ANTIMICROBIAL ACTIVITY OF EXTRACTS FROM *TEPHROSIA VOGELII* HOOK. F.

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ABSTRACT

The dichloromethane extracts of the root and leaf of *Tephrosia vogelii* Hook F. (Family: Papilionaceae) grown in Kenya, were subjected to antimicrobial assays against *Staphylococcus aureus*, *Escherichia coli* and *Fusarium phoseolida*. The minimum inhibition concentrations (MIC) ranged between 0.25 to 6.4 μg/ml for all the microorganisms tested. The crude root and leaf extracts were also toxic to the brine shrimps, organisms used as indicators. of toxicity (LC_{50}: 0.960; 0.958 μg/ml, respectively).

Chromatographic separation led to the identification of (6aβ, 12aβ, 5'β)-6a, 12a, 4',5'-tetra hydro-2,3-dimethoxy 5'-β- isopropenyl–furano (3',2': 8,9)-6H-rotoxen-12-one, (rotenone) in 9.75% yield, (roots), and 11.75% (leaves). Characterisation was achieved by use of spectroscopic techniques and comparison with rotenone standard and literature data. Novel HPLC conditions for purification of rotenoids were established. The MIC of rotenone against the tested microbes was found to be 5.2 and 1.0 μg/ml for *Staphylococcus aureus* and *Escherichia coli* respectively, while the LC_{50} against the brine shrimps was found to be 3.20 μg/ml. The findings indicate that the extracts of *T. vogelii* contain metabolites that have antimicrobial activity comparable to other medicinal plants.

Keywords: *Tephrosia vogelii*, Rotenone, *Staphylococcus aureus*, *Escherichia coli*, *Fusarium phoseolida*.

1.0 INTRODUCTION

1.1 Antimicrobial Agents

Antimicrobial agents are classified into three major types, antibacterial, antifungal and antiprotozoal. Since the discovery and appreciation of penicillin as an antibiotic, thousands of plant extracts have been screened for antimicrobial activities. Some plants have been found to contain compounds capable of acting as antibiotics, which have been isolated to the chemical state of purity (Gosh, 1994; Alindehou, *et al.*, 2002; Badria, 2004). Hot and humid climatic conditions found mainly in the tropics are highly favourable for the growth of bacteria and fungi; hence bacterial and fungal diseases of animals and plants are very common in these regions. In addition to the systemic diseases of man and animals, superficial infections of the skin are of common occurrence in tropical countries due to favourable growth conditions of pathogens. Gram-negative bacteria are among the major pathogens found in hospitals that have rapidly acquired resistance to multiple antibiotics in succeeding years (Greenwood, 1997).

Fusarium is a filamentous fungus widely distributed on plants and in the soil. It is found in normal mycoflora of commodities such as rice, bean, soybean and other crops. While most species are more common in tropical and subtropical areas, some inhabit the soil in cold climates. As well as being a common contaminant and a well-known plant pathogen, *Fusarium spp.* may cause various infections in humans. Most crops are invaded by one or more species of *Fusarium*. Members of this group of fungi are known for producing mycotoxins in cereal grains (Marin *et al.*, 1999).

1.2 *Tephrosia vogelii* Hook F. (Family: Papilionaceae)

Tephrosia vogelii is a small leguminous shrub which grows to a height of about three to four metres. It is a perennial shrub with mauve flowers and many pods. This species helps in the restoration of soil fertility (Chapman and Hall, 1994; Simute *et al.*, 1998, Ranga and Shanower, 1999). The plant's leaves, branches and seeds are used for stupefying fish, which remains fit for human consumption. The toxicity of *T. vogelii* is lower than that of other plant species used in the fish industry. *T. vogelii* is traditionally used for its ichthyotoxic, insecticidal and food parasiticidal properties (Ibrahim *et al.*, 2000; Lambert *et al.*, 1993; Lefournier *et al.*, 1980). The shrub is also used as a repellant

for mole crickets. Several iso-flavonoids have been isolated from *Tephrosia* species (Machocho *et al.*, 1995; Were *et al.*, 1990; Tarus *et al.*, 2002; Bashir *et al.*, 1992). The compounds isolated exhibited different effects ranging from anti-feedant, antibacterial, anti-repellant and insecticidal.

