical investigations from others Proteaceae showed their richness in the biosynthesis of tropane alkaloids, [4] naphtoquinones,[5] phenols and macrocyclic phenols, [6-8] aryl and cyanogenic glycosides.[9-12] These compounds displayed a wide spectrum of biological activities e.g., antimicrobial,[13-14] antioxidant,[15] cytotoxic,[8] anti-HIV,[16] anti-inflammatory [17] and antiplasmodial.[18]

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In our search of bioactive metabolites from plant extracts, the species *Dilobeia thouarsii* was selected from a screening to find new and active constituents and to explore further their antibacterial properties towards a wide range of Gram positive and Gram negative bacteria.

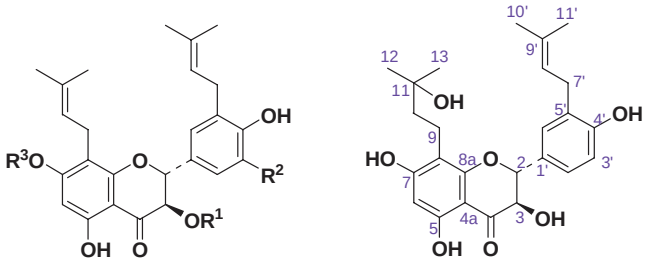
The current study reports the isolation and structural elucidation of seven new flavonol derivatives (**1-7**) from the crude extract as well as their *in vitro* antimicrobial activity against seven strains of Gram negative bacteria, four strains of positive ones and *Plasmodium falciparum*. Their cytotoxic activity was also evaluated.

Results and Discussion

Air-dried and ground leaves of male and female feet of *D. thouarsii* were extracted separately and successively with cyclohexane, EtOAc and MeOH. The two resulting EtOAc extracts displayed antimicrobial activity and showed comparable profile on TLC. Phytochemical investigation was done on EtOAc extract from female feet. Fractionation was performed over silica gel followed by Sephadex LH-20 to yield seven new dihydroflavonol (or flavanonol) derivative compounds **1-7**.

Dilobenol A (**1**) was isolated as a yellow amorphous powder. Its ESI-TOF HRMS spectrum showed a deprotonated molecular ion at *m/z* 439.1740 $[M-H]^-$ (calc. for $C_{25}H_{27}O_7$, 439.1742) corresponding to a molecular formula of $C_{25}H_{28}O_7$ which implied twelve degrees of unsaturation. The UV spectrum showed absorption maxima at 219 and 296 nm indicated a dihydroflavonol derivative.[19-20] The IR spectrum exhibited bands at 3394, 1635, 1292 cm^{-1} characteristic of hydroxyl, saturated carbonyl and olefinic groups.
The ^{13}C NMR spectrum showed the presence of 23 carbons suggesting some overlapped signals according to the molecular formula. They were assigned to a carbonyl carbon, eleven quaternary carbons including five oxygenated, seven methine carbons of which two are oxymetines, two methylene carbons and two methyl groups.
The ^1H spectrum performed in DMSO d_6 indicate the presence of an intense deshielded singlet at δ 11.84, three aromatic protons as

singlets at δ 6.74, 6.64 and 5.97, two olefinic protons at δ 5.23 (t, *J* = 7.2 Hz) and 5.10 (t, *J* = 7.0 Hz) and two oxymethine protons at δ 4.88 (d, *J* = 10.9 Hz) and 4.37(m). In addition, two methylenes were observed at δ 3.01 and 3.19 and the four signals between δ 1.49 and 1.66 are characteristic of methyl groups. Three phenolic hydroxyl protons were observed at δ 10.78, 9.28, 8.27 as broad singlets together with a hydroxyl group at δ 5.69 (d, *J* = 5.9 Hz) as suggested by their absence of correlation on HSQC spectrum. The presence of this deshielded proton at δ 11.84 is due to intramolecular hydrogen bond between hydroxyl and ketone group as indicated its HMBC connectivity with carbon at δ197.8. Two spin systems corresponding to isoprenyl groups (H_2-9, H-10, CH_3-12) and (H_2-7', H-8', CH_3-10') were identified on ^{1}H-^{1}H COSY as supported by long-range connectivities of methyl protons at δ 1.57 (H_3-12) with carbons at δ 25.2 (CH_3-12), 122.5 (C-10) and 130.2 (C-11) for the first. Additional spin system (H-2/H-3/OH-3) was revealed by the coupling between oxymethine protons H-2 and H-3 whereas H-3 is coupled with hydroxyl group at δ 5.69. All these data suggested that compound **1** is a prenylated dihydroflavonol derivative.



1 : R^1 = H, R^2 = OH, R^3 = H
3 : R^1 = Rha, R^2 = OH, R^3 = H
4 : R^1 = Rha, R^2 = H, R^3 = H
5 : R^1 = H, R^2 = OH, R^3 = Glu
6 : R^1 = Rha, R^2 = OH, R^3 = Glu
7 : R^1 = Rha, R^2 = H, R^3 = Glu

2

Figure 1. Structures of compounds **1**-**7** isolated from the leaves of *Dilobeia thouarsii*.

As aromatic protons resonated as singlets, the connectivities of H-2' (δ 6.74) with carbons at 127.5 (C-1'), 119.7 (C-6'), 143.1(C-4'), 144.4 (C-3') and 83.1 (C-2) established that the ring B was oxygenated at C-4' and C-3' and corresponding to a 1, 3, 4, 5-tetrasubstituted benzene. Further correlations of H-7' with C-4' and C-6' allowed its linkage with the isoprenyl group at C-5'.

The remaining proton at δ 5.97 (H-6) correlated with aromatic carbon at δ 95.5 on HSQC spectrum. The upfield shift of this carbon suggested the proximity of two oxygenated aromatic carbons as confirmed its long-range correlations with 160.9 (C-5) and 164.6 (C-7). It displayed also connectivities with two others quaternary carbons at δ 100.4 (C-4a) and 106.9 (C-8). The connection of hydroxyl proton at δ 11.84 with C-6, C-5 and C-4a proved the presence of a pentasubstituted benzene ring. Proton H-9 correlated with C-7 and the downfield aromatic carbon at δ 159.2 (C-8a) on the HMBC spectrum which indicated the attachment of prenyl moiety at C-8 of ring A. Vicinal coupling between H-2 and H-3 along with their connectivities with a ketone at δ 197.8 (C-4) defined the ring C. The junction of ring C to B was determined by the interaction of H-2' and H-6' to C-2 and by those of H-2 and H-3 to C-1'. A β-axial position was attributed to H-2 which developed a *trans*-diaxial relationship with H-3 as suggested the value of their coupling constant (*J* = 10.9 Hz). The NOE interaction between H-2 and OH-3 confirmed their orientation in the same side of the molecule. NOEs correlations between H-3, H-2' and H-6' indicated the proximity of these protons.

This compound is a prenylated derivative at position 8 and 5' of dihydroquercetin or taxifolin. From the above evidence, the structure of **1** was elucidated as 3,5,7,3',4'-pentahydroxy-8,5'-di-(3-methylbut-2-enyl)flavanone and was named dilobenol A.

Dilobenol B (**2**) was obtained as yellow amorphous powder, optically active $[\alpha]_D$ = -5,3 (*c* 0.33, MeOH). A molecular formula was deduced as $C_{25}H_{30}O_7$ by ESI-TOF HRMS at *m/z* 441.1904 $[M-H]^-$ compatible with eleven degrees of unsaturation. It displayed 2 uma higher than compound **1**. The IR absorption bands at 3416, 1715 and 1684 cm^{-1} revealed the presence of hydroxyl and carbonyl functionalities, including an α,β-unsaturated ketone.

The ^{1}H and ^{13}C NMR data showed a pattern of a prenylated flavanonol for compound **2** (Table 1 and 2).

Table 1. ^{13}C NMR data of compounds **1**-**7**

N°	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]	**6**[b]	**7**[b]
2	83.1	84.9	84.0	83.9	85.4	84.2	84.0
3	71.7	73.8	78.5	78.7	73.9	78.7	78.8
4	197.8	198.7	196.2	196.2	197.5	197.2	197.2
4a	100.4	102.2	102.6	102.5	103.2	103.9	104.0
5	160.9	162.9	163.2	163.2	163.1	163.1	163.2
6	95.5	96.8	96.7	96.8	96.7	96.7	96.7
7	164.6	166.5	166.3	166.4	164.9	164.8	164.8
8	106.9	110.0	109.2	109.2	110.0	111.3	111.3
8a	159.2	161.2	160.8	160.8	160.6	160.2	160.2
9	21.0	18.6	22.3	22.4	22.6	22.6	22.6
10	122.5	43.8	123.6	123.7	123.7	123.6	123.6
11	130.2	71.7	131.8	131.7	131.2	132.0	132.0
12	25.2	28.9	25.9	25.9	25.9	25.9	25.9
13	17.6	28.7	17.9	17.9	17.9	17.9	18.1
1'	127.5	129.4	128.5	128.6	129.0	128.3	128.4
2'	112.5	127.4	112.7	127.1	113.3	112.8	127.2
3'	144.4	115.6	143.8	115.8	144.0	146.0	115.8
4'	143.1	156.0	146.0	156.9	145.9	145.0	157.0
5'	133.0	129.4	129.8	129.6	129.5	129.8	129.7
6'	119.7	130.0	120.8	129.7	121.3	120.9	129.8
7'	28.2	29.4	29.1	29.1	29.3	29.1	29.2
8'	122.9	123.9	123.7	123.6	123.9	123.7	123.7
9'	130.9	133.1	133.8	133.4	132.3	133.3	133.4
10'	25.4	17.8	25.9	25.9	25.9	25.9	25.9
11'	17.7	17.7	17.9	17.9	17.9	17.9	18.1
1"			101.9	101.9		102.0	102.0
2"			71.8	71.9		71.8	71.8
3"			72.2	72.2		72.2	72.2
4"			73.9	73.9		73.8	73.8
5"			70.4	70.5		70.5	70.5
6"			17.8	17.8		17.8	17.9
1'"					101.5	101.4	101.5
2'"					78.3	78.2	78.3
3'"					74.9	74.8	74.9
4'"					78.3	78.2	78.3
5'"					71.2	71.1	71.2
6'"					62.4	62.4	62.4

[a] spectra were recorded in DMSO d_6 [b] spectra were recorded in CD_3OD.

In comparison with **1**, differences were noticed for the aromatic protons as well as the disappearance of an olefinic proton suggesting the modification of one of the prenyl moieties. The ^{1}H spectrum showed signals of three protons characteristic of trisubstituted aromatic ring at δ 7.23 (d, *J* = 2.2 Hz, H-6'), 7.18 (dd, *J* =2.2, 8.2 Hz, H-2') and 6.78 (d, *J* = 8.2 Hz, H-3'). The singlet observed at δ 5.97 (H-6) belongs to a pentasubstituted aromatic ring. The proton of a prenyl moiety was observed at δ 5.31 (t, *J* = 8.6 Hz) coupled to a multiplet at δ 3.31 and overlapped singlets at δ1.72. Two doublets were present at δ 4.45 and 4.93 (*J* = 11.2 Hz) corresponding to H-3 and H-2, two methyl groups at δ 1.13 and 1.12, a multiplet at δ 3.31 and a triplet δ at 2.51. As deuterated methanol (CD_3OD) was used as solvent for the realization of spectra, its exchanges with the hydroxyl groups prevent the observation of their signals.

The proton H-2' is shifted downfield and its connectivity with the carbon at δ 156.0 as well as its coupling with proton at δ 6.78 carried by carbon at δ 115.6 (C-3') were indicative of the presence

of a single oxygenated quaternary carbon for ring B instead of two ones in compound **1**.

