Department for Farm Animals and Veterinary Public Health

University of Veterinary Medicine Vienna

Institute of Animal Nutrition and Functional Plant Compounds

(Head: Univ.-Prof. Dr.dc.agr. Qendrim Zebeli)

Phytochemical investigation of an antimalarial plant:

Cochlospermum planchonii

Diploma thesis

Submitted by

Martina Mischka

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Contents

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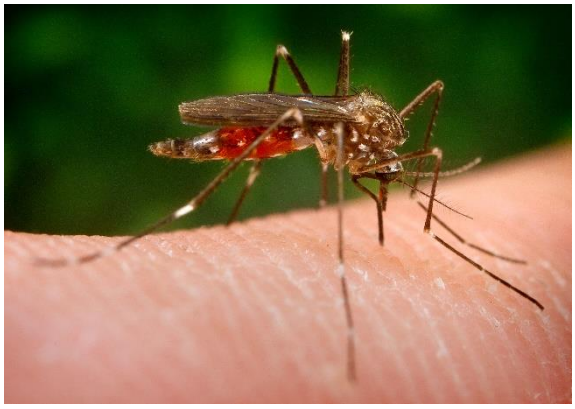
1. Introduction

1.1. Malaria – a portrait of the most deadly disease worldwide

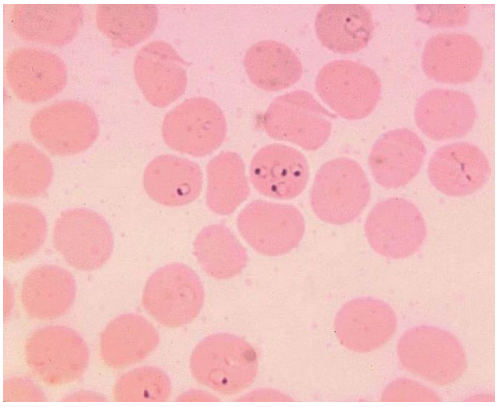
Malaria is a serious, life-threatening protozoan infection in humans with a typical feverish disease pattern. The main endemic areas are Sub-Saharan Africa, South –and Middle America, as well as South –and Southwest Asia. It is caused by an infection with a protozoan parasite of the genus *Plasmodium* and it is transmitted from human to human by a female mosquito of the genus *Anopheles* (GODDARD, 2000; LANG, 1996; DIESFELD and KRAUSE, 1997).

1.1.1. Anopheles and Plasmodium: the vector and the parasite

Anopheles (Figure 1) is one of the most widespread types of mosquitoes. It is not only limited to tropical –and subtropical areas, its worldwide spread even extends into arctic areas. The currently endemic areas are similar to the before mentioned malaria regions. *Anopheles* is the only possible natural vector for the human malaria pathogen *Plasmodium* (Figure 1) (GODDARD, 2000).



Anopheles gambiae



Plasmodium falciparum

Figure 1: Anopheles gambiae while biting a human (GATHANY and COLLINS, 2017) and Plasmodium falciparum with typical ring form in a blood smear under microscope (MAE, 2017).

There are four species of the protozoan parasite that can cause different forms of malaria: *Plasmodium vivax* and *Plasmodium ovale*, which are the pathogens for malaria tertiana and *Plasmodium malaria*, the pathogen for malaria quartana. All three of them are benign. The most important and dangerous one is *Plasmodium falciparum (P. falciparum)*, which is the pathogen

for the life-threatening malaria tropica. It predominates Sub-Saharan Africa (DIESFELD and KRAUSE, 1997).

The life cycle of *Plasmodium* proceeds as a sexual cycle in the mosquito, which is from a parasitological view the main host, and as an asexual cycle in the human body, where the maturation of eggs is happening (LANG, 2000).

When *Anopheles* infected with *Plasmodium* bites, *Plasmodium* sporozoites enter with mosquito-saliva the human bloodstream and infect liver cells. After 48 hours of growth, development and division, the infected cells rupture and release merozoites into the bloodstream. They invade red blood cells (=erythrocyte phase), where they grow and multiply asexually and cause the burst of the erythrocyte after 48-72 hours. Thereby a large number of new parasites is released, most of which enter new red blood cells. By that the cycle is reinitiated. Additionally to these asexual forms, some of the parasites develop into male and female gametocytes. These sexual forms of *Plasmodium* can be drawn up into the stomach of a susceptible, human blood feeding *Anopheles*. Now, the sexual part of the cycle, which takes 8-35 days, is happening inside the mosquito: In the stomach, the fertilization takes place and the result is a zygote that penetrates the gut wall and forms oocysts in the gut membrane. After some time, the oocytes rupture and release sporozoites, who migrate into the salivary glands. Then the *Anopheles* mosquito is infective and contagious for the next human, whose blood it's feeding on (GODDARD, 2000).

The symptoms occurring in humans infected with malaria tropica can be unspecific. The basic phenomenon of the pathology is a homeostatic problem, due to the infested erythrocytes, which tend to aggregate and form clumps in the fine vessels and lead to more or less heavy microcirculation problems. Further, all of the following symptoms can occur: fever (increasing progress, without a recognizable rhythm), chills, sweats, headaches, muscle pain, vertigo, vomiting, diarrhoea, malaise, abdominal pain, coughing and dyspnoea. Possible complications can be a coma (triggered by a cerebral form of malaria), repeated generalized convulsions, severe anaemia, renal insufficiency, oedema of the lungs, hypoglycaemia, Disseminated Intravascular Coagulation, haemoglobinuria and acidosis. Malaria tertiana –and quartana show all together much weaker symptoms. The fever tends to have a rhythmic pattern (DIESFELD and KRAUSE, 1997).

1.1.2. Importance of malaria (World Health Organisation - malaria report 2015)

Malaria is one of the most important parasitic infectious diseases worldwide. It is life-threatening and about 3.2 billion people, which is almost half of the world´s population, are at risk of malaria.

A report that depicts the enormous meaning of the disease and tells about the progress in the fight against malaria is published by the World Health Organisation (WHO) every year in December: *World Malaria Report*. It contains information about malaria-related policies in endemic countries, as well as the latest WHO recommendations for prevention and control of the disease (WHO (2016): World Malaria Report; http://www.who.int/malaria/publications/world_malaria_report/en/; accessed: 08.11.2016).

2015 was a milestone year for the fight against malaria and the *World Malaria Report* because of two special events: First it marked the end of the era of the "Millennium Development Goals", brought to life by the United Nations (UN). One of the eight goals was the combat of HIV/Aids, malaria and other diseases (UN (2016): Millennium Development Goals; http://www.un.org/millenniumgoals/; accessed: 10.11.2016). Secondly, 2015 was the target year for the malaria goals set by the World Health assembly and other global institutions (WHO, 2015).

The *World Malaria Report 2015* shows some very promising progresses since 2000: The number of countries with ongoing malaria transmission has decreased from 106 (in 2000) to 95 at the end of 2015. In 2000 there were 262 million malaria cases worldwide, in 2015 this number dropped to 214 million, which is a decline of 18 %. Deaths caused by malaria, fell from 839 000 in 2000, to 438 000 in 2015, a respectable decline of 48 %. Also malaria is no longer the leading cause of deaths in Sub-Saharan Africa among children under five years. It decreased from 723 000 globally in 2000 to 306 000 in 2015 (WHO, 2015).

A very central cognition was that 88 % of malaria cases and deaths in 2015, are estimated to have occurred in Africa. If one takes a closer look at this region alone, the number of malaria cases dropped only by 12 % (WHO, 2015). Therefore especially in this area a lot of work still has to be done in the fight against malaria.

1.1.3. Prophylaxis and therapy of malaria

The most important way for preventing malaria is the avoidance of mosquito-bites. Some of the possible prophylactic techniques are mentioned in the *World Malaria Report 2015*: sleeping under Insecticide-treated mosquito nets, indoor residual spraying and chemoprevention in pregnant woman and children. All women in malaria-endemic areas in their first or second pregnancy, should receive sulfadoxine-pyrimethamine (SP) as part of their antenatal care. For infants, in areas of moderate to high malaria transmission in Africa the WHO strongly recommends intermittent preventive treatment with SP, if this substance is still effective. SP is a combination antimalarial drug. Sulfadoxine is an antibiotic and pyrimethamine has an antiprotozoal effect (WHO-Guidelines for the treatment of malaria, 2015).

The WHO recommends diagnostic testing with a parasitological confirmation with either microscopy or rapid diagnostic test, in all patients with suspected malaria, before antimalarial treatment is started. Prompt treatment is always necessary, also if no testing is available within the next two hours after clinical suspicion (WHO (2016): Overview of malaria treatment; http://www.who.int/malaria/areas/treatment/overview/en/; accessed: 15.11.2016).

The choice of medicals for the therapy of acute malaria is adjusted to the form of clinical appearance and the resistance situation of the respective area. Next to supportive therapy (intensive care, exchange transfusion, etc.), there are a lot of synthetic antimalarial drugs available: chloroquine, quinine, mefloquine, halofantrine, sulfadoxine-pyrimethamine, primaquine, proguanil and doxycycline (DIESFELD and KRAUSE, 1997).

The WHO recommends for the treatment of both uncomplicated and severe malaria, caused by *Plasmodium falciparum* artemisinin-based combination therapies (ACTs). The advantage of using ACTs, instead of an artemisinin-monotherapy is that the risk of developing an artemisinin-resistance is much lower. Additionally, fixed dose formulations (meaning 2 different active ingredients co-formulated in one tablet) should be preferred over co-blistered, co-packaged or loose tablet combinations, which reduces the potential use of the individual components (WHO (2016); Overview of malaria treatment; http://www.who.int/malaria/areas/treatment/overview/en/; accessed 24.11.2016). The

following Table 1 shows the possible combinations for ACTs, which are recommended by the WHO (WHO-Guidelines for the treatment of malaria, 2015).

Table 1: ACTs, recommended by the WHO.

Artemisinin-derivate	Combined with
Artemether	Lumefantrine
Artesunate	Amodiaquine
Artesunate	Mefloquine
Dihydroartemisinin	Piperaquine
Artesunate	Sulfadoxine-pyrimethamine

ACTs are currently the most recommended antimalarial drugs by the WHO. They strongly call for the preservation of the efficacy of artemisinin, by withdrawing artemisinin-based monotherapies because there is no new class of antimalarial medicines expected to enter the market within the next few years (GLOBAL MALARIA PROGRAMME, 2014).

But despite all the therapeutic advantages of ACTs, they also have some not insignificant negative aspects. A big problem are the substantially higher costs for ACTs, especially in rural areas of Sub-Saharan Africa, where poverty is an issue. Also remote located communities have low access to functioning modern health services. Private pharmacies reported difficulties in the constant supply with these drugs because of the shortages at the wholesalers (BEIERSMANN et al., 2007). If antimalarial drugs in general are not available through legal sources, people are being forced to obtain the medicals for home treatment through illicit (market –and street vendors, shops) sources, where the danger of purchasing substandard anti-malarial drugs is huge. But even markets or licensed (public –and private pharmacies) sources are suffering under an increasing infiltration with fake medications. In a study from 2008, 42 % of antimalarial drug samples were found to be poor of quality. The drugs were collected from licensed sources, where 10.6 % were substandard and from illicit sources, were unfortunately 90 % were substandard (TIPKE et al., 2008).

Out of tradition but probably also due to all the difficulties that are connected with synthetic drugs, a lot of people use only or at least partly natural remedies for the treatment of malaria.

1.2. Antimalarial natural remedies in Africa

The WHO estimated in the year of 2002 that 80 % of the African population is using traditional medicine (meaning herbs, herbal materials, other plant materials and combinations) for their primary health care needs (WHO-traditional medicine strategy 2002 - 2005, 2002).

1.2.1. Monographs redaction and quality control of endangered antimalarial medicinal plants - (MEAMP)

Especially as an antimalarial treatment, herbal remedies have one big advantage over synthetic drugs: there are no records showing resistance of *Plasmodium* to these remedies, probably due to the different constituents in whole plant extracts. Also herbal medicines are better accessible (higher availability and cheaper) and produce fewer negative side effects (WILLCOX and BODEKER, 2000).

