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Evaluation of *Tarchonanthus camphoratus* plant extracts for antimicrobial activity against food-borne pathogens.

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ABSTRACT:

Food-borne pathogens are a major cause of illness, there are about 31 different pathogens known to cause food-borne illnesses. Meat products such as poultry meat, eggs, pork and beef are amongst the most important sources of food-borne illnesses. Antibiotics have also been used in animal feed to control food-borne pathogens however, resistance, which is an inevitable consequence of antibiotic; is an increasing public health problem. Plant secondary metabolites have been shown to exhibit antimicrobial activity against pathogens that cause food-borne diseases. This study evaluated the antimicrobial properties of *Tarchonanthus camphoratus*, the camphor bush, against three common poultry pathogens: *Salmonella typhi, Escherichia coli* and *Staphylococcus aureus.* Aqueous and organic extracts of the plant (leaves, stems and bark) were prepared using distilled water and 80% methanol. Phytochemical analysis revealed the presence of terpenoids and glycosides in both the extracts. Organic leaf extract exhibited the highest inhibitory activity against *S. typhi* (0.5 mg/ml, inhibition zone 19.3 mm in diameter), *S. aureus* (10 mg/m, 19.7mm) and *E. coli* (1 mg/ml, 7.7 mm) consistent with ethnomedicinal use. However, aqueous extracts exhibited no antimicrobial activity against the test microorganisms. *T. camphoratus* extracts exhibited antimicrobial activity against all the three tested food borne pathogens and its use should be considered in combination with conventional antibiotics.

Keywords:
Tarchonanthus camphoratus, *Salmonella typhi*, *Staphylococcus aureus, Escherichia coli,* phytochemical analysis, well diffusion assays, antimicrobial activity.

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INTRODUCTION

Food-borne pathogens are a major cause of concern in all parts of the world. With 31 different pathogens known to cause food-borne illnesses (CDC, 2011), various methods of bacterial growth control such as heat, sterilization and the use of antibiotics, have been employed to reduce the microbial populations and techniques and maintain them at levels below their infection rates. But even with improvements made during the past decade, the burden of food-borne illnesses still persists (Osterholm, 2011) causing human suffering and loss of productivity, and adding significantly to the cost of food production and healthcare.

Among many food types that can serve as sources of food-borne illness, meat products remain as one of the most important with poultry meat, eggs, pork and beef being more prominent in this regard (Petrovic *et al.,* 2010). In Europe, *Salmonella* and *Campylobacter* are the most frequently reported causes of food-borne illnesses with incidences of 38.2-51.6 cases per 100, 000 population being reported in 2005 (Petrovic *et al.,* 2010). While in the US, Centers for Disease Control and Prevention (CDC) estimates that each year roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of food-borne illnesses.

Of the various food-borne pathogens that have been identified as causative agents of food-borne illnesses, *Campylobacter, Norovirus, Listeria monocytogenes*, *Toxoplasma gondii, Clostridium perfringens, Salmonella* sp, *Staphylococcus aureus* and *Escherichia coli* O157:H7 have generally found to be the top five agents responsible for majority of food-borne illnesses, hospitalizations and deaths (CDC, 2011).

Pathogens such as *Staphylococcus aureus* (gram +ve), *Salmonella* (gram -ve) *and Escherichia coli* (gram -ve) are common in poultry and have been discovered to be a cause of food borne illnesses in humans (Mbata, 2005). *Staphylococcus aureus* are gram +ve bacteria that can be found living in the soil, on the skin or mucous membranes of humans and on the bodies of animals (Van Huyssteen, 2008). *E. coli* are the predominant gram -ve organisms living in the intestines of humans and animals and are known to cause diarrhea and urinary tract infections. They have also been found to be the causative agents of a variety of disease conditions in poultry such as colisepticemia, coligranuloma, air sacculitis, peritonitis, peicarditis, omphalitis and oophoritis, that account for about 5-50% mortality in poultry flocks (Sharada *et al.,* 2010). The presence of *E. coli* in foods is usually an indication of faecal contamination (FSIC, 2003).

Antibiotics have also been used in animal feed to control food-borne pathogens, such as in poultry, but this does not always guarantee the safety of the meat since in the long run the microbes may develop resistance to the antibiotics and the quality and taste of the meat may be affected. It is still possible that animal strains passing transiently through the human gut might transfer their resistance to human strains and cause human infections (Phillips *et al.,* 2004).

