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Antimicrobial activity of solvent extracts from the leaves of *Tarchonanthus camphoratus* (Asteraceae)

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ABSTRACT
The plant *Tarchonanthus camphoratus* belongs to the Asteraceae family. Its common name is wild sage or camphor bush. It is widely distributed, but has similar medicinal use throughout its range. It is taken orally or applied externally to relieve bronchitis, asthma, headache, inflammation, chilblains or abdominal pains. The aim of the study was to assess the antimicrobial activity of the extracts from the leaves of this plant. A powdered sample of the leaves weighing 500 g was sequentially extracted with hexane (1.5 L), dichloromethane (1.5 L) and methanol (3 x 1.5 L). The methanolic extract was partitioned between equal volumes (250 ml each) of distilled water and n-butanol. The n-butanol fraction was concentrated under reduced pressure followed by addition of diethyl ether, precipitating 20 g of a dry crude extract. The extract was successively fractionated using hexane, dichloromethane, chloroform, ethyl acetate, acetone, ethanol and methanol yielding 1.6 g, 1.4 g, 1.6 g, 7.0 g, and 1.4 g, 2 g and 4 g respectively of samples soluble in each solvent. Phytochemical tests revealed the presence of terpenoids in hexane, dichloromethane, and chloroform fractions. Ethyl acetate fraction contained terpenoids and phenolics. Tannins, phenolics and flavonoids were present in the acetone and ethanol fractions while tannins, phenolics and saponins were present in the methanol fraction. Antimicrobial activity tests of the extracts soluble in various solvents were then performed on *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Salmonella typhi, Candida albicans* and *Bacillus spp*. Ethyl acetate fraction was active against *E. coli, P. mirabilis, K. pneumoniae, S. typhi, and Bacillus spp* while the methanol fraction was active against *E. coli, K. pneumoniae, S. typhi, and Bacillus spp*. Minimum Inhibitory Concentration of Ethyl acetate fraction was 80 mg/ml, 67 mg/ml, 57 mg/ml and 200 mg/ml for *E. coli, P. mirabilis, Bacillus spp* and *S. typhi* respectively. The inhibitory bioactivity against a variety of bacteria validates the traditional usage of the plant as a remedy for various conditions.

Keywords: *Tarchonanthus camphoratus*, Phytochemicals, Antimicrobial activity.

1. Introduction
Nature has been a source of medicinal agents for thousands of years. Today, despite advances in pharmacology and synthetic organic chemistry, this reliance in natural products, particularly on plants, remains largely unchanged [1]. Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitates pharmacological studies of more potent drug with reduced toxicity [2].

The therapeutic or medicinal properties of plants are normally dependent upon the presence of certain active principles [3]. Plant based antimicrobials represent a vast untapped source of medicine with enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are associated with synthetic antimicrobials [4].

The plant kingdom can indeed be regarded as perhaps the largest potential source for the development of new drugs [5]. Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that appear to be active. These complementary components give the plant as a whole a safety and efficacy much superior to that of its isolated and pure active components [6]. The information obtained from ethno medicine is therefore being put on a scientific basis and it is very important to investigate the pharmacological and phytochemical aspects of different preparations from plant sources [7].

Tarchonanthus camphoratus (Asteraceae) [8] is a multi–stemmed shrub species naturally occurring in Africa and Arabia. Its common name is wild sage or camphor bush and local name Leleshwa (Kalenjin). These tree bushes form an excellent tree crop that provides a sustained source of slow burning high-grade charcoal in Kenya [9].

Phytochemical tests indicate the presence of tannins, saponins and reducing sugars, but not alkaloids, cardiac or anthraquinone glycosides [10]. A more recent investigation of antimicrobial activity of aqueous, ethanolic and hexane extracts of dried leaf did not demonstrate *in vitro* inhibitory effects against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli or Klebsiella pneumoniae* [11]. The current study was undertaken to assess the medicinal potential of the plant and validate its usage in traditional therapy.

