Characterization of geographical variation in antioxidative active *Eucalyptus camaldulensis* extracts

- for future use as biopesticides in Burkina Faso

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Abstract

The current use of pesticides in Burkina Faso has been described as problematic. An alternative to synthetically produced pesticides is products based on naturally occurring substances, e.g. from plants, so-called biopesticides. In this study aqueous and ethanol extracts from *Eucalyptus camaldulensis* collected at three different geographical locations in Burkina Faso are investigated with regard to flavonoid and polyphenol content, TLC fingerprint profile, and antioxidant activity by DPPH-radical scavenging. The result show high yields, high polyphenolic content and strong antioxidant activity, especially for the aqueous extracts. The activity of the extracts derived from leaves collected in the north and south-west of Burkina Faso have approximately three times higher antioxidant activity than those from the central part of the country. Furthermore, clear differences between the extract can be seen on TLC, especially for the aqueous extracts. In conclusion, water extracts from *E. camaldulensis* contain high amounts of antioxidant active polyphenols and are promising as active ingredients for a biopesticide formulation. However, the geographical variation in content and activity is a clear example of the issues with working on natural products and must be taken into consideration in the future work.

Sammanfattning

Användandet av kemiska bekämpningsmedel har uppmärksammats som problematiskt i Burkina Faso. Ett intressant alternativ till syntetiskt framställda bekämpningsmedel är så kallade biobekämpningsmedel, produkter gjorde på ämnen utvunna från t.ex. växter. Den här studien har undersökt geografiska skillnader i extrakt från *Eucalyptus camaldulensis*. Vatten- och etanolextrakt från träd som vuxit i tre olika regioner av Burkina Faso har jämförts gällande innehåll och antioxidativ förmåga. Resultatet visar att ångdestillering av *E. camaldulensis* plant material ger högt utbyte och antioxidativt aktiva extrakt med hög koncentration polyfenoler. Aktiviteten hos extrakten gjorda på blad från norra och sydvästra Burkina Faso är ungefär tre gånger högre än de gjorda på blad från den centrala delen av landet. Tydliga skillnader mellan extrakten kan ses vid analys via tunnskiktskromatografi, speciellt för vattenextrakten. Resultaten i den här studien har visat på eukalyptusextrakts potential som biobekämpningsmedel men även att det finns geografiska skillnader i aktiviteten hos dessa extrakt, ett tydligt exempel på en av svårigheterna med att arbeta med naturprodukter.

Table of content

1. Introduction and aim

A growing world population, climate change, and the spread of resistant pest populations are some of the challenges facing the agriculture industry globally. During the last decades, synthetic chemical pesticides have been the major go-to solutions for crop management. There are, however, several limitations to their use, especially in developing countries where lack of regulations, insufficient control, and knowledge increase the risk of misuse. Disadvantages include potential health risk for farmers, damage to the local environment, and high residual levels in crops that might restrict export possibilities. An alternative to chemical pesticides is so-called biopesticides. These are non-toxic products based on naturally occurring substances from e.g. plants or microorganisms and serve as biodegradable alternatives to conventional pesticides.

Burkina Faso is a landlocked country in West Africa with one of the world's lowest development indexes. The country is heavily relying on agriculture, and a large portion of the population work as small and self-sufficient farmers. Food security is regarded as one of the country's major challenges, with plant pests or diseases and the related misuse of pesticides being a part of the problem.

Eucalyptus camaldulensis (Fig.1) is an aromatic tree native to Australia but widely cultivated in Burkina Faso. Extracts from eucalyptus have previously been shown to contain high levels of polyphenolic compounds and high antioxidant activity (Ashraf 2015). It has also been suggested that these antioxidative active extracts might have antifungal properties. As the leaves of *E. camaldulensis* are a waste product from use of the stems in construction work in Burkina Faso they are a highly interesting raw material for future production of biopesticides.

When working with natural products, the term variability is of great importance. This thesis aims to investigate the geographical variation in flavonoid content and antioxidative activity of *E. camaldulensis* leaf extracts. Extracts obtained through hydrodistillation and subsequent ethanol extraction from leaves collected at three different geographical locations will be compared with regard to chemical content and antioxidant activity.

***Figure 1**. Picture of Eucalyptus camaldulensis in Burkina Faso.*

2. Background

2.1 Burkina Faso

Burkina Faso is a landlocked country in West Africa, about two-thirds the size of Sweden. The United Nations Development Program (UNDP) ranked it 185/188 among the world's countries according to the Human Development Index in 2015 (UN 2016). Today home to a population of 20 million, a number that is expected to have doubled by the year 2045 (UN 2017). According to the World Health Organization (WHO) "nutrition and food safety" have been one of the main focus area for the country's development during the last half decade (WHO 2009). As for many developing countries, a large part of the population is living in rural areas and agriculture is of great importance. It is estimated that up to 90 % of the workforce is being employed within this sector (FAO 2014), in many cases as small and self-sufficient farmers. Furthermore, it is estimated that the agricultural sector makes up approximately a third of the country's gross domestic product (GDP) (Ouédraogo 2011).

Agriculture and pesticide use

Pests and plant diseases have been reported to cause significant crop losses in Burkina Faso and numbers as high as 30 % have been suggested (Toé 2010). Hence, plant protection products are used to limit the losses, but the pesticide use in Burkina Faso has been reported to be problematic (Ouédraogo 2011, Lehmann 2017). Issues include for example, the presence of banned pesticides such as organo-chlorine pesticides, lack of proper labeling of pesticide containers, lack of knowledge amongst resellers and farmers, lack of proper protective clothing during application, etc. The resulting effects include the risk of acute and long-term poisoning of farmers, contamination of ground water and soil close to the application site and high levels of residual pesticides in vegetables. A study showed that the water in up to 30 % of traditional wells found in the field were considered unfit for drinking, and 36 % of the vegetables exceeded Maximum Residue Levels (MRLs) according to good agricultural practice (Lehmann 2017). Residual pesticides pose a serious health risk for the consumers and limit the export potential.

Geography and climate

Burkina Faso is found stretching between the so-called South Sudanian and Sahelian regions in West Africa. The country can be divided into four agro-ecological zones (Fig.2) differing in e.g. soil fertility and annual rainfall (Ouedraogo 2010). The north-Sahelian zone of the country have annual rainfall between 200 – 500 mm per year and generally low fertility of the soil. In the north-Sudanian and south-Sudanian regions the annual rainfall is 700 – 900 and > 900 mm respectively. The north-Sudanian and south-Sudanian zones have large portions of the country's arable land, and the main difference between the regions is the high population density in the north-Sudanian zone, the central parts of the country including the capital Ouagadougou.