The bioactive metabolites from plants have also been investigated for their possible use as anti-plant pathogenic substances or biological controls of agricultural pests. Natural products are more suitable in controlling plant pathogens in agriculture and horticulture than synthetic pesticides because they are selective in their mode of action, and biodegradable. Small-scale and organic farmers in Kenya are reportedly using the root and leaf extracts of *T. vogelii* to control diseases and pests on their crops. Since no comprehensive data is documented on this species growing in Kenya, there is need to screen the extracts from this plant with the aim of determining their bioactivities, isolating and characterising the chemical constituents in order to validate its use in agriculture.

2.0 MATERIALS AND METHODS

2.1 Sampling and Sample Pre-treatment

T. vogelii shrub was planted on Jomo Kenyatta University of Agriculture and Technology (JKUAT)-Thika farm. Roots and leaves were sampled during the vegetative and 50% flowering stages. The sampling was done randomly and destructively.

Roots and leaves were dried at room temperature under a shade or in the darkroom until completely dry. They were separately ground into fine powder using a motor laboratory grinding mill (Christy and Norris Ltd., Chemsford-England).

2.2 Extraction

Ground powder of roots (500 g) and leaves (500 g) were separately subjected to cold extraction in dichloromethane for three days. The mixtures were filtered and the filtrates collected. Excess solvent was evaporated in *vacuo* to yield crude samples (1.19 g and 2.40 g roots and leaves respectively) that were used for bioassays and chromatographic separation.

2.3 Brine Shrimp Lethality Test

Brine shrimp eggs were obtained from KenSalt Ltd, Malindi.

The brine shrimp toxicity tests were carried out according to Alkofahi *et al* (1989). Brine shrimp eggs were hatched in artificial seawater prepared by dissolving 40 g of sea salt in one litre of distilled water. After incubation for 48 hours at room temperature (22 - 29°C), the larvae were attracted to one side of the vessel with a light source and collected using a pipette. The nauplii were separated from eggs by aliquoting them three times in small beakers containing seawater (Solis *et al.*, 1992). Serial dilutions were made in 5 ml calibrated containers in triplicates. A suspension of larvae (10) was added to each container. The negative control contained only DMSO / seawater. All containers were then covered and transferred to a free bench at 24°C for 18 - 24 hrs. Lethal concentrations (LC) values were determined by taking average of the three experiments. Rotenone was used as the positive control.

2.4 Antimicrobial Assays

Glass petri dishes were cleaned and sterilised in the oven for 2 hours, then left to cool. Nutrient agar and potato dextrose agar were separately dissolved in distilled water, mixed by a magnetic stirrer in a microwave oven until clear solution was formed. The resultant solutions were autoclaved at 15 bars and 121°C for 15 minutes.

The test organisms *Staphylococcus aureus* (NCTC 8532) and *Escherichia coli* (NCTC 09001) were grown on nutrient agar (28 g/l) while *Fusarium phoseolida* (NCTC 8863) was grown on potato dextrose agar (39 g/l) for 24 hours. 'Wells' were made in the medium of the growing organisms (Madigan, 1997). The test organisms were obtained from Botany Department, Jomo Kenyatta University of Agriculture and Technology. The different concentration of various extracts were introduced and monitored for 24 hours, after which the inhibition diameters were measured. These were determined in triplicates. Penicillin and rotenone were used as controls.

