Chapter 8

Plant selection and antimicrobial activity of solvent – solvent fractions of leaf material

8.1. Introduction

No *in vitro* method is available to localise antiviral compounds present in crude plant extracts, but bioautography remains a useful tool in revealing compounds with antibacterial and antifungal activity, localized on TLC plates. The compounds isolated using this technique in bioassay-guided fractionation can subsequently be tested against viral pathogens. *Podocarpus henkelii* was selected for isolation of bioactive compounds using acetone as the extractant. This plant, from the preliminary screening study was selected for further investigation based on the following reasons, 1) no compounds have been isolated from this plant and assayed for biological activity, 2) the acetone and methanol extracts exhibited good antibacterial activity against *E. coli*, *P. aeruginosa* and *E. faecalis,* 3) the acetone extract had excellent antifungal activity against *C. albicans* and good activity against *C. neoformans* and 4) the acetone extract had good to moderate activity against CDV and LSDV in both the virucidal and attachment assays, while the methanol extract was active against LSDV in the virucidal assay.

8.1.1. Description of the plant *Podocarpus henkelii* stapt ex Dallim. & Jacks.

Podocarpus henkelii stapt ex Dallim. & Jacks. (Podocarpaceae) is a large tree that grows up to 20m or more in height and often occurs in moist, evergreen mountain forests and less commonly in coastal forests. The leaves are dark green, shiny, long and slender, up to 17 x 1 cm, drooping, gradually tapering to a narrow apex and base. The leaf margin is entire and finely and tightly rolled under. The bark is yellowish grey, brown or dark grey. The male cones of the plant are large, about 3 x 0.4 cm, while in the female cone, the receptacle is not well developed and remains green. The plant has large, oval olive-green seeds measuring up to 2.5 x 2cm.

8.1.2.Taxonomy

Seven main genera make up the family Podocarpaceae; namely *Podocarpus* L' Her. ex Pers., *Dacrydium* Sol. Ex Forst., *Phyllocladus* Rich. Ex Mirb., *Acmopyle* Pilg., *Microcachrys* Hook.f., *Saxegothaea* Lindl. and *Pherosphaera* W. Archer bis (= Microstrobos J. Garden & L.A.S. Johnson, nom. inval.: Brummitt *et al.,* 2004) and accounts for most of the diversity in the Podocarpaceae. While some of these traditional genera form actively evolving complexes (i.e. *Podocarpus*, *Dacrydium*), others are of remote relevance (e.g., *Microcachrys*, *Saxegothaea*). The heterogeneity of

the genera *Podocarpus* and *Dacrydium* was documented a long time ago, but this diversity was commonly expressed taxonomically by means of subgenera, sections, and subgroups (Endlicher, 1847; Bertrand, 1874; Pilger, 1903; Florin, 1940; Buchholz and Gray, 1948).

The genus *Podocarpus* was primarily subdivided into eight sections, with the division placing more emphasis on the structure of the leaf, namely: *Afrocarpus* J. Buchholz & N. E. Gray, *Dacrycarpus* Endl., *Eupodocarpus* Endl., *Microcarpus* Pilg., Nageia (Gaertn.) Endl., *Polypodiopsis* C. E. Bertrand, *Stachycarpus* Endl., and *Sundacarpus* J. Buchholz&N. E.Gray (Buchholz and Gray, 1948). The African taxa were placed in the *Podocarpus* section (Leistner, 1966). Later on, suggestions of raising the section *Podocarpus* to generic ranks was proposed by Quinn (1970) and accepted by De Laubenfels (1972), using data on embryology, gametophyte development, female cone structure and cytology. Page (1989) further raised some of the other sections to generic ranks. These changes have however brought about nomenclature complications as exemplified by the raising of section *Afrocarpus* to the rank of genus with resultant rejection by some botanists (Leistner *et al.*, 1995; Glen, 2000).

Subsequent to these studies, several phylogenetic classifications of the Podocarpaceae have been undertaken, based on both morphological and molecular (DNA sequence) data (Kelch, 1997; Conran *et al.*, 2000; Sinclair *et al.*, 2002; Barker *et al.*, 2004) in an effort to resolve these discrepancies in nomenclature. It thus appears from available reports that there is significant molecular and morphological proof in favor of the generic level recognition of *Afrocarpus* and the other genera as recommended by Page (1989). These studies have resulted in the change of nomenclature of species of *Podocarpus* (*P. falcatus*) to *A. falcatus* (Thunb) C.N.Page (Barker *et al.*, 2004). Similarly, the taxonomic statuses of *P. milanjianus* and *P. latifolius* have been in contention. However, recent studies (Barker *et al.*, 2004) invalidated these suppositions. However, there still remain other taxonomic complications within the African Podocarpaceae such as the delimitation of species pair and complex, which needs to be, resolved (Barker *et al.*, 2004). With these existing problems in nomenclature, care should be taken when selecting a plant or collecting literature on *Podocarpus* species for a particular study (Lourens *et al.*, 2008). A summary of traditional and proposed taxa in the Podoacarpaceae family is presented in Table 8.1.