It was also deduced from analysis of HSQC spectrum that carbon which resonates at δ71.7 is an oxygenated quaternary carbon. On the other hand, the proton at δ 2.51(H_2-9) coupled with those at δ 1.52 (H_2-10) correlated on HMBC spectrum with carbon at δ 71.7.

The connectivities of two methyl protons with the same carbon suggested that an hydroxylation of the prenyl moiety occurred at C-11. This 3-hydroxy-3-methylbutyl substituent was linked to aromatic ring A at position 8 as supported by long-range interaction of protons H_2-10 with C-8.

Table 2. ^{1}H NMR data for compounds **1-7**

N°	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]	**6**[b]	**7**[b]
	δH (*J* in Hz)	δH (*J* in Hz)	δH (*J* in Hz)	δH (*J* in Hz)	δH (*J* in Hz)	δH (*J* in Hz)	δH (*J* in Hz)
2	4.88, d (10.9)	4.93,d (11.2)	4.99,d (10.4)	5.09, d (10.4)	4.86, d (10.5)	5.01, d (10.7)	5.12, d (10.7)
3	4.37, m	4.45,d (11.2)	4.49,d (10.4)	4.53,d (10.4)	4.49, d (10.5)	4.56,d (10.7)	4.60, d (10.7)
6	5.97, s	5.97, s	5.96, s	5.96, s	6.33, s	6.33, s	6.32, s
9a	3.01, d (7.0)	2.51, t (9.7)	3.14, m	3.14, d (7.4)	3.31, m	3.31, m	3.31, m
9 b					3.13, m	3.15, dd (7.2, 13.9)	3.15, dd (7.3, 13.4)
10	5.1, t (7.0)	1.52, m	5.12, t (7.3)	5.14, t (7.4)	5.15, t (7.4)	5.13, t (7.2)	5.16, t (7.3)
12	1.57, s	1.13, s	1.61, s	1.61, s	1.60, s	1.61, s	1.61, s
13	1.49, s	1.12, s	1.54, s	1.53, s	1.53, s	1.57, s	1.55, s
2'	6.74, s	7.18, dd (2.2 ,8.2)	6.82, d (2.0)	7.15, dd (2.1, 8.2)	6.84, d (2.0)	6.83, d (1.9)	7.16, dd (1.8, 8.2)
3'	-	6.78, d (8.2)	-	6.79, d (8.2)	-	-	6.79, d (8.2)
6'	6.64, s	7.23, d (2.2)	6.71, d (2.0)	7.19, d (2.1)	6.76, d (2.0)	6.73, d (1.9)	7.21, d (1.8)
7'	3.19, d (7.2)	3.31, m	3.32, m	3.27, m	3.31, m	3.31, m	3.31, m
8'	5.23, t (7.2)	5.31, t (8.6)	5.30, t (8.7)	5.31, t (7.3)	5.32, t (7.2)	5.31, t (7.4)	5.32, t (7.3)
10'	1.66, s	1.72, s	1.73, s	1.75, s	1.73, s	1.74, s	1.75, s
11'	1.64, s	1.72, s	1.71, s	1.71, s	1.73, s	1.72, s	1.72, s
1"			4.08, d (1.7)	4.07, d (1.7)		4.05, d (1.2)	4.04, s
2"			3.56, dd (1.7, 3.3)	3.53, dd (1.7, 3.3)		3.51, dd (1.2, 3.2)	3.50, m
3"			3.65, dd (3.3, 9.5)	3.64, dd (3.3, 9.5)		3.65, dd (3.2, 9.6)	3.64, dd (3.6, 9.6)
4"			3.29, m	3.30, m		3.31, m	3.31, m
5"			4.22, m	4.19, dd (6.2, 9.5)		4.21 dd (6.2, 9.6)	4.19, m
6"			1.2, d (6.2)	1.17, d (6.2)		1.18, d (6.2)	1.17, d (6.2)
1"'					4.99, d (7.2)	5.00, d (7.0)	4.99, d (7.2)
2"'					3.47, m	3.46, m	3.47, m
3"'					3.48, m	3.37, m	3.47, m
4"'					3.48, m	3.46, m	3.47, m
5"'					3.40, m	3.37, m	3.40, m
6"'a					3.70, dd (5.2, 12.2)	3.70, dd (5.2, 12.2)	3.70, dd (5.1, 12.1)
6"' b					3.88, dd (2.0,12.2)	3.89, dd (1.7, 12.2)	3.88, d (1.5, 12.1)
OH-3	5.69, d (5.9)						
OH-5	11.84, s						
OH	10.78, s						
OH	9.28, s						
OH	8.27, s						

[a] spectra were recorded in DMSO d_6 [b] spectra were recorded in CD_3OD.

At the end, the HMBC correlations of H-6' with C-2, C-2', C-4' and C-7' substantiated that the prenyl group is located at C-5'. The coupling constant of 11.2 Hz established the *trans*-diaxial orientation of protons H-2 and H-3. Thus, compound **2** is a prenylated derivative at position 8 and 5' of dihydrokaempferol. Indeed, dilobenol B (**2**) was identified as 3,5,7,11,4'-pentahydroxy-8-(3-hydroxy-3-methylbutyl),5'-(3-methylbut-2-enyl)flavanone.

Dilobenol C (**3**) was isolated as an amorphous powder. The ESI-TOF HR-MS of **3** gave the molecular formula $C_{31}H_{38}O_{11}$ based on a pseudomolecular $[M-H]^-$ ion peak at *m/z* 585.2317 (calcd for $C_{31}H_{37}O_{11}$, 585.2314) involving thirteen degrees of unsaturation. The ^{13}C spectrum was similar to that of compound **1** and showed five additional oxymethine carbons between δ 70.4 and 101.9. Its ^{1}H spectrum exhibited also signals of five supplemental oxymethine protons at δ 3.29 and 4.22 together with a doublet at δ 1.20 which account for three protons. These NMR data suggested the presence of a sugar moiety as confirmed the correlation of the anomeric proton at δ 4.08 with carbon signal at δ 101.9 on the HSQC spectrum. Furthermore, the coupling of the methyl protons at δ 1.20 (d, *J* = 6.2 Hz) with carbon at δ 17.8 indicated the presence of a 6-deoxy sugar unit.

The ^{1}H-^{1}H COSY spectrum established the spin system H-1"/H-2"/H-3"/H-4"/H-5"/H_3-6". Proton H-5" correlated with carbons C-1", C-3", C-4" and C-6" while H-1" is connected with C-3, C-2" and C-5". This result suggested an ether linkage of the sugar with the flavanonol through C-3 and was supported by the downfield chemical shift of this oxymethine carbon (+7.0 ppm) in comparison to compound **1**.

The relative configuration for the sugar moiety was elucidated by NOESY experiment along with the analysis of vicinal ^{1}H-^{1}H coupling constants as shown in Figure 2.

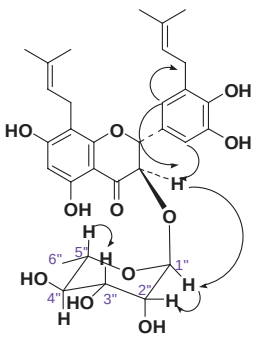


Figure 2. Selected NOE correlations of dilobenol C (**3**)

A β-axial position was attributed to H-3" due to large coupling constant (J = 9.5Hz) with H-4" whereas it showed a gauche coupling (J = 3.3 Hz) with H-2" eq. Protons H-4" and H-5" adopted a *trans*-diaxial orientation in agreement with the correlation of H-3" with H-5" sustained that they are both in the same side of the molecule. The proton H-1" adopted an α-equatorial position as shown the value of its coupling constant (J = 1.7 Hz) with H-2". The α-glycoside linkage was supported by the NOE correlation of H-1" and H-3. The sugar moiety was identified as α-rhamnose. The presence of L-rhamnopyranosyl unit was confirmed by acidic hydrolysis of **3** with TFA 2N followed by TLC analysis of components after their separation from the mixture by extraction with CH_2Cl_2.[21] The derivative which co-eluted with compound **1** (same R_f) was found in the CH_2Cl_2 layer and the sugar which was present in the aqueous fraction showed physical data (TLC analysis, MS and $[\alpha]^{20}_D$) identical to those of the monosaccharide L-rhamnose. Accordingly, the structure of compound **3** was determined as 3,5,7,3',4'-pentahydroxy-8,5'-di-(3-methylbut-2-enyl)flavanone-3-*O*-α-L-rhamnopyranoside.

Dilobenol D (**4**) was isolated as an amorphous powder. Its molecular formula $C_{31}H_{38}O_{10}$ was deduced from its ESI-TOF HRMS deprotonated molecular ion at *m/z* 569.2364 $[M-H]^-$. The ^1H and ^{13}C-NMR data (Table 1, 2) of **4** were closely related to those of **3** and the difference of 16 uma suggested that it is deoxygenated. The substitution patterns of aromatic benzene ring B for compound **4** seemed different and allowed to distinguish the two compounds. The signals of aromatic protons are typical to a trisubstituted ring-B with three protons at δ 7.19 (d, J = 2.1 Hz, H-6'), 7.15 (dd, J =2.1, 8.2 Hz, H-2') and 6.79 (d, J = 8.2 Hz, H-3') suggesting a dihydrokampferol derivative.

Careful analysis of two-dimensional NMR experiments (^1H-^1H COSY, HSQC and HMBC) established that the genuine of **4** corresponding to 3,5,7,4'-tetrahydroxy-8,5'-di-(3-methylbut-2-enyl)flavanone which is identical to lespedezaflavanone C isolated from *Lespedeza davidii*.[22] The coupling constant of 1.7 Hz between H-1" and H-2" was in good accordance with its linkage in α with 6-deoxy sugar unit corresponding to the rhamnopyranoside. From NOESY spectrum, it was observed that compound **4** shares the same relative configuration as **3**. Therefore, dilobenol D (**4**) was identified as 3,5,7,4'-tetrahydroxy-8,5'-di-(3-methylbut-2-enyl) flavanone-3-*O*-α-L-rhamnopyranoside.

The ESI-TOF HRMS spectrum of dilobenol E (**5**) shows a deprotonated molecular ion at *m/z* 601.2259 which is consistent with the molecular formula $C_{31}H_{38}O_{12}$ with 13 degrees of unsaturation. The ^1H and ^{13}C-NMR data were similar to those of **3**. The disappearance of the methyl doublet at δ 1.2 characteristic of rhamnose as well as the difference of molecular weight suggested the modification of the sugar moiety. Furthemore, the upfield chemical shift of the oxymethine carbon C-3 (- 4.6 ppm) in comparison to **3** indicated the presence of a free hydroxyl group at this position whereas H-6 was shifted downfield (+0.37 ppm).

Analysis of ^1H-^1H COSY spectrum established the spin system H-1'''/ H-2'''/ H-3'''/ H-4'''/ H-5'''/ H_2-6'''. The occurrence of two non-equivalent protons at δ 3.70 and 3.88 connected with carbon at δ 62.4 combined with that of a slightly downfield anomeric proton at δ 4.99 (d, J = 7.2 Hz) carried by a carbon at δ 101.5 were indicative of a pyranose moiety with β-configuration.

The linkage of the diprenylated dihydroflavonol with the glucosyl unit was evidenced by the long-range connectivities deduced from the HMBC. The anomeric proton H-1''' correlated with C-2''' and C-7 while H-4''' showed cross-peak with C-3''' and C-5'''. Further support was obtained by the NOESY experiment which revealed the interaction of H-6 and H-1". The coupling constant of 7.2 Hz at δ 4.99 is in agreement with a *trans*-diaxial between the protons H-1" and H-2" in a β attached D-glucopyranose. The lack of sufficient quantity didn't allow the acidic hydrolysis of this compound. Thus, Dilobenol E was characterized as 3,5,7,3',4'-pentahydroxy-8,5'-di-(3-methylbut-2-enyl)flavanone-7-*O*-β-D-glucopyranoside.