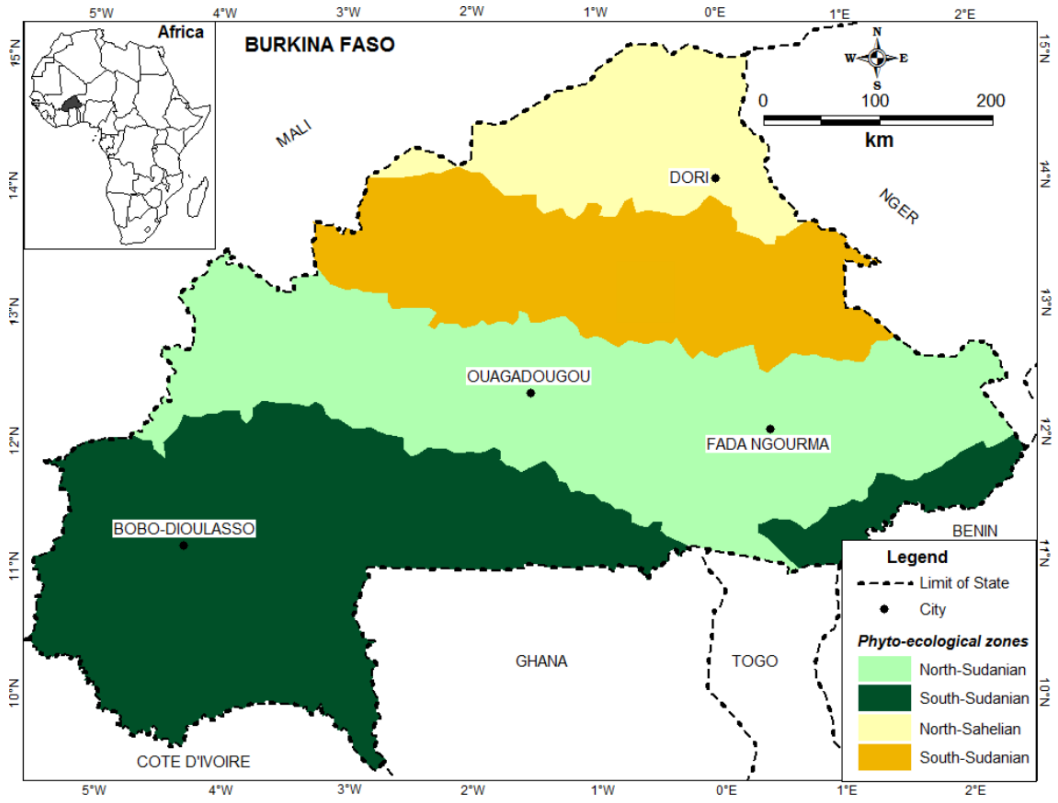

Figure 2. Phyto-ecological zones of Burkina Faso (Ouedraogo 2010).

The climate in Burkina Faso is a dry tropical climate marked by two main seasons, a dry season and a rainy season. Furthermore, the Sudano-Sahelian climate is characterized by unimodal rainfall curve, total absence of cool season (monthly average temperature above 18 ˚C) and decreasing aridity from the north to the south (Ouedraogo 2010, Lehmann 2017).

2.2 Biopesticides

Definition

The term biopesticides refers to a type of pesticide derived from natural materials such as animals, plants, bacteria, and/or certain minerals (EPA 2017). They can be further divided into three groups depending on their origin and mechanism of action: Microbial pesticides, plant-incorporated protectants and biochemical pesticides. Microbial pesticides are biopesticides consisting of living microorganisms that control pests. For example, several bacteria from the genus *Bacillus* have been used in biopesticide formulations. With the second group, plant-incorporated protectants, the pesticide active substances are being produced by the plants themselves after incorporation of external genetic material. Finally, the third group biochemical pesticides are naturally occurring substances derived from e.g. plants that can be extracted and then used to make a biopesticide formulation. As there are significant differences between these three types of biopesticides regarding challenges, possibilities, regulations etc., it is important to distinguish between them.

In this work, the term "biopesticides" will mainly be referring to the category biochemical pesticides. In order to qualify as a biochemical biopesticide the naturally occurring active ingredients must have

demonstrated a history of exposure to humans and the environment that show minimal toxicity (EPA 2017). Furthermore, and in contrast to many conventional pesticides, it must exhibit a non-toxic mechanism of action against the target pest.

Current situation and the example of tomato

Although biopesticides can be found on the market today, they only make up 2-5 % of the global pesticide market (Kumar 2015, Damalas 2018). In April 2016 there were 299 approved biopesticides and 1401 active product registrations in the United States (EPA 2018), suggesting an increasing interest for this type of products. This can also be seen as an increase in market share for biopesticides, while the trend for synthetic pesticides is decreasing (Thakore 2006).

Tomato (*Solanum lycopersicum*) is an important vegetable widely cultivated across the world. Tomatoes are easy to grow and have a high nutritional value, but a disadvantage is that there is a number of diseases/pests that are known to affect them. These include fungal diseases like tomato fusarium wilt, tomato late blight and powdery mildew, but also bacterial diseases like tomato bacterial spot or pest insects like tobacco whitefly (Sharma 2015, Song 2004). Traditionally, these diseases have been treated with conventional pesticides. However, issues regarding toxicity and development of resistant pest is making biopesticides an increasingly interesting alternative to conventional pesticides (Sharma 2015). Table 1 is showing five common tomato diseases and some biopesticides, as well as conventional pesticides, which can be used for the restriction of these.

***Table 1**. Table of common tomato diseases/pest and biopesticides and conventional pesticides that can be used to treat them.*

Tomato disease/pest	**Biopesticide**	**Conventional pesticide**
Tomato fusarium wilt (*Fusarium oxysporum f. sp. Lycopersici)*	Validamycin [a]	Prochloraz, Carbendazim and others [b]
Tomato late blight (*Phytophthora infestans*)	Oligosaccharins [c] and Validamycin [a]	Chlorothalonil, Mancozeb and others [d]
Powdery mildew of tomato (*Oidium lycopersicum*)	Validamycin [a]	Sulfur fungicides, Benomyl and others [e]
Bacterial spot on tomato (*Xanthomonas* and *Pseudomonas* families)	Kasugamycin [f]	Copper-containing bactericides [f]
Tobacco whitefly (*Bemisia tabaci*)	Neem formulations [g]	Pyrethroids, Organophosphates [h]

[a] (Ishikawa 2005)
[b] (Song 2004)
[c] (Focus Technology Co. 2018)
[d] (Zitter 2000)
[e] (LaMondia 1999)
[f] (Vallad 2010)
[g] (Sharma 2015)
[h] (Cahill 1995)

Challenges and possibilities

One of the major advantages of biopesticides is the environmentally friendly and biodegradable nature of these products (Kumar 2015). This significantly reduces the risk of harm being caused to the farmers, the consumers, or the local environment surrounding the cultivation site. In addition to this, reduced levels of residual pesticides in crops may give economic advantages as it enables export of e.g. vegetables.

Another potential economic advantage includes local production. In contrast to many conventional pesticides, the production of biopesticides may utilize locally available raw materials (Kumar 2015). In countries where production of chemical pesticides has not yet been established and the currently used products are imported, a local production of biopesticides could be profitable.

There are naturally challenges to the use of biopesticides. These include e.g. stability issues or slow speed of action, making the products unsuitable in the case of large outbreaks requiring fast pest management (Chandler 2011). Furthermore, some biopesticides are highly specific, requiring an exact identification of the target pest. However, this specificity could also be regarded as an advantage as it decreases the risk of damage being caused to other species than the target pest. Finally, one should consider the importance of current regulations, government policies, and the attitude of retailers and farmers. The use of synthetic pesticides is well established within the agricultural sector and in many cases the system for registration is not yet adapted for biopesticides (Chandler 2011, Damalas 2018).

2.3 *Eucalyptus camaldulensis*

Eucalyptus camaldulensis, is one of approximately 800 species within the genus Eucalyptus (ANBG 2004) and commonly known as red river gum tree. Although native to Australia, it is today found widely over the globe where it is either cultivated or spread naturally after introduction. The high occurrence of *E. camaldulensis* and other eucalyptus trees could be explained by their robust and fast-growing nature. The trees show good resistance to drought as well as to flooding.

Use in Burkina Faso

E. camaldulensis is found abundantly in Burkina Faso, growing in gardens and alongside roads, but is also cultivated in specific plantations (Fig.3A). The intended use of trees grown in these plantations is mainly in construction work. The stem of *E. camaldulensis* is used for stabilization of walls in larger building constructions (Fig.3C). Normally the leaves and branches are not utilized, but instead left at the site of harvest (Fig.3B) making them a cheap and easily accessible raw material in Burkina Faso.

A) B) C)

*Figure 3. Pictures of Eucalyptus Camaldulensis in Burkina Faso, **A)** trees grown in lines at a cultivation site, **B)** leaves left on the ground after felling of trees, **C)** use of the stems in construction.*

The essential oil of eucalyptus has found various applications, both in pharmaceutical, cosmetically and in various food product formulations. It has also been suggested to be useful as a biopesticide (Batish 2008). In the process of obtaining essential oil through hydrodistillation a large volume water/plant material crude remains. It has previously been shown in our laboratory that this crude still contains plant metabolites that are antioxidative active and utilization of these could be profitable.

