Chemical, biological and ethnopharmacological studies of two Malian medicinal plants:

Terminalia macroptera and *Biophytum umbraculum*

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Anh Thu Pham

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Manuscript

Abbreviations

2D-NMR	Two-dimensional nuclear magnetic resonance
BuOH	Butanol
^{13}C-NMR	Carbon nuclear magnetic resonance
CM	Cerebral malaria
COSY	Correlation spectroscopy
CVD	Cardiovascular diseases
DCM	Dichloromethane
DMT	Department of Traditional Medicine
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ET	Electron transfer
EtOAc	Ethyl acetate
GI	Gastrointestinal
^{1}H-NMR	Proton nuclear magnetic resonance
HAT	Hydrogen atom transfer
HHDP	Hexahydroxydiphenic
HMBC	Heteronuclear multiple bond correlation spectroscopy
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation spectroscopy
IC_{50}	Concentration to give 50 % inhibition
ICH_{50}	Concentration to give 50 % hemolysis
ITM	Improved Traditional Medicine
LC_{50}	Concentration to give 50 % lethality
LDL	Low density lipoprotein
LO	Lipoxygenase
LPS	Lipopolysaccharide
MeOH	Methanol
NO	Nitric oxide
NP	Normal phase
NMR	Nuclear magnetic resonance
OH	Hydroxyl group
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RP	Reverse phase
RS	Reactive species
RT	Room temperature
SEC	Size exclusion chromatography
STD	Sexually transmitted disease
TLC	Thin layer chromatography
UV	Ultraviolet
WHO	World Health Organization
XO	Xanthine oxidase

Abstract

The aim of this thesis was to investigate the chemical and biochemical properties of the Malian medicinal plants *Terminalia macroptera* and *Biophytum umbraculum*, with main focus on its phenolic substances. This thesis is a part of a research project in which the ultimate goal is to provide efficient, non-toxic and inexpensive medicines for the Malian population.

Extraction and purification of fractions from *T. macroptera* resulted in the isolation of several polyphenolic compounds such as hydrolyzable tannins and flavonoids. Chebulic acid trimethyl ester is a novel compound isolated from *T. macroptera*. *B. umbraculum* was found to be a good source of flavone-*C*-glycosides, and cassiaoccidentalin A, a rare natural product, was the major one. The crude extracts and pure compounds obtained were tested in various *in vitro* bioassays to determine their bioactivity as antioxidants, α-glucosidase inhibitors, antimalarial agents as well as their toxicity.

Ethnopharmacological studies revealed that 86 % of the traditional practitioners used *T. macroptera* for a variety of medical indications such as wounds, pain, cough, tuberculosis, hepatitis, and diabetes. The semipolar extracts, the ethyl acetate (EtOAc) extract in particular, were found to be very strong α-glucosidase inhibitors, strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavengers and 15-lipoxygenase (LO) inhibitors, and moderate xanthine oxidase (XO) inhibitors. None of the extracts or the isolated compounds seemed to be very toxic towards brine shrimp larvae.

B. umbraculum was used for treating cerebral malaria (CM) by more than 50 % of the interviewed traditional practitioners, and was thus tested in two CM-related *in vitro* assays in addition to the antiplasmodial activity assay. The semipolar plant extracts showed high complement inhibition of the classical pathway and dose-dependent inhibition of nitric oxide (NO) release from activated macrophages. Additionally, the EtOAc extract displayed moderate antimalarial activity against the erythrocyte stages of *Plasmodium falciparum.* The

antioxidant activity was also evaluated, and the semipolar extracts displayed strong radical scavenging and 15-LO inhibitory effects, but less activity towards XO.

1. Introduction

1.1 Mali

Mali is a landlocked country located in West Africa (Figure 1.1). With an area of approximately 1,240,000 km^2, it is actually one of the biggest countries in Africa, based on size. Despite the large area, the majority of the population lives in rural areas along the river Niger in the south since 2/3 of the land area is desert or semi-desert. The country is composed of various climatic zones and populated by a diversity of ethnic groups (Diallo & Paulsen, 2000; OperationWorld, 2013).

According to the Human Development Report of 2013, Mali is ranked as number 182 of 187 countries and categorized as a "low human development" country, whilst Norway in contrast is ranked as number one and "very high human development" (Malik, 2013). Being one of the poorest countries in the world, Mali faces numerous health challenges related to the high prevalence of infectious diseases and parasites in combination with low availability of medicines and a poor healthcare system. To meet their health care needs, the Malian people use both conventional and traditional medicine. And because of the price increase of conventional medicine in the local currency, the use of traditional medicine has further increased (Diallo et al., 2002).

Figure 1.1: Map of Mali (OperationWorld, 2013)

1.2 Traditional medicine

Traditional medicine is the total sum of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses (WHO, 2008). Traditional medicine is also known as alternative, non-conventional or complementary medicine, although none of these designations are fully equivalent to traditional medicine. However, traditional medicine is not an alternative to modern/conventional medicine, but in fact complementary in most developing countries (Iwu, 2002). In Mali, approximately 75 % of the population relies on traditional medicines for their primary health care (Robinson & Zhang, 2011). The traditional medicine is mainly based on plants, and many of these plants have never been investigated for their chemical and biological properties. It is therefore essential to evaluate scientifically the benefits, risks and limitations of Malian medicinal plants to provide a better health care system for the Malian people.

Since 1996, the Pharmacognosy section at the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo has collaborated with the Department of Traditional Medicine (DMT), Bamako, Mali. DMT is a collaborating center of the World Health Organization (WHO) for research in traditional medicine. One of the primary objectives of the collaboration is to assure that traditional medicine is complementary to conventional medicine, assuming that the medicines can be produced from local resources, especially from medicinal plants. The main activity of DMT is to collaborate with the traditional practitioners and to register their use of medicinal plants, in addition to research and development of improved traditional medicines (ITMs) from local plants. So far DMT have developed twelve ITMs, and seven of these are acknowledged as essential medicines in Mali. These ITMs have undergone extensive phytochemical, pharmacological and toxicological investigation and are standardized according to traditional administration regimes (Diallo & Paulsen, 2000).

The collaboration between the Section of Pharmacognosy and DMT has mainly focused on identifying bioactive polysaccharides from Malian medicinal plants used in the treatment of wounds (Austarheim et al., 2012; Diallo et al., 2002; Inngjerdingen et al., 2004). The

medicinal plants investigated in this collaborating project are chosen by the DMT as potential candidates for new ITMs. As plant polysaccharides are polar, it is usually the water extracts of the chosen plants that have been investigated, and little is known about the low molecular weight compounds of the lipophilic and semipolar extracts of these medicinal plants. It is therefore highly interesting to study the low molecular weight compounds in these extracts as possible active substances in the Malian medicinal plants.

1.3 Investigated plants

As mentioned earlier, the plants investigated are chosen by the DMT as potential candidates for new ITMs. In addition, limited chemical data has been reported on these species previously.

1.3.1 *Terminalia macroptera* Guill. & Perr. (Combretaceae)

Terminalia macroptera Guill. & Perr. (Combretaceae; syn. *Myrobalanus macroptera* Kuntze) is a tree, up to 20 meters high (Figure 1.2), which grows from Senegal to Cameroon, and as far east as Sudan. It is common and scattered, and thrives in savannahs and poorly-drained clay soils. According to Arbonnier (2004), the leaves have been used traditionally to treat gastritis, colic, fever, skin diseases, tuberculosis and high blood pressure. The roots are astringent and are used against cough, jaundice, syphilis, urinary infections among other diseases. Recent ethnobotanical and ethnopharmacological surveys have reported use against cough, snakebite (Sourabie et al., 2013), malaria, liver disorders, urinary retention, diarrhea, skin diseases, epilepsy, convulsion, gastric ulcer (Etuk et al., 2009; Ige, 2011; Nadembega et al., 2011; Oluranti et al., 2012; Traore et al., 2013), piles, constipation (Kayode et al., 2009a; Odugbemi, 2008; Wodah & Asase, 2012) and sexually transmitted diseases (STD) (Kayode et al., 2009b). *In vitro* studies have revealed antioxidant (Kone et al., 2012), antimicrobial (Silva et al., 1996; Silva et al., 1997), antifungal (Batawila et al., 2005), anti-inflammatory (Bernard et al., 2001), antiepileptic, anticonvulsant (Pedersen et al., 2009), antiplasmodial (Sanon et al., 2003), anti-*Neisseria gonorrhoeae* (Silva et al., 2002), anti-*Helicobacter pylori*

(Silva et al., 2012) as well as haemolytic (Karou et al., 2012) effects of *T. macroptera*. Chemical studies have shown that this tree is rich in hydrolyzable tannins, triterpenes, (Conrad et al., 2001a; Conrad et al., 1998; Conrad et al., 2001b; Kraus et al., 2002; Silva et al., 2000) benzoic and cinnamic acids (Kone et al., 2012), and flavonoids (Batawila et al., 2005). However, most of these studies are carried out on of the roots and bark of *T. macroptera*, and very few studies had been performed on the leaves until we started phytochemical studies on the leaves of this plant. It is very important if the leaves can be used rather than the roots and bark as this will help to ensure a more sustainable conservation of biodiversity. This is especially since *T. macroptera* is one of the more frequently used trees as a resource of fuelwood and timber, and is therefore in threat of extinction (Asase & Oteng-Yeboah, 2012; Lykke, 1998; Tee et al., 2009; Zida et al., 2008).

Figure 1.2: *Terminalia macroptera* Guill. & Perr. (Combretaceae). The local name is woloba (Photo: A.T. Pham).

1.3.2 *Biophytum umbraculum* Welw. (Oxalidaceae)

According to The Plant List (2013), *Biophytum petersianum* Klotzsch. is a synonym of the accepted name *Biophytum umbraculum* Welw. (Oxalidaceae). However, almost all scientific papers published have used the name *B. petersianum*, but the accepted name will be used throughout this thesis. *B. umbraculum* (*B. petersianum*) has also in many cases been used synonymous with *Biophytum sensitivum* (L.) DC., but these species are considered as two

distinct species (Farooqui et al., 1985; Lourteig, 1981). *B. umbraculum* is a slender annual herb with stems up to 25 cm long and leaves, which are very sensitive, in a terminal crown (Figure 1.3). The plant is widespread in tropical and subtropical regions of Africa, and across Asia to New Guinea (Burkill, 1997). *B. umbraculum* has been used traditionally in Nigeria for the treatment of wounds, gonorrhea, urinary stones and stomachache. In Gabon and Zaïre it is used as a purgative, in Togo for hypertension, and in New Guinea the plant is believed to increase fertility (Burkill, 1997; Mouzou et al., 2010). In an ethnopharmacological survey conducted in Mali in 2011 we found that *B. umbraculum* is used against cerebral malaria (Paper V). Two earlier ethnopharmacological surveys from Mali also reported the use against malaria (Grønhaug et al., 2008; Inngjerdingen et al., 2006). Additionally, the indications fever, wounds, and different types of pain were reported (Diallo et al., 2002; Grønhaug et al., 2008; Inngjerdingen et al., 2006).

Figure 1.3: Aerial parts of *Biophytum umbraculum* Welw. (Oxalidaceae). (Photo: D. Diallo)

In vitro studies demonstrated that *B. umbraculum* stimulated corticosterone and aldosterone secretion in rats (Kodjo et al., 2006) and exerted calcium antagonistic activity on vascular smooth cells in Wistar rats (Titrikou et al., 2007). Antagonistic effect on calcium release from sarcoplasmic reticulum in skeletal muscle cells has also been shown (Mouzou et al., 2010), and the mechanism underlying the hypotensive effect was not a result of sympathetic nervous system inhibition, but partially involved stimulation of the parasympathetic system (Titrikou et al., 2008) and peripheral vasodilatation (Titrikou et al., 1998). These results may explain the use of *B. umbraculum* as an antihypertensive in traditional medicine. Pectic polysaccharides from *B. umbraculum* have shown immunomodulatory activities in

biological screening assays, with effects varying from complement fixation (Diallo et al., 2002; Inngjerdingen et al., 2006) to potent activation of macrophages and of dendritic cells (Inngjerdingen et al., 2008), as well as impact on intestinal Peyer's patch cells (Grønhaug et al., 2011). A recent study showed that pectic polysaccharides isolated from aerial parts of *B. umbraculum* were able to protect against *Streptococcus pneumonia* infection in mice (Inngjerdingen et al., 2013).

1.4 General aspects of isolated polyphenols

Polyphenols are a large group of phytochemicals, with structural features characterized by the presence of one or more phenolic units (benzene ring with OH groups). Since this is a fairly common feature, this group is very diverse and contains several sub-groups, the major ones being flavonoids, phenolic acids, hydrolyzable tannins, stilbenes and lignans. Plant-based foods and beverages such as fruits, berries, vegetables, chocolate, tea and wine are rich sources of polyphenols. Most of the natural phenols are derived from secondary plant metabolism of the shikimic acid pathway, acetate-polymalonate pathway or both (Knaggs, 2001; Landete, 2012; Tsao, 2010). Shikimic acid is the key intermediate in the shikimic acid pathway and subsequently essential in the biosynthesis of many plant phenolics such as flavonoids (Herrmann & Weaver, 1999; Taiz & Zeiger, 2010). Shikimic acid is also a central precursor in the gallic acid biosynthesis, which in turn is a key intermediate for the synthesis of plant hydrolyzable tannins (Grundhofer et al., 2001; Muir et al., 2011; Werner et al., 2004).

1.4.1 Flavonoids

Flavonoids are a large group of naturally occurring polyphenolic compounds that are secondary metabolites, extensively spread throughout the plant kingdom. The structure of flavonoids consists of a C_{15} unit with two benzene rings A and B connected by a three-carbon chain. This chain is closed in most flavonoids, forming the heterocyclic ring C (Figure 1.4). The basic structure of a flavonoid allows a wide variety of different substitution

in the A, B and C rings, resulting in multiple subclasses. Classification of flavonoids into subclasses is based on the functional groups in the C-ring; e.g. flavones, flavonols, flavanones, flavan-3-ols, isoflavones and anthocyanidins (Habauzit & Morand, 2012). Flavonoids in nature are most often found as glycosides, methyl ethers and other conjugates. The sugar moiety helps to facilitate water solubility and transportability of the aglycone (Davies & Yáñes, 2013). Glycosides can either be O- or C- linked. The variation of flavonoid glycosides are based on the number of positions on the flavonoid for glycosylation, the level of glycosylation and the number of types of sugars involved in glycosylation (Ghasemzadeh & Ghasemzadeh, 2011).

