Phytochemical and Antifungal Analysis of Extracts of *Terminalia brownie* (Fresen. Mus. Senckenb) Shaf

By

Enass Yousif Abdelkarim Salih

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Supervisor

Dr. Hiba Abdel Rahman Ali

Department of Forest Products and Industries

Faculty of Forestry

University of Khartoum

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DEDICATION

To the sunshine in my life Mum

To Soul of my Father

To my Sisters and brothers

To my Colleagues

I dedicated this work

ACKNOWLEDGEMENTS

Thanks to Ulha who gave me strength, patience, health and insistence to complete my study.

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My most profound appreciation goes to my lovely family, Mohammed, Omima ,Nasir and Amel. Special thanks goes to the affectionate woman (mum) for her great impact in my life.

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ABBREVIATIONS

$CHCL_3$	Chloroform
CID	Collision induced dissociation
DAD	Diode array detector
DPPH	1, 1-Diphenyl-2-pierylhydrazyle
ESI	Electrospray Ionization
EtoAc	Ethyl acetate
HCl	Hydrochloric acid
HCO_2H	Formic acid
HPLC	High Performance Liquid Chromatography
H_2SO_4	Sulphuric acid
Marc	Residue of the extract
MeCN	Acetonitrile
MeOH	Methanol
MIC	Minimum inhibition concentration
MS	Mass spectroscopy
MS/MS	Tandem mass Spectroscopy
m/z	Mass to charge ratio
µl	Micromilliter
mm	Millimeter
NP	Normal phase
NPR	Natural products reagent
PE	Petroleum ether
PEG	Polyethylenglycol
R_f	Retardation factor
RP	Reverse phase
R_t	Retention time
S.D.A	Sabouroud dextrose agar
SPE	Solid Phase extraction
SR	Spray reagents
TLC	Thin layer chromatography
UV	Ultra Violet
ג	Wavelength (nm)

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Name: Enass Yousif Abdelkarim

Abstract:

Combretaceae is known for its medical uses in Africa and Asia. This study was conducted for phytochemical analysis of two different parts of *Terminalia brownii* (*Combretaceae*). Plant studied wood and bark extracts were subjected to biological and chemical screening implementing different chromatographic analytical methods (TLC, HPLC and LC-MS/MS)

Antimicrobial activity of the different extracts of *T. brownii* (wood, bark) was recorded against different plant pathogenic fungi. The aqueous extract of the wood of *T. brownii* exhibited the highest antifungal activity against *Aspergillus niger* (13mm), 11mm inhibitory zone against *A. flavus* and *Natrassia mangifera* and 12mm inhibition zone against *Fusarium moniliform*. The inhibitory zones of the aqueous extract of the bark were 14mm against *A. niger* and *A. flavus*, and 20mm against *N. mangifera* and *F. moniliform.* The ethyl acetate extract of the wood and bark of *T. brownii* gave similar growth inhibitory zones with the mean diameter of 15mm against *A. niger*, *A. flavus*, *N. mangifera* and *F. moniliform*.

Minimum inhibition concentration of the ethyl acetate extracts of the wood and the bark of *T. brownii* was measured against the four tested plants pathogens. MIC could not be determined because even at concentration as low as, 0.001g/ml antifungal activity was observed. MIC could be considered to be lower than 0.001g/ml. Oppositely; no effect was shown for the same extracts against *A. niger* even at the higher

concentration (0.05g/ml). This result demonstrated that the ethyl acetate extracts of the wood and the bark of *T. brownii* against *A. niger* are either not effective or they may have an MIC above 0.05g/m.

Thin layer chromatography (TLC) revealed absences of alkaloids in the extracts of the different parts. Flavonoids and stilbenes were mainly accumulated in the ethyl acetate fraction of both studied parts (wood and bark). Terpenoids were detected in all extracts screened.

Reverse phase HPLC coupled with UV detector (RP-HPLC-DAD) proved the presence of flavonoids and flavonoidal acids in the different parts studied. The flavonoids detected were mainly flavonones, flavonols and their derivatives. Similar compounds exist among the active extracts, namely the ethyl acetate phases of the wood and bark of the plant studied. Accordingly, the wood ethyl acetate fraction was subjected to further analysis for identification of the major compounds with special emphasis on flavonoids and stilbenes content.

Tandem mass spectrometry (MS/MS) led to the identification of ten compounds three of which were pentacyclic triterpenoidal acids namely masilinic acid, asistic acid and arjunic glycoside. Resveratrol 3-O-β-galloylglucoside was also identified in its *cis* and *trans* forms. Flavonoids identified in *T. browni* wood ethyl acetate extract include two Quercitin derivatives namely Quercitin 7-O-β- diglucoside and Quercitin 7-O-galloylglucoside. Naringenin 4'methoxy 7 arabinoside together with Naringenin 7 ellagic acid were detected in this extract. Additionally, 5,6 dihydroxy 3'4'7 trimethoxy flavone was among the identified flavonoids in this extract.

These results support the final aim of using the extracts of *T. brownii* wood and bark to biologically control plants pathogenic fungi.

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خلاصة الاطروحة

لها استعمالات طبية في قارتي افريقيا واسيا. هذه الدراسة خلصت *Combertaceace* عائلة الي التحليل الكيميائي لجزئين من الشاف .

دراسة مستخلص اللحاء والخشب فُعلت بيولوجيا وكيميائياً بتطبيق مختلف طرق التحليل الكروموتوغرافي (كروموتوغرافيا الطبقة الرقيقة ،وكروموتوغرافيا الضغط العالي ومكشاف الطيف الضوئي).

المكافحة الحيوية للمستخلصين (الخشب واللحاء). فُعلت ضَد كائنات نباتية ممرضة من انواع مختلفة. المستخلص المائي لخشب الشاف سجلَ أعلي منطقة لتثبيط النمو الفطري في فطر *A.favus* (13ملم)، و11ملم منطقة تثبيط ضَد *A.niger* و فطريات الموت التراجعي *Fusarium moniliform* . و12 ملم منطقة تثبيط ضَد *Nattrassia mangifera* و 20 *A.flavus* و *A.niger* منطقة منع النموالفطري في المستخلص المائي للحاء 14 ملم ضدً *F.moniliform* . و *N.mangifera* ملم منطقة تثبيط النمو في فطر

مستخلص خلات الايثيل لخشب ولحاء الشاف أعطي نتائج متشابه في منطقة تثبيط النمو الفطري مع متوسط قطر مقداره 10 ملم ضَد كل من *F.moniliform, A.flavus , A.niger* *N.mangifera,* .أقل تركيز في مستخلص خلات الاثيل لخشب ولحاء الشاف قيست ضَد أربع كائنات ممرضة نباتياً . أقل تركيز منع النمو الفطري لم يحدَد بعد لانه في أقل تركيز (0.001 جم ملم) لوحظ عدم النمو الفطري .أقل تركيز يمنع النمو الفطري يجب ان يكون اقل من 0.001 جم /ملم . إيجابياً يلاحظ نفس التأثير للمستخلص ضَد *A.niger* حتي في أعلي تركيز للمستخلص0.05جم/ مل . هذه النتيجة أظهرت أن مستخلص الخلات الايتيلي لخشب ولحاء الشاف ضَد *A.niger* إما ليس له تاثير أو يحدث تاثير عندما يكون أقل تركيز يثبط النمو الفطري أعلي من 0.05جم/ مل .

عند تتطبيق كروماتوغرافيا الطبقة الرقيقة لوحظ غياب القلويات في مستخلص الخشب واللحاء. لوحظ وجود الفلافونويد و الاستلبين بصورة اساسية في مستخلص الخلات الاثيلي بالخشب واللحاء . كل المستخلصات أظهرت وجود التبربنويدات .

كروماتوغرافيا الضغط العالي للطور العكسي جُمعت مع المكشاف الطيفي وبرهنت وجود الفلافونيد واحماض الفلافونيدل في مختلف اجزاء الدراسة . والفلافونيدات التي ظهرت هي المتشابة توجد في المستخلصات النشطة تحديداً الفلافونون والفلافونول ومشتقاتهم . المركبات مستخلص الخلات الاثيلي لخشب ولحاء الشاف. طبقاً لذلك جُزء مستخلص خلات الاثيل الخشبي وفعِّل لتحاليل ابعد للتعرف علي المركبات الرئيسية مع التركيز علي محتواها من الفلافونيد والاستلبين .

تجارب مطافية الضوء آدت الي التعرف علي عشرة مركبات كيميائية ثلاث منهم تحت رتبة مركبات التيربونيد الحلقية الخماسية وهم حمض الماسليك والاسيستك والعرجونك جلايكوسيدك. وعُرف في شكل سيس وترانس ارسفراترول -3- اوكسي بيتا جلايول جلايكوسيد وعُرف الفلافونيد في مستخلص خلات الايثيل بخشب الشاف وتتضمنت مشتقات الكوارستين تحديداً كوارستين-7- اوكسي بيتا داي جلايكوسيد و كوارستين -7- اوكسي .جلايول جلايكوسيد . تم تحديد نارجنين 4 برايم ميثوكسي- 7- اربنوسيد مع نارجنين -7- حمض الالاجك في هذا المستخلص بالاضافة الي 6،5 ، داي هيدروكسي 3 برايم 4 برايم -7- تراي ميثوكسي فلافونون .

هذه النتائج تدعَم الهدف النهائي لاستعمالات مستخلصات خشب ولحاء الشاف في المكافحة الحيوية ضَد الكائنات النباتية الممرضة .

CHAPTER ONE
INTRODUCTION

Many of the plant materials used in traditional medicine are readily available in rural areas and this has made traditional medicine relatively cheaper than modern medicine (Mann, *et.al*., 2008). Medicinal properties of plants are normally dependent on the presence of certain phytochemical principles such as alkaloids, anthraquinones, cardiac glycosides, saponins, tannins and polyphenols which are the bioactive bases responsible for the antimicrobial property (Mann, *et.al*., 2008).

Secondary metabolites are a source of new antimicrobial products and inexpensive starting materials for synthesis of many known medicine, insecticide, fungicide and drugs etc. Considering the great number of chemicals that have been derived from plants as antimicrobial compounds, scientific evaluation of plants used traditionally for the treatment of some diseases or that possess natural resistance to insect and fungi attacks seems to be logical step of utilizing the antifungal compound, which may be present in plants. Plants based antimicrobial compounds represent a vast untapped source of medicine and pesticide with great potential (Angeh, *et.al*., 2006). The use of chemicals to protect plants have an advantage of being quick and effective, however, there are disadvantages like toxic residues, extensive labour, pathogens develop resistance to chemicals and chemicals also kill natural enemies of pathogens (Eltahir, 2003). Consequently, the use of chemical protectants in forest tree diseases control has not been great (Rich, 1975).

Phytochemical analysis on *Terminalia spp.* (*Combretaceae*) started since early 1970s and extended well into present. Important secondary metabolites reported include, stilbenes, phenantherenes, terpenoids, flavonoids and tannins, (Conrad, *et al*., 1998). The cancer cell line active components were found to be gallic acid, ethyl gallate, and the flavone luteolin. Only Gallic acid was previously known to occur in this plant. Luteolin has a well-established record of inhibiting various cancer cell lines and may account for most of the rationale underlying the use of *T. arjuna* in traditional cancer treatments. Luteolin was also found to exhibit specific activity against the pathogenic bacterium (Conrad, *et al*., 1998). *T. stuhlmannii* stem and bark yielded two glycosides of hydroxyimberbic acid, one of which is reported for the first time, associated activities with these compounds are anticancer, antimalarial, anti-inflammatory, gastroprotective and antimicrobial activities. Bioassay-guided separation methods, led to the study of cancer cell growth inhibitory constituents residing in the bark, stem and leaves of *Terminalia spp.* The structure of the isolated compounds was elucidated by spectroscopic methods. Several compounds had antibacterial activity, imberbic acid showing particularly potent activity against *Muycobacterium fortuitum* and *staphylococcus aureus*. (http://www.sciencedirect.com. 2007).

Among the reasons for pursuing natural product chemistry resides in the actual or potential antimicrobial activity to be found in alkaloids, terpenoids, coumarins, flavonoid, lignans and other secondary metabolites. The use of plants derivatives as a source of antimicrobials has been virtually non-existent since the advent of antibiotics in the 1950s (Angeh, 2006).

Secondary metabolites content varies among wood species, between bark and wood of the same species. Differences between the species may be anticipated on the basis of differences in chemical composition. Abdelhameed (2003) found substantial differences between leaves, bark, wood, roots and flowers.

Terminalia brownii is distributed in wide range of Savanna zones on loamy soil in Darfor, Kordofan, Blue Nile, Kassala and South Sudan. *T. brownii* is one of the most resistant plant species to many pathogenic fungi that affects the savannah forests in those areas. Secondary metabolites found in the different parts of this plant was suggested to be responsible for its resistance i.e. *T. brownii* different extracts were expected to have antimicrobial activity.

1.1 Objectives of this study

To date no phytochemical studies are reported on *T. brownii.* The main objective of this study is to chromatographically profile and biologically screen *T. brownii* (wood and bark) extracts, elaborating their antimicrobial activity, isolating their active ingredients and elucidating their structure. The objectives could be achieved by:

- Biologically guided fraction of the different extracts obtained with special emphasis of their antimicrobial activity.
- Separation of the active fraction using high performance liquid chromatography (DAD. HPLC).
- On line identification of the major compounds residing in the active fractions using Tandem mass spectroscopy (LC MS MS).

CHAPTER TWO
LITERATURE REVIEW

2.1 Taxonomy of the genus *Terminalia*

Terminalias are a medium sized to large trees up to 20 m high. Bark grayish white, becoming very dark grey, scaly in old trees. Branches often drooping and slender. Leaves alternate, rarely opposite or subopposite; elliptic to ovate- lanceolate, 2 – 8 x 1.3 – 5 cm; densely silky becoming pubescent beneath. Inflorescence small, greenish-yellow globose head; petals absent. Fruits 2 winged fruit in globose or subglobose cone-like heads, coriaceous, broadly winged dark grey. Flowers in globose heads, small, greenish yellow, (Elamin, 1990). According to Carolus linnaeus the plant studied could be classified as: -

Kingdom: *Plantae: Plants*
Subkingdom: *Tracheobionta -- Vascular plants*
Superdivision: *Spermatophyta -- Seed plants*
Division: *Magnoliophyta -- Flowering plants*
Class*:* *Magnoliopsida -- Dicotyledons*
Subclass: *Rosidae*
Order: *Myrtales*
Family: *Combretaceae*
Genus: *Terminalia L.*
Species: *brownii*
S.N: *Terminalia brownii*
Vernacular name*:* *Al shaf, Al drot, Alsafraya, Alsobag*

2.2 Antimicrobial metabolites of plant origin

Plants have almost limitless ability to synthesize different types of secondary metabolites. Useful phytochemicals that have antimicrobial effects can be divided into several categories these include, simple phenols and phenolic acid e.g. cinnamic and caffeic acid which are effective against viruses, bacteria and fungi, (Cown, 1999). In addition more investigations revealed that terpenes are toxic for fungi and for bark beetles (Conrad, *et.al.,* 1998).

Contribution of secondary metabolites as antimicrobial activity also refer to active constituent namely triterpens and saponin like mollic acid, jessic acid and their derivatives, the sodium salt of mollic acids glycoside isolates from *C. molle* were found be toxic to *Biomphalaria glabrata* snails (Angeh, 2007). More recently series of stilbenes and dihydrostilbenes (combretastatin) with potent cytotoxic activity and acidictriterpenoids and their glycosides with molluscicidal, antifungal and anti-inflammatory activity have been isolated from *Terminalia* species

Some essential oils are effective against some higher organisms such as nematodes, helminthes and insects. Common active components of the essential oils include thymol, carvacol, camphor and terpinene-4-ol (Ncube, *et. al*., 2007). Many of the woods from which stilbenoids have been isolated are highly resistance to decay Stilbenes are involved in the protection of wood decay and are induced as phytoalexins (phenanthrenes and dihydro-phenantherenes) in response to pathogenic attacks (Seigler, 1998).

