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TLC SEPARATION, ANTIBACTERIAL AND ANTI-INFLAMMATORY ACTIVITY OF EXTRACTS DERIVED FROM *ZANTHOXYLUM HUMILE* ROOTS

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ABSTRACT
Internationally there is an increased need to come up with new therapeutics to counteract cases of microbial resistance which is diminishing successes of current pharmaceuticals. Traditional medicines have been in use since time memorial and they offer better options as a source of new lead compounds as compared to synthetic designs and libraries. The aim of this study was to evaluate antibacterial and anti-inflammatory activity of TLC isolated phytocompounds from *Zanthoxylum humile* roots. Disc diffusion method was employed to determine antibacterial activity while micro-plate serial dilution method was used to evaluate the minimum inhibitory concentration of crude fractions. Anti-inflammatory activity was ascertained by the nitrite assay. The present study results reveals that *Zanthoxylum humile* roots consist of antibacterial and anti-inflammatory activity against both gram positive and gram negative bacteria. The antibacterial and anti-inflammatory activity can be explained in terms of alkaloids, saponins, tannins and flavonoids present in the extracts. *Zanthoxylum humile* roots TLC isolates revealed significantly greater antibacterial and anti-inflammatory activity as compared to standard drugs cotrimoxazole and indomethacin. Results of the present study authenticate the use of *Zanthoxylum humile* roots in primary health care needs. Presence of phytocompounds such as flavonoids, saponins, tannins, alkaloids, terpenoids and steroids supports its use as medication for diarrhea, hypertension, diabetes, coughs and flu and as antivenins against snake bites. The presence of various bioactive compounds makes *Zanthoxylum humile* a promising plant as a source of new therapeutics that may be the answer to the current microbial resistance.
Keywords: Antibacterial, anti-inflammatory, *Zanthoxylum humile,* microbial resistance.

INTRODUCTION

Globally there is a dire need to come up with new therapeutics to counteract the ever increasing cases of multidrug resistance pathogens[1-5]. Synthetic libraries and drug designs can no longer adequately provide lead compounds for the development of new drugs and leaving natural product drug design as the only attractive option. The advent of high throughput screening methods has further made natural product drug design more feasible. WHO[6] report reveals that many people rely on traditional medicines in their primary health care needs. Markets for herbal medicines have been growing rapidly and significant economic rewards are being realized[7,8]. In the effort of providing a rationale behind the use of *Zanthoxylum humile* root extracts in traditional medicine this study was designed to investigate antimicrobial and anti-inflammatory activity of TLC separated extracts derived from *Zanthoxylum humile* roots. In traditional medicines *Zanthoxylum humile* has wide applications[7,8], its roots are fused to make a decoction that is taken orally and is believed to consist of healing activity against conditions such as erectile dysfunction, diarrhea, hypertension and diabetes. Aqueous leaf and root extracts are used to treat ailments such as, abdominal pain, including backache and chest pains, strengthening bones and flu. *Zanthoxylum humile* is also used as mouth anesthetic for toothache. Pulverized roots are added to water and drunk as antivenins against snake bites. Powdered leaves are also applied as poultice on wounds and burns and help in accelerating healing. The plant grows in the wild in many African countries such as Zimbabwe, Mozambique, Botswana, South Africa and Zambia. It falls under the family rutaceae. *Zanthoxylum humile* is currently not a protected species in many African countries. Protection may be supported primarily if the plant has shown livelihood and commercial value. Scientific researches may promote and provide the impetus for sustainable use of the species, which would otherwise become extinct. Reports appearing in literature have shown that other species in the same family such as *Zanthoxylum chalybeum*[9] and *Zanthoxylum chiloperone*[10] consist of antimicrobial activity. Despite its wide application[7,8] in traditional medicines in India and most Southern African countries such as Zimbabwe, Mozambique**,** South Africa and Namibia scientific researches on antimicrobial and inflammatory activity of *Zanthoxylum humile* is scarce therefore providing a rationale behind this study.

MATERIALS AND METHODS

Materials

All reagents including solvents used were of analytical grade purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant collection

Roots were collected in March 2013 according to WHO[6] guidelines from the Mashonal and East region, Goromonzi, Zimbabwe. The plant was identified with the help of local people and later validated by a taxonomist at Harare national herbarium and voucher specimen No. ZH2012/12 was deposited in the chemistry department (natural product Herbarium) of Bindura University for future reference purposes.