Flavonoids are important constituents of the human diet and are found in herbs, vegetables, fruits, cocoa, soybeans, berries, wine, and tea. Many plants are rich in flavonoids. Flavonoids are important not only for the plants, but also for humans and other animals. These compounds are acknowledged for having interesting medicinal properties, such as vasodilatory, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, anticarcinogenic, antiviral, antibacterial, antitumor, and enzyme inhibitory activities. They are also well known for their antioxidant and radical scavenging activities (Corradini et al., 2011; Cotelle et al., 1996; Rice-Evans et al., 1995).

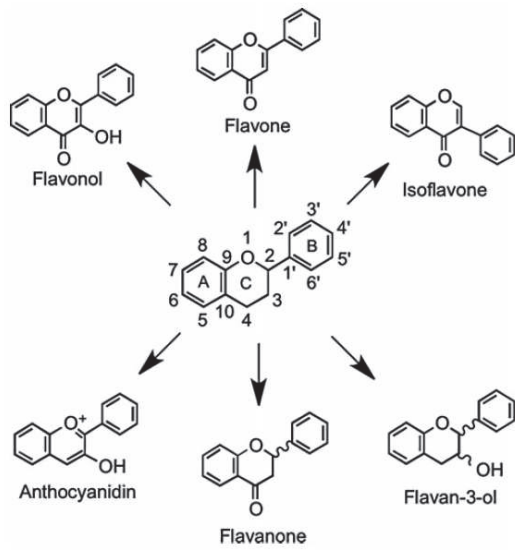

Figure 1.4: Structure of the flavonoid skeleton and some flavonoid subclasses (Del Rio et al., 2013).

1.4.1.1 Flavonols

Flavonols are a subclass of flavonoid compounds with a C2-C3 double bond and a hydroxyl group (OH) attached at the 3-position (3-hydroxyflavones) in the C-ring. These compounds are very widespread in higher plants where they usually occur in the leaves and outer parts of the plant. This is due to the dependence of light for their formation (Herrmann, 1976). About 450 different flavonol aglycones have been identified in plants (Corradini et al., 2011). In human diet, apples, plums, cranberries, strawberries, grapes, onions, broccoli and tomatoes are major food sources of flavonols. Beverages like red wine, tea and grape juice also contain large amounts (Habauzit & Morand, 2012). The majority of flavonols are present as *O*-glycosides, where one or more of the aglycone OH groups are bound to a sugar with formation of an O-C acid-labile acetal bond. The preferred position for glycosylation is 3-hydroxyl, and less frequently the 7-hydroxyl (Corradini et al., 2011; Herrmann, 1976). Flavonols have been identified as some of the best phenolics with antioxidant activity in wine, especially in white wines, although their antioxidant effects in red wines are usually exceeded by other more abundant phenolics, like flavan-3-ols and anthocyanins (Castillo-Munoz et al., 2009).

1.4.1.2 Flavones

Flavones are similar structurally to flavonols, except they lack oxygenation at C-3 (Figure 1.4). The flavones are not distributed as widely as flavonols, although substantial amounts have been detected in celery, parsley, sweet pepper, some herbs (Del Rio et al., 2013; Habauzit & Morand, 2012), as well as some medicinal plants (Du et al., 2010; Haraguchi et al., 2003; Wang et al., 2012). A wide range of substitutions is possible with flavones, including hydroxylation, methylation, *O*- and *C*-glycosylation, and alkylation (Del Rio et al., 2013; Habauzit & Morand, 2012). Flavones occur mainly as 7-*O*-glycosides (Hollman & Arts, 2000), but less frequently, glycosylation may take place by direct linkage of the sugar to the flavone basic nucleus, via an acid-resistant C-C bond, to form flavone-*C*-glycosides. Glucose is the most commonly encountered monosaccharide, whereas disaccharides and even higher saccharides are also found in association with flavones, the more common being the disaccharide rutinose (Corradini et al., 2011).

1.4.2 **Hydrolyzable tannins**

Plant tannins form one of the major groups of antioxidant polyphenols found in food and beverages. They have attracted considerable attention in recent years because of their multifunctional properties beneficial to human health (Yoshida et al., 2010). Tannins can be divided into four major groups based on their structural characteristics; gallotannins, ellagitannins, complex tannins and condensed tannins (proanthocyanidins) (Khanbabaee & van Ree, 2001) (Figure 1.5). Phlorotannins, condensed phloroglucinol derivatives, form a further group of tannins with limited distribution, being found in seaweeds (Singh & Bharate, 2006). Hydrolyzable tannins are polyesters of a sugar moiety and organic acids. The designation "hydrolyzable tannin" is due to the fact that these compounds undergo hydrolytic cleavage to the respective sugar and acid moiety upon treatment with diluted acids or they may be hydrolyzed by enzymes. If the acid component is gallic acid, the compounds are called gallotannins. Esters with hexahydroxydiphenic (HHDP) acid or ellagic acid are called ellagitannins (Serrano et al., 2009).

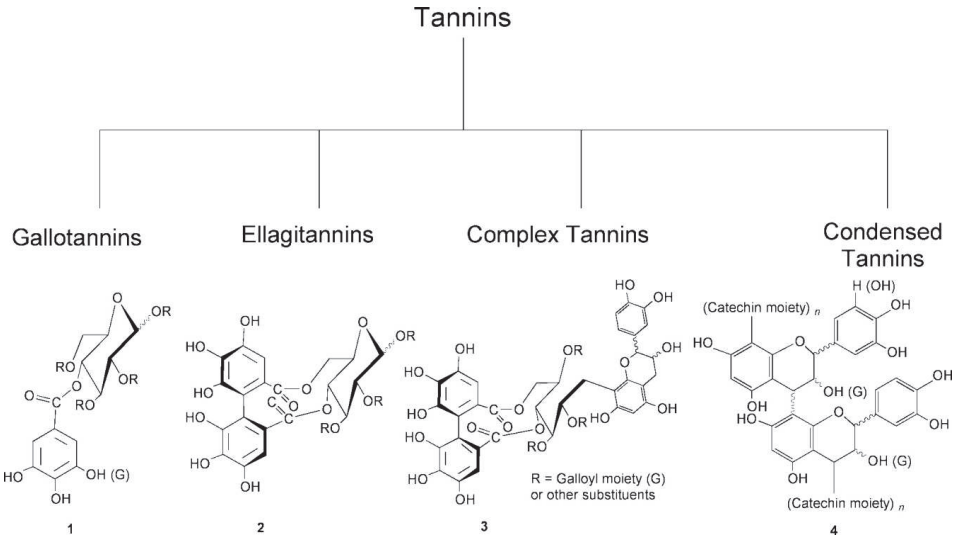

Figure 1.5: Classification of tannins. The hydrolyzable tannins consist of gallotannins, ellagitannins and complex tannins (Khanbabaee & van Ree, 2001).

1.4.2.1 Ellagitannins

Ellagitannins constitute a complex class of polyphenols characterized by one or more HHDP or ellagic acid moieties esterified to a sugar unit, usually glucose (Figure 1.6). HHDP moieties are constituted by oxidative biaryl coupling (C-C coupling) between suitably orientated neighbouring galloyl residues (Landete, 2011; Niemetz & Gross, 2005). Ellagitannins are widely distributed in the nature, with more than 500 natural products characterized so far, making ellagitannins the largest group of known tannins (Khanbabaee & van Ree, 2001). They have been reported in berries, fruits, nuts, peas and oak-aged wines (Serrano et al., 2009). Ellagitannins are hydrolyzed to ellagic acid under physiological conditions *in vivo* and ellagic acid is then gradually metabolized by the intestinal microbiota to produce different types of urolithins, that are considered as biomarkers for human exposure to dietary ellagitannins (Landete, 2011; Serrano et al., 2009). A wide range of significant biological activities beneficial to human health have been reported for ellagitannins. Antioxidant, astringent, antimicrobial, antitumor, antihypertensive, anti-inflammatory, enzyme-inhibitory and immunomodulatory properties are some of the effects demonstrated (Chen et al., 2009; Landete, 2011; Okuda, 2005; Okuda & Ito, 2011; Serrano et al., 2009; Vrhovsek et al., 2006; Yoshida et al., 2010). However, the evidence for the benefit of ellagitannins comes primarily from animal or *in vitro* studies, and additional clinical trials are needed to better understand their role as therapeutic agent, especially since there are very limited data on their bioavailability and metabolism in the human body.

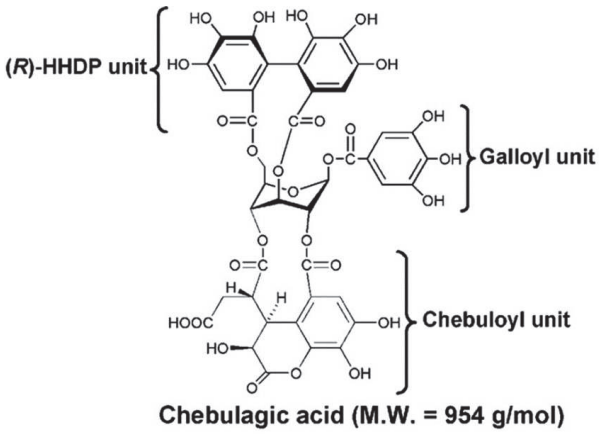

Figure 1.6: Example of a structure of an ellagitannin, chebulagic acid (Lin et al., 2011).

1.5 Biological activitites

1.5.1 Free radicals and antioxidant activity

There is a considerable interest in antioxidants, as they may exert beneficial effects in human health. It has been suggested that polyphenols such as flavonoids and tannins play a role as antioxidants by inhibiting lipid peroxidation including low density lipoprotein (LDL) oxidation and scavenging free radicals. These polyphenols may act as antioxidants because of the hydrogen-donating capacity of their phenolic groups (Rice-Evans et al., 1995). Suggested mechanisms are: (1) scavenging reactive species (RS) such as reactive oxygen species (ROS)/reactive nitrogen species (RNS); (2) suppressing ROS/RNS formation by inhibiting radical-forming enzymes (in particular xanthine oxidase, NADPH oxidase and lipoxygenases) or chelating trace metals (iron and/or copper) involved in free radical production; (3) inhibition of expression of inflammatory signalling molecules; (4) up regulating or protecting antioxidant defence (Ghasemzadeh & Ghasemzadeh, 2011; Mladenka et al., 2010).

Antioxidants are compounds that may protect human, animal and plant cells against the damaging effects of free radicals or other RS. Free radicals can be defined as any species containing one or more unpaired electrons in atomic or molecular orbitals and capable of independent existence (Halliwell, 2011). An imbalance between antioxidants and free radicals may result in oxidative stress and may lead to cellular damage. Because of this, oxidative stress has been suggested to be a major contributor to the pathogenesis of a number of human diseases as well as in the ageing process (Ghasemzadeh & Ghasemzadeh, 2011; Halliwell & Gutteridge, 2007; Valko et al., 2007). For this reason, antioxidant activity is one of the most commonly determined biological activities in extracts of plants (Clarke et al., 2013).

For a polyphenol to be defined as an antioxidant it must satisfy two basic conditions: first, when present in low concentration relative to the substrate to be oxidized it can delay, retard, or prevent the autoxidation or free radical-mediated oxidation; second, the resulting radical formed after scavenging must be stable (Rice-Evans et al., 1996). In order to determine

antioxidant activity, many tests use accelerated oxidative conditions which provoke oxidation.

Major antioxidant capacity assays can roughly be classified into two categories: the hydrogen atom transfer (HAT) reaction based assays and the single electron transfer (ET) reaction based assays. The ET-based assays measure an antioxidants' reducing capacity, while the HAT-based assays quantify hydrogen atom donating capacity (Huang et al., 2005).

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is a rapid, simple and accurate method for estimating the efficiency of substances as antioxidants. The assay is based on the spectrophotometric measurement (515-520 nm) of the DPPH concentration change resulting from the reaction with an antioxidant. The DPPH assay was believed to involve HAT reactions, but studies by Foti et al. (2004) and Litwinienko and Ingold (2004) suggested that an ET reaction is favored in the reaction between phenols having low pK_a values and DPPH in strong hydrogen-bond-accepting solvents, like methanol and ethanol.

The 15-lipoxygenase (15-LO) is an enzyme that catalyzes the stereospecific peroxidation of polyunsaturated fatty acids (PUFAs) and esters, which leads to the formation of hydroperoxides as well as active radical intermediates that are involved in pathological processes in animals and man (Khairullina et al., 2011). Inhibition of 15-LO is of interest, as the enzyme has been proposed to have a role in the oxidation of LDL, a process which is believed to be an important step in the development of atherosclerosis and cardiovascular diseases (CVD) (Lyckander & Malterud, 1996; Schneider & Bucar, 2005). Furthermore, 15-LO and their metabolites have been shown to be involved in a number of diseases such as cancer, psoriasis and diabetes (both type I and II) (Dobrian et al., 2011; Schneider & Bucar, 2005). Thus, 15-LO inhibitors may have a beneficial impact on the treatment of these disorders. The 15-LO inhibitory effects are assessed *in vitro* by using soybean 15-LO (catalyst), with linoleic acid as the substrate (Schneider & Bucar, 2005). This is a fairly stable, inexpensive and easy method measured spectrophotometrically (234 nm), and a good correlation between inhibitory activity towards mammalian and soybean-derived enzymes has been reported (Lyckander & Malterud, 1992; Lyckander & Malterud, 1996).

Xanthine oxidase (XO) is an enzyme that catalyzes the oxidation of hypoxanthine to xanthine, and produces uric acid and superoxide. Overproduction of uric acid is associated with gout and kidney stones, while increased superoxide generation may lead to the formation of other ROS, which can initiate oxidative stress (Van Hoorn et al., 2002). The superoxide anion radical generated by XO is involved in a series of pathological disorders such as hepatitis, inflammation, ischemia-reperfusion, carcinogenesis as well as aging (Nguyen et al., 2004). Moreover, possible beneficial effects of XO-inhibitors as adjuncts in the management of severe *Plasmodium falciparum* malaria have been reported (Iwalokun et al., 2006). Allopurinol is a XO-inhibitor used clinically in the treatment of gout, but due to unwanted side effects and allergic reactions it is desirable with other alternatives (Nguyen et al., 2004). Inhibition of XO may therefore be a therapeutic strategy to combat gout and other diseases associated with oxidative stress. In this *in vitro* assay, XO from cow's milk catalyzes the formation of uric acid and superoxide from the substrate hypoxanthine or xanthine, and the inhibition of XO can be measured spectrophotometrically around 290 nm.