2. Materials and methods

2.1 Collection and identification of the plants

The leaves of *Tarchonanthus camphoratus* were collected from the Egerton University Botanical Garden and identified by a plant taxonomist. The green leaves were dried under shade to constant weight and ground to a coarse powder using a domestic miller.

2.2 Extraction and fractionation of the plant material

A powder weighing 500 g of *Tarchonanthus camphoratus* leaves was extracted sequentially with hexane (1.5 L), dichloromethane (1.5 L) and methanol (3 x 1.5 L) after soaking the sample in each solvent for 24 hours. The methanol extract was filtered through a Buchner funnel fitted with a vacuum pump with a thin layer of activated charcoal, and then concentrated using a rotary evaporator and the solvent recovered. The crude methanol extract was partitioned between equal volumes (250 ml each) of distilled water and n-butanol, a procedure that targeted isolation of saponins. The n-butanol fraction was concentrated under reduced pressure followed by addition of diethyl ether, precipitating a crude sample, which was dried in desiccators after centrifugation at 3000 RPM for 15 minutes to obtain 20 g of amorphous powder. The dry sample was then extracted using hexane (4 x 200 ml), dichloromethane (4 x 200 ml), chloroform (4 x 200 ml), ethyl acetate (4 x 200 ml), acetone (4 x 200 ml), ethanol (4 x 200 ml) and methanol (4 x 200 ml). The solvents were recovered using a rotary evaporator to obtain 1.6 g, 1.4 g, 1.6 g, 7g, 1.4 g, 2 g and 4 g of dry hexane, dichloromethane, chloroform, ethyl acetate, acetone, ethanol and methanol soluble fractions respectively.

2. 3 Phytochemical screening of the fractions

The samples were tested for the presence of various phytochemicals.

2.3.1 Test for tannins /phenolic compounds

Dried samples weighing 0.2 g were boiled in 10 ml of distilled water in a test tube and then filtered. 2 ml of 0.1% $FeCl_3$ solution was then added to the filtrate. A positive test was confirmed by the formation of a characteristic blue, blue-black, green or blue-green color (tannins) and precipitate (phenolic compounds) [12].

2.3.2 Test for flavonoids

This was done by dissolving 0.2 g of each extract in 5 ml of distilled water and filtered. This was followed by the addition of 5 drops of 1% aluminium chloride solution to the aqueous filtrate. Formation of a yellow coloration indicated the presence of flavonoids [12].

2.3.3 Test for Steroids

The test was performed by dissolving 0.l g of each extract in 1 ml ethanol followed by the addition of 1 ml of acetic anhydride and 1 ml H_2SO_4. The presence of steroids was indicated by the change of color from violet to blue or green [13].

2.3.4 Test for terpenoids (Salkowski test)

0.1 g of each fraction was dissolved in 2 ml $CHCl_3$ followed by addition of 2 ml conc. H_2SO_4. A positive test was confirmed by the appearance of a red coloration at the interface [13].

2.3.5 Test for cardiac glycosides (Keller-Kiliani test)

Each fraction was mixed with 2 ml of glacial acetic acid containing one drop of 0.1% ferric chloride solution. The mixture was then underlayed with 1 ml of concentrated sulphuric acid. The formation of brown ring at the interface or a greenish ring in the acetic acid layer was considered a positive test [13].

2.3.6 Test for saponins

0.2 g of powdered sample of the extract was boiled in 10 ml of distilled water on a water bath and filtered. A fraction of the aqueous filtrate measuring 2 ml was mixed with 2 ml of distilled water and shaken vigorously for stable persistence froth. The frothing was mixed with a few drops of olive oil and shaken vigorously. Formation of an emulsion confirmed the presence of saponins [13].

