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Review

Pharmacological, pharmaceutical, cosmetic and diagnostic applications of sulfated polysaccharides from marine algae and bacteria

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Marine environment with rich biodiversity offer unlimited choice for novel biopolymers. Sulfated polysaccharides isolated from marine algae and bacteria constitute an important group in the marine-derived biomolecules and biopolymers. They possess unique structural features which can be exploited to their fullest potential in the development of new therapeutic molecules, design of nanocarriers and stimuli-responsive drug delivery systems, development of anti-aging and moisturizing creams and as molecular probes in diagnosis of cancers and cardiovascular diseases. The aim of the present review is to highlight the sources, characteristics and applications of sulfated polysaccharides and exopolysaccharides isolated from marine algae, cyanobacteria, extremophilic and halophilic bacteria. Detailed description of physicochemical properties and versatile applications of ulvan, fucoidan, galactofucan sulfate, laminarin, mauran, cyanobacterial exopolysaccharides and other lesser known exopolysaccharides of marine bacterial origin has been provided. In a nutshell, it can be concluded that sustainable exploitation of the renewable, diverse library of these unique and novel sulfated polysaccharides will unravel newer possibilities in future and will enrich the existing arsenal of biopolymers.

Key words: Exopolysaccharide, marine biopolymer, molecular probe, nanocarriers, stimuli-responsive drug delivery systems, sulfated polysaccharide.

INTRODUCTION

Around the world, demand for novel biopolymers and bioactive molecules with unique characteristics and improved functionalities has increased. Occupying almost ¾-ths of the Earth's surface, the oceans represent underexploited but sustainable source of biologically significant and relevant natural products which can also be used for various other industrial processes. Marine environment is a highly diverse environment which is a

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home to different species of bacteria, algae or seaweeds, invertebrates and vertebrate animals. Some particular marine environments offer wide biodiversity and hence chemi-diversity. It is often said that the marine microbial world can be regarded as the largest world of chemical diversity. Many of the natural products obtained from the marine ecosystem have been investigated for their potential applications and benefits in the field of medicine, pharmacy and diagnosis (Borresen et al., 2010). Compounds obtained from taxonomically diverse marine flora have exhibited antioxidant, anti-inflammatory, immune-stimulatory and antimicrobial properties. Moreover, they can control carcinogenesis by preventing oxidative damage of DNA, inducing apoptosis in cancer cells and therefore, can serve as lead compounds for anticancer agents (Boopathy and Kathirsen, 2010). Some of the most commonly exploited marine biomaterials in pharmaceutical field include agar, agarose, alginates, carrageenan, chitosan, chitin, collagen, hyaluronan etc. Advancements in biotechnological approaches and isolation techniques have created opportunities for exploring the vast world of marine flora for chemically diverse, novel bioactive molecules and biopolymers (Ige et al., 2012; Fink, 2015).

Seaweeds or marine algae are being used from time immemorial in China and Japan either as dietary components or as remedies because of their immense therapeutic benefits. They are rich sources of carotenoid pigments, omega-3-fatty acids, phycocyanin, fucosterols, iodine organic products, mannitol, macro- and micro-elements, vitamins A, B, C and E, unsaturated fatty acids, polyphenols, sulfated polysaccharides, essential amino acids, peptides and proteins (Boopathy and Kathiresan, 2010; Lee et al., 2013; Moghadamtousi et al., 2014; Menshova et al., 2016). Marine algae have been classified as Rhodophyta, Phaeophyta, Chlorophyta and Cyanophyta which are known as red, brown, green seaweeds and blue-green algae, respectively and till date, almost 680 species have been identified (Boopathy and Kathiresan, 2010; Silva and Alves et al., 2012). This classification has been done by botanists on the basis of the photosynthetic pigments present (Cunha and Grenha, 2016). Some of the compounds isolated from seaweeds that have been widely exploited in the pharmaceutical industry for commercial purposes include carrageenan (from red seaweed) and alginic acid (from brown seaweed). Crude extracts or seaweed as a whole have been traditionally used for amelioration of joint pain, inflammation and also for treatment of burns (Fitton et al., 2016). There are reports of anticancer effects of different species of brown algae, *Sargassaceae* sp., *Dictyota dichotoma,* and *Desmarestia ligulata* against varying cancer cell lines (human leukaemic T cell lymphoblast [Jurkat], human Burkitt's lymphoma [Daudi], human chronic myelogenous leukaemia [K562] cells, human breast adenocarcinoma [MCF-7] , human prostate cancer cells [DU 145, PC-3, and LNCa], murine colon cancer cell line [CT-26], human leukemia [THP-1], mouse melanoma [B-16], and human leukemia [U-937] cells (Moghadamtousi et al., 2014).

Bioactive metabolites of varying chemistry have been reported to be present in marine algae. They are brominated phenols, brominated oxygen heterocyclics, nitrogen heterocyclics, kainic acids, guanidine derivatives, phenazine derivatives, amino acids and amines, sterols, sulfated polysaccharides and prostaglandins. Most of the sulfated polysaccharides have been found to possess health-promoting effects (Lee et al., 2013). Polysaccharides are ubiquitous natural macromolecules consisting of osidic monomers. They can be classified as homopolysaccharides or heteropolysaccharides depending on the occurrence of single type of monosaccharide or different osidic residues. They are characterized by a regular back-bone structure with a repeating unit, which may be linear or branched, comprising up to 10 monomers and organic or inorganic substituents such as phosphate, sulfate, and lactic, succinic, acetic and pyruvic acids. Monosaccharide composition, linkages between the monosaccharide, sequence of the repeating units and non-carbohydrate substituents in the polysaccharide vary with the algal species, fermentation and harvesting conditions as well as the physiological state of the microbe. Types of glycosidic linkages between the monomeric units such as β-1,4 or β-1,3 provide a rigid backbone and α-1,2 or α-1,6 glycosidic linkages yield molecule with flexible regions (Ladrat et al., 2014). Polysaccharides have been reported to display an array of biological activities like antioxidant, anticoagulant, anticancer, antiviral, anti-allergic, anti-adhesive, anti-angiogenic and anti-inflammatory activities (Posocco et al., 2015; Cardoso et al., 2016). Biocompatibility, biodegradability, non-toxicity and stimuli-responsiveness of marine polysaccharides support their utilization in the fabrication of stimuli-responsive "smart" or "intelligent" drug delivery systems. Marine polysaccharides have found wide applications in the field of nanotechnology, in design of controlled release drug delivery systems for encapsulation of bioactive agents, in formulation of hydrogels, in gene delivery and also in regenerative medicine.

Sulfated polysaccharides obtained from marine algae and bacteria are unique in the sense that they possess different innovative and complex chemical structures and functionalities, with no parallels or equivalents being found in terrestrial organisms (Cunha and Grenha, 2016). Exopolysaccharides (EPS) are glycopolymers obtained from marine mesophilic, heterotrophic as well as extremophilic (psychrophilic, thermophilic and halophilic) bacteria and all of them are capsular polymers. These are attached to the cell membrane through lipopolysaccharides or proteins present in cell membrane, facilitate growth by promoting adhesion to solid surfaces,

protect the microorganisms against extreme environmental conditions of high or low temperature and/or salinity and lastly, against predators. Potential applications of marine bacterial exopolysaccharides include therapeutic applications as immunomodulator, antitumor, antiviral agents as well as adjuvants in vaccine delivery systems and diagnostic imaging agents. Extracellular polysaccharides of commercial importance have been produced from unicellular red algae, *Porphyridium cruemtum* and *P. aerugineum* and also from *Chlamydomonas mexicana* and certain species of green and blue-green algae. *Porphyridium* polysaccharide has the potential to replace the carrageenan in biomedical field (Laurienzo, 2010; Poli et al., 2010; Ladrat et al., 2014; Cardoso et al., 2016).

Numerous studies indicate pharmacological effects from chemically diverse compounds such as polyphenols, phlorotannins, alkaloids, polysaccharides, extracted from different species of marine alga and exopolysaccharides from marine bacteria. Overexploitation of petroleum resource for synthesis of additives and excipients used in pharmaceutical and cosmetic industry not only depletes the reserve but also adds to environmental risks and occupational hazards. Therefore, alternative and renewable source for less toxic and environment-friendly excipients is the need of hour. Among the different sulfated polysaccharides of marine algal origin investigated till date, number of studies has been carried out on various aspects of carrageenan (from red algae). Although, therapeutic potential of fucoidan (from brown algae) has been studied extensively, other applications of fucoidan in the design of drug delivery systems and as diagnostic tools have not been reported in a systematic manner. The situation is more or less similar with ulvan (from green algae) and other sulfated polysaccharides such as galactofucan, laminarin, mauran, species-specific sulfated polysaccharides and exopolysaccharides from different bacterial population. Thus, there is lack of comprehensive review on pharmacological, pharmaceutical, cosmetic and diagnostic applications of sulfated polysaccharides isolated from various species of marine algae and bacteria. The present review attempts to fill up this lacunae and focuses on the source, physicochemical characteristics and biofunctional activities of sulfated polysaccharides obtained from marine algae and bacteria, namely ulvan, fucoidan, galactofucan sulfate, laminarin, mauran, cyanobacterial exopolysaccharide and sulfated polysaccharides of not-so-frequent occurrence in different algae and bacteria.

PHYSICOCHEMICAL CHARACTERISTICS OF SULFATED POLYSACCHARIDES

Sulfated polysaccharides obtained from marine algae include sulfated fucans or fucoidans, sulfated galactans which include carrageenans and agarans, sulfated glucans and sulfated arabinogalactans. They may be classified as linear or branched polymers and classification can be on the basis of charge of the functional groups attached to the central sugar backbone (Raveendran et al., 2013a). Depending on the marine algal source and type of sugars present, commonly occurring sulfated polysaccharides can be divided into a. water-soluble fucan or fucoidan, obtained from brown algae, containing fucose, xylose, uronic acid, galactose with varying degrees of sulfation, b. linear galactans and carrageenans, obtained from red algae, composed of sulfated galactose and 3,6-anhydro galactose and lastly, c. water-soluble, ulvan which consists of sulfated rhamnose and sulfated aldobiouronic acid and obtained from green algae (Patel, 2012).

Chemical composition of the sulfated polysaccharides obtained from various families of marine algae is species-specific, depends on their ecophysiological origin and differs in the degree and distribution of the sulfate groups (Chiellini and Morelli, 2011). The soluble fibers found in abundance in brown seaweeds are alginates, fucans and laminarins. Similarly, the soluble fibers in red algae include amorphous polymers, sulfated galactans (agars and carrageenans), xylans and floridean starch. Starch, xylans, mannans and ionic sulfated polysaccharides are present in green algae. They also contain uronic acids, rhamnose, xylose, galactose, and arabinose (Raveendran et al., 2013a; Hamed et al., 2015).

Physical properties of sulfated polysaccharides such as aqueous solubility or ionic interactions and biological activity depend on their chemical structure, sequence of the monomeric units, and nature of linkages between the monomers. Nature of the substituents also governs the geometry and conformation of the polysaccharide molecules. Structural modification through de-polymerisation or over-sulfation results in development of new properties and improvement in polymer functionality (Chopin et al., 2014; Ladrat et al., 2014).

Ulvan is a branched sulfated anionic polysaccharide isolated from the green algae, *Ulva* and *Enteromorpha*, and consists of sugar residues-glucose, rhamnose, xylose, glucuronic acid, iduronic acid and sulfated rhamnose. Presence of iduronic acid and sulfated rhamnose makes ulvan unique from that of other marine polysaccharides and accounts for its similarity with mammalian glycosaminoglycans. It shows variations in molecular weight ($1.14\text{-}2 \times 10^6$), electronic density and charge distribution (Silva et al., 2012a; Cardoso et al., 2016). Composition of *Ulva*-derived sulfated heteropolysaccharide depends on taxonomic origin, growth conditions, period of collection and post-collection treatment (Chiellini and Morelli, 2011). Compounds such as 4-O-(β-D-glucuronsyl uronic acid)-L-rhamnose and small quantities of aldobiouronic acids, 3-O- and 4-O-(D-glucuronosylronic acid)-D-xylose could be detected in

high percentages in ethanolic extracts of *Ulva lactuca*. An acidic tetrasaccharide, D-glucuronic acid-(1→4)-L-rhamnosyl (1→3/4)-D-glucuronosyluronicacid-(1→3) D-glucose, was obtained on partial acid hydrolysis of desulfated and carboxy-reduced ulvan (Umapoorani et al., 2016). Net charge of the polysaccharide solution is governed by the pH and ionic strength of the medium in which it is dissolved. The pH of the aqueous solution has been found to be 7.5. Conformation change from ordered state to disordered structure occurs only at a critical charge density. Aqueous solution of ulvan displays low intrinsic viscosity (18-100 cps) attributed to the formation of necklace-type ultrastructure (Chiellini and Morelli, 2011; Cunha and Grenha, 2016).