Antimicrobial resistance in bacteria is an increasing public health problem, with resistance being an inevitable consequence of antibiotic use; the more antibiotics are used, the more resistant the bacteria become (Smith *et al.,* 2011). This poses a major threat to the continued effectiveness of antibiotics used to treat human and veterinary illnesses, which results in the emergence of antibiotic resistant zoonotic bacteria that can be transmitted to humans through the food chain (Walsh and Fanning 2008). The use of plants for their curative properties is not novel, (Cowan, 1999), the pharmaceutical industry is always seeking for new, better and natural drugs which can be obtained from medicinal plant extracts (Marius Hedimbi *et al.*, 2012). Studies on the roots, stems, leaves, seeds, flowers and fruits of many

plants have found that they possess antimicrobial characteristics (Anburaja *et al.,* 2011) and these antimicrobial characteristics have been found to be effective against different pathogens such as those that cause food-borne diseases. Leaf extracts of the plant *Senna siamae* (Kassod Tree), traditionally used for treating infectious diseases, have been found to possess antibacterial activity against *Salmonella typhi* (Doughari and Okafor, 2008). Stem bark extracts of *Ziziphus mucronata* (Buffalo Thorn) were tested against medically important pathogens such as *Escherichia coli* and *Staphylococcus aureus* and found to have significant antimicrobial activity against both the bacteria (Olajuyigbe and Afolayan, 2012). Therefore control of food-borne pathogens through the use of plant material with antibiotic properties may mitigate against the development of antibiotic resistance and the loss of product quality through the use of antibiotics. *Tarchonanthus camphoratus* (wild camphor bush), has been used in traditional settings to treat ailments such as bronchitis and inflammation, in addition to abdominal cramps and asthma (Van Wyk *et al.,* 1997). A study was conducted to investigate the presence of phytochemical compounds with known antimicrobial properties in *Tarchonanthus camphoratus*; as well as to evaluate the antimicrobial effects of *Tarchonanthus camphoratus* extracts against S. *typhi, E.coli* and *S. aureus.*

MATERIALS AND METHODS

Plant material

Fresh plant material of *Tarchonanthus camphoratus* was collected in the Omaheke region of Namibia in the month of April 2011. A voucher specimen was prepared and submitted to the National Botanical Research Institute (NBRI) of Namibia for identification. Specimens of plant stems, bark and leaves were also harvested for laboratory analysis. The plant material was air dried at room temperature for two weeks before being ground to a fine powder and stored in airtight containers at 4°C.

Extraction of Plant Material

For preparation of the crude organic plant extracts, 5 g of the powdered leaves, stems and bark parts of the plant were soaked in 50 ml of 80% methanol for 72 h at room temperature. The organic extracts were then filtered using Whatmann No. 1 filter paper, and concentrated using a rotary evaporator at 60°C and 85 rpm. Aqueous extracts were prepared using the same extraction procedure using distilled water as the solvent. All extracts were concentrated by rotary evaporation and then freeze dried overnight to remove all solvents and the yield of the extraction was weighed and recorded.

Stock solutions of each of the dry aqueous and organic extracts were prepared by adding the appropriate volume of dimethyl sulfoxide (DMSO) to each of them to make up concentrations of 20 mg/ml and stored at -20°C until needed.

Phytochemical Screening

Crude aqueous and organic extracts were prepared 72 h in advance for screening of phytochemical compounds. The crude extracts were qualitatively analyzed for the presence of glycosides according to the literature by Ndjoku and Obi (2009) and Fransworth (1966) and for the presence of terpenoids

Table 1 Total yields of the concentrated aqueous and organic leaf, stem and bark extracts of *Tarchonanthus camphoratus* plant

		Yield (g)	Percentage Yield %
Aqueous Extracts	Leaf	0.2147	4.294
	Stem	0.0390	0.780
	Bark	0.0735	1.470
Organic Extracts	Leaf	0.4664	9.328
	Stem	0.0245	0.490
	Bark	0.0473	0.946