2.4 Antimicrobial screening of the fractions

The anti-bacterial activity was done using the American Test Culture Collection (ATCC) and clinical isolates from the Kenya Medical Research Institute (KEMRI). The gram-negative bacteria used were *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae, Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* and *Salmonella typhi* while gram–positive ones were *Staphylococcus aureus* ATCC 25923 and *Bacillus spp.* The anti-fungal activity of the extracts was done on the pathogenic fungus *Candida albicans*. The antimicrobial activity test of the fractions was done using disc diffusion method [14].

2.5 Media preparation

The Mueller Hinton agar was prepared by dissolving 38 g of the solid media in distilled water to make a liter of solution, while Sabouraud Dextrose Agar (SDA) was prepared by dissolving 65 g of solid powder in a liter of solution. The solutions were then sterilized in an autoclave at 121 ^oC for 15 minutes, cooled then poured in Petri dishes. The solutions were then left to solidify.

2.6 Inoculation and incubation

Antibacterial activity was done on Mueller Hinton agar while antifungal activity on Subouraud Dextrose Agar (SDA). 1 ml of fungi/bacteria suspension was uniformly spread on the sterile Sabouraud Dextrose Agar/ Mueller Hinton Agar Petri dish. 0.4 g of each sample was dissolved in 1 ml of the respective fractionation solvents. Sterile filter paper disc (Whatman no.1, diameter 6mm) was soaked with 0.01 ml of the extract and the solvent allowed to dry. The disc was placed on the Sabouraud Dextrose agar/Mueller Hinton agar Petri dish inoculated with bacteria/fungi suspension and kept at 4 ^oC for 48 hours to allow the extracts to diffuse into the media.

The Petri dish was then placed in an incubator for 24 hours at 37 ^oC. At the end of the incubation period, the inhibition diameter was measured using calipers and expressed in millimeters. Chloramphenicol and Nystatin were used as standards. Positive antibacterial and antifungal activities were established by the presence of measurable zones of inhibition.

2.7 Minimum Inhibitory Concentration (MIC)

The MIC was performed on hexane, ethyl acetate and methanol fractions, which on screening, had been found to be moderately active against most of the microbes tested. A sample weighing

0.40 g of each fraction was dissolved in 1ml of the fractionating solvent to form a solution having a concentration of 400 mg/ml. Other concentrations were obtained by taking 0.1 ml of the solution containing 400 mg/ml and adding 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 ml of the solvent separately to obtain new solutions with concentrations 200 mg/ml, 133 mg/ml, 100 mg/ml, 80 mg/ml, 67 mg/ml, 57 mg/ml, 50 mg/ml and 44 mg/ml respectively. The concentration necessary to cause minimum inhibition was then determined (Tables 3, 4 and 5).

3. Results and Discussion

Table 1: Summary of phytochemical test results

Fraction	Tannins	Phenolic compounds	flavonoids	terpenoids	Steroids	Cardiac glycosides	saponins
Hexane	–	–	–	+	–	–	–
CH_2Cl_2	–	–	–	+	–	–	–
$CHCl_3$	–	–	–	+	–	–	–
EtOAc	–	+	–	+	–	–	–
Acetone	+	+	+	–	–	–	–
EtOH	+	+	+	–	–	–	+
MeOH	+	+	–	–	–	–	+

Key: + present, –Absent

Hexane, dichloromethane, and chloroform fractions contained terpenoids (Table 1). Ethyl acetate fraction contained terpenoids and phenolics. Tannins, phenolics and flavonoids were present in the acetone and ethanol fractions while tannins, phenolics and saponins were present in the methanol fraction.

3.1 Antimicrobial activity

Antimicrobial activity was performed with the aim of identifying the fraction(s) containing the active compound(s). The Minimal Inhibitory Concentration (MIC) was then performed on the fractions that exhibited reasonable activity. The extracts soluble in some solvents demonstrated moderate activity when tested against some Gram-positive and Gram-negative bacteria and fungus (Table 2). Hexane, ethyl acetate and methanol fractions showed higher activity against most microbes than other fractions.

