PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES ON *LUDWIGIA ADSCENDENS*, *TREWIA NUDIFLORA* AND *HYGROPHILA AURICULATA* FROM BANGLADESH

Thesis submitted by

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Signed:

Date: 08/10/09

Dedicated to my parents and wife

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Abstract

This thesis describes the isolation and structure elucidation of secondary metabolites of three selected medicinal plants from Bangladesh. The work also focused on the evaluation of the plant extracts and some of the isolated compounds for activity *in vitro* against a panel of Gram-positive and Gram-negative bacteria including *Mycobacterium aurum*. Compounds active against *M. aurum* were further tested in combination with selected antitubercular drugs.

A total of 30 compounds, including a mixture of two steroids were isolated from the three plants investigated. Two compounds were identified as novel natural products, namely (5α)-19-hydroxy sarmetogenin 3-*O*-α-L-rhamnopyranoside and 5α-sarmentogenin 3-*O*-[β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside].

Phytochemical investigation of *Ludwigia adscendens* led to the isolation of four triterpenes (squalene, betulinic acid, betulin, betulonic acid), a mixture of two steroids (6β-hydroxy-stigmast-4-en-3-one and 6β-hydroxy-stigmasta-4,22-dien-3-one), two ellagic acid derivatives (pteleoellagic acid and 3,3',4'-tri-*O*-methyl ellagic acid), one flavonol (quercetin), one dihydroflavonol (taxifolin), three flavonol glycosides (afzelin, quercitrin, myricitrin) and three simple phenolics (gallic acid, protocatechuic acid and methyl gallate). All compounds, except for quercitrin and myricitrin, are reported for the first time from this species.

Phytochemical investigation of the stem bark of *Trewia nudiflora* led to the isolation of a known cardenolide (alliotoxin) and two novel structures identified as (5α)-19-hydroxy sarmetogenin 3-*O*-α-L-rhamnopyranoside and 5α-sarmentogenin 3-*O*-[β-D-

glucopyranosyl-(1→4)-α-L-rhamnopyranoside]. The remaining eight compounds were characterised as three ellagic acid derivatives, namely 3,3'-di-*O*-methyl ellagic acid, 3,3'-di-*O*-methyl ellagic acid 4-*O*-α-L-rhamnopyranoside, 3-*O*-methyl ellagic acid 4'-*O*-α-L-rhamnopyranoside, one coumarin (scopoletin), one alkaloid (indole-3-carboxylic acid), two steroids (5α,8α-epidioxyergosta-6,22-dien-3β-ol and daucosterol) and one triterpene (3β-acetyl aleuritolic acid). Scopoletin and indole-3-carboxylic acid are reported for the first time from this species.

The ethyl acetate extract of *Hygrophila auriculata* afforded five simple phenolic compounds identified as 4-hydroxypthalide, 4-hydroxybenzoic acid, protocatechuic acid, gallic acid and caffeic acid. All compounds are reported for the first time from this species.

When screened for activity against a panel of Gram-positive and Gram-negative bacteria, squalene displayed activity against *Escherichia coli* (MIC 1.22 μM). Gallic acid, quercitrin, afzelin and 3,3',4'-tri-*O*-methyl ellagic acid were active against *Streptococcus pyogenes* (MIC values of 5.88, 2.23, 1.16, and 0.36 μM, respectively). The mixture of 6β-hydroxy-stigmast-4-en-3-one and 6β-hydroxy-stigmasta-4,22-dien-3-one was also active against *S. pyogenes* (MIC 125 μg/mL). The MIC values of myricitrin and protocatechuic acid against *Staphylococcus aureus*, *Enterococcus faecalis*, *S. pyogenes* and *Staphylococcus epidermidis* were in the range of 1.08-2.15 and 0.81-1.62 μM, respectively. Methyl gallate and protocatechuic acid were weakly active against *Pseudomonas aeruginosa* (MIC 5.88 and 6.49 μM, respectively). Daucosterol was active against *S. aureus*, *E. faecalis*, *S. pyogenes* and *E. coli* with

MIC values in the range of 0.22 to 0.44 μM. 3-*O*-methyl ellagic acid 4'-*O*-α-L-rhamnopyranoside was active only against *E. faecalis* (MIC 1.08 μM).

Among the ellagic acid derivatives tested against *Mycobacterium aurum*, pteleoellagic acid and 3,3'-di-*O*-methyl ellagic acid 4-*O*-α-L-rhamnopyranoside were found to be active with MIC values of 0.02 and 0.53 μM. Pteleoellagic acid potentiated the effect of the antitubercular drug rifampicin (FIC = 0.35) and showed partial synergy with isoniazid (FIC = 0.60) while 3,3'-di-*O*-methyl ellagic acid 4-*O*-α-L-rhamnopyranoside showed partial synergy with rifampicin (FIC 0.75) and additive effect with isoniazid (FIC = 1.25). This is first report of the activity of ellagic acid derivatives against *M. aurum* and their potentiating effect of antitubercular agents.

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List of Abbreviations

1D One Dimensional Nuclear magnetic Resonance Spectroscopy

2D Two Dimensional Nuclear magnetic Resonance Spectroscopy

3D Three Dimensional Structure

Acetone-d_6 Deuterated acetone

ASE Accelerated Solvent Extraction

ATCC American Type Cell Culture

CC Open Column Chromatography

$CHCl_3$ Chloroform

$CDCl_3$ Deuterated Chloroform

C_5D_5N Deuterated Pyridine

COSY Correlation Spectroscopy

DBE Double bond equivalence

DEPT Distortionless Enhancement by Polarisation Transfer

DMSO Dimethyl sulfoxide

DMSO-d_6 Deuterated dimethyl sulfoxide

ESI MS Electrospray Ionisation Mass Spectrocopy

EtOAc Ethyl acetate

FC Flash Chromatography

FIC Fraction Inhibitory Concentration

GF Gel Filtration

HRCI-MS High Resolution Chemical Ionisation Mass Spectrometry

HREI-MS High Resolution Electron Impact Mass Spectrocopy

HRESI-MS High Resolution Electrospray Ionisation Mass Spectrocopy

HRFAB-MS High Resolution Fast Atom Bombardment Mass Spectrometry

HMBC Heteronuclear Multiple Bond Coherence

HMQC Heteronuclear Multiple Quantum Coherence

MeOD Deuterated methanol

MeOH Methanol

MIC	Minimum Inhibitory Concentration
NMR	Nuclear magnetic Resonance
NOESY	Nuclear Overhauser Enhancement SpectroscopY
ROESY	Rotating frame nuclear Overhauser Enhancement SpectroscopY
RP	Reverse Phase column Chromatography with C-18 Column
TB	Tuberculosis
TLC	Thin layer chromatography
UV	Ultraviolet light
VLC	Vacuum Liquid Chromatography

Chapter 1

Introduction

1. Introduction

1.1 Drug discovery from natural sources

The "art" of using medicinal plants to treat illnesses has been recorded since the early steps of human civilisation. Some of the oldest monographs on herbal medication, for instance, are the 'Charaka-Samhita' of the Indian Subcontinent, 'Ebers Papyrus' of Egypt and 'Neijing Suwen' of China. During the ninth to twelfth century of the medieval age, the medical practitioners of the Mediterranean area took the advantage of worldwide trading trend and pioneered the compilation of knowledge on herbal practice from different parts of the world (Anderson, 1977). Drawing medicinal plants for identification purposes, preserving plant specimens, establishing botanical gardens and using binominal plant nomenclature made herbal practice more standardised. With the scientific achievements in chemistry and biology, the idea that plants contained some chemical agents responsible for the observed pharmacological properties became stronger (Lehane, 1977). This was exemplified with the isolation of morphine (**1**) from the opium poppy (*Papaver somniferum*) which became the first pure substance of natural origin to be commercialised as a drug (Goldstein *et al.*, 1974). Other examples of some natural products isolated from plants and their therapeutic importance include:

- Salicylic acid (**2**) from the bark of the willow tree or *Salix alba* (traditionally used in the management of pain) which led to the semisynthesis of aspirin, a drug extensively used as an analgesic and a prophylactic antithrombic agent (Khan and Mehta, 2005).

- Vincristine (3) and vinblastine (4) from the periwinkle plant (*Catharanthus roseus*) which are two cytotoxic agents used in cancer chemotherapy, particularly in leukaemia (Pearce and Miller, 2005).

- Paclitaxel (5) and a group of taxanes from the bark of the Pacific Yew tree (*Taxus brevifolia*). Paclitaxel is used in the treatment of breast, ovarian and lung cancer while its semisynthetic derivative docetaxel (6) is used in the treatment of breast and lung cancer (Cragg and Newman, 2005).

- Artemisinin (7) from the Chinese medicinal plant *Artemisia annua* (qinghao, sweet worm wood) and its semisynthetic derivatives which serve as the antimalarial drug in cases of chloroquine resistance (Woodrow *et al.*, 2005).

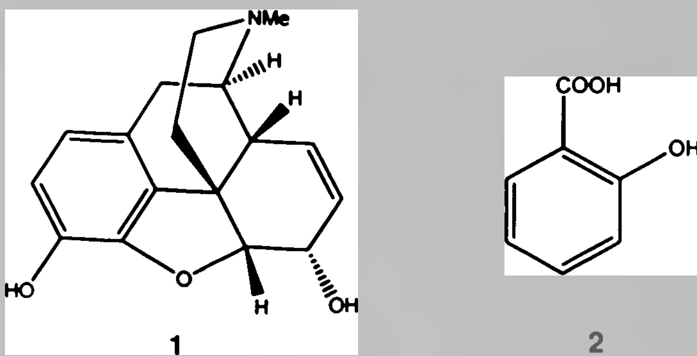
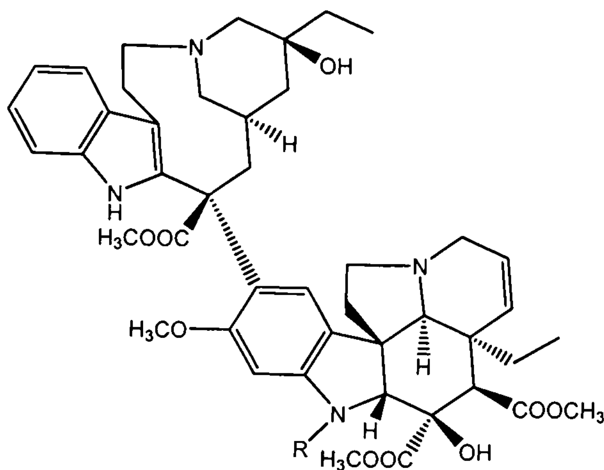
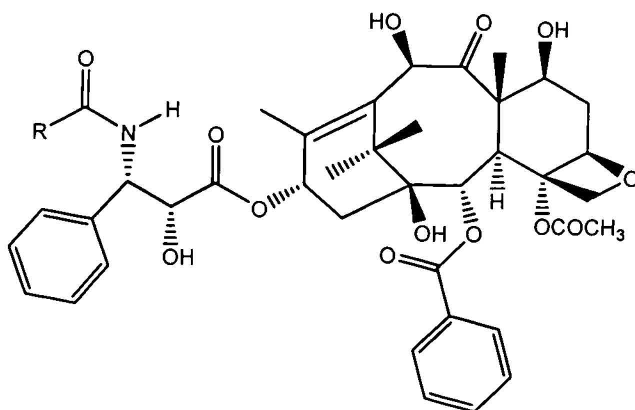


Fig 1.1: Examples of some therapeutic agents from plants



3 R = CHO
4 R = Me



5 R= C_6H_5
6 R= $OC(CH_3)_3$

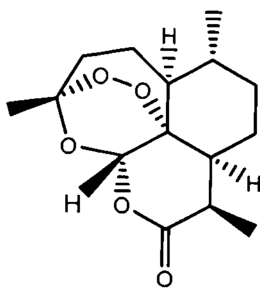


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Fig. 1.1: (cont.) Examples of some therapeutic agents from plants

1.2 Limitations of current antimicrobial therapy

The discovery of agents like penicillins, actinomycin and tyrothricin during 1939-1940 gave a way to the use of antibiotics in antimicrobial therapy (Swartz, 2000). Soon it was followed by the semisynthesis of a wide range of derivatives from their parent structures, *e.g.*, synthesis of methicillin from 6-amino penicillanic acid (Rolinson and Geddes, 2007). During the last few decades, a wide range of antimicrobial agents of natural, synthetic or semisynthetic origin active against bacteria, fungi or mycobacteria have been introduced (Cragg *et al.*, 1997; Theuretzbacher, 2009). The simplicity of the DNA present in microorganisms and their ability to mutate easily make it possible for them to become resistant towards antibiotics shortening the life span of antibiotics (Alanis, 2005).

In addition, toxicity associated with the current antimicrobial agents is another issue which requires to be addressed.

1.2.1 Antibacterial agents

1.2.1.1 Drug resistance

Gram-positive bacteria: Resistance of *S. aureus* to penicillin is as old as that of the introduction of penicillin (Alanis, 2005). Penicillinase-resistant penicillins (*e.g.* methicillin, cloxacillin, flucloxacillin) were first introduced in the 1960's but soon resistant strains were reported (Enright *et al.*, 2002). Up to the late 1980's, methicillin-resistant *S. aureus* (MRSA) was confined to hospitals and was the major causative agent for hospital-acquired infection. Community-acquired methicillin resistant *S. aureus* (CA-MRSA) is now the cause of skin infections particularly

affecting young people (Vandenesch and Etienne, 2004). Vancomycin is still the main drug for the treatment of MRSA infections but in recent years strains of *S. aureus* with resistance towards vancomycin have been detected (Sande and Ronald, 2003).

Gram-negative bacteria: Treatment options have become more limited with the increased number of strains producing extended spectrum beta-lactamases (ESBLs) (Livermore, 1995; Renniea *et al.*, 2003). Such strains of Gram-negative bacteria include *Enterobacter* spp. *Klebsiella pneumoniae, Escherichia coli* and are resistant towards carbapenems and cephamycins (Coque *et al.*, 2008). Unlike other Gram-negative bacteria, *Pseudomonas aeruginosa*, the second most frequently occurring pathogen in skin infections has also acquired resistance towards the quinolones and imipenem and some strains are resistant to all currently-available antibiotics (Lee *et al.*, 2005; NNIS report, 2003).

1.2.1.2 Toxicity

A major draw-back of the penicillins is hypersensitivity reaction. Penicillin-induced allergy includes rashes, fever, vasculitis, exfoliative dermatitis and may lead to anaphylactic shock (Waller *et al.*, 2001). Clavulanic acid, which is often given along with penicillins (*e.g.*, co-amoxiclav) leads to liver toxicity followed by cholestasis and vanishing bile duct syndrome (Geubel, 2001). Vancomycin, the drug of choice in treating MRSA infections has poor oral bioavailability, therefore can be given only intravenously (Waller *et al.*, 2001). Neurotoxicity, nephrotoxicity, ototoxicity, or

more minor side effects including gastrointestinal discomfort have also been associated with the use of antibiotics (Waller *et al.*, 2001).

1.2.2 Antimycobacterial agents

1.2.2.1 Drug resistance

The genus *Mycobacterium* comprises around 85 species. Amongst these, the most common human pathogens are *M. tuberculosis*, *M. leprae* and *M. ulcerans*. Tuberculosis (TB), caused mainly by *M. tuberculosis* and to a lesser extent by *M. bovis* and *M. africanum* remains one of the major diseases affecting one third of the total population of the world (Okunade *et al.*, 2004; WHO Report 2007). The mycobacterial cell has mycolic acid content of as high as 60% of their body weight. This extreme lipophilicity can inhibit the penetration of foreign molecules making the therapy difficult (Bhowruth *et al.*, 2008). Moreover, mycobacterial drug resistance occurs very quickly and multidrug therapy is required. In spite of all the precautions, cases of resistant strains are on the increase. Apart from the multidrug resistant strains (MDR-TB), newer strains of *Mycobacterium tuberculosis* termed as XDR-TB (extensively drug resistant) have been identified (Araújo-Filho *et al.*, 2008). While MDR-TB are resistant to the first-line agents, isoniazid and rifampicin, XDR-TB are resistant to isoniazid, rifampicin as well as to any of the fluoroquinolones and at least one of the three injectable second-line drugs (capreomycin, amikacin and kanamycin) (WHO Weekly Epidemiological Record, 2006). The emergence of XDR-TB cases raised the concern of having no treatment opportunity with the currently available drugs (CDC report, 2006). Since

mycobacteria can remain dormant for a long time, strong immunity is required in conjunction with chemotherapy which is also a major concern in immunocompromised patients (Bhowruth *et al.*, 2008). Long term use glycolytic phase inhibitors may also prove less satisfactory since strains from clinical specimens have shown to develop the glyoxylate shunt allowing them to survive longer (Okunade *et al.*, 2004). The emergence of non-tuberculus mycobacteria in recent years has worsened the situation since some of them are intrinsically resistant to one or more of the commonly available antitubercular drugs (Manzoor *et al.*, 1999; Sun and Zhang, 1999).

1.2.2.2 Toxicity

Isoniazid, rifampicin, pyrazinamide all are associated with hepatotoxicity. Thus they cannot be given to patients having hepatitis, carrying hepatitis virus or who have excessive alcohol consumption. Since streptomycin and ethambutol are excreted through the kidneys, the renal function of patients should be monitored. Thioacetazone should not be given to patients with renal failure since reducing the dose is complicated due to its very narrow margin of therapeutic and toxic dose (WHO, 2003).

1.3 The need for novel antimicrobials

The development of newer chemotherapeutic agents fails to keep pace with the growing drug-resistant microbes (Theuretzbacher, 2009). During the last few decades rational drug design and molecular modification has led to the synthesis of

newer derivatives of existing antimicrobial agents. But they are also prone to resistance since their target site is the same as that of their predecessors. This situation currently requires extensive research to discover some new antimicrobial agents from wider sources which can present a novel structural template and can work on a different site of action (*i.e.*, unlike current antibiotics). Drugs acting on different sites of action will be less prone to drug resistance (Cushnie and Lamb, 2005). Molecules acting on sites other than current antibiotics will not only prevent cross-resistance, but will also make them useful in combating strains resistant toward currently available drugs (Cheng *et al.*, 2007).

1.4 Higher plants as a source of novel antimicrobials

Plants have acquired evolutionary measures of defence mechanisms to combat "enemies" like herbivores, parasites and microbes. For example, a simple physical barrier like the cuticular wax on leaf surfaces acts as an effective defence mechanism (Wain, 1986). Plants also biosynthesise some defensive chemical substances. The latter are often also active against microbial pathogens causing diseases in humans. This gives a rationale for the use of plants in traditional medicine as remedies for various infections (Wallace, 2004; Ríos and Recio, 2005). Antimycobacterial agents are also widespread in plants. For example, in a study out of 182 randomly selected plants, eight extracts showed MIC values as low as 0.4 to 5 μg/mL (Okunade *et al.*, 2004).

There has been an increase in recent years in the search for antimicrobial agents from higher plants (Ncube *et al.*, 2008). While citations for antimicrobial activity of

medicinal plants in Pubmed reached 115 from 1966 to 1994, it was more than twice that number (307) from 1995 to 2004 (Ríos and Recio, 2005). Studies ranged from the screening of traditionally used medicinal plants to the isolation of active principles. They showed that a wide range of natural products with antimicrobial activity can be present even in a single plant species. Examples of antimicrobial compounds commonly found in plants are detailed in **Table 1.1**. While plants are still considered to be an important source of antimicrobial agents, several plant-derived compounds made their way to clinical trials as potential anti-infective agents during 2000-2006 (Saklani and Kutty, 2008). For example, calnolide A, a calnolide coumarin showed potent anti-HIV and antituberular activity and is now under preclinical trials by Sarawak Medichem and NCI (Xu *et al.*, 2004; Yu *et al.*, 2003). Crofelemer, an oligomeric proanthocyanidine from *Croton lecheri* latex has been found useful in the treatment of diarrhoea in patients with irritable bowel syndrome (Mangel and Chaturvedi, 2008). Based on its use in paediatrics and patients with acute diarrhoea, irritable bowel syndrome or HIV, four different crofelemer products are in different phases of development at Trine Pharmaceuticals Inc. and AsiaPharm Group Ltd. (Saklani and Kutty, 2008).

Table 1.1: Some antimicrobial compounds from plants

Chemical class	Compounds	Active against
Essential oil and terpenoids	Menthol	Gram-positive and Gram-negative bacteria (Cowan, 1999)
	Asiaticoside	*Mycobacterium leprae* (Cowan, 1999)
	Various monoterpenes present in 'tea tree oil'	Gram-positive and Gram-negative bacteria (Hammer *et al.*, 2003; Helkón and Milkus, 2004)
	Totarol	*P. acnes*, Gram-positive bacteria, *M. tuberculosis* (Constantine *et al.*, 2001)
Simple phenols	*P*-hydroxy benzoic acid	Gram-positive and Gram-negative bacteria (Cashman and Warshaw, 2005)
	Salicylic acid	*P. acne* (Cowan, 1999)
	Gallic acid, protocatechuic acid	Gram-positive and Gram-negative bacteria (Kubo *et al.*, 2003)
	Anthemic acid	*S. aureus, M. tuberculosis* (Cowan, 1999)
Tannins	Punicalagin	*Candida albicans, C. parapsilosis, C. krusei* (Liu *et al.*, 2009)
	Mallorepanin, mallotinic acid	*S. aureus, Corneybacterium accolans, C. albicans* (Fogliani *et al.*, 2005)
	Tannic acid	Fungus (Chung *et al.*, 1998a; Chung *et al.*, 1998b)
	Procyanidins	*A. actinomycetemcomitans, P. gingivalis, P. intermedia* (Ho, 2001)
Coumarins	Daphnetin, daphnin, daphnoretin	*E. coli* (Cottiglia *et al.*, 2001)
	Imperatorin, iso-imperatorin	*E. coli, Bacillus subtilis, Cladosporium herbarum, Aspergillus cand* (Kwon *et al.*, 1997)
Flavonoids	Apigenin, vitexin	Gram negative bacteria (Basile *et al.*, 1999)
	Luteolin, orientin, isoorientin	Bacteria (Cottiglia *et al.*, 2001)
	Glabrol	*S. aureus, M. tuberculosis* (Cowan, 1999)
Alkaloids	Berberine, hydrastine	General (Cowan, 1999)
	Piperine	Fungi, *Lactobacillus, Micrococcus, E. coli, E. faecalis* (Cowan, 1999)
Others		
Quinone	Lawsone	*M. tuberculosis* (Cowan, 1999)
Anthraquinone	Rhein	*S. aureus* (Cowan, 1999)
Sulfoxide	Allicin	General (Cowan, 1999)
Saponin	Acaciaside A and B	Bacteria and fungi (Mandal *et al.*, 2005)

1.5 Rationale for the selection of plants used in the present study

1.5.1 Plants traditionally used in folk medicine in Bangladesh

This is a common approach for drug discovery from plants. The traditional use is based on the result of a long history of trial and error. This could give some important and exciting results and is often recommended (Ríos and Recio, 2005). For reports on the traditional use, some recent books on the medicinal plants of Bangladesh have been consulted (Kirtikar and Basu, 1999; Yusuf *et al.*, 1994).

1.5.2 Plants of aquatic origin

Water is one of the prerequisites in the generation and existence of life. The aquatic environment is not only a favourable environment for the growth of plants, amphibians and fish; it is also suitable for the growth of fungi and bacteria. Some fungal genera called oomycetes, for example, require highly humid conditions and favour the fresh water environment for their growth. They can attack terrestrial plants growing in relatively high humidity. Interestingly, aquatic plants confined to fresh waters remain disease-free indicating that they might have some intrinsic mechanism to protect from fungal infection (Strobel *et al.*, 1999). The defence mechanism may be wide and is likely to include the production of phytochemicals. Often these chemical substances may also prove to be effective against human pathogens and show antimicrobial activity (Su, 1973). About 123 species of aquatic plants distributed in 67 genera under 35 families are found in Bangladesh. Some of these plants are often used for the treatment of various illnesses including skin and soft tissue infections (Yusuf *et al.*, 1994).

1.5.3 Selection of plants from the Bangladeshi flora

With the growing interest in investigating higher plants for antimicrobial activity, the natural Flora of several different countries (Argentina, Brazil, Cameroon, Colombia, Congo, Côte d'Ivoire, Ghana, India, Lebanon, Malaysia, Peru, Qatar, Thailand, Turkey, Uganda) has been investigated (Ríos and Recio, 2005). Reports on the antimicrobial natural products isolated from Bangladeshi higher plants have already been published (Ahmed *et al.*, 2005; Anjum *et al.*, 2002; Chowdhury *et al.*, 2003; Rahman *et al.*, 2007; Sadik *et al.*, 2003), the present work aimed to extend this type of work to the investigation of *Ludwigia adscendens* and *Hygrophila auriculata* which had previously been relatively unexplored chemically and/or biologically.

1.5.4 Plants showing activity in preliminary antibacterial screening

The antimicrobial activity observed for selected plant extracts indicated that they contained one or more active components. Small scale extraction was carried out with different plant materials and the extracts were tested against selected Gram-positive and Gram-negative bacteria. Active extracts were also chosen for further phytochemical studies. Despite extensive phytochemical and biological work on the seeds of *Trewia nudiflora*, the bark, which also enjoys its use in traditional medicine, had not been studied in both respects. Thus the antibacterial activity of *Trewia nudiflora* bark extract in the preliminary screening encouraged us to further investigate this species.

1.6 The family Onagraceae Juss.

The Onagraceae Juss. is a cosmopolitan family of flowering plants whose members are mostly present in temperate areas. The family consists of about 640 species distributed in 18 genera, mostly herbs, rarely shrubs or trees (Heywood, 1993). *Oneothera* (evening primrose) and *Epilobium* (willow herb) are the largest genera in this family (Hutchinson, 1959). Other genera include *Clerkia*, *Gaura, Fuchsia* and *Ludwigia* (Plowden, 1972).

1.7 The genus *Ludwigia* L.

1.7.1 Botany

Ludwigia L. (water primrose) is one of the most diverse genera in the Onagraceae. It consists of about 82 species grouped in 23 sections. Some of the sections of *Ludwigia* are *Brenania, Cryptosperma, Prieurea* of tropical Africa, *Nipponia* of east Asia and *Macrocarpon* of South America (Averett *et al.*, 1990). Plants belonging to the genus *Ludwigia* are branched or unbranched, creeping or floating herbs. The stems are often reddish tinged and glabrous to densely villous. The leaves are arranged alternatively along the axis. Flowers are borne singly in the axile or at the end of branches. The seeds are brown to pale brown in colour with a rounded or ellipsoidal shape (Khan and Halim, 1987).

1.7.2 Previous phytochemical reports on *Ludwigia*

Table 1.2: Flavonoids and flavonoid glycosides previously isolated from *Ludwigia*

Compound	Plant species	Reference
Quercetin 3-*O*-rhamnoside (**8**)	*L. adscendens*	Averett *et al.*, 1990
Quercetin 3-*O*-rutinoside (**9**)	*L. adscendens*	Averett *et al.*, 1990
Kaempferol 3-*O*-glucoside (**10**)	*L. adscendens*	Glaby, 1991
Myricetin 3-*O*-rhamnoside (**11**)	*L. adscendens*	Glaby, 1991
Myricetin 3-*O*-galactoside (**12**)	*L. adscendens*	Glaby, 1991
Luteolin (**13**)	*L. octovalvis*	Yan and Yang, 2005
Apigenin (**14**)	*L. octovalvis*	Yan and Yang, 2005
Quercetin (**15**)	*L. octovalvis*	Yan and Yang, 2005
Kaempferol (**16**)	*L. repens*	Marzouk *et al.*, 2007
Trifolin 2"-*O*-gallate (**17**)	*L. repens*	Marzouk *et al.*, 2007
Guaijaverin (**18**)	*L. repens*	Marzouk *et al.*, 2007
Reynoutrin (**19**)	*L. repens*	Marzouk *et al.*, 2007
Juglanin (**20**)	*L. repens*	Marzouk *et al.*, 2007
Avicularin (**21**)	*L. repens*	Marzouk *et al.*, 2007
Hyperin (**22**)	*L. repens*	Marzouk *et al.*, 2007
Trifolin (**23**)	*L. repens*	Marzouk *et al.*, 2007
Hyperin 2"-*O*-gallate (**24**)	*L. repens*	Marzouk *et al.*, 2007
Avicularin 2"-(4"'-*O*-*n*-pentanoyl)-gallate (**25**)	*L. repens*	Marzouk *et al.*, 2007
Orientin (**26**)	*L. prostrata*	Kim and Kim, 1997

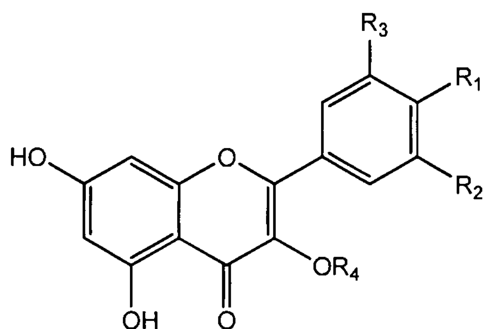
Table 1.3: Terpenoids and phytosterols previously isolated from *Ludwigia**

Compounds isolated	Reference
(23*Z*)-coumaroylhederagenin (**27**)	Chang *et al.*, 2004
(23*E*)-coumaroylhederagenin (**28**)	Chang *et al.*, 2004
(3*Z*)-coumaroylhederagenin (**29**)	Chang *et. al.*, 2004
Oleanolic acid (**30**)	Chang *et. al.*, 2004
Ursolic acid (**31**)	Chang *et al.*, 2004
2α-Hydroxy ursolic acid (**32**)	Yan and Yang, 2005
Tormentic acid (**33**)	Yan and Yang, 2005
β-Sitosterol (**34**)	Yan and Yang, 2005
Daucosterol (**35**)	Yan and Yang, 2005

* All the compounds were isolated from the species *L. octovalvis*

Table 1.4: Miscellaneous compounds previously isolated from *Ludwigia*

Compounds isolated	Plant species	Reference
3, 4, 8, 9, 10-Pentahydroxydibenzo[b,d]pyran-6-one (**36**)	*L. octovalvis*	Yan and Yang, 2005
Ellagic acid (**37**)	*L. octovalvis*	Yan and Yang, 2005
Gallic acid (**38**)	*L. prostrata*	Liu *et al.*, 1986
Triethyl chebulate (**39**)	*L. prostrata*	Liu *et al.*, 1986
Maltol (**40**)	*L. octovalvis*	Yan and Yang, 2005
Methyl brevifolincarboxylate (**41**)	*L. octovalvis*	Yan and Yang, 2005
Piperine (**42**)	*L. hyssopifolia*	Das *et al.*, 2007



Compound	R_1	R_2	R_3	R_4
8	OH	OH	H	α-L-Rhamnonsyl
9	OH	OH	H	Rutinosyl
10	OH	H	H	β-D-Glucosyl
11	OH	OH	OH	α-L-Rhamnonsyl
12	OH	OH	OH	β-D-Galactosyl
13	OH	OH	H	H
14	OH	OH	H	OH
15	OH	OH	H	H
16	OH	H	H	OH
17	OH	H	H	β-D-Galactosyl-2''-*O*-gallate
18	OH	OH	H	α-L-Arabinofuranosyl
19	OH	OH	H	β-D-Xylopyranosyl
20	OH	H	H	α-L-Arabinofuranosyl
21	OH	OH	H	α-L-Arabinofuranosyl
22	OH	OH	H	β-D-Galactopyranosyl
23	OH	H	H	β-D-Galactopyranosyl
24	OH	OH	H	β-D-Galactosyl-2''-*O*-gallate
25	OH	OH	H	α-L-Arabinofuranosyl-2''-(4'''-*O*-*n*-pentanoyl)-gallate

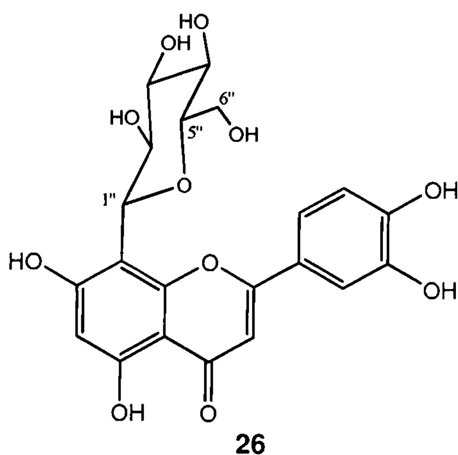


Fig. 1.2: Structures of flavonoids and flavonoid glycosides previously isolated from *Ludwigia*

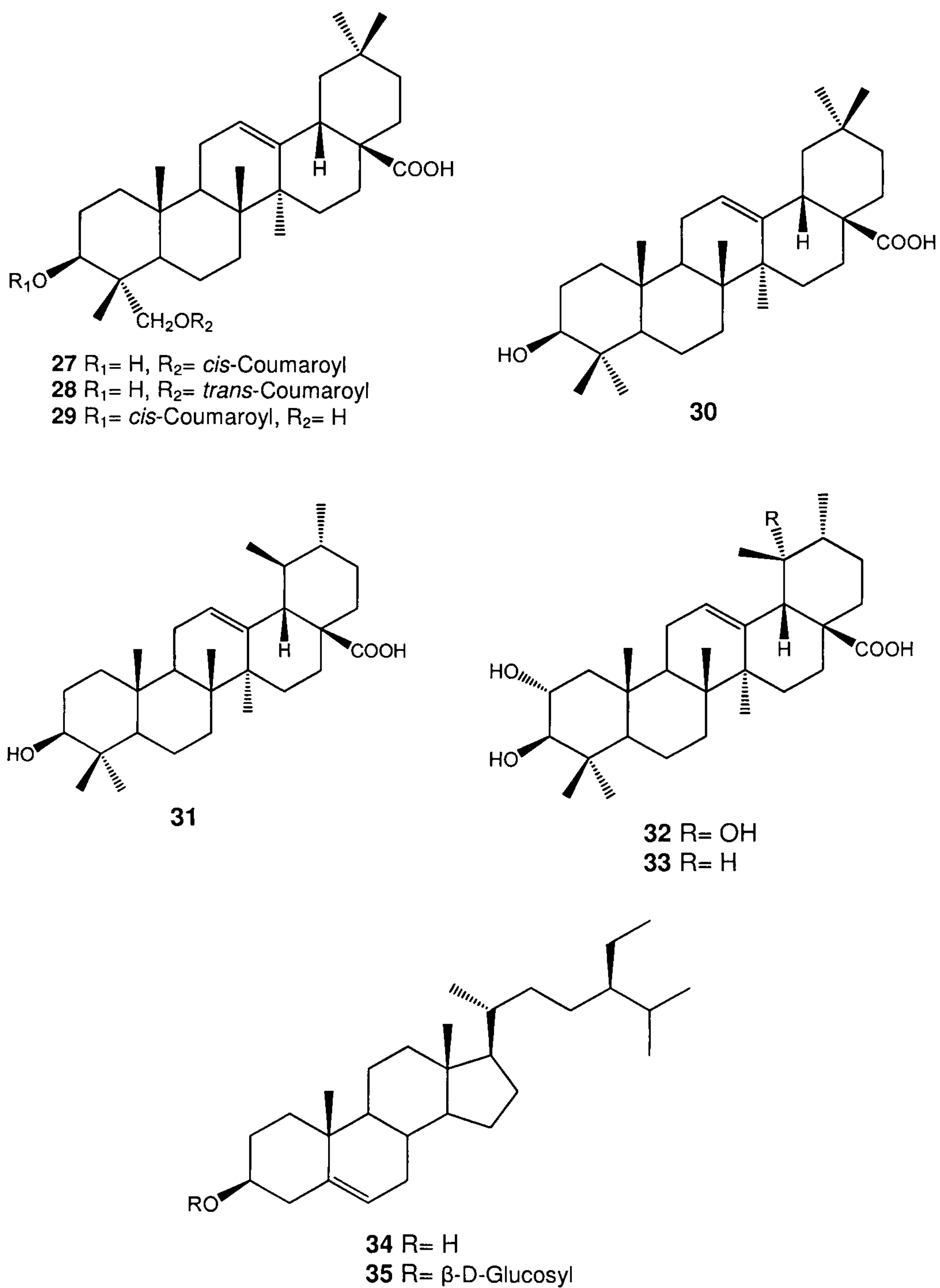
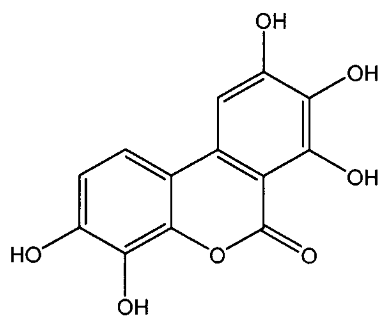
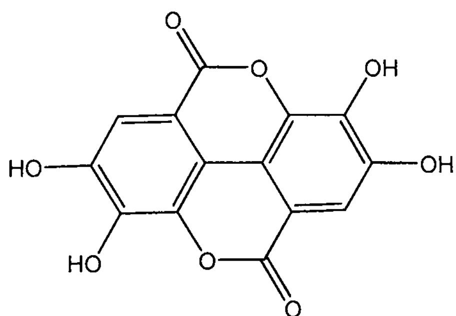
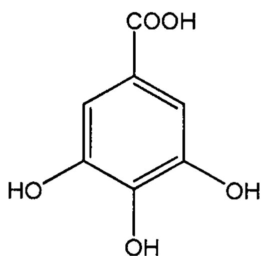
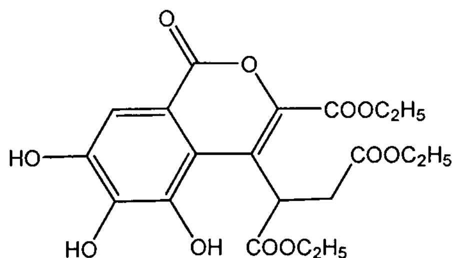


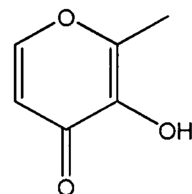
Fig. 1.3: Structures of terpenoids and phytosterols previously isolated from *Ludwigia*

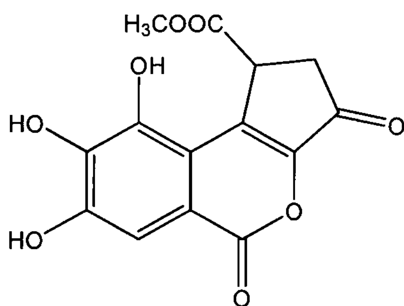












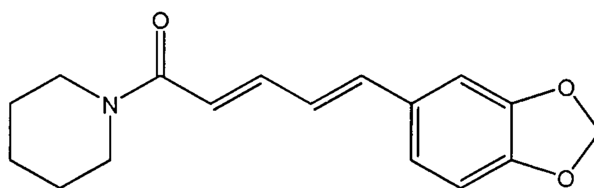


Fig. 1.4: Structures of miscellaneous compounds previously isolated from *Ludwigia*

1.7.3 Previous antimicrobial reports on *Ludwigia*

The *n*-hexane, ethyl acetate and methanol extracts of *L. hyssopifolia* exhibited antibacterial activity in a disk diffusion assay against *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus subtilis* (Das *et al.*, 2002).

Aqueous extract of *L. octovalvis* showed antibacterial activity against *Streptococcus mutans* serotype c and d with minimum inhibitory concentrations (MIC) of 3.9 and 5.9 mg/mL, respectively (Chen *et al.*, 1989).

The methanol extract of *L. peruviana* inhibited verotoxin production by enterohemorrhagic *Escherichia coli* (EHEC) with minimal inhibitory concentration (MIC) of 31.3 µg/mL (Sakagami *et al.*, 2001).

1.7.4 *Ludwigia adscendens* (L.) Hara

Synonyms: *Jussiaea adscendens* L. (Khan and Halim, 1987)

English name: Water primrose (Hutchinson, 1959)

Local names: Malcha, Mulcha, Mulsi, Keshardam (Bengali names) (Yusuf *et. al.*, 1994)

Family: Onagraceae

1.7.4.1 Botany and habitat

Ludwigia adscendens is a floating herb, rarely creeping and are often found in stagnant water. Floating branches are associated with rooting at the nodes of the stem. The plant floats on water with the help of white, spindle shaped

pneumatophores at the nodes. Usually the plants are glabrous but may also be densly villious if growing on dry land. Leaves are alternate, elliptic in shape with the main vein present. Surface of the leaves are shiny due to the presence of epicuticular wax. Sepals are glabrous to villous and deltiod in shape. Petals are obovate and round at the apex. Petals are creamy white colour with yellow coloration at the base. Stamens are twice as many as petals and are inserted with them. The capsules are glabrous to densely villous with 10 dark brown ribs. Pale brown seeds are arranged in a row in each locule and firmly embedded in coherent cubes of woody endocarp (Khan and Halim, 1987).

Ludwigia adscendens is an aquatic herb growing all over Bangladesh. It is also found in warmer regions of the world including India and Sri-Lanka extending through South China and Malaysia to North Australia (Khan and Halim, 1987; Kirtikar and Basu, 1999).

1.7.4.2 Traditional uses

The whole plant is used as a poultice in ulcers and other skin diseases (Yusuf *et al.*, 1994). In India and in China, it is used as an astringent, emetic, antidysenteric and for anthelmintic action (Kirtikar and Basu, 1999). In China, the pounded juice is used as diuretic. A toasted powdered leaf mixed with rapeseed oil is applied to boils, to wash wounds caused by fungal infection. It is also used as an antidote for intoxication and dog bites. In Taiwan, Indo-China and in Malay Peninsula, different preparations of the plant, (*e.g.*, mixed with oil, in combination with other plants or on

its own) are used to treat a wide variety of diseases such as skin, eye and throat complaints, swelling and diseases of the scalp (Perry, 1980).

Fig. 1.5: Free hand drawing of *Ludwigia adscendens* (L.) Hara

1.7.4.3 Previous phytochemical studies

See **Table 1.2**, **1.3** and **1.4**.

1.7.4.4 Previous biological studies

The methanol extract of *L. adscendens* (whole plant) showed antimicrobial activity against a range of Gram-positive and Gram-negative bacteria when tested in a disc diffusion assay (Ahmed *et al.*, 2005).

1.8 The family Euphorbiaceae Juss.

The Euphorbiaeceae Juss. is a cosmopolitan family which includes about 6,300 species mostly shrubs or trees and some are herbaceous, distributed in 245 genera (Hickey and King, 1988). Many plants of this family are cactus-like or possess phylloclades growing in the desert or in dry lands. Leaves are simple, alternate. Plants often produce latex such as from *Hevea brasiliensis* which is the main source of rubber. Plants from this family are generally poisonous. *Euphorbia* is the largest genus of this family consisting of about 2000 species (Hickey and King, 1988; Kulju *et al.*, 2007a).

1.9 The genus *Trewia* L.

1.9.1 Botany

Trewia L. is one of the smallest genera consisting of two species, *T. polycarpa* and *T. nudiflora* growing in the eastern region of the Indian subcontinent, Bangladesh and South China (Airy Shaw, 1975; Chamundeeswari *et. al*, 2003). The other species of *Trewia* resulted from misidentification of species belonging to genera including *Mallotus* and *Macaranga* and used as synonyms for the respective species (Hooker, 1890; Kulju *et al.*, 2007b). Due to their similarity in plastid (*trnL-F*) sequence and various nuclear markers (ITS, *ncpGS, phyC*), it has been suggested that the genus of *Trewia* be merged with *Mallotus* (Kulju *et al.*, 2007b).

1.9.2 Previous phytochemical reports on *Trewia*

Table 1.5: Maytansinoids from *Trewia**

Compound	**Reference**
Trewiasine (**43**)	Powell *et al.*, 1981
Dehydrotrewiasine (**44**)	Powell *et al.*, 1981
Demethyltrewiasine (**45**)	Powell *et al.*, 1981
Maytansine (**46**)	Powell *et al.*, 1981
Colubrinol (**47**)	Powell *et al.*, 1981
Maytanbutine (**48**)	Powell *et al.*, 1981
Treflorine (**49**)	Powell *et al.*, 1983
Trenudine (**50**)	Powell *et al.*, 1983
N-methyl trenudine (**51**)	Powell *et al.*, 1983
Trewsine (**52**)	Powell *et al.*, 1981
Maysine (**53**)	Powell *et al.*, 1981
10-epitrewiasine (**54**)	Powell *et al.*, 1983
Nortrewiasine (**55**)	Powell *et al.*, 1983

* All the compounds were isolated from the species *T. nudiflora*

Table 1.6: Cardenolides previously isolated from *Trewia**

Compounds isolated
Alliotoxin (**56**)
5α-Sarmentogenin (**57**)
5α-Gitoxigenin 3-*O*-α-L-rhamnopyranoside (**58**)
5α-Oleandrigenin 3-*O*-α-L-rhamnopyranoside (**59**)
5α-Oleandrigenin 3-*O*-[β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside (**60**)
11-Oxouzarigenin 3-*O*-α-L-rhamnopyranoside (**61**)
Frugoside (**62**)
Ascleposide (**63**)

* All the compounds were isolated from the species *T. nudiflora* and reported by Kang *et al.*, 2005

Table 1.7: Sterols and triterpenoids previously isolated from *Trewia*

Compounds isolated	Plant species	Reference
(22*E*,24*R*)-5α,8α-epidioxyergosta-6,22-dien-3β-ol (**64**)	*T. nudiflora*	Du and Shen, 2006
(22*E*,24*R*)-5α,8α-epidioxyergosta-6,9(11)22-trien-3β-ol (**65**)	*T. nudiflora*	Du and Shen, 2006
(22*E*,24*R*)-6-methoxyergosta-7,22-dien-3,5-diol (**66**)	*T. nudiflora*	Du and Shen, 2006
Stigmast-4-en-6β-ol-3-one (**67**)	*T. nudiflora*	Shaohua *et al.*, 2008
Stigmast-4-en-6α-ol-3-one (**68**)	*T. nudiflora*	Shaohua *et al.*, 2008
7β-Hydroxysitosterol (**69**)	*T. nudiflora*	Shaohua *et al.*, 2008
7α-Hydroxysitosterol (**70**)	*T. nudiflora*	Shaohua *et al.*, 2008
Schleicheol 2 (**71**)	*T. nudiflora*	Shaohua *et al.*, 2008
β-sitosterol (**38**)	*T. nudiflora*,	Du *et al.*, 2002
	T. polycarpa	Ahmad *et al.*, 2004
Daucosterol (**39**)	*T. nudiflora*,	Guohong *et al.*, 2004
	T. polycarpa	Ahmad *et al.*, 2004
Taraxerone (**72**)	*T. nudiflora*	Du *et al.*, 2004
3β-Acetylaleuritolic acid (**73**)	*T. nudiflora*	Du *et al.*, 2002
	T. polycarpa	Chamundeeswari, *et al.*, 2003

Table 1.8: Diterpenoids previously isolated from *Trewia**

Compounds isolated	Reference
3β,17-Dihydroxycleistantha-12,15-dien-2-one (**74**)	Du and Shen, 2006
17-Hydroxy-*ent*-atisan-19-oic acid (**75**)	Du *et al.*, 2004
17-Hydroxy-*ent*-atisan-19-oic acid methyl ester (**76**)	Du *et al.*, 2004
16α,17-Dihydroxy-*ent*-atisan-19-al (**77**)	Du *et al.*, 2004
Abbeokutone (**78**)	Shaohua *et al.*, 2008

* All the compounds were isolated from the species *T. nudiflora*

Table 1.9: Alkaloids and nitrogen-containing compounds previously isolated from *Trewia**

Compounds isolated	Reference
Ricinidine (**79**)	Ganguly, 1970
N-methyl-5-carboxamide-2-pyridone (**80**)	Sastry and Waller, 1972
Nudiflorine (**81**)	Mukherjee and Chatterjee, 1966
Caffeine (**82**)	Bingjun *et al.*, 1991

* All the compounds were isolated from the species *Trewia nudiflora*

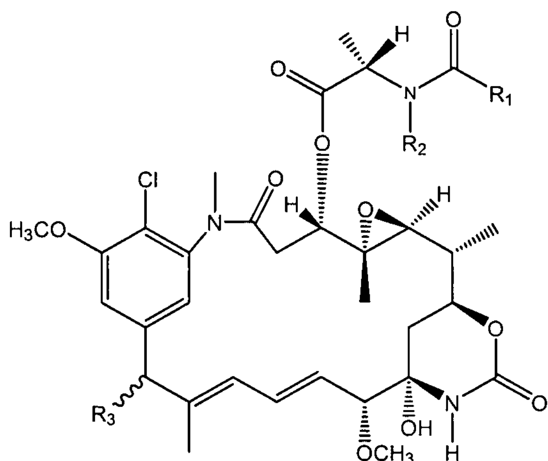
Table 1.10: Lignans previously isolated from *Trewia**

Compounds isolated	Reference
9'-Methyl-americanol A (**83**)	Guohong *et al.*, 2004
9'-Ethyl-americanol A (**84**)	Guohong *et al.*, 2004
9'-Butyl-americanol A (**85**)	Guohong *et al.*, 2004
Americanin (**86**)	Guohong *et al.*, 2004
9'-Methyl isoamericanol A (**87**)	Guohong *et al.*, 2004
Hierochin B (**88**)	Kang *et al.*, 2008
(+)-Balanophonin (**89**)	Kang *et al.*, 2008
Fisusal (**90**)	Kang *et al.*, 2008
1-(4-Hydroxy-3-methoxyphenyl)-2-(4-[2-formyl-(*E*)-vinyl]-2-methoxyphenoxy)-propane-1,3-diol (**91**)	Kang *et al.*, 2008
(+)-Dihydrodehydrodiconiferyl alcohol 4-O-β-(6"-O-galloyl)-glucopyranoside (**92**)	Kang *et al.*, 2008

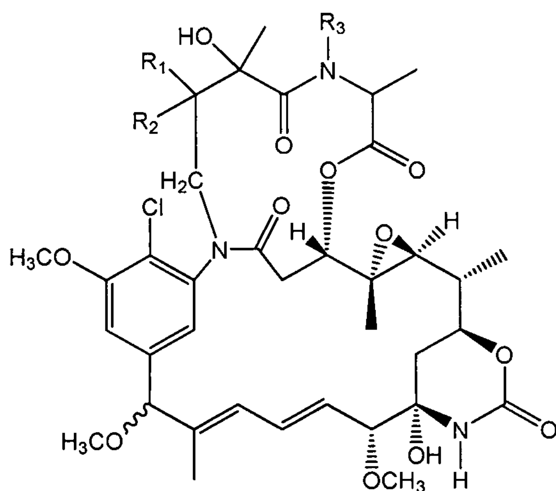
* All the compounds were isolated from the species *Trewia nudiflora*

Table 1.11: Miscellaneous compounds previously isolated from *Trewia*

Compounds isolated	Plant species	Reference
Trewinine (**93**)	*T. nudiflora*	Saha *et al.*, 1981
Gallic acid (**44**)	*T. nudiflora*	Du *et al.*, 2004
Ethyl gallate (**94**)	*T. nudiflora*	Du *et al.*, 2004
Protocatechuic acid (**95**)	*T. nudiflora*	Du *et al.*, 2004
trans-Cinnamic acid (**96**)	*T. nudiflora*	Du *et al.*, 2004
Ethyl *O*-β-(6'-galloyl)-glucopyranoside (**97**)	*T. nudiflora*	Kang *et al.*, 2008
3,4,4'-Tri-*O*-methylellagic acid (**98**)	*T. nudiflora*	Du *et al.*, 2004
3,3'-Di-*O*-methylellagic acid (**99**)	*T. nudiflora*	Kang *et al.*, 2008
3-*O*-Methylellagic acid 3'-*O*-α-L-rhamnopyranoside (**100**)	*T. nudiflora*	Kang *et al.*, 2008
3,3'-Di-*O*-methylellagic acid 4-*O*-α-L-rhamnopyranoside (**101**)	*T. nudiflora*	Kang *et al.*, 2008
4,4'-*O*-Dimethylellagic acid 3-(2"-*O*-acetlyl)-α-L-rhamnopyranoside (**102**)	*T. nudiflora*	Kang *et al.*, 2008
4-*O*-Methylellagic acid 3'-(4"-*O*-acetlyl)-α-L-rhamnopyranoside (**103**)	*T. nudiflora*	Kang *et al.*, 2008
Junipetrioloside A (**104**)	*T. nudiflora*	Kang *et al.*, 2008
Junipetrioloside B (**105**)	*T. nudiflora*	Kang *et al.*, 2008
α-Tocopherol (**106**)	*T. nudiflora*	Du *et al.*, 2004
O-Vanillyl alcohol (**107**)	*T. nudiflora*	Shaohua *et al.*, 2008
Isoliquiritigenin (**108**)	*T. nudiflora*	Peiji *et al.*, 2004
Dihydro-3-methoxy-4-(methylamino)-2,5-furandione (**109**)	*T. polycarpa*	Ahmad *et al.*, 2004

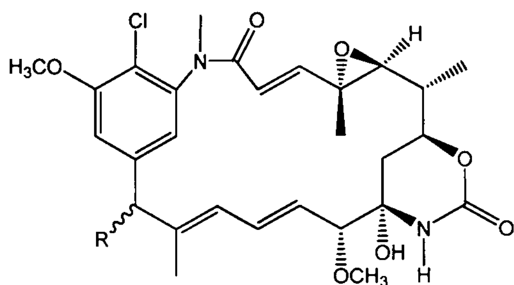


Compound	R_1	R_2	R_3
43	$CH(CH_3)_2$	CH_3	OCH_3
44	$CH(CH_3){=}CH_2$	CH_3	OCH_3
45	$CH(CH_3)_2$	H	OCH_3
46	CH_3	CH_3	H
47	$CH(CH_3)_2$	CH_3	OH
48	$CH(CH_3)_2$	CH_3	H



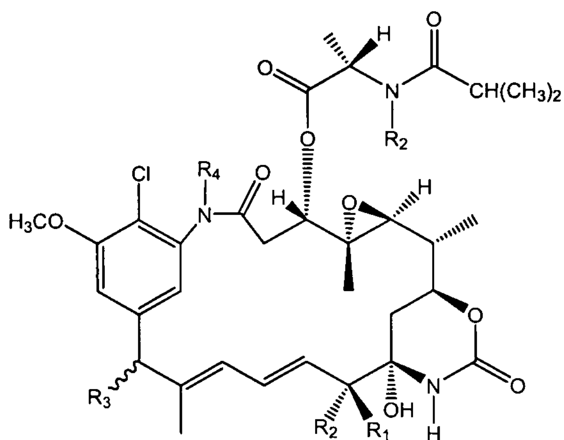
Compound	R_1	R_2	R_3
43	H	H	H
44	OH	H	H
48	H	H	CH_3

Fig. 1.6: Structures of maytansinoids previously isolated from *Trewia*



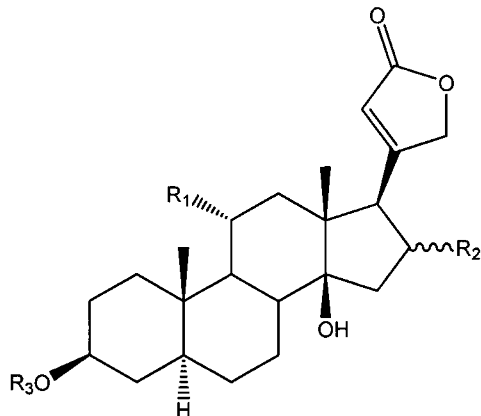
52 R= OCH_3

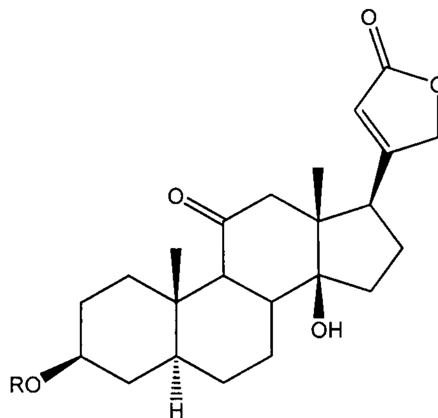
53 R= H



Compound	R_1	R_2	R_3	R_4
54	OCH_3	H	OCH_3	CH_3
55	H	OCH_3	OCH_3	H

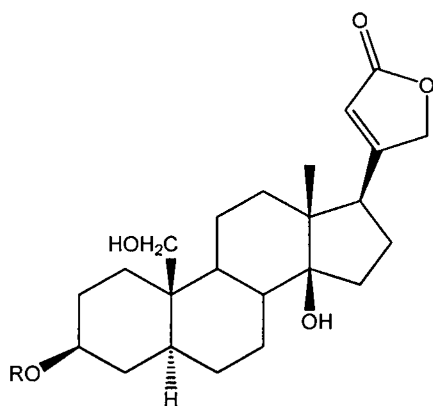
Fig. 1.6: (cont.) Structures of maytansinoids previously isolated from *Trewia*