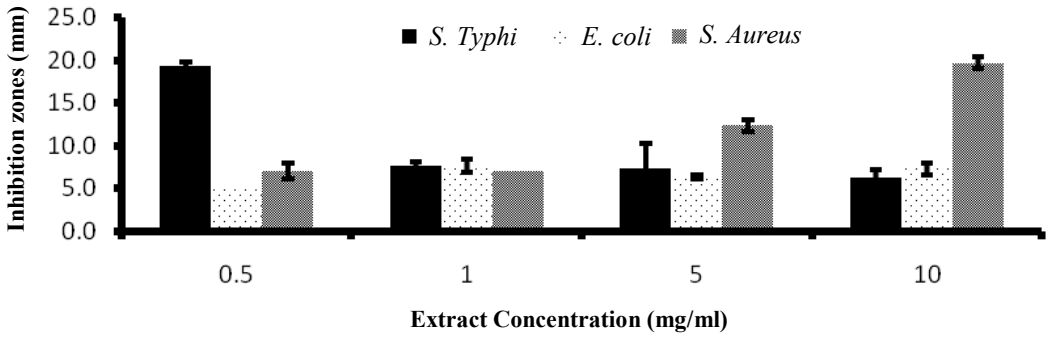

Figure 1 Mean Antimicrobial Activity of Organic Leaf Extracts. Mean diameter (mm) of the inhibition zones that formed in the different test organisms due to the activity of the organic stem extract

according to the literature by (Egwaikhide *et al.,* 2007). Brief description of the procedures used is as follows:

Glycosides

A weight of 0.5 g of each extract was mixed with 2 ml of chloroform. An equal volume of concentrated H_2SO_4 was carefully added to the chloroform-extract mixture and carefully shaken to mix. Formation of a reddish brown colouration at the interface indicated the presence of glycosides.

Terpenoids (Salkowski method)

A weight of 0.5 g of each extract was mixed with 2 ml of chloroform. An equal volume of concentrated H_2SO_4 was carefully added to the chloroform-extract mixture to form a layer. Formation of a reddish brown colouration at the interface indicated the presence of terpenoids.

Antimicrobial Assay

Three human pathogenic bacteria made up of two lab strains of multi-resistant *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923 and a field strain of *Salmonella typhi* were obtained from the Central Veterinary Laboratory of Namibia for the antimicrobial assay. The bacterial strains were used to inoculate freshly prepared nutrient broth from which streak plates on nutrient agar were prepared. Single colonies from the streak plates were then used to maintain cultures of the bacteria on nutrient agar plates.

The well diffusion method was used to test the plant extracts for antimicrobial activity. Concentrations of 0.5, 1, 5 and 10 mg/ml were first prepared from the 20 mg/ml aliquots of each of the aqueous and organic leaves, stem and bark extracts and set aside. Nutrient agar plates were prepared for each bacterial strain as per the manufacturer's instructions and incubated for 24 h to make sure of no contaminations. The plates were then divided into sets as par different extracts and their varying concentrations for each of the three pathogens. The plates were then inoculated with 100 µl of broth culture which was spread over the agar and allowed to dry. In the control plates, water and DMSO were used as negative controls and antibiotics, erythromycin and gentamycin, were used as positive controls. Assays were

Table 2 Phytochemical properties of aqueous and organic extracts of the leaves, stem and bark of the *Tarchonanthus camphoratus*

	Aqueous Extract			Organic Extracts		
	Leaves	**Stem**	**Bark**	**Leaves**	**Stem**	**Bark**
Terpenoids	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
	Present +			Absent –		