1.5.2 **α-Glucosidase inhibitory activity**

Diabetes mellitus is a group of metabolic diseases characterized by increased concentration of glucose in the blood (hyperglycaemia) resulting from defects in insulin secretion, insulin action, or both. Chronic hyperglycaemia is associated with long-term damage, dysfunction, and failure of various organs, especially eyes, kidneys, nerves, heart, and blood vessels (American Diabetes Association, 2006). According to WHO, 347 million people worldwide have diabetes, with an estimated 3.4 million deaths every year. Type II diabetes comprises 90 % of the people with diabetes around the world, and is largely the result of excess body weight and physical inactivity. The WHO projects that diabetes will be the seventh leading cause of death globally in 2030, and the deaths will double between 2005 and 2030 (WHO, 2013a) .

One therapeutic approach to treat diabetes type II is to retard the absorption of glucose via inhibition of digestive enzymes such as α-glucosidase and α-amylase. α-Glucosidase is a membrane bound enzyme located in the small intestine and also the key enzyme that catalyzes the final step in the digestive process of carbohydrates. Hence, α-glucosidase facilitates the absorption of glucose by catalyzing the hydrolytic cleavage of non-absorbable

oligosaccharides (e.g. starch and sucrose) into absorbable monosaccharides in the small intestine (Kim et al., 2005; Kumar et al., 2011). Inhibitors of α-glucosidase could therefore retard liberation of glucose from complex dietary carbohydrates and delay glucose absorption, thus suppressing postprandial hyperglycaemia. Currently, four α-glucosidase inhibitors are used clinically to treat diabetes mellitus type II: acarbose, miglitol, voglibose and emiglitate. Of these, acarbose is by far the most prescribed drug (Van de Laar et al., 2005). Although being effective by providing overall control of postprandial increase in blood glucose without causing any hypoglycaemia with a significant effect on the weight control, they usually cause bothersome gastrointestinal (GI) side effects such as flatulence, abdominal distension and diarrhea (Kumar & Sinha, 2012). These side effects, along with a stringent repetitive dosing regime at specified time intervals, lead to decreased patient compliance. More effective and safer α-glucosidase inhibitors are therefore desirable, and many efforts have been made to identify natural α-glucosidase inhibitors from plants as they may be lead compounds for treatment of diabetes (Gao et al., 2007; Kumar et al., 2011; Yoshikawa et al., 2002).

1.5.3 **Antimalarial activity**

Malaria is an infectious disease caused by *Plasmodium* parasites and is transmitted to humans via the bite of infected female *Anopheles* mosquitoes. According to the WHO, 3.4 billion people were at risk of being infected with malaria in 2012 (WHO, 2013b). Every year, about 250 million people are affected by malaria resulting in 1 million deaths. Most vulnerable are children under the age of five and pregnant women. Five *Plasmodium* parasite species can cause malaria in humans, with *Plasmodium falciparum* being responsible for most of the fatal cases. Due to the development of resistance towards existing treatment regimens, there is an urgent need for new antimalarial drugs or alternative strategies with the final aim of reducing the burden of this disease (Mimche et al., 2011; WHO, 2010).

Cerebral malaria (CM) is the most severe neurological complication of infection with *P. falciparum* malaria. The term ‘cerebral malaria’ implies the presence of neurological features, especially impaired consciousness. Hence, the clinical hallmark of CM is coma (Golenser et al., 2006; Idro et al., 2010). Mortality is high (15-20 %) and some surviving patients sustain brain injury that manifest as long-term neurocognitive impairments such as

agitation, psychosis, cerebellar ataxia, aphasia, seizures and impaired consciousness (from confusion to deep coma) (Mishra & Newton, 2009). The mechanisms behind these injuries and the pathogenesis of CM are incompletely understood and consequently a major hindrance to progress in CM research. Treatment of neurological complications is warranted, but trials conducted to date have not shown to improve the overall outcome of severe *P. falciparum* malaria. Combinations of adjuvant therapies (in addition to anti-parasitic drugs such as quinine or artemisinins) may be needed to improve the neurocognitive outcome (Idro et al., 2010; Mishra & Newton, 2009). It has therefore been suggested that the ultimate treatment of CM could possibly be a combination of an antimalarial and an immunomodulator as CM is considered to be an immunopathological event (Golenser et al., 2006; Waknine-Grinberg et al., 2010). A plant-based immunomodulator displaying dual antimalarial and immunomodulatory mechanisms of action could therefore become ideal candidates for antimalarial drug development.

1.5.4 **Toxicity**

Despite the extensive use of phytomedicines/traditional medicines in Mali (see section 1.2) and other developing countries, very little is known about their chemical and biological qualities which also include the toxicity. While a few structured systematic analyses on beliefs about toxicity among traditional practitioners have been done (e.g. (Maiga et al., 2005)), this is often not feasible due to lack of knowledge, infrastructure and resources among other factors. Simple preliminary toxicity studies of the plants used in traditional medicine is therefore an easy approach to obtain vital information to provide safe medicines to the Malian population.

One simple and widely used test for toxicity is the “brine shrimp assay”. Larvae of the brine shrimp *Artemia salina* are incubated with test substance solutions or solvent only, and the percentage of dead larvae after a predefined time period is registered. This test has been in use for more than thirty years (Meyer et al., 1982).

2. Aims of the Thesis

The ultimate goal for the Malian research project, of which this work is a part, is to provide efficient, non-toxic, available and affordable medicines to the population in Mali. The plants chosen for this thesis are *Terminalia macroptera* and *Biophytum umbraculum*. These two plants have been used in Mali in the treatment of a variety of diseases for long traditions, although the knowledge of their chemistry, toxicity and biological activities remains nearly unknown. It is therefore highly relevant to examine these features to ensure safe, efficient and cheap alternative medicinal products to the Malian people.

The specific objectives of the study were:

1. To perform an ethnopharmacological survey in order to obtain information on the traditional use of *T. macroptera* (Paper I).

2. To isolate and characterize the chemical constituents of the Malian traditional medicinal plants, *T. macroptera* and *B. umbraculum* (Paper II and IV)

3. To evaluate the *in vitro* antioxidant effects and other biological activities of *T. macroptera* and *B. umbraculum* extracts and their constituents (Paper II, III and IV).

4. To investigate possible antimalarial and immunomodulating activities of *B. umbraculum* extracts and constituents (Paper V).

3. Summary of Papers

Paper I. *Terminalia macroptera*, its current medicinal use and future perspectives

The aim of this paper was to identify the traditional use of the medicinal tree Terminalia macroptera. An ethnopharmacological investigation was carried out in the regions around Siby, Dogonland and Dioïla, Mali. The majority of the 78 traditional practitioners interviewed (86%) used T. macroptera in their practice, but the frequency of use varied between areas and between practitioners. More than 30 medical indications were reported. The most cited indications were sores and wounds, pain, cough, tuberculosis and hepatitis. The fidelity level among the traditional practitioners from the same area was calculated to compare results from different areas where the survey was performed. There was a high agreement among the traditional practitioners about the most frequently reported uses, 68% for the use against hepatitis in Dogonland, 38% for wounds in Siby and 28% for pain and rheumatism in Dioïla. The most commonly used tree parts were the stem bark and the roots, although all plant parts, even the parasitic Loranthus species growing on the tree, were employed. Most of the remedies were prepared as a decoction of the fresh tree material.

Paper II. DPPH radical scavenging and xanthine oxidase inhibitory activity of *Terminalia macroptera* leaves

The main objective of this study was to isolate and characterize the chemical composition of the leaves of *T. macroptera*. *Cis*-polyisoprene was the major non-polar constituent isolated from the DCM extract. The novel compound chebulic acid trimethyl ester, as well as the previously known ellagitannins corilagin, chebulagic acid, chebulinic acid, the flavonol-*O*-glycosides rutin and narcissin, methyl gallate and shikimic acid were identified in the MeOH

crude extract. However, it is likely that chebulic acid trimethyl ester and methyl gallate are artifacts of chebulic acid and gallic acid, respectively. The DPPH radical scavenging and XO inhibiting effects of the plant extracts and its isolated compounds were also investigated. The MeOH crude extract was found to be a very potent DPPH radical scavenger and a moderate XO inhibitor (IC_{50} = 6.2 ± 0.4 and 52 ± 5 μg/mL, respectively), and these activities are probably due to the high content of ellagitannins. Chebulagic acid and corilagin showed very high radical scavenging activity (IC_{50} = 3.5 ± 0.1 and 4.3 ± 0.5 μM, respectively), while rutin and chebulagic acid were the most potent compounds towards XO. The antioxidant and radical scavenging properties of some of the substances identified in this study may contribute to explain the medical use of this tree in West Africa.

Paper III. α-Glucosidase inhibition, 15-lipoxygenase inhibition, and brine shrimp toxicity of extracts and isolated compounds from *Terminalia macroptera* leaves

This paper is a continuation of the work on *T. macroptera*, and the α-glucosidase- and 15-LO inhibiting effects as well as the toxicity of the plant extracts and its isolated compounds were investigated. In the enzyme inhibiting assays, all extracts showed high activity. The EtOAc extract showed strongest inhibition of both α-glucosidase and 15-LO, with IC_{50} values of 0.40 ± 0.02 and 23.2 ± 0.5 μg/mL, respectively. Among the isolated compounds, the ellagitannin chebulagic acid was the strongest inhibitor of both enzymes, with an IC_{50} value of 0.05 μM towards α-glucosidase and 24.9 ± 0.4 μM towards 15-LO, which indicate that it is much more potent than the positive controls (acarbose: IC_{50} 201 ± 28 μM towards α-glucosidase, quercetin: 93 ± 3 μM towards 15-LO). Although chebulagic acid may be the strongest inhibitor, the ellagitannin corilagin is present in much higher amounts (more than 25 times, calculated from yield of substances) and may therefore be the most important contributor to the enzyme inhibitory activity of the extract. In addition, a simple brine shrimp toxicity test was carried out, and none of the extracts or the isolated compounds seemed to be toxic compared to the positive control podophyllotoxin. These results may substantiate

the fact that some traditional practitioners use *T. macroptera* leaves for the prevention or treatment of diabetes mellitus.

Paper IV. Bioactive flavone-*C*-glucosides of the African medicinal plant *Biophytum umbraculum*

The aim of this study was to explore the chemistry and to evaluate the biological activities of the plant *Biophytum umbraculum*. Fractionation and purification of components from extracts of the aerial parts of *B. umbraculum* followed by structural characterization by NMR resulted in the identification of three flavone-*C*-glycosides; cassiaoccidentalin A, isovitexin and isoorientin. Flavone-*C*-glycosides have not previously been reported in *B. umbraculum*. Whereas isovitexin and isoorientin are fairly common flavone glucosides, cassiaoccidentalin A is a very rare natural compound, only identified once before. Bioactivities were measured by DPPH radical scavenging and inhibition of the enzymes XO and 15-LO. The results of the antioxidant assays show higher activities in the EtOAc extract. This may be related to the bioactive flavone-*C*-glycosides isolated from this extract, especially isoorientin, as its structure is in good accord with previous structure-activity studies. Additional unidentified compounds may, however, be responsible for the XO inhibition. Thus, it was concluded that *B. umbraculum* might be a valuable source of flavone *C*-glycosides.

Paper V. *Biophytum umbraculum* as adjuvant treatment of cerebral malaria

In this paper, the antimalarial and the immunomodulating activities of *B. umbraculum* extracts and isolated compounds were investigated. Our ethnopharmacological investigations in Mali revealed that more than 50 % of the traditional practitioners used *B. umbraculum* against cerebral malaria. Two CM-related *in vitro* assays were chosen to assess the possible

immunomodulating activity. The plant extracts showed a complement inhibiting activity as well as dose-dependent inhibition of NO release from activated macrophages. The EtOAc extract was the most active in both assays. The EtOAc extract showed moderate antimalarial activity against the erythrocyte stages of *Plasmodium falciparum* strain NF54 and K1 (mean IC_{50} = 6.7 µg/mL and 5.6 µg/mL, respectively). Our results are comparable to the results reported for *Artemisia annua* (IC_{50} = 6.7 µg/mL), which is the source of artemisinins, the clinically used antimalarial drug. However, the isolated flavone-*C*-glycosides were inactive in all of the assays. The nature of the active compounds is thus so far unknown, and although the results are interesting, further investigations and clinical studies are needed before one can conclude whether *B. umbraculum* should be used as adjuvant treatment of cerebral malaria or not.

4. Results and Discussion

4.1 Ethnopharmacological research

Ethnopharmacology as a specifically designated field of research has a relatively short history, and the term was first used in 1967 in a book about hallucinogens. Nowadays, the term is more broadly defined by the statement "The observation, identification, description and experimental investigation of the ingredients and the effects of the ingredients and the effects of such indigenous drugs is a truly interdisciplinary field of research which is very important in the study of traditional medicine. Ethnopharmacology is here defined as the **interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man**" (Heinrich & Gibbons, 2001). Seeing that as much as 75 % of the Malian population relies on traditional medicine, it is highly important to do ethnopharmacological research to improve this type of medicines, in order to give a better life to the local people. This is particularly vital in remote regions where the availability of conventional medicine is limited because of poverty. Although the use of traditional medicine is well established and has very long traditions, only a small portion of information about these medicines has been documented scientifically. One of the reasons could be that since most of the people in Mali are illiterate, most of the information is not written down, but has been passed on orally for generations. And because this information is often held by the elderly, there is an urgent need to obtain and document valuable information on useful medicinal plants. In the aspect of bioprospecting and drug discovery it is also worth mentioning that many important drugs on the market today originate from plants with a long history of ethnopharmacological use. Examples include opiates, quinine, artemisinin and digitalis glycosides (DeSmet, 1997; Fabricant & Farnsworth, 2001; Heinrich & Gibbons, 2001).

In Paper I we investigated the traditional use of *Terminalia macroptera* in three different areas in Mali (Siby, Dioïla and Dogonland). We found that the traditional practitioners usually use a hot water decoction of the stem bark or the root. The plant was in most cases

administered orally or used for washing the body or the infected area. 86 % of the 78 practitioners interviewed used the plant to cover over 30 indications. The most frequent indications are wounds, infections, pain, cough, tuberculosis and hepatitis. Two different surveys performed during the years 1998-2002 reported *T. macroptera* as a wound healing remedy in the Bamako region (Diallo et al., 2002) and in the district of Bandiagara (Inngjerdingen et al., 2004) of Mali. In various surveys carried out in Africa (mainly West Africa), it has been reported that this plant is used to treat peptic ulcer, malaria, liver disorders, cough and various GI disorders (see section 1.3.1). It seems to be some extent of consensus between the indications covered in our survey and the indications reported in other West African countries. The use of the plant for wound healing seems widespread, so the plant might be antibacterial and perhaps astringent. This could be related to the presence of ellagitannins (see section 1.4.2.1).