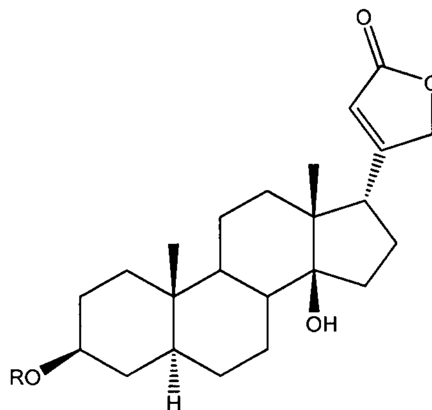


61 R= α-L-Rhamnopyranosyl

Compound	R_1	R_2	R_3
56	OH	H	α-L-rhamnopyranosyl
57	OH	H	H
58	H	OH	α-L-rhamnopyranosyl
59	H	CH_3COO	α-L-rhamnopyranosyl
60	H	CH_3COO	β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside

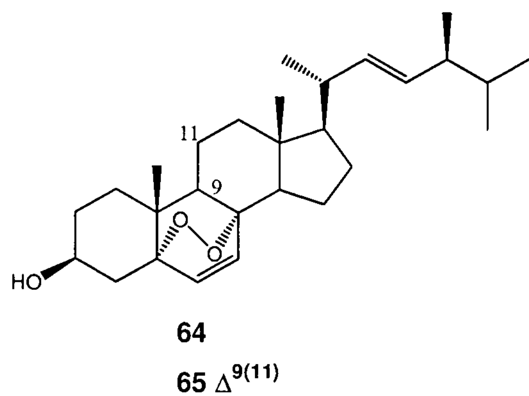


62 R= 6-Deoxy- β-D-allopyranosyl



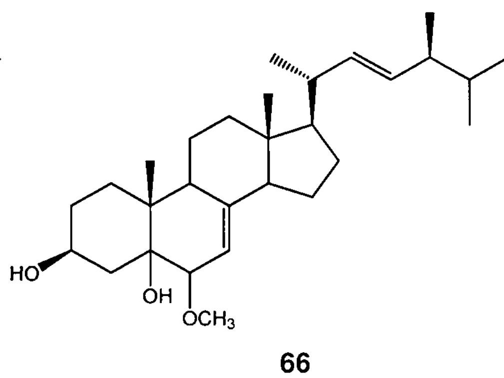
63 R= 6-Deoxy- β-D-allopyranosyl

Fig. 1.7: Structures of cardenolides and their glycosides previously isolated from *Trewia*



64

65 $\Delta^{9(11)}$



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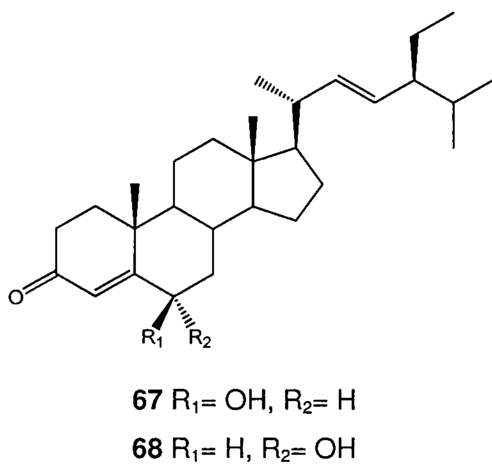
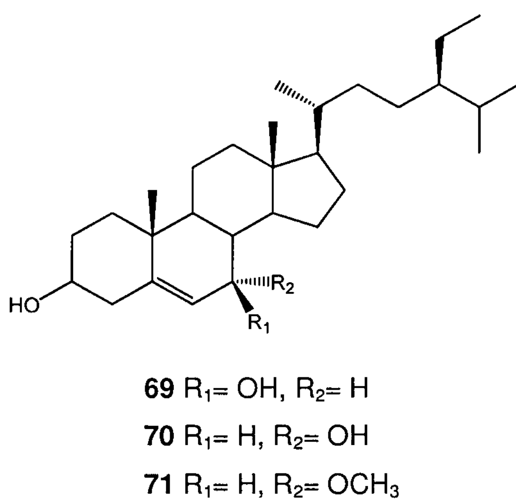


67 R_1= OH, R_2= H

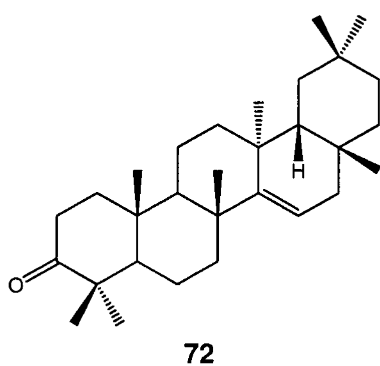
68 R_1= H, R_2= OH



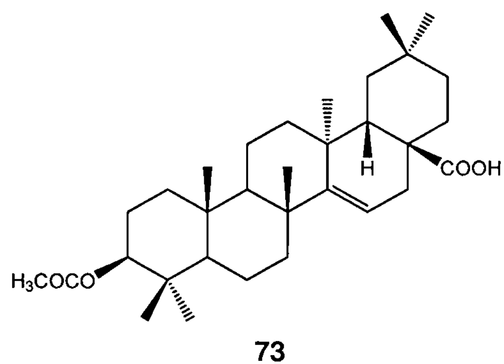
69 R_1= OH, R_2= H

70 R_1= H, R_2= OH

71 R_1= H, R_2= OCH_3



72



73

Fig. 1.8: Structures of phytosterols and triterpenoids previously isolated from *Trewia*

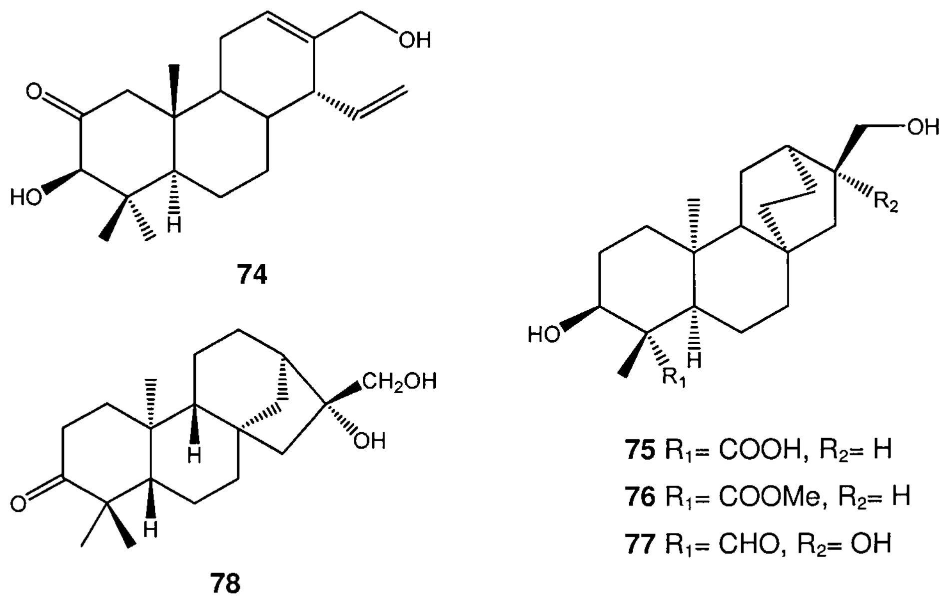


Fig. 1.9: Structures of diterpenoids previously isolated from *Trewia*

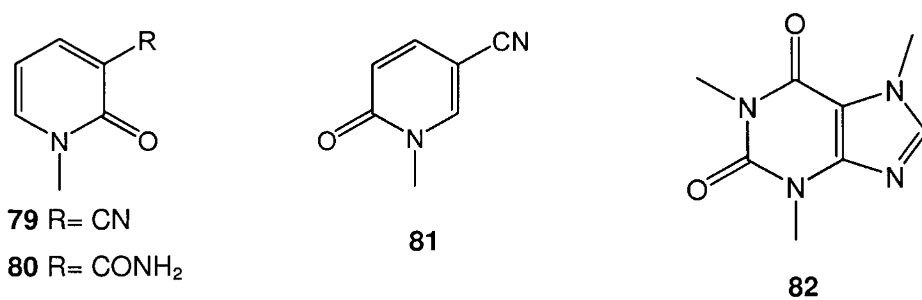
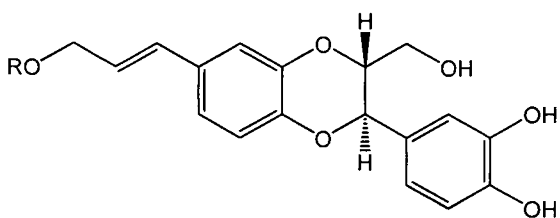
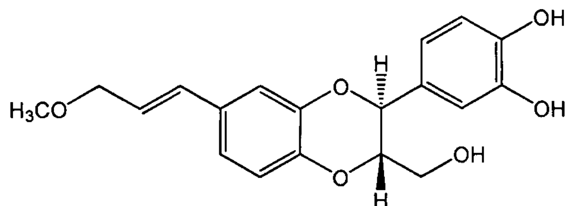


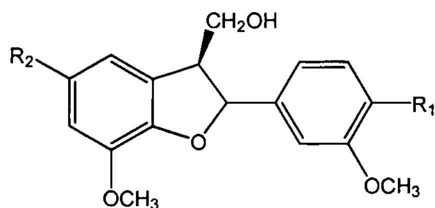
Fig. 1.10: Structures of alkaloids and nitrogen-containing compounds previously isolated from *Trewia*



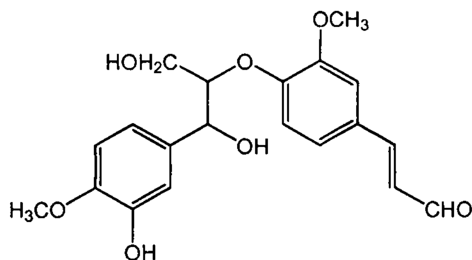
83 R= CH_3
84 R= CH_2CH_3
85 R= $CH_2CH_2CH_2CH_3$
86 R= CHO



87



88 R_1= OCH_3, R_2= CH=CHCHO
89 R_1= OH, R_2= CH=CHCHO
90 R_1= OH, R_2= CHO



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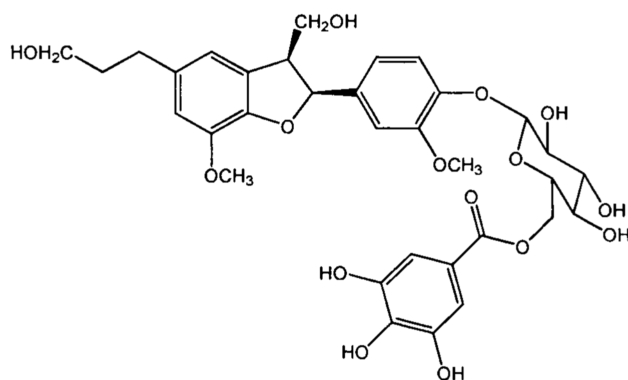
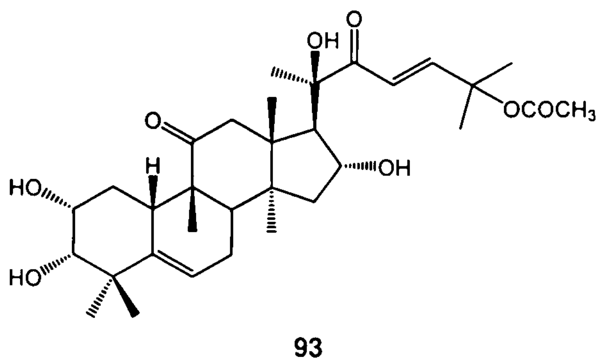
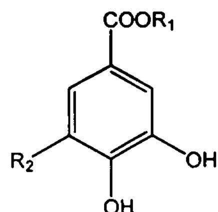
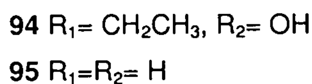


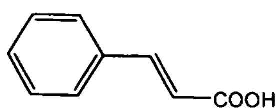
92

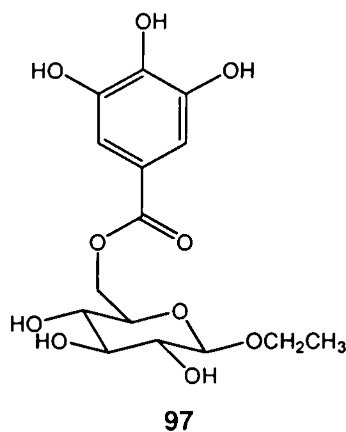
Fig. 1.11: Structures of neolignans and lignans previously isolated from *Trewia*

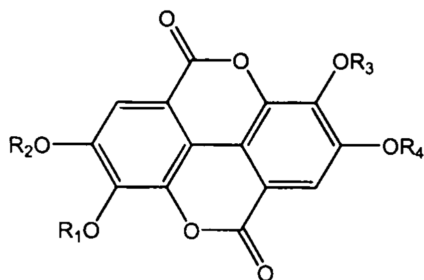


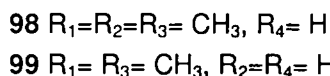


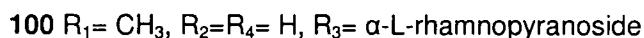


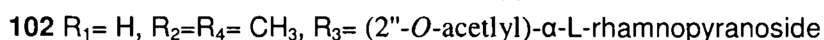


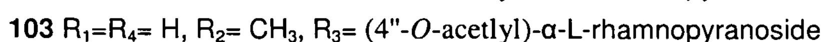


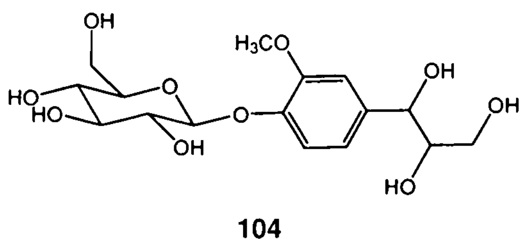












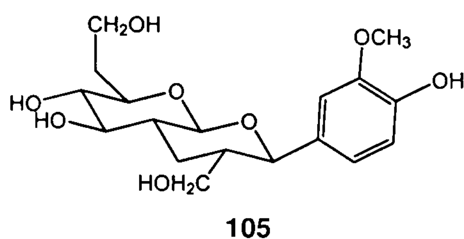


Fig. 1.12: Structures of miscellaneous compounds previously isolated from *Trewia*

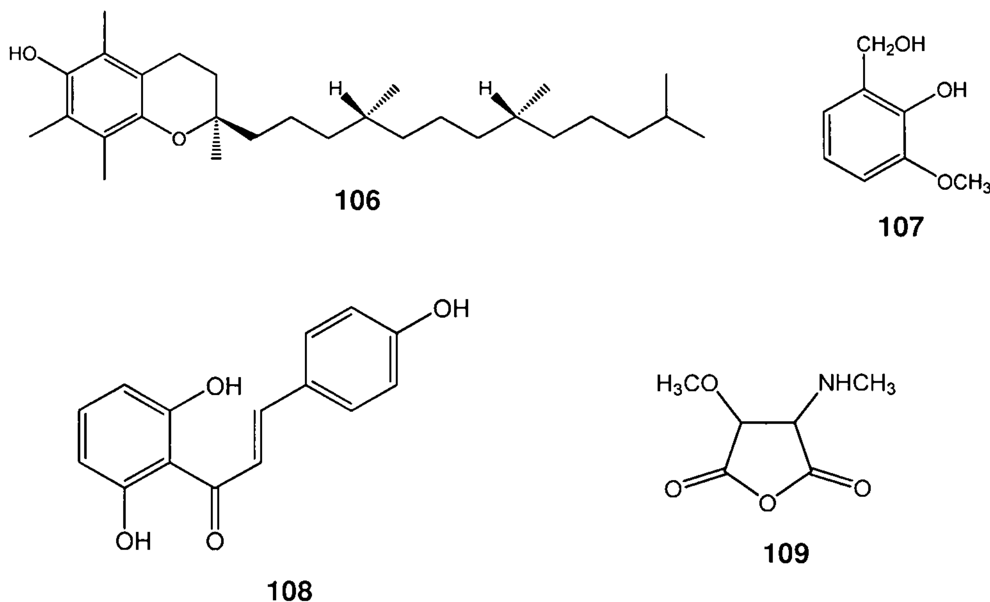


Fig 1.12: (cont.) Structures of miscellaneous compounds previously isolated from *Trewia*

1.9.3 Previous antimicrobial reports on *Trewia*

The ethanol extract of *T. polycarpa* roots, and the chloroform and aqueous phase derived from the ethanol extract showed activity against two Gram-positive (*S. aureus* and *S. pyogenes*), four Gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae*) and four fungi (*Candida albicans*, *Aspergillus niger*, *Cryptococcus neoformans*, *Penicillum* spp.) with MICs in the range of 0.313 to 5 mg/mL (Chamundeeswari *et al.*, 2004). In another study, 3β-acetyl aleuritolic acid (**73**) isolated from the *n*-hexane phase of the ethanol extract of the root bark was found to be active against *K. pneumoniae* (MIC 62.5 μg/mL). It was also active against *C. albicans* and *A. niger* at 125 μg/mL.

1.9.4 *Trewia nudiflora* L.

English name: False white teak (Yusuf *et al., 1994*)

Local names: Medda, Pitali, Meragota (Bengali names) (Yusuf *et al., 1994*)

Family: Euphorbiaceae

1.9.4.1 Botany and habitat

Trewia nudiflora is a white soft-wooded small to large deciduous tree. The leaves are broadly ovate-cordate and round shaped, 10-15 cm long and varies in proportion of length to breadth. *T. nudiflora* has 6-17 flowered pistillate inflorescences with flowers only in the apical part whereas *T. polycarpa* has 1-5 flowered pistillate inflorescences with flowers also in the basal part. The inflorescence of the male flowers is receme, *i.e.*, inflorescence appearing alternatively along the main axis with older flowers towards the base. Sepals are globular in shape and split into 3-4 broad concave. Numerous stamens grow on a convex receptacle and the filaments are free. Fruits are fleshy, 1.5 cm in diameter with dense finely tomentose surface and crustaceous pericarp. The fruits of *T. nudiflora* have 3-5 locules and differs from the fruits of *T. polycarpa* which have thinner pericarp and 2 locules. (Hooker, 1890; Kulju *et al.*, 2007b).

T. nudiflora grows throughout South Eastern Asia including India, Sri-Lanka, China, Indonesia, Malaysia and is common in Bangladesh (Kulju *et al.*, 2007b; Yusuf *et al.*, 1994).

1.9.4.2 Traditional uses

A decoction of the shoot is used to relieve swelling. The leaves are applied to swellings and to heal wounds. Leaves are also used in the treatment of flatulence, excessive bile and sputum. The root is used in the treatment of gout and rheumatism (Yusuf *et al.*, 1994).

Fig. 1.13: Stem with fruits and leaves of *Trewia nudiflora* L. (Picture provided by Prof. D.M. Ali)

Fig. 1.14: Cross sections of *Trewia nudiflora* fruits (Picture provided by Prof. D.M. Ali)

1.9.4.3 Previous phytochemical studies

See **Tables 1.5** to **1.11**.

1.9.4.4 Previous pharmacological studies

Two neo-lignans, namely 9'-butyl americanol A (**86**) and americanin (**87**), isolated from *Trewia nudiflora* seed endothelium were found to be active against *Staphylococcus aureus* and *Mycobacterium tuberculosis* with MIC values of 50 and 100 μg/mL, respectively (Guohong *et al.*, 2004). Isoliquiritigenin (**109**), isolated from the seed crust of *Trewia nudiflora*, also showed activity against the above microorganisms (Peiji *et al.*, 2004). In another study antibacterial activity was observed for a liquid disinfectant prepared from *T. nudiflora* seed oil (Mondal *et al.*, 2006).

During the screening for anticancer agents at National Cancer Institute (NCI), the seeds of *T. nudiflora* exhibited potent antitumor activity both *in vitro* and *in vivo*. Later on trewiasine (**43**) and other related maytansinoids were isolated as the active compounds. These maytansinoids have also been reported to possess antifungal and antifeedant activity (Du *et al.*, 2004; Powell, 2009).

In a study, three compounds (**93, 98** and **103**) isolated from the stem bark of *T. nudiflora* significantly prevented hydrogen-peroxide induced PC12 cell death at a concentration of 0.4 μM. The former one (**93**) also showed significant antioxidant activity in a DPPH assay (Kang *et al.*, 2008).

1.10 The family Acanthaceae Juss.

The Acanthaceae Juss. is a large family comprising of about 250 genera and 2700 species distributed mainly in tropical and subtropical regions of the world. Most plants are herbs or shrubs. Some are climbers and trees. Their habitat ranges from extremely dry climate to marshy wet land, but most of the plants grow in damp tropical forests. Plants of this family have simple leaves arranged in opposite pairs on the twigs. Flowers are bisexual and often appear in clusters. Individual flowers are enclosed by bracts which may be coloured and large (Shaw and Wills, 1973).

1.11 The genus *Hygrophila* Lindl.

1.11.1 Botany

Hygrophila Lindl. is the smallest genus in the Acanthaceae with about 25 species. There are two species of *Hygrophila* growing in Bangladesh, namely *H. auriculata* and *H. difformis* (Khan and Halim, 1987). As the name implies, plants of this genus grow in swampy or wet-land areas. Herbs are often branched from the base. Leaves of the plants belonging to the genus *Hygrophila* are opposite and often ovate, lanceolate or entire shaped. Inflorescences appear either at the top of the stem or at subsessile whorls (Khan and Halim, 1987).

1.11.2 Previous phytochemical reports on *Hygrophila*

Table 1.12: Iridoid glycosides previously isolated from *Hygrophila*

Compounds isolated	Plant species	Reference
Mussaenosidic acid (**110**)	*H. polysperma*	Jensen *et al.*, 1988
Isoaucubin (**111**)	*H. polysperma*	Jensen *et al.*, 1988
Hygrophiloside (**112**)	*H. diflormis*	Jensen *et al.*, 1988

Table 1.13: Triterpenoids and sterols previously isolated from *Hygrophila**

Compounds isolated	Reference
β-Sitosterol (**34**)	Sunita and Abhishek, 2008
Stigmasterol (**113**)	Ali and Tripathi, 2007
23-Ethylcholesta-11(12),23(24)-dien-3β-ol (**114**)	Ali and Tripathi, 2007
Lupeol (**115**)	Ali and Tripathi, 2007
Betulin (**116**)	Misra *et al.*, 2000
3β-Acetoxyurs-18-ene (**117**)	Ali and Tripathi, 2007

* All the compounds were isolated from the species *H. auriculata*

Table 1.14: Flavonoid glycosides and miscellaneous compound previously isolated from *Hygrophila**

Compound	Reference
Ferulic acid (**118**)	Surveswaran, *et al.*, 2007
Apigenin 7-*O*-glucoside (**119**)	Balraj and Nagarajan, 1982
Apigenin 7-*O*-glucuronide (**120**)	Balraj and Nagarajan, 1982

* All the compounds were isolated from the species *H. auriculata*

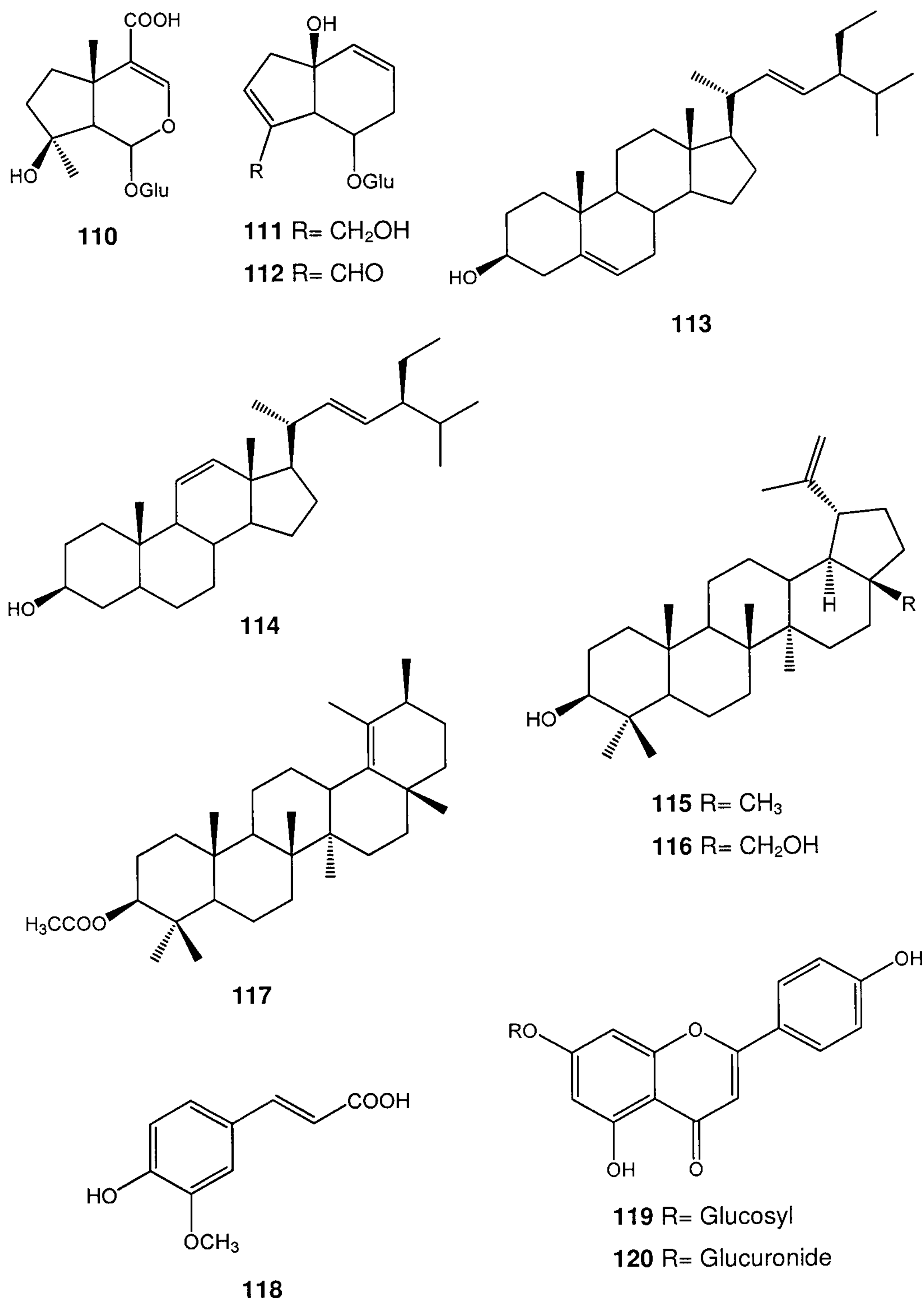


Fig 1.15: Structures of isolated constituents from *Hygrophila*

1.11.3 Previous antimicrobial reports on *Hygrophila*

The methanol extract and different partitions of the methanol extract of *H. stricta* (whole plant) exhibited antibacterial activity against a wide range of Gram +ve and –ve bacteria (Khan and Omoloso, 2002).

The methanol extract of *H. auriculata* leaf, at a concentration of 30 μg/disc was active against *S. aureus* and two different strains of *Burkholderia pseudomallei* in a disc diffusion assay (Samy, 2005).

1.11.4. *Hygrophila auriculata* Heine

Synonyms: ***Hygrophila spinosa*** **T. Anderson;** ***Asteracantha longifolia*** **Nees**

English name: Star thorn (Yusuf *et al.*, 1994)

Local names: Talmakhna, Kulekharha (Bengali names) (Yusuf *et al.*, 1994)

Family: Acanthaceae

1.11.4.1 Botany and habitat

Hygrophila auriculata Heine is an annual thorny stout herb with the average height of 0.6-1.5 m. Plants are usually unbranched but have numerous fasciculate subquadrangular stems. Six oblong-lanceolate or oblanceolate leaves vertical to the stem appear at the nodes. Two of the six leaves are large reaching a length of 18 cm and 1.3-3.2 cm width. The four inner leaves are about 3.8 cm long. Each of the six leaves have one straight sharp yellow spine (2.5-4.5 cm long). Flowers grow in a whorl of 8 (in 4 pairs) at each node. Each of the flowers has four sepals with the upper sepal 1.6-2 cm long and broad, the rest three 1.3 cm long, linear-lanceolate, coarsely hairy on the back with hyaline ciliate margins. The purple-blue corolla is

about 3.2 cm long and widely bilipped. Glabrous filaments are arranged in pair, one short and one long. Capsules are 0.8 cm long, linear-oblong, pointed, bearing 4-8 seeded. Seeds are flat or compressed with smooth surface (Warrier *et al.*, 1995).

This species grows throughout Bangladesh in marshy places including rice fields, canal banks or in tanks (Khan and Halim, 1987). It is also common in India, Sri-Lanka and in South Africa (Kirtikar and Basu, 2003).

1.11.4.2 Traditional uses

The seeds are used in the treatment for gonorrhoea and to relieve constipation (Kirtikar and Basu, 2003). Leaves are used in diarrhoea, dysentery, jaundice, urinary calculi, inflammation, biliousness, cough. The leaf paste is also applied externally for lumbago and rheumatism. A decoction of the root is diuretic (Warrier *et al.*, 1995; Yusuf *et al.*, 1994).

Fig. 1.16: Free hand drawing of *Hygrophila auriculata* Heine

1.11.4.3 Previous phytochemical studies

See **Tables 1.15**, **1.16** and **1.17**.

1.11.4.4 Previous pharmacological studies

The ethanol extract of *H. auriculata*, at the doses of 200, 400 and 600 mg/kg, showed significant diuretic activity in Wistar albino rats. The excretion of the Na^+, K^+ and Cl^- in the urine was comparable to that of the diuretic drug furosemide (Swamy *et al.*, 2007). The chloroform extract of *H. auriculata* leaves improved erythrocyte and haemoglobin counts and reduced cyclophosphamide-induced bone marrow supression in test mice treated for a period of 22 days (Pawar *et al.*, 2006). The aqueous extract of the whole plant protected the liver from the toxic effect of paracetamol and carbon-tetrachloride (Hewawasam *et al.*, 2003). The methanol extract of the seeds prevented diethylnitrosamine-induced hepatocarcinogenesis (Ahmed *et al.*, 2001).

1.12 Aims and objectives

The present work aimed to investigate three plants from Bangladesh in search for novel antimicrobial agents which could be active, alone or in combination with known antibiotics, against a range of microbial pathogens.

The objectives of the work were to

- extract selected plants and screen the extracts against a panel of Gram-positive and Gram-negative bacteria;
- apply various chromatographic procedures to isolate constituents present in the extracts;
- identify the isolated compounds using various spectroscopic techniques;
- screen compounds obtained in sufficient yield against selected bacteria and against *Mycobacterium aurum*;
- perform potentiation studies on selected active compounds in combination with commercially available antitubercular (anti-TB) agents;

Chapter 2

Materials and methods

2 Materials and methods

2.1 Solvents

2.1.1 Solvents for analytical and chromatographic purposes

Solvents listed below were used during different processes of extraction, chromatographic separation, analytical TLCs, crystallisation purposes.

- *n*-Pentane (HPLC grade)
- *n*-Hexane (HPLC grade)
- Diethyl ether (HPLC grade)
- Chloroform (HPLC grade)
- Ethyl acetate (Analytical grade, HPLC grade)
- Acetone (Analytical grade)
- DMSO (HPLC grade)
- Acetonitrile (HPLC grade)
- *n*-Butanol (Analytical grade)
- Ethanol (Analytical grade)
- Methanol (Analytical grade, HPLC grade)
- Acetic acid (Analytical grade)
- Water (HPLC grade)

Solvents were obtained in 2.5 L glass bottles from Fisher Scientific UK Ltd and VWR UK Ltd. Analytical grade solvents were distilled using in-house small scale distillation set up to remove any trace impurities including phthalates. All the solvents were kept in store at low temperature and transferred in 500 mL solvent

bottles for routine use. Chloroform was kept in amber glass bottle to prevent photodegradation.

2.1.2 Solvents for NMR analysis

Deuterated (99.9%) solvents were used for all the NMR analysis. Solvent shifts (in ppm) were used as the reference. Following is the list of solvent used and were obtained from Sigma-Aldrich UK Ltd:

Table 2.1: Deuterated solvents used for NMR analysis

Solvent	Chemical formula	^{1}H shift(s) in ppm (*multiplicity*)	^{13}C shift(s) in ppm (*multiplicity*)	Peak for trace of water in ppm
Deuterated acetone	$(CD_3)_2CO$	2.05 (*5*)	29.92 (*7*), 206.18 (*1*)	2.8
Deuterated chloroform	$CDCl_3$	7.25 (*1*)	77.00 (*3*)	1.5
Deuterated DMSO	$(CD_3)_2SO$	2.50 (*5*)	39.43 (*5*)	3.3
Deuterated methanol	CD_3OD	3.30 (*5*)	49.05 (*7*)	4.8
Deuterated pyridine	C_5D_5N	7.20 (*1*), 7.56 (*1*), 8.71 (*1*)	123.44 (*3*), 135.51 (*3*), 179.68 (*3*)	5.0

2.2 Reagents and chemicals

- *p*-Anisaldehyde (Phytochemistry research laboratory repository)
- Vanillin (Phytochemistry research laboratory repository)
- Sulphuric acid (VWR UK Ltd)
- Dragendorf's reagent (Phytochemistry research laboratory repository)

- TLC grade silica gel coated aluminium sheet (Merck Precoated Silica gel PF_{254})
- TLC grade silica gel (60H, Merck, Germany)
- Column grade silica gel (Silica gel 60, mesh size 20-200 μm (Merck, Germany)
- MTT dye (Sigma-Aldrich, UK)
- Ciprofloxacin (Sigma-Aldrich UK Ltd)
- Ethambutol (Sigma-Aldrich UK Ltd)
- Rifampicin (Sigma-Aldrich UK Ltd)
- Isoniazid (Sigma-Aldrich UK Ltd)
- Ellagic acid (≥95% HPLC, from tree bark) (Sigma-Aldrich UK Ltd)
- Cation-adjusted Mueller-Hinton broth with TES buffer (Trek Diagnostics)
- Tryptic Soya broth (Sigma-Aldrich, UK)
- Nutrient agar (Sigma-Aldrich, UK)

2.3 Plant material

Plant materials were collected from different locations in Bangladesh. A quick shade drying with sufficient aeration was carried out to prevent molding or any type of degradation. Garbling was carried out to remove contaminants and the plant was cut into small pieces. The dried plant material was ground in to a fine powder using a grinder. *Ludwigia adscendens* and *Hygrophila auriculata* were authenticated at the Bangladesh National Herbarium where voucher specimens have been submitted for future reference (**Table 2.1**). *Trewia nudiflora* was identified by Henry Noltie, Royal Botanic Garden Edinburgh. A voucher specimen has also been submitted to Phytochemistry Laboratory, Pharmacy Discipline, Khulna University, Bangladesh.

Table 2.2: Collection details of plant parts used

Species	Plant part	Location and time	Voucher no.
Ludwigia adscendens	Whole plant (Stem, root and leaves)	Khulna (Sept' 2005)	DACB 31255
Trewia nudiflora	Stem bark	Rajshahi (May' 2006)	PL 79
Hygrophila auriculata	Seeds	Purchased at Dhaka, Old town medicinal plant shop (Sept' 2005)	DACB 31257

2.4 Extraction and partitioning

The plant material was successively extracted with *n*-hexane, ethyl acetate and methanol. *Ludwigia adscendens* was extracted using a Soxhlet apparatus. Accelerated solvent extractor (ASE 100, Dionex UK Ltd.) which was later introduced in the phytochemistry laboratory was used for the extraction of other two plants. Operating conditions for the ASE comprises of four static cycles (one cycle 8 min) with oven temperature 100°C, flush volume 60%, purge time 150 s, pressure 1400-1500 psi. The extracts were evaporated at 40°C under vacuum using a rotary evaporator. Methanol extracts were dissolved in 2.5% ethanol in water and partitioned with chloroform and then with *n*-butanol using a separating funnel. Chloroform and *n*-butanol phases were first dried over anhydrous sodium sulphate and then evaporated using rotary evaporator. The remaining aqueous phase was freeze dried. All dried extracts and phases were transferred in screw-cap vials and stored at -20°C in air tight plastic bags.

2.5 Analytical techniques

2.5.1 Thin layer chromatography

Thin layer chromatography (TLC) is one of the simplest forms of chromatographic techniques. It gives a quick and easy way to analyse the components of a mixture, compare between mixtures or compare sample with standards. Plant extracts, fractions or pure compounds were dissolved in a suitable solvent (e.g. chloroform for non polar samples, ethanol for relatively more polar samples) and spotted approximately 1 cm above the bottom edge of a TLC grade silica gel coated aluminium sheet (Merck Precoated Silica gel PF_{254}). Spots were applied as bands to facilitate easy and accurate observation and the bands were kept as narrow as possible to reduce the overlapping of compounds. Based on the expected polarity of the extract or compound, a suitable solvent mixture was added to the TLC tank and left for a while to saturate the tank environment. Filter paper was put inside the tank to aid the saturation. Spotted TLC plates were then placed in the TLC tank to develop in an ascending direction. Care was taken that the solvent remained below the sample bands. Upon development, the TLC plates were taken out off the tank, the solvent front was marked and the plates dried immediately. They were then examined visually, then under UV light and finally sprayed with chemical reagents (Stahl and Mangold, 1975; Stock and Rice, 1974).

2.5.2 Selection of mobile phase

Mixtures of solvents were often used for the development of the TLC plates. Different solvent systems were tried until a desirable resolution between the spots

was obtained. For the analysis of fractions or pure compounds, a R_f value of 0.5 was satisfactory while a R_f value of 0.2 was desirable when choosing the starting solvent system for open column or flash chromatography. Distilled solvents were used in all phytochemical work.

2.5.3 Detection

2.3.3.1 Detection by UV light: Developed TLC plates were observed under UV lamp using short (λ= 254 nm) and long (λ= 366 nm) wavelengths. Short UV light is useful in detecting aromatic compounds whereas compounds with conjugated double bonds are visible under long UV light.

2.5.3.2 Detection by spray reagents: Anisaldehyde-sulphuric acid spray reagent was used routinely. Vanillin-sulphuric acid and Dragendorff's reagent were also used where necessary.

Modified anisaldehyde-sulphuric acid reagent: 0.5 mL of *p*-anisaldehyde was mixed with a mixture of 10 mL glacial acetic acid and 85 mL of methanol. Sulphuric acid (5 mL) of was added to it. The reagent was sprayed over the dried developed TLC plates and the plates were heated to 105-110° C for approximately 5 min (Waldi, 1965).

Vanillin-sulphuric acid reagent: 3 g of vanillin was dissolved in 100 mL of absolute ethanol followed by the addition of 0.5 mL of sulphuric acid. The reagent was

sprayed over the dried developed TLC plate and heated to 120° C until coloured spots appeared (Waldi, 1965).

Dragendorff's reagent (according to Munier and Macheboeuf): Equal parts of Solution A and solution B (10 mL each) were mixed with 20 mL of glacial acetic acid and 100 mL of water. (Solution A: Mixture of 2.125 g of bismuth subnitrate, 25 mL glacial acetic acid and 100 mL of water. Solution B: 40 g of potassium iodide in 100 mL water) (Stahl, 1969).

2.6 Chromatographic techniques

2.6.1 Vacuum liquid chromatography

Vacuum liquid chromatography (VLC) is one form of dry column chromatography which offers high resolution as that of a TLC. A sintered glass fitted funnel Büchner with a suction outlet was packed with TLC grade silica gel (60H, Merck, Germany) under vacuum to afford a layer of 5 to 6 cm thick with some space above to accommodate sample and a volume of solvent. A non polar solvent was allowed to pass through the column under vacuum to check the uniformity of the column. Sample to be fractionated was mixed with column grade silica gel (Silica gel 60, mesh size 20-200 µm (Merck, Germany) to get a free flowing powder which was applied as a thin layer on to the top of the packed VLC column. Elution was carried out with a non-polar solvent or non-polar solvent mixture first and then gradually with solvent mixtures of increasing polarity. A definite volume of solvent or solvent mixture was added to the top of the column each time and vacuum was applied until

the column dried up. Each fraction was collected, evaporated to dryness at 40° C under vacuum using a rotary evaporator. The fractions were analysed by TLC and pooled according to similar chemical profile (Coll and Bowden, 1986; Pelletier *et al.*, 1986).

2.6.2 Open column chromatography

Glass columns of appropriate sizes with porous plates at the bottom were used. A slurry of column grade silica gel (Silica gel 60, mesh size 20-200 μm (Merck, Germany) was prepared as the starting solvent system. One third of the column was filled with the same solvent and the slurry was applied as a thin stream, with a gradual flow of solvent. Once the column was packed with silica gel, the solvent was let to flow to get a homogeneous packing. Care was also taken to prevent the inclusion of any air bubble or drying of the column. The sample to be fractionated was dissolved in a suitable solvent, mixed with CC-grade silica gel and dried to get a free flowing powder. This was then applied to the top of the packed column. A cotton plug was applied over it to prevent any distortion while the solvent passes from the reservoir to the top of the column. Elution was carried out either isocratically or in a gradient manner. The collected fractions were checked by TLC and pooled according to similar chemical profile (Braithwaite and Smith, 1996; Ravindranath, 1989).

2.6.3 *Flash chromatography*

Flash chromatography is a simple and rapid chromatographic technique that can be used as a substitute for open column chromatography. In flash chromatography, the stationary phase consists of a smaller and narrower range of particle size to get a more efficient separation. Pressure is applied to overcome the reduced flow rate caused by the use of smaller particle size of the stationary phase. Thus, with a flow rate of 2 ml/min, 0.1 to 10 g of sample mixtures differing in their R_f value by 0.15 can be separated in 15 min, provided that TLC-mesh silica gel is used as the stationary phase (Braithwaite and Smith, 1996; Ravindranath, 1989). Normal phase silica gel or C-18 silica gel columns were obtained from Phenomenex to use on a Flashmaster Personal® (Biotage, UK).

2.6.4 *Size exclusion chromatography*

Gel filtration (GF) or molecular sieve chromatography is a form of liquid chromatography in which molecules can be separated according to their molecular size. In the present phytochemical work, a cross-linked dextran based resin (Sephadex® LH20100, Sigma-Aldrich, bead size 25-100 μm) was used to get an effective separation between molecules differing in their molecular size. When solvent passes through the crosslinked dextran molecules, smaller molecules have a greater tendency to diffuse into the porous gel particles and are thus eluted after the bigger molecules. Gel filtration is not only useful for removing chlorophyll or pigments; it also gives good separation between any molecules that differ in molecular size. For non-polar fractions, Sephadex® was slurry was prepared in 5% *n*-

hexane in chloroform and kept for overnight. A glass column of proper size with a sintered glass or cotton at the bottom was packed in such a way that slurry properly settles in the column but no solvent remains over the surface of the packed Sephadex®. The sample to be fractionated was dissolved in the same solvent system as the one used for in column and applied onto the top of the column. Once the sample diffused into the column, a cotton plug was inserted to prevent the escape of swollen gel in to the solvent above the column bed. The column was run isocratically first and then with 100% chloroform. Afterwards a small proportion of methanol was added in chloroform to elute more polar compounds. For mixtures of relatively polar compounds the Sephadex® was soaked in chloroform, and the column was run with chloroform first and then the polarity was increasing using methanol (Determann and Brewer, 1975; Kremmer and Boross, 1979).

2.7 Structure elucidation

Characterisation of the isolated compounds was carried out primarily using NMR spectroscopy and mass spectrometry. IR and UV spectroscopy, melting point determination, optical rotation or crystallographic analysis were also adopted depending on the need and opportunity.

2.7.1 UV-visible spectroscopy

UV spectra were recorded on a Unicam UV300 UV visible spectrophotometer. The spectrophotometer was switched on and allowed to stabilise for half an hour. A quartz cuvette was filled with the solvent to run a background and to determine the

solvent cut-off point. A 0.1 mg/mL sample solution was used to record the spectrum. Vision 1.05 software was used to record and process UV spectra.

2.7.2 IR spectroscopy

IR spectra were recorded on an ATI Mattson Genesis or Mattson Galaxy 5000 spectrometer. Approximately 230 mg of anhydrous potassium bromide and 0.9 to 1.1 mg of dry sample were taken in an agate mortar and mixed thoroughly with a pestle. The mixture was pressed (approx. 10 tonne) with a hydraulic pressure machine in to a disc. After running a background (500-4000 cm^{-1}), IR spectrum of the disc was recorded and processed using WinFirst software. Liquid samples were sandwiched between two sodium chloride discs to record the spectrum.

2.7.3 Mass spectrometry

EI, CI and FAB mass spectra were obtained on a JEOL JMS-700 high resolution mass spectrometer. EI experiment was carried out using a direct probe insert at 70 eV. For CI, samples were dissolved in isobutane and direct sample injection carried out at 180°C. Nitrobenzyl alcohol was used as matrix for positive ion FAB analysis (Temp 30°C). All the experiments were done by Mr. James Tweedie at Dept of Chemistry, University of Glasgow.

Positive ion and negative ion mode ESI experiment were carried out on a ThermoFinnigan LCQ-Decaiontrap or Orbitrap HRESI mass spectrometer (mass analyser set up at 100,000 ppm, externally calibrated at 3 ppm). Based on the polarity, samples were dissolved in acetonitrile, methanol or water (HPLC grade) or

in a binary mixture of these solvents to get a concentration of 100 μg/mL. Sample solution (10-20 μL) was injected along with a direct infusion of 0.1% formic acid in acetonitrile: water (90:10) at a flow rate of 200 μL/min. Key parameters for mass spectral analysis is presented in **Table 2.3** which may vary slightly for different experiments. All experiments were carried out by Dr. RuAngelie Edrada-Ebel and Dr. Tong Zhang.

Table 2.3: Key parameters for the ESI mass spectral analysis*

Attribute	Positive mode	Negative mode
Capillary Temp (°C)	220.00	
Sheath Gas flow (bar)	30.00	
Auxiliary Gas flow (bar)	10.00	
Source voltage (kV)	4.00	3.10
Source current (μA)	100.00	
Capillary voltage (V)	35.50	-48.00
Tube Lens (V)	90.00	-145.00

* May vary slightly from experiment to experiment

2.7.4 Polarimetry and MP determination

Samples were dissolved in a solvent as stated in the literature. For novel compounds a suitable solvent was used in which the sample is soluble. It was then transferred to a 10 mm cell and the Optical rotation was determined in Autopol V automatic polarimeter (Rudolph Research Analytical) or ADP 220 Polarimeter (Bellingham Stanley Ltd.). Specific rotation was calculated from the observed rotation using the following formula:

$$[\alpha] = \frac{rv}{nl}$$

where $[\alpha]$= specific rotation, r = observed rotation in degrees, v= volume in mL, n= weight of substance in g, l= length of the cell in dm.

Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected.

2.7.5 NMR spectroscopy

All NMR experiments were carried out on a JEOL (JNM LA400) 400 MHz or Bruker (Avance) 600 MHz machine. NMR tubes (5 mm) obtained from Wilmad-Labglass were used for routine NMR experiments. 535-PP-7 NMR tubes were used to record on a 600 MHz machine while 527-PP-7 NMR tubes were used to record on a 400 MHz machine. Samples were dissolved in 500-600 μL of suitable deuterated solvent and taken in the NMR tube. Too little or too much volumes were avoided to prevent shimming problem ((lineshape problem giving rise to sidebands) and long experimental hrs, respectively. NMR experiments of samples with low yield were carried out using Shigemi NMR tubes obtained from Sigma-Aldrich UK Ltd. Samples were dissolved in 200 μL of $CDCl_3$ or DMSO-d_6 and taken in a NMR tube that matched with the respective solvent.

The identification of pure compounds was carried out using one dimensional ^1H and ^{13}C NMR spectroscopy. Spectral data of known compounds were compared with published spectral data and thereby identified. Further 2D experiments were carried

of more complex molecules for accurate assignments of proton and carbon chemical shifts.

2.5.1.1 ^{1}H NMR

^{1}H NMR experiment was carried out for all compounds isolated and was used as the primary means of structural identification. In the ^{1}H NMR, the chemical shifts and integration indicate the number of each type of protons present in the molecule, the multiplicity and extent of coupling constant gives an idea of the adjacent protons and their spatial proximity. The purity of the compounds was also determined where possible (Breitmaier, 1993; Williams and Fleming, 2008). The ^{1}H NMR was also used successfully in determining the molar ratio of the components present in a mixture of two compounds.

2.5.1.2 ^{13}C NMR and DEPT

Broad band decoupled ^{13}C NMR was used to determine the number of carbons, their type and where necessary DEPT experiments were obtained to distinguish the carbons according to the extent of their proton attachments. DEPT spectrum is a pulse sequenced experiment that transforms the information of the CH signal multiplicity and spin-spin coupling into phase relationship. In the DEPT 135 spectrum, CH_3 and CH are directed towards the positive phase of the spectrum while CH_2 is facing the negative phase. The advantage of the DEPT spectrum over carbon spectrum is that it is 4 times more sensitive as it uses ^{1}H-^{13}C polarisation transfer (Breitmaier, 1993).

2.5.1.3 1D NOE Difference

The Nuclear Overhauser effect is based on the basis that a magnetic nucleus can interact with another magnetic nucleus through space. Thus, if two protons are apart from each other by no more than 2-4 Å, the irradiation of one of the protons at its resonance frequency leads to the increase in the intensity of the other proton signal(s). Selective 1D NOE experiment is an important tool for determining the relative stereochemistry of a selected proton (Breitmaier, 1993; Williams and Fleming, 2008).

2.5.1.4 COrrelation SpectroscopY (COSY)

This 2D experiment shows ^{1}H-^{1}H connectivities. The proton shifts are plotted on both axes with the contour plot along the diagonal of the square. Results called as cross peaks are arranged in the square symmetrically about the diagonal. Thus the cross peaks refer to the spin-spin coupled protons. The correlations observed are due to geminal (^{2}J) and vicinal (^{3}J) couplings. But ^{4}J and ^{5}J couplings e.g., 'W', zigzag or allylic couplings can also be observed in a COSY spectrum (Breitmaier, 1993; Williams and Fleming, 2008).

2.5.1.5 Heteronuclear Multiple Quantum Coherence (HMQC)

This 2D ^{1}H-^{13}C experiment identifies one-bond (^{1}J) connections. The pulse sequence used in this experiment uses a time delay set to $1/2J$ where J is the value similar to that of one-bond ^{1}H-^{13}C coupling. In a HMQC spectrum, the ^{1}H and ^{13}C (or DEPT) spectrum is plotted along the abscissa and ordinate, respectively (or vice versa).

Cross-peaks show protons and carbons that are directly connected to each other (Williams and Fleming, 2008).

2.5.1.6 Heteronuclear Multiple Bond Coherence (HMBC)

HMBC is another 2D ^{1}H-^{13}C experiment. The time delay (1/2*J*) used in the pulse sequence is kept such that the *J* value is in the range of $^{3}J_{CH}$ and $^{2}J_{CH}$. Unless otherwise stated, the HMBC experiments carried out for different samples in the present study used a time delay of 0.0625 s (*i.e.*, J_{CH} = 8 Hz). For highly-substituted compounds (e.g., ellagic acid derivatives) which lack sufficient protons to 'track' the carbons, a time delay of 0.25 s was used which allowed to find $^{4}J_{CH}$ correlations (*i.e.*, J_{CH} = 2 Hz). Since this pulse programme uses ^{1}H-^{13}C polarisation transfer, the detection is four times more sensitive than a ^{13}C NMR experiment. For this reason, all samples obtained in low yield were submitted for HMBC to determine carbon shifts (Breitmaier, 1993; Williams and Fleming, 2008).

2.5.1.7 Nuclear Overhauser Enhancement SpectroscopY (NOESY)

This is a 2D experiment which records all the ^{1}H-^{1}H NOE correlations occurring in a molecule. The spectrum is similar to a COSY spectrum, but in a correctly phased NOESY spectrum cross peaks represent NOE correlations between the respective protons. Since the mutual dipolar relaxation of protons falls off by a factor of $1/r^6$ (r= distance between protons), the intensity of the cross-peaks also falls off rapidly with the increase in the inter-proton distance. NOESY experiment is of importance to

establish the relative stereochemistry of a molecule (Williams and Fleming, 2008). A mixing time of 500 ms was set for all the NOESY experiments.

2.5.1.8 Rotating frame Overhauser Enhancement SpectroscopY (ROESY)

The ROESY experiment uses slightly different pulse sequence than that of the NOESY experiment. It is used to determine the NOE correlations in large molecules (≥500 Dalton). Although the shapes of the diagonal and cross peaks appearing in the ROESY spectrum are different than that of a NOESY spectrum, the interpretation is similar (Williams and Fleming, 2008).

2.8 Antimicrobial screening

2.8.1 Microorganisms used

Bacterial strains were purchased from Fisher Scientific (UK). The strains include four Gram-positive bacteria, namely, *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212) and *Streptococcus pyogenes* (ATCC 19615) and two gram-negative bacteria, namely, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). *Mycobacterium aurum* A+ (CIP 10482) was obtained from Pasteur Institute, Paris, France.

2.8.2 Evaluation of antibacterial activity

2.8.2.1 Sample and inoculum preparation

All the samples were dissolved in DMSO and diluted with broth (Cation-adjusted Mueller-Hinton broth with TES buffer, Trek Diagnostics) to use in the assay.

Bacterial cultures were transferred on to nutrient agar (Sigma-Aldrich, UK) slopes and incubated for 16-18 h at 37°C. Bacteria from the freshly grown culture were transferred in sterile Tryptic Soya broth (Sigma-Aldrich, UK) and incubated for 2-3 h. Bacterial suspensions were diluted to 0.9% NaCl solution by comparison with a 0.5 McFarland standard using the Biowave CO8000 cell density meter (Biochrom WPA®). Aliquots (110 μL) were added to 11 mL of broth.

2.8.2.2 Determination of minimum inhibitory concentrations (MICs)

The wells of a microtitre well plate were filled with 100 μL of broth. Sample suspensions (100 μL each) were added to the first well in a row. Serial dilution was carried out in the consecutive wells. Bacterial suspension (100 μL) was added in each well to get a bacterial concentration of 5×10^5 CFU/mL. The plate was incubated for 12-14 h at 37°C. Controls wells were also maintained and ciprofloxacin (Sigma-Aldrich, UK) was used as the standard antibiotic. After the end of the incubation period, 20 μL of MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, UK) in MeOH (5 mg/mL) was added to each well and placed back in the incubator for 20 min. A change in colour from yellow to blue/black indicated the presence of viable bacteria. The well with lowest concentration of the sample with no change in dye colour was considered as the minimum inhibitory concentration (MIC) for that sample. All the samples were screened in duplicate on two different days (Seidel *et al.*, 2008).