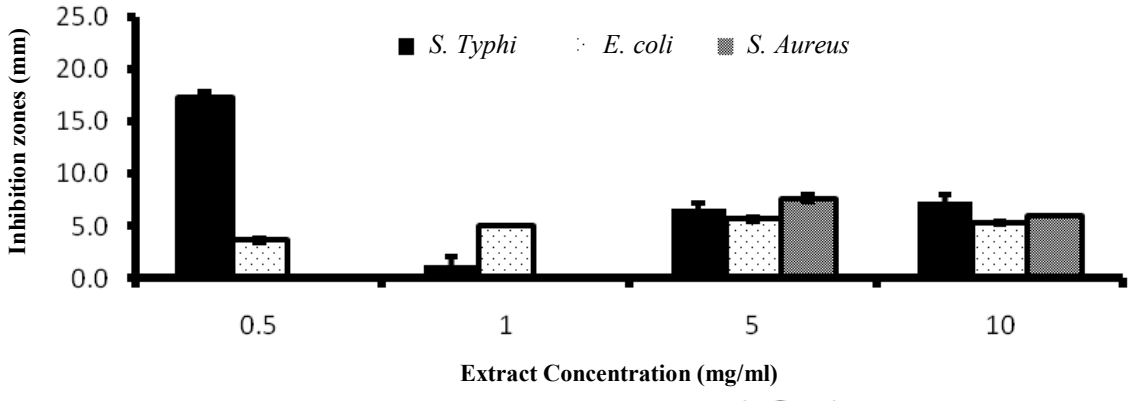

Figure 2 Mean Antimicrobial activity of Organic Stem Extracts. Mean diameter (mm) of the inhibition zones that formed in different test organisms due to the activity of the organic stem extract

carried out in triplicates whereby in each plate, three wells measuring 8 mm in diameter were made, one plate being designated to use for one concentration of any particular extract. The plates were allowed to settle down for thirty minutes to allow for absorption of the extracts into the agar before being incubated at 37°C. Antimicrobial activity was recorded following 24 h of initial incubation and again 24 h later (2days). A transparent ruler was used to measure the diameters of the zones of inhibition in millimeters for all three triplicates.

The minimum inhibitory concentration (MIC) of the plant extracts was determined for the test organisms using varying concentrations of 0.01, 0.1, 1 and 10 mg/ml. A volume of 0.6 ml of nutrient broth plus aqueous or organic plant extract was prepared in appropriately labeled glass screw tubes, taking into consideration dilution factors of both the broth and the extracts in order to obtain an exact dilution concentration at 0.6 ml per tube. The tubes were incubated at 37°C for 24 h. The test tube with the concentration of plant extract at which no detectable growth was observed was considered a the MIC. The activity indices, designated as AI, were calculated as the division of zone of inhibition of the extract by that of the standard drug (Singh *et al.,*2002).

RESULTS

The plant voucher specimen submitted to the NBRI was confirmed to be *Tarchonanthus camphoratus*. The yield of the leaf extracts (Table 1) was the highest for aqueous and organic extracts (4.3% and 9.3% respectively) followed by the bark (1.47% and 0.95%) and then the stem (0.78% and 0.49%).

Qualitative phytochemical tests conducted on both the aqueous and organic extracts of *T. camphoratus*

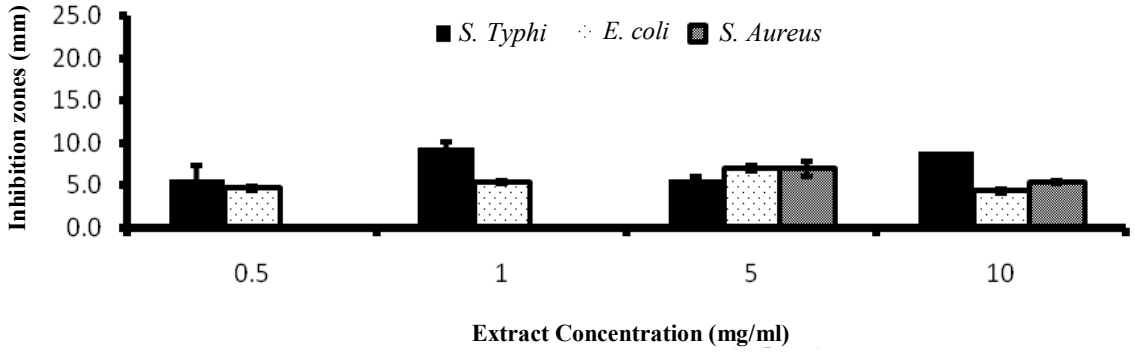

Figure 3 Mean Antimicrobial Activity of Organic Bark Extracts. Mean diameter (mm) of the inhibition zones that formed in different test organisms due to the activity of the organic bark extract