Traditional medicine in general has already proven its contribution to modern medicine in the past: more than 30 % of modern medicines are derived from medicinal plants. Famous examples in the field of antimalarial drugs are artemisinin and quinine (KASILO et al., 2010).

All these reasons lead to the conclusion that research on malaria combat must also include herbal remedies, which are already used since decades by traditional healers. Their potential for helping with new developments in malaria treatment and prophylaxis is huge. A lot of research has already been done but there are some fields, who still need more attention.

An example for encouraging cross border cooperation in research and education is the Austrian partnership programme in higher education and research for development (APPEAR), a research programme located in Austria. Their central ambition is to generate knowledge relevant to development-policy, by working across national, cultural and linguistic borders (OBRECHT, 2015).

One of their thematic focuses is the topic environmental and natural resources. As a result the idea was born, to identify plants that are used in traditional medicine by local healers in Burkina Faso against malaria. MEAMP is the research framework provided by APPEAR, in which the traditional antimalarial plants of Burkina Faso were investigated, with the University of Ouagadougou as a partner-university. By a combination of botanical and ethnopharmacological

research a list of local antimalarial plant species was established. First local healers were questioned about, which plants in their opinion are the most commonly used, have the best antimalarial effects and where they think scarcity of resources is a problem. Secondly the results of the healer-interviews were compared to a list of endangered plants of Burkina Faso, which confirmed the statements of the local healers. Therefore further studies were done on these five endangered plants:

- *Argemone mexicana*
- *Cochlospermum planchonii*
- *Pavetta crassipes*
- *Securidaca longepedunculata*
- *Zanthoxylum xanthoxyloides*

Two main goals were defined: contributing to a national pharmacopoeia in Burkina Faso by writing monographs of the five chosen plants and establishing a quality control for the remedies (LAMIEN-MEDA, 2015).

Cochlospermum planchonii (C. planchonii) takes up a very central position in the pool of traditional, antimalarial remedies in Burkina Faso. It is used as a single medicinal plant but also part of two of the main combination remedies, used against malaria: *Saye* (a combination remedy prepared from *C. planchonii*, *Cassia alata* and *Phyllantus amarus*) and *N´Dribala* (a root decoction of *C. planchonii*) (YERBANGA et al., 2012).

1.3. Research question and working hypothesis

Remedies made from the plant *C. planchonii*, which is still harvested wild, are widely available to buy at pharmacies in Burkina Faso. Since there are no established procedures for quality control, there is no assurance that people (even the pharmacists themselves) are purchasing a remedy in good condition, a pure form or even made from the right plant. If there would be a practical way for the pharmacists to inspect the plants, they could make sure to only retail good quality. On one hand it is necessary to obtain knowledge about the current situation on the market and on the other hand there should be a system for quality control that is easy, fast, cheap, valid and reliable.

The diploma thesis focuses on the question whether the quality of *C. planchonii* samples available at the market have the same quality as reference samples collected in the field. The hypothesis is that thin-layer chromatography with cochloxanthines (meaning the sum of cochloxanthine and dihydrocochloxanthine) as a reference may be a suitable method for quality control directly in pharmacies of Burkina Faso. Cochloxanthine was chosen as a marker because it has been reported to be contained in the rhizomes of *Cochlospermum tinctorium (C. tinctorium)* (BALLIN et al., 2002; DIALLO and VANHAELEN, 1988), which is a close relative to *C. planchonii* and also used as an antimalarial remedy. LAMIEN-MEDA et al. (2015) isolated cochloxanthine and an additional derivative dihydrocochloxanthine, as the main carotenoids from rhizomes of *C. planchonii* and optimised a TLC-method for separating these two main carotenoids. Also these carotenoids are suspected to be the main antiplasmodial substance of *C. planchonii* (LAMIEN-MEDA et al., 2015). Besides the two main carotenoids cochloxanthine and dihydrocochloxanthine, there are two further derivatives which only occur in a very small amount. Therefore in the following cochloxanthine and dihydrocochloxanthine are referred to as cochloxanthines. For a quantitative analysis of cochloxanthines a high-performance liquid chromatography was done. Additionally the antioxidative activity and the total phenolics of *C. planchonii* and *C. tinctorium* were investigated.

1.4. Cochlospermum planchonii – the yellow jack of all trades

Family: *Cochlospermaceae* (APG-System: *Bixaceae*)

Genus: *Cochlospermum*

Species: *Cochlospermum planchonii* Hook.f. (Figure 2)

C. planchonii-leaves

C. planchonii-roots

Figure 2: C. planchonii-leaves (DAKUYO et al., 2015) and C. planchonii-roots at the herbal-market in Bobo-Dioulasso (NOVAK, Johannes).

The *Cochlospermaceae*-family is quite small with fifteen species in two genera. The genus *Cochlospermum* contains 13 tropical species, where two of them are occurring wild in West Africa: *C. planchonii* and *C. tinctorium*. (BENOIT-VICAL et al., 2003).

C. planchonii is known under a lot of different vernacular names: False cotton in English and N´Dribala in Burkina Faso (OYEN, 2010). In the Hausan language it is called "*Bálàgándáá*" or "*Bálégándéé*". In western Nigeria it is known as *"Gbehutu"* or "*Feru*" (NAFIU et al., 2011b). The Malinke folk (Senegal, Mali and Ivory Coast) calls it "*Tourogbebourou*" (VONTHRON-SÉNÉCHEAU et al., 2003)

The closest relevant to *C. planchonii* is *C. tinctorium* but in Western Burkina Faso *C. planchonii* is more commonly used (LAMIEN-MEDA et al., 2015).

1.4.1. Botanical description of *C. planchonii* and its closest relevant *C. tinctorium*

C. planchonii is mainly a shrub of about 2-2.5 m in height with a woody subterranean rootstock. In the rainy season it produces up to 2.5 m tall leafy shoots The leafs bear three to five oblong lobes which are basally connate for the half to two thirds of their length. The shape of the leaves can be described as a mixture of cordate (shape of a heart) and cuneate (triangular shape). The upper surface is dark green and the lower surface is paler and has soft hairs. The stipules are subulated (meaning awl-shaped) and about 3 mm long, the petiole is up to 10 cm long and the blade measures 6-15 cm × 7-17 cm. *C. planchonii* flowers towards the end of the rainy season, with bright yellow flowers, who are actinomorphic (star-shaped or radial), pentamerous and

usually many-seeded. The black seeds are reniform (kidney-shaped), measure 6.5-7 mm × 3.5-4 mm and have loosely attached white hairs (OYEN, 2010; LAMIEN-MEDA, 2015).

C. tinctorium is a subshrub up to 80 cm tall and has a woody subterranean rootstock producing annual leafy shoots at the end of the rainy season as well. Compared to *C. planchonii*, the leaves are also three to five lobed but have a lanceolate to oblong shape and are basally connate for up to one fourth of their length. The margins are lined with asymmetric teeth pointing forward. In the dry season after the savanna burns, *C. tinctorium* flowers with golden-yellow flowers which are usually produced near the ground level (JANSEN, 2005; LAMIEN-MEDA, 2015)

The microscopic characteristic of *C. planchonii* for a definite identification could be the isodiammetrically shaped isolated sclereids (also called stone cells) with thick walls and lignified lumen showing striation (OLOTU et al., 2011).

1.4.2. Phytochemical constituents

The quantitative analysis of *C. planchonii*-rhizome powder showed a moisture content of 7.2 %, and an ash value of 16 %. The water soluble extractive is with a value of 3.9 % higher than the alcohol (ethanol) soluble extractive value (2.9 %). These are respectable values for a crude drug (OLOTU et al., 2011).

According to a phytochemical screening and a subsequent quantification, aqueous extracts made from roots contain saponins (7.5 %), phenolics (3.16 %), alkaloids (2.92 %), steroids (0.89 %), tannins (0.15 %), flavonoids (0.07 %), phlobatannins (0.03 %), triterpenes (0.09 %) and anthraquinones (0.19 %). Minerals occurring in the plant are potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), manganese (Mn), iron (Fe), selenium (Se), copper (Cu) and a trace amount of lead (Pb) (NAFIU et al., 2011b).

Essential oils of *C. planchonii*-roots, which are used as a protection against *Anopheles* exhibited a high rate of oxygenated components with predominance of ketones and esters (OUTTARA et al., 2007). Major constituents are 3-tetradecanone (24.7 %), ethyl tetradecanoate (11.4 %) and isoamyl dodecanoate (14.1 %). But the compound 2-tridecanone with insect repellent properties only occurs with 6.8 % (BOSSOU et al., 2013). Compared to the essential oil made from the rhizomes, the leaf-essential oils contain in a significant amount monoterpenoids and a smaller amount of sesquiterpenoids. On the other hand, essential oils made from leaves do not contain

a lot of sesquiterpenes, which are present in significant quantities in rhizome-essential oil (for example β-selinene, α-selinene, and 7-diepi-α-selinene) (USMAN et al., 2013).

1.4.3. Habitat and geographical distribution

C. planchonii occurs widely throughout the tropical regions of the world and is growing primarily in dry climates (OLOTU et al., 2011). It occurs from a sea level up to 1700 m and it is distributed from Senegal eastward to Chad (OYEN, 2010).

1.4.4. General usage

In northern Sierra Leone, northern Nigeria and Burkina Faso the fibre, yielded by the stem bark of *C. planchonii* is used for making strings and ropes. Also it is used for binding mats and the floss of the fruit can be used as a stuffing. The root shows a very bright yellow, which is used as a dye in Sudan and Nigeria. But also as a food *C. planchonii* can be used. For example in Benin a powder made from the roots is put into soups and sauces. In Burkina Faso not only the people are eating the flowers but also grazing cattle seem to like the plant (OYEN, 2010).

1.5. Cochloxanthine

Cochloxanthine and its derivative dihydrocochloxanthine are the main carotenoids of *C. planchonii* and are suspected to be the leading antiplasmodial compounds of the antimalarial plant. Next to these two, a six other cochloxanthine-derivatives were found but in a much smaller amount. (LAMIEN-MEDA et al., 2015).

The molecular formula of cochloxanthine is $C_{25}H_{32}O_4$ and it has a molecular weight of 396.527 g/mol. (PUBCHEM, 2017). The 2D structure can be seen in Figure 3. (PUBCHEM (2017): Cochloxanthine; https://pubchem.ncbi.nlm.nih.gov/compound/101202074 (accessed: 13.01.2017).

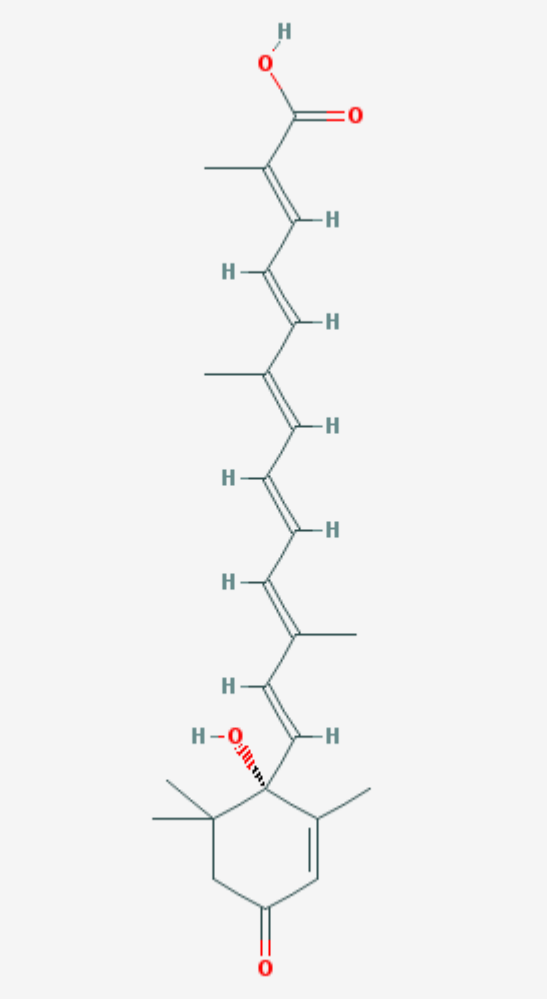


Figure 3: 2D-structure of cochloxanthine (PUBCHEM, 2017).