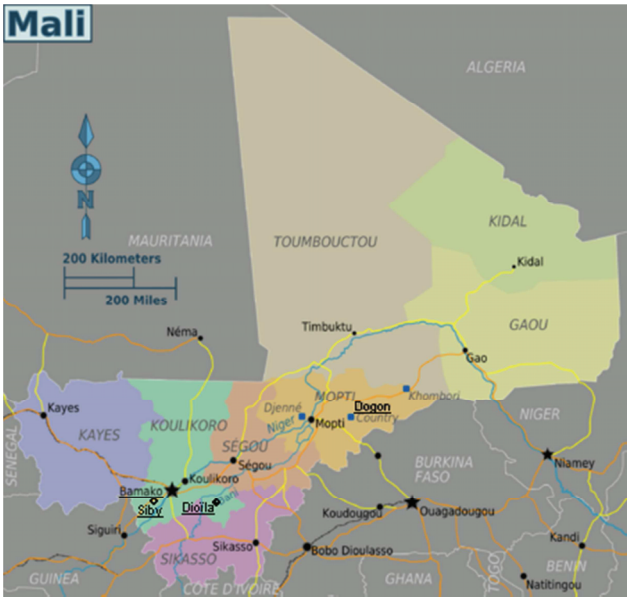

Figure 4.1: Map of Mali. The places where the ethnopharmacological surveys were carried out are underlined (Fitzgerald, 2013).

The traditional use of *Biophytum umbraculum* was investigated in Bamako, Siby and Dioïla (Paper V). Most of the practitioners (90 %) used the plant in their practice, and 50 % of the 34 interviewed practitioners used *B. umbraculum* against cerebral malaria. Other common

indications were various GI disorders. The aerial parts of the herb are usually prepared as a powder preparation or hot water decoction. Patients suffering from cerebral malaria are often in poor general condition (e.g. convulsions, seizures, and coma) which make it difficult to take the medicine orally, this could explain why the plant is usually prepared as a powder preparation for topical administration. These findings were similar to the results from the survey conducted by Grønhaug et al. (2008) where cerebral malaria was the main indication for *B. umbraculum* (58 %), while different types of pain and wounds were less common indications. In the survey carried out in the Bamako region by Diallo et al. (2002), *B. umbraculum* was identified as a wound healing plant. A limited survey conducted in Sikasso, Dioïla, Kolokani and Blendio, Mali, revealed that the plant was used against malaria, wounds and stomachache (Inngjerdingen et al., 2006). The traditional practitioners' consensus for the main indication is fairly high, and this supports the traditional use of *B. umbraculum* as a remedy to treat cerebral malaria.

Although being used as traditional medicines by many practitioners, these plants have undergone very limited chemical and/or pharmacological investigations. And the fact that traditional practitioners from regions that differ in geographic and ethnographic characteristics report the same plants for the treatment of the similar diseases further supports the medicinal use of these plants. It is therefore of great interest and relevance to study the structure and biological activities of these plants. Polar and semipolar substances of low molecular weight like tannins, flavonoids and other polyphenols were of particular interest as high molecular weight substances like polysaccharides have already been examined for e.g. wound healing properties and immunomodulating properties.

4.2 Extraction and isolation procedures

The studied plants were obtained fresh and then air-dried, milled and pulverized at DMT, Mali before shipping to Oslo, Norway. In general, the dried plant material was initially extracted with dichloromethane (DCM) followed by methanol (MeOH) in a Soxhlet apparatus or by maceration at room temperature (RT). The obtained crude extracts were then

extracted successively with solvents of increasing polarity (Figure 4.2). A rough separation of constituents with varying polarity is obtained with this procedure. Size exclusion chromatography (SEC) was the principle utilized to separate low and high molecular weight constituents. Sephadex LH-20 was chosen due to its high selectivity and adsorption effects for aromatic compounds, since most of the compounds studied in this work were of that kind. However, a drawback with this property is that it can lead to potential failure of size-based separation. Nevertheless, SEC is a low resolution chromatography as it does not discern similar species very well, so the chromatographic techniques chosen for further fractionation and purification was mainly based on polarity, pattern in NMR spectra and weight of the fractions. Reverse phase (RP) and normal phase (NP) silica gel were primarily used to separate compounds with different polarity. Additionally, preparative high performance liquid chromatography (HPLC) with a RP C-18 column was an effective method to obtain pure compounds. The pure compounds were obtained by collection of the peaks detected by a UV-detector. After a chromatographic run, the collected fractions were combined as indicated by thin layer chromatography (TLC). Spots were visualized by ultraviolet (UV) irradiation (254 nm and 366 nm), by spraying with $Ce(SO_4)_2$ in aqueous sulfuric acid followed by heating to detect organic compounds, or by spraying with methanolic DPPH radical solution to spot radical scavenging activity. ^{1}H-NMR and ^{13}C-NMR spectroscopy was regularly carried out to get an indication of the chemical composition of the extracts and the purified fractions.

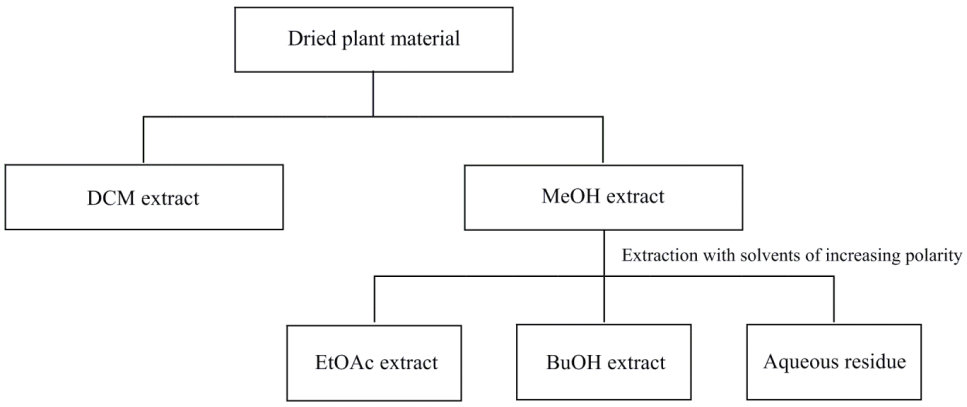

Figure 4.2: Illustration of the general extraction procedure.

Due to lack of material for biological studies, a second batch of *B. umbraculum* (Paper IV and V) was subjected to Soxhlet extraction to yield DCM and MeOH crude extracts, and subsequent ethyl acetate (EtOAc), butanol (BuOH) and aqueous fractions of the MeOH crude extract. Ideally, the second batch should have been extracted at RT as done with the first batch where the pure compounds originate from, since the Soxhlet system could cause thermal degradation of compounds. However, the NMR spectra were compared and no noteworthy dissimilarities were found.

4.3 Chemical characterization

T. macroptera leaves were found to contain a variety of ellagitannins and flavonoids (Paper II). Totally, nine compounds were isolated and identified; three ellagitannins (corilagin, chebulagic acid and chebulinic acid), two flavonol-*O*-glycosides (rutin and narcissin), rubber (poly-*cis*-isoprene), chebulagic acid trimethyl ester, methyl gallate and shikimic acid (Figure 4.3). Their structures were elucidated on the basis of NMR spectroscopy data. Optical rotation was also recorded as part of the characterization of novel compounds. Chebulic acid trimethyl ester was identified as a new chemical compound. Corilagin was found to be the main compound in the BuOH extract, and its NMR resonances were clearly visible in the ^{1}H-NMR spectrum of both the MeOH as well as the BuOH extract. Corilagin is a fairly rare ellagitannin which has obtained its name from the tree *Caesalpinia coriaria* (Leguminosae) (Schmidt & Lademann, 1951). The pods of this tree are a very good source of tannins (Pérez-Tello & Quintana-Hernéndez, 1995). Corilagin has, as far as we know, not been reported in *T. macroptera* previously, but has formerly been isolated from other *Terminalia* species. The other two ellagitannins, chebulagic acid and chebulinic acid, are also found in other *Terminalia* species (Pfundstein et al., 2010). The flavonol-*O*-glycosides rutin and narcissin have not previously been reported from the leaves of *T. macroptera*, but have previously been found in the flowers (Nongonierma et al., 1990). Narcissin is quite rare as compared with the very common flavonoid rutin. The biosynthetic precursor for flavonoids and hydrolyzable tannins, shikimic acid, was identified by comparison with published NMR data, as was methyl gallate, which is the methyl ester of gallic acid and possibly an artifact formed during extraction.

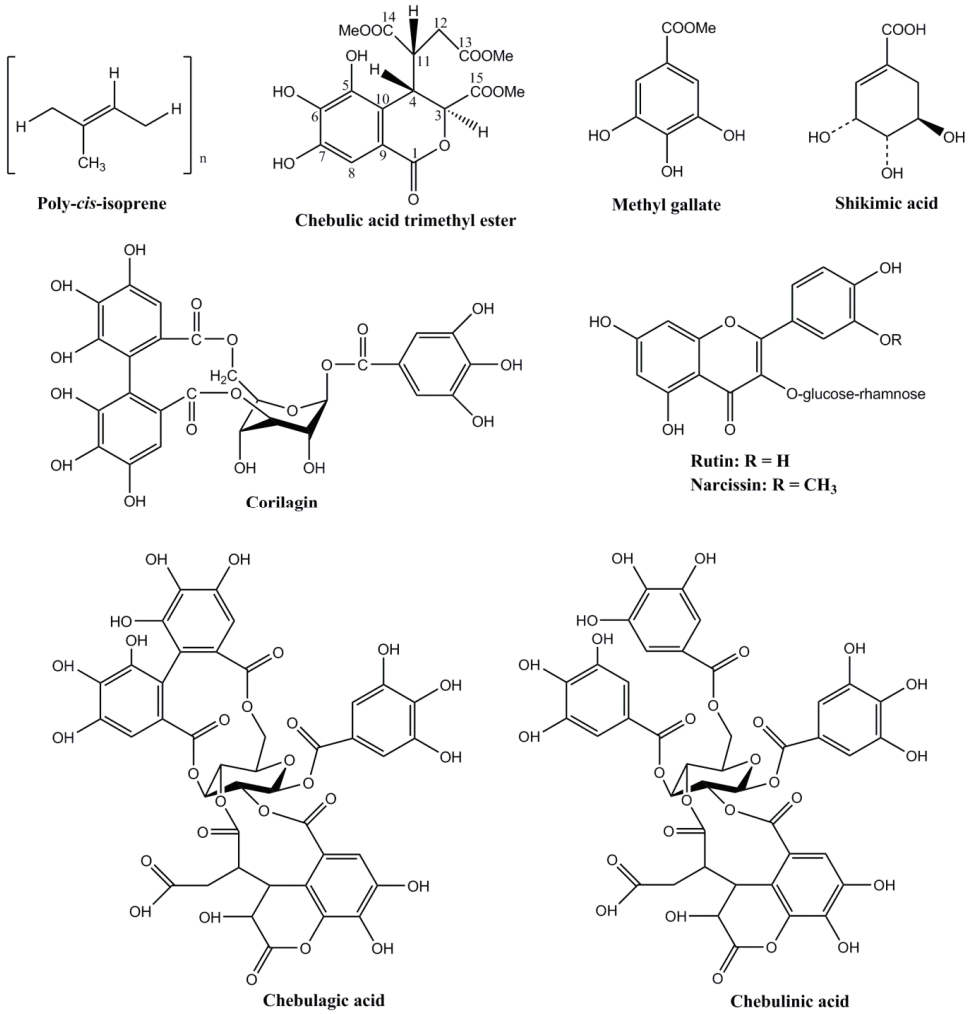

Figure 4.3: Chemical structures of the isolated compounds from *Terminalia macroptera*.

The NMR spectral data of chebulic acid trimethyl ester indicated a compound with three carboxyl groups, a carbonyl, three methyl esters and an aromatic ring with one proton and three hydroxyl groups. Assignments were confirmed by 2D-NMR techniques (COSY, HSQC and HMBC). Comparison with published data for chebulic acid (Ding et al., 2000; Lee et al., 2007) and chebulic acid triethyl ester (Yang et al., 2008) led to the identification of chebulic acid trimethyl ester. The main differences in the ^{1}H NMR spectra were the three singlets (3.54 – 3.68 ppm) for the three methoxyl groups of chebulic trimethyl ester lacking in the NMR spectrum of chebulic acid, whilst in the spectrum of chebulic acid triethyl ester,

Table 4.1: Comparison of ^{1}H NMR data of chebulic acid trimethyl ester with published data

Carbon No.	Chebulic acid trimethyl ester (300 MHz, $(CD_3)_2CO$)[a]	Chebulic acid triethyl ester (Yang et al. 2008)	Chebulic acid (Ding et al. 2000)
	δ_H (*J*, Hz)	δ_H (*J*, Hz)	δ_H (*J*, Hz)
1	-	-	-
2	-	-	-
3	5.27 (1H, d, 1.2)	5.23 (1H, d, 0.9)	5.28 (1H, d, 0.8)
4	3.90 (1H, dd, 1.2, 7.9)	3.88 (1H, dd, 0.9, 8.4)	3.90 (1H, dd, 0.8, 8.4)
5	-	-	-
6	-	-	-
7	-	-	-
8	7.12 (1H, s)	7.11 (1H, s)	7.09 (1H, s)
9	-	-	-
10	-	-	-
11	3.25 (1H, ddd, 4.8, 8.0, 10.0)	3.21 (1H, ddd, 4.8, 8.4, 9.9)	3.15 (1H, ddd, 4.4, 8.4, 10.4)
12a	2.87 (1H, dd, 10.0, 16.9)	2.86 (1H, dd, 9.9, 16.8)	2.90 (1H, dd, 10.4, 17.2)
12b	2.52 (1H, dd, 4.7, 16.9)	2.47 (1H, dd, 4.8, 16.8)	2.40 (1H, dd, 17.2, 4.4)
13	-	-	-
14	-	-	-
15	-	-	-
13 OCH_3	3.54 (3H, s)	[b]	[b]
14 OCH_3	3.68 (3H, s)	[b]	[b]
15 OCH_3	3.60 (3H, s)	[b]	[b]

[a] Assignments were confirmed by COSY, HSQC and HMBC experiments.