Another sulfated polysaccharide of interest is fucoidan which is obtained from brown algae, namely mozuku, komby, limu moui and from edible species such as *Fucus vesiculosis, Laminaria japonica*, *Analipus* sp., *Chordus* sp. and *Undaria pinnatifida*. Different types of fucoidans could be isolated from tropical brown sea weeds such as *Turbinaria turbinata*, *Sargassum filipendula*, *Dictyota caribaea* and *Padina perindusiata* and were found to be homofucan, heterofucan and galactofucan (Garcia-Rios et al., 2012). The molecular weight of fucoidan varies between 10-950 kDa. It usually exists in two forms: F-fucoidan comprising of l-fucose units and U-fucoidan consisting primarily of glucuronic acid units. The α-L-fucose units (also known as α-L-fucopyranose) may be sulfated at C-2, C-4 and sometimes at C-3 positions. Occurrence of fucoidan results in structural roughness and flexibility of the brown algae and also provides protection to the algal cell against UV rays and harsh environmental conditions. Other sugar components, including mannose, galactose, glucose, xylose, uronic acids and non-sugar acetyl groups are also present. Fucoidan isolated from *F. vesiculosis* consists primarily of fucose with low degree of acetylation (Moghadamtousi et al., 2014; Fitton et al., 2016). The aqueous solubility of the sulfated polysaccharide has been reported as 10 mg/ml. Although fucoidan is hygroscopic, its aqueous solution displays low viscosity. Rheological studies on fucoidan isolated from *F. vesiculosis* exhibited Newtonian behavior and highest viscosity compared to polysaccharides isolated from other species. Viscoelasticity of fucoidan is affected by the presence of electrolytes like sodium chloride and calcium chloride and also non-electrolytes like sugar. Stability of the polysaccharide is pH-independent (Silva et al., 2012; Kim and Venkatesan, 2015; Cunha and Grenha, 2016). It has an excellent oral safety profile in animals and humans and is GRAS according to FDA specifications (Dithmer et al., 2014; FDA GRAS Notice, 2014; Lean et al., 2015).

A water-soluble sulfated polysaccharide has been extracted from Korean green alga Maesaengi (*Capsosiphon fulvescens*). It is a glucuronogalactomannan, whose backbone consists of alternating sequence of 4-linked L-rhamnose-3-sulphate and D-xylose residues (ulvobiose U3s) having monomeric D-glucuronic acid or D-glucuronic acid-3-sulphate as the side chains on O-2 of some of the L-rhamnose-3-sulphate units (Synytsya et al., 2015). Sulfated polysaccharide is also present in another species of brown algae, *Eclonia cava*, which consists of high percentage of fucose and smaller percentages of galactose, xylose and mannose (Boopathy and Kathiresan, 2010). An acidic polysaccharide isolated from brown seaweed *Lobophora variegate* was found to possess a high level of sulfated fucose and galactose and is named as galactofucan sulfate (Castro et al., 2016).

Laminarin, which is a linear storage glucan has been isolated from some species of brown algae, *Ascophyllum nodosum, Laminaria hyperborea, Laminaria digitata, Ecklonia kurome, Saccharina sp. etc.* Laminarin consists of (1,3)-β-D-glucan having (1,3)-β-D-glucopyranose residues with some 6-*O*-branching in the main chain and some β-(1,6)-intrachain links. The molecular weight of the polysaccharide is approximately 5 kDa. It has a cloud-like and spongy appearance in the solid state. The aqueous solubility depends on the degree of branching (Silva and Alves et al., 2012; Burgess et al., 2015; Ji et al., 2015; Kadam et al., 2015). Its high solubility in both organic and aqueous solvents but low viscosity of the aqueous solution favor its easy handling for industrial applications (Custodio et al., 2016).

Exopolysaccharides (EPSs) are high molecular weight anionic or neutral heteropolysaccharides, abundant in marine microbes, as extracellular component. They contain three or four different monosaccharides arranged in groups of 10 or less to form the repeating unit and are usually linear with molecular weight in the range of 1-3kDa. Uronic acids (D-glucuronic acids, D-galacturonic acids) or ketal-linked pyruvate, succinate, acetate or inorganic residues such as phosphate or sulfate occur in the structure. Monosaccharide such as pentoses (as D-arabinose, D-ribose, D-xylose), hexoses (D-glucose, D-galactose, D-mannose, D-allose, L-rhamnose, L-fucose), amino sugars (D-glucosamine and D-galactosamine) are present in EPS (Poli et al., 2010).

Mauran is an anionic sulfated exopolysaccharide, isolated from a moderately halophilic bacterium, *Halomonas maura*. The polysaccharide has exhibited characteristic viscoelastic, pseudoplastic and thixotropic behavior (Raveendran et al., 2013b; Srivastava and Kowshik, 2015).

Exopolysaccharides produced from Cyanobacteria are released as soluble polysaccharides (RPS) in the culture medium and exhibit protective function by providing sheaths, capsules or slimes over the bacteria. They are unique in the sense that they are heteropolymers composed of 6-10 different monosaccharides, the most abundant being glucose. Variation in structure results in different architectures. Rheological properties of aqueous

solutions of RPS are unaffected by alterations in pH, temperature or ionic strength. Presence of uronic acid and sulfate groups imparts affinity for metal cations (Laurienzo, 2010).

The average molar mass of extracellular sulfated polysaccharide occurring in marine red microalgae, *Porphyridium* sp. has been found to be 2.3×10^6 g/mol. Increased sonication of aqueous solution of the polysaccharide resulted in significantly lower viscosity, indicated by transition from weak gel-state to liquid-like state (Geresh et al., 2002).

Two different EPS have been extracted from *Bacillus licheniformis* strain B3-15 and *Geobacillus* sp. 4004 EPS. The former is a tetrasaccharide repeating unit with sugars having a *manno-pyranosidic* configuration. In the latter, in the repeating saccharidic unit, two residues have a *gluco/galacto* configuration and three possess a *manno* configuration (Poli et al., 2010). The exopolysaccharide, HE 800, secreted by deep vent marine bacterium, *Vibrio diabolicus*, is structurally analogous to hyaluronic acid, possesses a linear backbone with a molecular weight of about 8 kDa and is regarded as glycosaminoglycan (Courtois et al., 2014).

THERAPEUTIC ACTIVITIES OF SULFATED POLYSACCHARIDES AND EXOPOLYSACCHARIDES FROM MARINE ALGAE AND BACTERIA

The unique structures and sulfation patterns of marine sulfated polysaccharides are responsible for their vast array of therapeutic activities including immune-modulatory and cytotoxic effects. Some of the molecules are already marketed as nutraceuticals and few others are in various stages of preclinical trials. They are known to possess low immunogenicity (Boopathy and Kathiresan, 2010; Glycomer, 2012). Molecular weight, sulfate content and distribution, introduction of other functional groups, monosaccharide composition and structure of the backbone of ulvan and fucoidan determine the anti-oxidant, anti-coagulant and anti-tumor efficacy of the sulfated polysaccharides. Higher molecular weight accounts for improved anti-coagulant action (Silva et al., 2012; Kim et al., 2015; Cardoso et al., 2016; Cunha and Grenha, 2016).

Ulvan shows diverse pharmacological actions such as antitumor, anti-hyperlipidemic, immune modulation, antibacterial, antiviral, laxative, antifungal, hepato-protective, antiprotozoal, leishmanicidal, anti-inflammatory, anti-nociceptive, antioxidant and anticoagulant actions. It can be used as a chelating agent owing to its ability to form complexes with metal ions and can prove beneficial in treatment of metal poisoning. Ulvan, isolated from *U. lactuca*, exhibited antimitotic activity during investigation on *Allium cepa* meristematic root tip whereas that from *Ulvan rigida* has demonstrated immunomodulatory activity on murine macrophages (Chiellini and Morelli, 2011; Silva et al., 2012; Cardoso et al., 2016; Umapoorani et al., 2016).

Fucoidan is known for its multifarious biological activities like anti-inflammatory, anti-coagulant, anticancer, anti-metastasis, antiviral (against herpes simplex virus type 1 [HSV-1], HSV-2 and human cytomegalovirus), anti-lymphangiogenesis and immunomodulatory actions (Silva et al., 2012; Kim and Venkatesan, 2015; Cunha and Grenha, 2016; Menshova et al., 2016). Various mechanisms have been postulated to explain the chemotherapeutic and chemopreventive action of fucoidan and also its anti-inflammatory activity (Moghadamtousi et al., 2014; Atashrazm et al., 2015; Lowenthal and Fitton, 2015; Choo et al., 2016). Role of fucoidans in inhibiting the attachment of *H. pylori* to gastric epithelial cells has been investigated which can be exploited in prevention of gastric cancer (Chua et al., 2015). Fucoidan is safe for use in age-related macular degeneration AMD (Dithmer et al., 2014). Intestinal inflammation and inflammatory bowel disease can be successfully controlled by oral administration of fucoidan preparation as nutraceutical (Lean et al., 2015). Use of fucoidan and its chemically modified derivatives in management of osteoarthritis are in various stages of development (Glycomer, 2012). Fucoidan is already available as a liquid nutritional supplement, FuCoyDon® and claimed to rejuvenate health (FuCoyDon® Factsheet). It also shows promise in treatment of fibrosis (Fitton, 2011). It can be exploited as an adjuvant in vaccine therapy (Fitton et al., 2015).

Galactofucan sulfate isolated from brown seaweed exhibited hepatoprotective effect in mice through lowering of serum levels of marker enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (γ-GT). It also affected sodium pentobarbital-induced sleep and demonstrated anti-oxidant and anti-inflammatory effects (Castro et al., 2016). It is being tested for its ability to control herpes virus infections (Fitton, 2011; Glycomer, 2012). Although the sulfated galactofucan demonstrated strong antithrombotic effect without causing any hemorrhage yet it did not exhibit anticoagulant activity. This might occur due to synthesis of highly sulfated heparin sulfate by the endothelial cells of the vascular wall as a result of the effect of the polysaccharide (Rocha et al., 2005). The polysaccharide isolated from *Capsosiphon fulvescens* was capable of stimulating the macrophage cells, RAW264.7 cell line, as evident from production of nitric oxide and prostaglandins and thus can be regarded as immunomodulators (Karnjanapratum et al., 2015). It could also stimulate rat intestinal epithelial cells (IEC-6) in a dose-dependent manner via increase in the expression of cyclinD1 and c-myc and induction of ERK1/2 phosphorylation (Hwang et al., 2011). It is reported to inhibit melanogenesis in B16 cells, induce apoptosis in

AGS gastric cancer cells, and lower cholesterol levels in hypercholesterolemic rats (Sun et al., 2016).

Laminarin is reported to possess anti-oxidant, anti-microbial, anti-apoptotic, anti-inflammatory, anticoagulant and immunostimulatory activities (Burgess et al., 2015; Kadam et al., 2015). Since, it is non-hydrolysable and non- digestible, it can reach the intestinal lumen in intact form and can regulate gene expression of pro- and anti-inflammatory cytokines in situation of ulcerative colitis and inhibit colonic Enterobacteriaceae. Moreover, colonic IL-6 mRNA level is also lowered (Shea et al., 2016). Laminarin was found to increase the release of hydrogen peroxide, calcium, nitric oxide, monocyte chemotactic protein-1, vascular endothelial growth factor, leukemia inhibitory factor, and granulocyte-colony stimulating factor in RAW 264.7 cells (Lee et al., 2012). It is known to induce apoptosis in human colon cancer cells by multiple pathways (Ji and Ji, 2014). It demonstrated protective effect against cisplatin-induced ototoxicity (Han et al., 2016). Oversulfated laminarin leads to improvement in antitumor activity (Ji et al., 2013).