2.8.3 Evaluation of antimycobacterial activity

The activity against *Mycobacterium aurum* was evaluated using a modification of a procedure previously reported (Seidel *et al.*, 2004). To obtain the desired inoculum, bacterial suspensions were mixed vigorously in normal saline to disrupt visible clumps, and left to settle for 5 min. Supernatants were diluted in normal saline to match the turbidity of a McFarland 0.5 standard and an aliquot (55 μL) was transferred to cation-adjusted Mueller Hinton broth (11 mL). Isoniazid, ethambutol and rifampicin (Sigma-Aldrich, UK) were used as positive controls. After the end of

the incubation period of 5 days, inhibition of growth was detected by addition (20 µL) of a methanolic solution (5 mg/mL) of MTT followed by incubation at 37 °C for 1 h.

2.8.4 Potentiation studies

Compounds active against *M. aurum* were selected for potentiation studies to investigate their effect on the MICs of antitubercular agents. Rifampicin and isoniazid were selected for these studies. The method adopts a varied concentration of the anti TB agents while active compounds were added at their subinhibitory concentrations (Mossa *et al.*, 2004).

Standard antibiotics were serially diluted in the 96-well microtitre plate. Sample suspensions (100 µL) were dispensed in each well. Following a proper mixing, 100 µL was discarded from each well. Finally bacterial inoculum (100 µL) was added to each well except the sterile control. After the incubation period MIC of the standard antibiotics alone and in the presence of test samples were recorded. The results were expressed as fraction inhibitory concentration index (FIC index) and were calculated as follows:

$$FIC\ index = \frac{MIC\ [A]\ combination}{MIC\ [A]\ alone} + \frac{MIC\ [N] combination}{MIC\ [N] alone}$$

where A and N are the anti-TB drug and natural product tested, respectively. The FIC index was interpreted as synergy if FIC ≤ 0.5, partial synergy if FIC ≥ 0.5 but <1.0, additive effect when FIC ≥ 1.0 and antagonism if FIC ≥ 2.0 (FIC) (Sato *et al.*, 2004).

Chapter 3

Results and discussion

Part I: Phytochemical studies

3.1.1 Cardiac glycosides

3.1.1.1 Characterisation of JS-1 as alliotoxin

A compound (**JS-1**) was isolated from the ethyl acetate extract of *Trewia nudiflora*. After treatment with *p*-anisaldehyde-sulphuric acid reagent and heating at 105°C, a greenish-blue spot appeared on the TLC.

The IR spectrum indicated the presence of an α,β-unsaturated five membered lactone ring (1754, 1735 cm^{-1}) and hydroxyl group(s) (3400 cm^{-1}) (Williams and Fleming, 2008). The positive mode HRESI-MS data showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 537.3066, suggesting a molecular ion of 536.2988 and thus a molecular formula of $C_{29}H_{44}O_9$ (DBE=8).

The ^1H NMR spectrum (**Spectrum 3.1, Table 3.1**) showed the presence of two methyl singlets at δ_H 0.77 and 0.82, a methine at δ_H 2.78, some oxymethines and oxygenated protons in the region of δ_H 3.14 to 4.91, and an olefinic proton at δ_H 5.92 (1H, *brs*). The proton spectrum also suggested the presence of a 6-deoxy sugar moiety with an anomeric proton at δ_H 4.67 (1H, *d* 1.5 Hz) (H-1') and a methyl doublet at δ_H 1.10 (J=6.2 Hz) (H-6'). With the aid of a COSY experiment, the proton signals for a rhamnosyl unit were identified. H-1' and H-2' were assigned as equatorial due to the small coupling constant observed for the anomeric proton (J=1.5 Hz). The H-4' proton of the rhamnose unit (δ_H 3.15, 1H, *dt* 5.6, 9.5 Hz) coupled with the 4'-OH proton. The other coupling constant (J= 9.5 Hz) indicated the *trans*-diaxial orientation of H-3'/H-4'/H-5' protons. The upfield shift of C-5' (δ_C 68.3)

further allowed the establishment of the relative configuration of the sugar unit as α-L-rhamnopyranoside (Kamel *et al.*, 2001).

The ^{13}C NMR (**Table 3.1**) and the DEPT 135 spectrum showed 29 carbons including three methyls, nine methylenes, twelve methines and five quaternary carbons.

In the HMBC experiment (**Spectrum 3.2**), the olefinic methine at δ_H 5.92 (H-22) showed ^2J correlations to a carbonyl at δ_C 173.8 (C-23) and to a highly deshielded olefinic quaternary carbon at δ_C 175.8 (C-20). It also showed ^3J correlation to a methylene at δ_C 73.1 (C-21). This established the presence of a butenolactone ring. The methylene protons of this lactone ring at δ_H 4.87 (1H, *dd* 1.5, 18.2 Hz) and 4.93 (1H, *dd* 1.5, 18.2 Hz) showed ^3J correlation to a methine carbon at δ_C 49.7 (C-17). The rest of the NMR data indicated the presence of a steroidal aglycone with two angular methyl groups. All these features suggested the structure typical of a cardenolide. The methyl group at δ_H 0.77 showed ^3J correlation to the methine at δ_C 49.7 (C-17) and was thus assigned as Me-18. Two additional hydroxy-protons at δ_H 4.13 (*d* 6.6 Hz) and 4.10 (*s*) were established to be at C-11 (δ_C 66.5) and C-14 (δ_C 83.7), respectively. The methine at δ_H 2.78 (H-17) and Me-18 showed ^3J correlation to C-14. The proton at δ_H 0.93 (1H. *t* 10.6 Hz) showed ^2J correlation to C-11 (δ_C 66.5). The large coupling constant of this proton (J =10.6 Hz) indicated that it was in an axial orientation (*i.e.*, in this case, on the α side of the ring plane).

The multiplicity and J values for H-3 were ambiguous due to signal overlapping in the ^1H NMR spectrum. However in the HMQC spectrum the cross-correlation peak for H-3/C-3 was relatively stretched (3.35-3.46 ppm or 44 Hz) to indicate that H-3

was axial (*i.e.*, on the α side of the ring plane). The *trans* junction between the A/B ring system was established following the presence of an upfield shift (δ_C 11.9) for Me-19 and a downfield shift for C-5 (δ_C 44.3). The chemical shifts observed from **JS-1** were in agreement with the ^{1}H and ^{13}C NMR assignments of 5α-rather than 5β-cardenolides (Hanada *et al.*, 1992a; Hanada *et al.*, 1992b; Ankli *et al.*, 2000). It was also supported by the NOESY spectrum where H-3 (δ_H 3.40) showed correlation to the H-5 proton (δ_H 1.03).

In the NOESY spectrum (**Spectrum 3.3**), H-11 (δ_H 3.56 1H, *m*) showed correlation to Me-18 and Me-19 and thus the 11-OH was placed on the α side. The NOESY spectrum also showed correlation between H-17 and H-12*ax* (δ_H 1.38) confirming that the C/D ring junction was *cis*. The above spectral analysis identified the aglycone as 5α-sarmentogenin. The mass spectrum also supported the finding with the presence of a fragment ion peak for [sarmentogenin+H]$^+$ at *m/z* 391.

Finally, the attachment of the rhamnose unit at C-3 of the agycone was established following a ^3J correlation between the anomeric proton (δ_H 4.67) of the sugar unit and C-3 (δ_C 74.4). Thus, **JS-1** was identified as 5α-sarmentogenin 3-*O*-α-L-rhamnopyranoside or alliotoxin. All spectral data were in agreement with previous reports (Kang *et al.*, 2005; Hanada *et al.*, 1992a). This compound was previously reported from the stem bark of *Trewia nudiflora* (Kang *et al.*, 2005).

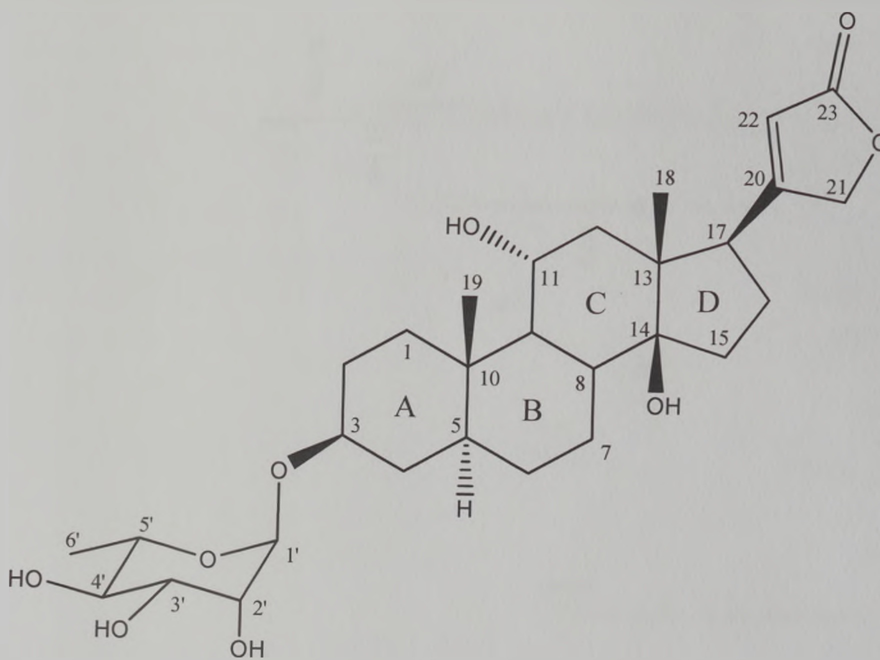


Fig. 3.1: Structure of JS-1

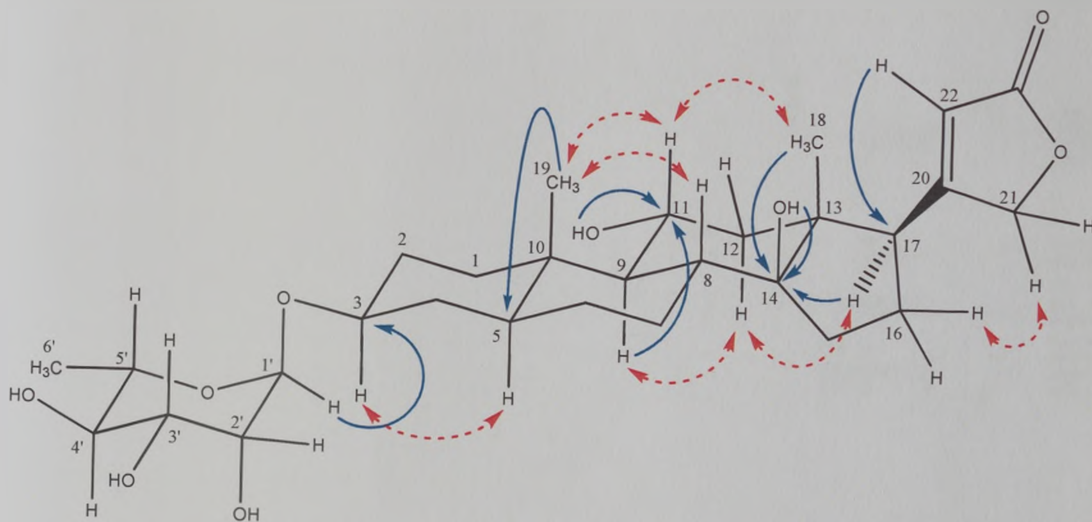
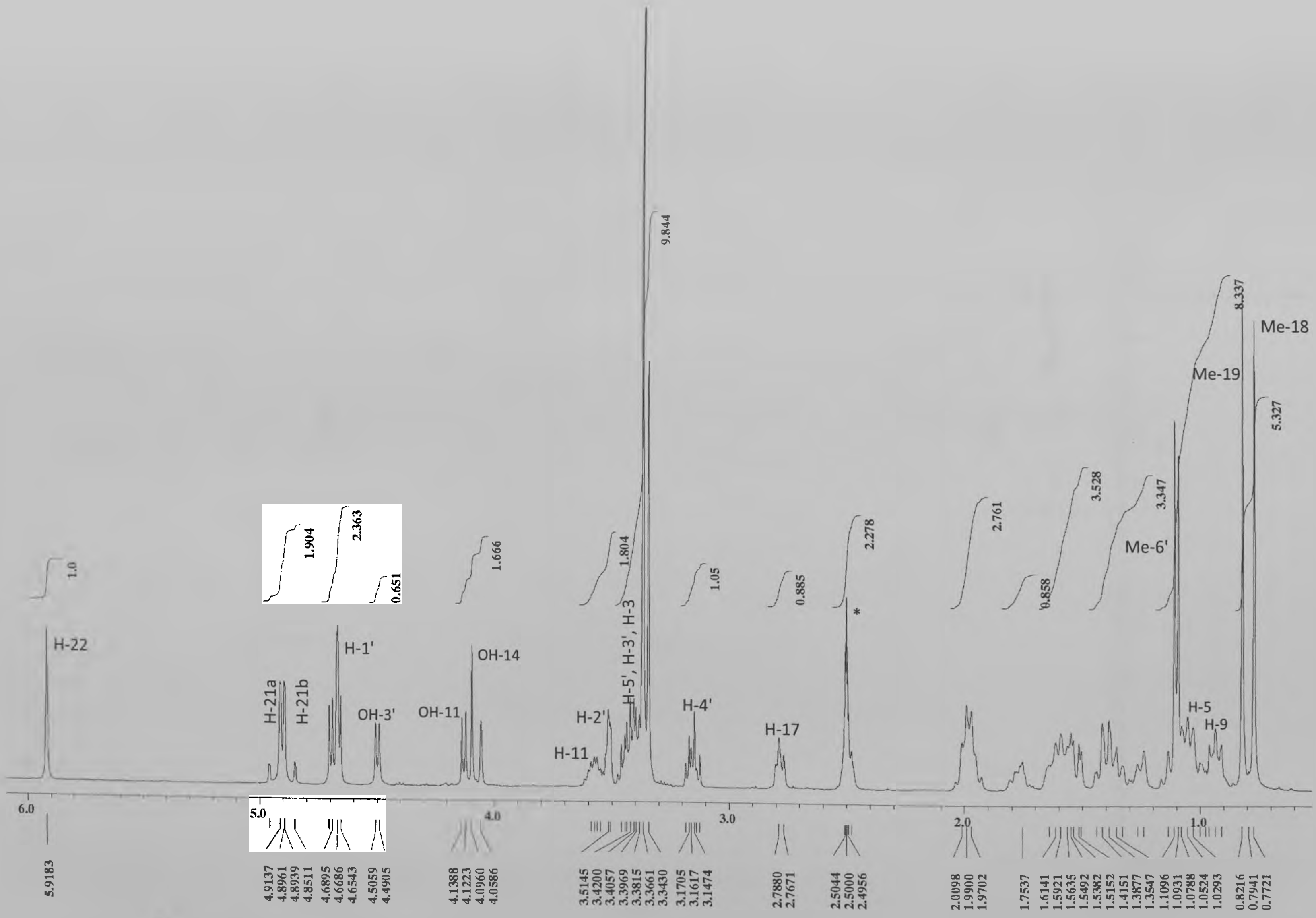
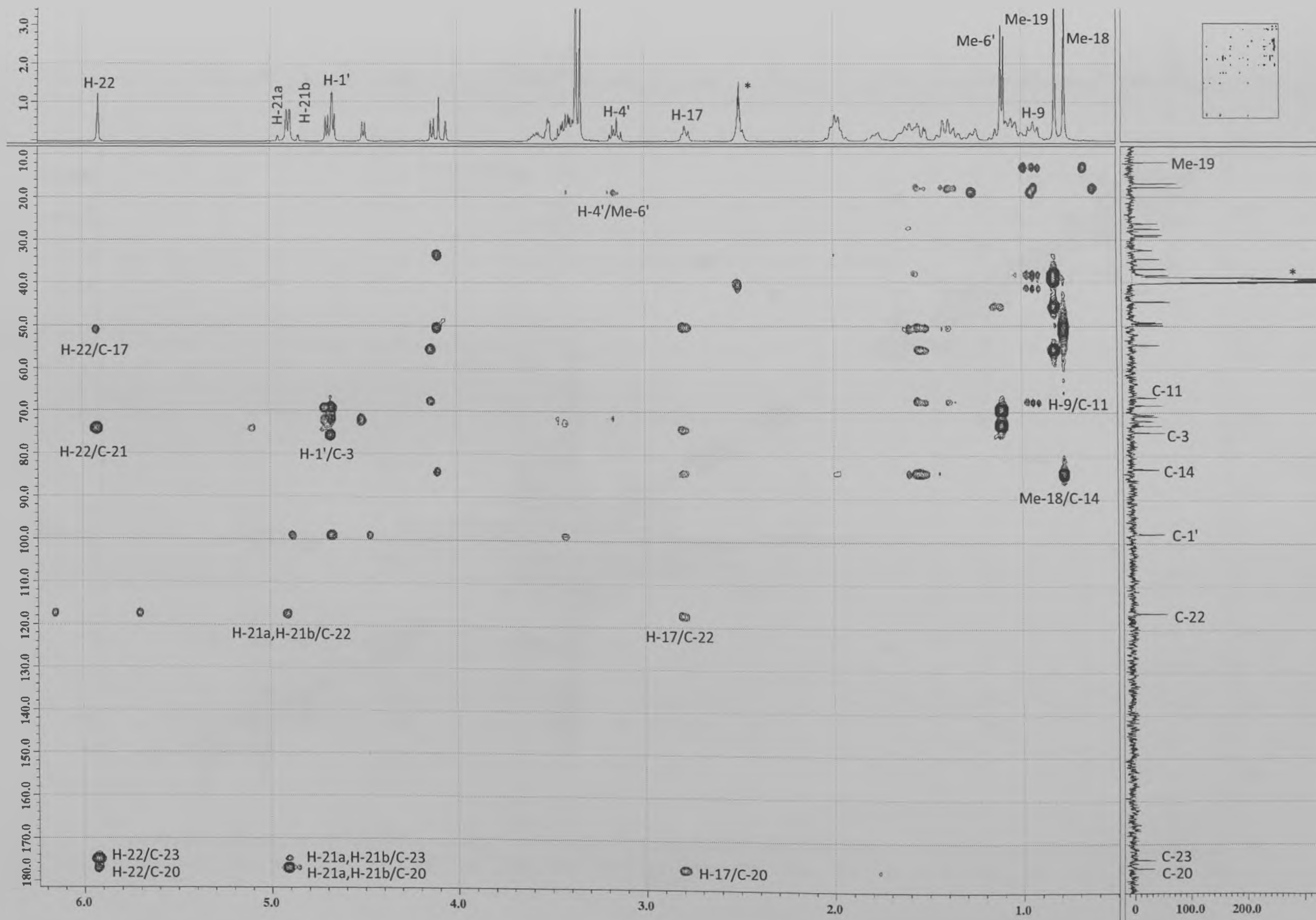


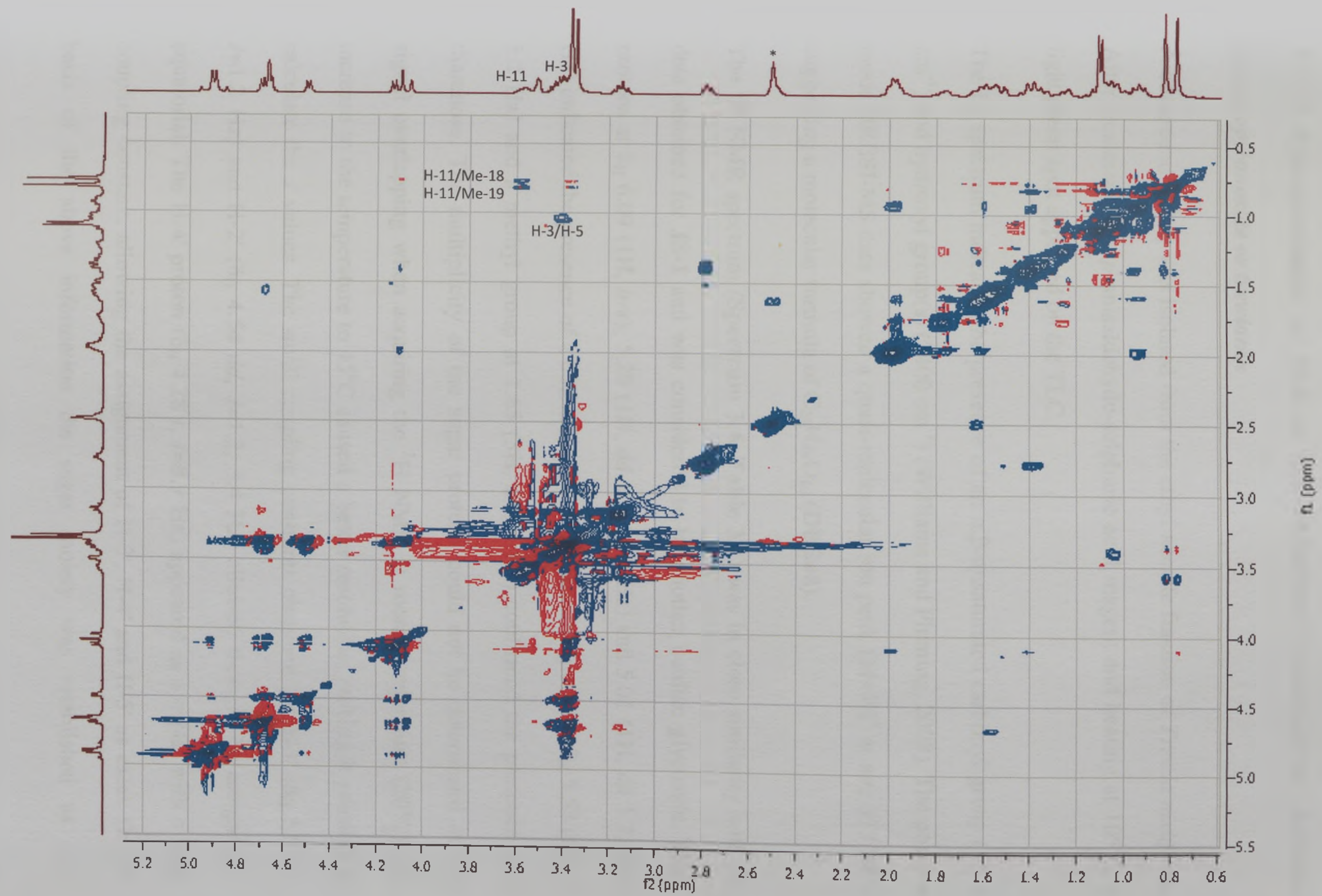
Fig. 3.2: Key HMBC (⟶) and NOESY (⟵--⟶) correlations observed in JS-1



Spectrum 3.1: ^{1}**H NMR (400 MHz, DMSO-**d_6***) spectrum of JS-1**



Spectrum 3.2: HMBC (400 MHz, DMSO-d_6*) spectrum of JS-1



Spectrum 3.3: NOESY (400 MHz, DMSO-d_6*) spectrum of JS-1

3.1.1.2 Characterisation of JS-2 as (5α)-19-hydroxy sarmentogenin 3-O-α-L-rhamnopyranoside or trewianin

A compound (**JS-2**) was isolated from the ethyl acetate fraction of *Trewia nudiflora*. After treatment with *p*-anisaldehyde-sulphuric acid reagent and heating at 105°C, a light green spot appeared on the TLC.

The IR spectrum indicated the presence of an α,β-unsaturated carbonyl group (1729 cm^{-1}) and hydroxyl group(s) (3400 cm^{-1}) (Williams and Fleming, 2008). The positive mode HRESI-MS data showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 553.2997 suggesting a molecular formula of $C_{29}H_{44}O_{10}$ (DBE=8).

The ^1H NMR spectrum (**Spectrum 3.4, Table 3.1**) was in close similarity with the data obtained for **JS-1** and was considered to be another cardiac glycoside. Thus, protons at δ_H 6.09 (1H, *brs*), 5.28 (1H, *dd* 1.7, 18.0 Hz) and 5.01 (1H, *dd* 1.7, 18.0 Hz) indicated the presence of a butenolactone ring while the proton at δ_H 5.53 (1H, *d* 1.2 Hz) and a methyl group at 1.65 (3H, *d* 5.7 Hz) confirmed the presence of a rhamnose. The multiplicity of the sugar protons could not be determined due to signal overlapping when acquiring the ^1H NMR at room temperature (20°C). An increase in the temperature to 45°C caused a better resolution, making it possible to calculate the *J* values. The small coupling constants observed for H-1' (δ_H 5.53 *d*, *J*=1.2 Hz) and H-2' (δ_H 4.54 *dd*, *J*=1.2, 3.4 Hz) allowed them to be assigned as equatorial. The H-4' proton (δ_H 4.28 *t*, *J*=8.9 Hz) appeared as a triplet with a large coupling constant, allowing the assignment of H-3', H-4' and H-5' as axial. On the basis of the above information the sugar moiety was established as α-L-

rhamnopyranose. The chemical shifts for the carbons resonances of the rhamnose unit were also in agreement with the literature data along with the upfield shift of C-5' (δ_C 69.7) which is true for the 6-deoxy sugars (Kamel *et al.*, 2001).

The ^{13}C NMR spectrum (**Spectrum 3.5, Table 3.1**) revealed the presence of 29 carbons which were identified with the aid of a DEPT 135 experiment as two methyls, ten methylenes, twelve methines and five quaternary carbons. In the HMBC spectrum, the methine proton at δ_H 2.89 (1H, *dd* 4.8, 9.2 Hz) showed ^3J correlation to the olefinic methine at δ_C 117.6 (C-22) of the lactone ring and was thus assigned as H-17. The methyl group at δ_H 1.12 (3H, *s*) showed ^3J correlation to C-17 and to an oxygenated quaternary carbon at δ_C 84.5. This methyl was identified as Me-18. The proton at δ_H 1.38 (H-9) appeared as a triplet and was assigned as axial due to its large coupling constant (J= 10.6 Hz). It showed ^2J correlation to the oxygenated carbon at δ_C 69.9 (C-11). The spectrum of **JS-2** lacked a methyl group compared to **JS-1**, but revealed an additional oxymethylene group at δ_H 4.16 (2H, *s*) which showed ^3J correlation to C-1 (δ_C 35.3), C-5 (δ_C 45.0), C-9 (δ_C 41.9) and ^2J correlation to C-10 (δ_C 41.4). This led to the conclusion of the presence of a hydroxymethyl at C-10 instead of an angular methyl group as observed in **JS-1**.

It has previously been reported that presence of a *trans* A/B junction in cardenolides can increase the C-5 shift of by 5-6 ppm (and up to 10-12 ppm) compared to A/B *cis* cardenolides. Thus, on the basis of the observed downfield shift of C-5 (δ_C 45.0) in **JS-2**, the A/B ring junction was assigned as *trans* with the orientation of H-5 (δ_H 1.18) as axial (*i.e.*, on the α face). The observed upfield shift of H-5 (δ_H 1.18) also

supported this *trans* orientation. All the above findings were consistent with the published literature on 5α-cardenolides, including structures with 19-hydroxymethyl group (Abe *et al.*, 1991; Ankli *et al.*, 2000; Pauli, *et al.*, 1999). The multiplet at δ_H 3.92 (1H, *dddd* 5.1, 5.5, 10.5, 11.0 Hz) was identified as the H-3 proton, which was and assigned as axial. In the ROESY spectrum (**Spectrum 3.7, Spectrum 3.8**), the H-3 proton showed correlation to H-4a at δ_H 1.79 while H-4b (δ_H 1.53) showed correlation to the 19-hydroxymethyl, allowing placement of H-3 on the α face. The H-3 proton also showed correlation to H-5 at δ_H 1.18, further proving that **JS-2** was a H-5α cardenolide. The multiplicity of H-11 (δ_H 4.54, 1H *td* 10.6, 3.5 Hz) was obtained from the ^{1}H NMR spectrum run at 45°C (**Spectrum 3.10**), assigning H-11 as axial due to its large coupling constant. In the ROESY spectrum, the H-11 proton showed correlation to Me-18 and the 19-hydroxymethyl and thus revealed that the 11-OH was on the α side. Furthermore the proton at δ_H 2.89 (H-17) showed correlation to the H-12a proton (δ_H 1.98) confirming the typical *cis* configuration of the C/D ring junction for cardenolides and placing the hydroxyl group at C-14 on the β side. On the basis of the above results, the aglycone was identified as (3β,5α,11α-form) 3,11,14,19-tetrahydroxycard-20(22)-enolide.

In the HMBC experiment the anomeric proton of the rhamnose unit showed ^3J correlation to the carbon of the aglycone at δ_C 75.6 establishing its attachement at C-3. Thus, **JS-2** was identified as (3β,5α,11α form) 3,11,14,19-tetrahydroxycard-20(22)-enolide 3-*O*-α-L-rhamnopyranoside. This cardenolide, for which we propose the trivial name trewianin, has never been reported before.

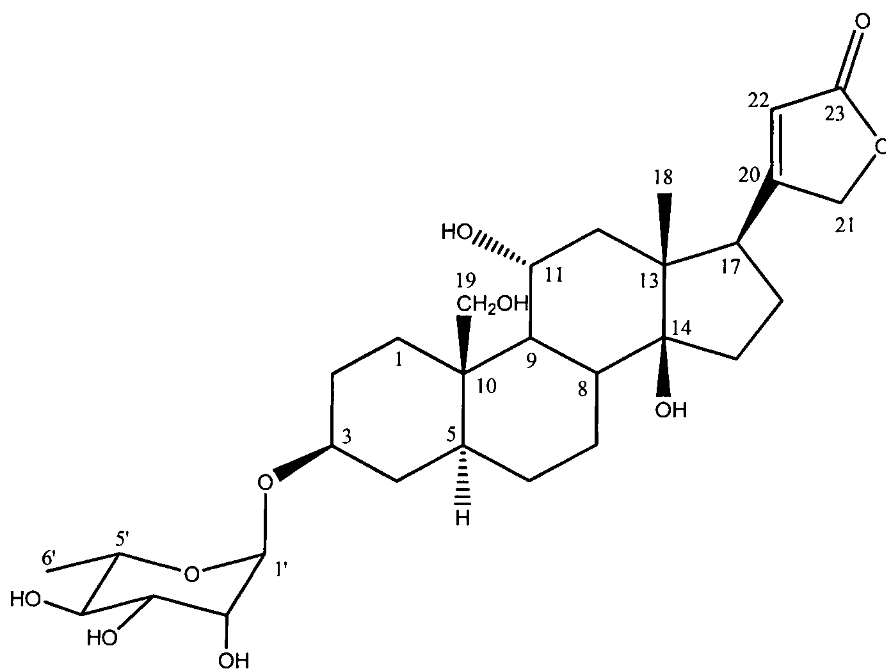


Fig. 3.3: Structure of JS-2

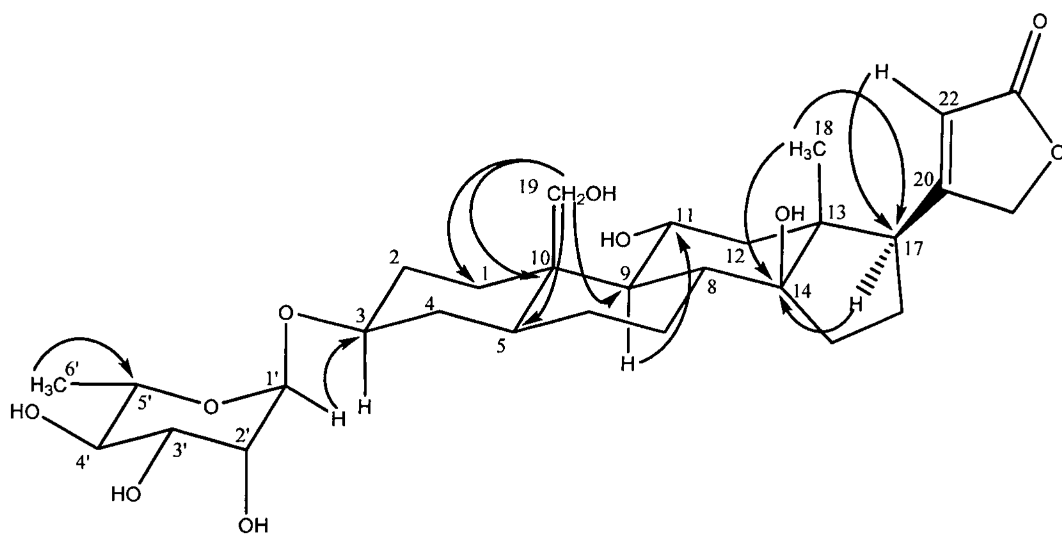


Fig. 3.4: Selected HMBC (⟶) correlations observed in JS-2

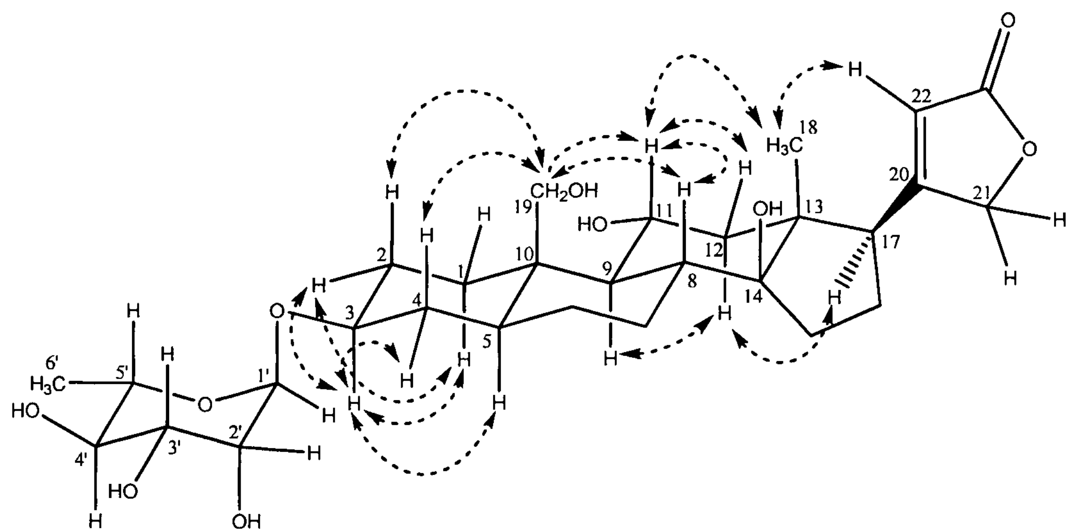
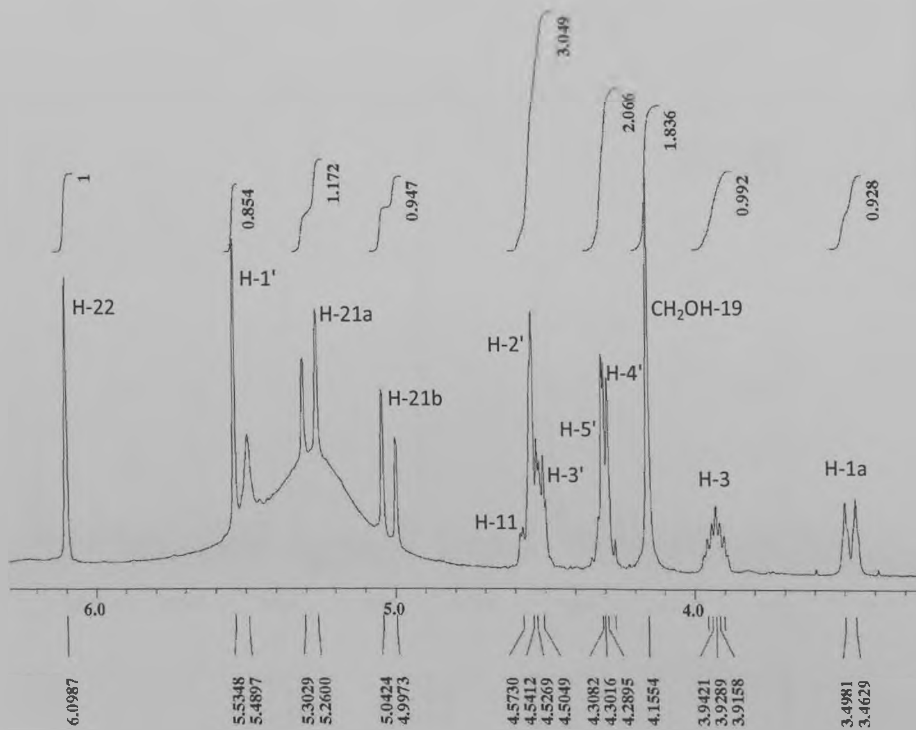
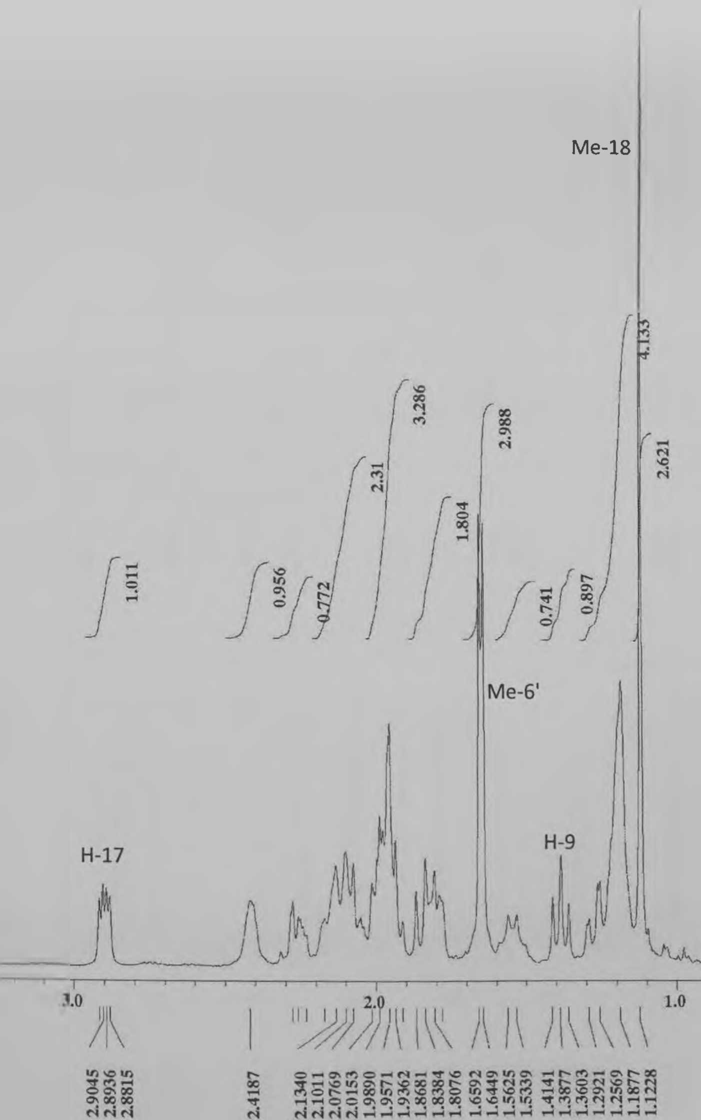
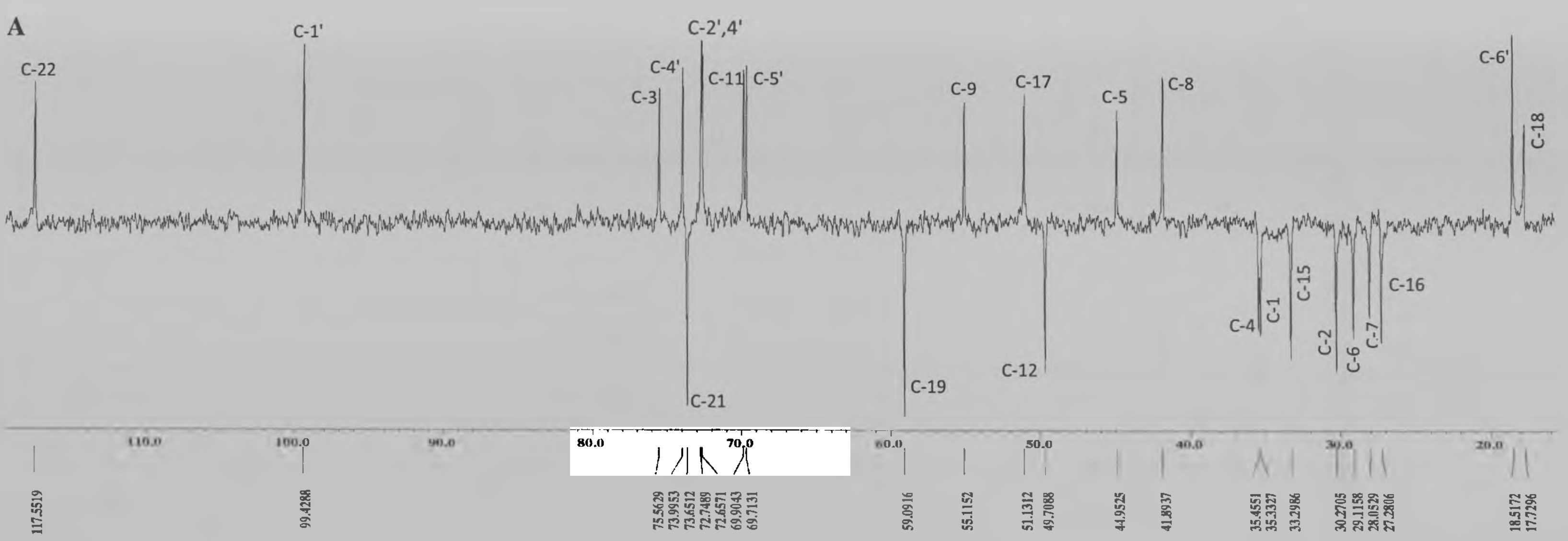


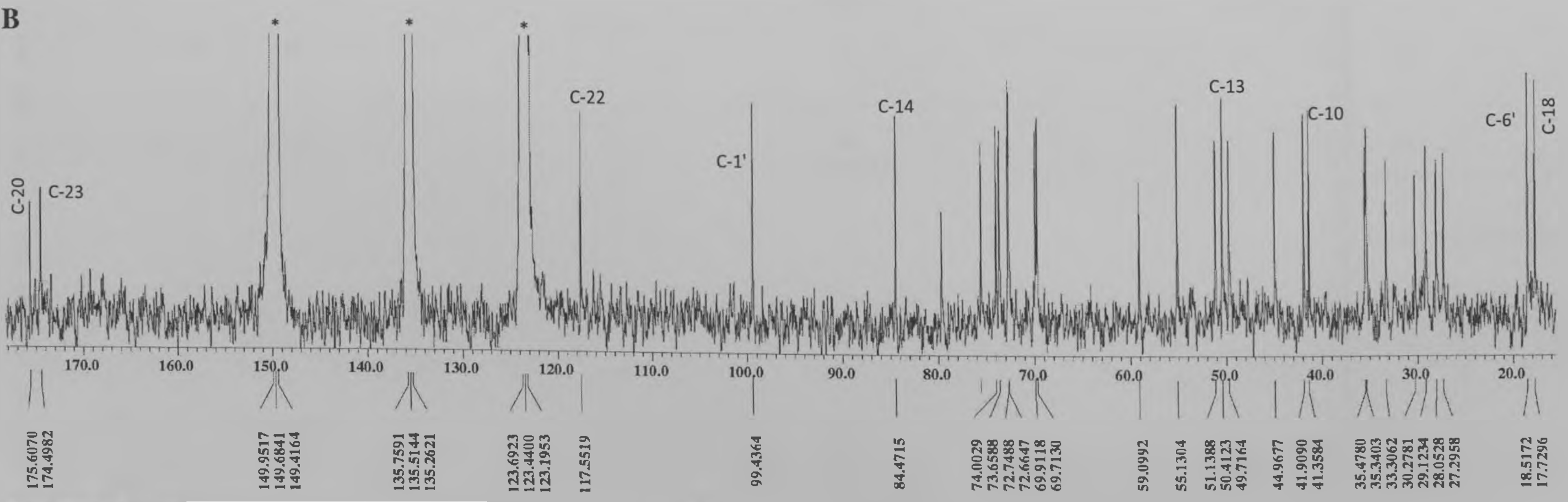
Fig. 3.5: Selected ROESY (◄- - ►) and 1D NOE (—►) correlations observed in JS-2



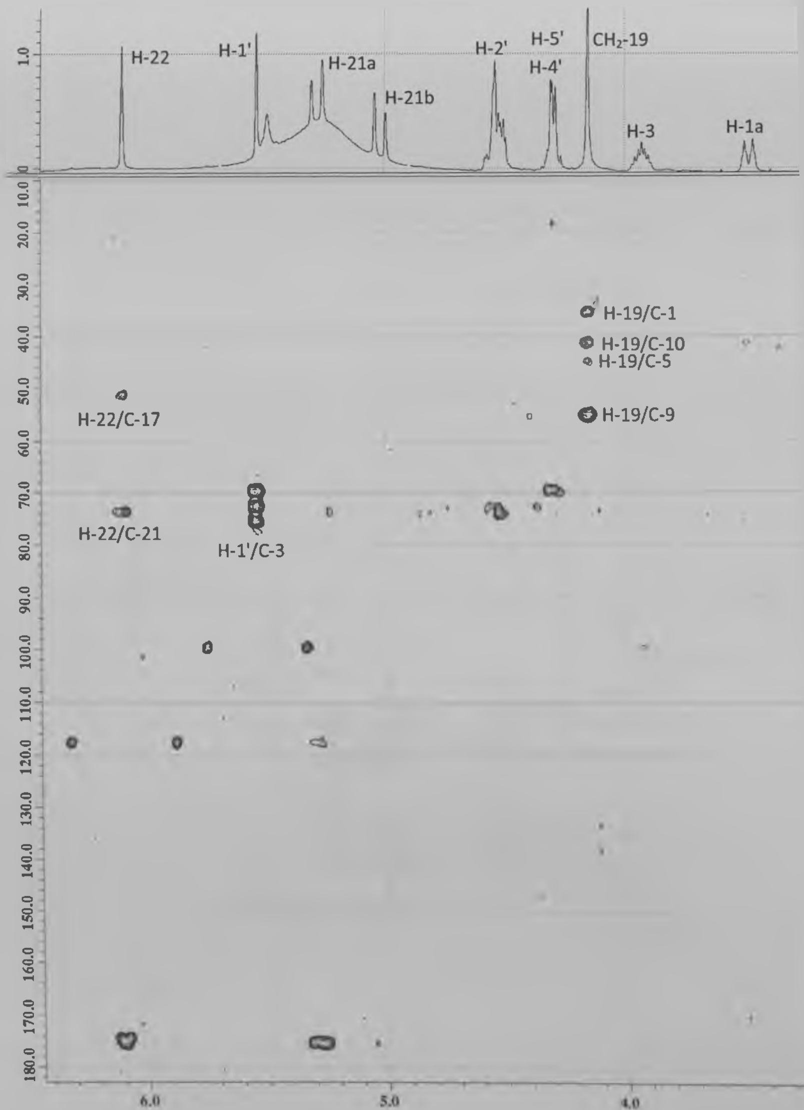
Spectrum 3.4: ^{1}**H (400 MHz,** C_5D_5N**) NMR spectrum of JS-2**


Me-18
4.133
3.286
2.988
2.31
2.621
1.804
1.011
0.956
0.772
0.741
0.897
Me-6'
H-17
H-9
3.0
2.0
1.0
2.9045
2.8936
2.8815
2.4187
2.1340
2.1011
2.0769
2.0153
1.9890
1.9571
1.9362
1.8681
1.8384
1.8076
1.6592
1.6449
1.5625
1.5339
1.4141
1.3877
1.3603
1.2921
1.2569
1.1877
1.1228

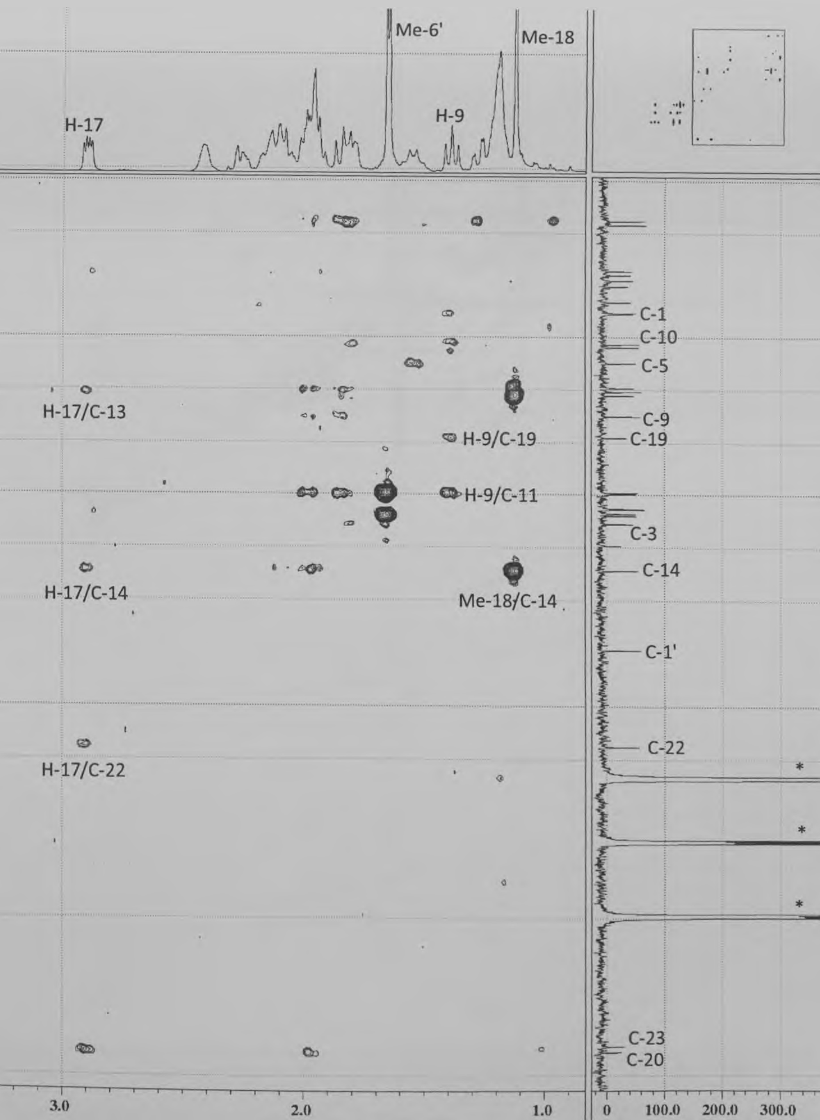


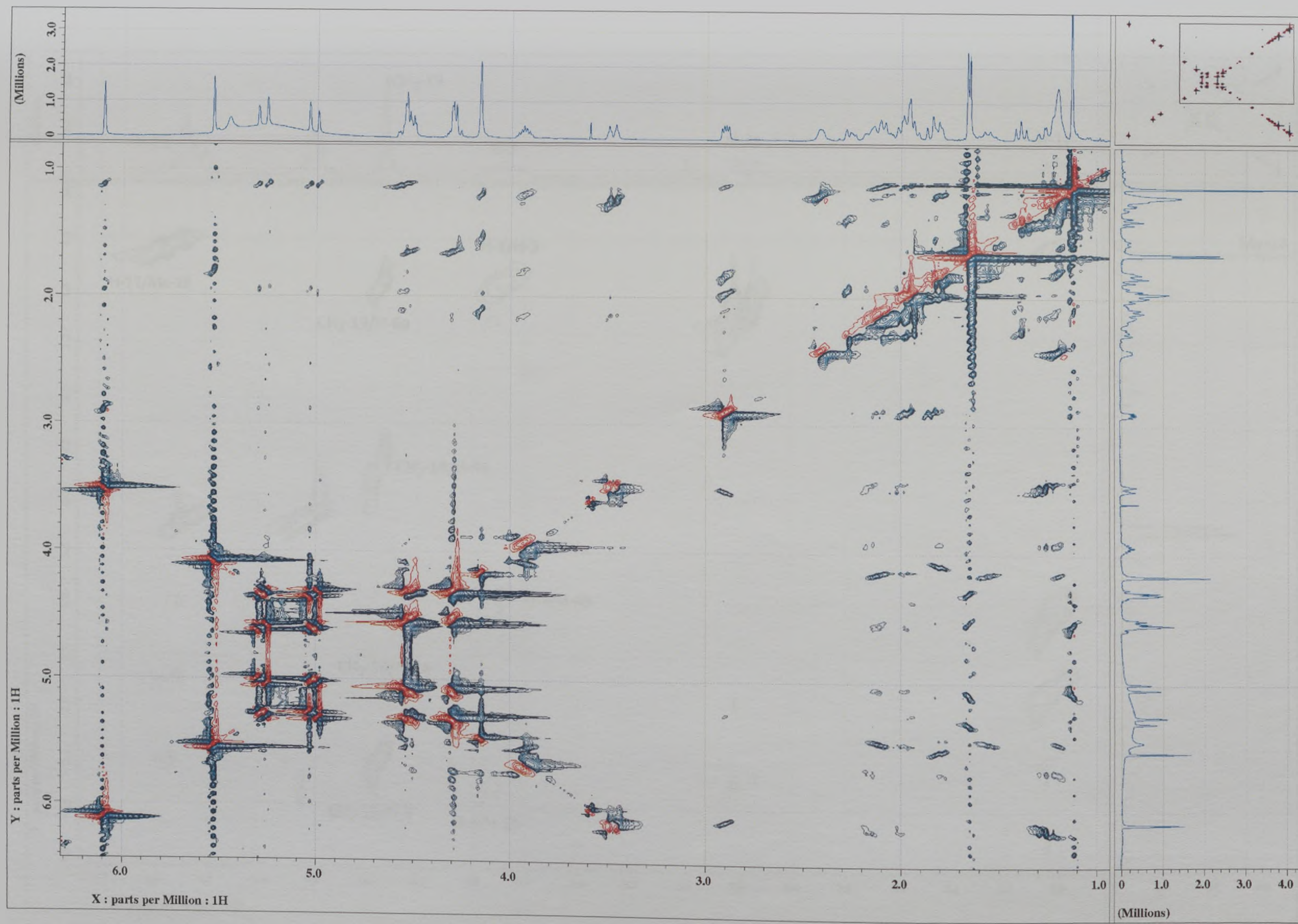


Spectrum 3.5: ^{13}C NMR (A) and DEPT 135 (B) (100 MHz, C_5D_5N*) spectra of JS-2

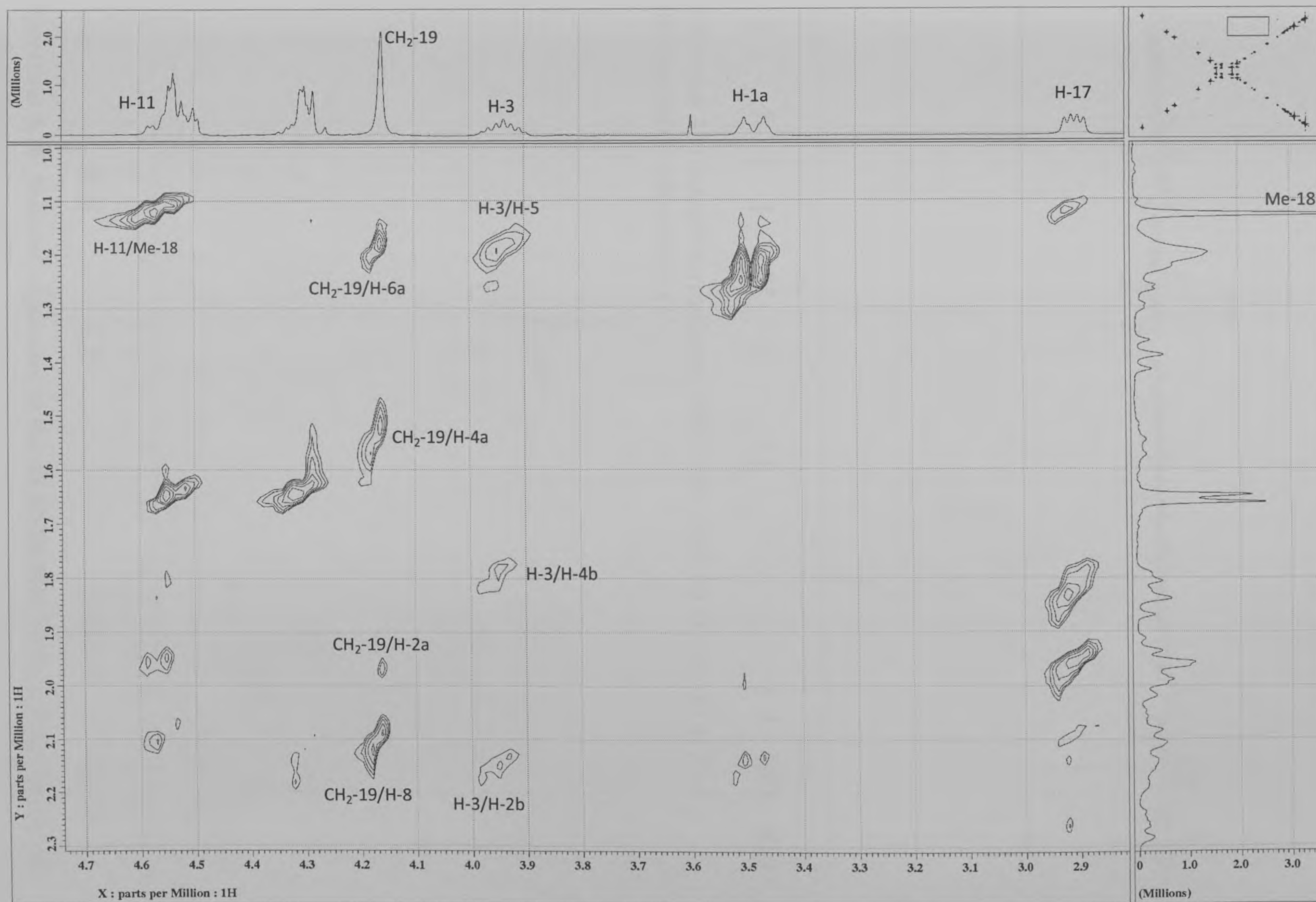


Spectrum 3.6: HMBC (400 MHz, C_5D_5N*) spectrum of JS-2

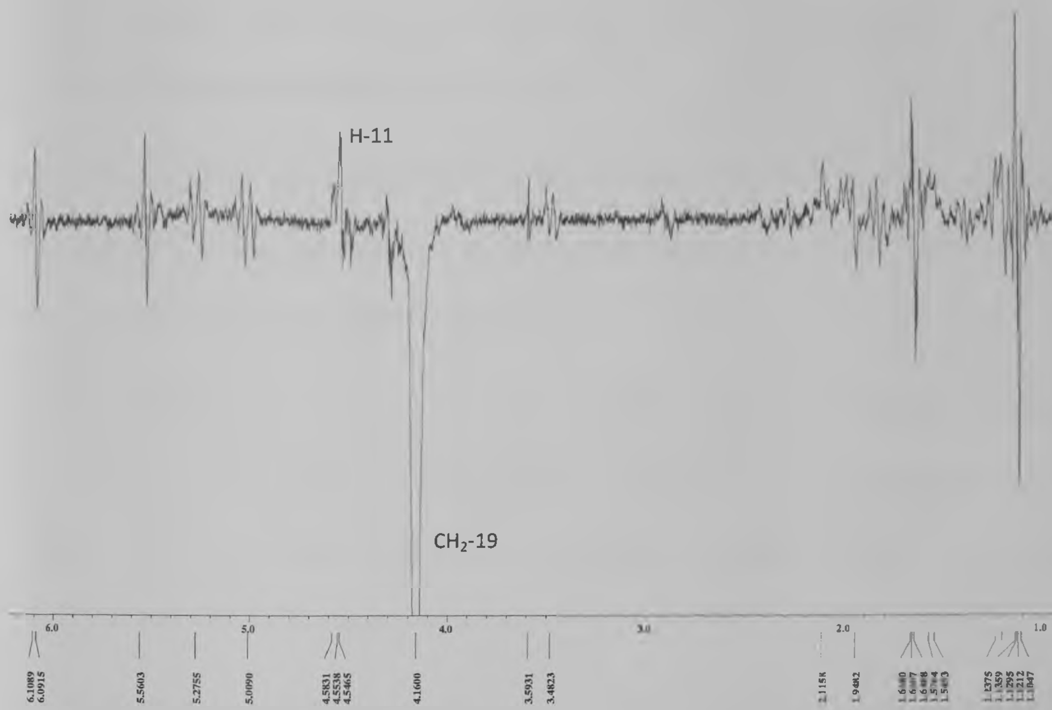

Me-6'
Me-18
H-17
H-9
H-17/C-13
H-9/C-19
H-9/C-11
H-17/C-14
Me-18/C-14
H-17/C-22
C-1
C-10
C-5
C-9
C-19
C-3
C-14
C-1'
C-22
C-23
C-20
*
*
*
3.0
2.0
1.0
0
100.0
200.0
300.0



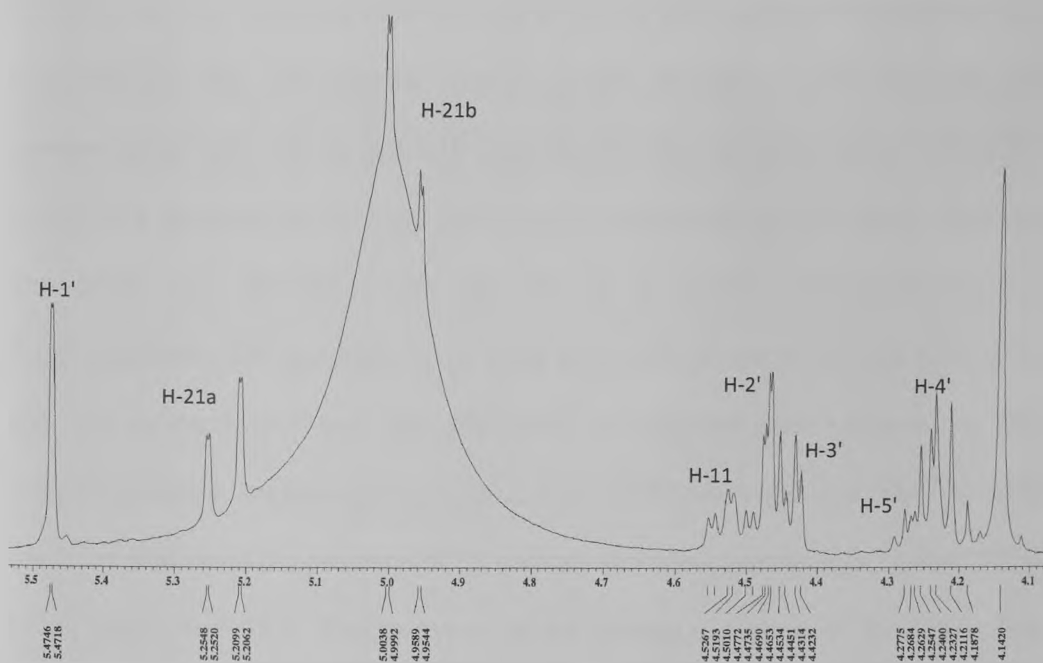
Spectrum 3.7: ROESY (400 MHz, C_5D_5N) spectrum of JS-2



Spectrum 3.8: Selected region of ROESY (400 MHz, C_5D_5N) spectrum of JS-2 with key correlations



Spectrum 3.9: Selective irradiation (400 MHz, C_5D_5N) of CH_2-19 of JS-2 showing NOE effect on H-11



Spectrum 3.10: Selected expansion of the ^1H NMR (400 MHz, C_5D_5N) spectrum of JS-2 obtained at 45°C

3.1.1.3 Characterisation of JS-3 as 5α-sarmentogenin 3-O-[β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside] or trewioside

A compound (**JS-3**) was isolated from the ethyl acetate extract of *Trewia nudiflora*. After spraying with *p*-anisaldehyde-sulphuric acid reagent and heating (105°C) for 5 min, a greenish-blue spot appeared on the TLC.