Dilobenol F (**6**) was isolated as an amorphous powder, optically active $[\alpha]_D$ = -35 (*c* 0.1, MeOH). Its HRESI-TOF spectrum exhibited a deprotonated molecular ion at *m/z* 747.2836 $[M-H]^-$ leading to a molecular formula of $C_{37}H_{48}O_{16}$ (calcd. 747.2834). The ^1H and ^{13}C of **6** displayed similarities to those of **3** (Table 1 and 2). It was noticed the presence of supplemental five oxymethine carbons and one oxymethylene carbon on ^{13}C spectrum compared to **3**. Furthemore the difference of 162 uma suggested that compound **6** is constituted of disaccharide moieties. Comparison of their ^1H spectra showed additional protons between δ 3.15 and 5.01 and H-6 was shifted downfield (+ 0.37 ppm) for **6**.

Analysis of HMBC and NOESY spectra allowed establishing the position where the two glycosyl moieties are linked with the diprenylated dihydroflavonol. The correlation of proton at δ 4.05 (H-1", d, J = 1.2 Hz) with C-3 together with NOEs interaction of H-1", H-2' and H-3' with H-3 revealed the α linkage of rhamnose at C-3. On the other hand, connectivity of H-1''' with C-7 along with the cross-peak of H-6 and H-1''' observed on the NOESY spectrum confirmed the β linkage of glucose at C-6. Acidic hydrolysis, described above, was performed also on compound **6**. It afforded the diprenylated dihydroflavanone corresponding to compound **1** and the monosaccharide components were identified as D-glucose and L- rhamnose. Finally, the structure of dilobenol F was established as 3,5,7,3',4'-pentahydroxy-8,5'-di-(3-methylbut-2-enyl)flavanone-3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside.

Dilobenol G (**7**), an amorphous powder, had a molecular formula of $C_{37}H_{48}O_{15}$ deduced from the deprotonated ion peak at *m/z* 747.2719 $[M-H]^-$ from the ESI-TOF HRMS. Its ^1H spectrum was quasi identical to that of dilobenol F (**6**) except the presence of signal of three aromatic protons instead of two. This result indicated the presence of trisubstituted aromatic ring-B as for compound **4**. The full assignments of all proton and carbon shifts of **7** was done by 2D NMR experiments. The signal for a β-glucoside (J = 7.2 Hz) moiety was observed in the ^1H and ^{13}C (Table 1 and 2). As consequence of a small coupling between H1"-H2" ($J \approx 0$ Hz), H-1" in equatorial position resonates as singlet suggesting an α-rhamnoside. Thus, compound **7** was identified as 3,5,7,4'-tetrahydroxy-8,5'-di-(3-methylbut-2-enyl)flavanone-3-*O*-α-rhamnopyranoside-7-*O*-β-glucopyranoside.

Compounds **1-7** were evaluated for their antibacterial activities against a panel of seven strains of Gram-negative bacteria including *Pseudomonas aeruginosa*, *Vibrio harveyi* and *V. fischeri*, *Salmonella antarctica* and *S.* Typhimurium, *Escherichia coli*, and *Klebsiella pneumoniae* being given that the crude EtOAc extract was active on these strains.

The activity was also determined against *Bacillus cereus*, *B. megaterium*, *Enterococcus faecalis* and *Staphylococcus aureus* which are Gram-positive bacteria. The results are summarized on Table 3. Hexane extract which was not active towards all tested strains has not been shown. The disc diffusion assay showed that the EtOAc extract was more effective than the MeOH extract for the majority of strains. [13-14] They both displayed similar effect towards *V. fisheri* and *Bacillus* spp. The EtOAc extract exhibited comparable activity towards *Vibrio harveyi* than the standard antibiotics gentamicin and tetracycline used as controls. Moreover, all isolated compounds were inactive against the most tested strains (Table 3).

All compounds except compounds **3** and **5** displayed moderate activity against *Vibrio harveyi*. Compounds **1**, **3**, **4**, **6** and **7** were also active against *V. fischeri*. On the other hand, compounds **1, 2** and **4** were active against *Bacillus cereus* and *Staphylococcus aureus* whereas **1** displayed some activity on *B. megaterium*. In comparison with controls, these compounds exhibited moderate antibacterial activity at the tested concentration (30 µg/disc). Perry and Brennan have shown that the inhibition diameter on solid cultures is dose dependent.[14] The active phenolic glycoside ester isolated from *Toronia toru* (Proteaceae) inhibited the growth of *B.*

subtilis, *E. coli* and *P. aeruginosa* at 120 µg/disc (inhibition diameters of 5, 4 and 3 mm respectively) but not at 30 µg/disc.

Table 3. Antibacterial activity of extracts and compounds 1-7[a]

Microorganism											
	Gram negative bacteria							Gram positive bacteria			
Samples[a]	Pa	Vh	Vf	ST	San	Ec	Kp	Bc	Bm	Ef	Sau
EtOAc ext.	7 ± 0.0	19± 0.0	11± 0.0	8 ± 0.1	7 ± 0.1	7 ± 0.4	7 ± 0.6	11 ± 0.0	11± 0.1	9 ± 0.6	10 ± 0.0
MeOH ext.	-	9 ± 0.1	10 ± 0.2	-	-	-	-	10 ± 0.0	10± 0.1	-	-
1	-	7 ± 2.2	7 ± 0.0	-	-	-	-	7 ± 2.0	8 ± 0.1	-	7 ± 1.0
2	-	7 ± 1.3	-	-	-	-	-	8 ± 0.0	-	-	8 ± 0.3
3	-	-	8 ± 0.0	-	-	-	-		-	-	-
4	-	7 ± 0.0	9 ± 1.5	-	-	-	-	7 ± 1.0	-	-	7 ± 0.0
5	-	-	-	-	-	-	-	-	-	-	-
6	-	9 ± 0.0	9 ± 1.6	-	-	-	-	-	-	-	
7	-	8 ± 0.0	8 ± 0.0	-	-	-	-	-	-	-	-
Gentamycin	19.0±0.0	13.0±0.0	19.5±0.0	19.0±0.0	26.0±0.0	30.5±0.5	19.5±0.5	25.5±0.5	22.5±0.2	20.0±0.0	20.0±0.5
Tetracycline	nd	19.5±0.0	-	14.0±0.0	22.0±0.0	11 ±0.2	13.0±0.5	20.5±0.5	18.0±0.5	8 ±0.5	22.0±0.5

[a] Samples: crude extract was tested at 1 mg/disc, compounds 1-7 at 30µg/disc, gentamycin at 10µg/disc, tetracycline at 30µg/disc. Results are expressed as zones of growth inhibititon (mm). (-) no zone of inhibition. nd: not determined. [b] Microorganisms: Pa : *Pseudomonas aeruginosa*, Vh: *Vibrio harveyi*, Vh: *Vibrio fischeri*, ST: *Salmonella* Typhimurium, San: *Salmonella antartica*, Ec: *Escherichia coli*, Kp: *Klebsiella pneumoniae* , Ef: *Enterococcus faecalis*, Bc: *Bacillus cereus*, Bm: *Bacillus megaterium*, Sau: *Staphylococcus aureus*

This species of Proteaceae contains other antibacterial compounds such as hydroquinone and tulipalin A that showed higher inhibition zone towards *B. subtilis* (9 and 10 mm respectively).

Globally, the crude extract, exhibited higher growth inhibition than the pure compounds **1-7** for all strains suggesting the occurrence of synergy between compounds. It has been reported in the literature that the inhibitory activity of a crude plant extract results from a complex interaction between its different constituents, which may produce, additive, synergistic or antagonistic effects, even for those present at lower concentrations. [21, 22]

As it whas been reported in the literature that some plants of Proteaceae family exhibited significant antiplasmodial and cytoxicity activities, [8, 16] all compounds were tested for their inhibitory capacity against the *in vitro* development of the chloroquine-resistant strain FcB1 of *Plasmodium falciparum* (Table 4).

Table 4. *In vitro* antiplasmodial and cytotoxic activities of compounds **1-7**

	IC_{50} ± SD (µg/ml) FcB1	IC_{50} ± SD (µM)FcB1	IC_{50} ± SD (µg/ml) L-6	IC_{50} ± SD (µM) L-6
1	10.9 ± 0.2	24.8 ± 0.5	28.3 ± 0.6	64.3 ± 1.0
2	15.2 ± 0.3	34.3 ± 0.6	27.8 ± 4.0	62.9 ± 9.0
3	9,40 ± 1.5	16.0 ± 2. 6	51.9 ± 3.6	88.6 ± 6.1
4	9 ± 0.8	15.8 ± 1.4	33.5 ± 0.2	58.8 ± 0.4
5	15.2 ± 0.6	25.3 ± 1.0	54.8 ± 3.4	91.0± 5.6
6	23 ± 2.2	30.8 ± 2.9	>100	> 134
7	15.2 ± 1.3	20.8 ± 1.8	>100	> 137
CQ	0.0532±0.008	0.103 ± 0.015	nd	nd

[a] Results are expressed as IC_{50} values (µM) ± standard deviations. All experiments were realised in triplicate. Chloroquine (CQ) was used as positive control for antiplamodial activity. nd: not determined

Compounds **3** and **4** were the most active with similar IC_{50} values (~16 µM). All the other compounds had IC_{50} value ranging between 20 and 34 µM. They all displayed moderate antiplasmodial activity in comparison with chloroquine used as control. The evaluation of their cytotoxicity against the rat cell line L-6 showed that they were also devoted of cytotoxicity with IC_{50} values up to 58.8 µM.

Conclusions

Seven new diprenylated flavanonol (dilobenol A-G) of which four dihydroquercetin and three dihydrokaempferol derivatives were isolated from the leaves of female feet of *Dilobeia thouarsii.* This is the first report of constituents from the genus *Dilobeia.*

Experimental Section

General Experimental Procedures

Optical rotations were measured on a Perkin Elmer model 341 polarimeter at 20 °C. IR spectra were taken on a Shimadzu FTIR-8400S spectrophotometer. Mass spectra data were recorded using an electrospray time of flight mass spectrometer (ESI-TOF-MS) operating in the positive mode (QSTAR Pulsar I of Applied Biosystems). ^{13}C NMR spectra were recorded on an AC 300 BRUKER spectrometer operating at 75.47 MHz (for ^{13}C). ^{1}H and 2D-NMR spectra were recorded on an Avance-400 BRUKER spectrometer operating at 400.13 MHz, equipped with ^{1}H-broad-band reverse gradient probe head. Temperature was controlled by a Bruker BCU-05 refrigeration unit and a BVT 3000 control unit. The ^{1}H and ^{13}C NMR chemical shifts are given in ppm relative to TMS, with coupling constants (*J*) reported in Hz. For the HMBC experiments, the delay (1/2 *J*) was 70 ms and for the NOESY experiments the mixing time was 150 ms. Analytical and preparative TLC were carried out on precoated Si gel 60 F_{254} plates (Merck). Spots were detected under UV (254 and 366 nm) before spraying with with vanillin-sulfuric acid solution in EtOH followed by heating the plate at 110 °C or with 2% ethanolic ferric chloride reagent. Column chromatography was performed on 200-response curve, and the results were expressed as the mean from three independent experiments. Chloroquine diphosphate (Sigma Aldrich Chimie SARL, St Quentin Fallavier, France) was used as positive control of the antiplasmodial activity.

400 mesh silica gel 60 (Merck) and on Sephadex LH-20 (25–100µm; Pharmacia Biotech Ltd). Preparative medium-pressure liquid chromatography (MPLC) was performed with a pump K-120 (Knauer) and Flashsmart cartridges (Si and C-18 gels 20-40 µm, AIT, France).