Extract preparation

The roots were shade dried at room temperature to constant mass. Dried plant materials were later ground to powder using a hand mill. One hundred grams of plant materials were defatted with hexane and extracted sequentially by maceration with n-hexane, dichloromethane, acetone and methanol. The crude extracts were preliminarily tested for antimicrobial activity and the crude extract showing the most activity was subjected to chromatograph extraction using a 2cm × 30cm silica gel 60 open column using a stepwise elution with ethyl acetate/methanol mixture (1:3). Collected extract was evaporated under vacuum and examined by preparative TLC based on the method reported by Ferreira et al[11] with minor modifications. A small volume of the extract was spotted on silica gel G plate and developed using different solvent regimes. The separated bands were identified using different revealing reagents. Fractions with similar R_f values and composition were combined by scratching into tubes and tubes were filled with methanol. The contents were filtered using a Whatman filter paper no. 1 and allowed to evaporate under a stream of cool air. Crystals were collected and washed with n-hexane. The percentage yield of extracts ranged from 12–22%w/w dry weight.

Anti-microbial assays

The antibiotic disc diffusion method was performed following a method reported by Bauer*, et al* [12] against four standard bacterial strains *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. A solution of the purified extract was prepared in methanol and filter paper discs were dipped into the solution. The sizes of the filter paper discs were 16mm ± 1. Filter paper discs soaked with the extract were placed on the inoculated plates and allowed to dry, allowing the extracts to diffuse into the media. The bacteria were then inoculated onto the media and then the plates were incubated at 37°C for 24hours. The antibiotic cotrimoxazole was used as a positive control while distilled water and methanol were used as the negative control. The diameters of the zones of inhibition were measured in millimeters (mm). The micro-plate serial dilution method proposed by Kobayashi et al[12] was applied to determine the minimum inhibitory concentration of crude fractions and purified compounds against four standard bacterial strains *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. Crude extracts (10mg/ml) or purified compounds (1mg/ml) were dissolved in acetone and serially diluted with sterile water in micro-plates. An equal volume of an actively growing culture of the test bacteria was then added to the different wells and cultures were grown overnight in 100% relative humidity at 37°C. The lowest concentration of the test solution that led to an inhibition of growth was taken as the MIC. Acetone was used as the negative control and depicted no influence on the growth of the micro-organisms.

Anti-inflammatory activity

This was performed by nitrite assay as described Bauer A.W. et al and Eloff JN et al by[13,14]. The test used to for anti-inflammatory activity of extracts involves evaluating their capacity to inhibit NO production in activated macrophages. Nitrite (NO_2^-) produced in the culture medium was measured as an indicator of NO production according to the colorimetric test based on the Griess reaction. A volume of 1ml of each plant extract was mixed with 1ml of Griess reagent and left to stand at room temperature for 30min. The nitrite concentration was determined using the calibration curve method by measuring the absorbance at 548nm using $NaNO_2$. Results were calculated as percentage of NO production compared to the control using the following equation

$$\%\ Inhibition = \frac{100 \times [NO_2^-]control - [NO_2^-]extract}{[NO_2^-]control}$$

Where $[NO_2^-]$ control is the concentration of nitrite released without addition of the extract and $[NO_2^-]$ extract is the concentration of nitrite released by the cells in presence of the plant extract.

Statistical Analyses

Results are presented as mean value ± standard deviation of three replicate experiments. Statistical analysis between treatments were determined at the significance level of P = 0.05 using ANOVA analysis followed by multiple comparisons using the least significant difference (LSD) test to separate the means. All statistical analyses were performed using SPSS 16 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Antibiotics agents are the most common therapeutic agents used to mitigate diseases as a result of pathogenic bacteria. The discovery of antibacterial agents gave hope for a possibility of eradicating infectious diseases such as tuberculosis, cholera and many sexually transmitted infections however the emergence of resistant strains has thwarted this hope. Emergence of resistant strains put a need to seek alternative medications. Since medicinal plants have been used for so many years hence these medicinal plants may be the answer to address this problem. Results of antibacterial activity of *Zanthoxylum humile* extracts are shown in Table 1, 2 and 4.

Table 1 shows that the solvent used in the analysis do not consist of antibacterial activity implying that any antibacterial activity observed is due to the extracts. Results of antibacterial activity of crude extracts are shown in Table 2. Methanol extract significantly exhibited the highest antibacterial activity followed by acetone and dichloromethane with ANOVA analysis $p > 0.05$. There was no significant difference between the antibacterial activity of acetone and dichloromethane. Hexane exhibited no antibacterial activity revealing that the bioactive compounds in *Zanthoxylum humile* are polar compounds supporting the use of aqueous decoctions in traditional medicines.