1.6. Ethnomedical use of *Cochlospermum planchonii*

C. planchonii-leaves –and rhizomes in different preparation forms, fare very common herbal remedies used by traditional healers for a wide spectrum of illnesses. In northern Sierra Leone a decoction of roots is drunk against gonorrhoea and in Mali it is used for the treatment of jaundice. Traditional healers in eastern Nigeria prescribe the plant against symptoms of AIDS. In Ivory Coast the root is used to treat back pains, fever and jaundice while in Senegal, it is used to treat jaundice, intestinal worms and hepatitis. A concoction of fresh root together with stem bark of *Erythrina senegalensis* is for the treatment of urinary tract infections and typhoid. Also for promoting the healing of burn wounds, dried powdered roots are applied on the skin and unprocessed roots are rubbed on snake bites. For palpitations, powdered dried leaves are

prescribed. Against fever, leaf decoctions are either drunk or used in washings. With gastrointestinal problems (diarrhoea, vomiting, dysentery, and intestinal worms) a tea made from leaves or roots is recommended. Also several preparation forms are used for the management of diabetes mellitus. A huge field of use, especially in sub-Saharan Africa is the treatment of malaria (BENOIT-VICAL et al., 2003; OYEN, 2010; TOGOLA et al, 2005).

In Burkina Faso *Saye*, a combination remedy for the treatment of malaria, is sold at pharmacies and markets. It is made from *C. planchonii*-rhizomes, *Cassia alata*-leaves and *Phyllantus amarus*-whole plant, in the ratio 2:2:1 (TRAORE et al., 2008). Also *N´Dribala* (a root decoction of *C. planchonii*) is a common traditional antimalarial medicine. Both are commercially produced by *Phyto Fla* which is a GMP-certificated laboratory, led by Dr. P. Dakuyo Zéphirin (Figure 4) and located in Banfora, Burkina Faso. Their products for the treatment of malaria are: herbal *Saye*, *Saye*-capsules and herbal *N´Dribala* (Figure 5).

Figure 4: Dr. Zéphirin Dakuyo demonstrating a self-constructed machine for producing homogenous mixtures of medicinal plants (DAKUYO et al., 2015) and the outside of the Phyto Fla laboratory.

Tisane Saye (herbal Saye) | *Gelules Saye* (Saye-capsules) | *Tisane N´Dribala* (herbal N´Dribala)

Figure 5: Products made from C. planchonii by Phyto Fla.

1.7. In vitro and in vivo therapeutic effects of C. planchonii

A respectable number of scientific studies to investigate diverse therapeutic activities of *C. planchonii* have been done on its rhizomes and leaves. The antiplasmodial effect is the main element in antimalarial research and was both *in vitro* and *in vivo* (in mice infected with *Plasmodium berghei*) tested and confirmed (VONTHRON-SÉNÉCHEAU et al., 2003; NERGARD et al., 2005; TRAORÉ, M. et al., 2008; YERBANGA et al., 2012; LAMIEN-MEDA et al., 2015). The herbal remedy even proved to be as efficient as chloroquine (although *C. planchonii* acted slower) for the treatment of uncomplicated malaria, in an area with low chloroquine-resistance (BENOIT-VICAL et al., 2003). Another important part for the protection against malaria is the control of vectors. Mosquitos have developed resistances to several synthetic insecticides, therefore the use of alternatives such as insect repellent herbals like *C. planchonii* is indicated (BOSSOU et al., 2013). Also *C. planchonii* is a promising source of new generations of trypanocidal agents, since the whole plant showed trypanocidal activity (ATAWODI, 2005). Analgesic –and anti-inflammatory effects were confirmed (AHMED, et al 2011; ANAGA and OPARAH, 2009) and lead to a lot of indications for prescribing *C. planchonii*. For example menstrual disorders, fever, back pains, urinary tract infects, etc. (OLOTU et al., 2011; USMAN et al., 2013). A wide spectrum of bacteria proved to be sensitive to *C. planchonii*. This antibacterial effect can be used for the treatment of diarrhoea, gastric ulcers, some sexual infections, urinary tract infections, fever, stomach disorders and typhoid fever. (OUTTARA et al., 2007; INNGJERDINGEN et al., 2014). The medicinal plant is also used in the treatment of infertility, since it was shown that an aqueous extract of the rhizome

has the ability to enhance spermatogenesis in male albino rats (ABU et al., 2012). Also confirmed are the antioxidative and hepatoprotective effects of *C. planchonii* (DA et al., 2014; NAFIU et al., 2011a).

2. Material and methods

2.1. Sample groups

The source material for the probed samples (n = 45) were dried parts of *C. planchonii* and *C. tinctorium*, more specifically their roots and leaves. Two main sample groups can be distinguished, by their provenance: market –and reference samples.

- Market samples (n = 28):

This part of the sample-collection was provided by the local herbal markets of Burkina Faso, from Ouagadougou and Bobo-Dioulasso and contains mostly of *C. planchonii*-roots (Table 2).

- Reference samples (n =17):

This sample group were wild-growing plants, collected by trained staff from the University of Ouagadougou in areas of the south west (Bougouriba, Bondjigu,), the upper basin (Kow, Koro, Dinderesso), the region of waterfalls (Wolokonto, Bounouna, Takaledougou) (Table 3).

Table 2: List of market-samples (with species, organ and origin).

Species	Organ	Origin
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Bobo-Dioulasso
C. planchonii	Root	Bobo-Dioulasso
C. planchonii	Root	Bobo-Dioulasso
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Leaf	Bobo-Dioulasso
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou

C. planchonii	Leaf	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Bobo-Dioulasso
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Bobo-Dioulasso
C. planchonii	Root	Ouagadougou
C. planchonii	Leaf	Bobo-Dioulasso
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Ouagadougou
C. planchonii	Root	Bobo-Dioulasso
C. planchonii	Root	Ouagadougou

Table 3: List of reference samples (with species, organ and origin).

Species	**Organ**	**Origin**
C. planchonii	Leaf	Bougouriba
C. planchonii	Root	Bougouriba
C. planchonii	Leaf	Kow
C. planchonii	Root	Koro
C. planchonii	Root	Takaledougou
C. tinctorium	Root	Dinderesso
C. tinctorium	Root	Bougouriba

C. tinctorium	Root	Wolokonto
C. tinctorium	Leaf	Takaledougou
C. tinctorium	Leaf	Bondjigu
C. planchonii	Leaf	Takaledougou
C. tinctorium	Leaf	Takaledougou
C. tinctorium	Leaf	Wolokonto
C. tinctorium	Leaf	Wolokonto
C. tinctorium	Leaf	Wolokonto
C. planchonii	Leaf	Bounouna
C. tinctorium	Leaf	Takaledougou

2.2. Preparation of the raw plant samples

In order to prepare the raw plants for the following testing procedures, they were extracted with five different solvents of differing polarity: Methanol (MeOH), ethanol (EtOH), acetone (DKM), dichloromethane (CH_2Cl_2) and water (H_2O). For extraction, they were first grinded in a mill and stored in labelled paper bags.

The extracts were filtered and stored in dark glass vials at -20°C.

2.3. Extraction

In order to transfer the finely powdered plants into the five extraction-forms the test tubes containing the samples were filled up with 10 ml of each solvent (methanol, ethanol, acetone, dichloromethane and distilled water (aqua dest.) (Sigma, Vienna, Austria) and closed with a pierced stopper.

After vortexing, the methanol-, ethanol-, acetone- and dichloromethane-dilutions were put in a sonication bath (Bandelin Sonorex RK 156 BH®, Germany) for one hour with cooling. The water-dilutions were put into a boiling water bath for thirty minutes after vortexing.

In order to get rid of the bigger plant material, the dilutions were leached through paper filters with the help of glass cones. The gained liquid was dropped into dark glasses, in which the extractions were stored at -20°C.

2.4. Thin-layer chromatography (TLC)

With TLC, a mixture of compounds can be separated into their different single components. By comparing the compounds (spots on the TLC plate) to those of known references, the spots in question can be identified or characterized.

2.4.1. Testing procedure

The different references used were cochloxanthine, ellagic acid, gallic acid and rutin. 10 mg of them was solved in 10 ml of methanol, to receive a concentration of 1 mg/ml.

In order to prepare the HPTLC Silica gel plates (glass plates 10 x 20 cm, Merck, Darmstadt, Germany) the references and samples (extractions) were applied on the plates with the help of a Linomat IV Sample Applicator (CAMAG, Muttenz, Switzerland) and syringes.

Only the water extractions needed an additional preparation step: 0.5 ml of the H_2O-samples were dried at 40°C for 12 hours and afterwards the residue was solved in 0.5 ml of 80 % methanol.

The parameter settings for the Linomat IV were:

- Plate width: 200 nm
- Start Position: 10 nm
- Band: 5 mm
- Space: 5 mm
- Sec/ml: 5 µl/g
- Volume: 15 µl for the sample-extracts and 5 µl for the references

Sorted by solvent, the sample-extracts were applied on the plates. Each plate also contained the spots of the references. After a few minutes of drying, the plates were labelled with a consecutive number and the date, and the strived mobile phase front marked. The already optimized solvent mixture for the mobile phase consisted of 77 % of ethyl acetate, 13 % of methanol and 10 % of H_2O. The plates were developed, until the mobile phase reached the before-marked strived front (at a height of 8 cm) and then dried on the TLC plate heater (CAMAG, Muttenz, Switzerland). In order to document the results, each plate was photographed. First under visible light and then under UV 254 nm and UV 366 nm.

For a check for possible alkaloids a few plates were sprayed with *Dragendorff´s reagent* (= solution of potassium bismuth iodide prepared from basic bismuth nitrate, tartaric acid and potassium iodide). Because it showed no data, this wasn´t continued for all the plates.

Every plate was sprayed with an anisaldehyde reagent preparation and also photographed under visible light and UV 254 nm and UV 366 nm. The anisaldehyde-spraying-reagent was prepared by the following pattern, whilst it is very important to mix the components in this given order:

- 1 ml of anisaldehyde-reagent
- 20 ml of 99 % acetic acid
- 170 ml of methanol
- 10 ml of concentrated sulfuric acid

The Retention factor (Rf) was then calculated for different compounds using the formula:

$Rf = D_x/D_s$

(D_x meaning the distance travelled by sample, D_s meaning the distance travelled by solvent.)

2.5. High-performance liquid chromatography (HPLC)

HPLC is a chemical method to analyse volatile and non-volatile liquid substances. The probes are not only separated into their different compounds but they can also be identified by comparison to references. By determining the concentration, it is also possible to do a quantitative analysis. In short, the HPLC has three main advantages: separation, identification and quantification of the chemical compounds of the plant samples. The HPLC-tests were

performed in collaboration with Dr. Aline Lamien-Meda and only the root-samples but not the leaf samples were examined.

2.5.1. Testing procedure

To prepare the extracts for HPLC, they had to be filtered one more time with the help of syringe filters (Rotilabo® syringe filter Nylon unsterilized, pore size 0.2 µm, Ø outer 33mm, Carl Roth GmbH, Karlsruhe, Germany). 500 µl of the sample were solved with 500 µl of methanol and then pressed through a filter. The new filtrate was put into small, flat-bottomed HPLC test vilas.