The IR spectrum indicated the presence of an α,β-unsaturated five membered lactone ring (1778, 1737 cm^{-1}) and hydroxyl group(s) (3430 cm^{-1}) (Williams and Fleming, 2008). The positive mode HRESI-MS data showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 699.3578, suggesting a molecular formula of $C_{35}H_{54}O_{14}$ (DBE=9).

The ^1H NMR spectrum (**Spectrum 3.11; Table 3.1**) was similar to the one obtained for **JS-1**. Thus, the olefinic methine at δ_H 6.10 (1H, *brs*) and methylene protons at δ_H 5.30 (1H, *dd* 1.7, 18.3 Hz), 5.03 (1H, *dd* 1.7, 18.3 Hz) indicated the presence of a butenolactone ring. The angular methyl groups belonging to the aglycone part appeared at δ_H 0.99 (3H, *s*) and 1.10 (3H, *s*) while the methyl group at 1.69 (3H, *d* 6.2 Hz) and anomeric proton at δ_H 5.47 (1H, *brs*) accounted for a 6-deoxy sugar unit. The latter was identified with the aid of a COSY experiment as α-L-rhamnopyranose. The presence of an extra anomeric proton at δ_H 5.26 (1H, d 8.4 Hz), one oxymethylene and four additional oxymethines were observed in **JS-3** compared to **JS-1**, suggesting the presence of an additional sugar unit. The ^{13}C NMR spectrum confirmed the presence of 35 carbons including signals at δ_C 174.4, 175.4, 117.6, 106.6 and 99.0. The presence of an anomeric carbon at δ_C 106.6, four oxymethines and an oxymethylene at δ_C 62.4 assigned the sugar unit as a hexose

moiety. The mass spectrum supported this finding since the molecular weight of **JS-3** was found to be 162 units higher than **JS-1**. This unit was identified as glucose with the help of COSY and HMBC data. As the anomeric proton (δ_H 5.26) appeared overlap with the signal of H-21a, its multiplicity could not be easily established. However, the observed multiplicity of H-2" (δ_H 4.14, *t* 8.4 Hz) assigned H-1", H-2" and H-3" in axial positions. The above information and especially with the carbon signals in close agreement with the literature, identified the extra unit as β-D-glucose (Kamel, *et al.*, 2001; Kang *et al.*, 2005). The downfield shift of the C-4' (δ_C 85.1) of the rhamnose unit and a ^3J correlation observed in the HMBC spectrum between the anomeric proton of the glucose unit (H-1") and C-4' established a (1→4) linkage between the two monosaccharides.

JS-3 was considered to be a 5α cardenolide on the basis of the downfield shift of C-5 (δ_C 44.8). It was not possible to establish the multiplicity of H-3 (δ_H 3.78) from the ^1H NMR spectrum due to its overlapping with H-5" (δ_H 3.81). A 2D *J*-resolved experiment (**Spectrum 3.14**), however, identified large coupling constants (*J*= 11.2, 11.5 Hz) establishing the axial orientation of H-3. In the ROESY spectrum, H-3 showed correlation to H-5 (δ_H 1.03) and therefore allowed assignment of both protons as axial (*i.e.*, on the α face). The proton at δ_H 2.91 (H-17) showed ROESY correlation to H-12a (δ_H 1.82) establishing the C/D-ring function as *cis*. The H-12ax proton also showed correlation to H-9 (δ_H 1.30).

On the basis of 2D NMR data obtained from COSY, HMBC, HMQC and ROESY experiments, the aglycone part of **JS-3** was identified as 5α-sarmentogenin. Major

HMBC and ROESY correlations are depicted in **Figure 3.7**. In the HMBC spectrum, the anomeric proton at δ_H 5.47 (H-1') of the rhamnose unit showed a ^3J correlation to δ_C 75.6 (C-3), establishing the attachment of the sugar moiety in position 3 of the aglycone. Thus, **JS-3** was identified as 5α-sarmentogenin 3-O-[β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside].

This compound, for which we propose the name trewioside, is reported for the first time.

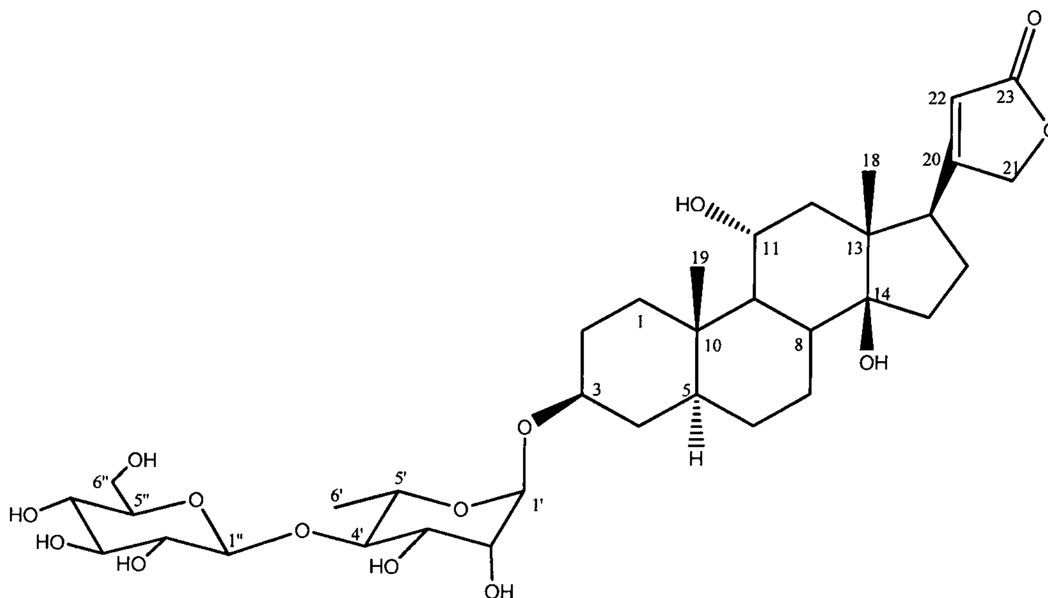


Fig. 3.6: Structure of JS-3

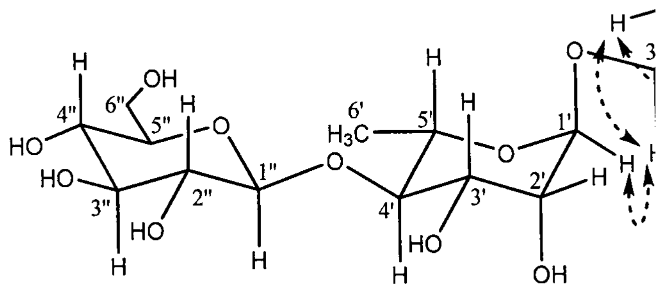
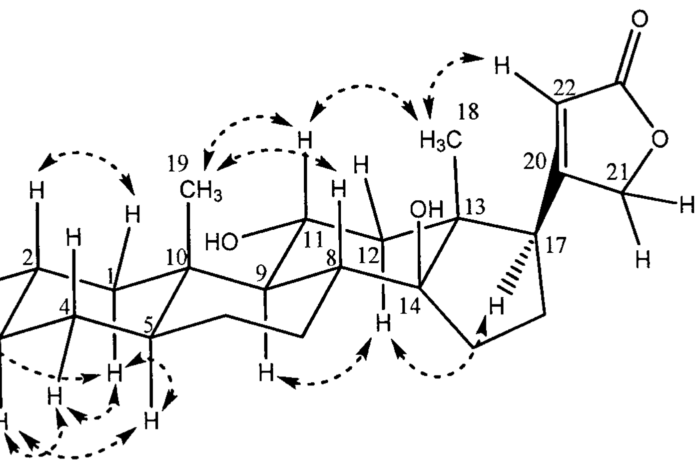
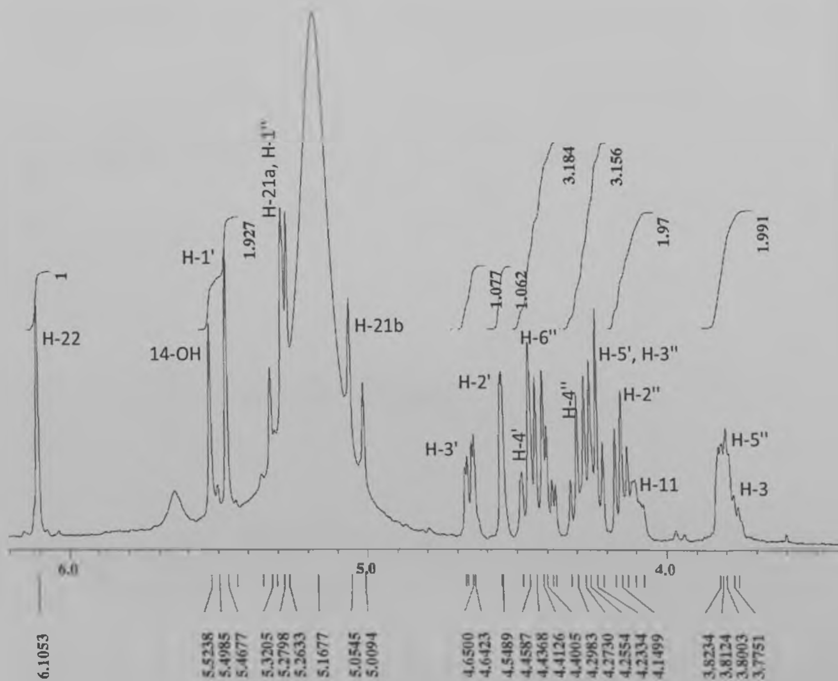


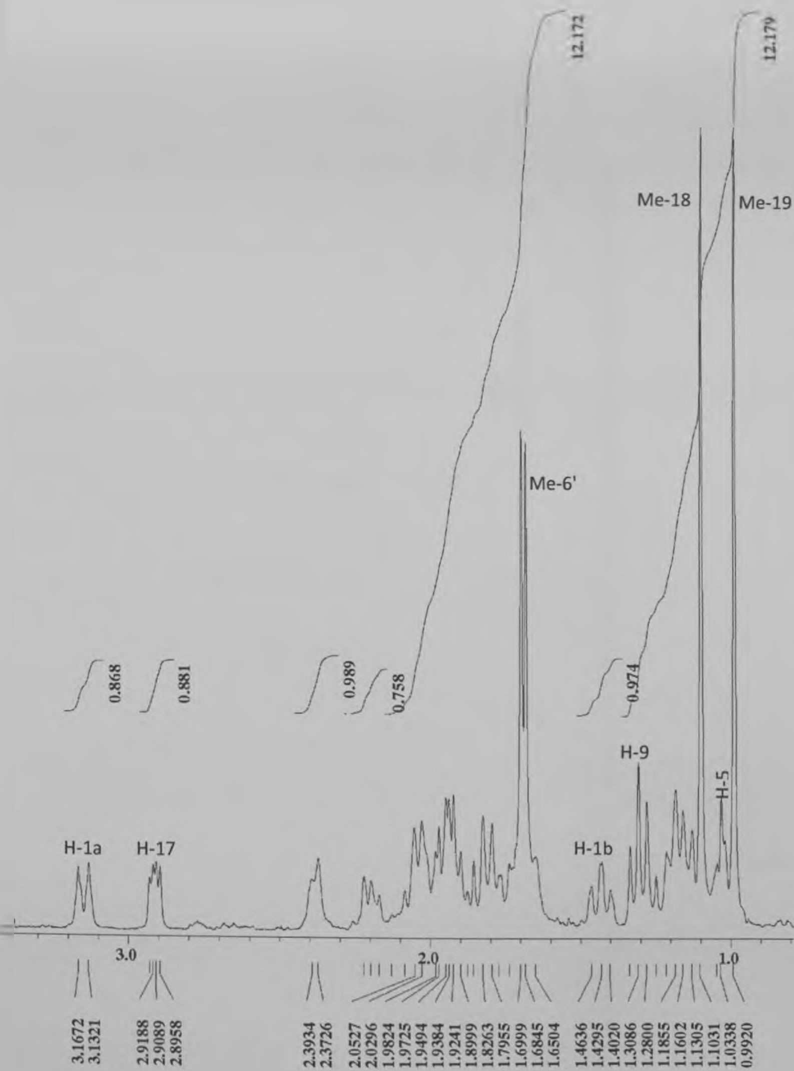
Fig. 3.7: Selected ROESY (

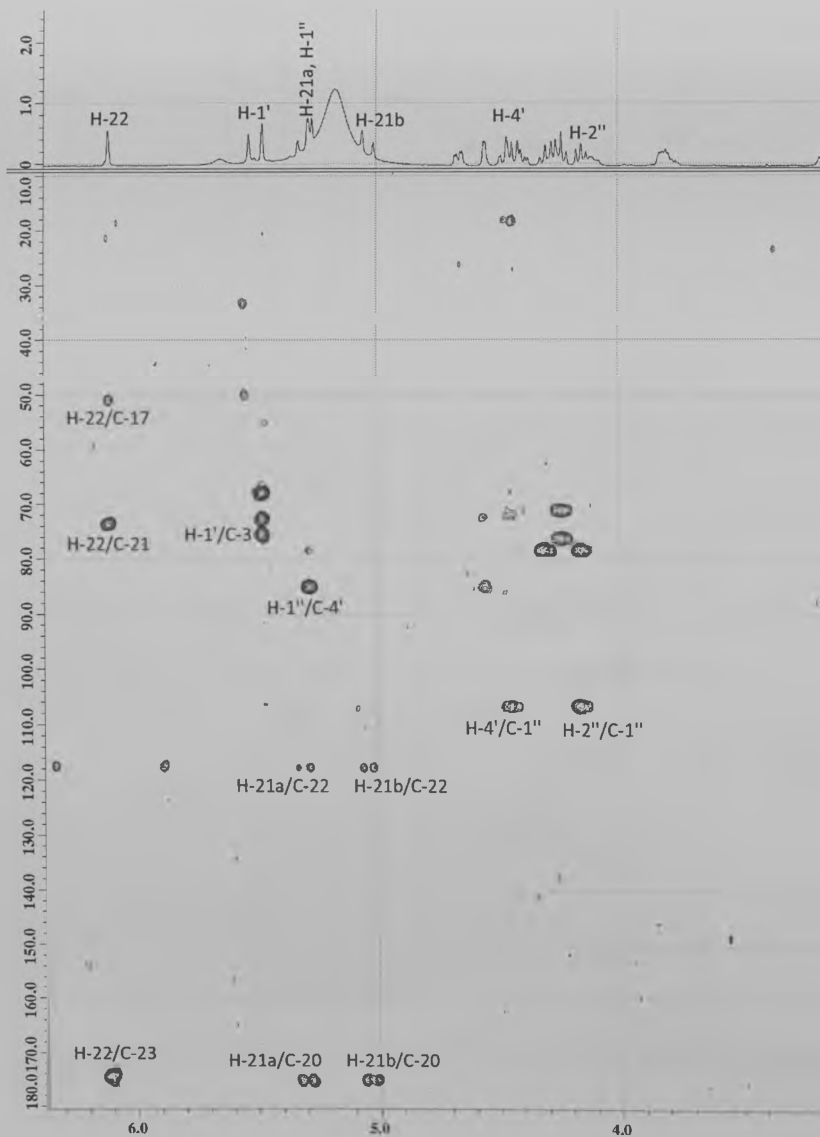


- - ►) correlations observed in JS-3

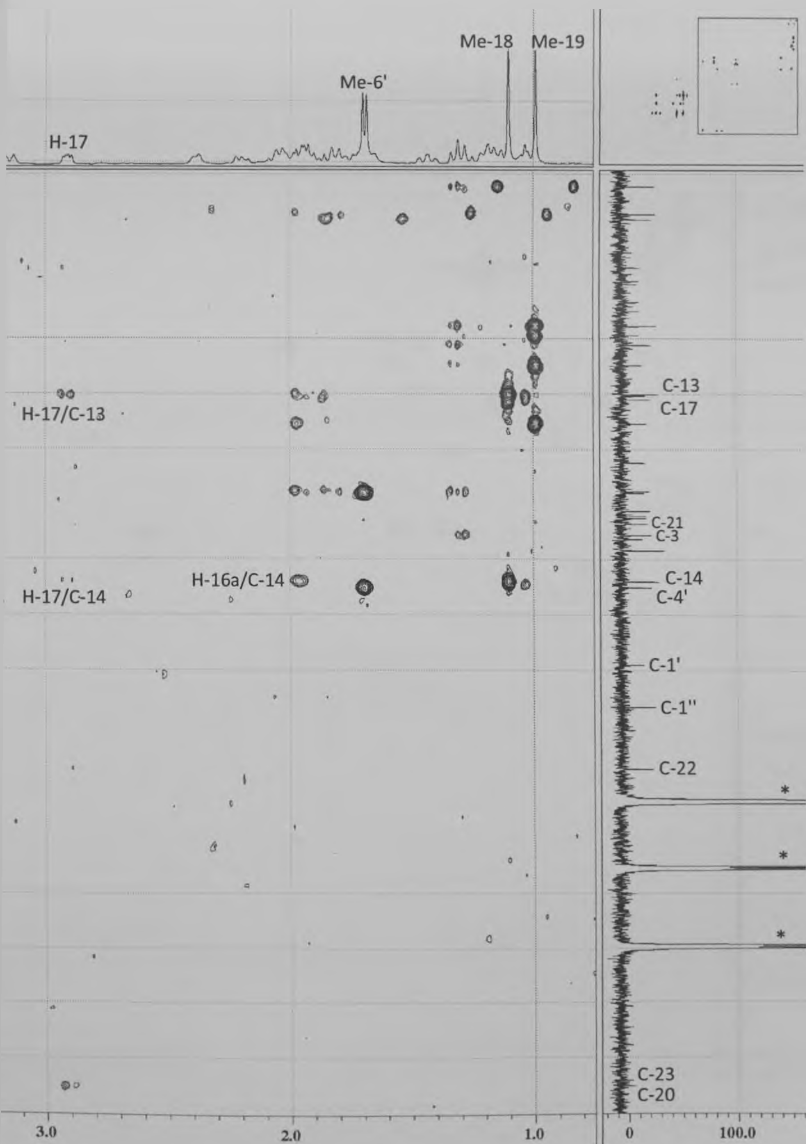


Spectrum 3.11: ^{1}H NMR (400 MHz, C_5D_5N) spectrum of JS-3

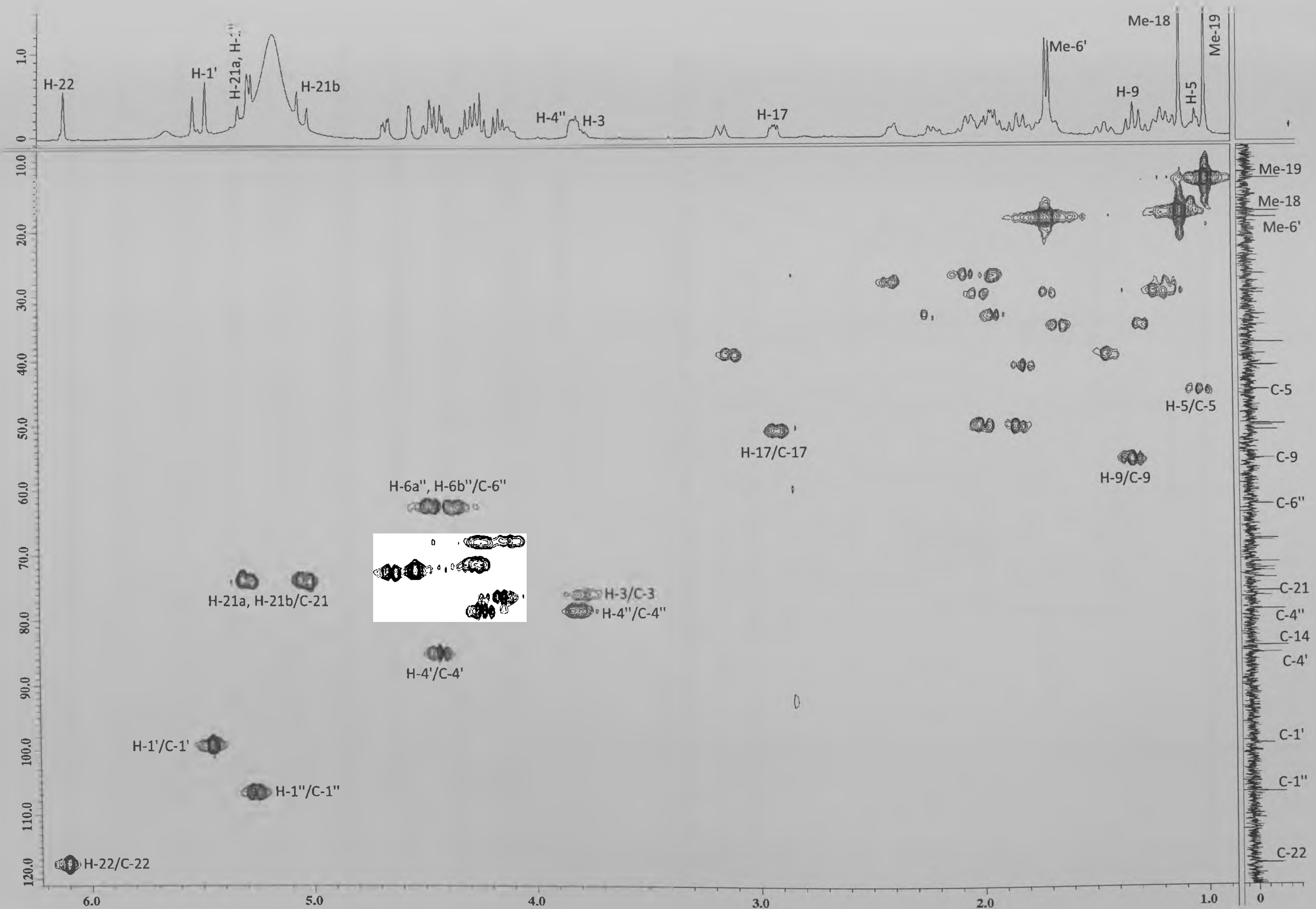

12.172
12.179
Me-18
Me-19
Me-6'
0.868
0.881
0.989
0.758
0.974
H-9
H-5
H-1a
H-17
H-1b
3.0
2.0
1.0
3.1672
3.1321
2.9188
2.9089
2.8958
2.3934
2.3726
2.0527
2.0296
1.9824
1.9725
1.9494
1.9384
1.9241
1.8999
1.8263
1.7955
1.6999
1.6845
1.6504
1.4636
1.4295
1.4020
1.3086
1.2800
1.1855
1.1602
1.1305
1.1031
1.0338
0.9920



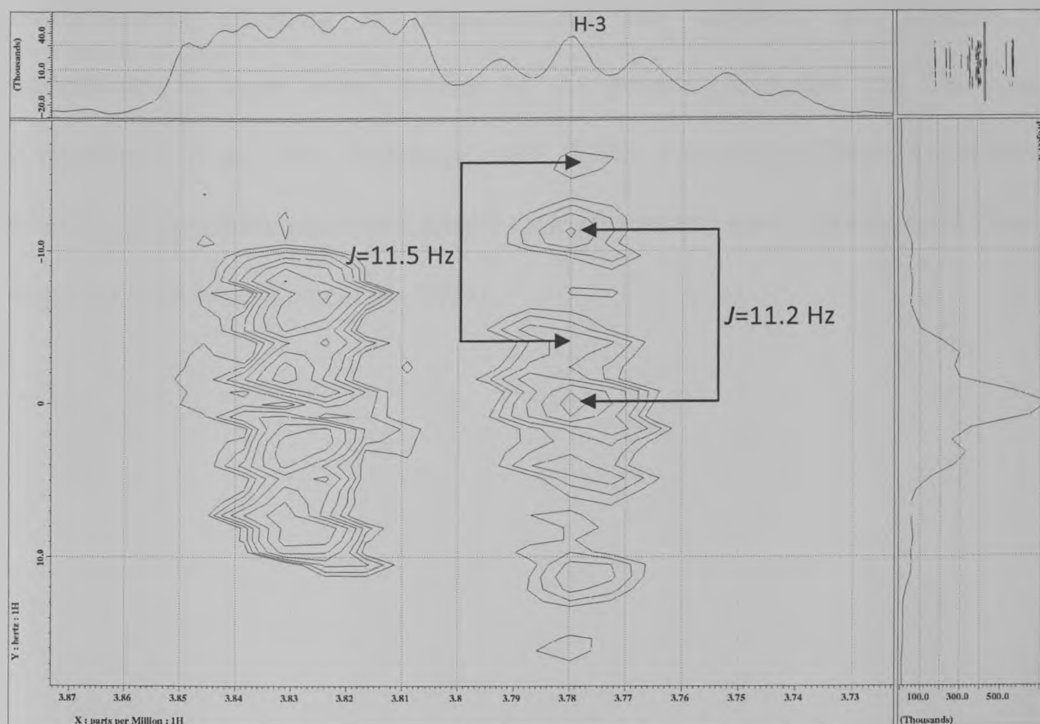
Spectrum 3.12: HMBC (400 MHz, C_5D_5N*) spectrum of JS-3



Me-18
Me-19
Me-6'
H-17
H-17/C-13
H-16a/C-14
H-17/C-14
C-13
C-17
C-21
C-3
C-14
C-4'
C-1'
C-1''
C-22
*
*
*
C-23
C-20
3.0
2.0
1.0
0
100.0



Spectrum 3.13: HMQC (400 MHz, C_5D_5N) spectrum of JS-3



Spectrum 3.14: Selected region of *J*-resolved spectrum (400 MHz, C_5D_5N) of JS-3

About 202 plant species in 55 genera and 12 Angiosperm families have been reported to contain cardenolides either in their genin form or as glycosides (Trigo, 2000). Cardenolide biosynthesis is more common in plants belonging to the families Asclepiadaceae and Euphorbiaceae (Becerra *et al.*, 2001). Extensive phytochemical investigation has been carried out on plants belonging to the families Asclepiadaceae (*e.g.*, *Cryptostegia* spp.), Apocynaceae (*e.g.*, *Strophanthus* spp.) and Scorphulariaceae (*e.g.*, *Digitalis* spp.) for their cardenolide content (Pauli *et al.*, 1999; Kamel *et al.*, 2001). A significant number of cardenolides have also been isolated from plants belonging to the family Euphorbiaceae including *Mallotus philippinensis*, *M. paniculatus* and *M. japonicas* (Chen, 1970; Okabe *et al.*, 1976).

Phytochemical screening in Euphorbiaceae has indicated the presence of cardenolides in some other species of the genera (*Acalypha* and *Codiaeum*) (Ogunwenmo *et al.*, 2007; Soladoye *et al.*, 2008). Previously alliotoxin and seven other 5α-cardenolides (aglycones and/or their glycosides) were reported from *Trewia nudiflora* stem bark (Kang *et al.*, 2005).

Table 3.1: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of the isolated cardiac glycosides in C_5D_5N

Position	JS-1*		JS-2		JS-3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.02 (1H, *m*) / 2.49 (1H, *m*)	38.3	1.27 (1H, *td* 14.0, 3.5) /3.48 (1H, *dt* 14.0, 3.5)	35.3	1.45 (1H, *td* 14.0, 3.5) / 3.14 (1H,*dt* 3.5, 14.0)	39.4
2	1.62 (1H, *m*) / 1.33 (1H, *m*)	29.2	1.96 (1H, *m*) /2.15 (1H, *m*)	30.3	1.97 (1H, *m*) / 1.65 (1H *m*)	30.0
3	3.40 (1H, *m*)	74.7	3.92 (1H, *dddd* 5.1, 5.5, 10.5, 11.0)	75.6	3.78 (1H, *dddd* 4.5, 4.6, 11.2, 11.5)	75.6
4	1.11 (1H, *m*) / 1.56 (1H, *m*)	34.4	1.53 (1H, *m*) / 1.79 (1H, *m*)	35.5	1.24 (1H, *m*) / 1.62 (1H)	34.9
5	1.03 (1H, *m*)	44.3	1.20 (1H, *m*)	45.0	1.03 (1H, *m*)	44.9
6	1.09 (1H, *m*) / 1.25 (1H, *m*)	28.9	1.22 (1H, *m*) / 1.16 (1H, *m*)	29.1	1.22 (1H, *m*) / 1.14 (1H)	29.5
7	0.93 (1H, *m*) / 1.98 (1H, *m*)	27.5	1.18 (1H, *m*) / 2.42 (1H, *m*)	28.1	2.38 (1H, *m*) / 1.16 (1H)	28.2
8	1.40 (1H, *m*)	40.1	2.11 (1H, *m*)	41.9	1.79 (1H, *m*)	41.1
9	0.93 (1H, *t* 10.4)	54.4	1.38 (1H, *t* 10.6)	55.1	1.30 (1H, *t* 11.4)	55.5
10	-	36.9	-	41.4	-	37.6
11	3.56 (1H, *m*)	66.5	4.54 (1H, *dt* 3.5, 10.6)	69.9	4.13 (1H, *m*)	67.7
12	1.38 (1H, *m*) / 1.52 (1H, *m*)	49.1	1.84 (1H, *m*) / 1.98 (1H, *m*)	49.7	1.82 (1H, *m*) / 2.01 (1H, *m*)	50.4
13	-	50.0	-	50.4	-	50.1
14	-	83.7	-	84.5	-	84.0
15	1.58 (1H, *m*) / 1.97 (1H, *m*)	32.3	1.96 (1H, *m*) / 2.27 (1H, *m*)	33.3	1.89 (1H, *m*) / 2.23 (1H, *m*)	33.3
16	1.78 (1H, *m*) / 1.94 (1H, *m*)	26.2	1.95 (1H, *m*) / 2.11 (1H, *m*)	27.3	2.03 (1H, *m*) / 1.93 (1H)	27.1
17	2.78 (1H, *dd* 5.1, 8.4)	49.7	2.89 (1H, *dd* 4.8, 9.2)	51.1	2.91 (1H, *dd* 4.7, 8.9)	51.0
18	0.77 (3H, *s*)	16.9	1.12 (3H, *s*)	17.7	1.10 (3H, *s*)	17.4
19	0.82 (3H, *s*)	11.9	4.16 (2H, *s*)	59.1	0.99 (3H, *s*)	12.3
20	-	175.8	-	175.6	-	175.4
21	4.87 (1H, *dd* 18.2, 1.5) / 4.93 (1H, *dd* 18.2, 1.5)	73.1	5.01 (1H, *dd* 1.7, 18.0) / 5.28 (1H, *dd* 1.7, 18.0)	73.7	5.03 (1H, *dd* 1.7, 18.3) / 5.30 (1H, *dd* 1.7, 18.3)	73.6
22	5.92 (1H, *brs*)	116.2	6.09 (1H, *brs*)	117.6	6.10 (1H, *brs*)	117.6
23	-	173.8	-	174.5	-	174.4
1′	4.67 (1H, *d* 1.5)	97.9	5.53 (1H, *d* 1.2)	99.4	5.47 (1H, *brs*)	99.0
2′	3.51 (1H, *dd* 1.5, 3.1)	71.0	4.54 (1H, *dd* 1.2, 3.4)	72.7	4.55 (1H, *dd* 1.5, 3.2)	72.2
3′	3.39 (1H, *m*)	70.6	4.50 (1H, *dd* 3.4, 8.9)	72.7	4.65 (1H, *dd* 3.2, 9.3)	72.6
4′	3.15 (1H, *dt* 5.6, 9.5)	72.0	4.28 (1H, *t* 8.9)	74.0	4.44 (1H, *m*)	85.1
5′	3.42 (1H, *m*)	68.3	4.29 (1H, *m*)	69.7	4.26 (1H, *m*)	67.9
6′	1.10 (3H, *d* 6.2)	17.8	1.65 (3H, *d* 5.7)	18.5	1.69 (3H, *d* 6.2)	18.3
1′′					5.26 (1H, *d* 8.4)	106.6
2′′					4.14 (1H, *t* 8.4)	76.3
3′′					4.23 (1H, *t* 8.8)	78.5
4′′					4.29 (1H, *m*)	71.2
5′′					3.81 (1H, *m*)	78.5
6′′					4.36 (1H, *m*) / 4.46 (1H, *m*)	62.4
11-OH	4.13 (1H, *d* 6.6)					
14-OH	4.10 (1H, *s*)				5.53 (1H, *s*)	
2′-OH	4.66 (1H, *d* 5.7)					
3′-OH	4.50 (1H, *d* 6.2)					
4′-OH	4.70 (1H, *d* 5.6)					

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz. *Run in DMSO-d_6.

3.1.2 Triterpenes and phytosterols

3.1.2.1 Characterisation of lupane derivatives

3.1.2.1.1 Characterisation of JS-4 as betulinic acid

This compound was isolated from the *n*-hexane extract of *Ludwigia adscendens*. A purple spot appeared on the TLC upon spraying *p*-anisaldehyde-sulphuric acid reagent followed by heating at 105°C.

The IR spectrum indicated the presence of a carbonyl group (1686 cm^{-1}), a hydroxy group (3467, 3436 cm^{-1}), exomethylene (3072, 1641, 882 cm^{-1}) (Williams and Fleming, 2008). The HREI-MS data showed a molecular ion $[M]^+$ at *m/z* 456.3605, suggesting for a molecular formula of $C_{30}H_{48}O_3$ and a double bond equivalence (DBE) of 7.

The ^1H NMR data (**Table 3.2**) showed the presence of two exomethylene protons at δ_H 4.93 (1H, *brs*) and 4.76 (1H, *brs*), one oxymethine at δ_H 3.45 (1H, *t* 7.9 Hz), one vinylic methine at δ_H 3.51 (1H, *m*) and six methyl singlets at δ_H 0.80, 1.00, 1.04, 1.06, 1.21 and 1.78. The downfield shift of the latter methyl indicated its presence next to an olefinic quaternary carbon. The ^{13}C NMR spectrum showed the presence of 30 carbons including a carbonyl carbon at δ_C 178.7, two olefinic carbons at δ_C 109.8, 151.2 and one oxygenated carbon at δ_C 78.0. The carbons were further assigned with the help of a DEPT 135 experiment as six methyls, eleven methylenes, six methines. Comparison of the DEPT spectrum with the ^{13}C NMR spectrum revealed a further 7 carbons as quarternaries. The above data suggested that **JS-4**

was a lupane-type triterpene. All proton and carbon resonances were further assigned using 2D NMR experiments. In the HMBC (**Spectrum 3.15**), the exomethylene protons at δ_H 4.93 and 4.76 (CH_2-29) showed ^3J correlations to the carbon at δ_C 47.6 (C-19) and to the methyl at δ_C 19.3 (Me-30), thus confirming the presence of the isopropenyl moiety. Furthermore, the methine at δ_H 1.74 (H-18) showed ^3J correlations to the carbon at δ_C 151.2 (C-20) and to the carbonyl at δ_C 178.7 (C-28) indicating C-17 as the attachment site of the carboxylic acid group. The two geminal methyls at δ_H 1.00 and 1.21 showed ^3J correlation of the oxymethine at δ_C 78.0 establishing the hydroxyl group at C-3.

Due to the large coupling constant, (J=7.9 Hz) H-3 proton was assigned to be axial. In the NOESY spectrum this oxymethine (δ_H 3.45) showed correlation to the one of the geminal methyl groups at δ_H 1.21 (Me-23) while the other methyl at δ_H 1.00 (Me-24) showed correlation to the methyl at δ_H 0.80 (Me-25) leading to the conclusion that the oxymethine was on the α side of the plane. Other NOESY correlations (**Fig. 3.8**) elucidated the relative stereochemistry of the chiral centres of **JS-4**.

On the basis of these results and by comparison with previously published data, **JS-4** was identified as 3β-hydroxy-20(29)-lupen-28-oic acid or betulinic acid (Mahato and Kundu, 1994; Ogunkoya, 1981; Reher and Budesinsky, 1992).

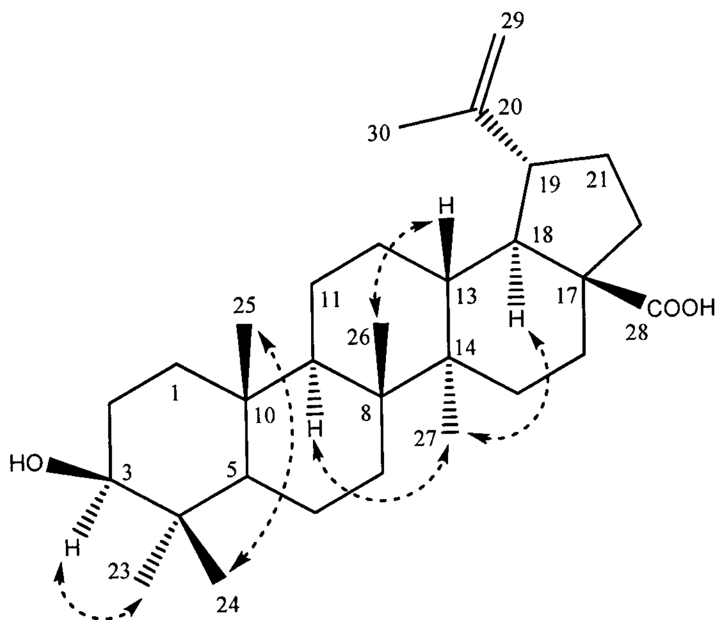
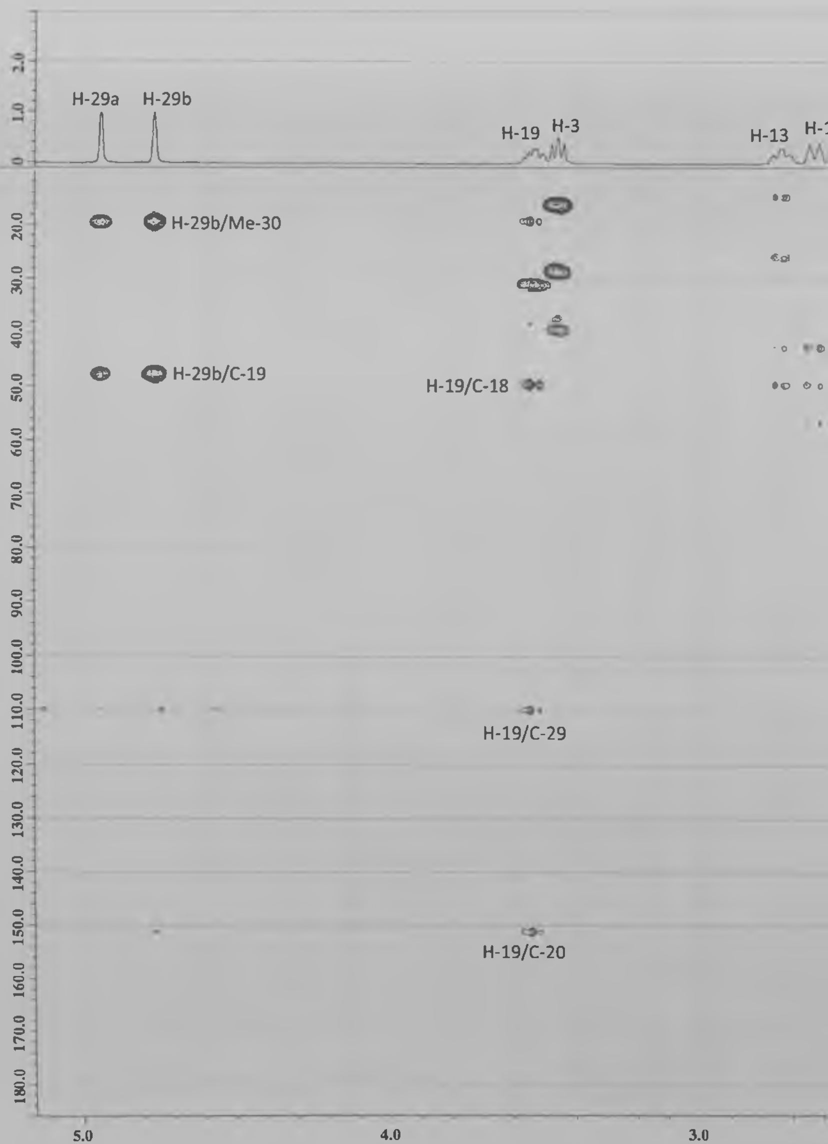
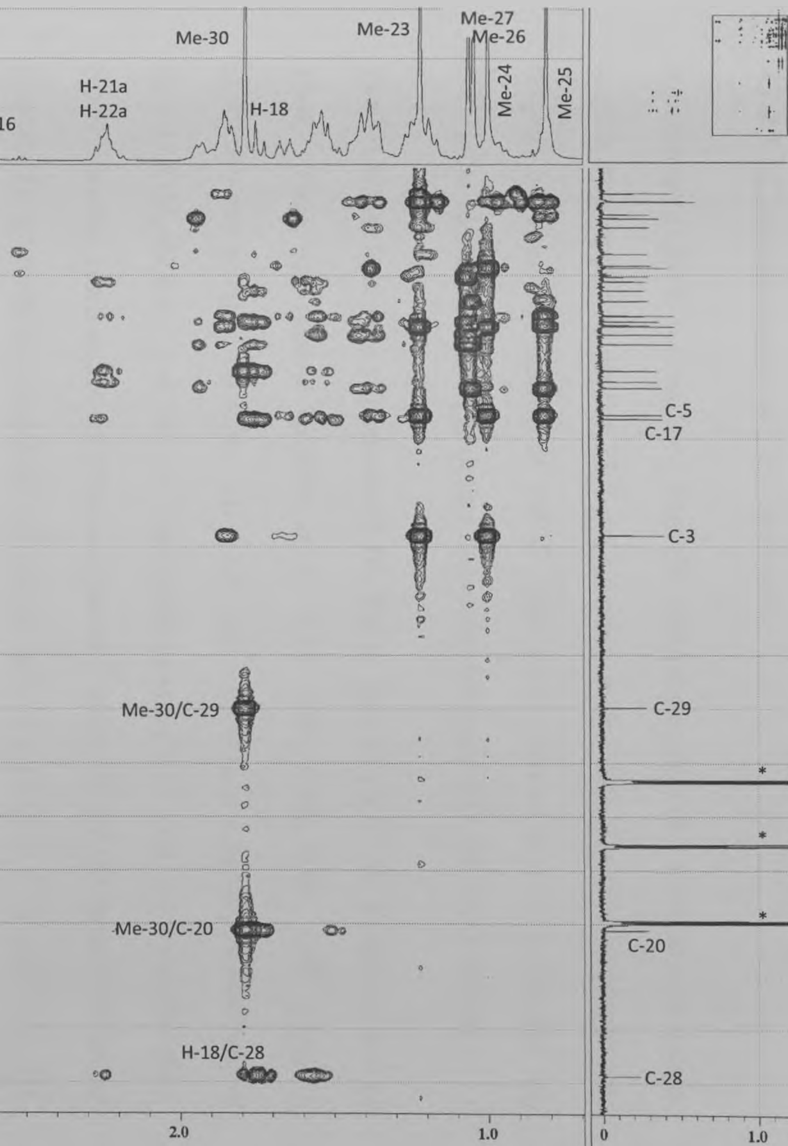


Fig. 3.8: Structure of JS-4 with key NOESY correlations



Spectrum 3.15: HMBC (400 MHz, C_5D_5N) spectrum of JS-4



Me-30
Me-23
Me-27
Me-26
H-21a
H-22a
H-18
Me-24
Me-25
16
C-5
C-17
C-3
Me-30/C-29
C-29
Me-30/C-20
C-20
H-18/C-28
C-28
2.0
1.0
0
1.0

3.1.2.1.2 Characterisation of JS-5 as betulin

A compound (**JS-5**) was isolated from the *n*-hexane extract of *Ludwigia adscendens*. A pink spot appeared on the TLC on spraying with *p*-anisaldehyde-sulphuric acid reagent followed by heating at 105°C. HRCI-MS showed a quasi-molecular ion $[M+H]^+$ at *m/z* 443.3884 suggesting a molecular formula of $C_{30}H_{50}O_2$ (DBE=6).

The ^1H NMR spectrum (**Spectrum 3.16, Table 3.2**) of **JS-5** was similar to that of **JS-4**. Thus, six methyl singlets at δ_H 0.74, 0.80, 0.95, 0.96, 1.00, 1.66, an exomethylene group at δ_H 4.56 (1H, *d* 1.8) and 4.66 (1H, *d* 1.8), one oxymethine at δ_H 3.17 (1H, *dd* 4.8, 11.4 Hz) and one methine at δ_H 2.37 (1H, *m*) were observed. Apart from the aforementioned common features, the spectrum exhibited an extra set of peaks for geminal oxymethylene protons at δ_H 3.32 (1H, *d* 10.3 Hz) and 3.78 (1H, *d* 10.3 Hz). The ^{13}C NMR spectrum revealed thirty carbons and was in close similarity with the data obtained for **JS-4** except for the absence of the carbonyl carbon and the presence of an oxymethylene at δ_C 60.5 suggesting that the carbonyl function had been reduced to a primary alcohol group in **JS-5**. 2D experiments including HMBC, HMQC and COSY were used to elucidate the structure. Due to the large coupling constant (*J*=11.4 Hz), H-3 was assigned as axial.

On the basis of the above data and by comparison with literature, **JS-5** was identified as 20(29)-lupen-3β,28-diol or betulin (Fig. 3). The spectral data obtained were in agreement with previous reports (Hironaka *et al.*, 1988; Patra *et al.*, 1988).

