Evaluation of Antimalarial Activity of Sesquiterpene Lactones of Wild Sudanese Basil (Ocimum basilicum L.) through Their in vitro Inhibition of Dihydrofolate Reductase (DHFR)

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Abstract

Sudan is one of the countries severely afflicted with malaria. The spread of resistance of the malaria parasite *Plasmodium falciparum* to currently used antimalarial drugs calls for continued efforts to discover new ones. Basil (*Ocimum basilicum* L), which grows widely as a rainy season wild annual plant, is used, limitedly though, as one of the folk remedies of malaria in Sudan. In this study, crude ethanol extracts of the leaves of wild basil caused 100%, 79.8% and 49% inhibition of the growth of *P. falciparum*, at doses of 500, 50 and 5 μg/ml, respectively, when tested *in vitro* using RPMI 1640 media. Subsequent work focused on Sesquiterpene lactones present in the ethanol extract of basil, isolated as a group by the method of Harborne (1989) and further separated by preparative TLC, yielding 11 individual compounds. These separated compounds were evaluated for potential antimalarial activity by assaying their *in vitro* inhibition of dihydrofolate reductase (inhibitors of this enzyme deprive protozoa, as well as bacteria, of reduced folates, carriers of one-carbon fragments required for the biosynthesis of nucleic acids). Inhibition of DHFR was carried out using a commercially available DHFR enzyme (Sigma Co., USA) and spectrophotometrically following the coupled conversion of NADPH to $NADP^+$. Of the eleven compounds, eluted from TLC plates and tested, two were inhibitory to DHFR, resulting in specific activities of 17.2 and 8.7 μmole/min/mg protein. Further work is underway.

Keywords: Antimalarial, *Ocimum basilicum*, Dihydrofolate reductase inhibitors, Sesquiterpene lactones

1.Introduction

Malaria remains one of the most prevalent infectious diseases in the world. In 2006, there were approximately 247 million cases of malaria and 3.3 billion people that were at risk of the disease. Nearly 1 million deaths, mostly of children under the age of 5, were caused by malaria. There are currently 109 malarious countries and territories, of which 45 are within the World Health Organization (WHO) African region. Although malaria is a curable and preventable disease, its prevalence increased in the 1980s and 1990s as the parasites developed resistance to the most frequently used anti-malarial drugs and the vectors became resistant to insecticides. During the 1990s, child deaths caused by malaria increased by up to two-fold in some parts of sub-Saharan Africa (WHO, 2008).

In Africa and elsewhere, plant extracts are still widely used in the treatment of malaria and other ailments, and up to 80% of the African population use traditional medicines for primary health care (WHO, 2002). In China, traditional herbal preparations account for 30 to 50% of the medicines consumed. In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of the children with high fevers, resulting from malaria, is the use of herbal medicines at home (WHO, 2003).

In Sudan, out of 21 compounds isolated from 9 medicinal plants used in traditional medicine, only gedunin and quercetin showed IC_{50} of 1 μM as anti-plasmodial activity when tested *in vitro* against *Plasmodium falciparum*. Moreover, an investigation of anti-plasmodial activity of selected Sudanese plants revealed that most plants from the family Meliaceae showed highly potent anti-plasmodial activity against the two tested strains (3D7-chloroquine and pyrimethamine sensitive and Dd2-chloroquine resistant and pyrimethamine sensitive *Plasmodium falciparum* strains). *Khaya senegalensis* (Mahogany), *Azadirachta indica* (Neem) and *Trichilia emetic* (Dabkar) showed $1C_{50}$ values less than 5 μg/ml when tested against *Plasmodium falciparum* (El-Hadi *et al*., 2010).

Since little scientific data exist to validate anti-malarial properties of these medicinal plants, it is important that their claimed anti-malarial properties are investigated, in order to establish their efficacy and determine their potential as sources of new anti-malarial drugs (such as, artemisinin isolated from *Artemisia annua*).