2.3 *Combretaceae* secondary metabolites and their biological significance

Species of *Combretaceae* contain compounds with potential antimicrobial properties (Angeh, 2006). There is a large variation in the chemical composition and antimicrobial activity among the different genera and species in the *Combretaceae*. Several species of *Combretaceae* used in traditional medicine in West Africa have been investigated for their antifungal activity against the pathogenic fungi. Phytochemistry screening revealed that these plants are particularly rich in tannins, and saponins, which might be responsible for their anti-fungal activity (Baba-Moussa, *et.al.*, 1999).

Stilbene aglycone are common in heartwood, living tissue often contents small amount of stilbenes glycoside (Figure 1-A), many of the wood from which stilbenoids have been isolated are highly resistance to decay (Seigler, 1998).

To date around 80 metabolites were reported from the genus *combretum* including stilbenes phenantherenes, terpenoids, cycloarenoids, macrolactones and flavonoids (Pettit, *et.al*., 1995; Jossang, *et al*., 1996; Adnyana, *et.al*., 2000, Ogan, 1972; Abdurzag, *et.al*., 1997). One of the most active stilbenes isolated is combretastatin A_4, which is in very late stages of clinical trials.

2.4 *Terminalia* important secondary metabolites

Phytochemical work on *Terminalia spp.* started since early 1970s and extended well into present. Important secondary metabolites reported include, stilbenes, phenantherenes, terpenoids, flavonoids and tannins, (Conrad, *et.al.,* 1998). Associated activities with these

compounds are anticancer, antimalarial, anti-inflammatory, gastroprotective and antimicrobial activities. By means of bioassay-guided separation methods, the cancer cell growth inhibitory constituents residing in the bark, stem and leaves of the Mauritius medicinal plant *Terminalia arjuna (Combretaceae)* were examined. The cancer cell line active components were found to be gallic acid, ethyl gallate, and the flavone luteolin. Only gallic acid was previously known to occur in this plant. Luteolin has a well-established record of inhibiting various cancer cell lines and may account for most of the rationale underlying the use of *T. arjuna* in traditional cancer treatments (http://www.sciencedirect.com. 2008). Luteolin was also found to exhibit specific activity against the pathogenic bacterium. *Terminalia stuhlmannii* stem and bark yielded two glycosides of hydroxyl imberbic acid, one of which is reported for the first time. The structure of the isolated compounds was elucidated by spectroscopic methods. Several compounds had antibacterial activity, imberbic acid showing particularly potent activity Against *Muycobacterium fortuitum* and *staphylococcus aureus* (http://www.sciencedirect.com. 2008).

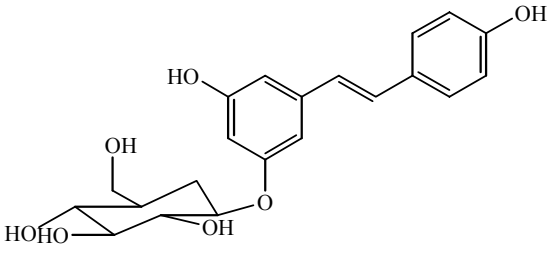
The root and bark of *Terminalia sericeae* yielded an unreported stilbene glycoside, 3'5'-dihydroxy-4- (2-hydroxy-ethoxy) resveratrol-3-O-β-rutinoside together with known compounds resveratrol-3-β-rutinoside glycoside, 3', 4,5'-Trihydroxystilbene (resveratrol) as shown in figure 1-A. Structure determination of the isolated compounds was achieved on the basis of spectroscopic measurements (Joseph, *et.,al*., 2007).

Many biologically active compounds were detected and their structures were elucidated, from the genus *Terminilia*, this includes:

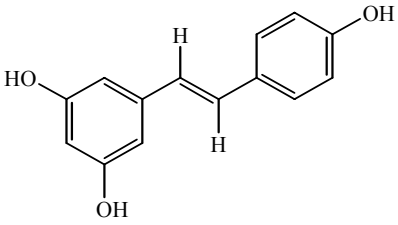
1,3-diarylpropanes,1-(4'-hydroxy-2'-methoxyphenyl)-3-(3"-methoxy 4"hydroxyphenyl)-propane and 1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3"-methoxy-4" -hydroxyphenyl)-propane, seven flavanones, naringenin, naringenin-4',7-dimethyl-ether, sakuranetin, isosakuranetin, liquiritigenin-4',7-dimethyl-ether, liquiritigenin-7-methyl-ether and liquiritigenin-4'-methyl-ether, two chalcones, isoliquiritigenin-4-methyl-ethe and isoliquiritigenin-4-methyl-ether, one flavan, 7,4'-dihydroxy-3'-methoxyflavan (Figure 1-B), nine triterpenes (Figure 1-C), arjunic acid, arjunetin, arjungenin, arjunglucoside I, arjunolic acid, arjunglucoside II, 23-galloylarjunglucoside II (isolated as its mono-, di- and tri-O-methyl derivatives after methylation with diazomethane), betulinic acid and ursolic acid acetate, along with gallic acid and sitosterol were isolated from the heartwood and bark of *Terminalia fagifolia* (http://www.scielob. 2008).

A new oleanane-type triterpene (3β, 6β, 23, 28-tetrahydroxyolean-12-ene) was isolated from the leaves of *Terminalia glabrescens*, together with ursolic, 2α-hydroxyursolic, oleanolic, maslinic, arjunolic, sumaresinolic and asiatic acids, squalene, phytol, sitosterol-3-O- β -D-glucopyranoside and n-alkanes. Friedelin, taraxerol, lupeol, lupenone, betulin, betulone, betulinic acid, stigmastane-3 β, 6 α -diol, - β sitosterol, catechin, . β-D-pyranotagatose, β-D-furanofructose and α-D-furanofructose were obtained from the trunk bark of *T. glabrescens* (www.scielo.br, 2008). A new cardenolide, 16,17-dihydroneridienone 3-O-β-D-glucopyranosyl- (1→6)-O-β-D-galactopyranoside, was isolated from the roots of *Terminalia arjuna*. (Yadav, *et.al.,* 2000). *T. calcicola* led to the isolation of two new cytotoxic xanthones, termicalcicolanone A and termicalcicolanone B. Both compounds

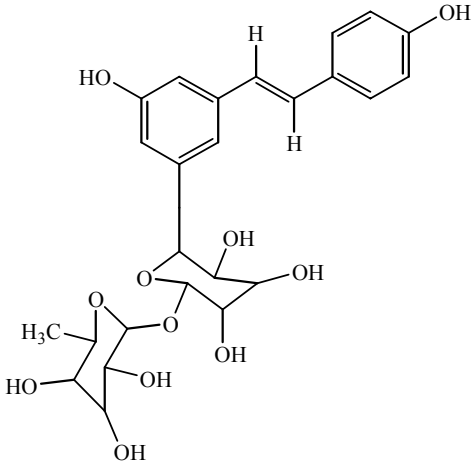
showed modest antiproliferative activity toward the human ovarian cancer cell line (Cao, *et.al.*, 2008).



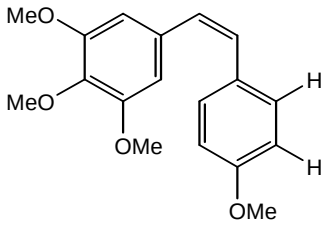
Revesatrol -beta-D-glycoside
T. sericeae



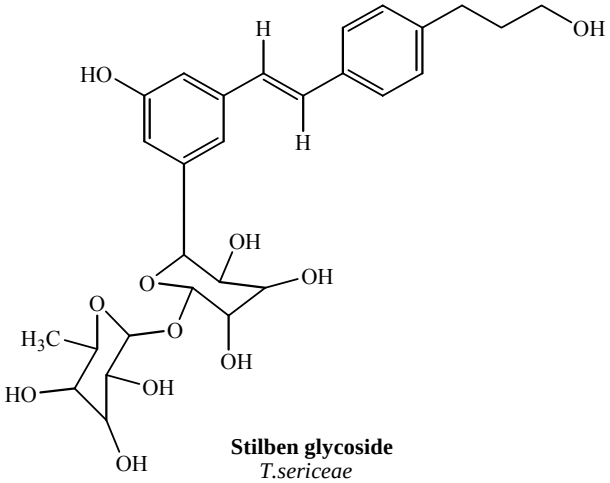
Resveratrol
T, sericeae



Reveratrol-3-ortho-beta-rutinoside
T.sericeae

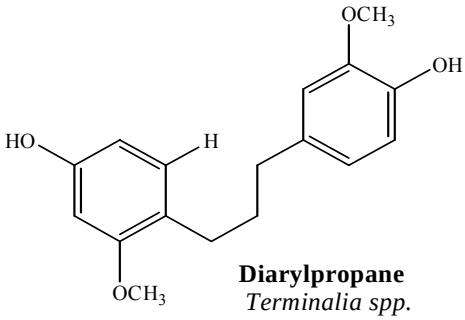


Combretastatin A_4
Combretum caffrum

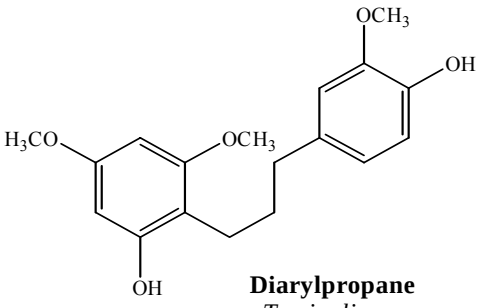


Stilben glycoside
T.sericeae

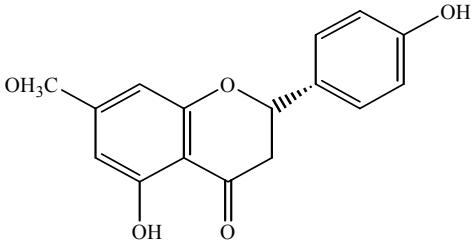
Figure 1.A: Stilbens reported in *Combretaceae*



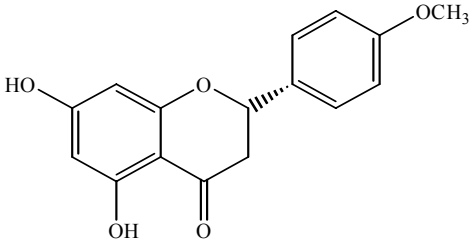
Diarylpropane
Terminalia spp.



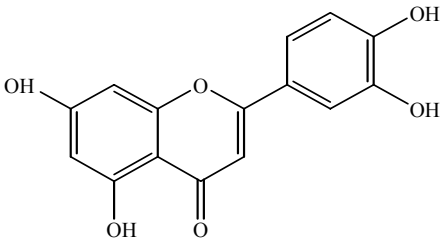
Diarylpropane
Teminalia spp.



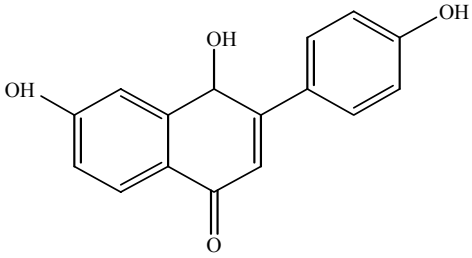
Sakuranetin
T. fagifolia



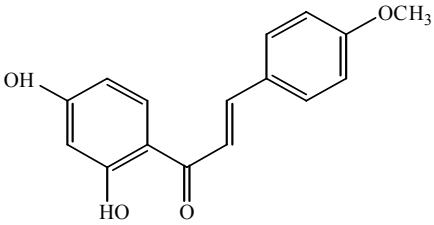
Isosakurantin(Naringenin-4'-methyl ether)
T.fagifolia



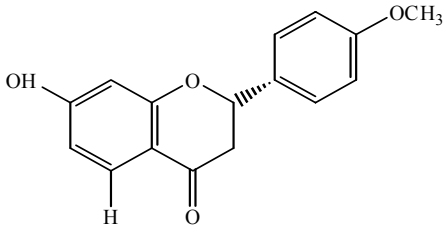
Luteolin
T. arjuna.



Isoliquiritigenin
T. fagifolia

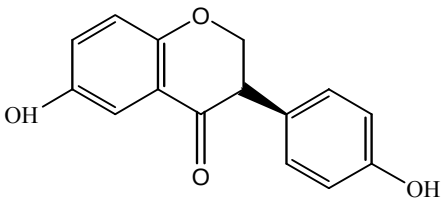


Isoliquiritigenin-4'-methyl ether (2,4 dihydroxy-4'-methoxy chalcone)
T. fagifolia

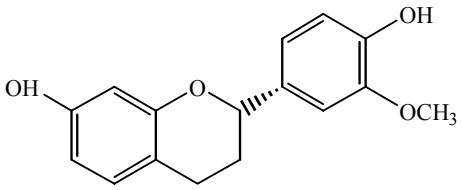


Liquiritigenin-4'-methyl ether
T. fagifolia

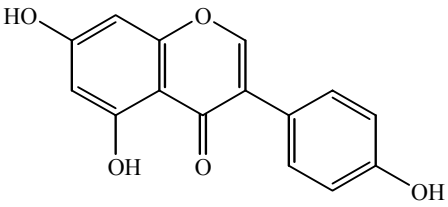
Figure 1.B.1: Flavonoids reported in *Terminalia spp*



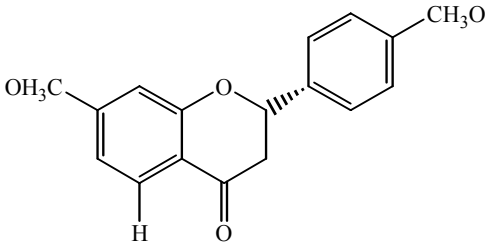
Liquiritigenin
T. fagifolia



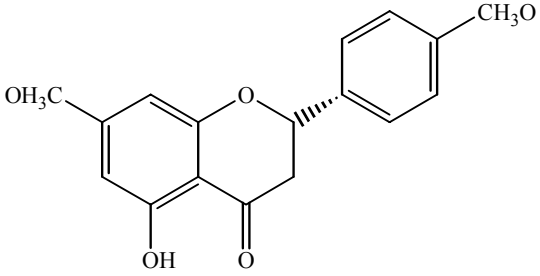
7,4' dihydroxy-3'-methoxy falvan
T. fagifolia and *T.aregentena*



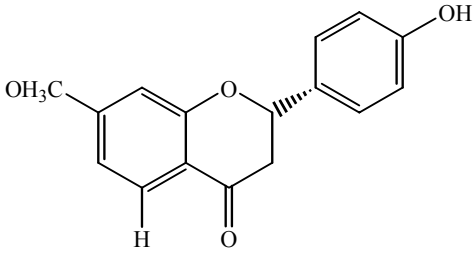
Genistien
T. arjuna



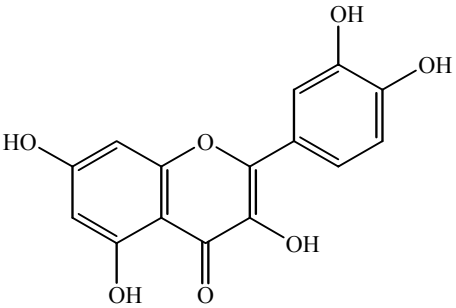
Liquiritigenin-4',7-dimethyl ether (7,4'-dimethoxy flavan)
T. fagifolia