The samples were put into the autosampler of the HPLC-machine (600 S Controller, 626 Pump, In-Line Degasser AF, 717 plus autosampler, 996 Photodiode Array Detector, Waters GmbH, Vienna, Austria). 10 µl of the sample was injected and separated trough a Luna C18 column (150 × 4.6 mm i.d.; 5 µm; Phenomenex, Torrance, CA, USA) at a flow rate of 1 ml/min. As a mobile phase 0.1% formic acid (solvent A) and MeOH (solvent B) were used.

The used gradient was: from 0-15 minutes a linear gradient of 10 % B till 50 % B was used und kept for five minutes. From 20-21 minutes an increasing gradient till 100 % B was used and kept for six minutes. From 27-28 minutes a decreasing gradient till 10 % B was used and kept for seven minutes.

UV-spectra of the compounds were recorded from 200 – 600 nm and chromatograms were registered at 254 nm.

2.6. Antioxidative activity with DPPH-testing

In this test procedure, the goal is to determine the antioxidative activity of the plant extracts. The basic principle is, that DPPH (2,2-Diphenyl-1-Picrylhydrazil), which is a stable radical, reacts in the presence of antioxidative substances with reduction and therefore with decolouration. A spectrophotometer is able to measure the change in colour objectively, compared to a calibration curve with trolox ((+-)-6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid) as a standard.

2.6.1. Testing procedure

For the 2.5 mM trolox-preparation, 6.3 mg of trolox (Sigma Aldrich, Missouri, USA) was weighted into a 10ml volumetric flask, filled up with 10 ml of ethanol (96 %) (Sigma, Vienna, Austria) and solubilized in an ultrasonication bath (Bandelin Sonorex RK 156 BH®, Germany) for three minutes. It can be stored in the refrigerator for a maximum of three days.

The DPPH-preparation can only be used fresh. 15.2 mg of DPPH (Sigma-Aldrich, Missouri, USA) was weighed into a 100-ml volumetric flask and filled up with 100 ml of concentrated methanol (Sigma, Vienna, Austria). The solution was wrapped up into aluminium foil for protection against light and put (with a magnetic stir bar inside) onto the magnetic stirring device. Thereby the dilution during the testing procedure, was preserved.

For the preparation of the calibration curve Trolox and methanol are pipetted into the microplate, according to the queue in Table 4. Each combination was pipetted four times and into each hole were added 100 µl of DPPH.

Table 4: Pipetting queue schema for the calibration curve.

Trolox (µl)	**MeOH conc. (µl)**
0	100
1	99
2	98
4	96
6	94
8	92
50 (zero value)	50 (zero value)

For the preparation of the samples, 15 µl of each extract were pipetted into the micro plate and into each hole were 85 µl of methanol and at last 100 µl of DPPH added. Also every sample was pipetted four times.

Because DPPH is very sensitive to light the micro plates were than covered with a parafilm and incubated thirty minutes in a dark space. After incubation time the colour range was measured in a microplate reader (iMark Microplate Absorbance Reader®, BioRad, California, USA), with the following settings:

Reading Mode: Endpoint

Reading Speed: Fast

Wavelength: 490 nm

Mix time: 20 seconds

Mix speed: L

The measured absorbances were imported and saved as an excel-file.

2.7. Antioxidative activity with ferric-reduction-testing

The aim of this test procedure is to determine the antioxidative activity of the plant extracts by means of the Fe^3-reduction-method.

2.7.1. Testing procedure

The required reagents were prepared according to the following pattern:

- Trolox-Standard: 6.2 mg of trolox (Sigma Aldrich, Missouri, USA) were diluted in 10 ml methanol and put into the ultrasonic bath for five minutes.
- 40 mM hydrochloride acid (HCl): 1 ml of HCl 36 % were solved in 299 ml of aqua dest.
- 300 mM acetic-acid-buffer: 1.55 g of Na-CH_3COOH x 3 H_2O were solved in 8 ml of CH_3COOH and filled up with 500 ml of aqua dest.
- 2,4,6-tripyridyl-s-triazine (TPTZ) solution: 31.2 mg of TPTZ (Sigma Aldrich, Missouri, USA) was diluted in 10 ml of 40 mM HCl, put into the ultrasonic bath (Bandelin Sonorex RK 156 BH® Germany) for five minutes and covered up with aluminium foil to protect it from light.
- $FeCl_3$-solution: 0.032 g $FeCL_3$ x 6 H_20 were diluted in 10 ml of aqua dest. and also covered up with aluminium foil to protect it from light.

To prepare the working reagent, 25 ml of the acetic-acid-buffer were mixed with 2.5 ml of the TPTZ-solution and 2.5 ml of the $FeCl_3$-solution.

In order to prepare the calibration curve a concentration series of Trolox was done, according to the following Table 5 and pipetted into eprouvettes.

Table 5: Pipetting-scheme for the Trolox concentration series.

Trolox-solution (µl)	**Concentrated MeOH (µl)**
0	1000
15	985
40	960
80	920
160	840
320	680
450	550
640	360

For the calibration curve from every concentration level 6 µl were pipetted into the microplate (four repeats per calibration point). Afterwards 18 µl of aqua dest. were added. The last step was adding 180 µl of the working reagent.

In order to prepare the plates for the samples 9 µl of the extract were pipetted into the plate and mixed with 15 µl of aqua dest. At last 180 µl of the working reagent were added.

The plates than were incubated for five minute in a dark space and after that read by a microplate reader (iMark Microplate Absorbance Reader®, Bio Rad, California, USA), with a mixing time of 20 seconds and a wavelength of 593 nm.

The received results were than imported and saved as an excel-file.

2.8. Total phenolics with FCR-testing

The goal of this test procedure is the identification of the total phenolic ingredients in the plant extractions. In an alkaline milieu the phenolics react with the FCR (Folin-Ciocalteu reagent) (Merck Millipore, Darmstadt, Germany). As a result the colour of the mixture changes into blue, which can be measured objectively with a photometer. The results were than evaluated with the help of a calibration curve in caffeic acid-equivalents (mg caffeic acid/g DW).

2.8.1. Testing procedure

For preparing the standard, 10 mg of caffeic acid were weighted into a 100 ml volumetric flask and filled up with aqua dest. The flask was sealed with a pierced parafilm and heated up in a 70 °C water bath for 45 minutes, until everything was dissolved. In order to set up an alkaline milieu a sodium carbonate-solution was prepared. 35 g of Na_2CO_3 were weighted into a 100 ml volumetric flask and filled up with aqua dest. This flask was also sealed with a pierced parafilm and heated up in a 70 °C water bath, until everything was dissolved. As a calibration curve, an increasing row of caffeic acid (listed in Table 6) was pipetted four times into a microtiter plate.

Table 6: Pipetting row for the calibration curve

Caffeic acid (0,1mg/ml in µl)	**Aqua dest. (µl)**
0 (Zero value)	110 (Zero value)
2	108
5	105
8	102
10	100
15	95
20	90
25	85

The average of these four measurements, minus the zero value, were used for the calibration curve. Thereby the amount of total phenolics in the plant sample extractions was estimated,

expressed in caffeic acid equivalents. Finally, the samples were prepared according to the following scheme, in this exact order:

- 100 µl of distilled water
- 10 µl of sample extract
- 5 µl of FCR
- 10 µl of sodium carbonate solution
- 125 µl of distilled water

After adding the FCR the microtiter plate was shook for three minutes, to make sure everything is mixed well. In order to get the zero value the 10 µl of sample extract were exchanged with only 10 µl of the respective solvent (MeOH, EtOH, Acetone, CH_2Cl_2 and H_2O). After the microplate was filled, it was covered with parafilm and put in the dark for one hour. The microplate reader (iMark Microplate Absorbance Reader®, Bio Rad, California, USA) was used with the following adjusted parameters:

Reading Mode: Endpoint

Reading Speed: Fast

Wavelength: 750 nm

Mix Time: 20 seconds

Mix Speed: L

The received results were imported and saved as an excel-file.

2.9. Statistical analysis

The statistical analysis was done with IBM SPSS Statistics Version 23 (IBM, Vienna, Austria). The used method was ANOVA (=analysis of variance).

3. Results

3.1. Identity specification with Thin-layer chromatography (TLC)

3.1.1. Cochloxanthines as a reference marker

A cochloxanthine-spot on the TLC-plates in visible light was found very clearly and with a consistent pattern in all the samples obtained from roots of *C. planchonii*, as well as from *C. tinctorium*, in all five different extraction solvents (MeOH, EtOH, CH_2Cl_2, Acetone and H_2O) (Figure 6). Only the extracts made with H_2O which is the most polar solvent of the five tested, produced much paler and sometimes not very easy detectible spots (for example see the TLC-plates with H_2O-extracts in Figure 6 and Figure 10).

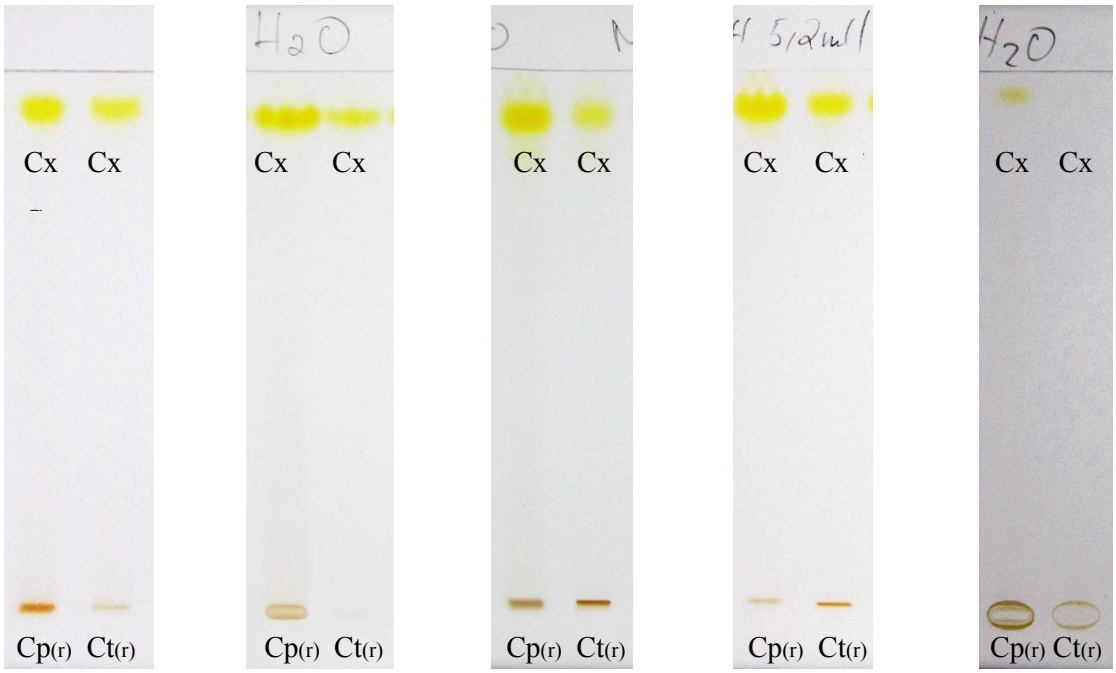
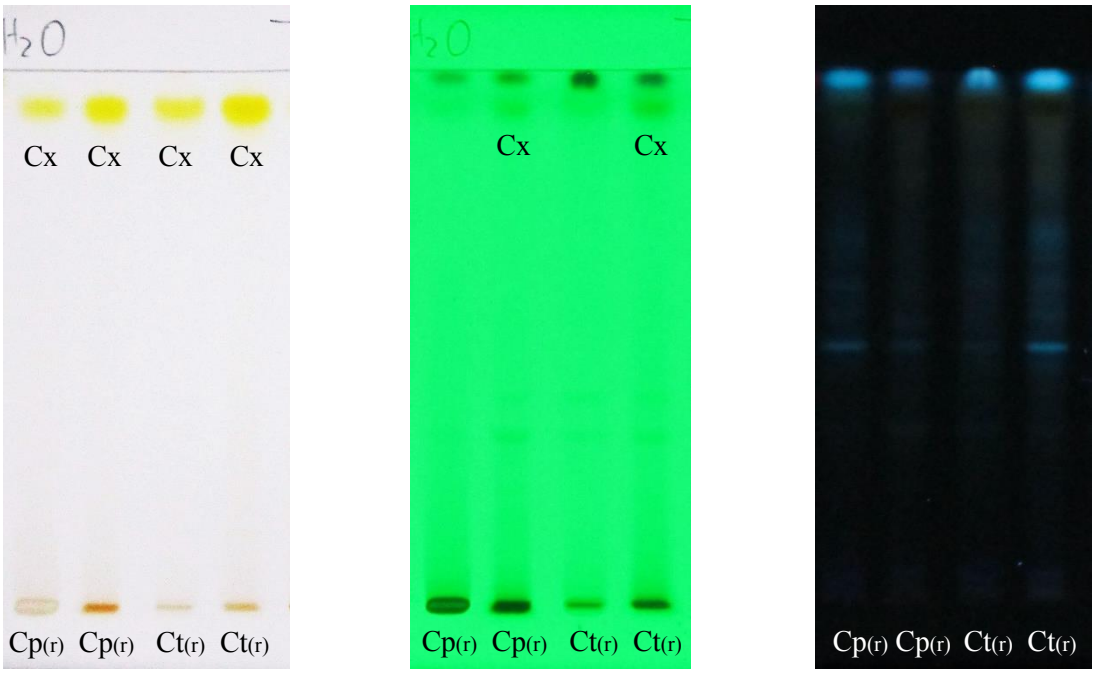


Figure 6: Comparison of cochloxanthine (Cx)-spots in five different extraction-forms: MeOH, EtOH, Acetone, CH_2Cl_2 and H_2O made from C. planchonii-root (Cp(r)) and C. tinctorium-root (Ct(r)).