Mauran has been reported to show immunomodulatory and anticancer effects owing to its high sulfate content (Srivastava and Kowshik, 2015). It also possesses antioxidant, antihemolytic and antithrombogenic activities (Sun et al., 2015).

Sulfated polysaccharide present in the cell walls of red microalgae, *Porphyridium* sp. have demonstrated hypocholesterolemic effect in Sprague-Dawley rats, through enhanced excretion of cholesterol and bile acid in the feces. Lower serum cholesterol levels, increase in the HDL/LDL ratio and enhanced levels of the hepatic enzyme hydroxymethyl glutaryl CoA reductase (HMG - CoA reductase) were also observed in the experimental animals (Dvir et al., 2009). Sulfated polysaccharide isolated from brown algae, *Eclonia cava*, was found to exhibit in vitro anti-proliferative activity on various cancer cells in a selective and dose-dependent manner. Results from Western blot analysis revealed marked effect on caspase 7 and 8, known to cleave protein substrates like PARP, thereby inducing apoptosis and subsequently DNA damage (Boopathty and Kathiresan, 2010). Non-digestible polysaccharide isolated from the brown alga *Saccharina latissima* has demonstrated antioxidant activity (Jimenez-Escrig et al., 2015).

Alteromonas macleodii subsp. *fijiensis* is an exopolysaccharide-producing aerobic, mesophilic bacterium isolated from a hydrothermal vent. The repeating unit is an hexasaccharide consisting of three uronosyl residues with branching at a galacturonosyl residue and a side chain. It can be used in bone healing and even in management of cardiovascular diseases. An EPS extracted from *B. licheniformis* strain (B3-15) exhibited immuno-modulating properties (Poli et al., 2010). SM1127 EPS demonstrated significant antioxidant activity (Sun et al., 2015). High molecular weight exopolysaccharide secreted from *Vibrio diabolicus*, resulted in bone and skin regeneration. It also exhibited antitumor, antiviral, and immunostimulant activities. “Heparin-like” or “heparin-mimetic” anticoagulant compound was obtained by sulfation of low-mass derivatives of depolymerised native exopolysaccharide, GY785 isolated from *Alteromonas infernos*, residing in deep hydrothermal vent of Guaymas region. It can promote wound healing and proliferation of human umbilical vein endothelial cells. Soluble exopolysaccharides isolated from blue-green algae, *Spirulina platensis* (RPS) have been reported to show antiviral actions owing to presence of high percentage of sulfate groups (Laurienzo, 2010; Sun et al., 2015).

PHARMACEUTICAL USES OF POLYSACCHARIDES

Two factors contribute to utilization of sulfated polysaccharides in the development of drug delivery systems. Glycosidic bonds, present in the molecule can facilitate enzyme-catalysed hydrolysis and thus biodegradation. Presence of negatively charged sulfate groups and hydroxyl groups render easy chemical modifications through introduction of several functionalities in addition to potentiation of interaction with negatively charged mucus membrane and thereby favoring muco-adhesion. Introduction of different moieties leads to modified sulfated polysaccharides of marine origin with customized properties.

Ulvan find applications in the fabrication of nanofibers and membranes and in the design of nanocarriers for drugs. Two-dimensional and three-dimensional platforms of ulvan have been developed by cross-linking, incorporating dexamethasone as model drug and have been investigated for use in wound healing and bone tissue regeneration with success**.** Modified ulvan has been tested for cytotherapy applications (Cardoso et al., 2016). Ulvan hydrogel formation requires the presence of boric acid and calcium ions at slightly alkaline pH. Ionic strength of the solution and alterations in pH can affect the stability of the thermo-reversible hydrogel. Mechanical strength of ulvan hydrogel has been improved by carrying out photopolymersiation using large excess of methacrylic anhydride in presence of cytocompatible photoinitiator and exposing to UV radiation for short duration thereby yielding ulvan methacrylate macromer. The hydrogel demonstrated good degree of swelling in phosphate buffered saline, good stability and excellent mechanical properties (Chiellini and Morelli, 2011).

Fucoidan alone has failed to produce gels even at 25% concentration. However, electrostatic interactions resulting from mixing polymers of opposite charges such as chitosan and poly(2-hydroxyethyl methacrylate) led to the formation of gels and films (Cunha and Grenha,

2016). Fucoidan-chitosan nanoparticles prepared by ionotropic-gelation method could protect the encapsulated curcumin from the acidic environment of the stomach and delayed the drug release till pH 7.0. Therefore, the polysaccharide-based nanoparticles could act as pH-sensitive carriers for oral delivery of curcumin (Huang and Lam, 2011). Self-assembly of sulfonated fucoidan, fucoidan-tauran with berberine and chitosan resulted in the formation of nanoparticles with high loading efficiency and fast release in simulated intestinal fluid. Thus they can be used in the local delivery of berberine for treatment of defective intestinal tight junction barrier (Wu et al., 2014).

Novel laminarin-based non-viral gene transfer vector has been designed to transfer B-cell-specific Moloney leukemia virus inset site 1 gene (*BMI-1*) targeting siRNA in breast cancer cells. Surface modification of laminarin nanoparticles by polyethyleneimine(PEI) reduced the toxicity and improved therapeutic efficacy (Ren et al., 2016). Biocompatible photo-cross-linkable methacrylate hydrogel has been synthesized for encapsulation of human-adipose-derived stem cells (Custodio et al., 2016).

Extremophilic bacterial polysaccharide, mauran has been complexed with cationic chitosan to form stable, biocompatible nanoparticles which have been used for encapsulation of 5-fluorouracil. Drug-loaded nanoparticles could kill the breast adenocarcinoma cells and glioma cells in a sustained and controlled manner (Raveendran et al., 2013; Raveendran et al., 2015). Magnetic nanoparticles coated with mauran have demonstrated biocompatibility and low cytocyoxicity towards normal cells. Application of magnetic hyperthermia along with administration of drug-loaded mauran naoparticles resulted in killing of 80% of cancer cells within a very short time (Balasubramanian et al., 2014). Mauran has been reported to stabilize ZnS:Mn^{2+} quantum dots(QDs) thereby improving biocomapatibility and lowering the cytotoxicity potential (Srivastava and Kowshik, 2015).

Although hydrophilic in nature, deoxysugars such as fucose and rhamnose present in cyanobacterial exopolysaccharides (RPS) are responsible for their hydrophobicity and their usefulness as emulsifying agents (Laurienzo, 2010).

A polysaccharide bioflocculant, MBSF17, extracted from halophilic bacterium, *Bacillus subtilis*, MSBN17, could yield spherical silver nanoparticles in reverse micelles through reduction of silver nitrate. Presence of carboxyl, hydroxyl and methoxyl groups in MBSF17 stabilised silver nanoparticles by forming a coating. Nanoparticles thus produced demonstrated broad spectrum antimicrobial effect (Srivastava and Kowshik, 2015).

Exopolysaccharides obtained from various marine extremophilic bacteria (*Geobacillus* sp. 400, *Halomonas* species, *Hahella chejuensis*, *Polaribacter* sp. SM1127) have been found to possess high viscosity and thus can be used as thickening agents. Most of them have been found to be highly active at the surface and thus can be employed as biosurfactants or emulsifiers. They are also reported to exhibit good pH stability and salt tolerance (Poli et al., 2010; Sun et al., 2015).

COSMETIC USES

Highly concentrated fucoidan extract (89% fucoidan) from *Undaria pinnatifida* is available commercially as Maritech Reverse™. It is known to protect skin against UV irradiation, prevent wrinkles and also act as soothing agent. The extract has been found to be non-sensitizing and non-allergenic to skin. Moreover, it is Halal Kosher certified (Fitton et al., 2016).

Oligosaccharides present in laminarin have been found to stimulate, regenerate and rejuvenate human fibroblasts and human epidermis keratinocytes (Yvin et al., 1999).

High molecular weight exopolysaccharide, HYD657, obtained from marine bacteria, *Alteromonas macleodii subsp. fijiensis biovar deepsane*, has been used for cosmetic purposes Poli et al., 2010). Water-binding capability of cyanobacterial EPS can be exploited in the development of cosmetic formulations (Laurienzo, 2010).

Owing to the presence of fucose in addition to large amounts of glucuronic acid and N-acetyl glucaosamine in the molecule, the exopolysaccharide, SM1127 EPS demonstrated superior water-retention and hence, humectant property compared to hyaluronic acid. Therefore, it can act as a moisturising agent in cosmetics and free radical scavenging activity makes it a good candidate for anti-aging preparations. It is safe to use and non-irritant to skin (Sun et al., 2015).

In a previous study on sulfated polysaccharide obtained from red alga, *Porphyridium cruentum*, it has been found to improve the amount of cornified envelope maturation in stratum corneum and reinforcement of the Dermal-Epidermal Junction (DEJ). Therefore, it seems to be a good choice for improving the skin characteristics of dry or aged facial skin and can prolong the effect of moisturizers when applied topically (Ghibaudo et al., 2014).

DIAGNOSTIC APPLICATIONS

Injectable, cross-linked dextran-pullulan microparticles functionalized with fucoidan and radiolabelled with Technetium 99m were developed as innovative SPECT diagnostic tool for abdominal aortic aneurysm, owing to the ability of fucoidan to target cell adhesion molecule, P-selectin (Bonnard et al., 2014). Reports of fucoidan as a molecular imaging probe for myocardial infarctions, it is

possible application in detection of thrombosis, myocardial ischemic memory, detection of inflammation in experimental autoimmune myocarditis exist in the literature (Cognet et al., 2014; Saboural et al., 2014; Chollet et al., 2016; Mikail et al., 2016). Doxorubicin-loaded fucoidan-capped gold nanoparticles can be exploited as a contrast agent in imaging of breast cancer using photoacoustic imaging technique (Manivasagan et al., 2016). Mauran-chitosan nanoparticles labeled with fluorescein isothiocyanate (FITC) could act as safe and non-toxic vehicles for imaging of cancer cells by employing confocal microscopic imaging and flow cytometry (Raveendran et al., 2013; Balasubramanian et al., 2014; Raveendran et al., 2015).

A sulfated polysaccharide, isolated from the red marine algae, *Solieria filiformi*s has been studied for its nociceptive and inflammatory effects in experimental animal models. Anti-nociceptive effects of the polysaccharide are mediated through a peripheral mechanism whereas, prostaglandins, nitric oxide and cytokines are responsible for edematogenic effects of the polymer. The results indicate that the tested sulfated polysaccharide can find use as a tool for studying the inflammatory processes associated with nociception (Araujo et al., 2011).

CHALLENGES IN COMMERCIALISATION OF NEWER SULFATED POLYSACCHARIDES

Marine algae and bacteria seem to produce a vast array of safe, biodegradable and biocompatible novel polysaccharides and exopolysaccharides with unique physicochemical properties, potential therapeutic benefits and diagnostic applications. Possibilities of being exploited in the pharmaceutical and cosmetic industry remain unlimited. However, only few have been marketed till date which include guar gum, alginate, pectin and carrageenan. Technical challenges in identification, ensuring product quality with high reproducibility, screening and isolation of active principles, high production cost, need for expensive chemicals for fermentation medium and poor handling properties of the newer polymers render them unsuitable for commercial scale utilization. Another important factor that hinders uninhibited research is secured access to the marine resources guided by intellectual property rights in order to protect the vast marine ecosystem biodiversity (Borresen et al., 2010).

FUTURE PROSPECTS

Numerous studies have been done on the applications of sulfated polysaccharides in their native, chemically modified forms or in combination with other natural polymers, primarily in the field of nanotechnology. Scope of their use in the field of targeted controlled drug delivery system, stimuli-responsive systems and in the fabrication of drug reservoir matrices is high. Theranostic applications are still unexploited. Design and synthesis of functional polymers for each of the new sulfated polysaccharide, with specific customized properties and uses is an unexplored area, which will also lower material usage. Till date, very few investigations have been done on the pharmacokinetic characterization of the novel sulfated polysaccharides obtained from marine algae and bacteria. Moreover, it has been observed that physicochemical characteristics and applications depend highly on the species from which the polymer has been extracted. Therefore, a detailed database needs to be constructed with information on every aspect of a marine polymer, obtained from different species of the same microorganism. Future studies in this regard and deep understanding of chemistry, fate and species-specific structural variability of the marine polymers will unravel a lot of possibilities for them (Fitton et al., 2015).