Table 1: Effect of solvent on test microorganisms

Solvent	Effect on microorganisms			
	E.coli	***S. aureus***	***P. aeruginosa***	***E. faecalis***
Methanol	-	-	-	-
n-hexane	-	-	-	-
Dichloro-methane	-	-	-	-
Acetone	-	-	-	-

No effect on growth of microorganisms

Table 2: Preliminary Antimicrobial activity test of crude extracts

	Average size of zones of inhibition (mm), n = 3			
Extract	***E.coli***	***S. aureus***	***P. aeruginosa***	***E. faecalis***
Methanol	29.3 ± 0.1^{a}	26.3 ± 0.1^{a}	20.7 ± 0.1^{a}	18.2 ± 0.1^{a}
n-hexane	-	-	-	-
Dichloro-methane	11.0 ± 0.3^{b}	8.3 ± 0.1^{b}	6.8 ± 0.1^{b}	-
Acetone	10.0 ± 0.2^{b}	9.3 ± 0.1^{b}	7.7 ± 0.1^{b}	17.9 ± 0.1^{a}

No diameter of zone of inhibition as there was no observable zone of inhibition;
Means in a column followed by different letters are significantly different ($p < 0.05$).

Table 3: TLC analysis of the methanol extract

	Tests							
R_f value	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**Phytochemicals present**
0.65	-	-	+	-	-	-	-	Tannins
0.71	+	-	-	-	-	-	-	Alkaloids
0.72	-	-	-	-	-	+	-	Flavonoids
0.77	-	-	+	-	+	+	-	Saponins, Tannins, Flavonoids
0.78	-	-	-	-	+	+	+	Saponins, Flavonoids, steroids, terpenoids

- = Negative results; + = Positive results; A = Dragendorffs test and Wagner`s reagent ; B = Borntrager`s test;
C = Ferric chloride test and Gelatin test; D = Keller-Killiani and Legal tests; E = Frothing test; F = Sodium hydroxide test;
G = Salkowski test Liberman-Burchard's test

Table 4: Antimicrobial activity of isolated compounds

R_f value	**Organisms**	**Average size of zones of inhibition at mg/ml dilution (mm) n = 3**								
		10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
0.65	*E.coli*	-	-	-	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-	-	-	-
	P. aeruginosa	-	-	-	-	-	-	-	-	-
	E. faecalis	-	-	-	-	-	-	-	-	-
0.71	*E.coli*	10.1^{a}	5.3^{b}	3.9^{b}	-	-	-	-	-	-
	S. aureus	3.3^{c}	-	-	-	-	-	-	-	-
	P. aeruginosa	-	-	-	-	-	-	-	-	-
	E. faecalis	-	-	-	-	-	-	-	-	-
0.72	*E.coli*	28.3^{a}	26.3^{b}	27.7^{a}	18.2^{a}	16.3^{b}	-	-	-	-
	S. aureus	24.0^{b}	27.3^{b}	16.5^{a}	15.7^{b}	-	-	-	-	-
	P. aeruginosa	18.0^{b}	17.3^{c}	15.6^{a}	13.9^{b}	-	-	-	-	-
	E. faecalis	18.0^{b}	17.3^{b}	16.5^{a}	15.4^{b}	-	-	-	-	-
0.77	*E.coli*	28.0^{a}	29.3^{b}	20.0^{a}	19.3^{b}	15.2^{b}	-	-	-	-
	S. aureus	19.3^{b}	17.3^{c}	19.3^{b}	17.3^{c}		-	-	-	-
	P. aeruginosa	20.0^{a}	19.3^{b}	20.0^{a}	19.3^{b}		-	-	-	-
	E. faecalis	19.3^{b}	16.3^{c}	15.3^{b}	13.6^{c}	13.4^{c}	-	-	-	-
0.78	*E.coli*	29.5^{a*}	28.3^{b}	25.0^{a}	22.3^{b}	18.2^{b}	10.4		-	-
	S. aureus	29.3^{b*}	27.3^{c}	22.3^{b}	19.6^{c}	12.0^{c}	10.5		-	-
	P. aeruginosa	20.0^{a*}	19.3^{b}	20.0^{a}	19.3^{b}	17.1^{b}	11.6	-	-	-
	E. faecalis	29.3^{b*}	27.3^{c}	21.3^{b}	18.8^{c}	16.8	12.3	-	-	-
cotrimoxazole	*E.coli*	23.0^{a*}	22.3^{b}	21.0^{a}	19.7^{b}	14.9^{b}	-	-	-	-
	S. aureus	17.3^{b*}	16.3^{c}	15.3^{b}	16.3^{c}	14.0^{c}	-	-	-	-
	P. aeruginosa	19.0^{a*}	17.3^{b}	15.2^{a}	13.3^{c}	-	-	-	-	-
	E. faecalis	18.4^{b*}	18.3^{c}	13.9^{b}	-	-	-	-	-	-

No diameter of zone of inhibition as there was no observable zone of inhibition;
Means in a column followed by different letters are significantly different ($p < 0.05$); a = 0.1; b = 0. 3; c = 0.5; * = differs significantly