2.4 Flavonoids and Other Polyphenols

Flavonoid is the general name of a compound containing the characteristic flavone backbone. The name flavonoid derives from the Latin word *flavus* meaning yellow, due to their characteristic color. There are over 5000 naturally occurring flavonoids that have been characterized, found in all parts of the plant kingdom. They possess a large variety of biological functions such as giving color and aroma to flowers, functioning as signal molecules and protecting against biotic and abiotic stress (Panche 2016).

The common characteristic of flavonoids is the flavone backbone, 2-phenyl-1,4-benzopyrone (Fig.4). It consists of three six carbon rings which are commonly referred to as A, B and C. The group flavonoids can be further divided into several subgroups differing in the 3-hydroxy substituent and saturation of the C ring. These groups include flavones, flavonols, flavanones and flavanonols (Fig.4). There are also similar structures that sometimes are counted into the flavonoid class such as isoflavones, neoflavanoids and anthocyanidins. The main substituents of flavonoids are hydroxyl groups, and the number, position and alkylation/acetylation of these are known to effect the chemical and biological activity (Ferreira 2015, Sarian 2017).

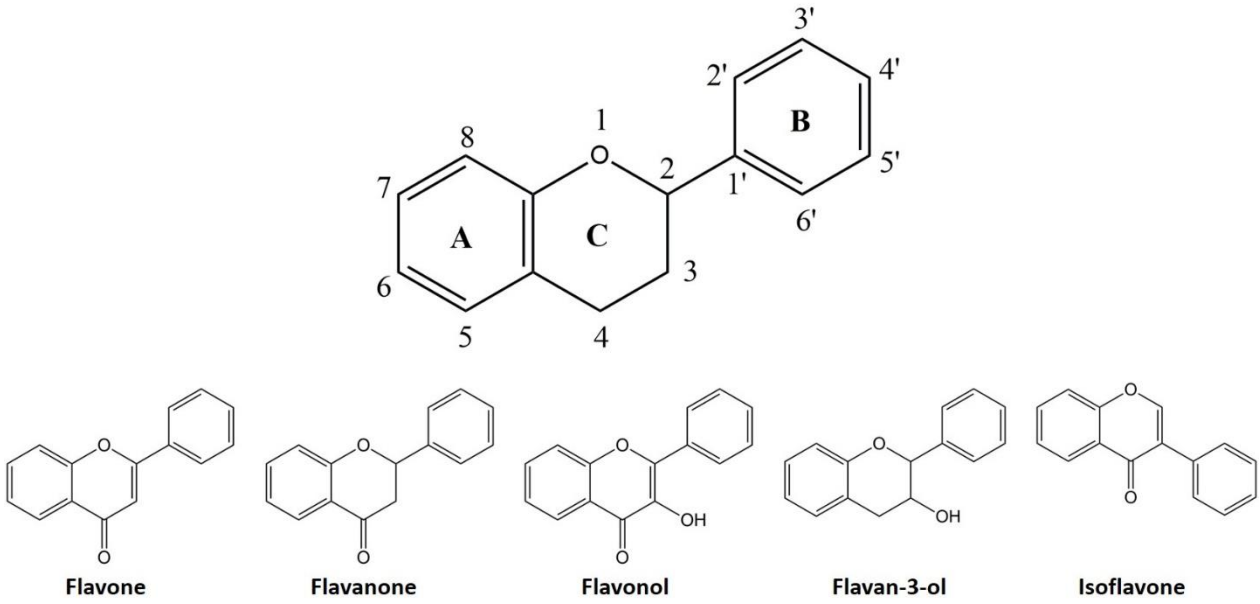

Figure 4. *Schematic picture of the flavone backbone including as well as the basic structure of common subclasses of flavonoids including flavone, flavanone, flavonol, flavan-3-ol and the sometimes included isoflavone.*

It is commonly known that external stimuli such as UV-light, drought, pathogens or nutritional depletion may trigger stress responses in plants, finally resulting in accumulation of secondary metabolites including flavonoids (Sudha 2002). It has been reported that geographical location caused variation in flavonoid contents of food products (Haytowitz 2013) and various flavonoid rich plant extracts (Dolkar 2017, Ismail 2017, Rimkiene 2017).

Polyphenols in E. camaldulensis

High polyphenol content as well as antioxidant, antimicrobial and antitumor activity have been reported for various eucalyptus extracts, including extracts from fruits, bark and leaves (Boulekbache-Makhlouf 2013, Ashraf 2015, González 2017). A wide range of eucalyptus species and extraction methods can be found in literature. Previously it has been shown that the antioxidant activity and the major constituents may vary with extraction method (El-Ghorab 2003, Fernández-Agulló 2015) and that antimicrobial activity and specificity may vary between the eucalyptus subspecies (Takahashi 2004). In the case of essential oil from *E. camaldulensis* both location and harvest time affected the obtained yield and the chemical composition (Moudachirou 1999).

3. Methods

3.1 Collection and Preparation of Plant Material

Collection procedure

Plant material consisting of leaves from *E. camaldulensis* was collected from three different geographical locations in Burkina Faso. In the northern part, outside the city of Dori, in the central part, outside the city of Ouagadougou, and in the south west part, outside the city of Bobo-Dioulasso. The collection was carried out in a similar manner at each collection point and within a period of 10 days in the end of March to the beginning of April (2018-03-28 and 2018-04-06). At each sampling point leaves from a minimum of three trees were collected, weighed and mixed in equal proportions. Trees with a stem circumference of 20-50 cm, measured 1 m of the ground, were chosen.

Collection included GPS coordinates and soil samples. GPS coordinates were noted using a GARMIN e-trex 30 GPS (satellite). At each collection point a soil sample were collected from 10-20 cm below the ground and the samples left to dry before being subjected to further analysis.

Washing, drying and pulverization

The collected leaves were washed in water to remove residual particles. Drying was carried out at approx. 40˚C for 7 days, in a closed room with ceiling fan. The wet samples from washing were turned 3 times/day until excess water was dried off. After drying the leaves were grinded into a fine powder and the powders were stored in a closed container at r.t. until further processing.

3.2 Soil and Plant Powder Characteristics

Plant powder moisture content

To determine the moisture content of the plant powders 1.0 g of each powder was dried in an oven at 105 ˚C for 3 h, left to cool in a desiccator before weighing. Samples were prepared and analyzed in triplicates.

Plant powder ash content

To determine the ash content of the plant powders 5.0 g of each powder was calcinated at 550 ˚C for 10 h, left to cool for an additional 10 h in the oven followed by 20 min in a desiccator before weighing. Samples were prepared and analyzed in duplicates. The ash content on dry basis was given in percentage calculated from the noted mass after calcination (m_{ash}) and the moisture content (MC) according to Eq.1;

$$Total\ ash\ content = \frac{m_{ash}}{5.0 * (1 - MC)} * 100 \qquad (Eq. 1)$$

Soil characteristics

The soil was analyzed with respect to grain size, soil pH, organic matter and total potassium, phosphor and nitrogen content. The analyses were carried out by an external operator at Bureau National des Sols (BUNASOL) in Ouagadougou, Burkina Faso.

Soil texture

101g of soil was sieved to < 2mm and air-dried before addition of 50 mL 5 % $(NaPO_3)_6$ - Na_2CO_3 and 250 of distilled water. The mixture was stirred for 2 h and the volume adjusted to 1.0 liter. After 1 min of vigorous shaking and 40 seconds of sedimentation the first reading was done using a hydrometer. This fraction is representing clay + silt particles (< 50 µm). The mixture was left to sediment for an additional 3 h before the second reading, representing only clay particles (< 2 µm). The fraction slit particles was calculated as the difference between the first and second reading.

Soil pH

20.0 g of soil (sieved to < 2 mm) with 50.0 mL of distilled water, stirring it for 30 min using a mechanical stirrer, and then measuring the pH of the solution after sedimentation of the solid particles.