The aim of this work was to evaluate the anti-malarial activity of wild Basil plant (*Ocimum basilicum* L.) using different methods of plant extraction in different anti-malarial bioassays as follows:

1- Crude ethanolic extracts (Hot Method)
2- Extraction of Sesquiterpene lactone (Harbone Method)
3- Extraction of alkaloid fraction (Wagner Method)
4- *In vitro* bioassay against malaria parasite using RPMI 1640
5- New sensitive anti-malarial method through *in vitro* inhibition on DHFR enzyme

1.1 Basil (Ocimum basilicum L.)

Ocimum basilicum is known by the vernacular names basil (English), basilica (French), rihan (Arabic), basilicum, basilienkraut (German), tulsi (Indian), basilica (Italian), Spanish (Spanish) (Simon *et al*., 1990). The Lamiaceae (Labiatae) to which the genus Basil belongs, is one of the ten largest families of flowering plants, comprising 9 subfamilies, 200 genera and about 3200 species (Politeo *et al*., 2007) and is widely distributed in tropical, subtropical and temperate zones of the world (Gupta, 2006).

Ocimum basilicum is a major essential producing-oil species belonging to the genus, with its oil showing a diversity of chemical structure (Grayer *et al*., 1996). Basil contains strong-scented essential oil composed primarily of chemical compounds such as eugenol, thymol, and estragol (Ozek *et al*., 1995). Basil also has what are known as chemotypes, minor variations among plants that contain significantly different mixes of constitute (Sajjadi, 2006). The exact components of basil oil vary widely, being affected not only by these chemotypes but also by factors such as the time of day of harvest (Randhawa and Gill, 1995; Vasconcelos *et al*., 1999). This may account for some of the variability in scientific and reports of medicinal efficacy of basil from culture to culture (Berger, 1985; Martin *et al*., 2003).

1.2 Traditional and Medicinal Uses

Leaves and flowering parts of *Ocimum basilicum* are traditionally used as antispasmodic, aromatic, carminative, digestive, galactogogue, stomachic, and tonic agents (Duke and Ayensu, 1985). They have been also used as a folk remedy to treat various ailments such as; feverish illnesses, poor digestion, nausea, abdominal cramps, gastro-enteritis, migraine, insomnia, depression, gonorrhea, dysentery and chronic diarrhea exhaustion (Chopra *et al*., 2002). Externally, they have been applied for the treatment of acne, loss of smell, insect stings, snake bites and skin infections (Martin and Ernest, 2004).

1.3 Biological Activities of Basil

Laboratory investigation carried out by Arthi *et al*., 2010, using the extract of plants like *Ocimum basilicum* and microbial pesticide spinosad against the malarial vector *Anopheles stephensi* Liston showed 85% mortality. Preliminary studies on essential oils of the most commonly grown ornamental type and wild-type of Sudanese basil showed strong anti-bacterial activity against *Escherichia coli, Staphylococcus auerus* and *Salmonella typhimurium* (Nour *et al*., 2009a). Methanol, ethanol and hexane extracts from *Ocimum basilicum* showed anti-candidal and anti-bacterial effects (Adiguzel *et al*., 2005). Also studies conducted by Farha (2007) on crude ethanol of Sudanese wild basil leaves showed that having anti-plasmodial activity on blood infected with *Plasmodium falciparum* tested *in vitro*. Mosquito larvicidal activity (Nour *et al*., 2009b) and mosquito repellency (Nour *et al*., 2009c) were also reported.

2. Materials and Methods

2.1 Collection of Plant Materials

The plant materials (leaves of *Ocimum basilicum* L.) were collected from the Demonstration Research Farm, University of Gezira, Wad Medani, Sudan at the period between April and June, 2010. This type of Sudanese basil grows in large pure stands during rainy season (Abdelrahman, 2007).

2.2 Preparation of Plant Materials

Basil leaves were collected and air-dried at room temperature (35±2ºC) for 3–5 days under shade. The plant materials (leaves) were ground in a laboratory electric blender just before extraction. Exposure to sunlight, during drying was avoided to prevent the loss of active compounds.

2.3 Extraction of Plant Materials

Hot extraction was carried out using absolute ethanol as a solvent in a Soxhlet apparatus for 4 hours after preparation of plant material (20 grams of dried leaves of basil) in powder form. The extract was concentrated in a rotary evaporator after filtration, and kept in refrigerator for further analysis.