With the chosen mobile phase (ethyl acetate, MeOH and H_2O) it was possible to separate the cochloxanthines very well. The calculated retention factor (Rf) for the cochloxanthine-spot was 0.91, which is close to the upper solvent line and the carotenoids appeared in a bright

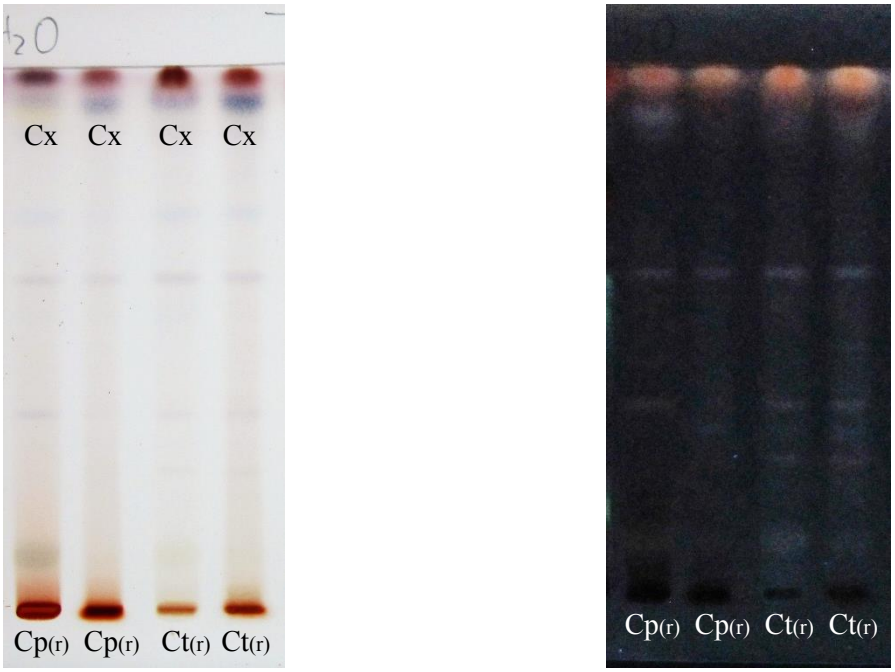
yellow colour. At 256 nm the cochloxanthine-spot appeared faint and at 366 nm not good to distinguish from other compounds (Figure 7). Therefore, observing under 256 and 366 nm didn´t provide more information.



A B C

Figure 7: MeOH-extracts of C. planchonii (Cp(r)) and C. tinctorium (Ct(r)) roots with cochloxanthine (Cx)-spots in visible light (A), in 254 nm (B) and in 366 nm (C).

After spraying the TLC-plate with anisaldehyde the spots for the cochloxanthines appeared in visible light in a pale light blue and also couldn´t be distinguished in 366 nm (Figure 8).

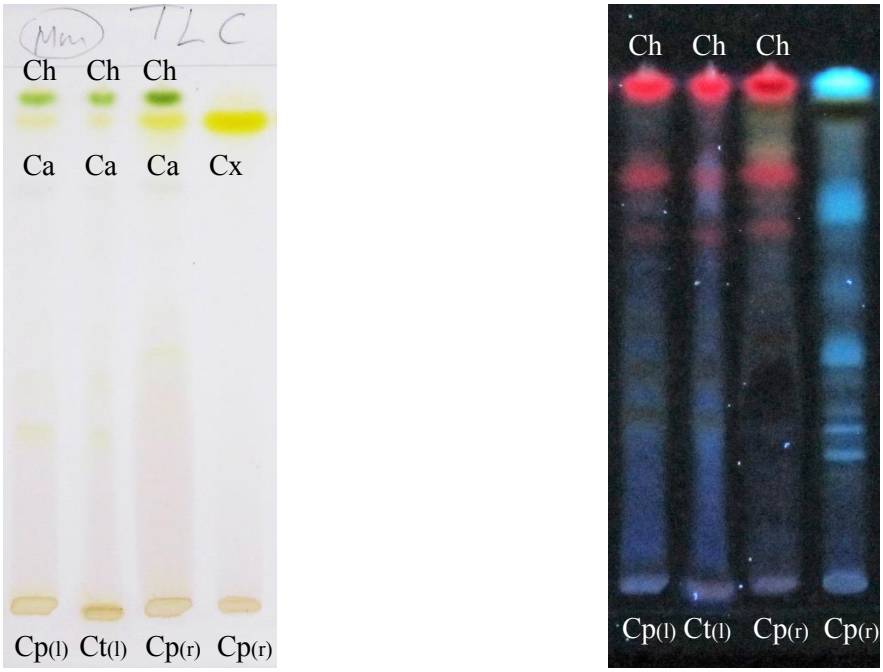


A B

Figure 8: MeOH-extracts of C. planchonii (Cp(r)) and C. tinctorium (Ct(r)) roots with cochloxanthine (Cx)-spots in visible light (A) and in 366 nm (B) after spraying with Anisaldehyde reagent.

The assumption that there are no carotenoids in the leaves of *C. planchonii* or *C. tinctorium* couldn´t be confirmed totally. In both of the *Cochlospermaceae* leaf extracts, there was a pale yellow spot with the same Rf-value as the cochloxanthine-spots in the root-samples. The fact of it being yellow, leads to the assumption that it could be an accumulation of various carotenoids. Furthermore, it had the same Rf-value as the cochloxanthine-reference. Therefore it is very likely that those spot is also a mixture of cochloxanthines (Figure 9).

The leave samples produced a spot close to the one of the cochloxanthines with only a slightly higher Rf-value: 0.95. The bright green colour in visible light and bright red in 366 nm suggests the spot to be chlorophyll. However, this spot was easy to distinguish from the cochloxanthine-spot because of the characteristic colours (Figure 9).



A B

Figure 9: MeOH-extracts of C. planchonii-leaves (Cp(l)) –and roots (Cp(r)) and C. tinctorium-leaves (Ct(l)) with carotenoids (Ca), cochloxanthine (Cx) and chlorophyll (Ch) in visible light (A) and in 366 nm (B).

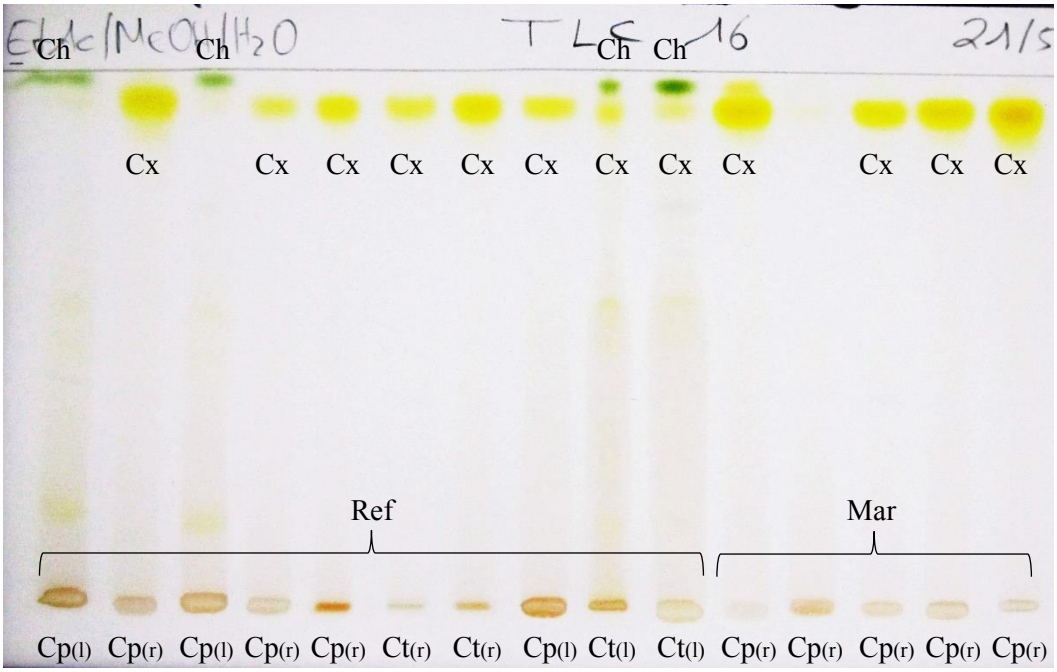
Besides cochloxanthines, other tested references were gallic acid, ellagic acid and rutin, in order to see if they could also be used to determine whether the investigated plant is a *Cochlospermaceae*. Neither of the three tested alternatives to cochloxanthines could be detected in root –or leaf samples of *C. planchonii* or *C. tinctorium*.

In conclusion cochloxanthines are a consistent marker for the identification of *C. planchonii* and *C. tinctorium*.