Soil nutrients

Total phosphor and total nitrogen content were analyzed using an autoanalyzer (Skalar) while total potassium content was analyzed with a flame photometer. Preparation of samples were done by mixing 1.0 g of air-dried soil (sieved to < 0.5 mm) with 5 mL of 0.7 % H_2SO_4-Se-$C_7H_6O_3$. The mixture was left to react at r.t. for 2 h (or overnight) before being heated to 100 ˚C for 1 h. 1.0 mL of hydrogen peroxide was added, the mixture was heated to 250˚C for 1 h, additionally 1.0 mL of hydrogen peroxide was added and finally the mixture was heated to 340˚C until the mixture became clear (2-4 h). The mixture was left to cool and distilled water was added to a final volume of 75 mL.

Soil organic matter

The amount organic matter in the soil is determined spectrophotometrically. To 0.5 g soil (sieved to < 0.5 mm) 2.5 mL $K_2Cr_2O_7$ (1 M) was added and mixed lightly before addition of 5 mL concentrated H_2SO_4. The mixture left to react at r.t. for 30 min before addition of 25 mL distilled water. The solution was filtrated and then subjected to colorimetric measurement at 620 nm.

3.3 Extraction Procedures

Hydro-distillation

Hydro-distillation was carried out with 100 g of plant powder in 1.0 liter of distilled water for 1 h using a Clevenger type apparatus. The volume of essential oil obtained was noted but the oil was not analyzed further. The remaining crude was filtered using a fabric tissue to obtain an aqueous solution and a solid residue. The volume aqueous extract was noted before being subjected to a second filtration using cotton. A sample of 100 or 200 mL of the resulting filtrate were frozen at -20˚C overnight and thereafter dried through lyophilization (LABCONCO FreeZone 2.5 Plus) to obtain a brown solid extract.

Ethanol extraction

The solid residue was dried at r.t. for 24 h before being subjected to ethanol extraction. 1.0 liter of 70 % ethanol was added to the residue and the mixture continuously stirred for 24 h at r.t. before a two-step filtration, first using a fabric tissue and then filter paper. The total volume was noted before 100 or 200 mL of the solution was processed further. The solvent was removed in vacuo, the remaining syrup re-dissolved

in water, frozen at -20˚C overnight and dried through lyophilization (LABCONCO FreeZone 2.5 Plus) to obtain a green solid extract.

3.4 Extract Characterization

Total polyphenolic content (Folin-Ciocalteu method)

Total polyphenolic content was determined using a modified version of the Folin-Ciocalteu method, adapted for microplate (Ainsworth 2007). 200 µL of Folin-Ciocalteu reagent, 1:10 dilution in distilled water, was added to 100 µL of plant extract, 0.5 mg/mL in distilled water, and mixed thoroughly before addition of 800 µL Na_2CO_3, 0.7 M in distilled water. Samples were transferred to a microplate, 200 µL/well in triplicates and incubated for 2 h at 28 ˚C. The absorbance at 765 nm were then measured (BMG Labtech SPECTROstar Nano) using pure reagents as blank. The absorbance was compared to a standard curve prepared with gallic acid (0.2-0.025 mg/mL) to give the total polyphenolic content as mg gallic acid equivalent (GAE)/g extract. All samples were prepared and analyzed in triplicates.

Extract fingerprint (TLC)

Extract composition were analyzed with thin layer chromatography (TLC) using an automatic TLC sampling system (LAMAG). 10 µL of ethanol extracts (10 mg/mL in MeOH) and 5 µL of water extract (20 mg/mL in distilled water) were loaded onto a silica plate (Sigma Aldrich) and run in a mobile phase of *n*-butanol : acetic acid : water = 65:15:10. The plate was left to dry in r.t. before the flavonoid specific NEU reagent (2-aminoethyl-diphenylborinate 1 % w/v in MeOH) was sprayed onto the plate followed by 5 % PEG-400 (5 % v/v in EtOH). The fluorescence at 365 nm was photographed using a manual development chamber.

Total flavonoid content ($AlCl_3$ method)

Total flavonoid content was analyzed using the $AlCl_3$-method (Pękal 2013) adjusted for microplate. 0.5 mL of $AlCl_3$ (2 % w/v in MeOH) was added to 0.5 mL of plant extract (1 mg/mL in MeOH) and the solution mixed well before being transferred to microplate in triplicates, 200 µL/well. The microplate was incubated for 10 min at 28˚C before reading the absorbance at 420 nm (BMG Labtech SPECTROstar Nano) using sample in pure MeOH as blank. The absorbance was compared to a standard curve prepared with quercetin (0.03-0.003 mg/mL) to give the flavonoid content as mg quercetin equivalent (QE)/g extract. All samples were prepared and analyzed in triplicates.

Antioxidant activity (DPPH-method)

The antioxidant activity of the extracts was analyzed using the DPPH-method adjusted for use in microplate (Cheng 2006) with minor adjustments. In short, samples were diluted in the microplate in triplicates to yield seven concentrations per sample, concentrations ranging from 0.1-0.0016 mg/mL for aqueous extracts and 0.25-0.0078 mg/mL for ethanol extracts. In each well, 100 µL of MeOH or 100 µL of freshly prepared DPPH (0.2 mM in MeOH) were added to 100 µL of sample. The microplate was incubated at 28˚C for 20 min before reading the absorbance at 515 nm. The percentage of DPPH inhibition was calculated for each concentration of sample according to Eq. 2. The percentage of inhibition was plotted against sample concentration and using linear regression the EC_{50}-value in µg/mL was calculated. Any data points with inhibition > 90 % was excluded from the linear regression as it indicated saturation. The antioxidant activity

of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was analyzed as standard. All samples were prepared and analyzed in triplicates.

$$\%\ DPPH\ inhibition = \frac{(A_{DPPH} - A_{MeOH}) - (A_{sample+DPPH} - A_{sample+MeOH})}{A_{DPPH} - A_{MeOH}} * 100 \quad (Eq. 2)$$

Extract stability

5 mL of an aqueous extract and an ethanol extract (1 mg/mL in MeOH) was stored either at r.t. or at +4˚C for 6 weeks. The antioxidant activity of the extracts after storage were analyzed and compared to the activity of the freshly prepared samples.

3.5 Methodology Studies

Sensitivity analysis

To determine the sensitivity of the analytical methods, one sample were analyzed repeatedly while varying physical or chemical analysis conditions. The included parameters were incubation temperature, incubation time and concentration major reagent and compared to the standard protocol, a high and a low value were used for each parameter (Tab.2). Unpaired, unequal variance t-test (Welch t-test) was used to determine if the changed parameter resulted in a significantly different value compared to the standard protocol.

***Table 2**. Analysis conditions for sensitivity analysis of the Folin-Ciocalteu Reagent-, the $AlCl_3$- and DPPH-method. Compared to standard setup a low and high value of the parameters incubation temperature, incubation time and concentration major reagent were tested.*

Analythical mehod	**Incubation temperature**			**Incubation time [min]**			**Reagent concentration**		
Folin-Ciocalteu Reagent	25˚C	28 ˚C	35 ˚C	90	120	150	1:11 dilution	1:10 dilution	1:9 dilution
$AlCl_3$				5	10	30	1.8 % v/w	2 % w/v	2.2 % w/v
DPPH				10	20	30	0.18 mM	0.2 mM	2.2 mM

Repeatability hydro-distillation

To analyze the repeatability of the extraction process, three separate hydrodistillations with following ethanol extraction were carried out using the plant powder from the central collecting point. The extracts were then analyzed with regard to total polyphenolic content, total flavonoid content, antioxidant activity and extract composition using TLC to evaluate the repeatability of the process.