The extraction of Alkaloids was carried out according to general procedure of Wagner *et al* (1984). Powdered of plant material (50 grams) were mixed thoroughly with 10% NH_4OH solution, then extracted by shaking for

about 5 minutes with 250 ml analar methanol at 60ºC using water bath. The filtrate was cooled and concentrated and kept for further analysis (Wagner *et al*., 1984).

The extraction of Sesquiterpene lactones was carried out according to the general procedure of Harbone (1998). Dried leaves (20 grams) were ground in a warring blender with 100 ml chloroform. The slurry was filtered under vacuum. The residue was dissolved in 25 ml of ethanol (95%) and 25 ml of aqueous lead acetate (40%) were added. Then the solution was filtered and the filtrate was concentrated. The water-oil mixture was extracted with chloroform and the extracts were kept for TLC analysis.

2.4 Phytochemical Screening of Plant Materials
A preliminary phytochemical analysis of the plant extracts was carried out using thin-layer chromatography (TLC). Standard screening tests using conventional protocol (Wagner *et al*., 1984) were utilized for detecting the major components.

2.5 UV Spectral Analysis
All spectra were measured according to the Continuous Spectrophotometric Rate Determination Method, described by Mathew *et al* (1963). This method was used for determination of purified isolates activities on inhibition of DHFR using an UV Gilford – 2000 attached to a Beckman DU Spectrophotometer at Central Laboratory, Faculty of Sciences, University of Cairo, Egypt.

2.6 *In vitro* Anti-plasmodium Assay
The petri-dish candle jar method was used in this study. The technique used was based on the work of Jensen and Trager (1977). Blood with erythrocytes infected with *Plasmodium falciparum* was collected from patients infected with malaria at Wad Medani Teaching Hospital. The degree of infection was determined according to the laboratory test carried out at the Teaching Hospital. The anti-plasmodium assay procedure consisted by mixing infected human blood with prepared culture medium in a ratio of 1:9 incubated with plant crude ethanolic extract with different concentrations were added to 100 μl of the blood/RPMI mixture in microtitre plate wells. A control treatment included the blood/RPMI mixture without plant extracts. The microtitre plate was placed in a desiccator containing two lighted-white candles and a stop cock partially closed. This was meant to provide optimum conditions for parasite growth of $2 - 3\%$ CO_2 and $5 - 10$ ml of water in the bottom of the desiccator to insure high humidity.

The desiccator was placed in an incubator at 37ºC for 48 hours. Thereafter, the red blood cells were harvested from the plates using pipettes. Thin and thick films were prepared in two rows of the same slide, and they were stained with Giemsa stain. The level of parasitemia was determined by microscopic counting of 1000 cells per field. The percentage inhibition of parasite growth was calculated according to the following equation:
% inhibition = (1 - A) × 100
Where: - **A** = Number of infected erythrocytes in treatment/ Number of infected erythrocytes in control

2.7 Enzymatic Assay of DHFR
The DHFR assay was carried out at a Central Laboratory, Faculty of Science, University of Cairo, Egypt. DHFR kit (Sigma Co.) was used.
 a – Principle: $DHF + \beta\text{-}NADPH + H^+ + DHFR \leftrightarrow THF + \beta\text{-}NADP^+$
b – Reagents used were Dihydrofolate Reductase (Product code D6566), Assay Buffer 10X for DHFR (Product code A5603), Dihydrofolic acid, DHFR substrate (Product code A6770) and Nicotinamide Adenine dinucleotide phosphate (NADPH) reduced tetrasodium salt) (Product code N6505).
c – Procedure of Assay for DHFR Activity:
Spectrophotometer was set at 340 nm and 25 °C, kinetic program (reading every 0.5 minutes for 2 minutes). Assay Buffer 1X was added to the test microcentrifuge tube according to the reaction scheme and to the test being performed. DHFR enzyme was added to the appropriate tube, and mixed well. For Inhibition assay only, the isolated compounds (inhibitors) were added and mixed well. The content of the tube was transferred to a quartz cuvette (1 ml). NADPH solution (6 ml) was added. The cuvette was covered using Parafilm and mixed by inversion. Dihydrofolic acid (5 ml) was added just before starting the reaction (dihydrofolic acid is the substrate of the reaction). After inversion immediately, the cuvette was inserted into the spectrophotometer. The kinetics program was started immediately. The absorbance at 340 nm decreased (due to decrease in NADPH concentration). 10-20 ml of the supplied DHFR enzyme usually gave a linear slope during the 2.0 minutes of the detection.