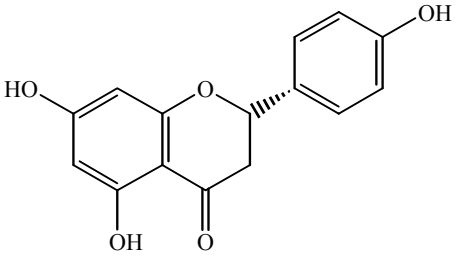
Narigenin-4',7dimethyl ether
T. fagifolia



liquiritigenin-7-methyl ether
T.fagifolia

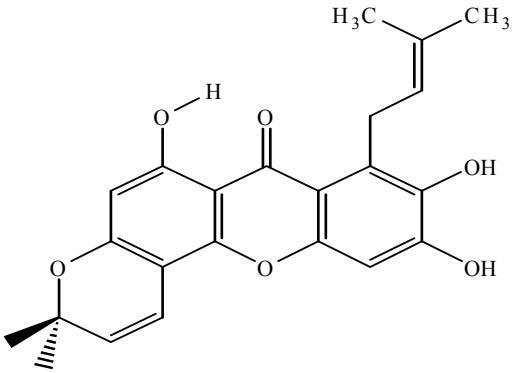


Quercetin
T. arjuna

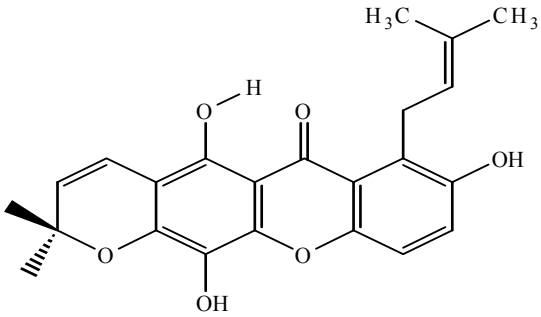


Naringenin
T. fagifolia

Figure 1.B.2: Flavonoids reported in *Terminalia spp* continued

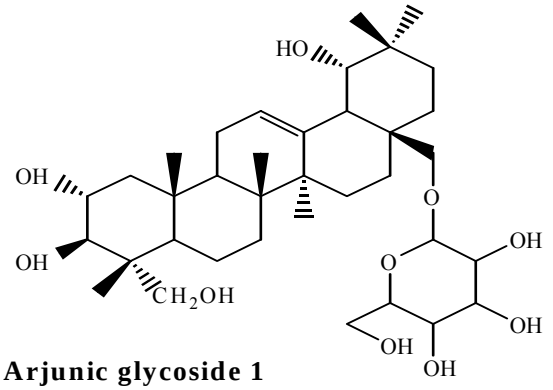


Termicalcicolanone B
T. Calcicola

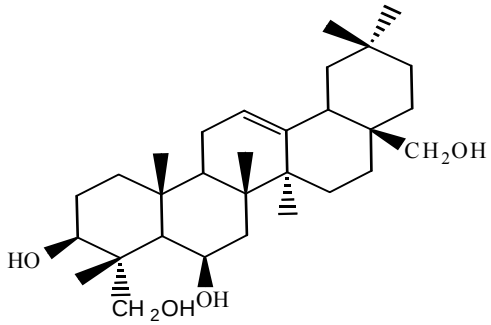


Termicalcicolanone A
T. calcicola

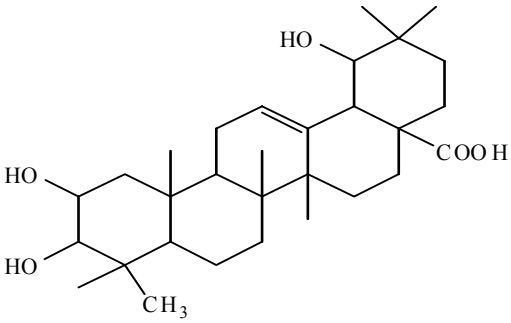
Figure 1.B.3: Flavonoids reported in *Terminalia spp* continued



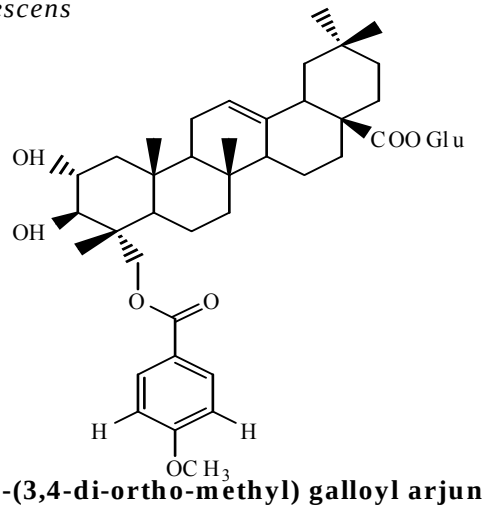
Arjunic glycoside 1
T. arjuna T.fagifolia



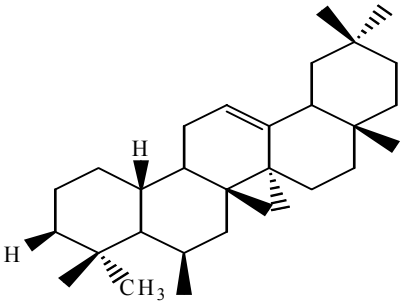
3beta,6beta,23,28 tetrahydroxyolean-12-ene
T. glabrescens



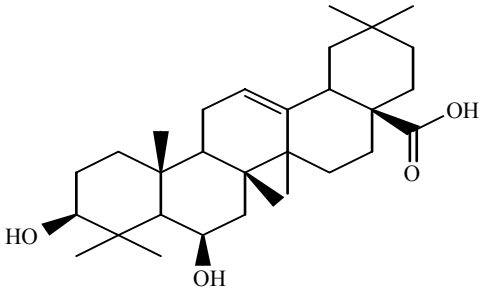
Arjunic acid
T. arjuna T. fagifolia



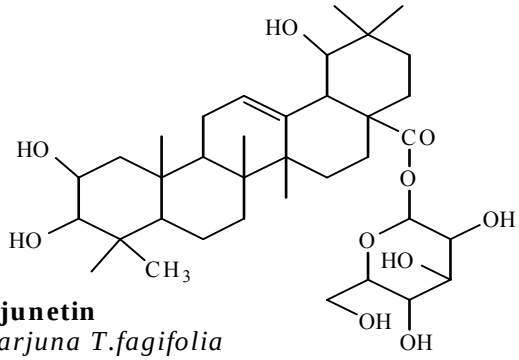
23-(3,4-di-ortho-methyl) galloyl arjun
T. fagifolia T. macropetra



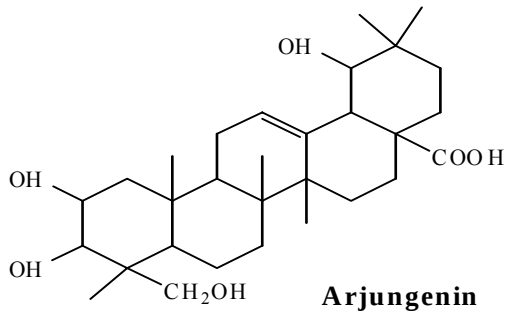
Squalene
T. glabresces



Sumarsonlic acid
Terminalia spp

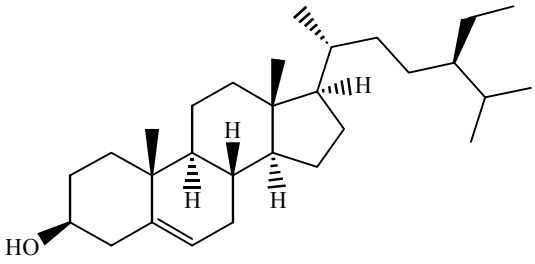


Arjunetin
T. arjuna T.fagifolia

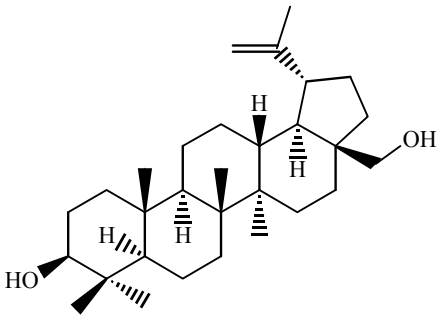


Arjungenin
T. arjuna

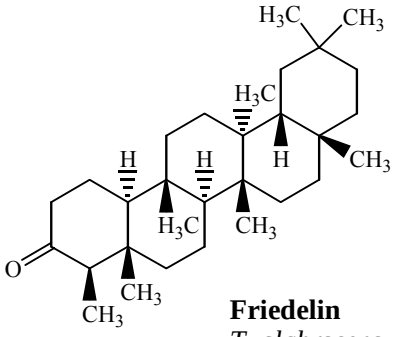
Figure 1.C.1: Terpenes reported in *Terminalia spp*



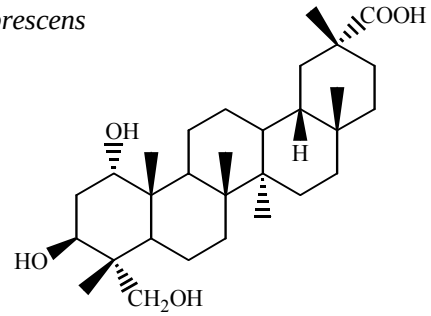
Sitostrol
T. glabrescen



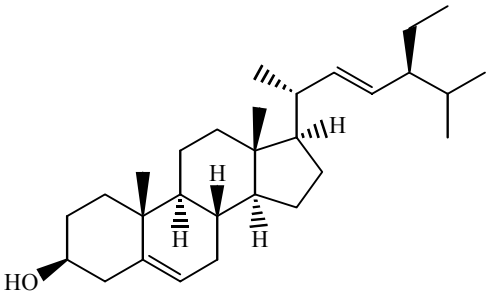
Betulin
T. glabrescens



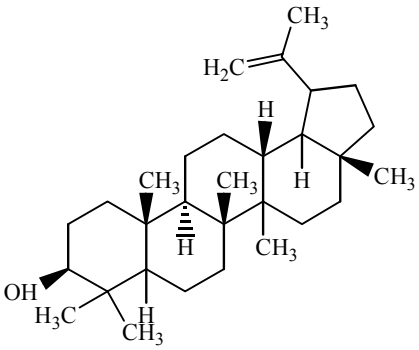
Friedelin
T. glabrscens



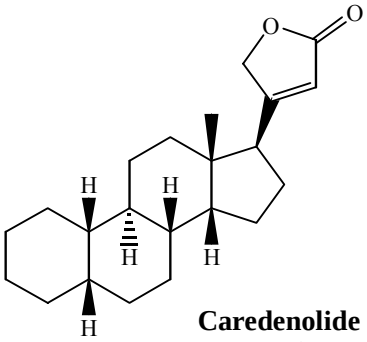
Imberbic acid
T.stuhlmanii



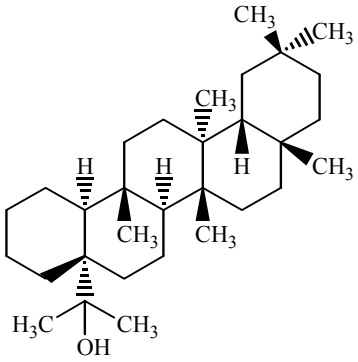
Stigmasterol
Terminalia spp.



Lupeol
T. glabrescens



Caredenolide
T.arjuna



Terminalin A
T. glaucescens

Figure 1.C.2: Terpenes reported in *Terminalia spp* continued

In *Terminalia macroptera* and *Terminalia belerica*, previous investigations have demonstrated that the leaves content of secondary metabolites changes after artificial inoculation with fungi. These modifications vary according to the resistance level of *Terminalia spp*. The study of protective responses of *Terminalia spp* to attack by pathogenic fungi is essential for developing new approaches to assess the resistance of trees to infectious diseases. In some cases, the rate of lignin accumulation can characterize the tolerance of a plant towards both fungal pathogens and for the insect.

Medicinally the barks are used for cough and bronchists and fumigant is used for rheumatism (El-Ghazali, *et.al*., 1997). The local knowledge in using species in body fumigation, cosmetic and folk medicine such as inflammatory and jaundice are well known to the forests-adjacent communities. Other *Terminlia spp* in Sudan and Africa and their traditional uses are summerized in Table 1

Table: 1 African and Sudanese traditional uses of *Terminalia spp*

Species	Part used	Claimed therapeutic uses
Terminalia albida	Leaf	Diarrhea
Terminalia avicennioids	Root	Conjunctivitis, phagedenic ulcer, wounds
	Leaf	Rheumatism
Terminalia basilie	Latex	Conjunctivitis, ear inflammation
Terminalia bauman	Root	Diarrhea
Terminalia brachystemma	Bark	Diarrhea, stomach pain
	Root	Hematuria, cholemesis
Terminalia brevipes	Bark	Jaundice, malaria
Terminalia brownii	Root	Cough
	Bark	Dysmenorrhagia, jaundice, yellow fever
Terminalia catappa	Leaves	Hypertension, diabetes
	Fruit	Sever diarrhea
Terminalia chebula (in Sudan)	Fruit	Asthma, cough, hypertension Chronic ulcer, laxative
Terminalia fatraea	Bark	Colic, indigestion
Terminalia glaucescens	Leaf	Burns, headache, stomach pain, cough,
	Root	Dental care
Terminalia ivorensis	Leaves	Wounds, hemostatic,hemorrhoids.malaria, yellow fever
Terminalia kaiserana	Root	Kidney pain, cough, headache,
Terminaliakilimandscharica	Bark	Asthma, cancer,
Teminalia laxiflora	Bark	Skin pustules, wounds, hemostatichemorrhoids
Terminalia macrocarpa	Leaves	Hemorrhoids
	Root	Jaundice,
Terminalia mollis	Bark	Wound, hemostatic, hemorrhoids
Terminalia monoceras	Leaves	Severe diarrhea
Terminalia orbicularis	Bark	Polymenorrea, cholera
Terminalia prunioides	Root	Gastrointestinal disorders, cougbronchitis
Terminalia sericea	Leaves	Diarrhea, stomach Dysentery, colic,
	Root	Wounds, diarrhea, skindisease,cough,stomache
	Bark	Diabetes, diarrhea, dysentery, colic
Terminalia spinosa	Leaves	Malaria
Terminalia stenostachya	Root	Epilepsy
Terminalia superba	Bark	Diuretic,insanity,cholemesis,dysentery,vomiting
Terminalia trichopoda	Bark	Abdominal pain
	Root	Stomach pain
Terminalia zambesiaca	Bark	Bloody diarrhea cancer, gastric ulcers
	Root	Cancer, gastric ulcers
Terminalia arjuna (in Sudan)	Bark	Astringent, tonic, febrifuge, diarrhea
Terminalia brownii (in Sudan)	Bark	Antinflammatory, cough bronchists
	Bark	Fumigant for rheumatism and cosmetics,
Terminalia belerica (in Sudan)	Fruit	Expectorant, antiseptic

Neuwinger, (2000), Ahmed, *et.al*., (1998), El-Ghazali, *et.al*., (1997)

2.5 Plant pathogen tested

Aspergillus niger (ATCC9763-8/29/2005) (aerobic fungi *Eurotiomycetes*) belongs to the class *Eurotiomycetes* order *Eurotiales* are filamentous fungi common in the environment. *Asp. niger* can cause the rotting of numerous fruits, vegetables, and other food products. *A. niger* causes black mould of onions. Infection of onion seedlings by *A. niger* can become systemic, manifested only when appropriate conditions are available. *A. niger* causes a common postharvest disease of onions, in which the black conidia can be observed between the scales of the bulb. The fungus also can infect in peanuts and grapes. *A. niger* damages surface layers of wood, raw cotton fibers and many other materials it is used to test the efficacy of preservative treatments (Jong, and Gantt, 1987). *A. niger* is a common laboratory contaminant and can causes disease of mycotoxins, where systemic infection is often fatal. (http://www.en.wikipedia.org/wiki/ 2008).