CONCLUSION

Marine-derived polysaccharides and their products possess immense potential to be alternative, renewable resource in synthesis of novel drug molecules with wide applications, in the design of patient-compliant novel controlled release site-specific drug delivery systems, in the field of cosmetic science and ultimately in diagnosis of cardiovascular diseases and cancers. Marine algae and bacteria possess a vast and valuable chemical library of unique polysaccharides. Sustainable exploration of this marine ecosystem will ensure both environmental and economic benefits and will ultimately enrich the medical, pharmaceutical, cosmetic and diagnostic field with availability of a range of non-toxic biopolymers of marine origin.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antipseudomonal potential of *Colophospermum mopane* and *Acrotome inflata*, medicinal plants indigenous to Namibia

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Plants with diverse therapeutic properties are indigenous to Namibia. Concoctions of *Colophospermum mopane* and *Acrotome inflata* are widely used traditionally in the management of respiratory, gastrointestinal and wound infections. Limited studies have validated these traditional uses particularly against resistant bacteria strains such as *Pseudomonas* spp. This study aimed to determine the antimicrobial activity and phytochemistry of extracts of *C. mopane* and *A. inflata*, medicinal plants indigenous to Namibia. Phytochemical analysis and antimicrobial testing were done on leaves and barks of *C. mopane* and *A. inflata* whole plant. Voucher specimens were collected from Omugulugoonime village, Oshikoto region and validated at the National Botanical Research institute, Windhoek. Crude extraction of dried plants was done by maceration with ethanolic and aqueous solvents. Phytochemical screening was done using methods described by Harborne (1998) and/or Tiwari et al (2011). The antimicrobial activity against wild types of *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtillis* and *Candida albicans* was done using the disc-agar-diffusion method. The mean diameters of the zones of inhibition (mm) for each extract were determined against each test organism. The antimicrobial activity (zones of inhibition) of ethanolic extracts (5 mg/ml) of *C. mopane* bark (10±0.6 mm) and leaves (10±1.2 mm) and *A. inflata* (8.7±0.6 mm) against *P. aeruginosa* is comparable to that of penicillin G (14±1.4 mm). Aqueous extracts leaves and bark of *C. mopane* showed activity against *P. aeruginosa* and *S. aureus.* The activity of the ethanolic extracts against *B subtillis* was*: C. mopane* leaves (7.3±0.6 mm), *C. mopane* bark (8.7±0.6 mm) and *A. inflata* (11.3±3.21 mm) and Penicillin G (26±1.4 mm). Both ethanolic and aqueous extracts did not have activity on *C. albicans. Aqeous extracts of A. inflata* had no activity on *Pseudomonas and B. subtillis.* Organic extracts of *Colophospermum mopane* and *A. inflata* exhibit antimicrobial potential against *Pseudomonas and Bacillus* species. The alkaloids, flavonoids and tannins should be further purified and characterized for antipseudomonal activity.

Key words: Antipseudomonal, Namibia, *Colophospermum mopane*, *Acrotome inflate.*

INTRODUCTION

The sub-Saharan Africa is indigenous to over 50,000 species flora and ethno-medicines (Clarkson et al., 2004; Cowan, 1999; Hosttetmann et al., 1996; Iwu, 2014). Namibia is home to about 8159 plant species with

therapeutic potential (Hedimbi and Chinsembu, 2012; Ilonga, 2012; Cunningham, 1993; Grote, 2003; Cheikhyoussef et al., 2011; Thomas, 2015). Over 80% of the world's population, mostly in low and middle income countries (LMIC) rely on traditional medicine for primary health care of common ailments (Fabricant and Farnsworth, 2001; Ganatra et al., 2012; Thirumurugan, 2010; WHO, Traditional Medicines, 2012). Despite the wide practice of ethno-medicine the sub-Saharan Africa, the region bares highest global burden of infectious diseases such as HIV/AIDS, tuberculosis and malaria. Out of the 500 000 estimated plant species found in nature, a limited proportion undergone phytochemicals, biological and pharmacological characterization (Mahesh and Satish,2008; Dushimemaria, 2014).

The antimicrobial potential of higher medicinal plants as sources for new drugs in Namibia is still largely unexplored. Recently, there is a rise in patients living with non-communicable diseases and the WHO has estimated that 3 out of 4 patients with hypertension reside in sub-Sahara region (WHO, 2016). This calls for action for further research for efficacious and cost-effective medicines from the indigenous plants and/or ethno-medicines. In addition, the global surge in resistance against essential antimicrobials has had its greatest impact on health care in the sub-Saharan region and is a public health concern (Wagate et al., 2009; Parekh and Chanda, 2007). As a result, antimicrobial resistance has led to use of more expensive medicines and or treatments, further constraining health care systems (Kamaraj et al., 2012).

Despite the global efforts to increase access to essential medicines, access to essential and alternative antimicrobial remains a challenge, particularly in remote settings where indigenous systems are commonly practiced as part of the primary health care (WHO, Traditional Medicines, 2012; Carlet et al., 2012). In recent years, the clinical efficacy of essential antibiotics such as cotrimoxazole and penicillin used in the sub-Saharan region against common pathogens has reduced tremendously (Mutembei et al., 2011; Monroe and Polk, 2000). Namibia has one of the highest case notification rates for multi-drug resistant tuberculosis (MDR-TB) in the world (World Health Organization, 2013). There is a growing burden of multi-drug resistance to bacterial pathogens in the public health care in Namibia including strains of *Escherichia coli, Enterococcal pneumoniae, Proteus mirabilis*, *Klebsiella* (Khan and Musharraf, 2004). The emergence of multi-drug resistant pathogens threatens the clinical efficacy of many existing antibiotics (Mutembei et al., 2011). In particular, there are high rates of resistance to amoxicillin, cotrimoxazole and nitrofurantoin. The resistance against amoxicillin is rising among strains of *E.coli* (79.6%), *Klebsiella* (96.72) and *Proteus* (55.91%). Similar patterns of resistance against cotrimoxazole have been documented on isolates of *E. coli* (78.6%), *Proteus* (57.85%) and *Klebsiella* (56.52%). The resistance against nitrofurantoin on *Proteus mirabilis* isolates is reported at 77.37% (Mengistu et al., 2014) . The rising burden of antimicrobial resistance is a wake-up call for further research and development of cost-effective medicines, particularly from the indigenous ethno-medicines of Namibia.

In Namibia, concoctions of *Colophospermum mopane* and *Acrotome inflata* are widely used traditionally in the management of infectious diseases (Musvoto et al., 2007; Cheikhyoussef et al., 2011; Bainbridge H, 2012). The traditional uses include cure and/or prevention of wound infections and treatment of syphilis, diarrhea, coughs, inflammatory diseases and fevers (Cheikhyoussef et al., 2011). However, despite the wide use of *C. mopane* and *A. inflata* in Namibia, the safety and efficacy profiles in the treatment of diseases have not been validated. *C. mopane* and *A. inflata* belong to the Fabaceae and Lamiaceae families, respectively. Secondary metabolites of these families including tannins, alkaloids, and flavonoids have exhibit antimicrobial activity (Cheikhyoussef et al., 2011; Mahesh and Satish, 2008; Lewis and Ausubel, 2006). Thus, extracts of these ethno-medicines from *C. mopane* and *A. inflata* may be a potential source of phytochemicals with antimicrobial activity conceivably with better safety profile and/or novel mechanisms against resistant bacteria (Thirumurugan, 2010; Parekh and Chanda, 2007; Tiwari et al., 2011; Cowan, 1999) Phytochemicals such as alkaloids, tannins, saponins and flavonoids have been reported to have antimicrobial activity (Heneman and Zidenberg-Cherr, 2008; Suliman, 2010). *C. mopane* (Figure 1) is a shrub or a small tree of the family *Fabaceae (Leguminosae)* and in Namibia, it is popularly known as Mopane or Omusati. *C. mopane* covers about 9% of Namibia's surface area and is more common in the Northern parts of Namibia (Dushimemaria, 2014). Decoctions of Mopane leaves, bark and gums are used by the Heikum Bushmen, Wambo, Damara and Himba communities for, management of gastrointestinal complaints, diarrhoea,coughs, wounds symptoms of inflammation, oral hygiene and diarrhoea (Von-Koenen, 2009; Van den Eynden et al., 1992). The roots of *C. mopane* are used to treat wounds; stomach problems, inflamed eye, and syphilis, and have been reported to contain tannins and resin (Von-Koenen E, 2009, 2001). *A. inflata* (Figure 2) belongs to Lamiaceae (Labiatae) family, it is a herb called tumbleweed and locally known

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Figure 1. Leaves of *Colophospermum mopane*.

Figure 2. *Acrotome inflata.*

as Etselyakuku in Ndonga (Sharma and Kumar, 2013). A. inflata is mostly distributed in northern part of Namibia including in Oshikoto, Oshana and Omusati regions (Van Rooyen, 1988; Walt and Riche, 1999; Nordenstam, 1970). The leaves of *A. inflata inflate* are used by the Damara communities as a tea to treat coughs and stomach upsets (Mukanganyama et al., 2011). The Wambo communities use the entire plant for treatment of malaria and as an indoor insecticide. In Kavango region, ashes of the dried and burnt tumbleweed flower are used for management of pain and scratches over the temples (Von-Koenen, 2009; Nafuka, 2014).

Limited studies provide scientific evidence of the antibacterial potential of these plants against commonly resistant bacteria such as *Pseudomonas*. Consequently, the aim of this study was to screen for phytochemicals and validate the use of *C. mopane* and *A. inflata*, in folk medicine in Namibia.

METHODOLOGY

Laboratory analysis of the phytochemistry and antimicrobial activity of crude extracts of *C.mopane* and *A. inflate* was done. The experiments were conducted at the laboratory of School of Pharmacy, University of Namibia.

Plant material preparation

Plant specimens of *A. inflata* and *C. mopane* were collected from the Omugulugoonime village, Omuntele constituency in the

Oshikoto region with the help of a local traditional healer. These specimens were subsequently validated at the National Botanical Research institute, Windhoek (voucher numbers: C.mopane-----, A. inflata -----). The plant samples, that is, the bark and leaves of *C. mopane* and the whole plant of *A. inflata* were rinsed with tap water, chopped into small pieces and air-dried at room temperature (23 to 26°C). The dry plant samples were then pulverized into fine powder using a laboratory mechanical mill. Crude extraction of the powders of the whole plant of *Ac. inflata* as well as the leaves and bark of *C. mopane* was done by maceration using ethanolic and aqueous solvents as described by Mutembei et al., (2011). This extraction method mimics the traditional processes for preparing concoctions of *C. mopane* and *A. inflata*. Aqueous extraction was done by soaking 10 g of the crude plant powder in 200 mL of distilled water in a conical flask. The aqueous mixture was subsequently heated at 70°C for 2 h using a hot plate. The aqueous extraction was done in triplet so as to obtain the mean percentage yield. The resulting mixture was cooled at room temperature and was subsequently filtered with Whatman Filter Paper No. 1 to remove the solvent and was subsequently concentrated by heating at 70°C. Organic extraction was done by macerating 10 g of the crude plant powders of *C. mopane* and *A. inflata* in 200 mL of ethanol at room temperature for 4 days. The organic extraction was also done in triplicate. The resulting mixture were filtered using Whatman Filter Paper No. 1. The filtrate was concentrated using a Rota evaporator at 40°C. The aqueous and ethanolic extracts were stored in the fridge until phytochemical and antimicrobial activity tests.

Test microorganisms

Phytochemical analysis

Phytochemical analysis of the aqueous and ethanolic extracts of *C. mopane* and *A. inflata* were performed according to standard methods described by Harbone (1989) and Tiwari et al., (2011). A mixture of aqueous (2 ml) extract and Ferling's solution (3 ml) was heated to near boiling, a color change indicated the presence of reducing sugars. To 2 ml of the aqueous extract were diluted with distilled water and 1-2 drops of 0.1% ferric chloride solution were added- a dark-green, blue-green or blue-black color indicated the presence of tannins.