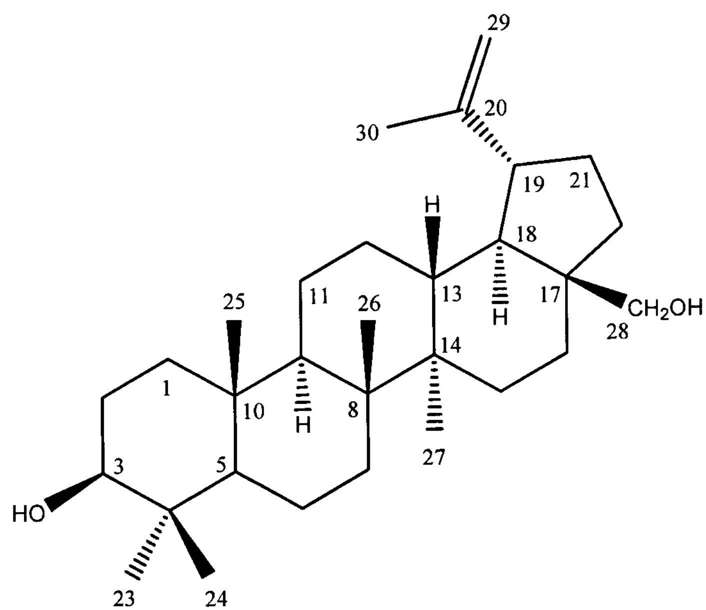
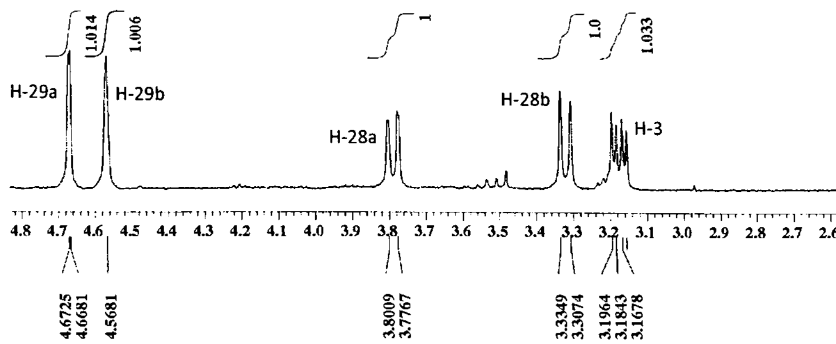
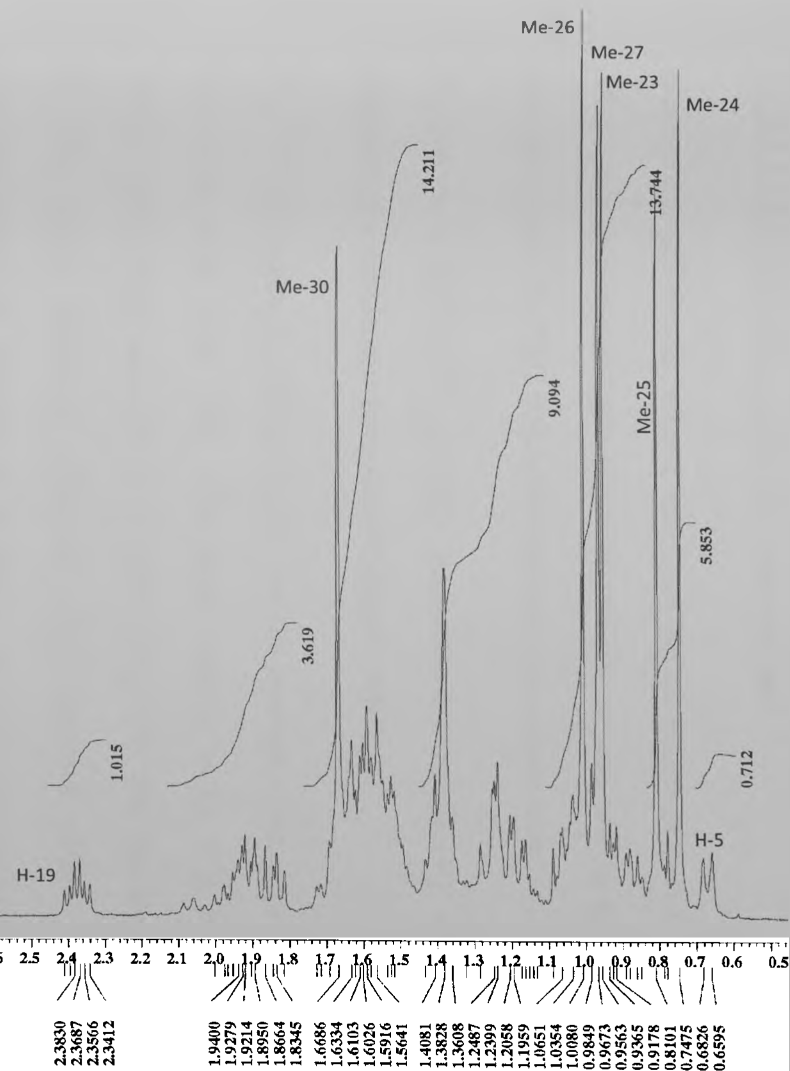


Fig.3.9: Structure of JS-5



Spectrum 3.16: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of JS-5


Me-26
Me-27
Me-23
Me-24
14.211
13.744
Me-30
9.094
Me-25
5.853
3.619
1.015
0.712
H-5
H-19
2.3830
2.3687
2.3566
2.3412
1.9400
1.9279
1.9214
1.8950
1.8664
1.8345
1.6686
1.6334
1.6103
1.6026
1.5916
1.5641
1.4081
1.3828
1.3608
1.2487
1.2399
1.2058
1.1959
1.0651
1.0354
1.0080
0.9849
0.9673
0.9563
0.9365
0.9178
0.8101
0.7475
0.6826
0.6595

3.1.2.1.3 Characterisation of JS-6 as betulonic acid

A compound (**JS-6**) was isolated from the *n*-hexane extract of *Ludwigia adscendens*. After spraying *p*-anisaldehyde-sulphuric acid reagent and heating at 105°C, a green spot appeared on the TLC which became blue on exposure to air.

Positive mode HRESI-MS showed a quasi-molecular ion $[M+H]^+$ at *m/z* 455.3520 suggesting for a molecular formula of $C_{30}H_{46}O_3$ (DBE=8).

The ^1H NMR spectrum (**Table 3.2**) showed the presence of six methyl singlets at δ_H 0.91, 0.96, 0.97, 1.00, 1.05, 1.67, broad singlets at δ_H 4.73 and 4.60 for a exomethylene group, and a vinylic methine at δ_H 2.98. The data closely resembled the other lupane triterpenes (**JS-4** and **JS-5**) discussed earlier. The ^{13}C NMR spectrum showed the presence of 30 carbons including two olefininc carbons at δ_C 150.2, 109.6 and a carbonyl at δ_C 180.6. However, the absence of the C-3 hydroxymethine and the presence of a carbon signal at δ_C 218.0 suggested that the C-3 hydroxyl group had been replaced by a ketone. This also correlated with the 8 degrees of unsaturation observed in **JS-6**. Further 2D experiments, including COSY, HMBC and HMQC confirmed the identity of **JS-6** as 3-oxo-20(29)-lupen-28-oic acid or betulonic acid and all spectral data obtained showed good agreement with previous reports (Patra *et al.*, 1988).

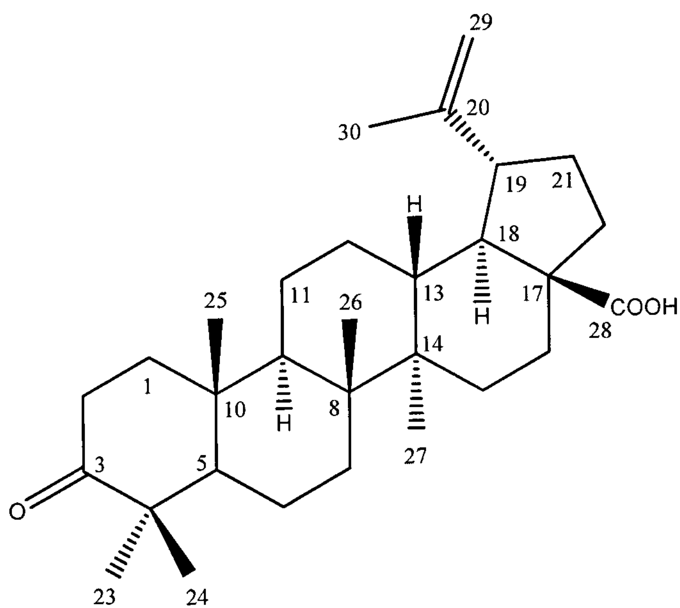


Fig. 3.10: Structure of JS-6

Table 3.2: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of isolated lupane triterpenes in $CDCl_3$

Position	JS-4*		JS-5		JS-6	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.65 (1H, *m*) / 0.96 (1H, *m*)	39.4		38.6	2.51 (1H, *m*) / 1.90 (1H, *m*)	39.5
2	1.82 (1H, *m*) / 1.18 (1H, *m*)	28.2		27.3	2.36 (1H, *m*) / 1.46 (1H, *m*)	34.1
3	3.45 (1H, *t* 7.9 Hz)	78.0	3.17 (1H, *dd* 4.8, 11.4 Hz)	78.9	-	218.0
4	-	37.5	-	38.8	-	47.3
5	0.81 (1H, *m*)	55.8	0.66 (1H, *m*)	55.2	1.30 (1H, *m*)	54.9
6	1.56 (1H, *m*) / 1.38 (1H, *m*)	18.6	1.38 (1H, *m*) / 1.51 (1H, *m*)	18.2		19.6
7	1.51 (1H, *m*) / 1.39 (1H, *m*)	34.7	1.38 (1H, *m*) / 1.03 (1H, *m*)	34.2		33.5
8	-	41.0	-	40.8	-	40.6
9	1.35 (1H, *m*)	50.8		50.3	1.36 (1H, *m*)	49.8
10	-	39.1	-	37.1	-	36.8
11	1.44 (1H, *m*) / 1.18 (1H, *m*)	21.1		20.8		21.3
12	1.92 (1H, *m*) / 1.20 (1H, *m*)	26.0		25.2		25.4
13	2.72 (1H, *m*)	38.4		37.2		38.4
14	-	42.7	-	42.6	-	42.4
15	1.86 (1H, *m*) / 1.25 (1H, *m*)	30.1		27.0		30.5
16	2.61 (1H, *m*) / 1.54 (1H, *m*)	32.8		29.1		32.0
17	-	56.5	-	47.7	-	56.2
18	1.74 (1H, *t* 11.4)	49.6	1.57 (1H, *m*)	48.7	1.61 (1H, *m*)	49.1
19	3.51 (1H, *m*)	47.6	2.37 (1H, *ddd* 5.7, 11.0, 11.0)	47.7	2.98 (1H, *m*)	46.8
20	-	151.2	-	150.4	-	150.2
21	2.23 (1H, *m*) / 1.51 (1H, *m*)	31.1		29.7		29.3
22	2.24 (1H, *m*) / 1.55 (1H, *m*)	37.4		33.9		37.0
23	1.21 (3H, *s*)	28.5	0.95 (3H, *s*)	27.9	1.05 (3H, *s*)	26.6
24	1.00 (3H, *s*)	16.2	0.74 (3H, *s*)	15.3	1.00 (3H, *s*)	20.9
25	0.80 (3H, *s*)	16.3	0.80 (3H, *s*)	16.0	0.91 (3H, *s*)	15.9
26	1.04 (3H, *s*)	16.3	1.00 (3H, *s*)	15.9	0.96 (3H, *s*)	15.8
27	1.06 (3H, *s*)	14.8	0.96 (3H, *s*)	14.7	0.97 (3H, *s*)	14.6
28	-	178.7	3.78 (1H, *d* 10.8) / 3.32 (1H, *d* 10.8)	60.5	-	180.6
29	4.94 (1H, *brs*) / 4.76 (1H, *brs*)	109.8	4.66 (1H, *d* 1.8) / 4.56 (1H, *d* 1.8)	109.7	4.60 (1H, *brs*) / 4.73 (1H, *brs*)	109.6
30	1.78 (3H, *s*)	19.3	1.66 (3H, *s*)	19.0	1.68 (3H, *s*)	19.3

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz. *Run in C_5D_5N.

3.1.2.2 Characterisation of JS-7 as 3β-acetyl aleuritolic acid

A compound (**JS-7**) was isolated from the ethyl acetate extract of *Trewia nudiflora*. After treatment with *p*-anisaldehyde reagent and heating (105°C) for a few minutes, a purple spot appeared on the TLC.

The IR spectrum indicated the presence of two carbonyl groups (1734, 1689 cm^{-1}), hydroxyl groups (3447 cm^{-1}) (Williams and Fleming, 2008). The HRESI-MS data obtained showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 499.3695 suggesting a molecular formula of $C_{32}H_{50}O_4$ (DBE=8). The mass spectrum also suggested the presence of a carboxylic and an acetyl group with the fragment ions at *m/z* 471 $[(M–CO)+H]^+$ and *m/z* 439 $[(M–CH_3COOH)+H]^+$ (rel. int. 90%), respectively.

The ^1H NMR (**Table 3.3**) spectrum showed the presence of eight methyl singlets at δ_H 0.83, 0.86, 0.89, 0.90, 0.93, 0.94 and 2.02. The downfield shift of the latter methyl indicated it to be a part of an acetyl group. The spectrum also revealed the presence of one olefinic and one oxymethine proton at δ_H 5.50 (1H, *dd* 3.5, 8.0 Hz) and 4.44 (1H, *dd* 5.7, 10.1 Hz) respectively.

The ^{13}C NMR (**Spectrum 3.17, Table 3.3**) spectrum showed the presence of 32 carbons including two carbonyls at δ_C 184.2, 171.0, one olefinic quaternary at δ_C 160.5, one olefinic methine at δ_C 116.8 and an oxymethine at δ_C 80.9. With the help of the DEPT 135 experiment, the carbon signals were further assigned as eight methyls, ten methylenes, five methines and nine quarternaries. These data suggested for the presence of an ester of either an oleanene or of a taraxarene type triterpene.

The proton and carbon signals were further analysed with the help of 2D experiments including COSY, HMBC and HMQC. In the HMBC experiment, the oxymethine at δ_H 4.44 (H-3) showed ^3J coupling to two geminal methyls at δ_C 27.9 (Me-23) and 16.5 (Me-24). The hydroxyl at C-3 was found to be esterified by an acetyl group since both the H-3 and methyl of the acetyl group at δ_H 2.02 showed ^3J and ^2J correlation to the carbonyl (δ_C 171.0), respectively. Methyl groups at δ_H 0.90 (Me-27) and 0.93 (Me-26) showed ^3J correlation to the carbon at δ_C 160.5 (C-14). The methylene protons at δ_H 1.90 (H-16a) and 2.35 (H-16b) showed ^3J correlation to carbons at δ_C 160.5 (C-14) and 184.2 (C-28) establishing the presence of a double bond at Δ14,15 and a carbocylic acid group at C-28. Another set of geminal methyls (δ_H 0.89 and 0.92) were found to be attached to a quaternary carbon at δ_C 29.2 (C-20).

The oxymethine at δ_H 4.44 (H-3) was considered to be axial due to the large coupling constant observed (J=5.7, 10.1 Hz). In the NOESY spectrum, the H-3 proton also showed correlation to the methyl at δ_H 0.83 (Me-23) further confirming its position on the α side of the ring plane.

On the basis of the above data, **JS-7** was identified as 3β-acetyltaraxarane-14-en-28-oic acid or 3β-acetyl aleuritolic acid. All spectral data obtained matched previous reports (McLean *et al.*, 1987; Mahato and Kundu, 1994). This compound has already been reported from the stem bark of *Trewia nudiflora* (Du *et al.*, 2002).

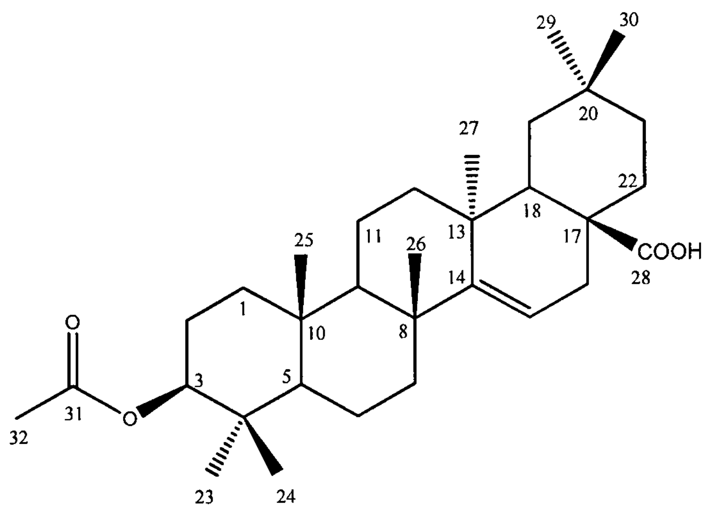
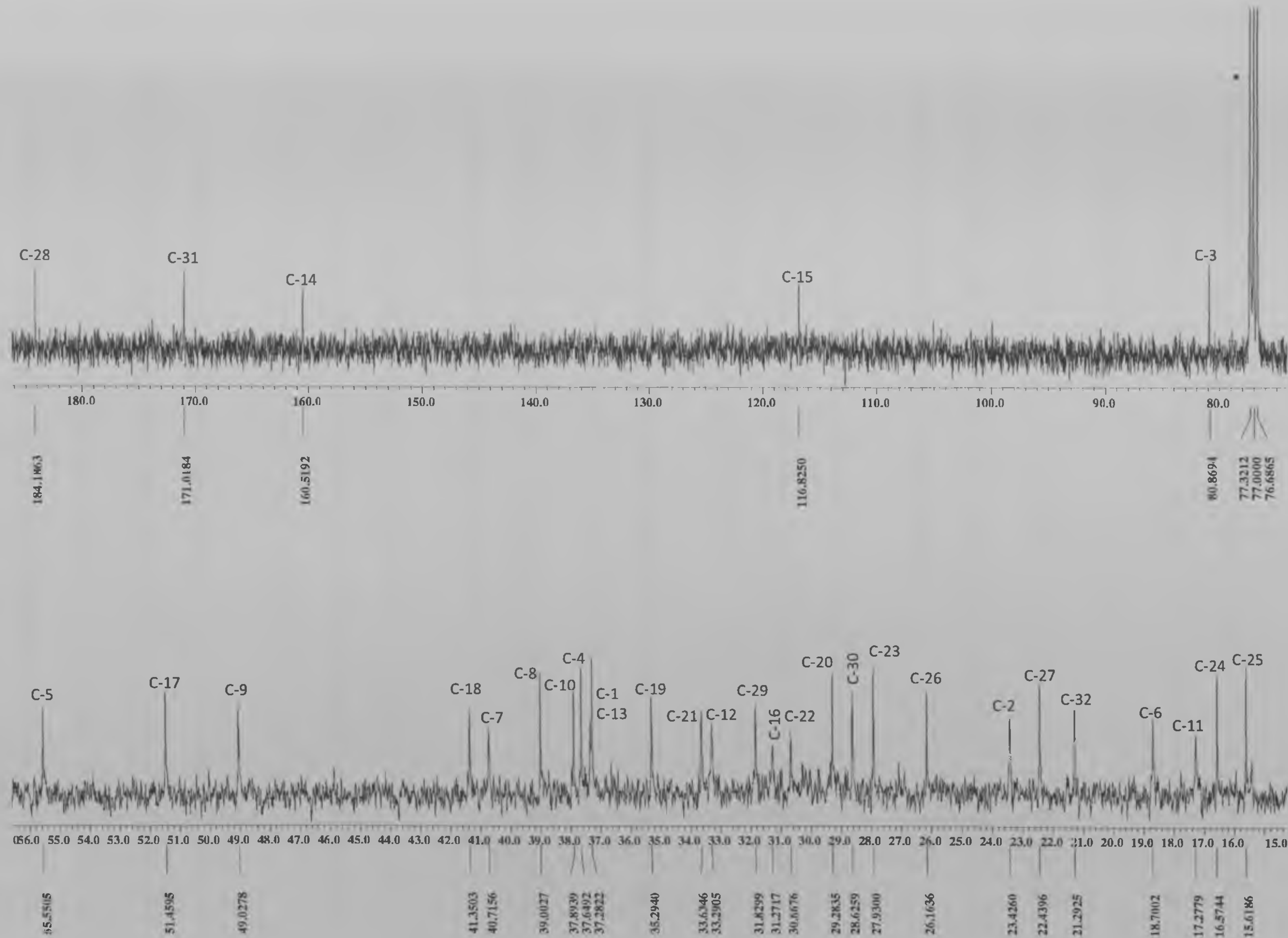


Fig. 3.11: Structure of JS-7

Table 3.3: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of JS-7 in $CDCl_3$

Position	δ_H	δ_C	Selected HMBC correlation
1	1.01 (1H, m) / 1.60 (1H, m)	37.3	
2	1.59 (1H, *m*) / 1.64 (1H, *m*)	23.4	
3	4.44 (1H, *dd* 5.7, 10.1)	80.9	C-31, Me-23, Me-24
4	-	37.6	
5	0.85 (1H, *m*)	55.5	
6	1.46 (1H, *m*) / 1.61 (1H, *m*)	18.7	
7	1.26 (1H, *m*) / 1.94 (1H, *m*)	40.7	
8	0.93 (1H, *m*)	39.0	
9	1.40 (1H, *m*)	49.0	C-8, C-11, Me-26
10	-	37.9	
11		17.3	
12	1.58 (1H, *m*) / 1.75 (1H, *m*)	33.3	C-14
13	-	37.3	
14	-	160.5	
15	5.50 (1H, *dd* 3.5, 8.0)	116.8	C-13
16	1.90 (1H, *m*) / 2.35 (1H, *m*)	31.3	C-14, C-28
17	-	51.4	
18	2.26 (1H, *m*)	41.4	C-28
19	1.09 (1H, *m*) / 1.23 (1H, *m*)	35.3	
20	-	29.3	
21	1.03 (1H, *m*) / 1.15 (1H, *m*)	33.6	
22	1.43 (1H, *m*) / 1.69 (1H, *m*)	30.7	
23	0.83 (3H, *s*)	27.9	C-3, C-5
24	0.86 (3H, *s*)	16.6	C-3, C-5
25	0.93 (3H, *s*)	15.6	C-1, C-5, C-9, C-10
26	0.93 (3H, *s*)	26.2	C-8
27	0.90 (3H, *s*)	22.4	C-12, C-13, C-14, C-18
28	-	184.2	
29	0.92 (3H, *s*)	31.8	Me-30, C-19, C-20, C-21
30	0.89 (3H, *s*)	28.6	Me-29, C-19, C-20, C-21
31	-	171.0	
32	2.02 (3H, *s*)	21.3	C-31

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz.



Spectrum 3.17: ^{13}C NMR (100 MHz, $CDCl_3$*) spectrum of JS-7

3.1.2.3 Characterisation of JS-8 as squalene

A compound (**JS-8**) was isolated from the *n*-hexane extract of *Ludwigia adscendens*. A pink spot appeared on the TLC after treatment with *p*-anisaldehyde-sulphuric acid followed by heating (105°C).

The IR spectrum indicated the presence of alkyl (2959, 2932, 2867 cm^{-1}) (Williams and Fleming, 2008). The HREI-MS gave a molecular ion peak $[M]^+$ at *m/z* 410.3915 suggesting a molecular formula of $C_{30}H_{50}$ (DBE=6).

The ^1H NMR spectrum (**Table 3.4**) showed three methyls at δ_H 1.60 (9H, *s*) and one methyl 1.68 (3H, *s*), three olefinic methines in the range δ_H 5.08-5.15 (*m*), and five allylic methylenes in the range δ_H 1.95 to 2.09 (*m*).

The ^{13}C NMR spectrum showed 15 carbon signals. The molecular formula, however, indicated 30 carbons. This suggested that **JS-8** is a symmetric compound. With the help of the DEPT 135 experiment, the carbons were subdivided into eight methyls, ten methylenes, six methines and six quaternary carbons.

On this basis, the ^1H NMR spectrum was reintegrated for showed six methyls at δ_H 1.60 (18H, *s*) and two methyls 1.68 (6H, *s*), six olefinic methines in the range δ_H 5.08-5.15 (*m*), and ten allylic methylenes in the range δ_H 1.95 to 2.09 (*m*).

The two terminal geminal methyls groups appeared at δ_C 25.9 indicating their *cis* orientation with the double bonds while the rest six methyls appeared at δ_C 16.2 and 17.9 referring to their *trans* confirmation. This confirmed the *trans* configuration of all the double bonds present in the molecule. Five olefinic methines appeared at δ_C

124.5 and 125.6 and five olefinic quaternary carbons at δ_C 131.4, 135.1 and 135.3. All the above spectral data suggested **JS-8** to be an acyclic triterpene. This was further supported in the mass spectrum by the base peak at *m/z* 69 $[C_5H_9]^+$ indicative of the presence of isoprenyl units. All the above information led to the identification of **JS-8** as all-*trans* 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosa hexaene or (All-*E*)-squalene. All the spectral data were in agreement with previous reports (He *et al.*, 2002; Nishiyama *et al.*, 1996; Pouchert and Behnke, 1993; Tornabene *et al.*, 1969). This is the first report of the isolation of squalene from *Ludwigia adscendens*.

Apart from shark liver oil, which is the major source of squalene, this triterpene is also found in yeasts and in plants including olive oil, wheat germ and rice bran oils (He *et al.*, 2002; Kelly, 1999).

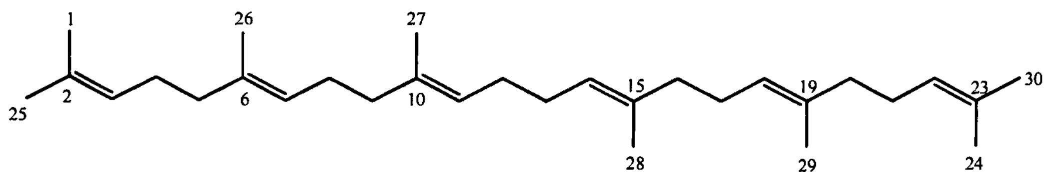


Fig. 3.12: Structure of JS-8

Table 3.4: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of JS-8 in $CDCl_3$

Position	JS-8	
	δ_H	δ_C
1, 24	1.60 (6H, *s*)	17.9
2, 23	-	131.4
3, 22	5.12 (2H, *m*)	124.6
4, 21	2.09 (4H, *m*)	28.5
5, 20	2.02 (4H, *m*)	39.9
6, 19	-	135.1#
7, 18	5.12 (2H, *m*)	124.5
8, 17	2.09 (4H, *m*)	26.9
9, 16	2.02 (4H, *m*)	39.9
10, 15	-	135.3#
11, 14	5.12 (2H, *m*)	124.5
12, 13	2.02 (4H, *m*)	29.9
25, 30	1.68 (6H, *s*)	25.9
26, 29	1.60 (6H, *s*)	16.2
27, 28	1.60 (6H, *s*)	16.2

Chemical shifts are in ppm. #Values are interchangeable in the same column

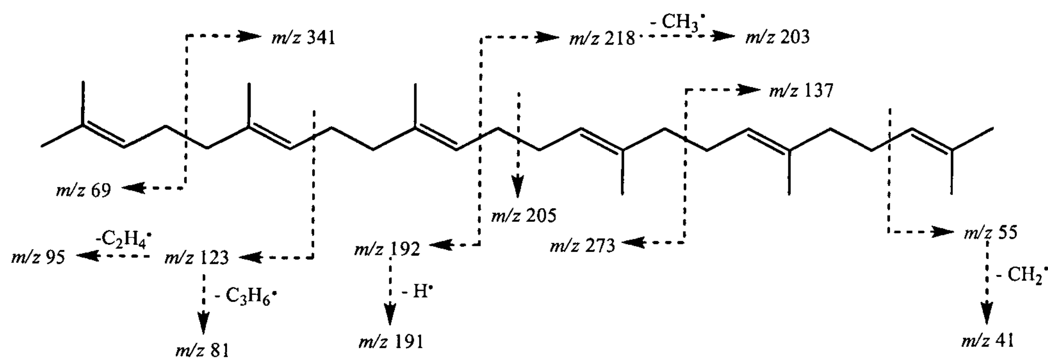


Fig. 3.13: Possible mass fragmentation for JS-8

3.1.2.4 Characterisation of JS-9 as daucosterol

A compound (**JS-9**) was isolated from the *n*-hexane extract of *Trewia nudiflora*. A purple spot appeared on the TLC when treated with *p*-anisaldehyde-sulphuric acid reagent followed by heating (105°C).

Negative mode ESI-MS showed a quasi-molecular ion $[M-H]^-$ at *m/z* 575 suggesting a molecular formula of $C_{35}H_{60}O_6$ (DBE=6).

The ^1H NMR spectrum indicated the presence of a phytosterol skeleton and a hexose unit. The phytosterol was identified as β-sitosterol with two tertiary methyls at δ_H 0.65 and 0.91, three secondary methyls at δ_H 0.86, 0.88 and 0.98 and one primary methyl at δ_H 0.90. The spectrum also revealed an olefininc proton at δ_H 5.33 (1H, *brs*). The sugar unit was identified as β-D-glucose with an anomeric proton δ_H 5.05 which appeared as a doublet (*J*=7.5 Hz) indicating a H-1'/H-2' *trans* diaxial configuration. The ^{13}C NMR spectrum revealed 35 carbons including the anomeric carbon at δ_C 102.5 (C-1') and olefinic carbons at δ_C 121.8, 140.8. In the HMBC spectrum, the anomeric proton showed a ^3J correlation to the carbon at δ_C 78.1 (C-3). The above spectral data led to the identification of **JS-9** as β-sitosterol 3-*O*-β-D-glucoside or daucosterol (Kojima *et al.*, 1990). This compound has previously been reported from the stem bark of *Trewia nudiflora* (Guohong *et al.*, 2004).

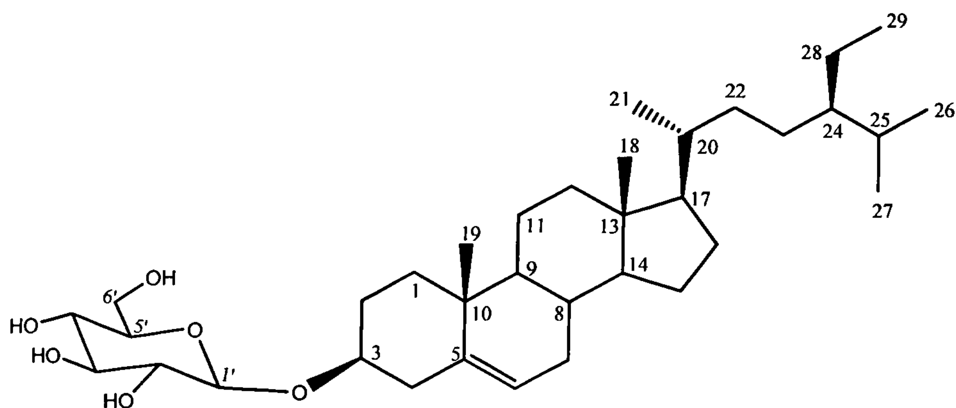


Fig 3.14: Structure of JS-9

3.1.2.5 Characterisation of JS-10 as 5,8-epi-dioxyergosta-6,22-dien-3-ol

A compound (**JS-10**) was isolated from the *n*-hexane extract of *Trewia nudiflora*. On treatment with *p*-anisaldehyde-sulphuric acid reagent followed by heating (105°C), a grey spot appeared on the TLC.

Positive mode HRCI-MS showed a quasi-molecular ion $[M+H]^+$ at *m/z* 429.3371 suggesting a molecular formula of $C_{28}H_{44}O_3$ (DBE=7).

The ^1H NMR spectrum (**Spectrum 3.18, Table 3.5**) showed the presence of two *cis* olefinic methines at δ_H 6.23 (1H, *d* 8.8 Hz), 6.49 (1H, *d*, 8.8 Hz) and two *trans* olefinic methines at 5.12 (1H, *dd* 15.4, 7.5 Hz) and 5.21 (1H, *dd* 15.4, 8.4 Hz). The spectrum also showed one oxymethine at δ_H 3.95 (m) along with six methyls at δ_H 0.79 (3H, *d*), 0.80 (3H, *s*), 0.81 (3H, *d*), 0.87 (3H, *s*), 0.98 (3H, *d*), 0.89 (3H, *d*). This suggested the presence of a steroid skeleton.

The ^{13}C NMR spectrum (**Table 3.5**) confirmed the presence of 28 carbons. The *J* modulated ^{13}C NMR spectrum showed six methyls, seven methylenes, eleven methines and four quaternary carbons. Two quaternary carbons at δ_C 79.4 and 82.1 indicated the presence of two oxygen-bearing centres as part of a peroxide bridge. This extra cycle in the molecule satisfied the calculated degree of unsaturations (7). In the HMBC spectrum, the olefinic methine at δ_H 6.23 (H-6) showed ^2J and ^3J correlations to carbons at δ_C 79.4 (C-8) and 82.1 (C-5). The olefinic methine at 6.49 (H-7) showed ^3J and ^2J correlations to the same carbons. The C-4 methylene protons (δ_H 2.10 and 1.89) showed ^2J correlations to the carbons at δ_C 66.5 (C-3), 82.1 (C-5) and ^3J correlations to carbons at δ_C 135.3 (C-6), 36.8 (C-10). The above correlations

established the *cis* double bond at C-6 and C-7 with the presence of the oxygen bridge between C-5 and C-8. The methyl group at δ_H 0.98 (Me-21) showed ^3J correlation to carbons at δ_C 56.1 (C-17) and 135.1 (C-22) whereas the methyl at δ_H 0.89 (Me-28) showed ^3J correlation to carbons at δ_C 132.3 (C-23) and 33.0 (C-25). This suggested that a methyl group was at C-24 and that **JS-10** was an ergosterol derivative.

The multiplet at δ_H 3.95 (1H, *dddd* 4.8, 5.3, 11.4, 11.4 Hz) was identified as the H-3 proton and assigned as axial and on the α side of the plane. In the NOESY spectrum, the H-3 proton showed correlation to the 4-Ha protons (δ_H 2.10) while 4-Hb (δ_H 1.89) showed correlation to the methyl at δ_H 0.87 (Me-19). Furthermore, Me-19 showed NOE effect to the proton at δ_H 6.23 (H-6) indicating that the peroxide bridge was on the α side of the plane. The δ_C value of C-24 was considered to be *R* according to the literature (Yue *et al.*, 2001). The above structural information led to the identification of **JS-10** as 22*E*,24*R*-5α,8α-epidioxyergosta-6,22-dien-3β-ol or ergosterol endoperoxide. All spectral data obtained were in agreement with previous reports (Gauvin *et al.*, 2000; Yue *et al.*, 2001). This compound was previously reported from the pericarp of *Trewia nudiflora* seeds (Du and Shen, 2006). This is the first report of this compound from the stem bark.

Ergosterol endoperoxide has been reported from different natural sources including plants (Cantrell *et al.*, 1999; Prakash and Basak, 1976), marine sponges and fungi (Gauvin *et al.*, 2000; Yue *et al.*, 2001). The latter seem to contain 5,8-epidioxy

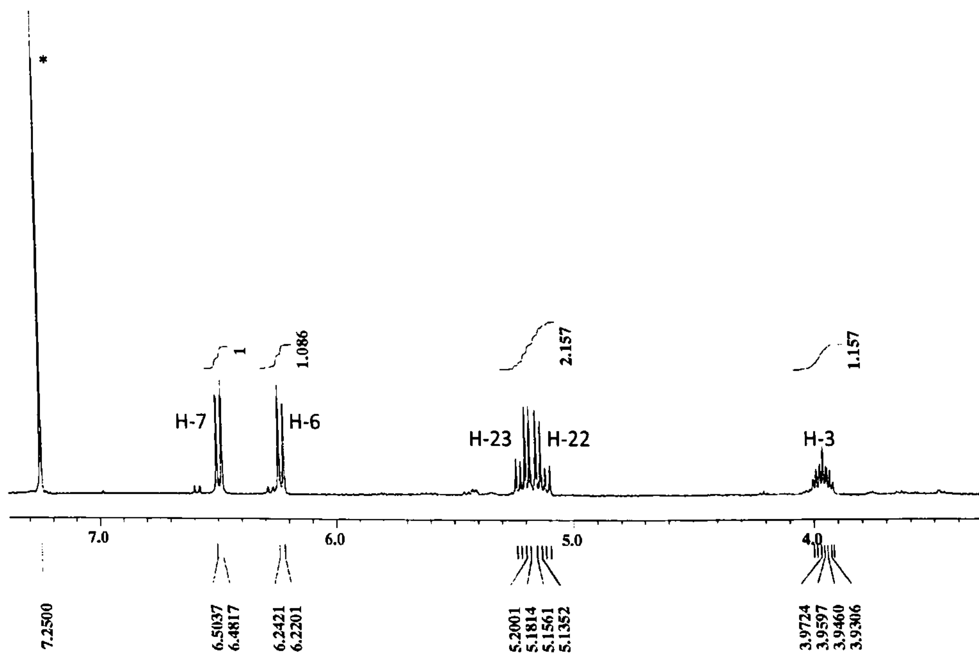
sterols rather than commonly occurring 3β-hydroxy phytosterols (Gauvin *et al.*, 2000).

Table 3.5: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for JS-10 in $CDCl_3$

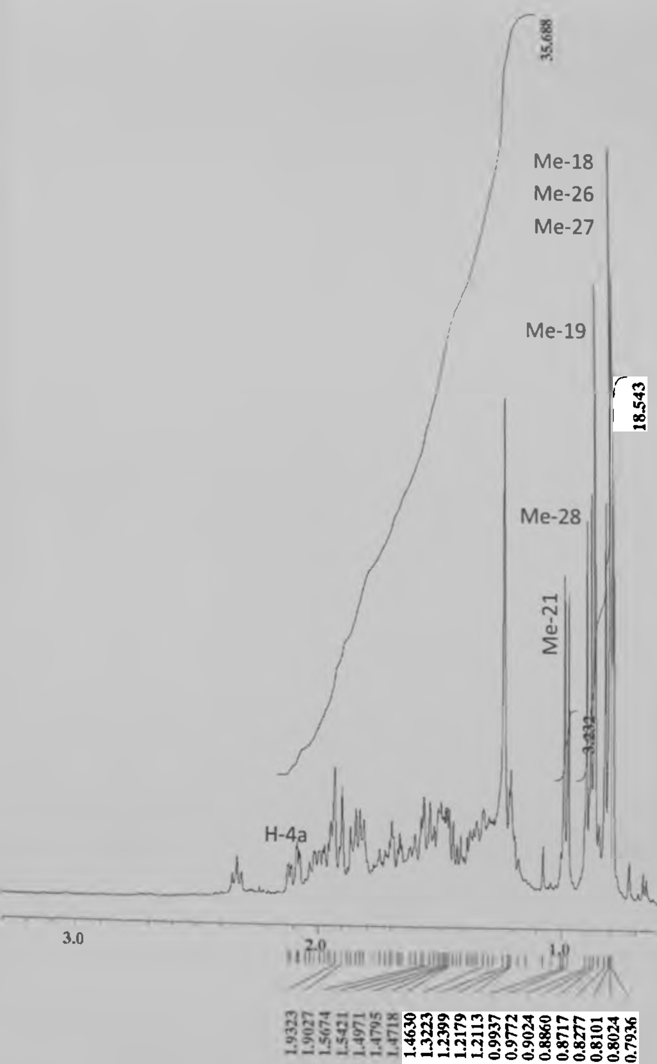
Position	δ_H	δ_C	Selected HMBC correlations
1	1.94 (1H, *m*) / 1.67 (1H, *m*)	34.6	
2	1.82 (1H, *m*) / 1.52 (1H, *m*)	30.0	
3	3.95 (1H, *dddd* 4.8, 5.3, 11.4, 11.4)	66.5	
4	2.10 (1H, *m*) / 1.89 (1H, *m*)	36.8	C-2, C-3, C-5, C-6, C-10
5	-	82.1	
6	6.23 (1H, *d* 8.8)	135.3	C-5, C-8, C4/10
7	6.49 (1H, *d* 8.8)	130.7	C-5, C-8
8	-	79.4	
9	1.49 (1H, *m*)	51.0	
10	-	36.8	
11	1.21 (1H, *m*) / 1.95 (1H, *m*)	23.3	
12	1.23 (1H, *m*) / 1.93 (1H, *m*)	39.3	
13	-	44.5	
14	1.54 (1H, *m*)	51.6	
15	†	20.5	
16	†	28.6	
17	1.2 (1H, *m*)	56.1	C-18
18	0.80 (3H, *s*)	12.8	
19	0.87 (3H, *s*)	18.2	C-5
20	2.00 (1H, *m*)	39.7	
21	0.98 (3H, *d* 6.6)	20.8	C-17
22	5.12 (1H, *dd* 15.4, 7.5)	135.1	C-17
23	5.21 (1H, *dd* 15.4, 8.1)	132.3	C-20, C-28
24	1.83 (1H, *m*)	42.7	C-22, C-23
25	1.44 (1H, *m*)	33.0	
26#	0.79 (3H, *d* 7.0)	19.9	C-24
27#	0.81 (3H, *d* 7.0)	19.6	C-24
28	0.89 (3H, *d* 6.6)	17.5	C-23

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz. #Values are interchangeable. † δ_H values for 15 and 16 methylene protons were not determined due to signal overlapping.

Fig. 3.15: Structure and selected NOESY correlations of JS-10



Spectrum 3.18: ^{1}H NMR (400 MHz, $CDCl_3$*) spectrum of JS-10



35.688
Me-18
Me-26
Me-27
Me-19
18.543
Me-28
Me-21
H-4a
3.0
2.0
1.0
1.9323
1.9027
1.5674
1.5421
1.4971
1.4795
1.4718
1.4630
1.3223
1.2399
1.2179
1.2113
0.9937
0.9772
0.9024
0.8860
0.8717
0.8277
0.8101
0.8024
0.7936

3.1.2.6 Characterisation of JS-11 as a mixture of 6β-hydroxy-stigmasta-4-en-3-one (JS-11a) and 6β-hydroxy-stigmasta-4,22-dien-3-one (JS-11b)

These compounds were isolated as a mixture from the ethyl acetate extract of *L. adscendens*. On TLC, **JS-11** showed quenching under short UV but no fluorescence under long UV light. Upon spraying *p*-anisaldehyde-sulphuric reagent and heating at 105°C, it gave a bright orange colour which faded on exposure to air.

The IR spectrum indicated the presence of α,β-unsaturated carbonyl (1691 cm^{-1}) and hydroxyl group (3500 cm^{-1}). Positive mode HRESI-MS showed two quasi-molecular ions $[M+H]^+$ at *m/z* 429.3728 and 427.3572 suggesting the molecular formula of $C_{29}H_{48}O_2$ (DBE=6) and $C_{29}H_{46}O_2$ (DBE=7), respectively.

The ^1H NMR spectrum (**Table 3.6**) showed the presence of olefinic methines at δ_H 5.81 (1H, *brs*), 5.12 (*dd* 8.3, 15.0 Hz), 5.00 (*dd* 8.8, 15.0 Hz) and an oxymethine at δ_H 4.33 (1H, *t* 2.6 Hz). When calculating, the integration for the methines at δ_H 5.81 and 4.33, which was approximately 2.5 fold larger than that of the methines at δ_H 5.12 and 5.00, the presence of two compounds was established. Except for one methyl singlet at δ_H 1.36, all methyl groups were found between δ_H 0.72 to 1.00. These data suggested the presence of phytosterol skeletons.

In the ^{13}C NMR spectrum (**Table 3.6**), the bulk of the carbon signals were clustered in the region of δ_C 11.9 to 55.9. Additionally, the spectrum showed the oxymethine carbon at δ_C 73.2, olefinic carbons at 126.3, 129.4, 138.1, 168.5 and a ketone at δ_C 200.5. In the HMBC experiment, the olefinic methine singlet at δ_H 5.81 (H-4) showed a ^3J correlation to the oxymethine at δ_C 73.2 (C-6). The most downfield

methyl (δ_H 1.36, Me-19) showed a ^3J correlation to the quaternary olefinic carbon at δ_C 168.5 (C-5) and to the methylene carbon at δ_C 37.0 (C-1). The above data suggested a hydroxyl group at C-6 and a double bond at Δ4,5 on the phytosterol skeleton. The presence of a keto group at C-3 was established from the presence of a ^3J correlation between the methylene proton at δ_H 1.95 (H-1a) and the carbon at δ_C 200.5. Further analysis of 2D experiments including COSY, HMBC and HMQC allowed the identification of **JS-11** as a mixture of 6β-hydroxy-stigmasta-4-en-3-one (**JS-11a**) and 6β-hydroxy-stigmasta-4,22-dien-3-one (**JS-11b**) in a ratio of approximately 1.0:0.4. Both compounds shared similar chemical shifts for most carbons and protons except for the side chain which was different due to the presence of a double bond (Δ22,23) in compound '**JS-11b**'.

The β-orientation of the C-6 hydroxyl group in both compounds was established from the NOESY spectrum where H-6 did not show any correlation with Me-19, thus placing H-6 on the α side of the ring plane. A NOESY correlation observed between H-4 and H-6 also supported this fact since a model with H-6 on the α side showed a H4-H6 distance of 2.275 Å (**Fig. 3.18**). The downfield shift of Me-19 (δ_H 1.36) also confirms the presence of a 6β-hydroxyl group (Nair and Chang, 1973). The stereochemistry at C-24 was assigned as *R* because of the chemical shift of C-24 which was in agreement with the literature on C-24*R* form steroids (Arai, *et al.*, 1998). The above data led to the identification of **JS-11a** and **JS-11b** as (24*R* form) 6β-hydroxy-stigmasta-4-en-3-one and (22*E*,24*R* form) 6β-hydroxy-stigmasta-4,22-dien-3-one, respectively and were in agreement with previous reports. Both compounds are reported for the first time from *Ludwigia adscendens*.

Both compounds have previously been isolated as a mixture from several other plants. They are believed to be genuine natural products rather than artefacts formed by oxidation during the isolation process (Fernandez, *et al.*, 1983; Hui, *et al.*, 1975; Wu *et al.*, 1995).

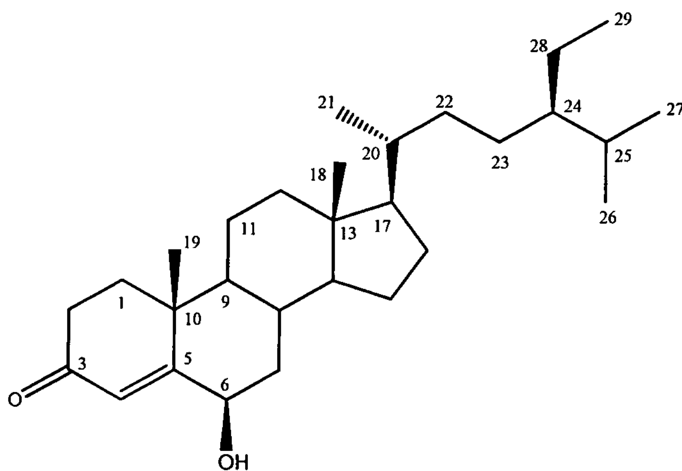


Fig. 3.16: Structures of JS-11a and JS-11b (Δ^{22})

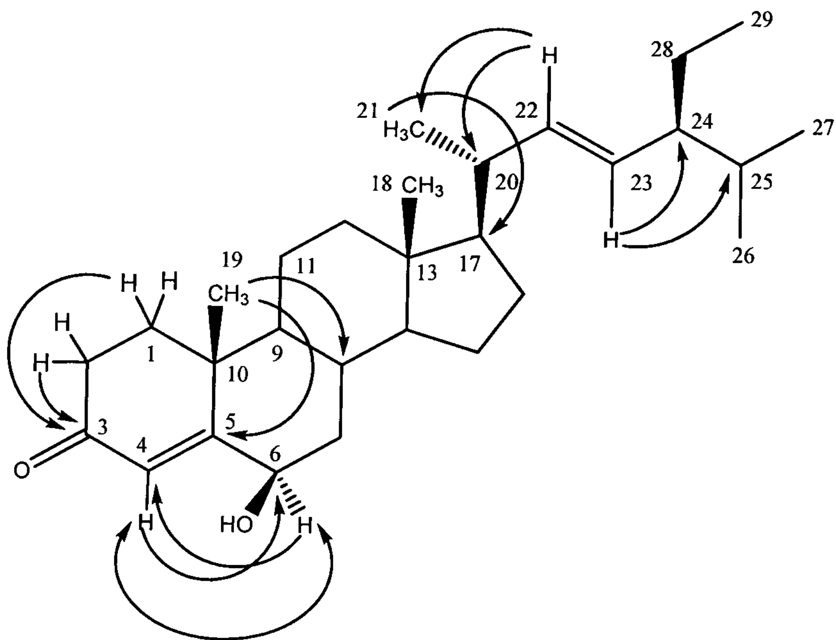


Fig. 3.17: Selected HMBC (→) and NOESY (↔) correlations observed in JS-11b

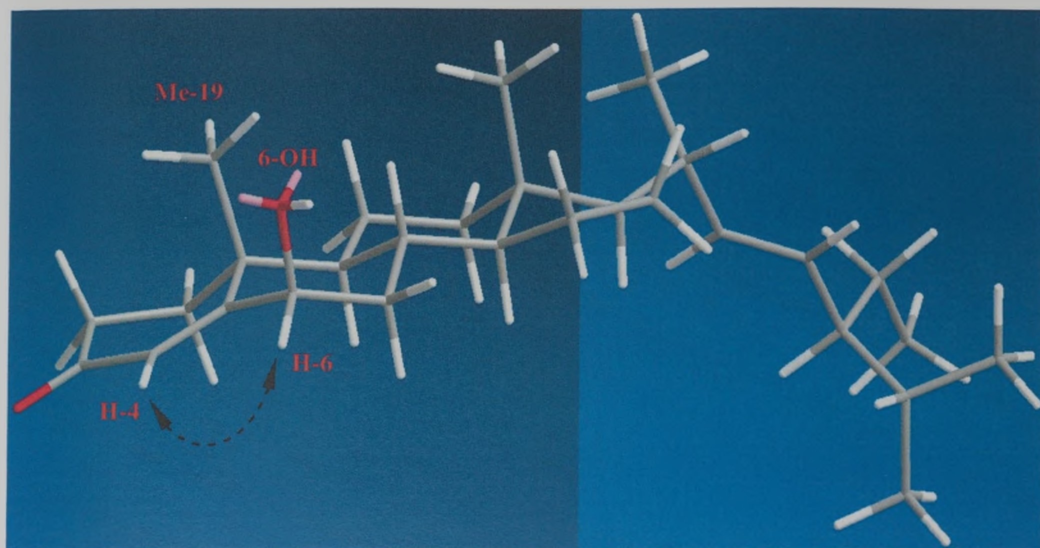


Fig.3.18: Energy minimised 3D structure of JS-11b using Chemdraw (◄··► shows H-4 and H-6 NOESY correlation)

Table 3.6: ^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data of JS-11a and JS-11b

Position	δ_H		δ_C	
	JS-11a	JS-11b	JS-11a	JS-11b
1	1.95 (1H, *m*), 1.67 (1H, *m*)		37.0	
2	2.36 (1H, *m*), 2.51 (1H, *m*)		34.4	
3	-		200.5	
4	5.81 (1H, *brs*)		126.2	
5	-		168.5	
6	4.33 (1H, *t* 2.60)		73.2	
7	1.22(1H, *m*), 1.98 (1H, *m*)		38.5	
8	1.93 (1H, *m*)		29.6	
9	0.90 (1H, *m*)		53.5	
10	-		37.9	
11	1.47 (1H, *m*), 0.80 (1H, *m*)		20.9	
12	†	1.14 (1H, *m*), 2.01 (1H, *m*)	39.5	39.4
13	-	-	42.4	42.3
14	†	†	55.8	55.9
15	†	†	24.1	
16	†	†	28.2	28.8
17	†	†	55.9	55.8
18	0.72 (3H, *s*)	0.74 (1H, *s*)	11.9	12.1
19	1.36 (3H, *s*)		19.4	
20	†	2.03 (1H, *m*)	36.0	40.5
21	0.90 (3H, *d* 6.6)	1.00 (3H, *d* 6.5)	18.7	21.1
22	†	5.12 (1H, *dd* 8.3,15.0)	33.8	138.1
23	†	5.00 (1H, *dd* 8.8,15.0)	25.9	129.4
24	0.91 (1H, *m*)	1.52 (1H, *m*)	45.7	51.3
25	1.66 (1H, *m*)	†	29.0	31.8
26	0.83 (3H, *d* ‡)	0.84 (3H, *d* ‡)	19.8	21.1
27	0.79 (3H, *d* ‡)	0.77 (3H, *d* ‡)	19.0	19.0
28	1.26 (1H, *m*), 1.20 (1H, *m*)	†	23.0	25.4
29	0.83 (3H, *m*)	0.79 (3H, *m*)	11.9	12.2

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz. Unassigned proton shifts (†) or coupling constants (‡) due to overlapping or because of ambiguity in determining the exact chemical shift or the coupling constants.

3.1.3 Ellagic acid derivatives

These compounds were isolated from the ethyl acetate and the methanol extracts of *Ludwigia adscendens* and *Trewia nudiflora*. All showed quenching under short UV and weak fluorescence under long UV light. After treatment with *p*-anisaldehyde-sulphuric acid reagent and heating at 105°C **JS-12, JS-13** and **JS-14** did not show any colour change while **JS-15** and **JS-16** turned pale yellow.

The IR spectra of all compounds showed one or two absorption bands in the region of 1752-1700 cm^{-1} supporting the presence of α,β-unsaturated carbonyl groups (Williams and Fleming, 2008). The IR spectra also indicated the presence of hydroxyl group(s) with the presence of absorption bands in the region of 3274-3435 cm^{-1} (Williams and Fleming, 2008). The UV spectra showed a similar pattern with absorption maxima at or around 247 and 370 nm.

Their ^1H NMR spectra (**Table 3.7 and 3.8**) showed the presence of two singlets in the region of δ_H 7.47 to 7.58 accounting for two aromatic protons. One exception was for **JS-14** which showed both aromatic protons together due to symmetry in the structure. These protons were found at δ_H 8.04/8.05 and δ_H 8.43/8.47 for some of the samples run in pyridine-d_5 instead of DMSO-d_6. The ^1H NMR also showed the presence of one or more methoxy groups in the region of δ_H 4.0-4.19.

In the ^{13}C NMR spectra (**Table 3.7 and 3.8**), all compounds showed two highly shielded carbonyls (δ_C 157.3-159.8) and 12 aromatic carbons. Among the 12 aromatic carbons, 8 appeared in the region of δ_C 130.7 to 154.3 indicating they were oxygen-bearing quaternary carbons. These data suggested that the above compounds

were highly oxygenated biphenyl derivatives possessing two carbonyl groups present in lactone rings as in the structure of ellagic acid. The isolated compounds only differed from each other by the presence of one or more than one methoxy, methylenedioxy, and/or rhamnose substituent(s) at some of the four hydroxyl functions. The nature of the substituent(s), their attachment site(s) as well as complete proton and carbon assignments for these ellagic acid derivatives were accomplished with the help of mass spectrometry and various 1D and 2D NMR experiments.

3.1.3.1 Characterisation of JS-12 as 3,3',4'-tri-O-methylellagic acid

This compound was isolated from the ethyl acetate extract of *Ludwigia adscendens*. The positive mode HRESI-MS data obtained for **JS-12** showed a quasi-molecular ion $[M+H]^+$ at *m/z* 345.0605 suggesting a molecular formula of $C_{17}H_{12}O_8$ (DBE=12).

In the ^{1}H NMR spectrum (**Spectrum 3.19, Table 3.7**) signals at δ_H 7.52 (1H, *s*) and 7.58 (1H, *s*) accounted for two aromatic protons of the ellagic acid structure. The spectrum also showed the presence of three methoxy groups at δ_H 4.00 (3H, *s*), 4.07 (3H, *s*) and 4.08 (3H, *s*). The ^{13}C NMR spectrum (**Spectrum 3.19, Table 3.7**) revealed three methoxy carbons at δ_C 56.4, 60.5 and 60.7 in addition to 14 carbon signals characteristic of the ellagic acid moiety. The downfield shift of two of the methoxy carbons (δ_C 60.5 and 60.7) indicated their attachment at sterically-hindered site(s). In the HMBC spectrum, the proton at δ_H 7.52 (H-5) and the methoxy group at δ_H 4.08 (3-OMe) showed ^3J correlation to the carbon at δ_C 140.0 (C-3). In the 1D

NOE difference experiment, irradiation of the signal at δ_H 7.52 (H-5) further showed an NOE effect to the hydroxyl proton at δ_H 3.07 (4-OH). This established the position of one of the methoxy groups at C-3. The proton at δ_H 7.58 (H-5') and the methoxy group at δ_H 4.07 (3'-OMe) showed a ^3J correlation to the carbon at δ_C 140.9 (C-3'). The methoxy group at δ_H 4.00 (4'-OMe) showed a ^3J correlation to the carbon at δ_C 153.4 (C-4') while H-5' showed a ^2J correlation to the same carbon. The irradiation of the signal at δ_H 7.58 (H-5') showed an NOE effect to 4'-OMe (δ_H 4.00). This clearly helped to establish the positions and assignments of the two remaining methoxy groups at C-3' and C-4'. The assignments of C-1/C-1' and C-6/C-6' were readily established from several HMBC experiments acquired using different evolution times. When the d6 (1/2*J*) value was set to 0.071 s, H-5 and H-5' showed weak ^4J 'W' coupling to C-1' and C-1, respectively (**Spectrum 3.20**). When the d6 value was set to 0.625 s, H-5 and H-5' showed more prominent ^2J correlation to C-6 and C-6', respectively. Thus, with the above data, **JS-12** was identified as 3,3',4'-Tri-*O*-methylellagic acid. Upon comparison of the assignments with those published in the literature, a discrepancy was observed for C-2'/C-3' and C-1'/C-6' assignments (Khac *et al.*, 1990).

This is the first report of 3,3',4'-tri-*O*-methylellagic acid from *Ludwigia adscendens* as well as from the genus *Ludwigia*.