Table 8.1. Traditional and proposed taxa in the Podocarpaceae (Adapted from Kelch, 1997)

Adapted from Dallimore *et. al.*, 1966; Buchholz and Gray, 1948c; Florin, 1931	Adapted from Page, 1990	Present geographical distribution
Acmopyle Pilger	*Acmopyle* Pilger	New Caledonia, western Pacific (Fiji)
Dacrydium Sol. ex Lam.		
group A	*Falcatifolium* De Laubenf	New Caledonia, Malesia
group B	*Dacrydium* Sol. ex Lam	New Zealand, New Caledonia, western Pacific, Malesia, Philippines, Southeast Asia
group C	*Halocarpus* Quinn	New Zealand
group C	*Lagarostrobos* Quinn	New Zealand, Tasmania
group C	*Lepidothamnus* Phil..	New Zealand, southern South America
Microcachrys Hook. f.	*Microcachrys* Hook. f.	Tasmania
Microstrobos Gard. et Johns	*Microstrobos* Gard. et Johns.	Tasmania, New South Wales
Phyllocladus Rich. ex Mirbel	*Phyllocladus* Rich. ex Mirbe	New Zealand, Tasmania, Malesia
Podocarpus L, Her. ex Persoon		
P. sect. *Podocarpus* Endl.	*Podocarpus* L, Her. ex Persoon	
subsect. A, C, D, & E	P. subg. *Podocarpus*	New Caledonia, Australia, Tas- mania, New Zealand, Africa, Madagascar, Central and South America, Mexico, Caribbean
subsect. B & F	P. subg. Foliolatus De Laubenf.	Southeast Asia, Japan, Malesia, Philippines, western Pacific, New Caledonia, Australia
P. sect. Nageia Endl.	Nageia Gaertn. N. sect. Nageia (Endl.) De Laubenf.	Southeast Asia, India, Malesia, Philippines, Japan
P. sect. Afrocarpus Buchh. et Gray	N. sect. Afrocarpus (Buchh. et Gray) De Laubenf.	Africa
P. sect. Polypodiopsis Bertr.	N. sect. Polypodiopsis (Bertr.) De Laubenf.	South America, New Caledonia, western Pacific, Malesia
P. sect. Dacrycarpus Endl.	Dacrycarpus (Endl.) De Laubenf.	Malesia, New Zealand, New Caledonia, western Pacific, Philippines
P. sect. Microcarpus Pilger	Parasitaxis DeLaub.	New Caledonia
P. sect. Stachycarpus Endl. p.p.	Prumnopitys Phil.	South America, New Zealand, Queensland, New Caledonia
P. sect. Sundacarpus Buchh. et Gray	Sundacarpus (Buchh. et Gray) Page	Malesia, Queensland
Saxegothaea Lindl.	Saxegothaea Lindl.	southern South America

8.1.3. Chemotaxonomy

Plants contain an array of chemical constituents, some of which occur in abundance or are unique to a particular species. Hence, the presence or absence of certain classes of compounds can be used as chemotaxonomic markers in plants. One such class of compounds is the biflavonoids. Biflavonoids are dimers of flavonoids, linked by a C–O–C or C–C bond. In nature, very few plants contain biflavonoids as major constituents, and they can be found in *Selaginella* species, *Ginkgo biloba* and *Garcinia kola* (Kim *et al*., 2008). These classes of compounds are the chemotaxonomic markers in a majority of families from the Gymnospermae, including the families Taxaceae and Ginkgoaceae (Geiger and Quinn, 1988). Consequently, the *Podocarpus* species contain a simple pattern of derivatives based on amentoflavone and hinokiffavone. Presence of the biflavonoids amentoflavone and hinokiflavone including nor- and bisnorditerpenes in *Podocarpus* has been shown to be a good taxonomic marker in these species (Cambie and James, 1967; Ito and Kodama, 1976; Roy *et al*., 1987). Podocarpusflavone A is present in every species of *Podocarpus* so far investigated, except *P. latifolius*. Of equal importance is the recognition of the *Podocarpus* segregated genera by the presence or absence of different monomer flavonoid glycosides. For instance, *Dacrycarpus* is differentiated by the presence of 3-methoxyflavones, while *Prumnopitys* and *Podocarpus* are characterized by the predominance of flavonol 3-O-glycosides and flavone C-glycosides respectively (Markham *et al.,* 1985). The phytochemistry of *Podocarpus* s.l. has been extensively reviewed by Abdillahi *et al*., (2010) and a summary of chemical structures and pharmacological activities of some compounds isolated from species of *Podocarpus* is presented in Fig. 8.1.