Plant material

The leaves and stem bark of *Dilobea thouarsii*, Proteaceae were collected in the Mandraka area at 70 Km of Antananarivo in April 2008 and November 2008. This species was identified by Dr Rabarison Harison, Department of biology and vegetal ecology, University of Antananarivo. A voucher specimen was deposited in the Herbarium of the University under number HERB/DBEV/4708.

Extraction and isolation

Air-dried powdered leaves of *Dilobeia thouarsii* (275 g) were extracted successively with cyclohexane, EtOAc and MeOH to afford after evaporation of solvent 1.78 g, 9.75 g and 32 g of corresponding extracts, respectively. A portion (6 g) of the EtOAc extract was chromatographed on a silica gel column using a mixture of cyclohexane/EtOAc / MeOH of increasing polarity as eluant to give 12 fractions. Fraction F4 (102 mg) was purified by silica gel eluted with CH_2Cl_2/MeOH (98:2 to 90:10) gradient to yield 14 subfractions. Subfractions F4-5 (21 mg) and F4-8 (8 mg) were subjected to Sephadex® LH-20 eluted with MeOH to furnish compounds **1** (2 mg) and **2** (4mg). Fraction F6 (239 mg) was submitted successively to MPLC eluted with CH_2Cl_2/MeOH (95:5 à 90:10) gradient and to silica gel eluted with CH_2Cl_2/MeOH (95 :5) to afford dilobenol D (**4**, 4 mg). Fraction F8 (73 mg) was chromatographed on silica gel eluted with CH_2Cl_2/MeOH (90:10, 80:20) gradient to give **3** (10 mg). Fraction F9 (1156 mg) was subjected to silica gel chromatography eluted with increasing gradient of CH_2Cl_2/MeOH to provide 18 fractions. Compounds **7** (7 mg) and **5** (2 mg) were obtained from F9-16 (245 mg) through Sephadex® LH-20 eluted with MeOH/H_2O (90:10) followed by MPLC on RP-18 silica gel eluted with MeOH/H_2O (30:70 to 80/20). Chromatography of subfraction F9-17 (200 mg) by repeated Sephadex® LH-20 eluted with MeOH/H_2O (90:10) furnished **6** (40 mg).

Dilobenol A (1): Yellow amorphous powder; $[\alpha]^{20}_D$ -23.3 (*c* 0. 12, MeOH); UV (MeOH) λ_{max} (log ε) 219 (4.04), 296 (3.79), 345 (sh) nm ; IR ($CHCl_3$) ν_{max} 3394, 2924,1635, 1446, 1292, 1080, 999 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1 and 2; HRESI-MS *m/z*: 439.1740 $[M-H]^-$ (calcd for $C_{25}H_{27}O_7$, 439.1742).

Dilobenol B (2): Yellow amorphous powder; $[\alpha]^{20}_D$ -5.3 (*c* 0.325, MeOH); UV (MeOH) λ_{max} (log ε) 220 (3.75), 294 (3.52), 347 (sh) nm; IR ($CHCl_3$) ν_{max} 3360, 2924,1635, 1508, 1439, 1261, 1134, 1076 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1 and 2; HRESI-MS *m/z*: 441.1904 $[M-H]^-$ (calcd for $C_{25}H_{29}O_7$, 441.1898).

Dilobenol C (3): White amorphous powder $[\alpha]^{20}_D$ -25.5 (*c* 0.26, MeOH); UV (MeOH) λ_{max} (log ε) 219 (4.03), 294 (3.77), 347 (sh) nm; IR ($CHCl_3$) ν_{max} 3410, 2924,1640, 1446, 1284, 1080 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1 and 2; HRESI-MS *m/z*: 585.2317 $[M-H]^-$ (calcd for $C_{31}H_{37}O_{11}$, 585.2314).

Acid hydrolyse of 3:

Compound 3 (6 mg) was refluxed in 2N aqueous CF_3COOH (2 mL) for 3 h. After cooling, the reaction mixture was diluted with 2 mL of H_2O and extracted with CH_2Cl_2. The organic layer was washed with a saturated solution of $NaHCO_3$, dried with Na_2SO_4, filtered, concentrated under reduce pressure. The acidic aqueous layer was co-evapored twice with MeOH/$H_2$0 (1/1) until neutrality to afforded 2 mg of L-rhamnose. The obtained compounds were analysed by TLC with CH_2Cl_2-MeOH (90-10) and MS by comparison with authentic samples.

L- Rhamnose $[\alpha]^{20}_D$ +15 (*c* 0.10, MeOH)

Dilobenol D (4): White amorphous powder $[\alpha]^{20}_D$ -12.7 (*c* 0.23, MeOH) ; UV (MeOH) λ_{max} (log ε) 220 (3.82), 296 (3.57), 347 (sh) nm ; IR ($CHCl_3$) ν_{max} 3371, 2920,1635, 1438, 1261, 1080 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1 and 2; HRESI-MS *m/z*: 569.2371 $[M-H]^-$ (calcd for $C_{31}H_{37}O_{10}$, 569.2366).

Dilobenol E (5): Colorless amorphous powder; $[\alpha]^{20}_D$ -24 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 218 (3.84), 289 (3.53), 347 (sh) nm ; IR ($CHCl_3$) ν_{max} 3379, 2924, 1635, 1446, 1076 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1 and 2; HRESI-MS *m/z*: 601.2259 $[M-H]^-$ (calcd for $C_{31}H_{37}O_{12}$, 601.2262).

Dilobenol F (6): Colorless amorphous powder $[\alpha]^{20}_D$ -35 (*c* 0.1, MeOH) UV (MeOH) λ_{max} (log ε) 220 (4.19), 290 (3.95), 344 (3.27) nm ; IR ($CHCl_3$) ν_{max} 3371, 2920, 1635, 1585, 1438, 1072 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1 and 2; HRESI-MS *m/z*: 747.2836 $[M-H]^-$ (calcd for $C_{37}H_{47}O_{16}$, 747.2834).

Acid hydrolyse of 6: was performed as for compound 3 and 10 mg of 6 was used. The organic soluble fraction (4 mg) was purified by on Sephadex® LH-20 eluted with MeOH to afford D-glucose (1.9 mg) and L-rhamnose (1.4 mg). The optical rotations taken were similar with those of authentics samples.

D-glucose : $[\alpha]^{20}_D$ + 120 (*c* 0.19, MeOH)

Dilobenol G (7): White amorphous powder; $[\alpha]^{20}_D$ -27.9 (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ε) 219 (3.93), 290 (3.66), 340 (3.03) nm ; IR ($CHCl_3$) ν_{max} 3379, 2924, 1635, 1585, 1438, 1373, 1072 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1 and 2; HRESI-MS *m/z*: 731.2888 $[M-H]^-$ (calcd for $C_{37}H_{47}O_{15}$, 731.2886).

Biological activities

Antimicrobial assays

Four Gram-positive (*Bacillus cereus* LMG 6910, *Bacillus megaterium* LMG 7127, *Staphylococcus aureus* ATTC 25920, *Enterococcus faecalis* ATTC 29212) and seven Gram-negative bacteria (*Vibrio harveyi* ATCC 14126, *Vibrio fischeri* ATCC 49387, *Salmonella* Typhimurium ATCC 14028, *Salmonella antarctica* LMG 3264, *Escherichia coli* CCM 451, *Klebsiella pneumoniae* ATTC 13883 and *Pseudomonas aeruginosa* LMG 1242) were used to study the antibacterial activity. The bacteria were obtained from the collections of both the University of La Réunion (LCSNSA: Laboratoire de Chimie des Substances

naturelles et des Sciences des aliments, Saint Pierre) and Cirad (Montpellier, France). Susceptibility screening test using disc diffusion method was used.[25] Each microorganism was suspended in brain heart infusion (BHI) (Difco, Detroit, MI) broth and diluted with peptone water to provide initial cell counts of the inoculum of about 10^6CFU/ml. Bacterial strains were inoculated on duplicate plates of Marine agar for vibrios and Mueller-Hinton agar for the other strains. Sterilized filter paper discs of 6 mm (Biomérieux. Marcy l'Etoile, France) were saturated with 10µL of the ethyl acetate, methanol extract and the pure compounds **1-7** (in distillate water). The 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 were prepared using the same solvents employed to dissolve the plant extracts samples. Each assay was repeated three folds. The results were expressed as the mean value (mm ± SD). The reference antibiotics Tetracycline and Gentamycin (30µg Bio-Rad, Marnes-la-Coquette, France) were used as positive controls.

In vitro antiplasmodial assay

The *in vitro* antiplasmodial tests, based on the inhibition of $[^3H]$-hypoxanthine uptake by *P. falciparum* cultured on human red blood cells, were performed as previously described.[26] The concentration causing 50% of growth inhibition (IC_{50}) was obtained from the drug concentration-response curve, and the results were expressed as the mean from three independent experiments. Chloroquine diphosphate (Sigma Aldrich Chimie SARL, St Quentin Fallavier, France) was used as positive control of the antiplasmodial activity.

In vitro cytotoxicity assay on mammalian cell

The cytotoxicity was evaluated using a rat myoblast-derived cell line (L-6) as previously described.[26] Cells were obtained from ATCC (Rockville, Maryland, USA). They were maintained 5 days in culture in presence of drug and the cytotoxicity was

determined using the colorimetric MTT assay according to the manufacturer's recommendations (cell proliferation kit I, Roche Applied Science, France). The IC_{50} was obtained from the drug concentration-response curve, and the results were expressed as the mean from three independent experiments.

Supporting Information (see footnote on the first page of this article): … . ^{1}H NMR and HMBC spectra of compounds **1**-**7**.

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Entry for the Table of Contents

Layout 1:

((Key Topic))

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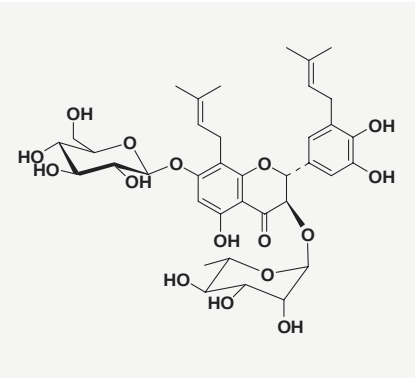
Dilobenol A-G, Diprenylated dihydroflavonols from the leaves of *Dilobeia thouarsii*

Keywords: Natural products / Prenylated dihydroflavonols / Structure elucidation / Antimicrobial activity / Cytotoxicity

Seven new diprenylated flavanonols have been isolated from the leaves of *Dilobeia thouarsii*. Among them, five are glycosylated. Their structures were established through analyses of their spectroscopic data. The evaluation of their antibacterial, antiplasmodial and cytotoxic activities is reported.



Supporting Information

Dilobenol A-G, Diprenylated dihydroflavonols from the leaves of *Dilobeia thouarsii*

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Antimicrobial activities of *Dilobeia thouarsii* Roemer and Schulte, a traditional medicinal plant from Madagascar

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Abstract

The leaves of *Dilobeia thouarsii* (Roemer and Schulte), a tree that is endemic to Madagascar (Proteaceae), are used in traditional Malagasy medicine to treat bacterial skin infections and wounds. This study investigated the *in vitro* antibacterial activities of *D. thouarsii* leaf extracts and identified the bioactive compounds with the aim of providing a scientific basis for its use against skin diseases. Using broth microdilution method for leaf crude extract and its compounds, we investigated inhibition of the growth of *Bacillus cereus, Bacillus megaterium, Staphylococcus aureus, Enterococcus faecalis, Vibrio harveyi, Vibrio fisheri, Salmonella* Typhimurium, *Salmonella antarctica, Escherichia coli,* and *Klebsiella pneumoniae*. The two purified phenolic compounds from leaf ethyl acetate extracts (**1**, **2**) were found to be more active than the crude extract itself. The structure of the two compounds was elucidated by NMR and mass spectrometry: compound **1** was identified as 4-aminophenol and compound **2** as 4-hydroxybenzaldehyde. A marked inhibitory effect (MIC < 0.1mg/ml) was found against *Staphylococcus aureus,* which is a major agent in skin infections. We observed moderate activities (MIC values of between 0.1 and 0.5 mg/ml) for *Enterococcus faecalis, Vibrio* spp., and *Bacillus* spp. Neither compound was active against *Salmonella* spp., *Escherichia coli* and *Klebsiella pneumoniae* (MICs > 1 mg/ml). To conclude, the high antimicrobial activity of *Dilobeia thouarsii* leaf extracts against *Staphylococcus aureus* supports its traditional use to treat skin infections.