Table 2: Antimicrobial activity screening of the fractions

		Inhibition Zones (mm)							
Microorganism		Sample (400 mg/ml)							
Gram-negative bacteria	**Culture collection and Ref. No.**	**Hexane**	**CH_2Cl_2**	**$CHCl_3$**	**EtOAc**	**Acetone**	**EtOH**	**MeOH**	**Standard**
E. coli	ATCC 25922	14	0	9	15	0	0	14	30
S. typhi	KEMRI	10	0	0	9	0	0	12	30
K. pneumoniae	KEMRI	8	0	0	7	0	0	10	20
P. mirabilis	KEMRI	0	0	8	16	0	8	0	25
P. aeruginosa	ATCC 27853	0	0	0	0	0	0	0	0
Gram-positive bacteria									
S. aureus	ATCC 25923	11	0	0	0	0	0	0	30
Bacillus spp	KEMRI	14	0	0	18	8	0	7	18
Fungus									
C. albicans	KEMRI	10	0	0	0	0	0	10	18

3.1.1 Hexane fraction

The hexane fraction demonstrated reasonable activity (Table 2) against *E. coli, S. typhi, K. pneumoniae, S. aureus, Bacillus spp* and *C. albicans* while *P. aeruginosae* and *P. mirabilis*, were resistant. *P. aeruginosae* was resistant even to the standard chloramphenicol drug. *P. aeruginosa* and *P. mirabilis* are both gram-negative bacteria which are less susceptible to antimicrobial agents than gram–positive ones because they possess outer membrane surrounding the cell wall [15]. A previous study on the aqueous, ethanol and hexane extracts of the same plant species did not demonstrate in vitro inhibitory effects against *S. aureus, Bacillus subtilis, E. coli and K. pneumoniae* [11]. The differences in the antimicrobial properties of the same plant species may be due to the fact that geographical areas of the plants play a role in the availability of the bioactive secondary metabolites in plants [16]. The active metabolites may also be generated only during

specific developmental period of the plant, and also these compounds partition exclusively in particular solvents [17].

3.1.2 Dichloromethane, chloroform, acetone and ethanol fractions

Almost all the microorganisms were resistant to these fractions (Table 2). The medicinal properties of plant extracts normally depend upon the presence of active compounds [2] possessing specific functional groups that are soluble only in solvents of particular polarity. The active compounds in the extract of the leaves of *Tarchonanthus camphoratus* were therefore not soluble in these solvents.

3.1.3 Ethyl acetate fraction

Ethyl acetate fraction exhibited the highest activity against *E. coli*, *P. mirabilis* and *Bacillus spp* compared to all other fractions. The activity of 4 X 10^3 µg of the extract against *P. mirabilis* and *Bacillus spp* compared favorably with that of 30 µg of the standard chloramphenicol. The big difference with the reference antibiotic could be due to the fact that the active compound was only a small percentage of the extract since no purification had been done at this stage. The fraction was moderately active against *E.* coli, *P. mirabilis* and *Bacillus spp* and weakly active against *S. typhi* as compared to the standard (see Table 2). The gram-positive bacteria, *S. aureus*, the gram-negative bacteria, *P. aeruginosa* and the fungus, *C. albicans* were resistant to the ethyl acetate fraction (see Table 2).

3.1.4 Methanol fraction

The fraction representing 4 X 10^3 µg showed lower activity than 30 µg of the standards (Table 2) with the highest activity being recorded against *E. coli* and the lowest activity against *Bacillus spp*. On the other hand, *S. aureus*, *P. aeruginosa* and *P. mirabilis* were all resistant to the fraction.

3.2 Minimum Inhibitory Concentration (MIC)

The MIC was performed on hexane, ethyl acetate and methanol fractions, which on screening, had been found to be moderately active against most of the microbes.