Saponin test

3 ml of the aqueous extract was shaken vigorously in a stoppered test tube- the persistence of froth for at least 5 min indicated presence of saponins. To 3 ml of the aqueous extract, dilute ammonia solution (3 ml) and sulphuric acid (1 ml) were added- a yellow color that disappeared on standing indicated the presence of flavonoids.

Test for cardiac glycosides

To a test tube containing 5 ml of the aqueous extract, 2 ml of glacial acetic acid added followed by a drop wise addition of 1 ml concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxy-sugars.

Anthraquinones test

To a test tube containing 2 ml of the ethanolic extract, a drop wise addition of 1 ml of dilute ammonia was done. A reddish color in the upper layer indicated the presence of anthraquinones.

Alkaloids test

A thin layer chromatogram (TLC) separation was done on the ethanolic extract. The dried TLC spots were subsequently were sprayed with Dragendorff reagent. A pink color indicated the presence of alkaloids.

Salkowski testfor sterols and steroid

To the 2 ml of the ethanolic extract, a drop wise addition of 3 ml of concentrated sulphuric acid was done. A reddish brown color at the interface indicated the presence of phytosterols.

Test for coumarins

1 ml of the ethanolic extract was spotted on the TLC plate for separation. Also, a mixture of 0.5 ml of diluted ammonia solution and the ethanolic extract was spotted on the other end of the TLC plate. The two spots were observed under UV light. Intense fluorescence indicated the presence of coumarins.

Antimicrobial activity analysis

The antimicrobial activities of the extracts were tested on four human pathogenic organisms: *P. aeruginosa, B. subtillis, S. aureus* and *C. albicans* (yeast). The test organisms were obtained from an accredited national laboratory, the Namibia Institute of Pathology (NIP) laboratory. The bacterial strains- *P. aeruginosa, B. subtillis* and *S. aureus* were cultured in a nutrient broth at 37°C for 24 h. The fungal strain- *C. albicans* was cultured on the potato dextroxe agar at 25°C for 24 h.

The antimicrobial activity of the extracts were determined with the mean zones of inhibition using Filter-paper disc-agar diffusion procedure as described by Kirby-Bauer (Bauer et al., 1966). In the Kirby-Bauer method, Whatman's filter papers were punched into discs of diameters of 6 mm and 10 µL of varying concentrations (20, 10 and 5 mg/ml) of aqueous and ethanolic extracts incorporated using a micropipette. The concentrations were prepared by dissolving the respective quantities of dried ethanolic extract of *C. mopane and A. inflata* in the dimethyl sulfoxide (DMSO). The discs were allowed to dry and were subsequently stored at room temperature. A volume of 25 ml of sterilized nutrient agar wad added on sterile Petri-plates and allowed to solidify. A volume of 100 µl of fresh culture of human pathogens- *P. aeruginosa, B. subtillis* and *S. aureus* were separately applied on the nutrient Agar using a sterile spreader. Whatman's paper discs with varying concentration of the extracts (20, 10 and 5 mg/ml) were placed on separate petri-plates containing the cultured micro-organisms using sterile forceps. The plates were then incubated at 37°C for 24 h, except the *C. albicans* which was incubated at 25°C for 24 h.

The zones of inhibition of each extract on each test organism at the three different concentrations were measured for three replicates. Penicillin G and streptomycin were used as positive controls for the antimicrobial susceptibility tests for bacterial activity and antifungal activity, respectively.

DMSO was used as the negative control for all experiments. The mean zone of inhibition was determined as a mean ± standard deviation.

Data analysis

The qualitative and quantitative methods were used to analyze the

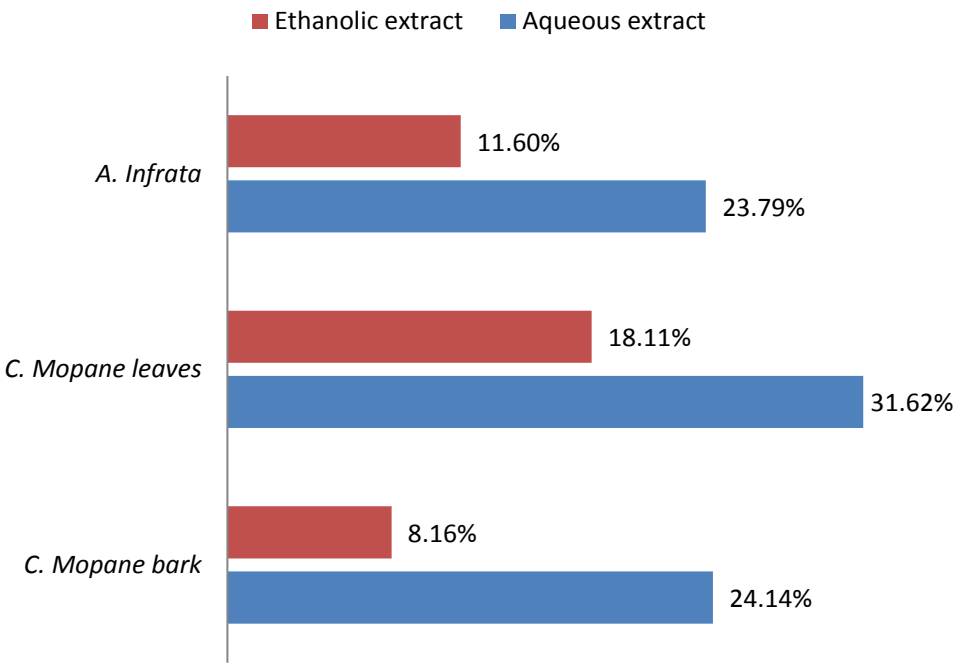

Figure 3. Percentage yield of aqueous and ethanolic extracts of *C. mopane* and *A. inflate.*

data. The percentage yield was calculated by using the formula: percentage yield = amount of extract/amount of starting material. The mean for zone of inhibition was calculated. The plant extracts that inhibited the growth of bacterial, indicated that the plant was effective and can be synthesized further to produce the antimicrobial agent. The comparison between the medicinal plants extracts and synthetic antibiotics was investigated. Aqueous and ethanol extracts were used to screen for phytochemicals which indicated certain colour change if there was any kind of phytochemical present.

Ethical considerations

The study was approved by the Faculty of Health Sciences, University of Namibia ethics review board, UNAM laboratory management and local leadership of Omuntele constituency council.

RESULTS

Percentage yield of crude extracts

Aqueous extracts gave higher percentage yields (23.79 to 31.62%) as compared to ethanolic extracts (8.16 to 18.11%).
Leaves of *C. mopane* gave the highest yield for both the aqueous and ethanolic extracts, as compared to the bark of *C. mopane* and whole plant of *A. inflata* (Figure 3).

Phytochemical profile

Table 1 show the phytochemicals identified in extracts of *C. mopane* and *A. inflata*. Aqueous extracts of *inflate* leaves and bark *C. mopane* gave positive tests for tannins, saponins, flavonoids and cardiac glycosides. The test was more reactive with the bark as compared to the leaf extracts. Aqueous extract of *A. inflata* tested positive for tannins and cardiac glycosides. The test for reducing sugars in the *C. mopane* and *A. inflata* were negative. Only the ethanolic extracts of the bark of *C. mopane* were positive for alkaloids, sterols and steroidsand coumarins. The enthanolic extracts of leaves of *C. mopane* and the whole plant of *A. inflata* were also positive for coumarins (Table 1).

Antimicrobial potential of *C. mopane* and *A. inflata*

Table 2 and Figure 4 show the mean zone of inhibition (±SD) of the aqueous and ethanolic extracts of *C. mopane* and *A. inflata*. Generally, the ethanolic extracts showed higher antimicrobial activity than the aqueous extracts on all the bacterial test organisms. Antimicrobial activity against *S. aureus* was higher with the aqueous (10.7±1.3 mm) than the ethanolic leaf (9.7±2.1 mm) and bark (9.0±1.0 mm) extracts of *C. mopane*. This was however lower than the positive controls. The aqueous and ethanolic extracts of *A. inflata* had no activity against *S. aureus*. There was antimicrobial activity against strains of *P. aeruginosa* with the aqueous extract of the bark of *C. mopane* (10.7±3.1 mm), ethanolic extract of the leaves (12.7±0.6 mm) and bark (11.3±0.6 mm) of *C. mopane,* as well as the ethanolic extract of A. inflata (11.7±2.1 mm).

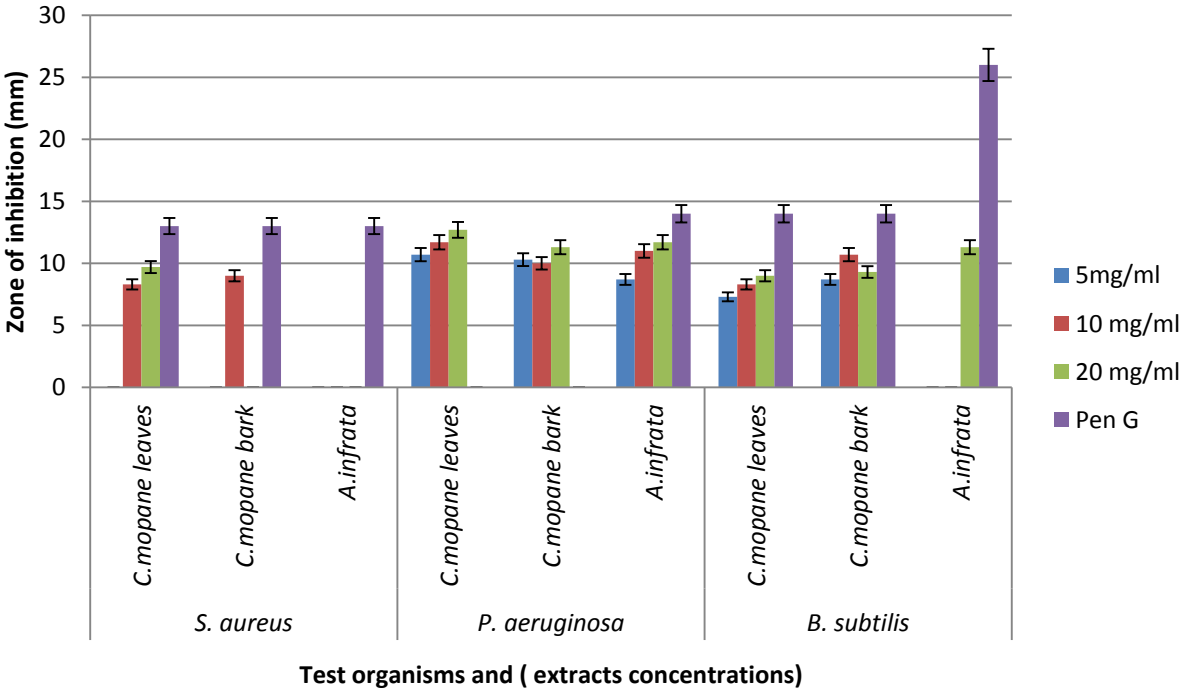

Figure 4. Antibacterial activity of the ethanolic extracts of *C. mopane* and *A. inflate.*

Table 1. Phytochemical profiles of the aqueous and organic extracts.

Phytochemical constituents	*Colophospermum mopane*-bark	*Colophospermum mopane*-leaves	*Acrotome inflata*
Aqueous extracts			
Reducing sugars	-	-	-
Tannins	++	+	+++
Saponins	+++	++	-
Flavonoids	++	+	-
Cardiac glycosides	++	+	+
Ethanolic extracts			
Anthraquinones	-	-	-
Sterols and steroids	++	-	-
Alkaloids	+	-	-
Coumarins	++	+	+

(-) = Absent; (+) = present; (++) = moderate present; (+++) = highly present.