[b] Not applicable

occurred as quartets (3.97 – 4.17 ppm) and triplets (1.05 – 1.23 ppm) for the CH_2 and CH_3 in the ethyl groups, respectively, were observed (Table 4.1 and 4.2). Although being a novel compound not previously reported in scientific literature, it is likely that chebulic acid trimethyl ester is an artifact formed by esterification between methanol and COOH groups of chebulic acid during extraction. Analogously, *Terminalia chebula* extraction with ethanol yielded chebulic acid triethyl ester (Yang et al., 2008). Additionally, chebulic acid has also been reported from *T. chebula* (Lee et al., 2007). Ellagitannins with a $^{1}C_4$-glucopyranose core and a unique chebuloyl group, such as chebulagic acid and chebulinic acid, and their co-occurrence with punicalagin (a fairly rare tannin reported in *T. macroptera* (Silva et al., 2000)) and punicalin is a chemotaxonomic feature of the *Terminalia* species (Yoshida et al., 2010). These so-called chebulic ellagitannins belong to a relatively small group of metabolites of the hexahydroxydiphenoyl ester class in which one of the aromatic rings has undergone hydrolytic cleavage to generate one or more additional carboxylate groups (Figure 4.4) (Haslam, 1998; Pfundstein et al., 2010). Strictly speaking, chebulinic acid is a

Table 4.2: Comparison of ^{13}C NMR data of chebulic acid trimethyl ester with published data

Carbon No.	Chebulic acid trimethyl ester (300 MHz, $(CD_3)_2CO$)[a]	Chebulic acid (Gang et al. 2001)	Chebulic acid (Lee et al. 2007)
	δ_C	δ_C	δ_C
1	163.87	166.87	163.3
2	-	-	-
3	77.69	78.76	76.4
4	36.68	37.33	35.6
5	143.29	143.98	142.7
6	139.00	140.58	138.7
7	145.99	146.66	145.3
8	108.99	109.33	107.6
9	116.95	116.29	114.8
10	117.07	118.12	116.6
11	44.76	45.49	43.7
12	34.55	35.23	34.2
13	172.39	175.55	172.9
14	173.97	176.97	174.4
15	170.57	172.84	170.9
13 OCH_3	51.85	[b]	[b]
14 OCH_3	52.46	[b]	[b]
15 OCH_3	53.00	[b]	[b]

[a] Assignments were confirmed by COSY, HSQC and HMBC experiments.

[b] Not applicable

gallotannin, although it is closely associated with the ellagitannins (and chebulagic acid, which may be characterized as dehydrochebulinic acid) (Haworth, 1961), and is nowadays regarded as a chebulic ellagitannin (Pfundstein et al., 2010).

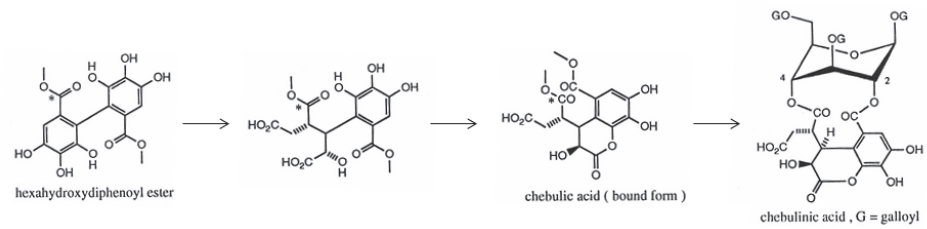

Figure 4.4: The putative biogenetic pathway to "ring opened" HHDP ester; chebulic acid, a component of chebulinic acid (Haslam, 1998) and chebulagic acid.

During the chemical characterization of *Biophytum umbraculum*, the herb was found to contain high amounts of flavonoids (Paper IV). The three flavone-*C*-glycosides

cassiaoccidentalin A, isovitexin and isoorientin were isolated from the EtOAc extract of *B. umbraculum* (Figure 4.5). Their structures were established on the basis of spectroscopic data. Cassiaoccidentalin A turned out to be the main compound in the EtOAc extract, and traces of it are also seen in the BuOH extract, as evidenced by NMR. Cassiaoccidentalin A is a very rare flavone glycoside which has only been identified once before, in *Cassia occidentalis* (Hatano et al., 1999). Interestingly, *C. occidentalis* happens to be the principal plant accounting for 62 % (together with 32 % *Lippia chevalierii* and 6 % *Spilanthes oleracea*) of the phytoremedy Malarial (Figure 4.6), one of the ITMs developed by the DMT for the treatment of malaria and recognized as an essential and effective medicine in Mali (Diallo & Paulsen, 2000). It is therefore highly relevant so see if cassiaoccidentalin A could be one of the components contributing to the antimalarial activity of Malarial. The flavone glucosides isovitexin and isoorientin are more common and have previously been found in another *Biophytum* species, *Biophytum sensitivum* (Bharati & Sahu, 2012).

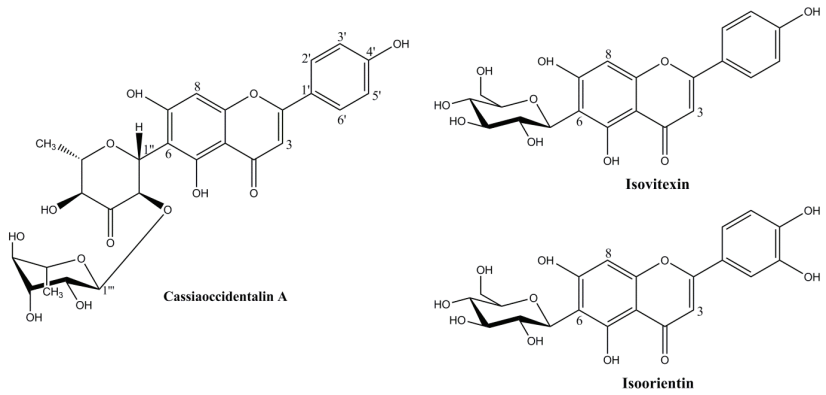

Figure 4.5: Chemical structures of the flavone-*C*-glycosides isolated from *Biophytum umbraculum*.

Figure 4.6: The improved traditional medicine (ITM) Malarial (Photo: A.T. Pham).

4.4 Biological activities

4.4.1 Antioxidant activity

The antioxidant activity of the plant extracts and isolated compounds was evaluated in Paper II, III and IV. Three different methods were employed because one single method is not sufficient to evaluate the total antioxidant activity due to the complexity of phytochemicals as well as of oxidative processes (Wangensteen et al., 2004); scavenging of DPPH radical, inhibition of 15-LO and inhibition of XO. The results are summarized in Table 4.3 and 4.4.

It was observed that the lipophilic crude extracts were generally inactive as antioxidants, in contrast to the more polar extracts which possessed high or moderate antioxidant activities. In all three bioassays, the EtOAc extracts were, in particular, the most potent of all the extracts, whereas the aqueous residues were the least potent. This might suggest that ethyl acetate has a higher selectivity for phenolics and antioxidant components. Chyau et al. (2002) reported similar results from their work on *Terminalia catappa* leaves, as did Jayaprakasha et al. (2008) on their studies on extraction efficiency of different solvents on the antioxidant capacities of pummelo and navel oranges. Although the DCM extract of *T. macroptera* was not tested for XO and 15-LO inhibitory activity due to low solubility or precipitation in the test systems, it would probably be inactive as it was inactive in the DPPH radical scavenging assay (Table 4.3). Moreover, it was observed that the number of OH groups is important for radical scavenging activity. Consequently, monophenolic compounds were generally weaker as antioxidants compared to polyphenols. In Paper II, the DPPH radical scavenging activity of the flavonol glycosides rutin and narcissin (IC_{50} values 22 ± 2 μM and > 83 μM, respectively) were studied. The lower IC_{50} value of rutin indicates that the 3',4'-dihydroxy structural element is important for radical scavenging activity. The same phenomenon was seen with the flavone-*C*-glycosides isovitexin and isoorientin (IC_{50} values 96 ± 3.6 μM and 18.1 ± 1.1 μM, respectively) in Paper IV. Our results are consistent with previous reports on antioxidant activity of flavonoids being dependent on the number and position of substituted OH groups. (Farkas et al., 2004; Heim et al., 2002; Selloum et al., 2001).

In Paper III and IV, the inhibitory potency of the plant extracts and its isolated constituents towards peroxidation of linoleic acid catalysed by soybean 15-LO was studied. Extracts of both *T. macroptera* and *B. umbraculum* were active as inhibitors of 15-LO, but extracts of *T. macroptera* showed generally higher activity, on the same order as the positive control, quercetin (IC_{50} = 33.4 ± 0.3 µg/mL). The high content of ellagitannins and flavonoids may explain the results, seeing that some of these showed considerably higher effects than the positive control, as was the case with chebulagic acid, corilagin and narcissin. Interestingly, in our results, narcissin was more potent than rutin in 15-LO inhibition, as opposed to the results by Robak et al. (1988), who reported narcissin to be inactive. The deviating results may be due to differences in the experimental setup or to different enzyme sources. The reversed activity of rutin and narcissin in the DPPH versus the 15-LO assays might seem unexpected. It has, however, been shown that DPPH scavenging and 15-LO inhibition are not necessarily correlated (Malterud et al., 1993).

The apparent 15-LO inhibitory activity of narcissin is probably explained by other factors than the C2-C3 double bond in conjunction with a 4-oxo function in the C-ring alone. This reversal of activity was not seen in the case of isovitexin and isoorientin, where isoorientin was a stronger antioxidant in all of the three antioxidant tests.

The inhibitory effects towards the superoxide-producing enzyme XO from cow's milk were investigated. *T. macroptera* crude extract was moderately active as XO inhibitor (Paper II). Among the isolated compounds, only rutin and chebulagic acid possessed enzyme inhibitory activity, while the remaining compounds were inactive at the highest measured concentration. However, all samples showed lower activities than the reference compound, quercetin. Rutin has previously been reported to be a XO inhibitor (Choi et al., 2002), in good accord with our findings. *B. umbraculum* showed moderate activity towards XO (Paper IV). This activity cannot be attributable to any of the isolated compounds, suggesting that the EtOAc extract may contain additional unidentified XO inhibitors. Among the isolated flavone-*C*-glycosides, isoorientin was the most potent antioxidant. Cotelle et al. (1996) have previously reported that flavones with both a 7-OH substitution and a catechol (or 3',4',5'-pyrogallol) function on the B-ring, are the most effective inhibitors of XO, and our findings seems to concur with the suggested theory. Due to lack of material, chebulinic acid was not tested for antioxidant activity at all.

Table 4.3: Antioxidant activity of *T. macroptera* and *B. umbraculum* extracts tested by DPPH radical scavenging, 15-LO inhibition and XO inhibition. IC_{50} values ± S.D. (in µg/mL) are shown.

Extract	DPPH	XO	15-LO
Terminalia macroptera:			
DCM crude extract	Inactive	[a]	[a]
MeOH crude extract	6.2 ± 0.4	52 ± 5	27.9 ± 1.5
EtOAc extract	3.7 ± 0.2	26 ± 3	23.2 ± 0.5
BuOH extract	6.5 ± 0.4	64 ± 8	30.0 ± 2.3
Aqueous residue	12.4 ± 0.6	146 ± 15	[a]
Quercetin (positive control)	3.0 ± 0.2	0.6 ± 0.1	28.1 ± 0.8
Biophytum umbraculum:			
DCM crude extract	> 167	> 167	> 167
MeOH crude extract	13.4 ± 0.6	59.7 ± 6.1	68.9 ± 5.0
EtOAc extract	6.8 ± 0.6	ca. 21	43.0 ± 3.6
BuOH extract	12.5 ± 1.9	102.6 ± 5.6	53.4 ± 2.1
Aqueous residue	29.8 ± 3.5	> 167	> 167
Quercetin (positive control)	4.4 ± 0.4	2.33 ± 0.09	33.4 ± 0.3

[a] Not tested

Table 4.4: Antioxidant activity of isolated compounds from *T. macroptera* and *B. umbraculum* tested by DPPH radical scavenging, 15-LO inhibition and XO inhibition. IC_{50} values ± S.D. (in µM) are shown.

Compound	DPPH	XO	15-LO
Terminalia macroptera:			
Chebulic acid trimethyl ester	11.8 ± 1.0	> 419	85.3 ± 1.2
Methyl gallate	12.1 ± 0.4	> 83	96 ± 5
Shikimic acid	529 ± 46	> 958	> 957
Corilagin	4.3 ± 0.5	> 83	41 ± 4
Rutin	22 ± 2	ca. 40	97 ± 7
Narcissin	> 83	> 83	45 ± 2
Chebulagic acid	3.5 ± 0.1	53 ± 8	24.9 ± 0.4
Chebulinic acid	[a]	[a]	[a]
Quercetin (positive control)	9.4 ± 0.6	1.9 ± 0.4	93 ± 3
Biophytum umbraculum:			
Cassiaoccidentalin A	> 167	149.5 ± 7.4	99.9 ± 2.5
Isovitexin	96.0 ± 3.6	> 167	107.1 ± 2.1
Isoorientin	18.1 ± 1.1	117.2 ± 13.5	86.4 ± 0.5
Quercetin (positive control)	13.7 ± 1.3	7.7 ± 0.3	110.6 ± 1.0

[a] Not tested

4.4.2 **α-Glucosidase inhibitory activity**

In Paper III, *Terminalia macroptera* extracts and components were assessed for their abilities to inhibit α-glucosidase from yeast, since diabetes was one of the indications mentioned in our ethnopharmacological survey carried out previously (Paper I). All the tested extracts were strong inhibitors of α-glucosidase, more than 250 times stronger than the positive control acarbose, which is a widely used antidiabetic drug that is used clinically today. The lipophilic DCM extract was not tested due to low solubility. The observed α-glucosidase inhibitory activity may be ascribed to the high content of polyphenols, which have been shown to inhibit the activities of digestive enzymes due to their ability to bind to proteins (McDougall & Stewart, 2005). Previous studies have shown that ellagitannins possess α-glucosidase inhibitory activity, and it has been proposed that an increasing number of galloyl units in the molecule might lead to an increase in inhibitory activity (Omar et al., 2012; Toda et al., 2000). However, it is unclear whether only the number of free galloyl groups is responsible for the activity, or that the position of HHDP or galloyl substitution might also affect the activity (Toda et al., 2000). Based on our results, we cannot draw any conclusions about the relevance of the galloyl- and HHDP groups for the inhibitory activity because only corilagin and chebulagic acid was tested, and both of them are comprised of one galloyl and one HHDP group. In chebulinic acid, the HDDP group has been replaced by two galloyl groups, and it has previously been reported that chebulinic acid is a slightly better inhibitor of α-glucosidase than chebulagic acid (Gao et al., 2007). Since chebulagic acid is 50 times more active than corilagin, the chebuloyl group would seem to be important for inhibition of this enzyme, while the relative importance of galloyl and HHDP groups remains unclear. It should be noted that inhibitory activity may vary widely between α-glucosidases from different sources (Gao et al., 2008). Further investigations are needed to follow this up. Although being less active, corilagin is present in much higher amounts and may therefore be more responsible for the very high inhibitory activity of the extract. Ryu et al. (2010) have reported that flavonoids have shown high α-glucosidase inhibitory activity, and structure-activity relationship studies by Tadera et al. (2006) revealed that the inhibitory activity of flavonoids increased considerably with an increase in the number of the hydroxyl groups on the B-ring, unsaturated C-ring, hydroxylation at the 3 and 5 positions of the flavone skeleton, 2,3-double bond and 4-CO in the C-ring. Rutin, which is the most potent flavonol glycoside in our studies, has many of the structural features mentioned above, and

that probably explains its high inhibitory activity towards α-glucosidase, in contrast to the less active narcissin, which lacks an OH group on the B-ring compared to rutin.