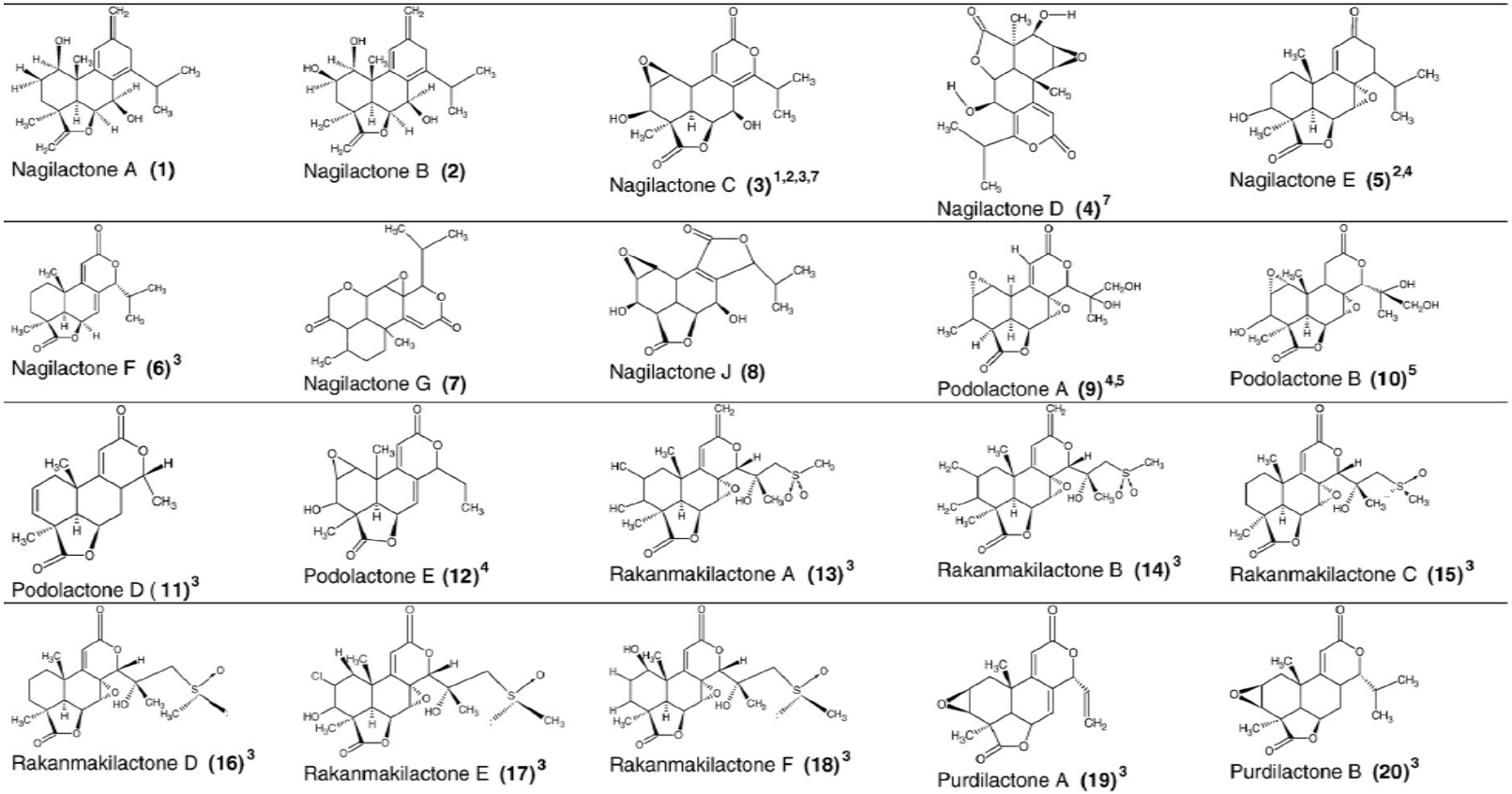

Figure. 8.1. Chemical structures and pharmacological activities of some compounds isolated from species of *Podocarpus* and revised genera. [1]antibacterial; [2]antifungal; [3]antitumor/cytotoxic/anticancer; [4]plant growth regulatory; [5]insect growth regulatory; [6]anti-inflammatory; [7]insecticidal; [8]antioxidant; [9]molluscidal; [10]larvicidal; [11]gastroprotective; [12]hypocholesterolemic; [13]anti-tyrosinase/melanin inhibition. (Adapted from Abdillahi *et al*., 2010)