Nattrassia. mangiferae (Eltahir, 2003) (Imperfect fungi, *Deuteromycetes*) belongs to the class *Coellomycetes* order *Sphaerospidales* causing cankering of the main stem, wilting of the branches is associated with decline of trees. (Eltahir, 2003).

Nattrassia mangiferae is a common fungus that causes branch wilt in Sudan. It has a wide host range consisting of fruit trees, shade trees, and ornamental trees. *N. mangiferae* infection causes about 33.3-95.34% and 79.16-100% mortality in *Ficus nitida and Ficus benjamina,* respectively. The first infection by *N. mangiferae* on humans had been reported in India in 1970. It causes dry, scaling skin disease. Infection of humans is thought to occur by contact with infected soil, although in some cases people become infected by direct contact with splinters of wood. Human infection by this

fungus is probably most common than is reported, because it could easily be mistaken with other diseases. Infection of humans usually occurs in tropical and subtropical areas where the fungus is endemic (Eltahir, 2003).

Aspergillus flavus (N.H.L, 2006) (aerobic mould fungi *Eurotiomycetes*) belongs to the class *Eurotiomycetes* order *Eurotiales*. The mold damage *A. flavus* is one of the most important causative agent of damage for corn and peanuts, and it is the one of several species of moulds known to produce aflatoxin. Aflatoxin in human causes acute hepatitis, immuno-suppression, and hepatocellular carcinoma. (http://www.wikipedia.odjechane. 2008). It is a pathogen, associated with aspergillosis of the lungs and sometimes believed to cause Corneal, Otomycotic, and nasoorbital infections. (http://www.wikipedia.odjechane. 2008).

Fusarium moniliforme (PPI/PPA/MA, 2006) (filamentous fungus *Hypocreaceae*) belong to the class *Sordariomycetes* order *Hupocreales,* it is fungus infecting soybean, bean and other crops. It causes bakanae disease in rice seedlings, by overloading them with the phytohoromone, gibberellins (http://www.wikipediaodjechane.net. 2008). Southern forest pine heavily infected with *Fusarium species* are predisposed to be killed by the Cankers (George, 2004). The inflorescences of Mango (*Mangifera indica***)** when attacked by *Fusarium moniliform* are commonly followed by other fungi like *Aspregillus niger*. Fungal mycelia gradually invade the xylem tissues from the top of the branches and spread basipetally ultimately causing death of the infected branches (Rajput, and Rao, 2004).

2.6 Biosynthesis of Flavonoids

Flavonoids are member of a class of natural compounds (phenylpropanoids) with widespread occurrence in plant kingdom. Plant phenols are being regarded as those substances derived from the shikimate pathway and phenyl propanoid metabolism. The flavonoids are built upon a C_6 -C_3-C_6 flavones skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen (Robards, and Antolovich, 1997). The biosynthesis of flavonoids compounds are derived from a branch of the flavonoid pathway (Figure 2), for which chalcone synthase (CHS) provides the first committed step by condensing one molecule of p-coumaroyl-CoA with three molecules of malonyl-CoA to produce tetrahydroxychalcone (a chalcone, Figure 2). Chalcone provides the precursor for all classes of flavonoids, which include the flavones, flavonols, flavan-diols, flavan 4- ols, proanthocyanidins (condensed tannins), isoflavonoids, and anthocyanins. The closure of the C-ring, resulting in the formation of flavanones, is carried out by chalcone isomerase (CHI). Flavanones (e.g., naringenin) provide a central branch point in the flavonoid pathway and can serve as substrates for enzymes that introduce–OH groups at the 3' and 5' positions of the B-ring, or for the hydroxylation of the C-ring by flavanone 3-hydroxylase. Dihydroflavonol 4-reductase (DFR) provides one entry step to the biosynthesis of anthocyanins, and depending on the plant species, it can utilize as a substrate any one or all three of the possible dihydroflavonols, dihydromyricetin, dihydrokaempferol, or dyhydroquercetin.

The leucoanthocyanidins are converted into the corresponding anthocyanidins by the action of a leucoanthocyanidin dioxygenase/anthocyanidin synthase. (Grotewold, 2006)

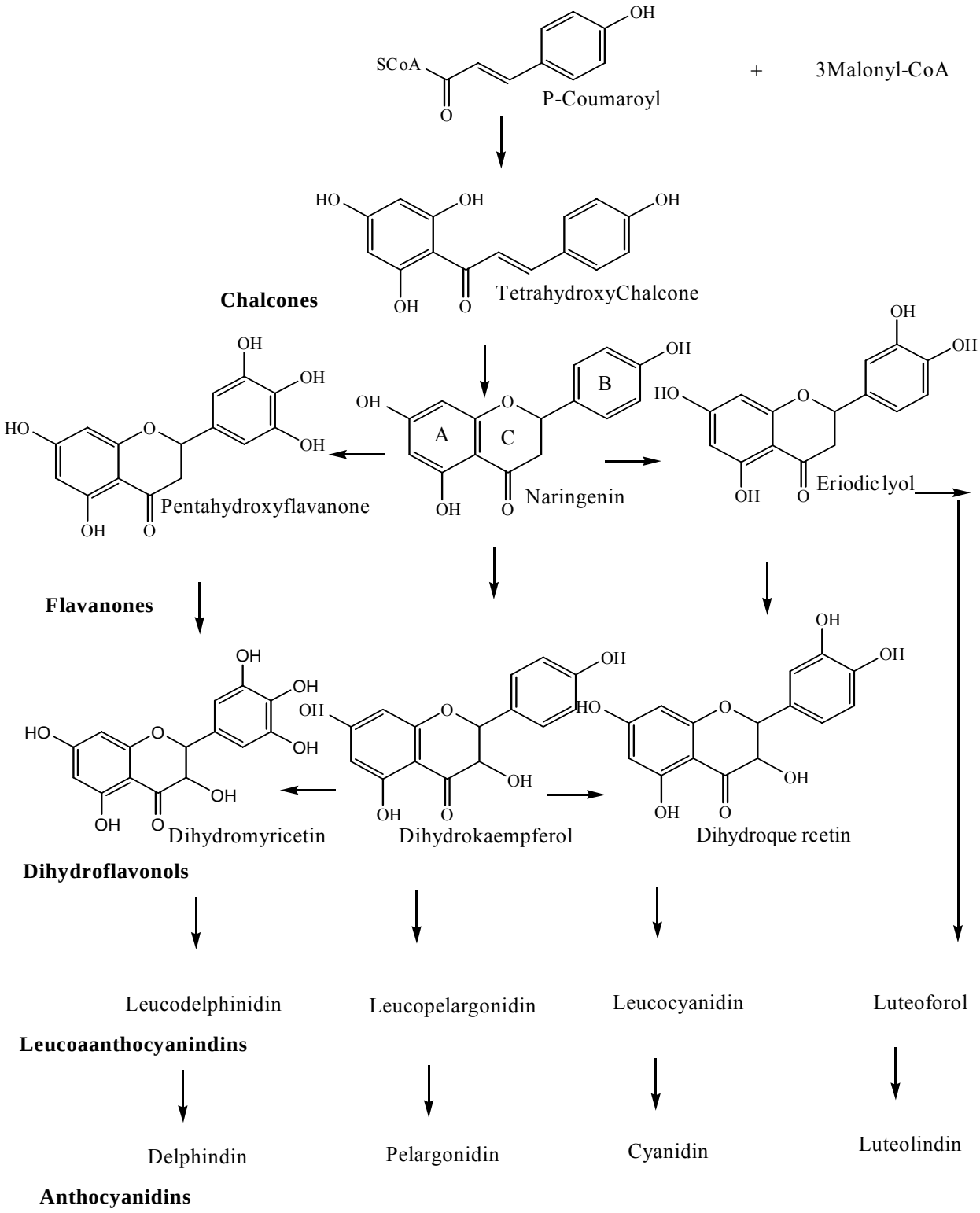


Figure 2: Biosynthesis of the different classes of flavonoids

2.7 Chromatographic analysis of flavonoids

In both profiling and quantification studies of flavonoids, the most successful approaches to date have been based on chromatography. Thin layer chromatography is an ideal technique for the screening of antimicrobial activity because of it is low cost, easy maintenance and selectivity of detection reagents. TLC on silica gel is very favorable for the analysis of flavonoids, (Stobiecki, 2000, Robard and Antolovich, 1997). The selection of the suitable stationary phase and solvent depend on the class of flavonoids to be examined.

High performance liquid chromatography (HPLC) and Gas liquid chromatography (GLC) are important where identification is required. Liquid chromatography (LC) of flavonoid is usually carried out in the reversed-phases (RP) mode. On C8-or C18-bonded silica columns. Gradient elution is generally performed with binary solvent systems, i.e. with water containing acetate or formate buffer and methanol or acetonitrile as organic modifier. LC is usually performed at room temperature, but temperature up to 40° C are sometimes recommended to reduce the time of analysis and because thermostated columns give more repeatable elution times. If the main aim of the study is to determine the major flavonoids in a sample, run times of 0.5-1 h usually suffice to separate five to ten compounds of interest (Rijke, *et.al.*, 2006). Flavonoid detection is carried out at 250, 265, 290, 350, 370, and or 400nm (with an added wavelength in 500-525nm ranges if anthocyanidins are included. (Rijke, *et.al.*, 2006).

2.8 Stilbenes and their biosynthetic pathway

There are two major groups of the stilbenes, resveratrol (stilbenes), and the phenanthrenes, together with their respective dihydro derivatives are characteristics in *Combretaceae* family (Seigler, 1998).

The stilbenes are often in plants that are not routinely consumed for food or in the nonedible tissue (Cassidy, *et.al*., 2000), and are usually assumed that the resistance of these woods to fungal attack is due to presence of these phenolic materials. Other higher plant source includes *Combretaceae* (Harborne, *et.al.*, 1999). Stilbenoids are widely distributed in higher plants, 29 in monomeric form and as dimeric, trimeric and polymeric stilbenes, the so-called viniferins. Among monomeric stilbenes, trans-resveratrol has been identified as the major active compound, and most of the studies in the literature about the physiological activity have focused on it; however, there are also some studies of the 3-β-glucoside of transresveratrol, the so-called piceid or polydatin, and the viniferins. (Cassidy, *et.al*., 2000).

Phenanthrenes are rather uncommon class on aromatic metabolites, where as presumably formed by oxidative coupling of the aromatic rings of stilbene precursors. Besides these stilbene derived compounds, phenanthrenes most likely originated from diterpenoid precursors (Cassidy, *et.al*., 2000). Biosynthesis of dihydrophenanherenes is similar to that of stilbenes, but appears to involve dihydrocinnamic acids and the enzyme bibenzyl synthase, where as the biosynthesis of phenantherenes involves the corresponding unsaturated acids (Seigler, 1998).

The phenanthrenes classified into three major groups. Monophenanthrenes, diphenanthrenes and triphenanthrenes. Monophenanthrene are subdivided according to the number and type of the structural moieties, while the type of connection of the phenanthrene units can classify diphenanthrene, Tricyclic 9,10dihydrophenanthren originate from phenylpropane derivatives by chain elongation and cyclization according to the polyacetate rule. Bibenzyls are bicyclic intermediates, and O-methylation is a prerequisite for their conversion into dihydrophenanthrenes. (Preisigmuller, *et.al.,* 1995). Up to present only one compound of the triphenanthrene group was described. (Kovacs, *et.al*., 2007). Large number of biological activities of differently substituted phenanthrene has been reported to occur in plants and has been demonstrated to possess various active compounds, Phenanthrenes have been studied for their cytotoxicity, antimicrobial, spasmolytic, anti-inflammatory, antiplatelet aggregation, antiallergic activities and phytotoxicity, most natural phenanthrenes occur in monomeric form, this group consist about 210 compounds, almost 100 are only hydroxy- and/or methoxy- substituted, and equally 9, 10-dihydro or dehydro-derivatives. Their great structural diversity stems from the number and position of their oxygen functions. The hydroxyl and methoxy moieties number are between 3 and 6, and can usually be found on C-2, C-3, C-5, and C-6 or C-7. Besides hydroxyl and methoxy groups, further substituents can be found in monomeric phenanthrenes, such as methyl, hydroxylmethyl, carboxy, formyl, prenyl and vinyl. Another type of monomeric phenanthrenes is the group of phenanthraquinones; altogether 19 compounds belong to this group. They are usually hydroxyls, methoxy or methyl substituted (Kovacs, *et.al*., 2007).

Stilbenes are 1, 2-diarylethenes (Figure 3), Ring A usually carries two hydroxyl groups, while ring B is substituted by hydroxy and methoxy groups in the 0-, m- and/or p-position. The stilbenoids are group of phenolic compound biosynthetically inter related through their common origin from a C6-C2-C6 intermediate. Like flavonoids, they are formed from the condensation of a P-hydroxycinnamic acid (C6-C3) precursor with three molecules of malonyl coenzyme A, but they differ in that one carbon atom is lost by (decarboxylation) in the process. Biosynthetic activities require 4-coumaroyl-CoA and three malonyl-CoA; these are present in all plants (Harborne, *et. al*., 1999). The reactions of resveratrol synthase and chalcone synthase are very similar, and only the final ring-folding is different in resveratrol synthase. Resveratrol synthase and chalcone synthase are condensing enzymes; they use three sequential condensation reactions with malonyl-CoA to produce an enzyme-bound tetraketide intermediate (Harborne, *et. al*., 1999).