This was however lower than the activity of the positive controls against *Pseudomonas.*

None of the aqueous extracts of the leaves and bark of *C. mopane* as well as *A. inflata* had activity against *B. subtilis.* All the ethanolic extracts of *C. mopane* leaves (9.0±1.0 mm) and bark (10.7±1.2 mm) as well as *A. inflata* (11.3±3.2 mm) had activity against strains of *B. subitlis. C. albicans* was not susceptible to any of the aqueous and ethanolic extracts.

Ethanolic extracts of *C. mopane* leaves and bark had significant antimicrobial activity against *P. aeruginosa*, *S. aureus* and *B. subtilis* (Table 2).

The antimicrobial activities of ethanolic extracts against *Pseudomonas* were comparable to the positive control penicillin G. The zone of inhibition for Penicillin G was highest with *B. subtilis* (26 mm) and lowest with *S. aureus*. Econazole had minimum zone of inhibition against *C. albicans*.

Table 2. Antimicrobial activity of the plant extracts (zone of inhibition (mean± SD).

Plant/test organism	Aqueous extracts			Ethanolic extracts			Controls		
	5 mg/ml	10 mg/ml	20 mg/ml	5 mg/ml	10 mg/ml	20 mg/ml	Streptomycin	Pen G	Econazole
Mopane leaves									
S. aureus	-	-	10.7±1.3	-10.67±1.2	8.3±0.6	9.7±2.1	16.0±1.4	13.0±1.4	-
P. aeruginosa	-	-	-	7.33±0.6	11.7±0.6	12.7±0.6	15.5±5.0	14.0±1.4	-
B. subtilis	-	-	-	-	8.3±0.6	9.0±1.0	14.0±1.4	26.0±1.4	-
C. albicans		-	-		-	-	-	-	13.0±0
Mopane bark									
S. aureus	-	-	-	-	9.0±1.0	-	16.0±1.4	13.0±1.4	-
P. aeruginosa	9.0±1.0	10.7±3.1	-	10.3±0.6	10.0±1.0	11.3±0.6	15.5±5.0	14.0±1.4	-
B. subtilis	-	-	-	8.7±0.6	10.7±1.2	9.3±0.6	15.0±2.8	26.0±1.4	-
C. albicans		-	-	-	-	-	-	-	13.0±0
A. Inflata									
	-	-	-	-	-	-	16.0±1.4	13.0±1.4	
	-	-	-	8.7±0.6	11.0±1.7	11.7±2.1	15.5±4.9	14.0±1.4	
	-	-	-	8.7±0.6	11.0±1.7	11.7±2.1	15.0±2.8	26.0±1.4	
	-	-	-	-	-	11.3±3.2			
	-	-	-	-	-	-	-	-	13.0±0

DISCUSSION

C. mopane and *A. inflata* gave significant yields of crude extracts. *C. mopane* and *A. inflata* are both higher plant and cover a wide area of Namibia and thus cultivation of these plants on large scale is commercially viable (Dushimemaria, 2014).

The phytochemical analysis revealed the presence of tannins, saponins, flavonoids and cardiac glycosides in the leaf and bark aqueous extracts of *C. mopane.* The aqueous extracts of *A. inflata* were positive for tannins and cardiac glycosides (Table 1). The findings in this study are similar to a study on *Sphenostylis stenocarpa*, both belonging to the Fabaceae family, also tested positive for presence of flavonoids, tannins and alkaloids (Nyananyo and Nyingifa, 2011). Similar studies have associated antimicrobial and/or activity of certain plants to the presence oftannins (Athanasiadou, 2001) and saponins (Avato et al., 2006) have been reported to have antimicrobial properties. The organic extract of the bark of *C. mopane* was positive for alkaloids, steroids and sterols and coumarins. Alkaloids have been documented to have several pharmacological properties including antimicrobial activity, antioxidant activity and analgesic properties. A study by Tiwari et al. (2011) has associated flavonoids, coumarins and tannins with antimicrobial activities in medicinal plants (Tiwari et al., 2011). This may explain why the bark of *C. mopane* is widely used as a folk medicine to manage inflammatory conditions as well as acute infections of the gastrointestinal, respiratory systems and well as wounds. However, the presence of coumarins may have safety implications as they have been documented to have anticoagulation effects that may aggravate bleeding. This calls for further investigation of the safety profile of preparations of *C. mopane* used in folk medicine in Namibia.

The extracts of *C. mopane* had antibacterial activity against *S. aureus, Pseudomonas* and *B. subtillis*. The plant extracts exhibited more antimicrobial activity against *P. aeruginosa* more than *B. subtillis* and *S. aureus.* The antimicrobial activity of the ethanolic extracts of *C. mopane* on *Pseudomonas* strains was comparable to penicillin G and streptomycin, the positive control. This result suggests that phytochemicals in *C. mopane* have activity against both aerobic Gram positive and facultative Gram negative bacteria. This may explain why preparations of *C. mopane* are widely used in the management of wounds, diarrhea, coughs and inflammatory diseases. Thus, further research is required to purify, characterize and test for antibacterial activity of the saponins, tannins and alkaloids in *C. mopane* that have been associated with antimicrobial activity (Table 1). Extracts of *A. inflata* showed antimicrobial activity against *P. aeruginosa* and *B. subtillis* but not *S. aureus* (Figure 4). This finding suggests that *A. inflata* has major activity on facultative Gram negative bacteria and anaerobic Gram positive bacteria limited activity on Gram positive bacteria. This explains the use of preparations of *A. inflata* in management of stomach upsets that are mainly attributed to Gram negative bacteria. The

antimicrobial activity of extracts from *C. mopane* and *A. inflata* on all the test organisms was lower than the penicillin G, the positive controls (Figure 4) (this is attributed to the insufficient quantities of phytochemicals responsible for antimicrobial activity in the crude extracts, particularly, if the activity is dose dependent.

The organic extracts had more activity against the test organisms than the aqueous extracts (Table 2). Higher activity of organic extracts has been attributed to the ability of organic solvent systems to isolate a variety of bioactive compounds and activity (Fouche et al., 2008). However, the percentage yield was higher than the aqueous extraction than the ethanolic extract (Figure 3). The water preparations of *C. mopane* and *A. inflata* are widely used traditionally in Namibia. However, in traditional practice, *C. mopane* leaves are chewed and swallowed or applied on the wound and not boiled. Traditional therapies of *A. inflata* are prepared by boil in water for few minutes or the fresh plant material is soaked in hot water to make tea.

Thus, the methods used aqueous extraction using boiling may have had an effect on the antimicrobial activity of *C. mopane* (Pradeepa et al ., 2016; Clarkson et al., 2004). Further research is required to isolate most of the active phytochemicals using more efficient solvent systems with varying polarities and less thermolabile techniques.

The study concludes that aqueous and organic extracts of *C. mopane* and *A. inflata* contain phytochemicals with antimicrobial activity (Jäger et al., 2005). The extracts have antibacterial activity on *P. aeruginosa*, *B. subtilis* and *S. aureus* but lack antifungal. The lack of antifungal activity of *A. inflata* is contrary to the findings in Botswana that found antifungal activity (Mukanganyama etal., 2011). This is mainly because the study in Botswana used extracts from the leaf unlike our study that assessed the plant as a whole activity. Organic extracts have better antimicrobial activity as compared to the aqueous extracts for *C. mopane* and *A. inflata* and have a wide activity against aerobic Gram positive bacteria (*S. aureus*), Gram positive anaerobes (*B. subtilis*) and facultative Gram negative bacteria (*Pseudomonas*). The organic extracts of *C. mopane* have superior antibacterial activity against pseudomonas than other test organisms.

Consequently, this study provides evidence for further research on the antimicrobial potential against resistant bacterial strains including *P. aeruginosa* and *B. subtilis* of ethanolic extracts of *C. mopane* leaves and bark as well as *A. inflata*. Future studies should also focus on optimizing the extraction of phytochemicals using different solvent systems in order to achieve a greater yield of bioactive compounds. The phytochemicals with antimicrobial activity should be purified and characterized for antipseudomonal activity of these plant extracts. There is need to perform acute and chronic toxicity studies on the extracts of *C. mopane* and *A. inflata* so as to outline the safety of these plants.

Limitations

In this study, only aqueous and ethanol solvents were used to isolate phytoconstituents. In future, organic solvent systems such as methanol and chloroform should be used. The use of aqueous and ethanolic extracts was used in order to mimic the solvents and processes used to prepare the medicines in practice. Secondly, antimicrobial activity was not tested on *E. coli*, a common organism involved in gastrointestinal and urogenital infections. Specimens of *E. coli* were not accessible from the Namibia Institute of Pathology (NIP) at the time of the study. However, the use of strains of *Pseudomonas* represented the facultative Gram negative bacteria to which *E. coli* belongs.

Conflict of Interests

The authors have not declared any conflict of interests.

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Antiplasmodial activity of *Vernonia cinerea* Less (Asteraceae), a plant used in traditional medicine in Burkina Faso to treat malaria

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Research and development of new antiplasmodial molecules in plant is a very important way for the development of new anti-malarial drugs. In this study, *Vernonia cinerea* Less (Asteraceae) was selected for its promising antiplasmodial activity because it is traditionally used in Burkina Faso to treat malaria. The aim of this study was to investigate the antiplasmodial activity of this whole plant. Five crude extracts of *V. cinerea* Less were prepared from the solvents of increasing polarity (CH_2Cl_2, CH_3OH, CH_3OH/H_2O (1/1), H_2O and alkaloids extracts). The method of Ciulei (1982) and thin layer chromatography were used for chemical characterization. The p-LDH technique was used *in vitro*. Extracts were evaluated *in vitro* for efficacy against the *Plasmodium falciparum* strain K1, which is resistant to chloroquine, and 3D7, which is sensitive to chloroquine. The crude extracts of alkaloids showed the IC_{50}=4.25 µg/ml with the strains 3D7 and IC_{50}=2.56 µg/ml with the K1 strains. The CH_2Cl_2 extracts showed the IC_{50}= 8.42 µg/ml and IC_{50}=5.85 µg/ml on strains 3D7 and K1, respectively. The CH_3OH extracts showed the IC_{50}=21.08 µg/ml, CH_3OH/H_2O extracts gave 41.56 µg/ml and H_2O extracts gave 37.17 µg/ml on strains of *P. falciparum* K1. The present study highlighted the very promising antiplasmodial activity of *V. cinerea* Less. The most antiplasmodial activity of this plant extracts merit further study about its *in vivo* antiplasmodial activity in *Plasmodium berghei* infected mice.

Key words: *Vernonia cinerea* Less, alkaloids, triterpenes, antiplasmodial activity, *Plasmodium falciparum.*

INTRODUCTION

Malaria is a potential fatal parasitic disease that remains a public health concern in tropical countries, thus also in Burkina Faso. Globally, 198 million cases of malaria are registered per year, causing about 438 000 deaths (WHO, 2015). The burden is particularly heavy in Africa where 90% of all fatal cases occur, of which 78% occurs in children under the age of five (WHO, 2014). Efforts for malaria eradication have been made from 2000 to 2013

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(WHO, 2014), but this is hampered by the resistance of *Plasmodium falciparum* to antimalarial drugs available (Dondorp et al., 2012). The history of malaria has shown that plants are a source of new molecules. This is the case of quinine that has been isolated from *Chincona* and artemisinin that has been isolated from *Artemisia annua* (Batista et al., 2009; Bero and Joëlle, 2011; Bero et al., 2009; Kaur et al., 2009; Nogueira and Lopes, 2011; Phillipson and Wright, 1991). Over 80% of the world's population use medicinal plants for healing (WHO, 2008, 2010). In Burkina Faso, the majority of the population use medicinal plants as the first therapeutic means (Bero et al., 2009; Traoré et al., 2009) in order to contribute to find new antimalarial molecules having a wide margin of safety and efficiency. In traditional medicine, *Vernonia cinerea Less* (Asteraceae) has many therapeutic uses. Its vernacular name is "little ironweed (USA)" and the whole plant is used in therapeutic. It is used to treat malaria fever, vomiting, inflammation, infections, diuresis, cancer, abortion and gastrointestinal (Jain and Puri, 1984). The decoction is used to treat cardiac pathologies, wounds, colic and diarrhea (Rivière et al., 2005). In Burkina Faso, the plant is recommended in the care of malaria and for the care of dysentery and wounds in therapeutic use. The dosage used is usually a decoction of 50 g/l (Http://www.jardinsdumonde.org). In the face of resistance to artemisinin which is the core molecule of ACT, which extracted from *A. annua* (Asteracea), the priority is to research of new active molecules against emerging resistant strains. It was in this context that we chose *V. cinerea* Less which is a plant of the Asteracea family. The aim of the study was to evaluate the antimalarial activity of extracts from *V. cinerea* Less (Asteraceae) that is used in traditional medicine in Burkina Faso to treat malaria.