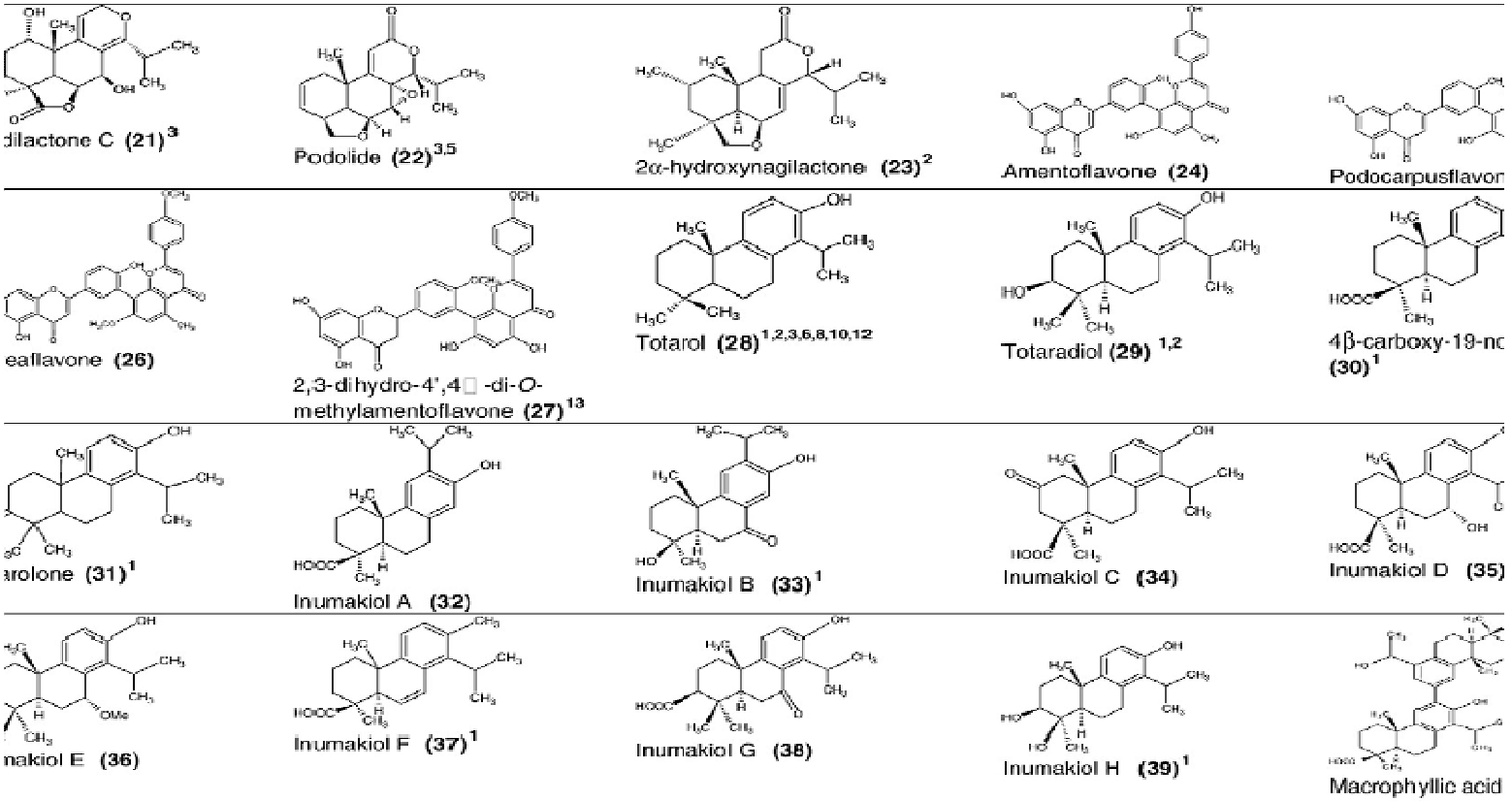

Fig. 8.1. cont. Chemical structures and pharmacological activities of some compounds isolated from species of *Podocarpus* and revised genera. [1]antibacterial; [2]antifungal; [3]antitumor/cytotoxic/anticancer; [4]plant growth regulatory; [5]insect growth regulatory; [6]anti-inflammatory; [7]insecticidal; [8]antioxidant; [9]molluscidal; [10]larvicidal; [11]gastroprotective; [12]hypocholesterolemic;[13]anti-tyrosinase/melanininhibition. (Adapted from Abdillahi *et al.*, 2010)