4. Result

4.1 Collection, Soil and Plant Material

Collection

Samples of *E. camaldulensis* leaves were collected at three different geographical locations of Burkina Faso (Fig.5A). In the northern part of the country outside the city of Dori (14˚04'22"N, 0˚08'48"W), in the central part of the country outside the city of Ouagadougou (12˚25'30"N, 1˚40'44"W) and in the south-west part of the country outside the city of Bobo-Dioulasso (11˚11'44"N, 4˚23'09"W). The collections were done in a plantation setting outside the respective city (Fig.5B-D) in the middle of peak dry season, 2018/03/28-2018/04/06.

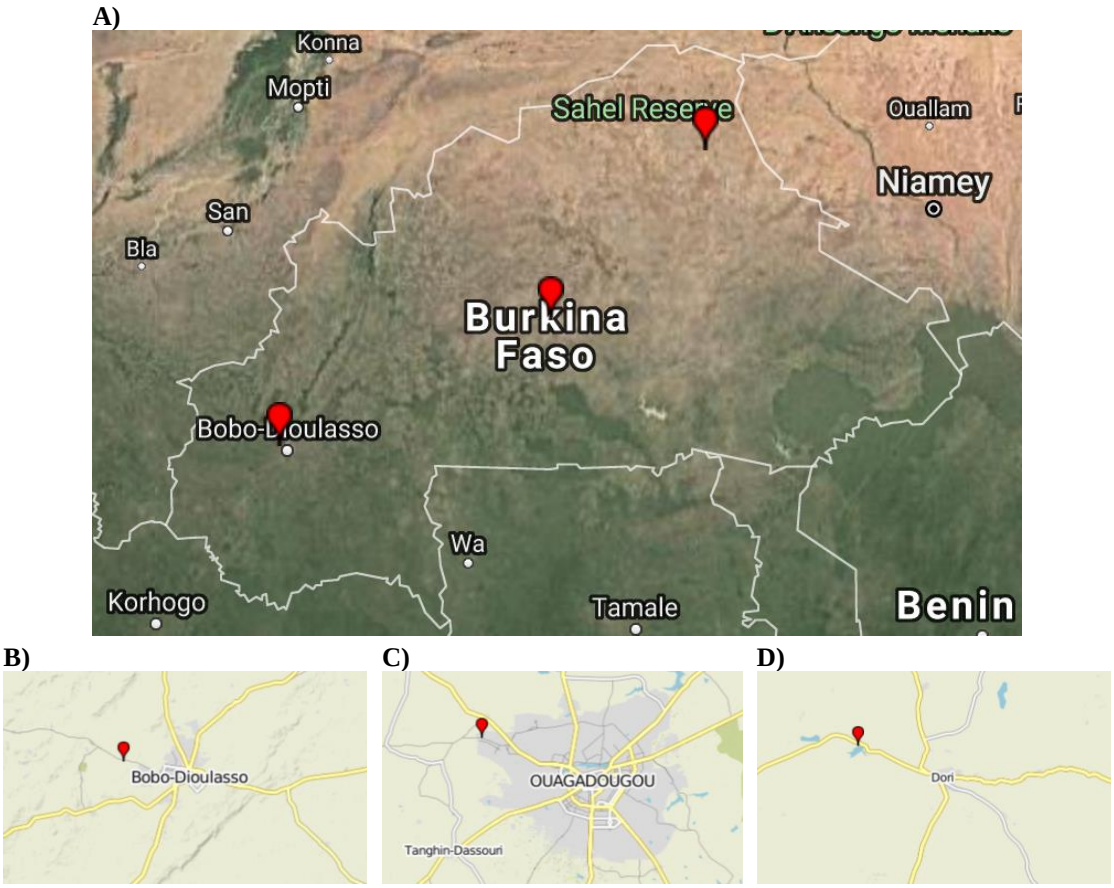

Figure 5. *Map showing the collection points of E. camaldulensis leaves used for extraction.* ***A)*** *Picture of collection points on a map of Burkina Faso,* ***B-D)*** *collection locations in relation to the closest city.*

Soil characteristics

The analysis of soil collected at each collection point showed an overall high sand content (Tab.3). Soil from the north and south-west collection points were characterized as sandy soils, while soil from the central collection point were identified as sandy loam, meaning a larger part slit and clay portion. Furthermore, the pH of the soil were in the range 5.6-5.9 suggesting moderately acidic soils, somewhat unfavorable for microbiological activity (Peverill 1999), but within the range of what is normal in Burkina Faso (Kissou 2018).

Percentage organic matter in the soils were below 1 % at all three points, which is to be regarded as a low value. The total amount of nitrogen, potassium and phosphor were analyzed and found to be within the expected range for soils in Burkina Faso (Kissou 2018), with the most pronounced result being the relatively high level of phosphor in the soil from the north collection point.

Table 3. *Soil characteristics including soilt texture, pH and major nutrients for three different geographical locations in Burkina Faso. Soil collected in close proximity to E. camaldulensis trees used for extraction.*

Collection point	Soil type	Sand [%]	Slit [%]	Clay [%]	pH	Organic matter [%]	Total carbon [%]	Total nitrogen [%]	Total potassium [ppm]	Total phosphor [ppm]
North	Sandy	96.1	2.0	2.0	5.78	0.271	0.018	0.018	1132	175
Central	Sandy Loam	76.5	15.7	7.8	5.87	0.717	0.044	0.044	756	349
South-West	Sandy	88.2	7.8	3.9	5.58	0.334	0.194	0.027	660	262

Plant Material

The three plant materials were similar in terms of moisture content and ash content (Tab.A, Appendix). The moisture content was approximately 5 % for all three powders. The total ash content was approximately 6 %, with the powder from the north collection point slightly higher than the central and south-west. The numbers were 6.2 %, 5.6 % and 5.8 % respectively.

4.2 Extract Characteristics

Extraction yield

The aqueous phase after hydro-distillation gave approx. 30 g dry extract per 100 g of plant powder processed (Tab.3). The south-west collection point gave a slightly higher yield than the central and the north, the numbers were 33.6 g versus 31.0 and 26.4 g respectively. Further ethanol extraction of the solid residue remaining after hydro-distillation gave a yield of about 10 g ethanol extract per 100 g plant material originally processed. This number was slightly lower for the central collection point, 8.5 g versus 10.0 g and 10.1 g for north and south-west.

Polyphenolic content

The total polyphenolic content was found to be generally higher in the aqueous extracts than in the ethanol extracts (Tab.3). Furthermore, they were found to be lower in the extracts from the central collection point, while the south-west and north showed similar values. For the water extracts the numbers were 171 ± 12 GAE/g, 266 ± 4 GAE/g and 251 ± 13 mg GAE/g, and for the ethanol extracts 68 ± 3 GAE/g, 129 ± 9 GAE/g and 123 ± 7 GAE/g respectively.

Flavonoid content

The ethanol extracts generally showed a higher amount of total flavonoid content compared to the aqueous extracts (Tab.3). Between the different collection points, the extracts from the northern collection point had

generally low amounts of flavonoids. For the aqueous extracts the numbers were 8.2 ± 1.8 mg QE/g versus 11.1 ± 1.1 mg QE/g and 11.4 ± 1.2 mg QE/g for central and south-west respectively while for the ethanol extracts the same numbers were 30.1 ± 1.3 mg QE/g versus 37.4 ± 2.1 mg QE/g and 38.3 ± 4.3 mg QE/g.

Antioxidant activity

The aqueous extracts from the north and South-west collection point showed the highest antioxidant activity (Tab.4, Fig.6), with EC_{50}-values of 9.3 µg/mL and 8.3 µg/mL which is comparable to that of the standard Trolox, 6.3 µg/mL in this assay. The aqueous extract from the central collection point showed lower antioxidant activity, seen as a higher EC_{50}-value of 27.8 µg/mL. Furthermore, for the ethanol extracts the north and south-west had a higher antioxidant activity than the central collection point, the numbers were 20.0 µg/mL, 26.4 µg/mL compared to 60.3 µg/mL.