Keywords: *Dilobeia thouarsii*; Proteaceae; Medicinal plant; Antibacterial; Plant extract; Phenolic compound.

1. Introduction

Traditional medicine is an important component of the health care system in Madagascar and a large number of plants remain to be studied, including *Dilobeia thouarsii*, a tree that belongs to the family Proteaceae and is endemic to Madagascar (Boiteau, 1986). This species is widely distributed in the Central, Eastern, South-Eastern regions and in the high Matsiatra Fianarantsoa in Madagascar (Bosser and Rabevohitra, 1991) and is known by the common names of Vivaona, Hazontavolo and Tavolohazo (Rabesa, 1986). In southern Madagascar, decoctions of the leaves and bark of *Dilobeia thouarsii* are used for abortion, or as an anthelmintic, or a diuretic (Beaujard, 1988; Rabesa, 1986). Concerning the East coast of Madagascar (Mandraka region), our ethnobotanical investigations confirmed the use of the leaves in traditional medicine to treat bacterial skin infections and wounds (Razafintsalama, 2012).

In vitro assays have shown that phenolic compounds are often responsible for the antimicrobial activities of different plant extracts (Shikanga et al., 2010; Tepe et al., 2005; Zampini et al., 2005). Several species belonging to the family Proteaceae, such as *Grevillea robusta*, *Toronia toru*, *Gevuina avellana*, *Kermadecia elliptica*, *Protea obtusifolia* or *Lomatia hirsuta* contain phenolic compounds (Ahmed et al., 2000; Chuang and Wu, 2007; Moure et al., 2001; Perry and Brennan, 1997; Simonsen et al., 2006; Verotta et al., 1999). In addition, species belonging to this family display antimicrobial activities against different microorganisms. *Lomatia hirsuta*, which is used in traditional medicine in Chile, is active against the pathogenic fungus *Candida albicans* (MIC = 8 µg/mL) (Simonsen et al., 2006). A phenolic glycoside ester isolated from the New Zeland tree *Toronia toru* is active against *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus subtilis* (Perry and Brennan, 1997). A glycoside compound isolated from *Persoonia linearis* x *pinifolia*, a crosshybrid of *P. pinifolia* and *P. linearis*, displays antimicrobial activity against *Escherichia coli* and *Phytophthora cinnamoni* (MacLeod et al., 1997). An extract made from leaves of *Protea simplex*, a plant used in South Africa against human dysentery and diarrhea, provides good antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans* (Fawole et al., 2009).

To the best of our knowledge, no report has been published on the chemical composition and the biological activities of *Dilobeia thouarsii* (Bosser and Rabevohitra, 1991). In the present study, we investigated the antibacterial activity of *D. thouarsii* and identified bioactive compounds in order to provide a scientific basis for its traditional use, and to characterize the potential of this medicinal plant in Madagascar. Bioassay fractionation enabled isolation of two phenolic compounds that were identified on the basis of spectroscopic data including 1D NMR and mass spectrometry (MS).

2. Material and methods

2.1 Plant material

The leaves of *Dilobeia thouarsii* were harvested in Mandraka region, in the eastern part of Madagascar, 70 km from Antananarivo. Leaves were collected in April 2008. The plant was identified by Dr. Rabarison HARISON from the Botany Department of Antananarivo Faculty of Sciences. Reference specimens (HERB/DBEV/4708) were deposited in the herbarium of the same department of the University of Antananarivo.

2.2 Extraction of *Dilobeia thouarsii* leaves

Plant materials were dried at room temperature and ground to a fine powder. The obtained powder (100 g) was extracted successively through a maceration process using 500 mlx6 of solvents of increasing polarity (hexane, ethyl acetate and methanol). Each combined extract was evaporated under reduced pressure to yield crude hexane extract (0.7 g), EtOAc extract (5 g), MeOH extract (10 g), respectively. Extracts were stored at room temperature until use.

2.3 Bioassay-guided extract

Part of the ethyl acetate extract (1.5 g) was subjected to flash chromatography on a silica gel 60 (10-40 µ) column (CC) eluted with 0-100% gradient of EtOAc in hexane followed by MeOH in EtOAc. Fourteen 100 ml fractions were collected: Hex–EtOAc 80:20 (1-4), Hex–EtOAc 40:60 (5–8), Hex– EtOAc 20:80 (9–12), EtOAc (13), MeOH (14). On the basis of the analytic TLC, and according to the antimicrobial assay, similar active fractions 5-8 (0.15 g) were combined and rechromatographed on the same support using the same solvent system. Fourteen new fractions were obtained, but only two displayed antibacterial activity. Each active fraction was treated with 75% ethanol and concentrated to yield compounds **1** (100 mg) and **2** (40 mg). The antimicrobial activity of these compounds was evaluated on Gram-positive and Gram-negative bacteria.

2.4 Antimicrobial assays

2.4.1 Microorganism strains

Four Gram-positive (*Bacillus cereus* LMG 6910, *Bacillus megaterium* LMG 7127, *Staphylococcus aureus* ATTC 25920, *Enterococcus faecalis* ATTC 29212) and six Gram-negative bacteria (*Vibrio harveyi* ATCC 14126, *Vibrio fisheri* ATCC 49387, *Salmonella* Typhimurium ATCC 14028, *Salmonella antarctica* LMG 3264, *Escherichia coli* CCM 451, *Klebsiella pneumoniae* ATTC 13883) were used to study antibacterial activity. The bacteria were obtained from the collections of both the University of La Réunion (LCSNSA: *Laboratoire de Chimie des Substances naturelles et des Sciences des aliments*, Saint Pierre) and Cirad (Montpellier, France).

2.4.2 MIC and MBC determination

The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) were evaluated using the microdilution method described by Kuete et al. (2009). The samples were first dissolved in sterile distilled water. The concentration of the resulting solutions was adjusted to 7 mg/ml. This was serially diluted twofold to obtain concentration ranges of 0.027-7mg/ml. Next, 100 µl of each concentration was added in a well (96-well microplate) containing 95 µl of Zobell medium for vibrios (1g/l yeast extract, 4 g/l peptone, 30 g/l NaCl) or Mueller-Hinton broth for the other microorganisms and 5 µl of inoculum (standardized at 1.5×10^6 cfu/ml by adjusting the optical density to 0.125 at 600 nm). A positive control containing the bacterial culture without the extract and a negative control containing only the medium were also analyzed. The plates were covered with sterilized aluminum foil, and then incubated for 24 h at 25 °C for *Vibrio* sp. and at 37 °C for the other strains. The assay was repeated three times. The MIC of each compound was defined as the lowest concentration that inhibited the microorganism growth. Bacterial growth was visually evaluated based on the degree of turbidity (Kil et al., 2009).

For the determination of MBC, 5 µl from each well not showing turbidity was placed on Mueller-Hinton agar and incubated at 37 °C for 24 h. The lowest concentration at which no growth occurred on the agar plates after 24 h of incubation at 37°C corresponded to the MBC.

3. Results and Discussion

3.1 Active compounds identified

Compound **1** (Fig. 1) was isolated as an amorphous powder. HRESI-TOF performed in the negative mode exhibited a deprotonated molecular ion at *m/z* 108.0435 $[M-H]^-$ indicating a molecular formula of C_6H_7NO (calcd. 108.0447) requiring four degrees of unsaturation. The ^{13}C NMR spectrum revealed the presence of an oxygenated quaternary carbon at δ 151.2, another quaternary carbon at δ 117.4 and a methine carbon at δ 115.8.
The ^1H NMR spectrum of this small molecule displayed an intense signal of four aromatic protons at δ 6.49. As the spectra were realized in CD_3OD, the three remaining protons not observed as suggested the molecular formula are exchangeable protons. Comparison with RMN data of the sample indicated that compound **1** was a 4-aminophenol (Sigma-Aldrich catalogue).

The molecular formula of compound **2** (Fig. 1) was deduced as $C_7H_6O_2$ from the deprotonated molecular ion peak at *m/z* 122.0368 $[M-H]^-$ observed in the HRESI-MS compatible with four degrees of unsaturation. Its ^1H NMR spectrum showed the presence of an aldehyde proton at δ 9.73 and two doublets at δ 6.9 (2H, *J* = 8.4 Hz, H-3; H-5) and 7.8 (2H, *J* = 8.4 Hz, H-2; H-6). In comparison with **1**, the difference of 13 uma suggested that the amino group was replaced by an aldehyde group (Chen et al., 1999). Consequently, compound **2** was identified as 4-hydroxybenzaldehyde.

3.2 Antibacterial activity

This is the first time that antimicrobial activity of *D. thouarsii* extracts has been reported. The two phenolic compounds determined from the leaf ethyl acetate extract (4-aminophenol and 4-hydroxybenzaldehyde) were more active against both Gram-positive and Gram-negative bacteria than the crude extract itself (Table 1). MIC and MBC values varied with the extracts and compounds tested. *Staphylococcus aureus* was the most sensitive strain. According to Oussou et al. (2008), the ratio observed for MBCs and MICs (MBC:MIC<4) indicated that the bactericidal effect of the compounds on the majority of strains tested could be expected. Globally, Gram-positive bacteria were more sensitive to these compounds than Gram-negative ones (Table 1).

MIC values obtained with the two compounds for *Staphylococcus aureus* were lower than those of leaves and bark extracts of *Protea simplex* (a Proteaceae from South Africa) which ranged between 0.147 and 0.780 mg/ml (Fawole et al., 2009). MIC values of our extracts were lower than 0.1 mg/ml for *Staphylococcus aureus*, which, according to Holetz et al. (2002), can be considered as good antimicrobial activity. *Staphylococcus aureus*, which is a major agent in skin infections, was sensitive to other African plant extracts of species including *Combretum vendae* (Combretaceae), *Commiphora harveyi* (Burseraceae), *Khaya anthotheca* (Meliaceae), *Kirkia wilmsii* (Kirkiaceae), *Loxostylis alata* (Anacardiaceae), *Ochna natalitia* (Ochnaceae), *Protorhus longifolia* (Anacardiaceae), *Lippia* spp., *Garcinia smeathmannii* (Clusiaceae) and *Ficus ovata* (Moraceae) (Kuete et al., 2009; Shikanga et al., 2010; Suleiman et al., 2010). Sato et al. (1997) examined the activity of three extracts from the fruiting bodies of *Terminalia chebula* RETS against methicillin-sensitive and methicillin-resistant *S. aureus* as well as 12 other Gram-negative and Gram-positive bacteria. The two compounds isolated from the Et_2O soluble part material, gallic acid and its ethyl ester

derivative proved to be more effective against both types of *S. aureus* than against other species. It appears that the ability to inhibit respiratory electron transport systems plays an essential role in the antibacterial activity of alkyl gallates against Gram-positive bacteria.
The MIC values of between 0.1 and 0.5 mg/ml that we observed for *Enterococcus faecalis, Vibrio* spp., and *Bacillus* spp. can be considered as moderate antimicrobial activity according to Holetz et al. (2002) (Table 1). Neither purified compound (**1**, **2**) was shown to be active against *Salmonella* spp., *Escherichia coli* and *Klebsiella pneumoniae* (MICs > 1 mg/ml).