Specific activity was calculated by the formula:
$$\text{Units/mg P} = \frac{(\Delta OD/min_{sample}) - (\Delta OD/min_{blank}) \times d}{12.3 \times V \times mg\ P/ml}$$

Units/mg P: Specific activity in µmol/min/mg protein
Unit definition: One unit will convert 1.0 µmole of dihydrofolic acid to tetrahydrofolic acid in one minute at pH 7.5 at 25°C. (This is equivalent to the conversion of NADPH to $NADP^+$).
The equation referred to a reaction volume of one ml. Specific activity was expressed as µmole/min/mg protein. Where by:-
V = 10µl = 0.01ml, d = 1 and mg P/ml = 0.032 mg/ml

3. Results and Discussion

Drugs for malaria treatment are commercially available in pharmacies. However, development of resistance to those available drugs by malaria-parasite is the main problem, in addition to their undesirable side-effects.
Natural products from plants represent cheap, effective and safe alternative drugs. Moreover, Sudanese flora is rich and varied as well as people's experiences and beliefs on medicinal plants varied with different ethnic groups.

The main basis of selection of basil plant (*Ocimum basilicum* L.) is depended on its uses in folk medicine. In some areas of Southern Sudan is used as anti-malarial while in different other parts of Sudan is used to treat other human diseases.

3.1 Anti-malarial Activity of Crude Ethanolic Extract:

The plant material was extracted with ethanol (98.9%) in a Soxhlet extractor. After filtration, the dry extracts were weighed and different concentrations were prepared according to W/V (i.e. weight of plant material extracts in a volume of ethanol).

Screening of anti-malarial activity of basil leaves with different concentrations against *Plasmodium falciparum* was carried on RPMI 1640 medium. The activity was increased with increasing of extract concentration as shown in Table (3.1). The high concentration of basil extract (500µg/ml) gave 100% growth inhibition followed by 79.8% inhibition for 50µg/ml concentration. However, the low concentration (5µg/ml) gave 49% growth concentration in this *in vitro* study. Farha (2007), reported on the activity of *in vitro* study of crude ethanolic extract of basil leaves on RPMI 1640. The non-defatted gave the same percentage growth inhibitions that agree with our results i.e. 5, 50 and 500µg/ml gave 50, 80 and 100% parasite inhibition respectively. Moreover, Kayembe *et al* (2010) found that 12.5µg/ml crude extract resulted in 78% inhibition of malaria-parasite; it is also within the range of our results. Further researches of modern methods of enzyme inhibitor were applied as discussed below.

Table 3.1: *In vitro* Anti-malarial Activity of Crude Ethanolic Extract (3 concentrations)

Concentration (µg/ml)	Growth inhibition (%)
5	49
50	79.8
500	100

3.2 Studies of Purified Isolates of Sesquiterpene Lactones Using DHFR Inhibitors

Dihydrofolate reductase, DHFR, that role is to regenerate folic acid into its reduced form tetrahydrofolate, is necessary for bacteria, Plasmodia and normal cancerous human cells. Inhibitors of DHFR have antibiotic, anti-malarial and anti-neoplastic properties. The mechanism of action according to Nicola *et al* (2007) showed that, folate and its derivatives are important co-factors in synthesis of nucleotides (DNA) for all organisms (man, Plasmodia, etc.). Synthesizing nucleotides (DNA) required tetrahydrofolate. THF is from DHF by action of DHFR. Thus, any drug that can selectively inhibit the protozoa DHFR can inhibit and kill the protozoa. Malarial dihydrofolate reductase is 2000 times more sensitive than mammalian DHFR.