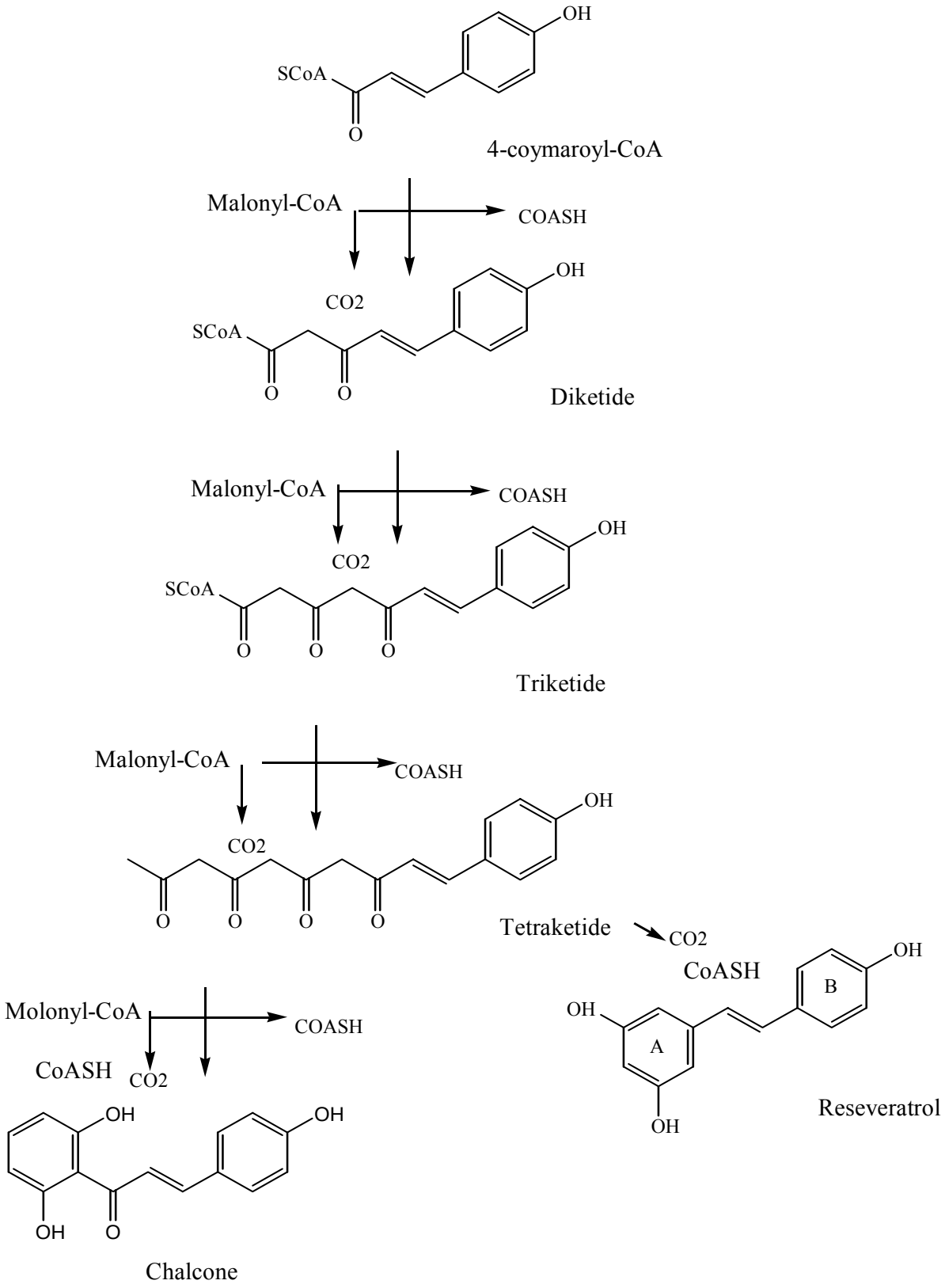


Figure 3: Stilbenes biosynthesis (Schroder, 1990)

2.9 Chromatographic analysis of stilbenes and phenantherenes

Stilbenes and related compounds can be separated with a number of techniques such as thin-layer (TLC), gas liquid chromatography (GLC), and high performance liquid (HPLC) chromatography, characterization and identification of these compounds can best be accomplished by nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (Seigler, 1998).

To investigate the presence of stilbenes in red wine a new reversed-phase (RP) high-performance liquid-chromatographic (HPLC) method with enhanced separation efficiency and improved selectivity, sensitivity, and speed has been established for determination of the stilbenes cis- and trans-resveratrol, in a single run. UV-absorbance, fluorescence (FLD), and mass-spectrometric (MS) detection were also evaluated. UV-absorbance detection was adjusted at 320 nm for stilbenes (Laszlo, *et.al*., 2005).

To quantify and qualify phenantherenes in biological matrices, detection and identification of the anylte (phenantherenes) were achieved using gas chromatography coupled to mass spectroscopy (Grova, *et.al*., 2005). RP-HPLC, column, elution systems of mobile phase and the detectors effected the separation of phenantherenes (Arbabi, *et.al*., 2004). In the other case phenanthrene concentration in contaminant soil was measured by HPLC, with chromatographic conditions as follows: Analytical column C18 Ultra Sep ES PAH QC Speica, 60 × 2 mm ID. Flow rate (ml/min) 0.5, injection rate 50 µl, Elute acetionitirl/water: 40-100%, UV detector wavelength: 254 nm according to pre-test results the optimum elute condition for phenanthrene was determined at 60/40 (acetonitril/water) (Arbabi, *et.al*., 2004)

CHAPTER THREE
MATERIALS AND METHODS

3.1 Plant material collection

The plant material (wood and bark) used in this study was collected from El Nour Natural Forest Reserve, southeast of El Damazine district-Sudan. Geographically located between latitude 11˚ 52.5` and 11˚ 48` N; longitude 34˚ 30`and 34˚ 29.5` E. Wood and bark pieces were collected separately from *Terminallia brownii* (*Combretaceae*) trees free from diseases, accumulation of knots, resin galls and gums. A voucher specimen was made for the plant studied and identified by taxonomist in the department of siliviculture, Faculty of Forestry, University of Khartoum. The herbarium was deposited at the Department of Biochemistry, Commission of Biotechnology and Genetic Engineering, National Center for Research. Collection data categorized under place, date and collector, an asterisk indicating a herbarium sample are as follows: *Terminallia brownii* (1*), El Nour Natural Forest Reserve, May – 2006, Enass, (Figure 4).

A

B

C

D

Figure 4: *Terminalia brownii* parts

A. Whole Plant

B. Bark

C. Leaves

D. Inflorescence

3.2 Plant material preparations and extractions

Samples were taken according to Koch (1985) from healthy old trees growing in that natural forest 50 cm long logs (diameter 20-42 cm) above 180cm from the ground All logs were manually debarked; samples were air dried under shade. The wood and barks samples were chipped to small chips using sawmill followed by hammer mill (mesh.) to obtain finely grounded wood powder which were stored separately in paper bags. Hundred grams of the air dried species were extracted sequentially using solvent of increasing polarities. The plant material was extracted using petroleum ether (PE), marc was then extracted with chloroform (Ch) and finally the marc was extracted using 80% methanol (MeOH). The methanolic extract was then fractionated (liquid/liquid) using ethyl acetate (EtoAc). Extracts were concentrated to dryness by evaporating the solvent at room temperature, (Figure 5).

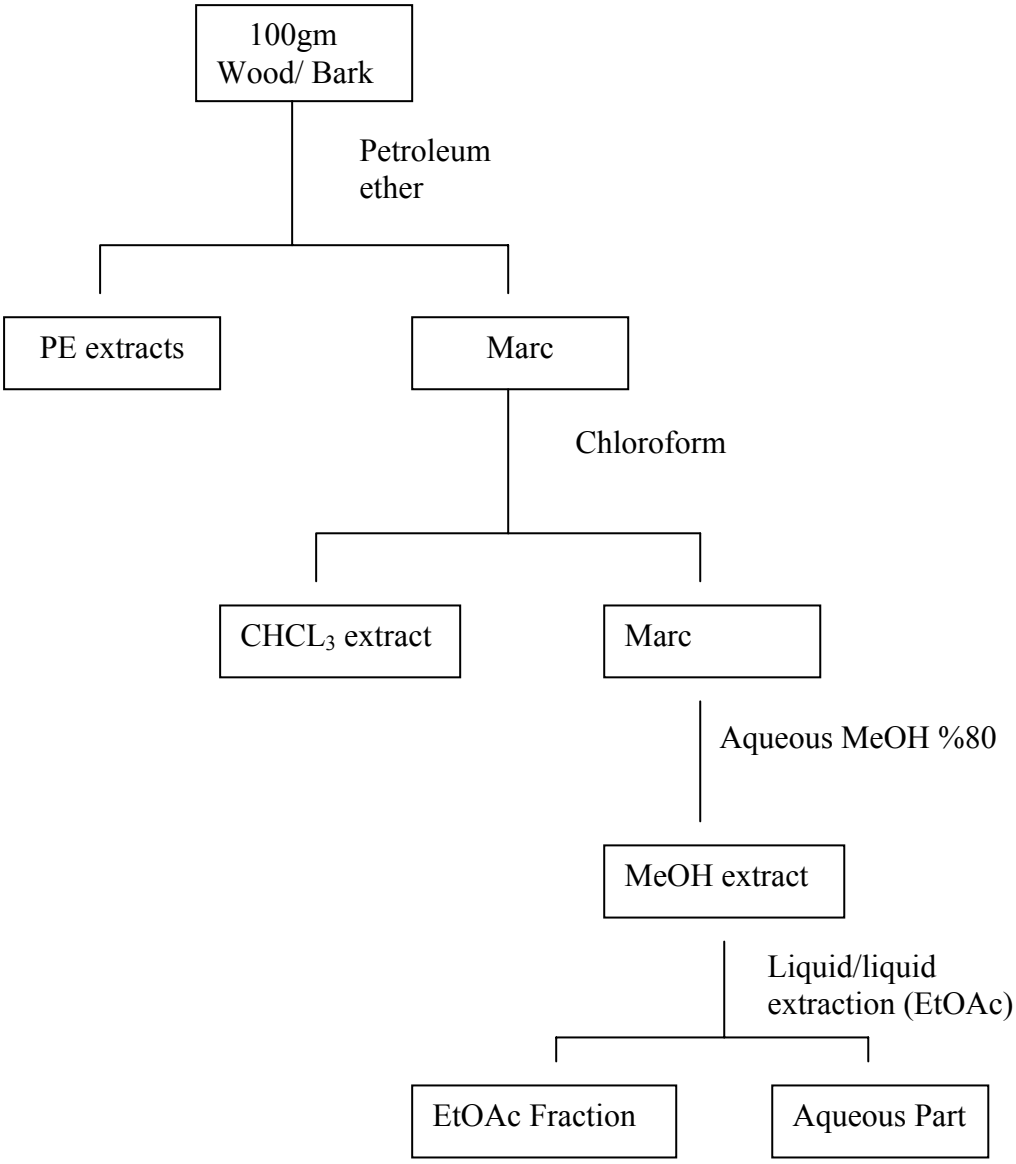


Figure 5: Schematic diagram of *T. brownii* wood and bark extraction

3.3 Chromatography

3.3.1 Thin Layer Chromatography (TLC) analysis for plant extracts

Thin layer chromatography was carried out using silica gel plates $60F_{254}$ (Merck5554) or pre-coated TLC plates SIL RP-18W/UV 254 (Macherey-Nagel). Chromatograms of the plant extracts were prepared by applying 20µl solution (5mg/ml) to the silica gel plate and developing it in different solvent systems depending on the type of the extract, (Table 2). Chromatograms were detected under UV light UV lamp (Camag), (254 and 366nm) and sprayed with diagnostic reagents which include vanillin-H_2SO_4reagent, Aluminium chloride and Natural Products reagent.

Table 2: Developing Solvent systems used in TLC

Developing system	Ratio of Solvents
Methanol/ Water/ Acetic acid RP	8:1:1
Methanol/ Water/ Acetic acid RP	7:1:2
Methanol/ Water/ Acetic acid RP	6:2:2
Toluene / Ethyl acetate/ Formic acid NP	8:1:1
Toluene/ Ethyl acetate/Formic acid NP	5:4:1
Toluene/ Ethyl acetate/ Formic acid NP	4:5:1

3.3.1.1 Spray reagents

- *Vanillin H_2SO_4* : was prepared as follows: 1gm of vanillin powder dissolved in 90ml methanol to which 10 ml sulphuric acid was added carefully. Sprayed TLC plates were examined after heating at 120 ^{0}C.
- *Dragendorff Reagent*: Composed of two solutions:
  - Solution A: 0.3 g bismuthsubnitrate in 1 ml of 25% HCL and 5 ml H_2O
  - Solution B: 3 g potasium iodide in 5 ml H_2O

  The spray reagent was composed of 5 ml (A) + 5 ml (B) + 5 ml of 12.5% HCL + 100 ml H_2O.
- *Natural Products (polyethylenglycol)(NP/PEG) Reagent:* Plates were sprayed with 1% methanolic diphenylboric acid (NP), followed by 5% ethanolic polyethyleneglycol – 4000 (PEG) (10 ml and 8 ml, respectively).

3.4.2 Solid Phase Extraction (SPE)

Sorbents for SPE were LC-18 reversed phase packings supplied by Supelco. Before applying the sample, the column was equilibrated with the first designated eluent. For LC-18 silica the starting eluent was 100 % H_2O, 50% H_2O: MeOH and the column was finally washed with 100 % MeOH.

3.4.3 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

The Agilent 1100 series HPLC system was composed of Agilent series 1100 thermostated column compartment, Agilent series 1100 autosampler, binary Agilent 1200 series Bin pumps, Agilent series 1100 vacuum degasser and Agilent series 1100 DAD detector. Plants extracts and fractions were separated on a (Varian LC-18, 4.6 mm x 250 mm x 5 μm USA) reverse-phase column at 30^{o}C and a flow rate of 0.5 mL/min. The column was eluted with a gradient mobile phase consisting of 1% acetic acid in H_2O (phase A) and 100% acetonitrile (Solvent B) using the gradient programs presented in Table 1. UV detection was performed at 320-380 nm for flavonoids and stilbenes, respectively.

3.4.4 LC-triple quadruple spectrometric analysis (LC-MS/MS)

The HPLC was joined with a HTC ultra-Bruker Daltonics - Advanced Mass Spectrometry Instrumentation (Germany) with Electrospray Ionization (ESI) interface at alternative ion mode. The capillary temperature was set to 280^{o}C and the spray voltage was set to 5000 V. Nitrogen was used as sheath gas, and the flow was set to 40 U. Helium was used as collision gas at 0.8 mTorr. Collision Induced Dissociation (CID) or IT-MS^{n} experiments were performed for fragmentation of the compounds studied. Neutral loss scan were investigated with scan range from m/z 50 to 1000 at collision energy of 15 and 30 ev.

3.5 Antimicrobial activity

The extracts of *T. brownii* were tested for antifungal activity; the method used was cup plate agar diffusion method (Kavanagh, 1972) with minor modifications. Plant extracts were tested against plant pathogens *Aspergillus niger* (ATCC9763-8/29/2005), *Nattrassia mangiferae* (Eltahir, 2003), *Aspergillus flavus*, (N, H, L, 2006). *Fusarium moniliforme*, (PPL/PPA/MA, 2006).

3.5.1 Preparation of Fungal suspensions

One ml aliquots of a 24 hours broth culture of test fungus were aseptically distributed into sabouraud dextrose agar slant and incubated at 25oC for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100ml of normal saline and the suspension was stored in the refrigerator of 4o C until used (Kavanagh, 1972).

3.5.2 Testing for the antifungal activity, cup-plate agar diffusion method

The total activity of *T. brownii* crude extract was carried out by cup-plate agar diffusion method to assess the antifungal activity of the prepared extracts. 20 ml of aliquots of the inoculated sabouraud dextrose agar were distributed into sterile Petri dishes. The agar was left to set and in each of these plates 4 cups (10 mm in diameter) were cut using borer tool 10 mm in diameter. Alternative cups were filled with 0.1ml from the different diluted extracts (petroleum ether, Chloroform, and methanolic extracts) using automatic micropipette. Five replicates for each extracts of the tested fungi were made each test. Two different concentrations of

the Plant extracts were used: 1mg in 1ml solvent and 5mg in 1ml solvent. The extracts were allowed to diffuse at room temperature for two hours. The plates were then incubated at 25º C for 24 hours.

After incubation the diameters of the resultant growth inhibition zones were measured, average was taken and the mean values were tabulated. The solvents used for extraction and dissolution were used as negative controls by adding them to the media instead of the extracts in another set of experiment to confirm that they have got no effect on the growth of the fungi. Further investigations were conducted with concentration on the ethyl acetate extract only.

3.5.3 The antifungal activity by determining the minimum inhibitory concentration (MIC)

To quantifying the activity of the extracts, the modified serial dilution method was used to determine the minimum inhibitory concentration (MIC) (Abdalla, 2004). The minimum inhibitory concentration (MIC) of the extracts was estimated for each of the test organisms in triplicates. Two grams of the extract were dissolved in 20ml-distilled water to end with the one of 0.1g/ml, five serially dilution of the one were made to have the following concentration 0.05g/ml, 0.025g/ml, 0.0125g/ml, 0.0062g/ml and 0.0031g/ml. The 10ml serially diluted extracts were added to 10ml sabouraud dextrose agar in petridishes. Each dish was then inoculated with 0.1ml of the spore suspension of *A. niger*, *A. flavus, N mangiferae* and *F. moniliforme*, Petridishes were incubated at 250 C and examined for growth after 24h. The least concentration of the plant extracts that does not permit any visible

growth of the inoculated test organism in the broth medium was regarded as the MIC in each case. Control experiments were performed using the solvents only without the plant extracts.