4. Conclusion

Its antimicrobial activity against *Staphylococcus aureus*, a major agent in skin infections, provides a scientific basis for the traditional Malagasy use of *Dilobeia thouarsii* (Roemer and Schulte) in the treatment of skin infections. The two purified phenolic compounds from leaf ethyl acetate extracts involved in this antimicrobial activity were 4-aminophenol and 4-hydroxybenzaldehyde. Consequently, leaf ethyl acetate extract could be used in further investigations to identify the other molecules present in this plant.

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Table

Table 1: The minimum inhibitory concentration (MIC mg/ml) and minimum bactericidal concentration (MBC mg/ml) of *Dilobeia thouarsii* leaf ethyl acetate extract against bacteria tested in microdilution assays.

Figure captions

Fig. 1: Structure of the two purified compounds **1** and **2** from the leaf ethyl acetate extract of *Dilobeia thouarsii.*

Marked toxicity of *Albizia bernieri* extracts on embryo-larval development in the medaka fish (*Oryzias latipes)*

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Abstract

Previous phytochemical studies have shown that the plants of the *Albizia* genus (Fabaceae) contain bioactive saponins, lignans, spermine alkaloids, flavonoids, glycosides phenols and pyridoxine derivatives. Their extracts sometimes display medical properties, but can have also toxic effects. The purpose of our study was to determine the *in-vivo* toxicity of *Albizia bernieri* seeds in the experimental model of the medaka fish embryo, which is recommended for use in toxicity studies. Our results show clearly that incubating the embryos or larvae of the medaka fish in a medium containing *Albizia bernieri* extracts caused a dose-dependent reduction in embryo survival. Embryos exposed to an extract of *A.bernieri* displayed cerebral lesions, such as cell lysis and the emergence of lysosomes in the glial tissue. We conclude that *Albizia* species display unusually high toxicity compared to other plants as evaluated using the same animal model.

1. **Introduction**

Previous phytochemical studies have shown that the plants of the *Albizia* genus which belongs to the Fabaceae family contain bioactive saponins (Krief et al., 2005; Zheng et al., 2006; Melek et al. 2007), lignans (Ito et al., 1994), spermine alkaloids (Mar et al, 1991), flavonoids (Kang et al. 2000), phenolic glycosides (Jung et al., 2004) and pyridoxine derivatives known as ginkgotoxins (Higuchi et al., 1992; Leistner and Drewke, 2010). Their extracts sometimes display medical properties, for instance, aqueous extracts of *Albizia anthelmintica* bark administered at 10-150 g/kg p.o. displayed marked anthelmintic activity (68-100%) against experimental *Hymenolepis diminuta* infection in albino rats, but no toxicity (Galal et al., 1991). An aqueous extract of *A. lebbeck* bark was less effective at 10-100 g/kg p.o. against this cestode, and was toxic to rats at the highest dose tested (150 g/kg) (Galal et al., 1991).

The leaves and bark of *A. lebbeck* have been in clinical use for centuries in the Ayurvedic system of medicine. Decoctions of stem bark are used in the treatment of bronchial asthma. Tripathi and Das (1977) have reported that this species has anti-asthmatic and anti-anaphylactic activity, and *A. lebbeck* increases plasma cortisol levels in patients with bronchial asthma (Tripathi et al., 1978). It has also been reported that *A. lebbeck*, unlike disodium chromoglycate, displays antianaphylactic activity in guinea pigs (Tripathi and Shukla, 1979).

In addition, it appears to inhibit the early stages in the sensitization and synthesis processes of reaginic-type antibodies. If *A. lebbeck* is given during the first week of sensitization, it markedly inhibits the early stages of the sensitizing process, whereas given during the second week it suppresses antibody production throughout the period of drug administration. The effect of *A. lebbeck* has been studied on the degranulation rate of sensitized peritoneal mast cells of albino rats when challenged with an antigen (horse serum) and has been shown to have a significant cromoglycate-like action on mast cells (Tripathi et al. 1979a,b). Ethanol extracts of the leaves of *A. lebbeck* have also been shown to exhibit anticonvulsant activity (Katsure et al., 2000). The extracts increased brain levels of gamma-aminobutyric acid (GABA) and serotonin, and were found to be anxiogenic and general central nervous system depressants (Kasture et al., 2000).

The aqueous-methanol extract of *A. lebbeck* seeds (2.5-5 mg/kg i.p.) has also been shown to have anti-diarrhoeal activity (Gomes et al., 2002).

Five sheep were reported to have developed severe neurological signs after being drenched with *Albizia versicolor* pod-material (Gummow et al., 1992), and subsequently an outbreak of *A. versicolor* poisoning was reported in sheep and goats in Malawi (Soldan et al., 1996). Approximately 800 animals were estimated to have died over a 9-year period on a government farm near Lake Malawi. The deaths all occurred between August and December, when the animals were ingesting dry ripe pods that had fallen on the ground. The major clinical signs were hyperaesthesia, wild running, lateral recumbence with rapid leg movements, nystagmus and rapid blinking. A series of experiments was conducted, and all the animals given 6.4 g/kg or more of dry pods died exhibiting typical clinical signs. Although *A. versicolor* is familiar to the local population, they appeared to be unaware of its toxicity. Such poisoning events are probably rare in traditionally-managed flocks.

The Chinese Pharmacopoeia includes the stem bark of *A. julibrissin* as a sedative and anti-inflammatory agent, and indicates its use to treat injuries from falls and remove carbuncles (Yu et al. 2004, Pharmacopoeia of China 2005). *A. zygia* is found in the South-Western province of Cameroon, where it is used as a commercial source of timber and in the treatment of various disorders including fever and eczema. Remedies based on the root bark are also used to treat venereal diseases (Laird et al., 1997). The seeds are eaten by chimpanzees, and are known to be a source of a gum exudate rich in mineral salts (Ushida et al., 2006).

A. procera is a tree cultivated in streets and public gardens in Egypt. In folk medicine, the bark is considered useful in pregnancy and in stomach ache. It is also used as a medicine for water buffalo and is given with salt (Melek et al., 2007).

A.bernieri is endemic in Madagascar, and is widespread in the Western part of the island. *A. bernieri* generally flowers from October to December. It can be found in cleared forests and in the bush up to altitudes of 100 m, in wet situations, such as argillaceous depressions and beside rivers. The bark is used by the local population to make clothes and ropes, and also as a detergent and for tanning leather. Its wood is used for the construction of dwellings locally and to produce timber for the construction industry (Du Puy et al., 2002). The seed extracts have been shown to be toxic to mice, and to display a haemolytic effect on the red blood cells of sheep; it is also reported to have antibacterial action.

The purpose of our study was to investigate the *in-vivo* toxicity of *Albizia bernieri* seeds using the experimental model of the medaka fish embryo, which is currently recommended for toxicity studies.

2. Materials and methods

2.1 Sample collection

Dried fruits of *Albizia bernieri* were collected from the wild in the region of Mampikony in the North-western part of Madagascar in July 2010.

As usual, the systematic identification of the plant was done by consulting the herbarium of the Department of Forest and Piscicultural Research of the FOFIFA Antananarivo: it is indexed under the herbarium reference no. 29133-SF.

2.1 Extraction of compounds for the toxicological studies

The peeled seeds from dried fruits of *Albizia bernieri* were finely ground and pulverized. A fraction of the powder obtained (262 g) was defatted in cyclohexane (3 x 500 mL) and extracted successively with MeOH (2.2 L) and MeOH-H_2O (1/1, 1.5 L) at room temperature. Each combined extract was evaporated under reduce pressure to yield a crude cyclohexane extract (5 g), a MeOH extract (48.4 g), and a MeOH-aqueous extract (23.6 g). A portion of the crude MeOH extract (38.4 g), which displayed toxicity, was suspended in H_2O (100 mL) and partitioned successively with EtOAc and *n*-butanol (v/v). The concentration of solvent

used yielded the corresponding EtOAc (1.49 g), nBuOH (6.2 g) and H_20 (26.8 g) soluble extract. Our study was performed with the MeOH and *n*-BuOH extracts.

2.3. Oryzias latipes *breeding and treatment*

Oryzias latipes progenitors were kept in 8-liter glass aquaria at 26-28°C under an artificial lighting regime (10 hrs Light -14 hrs Dark cycle) conducive to breeding. Fertilized egg clusters were carefully removed from the female progenitors and 650 post-fertilized (pf) eggs were collected and placed in Petri dishes containing Yamamoto's embryo rearing medium (Iwamatsu 2004).

The *O. latipes* embryos were then used in incubation experiments. To do this, Yamamoto's embryo rearing medium was prepared containing several different concentrations of a given extract. The development of *O. latipes* embryos has been very clearly characterized and 39 stages have been defined over the period of 10 days between fertilization and hatching. The effects of the test extract, E, were tested 1) 24h after fertilization, corresponding to stage 17 (test 1); 2) at the end of embryo development with exposure to the compound from 24h just before hatching until 3 days post hatching (test 2) and 3) during larval development by exposing hatched animals from hatching until 4 days post-hatching (test 3) as previously described (Rakotobe et al., 2009).

Five embryos (in 2 mL medium) in 12-well microplates were raised under the same conditions as described above (25°C, LD: 10-14 hrs), observed daily, and photographed (Leica) until 3 or 4 days post-hatching. The toxic or control media were changed every 48h during treatment. The animals were handled in accordance with European Union regulations concerning the protection of experimental animals (Dir. 86/609/EEC).

2.4 Anatomopathological studies

At the end of the incubation experiments, the surviving hatched embryos (*A. bernieri*-extract treated and untreated) were fixed. The samples used for photon microscopy were fixed with a mixture of paraformaldehyde (2%), glutaraldehyde, 0.5%, picric acid 0.5%, and sucrose (0.18M) in 0.1M, pH 7.4, Sørensen buffer. For the ultrastructural observations the same fixing mixture was used, but containing a higher level of glutaraldehyde (1%), and fixing was followed by postfixing with osmium tetroxide (1%). In both cases, the samples were washed three times with Sørensen phosphate buffer (0.1 M, pH 7.4) in three successive 10-min baths,

and were then dehydrated in ethanol (50%, 70%, 90% and 100%), with three successive baths at each dehydration step, before being embedded in a epoxy mixture (Spurr's resin). Medium and ultrathin sections were sliced with diamond knives (Diatome) on a Reichert-Jung Ultracut microtome. The semi-thin sections were stained with toluidine blue (1%) and sodium borate (1%) in ethanol (70%) or with methylene blue-basic fuchsin staining, and examined under a Nikon photon microscope. The ultrathin sections were stained with a saturated solution of uranyl acetate in 50% alcohol, and then observed under a transmission electron microscope (TEM), (Hitachi H-7100). The pictures were taken using Hamamatsu CCD camera, and further processed using Adobe Illustrator 10 software.

2.5 Statistical methods

One-factor ANOVA (Graphpad Prism 5 Software, http://www.graphpad.com) was used to test the differences between the residual survival rates of each incubation series (control vs. treated embryos). Tukey-Kramer's multiple comparison test was then used to compare the responses of the embryos and controls at each dose level.