2.5 Determination of Minimum Inhibition Concentrations (MIC)

Minimum inhibition concentrations were determined by considering the lowest concentration of each extract completely inhibiting the growth of test organisms. Tube

dilution method was used to determine the MIC (Clark *et al.*, 1981; Hufford *et al.*, 1975). Nutrient broth for culturing the bacteria strain and potato dextrose broth for fungi, were prepared as described in Section **2.4**. A known concentration of the sample was dissolved in DMSO. Ten test tubes of the appropriate sterile culture broth were prepared for each MIC determination. The first tube in the series contained 4.5 ml of the culture broth and the remainder contained 2.5 ml each. The test tube sample solution (0.5 ml) was then added to the first culture of broth and mixed thoroughly. The contents of the first tube were withdrawn and added to the second tube and mixed thoroughly such that a two-fold serial dilution was accomplished through the series of tubes on repeating the treatment down the line. Each tube was then inoculated with a suspension of the appropriate test organism and incubated accordingly. Rotenone was used as a reference.

After incubation period, growth in each tube was examined visually by comparing it with that in the control. The lowest concentration of each compound completely inhibiting the growth of test organism was considered as MIC against that organism.

2.6 General Experimental Procedures

The standard compound (synthetic rotenone), was obtained from Sigma-Aldrich (98% purity). Solvents and reagents used were purchased locally (Kobian Kenya Ltd and Chemrectic Co.), and were of analytical grade.

Melting point, uncorrected, was measured on a Yamato melting point apparatus, model m.pt-21°C. Ultraviolet (UV) spectra were recorded on a Shimadzu double beam spectrophotometer (UV-180). Preliminary TLC was done on pre-coated Kieselgel 60 F_{254} plates, DC-Alufolien, (0.2mm thickness, Merck), visualised with a spray reagent (98% MeOH in H_2SO_4). Preparative chromatography was carried out using silica gel [(Merck, 60(0.040:0.063 mm)]. Column chromatography was performed on a normal column using silica gel 60, Merck {particle size 0.040-0.063 mm, (230-400 mesh ASTM)} stationary phase.

Preparative HPLC was performed on a VARIAN 5000 LC, equipped with UV detector (λ 254 nm), using a Beckman ultrasphere C-8 column (250 mm x 10 mm) at a flow rate of 2.5 ml/min and solvent composition of 78% acetonitrile and 22% water.

Analytical HPLC was performed on a Beckman system Gold 126 equipped with a 168 diode array detector module using Beckman Ultrasphere C-8 column (250 mm x 4.6 mm) at a flowrate of 1 ml/min, and solvent composition of 78% acetonitrile and 22% water.

The ^{1}H NMR. spectra were recorded in $CDCl_3$ on Bruker AM (200 MHz) and NABSA NMR (300 MHz) spectrometers using the UNIX data systems. ^{13}C NMR spectra were also recorded on Bruker AM (200 MHz) and NABSA NMR (300 MHz) spectrometers using the UNIX data systems and are reported in ppm downfield from TMS. The correlated two dimensional spectra (^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC and HMBC) spectra were recorded on a NABSA NMR (300 MHz) spectrometer. The electro spray ionisation mass spectra (ESIMS) were recorded on VG Autospec mass spectrometer with an ionising potential of 70 eV.

2.6.1 Isolation and Characterisation of (6aβ, 12aβ, 5'β)-6a, 12a, 4', 5'-tetra hydro-2, 3-dimethoxy 5'-β- isopropenyl -furano (3',2':8,9)-6H-rotoxen-12-one (rotenone)

The DCM extract (1.10 g, roots and 2.00 g, leaves.) was washed with water (20 ml, x 3) and evaporated in *vacuo* to yield crude solids (0.90g, 1.80 g, roots and leaves respectively) these were subjected to column chromatography (n-hexane/ethyl acetate 7:3). A major fraction was isolated and further subjected to Prep-TLC (R_f = 0.42) and Prep-HPLC. Analytical HPLC was finally carried out to yield a solid that was re-crystallised from diethyl ether to afford rotenone as colourless needles (9.75, 11.75%, roots and leaves respectively, R_T = 12.38 minutes), m.p. 163.5-164.5°C (lit. 165.5-166.5°C, Crombie *et al.*,1975). UV, λ_{max} (MeOH): 284, 295 nm. There were other minor unidentified fractions.