Table 4. *Yield and chemical characteristics of E. camaldulensis dried leave extracts from three geographical locations in Burkina Faso. Extracts derived from hydrodistillation and following ethanol extraction.*

Extract	Collection point	Yield [g/100 g powder]	Polyphenolic content [mg GAE/g]	Flavonoid content [mg QE/g]	Antioxidant EC_{50} [µg/mL]
Water	North	26.4	251 ± 13	8.2 ± 1.8	9.3
	Central	31.0	171 ± 12	11.1 ± 1.1	27.8
	South-West	33.6	266 ± 4	11.4 ± 1.2	8.3
Ethanol	North	10.0	123 ± 7	30.1 ± 1.3	20.0
	Central	8.5	68 ± 3	37.4 ± 2.1	60.3
	South-West	10.1	129 ± 9	38.3 ± 4.3	26.4

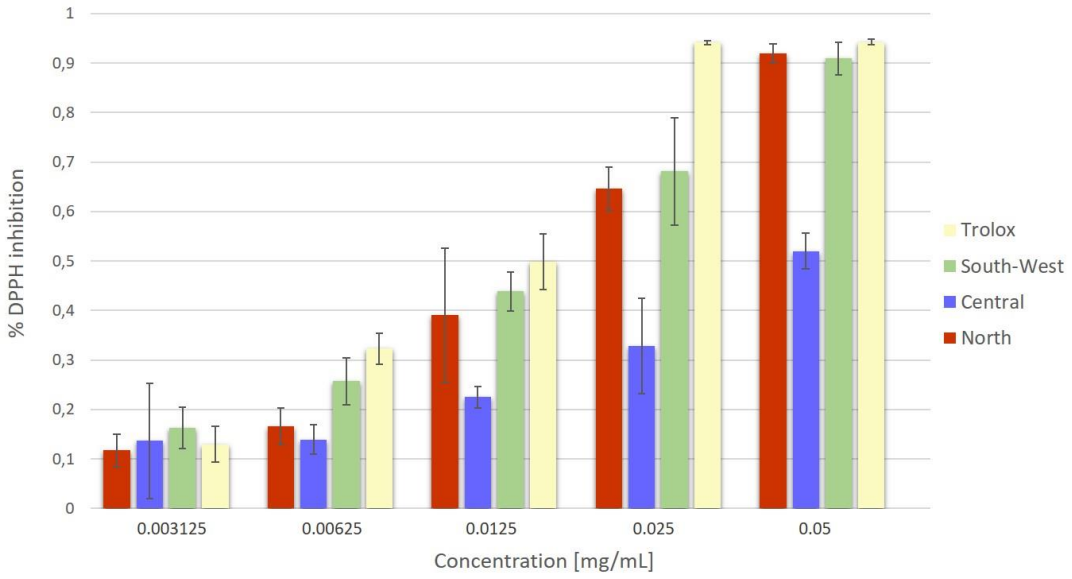

Figure 6*. Antioxidant activity in form of % DPPH-radical scavenging. Data for various concentrations of aqueous extracts from E. camaldulensis and Trolox (standard). Extracts based on leaves from three different geographical locations in Burkina Faso. Data used for calculation of EC_{50} values using linear regression.*

Correlation flavonoid or polyphenol content and antioxidant activity

Antioxidant activities were plotted against either total polyphenolic or total flavonoid content to examine the potential correlation. The results (Fig.7A) shows a weak positive correlation (Pearson's correlation coefficient = 0.66) between flavonoid content of the extracts and their antioxidant activity given as EC_{50}-values. This indicates that a high level of flavonoids does not correlate with a strong antioxidant activity. The correlation between total polyphenolic content and antioxidative activity (Fig.7B) is negative and stronger (Pearson's correlation coefficient = -0.85), suggesting that a high polyphenolic content correlates with a high antioxidative activity for both ethanolic and water extracts.

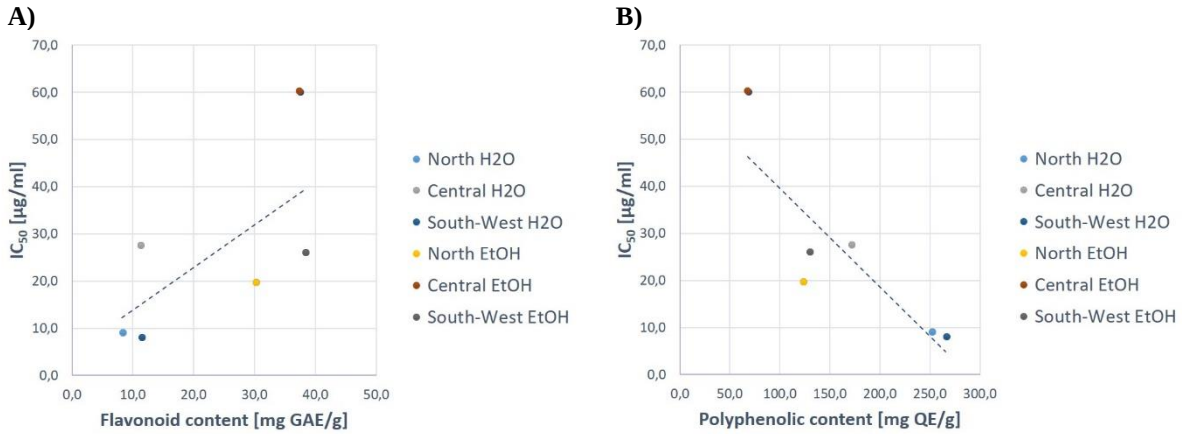

***Figure 7**. Correlation between antioxidative activity of E. camaldulensis extracts from three different geographical locations in Burkina Faso, given as EC_{50} value, and **A)** flavonoid content or **B)** polyphenolic content.*

Extract fingerprint (TLC)

Extract fingerprint were analyzed through TLC and the result (Fig.8) and show several bands: blue, orange, green and yellow, after derivatization with NP/PEG reagent, suggesting the presence of flavonoids. Clear differences can be seen between the extracts, especially in intensity of the orange band at $R_f \approx 0.30$, which is highly visible for the water extracts, but can also be seen in the ethanol extracts. Furthermore, the blue band at $R_f \approx 0.45$, which can be seen primarily in the water extracts, is present in all three extracts with similar intensity but show a slightly different degree of migration, suggesting slightly different polarity. In both the water and ethanol extracts, a green smear around $R_f \approx 0.5$ is visible in the north and south-west extracts but to a much lesser extent in the central extract. In general, the same bands are visible in the ethanol extracts as in the water extracts but with a lower intensity. The difference is mainly the large yellow/orange and red spots around $R_f \approx 0.9$.

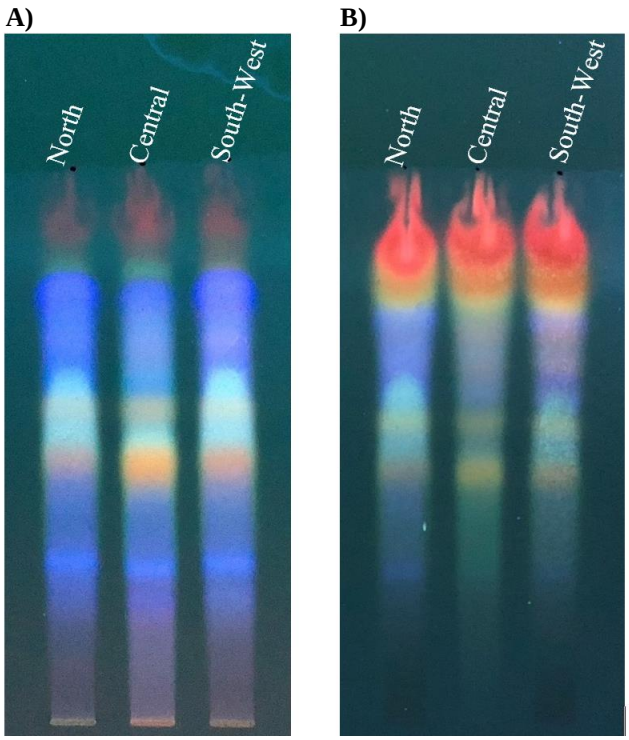

***Figure 8**. TLC analysis of **A)** water extracts or **B)** ethanol extracts from E. calmadulensis collected three different locations in Burkina Faso. Normal phase TLC with mobile phase butanol : acetic acid : water = 65:5:10, derivatizing reagent (NP/PEG) and visualization at 365 nm.*

Extract stability

The antioxidant activity of the aqueous extract did not decrease significantly after 6 weeks at either 4˚C or approx. 35˚C (Tab.5), suggesting satisfactory stability under given conditions. However, the ethanol extracts partly lost its activity, as can be seen by an increase in the EC_{50} of the different extracts. The loss was greater for the extract stored at approx. 35 ˚C, compared to that stored at 4˚C. The EC_{50} values were 120.7 µg/mL and 85.5 µg/mL respectively, compared to 60.3 µg/mL of the original sample.