Even though *T. macroptera* seem to be a good potential source of highly active α-glucosidase inhibitors, additional *in vitro* and *in vivo* testing is needed, especially since yeast α-glucosidase is distinct from the small intestinal α-glucosidase. It would therefore be relevant to first test against rat small intestinal α-glucosidases and the closely related digestive enzyme α-amylase for inhibitory activity. Furthermore, the potential to reduce glucose concentration postprandial in patients with type II diabetes should be investigated in clinical studies.

4.4.3 Antimalarial activity

4.4.3.1 *In vitro* antiplasmodium assay

Extracts and purified compounds from *Biophytum umbraculum* were screened against two strains of *Plasmodium falciparum*, the chloroquine-sensitive strain NF54 and the multidrug resistant strain K1 (Paper V). Human erythrocytes served as host cells. The highest measured concentration was 10 µg/mL, since IC_{50} values above this concentration were considered inactive. Of the semipolar extracts, it was only the EtOAc extract which possessed a moderate antiplasmodial activity against both the NF54 and the K1 strains of *P. falciparum* (IC_{50} = 6.7 and 5.6 µg/mL, respectively). The IC_{50} values are comparable to the IC_{50} value (3.9 µg/mL) reported for the ethanolic extracts of *Artemisia annua* (Phillipson & Wright, 1991; Vonthron-Senecheau et al., 2003) and other active plant extracts (Abiodun et al., 2011; Bah et al., 2007; Irungu et al., 2007; Ramazani et al., 2010). These results are interesting and warrant further investigations, since traditional medicines have been used to treat malaria since ancient times, and are the source of two main groups (artemisinins and quinine derivatives, from *A. annua* and *Cinchona* back, respectively) of modern antimalarial drugs (Irungu et al., 2007). The lipophilic DCM extract showed activity against the K1 strain only (IC_{50} = 7.4 µg/mL). All the isolated flavone-*C*-glycosides, including cassiaoccidentalin A, were inactive towards both strains. The observed antiplasmodial activity of the EtOAc extract is therefore likely to come from other unidentified substances, or possibly by synergistic action.

Even though there have been some reports on the antiplasmodial activity of flavonoids (Andayi et al., 2006; Kraft et al., 2003), the observed effects are usually low compared to the positive controls which are often artemisinins or chloroquine. The most active antimalarial natural products are generally alkaloids, polyacetylenes, xanthones, quinones, polyketides or terpenoids (Nogueira & Lopes, 2011). Searches through scientific databases such as SciFinder and Web of Science did not show any of these classes of substances in *B. umbraculum* or in other *Biophytum* species.

There are several ways to measure the *in vitro* antimalarial activity. The method used is standard [$_3$H]hypoxanthine incorporation assay. It is the most commonly used method in well-equipped laboratories, and the advantage is high reproducibility. However, the handling of radioactive material is costly, hazardous and requires the availability of trained staff as well as appropriate technology. A potential alternative, especially in resource-limited environments, is the novel aldolase ELISA assay, which is highly reproducible, less hazardous (involves no radioactivity) and required little and cheap technical equipment. It is a userfriendly assay and can be operated by relatively unskilled personnel (Tritten et al., 2009).

4.4.3.2 Immunomodulating activity

In regard to the possible immunomodulating activities of *B. umbraculum*, the extracts and isolated compounds were tested in two immunological *in vitro* test systems (Paper V). The two systems focus on different parts of the immune system, the complement system and the macrophages, which both are parts of the innate immune system. Owing to the fact that cerebral malaria (CM) can be associated with a high complement activation and inflammation, complement inhibitory and anti-inflammatory agents may therefore be useful in the treatment of this disease (Goto & Sanchez, 2013; Mimche et al., 2011; Waknine-Grinberg et al., 2010).

All of the semipolar extracts were shown to be highly active complement inhibitors of the classical pathway, with the EtOAc extract as the most potent inhibitor (ICH_{50} = 5 µg/mL). It is twelve times more active than well-known complement inhibitor, rosmarinic acid (ICH_{50} = 64 µg/mL) (Sahu et al., 1999). Despite the high complement inhibition observed for the

EtOAc extract, the major isolated flavone-*C*-glycosides, isovitexin and isoorientin, exhibited weak complement activating properties. Previous studies have shown that flavone aglycones and flavone-*O*-glycosides have anti-complementary activity (Pieroni et al., 2000). Isovitexin and isoorientin account for only 2 % of the extract, and it is unlikely that they would have a high impact on the activity of the EtOAc extract. Possible synergistic effect between some of the components or other highly active, so far unidentified components with higher impact, may explain the complement inhibitory effects presented. The DCM extract displayed both inhibiting and activating properties indicating that it contains both activating and inhibiting constituents.

The EtOAc extract gave the highest inhibition of nitric oxide (NO) production in RAW 264.7 macrophages activated by lipopolysaccharide (LPS). The potency order of the extracts is the same as in the complement assay; EtOAc > BuOH > MeOH. And somewhat similarly, all of the isolated compounds were inactive as well, and therefore it is anticipated that other, so far unidentified, substances are responsible for the anti-inflammatory activity observed.

The complement inhibitory effects as well as the ability to inhibit NO production by activated macrophages may have the potential to lead to improved outcomes in CM. Although *in vitro* anti-inflammatory activity may not be clinically relevant *in vivo*, these findings merit additional research to see if *B. umbraculum* could be used in adjuvant therapy together with an antimalarial medicine to treat CM.

4.4.4 **Toxicity**

The brine shrimp lethality assay is a simple, effective and inexpensive method to screen pure compounds and plant extracts for potential toxic activity. In addition, it has the advantage over cytotoxicity assays of not requiring higher animal serum, and is faster and cheaper. In Paper III, tested compounds and extracts of *T. macroptera* showed low toxicity (LC_{50} > 200 µM for pure compounds and LC_{50} > 100 µg/mL for extracts) against brine shrimp larvae compared to the positive control, podophyllotoxin (which had 87 % lethality at 50 µg/mL). According to Conrad et al. (2001b), two other tannins, 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose and punicacortein C, isolated from *T. macroptera* bark were found to be cytotoxic

towards 5637 cells (human primary bladder carcinoma). The extracts were, however, not tested.

B. umbraculum extracts were tested for cytotoxic effects against RAW 264.7 cells using the MTS-assay in Paper V. The highest measured concentration was 50 µg/mL. The DCM extract showed cytoxicity (37 % cell viability compared to LPS control at T = 130 min) and was therefore not included in the NO-assay. Moreover, the highest EtOAc concentration showed some cytotoxicity (69 % cell viability at T = 130 min) as well, but not enough to explain the decrease in NO production. *B. umbraculum* has not been investigated for toxicity before, but previous studies on *B. sensitivum* have shown cytotoxicity and inhibition of NO production as well (Guruvayoorappan & Kuttan, 2007).

Maiga et al. (2005) conducted a survey of toxic plants on the market in the district of Bamako, Mali. A total of 106 traditional practitioners were interviewed and 19 plants were reckoned to be toxic. Neither *T. macroptera* nor *B. umbraculum* were among the possible toxic plants mentioned. Interestingly, the plants used to treat the toxic effects were plants known to contain tannins or belong to families containing tannins. Tannins in food may act as digestion inhibitors with the resultant suppression of uptake from the GI tract (Aganga & Mosase, 2001). *Guiera senegalensis*, which comes from the same family as *T. macroptera*, Combretaceae, is one of the most frequently used plants by the traditional practitioners for intoxication. *T. macroptera* should perhaps be investigated for possible detoxification effects due to its tannin content, as diarrhea, which is one of the common adverse signs of intoxication, is also one of the indications reported in previous ethnopharmacological studies of *T. macroptera*.

5. Conclusions

As modern health care services are not adequate for the majority of the Malian population, and most people have limited economic means to buy Western conventional medicine, traditional medicine prepared from plants remains the most popular medicine in solving health problems in Mali. Although being used by 75 % of the population, the knowledge of the plants' chemistry, toxicity and biological activities is close to unknown. This study provides a better understanding of two of the plants, *Terminalia macroptera* and *Biophytum umbraculum*, with the main focus on the phenolic compounds and the antioxidant potential, as the health benefits of antioxidants have acquired considerable interest in recent years. Most of the semipolar extracts displayed high antioxidant activity, and this activity is attributable to the presence of tannins and flavonoids. In addition to antioxidant activity, strong α-glucosidase inhibitory activity was seen for *T. macroptera*, and significant antimalarial and immunomodulating activities were observed for *B. umbraculum*. Even though the results are promising and may to some extent give a rationale for the traditional use, they are derived from *in vitro* experiments and need to be followed up with *in vivo* tests and clinical trials. This should be subjects for future research.

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Papers

Paper I **Anh Thu Pham, Christina Dvergsnes, Adiaratou Togola, Helle Wangensteen, Berit Smestad Paulsen, Drissa Diallo and Karl Egil Malterud**
Terminalia macroptera, its current medicinal use and future perspectives
Journal of Ethnopharmacology **2011**, *137*, 1486-1491

Paper II **Anh Thu Pham, Karl Egil Malterud, Berit Smestad Paulsen, Drissa Diallo and Helle Wangensteen**
DPPH Radical scavenging and xanthine oxidase inhibitory activity of *Terminalia macroptera* leaves
Natural Product Communications **2011**, *6*, 1125-1128

Paper III **Anh Thu Pham, Karl Egil Malterud, Berit Smestad Paulsen, Drissa Diallo and Helle Wangensteen**
α-Glucosidase inhibition, 15-lipoxygenase inhibition and brine shrimp toxicity of extracts and isolated compounds from *Terminalia macroptera* leaves
Pharmaceutical Biology **2014**, *52*, 1166-1169

Paper IV **Anh Thu Pham, Celine Nguyen, Karl Egil Malterud, Drissa Diallo and Helle Wangensteen**
Bioactive flavone-*C*-glycosides of the African medicinal plant *Biophytum umbraculum*
Molecules **2013**, *18*, 10312-10319, doi: 10.3390/molecules180910312

Paper V **Ingvild Austarheim, Anh Thu Pham, Celine Nguyen, Yuan-Feng Zou, Sibylle Sax, Sergio Wittlin, Karl Egil Malterud, Drissa Diallo and Helle Wangensteen**
The Malian medicinal plant *Biophytum umbraculum* as adjuvant treatment of cerebral malaria
Manuscript

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II

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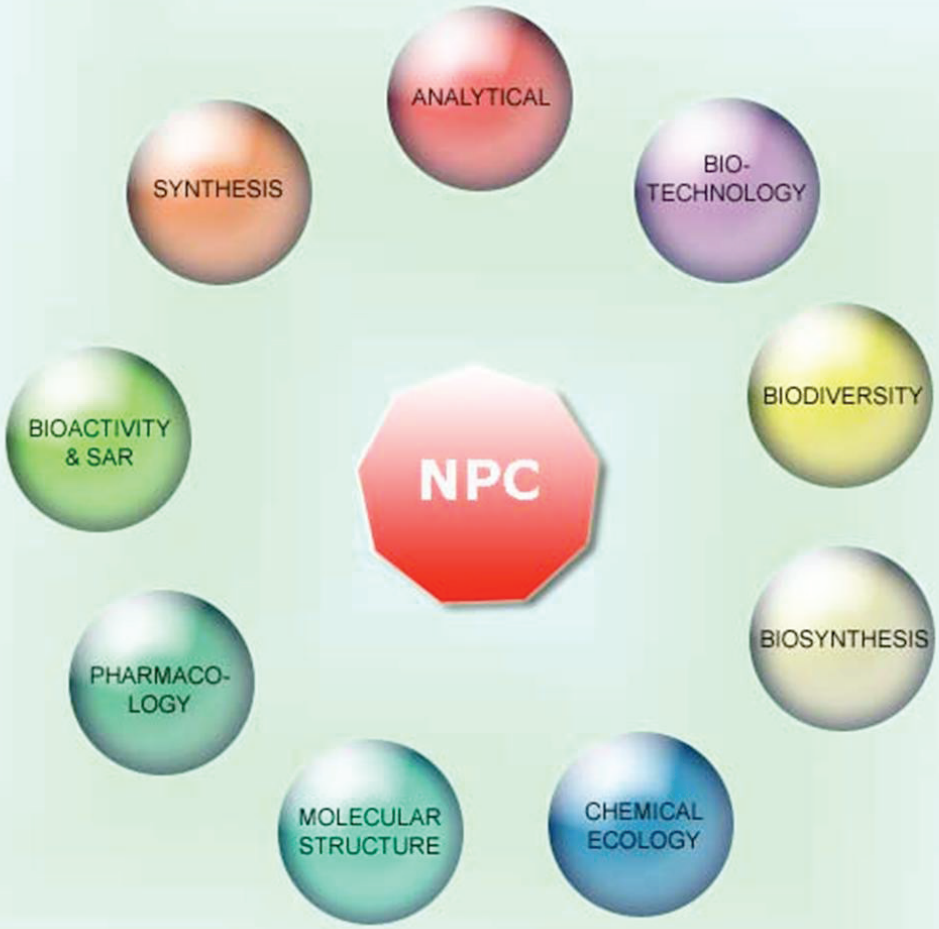

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DPPH Radical Scavenging and Xanthine Oxidase Inhibitory Activity of *Terminalia macroptera* Leaves

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From a methanol extract of the leaves of the Malian medicinal tree *Terminalia macroptera, cis*-polyisoprene (**1**), chebulic acid trimethyl ester (**2**), methyl gallate (**3**), shikimic acid (**4**), corilagin (**5**), rutin (**6**), narcissin (**7**), chebulagic acid (**8**) and chebulinic acid (**9**), were isolated. *Cis*-polyisoprene (**1**) was the major non-polar constituent. The novel compound **2** showed high radical scavenging activity (IC_{50} 4.7 µg/mL), but was inactive as xanthine oxidase inhibitor. The major substituent of the crude extract, substance **5**, showed a high radical scavenger effect (IC_{50} 2.7 µg/mL) and weak xanthine oxidase inhibition (IC_{50} ca 105 µg/mL). The antioxidant and radical scavenging effects of some of the substances identified in this study may to some extent explain the medical use of this tree in West Africa.