CHAPTER FOUR

RESULTS AND DISSCUSION

4.1 Thin layer chromatography of the bark and the wood of *T. brownii*

All analyzed extracts gave negative reaction with Dragendorff reagent suggesting the absence of alkaloids. Alkaloid develops brown or orange visual day light zones immediately on spraying (Wagner, *et.al.*, 1984). Typical pink to purple colours were developed upon spraying with vanillin H_2SO_4 (heat 110˚C) in all studied extracts (Table 3) Flavonoids were mainly accumulated in the ethyl acetate fraction of both studied parts (wood and bark). The selection of a suitable stationary phase and solvents depends on the class of fractions. Vanillin H_2SO_4 is a universal reagent that detects components of essential oils, terpenoids, stilbenes, phenols etc. (Wagner, *et.al*., 1984). Metabolites to be examined (Robards, and Antolovich, 1997). Best separation was obtained using RP-TLC (Merck), and flavonoids were detected using natural product reagent (NPR) (Fig 6. Table 4). The presence of flavonoids was confirmed by their colour change from quenching fluorescence (366 nm) to yellow or orange colour and prominent blue color in case of flavonoidal acids or other phenolic acids (366) after spraying with natural product reagent (NPR) (Fig 6 Table 4). Fluorescence behavior of flavonoids in response to (NPR) is structure dependent. Flavonols e.g. glycosides of quercetin and myricitin develops orange color and those of kaempferol and isorhamnetin yellow to green color flavones glycosides of luteolin

develops orange colour and those of apigenin yellow to green (Wagner, *et.al*., 1984).

Table: 3 TLC profile for the ethyl acetate fraction of *T. brownii* bark and wood after vanillin H_2SO_4 Reagent

Spot No.	R_f value	UV reaction λ_{max}		Vanillin	Expected metabolite
		254nm	366nm		
w1in RP	0.571	Quenching	Blue f	Dark spot	Terpenoids
W2in RP	0.557	Quenching	Blue f	Blue	Flavonoids
W3in RP	0.357	Quenching	Brown	Purple	Terpenoids
b1in RP	0.871	Quenching	Yellow	Pink	Terpenoids
b2in RP	0.571	Quenching	Blue f	Quenching	Terpenoids
b3in RP	0.557	Quenching	Blue	Blue	Flavonoids
b4in RP	0.357	Quenching	Brown	Purple	Terpenoids
w1in NP	0.870	Quenching	Blue f	Pink	Flavonoids
w2in NP	0.797	Quenching	Blue f	-	Flavonoids
w3in NP	0.565	Quenching	Yellow	Blue	Flavonoids
w4in NP	0.507	Quenching	Yellow	Purple	Terpenoids
b1in NP	0.797	Quenching	Blue f	-	Flavonoids
b2 in NP	0.565	Quenching	Blue f	Blue	Flavonoids
b3 in NP	0.507	Quenching	Yellow	Purple	Flavonoids
b4 in NP	0.362	Quenching	Yellow	-	Flavonoids

w in NP = wood normal phase b in NP = bark normal phase

w in RP= wood in reverse Phase b in RP = bark in reverse phase

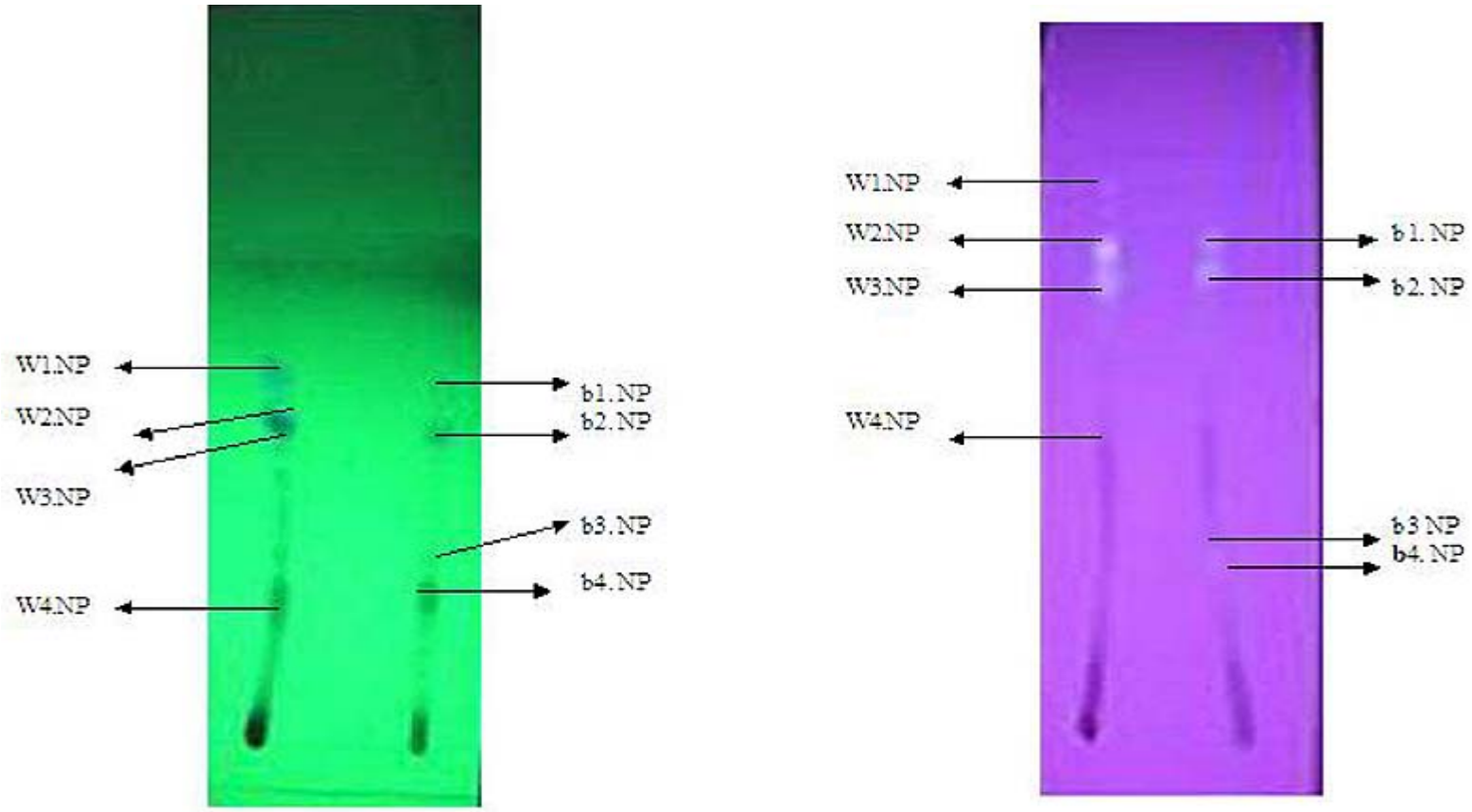
Table: 4 TLC profile for the ethyl acetate fraction of *T. brownii* bark and wood after NPR Reagent

Spot no	R_f Value	UV Reaction λ_{max}		NPR (In366nm)	Expected Metabolite
		254nm	366nm		
w1 in RP	0.931	-	Blue f	Blue f	Flavonoidacid
w2 in RP	0.857	Quenching	Yellow f	Yellow f	Flavonoid
w3 in RP	0.714	Quenching	Blue f	Blue f	Flavonoid
w4 in RP	0.571	Quenching	Blue f	Blue f	Flavonoid
b1 in RP	0.857	Quenching	Yellow f	Yellow f	Flavonoid
b2 in RP	0.743	Quenching	Blue f	Blue f	Flavonoid
b3 in RP	0.571	Quenching	Blue f	Blue f	Flavonoid
w1in NP	0.857	Quenching	Blue f	Blue f	Flavonoid
w2in NP	0.814	Quenching	Blue f	Blue f	Flavonoid
w3in NP	0.671	Quenching	Blue f	Pink	Flavonoid
w4in NP	0.571	Quenching	Blue f	Blue f	Flavonoid
b1 in NP	0.857	Quenching	Blue f	Blue f	Flavonoid
b2 in NP	0.814	Quenching	Blue f	Blue f	Flavonoid
b3 in NP	0.329	Quenching	Blue f	Pink	Flavonoid
b4 in NP	0.571	Quenching	Blue f	Blue f	Flavonoid

NPR= Natural products reagent

w in NP = wood in normal phase b in NP = bark in normal phase

w in RP = wood in reverse Phase b in RP = bark in reverse phase



A B

Figure 6-A: TLC Profile in normal phase (NP) Ethyl acetate phases of the studied parts of *T. brownii*. Sprayed with NPR (A) 254 and (B) 366nm

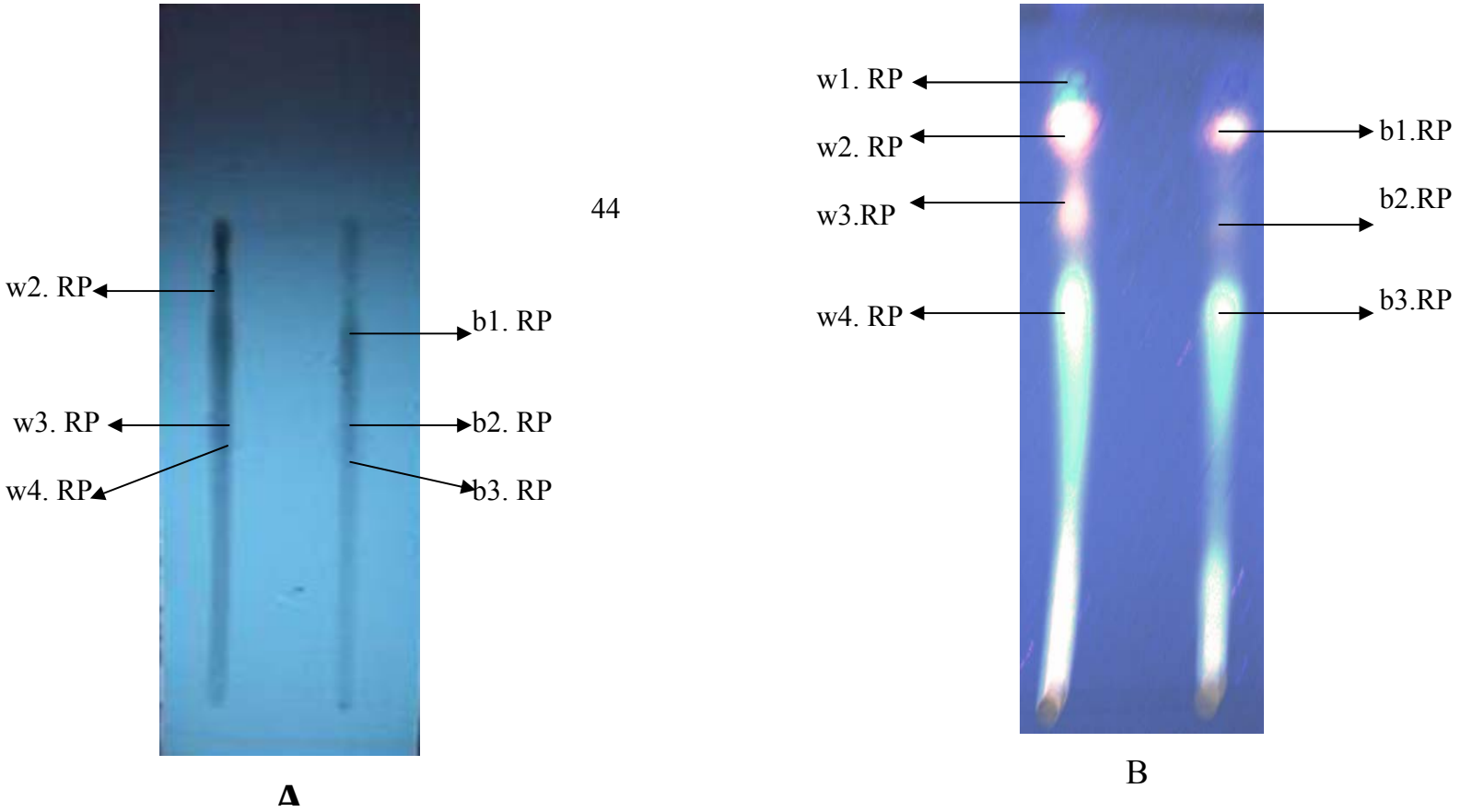


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Figure 6-B: TLC Profile in Reversed phase (RP) Ethyl acetate phases of the studied parts of *T. brownii.* (A) 254 nm and (B) Sprayed with NPR at 366nm

4.2 Antimicrobial activity of *T. brownii*

Cup-plate agar diffusion technique was used to determine the minimum inhibitory concentration to evaluate the effects of the extracts on the four standard organisms.

The effects of varying the concentration of the extracts, the type of the solvent used in the extraction method and the four standard organisms were the three variables examined for the *in vitro* antifungal activity of the wood and the bark of *T. brownii* extracts.

The wood and the bark extracts of *T. brownii* at the concentration of 1mg/ml against the four standard organisms gave inhibitory zone for the four solvents used except for the PE extract which exhibited no inhibition zone; the mean diameters were 12mm in ethyl acetate extract, 13mm in aqueous extract while it was less than1mm in the case of Ch extract (Table 5).

The following experiments were performed using the plant extract of the different solvents at the concentration of 5mg/ml:

All the four standard organisms not affected by petroleum ether extract of the wood and the bark of *T. brownii*, which indicates that petroleum ether extract, might not have an antifungal activity.

The average of four readings for the inhibitory zone of Ch extract of the wood of the plant used was as follows:

12.5mm in *A. niger*, 14mm in *A. flavus*, 13mm in both *Nattrassia mangifera*, and *Fusarium moniliform*, but the bark extract of the same solvent gave inhibitory zone diameter of 11mm against *A. niger*, *A. flavus* and *Fusarium moniliform* and 10.5mm against *Nattrassia mangifera* as shown in table 5.

The aqueous extract of the wood of *T. brownii* exhibited the highest antifungal activity against *A. niger* which 13mm inhibitory zone diameter, while it was 11mm against *A flavus* and *Natrassia*

mangifera and 12mm diameter in the case of *Fusarium moniliform*, the inhibitory zones of the aqueous extract of the bark were 14mm against *A. niger* and *A. flavus*, and 20mm against *Nattrassia mangifera* and *Fusarium moniliform* (Table 5)

Likewise, the EtoAc extracts of the wood of *T. brownii* give growth inhibitory zones with the mean diameter of 15mm *against A. niger*, *A. flavus*, *Nattrassia mangifera* and *Fusarium moniliform*, the EtoAc extract of the bark was found to give antifungal activity represented by the inhibitory zones of 15mm against *A. niger*, *Fusarium moniliform* and *Nattrassia mangiferae*, and 14.5mm a ganist *A. flavus* (Table 5)

The results of the antifungal effects of the eight extracts of *T. brownii* species against the four standard organisms Showed that the wood extract generally gave more antifungal effects than the bark extracts (Table 5).

Substantial antifungal effects were found at the concentration of 5 mg/ml compared with 1mg/ml for the different solvents extracts.

The solvents used for extraction and dissolution used as negative controls instead of the extracts confirm that they have got no effect on the growth of the fungi.