The structure of rotenone was confirmed by NMR (Nuclear Magnetic Resonance) studies, comparison with rotenone standard and literature data (Carlson *et al.*, 1973; Crombie and Whiting, 1975; Fukami and Nakajima, 1971). Two dimensional experiments were performed on the compound in order to confirm the structure.

2.7 Data Analysis

Data from brine shrimp toxicity tests was analysed using Genstat, logistic regression option. The antibacterial bioassay data were analysed using Microsoft Excel.

3.0 RESULTS AND DISCUSSION

3.1 Brine Shrimp Toxicity Tests

This assay is considered a useful tool for preliminary assessment of toxicity. It is widely used for pharmacological activities in plant extracts based on their ability to kill laboratory cultured *Artemia nauplii* brine shrimp (Fatope *et al.*, 1993). Generally, LC_{50} of 30 µg/ml is considered to be lethal. From Table 1, the crude extract from the leaves was more lethal towards brine shrimps than that from the roots. This could be attributed to the presence of a higher concentration of the toxic bioactive compounds in the leaves than in the roots. However, the F test indicates no significant difference between the two extracts.

The purified compound, rotenone, was less active than the crude extracts. This could be due to additive effects of minor compounds present in the crude extracts, which might have enhanced the activity.

3.2 Microbial Assays

The crude plant extracts, rotenone, penicillin were tested against two bacteria strains. The sensitivity, expressed as a measure of the diameter of zone of inhibition observed, is summarised in Table 2. The crude root extract was the most active, implied by the inhibition of growth of the Gram-positive bacteria, *S. aureus*, at 0.5 µg/ml (1.25 mm). The root extract may contain a high concentration of active biochemicals. However, both extracts had similar inhibition on the Gram-negative, *E. coli* (0.5 - 8.50 mm). Rotenone had lower inhibition activity than both extracts. This also indicates the blending effects of compounds in the crude extracts, which diminishes with purification. The observed antimicrobial activities of *T. vogelii* may account for the use of this species to treat stomach ache, ear infections and wounds by various communities (Ghazanfar and AL-Sabahi, 1993; EL-Kheir and Salih, 1980).

The MIC value gives the lowest concentration of each extract, completely inhibiting the growth. From Table 3, there was no significant difference between the activity of the crude root and leaf extracts against *E. coli*. However, there was a slight variation in MIC values of the two extracts against the *S. aureus*. The activity obtained for the isolated rotenone compares well with that reported in literature of 1.3 - 4.0 μg/ml (Nagata *et al.*, 2001; Mills, *et al.*, 2004) on various microbes. However, both crude extracts had higher activity than the two controls (penicillin and rotenone). This again could be due to additive or complementary effects from the minor compounds in the crude extracts. Rotenone was more active towards the Gram-positive bacteria and less active on the Gram-negative bacteria, though the difference was not significant statistically
(F test, $P > 0.05$).

F. phoseolida was readily available and used to determine the antifungal activity. The fungi were more sensitive to crude extracts than to the purified compound (Table 4). The crude root extract was generally more active than the crude leaf extracts (0.65mm and 1.5 mm respectively). The inhibition of rotenone was comparable to that of the crude leaf extract. The MIC range was found to be 2.5 - 5.0 μg/ml, though again the root extract had a higher activity. These ranges fall within the reported data (1.0 - 8 μg/ml) for various antifungals on *F. solani*, a species commonly recovered from humans, animals and plants.