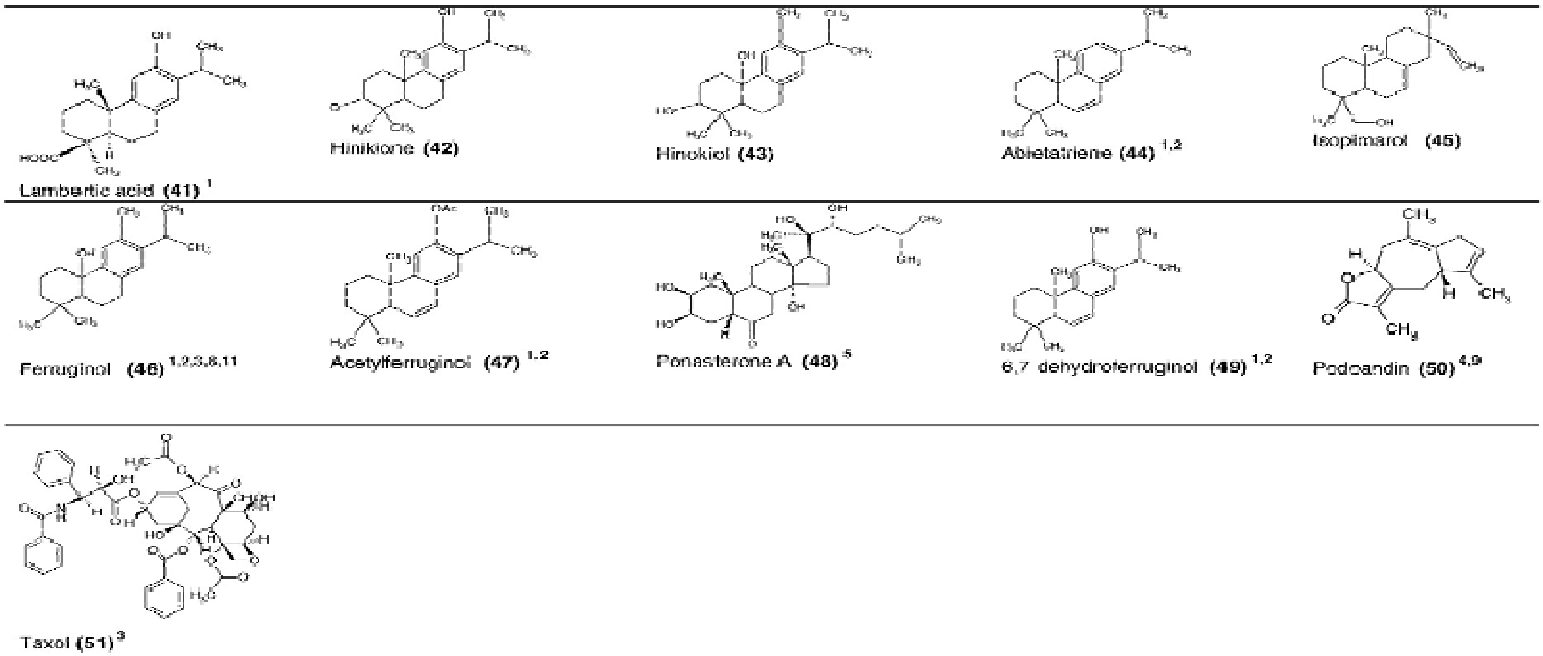

Fig. 8.1. cont. Chemical structures and pharmacological activities of some compounds isolated from species of *Podocarpus* and revised genera. [1]antibacterial; [2]antifungal; [3]antitumor/cytotoxic/anticancer; [4]plant growth regulatory; [5]insect growth regulatory; [6]anti-inflammatory; [7]insecticidal; [8]antioxidant; [9]molluscidal; [10]larvicidal; [11]gastroprotective; [12]hypocholesterolemic; [13]anti-tyrosinase/melanin inhibition (Adapted from Abdillahi *et al*., 2010)

8.1.4. Medicinal uses

Several species of *Podocarpus* s.l. have been used in different cultures around the world as a remedy for various ailments in human and animals. Depending on the type of ailments, different plant parts such as leaves, bark and fruit have commonly been used. A summary of the medicinal use of species of *Podocarpus* s.l is presented in Table 8.2. This species of plant has proved to be useful in the treatment of fevers, asthma and coughs (Chopra *et al*., 1986; Riley, 1994), cholera, heart ailments, kidneys, lungs, stomach diseases and for sweaty feet, worms and blood disorders (Duke and Ayensu, 1985). Other authors have reported their use in the treatment of rheumatism and painful joints (Chopra *et al*., 1986), as antitumor agent and in pest control (Nakanishi, 2006), treatment of distemper in dogs and gall sickness in cattle (Dold and Cocks, 2001; Masika and Afolayan, 2003), chest complaints and stomach ache (Watt and Breyer-Brandwijk, 1962; Beentje, 1994) and gonorrhoea (Pankhurst, 2000).

Table 8.2. Medicinal uses of *Podocarpus* species (Adapted from Abdillahi *et al.,* 2010)

Species	Geographical distribution	Plant part	Medicinal uses	References
Podocarpus henkelii Stapf ex Dallim. & Jacks.	South Africa	Sap	chest complaints	(Watt and Breyer-Brandwijk,1962; Hutchings *et al.,* 1996)
Podocarpus falcatus (Thunb.) R. Br. Ex Mirb.	South Africa, East Africa	bark sap Oil	gallsickness in cattle,distemper in dogs, head ache chest complaints gonorrhoea	Watt and Breyer-Brandwijk,1962; Sindiga, 1995;Hutchings *et al.*, 1996; Venter and Venter, 1996;Pankhurst, 2000; Dold and Cocks, 2001)
Podocarpus ferrugineus Don. (Miro)	New Zealand	Gum		(Uphof, 1968; Johnson, 1999)
Podocarpus latifolius (Thunb.) R. Br. Ex Mirb.	South Africa, East Africa	bark Sap	gallsickness in cattle,distemper in dogs stomachache, screeaned for anticancer and AIDS. chest complaints	(Watt and Breyer-Brandwijk,1962;Cunningham ,1993; Beentje, 1994;Sindiga,1995; Hutchings *et al*.,1996; Dold and Cocks, 2001)
Podocarpus macrophyllus (Thunb.) Sweet	China, Japan, E. Asia	stem bark fruit	ringworms and blood disorders tonic for heart, kidneys, lungs and stomach .	Duke and Ayensu (1985)
Podocarpus nagi (Thunb.) Zoll. & Moritz.	East Asia, Japan, Mexico, New Zealand	bark stem bark fruit seed	antiseptic,astringent, carminative and treatment of fevers, asthma, coughs, cholera arsenic poisoning, skin diseases and ulcers carminative, pectoral and stomachic. cholera, heartailments, stomach diseases and sweaty feet.	(Chopra *et al*., 1986; Duke and Ayensu, 1985)
Podocarpus nakaii Hayata	Taiwan		antitumor agent and pest control	Nakanishi (2006)
Podocarpus neriifolius D. Don.	Papua New Guinea, Himalayas and China	leaves	rheumatism and painful joints.	Chopra *et al.*, (1986)
Podocarpus totara G. Bennett ex D. Don	New Zealand	bark leaves berries	gonorrhoea and syphilis, splints on limbs, fever piles, sores and lesions laxative,constipation in women .	Riley (1994)
Podocarpus sp.	Java, Malaya		arthritis and rheumatism.	Johnson (1999)

8.2. Materials and Methods

8.2.1. Solvent-solvent fractionation of leaf material

Ground material (500 g) was extracted using acetone (1 g/10 ml) for 24 hours. The supernatant was filtered through Whatman No 1 filter paper using a Büchner funnel. The dried acetone extract (43 g) was subjected to solvent-solvent fractionation as described by Suffness and Douros (1979) and adapted by Eloff (1998a) to fractionate the components based on polarity (section 3.6). The components of the crude extract were separated into the n-butanol, hexane, ethyl acetate, carbon tetrachloride, chloroform and methanol: water fractions.