***Table 5**. Stability of aqueous and ethanol extracts from E. camaldulensis after storage for 6 weeks in methanol, either at + 4˚C or approx. 35˚C. Antioxidative activity given as EC_{50} concentration and % DPPH inhibition by 0.025 mg/mL aqueous extracts or 0.0625 mg/mL ethanol extracts.*

Extract	**Conditions**	**Antioxidant EC_{50} [µg/mL]**	**DPPH inhibition**
Water	Day 1	27.8	52 ± 4 %
	6 weeks +4˚C	26.3	57 ± 4 %
	6 weeks approx. 35˚C.	23.6	60 ± 6 %
Ethanol	Day 1	60.3	55 ± 3 %
	6 weeks +4˚C	85.5	41 ± 5 %
	6 weeks approx. 35˚C	120.7	32.6 ± 6 %

4.3 Methodology studies

Repeatability of hydro-distillation

The repeatability of the extraction methods, hydro-distillation followed by ethanol extraction of the remaining solid residue, was studied by repeating the complete extraction process three times using the same plant material. The result shows an overall robust process in terms of yield and composition of the resulting extracts (Tab.6). Notably one of the ethanol extracts showed a slightly lower polyphenolic content and antioxidative activity. The same extract gave a weaker visual on TLC (Fig.9), especially with regard to the intense yellow band at R_f = 0.45.

Table 6. *Yield and chemical characteristics of E. camaldulensis dried leave extracts from three geographical locations in Burkina Faso. Extracts derived from hydrodistillation and following ethanol extraction.*

Extract	**Rep**	**Yield [g/100 g powder]**	**Polyphenolic content [mg GAE/g]**	**Flavonoid content [mg QE/g]**	**Antioxidant EC_{50} [µg/mL]**
Water	1	31.0	171 ± 12	11.1 ± 1.1	27.8
	2	29.8	179 ± 3	12.3 ± 1.5	25.9
	3	30.4	194 ± 4	10.4 ± 1.0	23.9
Ethanol	1	8.5	68 ± 3	37.4 ± 2.1	60.3
	2	10.1	90 ± 10	38.7 ± 1.9	51.9
	3	8.4	111 ± 6	36.9 ± 1.9	54.6

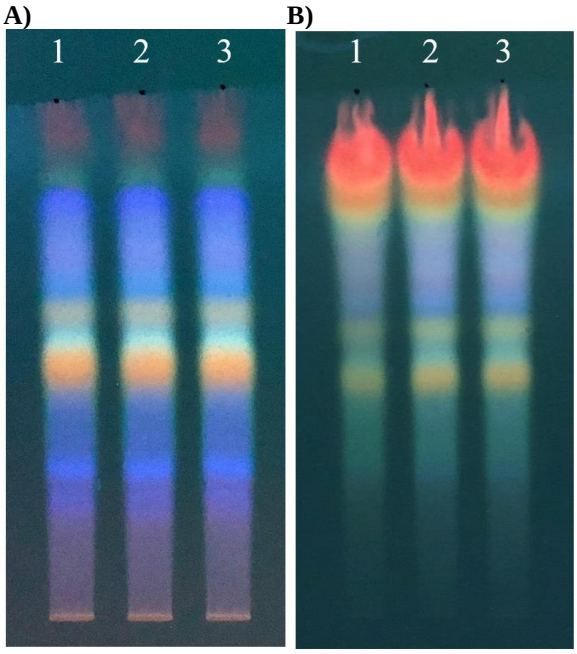

Figure 9. *Picture of TLC analysis of extracts from repeated hydrodistillations and ethanol extractions of E. camaldulensis dried leaved collected in Burkina Faso. Normal phase TLC with mobile phase butanol : acetic acid : water = 65:5:10, derivatizing reagent (NP/PEG) and visualization at 365 nm.*

Sensitivity analysis analytical methods

The same sample were analyzed under varying conditions such as temperature, incubation time and concentration of major reaction reagent to investigate the sensitivity of the spectrophotometric methods used. The results show an overall satisfactory robustness of all three methods studied, with some parameters affecting the result more than others. An increased incubation temperature, 35˚C compared to the standard setup using 28˚C, was the only parameter affecting all the methods. It resulted in an apparently higher content flavonoids and antioxidant activity, but apparently lower polyphenolic content. Increased incubation temperature was the only parameter affecting the result of the $AlCl_3$-method significantly (Fig.10A), but overall the assay gave low values for flavonoid content with relatively high variability.

In addition to increased incubation temperature, the result of the Folin-Ciocalteu-method showed a significantly higher polyphenol content when a lower amount of reagent was used (Fig.10B). The DPPH-method for analyzing antioxidative activity turned out to be the most sensitive method (Fig.10C). The incubation time affected the result significantly, a shorter incubation time resulted in a lower antioxidative activity and a longer incubation time resulted in a higher antioxidative activity. Furthermore, an increase in reagent concentration resulted in a slightly lower percentage DPPH-scavenging.

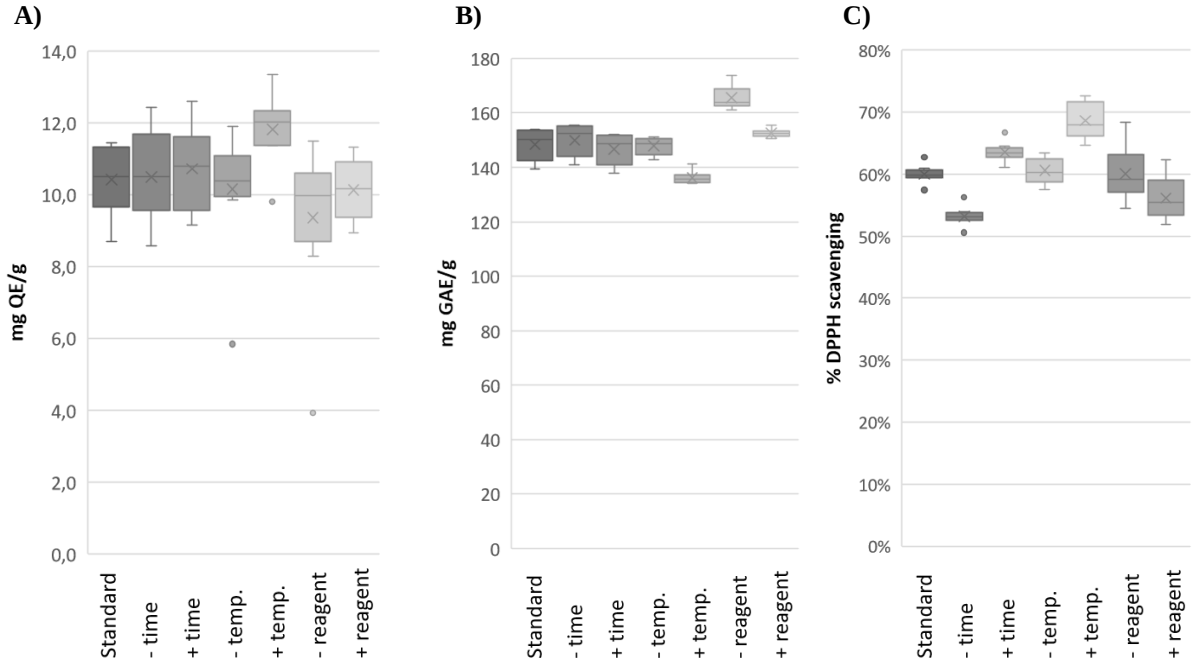

Figure 10. *Boxplots showing the sensitivity of three analytical methods, the $AlCl_3$-, Folin-Ciocalteu- and DPPH-methods to varying physical and chemical reaction conditions. Compared to the standard setup, a high and low value were analyzed for each parameter. The parameters included incubation time, incubation temperature and concentration major reagent.*