Keywords: *Terminalia macroptera*, DPPH, xanthine oxidase, ellagitannins.

Terminalia macroptera Guill. & Perr. (Combretaceae) is a tree, up to 20 m high, distributed from Senegal to Sudan. All parts of the tree are widely used in traditional medicine. The root is used against hepatitis, gonorrhea and sexually transmitted diseases [1,2]. Preparations from the leaves are employed in the treatment of hepatitis, ringworm, skin diseases [3] as well as gastritis, colic, high blood pressure, fever and tuberculosis [4]. A healer in Mali has reported that the leaves, roots and stem bark are used against diabetes. The flowers contain flavonoids [5,6]. Triterpenoids, tannins and other phenolics are found in the bark [1,7-9]. Phenolic acids, flavonoids and ellagitannins have been identified in the leaves [2,9]. The leaves have antibacterial [2] and antifungal activities [10]. As part of our research on Malian medicinal plants [11], we have isolated and identified antioxidant compounds from *T. macroptera* leaves.

The dichloromethane (DCM) crude extract was inactive as DPPH scavenger (IC_{50} > 167 µg/mL). Fractionation over Si gel gave *cis*-polyisoprene (**1**) [12] (Fig. 1) with a mean chain length of 25 isoprene units, calculated from ^{1}H NMR. This is the first report of rubber as a constituent in *T. macroptera*. The MeOH crude extract of *T. macroptera* leaves showed high radical scavenger activity and moderate xanthine oxidase (XO) inhibition (Table 1). The MeOH crude extract was partitioned by liquid-liquid extraction to obtain EtOAc and *n*-BuOH extracts as well as an aqueous residue. The EtOAc extract showed high DPPH radical scavenging effect and XO inhibition. Separation of the EtOAc extract afforded chebulic acid trimethyl ester (**2**) and methyl gallate (**3**) [13]. The BuOH extract was fractionated to afford shikimic acid (**4**) [14] and corilagin (**5**) [15]. Compound **5** is a major constituent of the methanolic extract. Additionally, purification with preparative HPLC gave rutin (**6**) [16] and narcissin (**7**) [17]. Chebulagic acid (**8**) and chebulinic acid (**9**) [18] were also isolated from the EtOAc and BuOH extracts. The activity of isolated compounds as DPPH scavengers and XO inhibitors is shown in Table 2. Compound **2** is a novel compound. However, it could be an artifact created by esterification between COOH groups of chebulic acid and methanol during extraction and therefore may not come directly from the plant itself. Analogously, chebulic acid triethyl ester has been reported from *Terminalia chebula*

Table 1: DPPH radical scavenging and xanthine oxidase inhibition of *T. macroptera* extracts.

Extract	DPPH radical IC_{50} ± S.D. (µg/mL)	Xanthine oxidase IC_{50} ± S.D. (µg/mL)
DCM crude extract	Inactive	[a]
MeOH crude extract	6.2 ± 0.4	52 ± 5
EtOAc extract	3.7 ± 0.2	26 ± 3
BuOH extract	6.5 ± 0.4	64 ± 8
Aqueous residue	12.4 ± 0.6	146 ± 15
Quercetin (positive control)	3.0 ± 0.2	0.6 ± 0.1

[a] Not tested

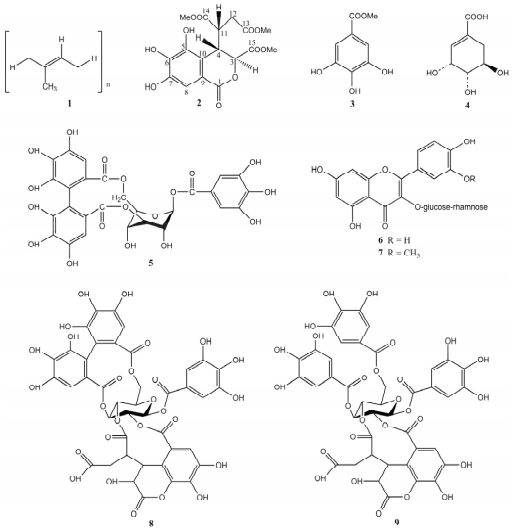

Figure 1: Chemical structures of compounds **1-9**.

Table 2: Effects of the isolated compounds from *T. macroptera* on scavenging of DPPH radical and XO activity.

Compound	DPPH radical IC_{50} ± S.D. (μM)	Xanthine oxidase IC_{50} ± S.D. (μM)
1 Poly-*cis*-isoprene	[a]	[a]
2 Chebulic acid trimethyl ester	11.8 ± 1.0	> 419
3 Methyl gallate	12.1 ± 0.4	> 83
4 Shikimic acid	529 ± 46	> 958
5 Corilagin	4.3 ± 0.5	> 83
6 Rutin	22 ± 2	ca. 40
7 Narcissin	> 83	> 83
8 Chebulagic acid	3.5 ± 0.1	53 ± 8
9 Chebulinic acid	[a]	[a]
Quercetin (positive control)	9.4 ± 0.6	1.9 ± 0.4

[a] Not tested

after extraction with ethanol [19]. The structure elucidation and stereochemistry are based on comparison with published data for chebulic acid [20] and chebulic acid triethyl ester [19]. Chebulic acid itself has previously been isolated from other *Terminalia* species, but never from *T. macroptera*. Compound **2** was found to have strong DPPH scavenging activity, but was inactive as an XO inhibitor. Compound **3** may be an artifact from esterification of gallic acid with methanol, but has previously been found in other *Terminalia* species [21]. Gallic acid has previously been identified in *T. macroptera* roots [1], but not in the leaves. Compound **3** was found to have strong DPPH scavenger activity, a result also reported by Lee et al. [13]. It had no XO inhibitory activity; this is supported by previous results [22]. This is the first identification of compound **4** in *T. macroptera*. However, shikimic acid has been identified in other *Terminalia* species [23]. In our study, shikimic acid was inactive as XO inhibitor, and it showed weak DPPH scavenger effect. The flavonoid glycosides rutin (**6**) and narcissin (**7**) have not previously been reported from *T. macroptera* leaves, but have previously been found in the flowers [5]. Their DPPH scavenger activities clearly show that the 3',4'-dihydroxy structural element of rutin is of importance for radical scavenging effect. Rutin was also more active than narcissin as XO inhibitor.

Ellagitannins, the class of natural products to which corilagin belongs, are found in many medicinal plants and have numerous biological activities , including antioxidant, antiatherosclerotic, α-amylase inhibiting and anticancer properties [24]. It should, however, be realized that little is known about clinical properties of ellagitannins. For corilagin, **5**, not previously reported from this plant, several interesting biological properties have been reported. Some of these are, in addition to the known antioxidant properties, antiinflammatory activity [25], antihepatotoxic activity [26], antiviral activity [27], antitumor properties [28], and antimicrobial effects [29]. Corilagin appears to potentiate the activity of β-lactam antibiotics against methicillin-resistant strains of *Staphylococcus aureus* [30]. Most of these are, however, *in vitro* studies.

The high radical scavenging activity of polar and semi-polar fractions from leaves of *T. macroptera* is probably due to its high content of ellagitannins, and corilagin is a significant contributor to this activity.

Experimental

^{1}H and ^{13}C NMR spectroscopy was conducted on Varian Gemini 200, Bruker DPX 300 or Bruker AVII 400 instruments. EI-MS was accomplished on a Micromass Prospec Q instrument. Optical rotation was measured on a Perkin Elmer model 341 polarimeter. Sephadex LH-20 (Pharmacia), MCI gel CHP 20P (Supelco) and VersaPak C18 and silica cartridges (Sigma-Aldrich) were used in CC. Foils coated with Si gel 60 F_{254}, 0.2 mm or RP-18 F_{254S} (Merck) were used for analytical TLC. Fractions from CC were combined as indicated by TLC. In analytical TLC, spots were visualized by UV irradiation (254 and 366 nm), by spraying with $Ce(SO_4)_2$ (1 % in 10 % aqueous H_2SO_4) followed by heating (100 ˚C, 10-15 min) or by spraying with DPPH (0.04 % (w/v) solution in MeOH). Preparative HPLC was carried out on a Varian ProStar Polaris system, with a Microsorb 60-8 C18 (21.4 × 250 mm) column (254 nm UV-detection, 20 mL/min). For UV/VIS measurements, a Biochrom Libra S32 PC or a Shimadzu UV-160A instrument was employed.

Plant material: Leaves of *Terminalia macroptera* (Combretaceae) were collected in Blendio, Mali, in December 2007, and identified by the Department of Traditional Medicine (DMT), Bamako, Mali, where a voucher specimen (No. 2468 DMT) is deposited.

Extraction and isolation: *T. macroptera* air-dried and milled leaves (1116 g) were extracted (Soxhlet) with

DCM (3500 mL), yielding 59.8 g (5.4 % of total amount) of extract. The plant residue was extracted similarly with MeOH (4000 mL) to yield 398 g (35.7 %) of MeOH extract. The MeOH extract (387 g) was suspended in 2 × 500 mL distilled water and then extracted successively with EtOAc (6 × 500 mL) and BuOH (4 × 500 mL). The solvents were removed *in vacuo*, resulting in an EtOAc extract (85.4 g), a BuOH extract (155.6 g) and an aqueous residue (ca. 75 g). The DCM extract (2 g) was flash chromatographed over Si gel (Versapak, 4 × 15 cm) eluting with a DCM/EtOAc gradient to yield **1** (0.77 g). The EtOAc (E) extract (30 g) was chromatographed over Sephadex LH-20 (4.5 × 36 cm) with MeOH/H_2O (25:75 to 100:0) as eluent, and finally with acetone/H_2O, 70:30. Fractions E4 (2.29 g), E5 (2.76 g) and E8 (4.51 g) were flash chromatographed over RP C-18 Si gel (VersaPak, 4 × 15 cm) eluting with a MeOH/H_2O gradient. E4.2 (0.83 g), E4.3 (0.91 g) and E4.4 (0.13 g) were combined and rechromatographed over MCI gel CHP 20P (2.5 × 24 cm) with MeOH/H_2O as solvent to yield **2** (0.92 g). Flash chromatography of E5 yielded **3** (0.01 g). E8 was rechromatographed repeatedly using flash chromatography followed by MCI gel CHP 20P as described above to yield E8.1-3.B-D2.3 (775.1 mg).

The BuOH (B) extract (28.6 g) was chromatographed over Sephadex LH-20 (4.5 × 38 cm), eluting with MeOH/H_2O (from 25:75 to 100:0). Fractions B3 (1.11 g) and B10 (3.22 g) were flash chromatographed as above to yield **4** (0.4 g) and **5** (1.51 g), respectively. B7 (0.31 g) was also purified using this system. Subfraction B7.5 (0.05 g) was further purified by preparative HPLC (linear gradient of MeOH/H_2O). This gave **6** (2.2 mg) and **7** (11.7 mg). B12, B13 and B14 (2.38 g) were combined and flash chromatographed as above. B12-14.1 (1.9 g) was purified on MCI gel CHP 20P (2.5 × 23.5 cm) with increasing amounts of MeOH in H_2O. B12-14.1.9 (775.9 mg) was purified on MCI gel CHP 20P to yield B12-14.1.9C (564.1 mg). On the basis of similar NMR-spectra, B12-14.1.9C (524.1 mg) was combined with E8.1-3.B-D2.3 (775.1 mg) from the EtOAc extract and subjected to repeated flash chromatography to afford **8** (61.1 mg) and **9** (23.7 mg).

DPPH scavenging: Test substances were dissolved in DMSO, and the assay was carried out as reported previously [31]. Quercetin was used as positive control. All samples were analyzed in triplicate and results are given as averages ± SD.

Inhibition of xanthine oxidase: Test substances were dissolved in DMSO. Enzyme solution containing xanthine oxidase from cow's milk (Sigma-Aldrich) in phosphate buffer 0.05 M, pH 7.5 (1.8 units/mL), was made immediately before use and kept on ice during the experiment. Substrate, hypoxanthine in distilled water (0.02 mg/mL), was prepared immediately before use. The assay mixture consisted of 1.85 mL of phosphate buffer, 0.1 mL of enzyme solution and 0.05 mL of test solution. The reaction was initiated by adding 1.0 mL of substrate solution, and the absorbance of the assay mixture was measured at 290 nm. In blanks, 0.05 mL DMSO was added instead of test solution. The XO inhibitory activity was expressed as the percentage inhibition of XO, calculated as $100 \times (A_2 - A_1)/A_2$, where A_1 and A_2 are values for increase in A_{290} for sample with and without test substance, respectively. Quercetin was used as positive control. All samples were analyzed in triplicate and results are given as averages ± SD.

Chebulic acid trimethyl ester

Yellow-brown solid.
$[\alpha]_D^{22}$: +14.8 (*c* 1.00, MeOH).
UV λ_{max} (MeOH-H_2O): 290 nm.
EI-MS: m/z 398.0839 $[M]^+$ (calcd. for $C_{17}H_{18}O_{11}$, 398.0849); EI-MS: m/z (%): 398 $[M]^+$ (14), 366 $[M - 32\ (CH_3OH)]^+$ (43), 334 $[M - 64\ (2 \times CH_3OH)]^+$ (33), 307 $[M - 91]^+$ (45), 293 $[M - 105]^+$ (100).
^1H and ^{13}C NMR data: Table 3.

Table 3: ^1H and ^{13}C NMR of **2** (300 MHz, $(CD_3)_2CO$)[a].

No.	**2**	
	δ_H (*J*, Hz)	δ_C
1		163.87
2		-
3	5.27 (1H, d, 1.2)	77.69
4	3.90 (1H, dd, 1.2, 7.9)	36.68
5		143.29
6		139.00
7		145.99
8	7.12 (1H, s)	108.99
9		116.95
10		117.07
11	3.25 (1H, ddd, 4.8, 8.0, 10.0)	44.76
12	2.52 (1H, dd, 4.7, 16.9) and 2.87 (1H, dd, 10.0, 16.9)	34.55
13		172.39
14		173.97
15		170.57
13 OCH_3	3.54 (3H, s)	51.85
14 OCH_3	3.68 (3H, s)	52.46
15 OCH_3	3.60 (3H, s)	53.00

[a]Assignments were confirmed by COSY, HSQC and HMBC experiments.