MATERIALS AND METHODS

Plant collection

The plant was collected in the Comoé region (West Burkina Faso), GPS 10°38'N, 4°45'W in August 2010 and authenticated by a botanist M. Madou Ouedraogo from the Comoé Regional Forestry Department. After collection, a voucher specimen of plant was deposited in the herbarium of Centre National de Recherche et de Formation sur le Paludisme (CNRFP) in Burkina Faso (Ouagadougou). Then the plant was sprayed and the raw material obtained was sent to the laboratory of pharmacognosy for preparation of crude extracts.

Preparation of plant extracts

Five (5) types of extracts were prepared from the plant powder. We obtained three organic extracts, one aqueous with water and one crude alkaloid. Crude organic extracts were prepared by maceration for 16 h successively with dichloromethane (CH_2Cl_2), methanol (CH_3OH) and water-methanol (CH_3OH/H_2O) solvents. Plant powder (10 g) was used for these organic extraction methods with 100 ml of each solvent. CH_2Cl_2 extract was air dried at room temperature. CH_3OH and CH_3OH/H_2O extracts were freeze-dried with lyophilisator (Brand) after total evaporation of solvents. Aqueous extracts were prepared by boiling 10 g of plant powder in 100 ml of purified water for 30 min. After cooling, solutions were filtered on cotton wool and freeze-dried. Crude alkaloid extracts were obtained by alkalization with 28% NH_4OH to pH9 of the plant powder and extraction with CH_2Cl_2 for 24 h. Plant powder (10 g) was used by applying the classical alkaloids extraction method (Sanon et al., 2003). After 16 h of maceration with ammoniac and CH_2Cl_2, a percolation was made with CH_2Cl_2 solvent. Then 100 ml of CH_2Cl_2 layer was concentrated under vacuum and then extracted with a 3% solution of H_2SO_4 to pH3. The aqueous acid solution was alkalinized again with NH_4OH and extracted with CH_2Cl_2, and a crude alkaloid extracts was obtained by concentration. The yields were calculated using the following:

$$\text{Extracts Yields} = \frac{\text{Mass of the crude extract obtained}}{\text{Test portion of the powder} - \text{plant}} \text{X } 100 \quad (1)$$

Characterization of chemical groups

Phytochemical screening

For characterizing the major chemical groups, non-hydrolyzed extracts and hydrolyzed extracts were prepared. 1 g of each lyophilizate was weighed and mixed with 100 ml of distilled water then decanted into a bottle to get non-hydrolyzed extracts. To obtain hydrolyzed extracts, 25 ml of non-hydrolyzed extracts were removed and mixed with 15 ml of 10% HCl, this was heated under reflux for 30 min. After cooling, the mixture was transferred to a separating funnel. The liquid-liquid partition was done by the addition of 3×10 ml of dichloromethane. The organic phase was recovered and filtered and then stored in vials.

Phytochemical screening of plant extracts was made according to Ciulei method (1982). Chemical groups were identified by liquid medium characterization tests of the extract. Triterpenes and sterols were identified with Liebermann-Büchard test. Tannins presence has been highlighted by the reaction of $FeCl_3$ 1% test tubes. Saponins were identified with the observation of persistent foam column. Coumarins were detected with NH_4OH 10%, UV (366 nm). Emodols and anthracenosids were identified with the Bornträger test. Carotenoids were detected with the H_2SO_4.

Chromatographic analysis thin layer (TLC)

Five microliters of each of the dichloromethane extracts, non-hydrolyzed extracts and hydrolyzed extracts were deposited on chromatography plates (silica gel G60, Merck). The plates were dried in ambient air and placed in migration vats covered before hand containing appropriate solvent systems. The distance covered of the eluent (solvent front) is predefined at 8 cm from the extracts of the deposition line. At the end of migration, the plates were removed and oven dried, and then the UV (254 or 366 nm) was read and after visualized with a reagent specific to the desired chemical groups. Alkaloids were identified with migration solvent toluene-ethyl acetate-diethylamine (17.5: 5: 2.5) and revealed with Draggendorf test. Triterpenes and sterols were identified with migration solvent n-hexane-ethyl acetate-toluene (6: 2: 4) and revealed with sulfuric acid 3% in ethanol.

Tannins has been highlighted by the solvent migration ethyl acetate-methanol-water (2: 1: 1) and revealed with aqueous solution of ferric chloride to 1%. Coumarins, emodols and anthracenosids were detected with migration solvent n-hexane-

ethyl acetate-toluene to 6: 2: 2 and revealed with the KOH solution (1 N).

Strains of *P. falciparum*

The biological material used was strains of *P. falciparum*, the species responsible for the majority of malaria cases in Africa. Strains of *P. falciparum* resistant to chloroquine K1 and sensitive to chloroquine 3D7 were used. The K1 parasites were provided by the laboratory Warhust, London School of Hygiene and Tropical Medicine, London, England, United Kingdom (LSHTM). The 3D7 parasites were provided by the Laboratory Nuguchi Memorial Institute on Medical Research (NMIMR) (Ghana). They were maintained in continuous culture in human blood in the Laboratory of "Centre National de Recherche et de Formation sur le Paludisme" (CNRFP) in Burkina Faso (Ouagadougou).

Continuous culture of parasites *in vitro* by the method of Trager

The strains in continuous culture were maintained using the technique of Trager and Jensen (1976) and we renewed the culture medium every 24 h. Parasites were thawed and cultured in flasks containing complete culture medium composed of RPMI 1640, L-glutamine 2%, Stamps Hepes 2%, Gentamicin 0.5%, Albumax 5% and Hypoxanthine 0.5%. The flasks containing the culture were aerated with mixed gas composed of 2% O_2, 5% CO_2, and 93% N_2. The flasks were then incubated in the CO_2 incubator at 37°C under conditions for maximal growth. Parasitaemia was controlled by making blood smears after the renewal of the culture medium. When parasitaemia reached 6%, a subculture was made using fresh blood without the interference from blood group A+.

***In vitro* evaluation of antiplasmodial activity**

The antiplasmodial activity of extract from *V. cinerea* Less was evaluated using the technique of *Plasmodium* Lactate Deshydrogenase (pLDH). Reference products (Dihydroartemisinin and Chloroquine) and extracts were dissolved in dimethylsulfoxide (DMSO) or in distilled water. The starting concentration of the extracts was 10 mg/ml that was further diluted to reach a final concentration of 100 µg/ml. The tests were performed on 96-well plates filled with a fixed volume of parasitized erythrocytes (2% parasitaemia). Samples were serially diluted with complete culture media (RPMI 1640 with albumax) to achieve the required concentration with DMSO concentration < 0.5%. Each extract was applied in a series of duplicate dilutions (final concentrations ranging from 0.78 to 50 µg/ml) on two rows. Dihydroartemisinin was used to validate the malaria test and chloroquine diphosphate salt (Sigma Aldrich) was used to validate the real chloroquine resistance of malaria strain K1. Infected and uninfected erythrocytes A+ were used as positive and negative controls, respectively.

After 72 h of incubation, the plates' counterpart's tests plates were prepared and the various substrates and coenzyme were then added. 100 µl MALSTAT (160 ml distilled H_2O, 200 µl Triton X100, 2 g of L-Lactate, 0.66 g Trizma base, 66 mg 3-acétylpiridine adenine di-nucleotide (APAD), at pH 9), 25 µl NBT/PES (100 ml of distilled water, 160 mg of NTB and PES 8 mg) and 20 µl of blood from the test plate was dispensed in each well including positive and negative controls. After 10 min of incubation, the plates were read on a spectrophotometer at a wavelength of 650 nm. Data were scored and analyzed using Microsoft Excel 2007. The mean optical density of negative controls was subtracted from that of each product to obtain the percentage of viability.

$$\% \text{ viability} = \frac{\text{OD product} - \text{OD negative control}}{\text{OD positive control}} \text{ X } 100 \quad (2)$$

The 50% inhibitory concentrations (IC_{50}) were calculated graphically with the Table Curve 2D v.5.0 software using the percentages of viability or cells proliferation versus log concentration. The IC_{50} of various extracts obtained were analyzed according to the criteria Deharo (Deharo et al., 2001); good activity IC_{50}<= 5 µg/ml, moderate activity 5<IC_{50}≤10 µg/ml, and inactive IC_{50}>10 µg/ml.

RESULTS AND DISCUSSION

Five types of extracts were prepared. Phytochemical screening of *V. cinerea* Less revealed the presence of alkaloids, triterpenes and sterols, saponins, tannins, emodols, anthracenosids, coumarins, and carotenoids (Tables 1 and 2). The *in vitro* antiplasmodial activity of the crude extracts on strains reference K1 and 3D7 was assessed by using five crude extracts of plant prepared. Amongst the 5 extracts tested, alkaloids extracts were identified as having good antimalarial effects (IC_{50}< 5 µg/ml), CH_2Cl_2 with moderate effects (5 µg/ml ≤ IC_{50}< 10 µg/ml), and CH_3OH, CH_3OH/H_2O and H_2O as inactive (IC_{50} ≥ 10 µg/ml) (Table 3) according to Deharo et al. (2001). The best antimalarial effects were obtained with alkaloids extracts of plant (Figures 1 and 3).

The crude alkaloids extracts from the whole plant showed good antimalarial effects against the chloroquine-resistant strain K1, with IC_{50} values 2.56 µg/ml. The moderate antimalarial effects were obtained with dichloromethane extracts against K1, with IC_{50} values 5.85 µg/ml (Figures 2 and 4).

In Cambodia, a similar study showed that dichloromethane extracts of *V. cinerea* had an IC_{50}= 18.3 µg/ml with the W2 malaria strain chloroquine-resistant (Hout et al., 2006). Although, our results are different from those of Simonsen et al. (2001) in India on ethanol extracts (82 µg/ml) tested on 3D7.

Based on Deharo's efficiency criteria, results from Cambodia and India are the same as our findings. These differences may be related to many parameters, including the local environment and the collection periods, which contribute to the variation of the chemical components of the plant as shown in a previous study on seasonal effects on bioactive compounds (Aires et al., 2011).

In Burkina Faso, a previous study showed that alkaloids extracts of bark of *Terminalia avicennoides* had an IC_{50}= 2.9 µg/ml with the K1 malaria strain (Sanon et al., 2013). Based on Deharo's efficiency criteria, results from this plant are a same from our findings.

The previous study was conducted with *Dicotoma tomentosa* (Asteracea) also collected in Burkina Faso, and in a different area. The antiplasmodial activity obtained with this plant (IC_{50}= 1.9 ± 0.2 µg/ml) was different from our dichloromethane extracts based on Deharo's efficiency criteria (Jansen et al., 2012).

In summary, our study confirms the pharmacological

Table 1. Summary of phytochemical screening of plant extracts was made according to Ciulei method (1982).

Chemical groups	Hydrolyzed extracts			Crude non-hydrolyzed extracts			
	CH_3OH	CH_3OH/H_2O	H_2O	CH_3OH	CH_3OH/H_2O	H_2O	CH_2Cl_2
Alkaloids	nr	nr	nr	–	–	–	–
Saponosids	nr	nr	nr	+	+	+	nr
Flavonoids	–	–	–	nr	nr	nr	–
Triterpenes/Sterols	+	+	+	nr	nr	nr	+
Tannins	nr	nr	nr	+	+	+	nr
Anthracenosids	+	–	–	nr	nr	nr	nr
Anthocyanosids	–	–	–	nr	nr	nr	nr
Emodols	nr	nr	nr	nr	nr	nr	+
Carotenoids	nr	nr	nr	nr	nr	nr	+
Coumarins	+	+	–	nr	nr	nr	+
Reductors compounds	nr	nr	nr	–	–	–	nr

(+): Presence ; (–) : not detected; (nr): not researched

Table 2. Chemical groups showed by chromatographic analysis thin layer.