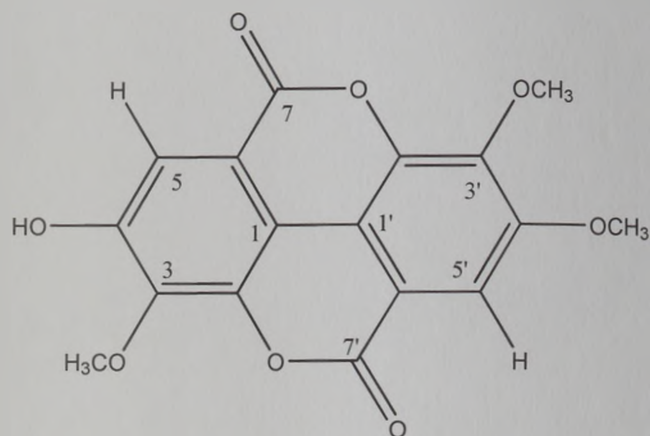


Fig. 3.19: Structure of JS-12

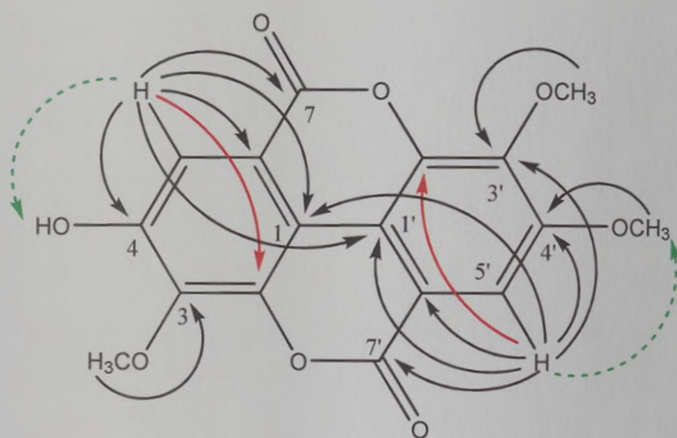
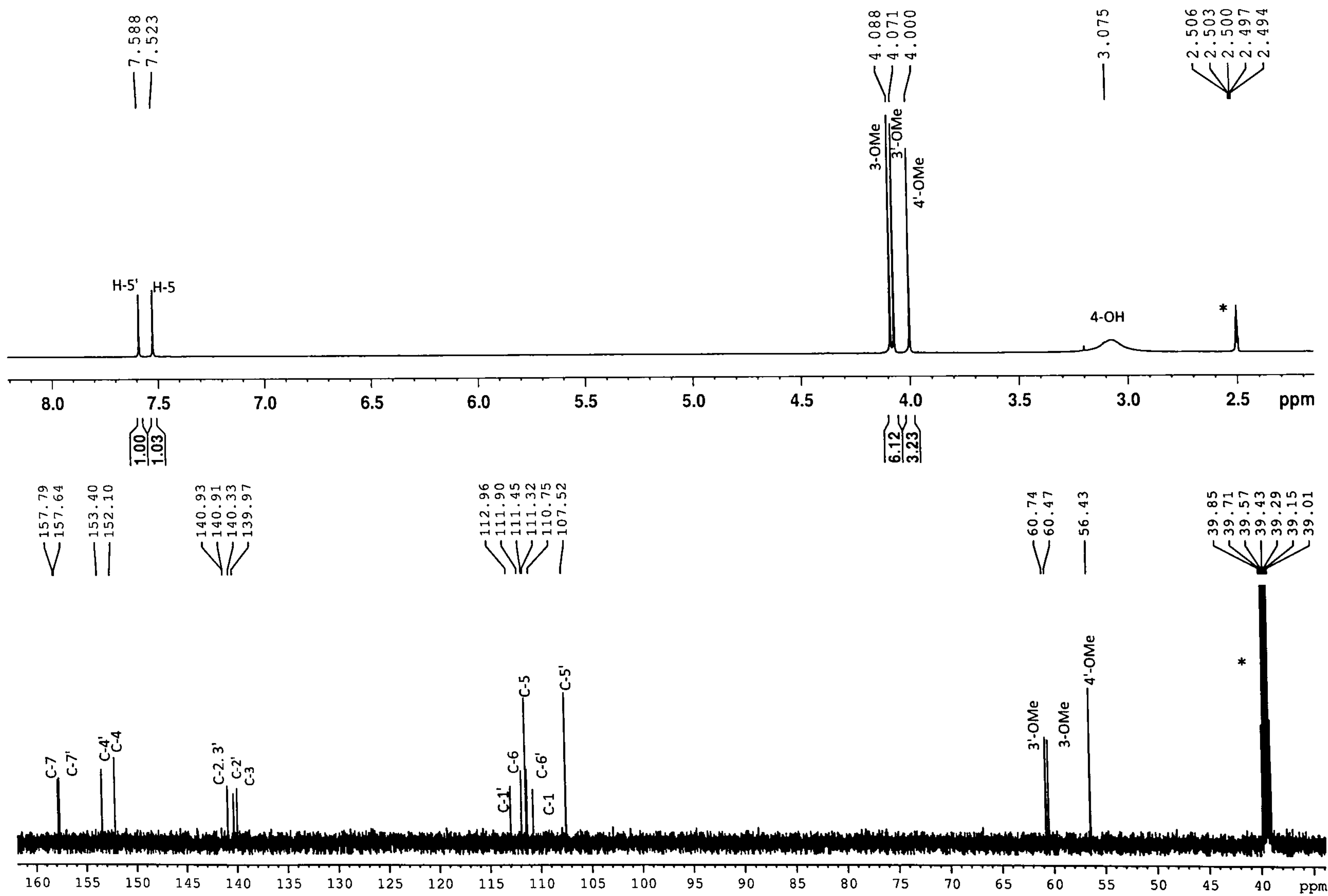
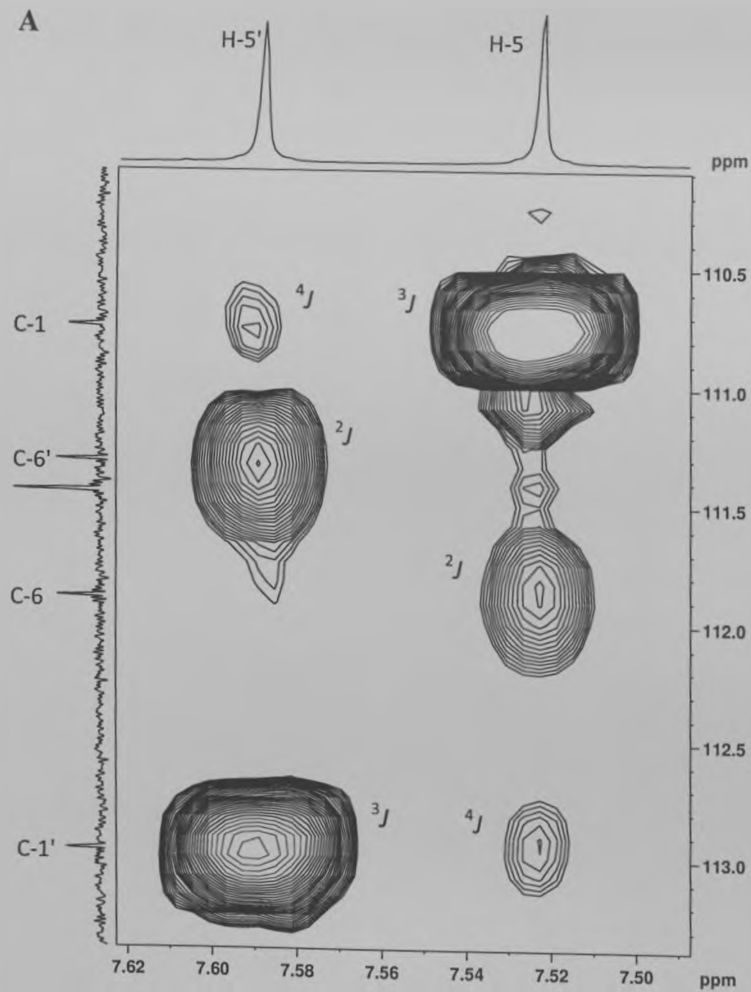
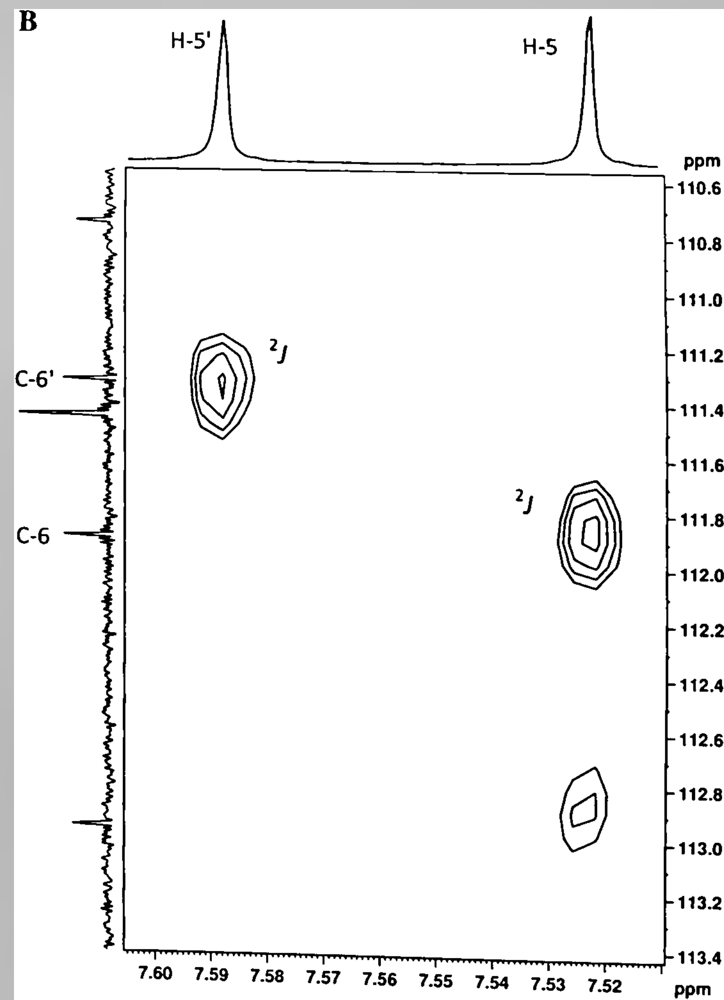


Fig. 3.20: Correlations observed for JS-12 in 1D NOE Difference (- - -➤) and in HMBC experiments with different evolution time (d6= 0.071 s ⟶), (d6=0.625 s ⟶).



Spectrum 3.19: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra of JS-12 in DMSO-d_6*





Spectrum 3.20: HMBC spectra of JS-12 (600 MHz, DMSO-d_6) with d6 set to 0.071 s (A) and 0.625 s (B) showing different intensities for ^2J, ^3J and ^4J correlations

3.1.3.2 Characterisation of JS-13 as pteleoellagic acid

This compound was isolated from the ethyl acetate extract of *Ludwigia adscendens*. The negative mode HRESI-MS data gave a quasi-molecular ion $[M-H]^-$ at *m/z* 327.0149 suggesting a molecular formula of $C_{16}H_8O_8$ (DBE=13).

The ^1H NMR spectrum (**Spectrum 3.21, Table 3.7**) of **JS-13** showed two aromatic protons at δ_H 7.50 (1H, *s*) and 7.52 (1H, *s*), a methylenedioxy and a methoxy group at δ_H 6.37 (2H, *s*) and 4.05 (3H, *s*), respectively. The ^{13}C NMR spectrum (**Spectrum 3.21, Table 3.7**) showed the presence of 16 carbons, *i.e.*, two more carbon signals than that of ellagic acid itself, which were attributed to the methylenedioxy (δ_C 104.2) and the methoxy group (δ_C 60.9). This was confirmed in the DEPT spectrum. The downfield shift of the methoxy carbon at δ_C 60.9 indicated its attachment to a sterically-hindered site. In the HMBC spectrum, the methylenedioxy protons at δ_H 6.37 showed a ^3J coupling to carbons at δ_C 138.2 (C-3) and δ_C 150.0 (C-4). The double bond equivalence of 13 (one extra compared to **JS-12**) further supported the presence of the methylenedioxy bridge. The location of the methylenedioxy was further confirmed by 1D NOE Difference experiment where irradiation of the signal at δ_H 7.50 (H-5) led to the enhancement of the methylenedioxy protons at δ_H 6.37.

The downfield shift of the methoxy carbon (δ_C 60.9) supported its preferred attachment site at C-3'. In the 1D NOE Difference experiment, irradiation of the signal δ_H 7.52 (H-5') showed enhancement of the hydroxyl group at δ_H 3.32 proving the presence of the free hydroxyl group at C-4' (**Spectrum 3.22**). Thus, **JS-13** was

identified as 3,4-methylenedioxy-3'-*O*-methyl-ellagic acid or pteleoellagic acid. All spectral data were in agreement with previous reports (Khallouki *et al.*, 2007).

Although ellagic acid has already been isolated from *Ludwigia octovalvis*, and its derivatives are widespread in plants, the occurrence of pteleoellagic acid is quite rare and has never been reported from *Ludwigia adscendens* (Correa *et al.*, 1975; Khac *et al.*, 1990; Lowry, 1968; Öksüz *et al.*, 2002; Rahman *et al.*, 2001; Yan and Yang, 2005).

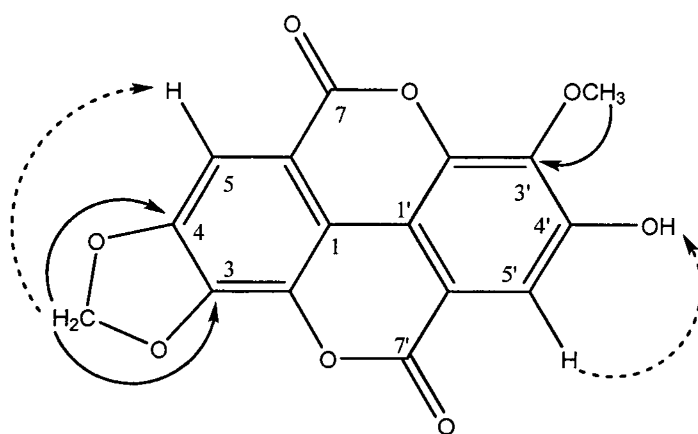
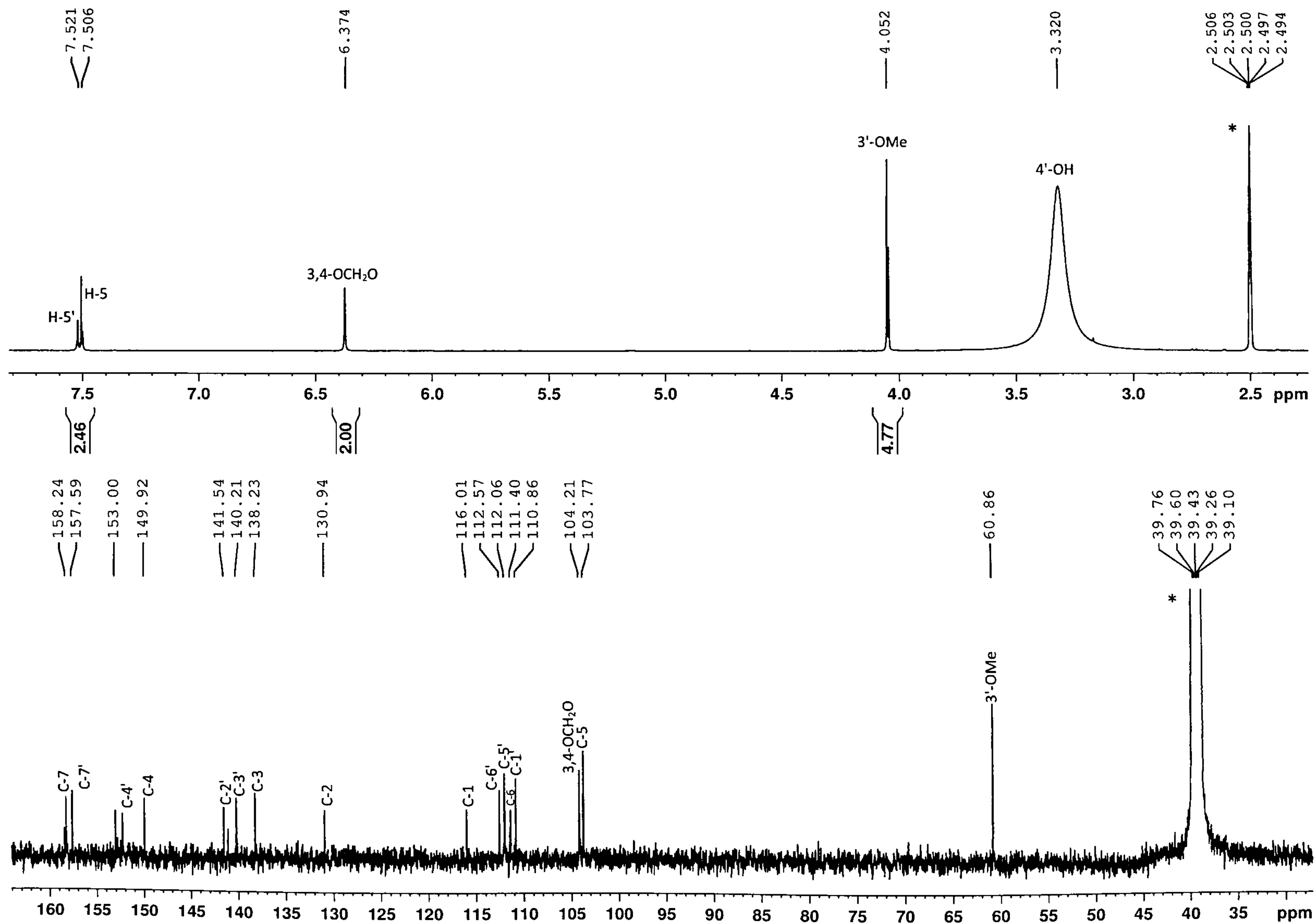
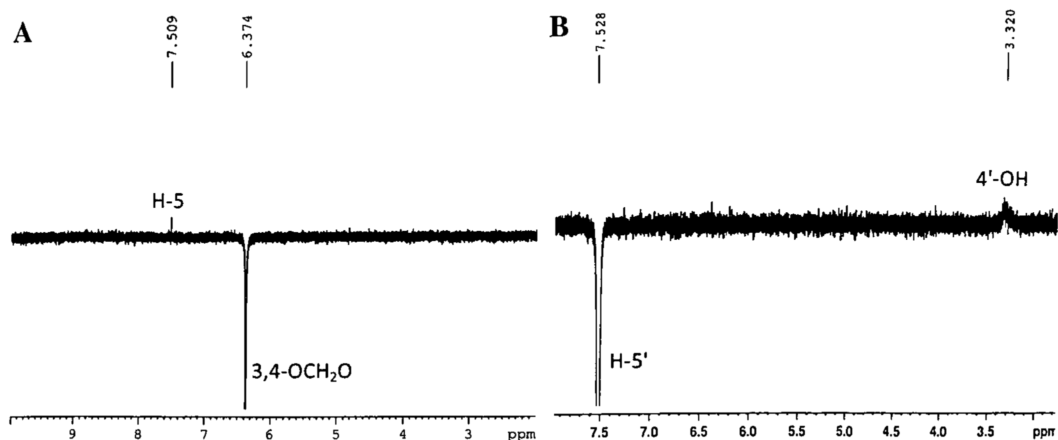


Fig. 3.21: Structure of JS-13 and key HMBC (——►) and 1D NOE (---►) correlations



Spectrum 3.21: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra of JS-13 in DMSO-d_6*



Spectrum 3.22: Selective irradiation (600 MHz, DMSO-d_6) of 3,4-OCH_2O (A) and H-5' (B) protons of JS-13

3.1.3.3 Characterisation of JS-14 as 3,3'-di-O-methylellagic acid

This compound was isolated from the ethyl acetate extract of *Trewia nudiflora*. The negative mode HRESI-MS data obtained for **JS-14** showed quasi-molecular ion $[M-H]^-$ at *m/z* 329.0297 suggesting for a molecular formula of $C_{16}H_{10}O_8$ (DBE=12).

The ^1H NMR spectrum (**Table 3.7**) showed structural symmetry with the presence of a signal at δ_H 7.47 (2H, *s*) and two methoxy groups at δ_H 4.04 (6H, *s*). The ^{13}C NMR spectrum (**Table 3.7**) showed the presence of eight carbons for a symmetrical structure with 16 carbons. The DEPT 135 spectrum, disclosed two methoxy groups, two aromatic methines and twelve quaternary carbons. The chemical shift for the methoxy carbons (δ_C 60.6) suggested their preferred positions as C-3 and 3'. In the HMBC experiment (**Spectrum 3.23**), the methoxy groups at δ_H 4.04 and the protons at δ_H 7.47 (H-5/5') showed ^3J correlation to carbons at δ_C 140.0 (C-3/3'), thus

establishing the position of the two methoxy groups in 3/3'. On this basis, **JS-14** was assigned as 3,3'-di-*O*-methyl ellagic acid. All spectroscopic data were in accordance with previous reports (Khallouki *et al.*, 2007; Sato, 1987).

This compound has already been reported from the stem bark of *Trewia nudiflora* and from other plants belonging to the Euphorbiaceae family including *Cleidion brevipetiolatum*, *Euphorbia sororia* and *Euphorbia soongarica* (Kang *et al.*, 2008; Lu *et al.*, 2007; Shi *et al.*, 2006; Zhang *et al.*, 2008).

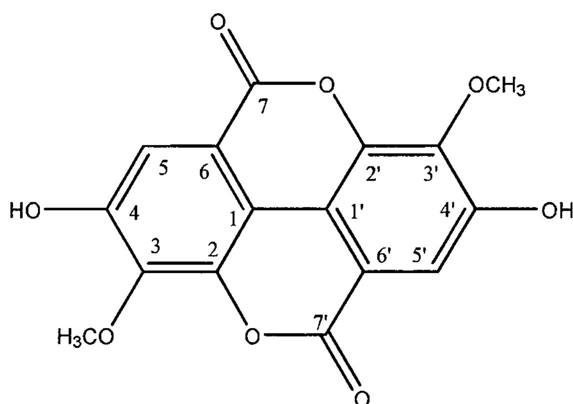
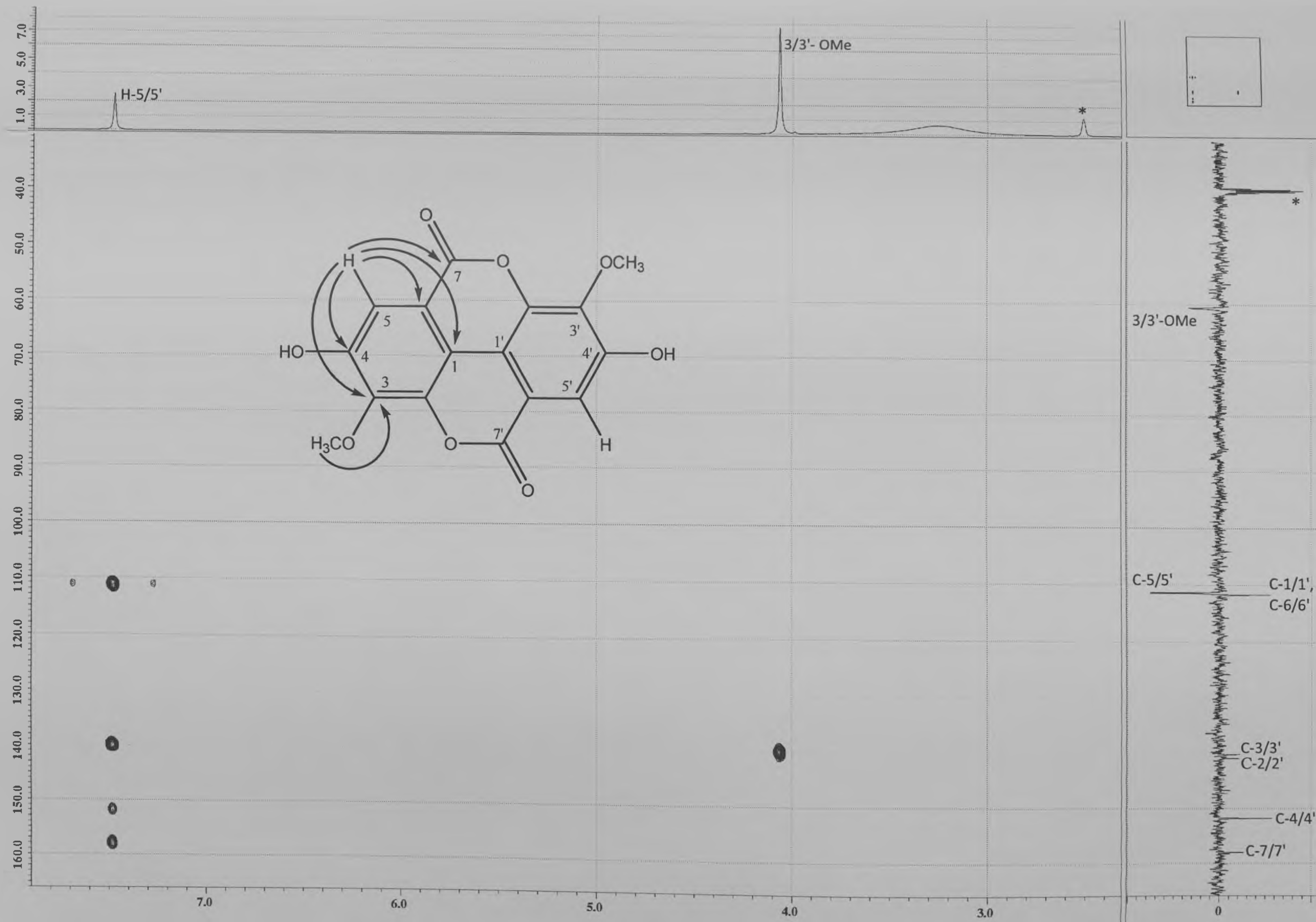


Fig. 3.22: Structure of JS-14



Spectrum 3.23: HMBC spectrum (400 MHz, 1/2J= 0.0625 s, DMSO-d_6*) and key HMBC correlations of JS-14

3.1.3.4 Characterisation of JS-15 as 3,3'-di-O-methylellagic acid 4-O-α-L-rhamnopyranoside

This compound was isolated from the methanol extract of *Trewia nudiflora.* The negative mode HRESI-MS showed a quasi-molecular ion $[M-H]^-$ at *m/z* 475.0865 suggesting a molecular formula of $C_{22}H_{20}O_{12}$ (DBE=13).

The ^{1}H NMR spectrum (**Spectrum 3.24; Table 3.8**) showed the presence of two aromatic protons at δ_H 8.05 (1H, *s*), 8.43 (1H, *s*) and two methoxy groups at δ_H 4.07 (3H, *s*) and 4.19 (3H, *s*). The spectrum also indicated the presence of a sugar unit with an anomeric proton at δ_H 6.33 (1H, *brs*), oxymethines at δ_H 4.85 (1H, *dd* 1.8, 3.3 Hz), 4.74 (1H, *dd* 3.3, 9.2 Hz), 4.43 (1H, *t* 9.2 Hz), 4.39 (1H, *m*) and a methyl group at δ_H 1.66 (3H, *d* 6.2 Hz). The H-1" and H-2" protons were assigned as equatorial due to the small coupling constant observed. The H-3", H-4" and H-5" protons were assigned as axial since H-4" appeared as a triplet with a coupling constant of 9.2 Hz. On the basis of these data, the sugar unit was identified as α-L-rhamnopyranoside. The ^{13}C NMR spectrum (**Table 3.8**) showed the presence of 22 carbons, of which 16 carbons accounted for the ellagic acid moiety and the remaining 6 to the sugar unit. The downfield shift of the two methoxy carbons (δ_C 61.2 and 61.6), indicated that they were between bulky substituents. In the HMBC experiment, the methoxy groups at δ_H 4.07 (3-OMe) and 4.19 (3'-OMe) showed ^3J correlation to C-3 (δ_C 141.3) and C-3' (δ_C 143.1), respectively. The anomeric proton at δ_H 6.33 showed ^3J correlation to C-4 (δ_C 151.3) establishing the position of the rhamnose moiety at C-4. The above data led to the identification of **JS-15** as 3,3'-di-

O-methylellagic acid 4-*O*-α-L-rhamnopyranoside. This compound has previously been reported from the stem bark of *Trewia nudiflora* (Kang *et al.*, 2008).

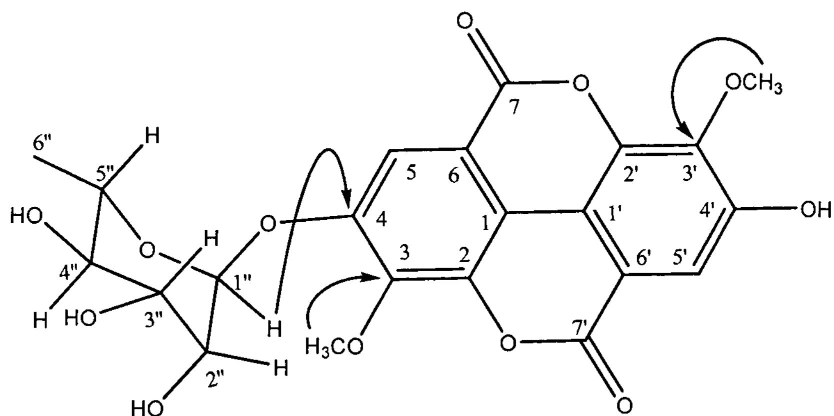


Fig. 3.23: Structure of JS-15 and key HMBC (→) correlations

3.1.3.5 Characterisation of JS-16 as 3-O-methylellagic acid 4'-O-α-L-rhamnopyranoside

This compound was isolated from the methanol extract of *Trewia nudiflora*. The positive ion mode HRESI-MS data showed quasi-molecular ions $[M+H]^+$ at *m/z* 463.0863 and $[M+Na]^+$ at *m/z* 485.0681 suggesting a molecular formula of $C_{21}H_{18}O_{12}$ (DBE=13).

The ^1H NMR spectrum (**Spectrum 3.24; Table 3.8**) showed two aromatic protons at δ_H 8.05 (1H, *s*) and 8.47 (1H, *s*) accounting for the H-5 and H-5' protons of the ellagic acid moiety, respectively. The presence of a rhamnose unit was detected with its anomeric proton at δ_H 6.43 (1H, *brs*), oxymethines at δ_H 4.92 (1H, *brs*), 4.78 (1H, *dd* 3.0, 9.2 Hz), 4.41 (1H, *t* 9.2 Hz), 4.60 (1H, *m*) and a methyl group at δ_H 1.65 (3H, *d* 6.2 Hz). Compound **JS-16** differed from **JS-15** by the presence of only one methoxy group at δ_H 4.19 (3H, s) instead of two. The ^{13}C NMR spectrum (**Table 3.8**) indicated the presence of 21 carbons including a methoxy carbon at δ_C 61.2. The downfield shift of this methoxy carbon indicated it to be flanked by two bulky substituents. The attachment site of the methoxy group and the rhamnose unit were determined with the help of HMBC and HMQC experiments. This led to the identification of **JS-16** as 3-*O*-methylellagic acid 4'-*O*-α-L-rhamnopyranoside. All spectral data were in agreement with previous reports (Liu *et al.*, 2009). This compound has previously been reported from the stem bark of *Trewia nudiflora* (Kang *et al.*, 2008).

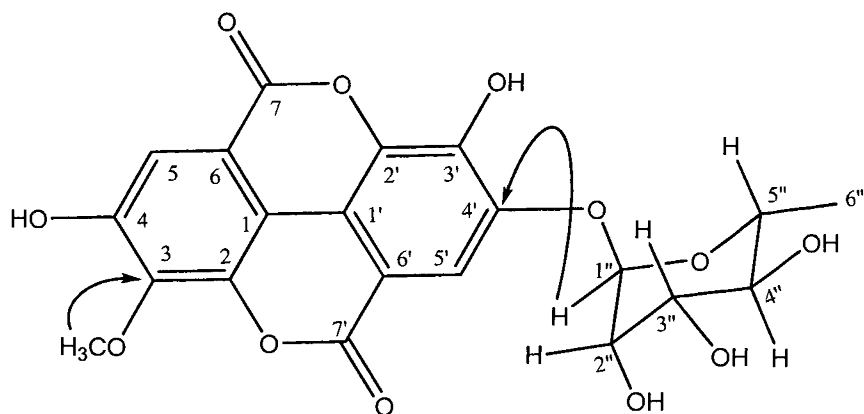
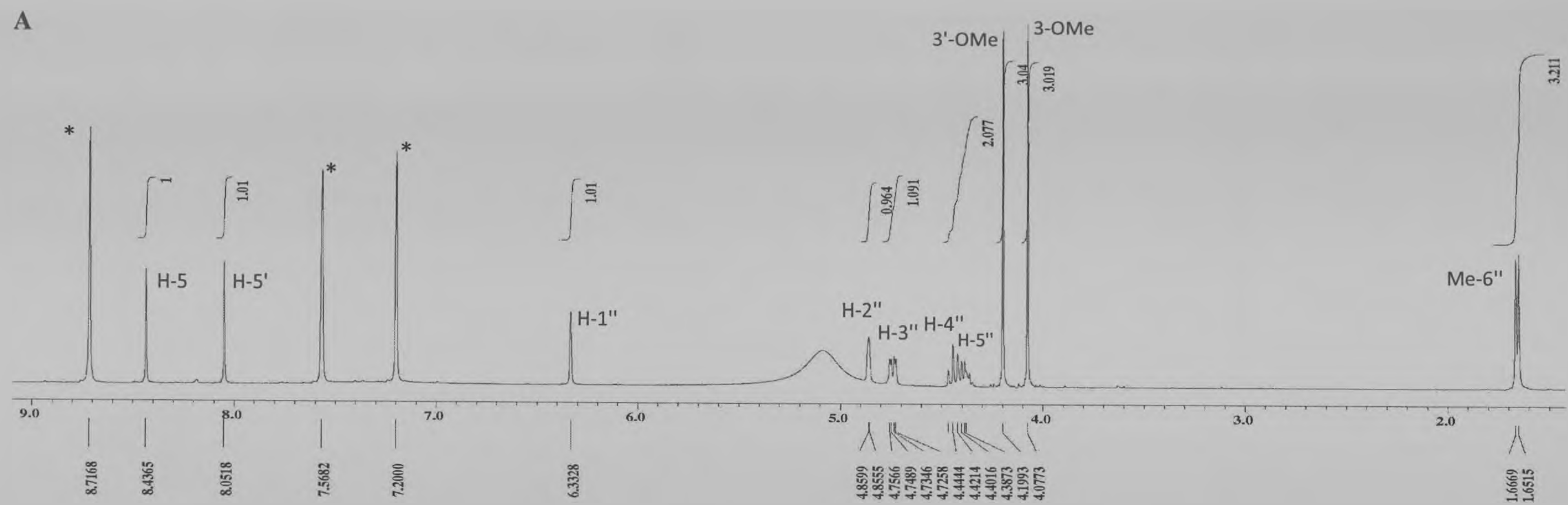
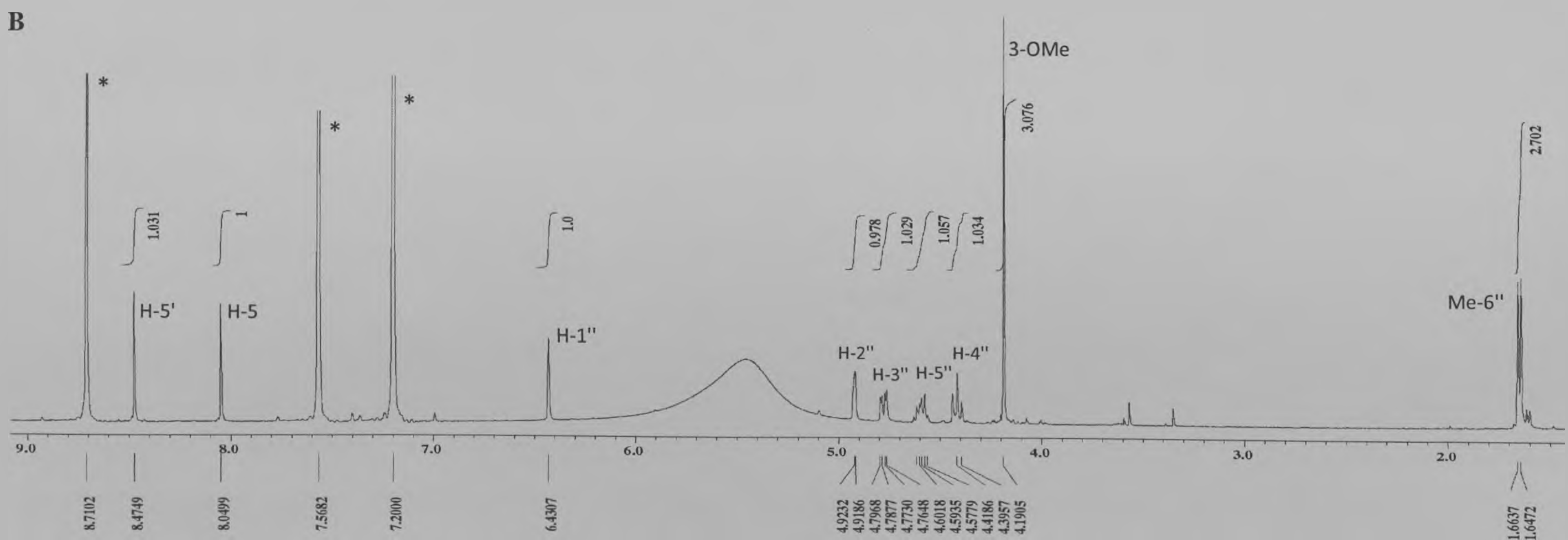


Fig. 3.24: Structure of JS-16 and key HMBC (→) correlations





Spectrum 3.24: ^{1}H NMR (400 MHz, C_5D_5N*) spectra of JS-15 (A) and JS-16 (B)

Table 3.7: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of JS-12, JS-13 and JS-14 in DMSO-d_6

Position	JS-12		JS-13		JS-14[§]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		110.7		116.0		111.6
2		140.9		130.9		140.8
3		140.0		138.2		140.0
4		152.1		150.0		151.8
5	7.52 (1H, *s*)	111.4	7.50 (1H, *s*)	103.8	7.47 (1H, *s*)	111.3
6		112.0		111.4		111.6
7		157.6		158.2		158.0
1'		113.0		110.9		111.6
2'		140.3		141.5		140.8
3'		140.9		140.2		140.0
4'		153.4		153.0		151.8
5'	7.58 (1H, *s*)	107.5	7.52 (1H, *s*)	112.1	7.47 (1H, *s*)	111.3
6'		111.3		112.6		111.6
7'		157.8		157.6		158.0
3-OMe	4.08 (3H, *s*)	60.5			4.04 (3H, *s*)	60.6
4-OMe						
3'-OMe	4.07 (3H, *s*)	60.7	4.05 (3H, *s*)	60.9		
4'-OMe	4.00 (3H, *s*)	56.4			4.04 (3H, *s*)	60.6
3,4-OCH_2O			6.37 (2H, *s*)	104.2		

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz. All NMR data were obtained at 60°C. [§] ^{1}H and ^{13}C were run at 400 MHz and 100 MHz, respectively.

Table 3.8: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of JS-15 and JS-16 in C_5D_5N

Position	JS-15		JS-16	
	δ_H	δ_C	δ_H	δ_C
1		114.9		112.3
2		141.8[#]		142.8
3		141.3		141.3
4		151.3		154.1
5	8.43 (1H, *s*)	113.0	8.05 (1H, *s*)	112.7
6		112.9		114.2
7		158.8		159.8
1'		111.6		115.5
2'		142.3[#]		137.6
3'		143.1		145.1
4'		154.3		148.1
5'	8.05 (1H, *s*)	113.2	8.47 (1H, *s*)	115.0
6'		113.8		107.2
7'		158.8		159.4
3-OMe	4.07 (3H, *s*)	61.6	4.19 (3H, *s*)	61.2
3'-OMe	4.19 (3H, *s*)	61.2		
1"	6.33 (1H, *brs*)	101.6	6.43 (1H, *brs*)	102.1
2"	4.85 (1H, *dd* 1.8, 3.3)	71.7	4.92 (1H, *brs*)	71.8
3"	4.74 (1H, *dd* 3.3, 9.2)	72.4	4.78 (1H, *dd* 3.0, 9.2)	72.5
4"	4.43 (1H, *t* 9.2)	73.4	4.41 (1H, *t* 9.2)	73.7
5"	4.39 (1H, *m*)	71.6	4.60 (1H, *m*)	71.4
6"	1.66 (3H, *d* 6.2)	18.3	1.65 (3H, *d* 6.2)	18.5

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz. All NMR data were obtained at 40°C. [#] Values are interchangeable.

3.1.4 Flavonoids

3.1.4.1 Characterisation of JS-17, JS-18 and JS-19 as flavonol 3-*O*-α-L-rhamnopyranosides

These compounds, except for **JS-19** which was isolated from the methanol extract, were isolated from the ethyl acetate extract of *Ludwigia adscendens*. On TLC, the compounds showed quenching under short UV and light yellow fluorescence under long UV light. After treatment with *p*-anisaldehyde-sulphuric acid reagent and heating at 105°C, all compounds turned yellow.

Their ^{1}H NMR spectra (**Table 3.9**) showed the presence of two aromatic spin systems with one system common in all compounds showing two highly-shielded *meta* coupled protons in the region of δ_H 5.87 to 6.54 (*i.e.*, 5,7 disubstituted A ring). The other aromatic system (B ring) exhibited various substitution patterns, including 4'-monosubstituted, 3',4'-disubstituted and 3',4',5'-trisubstituted systems. **JS-17**, **JS-18** and **JS-19** also showed the presence of a 6-deoxy sugar with an anomeric proton at δ_H 5.30-5.36 and a methyl doublet at δ_H 0.91-0.95.

Their ^{13}C NMR spectra (**Table 3.9**) indicated the presence of 21 carbons with 6 belonging to a hexose sugar unit. They showed a carbonyl carbon at δ_C 179.6-180.3 with the remaining carbons in the region of 94.7-166.5. Thus, the above data suggested that these compounds had a parent skeleton typical for flavonol glycosides.

3.1.4.1.1 Characterisation of JS-17 as afzelin

The negative mode HRESI-MS data obtained for **JS-17** showed a quasi-molecular ion $[M-H]^-$ at *m/z* 431.0979 suggesting a molecular formula of $C_{21}H_{20}O_{10}$ (DBE=12).

In the ^1H NMR spectrum (**Spectrum 3.25; Table 3.9**), the 1,2,3,5-tetrasubstituted ring A of the flavonoid structure was identified with the *meta*-coupled protons at δ_H 6.19 (1H, *d* 2.0 Hz), 6.36 (1H, *d* 2.0 Hz) while two *ortho*, *meta*-coupled doublets at δ_H 6.92 (2H, *dd* 8.8, 2.0 Hz), 7.75 (2H, *dd* 8.8, 2.0 Hz) suggested for a 1,4-*para* disubstituted B ring. The sugar unit was detected with an anomeric proton at δ_H 5.36 (1H, *d* 1.8 Hz) and methyl doublet at δ_H 0.91 (3H, *d* 5.7 Hz). Although the multiplicity of H-4" could not be calculated due to signal overlapping, the large coupling constant (*J*=9.2 Hz) observed for H-3" led to the assignment of H-3" and H-4" as axial protons and thus the sugar unit was identified as α-L-rhamnose.

In the HMBC spectrum, both the *meta*-coupled protons at δ_H 6.19 (H-6) and 6.36 (H-8) showed ^2J correlation to three highly-deshielded oxygen-bearing quaternary carbons at δ_C 163.2 (C-5), 166.0 (C-7) and δ_C 158.6 (C-9), 166.0 (C-7), respectively. The proton at δ_H 6.36 (H-8) also showed a ^4J 'W' coupling to the carbonyl carbon. The protons at δ_H 7.75 (H-2'/6') and 6.92 (H-3'/5') showed ^3J and ^2J correlation respectively to one oxygen-bearing carbon at δ_C 161.6 (C-4') establishing the presence of a OH substituent at C-4' on the B-ring. Protons at δ_H 7.75 (H-2'/6') also showed ^3J correlation to another oxygen-bearing deshielded carbon at 159.3 (C-2). The attachment site of the rhamnose unit was established from the HMBC spectrum

where the anomeric proton at δ_H 5.36 (H-1") showed a ^3J correlation to C-3 (δ_C 136.2) on the C-ring. On the basis of these results and by comparison with previously published data, **JS-17** was identified as kaempferol 3-*O*-α-L-rhamnopyranoside or afzelin (Kim *et al.*, 2004).

3.1.4.1.2 Characterisation of JS-18 as quercitrin

The positive ion HRFAB-MS for **JS-18** showed a quasi-molecular ion $[M+H]^+$ at *m/z* 449.1081 suggesting a molecular formula of $C_{21}H_{20}O_{11}$ (DBE=12). The IR spectrum indicated the presence of carbonyl (1654 cm^{-1}) and hydroxyl (3399 cm^{-1}) groups (Williams and Fleming, 2008).

The ^1H NMR spectrum (**Spectrum 3.25; Table 3.9**) of **JS-18** followed a similar pattern to that of **JS-17** with the presence of *meta*-coupled protons at δ_H 6.17 (1H, *d* 2.0) and δ_H 6.33 (1H, *d* 2.0) accounting for H-6 and H-8 protons of ring A, respectively. The presence of a α-L-rhamnopyranosyl unit was detected with an anomeric proton at δ_H 5.34 (1H, *d* 1.5 Hz) (H-1"), oxymethines at δ_H 4.22 (1H *dd* 1.5, 3.4 Hz) (H-2"), 3.75 (1H, *dd* 3.4, 9.3 Hz) (H-3"), 3.34 (1H, *t* 9.3 Hz) (H-4"), 3.41 (1H, *m*) (H-5") and a methyl group at δ_H 0.93 (3H, *d* 6.3 Hz) (H-6"). The B-ring followed a 1,3,4-substitution pattern with *ortho*-coupled, *ortho*, *meta*-coupled and *meta*-coupled protons at δ_H 6.89 (1H, *d* 8.3 Hz), 7.29 (1H, *dd* 2.0, 8.3 Hz) and 7.32 (1H, *d* 2.0 Hz), respectively. Thus, the ^1H NMR was found to be in close similarity to that of quercitrin.

In the ^{13}C NMR the carbonyl appeared at δ_C 180.3 and six oxygen-bearing aromatic quarternary carbons at δ_C 147.1, 150.5, 159.2, 160.0, 163.9 and 166.5. The downfield shift of C-3 (δ_C 136.2) also indicated the presence of an oxygen-bridge at C-3 connecting to the sugar unit. The above spectral data for **JS-18** were found to match with those of quercetin 3-*O*-α-L-rhamnopyranoside or quercitrin (Jung *et al.*, 2007).

3.1.4.1.3 Characterisation of JS-19 as myricitrin

Positive ion HRFAB-MS of **JS-19** showed a quasi-molecular ion $[M+H]^+$ at *m/z* 465.1035 suggesting a molecular formula of $C_{21}H_{20}O_{12}$ (DBE=12).

The ^{1}H NMR spectrum (**Spectrum 3.25; Table 3.9**) revealed an A ring identical to that of **JS-17** and **JS-18** with protons at δ_H 6.19 (1H, *d* 2.0 Hz) and 6.35 (1H, *d* 2.0 Hz) assigned as H-6 and H-8, respectively. The substitution pattern for B ring was suggested to be 1,3,4,5 due to the presence of a broad singlet at δ_H 6.94 which integrated for two protons. The sugar moiety was also established as α-L-rhamnopyranoside with an anomeric proton at δ_H 5.30 (1H, *d* 1.8 Hz) (H-1''), oxymethines at δ_H 4.21 (1H, *dd* 1,8, 3.1 Hz) (H-2''), 3.78 (1H, *dd* 3.1, 9.2 Hz) (H-3''), 3.33 (1H, *t* 9.2 Hz) (H-4''), 3.51 (1H, *m*) (H-5'') and a methyl group at δ_H 0.95 (3H, *d* 6.2 Hz) (H-6''). In the ^{13}C NMR spectrum (**Table 3.9**) the carbonyl appeared at δ_C 179.7 while two signals were of double intensity (δ_C 109.6 and 146.9). In the HMBC spectrum the protons at δ_H 6.94 (H-2'/6') showed direct as well as ^3J correlation to the carbon at δ_C 109.6 (C-2'/6'). These protons also showed ^2J correlation to the oxygen-bearing quaternary carbons at δ_C 146.9 (C-3'/5') and ^3J correlation to the

carbon at δ_C 137.9 (C-4'). This led to the consideration of the presence of three hydroxyl groups at C-3',4',5' positions. The protons at δ_H 6.94 (H-2'/6') also showed ^3J correlation to the carbon at δ_C 159.5 assigned as C-2. The anomeric proton of the rhamnose unit showed ^3J correlation to C-3 (δ_C 136.3). These data identified **JS-19** as myricetin-3-*O*-α-L-rhamnopyranoside or myricitrin in agreement with previous reports (Agarwal, 1989; David *et al.*, 1996).

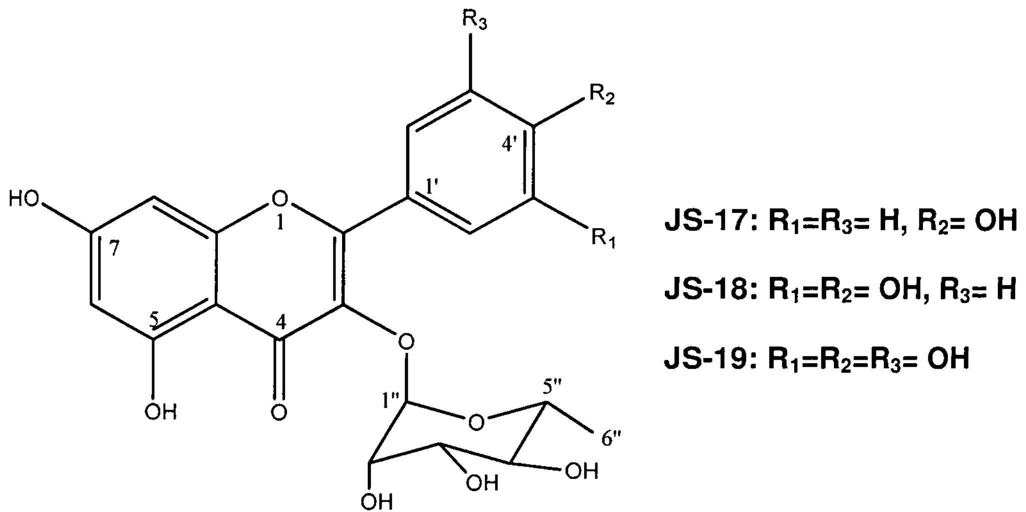
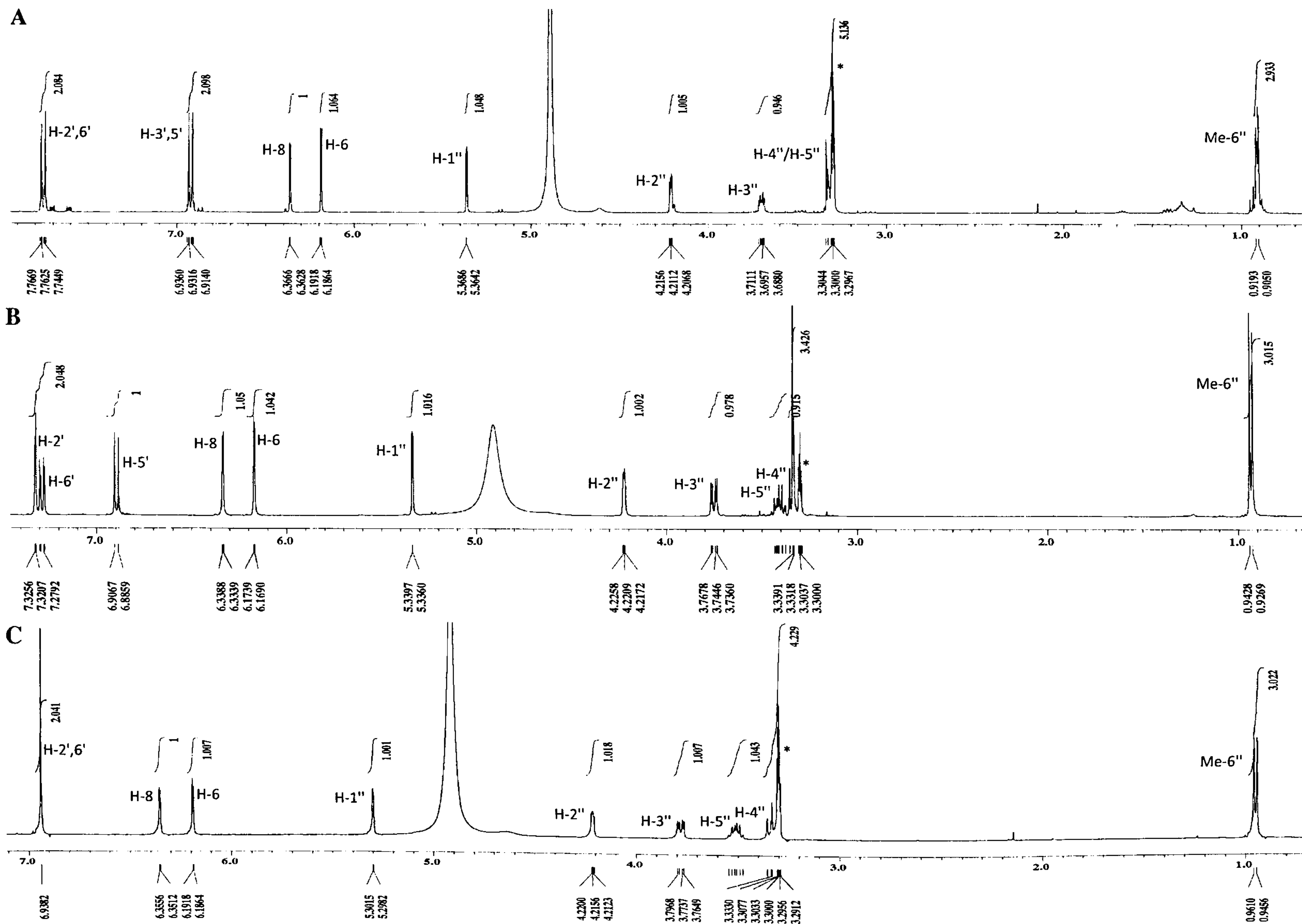


Fig. 3.25: Structures of JS-17, JS-18 and JS-19

Table 3.9: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of flavonol glycosides in CD_3OD

Position	JS-17		JS-18		JS-19	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	-	159.3	-	159.2	-	159.5
3	-	136.2	-	137.0	-	136.3
4	-	179.6	-	180.3	-	179.7
5	-	163.2	-	163.9	-	163.2
6	6.19 (1H, *d* 2.0)	99.9	6.17 (1H, *d* 2.0)	100.5	6.19 (1H, *d* 2.0)	99.8
7	-	166.0	-	166.5	-	165.9
8	6.36 (1H, *d* 2.0)	94.8	6.33 (1H, *d* 2.0)	95.5	6.35 (1H, *d* 2.0)	94.7
9	-	158.6	-	160.0	-	158.5
10	-	105.9	-	106.6	-	105.9
1'	-	122.7	-	123.7	-	121.9
2'	7.75 (1H, *dd* 8.8, 2.0)	131.9	7.32 (1H, *d* 2.0)	117.1	6.94 (1H, *s*)	109.6
3'	6.92 (1H, *dd* 8.8, 2.0)	116.6	-	147.1	-	146.9
4'	-	161.6	-	150.5	-	137.9
5'	6.92 (1H, *d* 8.8, 2.0)	116.6	6.89 (1H, *d* 8.3)	117.7	-	146.9
6'	7.75 (1H, *d* 8.8, 2.0)	131.9	7.29 (1H, *dd* 2.0, 8.3)	123.6	6.94 (1H, *s*)	109.6
1"	5.36 (1H, d 1.8)	103.5	5.34 (1H, *d* 1.5)	104.2	5.30 (1H, *d* 1.8)	103.7
2"	4.21 (1H, dd 1.8, 3.3)	72.1	4.22 (1H, *dd* 1.5, 3.4)	72.7	4.21 (1H, *dd* 1.8, 3.1)	72.0
3"	3.70 (1H, dd 3.3, 9.2)	72.1	3.75 (1H, *dd* 3.4, 9.3)	72.7	3.78 (1H, *dd* 3.1, 9.2)	72.0
4"	3.32 (1H, *m*)	73.2	3.34 (1H, *t* 9.3)	72.8	3.33 (1H, *t* 9.2)	73.4
5"	3.32 (1H, *m*)	71.9	3.41 (1H, *m*)	72.6	3.51 (1H, *m*)	72.0
6"	0.91 (3H, d, 5.7)	17.7	0.93 (3H, *d* 6.3)	18.4	0.95 (3H, *d* 6.2)	17.7

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz.