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Article

Bioactive Flavone-*C*-Glycosides of the African Medicinal Plant *Biophytum umbraculum*

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Abstract: Three flavone-*C*-glycosides—cassiaoccidentalin A (**1**), isovitexin (**2**) and isoorientin (**3**)—were isolated from the ethyl acetate (EtOAc) soluble fraction of the methanol crude extract of the African medicinal plant *Biophytum umbraculum*, This is the first report of these compounds in this plant. All compounds were identified by spectroscopic analysis and comparison with published data. Isoorientin (**3**) and the EtOAc extract showed the greatest antioxidant activity in the DPPH assay as well as the strongest inhibition of xanthine oxidase (XO) and 15-lipoxygenase (15-LO). From these results, the extract of *B. umbraculum* might be a valuable source of flavone *C*-glycosides.

Keywords: *Biophytum umbraculum*; flavone-*C*-glycosides; DPPH; xanthine oxidase; 15-lipoxygenase

1. Introduction

Biophytum umbraculum Welw. (common syn. *Biophytum petersianum* Klotzsch) (Oxalidaceae) is a slender annual herb distributed in tropical and subtropical Africa, and across to Asia and New Guinea. The aerial parts of the plant have several medicinal uses in Mali and other African countries [1].

Ethnopharmacological surveys on the use of *B. umbraculum* by practitioners in different districts in Mali (Bamako, Siby and Dioila) show that the plant is frequently used against cerebral malaria, but also against hemorrhoids, colonic ailments, wounds, stomach ache and fever [2–4]. In Nigeria the plant has been used against wounds, gonorrhea, urethral stones and stomach ache [1]. Other indications reported are constipation, hypertension, migraine, epilepsy, breathing difficulties and lack of fertility [1,5,6]. Various *in vitro* studies indicate that extracts of *B. umbraculum* may exert beneficial pharmaceutical effects on hypertension [5,7–10]. In addition, pectic polysaccharides isolated from *B. umbraculum* have demonstrated diverse effects on the immune system by virtue of complement fixation [3,4], activation of macrophages and dendritic cells [11], and modulation of intestinal Peyer's patch cells [12]. Phytochemical investigations on *B. umbraculum* have so far been restricted to polysaccharides [3,4,11,12], although saponins of unknown structure have been stated to be present in aqueous extracts of the plant [13].

In the past decades, there has been a growing interest in antioxidants and free radical scavengers as they may have an important role in the prevention of pathologies in which reactive oxygen species (ROS) or free radicals are implicated, such as atherosclerosis, cardiovascular diseases (CVD), ischemia/reperfusion injury, neurodegenerative diseases and cancer [14,15]. As a part of an ongoing project on Malian medicinal plants [16–18], in the present study chemical characterization and investigation of antioxidant activity were performed on *B. umbraculum*.

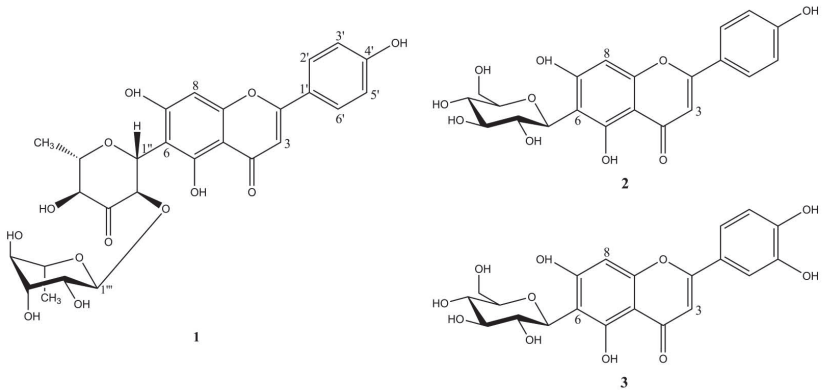
2. Results and Discussion

2.1. Isolation and Structural Elucidation

Compounds **1**, **2** and **3** were isolated from the EtOAc soluble fraction of the MeOH crude extract of *Biophytum umbraculum* by column chromatography (CC). Based on the weights of purified fractions, estimated concentrations of substances in the crude MeOH extract are 0.45% (**1**), 0.37% (**2**) and 0.17% (**3**). The compounds were identified as cassiaoccidentalin A (**1**), isovitexin (**2**) and isoorientin (**3**), respectively, by comparing their ^{1}H-NMR and ^{13}C-NMR spectroscopic data with those reported in the literature [19–21]. Besides, the COSY spectrum revealed ^{1}H-^{1}H couplings and helped assign proton resonances, especially those of the inner sugar of **1**. The HSQC spectrum showed all ^{1}J direct ^{1}H-^{13}C correlations and thus confirmed the assignments of all signals arising from the CH- and CH_2- groups. Among all the ^{1}H-^{13}C long-range correlations observed in the HMBC spectrum, the most important were the correlations from the anomeric proton of the inner sugar (H-1′) to C-6 (^{2}J), C-5 (^{3}J) and C-7 (^{3}J) of the aglycone, which confirmed the *C*-glycosylation at C-6.

The chemical structures of the three flavone-*C*-glycosides are shown in Figure 1. To our knowledge, this is the first identification of flavone-*C*-glycosides in *B. umbraculum*. Compound **1** is a very rare flavone-*C*-glycoside which has only been identified once before, in *Cassia occidentalis* [19], whereas the more common flavone glucosides **2** and **3** have been identified in another *Biophytum* species, namely *Biophytum sensitivum* [22].

Figure 1. Chemical structures of the three flavone-*C*-glycosides **1**–**3**.



2.2. Antioxidant Activity

The dichloromethane (DCM) crude extract of *B. umbraculum* was virtually inactive as a DPPH radical scavenger, xanthine oxidase (XO) and 15-lipoxygenase (15-LO) inhibitor (IC_{50} > 167 µg/mL). The MeOH crude extract and the semi-polar extracts obtained by partitioning the MeOH extract showed fairly high radical scavenger activity and 15-LO inhibition (Table 1). The extracts showed moderate or low inhibitory activity towards XO compared to the positive control quercetin. The EtOAc extract exhibited the highest activity in all three assays.

Table 1. DPPH radical scavenging, xanthine oxidase (XO) inhibition and 15-lipoxygenase (15-LO) inhibition of *Biophytum umbraculum* extracts. IC_{50} values ± SD (in µg/mL) are shown.

Extract	DPPH	XO	15-LO
DCM crude extract	>167	>167	>167
MeOH crude extract	13.4 ± 0.6	59.7 ± 6.1	68.9 ± 5.0
EtOAc extract	6.8 ± 0.6	*ca.* 21	43.0 ± 3.6
BuOH extract	12.5 ± 1.9	102.6 ± 5.6	53.4 ± 2.1
Aqueous residue	29.8 ± 3.5	>167	>167
Quercetin (positive control)	4.4 ± 0.4	2.33 ± 0.09	33.4 ± 0.3

The activity of the isolated compounds as DPPH scavengers, XO- and 15-LO inhibitors is shown in Table 2. Flavone-*C*-glycosides were found to contribute both as DPPH scavengers and 15-LO inhibitors in the EtOAc extract. This is in good accordance with previous structure-activity studies indicating the importance of a 2,3 double bond in conjugation with a 4-oxo function in the C-ring and *o*-dihydroxy structure in the B-ring [23,24]. The strongest inhibitor of XO and 15-LO and the best DPPH scavenger was isoorientin. The strong DPPH radical scavenging activity of isoorientin and the much weaker activity of isovitexin are supported by previous results [25,26]. This antioxidant activity clearly shows that the 3′,4′-dihydroxy structural element of isoorientin is of importance for activity, isoorientin having an IC_{50} value similar to the positive control quercetin. This is in accordance with a

previous report [27]. The EtOAc extract may contain additional unidentified XO inhibitors, since XO inhibition of the isolated compounds does not account for the activity observed in the EtOAc extract. It has previously been suggested that flavones without a glycosyl group were relatively strong inhibitors of XO, as the presence of a glycosyl group would decrease the inhibitory activity [28,29]. XO and 15-LO inhibitors may be beneficial for diseases and conditions such as ischemia/reperfusion, gout, renal stones, inflammation, arteriosclerosis, neurodegenerative diseases, cancer, aging, *etc.* [28,30–32]. Additionally, XO-inhibitors may have beneficial effects as adjuncts in the management of severe *Plasmodium falciparum* malaria [33].

Since the solubility in water of the flavonoids reported here is unknown (although a calculation, as given on the SciFinder website, gives theoretical values of between 0.12 and 0.15 mg/mL), an analysis of aqueous extracts of the plant may be relevant for the evaluation of its ethnopharmacological use, and might thus represent a useful continuation of the present work.

These results imply that the extracts from *B. umbraculum* are a rich source of flavone-*C*-glycosides, and that herbal remedies obtained from this plant may have an effect against inflammations or other diseases related to oxidative stress. The results from our study would seem to be in accord with several of the reported ethnopharmacological usages of the plant.

Table 2. Effects of isolated compounds from *B. umbraculum* on DPPH radical scavenging, xanthine oxidase (XO) inhibition and 15-lipoxygenase (15-LO) inhibition. IC_{50} values ± SD (in μM) are shown.

Isolated compound	DPPH	XO	15-LO
Cassiaoccidentalin A (**1**)	>167	149.5 ± 7.4	99.9 ± 2.5
Isovitexin (**2**)	96.0 ± 3.6	>167	107.1 ± 2.1
Isoorientin (**3**)	18.1 ± 1.1	117.2 ± 13.5	86.4 ± 0.5
Quercetin (positive control)	13.7 ± 1.3	7.7 ± 0.3	110.6 ± 1.0

3. Experimental

3.1. General

1D and 2D NMR spectra were recorded on Bruker DPX 300 or Bruker AVII 400 instruments with CD_3OD as solvent and TMS as internal standard. CC was done over Sephadex LH-20 (Pharmacia) or reverse phase (RP) silica (VersaPak C18 cartridges; Supelco). Fractions from CC were combined as indicated by TLC. Foils coated with Si gel RP-18 F_{254S} (Merck) were used for analytical and preparative TLC. In analytical TLC, spots were visualized by UV irradiation (254 and 366 nm) and by spraying with $Ce(SO_4)_2$ (1% in 10% aqueous H_2SO_4) followed by heating (100 °C, 10–15 min). For UV/VIS measurements, a Biochrom Libra S32 PC instrument was employed.

3.2. Plant Material

Flowering, whole aerial parts of *Biophytum umbraculum* Welw. (Oxalidaceae) were collected in Blendio, Mali. The plant was identified by one of the authors, Prof. Drissa Diallo. A voucher specimen

(NO. 2653 DMT) was deposited in the herbarium at the Department of Traditional Medicine (DMT), Bamako, Mali.

3.3. Extraction and Isolation

The dried and powdered aerial parts of *B. umbraculum* (305 g) were extracted at RT with DCM (4 × 2.5 L), each time for 24 h, yielding 5.1 g of DCM extract (1.7% yield). The plant residue was then extracted similarly with MeOH (5 × 2.5 L) to yield 17.7 g (5.8%) of MeOH extract. The MeOH extract was suspended in 3 × 100 mL distilled water and successively partitioned with EtOAc (6 × 300 mL) and BuOH (5 × 200 mL). The solvents were removed under vacuum, affording an EtOAc extract (4.6 g), a BuOH extract (5.3 g) and an aqueous residue (5.0 g). The EtOAc (E) extract was chromatographed over Sephadex LH-20 (2.5 × 74 cm) with a gradient of H_2O/MeOH (25%–100% MeOH) to yield 15 subfractions (E1-E15). E9 (334 mg), E10 (1,029 mg) and E11 (555 mg) were chosen for isolation of bioactive compounds based on fraction weights, pattern in NMR spectra and results from activity assays. E9 was flash chromatographed over RP C-18 Si gel (VersaPak, 2.3 × 11 cm) eluting with a H_2O-MeOH gradient (40%–100% MeOH) to give **1** (57 mg). E10 was applied to a RP C-18 Si gel VersaPak column (4 × 15 cm) and eluted with a gradient of H_2O-MeOH (20%–100% MeOH) to afford E10.1-E10.9. E10.6 (42 mg) and E10.7 (24 mg) were combined and rechromatographed over a smaller VersaPak column (2.3 × 11 cm) with a H_2O-MeOH gradient (20%–100% MeOH) followed by preparative TLC to give **2** (8.8 mg). E11 was flash chromatographed with the same column as used for E10 and eluted with a gradient of H_2O-MeOH (40%–100% MeOH) to yield E11.1-E11.13. E11.2 (50 mg) and E11.3 (21 mg) were combined and purified using the same system to give **3** (6.5 mg). A second batch of plant material (225 g) was subjected to Soxhlet extraction (2 L DCM for 48 h, followed by 3 L MeOH for 48 h). Yield of DCM extract was 7.5 g (3.3%), and of MeOH extract 17.2 g (7.6%). EtOAc extract (4.6 g), BuOH extract (3.3 g) and aqueous residue (5.1 g) were obtained similarly as above.

3.4. DPPH Radical Scavenging

Pure substances and crude extracts (Soxhlet) were dissolved in DMSO, and the assay was carried out as reported previously [34]. Quercetin (Sigma-Aldrich) was used as positive control.

3.5. Inhibition of Xanthine Oxidase (XO)

Pure substances and crude extracts (Soxhlet) were dissolved in DMSO, and the assay was carried out as reported previously [35]. Quercetin (Sigma-Aldrich) was used as positive control.

3.6. Inhibition of 15-Lipoxygenase (15-LO)

Pure substances and crude extracts (Soxhlet) were dissolved in DMSO, and the assay was carried out as reported previously [34]. Quercetin (Sigma-Aldrich) was used as positive control.

3.7. Statistical Analysis

All samples were analyzed in triplicates and the results are shown as mean ± standard deviation (SD).

4. Conclusions

Our phytochemical study led to the isolation and characterization of three flavone-*C*-glycosides from the aerial parts of *B. umbraculum*. This is the first report on this type of compound from this plant. Compound **3** and the EtOAc extract of the plant revealed strong antioxidant activity towards DPPH radical and 15-LO, and moderate activity towards XO. Further studies on these extracts with respect to antioxidant properties *in vivo* are needed.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds **1**–**3** are available from the authors.