Table: 5 Antimicrobial activity of the wood and bark extracts of *T. brownii*

Plant Parts	Extract Concentration	Extracts	Measurment of inhibitory zones diameter MIZD for Fungi (mm)			
			A.n	*A.f*	*N.m*	*F.m*
Wood	1mg/ml	Petroleum ether	-	-	-	-
		Chloroform	>1	>1	>1	>1
		Ethyl acetate	12	12	12	12
		Aqueous	13	13	13	13
Wood	5mg/ml	Petroleum ether	-	-	-	-
		Chloroform	12.5	14	13	13
		Ethyl acetate	15	15	15	15
		Aqueous	13	11	11	12
Bark	1mg/ml	Petroleum ether	-	-	-	-
		Chloroform	>1	>1	>1	>1
		Ethyl acetate	12	12	12	12
		Aqueous	13	13	13	13
Bark	5mg/ml	Petroleum ether	-	-	-	-
		Chloroform	11	11	10.5	11
		Ethyl acetate	15	14.5	15	15
		Aqueous	14	14	20	20

A..n = *Aspergillus niger*. ***A .f*** = *Aspergillus flavus.* ***N.m*** = *Nattrassia mangifera* and ***F.m*** = *Fusarium moniliform*.

MIZD (mm): >18mm: sensitive

14-18: intermediate

<14mm: Resistant

4.3 Minimum inhibitory concentration of *T.brownii* extracts (MIC)

Another set of experiments was performed for the evaluation of the effects of the extracts against the four standard organisms by determining the minimum inhibitory concentration (MIC).

The modified serial dilution technique was used (Abdalla, 2004).

The ethyl acetate extracts of the bark and the wood of *T. brownii* at the concentrations ranging from 0.001g/ml to 0.05g/ml against the four standard test organisms demonstrated activity against *A. flavus, Nattrassia mangiferae* and *Fusarium moniliforme*, but against *A. niger* no effect was obtained even at the higher concentration used.

The MIC of the ethyl acetate extracts of the wood and the bark of the plant used against *A. flavus, Nattrassia mangiferae* and *Fusarium moniliforme* could not be determined because even at concentration as low as, 0.001g/ml antifungal activity was observed, So the MIC could be considered to be lower than 0.001g/ml. But the extracts against *A. niger* even at the higher concentration (0.05g/ml) no effect was shown. This result demonstrated that the ethyl acetate extracts of the wood and the bark of *T. brownii* against *A. niger* are either not effective or they may have an MIC above 0.05g/ml.

4.4 RP-HPLC-DAD of *T. brownii* wood ethyl acetate phase

Reverse phase HPLC-DAD of the ethyl acetate phases of the wood and bark are presented in figure (7). Stilbenes and flavonoids are mainly accumulated in the ethyl acetate extract of the wood, (Fig. 9). (Fig. 10) Show that the stilbenes are best detected at 320 nm. The utility of RP HPLC separation for more specific and selective identification of stilbene and flavonoid derivatives was greatly enhanced by mass-spectrometric detection; in particular the use of MS–MS enabled the safe identification of co-eluting peaks in the complex biological matrix, (Stecher, *et.al.,* 2001).

A comparison RP-HPLC (λ_{max} 254nm and 320 -370) chromatograms are presented in (Fig. 7 and 8). This UV range enabled the detection of the metabolites classes of interest (flavonoids and stilbenes). It is clear from these chromatograms that similar compounds exists among the active extracts, namely the ethyl acetate phases of the wood and bark of the plant studied. Accordingly, the wood ethyl acetate fraction was subjected to further analysis for identification of the major compounds with special emphasis on its flavonoids and stilbenes content. Solid phase extraction led to a further step in the purification of the wood ethyl acetate phase.

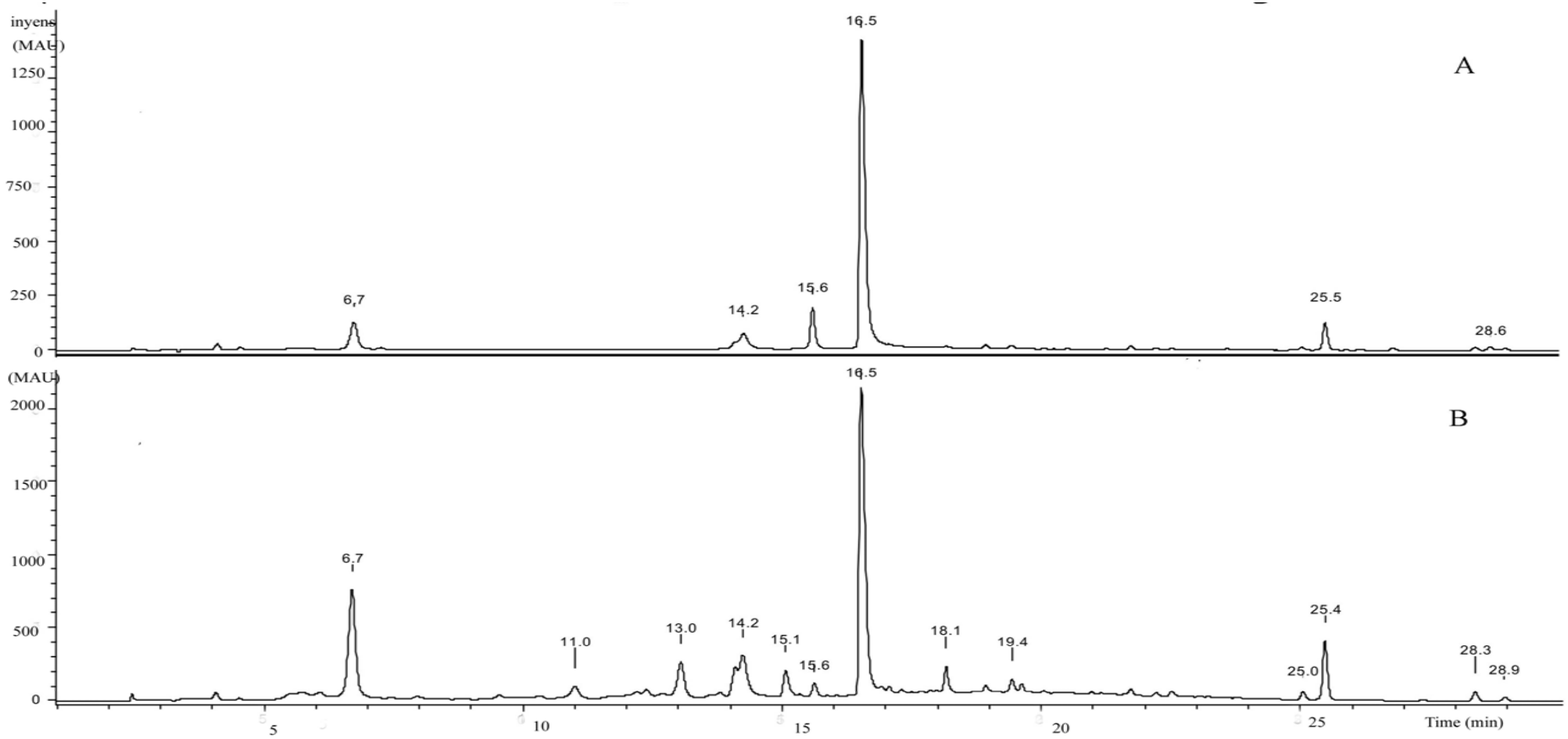

Figure: 7 RP-HPLC-DAD Chromatogram of ethyl acetate fraction of *T. brownii* wood (A) and bark (B) recorded at λ_{max} 254nm

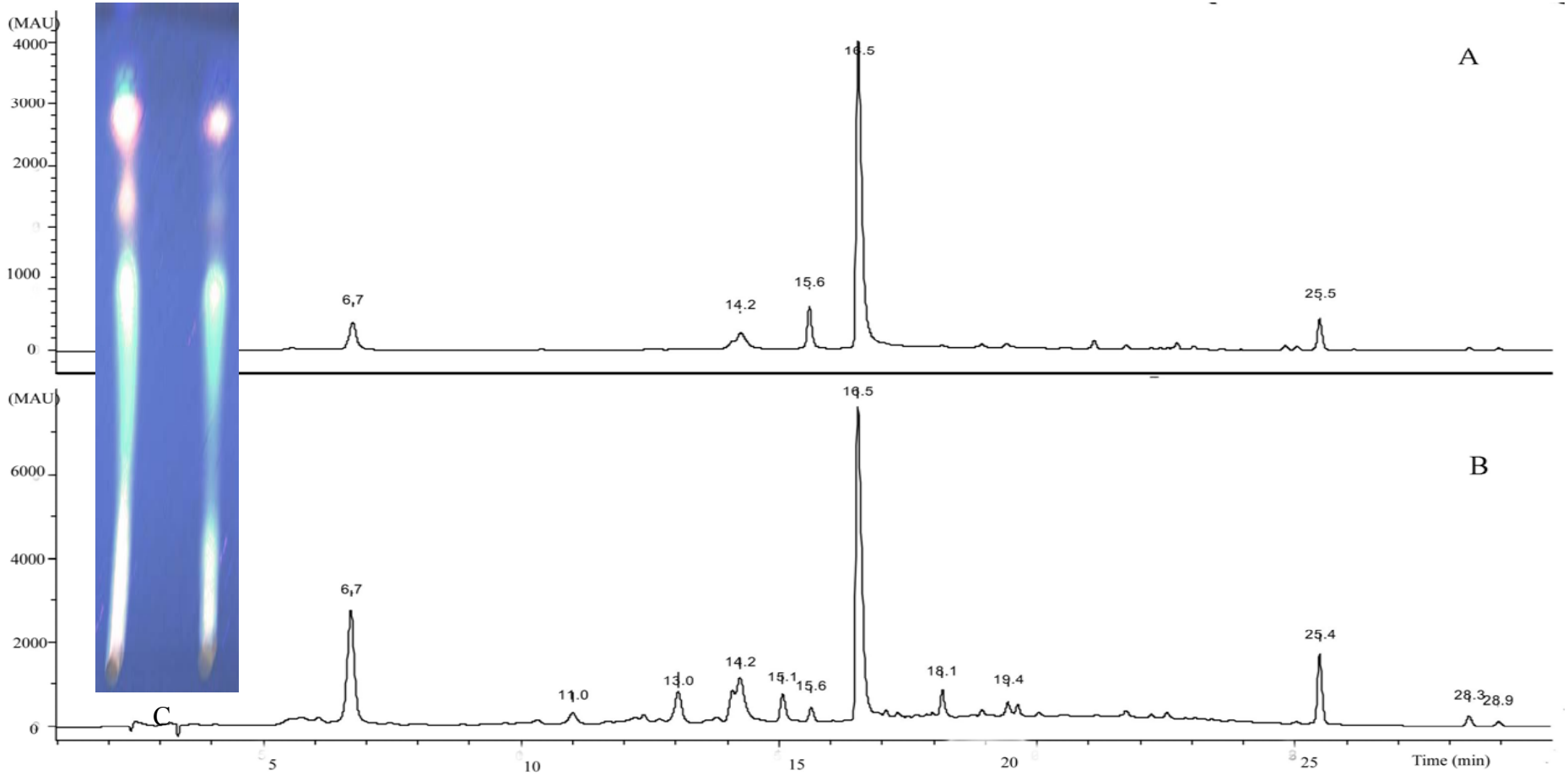

Figure: 8 RP-HPLC-DAD Chromatogram of ethyl acetate fraction of *T. brownii* bark (A) and wood (B) recorded at λ_{max} 320-380 nm, (C) Figure 6.B.B

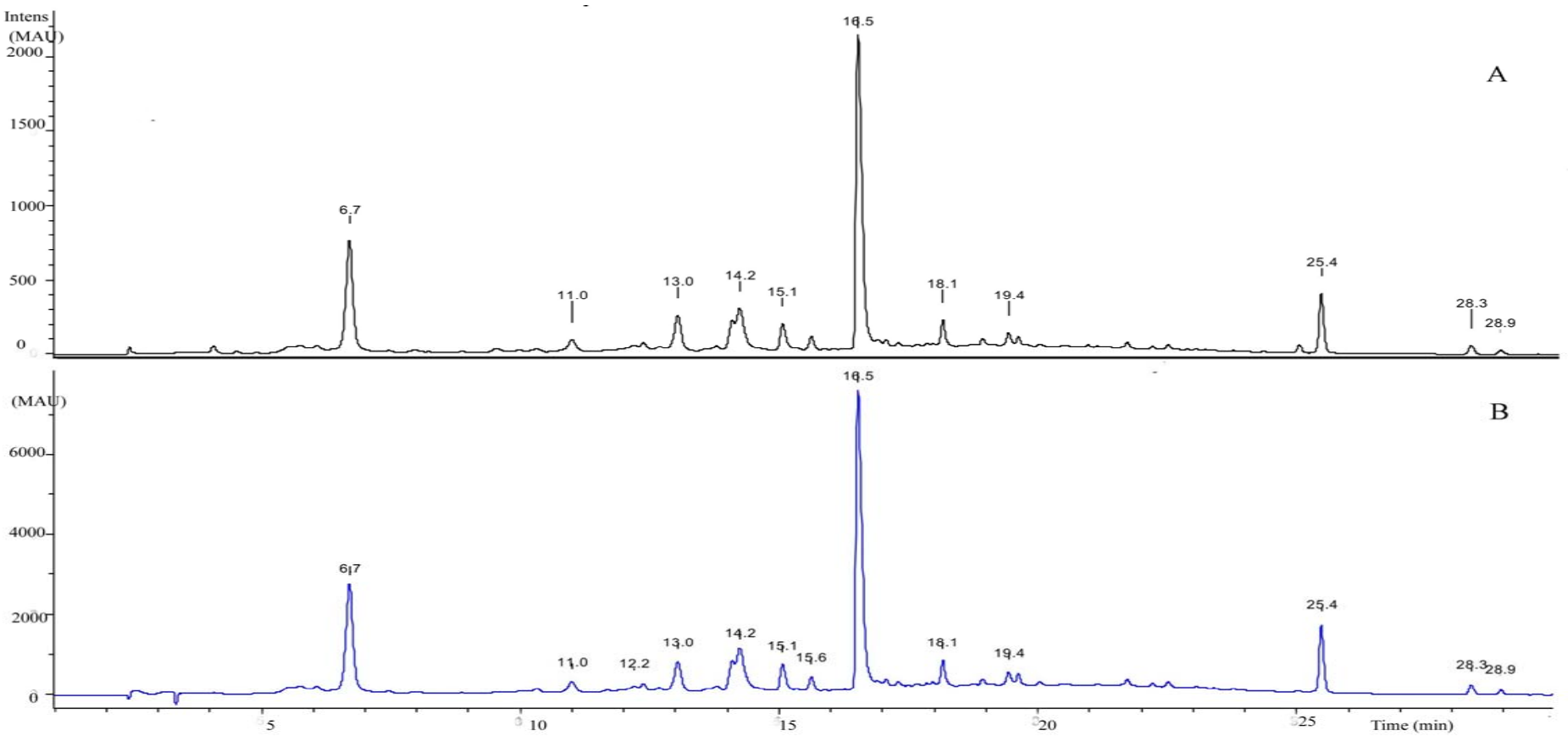

Figure 9: RP- HPLC-DAD chromatogram of ethyl acetate fraction of *T. brownii* wood recorded at λ_{max} 254(A) and 320(B) nm

4.4.1 Identification of compounds in *T. brownii wood* ethyl acetate phase by LC–triple quadruple mass spectrometric analysis (LC-MS/MS)

Flavonoids were identified using UV profile at 360 nm and their mass fragmentation pattern using LC-MS/MS in comparison with reported data. The most useful fragmentations in terms of flavonoid aglycone identification are those that require cleavage of two C-C bonds of the C-ring resulting in structurally informative $^{i,j}A^+$ and $^{i,j}B^+$ ions (Fig. 11). These ions can be rationalized by retro-Diels-Alder (RDA) reactions and are the most diagnostic fragments for flavonoid identification since they provide information on the number and type of substituents in the A- and B- rings, Cuyckens and Claeys (2004). For free aglycones, the $^{i,j}A$ and $^{i,j}B$ labels refer to the fragments containing intact A- and B-rings, respectively, in which the superscripts *i* and *j* indicate the C-ring bonds that have been broken. The major routs of fragmentation resulting in A and B ions require cleavage of the C-C bonds at positions 1/3, 0/2, 0/3, 0/4 or 2/4 of the C-ring (Fig. 10). The fragmentation pathways depend strongly on the substitution pattern and the class of flavonoids studied, e.g. the additional hydroxyl group in position 3 of flavonols results in more and different possibilities for fragmentations compared with flavones. $^{0,2}A^+$, $^{0,2}A^+$ - CO, $^{1,4}A^+$ + 2H, and $^{1,3}B^+$ - 2H are typically observed for flavonols, while $^{1,3}B^+$, $^{0,4}B^+$, and $^{0,4}B^+$ - H_2O are found in flavones. Product ions from glycoconjugates are denoted according to the nomenclature reported by Cuyckens and Claeys (2004). Ions containing the aglycone are labeled $^{k,l}X_j$, Y_j, and Z_j, where *j* is the number of the interglycosidic bond broken, counting from the aglycone, and the

Superscripts *K* and *l* indicate the cleavages within the carbohydrate rings. The glycosidic bond linking the glycan part to the aglycone is numbered 0.