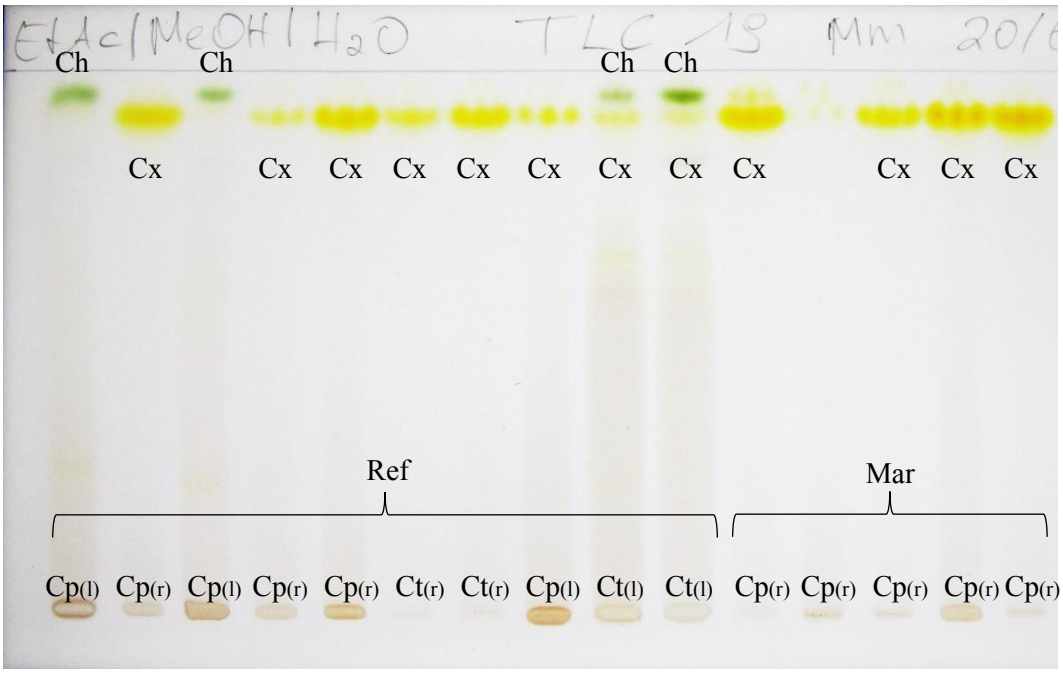
3.1.2. Quality of market samples compared to reference samples in TLC

The TLC-plates for the samples, collected from trained persons in the field (= reference samples) and the samples purchased at the various local pharmacies (= market samples) were compared to each other. Both sample-groups, independently of the extraction form or the wavelength of light the plates were viewed in showed the same consistent pattern for cochloxanthine-spots in the TLC. Therefore, the market samples are not different to the reference samples, regarding the TLC. Since cochloxanthine is characteristic for

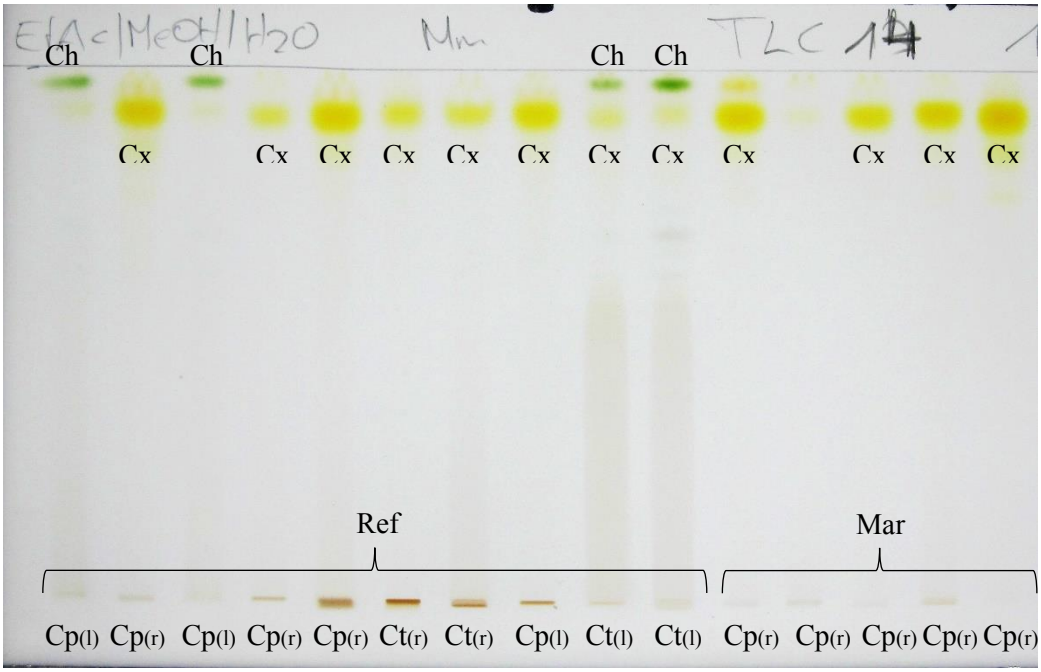
Cochlospermaceae there is a certainty that the market samples are in fact *C. planchonii* or *C. tinctorium* (Figure 10). Only two *C. planchonii*-root samples from the market (market-samples *n*=28) in Ouagadougou did not produce a spot for cochloxanthines.


Ch
Ch
Ch
Ch
Cx
Ref
Mar
Cp(l) Cp(r) Cp(l) Cp(r) Cp(r) Ct(r) Ct(r) Cp(l) Ct(l) Ct(l) Cp(r) Cp(r) Cp(r) Cp(r) Cp(r)

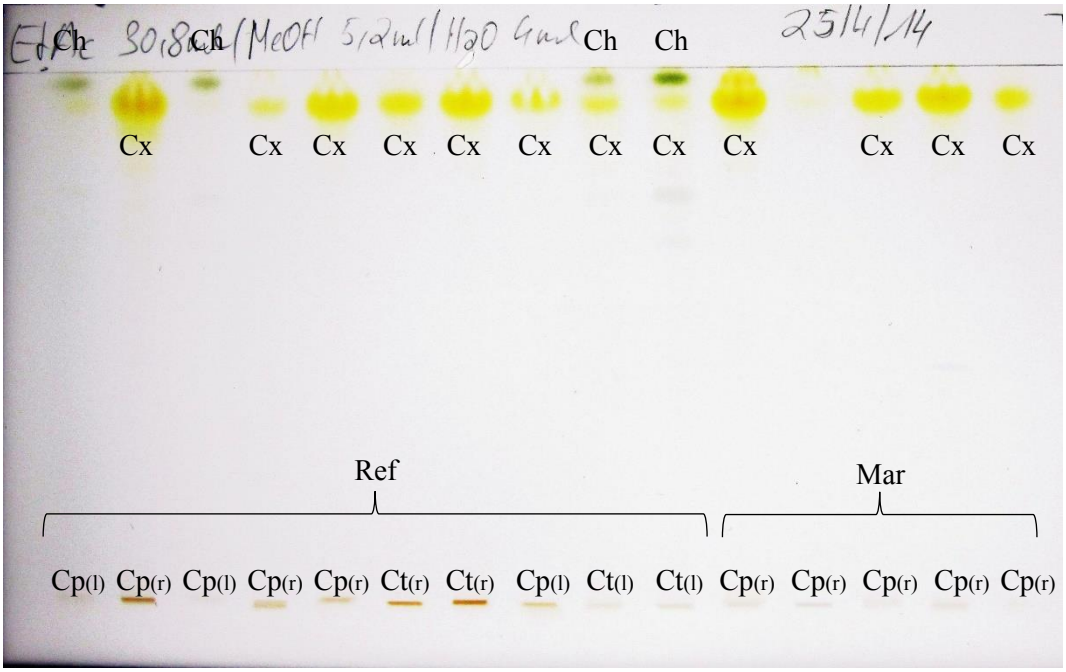
MeOH


Ch
Ch
Ch
Ch
Cx
Ref
Mar
Cp(l) Cp(r) Cp(l) Cp(r) Cp(r) Ct(r) Ct(r) Cp(l) Ct(l) Ct(l) Cp(r) Cp(r) Cp(r) Cp(r) Cp(r)

EtOH



Acetone



CH_2Cl_2

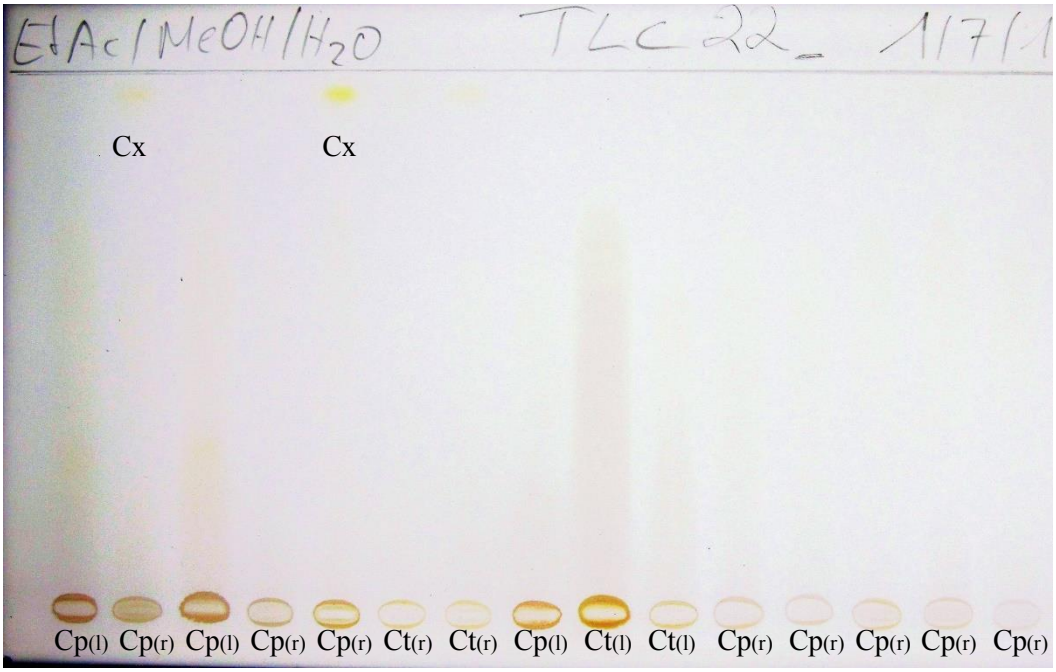


Figure 10: Comparison of market (Mar) –and reference (Ref) samples made from MeOH, EtOH, acetone, CH_2Cl_2 –and H_2O-extracts of C. planchonii-roots (Cp(r)), C. planchonii-leaves (Cp(l), C. tinctorium-roots (Ct(r)) and C. tinctorium-leaves (Ct(l)) with cochloxanthine (Cx)-spots and chlorophyll-spots in visible light.

3.2. HPLC

Only the root samples but not the leaf samples were tested with HPLC.

3.2.1. Extractability for cochloxanthines

The solvents were tested for their extractability for cochloxanthine, dihydrocochloxanthine and six other found cochloxanthine-derivatives which are contained in a much smaller proportion than the two main carotenoids. Since MeOH showed the best results in preliminary studies, its extractability was chosen to be 100 %. As can be seen in Figure 11 acetone, CH_2Cl_2 and EtOH showed good results, similar to MeOH for all of the carotenoids. Acetone delivered extractabilities for the sum of cochloxanthines (cochloxanthine, Dihydrochloxanthine and six cochloxanthine derivatives) with a mean value of 92% (± 0.114), CH_2Cl_2-extracts 97% (± 0.080) and EtOH-extracts 100%. However, the H_2O-extracts only were able to extract a small percentage of cochloxanthines with a mean value of 4.4% (± 0.035).

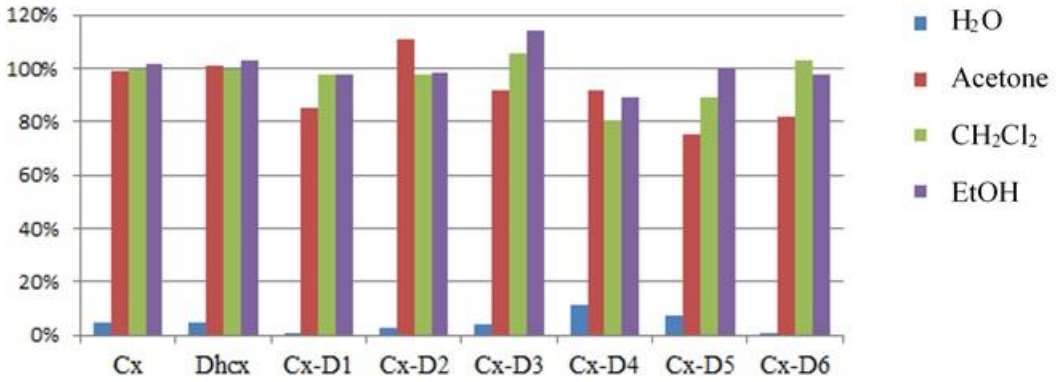


Figure 11: Extractability of different solvents (H_20, Acetone, CH_2CL_2 and EtOH) for Cochloxanthine (Cx), Dihydrocochloxanthine (Dhcx) and various Cochloxanthine-derivatives (Cx-D1-Cx-D6).