8.2.2. Analysis and concentration of fractions

All fractions were collected in glass jars following solvent-solvent fractionation and concentrated under a stream of air. Fractions were reconstituted to 10 mg/mℓ for TLC analysis and bioassay. For TLC analysis, fractions were spotted onto TLC plates, eluted in suitable solvent systems (section 3.4), viewed under UV light and sprayed with vanillin sulphuric acid reagent prepared as described (section 3.4) and heated at 100°C for five minutes to allow for colour development. Those fractions with similar TLC profiles were combined. The minimum inhibitory concentrations of the fractions were determined as well as bioautography using the different organisms. Plates for bioautography were not sprayed with the chromogenic spray reagent prior to the assay.

8.2.3. Bioassay–guided fractionation

The different fractions were spotted onto TLC plates and sprayed with concentrated suspensions of bacterial and fungal pathogens as described (Sections 3.7.1 and 3.8.1) while MIC was determined as described (section 3.7.2. and 3.8.2.). The retardation factor (R_f) values of compounds showing zones of inhibition were recorded.

8.3. Results and Discussion

The acetone leaf extract of *Podocarpus henkelii* was partitioned into six fractions and bioautography was carried out to identify active antibacterial constituents using *S. aureus* and *E. coli*, and antifungal constituents using *C. albicans, C. neoformans* and *A. fumigatus*. The carbon tetrachloride fraction had more active compounds, followed by chloroform, ethyl acetate and hexane fractions in that order against *S. aureus*. The n-butanol and methanol: water fraction had less active compounds (Fig. 8.2). The R_f values of compounds active against *S. aureus* in the carbon tetrachloride fraction were 0.5, 0.62, 0.8 and 0.9, for chloroform they were 0.6, 0.62 and 0.9, for ethyl acetate 0.6 and 0.62, and for the hexane fraction 0.8. On the other hand, R_f values for compounds active against *E.coli* for the carbon tetrachloride fraction were 0.43, 0.53, 0.62 and 0.9, for chloroform 0.43, 0.53, 0.62 and 0.9, for ethyl acetate 0.6 and 0.62, and for the hexane fraction 0.9 (Table 8.3a).

For the antifungal activity, the chloroform fraction had more active compounds against the three fungal pathogens, followed by the carbon tetrachloride and ethyl acetate fractions, while the n-butanol and methanol: water fractions had fewer active compounds (Fig 8.3). The R_f values for compounds in the carbon tetrachloride fraction active against *A. fumigatus* were 0.63 and 0.9, and against *C. albicans* 0.93. On the other hand, the chloroform fraction had compounds active against *A. fumigatus* with R_f values of 0.54, 0.63 and 0.9, against *C. albicans*, R_f values were 0.93, 0.6 and 0.7 and against *C.neoformans*, R_f values were 0.6 and 0.63. The ethyl acetate fraction had one compound active against *A.fumigatus* (R_f = 0.9) and *C. albicans* (R_f = 0.93) (Table 8.3b).