3.2.1 Hexane fraction

Table 3: Hexane fraction MIC

	Inhibition Zones (mm)									
	Concentration (mg/ml X 10^2)									
Microorganism **Gram-negative bacteria**	**4.00**	**2.00**	**1.33**	**1.00**	**0.8**	**0.67**	**0.57**	**0.50**	**0.44**	**STD (mg/ml)**
E. coli	14	9	8		0	0	0	0	0	25.0
S. typhi	10	8		0	0	0	0		0	25.0
Gram-positive bacteria									0	
S. aureus	11	9		0	0	0	0	0	0	31.3
Bacillus spp	14	11	8		0	0	0	0	0	26.3
Fungus										
C. albicans	10	9		0	0	0	0	0	0	-

▨ -Minimum inhibitory concentration

The MIC of the hexane fraction was 100 mg/ml against *E. coli* and *Bacillus spp*, whereas against the *S. aureus*, *S. typhi* and *C. albicans* the MIC was 133 mg/ml. The hexane fraction was therefore most active against *E. coli* and *Bacillus spp.* (Table 3). The MIC of the standard chloramphenicol for the same microorganisms was in the range of 25 mg/ml to 31.3 mg/ml. The standard was therefore approximately four times more active than the hexane-soluble fraction.

3.2.2. Ethyl acetate fraction

Table 4: Ethyl acetate fraction MIC

	Inhibition Zones (mm)									
	Concentration (mg/ml X 10^2)									
Microorganism **Gram-ve bacteria**	**4.00**	**2.00**	**1.33**	**1.00**	**0.8**	**0.67**	**0.57**	**0.50**	**0.44**	**STD (mg/ml)**
E. coli	15	13	11	9		0	0	0	0	25.0
P. mirabilis	16	15	13	12	8		0	0	0	_
S. typhi	9		0	0	0	0	0	0	0	25.0
Gram-positive bacteria										
Bacillus spp	18	15	14	12	10	9		0	0	26.3

▨ -Minimum inhibitory concentration

The MIC of Ethyl acetate fraction (Table 4) ranged from 57 mg/ml to 200 mg/ml as compared to the standard values of between 25 mg/ml and 26.3 mg/ml for the same microorganisms. The MIC of the fraction for *E. coli* was 80 mg/ml compared to 25.0 mg/ml of the standard. *Bacillus spp.* was most susceptible to the fraction with a MIC of 57 mg/ml compared to 25.0 mg/ml for the standard. The standard was therefore only twice more active than the fraction against *Bacillus spp.* The fraction was least active against *S. typhi* with a MIC of 200 mg/ml compared to 25.0 mg/ml of the standard.

3.2.3 Methanol fraction.

Table 5: Methanol fraction MIC

	Inhibition Zones (mm)									
	Concentration (mg/ml X 10^2)									
Microorganism / **Gram-ve bacteria**	**4.00**	**2.00**	**1.33**	**1.00**	**0.8**	**0.67**	**0.57**	**0.50**	**0.44**	**STD (mg/ml)**
E. coli	14	10	11	9	▨	0	0	0	0	25.0
K. pnemoniae	10	9	▨	0	0	0	0	0	0	22.5
S. typhi	12	10	8	▨	0	0	0	0	0	25.0
Fungus										
C. albican	10	▨	0	0	0	0	0	0	0	-

▨ -Minimum inhibitory concentration

The Minimum Inhibitory Concentrations for *E. coli, K. pneumoniae, S. typhi* and *C. albican* were 80 mg/ml, 133 mg/ml, 100 mg/ml and 200 mg/ml respectively (Table 5). The MIC of the methanol fraction was between 80 mg/ml and 200 mg/ml compared to 22.5 mg/ml and 25.0 mg/ml for the standard (Table 5). The fraction was most active against *E. coli* with MIC of 80mg/ml and least active against *C. albicans* with MIC of 200 mg/ml.

4. Conclusions

The antimicrobial activity tests results contradicted the findings of a previous study, which showed that the extracts of the leaves of this plant are not active against most Gram-positive and Gram-negative bacteria. The findings of this work support, at least in part, the validity of the use of *Tarchonanthus camphoratu*s in traditional medicine.

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