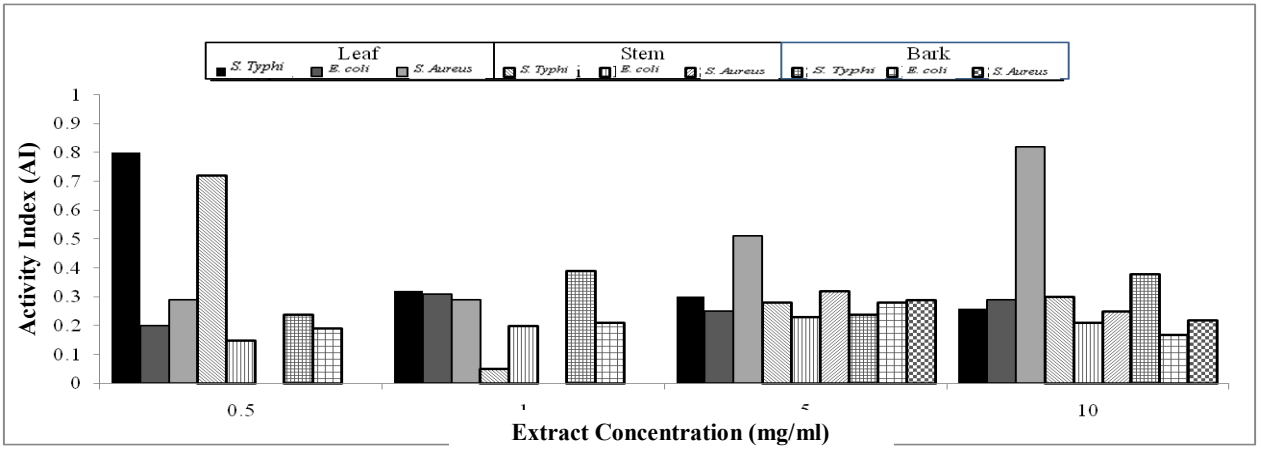

Figure 4 Activity index of all the three organic extracts of *Tarchonanthus caphoratus* against the standard drug gentamycin Organic leaf extracts had the highest activity index against *S. typhi* and *S. aureus*, AI = 0.8, 0.82 at 0.5 and 10 mg/ml respectively), followed by the stem extract activity against *S. typhi* with AI = 0.72 at 0.5 mg/ml. Equal or sometimes higher activities were observed between the stem and bark extracts at concentration 5 mg/ml with AI ranging from 0.23-0.32.

plant showed the presence of test compounds (Table 2), terpenoids and glycosides in all the aqueous extracts of the leaves, stem and bark. The same result was obtained for the organic extracts with terpenoids and glycosides being detected in extracts prepared from the leaves, stem and bark of the plant.

Aqueous extracts prepared from the leaves, stem and bark showed no antibacterial activity in the well diffusion assays as no inhibition zones were formed in all the plates with *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923 and a field strain of *Salmonella typhi*. The organic leaf, stem and bark extracts, however, all had varying levels of antimicrobial activity against all the three test organisms; zones of inhibition were defined as bacteriostatic that is zones with partial inhibition of microbial growth, and antimicrobial, that is formation of defined growth inhibition zones (Figure 1-3). The organic leaves and bark extracts both exhibited bacteriostatic activity against the three test microorganisms with the formation of large zones of partial inhibition of microbial growth occurring while the organic stem extracts exhibited antimicrobial activity against the test microorganism with the formation of more defined inhibition zones.

The organic leaf extract exhibited the highest inhibitory activity against all the three test organisms, compared to the other two organic extracts, as larger inhibition zones were observed in the plates. The highest activity was detected against *S. typhi* at a concentration of 0.5 mg/ml, with the inhibition zones measuring an average of 19.3 mm in diameter, and against *S. aureus*, at a concentration of 10 mg/ml, with the inhibition zones measuring an average of 19.7 mm in diameter. Antimicrobial activity of the organic leaves extract against *E. coli* was fairly consistent at all concentrations with the diameter of the inhibition zones measured ranging between 5-7.3 mm, a difference of 2.3 mm.

The organic stem extract showed the most potency against *S. typhi* at 0.5 mg/ml, with the inhibition zones measuring an average of 17.3 mm in diameter (Figure 2). Antimicrobial activity against *S. aureus* was only observed at 5 and 10 mg/ml with the diameter of the inhibition zones measured ranging between 6-7.7 mm, a difference of 1.7 mm, with none occurring at 0.5 and 1 mg/ml. Antimicrobial activity of the organic stem extracts against *E. coli* was fairly consistent at all concentrations with the diameter of the inhibition zones measured ranging between 3.7-5.7 mm, a difference of 2 mm.