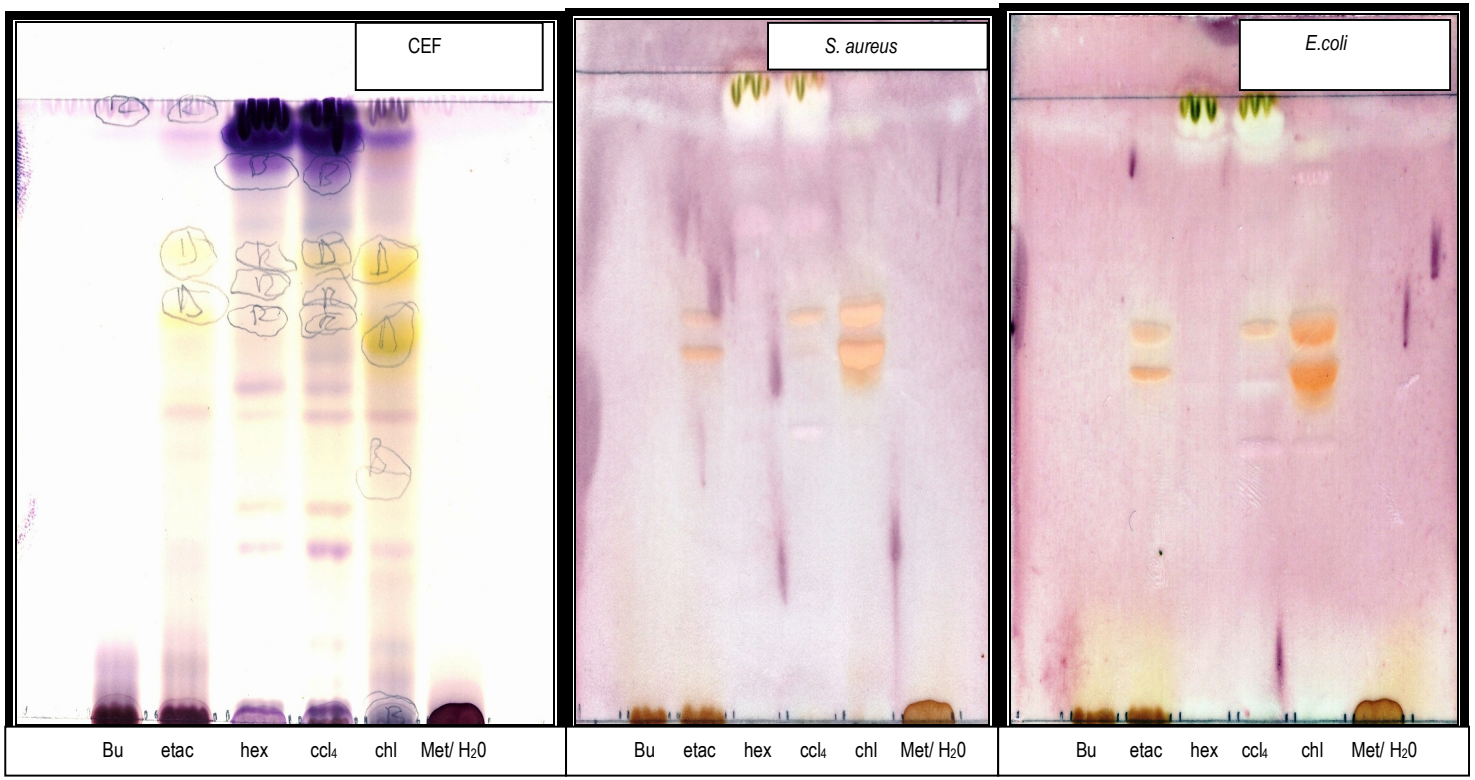

Figure 8. 2. Bioautography of solvent-solvent fractions indicating zones of inhibition on TLC plates against bacterial pathogens. Zones of inhibition against a purple background indicate activity of separated compounds on TLC plates eluted in CEF against *S. aureus* and *E. coli,* Bu = butanol, etac = ethyl acetate, hex = hexane, ccl4 = carbon tetrachloride, chl = chloroform, met = methanol