Spectrum 3.25: ^{1}H NMR (400 MHz, CD_3OD*) spectra of flavonol glycosides JS-17 (A), JS-18 (B) and JS-19 (C)

3.1.4.2 Characterisation of flavonol (JS-20) and dihydroflavonol (JS-21)

These compounds were isolated from the ethyl acetate extract of *Ludwigia adscendens*. On TLC the compounds showed quenching under short UV and light yellow fluorescence under long UV light. After treatment with *p*-anisaldehyde-sulphuric acid reagent and heating at 105°C, **JS-20** turned yellow while **JS-21** turned yellow to orange.

3.1.4.2.1 Characterisation of JS-20 as quercetin

The positive mode HRESI-MS of **JS-20** showed a quasi-molecular ion $[M+H]^+$ at 303.0494 suggesting a molecular formula of $C_{15}H_{10}O_7$ (DBE=11).

The ^1H NMR spectrum (**Spectrum 3.26; Table 3.10**) was identical to that of the flavonol glycoside **JS-18** except for the absence of the sugar unit. Thus, the protons at δ_H 6.26 (1H *d* 2.0 Hz) and 6.53 (1H, *d* 2.0 Hz) accounted for the A-ring H-6 and H-8 protons, respectively. Protons at δ_H 6.87 (1H, *d* 8.6 Hz), 7.62 (1H, *dd* 8.6, 2.2 Hz), 7.72 (1H *d* 2.2 Hz) represented the B ring of this molecule. The ^{13}C NMR spectrum (**Spectrum 3.26; Table 3.10**) indicated the presence of 15 carbons including a carbonyl at δ_C 177.3. These data were in agreement with those published for quercetin (Ternai and Markham, 1976).

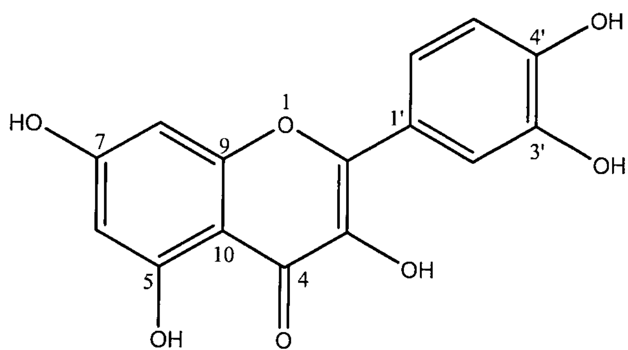
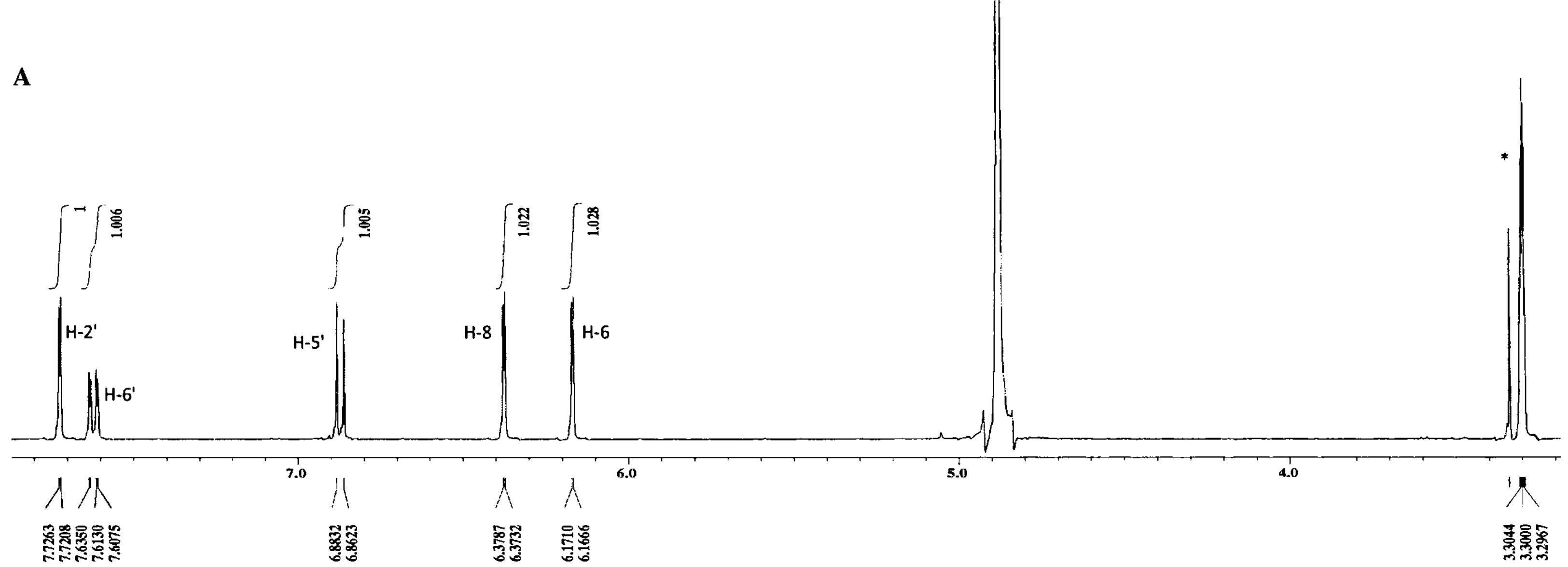
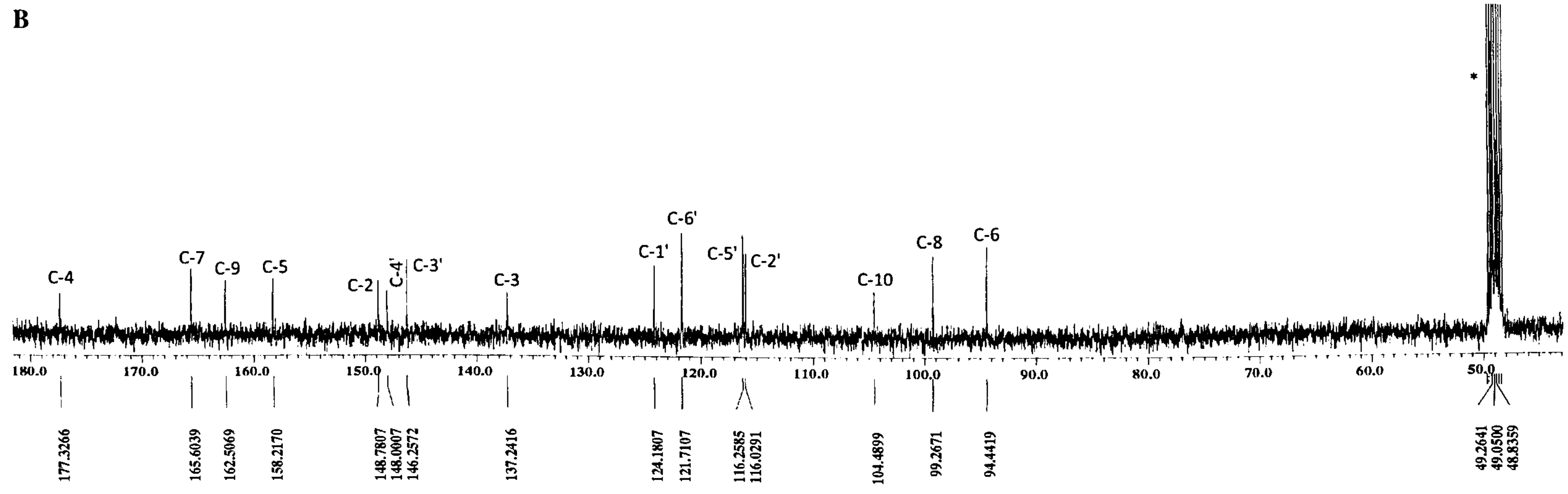


Fig. 3.28: Structure of JS-20

A



B



Spectrum 3.26: ^{1}H (400 MHz) (A) and ^{13}C (100 MHz) (B) NMR spectra of JS-20 in CD_3OD*

3.1.4.2.2 Characterisation of JS-21 as (+) trans taxifolin

Negative mode HRESI-MS of **JS-21** showed a quasi-molecular ion $[M-H]^-$ at 303.0515 suggesting a molecular formula of $C_{15}H_{10}O_7$ (DBE=11).

The ^{1}H NMR spectrum (**Spectrum 3.27; Table 3.10**) of **JS-21** showed the A-ring H-6 and H-8 protons at δ_H 5.87 (1H, *d* 2.2 Hz) and 5.91 (1H, *d* 2.2 Hz), respectively, while H-2', H-5' and H-6' of ring B appeared at δ_H 6.95(1H, *d* 2.2 Hz), 6.79 (1H, *d* 8.4 Hz) and 6.84(1H, *dd* 2.2, 8.4 Hz), respectively. An extra set of doublets at δ_H 4.91 (1H, *d* 11.4 Hz) and 4.50 (1H, *d* 11.4 Hz) suggested for the presence of a saturated double bond between C-2 and C-3. The large coupling constant of these protons accounted for their *trans*-diaxial configuration. Due to the low yields obtained for this compound, the carbon shifts were extracted from the HMBC and HMQC experiments. The saturation of the double bond caused an upfield shift of C-2 and C-3 while lack of conjugation made C-4 more deshielded (δ_C 199.2) (Pelter *et al.*, 1976). Measurement of the optical rotation confirmed that **JS-21** was (2*R*,3*R*)-dihydroquercetin or (+) *trans* taxifolin (Dok-Go *et. al.*, 2003).

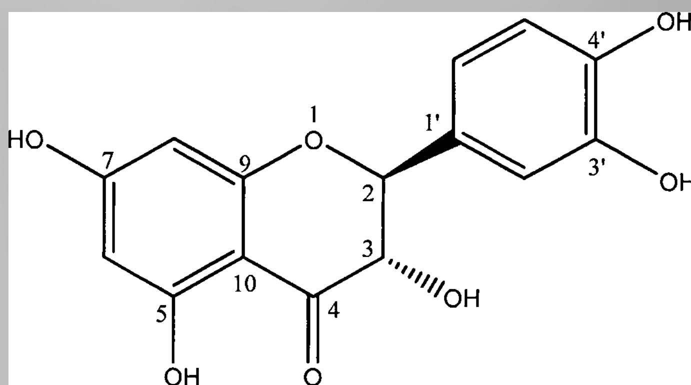
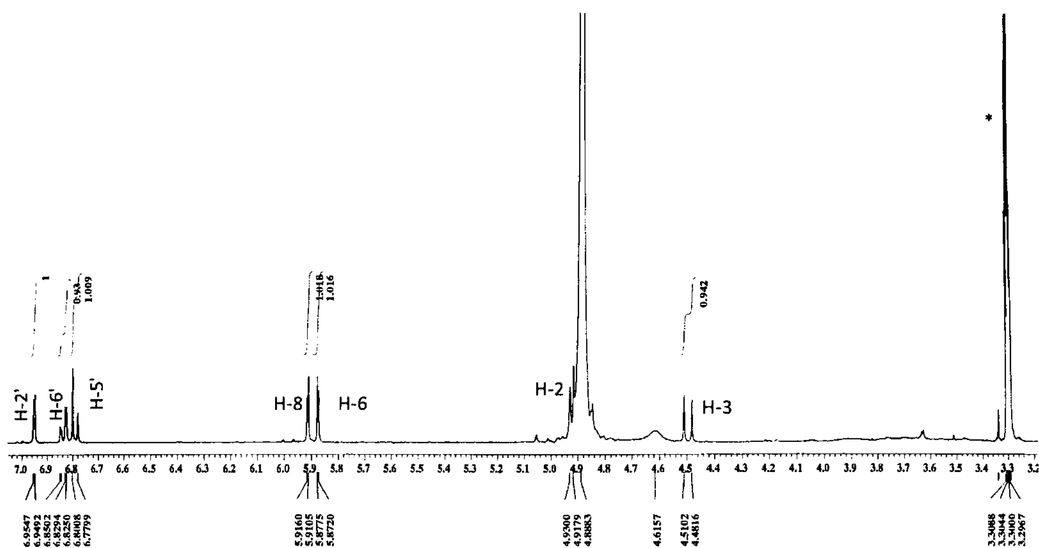


Fig. 3.29: Structure of JS-21



Spectrum 3.27: ^{1}H NMR (400 MHz, CD_3OD*) of JS-21

Table 3.10: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of JS-20 and JS-21 in CD_3OD

Position	JS-20		JS-21	
	δ_H	δ_C	δ_H	δ_C
2	-	148.8	4.91 (1H, *d* 11.4)	84.3
3	-	137.2	4.50 (1H, *d* 11.4)	72.7
4	-	177.3		199.2
5	-	158.2		163.8
6	6.17 (1H, *d* 2.0)	94.4	5.87 (1H, *d* 2.2)	95.6
7	-	165.6		167.9
8	6.54 (1H, *d* 2.0)	99.3	5.91 (1H, *d* 2.2)	96.4
9	-	162.5		164.5
10	-	104.5		101.0
1'	-	124.1		129.0
2'	7.72 (1H, *d* 2.2)	116.0	6.95 (1H, *d* 2.2)	115.2
3'	-	146.2	-	145.4
4'	-	148.0	-	146.4
5'	6.87 (1H, *d* 8.6)	116.2	6.79 (1H, 8.4)	115.0
6'	7.62 (1H, *dd* 8.6, 2.2)	121.7	6.84 (1H, *dd* 2.2, 8.4)	119.8

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz.

3.1.5 Simple phenolics

3.5.1. Characterisation of hydroxylated benzoic acid derivatives

These compounds were isolated from the ethyl acetate and the methanol extract of *Ludwigia adscendens* and the ethyl acetate extract of *Hygrophila auriculata.* When analysed on TLC, they showed quenching under short UV and faint glow under long UV light. After treatment with *p*-anisaldehyde-sulphuric acid reagent and heating at 105°C, the spots turned grey.

Their ^{1}H NMR spectra (**Table 3.11**) showed the presence of an aromatic ring with 1,4; 1,2,3; 1,3,4 or 1,3,4,5-substitution patterns. Their ^{13}C NMR spectra (**Table 3.12**) revealed the presence of 7 to 9 carbons including a carbonyl (δ_C 166.4-169.6). The presence of one or more than one hydroxyl functions on the aromatic ring was evidenced with some highly-deshielded aromatic quaternary carbons in the region of δ_C 137.8-162.3. The above spectral characteristics identified **JS-22**, **JS-23**, **JS-24** and **JS-25** as benzoic acid derivatives substituted with one or more than one hydroxyl groups.

3.1.5.1.1 Characterisation of JS-22 as methyl gallate

This compound was isolated from the methanol extract of *Ludwigia adscendens.* The HREI-MS for **JS-22** showed a molecular ion $[M]^+$ at *m/z* 184.0373 suggesting a molecular formula of $C_8H_8O_6$ (DBE=5). The IR spectrum indicated the presence of a carbonyl (1695 cm^{-1}) and hydroxyl groups (3468, 3316 cm^{-1}).

The ^1H NMR spectrum (**Table 3.11**) consisted of aromatic protons at δ_H 7.12 (2H, *brs*) and an esterified methyl group at δ_H 3.78 (3H, *s*). The ^{13}C NMR spectrum (**Table 3.12**) exhibited the presence of 8 carbons including a carbonyl at δ_C 166.4, three oxygen-bearing aromatic quaternaries at δ_C 145.2 (×2) and 138.0. In the HMBC spectrum, the protons at δ_H 7.12 (H-2/6) showed direct as well as ^3J correlation to the same carbon indicating their relative *meta* positions. They also showed ^3J correlation the carbonyl and one of the oxygen-bearing carbon (δ_C 137.9), and ^2J correlation to the other oxygen-bearing carbons (δ_C 145.2). The methyl group showed ^2J correlation to the carbonyl carbon. All the above information led to the conclusion that **JS-22** was the methyl ester of gallic acid or methyl gallate. All spectral data were in agreement with previous reports (Wang *et al.*, 2007). This is the first report of methyl gallate from *Ludwigia adscendens*.

3.1.5.1.2 Characterisation of JS-23 as gallic acid

This compound was isolated from the ethyl acetate extract and the methanol extract of *Ludwigia adscendens* as well as from the ethyl acetate extract of *Hygrophila auriculata*. The HREI-MS showed molecular ion $[M]^+$ at *m/z* 170.0212 suggesting a molecular formula of $C_7H_6O_5$ (DBE=5).

The ^1H and ^{13}C NMR (**Table 3.11 and 3.12**) spectrum followed the same pattern as that of **JS-22**. A signal at δ_H 7.14 (2H, *s*) accounted for H-2/6 protons. The carbonyl carbon appeared at δ_C 167.8 (C-7), and three oxygen-bearing quaternary aromatic carbons were observed at δ_C 146.0 (×2) and 138.6 (C-3/5 and C-4, respectively). The

spectral data of **JS-23** were in agreement with those published for gallic acid (Owen *et al.*, 2003; Wang *et al.*, 2007).

Gallic acid has been isolated as an anti-dysentery compound from *Ludwigia prostrata* (Liu *et al.*, 1986). This is the first report of the isolation of gallic acid from *Ludwigia adscendens* and from *Hygrophila auriculata.*

3.1.5.1.3 Characterisation of JS-24 as protocatechuic acid

This compound was isolated from the ethyl acetate extracts of *Ludwigia adscendens* and *Hygrophila auriculata.* The HRCI-MS of **JS-24** showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 155.0336 suggesting a molecular formula of $C_7H_6O_4$ (DBE=5).

The ^1H NMR spectrum (**Table 3.11**) revealed a 1,3,4-trisubstituted aromatic ring with the presence of proton signals at δ_H 6.79 (1H, *d* 8.0 Hz), 7.42 (1H, *dd* 8.0, 1.8 Hz), 7.44 (1H, *d* 1.8 Hz). The ^{13}C NMR spectrum (**Table 3.12**) showed 7 carbons including a carbonyl at δ_C 169.6 and two oxygen-bearing aromatic quaternaries at δ_C 145.1 and 150.5. With the above data, **JS-24** was identified as 3,4-dihydroxy benzoic acid or protocatechuic acid. The ^1H, ^{13}C NMR and mass spectral data were in agreement with those published in the literature (Gutzeit, *et al.*, 2007). This is the first report of this compound from *Ludwigia adscendens* and from *Hygrophila auriculata.*

3.1.5.1.4 Characterisation of JS-25 as 4-hydroxy benzoic acid

This compound was isolated from the ethyl acetate extract of *Hygrophila auriculata*. The HRCI-MS for **JS-25** showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 139.0396 suggesting a molecular formula of $C_7H_6O_3$ (DBE=5).

The ^1H NMR spectrum (**Table 3.11**) indicated the presence of a 1,4-disubstituted aromatic ring with proton signals at δ_H 6.80 (2H, *d* 8.8 Hz), 7.86 (2H, *d* 8.8 Hz). Due to the low yield obtained for this compound, it was not possible to record its ^{13}C NMR spectrum. Instead, carbon assignments were extracted from the HMBC experiment. With seven carbons including one carbonyl (δ_C 169.5) and one highly-deshielded oxygen-bearing quaternary aromatic carbon (δ_C 162.3), **JS-25** was found to be structurally similar to the aforementioned benzoic acid derivatives. Its spectral data indicated the presence of one hydroxyl group in *para* position relative to the carboxylic acid function and **JS-25** was identified as *para* hydroxy benzoic acid or 4-hydroxy benzoic acid (4-HBA). The above spectral data were in agreement with previous reports (Cho *et al.*, 1998). This is the first report of 4-HBA from *Hygrophila auriculata*.

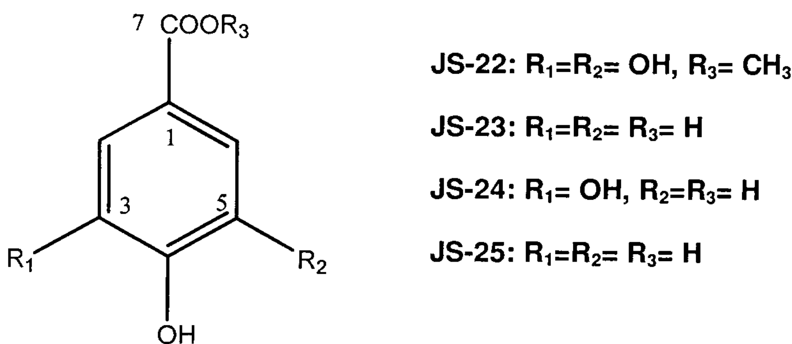


Fig. 3.30: Structures of JS-22, JS-23, JS-24 and JS-25

3.1.5.2 Characterisation of JS-26 as caffeic acid

This compound was isolated from the ethyl acetate extract of *Hygrophila auriculata*. The HREI-MS for **JS-26** showed a molecular ion peak $[M]^+$ at *m/z* 180.0426 suggesting a molecular formula of $C_9H_8O_4$ (DBE=5).

The ^1H NMR spectrum (**Table 3.11**) indicated the presence of 1,3,4-trisubstituted aromatic ring with proton signals at δ_H 6.77 (H, *d* 8.4 Hz), 6.92 (1H, *dd* 1.8, 8.4 Hz) and 7.03 (1H, *d* 1.8 Hz). The spectrum also showed the presence of *trans* olefinic protons at δ_H 6.21 (1H, *d* 16.0 Hz) and 7.52 (1H, *d* 16.0 Hz) suggesting a phenylpropanoic acid-type structure. ^{13}C NMR assignments (**Table 3.12**) were extracted from the HMBC spectrum. A total of 9 carbons were identified including a carbonyl at δ_C 170.2. The olefinic protons at δ_H 6.21 (H-8) and 7.52 (H-7) showed ^2J and ^3J correlation to the carbonyl (δ_C 170.2), respectively. The H-7 proton also showed ^3J correlation to two aromatic carbons at δ_C 114.2 (C-2) and 122.1 (C-6). The protons at δ_H 7.03 (H-2) and 6.92 (H-6), showed ^3J and ^2J correlation to oxygen-bearing quaternary aromatic carbons at δ_C 148.8 (C-4) and 146.0 (C-3), respectively. The above data suggested **JS-26** to be 3,4-dihydroxy (*E*)-cinnamic acid or (*E*)-caffeic acid. The spectral data were consistent with those published in the literature (Dürüst *et al.*, 2001; Hussein *et al.*, 2005).

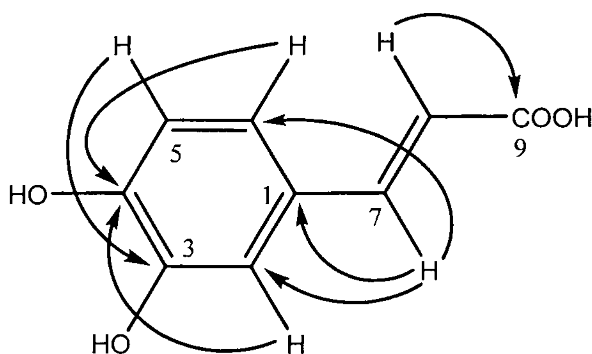


Fig. 3.31: Structure of JS-26 with key HMBC correlations

3.1.5.3 Characterisation of JS-27 as 4-hydroxyphthalide

This compound was isolated from the ethyl acetate extract of *Hygrophila auriculata*. On TLC, it showed quenching under short UV light and no fluorescence under long UV light. On spraying *p*-anisaldehyde sulphuric acid reagent and heating at 105°C, the spot did not show any colour change.

Negative mode HRESI-MS for **JS-27** showed a quasi-molecular ion peak $[M{-}H]^-$ at *m/z* 149.0244 suggesting a molecular formula of $C_8H_6O_3$ (DBE=6).

The ^{1}H NMR spectrum (**Spectrum 3.28; Table 3.11**) showed a 1,2,3-trisubstituted aromatic ring with protons at δ_H, 7.08 (1H, *d* 7.7 Hz), 7.32 (1H, *d* 7.7 Hz) and 7.39 (1H, *t* 7.7 Hz). The spectrum also showed the presence of a methylene group at δ_H 5.29 (2H, *s*). The ^{13}C NMR spectrum (**Spectrum 3.28; Table 3.12**) accounted for eight carbons including a carbonyl at δ_C 173.8, an oxygen-bearing quaternary aromatic carbon (δ_C 153.8) and another oxygen-bearing carbon at δ_C 69.7. A DEPT

135 experiment established the presence of three methines, one methylene. Comparison of DEPT spectrum with the ^{13}C NMR spectrum further revealed four carbons as quaternaries. In the HMBC experiment, the protons at δ_H 7.39 (H-6) and the methylene group at δ_H 5.29 (3-CH_2) showed ^3J correlation to the carbon at δ_C 153.8 (C-4). The proton at 7.08 (H-5) showed ^2J correlation to the same carbon. The proton at δ_H 7.32 (H-7) and the methylene group at δ_H 5.29 (3-CH_2) showed ^3J correlation to the carbonyl. This suggested the presence of a lactone ring on adjacent aromatic carbons. The presence of this extra ring also satisfied the 6 degree of unsturations calculated from the molecular formula. This led to the identification of **JS-27** as 4-hydroxy-1(3)-isobenzofuranone or 4-hydroxyphthalide. Further crystallographic analysis of **JS-27** confirmed this structure. All spectral data were in agreement with previous reports (Nobuhiro and Yasumasa, 2001).

This compound has been reported from some other plants (including oats) (Knights, 1966). This is the first report of its isolation from *Hygrophila auriculata*.

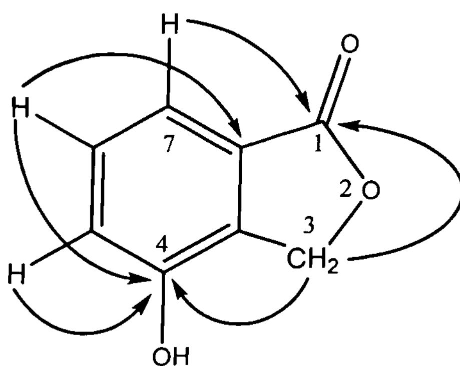


Fig. 3.32: Structure of JS-27 with selected HMBC correlations

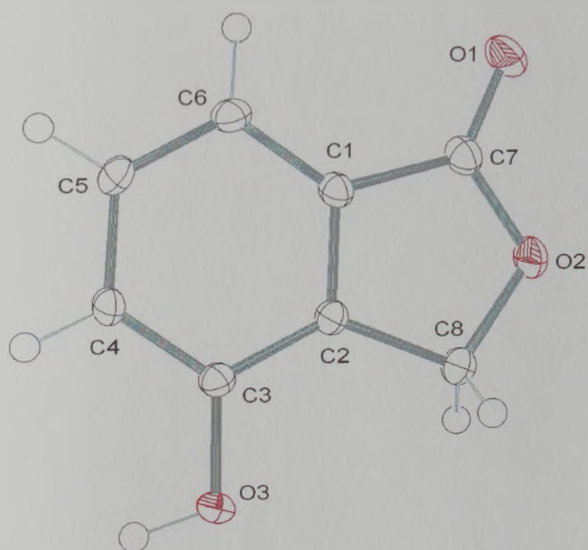
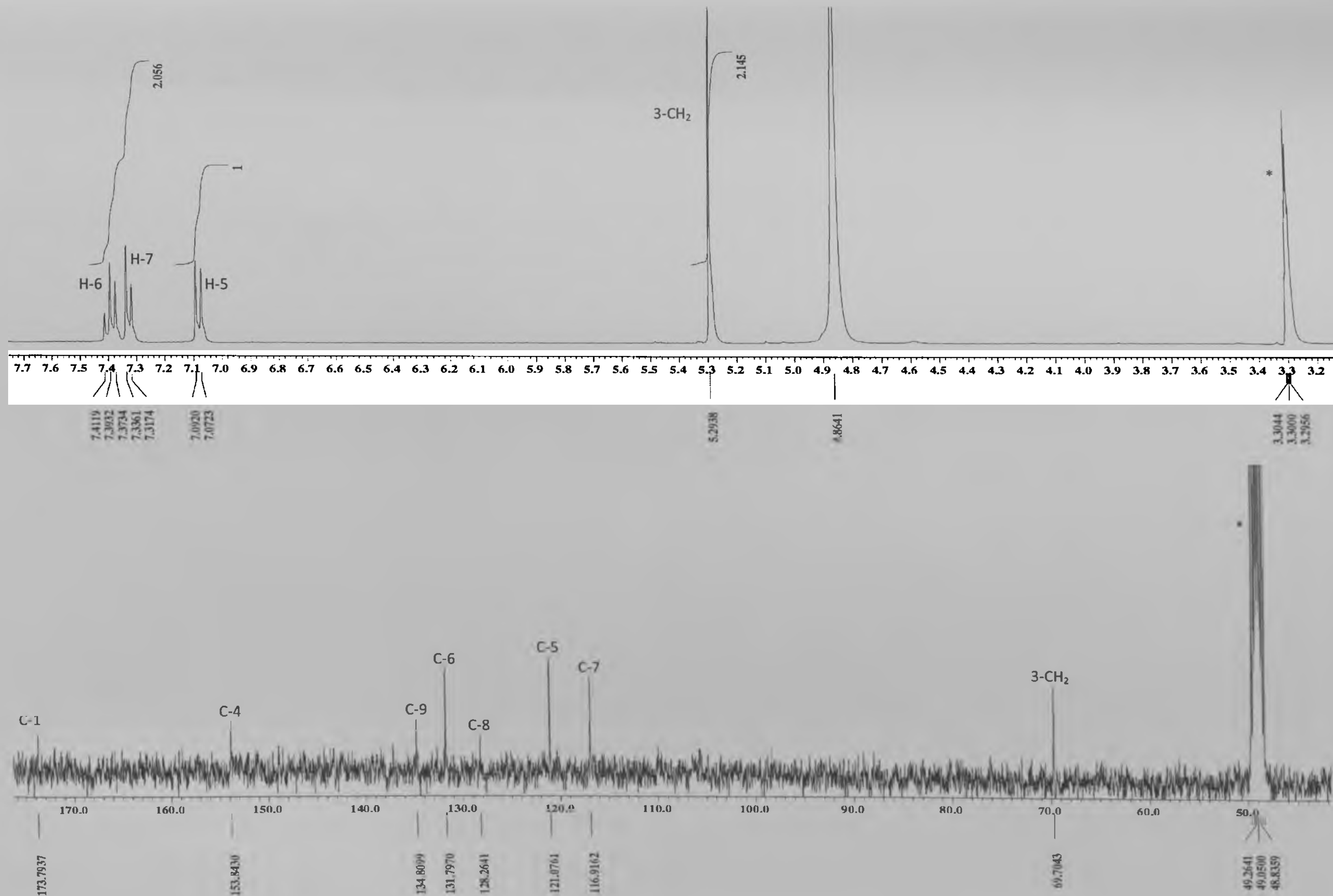


Fig. 3.33: ORTEP representation of the crystal structure of JS-27



Spectrum 3.28: ^{1}H (400 MHz) (A) and ^{13}C (100 MHz) (B) NMR spectra of JS-27 in CD_3OD*

Table 3.11: ^{1}H (400 MHz) NMR data of isolated simple phenolics in CD_3OD

Position	JS-22*	JS-23*	JS-24	JS-25	JS-26	JS-27
2	7.12 (1H, *brs*)	7.14 (1H, *s*)	7.44 (1H, *d* 1.8)	7.86 (2H, *d* 8.8)	7.03 (1H, *d* 1.8)	-
3	-	-		6.80 (2H, *d* 8.8)	-	5.29 (2H, *s*)
4	-	-		-	-	-
5	-	-	6.79 (1H, *d* 8.0)	6.80 (2H, *d* 8.8)	6.77 (1H, *d* 8.4)	7.08 (1H, *d* 7.7)
6	7.12 (1H, *brs*)	7.14 (1H, *s*)	7.42 (1H, *dd* 8.0, 1.8)	7.86 (2H, *d* 8.8)	6.92 (1H, *dd* 1.8, 8.4)	7.39 (1H, *t* 7.7)
7	-	-	-	-	7.52 (1H, *d* 16.0)	7.32 (1H, *d* 7.7)
8	3.78 (3H, s)				6.21 (1H, *d* 16.0)	-

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz. *Run in Acetone-d_6

Table 3.12: ^{13}C (100 MHz) NMR data of isolated simple phenolics in CD_3OD

Position	JS-22*	JS-23*	JS-24	JS-25	JS-26	JS-27
1	121.0	122.1	122.6	122.1	127.0	173.8
2	109.0	110.1	116.9	132.2	114.2	-
3	145.2	146.0	145.1	115.2	146.0	69.7
4	137.9	138.6	150.5	162.3	148.8	153.8
5	145.2	146.0	114.8	115.2	115.7	121.1
6	109.0	110.1	123.1	132.2	122.1	131.8
7	166.4	167.8	169.6	169.5	146.0	117.0
8	51.1				114.7	128.3
9					170.2	134.8

Chemical shifts are in ppm. *Run in Acetone-d_6

3.1.6 Miscellaneous compounds

3.1.6.1 Characterisation of JS-28 as scopoletin

This compound was isolated from the ethyl acetate extract of *Trewia nudiflora*. On TLC, **JS-28** showed quenching under short UV and a bright bluish glow under long UV light. The spot turned yellow when sprayed with *p*-anisaldehyde-sulphuric acid reagent and heated at 105°C.

The IR spectrum indicated the presence of a carbonyl (1701 cm^{-1}) and a hydroxyl group (3338 cm^{-1}) (Williams and Fleming, 2008). The HRCI-MS spectrum showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 193.0505 corresponding to a molecular formula of $C_{10}H_8O_4$ (DBE=7).

The ^1H NMR spectrum (**Table 3.13**) showed the presence of *cis* olefinic methines at δ_H 7.59 (1H, *d* 9.5 Hz) and 6.26 (1H, *d* 9.5 Hz), two aromatic proton singlets at δ_H 6.90 (1H, *s*), 6.83 (1H, *s*) and a methoxy group at δ_H 3.94 (3H, *s*). The ^{13}C NMR spectrum (**Table 3.13**) showed the presence of 10 carbons including a highly shielded carbonyl carbon at δ_C 161.4, two oxygen-bearing aromatic quaternary carbons (δ_C 144.0, 150.2) and a methoxy carbon at δ_C 56.4. All these features were in close similarity to that of a hydroxylated coumarin with a methoxy substituent. Thus, the *cis* olefinic methines were assigned as H-3 (δ_H 6.25) and H-4 (δ_H 7.59) of the pyranolactone ring, respectively. The aromatic protons were assigned as *para* since they appeared as singlets. In the HMBC spectrum, the proton at δ_H 7.59 (H-4) showed a strong ^3J coupling to the carbon at δ_C 107.5 (C-5). It also showed a weak ^4J 'W' coupling to the carbon at δ_C 103.2 (C-8). In the NOESY spectrum (**Spectrum**

3.29), the H-5 proton showed correlation to H-4 and to the methoxy group at δ_H 3.94, confirming the presence of the methoxy group at C-6 (δ_C 144.0). On the basis of the above data, **JS-28** was unambiguously identified as 7-hydroxy-6-methoxycoumarin or scopoletin. The spectral data were in agreement with previous reports (Öksüz *et al.*, 2002).

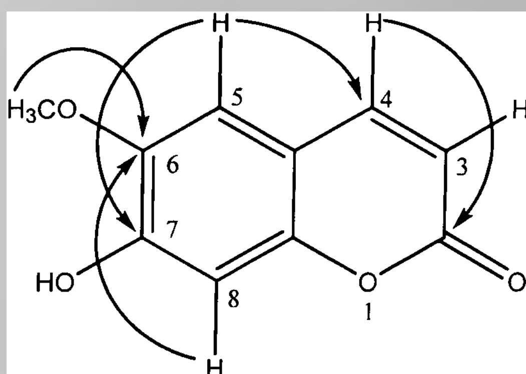
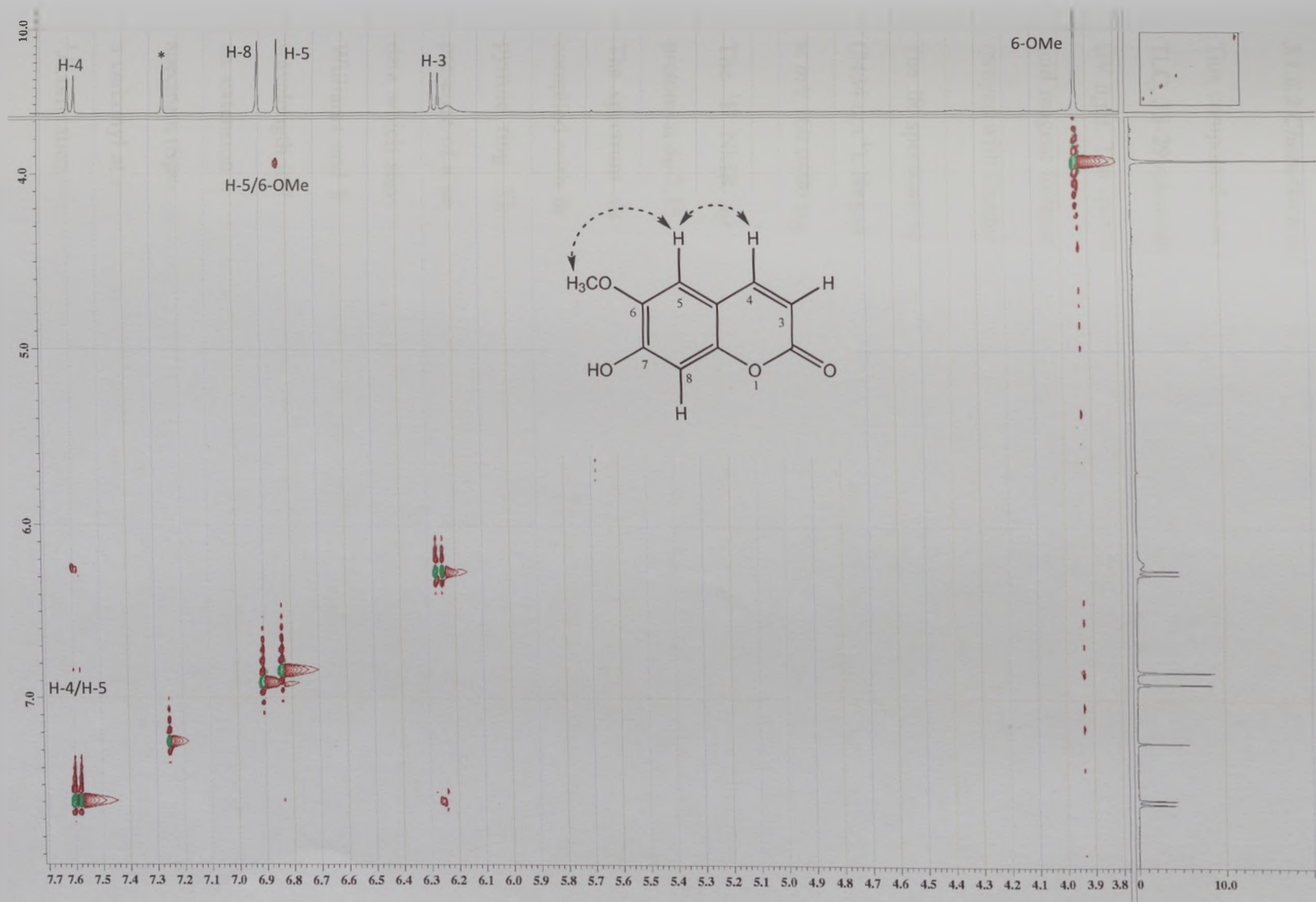


Fig. 3.34: Structure of JS-28 with selected HMBC correlations

Table 3.13: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of JS-28 in $CDCl_3$

Position	δ_H	δ_C
2	-	161.4
3	6.25 (1H, *d* 9.5)	113.4
4	7.59 (1H, *d* 9.5)	143.3
5	6.83 (1H, *s*)	107.5
6	-	144.0
7	-	150.2
8	6.90 (1H, *s*)	103.2
9	-	149.7
10	-	111.4
6-OMe	3.94 (3H, *s*)	56.4

Chemical shifts are in ppm. Coupling constants in the parentheses are in Hz.



Spectrum 3.29: NOESY spectrum (400 MHz, $CDCl_3$*) and key NOE correlations of JS-28

3.1.6.2 Characterisation of JS-29 as indole-3-carboxylic acid

This compound was isolated from the ethyl acetate extract of *Trewia nudiflora*. On TLC, **JS-29** quenched under short UV but did not show any fluorescence under long UV light. The spot turned bright orange when treated with *p*-anisaldehyde-sulphuric acid reagent followed by heating at 105°C. It did not give any positive result when sprayed with Dragendorff's reagent.

The IR spectrum indicated the presence of a carbonyl (1641 cm^{-1}) and a hydroxyl (3434 cm^{-1}). Negative mode HRESI-MS data showed a quasi-molecular ion $[M-H]^-$ at *m/z* 160.0400 suggesting a molecular formula of $C_9H_7O_2N$ (DBE=7).

The ^1H NMR spectrum (**Spectrum 3.30; Table 3.14**) showed the presence of protons at δ_H 7.15 (1H, *m*), 7.18 (1H, *m*), 7.46 (1H, *dd* 1.5, 7.0 Hz) and 8.00 (1H, *m*). The spectrum also showed a singlet at 7.99 (1H, *s*). Thus, the proton spectrum complied with that of an indole ring structure with one substituent attached to the pyrrole ring. This statement was supported by the IR data which showed the presence of a secondary amine group of an indole ring (3308 cm^{-1}, sharp) and UV data which showed absorption bands at 275, 355 and 371 nm (Bano *et al.*, 1987; Williams and Fleming, 2008). The appearance of UV absorption bands at higher wavelength indicated the presence of a carbonyl group in the pyrorole ring causing an extension of the conjugation (Williams and Fleming, 2008). The ^{13}C NMR spectrum (**Spectrum 3.30; Table 3.14**) showed the presence of 10 carbons including a carbonyl at δ_C 165.9. In the HMBC spectrum, the proton at δ_H 7.46 (H-4) showed ^3J correlation to carbons at δ_C 107.3 (C-3) and 136.0 (C-8) while the signal at δ_H 7.99

(H-2) showed ^2J and ^3J correlations to C-3 and C-8, respectively. The upfield shift of C-3 justified the attachement site of the carbonyl carbon. The lack of a positive alkaloid test indicates the presence of an acidic group in **JS-29** which was in agreement with the presence of a hydroxyl group present in the IR spectrum. On this basis, **JS-29** was identified as indole-3-carboxylic acid. All spectral data were in agreement with those published in the literature (Bano *et.al.*, 1987). This is the first report of this compound from *Trewia nudiflora*.

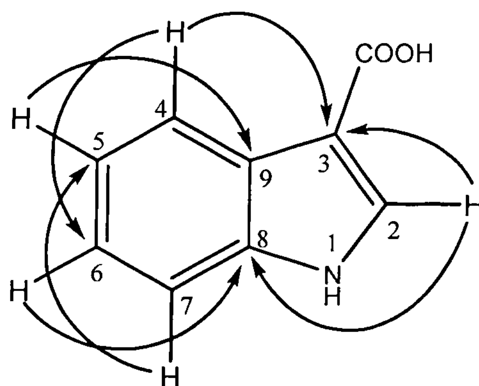
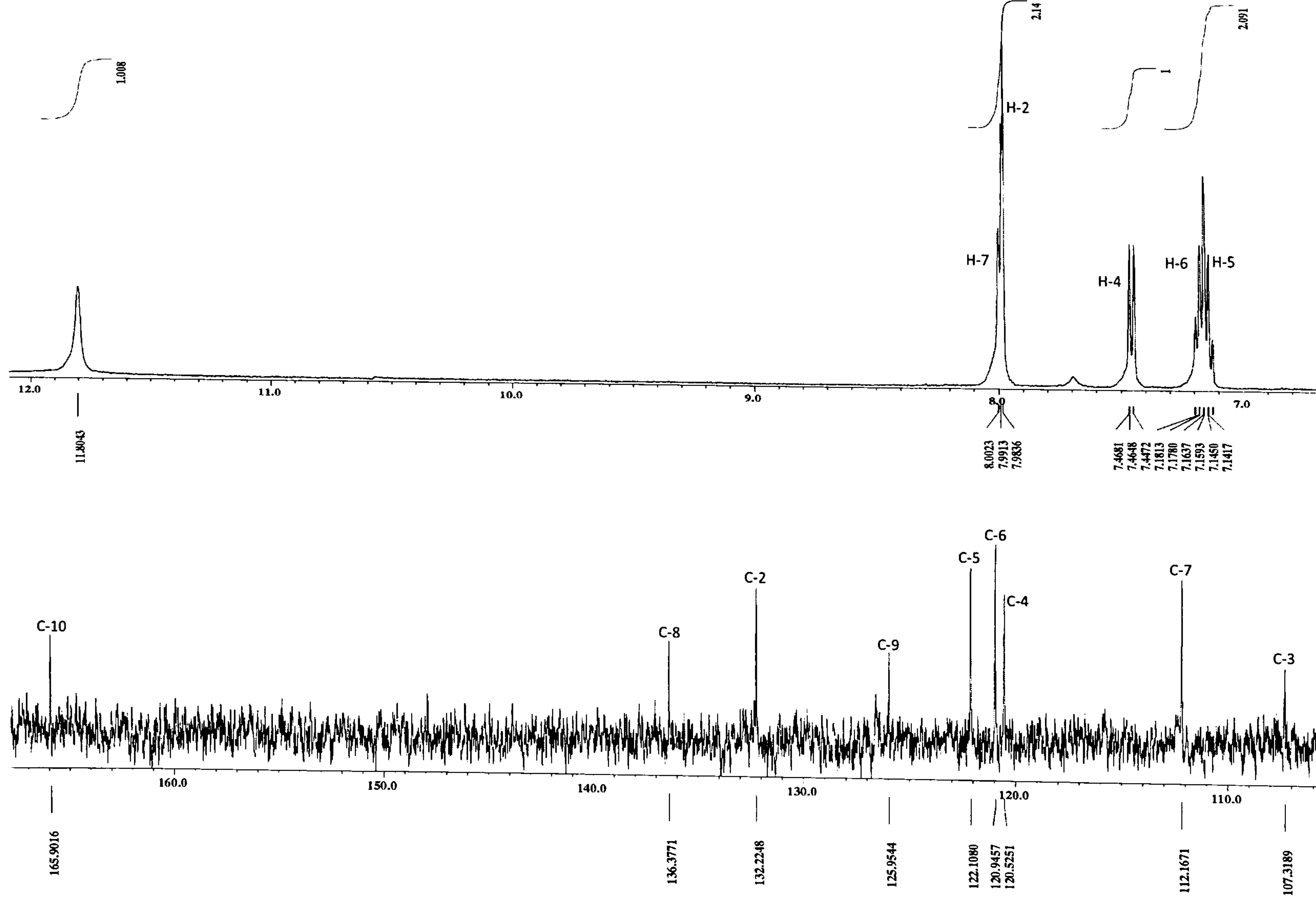


Fig. 3.35: Structure of JS-29 with selected HMBC correlations

Table 3.14: ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of JS-29 in $CDCl_3$

Position	δ_H	δ_C
2	7.99 (1H *s*)	132.2
3	-	107.3
4	7.46 (1H *dd* 1.5, 7.0 Hz)	120.5
5	7.15 (1H *m*)	122.1
6	7.18 (1H *m*)	121.0
7	8.00 (1H *m*)	112.2
8	-	136.0
9	-	126.0
10	-	165.9

Chemical shifts are in ppm.



Spectrum 3.30: ^{1}H (400 MHz) (A) and ^{13}C (100 MHz) NMR spectra of JS-29 in DMSO-d_6

Chapter 3

Results and discussion

Part II: Antimicrobial studies

3.2.1 Antibacterial screening

3.2.1.1 *Ludwigia adscendens*

Results of the activity of *Ludwigia adscendens* extracts and constituents against selected bacteria are presented in **Table 3.15**. The ethyl acetate and methanol extracts showed weak activity against the two Gram-positive bacteria and no activity against the two Gram-negative bacteria. The *n*-hexane extract was inactive against all microorganisms at the highest concentration used in the assay (>500 μg/mL). Subsequent screening of the various phases obtained from the methanol extract revealed that the chloroform phase was most active with an MIC of 62.5 μg/mL against the two Gram-positive bacteria. The *n*-butanol and water phases were only weakly active against the Gram-positive bacteria with MIC values of 250 and 500 μg/mL, respectively. None of the phases were active against the two Gram-negative bacteria. The MIC values of ciprofloxacin against the bacteria used in this study were consistent with those cited in the literature (Andrews, 2001).

3.2.1.1.1 Triterpenes and sterols

Among the compounds isolated from *Ludwigia adscendens*, the acyclic triterpene squalene (**JS-8**) only showed weak activity against *E. coli* (MIC 1.22 μM or 500 μg/mL). Among the other triterpenes isolated from the *n*-hexane extract, only betulinic acid (**JS-4**) was screened for activity and was inactive against all the organisms used. In a previous study betulinic acid was also inactive against *S. aureus*

and *P. aeruginosa* but unlike the previous report it was inactive against *E. coli* (>1000 μg/mL) (Kuete, *et al.*, 2007).

The mixture of two phytosterol derivatives 6β-hydroxy-stigmasta-4-en-3-one and 6β-hydroxy-stigmasta-4,22-dien-3-one (**JS-11**) (M:M = 1.5:1.0, w:w = 644.06: 427.36) was active against *S. pyogenes* at a concentration of 125 μg/mL. Using the molar ratio, the individual amount of the samples were calculated to be 75 (0.18 μM) and 50 (0.12 μM) μg/mL, respectively. For this sample, it is not clear whether the activity is due to a single compound or to the combined effect of both.

Several triterpenes and phytosterols were either isolated or detected in all the extracts. An increased concentration of these compounds may interfere with the sterol synthesis, affecting the integrity of bacterial cell wall which may in turn offer better membrane permeability towards the natural antibacterial products present in the extract (Georgopapadakou and Bertasso, 1992, Ryder *et al.*, 1984).

3.2.1.1.2 Flavonoids

The three flavanol glycosides isolated from *Ludwigia adscendens* were obtained in sufficient yield to screen them for antibacterial activity. Afzelin (**JS-17**) and quecetrin (**JS-18**) were active only against *S. pyogenes* (MIC= 1.16 and 2.23 μM, respectively). Myricitrin (**JS-19**) was active against all Gram-positive bacteria (MICs of 1.08 μM (500 μg/mL) except *S. aureus*, for which the MIC was 2.15 μM (1000 μg/mL).

These above results suggest that the replacement of a 3',4'-dihydroxy B-ring with a 3',4',5'-trihydroxy B-ring is important for activity as reported in the literature (Mori *et al.*, 1987). In a study reported by Fukunaga *et al.* (1989), when quercetin and quercitrin were tested against *S. aureus*, *E. coli* and *P. aeruginosa*, only quercetin showed inhibition of *S. aureus* at the highest concentration of 400 µg/mL. Consulting other reports led to the conclusion that while quercetin is antibacterial at a relatively low concentration, quercitrin requires higher concentration to exhibit antibacterial activity (Basile *et al.*, 2000; Waage and Hedin, 1985). In the present study, afzelin (**JS-17**), the 3-*O*-rhamnosyl glycoside of kaempferol failed to show activity against the test organisms except *S. pyogenes*. In the study reported by Basile *et al.*, (2000), kaempferol was inactive against any of the test organisms. Flavonoid glycosides irrespective of their attached sugar units are less active than their free flavonoid (Mandalari, *et al.*, 2007; Sohn *et al.*, 2004). It is well-known that flavonoids can be synthesised in plants due to a response against microbial attack. Plants tend to store flavonoids as glycosides to reduce their intrinsic activity (Rice-Evans *et al.*, 1997). It has been demonstrated that prenylated flavonoids showed promising antibacterial activity with MIC values of as low as 5 to 20 µg/mL (Sohn *et al.*, 2004). The high lipophilicity of such derivatives may result in a better cellular intake, increased concentration at the active site and better antimicrobial activity than for free flavonoids or their glycosides. One of the possible mechanisms by which flavonoids exert their activity is by inhibiting bacterial DNA-gyrase which stops DNA and RNA synthesis (Mori *et al.*, 1987; Ohemeng *et al.*, 1997). Flavonoid glycosides may also

induce topoisomerase IV-dependent DNA cleavage and have been shown to block the decatenation activity of topoisomerase IV in *E. coli* (Bernard *et al.*, 1997).

Whilst the activity observed for the ethyl acetate and methanol extracts were promising, none of the isolated compounds showed better activity. Synergism between flavonoids present in the extracts can also play an important role in reducing the MIC of the extracts or their phases (Alvarez *et al.*, 2008).

3.2.1.1.3 Simple phenolics and ellagic acid derivatives

Pteleoellagic acid (**JS-13**) obtained in low yield and for this reason it was tested only against *S. aureus* at a starting concentration of 250 μg/mL. Results revealed that it was inactive at that concentration. Pteleoellagic acid has been reported to be active against both Gram-positive and Gram-negative bacteria including *S. aureus*, *P. aeruginosa* and *E. coli* but the literature did not report any MIC values (Rahman *et al.*, 2001). The other ellagic acid derivative, 3,3',4'-tri-*O*-methyl ellagic acid (**JS-12**) was active against *S. pyogenes* with an MIC of 125 μg/mL (0.36 μM). It was not active against *S. aureus* and *E. coli* at the highest test concentration used (250 μg/mL). In a previous study it was found to be active against these two organisms at a concentration of 70 mg/mL (Ndukwe and Zhao, 2007). The observed activity may be due to remarkably high concentration used in the assay.

Among the three simple phenolics isolated from *L. adscendens*, protocatechuic acid (**JS-24**) showed a wide spectrum of activity. It was least active against *P. aeruginosa*

with an MIC of 1000 μg/mL (6.49 μM) and inactive against against *E. coli.* The highest activity was ovserved against *S. epidermidis* with an MIC of 125 μg/mL (0.81 μM). For the rest three Gram-positive bacteria (*S. aureus*, *S. pyogenes* and *E. faecalis*) the MIC was 1.62 μM (250 μg/mL).

Ellagic acid derivatives and other simple phenolic acids are weak acids which dissociate at the membrane pH of 6.8-7.2. This increases H^+ ion concentration across the bacterial cell, which can alter the resting membrane potential leading to the interference with the function of ion pumps. A disruption in the Ca^+ homeostasis can lead to microbial cell death. The antimicrobial activity of phenolic compounds may also be due to their various effects on the cell including proteolytic action (Vattem and Shetty, 2005).