3.2.1 Sesquiterpene Lactones Activity

Sesquiterpene lactones isolated from Indigenous wild basil were selected to test their anti-malarial activities. These compounds were extracted as a group from dry leaves of wild basil according to Harbone Method. Thin Layer Chromatography (PTLC) separated eleven compounds. The separated lactones were eluted from TLC plates individually and they were evaluated for their *in vitro* inhibition to DHFR. Only five compounds, out of eleven have been shown to possess anti-malarial specific activity with their inhibition on DHFR. The five compounds results in inhibition of more than one µmol/min/mg protein (Table 3.2).

In Table (3.3), three separated compounds of lactones 8, 9 and 10 showed the same low activity (1.7784 µmol/min/mg protein), followed by high activity associated with the compound-5, reaching up to 8.7195 µmol/min/mg protein. However, the greatest activity associated with compound-6 giving a value of 17.2174 µmol/min/mg protein. According to Nicola *et al* (2007) Methods on reductase in *Plasmodium falciparum* by structure-based screening, it seems that Sesquiterpene isolates of basil possessing high specific activity in an *in vitro* study on inhibition of DHFR varying from 1 up to 17µmol/min/mg protein.

Table 3.2: Anti-malarial Specific Activity* of Sesquiterpene Lactones Separated by TLC on Inhibition of DHFR

Bands (TLC-plate from origin)	R_f % values	Absorbance at 340 nm with time intervals in minutes				Mean specific activity (µmole/min/mg protein)
		0.5	1.0	1.5	2.0	
0 **	0.0	0.0980	0.0985	0.1003	0.0994	-***
1	7.5	0.0635	0.0644	0.0640	0.0664	-
2	11.8	0.0024	0.0045	0.0054	0.0050	-
3	15.7	0.1624	0.1645	0.1654	0.1650	-
4	52.7	0.1394	0.1415	0.1424	0.1420	-
5	56.5	0.2007	0.1777	0.1652	0.1560	8.71935
6	70.0	0.2376	0.2004	0.1685	0.1406	17.21714
7	70.9	0.3191	0.3215	0.3257	0.3285	-
8	78.9	0.2707	0.2707	0.2707	0.2637	1.7846
9	93.9	0.1330	0.1330	0.1330	0.1260	1.7846
10	96.9	0.1246	0.1246	0.1246	0.1176	1.7846

(Sample w/v was determined according to Sigma Method i.e. 0.032 mg/ml)
*Specific activity =one unit will convert 1.0 ≥mole of DHF to THF in one minute at pH 7.5 at 25 °C.
**0 = Origin
 *** = Not detected

Table 3.3: Mean of Specific Activity of Most Active Lactones Components Separated on TLC-plate

Compounds (TLC bands No.)	Time interval per minute	OD (Sample activity at 340 nm/min)	ΔOD (Sample - Blank)	Mean specific activity (µmole/min/mg protein)
5	0.5	0.2007		
	1.0	0.1777	0.0230	
	1.5	0.1652	0.0355	
	2.0	0.156	0.0447	8.71935
6	0.5	0.2376		
	1.0	0.2004	0.0372	
	1.5	0.1685	0.0691	
	2.0	0.1406	0.0970	17.21714
8	0.5	0.2707		
	1.0	0.2707	0	
	1.5	0.2707	0	
	2.0	0.2637	0.007	1.77846
9	0.5	0.1330		
	1.0	0.1330	0	
	1.5	0.1330	0	
	2.0	0.1260	0.007	1.77846
10	0.5	0.1246		
	1.0	0.1246	0	
	1.5	0.1246	0	
	2.0	0.1176	0.007	1.77846

(Inhibition of DHFR was calculated according to Sigma Method)
No literature available on research of basil extracts anti-malarial activity used for the inhibition of DHFR enzyme.

3.2.2 Studies of Basil-Alkaloid on Inhibition of DHFR Enzyme
Only one alkaloid compound was separated from dry leaves of wild basil according to Wagner *et al* (1984). The separated alkaloid compound was eluted from PTLC plate and tested for *in vitro* using enzyme DHFR inhibitor. No specific activity detected on inhibition of DHFR with basil-alkaloid.

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