3.2.2. Amount of the carotenoids cochloxanthine, dihydrocochloxanthine and other cochloxanthine-derivatives

Dihydrocochloxanthine and cochloxanthine proofed to be the major carotenoids in *C. planchonii* and *C. tinctorium*. They showed a high accumulation-variability (Table 7).

*Table 7: Mean contents of Dihydrocochloxanthine (mg/100 g), Cochloxanthine (in mg/100 g) and Cochloxanthine-derivatives of C. planchonii-reference-samples (Ref(Cp)), C. tinctorium-reference-samples (Ref(Ct)), market-samples from Bobo Dioulasso (Mar(b)) and market-samples from Ouagadougou (Mar(o)) in five different solvents (MeOH, EtOH, Acetone, CH_2Cl_2 and H_2O). *Cochloxanthine-derivatives were estimated in cochloxanthine equivalent per 100 g of dry weight.*

Solvent	Origin	Dihydro-cochloxanthine (mg/100 g)	Cochloxanthine (mg/100 g)	Cochloxanthine -derivatives*
MeOH	Ref (Cp)	171.4	57.9	1.3
	Ref (Ct)	64.2	38.3	3.7
	Mar (b)	145.7	61.5	3.0
	Mar (o)	112.6	48.7	1.8
EtOH	Ref (Cp)	272.2	85.3	3.2
	Ref (Ct)	58.2	34.0	3.6
	Mar (b)	116.0	39.6	3.1
	Mar (o)	112.0	50.5	2.0
Acetone	Ref (Cp)	190.3	68.3	4.6
	Ref (Ct)	68.7	40.9	4.1
	Mar (b)	127.2	42.5	3.4
	Mar (o)	124.5	60.0	1.9
CH_2Cl_2	Ref (Cp)	129.5	37.4	4.1
	Ref (Ct)	53.42	31.7	3.7
	Mar (b)	193.2	76.2	3.7
	Mar (o)	152.3	76.0	1.6
H_2O	Ref (Cp)	33.2	12.8	2.2
	Ref (Ct)	10.3	9.0	1.0
	Mar (b)	2.3	3.0	0.4
	Mar (o)	0.9	2.1	tr.

Dihydrocochloxanthine was the major carotenoid in the reference-samples, as well as in the market-samples, independent of the solvent. The mean value (calculated from all five extraction forms of the root samples collected in the field and from the market) of the dihydrocochloxanthine was with 107 mg/100 g more than two times higher than the cochloxanthine (44 mg/100 g). The only exception where cochloxanthine was contained in a higher amount, than dihydrocochloxanthine were the market-samples in H_2O-extracts from Bobo Dioulasso and Ouagadougou.

Regarding the species, it was obvious that the sum of carotenoids was higher in *C. planchonii* (with a mean value of 72 mg/100 g) than in *C. tinctorium* (with a mean value of 28 mg/100 g).

Between the solvents there were only light differences in the sum of carotenoids which approximately correlate with the increase of the solvent-polarity (Table 8). Again, only the H_2O-extracts delivered much smaller amounts, than the other four solvents. In Table 8 the solvents are listed in order of their increasing polarity: CH_2Cl_2, Acetone, EtOH, MeOH and H_2O.

Table 8: Solvents listed in order of their increasing polarity with the mean values for the sum of carotenoids in mg/100 g.

Solvent	**Carotenoids (mg/100 g)**
CH_2Cl_2	63.57
Acetone	61.37
EtOH	64.98
MeOH	59.18
H_2O	6,43

3.3. Antioxidative activity (with DPPH and Ferric-Reduction) and total Phenolics

The mean values for all three test procedures were: for ferric reduction 0.04 - 5.86 mg Trolox/g, for the capacity of DPPH radical scavenging 0.04 - 1.36 Trolox/g and for total phenolic content 0.51 - 3.7 caffeic acid equivalents/g. The DPPH –and Ferric-reduction methods are both testing for the antioxidative activity but delivered disparate results for the same samples. They are not correlating with each other because those two methods are measuring the antioxidative activity on different chemical levels.

All together the leaf samples showed a higher antioxidative activity with ferric-reduction testing, as can be seen in Table 9. Also, the H_2O-extracts showed significantly higher values than the other four (less polar) solvents (Table 9 and 10). *C. planchonii* and *C. tinctorium* both showed moderate antioxidative activity.

Table 9: Fe3-reduction (mg Trolox/g), DPPH (Trolox/g) –and total phenolics (mg caffeic acid/g) mean values and standard derivation (s.d.) of the reference samples of C. planchonii-roots –and leaves and C. tinctorium-roots –and leaves. No DPPH-test were done with MeOH-extracts.

Solvent	**Species**	**Organ**	***n***	**Fe^3 ± s.d.**	**DPPH ± s.d.**	**Total phenolics ± s.d.**
MeOH	*C. planchonii*	Roots	3	0.994 ± 0.343	-	1.213 ± 0.207
	C. planchonii	Leaves	4	1.386 ± 0.672	-	1.211 ± 0.672
	C. tinctorium	Roots	3	0.942 ± 1.071	-	1.995 ± 1.393
	C. tinctorium	Leaves	7	1.155 ± 1.165	-	1.558 ± 0.991
EtOH	*C. planchonii*	Roots	3	1.213 ± 0.371	1.285 ± 0.789	3.700 ± 2.910
	C. planchonii	Leaves	4	0.624 ± 0.146	1.339 ± 1.688	1.882 ± 1.669
	C. tinctorium	Roots	3	1.231 ± 1.676	0.513 ± 0.080	1.170 ± 0.744
	C. tinctorium	Leaves	7	1.145 ± 1.285	0.210 ± 0.110	3.698 ± 2.889
Acetone	*C. planchonii*	Roots	3	0.935 ± 0.381	1.286 ± 0.790	1.121 ± 1.065
	C. planchonii	Leaves	4	1.990 ± 0.623	1.320 ± 1.706	1.164 ± 0.513
	C. tinctorium	Roots	3	0.982 ± 0.938	0.513 ± 0.080	1.021 ± 0.799
	C. tinctorium	Leaves	7	2.983 ± 1.531	0.181 ± 0.130	2.304 ± 1.320
H_2O	*C. planchonii*	Roots	3	2.834 ± 0.317	0.681 ± 0.330	1.079 ± 0.162
	C. planchonii	Leaves	4	5.858 ± 2.043	1.359 ± 1.936	2.375 ± 1.725
	C. tinctorium	Roots	3	1.628 ± 0.989	0.173 ± 0.077	0.509 ± 0.318
	C. tinctorium	Leaves	7	3.834 ± 4.6039	0.117 ± 0.121	1.547 ± 1.851

Table 10: Fe3-reduction (mg Trolox/g), DPPH (Trolox/g) –and total phenolics (mg caffeic acid/g) mean values and standard derivation (s.d.) of the market samples (from Ouagadougou and Bobo-Dioulasso) of C. planchonii-root samples. No DPPH-tests were done with MeOH-extracts.

Solvent	Origin	n	Fe^3 ± s.d.	DPPH ± s.d.	Total phenolics ± s.d.
MeOH	Ouagadougou	19	1.518 ± 0.901		0.974 ± 0.802
	Bobo-Dioulasso	6	1.424 ± 0.735		0.969 ± 0.523
EtOH	Ouagadougou	19	0.039 ± 0.391	0.039 ± 0.069	1.724 ± 1.616
	Bobo-Dioulasso	6	1.434 ± 1.003	0.053 ± 0.041	2.481 ± 2.756
Acetone	Ouagadougou	19	0.428 ± 0.279	0.044 ± 0.090	1.236 ± 0.833
	Bobo-Dioulasso	6	1.055 ± 0.743	0.038 ± 0.018	1.058 ± 0.478
H_2O	Ouagadougou	19	1.407 ± 0.846	0.044 ± 0.031	0.879 ± 0.720
	Bobo-Dioulasso	6	1.228 ± 0.561	0.059 ± 0.034	0.544 ± 0.309

4. Discussion

Various studies were already done to prove the antiplasmodial activity of *C. tinctorium* and *C. planchonii*. For example, LAMIEN-MEDA, et al. (2015) were able to show that extracts from both the plants and also the two isolated carotenoids cochloxanthine and dihydrocochloxanthine possess antiplasmodial activity. Since *C. planchonii* and a little smaller amount of *C. tinctorium* are widely sold at markets in Burkina Faso as antimalarial remedies, there is a need for a quality surveillance system to determine on one hand the identity of the plants and on the other hand the presence of antiplasmodial carotenoids. Since dihydrocochloxanthine and cochloxanthine are specific for the genus *Cochlospermaceae*, cochloxanthine was chosen as a marker.

The TLC-system as described in this thesis proved to be a practical method for a quality control of the medicinal plant *C. planchonii*. The cochloxanthine-spots are a clear characteristic for *C. planchonii*-roots and easy to obtain, since the spots appear in a bright yellow with a consistent Rf-value. *C. tinctorium*-roots produce exactly the same cochloxanthine-spots as *C. planchonii*-roots, therefore it is not possible to distinguish these two *Cochlospermaceae* by means of cochloxanthine as a marker from each other. This can be overlooked simply because only these two species can be found in the sub-Saharan area and both of them contain antiplasmodial carotenoids and therefore have a therapeutic effect on malaria patients. Also, the leaves of the two antimalarial plants produce the same cochloxanthine-spot but the spots were much paler due to very low amounts. Therefore it is possible to identify leaves of *C. planchonii* and *C. tinctorium* as well but because of the paleness of the spot, mistakes could be made.

The estimated Rf-value 0.91 for the cochloxanthine-spots in this TLC-system is clearly higher than described in earlier literature (for example 0.53 for cochloxanthine and 0.49 for dihydrocochloxanthine in LAMIEN-MEDA et al., 2015) because a different solvent-system was used in this study: 10 % aqua dest., 13 % methanol and 77 % ethyl acetate. The advantage of this composition is its ability to separate cochloxanthines very well from other plant compounds. On the other hand a disadvantage is the Rf-value being close to the solvent front. Therefore the cochloxanthines are accumulating in one spot (most likely consisting of the two

main carotenoids cochloxanthine and dihydrocochloxanthine and a small amount of other carotenoids) and not separated further.

The TLC-method as a quality control is not just suitable for a modern laboratory but can also simply be done by remedy-selling pharmacists in Burkina Faso. The only required materials are TLC-plates, pipettes and the ingredients for the described solvent (MeOH, ethyl acetate and H_2O), which are easy to procure. Furthermore, TLC is a fast, cheap, valid and reliable method and can be learned also by people who are not specialists in the field. Solely the reference marker cochloxanthine in a pure form could pose a problem because it is not commercially available in West Africa. This problem could be solved either by the government providing it or by transferring knowledge about how this reference looks on a TLC-plate. For example the pictures and the estimated Rf-value in this diploma thesis could be used as a template of how cochloxanthines appear on a TLC-plate if the described extract-concentration and solvent-composition are used.

It should be considered that the system was just tested for N´Dribala (*C. planchonii*-roots) and *C. planchonii*-leaves and that it is not applicable for the combination remedy Saye, so only the *Cochlospermum*-part can be tested with cochloxanthines, not the other two plants (*Cassia alata* and *Phyllantus amarus*).

The polarity of the chemical used for the extraction plays a big role for the quality of the TLC-results. Also the lower boiling temperature a chemical has, the faster it evaporates at room temperature which is an advantage for producing clear spots. Due to evaporation, a reduced amount of the chemical used for extraction is running with the solvent-system in the TLC and can´t interfere with the results. Cochloxanthines are better soluble in more apolar substances because carotenoids are polyhydrocarbons and therefore mostly apolar. Out of the five tested extraction-forms (MeOH, EtOH, Acetone, CH_2Cl_2 and H_2O), CH_2Cl_2 is the most apolar one ((Eτ(30): 171.8 kJ/mol) and also has the lowest boiling point (40 °C). H_2O on the other hand is very polar ((Eτ(30): 263.8 kJ/mol) and has the highest boiling temperature (100 °C), which explains why it has the lowest extractability for carotenoids. Acetone is close to CH_2Cl_2 ((Eτ(30): 176.4 kJ/mol and 56 °C) and EtOH and MeOH are ranging in the middle of this polarity row (EtOH: Eτ(30): 216.9 kJ/mol and 78.2 °C; MeOH: Eτ(30): 232.0 kJ/mol and 64.6 °C) (REICHARDT, 2016; CHEMIDPLUS, 2016). CH_2Cl_2 and Acetone are not easily

available in Burkina Faso and H_2O has the least suitable chemical characteristics. EtOH and MeOH combine good availability and acceptable chemical characteristics and are therefore the most practically solvents on set. Solely the expiration date with EtOH or MeOH should be considered, since cochloxanthines tend to decompose.