Table 3.15: Antibacterial activity of *Ludwigia adscendens* extracts and constituents

Sample	MIC µg/mL (µM)					
	S. aureus	*E. faecalis*	*S. pyogenes*	*S. epidermidis*	*P. aeruginosa*	*E.coli*
n-Hexane	>500	nd	nd	>500	>500	>500
EtOAc	250	nd	nd	500	>500	>500
MeOH	500	nd	nd	250	>500	>500
$CHCl_3$ phase	62.5	nd	nd	62.5	>500	>500
BuOH phase	250	nd	nd	250	>500	>500
MeOH phase	500	nd	nd	500	>500	>500
JS-8	>500	>500	nd	>500	>500	500 (1.22)
JS-11	>500	>500	125	>500	>500	>500
JS-4	nd	>1000	>1000	nd	>1000	>1000
JS-12	>250	>250	125 (0.36)	>250	>250	>250
JS-13	>250	nd	nd	nd	nd	nd
JS-17	>500	>500	500 (1.16)	>500	>500	>500
JS-18	>1000	>1000	1000 (2.23)	>1000	>1000	>1000
JS-19	1000 (2.15)	500 (1.08)	500 (1.08)	500 (1.08)	>1000	>1000
JS-23	>1000	>1000	1000 (5.88)	>1000	>1000	>1000
JS-22	>1000	>1000	>1000	>1000	500 (2.72)	>1000
JS-24	250 (1.62)	250 (1.62)	250 (1.62)	125 (0.81)	1000 (6.49)	>1000
Ciprofloxacin	2.0 (6.04×10^{-3})	0.5 (1.6×10^{-3})	1.0 (3.02×10^{-3})	0.25 (0.8×10^{-3})	1.0 (3.02×10^{-3})	0.5 (1.6×10^{-3})

nd: not determined.

3.2.1.2 *Trewia nudiflora*

Results of the antibacterial activity of *Trewia nudiflora* extracts and its constituents against selected test organisms are presented in **Table 3.16.** Only the methanol extract of *Trewia nudiflora* was active against *S. epidermidis* (MIC of 250 μg/mL). Daucosterol (**JS-9**), isolated from the *n*-hexane extract, showed activity against both Gram-positive and negative bacteria including *S. aureus* (125 μg/mL), *S. pyogenes* (125 μg/mL) and *E. coli* (250 μg/mL) and were in agreement with those published in the literature (Lai *et al.*, 2003; Rashid *et al.*, 2006).

Of the ellagic acid derivatives, 3-*O*-methyl-ellagic acid-3'-*O*-rhamnopyranoside (**JS-70**) was active against *E. faecalis* at an MIC of 500 μg/mL (1.08 μM).

Daucosterol was active against some Gram-positive bacteria including *E. faecalis* at concentrations as low as 0.22 μM compared to JS-70 (1.08 μM).

None of the cardiac glycosides tested were active against any of the organisms tested. Scopoletin (**JS-28**), the only coumarin isolated from *T. nudiflora*, was inactive at the highest test concentration of 500 μg/mL. In a previous study using disc diffusion assay scopoletin showed a 5 mm zone of inhibition against *S. aureus* at a concentration of 400 μg/disc. In another study scopoletin was reported to be active against *S. aureus*, *P. aeruginosa*, and *E. Coli* (Kayser and Kolodziej, 1998). The inoculum size used in that assay was much lower (2×10^5 CFU/mL) than the one used in this study (5×10^5 CFU/mL).

Table 3.16: Antibacterial activity of *Trewia nudiflora* extracts and constituents

Sample	MIC μg/mL (μM)					
	S. aureus	*E. feacalis*	*S. pyogenes*	*S. epidermidis*	*P. aeruginosa*	*E. coli*
n-Hexane	>500	nd	nd	>500	>500	>500
Ethyl acetate	>500	nd	nd	>500	>500	>500
Methanol	>500	nd	nd	250	>500	>500
JS-9	125 (0.22)	125 (0.22)	125 (0.22)	>1000	>1000	250 (0.44)
JS-14	>1000	>1000	>1000	>1000	>1000	>1000
JS-15	>500	>500	>500	>500	>500	>500
JS-16	>500	500 (1.08)	>500	>500	>500	>500
JS-1	>500	>500	>500	>500	>500	>500
JS-2	>500	nd	nd	>500	>500	>500
JS-28	>500	>500	>500	>500	>500	>500
Ciprofloxacin	2.0 (6.04×10^{-3})	0.5 (1.6×10^{-3})	1.0 (3.02×10^{-3})	0.25 (0.8×10^{-3})	1.0 (3.02×10^{-3})	0.5 (1.6×10^{-3})

nd: not determined.

3.2.1.3 *Hygrophila auriculata*

Only the ethyl acetate extract of *Hygrophila auriculata* was active against the two Gram-positive bacteria at a concentration of 250 µg/mL (**Table 3.17**). All extracts were inactive against the two Gram-negative bacteria.

Several simple phenolic compounds such as gallic acid (**JS-23**), methyl gallate (**JS-22**), protocatechuic acid (**JS-24**), caffeic acid (**JS-26**), *para* hydroxy benzoic acid (**JS-25**) were isolated from the ethyl acetate extract.

Numerous studies have reported the antibacterial activity of such compounds against various microbial pathogens (Cho *et al.*, 1998; Garrote *et al.*, 2004; Taguri *et al.*, 2006). Thus, the broad spectrum antibacterial activity of *para*-hydroxy benzoic acid or 4-hydroxy benzoic acid (4-HBA) led to the synthesis of a series of alkyl esters commonly known as parabens (Cashman and Warshaw, 2005). The activity of this class of compounds has been associated with the hydroxylated benzoic acid part whereas the alkyl chain affects the pharmacokinetic properties by increasing bioavailability at the site of action and optimising its lipophilicity (Kubo *et al.*, 2003). The substitution of the hydroxyl group can lead to a reduction or lack of activity (Baek, 2004). In the present study, gallic acid (**JS-23**) and protocatechuic acid (**JS-24**) isolated from *Ludwigia adscendens* has shown antibacterial activity against various organisms. Kubo and co-workers (2003) carried out some anti-staphylococcal assays with a number of gallic acid, protocatechuic acid and their various alkyl esters and found them to be active. However addition of an alkyl chain

can reduce the MIC which may be due to better membrane permeability of the lipophilic esters.

In conclusion, it is very likely that the activity observed for *Hygrophila auriculata* ethyl acetate extract is due to these simple phenolics. This would also justify the traditional use of *Hygrophila auriculata* seeds.

Table 3.17: Antibacterial activity of *Hygrophila auriculata* extracts

Sample	MIC (μg/mL)			
	S. aureus	*S. epidermidis*	*P. aeruginosa*	*E. coli*
***n*-Hexane**	>500	>500	>500	>500
Ethyl acetate	250	250	>500	>500
Methanol	>500	>500	>500	>500
Ciprofloxacin	1.25	0.31	0.63	0.31

3.2.2 Antimycobacterial screening and potentiation studies

3.2.2.1 Antimycobacterial screening

The preliminary antimycobacterial screening was carried out by **Andréa Y. Gordien** (PhD student, Phytochemistry Research Laboratory) on pteleoellagic acid (**JS-13**) in which it turned to be active with an MIC of 62.5 μg/mL (0.02 μM). This finding prompted us to test other ellagic acid derivatives to test against *M. aurum*. Commercially available ellagic acid (Sigma-Aldrich UK Ltd) was also included in the study.

Results of the antimycobacterial screening of the isolated ellagic acid derivatives is presented in **Table 3.18**. Pteleoellagic acid (**JS-13**) was the most active. The other active compound was 3,3'-di-*O*-methyl ellagic acid-4-*O*-α-L-rhamnopyranoside (**JS-15**) with an MIC of 250 μg/mL (0.53 μM). All other derivatives, including ellagic acid itself, were inactive at the highest concentration tested.

Mycobacterum tuberculosis is a slow growing and highly infectious microorganism. Several studies have used *M. aurum* as a surrogate to *M. tuberculosis* when screening candidate compounds for possible antitubercular activity (Chung *et al.*, 1995).

Although ellagic acid has previously shown antifungal and antibacterial activity (Akiyama, *et al.*, 2001; Fogliani, 2005; Thiem and Goślińska, 2004), in the present study ellagic acid was tested for the first time against *Mycobaterium aurum* and was inactive at the highest test concentration of 1000 μg/mL.

It has been found that several methyl ether derivatives of ellagic acid can interfere with the mycolic acid synthesis causing mycobacteria to lose its acid fastness (Kondo *et al.*, 1979). Several mechanisms have been put forward to justify the antimicrobial activity of ellagic acid derivatives. One of the most commonly accepted mechanisms is the ability to increase the H^+ concentration in the cell vicinity affecting the function of Na^+/K^+ or Ca^+ pump and inactivate ion channels and enzymes by interacting with the oxidoreductases of the protein moiety of these macromolecules (Brantner *et al.*, 1996; Vattem and Shetty, 2005). They can also exhibit a proteolytic action by reacting with sulfhydryl groups and through non-specific interaction with proteins (Cowan, 1999). Like other phenolics, ellagic acid derivatives are good electron scavengers, *i.e.*, it can quench electrons from the electron transport chain (ETC) and delocalise them in its ring system (Rice-Evans *et al.*, 1997). A disruption in the flow of electron transport in cytochrome level can block phosphorylation (Vattem and Shetty, 2005). Plant polyphenols have also been reported to form chelate with metal ions. Precipitation of biologically important metal ions in microbial cell can lead to the inhibition of critical cell functions (McDonald, 1996).

Table 3.18: Effect of ellagic acid and its derivatives on the growth of *M. aurum*

Test sample	MIC values in µg/mL (µM)
Ellagic acid (EA)	>1000
3,4,3'-tri-*O*-methyl ellagic acid (JS-12)	>500
Pteleoellagic acid (JS-13)	62.5 (0.02)
3,3'-di-*O*-methyl ellagic acid (JS-14)	>1000
3,3'-di-*O*-methyl ellagic acid-4-*O*-rhamnopyranoside (JS-15)	250 (0.53)
3-*O*-methyl ellagic acid-4'-*O*-rhamnopyranoside (JS-16)	>250
Rifampicin	1.0 (1.22×10^{-3})
Isoniazid	0.25(1.82×10^{-3})
Ethambutol	2.0 (9.80×10^{-3})

3.2.2.2 Potentiation studies with active ellagic acid derivatives

As some of the ellagic acid derivatives isolated in the present study were active against *M. aurum*, it was of interest to find out whether they could potentiate the activity of some currently available anti-TB drugs. Rifampicin and isoniazid were chosen for this purpose. The effect of pteleoellagic acid (**JS-13**) and 3,3'-di-*O*-methyl ellagic acid-4-*O*-rhamnopyranoside (**JS-15**) on the MICs of these two anti-TB agents against *M. aurum* is presented in **Table 3.19**.

When tested, pteleoellagic acid (**JS-13**), at a subinhibitory concentration (6.25 µg/mL), showed synergistic effect with rifampicin (FIC= 0.35) and partial synergy with isoniazid (FIC= 0.60), while 3,3'-di-*O*-methyl ellagic acid 4-*O*-rhamnopyranoside (**JS-15**), at its subinhibitory concentration (62.5 µg/mL) showed

partial synergy with rifampicin (FIC= 0.75) and additive effect with isoniazid (FIC= 1.25).

Future work is required to establish the exact mode of action of pteleoellagic acid (**JS-13**) on the *M. tuberculosis* and determine whether the synergism occurs with other anti-TB drugs. Several ellagic acid derivatives with various structural features could be synthesised or isolated from natural sources and further tested to gain more insight into the structure activity relationships. A series of ellagic acid derivatives bearing alkyl chains of different length on hydroxyl functions could be synthesised to quantify the correlation between lipophilicity and antimycobacterial activity. Testing ellagic acid derivative with methylenedioxy bridges at 3,4 and 3',4' may also be useful to find out whether a free hydroxyl function is necessary for the activity or not. Prenylated flavonoids and coumarins are known to be superior over their parent structure in terms of antimicrobial activity (Sohn *et al.*, 2004). No prenylated ellagic acid derivatives have been isolated so far from natural sources. It would be of interest to synthesise such compounds to compare their antimicrobial activity.

Table 3.19: Effect of ellagic acid derivatives on the MIC of isoniazid and rifampicin against *Mycobacterium aurum*

Combination of drugs	**MIC (µg/mL)**		**FIC Index**
	Individual	**Combination**	
JS-13/Rifampicin	62.5/1.0	6.25/0.25	0.35
JS-13/Isoniazid	62.5/0.5	6.25/0.25	0.60
JS-15/ Rifampicin	250/1.0	62.5/0.5	0.75
JS-15/ Isoniazid	250/0.5	62.5/0.5	1.25

Chapter 3

Results and discussion

Part III: Conclusion and future work

3.3 Conclusion and future work

The phytochemical investigation of *Ludwigia adscendens* led to the isolation of several flavonoids and phenolics. This was in good agreement with previous reports on the biosystematics of the family Onagraceae (Averett *et al.*, 1990). Ethyl acetate and methanol extract of *Ludwigia adscendens* further showed antimicrobial activity. Different phenolics and flavonoids isolated from these two extracts also showed varying degrees of antimicrobial activity against a range of Gram positive and Gram negative bacteria. It is possible that since this species was collected during the monsoon season, a moist and warm climate would have favoured the growth of pathogens and played a role in the excessive production of such compounds having antimicrobial activity. The antimycobacterial activity of pteleoellagic acid (**JS-13**) against *M. aurum* (Write in full) is an important finding which extends the therapeutic potential of ellagic acid derivatives. It would be interesting to see whether foods rich in ellagic acid derivatives, such as pomegranate, berries can be beneficial to patients receiving anti-TB therapy.

The isolation of two novel cardenolides namely (5α)-19-hydroxy sarmetogenin 3-*O*-α-L-rhamnopyranoside (**JS-2**) and 5α-sarmentogenin 3-*O*-[β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside] (**JS-3**) and other compounds belonging to six different classes from *Trewia nudiflora* demonstrates that this species has rich biosynthetic pathways. Although *Trewia nudiflora* bark is traditionally used topically (Yusuf *et al.*, 1994), it is advisable to include some toxicity warnings to prevent health hazards resulting from a possible absorption of alliotoxin (**JS-1**) to the

systemic circulation. Further work should be carried out on the two newly isolated cardenolides **JS-2** and **JS-3** to establish their effect on the cardiac muscle.

The seeds of *Hygrophila auriculata* are extensively used in South-East Asian traditional medicine as part of herbal preparations taken orally (Ahmed *et al.*, 2001). The present phytochemical investigation led to the isolation of antimicrobial compounds that are commonly present in daily diets (Hussain *et al.*, 2009; Knights, 1966; Puupponen-Pimiä *et al.*, 2005). Previous reports revealed that *Hygrophila auriculata* did not show any toxicity in animal models (Shanmugasundaram, and Venkataraman, 2006).

A lack of time prevented us from testing other isolated compounds against *M. aurum* and as well as against other mycobacterial species. Thus these compounds can be tested to establish their role on the growth of various mycobacteria.

In conclusion, the present investigation resulted in some interesting outcome in the field of natural products with the isolation of a wide range of secondary metabolites, some of them presenting antimicrobial activity. However, further toxicity studies need to be carried out on individual compounds to establish their safety before any attempt of further exploitation of their biological activity is carried out.

Appendix I

Properties of isolated compounds

Alliotoxin (JS-1): Brownish needles (MeOH/C_5H_5N). Mp 258-262° (Lit. Mp 262-272°, Kang *et. al.*, 2005) Analysed on TLC with $CHCl_3$:MeOH 4:1 ($R_{f.}$: 0.42). IR ν_{max} (KBr disc) cm^{-1}: 3390 (O-H), 2969, 2931, 2860, 1754 and 1735 (butenolactone), 1620, 1449, 1383, 1285, 1049, 981. Found $[M+H]^+$ at *m/z* 537.3066 ($C_{29}H_{45}O_9$ requires for 537.3064). HRESI-MS *m/z* 537, 519, 503, 501, 492, 491, 449, 391, 373, 355, 337, 282. $[\alpha]_D^{23}$ -17° (*c* 1.0, Pyr), Lit. $[\alpha]_D^{20}$ -5.2° (*c* 0.42, Pyr) (Kang *et al.*, 2005).

(5α)-19-Hydroxy sarmentogenin 3-O-α-L-rhamnopyranoside or trewianin (JS-2): White amorphous solid. Mp 217-227° Analysed on TLC with $CHCl_3$:MeOH 4:1 ($R_{f.}$: 0.3). IR ν_{max} (KBr disc) cm^{-1}: 3400 (O-H), 2930, 1729, 1619, 1449, 1383, 1259, 1031. Found $[M+H]^+$ at *m/z* 553.2997 ($C_{29}H_{45}O_{10}$ requires for 553.3012). HRESI-MS *m/z* 553. $[\alpha]_D^{25}$ +92.5° (*c* 0.24, MeOH).

5α-Sarmentogenin 3-O-[β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside] or trewioside (JS-3): White needle-like crystals (CH_3Cl/MeOH). Mp 181-184° Analysed on TLC with $CHCl_3$:MeOH 7:3 ($R_{f.}$: 0.36). IR ν_{max} (KBr disc) cm^{-1}: 3430, 2930, 2866, 1778, 1737, 1624, 1450, 1386, 1262, 1032. Found $[M+H]^+$ at *m/z* 699.3578 ($C_{35}H_{55}O_{14}$ requires for 699.3585). HRESI-MS *m/z* 699, 664, 613, 546, 537, 481, 427, 388, 349, 331, 288, 248, 202, 166, 149, 85. $[\alpha]_D^{23}$ -30° (*c* 1.0, MeOH).

Betulinic acid (JS-4): White amorphous solid. Analysed on TLC with *n*-Hexane:EtOAc 3:2 ($R_{f.}$: 0.56). IR ν_{max} (KBr disc) cm^{-1}: 3467, 3436, 3072, 2942, 2869, 1686, 1641, 1452, 1388, 1376, 1236, 1191, 1043, 1032, 882. Found $[M]^+$ at *m/z* 456.3605 ($C_{30}H_{48}O_3$ requires 456.3603). HREIMS *m/z* (Rel. int. %) 456 (12),

438 (4), 410 (3), 248 (9), 207 (10), 189 (16), 79 (100), 52 (63). $[\alpha]_D^{20}$ +6.7° (*c* 0.3, Pyr), Lit. $[\alpha]_D^{23}$ +7.9° (*c* 0.57, Pyr) (Robinson and Martel, 1970).

Betulin (JS-5): White amorphous solid. Analysed on TLC with *n*-Hexane:EtOAc 1:1 (R_f.: 0.54). Found $[M+H]^+$ 443.3884 ($C_{30}H_{51}O_2$ requires for 443.3888). HRCI-MS *m/z* (Rel. int. %) 443 (10), 425 (100), 407 (10), 395 (5), 235 (5), 217 (8), 191 (6), 141 (8), 69 (22). $[\alpha]_D^{23}$ +19° (*c* 1.0, Pyr), Lit. $[\alpha]_D^{23}$ +16° (*c* 1.2, $CHCl_3$) (Lawrie *et. al.*, 1964).

Betulonic acid (JS-6): White amorphous solid. Analysed on TLC with *n*-Hexane:EtOAc 3:2 (R_f.: 0.60). Found $[M+H]^+$ at *m/z* 455.3520 ($C_{30}H_{47}O_3$ requires for 455.3525).

3β-Acetyl aleuritolic acid (JS-7): White needle-like crystals (Chloroform). Analysed on TLC with *n*-Hexane:EtOAc 7:3 (R_f.: 0.61).IR ν_{max} (KBr disc) cm^{-1}: 3447, 3051, 2935, 2920, 2851, 1734, 1689, 1635, 1467, 1377, 1365, 1245, 1028. Found $[M+H]^+$ 499.3695 ($C_{32}H_{51}O_4$ requires 499.3787). HRESIMS *m/z* 499, 471 $[(M-CO)+H]^+$ (rel. int. 100%), 439 $[(M-CH_3COOH)+H]^+$ (rel. int. 90%). $[\alpha]_D^{21}$ +80° (*c* 2.5, $CHCl_3$), Lit. $[\alpha]_D^{23}$ +23.1° (*c* 0.6, $CHCl_3$) (Woo and Wagner, 1977).

Squalene (JS-8): Pale yellow oil. Analysed on TLC with *n*-hexane:EtOAc 9:1 (R_f 0.67). IR ν_{max} (KBr disc) cm^{-1}: 2959, 2932, 2867, 1465, 1381, 1061. Found $[M]^+$ at *m/z* 410.3915 ($C_{30}H_{50}$ requires 410.3913). HREIMS *m/z* (Rel. int. %) 410 (2), 341 (2), 149 (5), 137 (12), 123 (6), 121 (8), 109 (6), 107 (5), 95 (12), 81 (42), 69 (100), 55 (12), 57 (13), 43 (12), 41 (36).

Daucosterol (JS-9): White amorphous powder. Found $[M-H]^-$ at *m/z* 575 ($C_{35}H_{60}O_6$ requires 575). ESI-MS *m/z* 575. ^1H NMR (400 MHz, C_5D_5N) δ_H: 0.65 (3H, *s*, Me-18), 0.86 (3H, *d*, Me-27), 0.88 (3H, *d*, Me-26), 0.90 (3H, *t*, Me-29), 0.91 (3H, *s*, Me-19), 0.98 (3H, *d* 6.6 Hz, Me-21), 3.94 (1H, *m*, H-3), 5.05 (1H, d 7.5 Hz, H-1'), 5.34 (1H, *brs*, H-6). ^{13}C NMR (100 MHz, C_5D_5N) δ_C: 11.9 (C-18), 12.1 (C-29), 18.9 (C-21), 19.1 (C-19), 19.3 (C-26), 19.9 (C-27), 21.2 (C-11), 23.3 (C-28), 24.4 (C-15), 26.3 (C-23), 28.4 (C-16), 29.4 (C-25), 30.1 (C-2), 32.0 (C-8), 32.1 (C-7), 34.1 (C-22), 36.3 (C-20), 36.8 (C-10), 37.4 (C-1), 39.2 (C-4), 39.9 (C-12), 42.4 (C-13), 46.0 (C-24), 50.3 (C-9), 56.1 (C-17), 56.7 (C-14), 62.7 (C-6'), 71.6 (C-4'), 75.2 (C-2'), 78.1 (C-3), 78.3 (C-5'), 78.5 (C-3'), 102.5 (C-1'), 121.8 (C-6), 140.8 (C-5).

5,8-Epidioxyergosta-6,22-dien-3-ol (JS-10): White amorphous solid. Analysed on TLC with *n*-hexane:EtOAc 7:3 (R_f: 0.17). $[\alpha]_D^{23}$ -6 (Pyr, *c* 1.0), Lit. $[\alpha]_D^{20}$ -3.8 ($CHCl_3$, *c* 0.16) (Ioannou *et. al.*, 2009). Found $[M+H]^+$ at *m/z* 429.3371 ($C_{28}H_{45}O_3$ requires 429.3369). HRCI-MS *m/z* (Rel. int. %) 429 (100), 411 (95), 396 (50), 377 (65), 223 (25), 85 (78), 69 (100).

Mixture of 6β-hydroxy-stigmasta-4-en-3-one (JS-11a) and 6β-hydroxy-stigmasta-4,22-dien-3-one (JS-11b): Colourless needles (Pyridine or $CHCl_3$). Analysed on TLC with *n*-Hexane:EtOAc 3:2 (R_f 0.33). IR ν_{max} (KBr disc) cm^{-1}: 3465, 2957, 2868, 1691, 1616, 1466, 1384,1245, 1231, 1193, 1038, 1016, 971, 878. Found $[M+H]^+$ at *m/z* 427.3572 ($C_{29}H_{47}O_2$ requires 427.3571) and $[M+H]^+$ at *m/z* 429.3728 ($C_{30}H_{49}O_2$ requires 429.3727).

3,3',4'-Tri-O-methylellagic acid (JS-12): Light brown amorphous solid. Analysed on TLC with $CHCl_3$:MeOH 9:1 (R_f.: 0.57). IR ν_{max} (KBr disc) cm^{-1}: 3435, 2953, 2851, 1752, 1727, 1607, 1577, 1493, 1434, 1411, 1357, 1113, 1090, 988, 914. λ_{max}^{MeOH} (nm) 247, 371. Found $[M+H]^+$ at *m/z* 345.0605 ($C_{17}H_{13}O_8$ requires 345.0610).

Pteleoellagic acid (JS-13): Light green needles ($CHCl_3$/MeOH). Analysed on TLC with $CHCl_3$:MeOH 9:1 (R_f.: 0.80). IR ν_{max} (KBr disc) cm^{-1}: 3434, 3068, 2917, 1741, 1640, 1606, 1497, 1477, 1364, 1108, 1022, 910. UV λ_{max}^{MeOH} nm: 250, 370. Found $[M-H]^-$ at *m/z* 327.0149 ($C_{16}H_7O_8$ requires 327.1464).

3,3'-Di-O-methylellagic acid (JS-14): Light brown amorphous solid. Analysed on TLC with $CHCl^3$:MeOH 9:1 (R_f.: 0.48). IR ν_{max} (KBr disc) cm^{-1}: 3274, 2946, 1725, 1700, 1609, 1579, 1489, 1441, 1353, 1285, 1211, 1171, 1106, 1067, 989, 915. UV λ_{max}^{MeOH} nm: 247, 374. Found $[M-H]^-$ at *m/z* 329.0297 ($C_{16}H_9O_8$ requires 329.0292).

3,3'-Di-O-methylellagic acid 4-O-α-L-rhamnopyranoside (JS-15): Light brown amorphous solid. Analysed on TLC with $CHCl_3$:MeOH 9:1 (R_f.: 0.18). IR ν_{max} (KBr disc) cm^{-1}: 3412, 2953, 2937, 1745, 1608, 1486, 1438, 1414, 1358, 1106, 1061, 971, 914, 757. UV λ_{max}^{MeOH} nm: 246, 369. Found $[M-H]^-$ at *m/z* 475.0865 ($C_{22}H_{19}O_{12}$ requires 475.0871).

3-O-Methylellagic acid 4'-O-α-L-rhamnopyranoside (JS-16): Light yellow amorphous solid. Analysed on TLC with $CHCl_3$:MeOH 7:3 (R_f.: 0.38). IR ν_{max} (KBr disc) cm^{-1}: 3376, 2935, 1718, 1606, 1577, 1491, 1441, 1355, 1278, 1208, 1099, 1053, 974, 916. UV λ_{max}^{MeOH} nm: 252, 353. Found $[M+H]^+$ at *m/z* 463.0863

($C_{21}H_{19}O_{12}$ requires 463.0871) and $[M+Na]^+$ at *m/z* 485.0681 ($C_{21}H_{18}O_{12}Na$ requires 485.0690).

Afzelin (JS-17): Light yellow amorphous solid. Analysed on TLC with Choloroform:MeOH 8:2 (R_f.: 0.32). Found $[M-H]^-$ at *m/z* 431.0979 ($C_{21}H_{19}O_{10}$ requires 431.0984).

Quercitrin (JS-18): Yellow crystals (MeOH). Analysed on TLC with EtOAc:MeOH 9:1 (R_f.: 0.45), Found $[M+H]^+$ at *m/z* 449.1081 ($C_{21}H_{21}O_{11}$ requires for 449.1084). HRFAB$^+$ MS *m/z* (Rel. int. %) 449 (21), 329 (10), 307 (24), 303 (55), 302(45), 289 (12), 232 (25), 176 (14), 155 (100), 138 (50), 137 (65), 81 (56).

Myricitrin (JS-19): Yellow crystals (MeOH). Analysed on TLC with EtOAc:MeOH:Acetic acid 6:4:2drops (R_f.: 0.60). Found $[M+H]^+$ at *m/z* 465.1035 ($C_{21}H_{21}O_{12}$ requires 465.1033). HRFAB$^+$ MS *m/z* (Rel. int. %) 465 (5), 464 (2), 419 (2), 385 (2), 319 (5), 318 (3), 254 (2), 232 (17), 214 (3), 157 (50), 101 (4), 79 (100), 61 (4).

Quercetin (JS-20): Yellow amorphous solid. Analysed on TLC with n-hex:EtOAc:Acetic acid 4:6:2drops (R_f.: 0.25). Found $[M+H]^+$ at *m/z* 303.0494 ($C_{15}H_{11}O_7$ requires 303.0499).

(+) trans-Taxifolin (JS-21): Yellow amorphous solid. Analysed on TLC with *n*-hexane:EtOAc 6:4 (R_f.: 0.30). $[\alpha]_D^{23}$ +9.5° (*c* 1.05, MeOH). Lit. $[\alpha]_D^{24}$ +22.0° (*c* 1.68, MeOH) (Dok-Go *et. al.*, 2003).

Methyl gallate (JS-22): White crystals (MeOH). Analysed on TLC with $CHCl_3$:MeOH 8:2 (R_f.: 0.32). Found $[M]^+$ at *m/z* 184.0373 ($C_8H_8O_6$ requires 184.0372). HREI-MS *m/z* (Rel. int. %) 184 (62), 153 (100), 125 (27), 107 (8), 79 (20), 51 (11), 43 (20).

Gallic acid (JS-23): Yellow amorphous solid. Analysed on TLC with *n*-hexane:EtOAc 6:4 (R_f.: 0.30). Found $[M]^+$ at *m/z* 170.0212 ($C_7H_6O_5$ requires 170.0215). HREI-MS *m/z* (Rel. int. %) 170 (100), 153 (88), 135 (11), 133 (10), 125 (23), 103 (33), 85 (10), 73 (68), 61 (58), 43 (46).

Protocatechuic acid (JS-24): Brown amorphous solid. Analysed on TLC with *n*-hexane:EtOAc 6:4 (R_f.: 0.30). Found $[M+H]^+$ at *m/z* 155.0336 ($C_7H_7O_4$ requires 155.0344). HRCI-MS *m/z* (Rel. int. %) 155 (42), 113 (43), 73 (100).

4-Hydroxy benzoic acid (JS-25): Brown amorphous solid. Analysed on TLC with *n*-hexane:EtOAc 6:4 (R_f.: 0.30). Found $[M+H]^+$ at *m/z* 139.0396 ($C_7H_7O_3$ requires 139.0395). HRCI-MS *m/z* (Rel. int. %) 139 (100), 113 (17), 97 (20). 85 (67), 69 (75).

4-Hydroxyphthalide (JS-26): Colourless crystals (MeOH). Analysed on TLC with *n*-hexane:EtOAc 4:6 (R_f.: 0.44). Found $[M-H]^-$ at *m/z* 149.0244 ($C_8H_5O_3$ requires 149.0244).

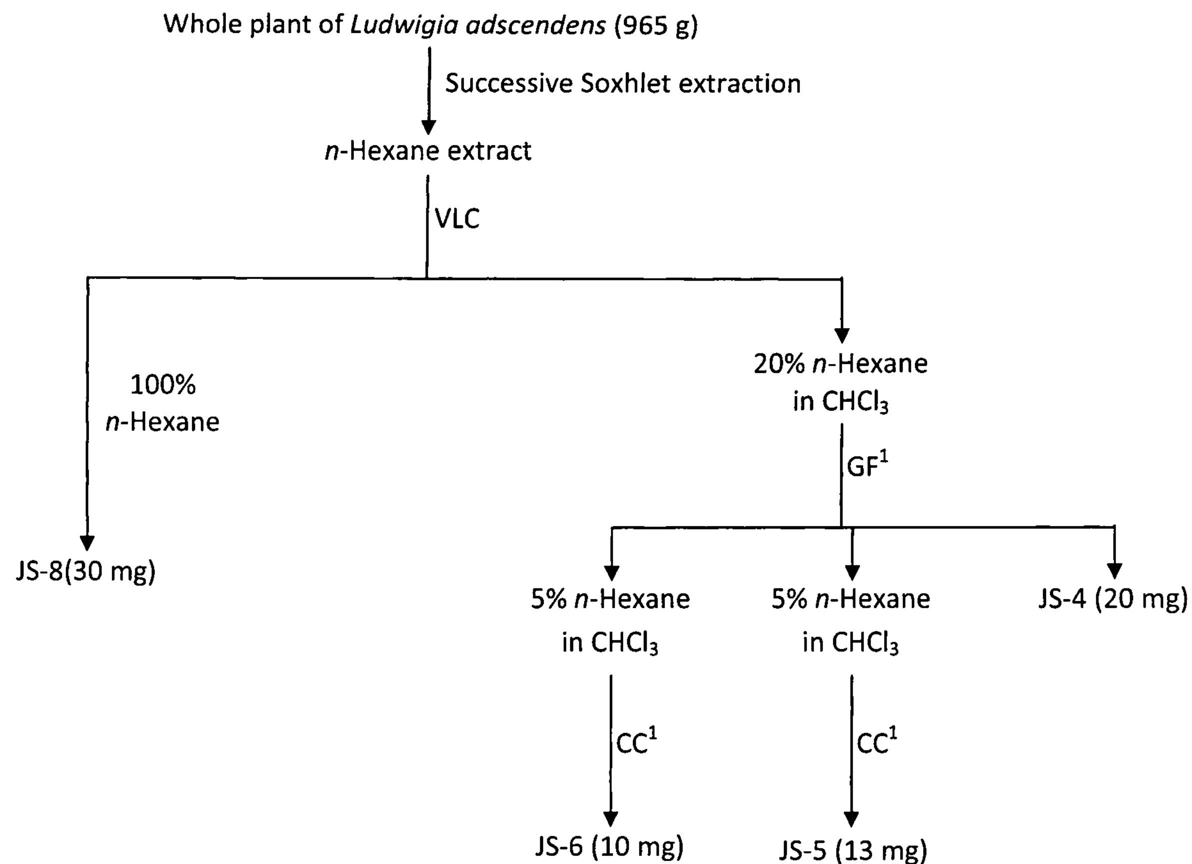
Caffeic acid (JS-27): Brown amorphous solid. Found $[M]^+$ at *m/z* 180.0426 ($C_9H_8O_4$ requires 180.0423). HREI-MS *m/z* (Rel. int. %) 180 (20), 154 (83), 137 (100), 136 (70), 109 (30), 89 (30), 63 (32), 44 (31).

Scopoletin (JS-28): Yellow crystals. Analysed on TLC with *n*-hex:EtOAc 1:1 (R_{f}.: 0.18). IR ν_{max} (KBr disc) cm^{-1}: 3338, 2946, 1710, 1566, 1509, 1435, 1209, 1140, 1019, 922, 861. Found $[M+H]^{+}$ at *m/z* 193.0505 ($C_{10}H_{9}O_{4}$ requires 193.0501). HRCI MS *m/z* (Rel. int. %) 193 (100), 159 (3), 145 (3), 85 (8), 69 (8).

Indole-3-carboxylic acid (JS-29): White crystals ($CHCl_3$/MeOH). Analysed on TLC with *n*-hex:EtOAc 2:3 (R_{f}.: 0.35). IR ν_{max} (KBr disc) cm^{-1}: 3400, 3300, 3112, 2928, 1641, 1581, 1522, 1445, 1310, 1197, 1129, 1035. UV λ_{max}^{MeOH} nm: 275, 355, 371. Found $[M-H]^{-}$ at *m/z* 160.0400 ($C_{9}H_{6}O_{2}N$ requires 160.0393). HRESI MS *m/z* 160.0400.

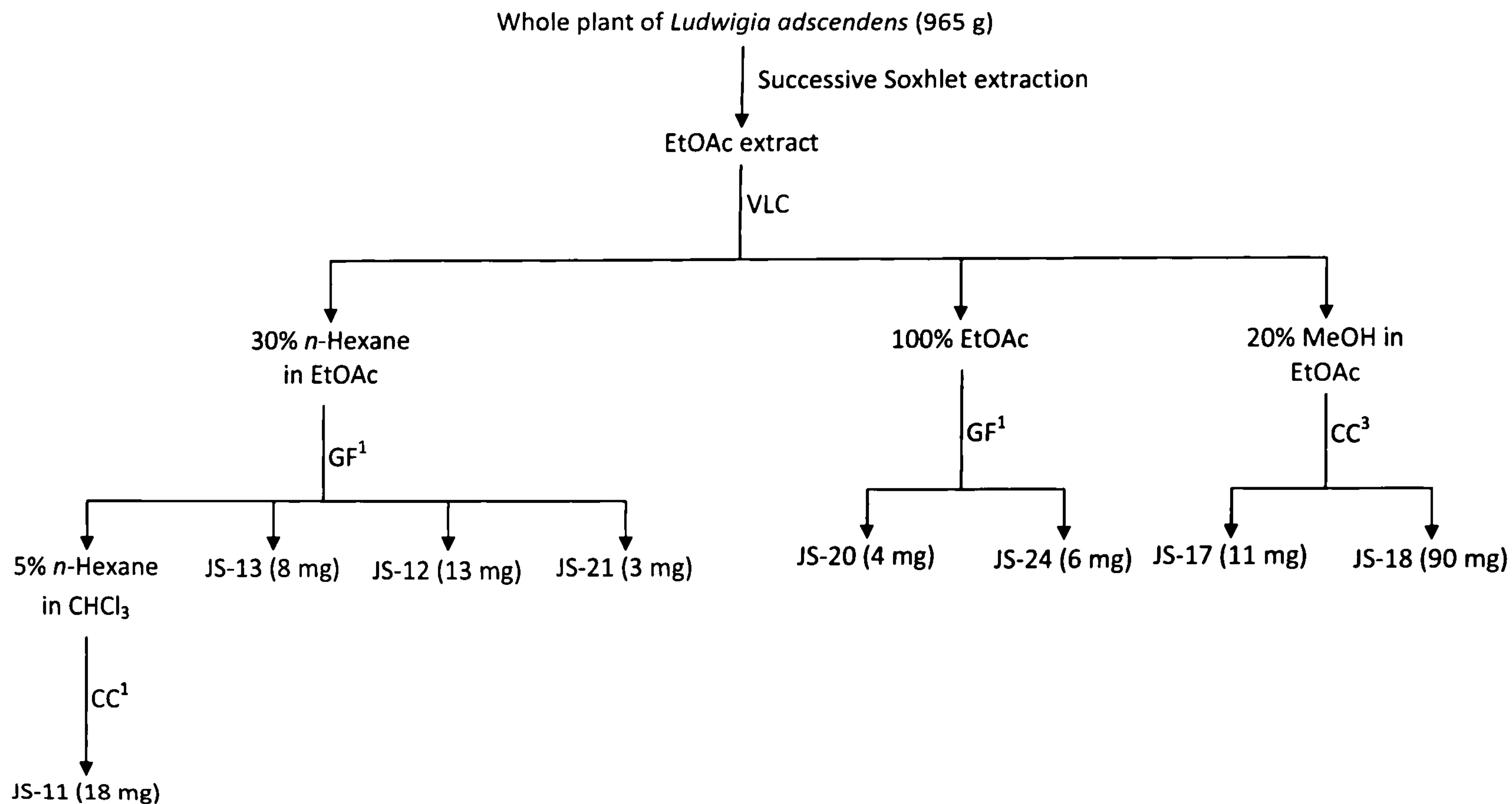
Appendix II

Isolation schemes



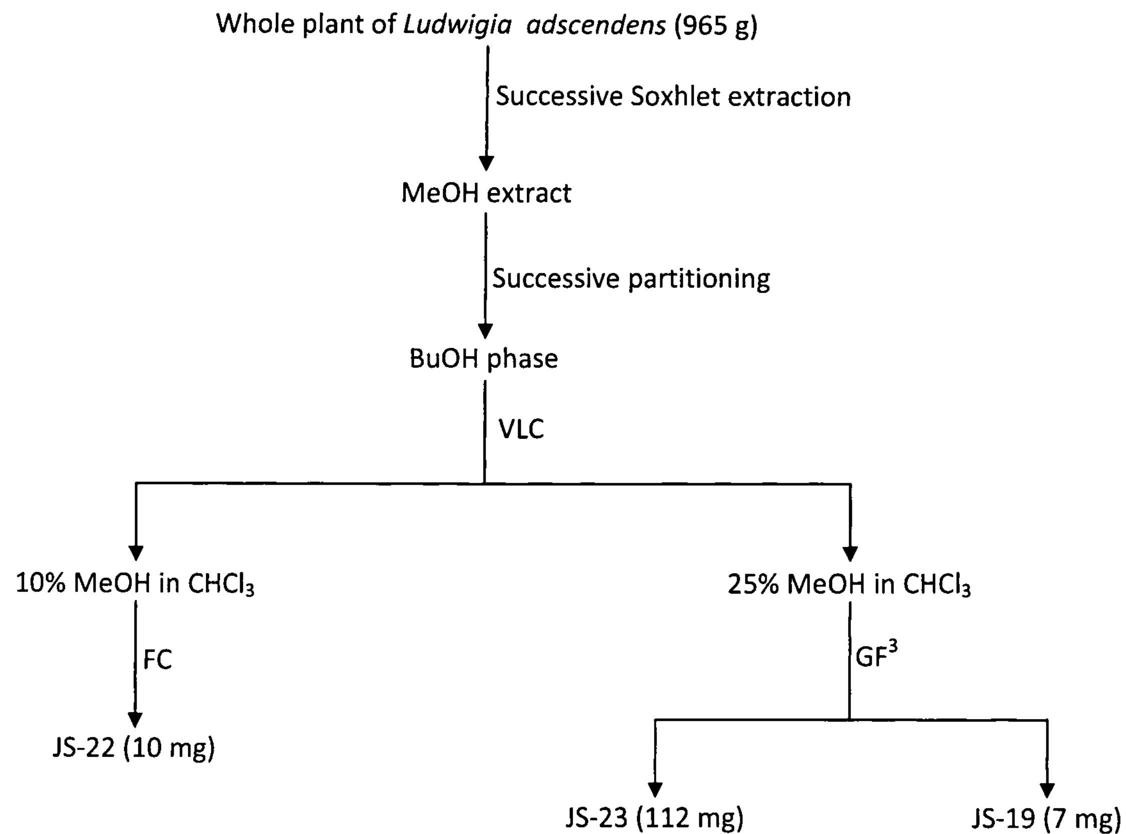
GF[1]: Gel filtration elued with 5% *n*-hexane in $CHCl_3$, with the gradual increase of $CHCl_3$, followed by the increase of MeOH; CC[1]: Gradient Si. gel column eluted with 100% *n*-hexane, increasing polarity by gradual increase in EtOAc.

Scheme 1: Isolation of compounds from the *n*-hexane extract of *Ludwigia adscendens*



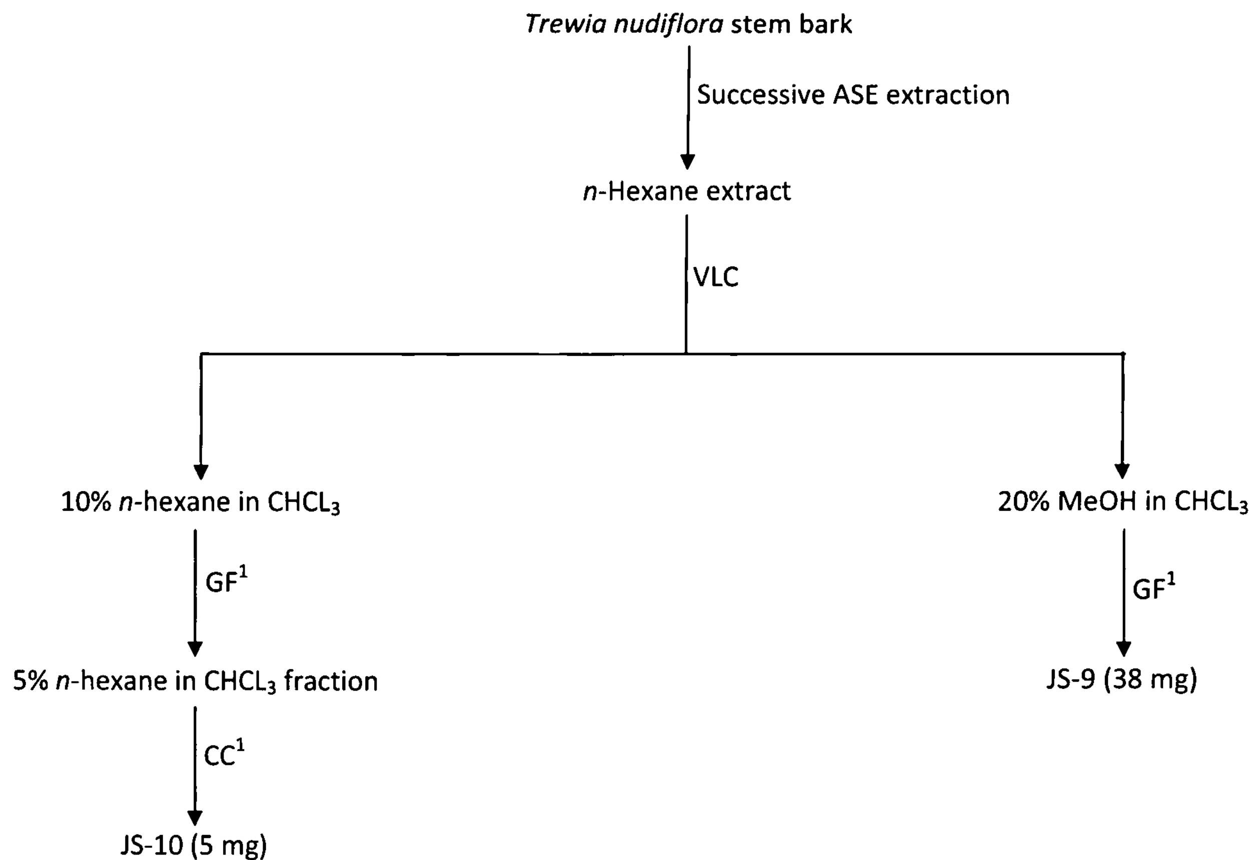
GF[1]: Gel filtration started with 5% *n*-hexane in $CHCl_3$, with the gradual increase of $CHCl_3$, followed by the increase of MeOH; CC[3]: Gradient Si gel column, starting with 100% $CHCl_3$ with gradual increase of MeOH

Scheme 2: Isolation of compounds from the EtOAc extract of *Ludwigia adscendens*



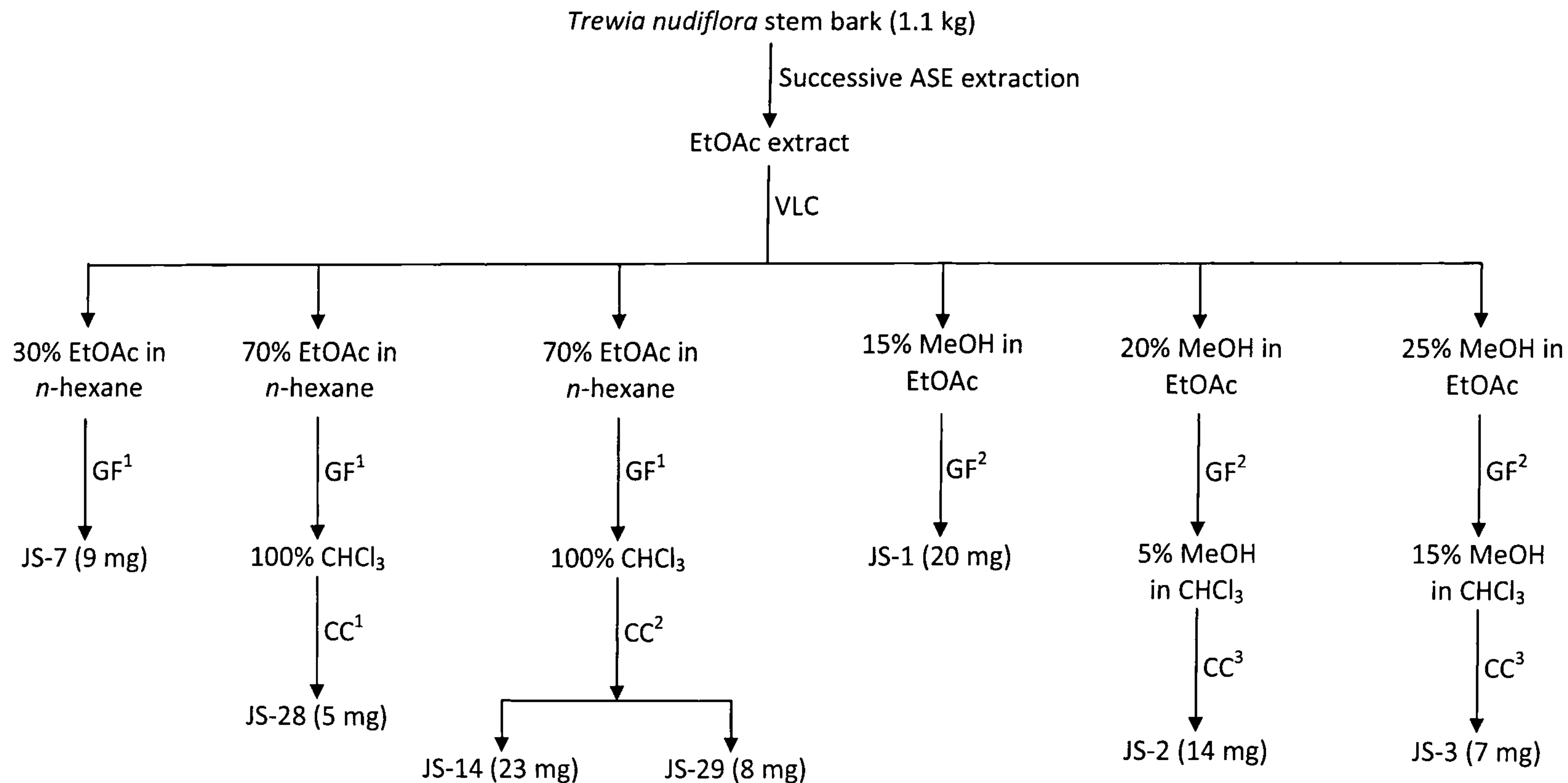
FC: Flash chromatography using Si. gel column. Eluted with 100% $CHCl_3$ with gradual increase of MeOH; GF[3]: Gel filtration eluted with 100% MeOH.

Scheme 3: Isolation of pure compounds from the MeOH extract of *Ludwigia adscendens*



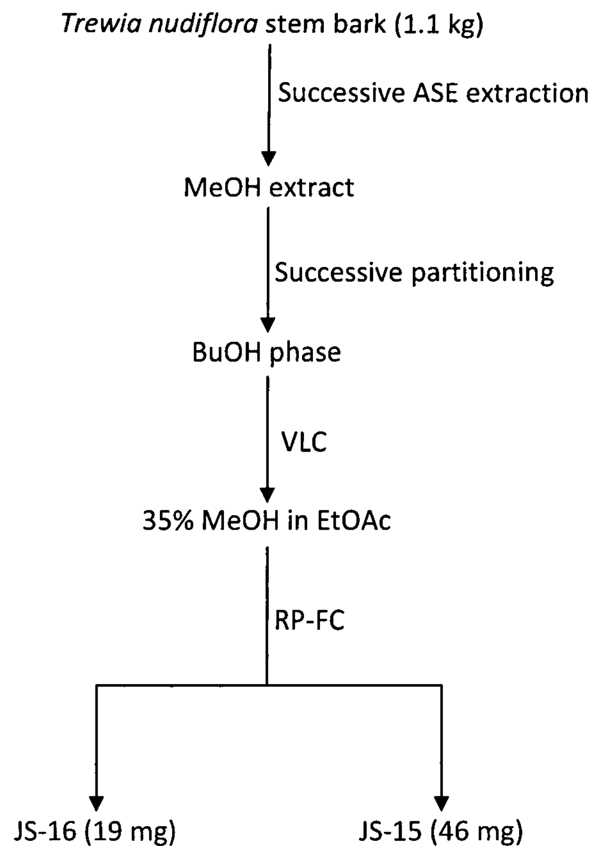
GF[1]: Gel filtration eluted with 5% *n*-hexane in $CHCl_3$, with the gradual increase of $CHCl_3$, followed by the increase of MeOH; CC[1]: Gradient column eluted with 100% n-hexane, increasing polarity by the addition of EtOAc.

Scheme 4: Isolation of pure compounds from the *n*-hexane extract of *Trewia nudiflora*



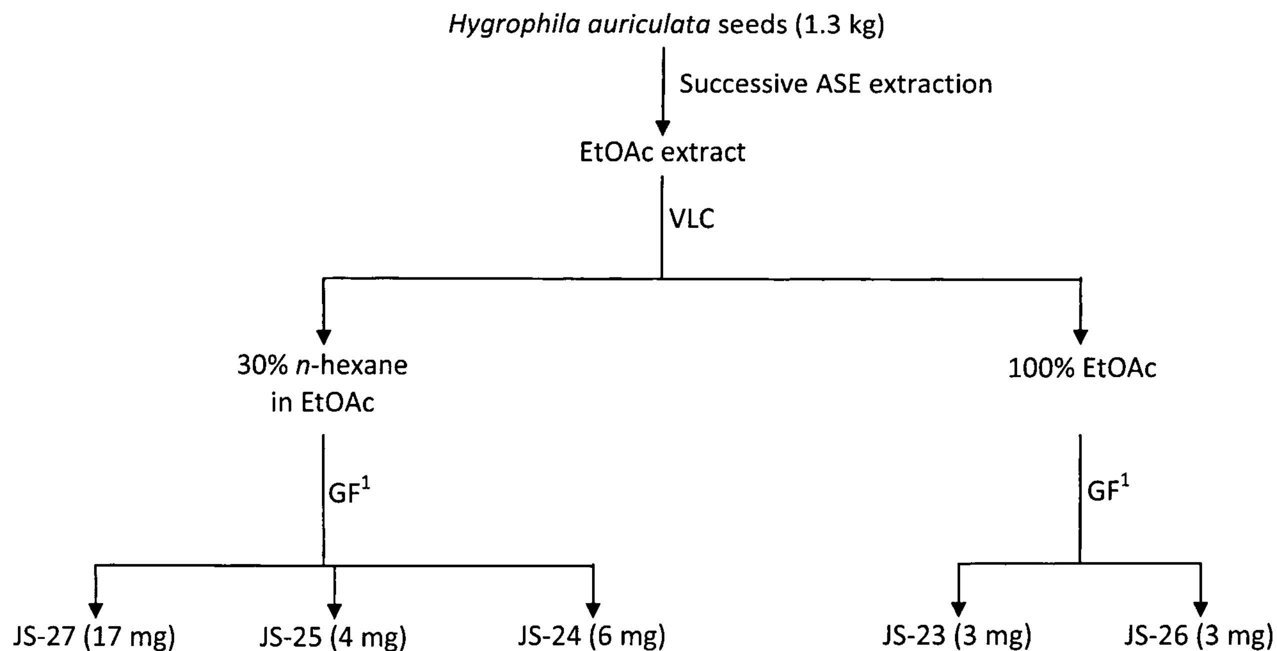
GF^1: Gel filtration eluted with 5% *n*-hexane in $CHCl_3$, with the gradual increase of $CHCl_3$, followed by the increase of MeOH; GF^2: Gel filtration eluted with 100% $CHCl_3$ with gradual increase of MeOH. CC^1: Gradient Si gel column, eluted with 100% *n*-hexane with gradual increase of $CHCl_3$; CC^2: Gradient Si gel column, eluted with 100% *n*-hexane with gradual increase of EtOAc; CC^3: Gradient Si gel column, eluted with 100% $CHCl_3$ with gradual increase of MeOH

Scheme 5: Isolation of compounds from EtOAc extract of *Trewia nudiflora*



RP-FC: Flash chromatography using C-18 Si. gel column. Eluted with 100% water with gradual increase of acetone

Scheme 6: Isolation of pure compounds from the MeOH extract of *Trewia nudiflora*



GF[1]: Gel filtration eluted with 5% *n*-hexane in $CHCl_3$, with the gradual increase of $CHCl_3$, followed by the increase of MeOH

Scheme 7: Isolation of compounds from EtOAc extract of *Hygrophila auriculata*

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