Table 5: Anti-inflammatory activity of isolated compounds

R_f value	Percentage inhibition at dilution mg/ml dilution n = 3								
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
0.65	18.2^{a}	18.3^{b}	12.8^{b}	5.2^{a}	-	-	-	-	-
0.71	-	-	-	-	-	-	-	-	-
0.72	38.6^{b}	36.7^{a}	36.0^{a}	32.3^{a}	30.6^{a}	22.4^{b}	18.3^{c}	8.4^{b}	2.3^{a}
0.77	42.2^{a}	38.9^{a}	36.7^{b}	33.5^{b}	31.0^{c}	26.5^{b}	20.3^{c}	13.6^{c}	9.8^{c}
0.78	68.9^{a*}	66.4^{c*}	63.2^{c*}	62.9^{a*}	59.0^{b*}	55.6^{b}	33.4^{c}	20.2^{a}	16.7^{b}
Indomethacin	56.8^{c*}	55.9^{a*}	53.1^{c*}	49.3^{a}	45.1^{c*}	49.0^{c}	29.9^{a}	19.6^{c}	15.7^{c}

a = 0.3; b = 0.1; c = 0.5
* = significantly different

Methanol turns to extract compounds with polar groups such as hydroxyl and amines that can participate in hydrogen bonding. Antibacterial activity of the compound at R_f value 0.78 significantly exhibited greater antioxidant activity as compared to standard antibiotic cotrimoxazole. This can be rationalized by the fact that these compounds consist of four bioactive compounds, saponins, flavonoids, steroids and terpenoids. The minimum inhibitory concentrations achieved in this study are10^{-6} and 10^{-5} for *Zanthoxylum humile* extract and cotrimoxazole respectively.

TLC analysis of methanolic extract table 3 revealed 5 spots with R_f values of; 0.65, 0.71, 0.72, 0.77, 0.78. Analysis of the five spots with different standard revealing agents depicted that the bioactive compounds in *Zanthoxylum humile* are tannins, alkaloids, flavonoids, saponins, steroids and terpenoids. Cardiac glycosides were not found in a *Zanthoxylum humile* methanolic extracts. Compounds such as flavonoids, saponins, steroids and terpenoids consist of various medicinal properties. Flavonoids and Saponins have been reported to consist of antioxidant activity against free radicals which are implicated in the etiology of cancers, cardiovascular diseases and diabetes[15-17]. According to Ebana, *et al*[18], plant phytocompounds can inhibit pathogenic bacteria and tannins plays an important role in treating wounds and arrests bleedings. Presence of tannins in *Zanthoxylum humile* confirms its use for wound treatment in primary health care. In a study conducted by Lima L.M. et al[10] tannins exhibited antibacterial activity against bacteria strains, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212. The mode of action of tannins is believed to complex with proteins of the pathogenic bacteria. The present findings are encouraging as they indicate that *Zanthoxylum humile* may be a source of compounds that can serve as natural antibiotics. Such compounds may be used synergistically with other drugs to fight microbial resistance. The extracts can act against both gram negative and gram positive bacterial.

According to recent studies by Eloff J.N. et al, Francois M.N. et al and Diaz P et al[14-16] inflammation has been reported as a major risk factor for various human ailments. Inflammation maybe characterized by infiltration of inflammatory cells, excessive production of cytokines, dysregulation of cellular signaling and loss of barrier function[19,20]. Finding ways of reducing chronic inflammation conditions to curb occurrence of several human diseases is very important. Flavonoids from medicinal plants and in the diet have been shown to exhibit a broad spectrum of biological activities including anti-inflammatory activity. The mode of action is through a variety of mechanisms that prevent and attenuate inflammatory responses and serve as potential cardioprotective, neuroprotective and chemopreventive agents. The present study has shown that the extracts of *Zanthoxylum humile* consist of ant-inflammatory activity (Table 5). The ant-inflammatory activity follows a dose response relationship. Prostaglandins and nitric oxide biosynthesis processes are involved in inflammation condition. Isoforms of inducible nitric oxide synthase and of cyclooxygenase produce great amount of these mediators. Flavonoids are able to inhibit these enzymes by complexing with metals responsible for regulating the three dimensional conformation. Phytochemical compounds with R_f value of 0.78 exhibited significantly greater anti- inflammatory activity than Indomethacin. Presence of saponins and flavonoids at this isolate as shown by revealing agents, Table 3 may be responsible for the increased activity.

CONCLUSION

Results of the present study authenticate the use of *Zanthoxylum humile* in primary health care needs. It has antibacterial and anti-inflammatory activity. The plant consist of various bioactive compounds including; alkaloids, tannins, flavonoids, saponins, steroids and terpenoids which have been shown to consist of medicinal properties.

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