3. Results

3.1. Effects of the methanol extract of Albizia bernieri *on medaka embryo-larval development*

The incubation of medaka fish embryos in a medium containing a methanol extract of *Albizia bernieri* starting the incubation with the extract soon after fertilization (1 day post fertilization corresponding to stage 17: test 1). Dose dependent toxicity was observed (Fig. 1). Below 10 $\mu g.mL^{-1}$ no toxic effect (100% survival rate) was detected. Concentrations of 15 to 30 $\mu g.mL^{-1}$ reduced the larval survival rate by 50 to 100 %. The LC_{50} was estimated to be 19.05 $\mu g.mL^{-1}$ extract (Table 1).

Similar experiments were conducted using the same *Albizia bernieri* extract, but starting incubation of the embryos with the extract from stage 39 of embryonic development (24 h before hatching: test 2) (Fig.2). Below 10 $\mu g.mL^{-1}$ no toxic effect was observed. Concentrations from 18 $\mu g.mL^{-1}$ reduced the survival rate by 80 to 100%. The LC_{50} was estimated to be 19.4 $\mu g.mL^{-1}$ (Fig. 2, Table 1). Severe effects on embryo hatching rates (Fig.3) were also observed. Administration of *Albizia bernieri* extract at a concentration of 20 $\mu g.mL^{-1}$ altered the timing of hatching in the surviving embryos. In these groups, hatching occurred

earlier in the toxin-treated embryos with up to 50% hatching after 11 days and 100% after 13 days, which is 2 days earlier than in controls. Lower concentrations (10, 15 and 18 µg.mL-1) of *Albizia bernieri* also resulted in early hatching, but only one day in advance of controls. Lower concentrations had no effect on the timing of hatching. A third type of experiments was conducted using the same *Albizia bernieri* extracts, but starting the incubation with the extract at hatching (day 12 post fertilization: test 3). This also resulted in dose-dependent toxicity, and fairly similar dose responses were obtained, with the 30, 25 and 20 $\mu g.mL^{-1}$ concentrations being lethal (Fig.4). The $LC_{50,}$ 16.65 $\mu g.mL^{-1}$ (Table 1), was similar to that found for the embryos treated in tests 1 and 2.

3.2 Effects of butanol extracts of Albizia bernieri *on medaka embryonic-larval development*

Embryos and hatchlings were treated with increasing concentrations of butanol extracts of *Albizia bernieri* under the same conditions as previously, 1 day post fertilization; test 1; 1 day before hatching; stage 37, test 2, and at hatching, test 3. The toxic effects observed were very similar to those seen following treatment with the methanol extract. The LC_{50} values found for tests 1, 2 and 3 were 15.34, 16.15 and 10.13 $\mu g.mL^{-1}$ respectively (Table 1), which is of the same order as those found using the methanol extract.

3.3 *Anatomopathological studies*

In order to find out more about *Albizia bernieri* toxicity, experiments were conducted at the end of embryo-larval development. Embryos exposed to an extract of *A.bernieri* at a concentration of 19 µg/mL showed cerebral lesions, such as cell lysis and the emergence of lysosomes in the glial tissue. Hepatic lesions were also observed, and indeed the hepatocytes lost their structural organization and numerous vesicles in were observed in the liver tissue. (Figure 5)

4. Discussion

The experiments show clearly that incubating the embryos or larvae of medaka in a medium containing *Albizia bernieri* causes a dose-dependent reduction in embryo survival.

In conclusion, these experiments show that the embryos of medaka can be used to investigate the toxicity of plants, in addition to its current use for investigating toxicity due to cyanotoxins (Jacquet et al., 2004) or food toxins (Rakotobe et al., 2009).

Dose-response curves indicating the level of toxicity for aquatic animals have been obtained. The pathology studies identified severe lesions in the brain, where zones of lysis and the presence of lysosomes were observed, and also in the liver, where vesiculation of the hepatic tissue was observed. Similarly, 0.5 mg/mL of aqueous extract of tubers of *Dioscorea antaly* caused lesions of the kidneys and liver in the medaka fish, in particular widening of the sinusoidal capillaries (Rakotobe et al. 2010).

Medaka embryos treated with an aqueous extract of bulbs of *Rhodocodon. madagascariensis* display zones of encephalic congestion (Rakotobe et al., 2009) after being treated with a concentration of 0.5 mg/mL. It should also be noted that convulsive alkaloids have been isolated from *Dioscorea dumetorum* (David at al., 1985). Furthermore, extracts of *Albizia lebbeck* have been shown to increase brain levels of gamma-aminobutyric acid (GABA) and serotonin and to be anxiogenic and general depressants of the central nervous system (Kasture et al., 2000). Finally, sheep and goats display severe neurological signs after being drenched with *Albizia versicolor* pod-material (Gummow et al., 1992).

The LC_{50} values found for *Rhodocodon madagascariensis* in the tests of medaka development were 1070 µg/mL and 250 µg/mL for tests 2 and 3 respectively (Rakotobe et al., 2009). The LC_{50} determined in development test 1 using an aqueous extract of the tubers of *Dioscorea. antaly* was 860 $\mu g.mL^{-1}$ (Rakotobe et al. 2010). With LC_{50} values ranging from 10 to 19 $\mu g.mL^{-1}$, *Albizia* species therefore display unusually high toxicity compared to other plants.

Phytochemical investigations are undertaken on these extracts for the identification of compounds responsible of the toxicity.

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Figures legends

Figure 1. Survival rate of medaka embryos incubated with concentrations of *Albizia bernieri* ranging from 7.8 to 30 µg.mL^{-1} . Embryos were exposed soon after fertilization (1 day post fertilization corresponding to stage 17: test 1). Bars represent mean+/- s.e.m of 3 to 6 experiments. * denotes a significant difference from controls ($p<0.05$).

Figure 2. Survival rate of medaka embryos incubated with different concentrations of *Albizia bernieri* ranging from 7.8 to 30 µg.mL^{-1} . Embryos were exposed from 24h before hatching to day 5 post hatching. Bars represent mean+/- s.e.m of 3 to 6 experiments. * denotes a significant difference from controls (*$p<0.05$,).

Figure 3. Dose-response curve of mortality associated with *Albizia bernieri* treatment. Bars represent the mean+/- s.e.m of 3 to 6 experiments.

Figure 4. Hatching of medaka embryos incubated with increasing concentrations of *Albizia bernieri.* Embryos were incubated from 24h before hatching.

Figure 5.Toxic effects of *A. bernieri* on medaka fish

A) Brain section of a control medaka: normal white (arrow) and grey substance (arrowhead) are observed. Photonic microscopy, semi thin section, scale bar 200µm

B) Brain section of a treated medaka with a methanol extract of *A.bernieri*: note the presence of vesicles (arrow) both in the white and grey substance. Photonic microscopy, semi thin section, scale bar 200µm

C) Control brain section (TEM): the white substance is homogeneous (arrowhead), the neuron nuclei (arrow) and the cytoplasms are well preserved.

D) Brain section of medaka treated with a methanol extract of *A.bernieri* methanol: note the presence of numerous lysosomes in the cytoplasm (arrows), cellular lysis (arrowhead).

E) Control liver section (arrow), photonic microscopy, semi-thin section, scale bar 200µm. Abbreviations: L, liver; O, oesophagus; S, spleen; I, intestine; Vi, vitellus.

F) Treated liver section: the hepatocytes have lost their characteristic organisation; a large number of vesicles can be seen in the hepatic tissue (arrow). Photonic microscopy, semi-thin section, scales bar 200µm.

A. bernieri extract	Methanol extract			Butanol extract		
Experimental group	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
LC_{50} (µg.mL^{-1})	19.05	19.40	16.65	15.34	16.15	10.13

Table 1

[SL 9B] *In vitro* Effects of Extracts from five Malagasy Endemic Species of *Albizia* (Fabaceae) on Vegetable Seeds Germination

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Key-words: Albizia, crude extracts, purified extracts, saponins, alkaloids, inhibition, germination.

Introduction

Trees belonging to the genus *Albizia* (Fabaceae) grow in tropical areas such as Africa, Asia and South-America where they are widely used in traditional medicine (Kang et al., 2000; Zou et al., 2006; Rukunga et al., 2007). Chemical and pharmacological investigations on number of them have led to the isolation of novel structures with various properties, indicating the efficacy of the healers herb preparations.

Thus, ethanolic extract from *A. lebbeck* exhibited anticonvulsive activity (Kasture et al., 2000). The structure of cytotoxic triterpenoidal saponins from *Albizia julibrissin* was established (Zou et al., 2006). Sedative activity of flavonol glycosides from this species was reported (Kang et al., 2000). Antiplasmodial spermin alkaloids were isolated from *Albizia gummifera* (Rukunga et al., 2007). Antimicrobial activity of several species such as *A. ferruginea, A. gummifera, A. lebbeck,* was reported (Agyare et al., 2005; Geyid et al., 2005; Sudharameshwari et al., 2007). It is also of importance to note that some *Albizia* species are toxic (Gummow et al., 1992; Agyare et al., 2005)

However, reports on the effects of these species on other plants are rare. Now, high application of herbicides leads to resistance of many weeds.

In Madagascar, *Albizia* is represented by 25 endemic species. No previous report on both the chemical constituents and the pharmacological activities of these plants could be found in the literature.

The purpose of this study was to carry out assessment of the effects of extracts from five Malagasy species of *Albizia*: A_2, A_4, A_5, A_6 and A_7 on Monocotyledons and Dicotyledons. Vegetables were used in germination assays since calibrated seeds were available.

Materials and Methods

Plant materials

Collection and processing of plants

Five species of *Albizia* encoded A_2, A_4, A_5, A_6 and A_7 were used in this study. Plant parts were collected in western and southern regions of Madagascar. The organs used for each plant are shown in Table 1.

Table 1: Organs used for the 5 species

Species	**A_2**	**A_4**	**A_5**	**A_6**	**A_7**
Organs	Teguments	Empty pods	Seeds	Leaves	Seeds

Seeds (for A_2, A_5, A_7) were washed and all plant materials (seeds, pods and leaves) were sun-dried. Dried seeds (for A_5, A_7), empty pods (for A_4) and leaves (for A_6) were ground into a fine powder, using a microgrinder Culatti. For A_2, teguments were separated from almond by several cycles of grinding/sieving. Thereafter, teguments were washed to remove almond residues, sun-dried and ground into a fine powder.

Tests-seeds

Calibrated vegetable seeds used for germination tests came from the collection of *Foibe Fikarohana momba ny Fambolena* (Fofifa, Antananarivo) seed bank. For each test, experiments were carried out with one representative of Monocotyledons and one representative of Dicotyledons.

Extracts preparation

Crude extracts

Cold extraction (A_2, A_4, A_7)

Powdered dried teguments (A_2), pods (A_4) and seeds (A_7) were extracted with 75% ethanol, distilled water and 50% ethanol, respectively. Prior to extraction, A_7 powdered dried seeds were defatted by Soxhlet extraction with hexan at 45°C for 18 h.

Hot extraction (A_5, A_6)

Powdered dried seeds (A_5) were defatted by extraction with petroleum ether (60-80°C) in a Soxhlet's extractor. Using the same procedure, powdered dried leaves (A_6) were depigmented with acetone. Both defatted powdered seeds and depigmented powdered leaves were extracted with absolute ethanol, using a reflux heating system.

Purified extracts

A_2, A_5, A_6 and A_7 crude extracts were purified using methods based on solubility, molecular weight or electric charge properties of active principles.

Phytochemical screening

Extracts were subjected to preliminary phytochemical testing for the major chemical groups (Fransworth, 1966; Marini-Bettolo et al., 1981).

Assays on plants

Assays on seedlings growth

The effects of the crude (A_2, A_4, A_7) or purified (A_5, A_6) extracts were studied on epicotyl and hypocotyl growth. Seven batches of 10 seeds were soaked for 48 h at 30°C in darkness, then transfered onto Petri dishes layered with cotton. Six batches among the seven ones were germinated and regularly watered with different concentration levels of the tested extract. One

batch was watered with distilled water as a control. Epicotyl and hypocotyl lengths were measured at 2 days intervals during 14 days.