The isolated rotenone is a well known isoflavone reportedly isolated from *Tephrosia* species, and this is the first time it is being reported from *T. vogelii* grown in Kenya. However, there were other minor compounds in the DCM extract and studies are underway to fully characterise these compounds. Rotenone is one of the more acutely toxic botanical insecticides, with an LD_{50} of 60 –1,500 mg/kg, (van Andel, 2000; Andrei *et al.*, 2002). This botanical insecticide works both as a contact and a stomach poison, and has a short residual activity, degrading rapidly in air and sunlight. It is active on many insect and mite pests, including leaf-feeding beetles, caterpillars, thrips, aphids and spider mites. This supports the use of the species by small-scale farmers to control pests and diseases in their farms.

4.0 CONCLUSIONS

The results of the study indicate that *T. vogelii* root and leaf dichloromethane extracts have antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Fusarium phoseolida*. The MIC range of 0.25 - 6.4 μg/ml obtained is within the reported range for various antimicrobials. The extracts contained rotenone, just like it is reported for other *Terphrosia spp*. The presence of rotenone and the antimicrobial activity of the extracts support the use of *T. vogelii* extracts by small scale farmers to control pests and diseases in the farms.

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Tables

Table 1: Lethal concentration (LC ± s.d) values for crude extracts and rotenone

LC values	*Crude root extract (µg/ml)*	*Crude leaf extract (µg/ml)*	*Rotenone (µg/ml)*
25	0.963 ± a	0.750 ± b	1.22 ± c
50	0.960 ± a	0.9 ± b	3.20 ± c
75	1.20 ± a	1.23 ± b	5.30 ± c
100	1.71 ± a	2.00 ± b	7.35 ± c

a = 0.0003; b = 0.0001; c = 0.0002

Table 2: Average inhibition zones for the crude leaf, root and rotenone extracts of T. vogelii against two strains of bacteria

	Leaf extract	*Root extract*	*Penicillin*	*Leaf extract*	*Root extract*	*Rotenone*		*Penicillin*
Conc (µg/ml)	IZ-S (mm)	IZ-S (mm)	IZ- S (mm)	IZ-E (mm)	IZ-E (mm)	IZ-*S* (mm)	IZ-E (mm)	IZ-E (mm)
0.00	**	**	**	**	**	**	**	**
0.5	**	1.25	**	**	**	**	**	**
1	**	2.50	**	0.50	0.50	**	**	**
2	**	4.98	**	1.00	1.05	0.10	0.95	**
4	3.00	10.00	1.00	2.00	2.11	1.30	2.10	**
8	6.00	18.00	2.00	4.00	4.250	4.25	4.03	**
16	11.00	32.00	4.00	7.99	8.50	5.06	8.07	**
32	--	--	--	--	--	10.60	16.33.0	

Key

IZ-S: Average inhibition zone for *Staphylococcus aureus* bacteria

IZ-E: Average inhibition zone for *Escherichia coli* bacteria

¯not tested

**sensitivity to microorganism not observed

Table 3: MIC values for the crude leaf root and rotenone extracts against the S. Aureus and E. coli strains of bacteria

Extract	*MIC (S) (µg/ml)*	*MIC (E) (µg/ml)*
Root extract	1.0	0.50
Leaf extract	2.0	0.50
Rotenone	5.2	1.0

Table 4: Average inhibition zones for root, leaf extracts of T. vogelii and rotenone against F. phoseolida

	Root extract	*Leaf extract*	*Rotenone*	*Penicillin*
Conc (µg/ml)	IZ-F (mm)	IZ-F (mm)	IZ-F (mm)	IZ-F (mm)
0	**	**	**	**
4	1.50	0.65	0.26	**
8	3.00	1.30	0.63	**
16	5.90	2.60	1.30	**
32	12.00	5.19	3.25	**
64	--	--	7.50	**

Key

IZ-F: Average inhibition zone measured on *Fusarium phoseolida* fungi

¯not tested; ** sensitivity to *F. fusarium* not observed