Table 3 Activity index of all three organic extracts of *Tarchonanthus caphoratus* against the standard drug gentamycin. the standard drug gentamycin

	Organic Extracts - Activity Index (AI)								
	Leaf			Stem			Bark		
[Plant extract] mg/ml	*S. typhi*	*E. coli*	*S. aureus*	*S. typhi*	*E. coli*	*S. aureus*	*S. typhi*	*E. coli*	*S. aureus*
0.5	0.8	0.2	0.29	0.72	0.15	0	0.24	0.19	0
1	0.32	0.31	0.29	0.05	0.2	0	0.39	0.21	0
5	0.3	0.25	0.51	0.28	0.23	0.32	0.24	0.28	0.29
10	0.26	0.29	0.82	0.3	0.21	0.25	0.38	0.17	0.22

The organic bark extract was least potent against the three test microorganisms with the largest observed inhibition zones measuring an average of 9.3 mm in diameter for *S. typhi* at a concentration of 1 mg/ml and no antimicrobial activity occurring at 0.5 and 1 mg/ml against *S. aureus* (Figure 3). Again, the antimicrobial activity of the organic bark extract against *E. coli* was fairly consistent at all concentrations with the diameter of the inhibition zones measured ranging between 4.7-7 mm, a difference of 2.3 mm.

Antimicrobial Activity Index

All the three organic extracts of *Tarchonanthus camphoratus* exhibited varying degrees of antimicrobial activities against the three test organisms.

DISCUSSION

The phytochemical compounds that were tested for glycosides and terpenoids, are significant antibiotic constituents and have not been previously tested for in *T. camphoratus*. Phytochemical testing done on the plant extracts revealed the presence of tannins, saponins and reducing sugars (Mwangi and Achola, 1994) but not alkaloids, or cardiac and anthraquinone glycosides. Various flavanones such as luteolin, apigenin, nepetin, and hispidulin have also been identified from Egyptian collections as well as the sesquiterpine lactone, parthenolide and a quaternary alkaloid, tarchonanthine (Scott and Springfield, 2005). The volatile oil has a characteristic camphor-like aroma but the plant only contains a small amount of camphor. It is the part of the plant which is said to be responsible for the reported analgesic, decongestant, diaphoretic and analgesic effects (Bruneton, 1995). Glycosides form an important compound called aminoglycosides from which antibiotics such as streptomycin and gentamicin are made. Terpenoids are oxygen-containing derivatives of terpenes, many of which have been found to be effective against many types of bacteria. The presence of these compounds may be significant for antibiotic activity.

The medicinal uses of the wild camphor bush are remarkably similar throughout its geographical range. Fresh or dried plant leaves and branches are usually crushed and burnt and the smoke is inhaled by the patient (Watt and Breyer-Brandwijk, 1962; Hutchings and van Staden, 1994). Along with the preparation of tinctures and infusions, from leaves and twigs, which are either taken orally or chewed to produce a therapeutic effect, (Van Wyk *et al*., 1997) reported the traditional use of such preparations as a diaphoretic and for the treatment of abdominal complaints, headaches, toothache, asthma, bronchitis and inflammation. The ethnomedicinal preparation of *T. camphoratus*, which is using aqueous solvents such as water, was shown to be ineffective against all three pathogens in the well diffusion assays in contrast to the organic extracts. This may have been a concentration effect with antimicrobial compounds not dissolving freely in the aqueous solvent during extraction. Extraction at higher temperatures or a shorter period may have resulted in a greater yield of active compounds. The organic extracts, may have shown more activity due to the increased solubility of the plant compounds in the methanol used to prepare the organic extracts. Controls using the solvent, methanol alone did

not show any antibiotic activity. The antimicrobial activities of the plant's organic leaf and bark extracts seemed to be more of a bacteriostatic nature against the test microorganisms *Salmonella typhi, Escherichia coli* and *Staphylococcus aureus,* whilst the organic stem extract's activity appeared to be more of an antimicrobial nature.

CONCLUSION

The ethnomedicinal properties of *T. camphoratus* as an antibiotic was validated in this study and it may have been due to the presence of glycosides and terpenoids in the extracts. The use of this plant can be recommended as a natural antibiotic supplement to other drug treatments set up for poultry animals against tested microorganisms once further studies and trial runs have been done on the plant.

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