Chemical groups	Hydrolyzed extracts			Crude extracts non-hydrolyzed				
	CH_3OH	CH_3OH/H_2O	H_2O	CH_3OH	CH_3OH/H_2O	H_2O	CH_2Cl_2	Alkaloids extracts
Alkaloids	nr	nr	nr	nr	nr	nr	nr	+
Tannins	nr	nr	nr	+	+	+	nr	nr
Triterpenes/Sterols	+	+	+	nr	nr	nr	+	nr
Coumarines	–	–	nr	nr	nr	nr	+	nr
Emodols	nr	nr	nr	nr	nr	nr	+	nr
Anthracenosids	+	nr	nr	nr	nr	nr	nr	nr
Saponosids	nr	nr	nr	–	–	–	nr	nr

(+): Presence ; (–) : not detected; (nr): not researched

Table 3. *In vitro* antiplasmodial activity of crude extracts obtained from *Vernonia cinerea* Less.

Plant	Family	Herbarium voucher	Part of plant	Extracts	Yield (in the plant) (%)	IC_{50} 3D7 (µg/ml)	IC_{50}K1 (µg/ml)
***Vernonia Cinerea* Less**	Asteracea	Cnrfp10Vc	Whole plant	CH_2Cl_2	2.57	8. 42	5.85
				CH_3OH	5.07	26.43	21.08
				CH_3OH/H_2O (1/1)	7.11	>50	41.56
				H_2O	23.7	>50	37.17
				Alkaloids extracts	0.2	4. 25	2.56
Chloroquine	-	-	-	-	-	0. 045	0.126
Dihydro-artemisinin	-	-	-	-	-	0. 0015	0. 002

properties of this plant species shown by its antibacterial activity with petroleum ether and ethanol extracts (Somasundaram et al., 2010), antipyretic, analgesic and anti-inflammatory activity (Iwalewa et al., 2003). Another study showed a good antiplasmodial activity of vernolide C and D molecules against W2 (Chea et al., 2006).

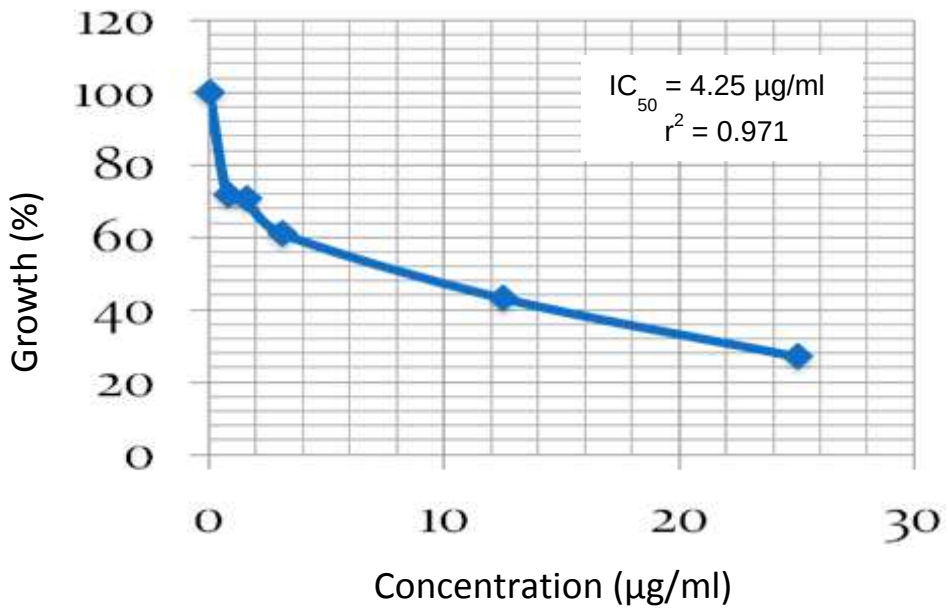

Figure 1. Curve of growth inhibition 3D7 of crude alkaloids.

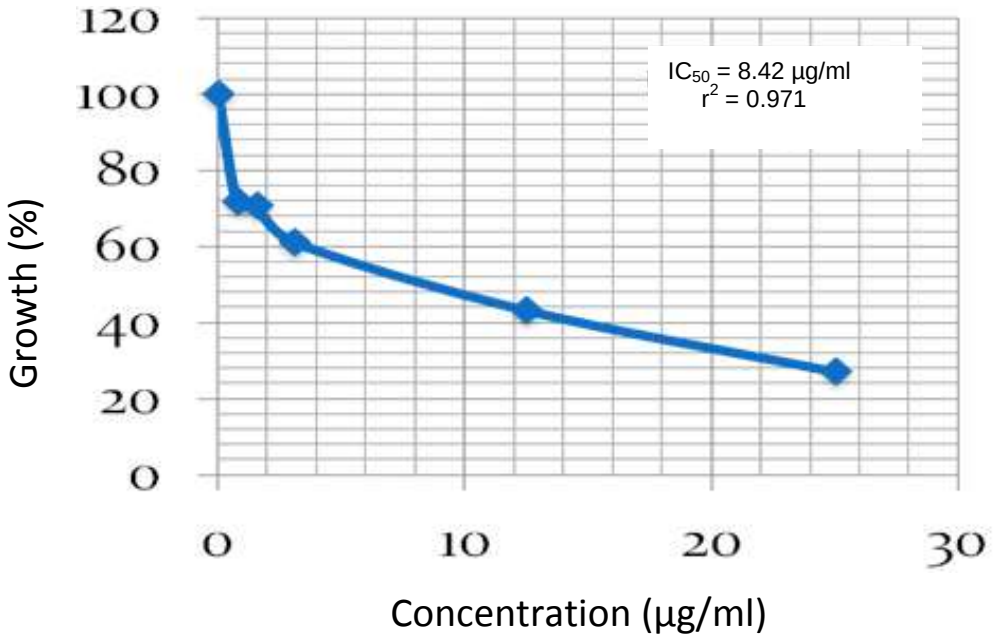

Figure 2. Curve of growth inhibition 3D7 of CH_2Cl_2 extracts.

This activity could be due to the presence of alkaloids (Bruneton, 1993) and triterpenes in the plant which mentioned by Chea et al. (2006). Alkaloids are one of the most important classes of natural products providing drugs since ancient times. The outstanding example is quinine from *Cinchona succirubra* (Rubiaceae) used for the treatment of malaria for more than three centuries (Kaur et al., 2009). Several plants of the Asteraceae family have been revealed as a good source for antimalarials. The most famous one is *A. annua*, the Chinese herb from which artemisinin (qinghaosu) was isolated (Liu et al., 1992). The good activity observed from the present investigation with *V. cinerea* Less which is a member of this family, thus supports the use of this

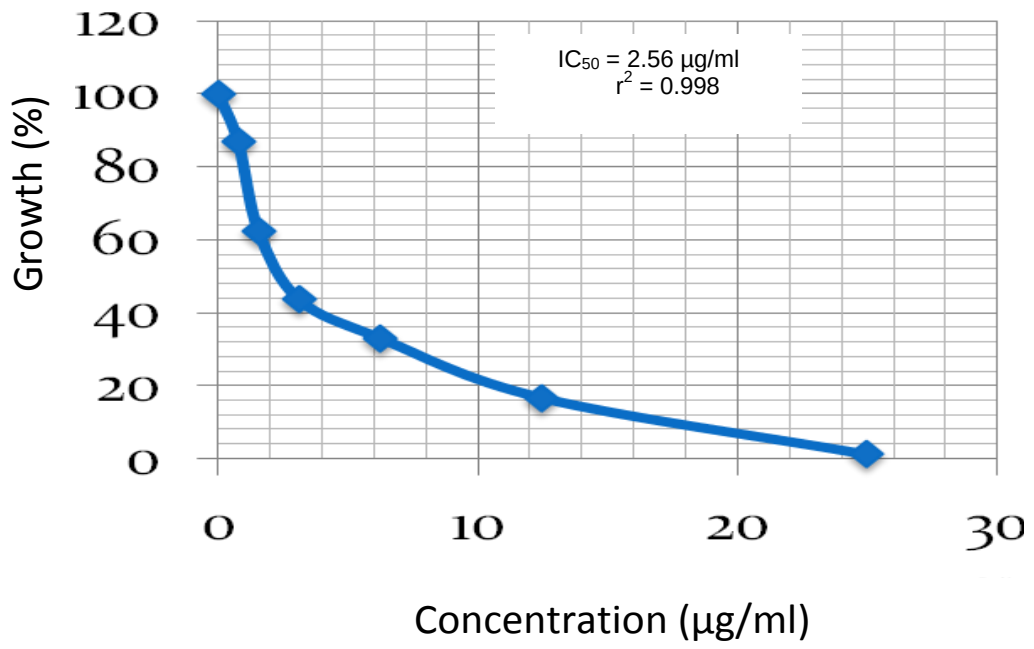

Figure 3. Curve of growth inhibition K1 of crude alkaloids extracts.

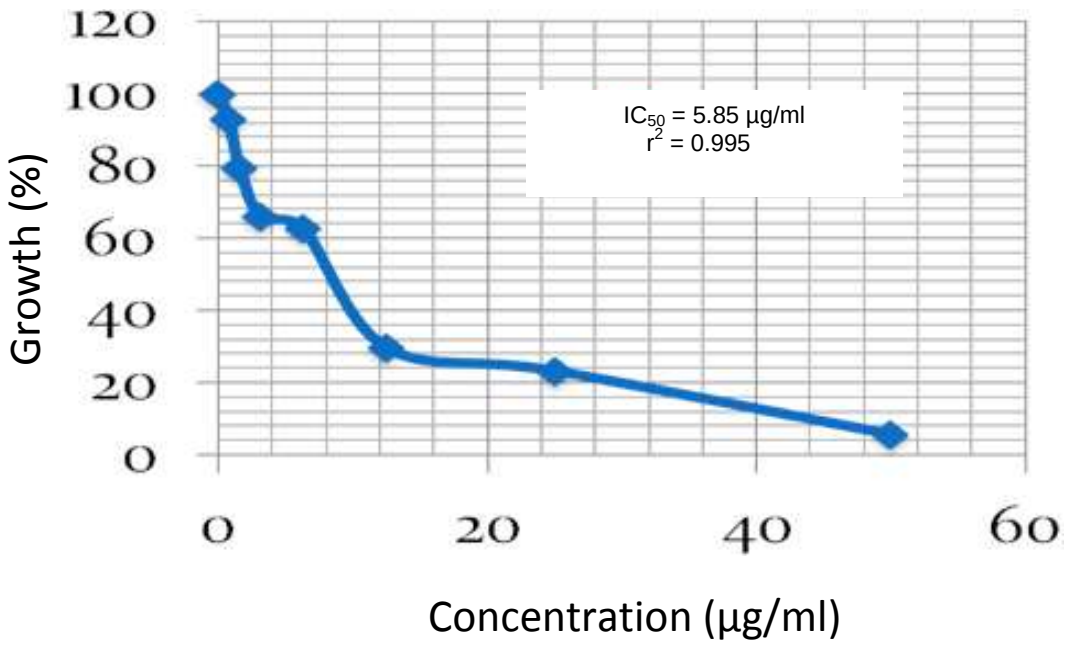

Figure 4. Curve of growth inhibition K1 of CH_2Cl_2 extracts.

plant for malaria in traditional medicine. This plant can be used in traditional medicine by paying close attention to the dosage. Further investigations are needed to evaluate the antiplasmodial activity *in vivo* in mice infected with *Plasmodium berghei* and to study the acute toxicity of plant extracts of *V. cinerea* Less, for the development of new drugs.

CONCLUSION

In vitro tests conducted on the parasites in the present

study have shown that *V. cinerea* Less has a very promising antiplasmodial activity. The CH_2Cl_2 extracts and crude alkaloids allowed us to get good IC_{50} on plasmodial strains. These results support the traditional use of this plant in traditional medicine for the treatment of malaria. Further studies will be needed, in particular *in vivo* tests on mice infected with *P. berghei* to assess antiplasmodial activity.

Conflict of interests

The authors have not declared any conflict of interests.

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