For examining the spots on the TLC plates the view under visible light provided enough information and the view under UV 254 and UV 366 didn´t deliver more data. Also spraying the plate with anisaldehyde was not necessary to offer more info.

Hence, regarding the TLC for quality control on site in Africa, the system can and should be kept simple: EtOH –or MeOH-extracts of the samples and a view under solely visible light for evaluating the results.

At the markets in Burkina Faso not only *C. planchonii* –and *C. tinctorium*- roots are purchasable but also dried leaves or mixtures of roots and leaves of these plants are sold as antimalarial remedies. Unexpectedly the leave-samples seem to contain cochloxanthines respectively carotenoids because they produced the same yellow spot with the same Rf-value as the root-samples in the TLC, only much paler. Because of that fact and because cochloxanthines provide the antiplasmodial activity of the plant, it is acceptable to allow selling leaves as remedies as well. All though it seems likely that the roots contain a much bigger amount of cochloxanthines and should therefore be preferred.

The market-samples investigated in this study showed the same good quality, as the reference-samples. They all produced the same spots for cochloxanthines in the TLC, therefore those samples were definitely identified as *C. planchonii* or *C. tinctorium*. Only two claimed *C. planchonii*-root samples, obtained from a market in Ouagadougou couldn´t be identified as a *Cochlospermaceae* because no cochloxanthine-spot appeared in all five different extraction-forms. Also in HPLC the market-samples contained approximately the same amount of cochloxanthine and dihydrocochloxanthine as the reference-samples.

This currently good quality found in the market samples is very reassuring, especially because some synthetic antimalarial drugs were found to be of poor quality (TIPKE et al., 2008). In order to maintain the good state of antimalarial plants, there should be an obligatory procedure for a uniform and comprehensive monitoring system. Otherwise there is a risk of decreasing

quality, due to illicit trades or simply because of unintentional misidentification of the *Cochlospermaceae*. Since the plants are not easy to identify macroscopic, as soon as they are dried and processed, the TLC-system described in this diploma thesis offers a safe method for quality surveillance. Efforts by the government in this regard are already in progress. The HPLC-system used in this study, proved to be a good method for quantifying cochloxanthines in the plants but is not necessary if the goal is to just determine the quality of *Cochlospermaceae*. Also the HPLC is not practically for daily use in Burkina Faso because the required machines are not easily available on site, experts are needed for performing it and the method all together is much more expensive than a TLC.

In Burkina Faso the remedies *C. planchonii* and *C. tinctorium* are mostly sold as dried plant material and are prepared as a water decoction for malaria patients. But since the HPLC showed that H_2O has by far the lowest extractability for cochloxanthines, compared to the other tested solvents, the preparation as a tee could be reconsidered. Water decoction, done with good quality plants probably contain a big enough amount of cochloxanthines to gain a therapeutic dosage but with for example a preparation on the basis of ethanol it would be safer and have a more stable content of the antiplasmodial compounds. Also the HPLC showed, that the amount of carotenoids in the two medicinal plants is very variable in all five extract-forms. Since the carotenoids are claimed to be responsible for the antiplasmodial effects, it would be good to try and standardise the contents of cochloxanthines.

In the antioxidative assay of *C. planchonii* and *C. tinctorium*, the two different testing systems DPPH and FRAP delivered dissimilar results because they both work on other chemical levels. DPPH is a stable radical and reacts in the presence of antioxidative potent substances with reduction and therefore with decolouration, measured at 490 nm. When the results are evaluated, it should be taken into consideration that antioxidant compounds which absorb at a similar wavelength could interfere with the results. FRAP works without any free radicals but the reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidants is measured at a wavelength of 593 nm (PRIOR, et al., 2005; HUANG, et al., 2005). Regarding the antioxidant activity of *C. planchonii* and *C. tinctorium*, H_2O-extracts had the highest potential to inhibit oxidation. H_2O is the most polar solvent of the five tested ones and molecules with antioxidative activity are also mostly polar, therefore a water decoction is able to extract the most antioxidants. But

since the purpose of *C. planchonii* and *C. tinctorium*-remedies is not focused on their antioxidative properties but on their antiplasmodial effects, this should not be a reason to prefer H_2O-extracts.

It seems very realistic that cochloxanthine and dihydrocochloxanthine are not the only antiplasmodial active substances. With the HPLC six other minor cochloxanthines-derivatives were found and it seems very likely that they also possess antiplasmodial activity. Perhaps these minor carotenoids are occurring in the leaves of the plants and are responsible for the paler cochloxanthine-spots in the TLC. Also the possible existence of other synergistic substances (helping or amplifying) cannot be discarded, especially because in the study of LAMIEN-MEDA, et al. (2015), the CH_2Cl_2-extracts had a better IC50 than isolated cochloxanthine and chloroquine. For better understanding the antimalarial mechanisms further studies should be done on these unknown substances.

If one looks at the various areas of use for the medicinal plant *C. planchonii* and its already proven therapeutic effects, such as analgesic, anti-inflammatory, antibacterial –and hepatoprotective effects, it raises the question whether the plant is simply treating malaria with eradicating the *Plasmodia* or also with treating the symptoms of malaria.

The idea for studying *C. planchonii* emerged because local traditional healers in the area of Burkina Faso claimed it to be a strong antimalarial remedy. The dialogue and knowledge transfer between traditional healers and scientists should be promoted and encouraged, since the two parties can learn a lot from each other. There is a huge need for new ways of treating and preventing the dreadful disease malaria and maybe nature can provide the desired answers.

5. Summery/Zusammenfassung

The pathogen of malaria *Plasmodium falciparum* is developing more and more resistances for current synthetic drugs and therefore there is a huge need for alternative ways of treating the deadly disease. *Cochlospermum planchonii* is an antimalarial remedy, sold at herbal markets and pharmacies in Burkina Faso. The aim of this study was to develop a quality control system for identification of the plant and verification of it containing the antiplasmodial carotenoids cochloxanthine and dihydrocochloxanthine. The tested plant samples were dried roots and leaves of *C. planchonii* and *C. tinctorium* obtained from herbal markets in Ouagadougou and Bodo Dioulasso (n=28) and collected by experts in the field (n=17), each one prepared in five different extract-forms: Methanol (MeOH), ethanol (EtOH), acetone, dichloromethane (CH_2Cl_2) and water (H_2O). The Thin-layer chromatography (TLC) with cochloxanthine as a reference marker proved to be a practical and clear method for verifying the plant sample in question being a *Cochlospermaceae*. With the High-performance liquid chromatography (HPLC) the amount of the main carotenoids cochloxanthines, dihydrocochloxanthines and six minor cochloxanthine-derivatives was measured in the root-samples. In general, the carotenoids showed a high variability in accumulation and the most polar solvent H_2O had by far the lowest extractability for cochloxanthines. Furthermore, an antioxidative assay was done with DPPH and FRAP and the amount of total phenolics were measured.

The developed TLC-system can also easily be done by pharmacists on site in Burkina Faso. In order to obtain the currently found good quality of the herbal remedies *C. planchonii* and *C. tinctorium* it should be considered as an obligatory and comprehensive quality monitoring system.

Der Erreger der Malaria *Plasmodium falciparum* entwickelt immer mehr Resistenzen gegenüber gängiger synthetischer Medikamente und dadurch ist ein großer Bedarf an alternativen Therapiemöglichkeiten für die tödliche Krankheit entstanden. *Cochlospermum planchonii* ist ein pflanzliches Heilmittel gegen Malaria, das in Apotheken und Kräutermärkten in Burkina Faso verkauft wird. Das Ziel dieser Studien war es, ein System zur Qualitätskontrolle zu entwickeln, um die eindeutige Identifikation der Pflanze und die Anwesenheit von Cochloxanthinen und Dihydrocochloxanthinen nachzuweisen. Die getesteten

Pflanzenproben waren getrocknete Wurzeln und Blätter von *C. planchonii* und *C. tinctorium*, einerseits bei Kräutermärkten in Ouagadougou und Bobo-Dioulasso erhältliche (*n*=28) und andererseits von Experten in der Wildnis gesammelte (*n*=17). Jede Probe wurde in fünf verschiedenen Extraktionsformen getestet: Methanol (MeOH), Ethanol (EtOH), Azeton, Dichlormethan (CH_2Cl_2) und Wasser (H_2O). Die Dünnschichtchromatographie (DC) mit Cochloxanthin als Referenzmarker stellte sich als praktische und eindeutig Methode heraus, um die fraglichen Pflanzenproben als *Cochlospermaceae* zu identifizieren. Mit der Hochleistungsflüssigkeitschromatographie (HPLC) wurde der Gehalt an den Hauptcarotenoiden Cochloxanthin, Dihydrocochloxanthin und sechs kleineren Cochloxanthin-Derivaten in den Wurzel-Proben gemessen. Es stellte sich heraus, dass die Carotenoide eine hohe Variabilität in ihrer Anhäufung in der Pflanze haben und dass H_2O eine viel schlechtere Extraktionsfähigkeit für Cochloxanthin hat als die anderen vier Lösungsmittel. Zusätzlich wurden zwei antioxidative Essays (die DPPH- und die Eisen-Reduktionsmethode) durchgeführt und der Gehalt der Gesamtphenole bestimmt.

Die entwickelte DC-Methode kann jederzeit mit geringem Aufwand von Pharmazeuten vor Ort in Burkina Faso durchgeführt werden. Um die aktuell gute Qualität der pflanzlichen Heilmittel *C. planchonii* und *C. tinctorium* aufrecht zu erhalten, sollte diese Methode als verpflichtendes und flächendeckendes Qualitätsprüfungssystem in Betracht gezogen werden.

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8. List of Figures

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10. Abbreviations

ACT	Artemisinin-based combination therapy
APPEAR	Austrian partnership programme in higher education and research development
Aqua dest.	Distilled water
C. planchonii	*Cochlospermum planchonii*
C. tinctorium	*Cochlospermum tinctorium*
Ca	Carotinoids
Ch	Chlorophyll
CH_2Cl_2	Dichlormethan
Cp(l)	*Cochlospermum planchonii*-leaves
Cp(r)	*Cochlospermum planchonii*-roots
Ct(l)	*Cochlospermum tinctorium*-leaves
Ct(r)	*Cochlospermum tinctorium*-roots
Cx	Cochloxanthines
Cx-D	Cochloxanthine-derivatives
Dhcx	Dihydrochloxanthine
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
D_s	Distance travelled by solvent
D_x	Distance travelled by sample
EtOH	Ethanol
Eτ(30)	Solvent-polarity
H_2O	Water
HCl	Hydrochloride acid
HPLC	High-performance liquid chromatography
Mar	Market samples
Mar(b)	Market-samples from Bobo Dioulasso
Mar(o)	Market-samples from Ouagadougou
MEAMP	Monographs redaction and quality control of endangered antimalarial medicinal plants

MeOH	Methanol
P. falciparum	*Plasmodium falciparum*
Ref	Reference samples
Ref(Cp)	Reference-samples of *C. planchonii*
Ref(Ct)	Reference-samples of *C. tinctorium*
Rf	Retention factor
SP	Sulfadoxine-pyrimethamine
TLC	Thin-layer chromatography
TPTZ	2,2-diphenyl-1-picrylhydrazyl
UN	United Nations
WHO	World health organisation