Assays on axillary bud growth

Assays were carried out on 15-day-old pea seedlings previously sectionned above the second axillary bud. Effects of extracts were compared with those of the plant growth regulators giberellin and auxin. Tested solutions (1µl) were mixed with lanolin and deposited on the top of the sectionned part. Five groups of 5 plants each were studied: group 1received 50 µg giberellin; group 2: 50 µg of auxin; groups 3 and 4: 50µg of extracts and group 5: 1µl distilled water. Axillary bud growth was measured at 2-days intervals during 10 days.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Newman Keuls comparison test with Statitcf® software were used for statistical analysis. Statistical estimates were made at confidence interval of 95%.

Results and discussion

The extracts used, corresponding to the different species of *Albizia*, and the vegetable seeds tested in germination assays are shown in Table 2.

Table 2: Extracts from for the 5 species and vegetable seeds used

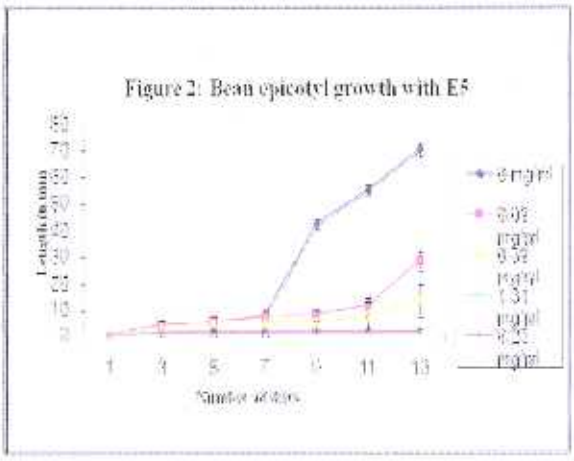
Plants	**A_2**	**A_4**	**A_5**	**A_6**	**A_7**
Extracts	CE = E_{23} PE = E_{24}	CE = E_{41}	PE = A_5	PE = E_6	CE = E_{71} PE = E_{72}
Seeds	Rice/Bean	Rice/Bean	Rice/Bean	Maize/Pea	Rice/White tissam

CE = Crude extract; PE = Purified extract

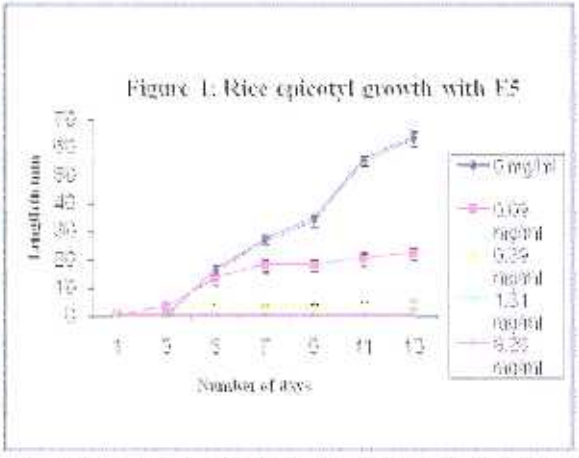
Vegetable seeds (from Monocotyledons and Dicotyledons) were chosen among those which did not germinate during preliminary tests with extracts at 1 mg/ml.

All extracts inhibited the epicotyl and hypocotyl growth of seedlings tested at the used concentration levels. However, a slight stimulation effect was exhibited by some extracts at low concentrations, such as 0.23 mg/ml and 0.46 mg/ml for E_{23} or 0.035 mg/ml for E_4. Above these concentrations, dose-effect was observed ($p<0.05$). In some cases (A_5 and A_6), epicotyl appeared to be more susceptible than hypocotyl.

E5 at 1.31 mg/ml was the most active extract as demonstrated by the total inhibition of growth noted for epicotyl of rice and beans seedlings (Figures 1 and 2).



Crude extract E_2 slightly inhibited axillary bud growth. This effect was lower than auxin at the same level. On the contrary, purified extract E_{24} exhibited no effect. Active principles were probably removed by purification.



The inhibition activity of the extracts from the various parts of the investigated plants appears to be due to saponins (A_4, A_5, A_7) or alkaloids (A_2, A_6) identified by phytochemical screening (Table 3).

In conclusion, these natural products were demonstrated to be toxic on seeds at the levels used in this study. On the other hand, these substances could be involved in plant-plant interactions, warranting studies of relationships between plants and their environment. Further investigations are necessary to determine their action mechanism, before undertaking research for the purpose of their probable use as alternatives for herbicides.

Table 3 : Phytochemical screening of extracts from species A_2, A_4, A_5, A_7, A_6

Phytochemical compounds	**Extracts**						
	E_{23}	E_{24}	E_{41}	E_5	E_6	E_{71}	E_{72}
Alkaloids	+	+	–	–	+	–	–
Flavonoids	–	–	–	–	+	–	–
Anthocyanins	+	–	–	–	–	–	–
Phenols	–	–	–	–	–	–	–
Quinons	–	–	–	–	–	–	–
Unsaturated sterols	+	+	–	+	–	+	+
Triterpenes	+	+	+	+	–	+	+
Deoxysugars	–	–	+	+	–	+	+
Saponins	–	–	–	+	–	+	+

Legend for extracts : see Table 2

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[PS 16] *In vitro* Antimicrobial Activity of Extracts from five Malagasy Endemic Species of *Albizia* (Fabaceae)

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Key-words: *Albizia*, seeds, extracts, antimicrobial, MIC, MBC.

Introduction

For centuries, most of the population in many developing countries have relied on a system of traditional medicine in which plants constitute the principal element of therapy. Plants belonging to the genus *Albizia* (Fabaceae) are trees distributed in African, Asian and South-American countries where they are widely used in indigenous pharmacopoeia (Agyare et al., 2005; Geyid et al., 2005; Murugan et al., 2007; Rukayadi, 2008). *Albizia* species have been the subject of several chemical and pharmacological studies. Thus, many structures (heterosids, alkaloids, ...) were elucidated (Zou et al., 2006; Rukunga et al., 2007) and various activities such as anthelmintic (Githiori et al., 2003), cytotoxic (Zou et al., 2006), larvicidal (Murugan et al., 2007) or antimicrobial (Agyare et al., 2005; Geyid et al., 2005; Sudharameshwari et al., 2007) were found.

In Madagascar, *Albizia* is represented by 25 endemic and 2 introduced species. No previous report on both the chemical constituents and the pharmacological activities of these plants could be found in the literature. Since infectious diseases account for the significant proportion of health problems, antimicrobial principles from five Malagasy species of *Albizia* encoded A_1, A_2, A_3, A_4 and A_5, were studied in this work. They were purified and the major secondary metabolites were identified by phytochemical screening. Extracts or pure compounds were tested *in vitro* against two Gram positive bacteria, three Gram negative bacteria and one yeast *Candida albicans*. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) were determined on susceptible germs.

Materials and methods

1- Plant materials

Seeds of plants A_1, A_2, A_3, A_4 and A_5 were used in this study. Fruits were collected in western and southern regions of Madagascar.Seeds were washed, sun-dried and ground into a fine powder, using a microgrinder Culatti.

2- Microorganisms

The pathogenic microorganisms consisted of two Gram positive bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, three Gram negative bacteria: *Klebsiella pneumoniae*, *Escherichia coli Salmonella*

typhi and one yeast *Candida albicans*. They were isolated and identified from heterogeneous cultures available in Institut Pasteur de Madagascar.

3- Extracts preparation

3.1- *Extraction*

Powdered dried seeds were defatted by extraction with petroleum ether (60-80°C) in a Soxhlet's extractor, then extracted with distilled water, 50% ethanol or 75% ethanol.

3.2- *Purification*

Crude extracts were purified using methods based on solubility, molecular weight or electric charge properties of active principles.

4- Phytochemical screening

Extracts were subjected to preliminary phytochemical testing for the major chemical groups (Fransworth, 1966; Marini-Bettolo et al., 1981).

5- Assays on microorganisms

The antimicrobial tests were carried out by disc diffusion method in Mueller Hinton agar (Rios et al., 1988).MIC was determined by broth dilution method (Duval et Soussy, 1990; Ferron, 1994). Each medium showing no visible growth is subcultured on Mueller Hinton agar plates. After 24 hours at 37°C, MBC was the corresponding concentration required to kill 99.9% of the cells (Duval et Soussy, 1990; Ferron, 1994).

6- Statistical analysis

One-way analysis of variance (ANOVA) followed by Newman Keuls comparison test with Statitcf® software were used for statistical analysis. Statistical estimates were made at confidence interval of 95%.

Results and Discussion

1- Phytochemical screening

The major secondary metabolites identified in extracts are shown in Table 1

Table 1: Phytochemical screening of extracts from 5 Malagasy species of *Albizia* (A_1 to A_5)

Phytochemical compounds	**Extracts**					
	E_1	E_{21}	E_{22}	E_3	E_4	E_5
Alkaloids	–	–	–	–	–	–
Flavonoids	–	–	–	–	–	–
Anthocyanins	–	–	–	–	–	–
Phenols	–	–	–	–	–	–
Quinons	–	–	–	–	–	–
Unsaturated sterols	+	+	+	+	+	+
Triterpenes	–	+	+	+	+	+
Deoxysugars	+	+	+	+	+	+
Saponins	+	+	+	+	+	+

–: negative test +: positive test

E_1, E_3, E_4, E_5: purified extracts from plants A_1, A_3, A_4 and A_5 respectively

E_1, E_{21}, E_{22}: pure compounds from plant A_1, A_2 respectively Except A_1 which didn't contain triterpenes, all extracts showed the presence of unsaturated sterols, triterpenes and deoxysugars, indicating glycosidic nature of active principles. The presence of saponins, in addition with positive foam test and hemolytic effect (not shown) mean that antimicrobial compounds may be saponins.

Saponins and other glycosides were isolated and identified from other species of *Albizia* (Pal et al., 1995; Debella et al., 2000; Zou et al., 2006).

2- Antimicrobial activity

According to these results, the extracts E_3 and E_5, respectively from **A3** and **A5**, showed activity against all the tested germs. *Bacillus subtilis* seemed to be the most susceptible bacterium (13 mm inhibition zone for E_3 and 16 mm for E_5) to these extracts. On the other hand, all the extracts inhibited the growth of *Staphylococcus aureus* and *Candida albicans* at the tested concentrations. E_{21} (pure compound) exhibited the strongest activity against the fungus (20 mm). In a general manner, Gram positive germs, including *Candida albicans,* were more susceptible than Gram negative ones.

Similar results were obtained with some other species of *Albizia* (Mbosso et al., 2010; Rukayadi, 2008; Sudharameshwari et al., 2007).

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determined on susceptible germs are given in Table 2.

Table 2: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of extracts from 5 Malagasy species of *Albizia*

Extracts	Sensitive germs	MIC (µg/ml)	MBC (µg/ml)
E_1	*Staphylococcus aureus*	320	2500
E_{22}	*Candida albicans*	6.25	100
E_{22}	*Klebsiella pneumoniae*	50	800
E_3	*Escherichia coli*	2500	10 000
E_4	*Staphylococcus aureus*	625	10 000
E_4	*Escherichia coli*	1250	20 000
E_5	*Escherichia coli*	12 500	12 500

Pure compound E_{22} from the plant A_2, showed the lowest **MIC (6.25 µg/ml)** and **MBC (100 µg/ml)** against *Candida albicans*. With MIC values respectively corresponding to 100 µg/ml and 12.5 µg/ml, *Albizia myriophylla* and *Albizia gummifera* (Mbosso et al., 2010; Rukayadi, 2008) showed lower activity than A_2 against this germ.

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