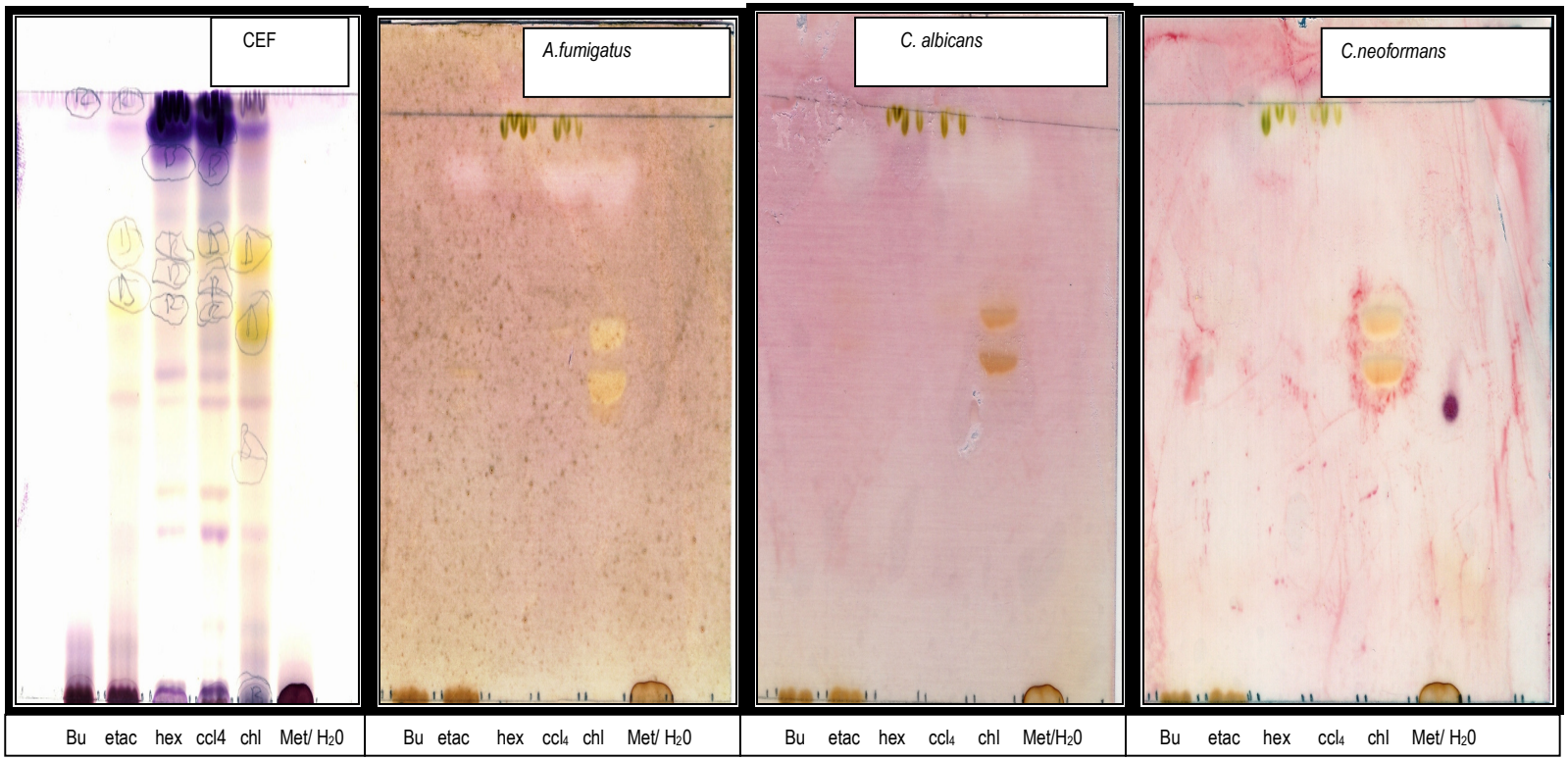

Figure 8. 3. Bioautography of solvent-solvent fractions indicating zones of inhibition on TLC plates against fungal pathogens.

Zones of inhibition against a purple background indicate activity of separated compounds on TLC plates eluted in CEF against *A. fumigatus, C. albicans* and *C. neoformans,* Bu = butanol, etac = ethyl acetate, hex = hexane, ccl4 = carbon tetrachloride, chl = chloroform, met = methanol

Table 8.3a. R_f values of compounds in solvent-solvent fractions active against bacterial pathogens

Organism	CCL_4	Ethyl acetate	$CHCL_3$	Hexane
S. aureus	0.5	0.6	0.6	0.8
	0.62	0.62	0.62	
	0.8		0.9	
	0.9			
E. coli	0.43	0.6	0.43	0.9
	0.53	0.62	0.53	
	0.62		0.62	
	0.9		0.9	

Table 8.3b. R_f values of compounds in solvent-solvent fractions active against fungal pathogens

Organism	CCL_4	Ethyl acetate	$CHCL_3$
A. fumigatus	0.63	0.9	0.54
	0.9		0.63
			0.9
C. albicans	0.93	0.93	0.93
			0.6
			0.7
C. neoformans			0.6
			0.63

The MIC values for the different fractions are presented in Tables 8.3a and 8.3b) against bacterial and fungal pathogens. In some fractions, variation was observed in susceptibility of pathogens with time of incubation. The hexane fraction against *S. aureus* for instance had a low MIC of 0.08 mg/mℓ at 12 hour but following prolonged incubation, a higher MIC of 0.16 mg/mℓ was obtained. This variation in susceptibility may suggest the ability of the organism to revive itself at lower concentrations and susceptibility to the extract at higher concentrations. The lowest MIC of 0.08 mg/mℓ was obtained for the carbon tetrachloride and ethyl acetate fractions against *S. aureus* followed by the chloroform and hexane fractions having an MIC of 0.16 mg/mℓ. With this pathogen, the presence of a high number of active compounds on bioautography correlated with a good MIC value. Unlike *S. aureus*, *E. coli* was more sensitive to the hexane extract with an MIC of 0.04 mg/ml, followed by the carbon tetrachloride and ethyl acetate fractions respectively. Although the chloroform fraction had active compounds comparable to those in the ethyl acetate fraction, the MIC value were higher (0.16 mg/mℓ) when compared to those obtained for the ethyl acetate fraction. The low activity obtained for the chloroform fraction suggests the presence of compounds in this fraction that may antagonize the activity of one another in the fraction. *E. faecalis* on the other hand was more sensitive to the carbon tetrachloride fraction, while the same MIC value of 0.16 mg/mℓ was obtained for both the ethyl acetate and hexane fractions against this pathogen. With *P. aeruginosa* the organism was most susceptible to the carbon tetrachloride fraction with an MIC of 0.08 mg/mℓ. Apart from the carbon tetrachloride fraction, the susceptibility of *P. aeruginosa* to the other fraction seems to require a high concentration with prolonged exposure time. Overall, the carbon tetrachloride and ethyl acetate fractions were the most active against the test bacterial pathogens.

In the antifungal assay, *C. neoformans* was the most susceptible of the three pathogens tested with MIC of 0.08 mg/mℓ obtained for the carbon tetrachloride and chloroform fractions followed by *A. fumigatus* with an MIC of 0.16 mg/mℓ (Table 8.3b). Comparing the presence of active constituents on bioautography in the different extracts, the susceptibility of *C. neoformans* to the carbon tetrachloride fraction does not appear to be related to the presence of single active components in the fraction. A similar effect was observed with this fraction against *C. albicans*. Although no active compound was identified in the carbon tetrachloride fraction against this pathogen, the low MIC obtained suggests the presence of compounds with synergistic effects in the fraction responsible for the activity observed.

8.4. Conclusion

Of the different fractions evaluated, the ethyl acetate, carbon tetrachloride and chloroform fractions contained the highest numbers of antibacterial and antifungal compounds that were active against one or more organisms tested in the study. The study also suggests that synergism and antagonism between different compounds contained in a fraction may potentiate or reduce the activity of the fraction. The fractions identified containing active compounds will be combined. The bioactive compounds will be isolated and evaluated for biological activity.