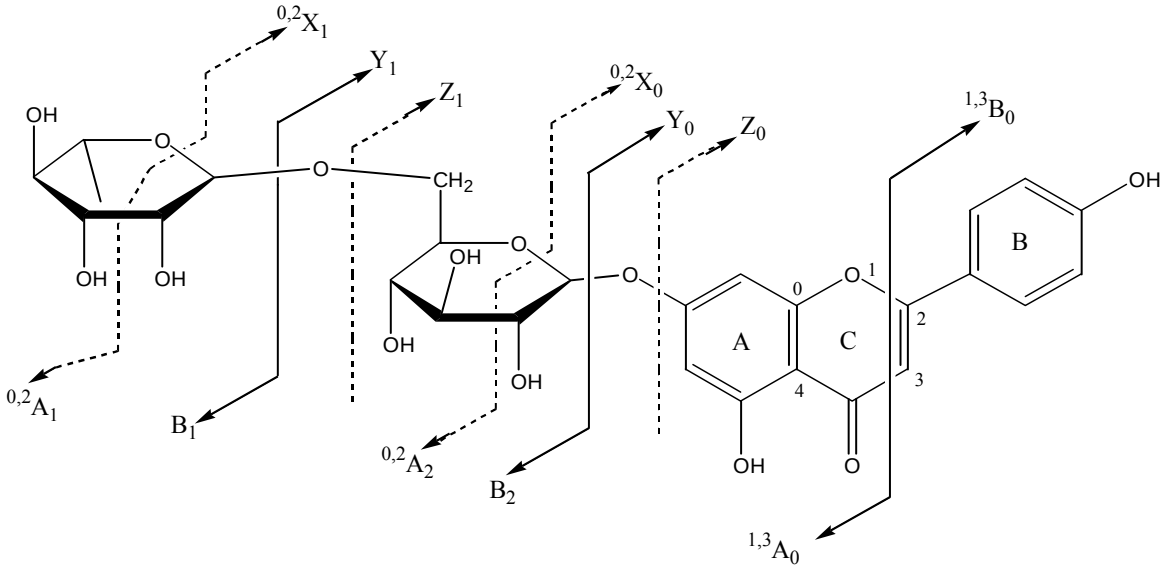

Figure: 10 Fragmentation pathways for flavonoid glycosides (illustrated on apigenin-7-O-rutinoside) (Cuyckens and Claeys, 2004).

4.4.2 Compounds structures assignment in the ethyl acetate phase of the wood of *T. brownii*

Assignment of structures of the metabolites recorded in the wood ethyl acetate phase was done by studying the results of LC-MS/MS CID experiments fragments and comparing them to the reported data or to injected standards when available (Table 6. Fig. 11)

The overall polarity and stereochemistry of the compound are key factors governing their chromatographic behaviour. It has been found that sugars with a D-configuration namely glucose, galactose, xylose and glucuronic acid are usually linked to the aglycone by β bonds, whilst α linkages occur to L- arabinose and L-rhamnose (Cuyckens and Claeys, 2004).

Compound 1,**2** (m/z 469,491) are most polar compound (6.8min). Fragmentation of the ({M-H}$^-$ - 44) from the main molecule suggests the loss of COOH group. According to the fragmentation pattern of the product ion after the loss of the acidic group and in comparison with published data (Angelika, *et.al*., 2002, Van der Doele 1998) **Compound 1** was assigned masilinic acid and **compound 2** asistic acid. Both pentacyclic terpenoid acids were reported previously from the genus *Terminalia* (Garcez, *et.al*., 2003).

Compound 3 and **4** (m/z 541) showed similarity in fragmentation pattern with retention time 11.1min and 13.4 min, respectively. Both compounds possessed of a product ion of m/z 227 ({M-H}$^-$ after the loss of a 314 fragment attributed to a galloylhexose molecule. Fragmentation pattern of this product ion {m/z 227} was in agreement with that of the stilbene resveratrol. Hence, these compounds were assigned resveratrol 3-O-β-galloylglucoside. Differences in their retention times suggest them to be isomers of a

cis (**compound 3**) and trans (**compound 4**) form, (Joseph, *et.al*., 2007). Resveratrol was previously isolated from *Terminalia sericeae* (Joseph, *et.al*., 2007) but resveratrol 3-O-β-galloylglucoside is reported for the first time in this genus.

Loss of the fragment of m/z 302 form **compound 5** [Rt14.4min (601m/z)] gave products ion of (Y^0=271). Fragmentation pattern of the products ion was in agreement with that of a flavonone (Lee, *et.al.,* 2002). The compound was suggested to be Flavellagic acid. The compound was tentatively assigned the structure (appendix 5).

Compound 6 (433 m/z, Rt 15.3 min) first fragmentation pattern (433{M-H}$^-$ -132) gave the products ion (Y^0=271) after the loss of methyl group 15 units. The product ion fragmentation was again that of naringenin (Lee, *et.al.,* 2002**).** Regarding the intensity of the product ion **compound 6** was assigned naringenin 4' methoxy7arabinoside.

Compound 7 (625 m/z, 16.5 min) is the major compound in the ethyl acetate extract of *T.brownii* wood and bark. Loss of two glucose molecules ({M-H}$^-$ -162-162) gave the main peak of the product ion a glycone (m/z 301). Intensity of the glycone product ion suggests **compound 7** to be quercetin 7-β-0-diglucoside, Cuyckens and Claeys (2004).

The MS/MS data **compound 8** (633m/z, 18.2min) is presented in Table 8. Loss of a glucose molecule ({M-H}$^-$ -162) in addition to the fragmentation pattern of the product ion suggest the compound to be. arjunic glycoside. Arjunic glycoside was previously reported in *Terminalia arjuna* (Ghosh, *et.al*., 2008).

Compound 9 MS/MS data are shown in Table 4 (585 m/z.18.4 min) a glycone product ion peak (301m/z) was obtained after the loss of a pentose sugar molecule and a galloyl molecule {(M-H}$^-$ -

132-153}. C**ompound 9** was suggested to be Quercetin derivatives. Comparing the fragmentation pattern of the aglycone product ion to reported data (Ram, *et.al.,* 1997) and the intensity of this ion suggest **compound 9** to be Quercetin 7-0 - galloyl glucoside.

Loss of 3-methyl molecule for **compound 10** (m/z 343. Rt 25.5min) ({M-H}$^{-}$ -15 x 3) with the intensity of the product ion suggest it to be 5,6 dihydroxy 3', 4', 7 trimethoxy flavone.

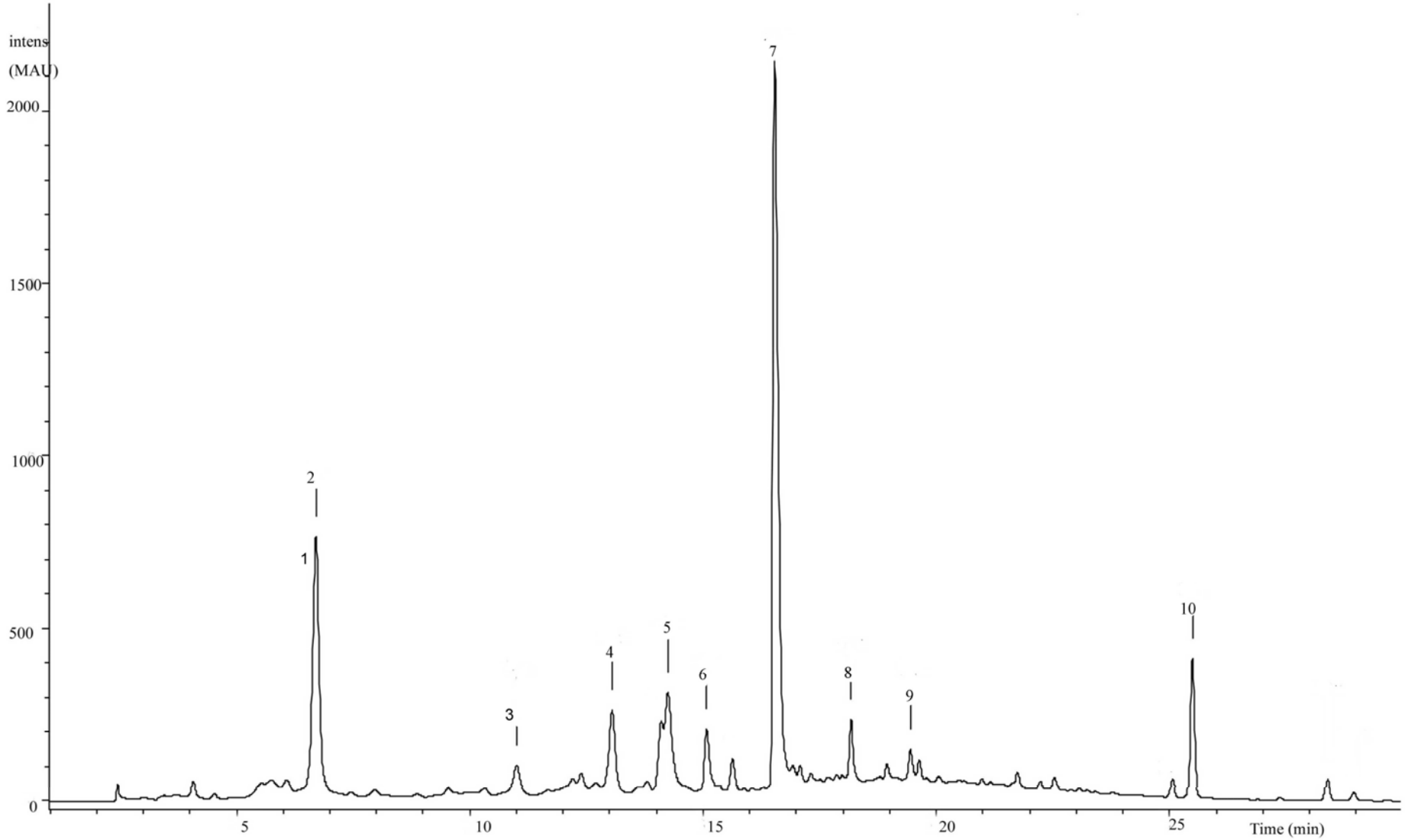

Figure 11: RP-HPLC-DAD Chromatogram of ethyl acetate fraction of *T.brownii* wood extract at λ_{max} 320-380 nm

Table (6) Peak No. (**Fig 11**), RP- HPLC data (R_t), molecular weight *(m/z)*, MS/MS data *(m/z)* and assigned structures of the wood of *T. brownii* ethyl acetate fraction

Compound Peak	R_t (min)	M+H *(m/z)*	CID M^n Main fraction ions (m/z)	Expected compound
1	6.8	469	425,407,379,353,300,271	Masilinic acid
2	6.8	491	447, 429, 410, 301	Asistic acid
3	11.1	541	532,424,300,273,227,199,169	Resevertrol derivative cis
4	13.4	541	532,424,299,275.227	Resevertrol derivative trans
5	14.4	601	296,270.7,242.8,214.8,	Flavellagic acid ester
6	15.3	433	314,229,271	Naringenin 4'methoxy 7arabinoside
7	16.5	625	300,256.7,229,185,129	Querctin 7- ß -0 -diglycoside
8	18.2	633	481,463,421,387,305,211	Arjunic glycoside
9	18.4	585	301,283,256,228,785	Quercetin7-0 -galloyl glycoside
10	25.5	343	327,313,298,285	5,6 dihydroxy 3',4',7 trimethoxy flavone

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CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

Combretaceae is known for its medical uses in Africa and Asia. To date no phytochemical records are available for *Terminalia brownii.* This study was conducted for phytochemical analysis of two different parts (wood, bark) of *T. brownii*. Plant studied different parts extracts were subjected to biological and chemical screening implementing different chromatographic analytical methods (TLC, HPLC and LC-MS/MS). The main objectives of this study were the chemical and biological screening of the parts of the plant studied.
Regarding their well documented biological activities, flavonoids, stilbenes and phenantherenes were the targeted metabolites in this study. These metabolites were mainly traced in the most active extracts of the wood of *T. brownii.*

5.1 Conclusions

- The results of the antifungal effects of the extracts of *T. brownii* showed that the wood extract generally gave more antifungal effects than the bark extracts. No significant activity was observed from the petroleum ether extracts of the bark and wood against four tested organisms
- Substantial antifungal effects were found at the concentration of 5 mg/ml compared with 1mg/ml for the different solvents extracts. The ethyl acetate extracts of the different parts tested is the most active among other extracts.
- The MIC of the ethyl acetate extracts of the wood and the bark of *T. brownii* against *Aspergilus niger* are either not effective or they may have an MIC above 0.05g/ml.

- The MIC of the ethyl acetate extracts of the wood and the bark could be considered to be lower than 0.001g/ml against *A. flavus, Nattrassia mangiferae* and *Fusarium moniliforme*.
- RP-HPLC-DAD coupled with tandem mass spectrometry was used for qualitative and quantitative determination of stilbenes, flavonoids and phenantherenes in ethyl acetate extracts of wood and bark of *T. brownii*
- Flavonoids identified include quercitin and naringenin derivatives and a methoxylated flavone
- Resveratrol galloylglucoside in a cis and trans form was also identified in the active extract
- Among the compounds identified were pentacyclic terpenoidal acids which were previously reported in the genus *Terminalia*
- Present study will help us in promoting and utilizing eco-friendly preservative in appropriate quantity for specialized and uses.

5.2 Recommendations

- It is expected that the major area of use will be for prevention purpose, especially in Implementing wood preservation
- Use of active extracts for biological control in forest products.
- Running similar analysis on the roots, leave, seeds of the plant.
- Conserve the biological and genetic diversity of important natural resources.
- Genomic mapping of the plant studied.

CHAPTER SIX

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Appendix

ESI MS/MS spectra of compounds isolated from *Terminalia brownii* wood:

1. Masilinic acid

2. Asistic acid

3. *cis* Resveratrol 3-O- ß- galloyl glucoside

4. *trans* Resveratrol 3-O- ß- galloyl glucoside

5. Flavellagic acid ester

6. Naringenin 4' methoxy 7 arabinoside

7. Quercitin 7- ß- O diglucoside

8. Quercitin 7-O- galloyl glucoside

9. Arjunic glycoside

10. 5,6 dihydroxy 3' 4' 7 trimethoxy flanvon