5. Discussion

One of the major difficulties in working with natural products is their large variation. This work exemplified this difficulty by showing the variation in antioxidative activity of *E. camaldulensis* leaf extracts from three different geographical locations in Burkina Faso. The result show that extracts from leaves collected in the north and south-west parts of the country are similar in activity and approximately three times more active than the extracts from the central collection point. The difference was similar for aqueous and ethanol extracts, although the activity of the ethanol extracts is generally lower than of that of the aqueous extracts. The differences in activity correlated well with polyphenol content and to some extent also with visual appearance on TLC.

When discussing extract variation, it is important to also consider the effect of harvest period. In this study the collection took place within a period of 10 days in the middle of peak dry season in Burkina Faso. Daytime temperatures during this period can reach above 40˚C and there was very limited, or no rain fall the weeks prior to collection. As harvest time has previously been shown to affect the yield and composition of essential oil from *E. camaldulensis* (Moudachirou 1999), it should be considered that the differences seen in this study might not be valid for a different period for harvest.

The water extracts are resulting in both around three times better yield of extraction, as well as a higher antioxidant activity per g of extract. When considering this, in combination with the availability and suitability of ethanol use in a rural area, one could argue that only hydrodistillation was sufficient, or even preferable as extraction method. However, it is important to remember that the result of this study was only considering the chemical characteristics of the extracts, such as polyphenol content and DPPH-scavenging activity. Although it has been suggested that there is a correlation between these characteristics and antifungal activity, it is still not fully established (Dambolena 2010) and investigating the biological activity of both extracts would be relevant.

The lack of correlation between flavonoid content and antioxidant activity was apparent. When plotted, the results even show a week positive correlation between flavonoid content and antioxidative activity given as EC_{50}, suggesting that a lower amount of flavonoid would result in a higher antioxidative activity. Furthermore, the results in this study showed generally low flavonoid content, especially for the water extracts. Lately, some criticism against the $AlCl_3$ method for determination of total flavonoid content has been raised (Mammen 2012, Pękal 2013). One study suggested the method to be specific for flavonols and the flavone luteolin, rather than general for all flavonoids (Pękal 2013). A possible explanation for the low flavonoid contents of the extracts in this study, and the poor correlation to antioxidant activity, could be major components other than a flavonol/lueotin. Meaning it is a weakness of the method, rather than absence of flavonoids in the extracts, that is seen. This is somewhat supported by the visual appearance of yellow/orange and green bands on TLC, as well as by previously reports of major constituents of *E. camaldulensis* extracts including flavonoids (El-Ghorab 2003, Singab 2011, Ashraf 2015). These results suggest that the $AlCl_3$-method as used here might not be a suitable method for characterization or comparison of these extracts, and other methods or standards should be considered.

Soil analysis

Overall the soil analysis showed soils typical for Burkina Faso. The soils were identified as being sandy soils at the north and south-west collection point while sandy loam soil at the central collection point. Sandy

soils are generally well drained, well aired and with low fertility (Peverill 1999). This generally leads to low amount organic matter, which is keeping with the result obtained here. Regarding the soil nutrients, nitrogen, potassium and phosphor, the result in this study is showing the total amount of these elements in the soil. Hence, it is difficult to really draw conclusions about soil nutrients levels as it could be that they are not in a form available for plants.

Extract stability

Regarding the stability of extracts, the antioxidant activity of the water extract did not decrease during 6 weeks of storage, even at approx. 35˚C, while the ethanol extract did lose some of its activity. Worth pointing out is the fact that the stability screening was carried out in methanol, as this is the solvent used in the DPPH-method for determining antioxidative activity. It would be necessary to study the stability in more formulation realistic solvent such as water, or water-excipients solution, and then adjust the antioxidative assay or biological test accordingly. In the future work of developing a biopesticide formulation stability is indeed of great importance.

Methodology studies

The process of hydro-distillation followed by ethanol extraction showed good repeatability. Both water and ethanol extracts gave similar results when analyzed with regard to content and antioxidant activity. This means that the differences seen between the three extracts from different locations is not a result of variability in the processing. Interestingly, in one of the ethanol extracts a potential correlation between a slightly weaker TLC intensity and slightly lower antioxidative activity could be seen. However, this would need to be confirmed further.

The spectrophotometric methods showed an overall acceptable robustness with high incubation temperature being the single most important parameter. This could partly be explained by the fact that two of the assays were being run using methanol, which is a volatile solvent. It is important to take this into consideration during preparation of samples and all incubation of samples should be carried out with covered microplates. If high temperatures during preparation and incubation continued to be an issue, perhaps a less volatile solvent such as ethanol should be considered. The most sensitive of the three assays were the DPPH-method, and especially important was the incubation time, where both shorter and longer time affected the results significantly. The high sensitivity of this method could be explained by it depending on a radical reaction, rather than complex-formation such as the $AlCl_3$ and Folin-Ciocalteu Reagent (FCR) methods.

Environment and ethics

An effective, locally produced, biopesticide formulation based on *E. camaldulensis* would have clear environmental and public health benefits. Using eucalyptus leaves instead of other aromatic plants that has been considered for biopesticide formulation, such as e.g. *Mentha piperita* and *Cymbopogon citratus*, has its advantages. It would be to utilize an already existing waste product from the usage of stems for construction work in Burkina Faso. Additionally, *E. camaldulensis* is both robust and fast growing, meaning it is not occupying fertile arable land for its cultivation. However, criticism against eucalyptus trees for soil nutrient depletion and excessive soil water utilization has been raised (Aweto 2005), suggesting it to be unbeneficial for other species. If leaves from *E. camaldulensis* are to be used in a large scale production, it is important to ensure sustainable cultivation and to counteract any potential negative effect it might have on local flora or fauna.

6. Conclusions

Aqueous and ethanolic extracts from *E. camaldulensis* leaves collected in Burkina was shown to contain polyphenols and exhibit antioxidative activity in the form of DPPH-radical scavenging. The aqueous extracts generally gave both higher yields and showed greater antioxidant potential compared to the ethanol extracts. The extract from leaves collected in central Burkina Faso showed an approximately three times lower antioxidant activity than that from leaves collected in the north or south-west parts of the country.

Flavonoid content in the extracts was low and correlated poorly with antioxidative activity. Polyphenolic content and extract composition analysis using TLC are likely to be more suitable assays, at least for this type of extracts. Finally, a stability screening was carried out for the extracts and the result showed a good stability over a period of 6 weeks, especially the water extracts.

To understand the importance of the results in this study, it would be necessary to study the correlation between chemical characteristics and biological activity of these extracts. A thorough analysis of this correlation would give important information on the usefulness of *E. camaldulensis* extracts as biopesticides, and help determine what type of chemical characterization that would be important to include in any future work

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9. Appendix

Table A. *Characteristics of powdered E. camaldulensis leaves from three different collection points in Burkina Faso, including moisture content and ash content given as percentage.*

Collection Point	**Moisture Content [%]**	**Ash Content [%]**
North	5.2 ± 0.4	6.2 ± 0.0
Central	4.9 ± 0.3	5.6 ± 0.2
South-West	5.0 ± 0.1	5.8 ± 0.0