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Research Article

Antimicrobial activity and phytochemical screening of *Antidesma venosum* root and stem bark ethanolic extracts

Denis Thobias Mwangomo*, Mainen Julius Moshi, Joseph Jangu Magadula

Institute of Traditional Medicine, Muhimbili University of Health and Allied Science P.O Box 65001, Dar Es Salaam, Tanzania

ABSTRACT

Antidesma venosum E. Mey. ex Tull root and stem bark ethanol extracts and their dichloromethane, petroleum ether and methanol fractions exhibited strong antibacterial activity against 5 Gram positive bacteria including *Staphylococcus aureus* (ATCC25923), *Bacillus anthracis* (NCTC10073), *Bacillus subtilis* (clinical isolate), *Streptococcus faecalis* (clinical isolate) and *Bacillus cerius* (clinical isolate) with MICs ranging from 0.0195 to 0.7812 mg/ml. The crude ethanol extracts of both the stem bark and roots were inactive against Gram negative bacteria except for *Pseudomonas aeruginosa* (ATCC 29953) against which they both exhibited weak activity MIC 1.25 and 5.0 mg/ml, respectively, *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 700603) which gave MICs between 2.50-5.00 mg/ml. The Petroleum ether and dichloromethane fractions of the root ethanol extract showed weak activity against almost all the Gram negative bacteria with MICs between 2.5 and 5.0 mg/ml. Whereas the crude extracts did not show antifungal activity, the petroleum ether and dichloromethane fractions of both the root and stem bark ethanol extracts showed weak activity against *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (clinical isolate), MIC 2.5-5.0 mg/ml. The extracts of the stem bark and roots exhibited mild to moderate toxicity against brine shrimp larvae with LC50 ranging between 25.56-40.93 µg/ml for the stem bark and 62.97-80.26 µg/ml for the root ethanol extract. Phytochemical screening revealed the presence of terpenoids, tannins and steroids in both the root and stem bark extracts. These results show promising activity against Gram positive bacteria, especially by the petroleum ether and dichloromethane fractions of the roots and thus support the popular use of this plant for the treatment of conditions associated with bacterial infections such as cut wounds, chest infections and some types of diarrhoea. Further studies are ongoing to identify the active compounds.

Keywords: *Antidesma venosum*; Antimicrobial activity; Brine shrimp toxicity; Phytochemical screening

INTRODUCTION

Infectious diseases are among the greatest public health problems the world is currently facing, especially among the developing countries. This problem has, in recent years, been escalated by the occurrence of resistance to most of the currently in use antimicrobial drugs (Alanis, 2005; David and Daum, 2010; Abdallah, 2011). Thus there is an urgent need to search for new antimicrobial drugs with broader spectrum, less side effects and without cross resistance (Singh and Pandeya, 2010).

Natural products from higher plants, due to their chemical and biological diversity have played a great role in treating and preventing human diseases (Chin et al., 2006; Huang et al., 2009). Although many reports indicate a growing interest in the use of herbal medicines, only a small percentage of medicinal plants have been evaluated for safety and efficacy (Chariandy et al, 1999; WHO, 2002).

A. venosum is used in traditional medicine to treat a diversity of conditions, including hookworm infestation (Kokwaro, 1976), gonorrhoea, malaria and bilharzias (Chhabra et al., 1993), schistosomiasis (Sparg et al., 2000), abdominal disorders, dysentery (Hunchings et al., 1996) and cut wounds (De Boer, 1989). Extracts of this plant are reported to possess anti-inflammatory activity (Fawole et al., 2009), and have also been used for the treatment of diarrhoea, anaemia and lack of appetite, tuberculosis and Candida infections (Chinsembu and Hedimbi, 2010; Kisangau et al., 2007).

This study aimed to evaluate extracts of A. venosum for antimicrobial activity to validate its use for managing infectious diseases. An attempt was also made to make a phytochemical profile of the plant extracts and establish potential for toxicity using the brine shrimp lethality test.

MATERIALS AND METHODS

Collection and extraction of plant material

Roots and stem barks were collected from Tanga region, northeastern Tanzania, in October 2011. Identi-

* Corresponding Author
Email: ngomodp@gmail.com
Contact: +255 782 660704
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Table 1: Minimum inhibitory concentrations

Bacteria	Minimum inhibitory concentrations (mg/ml)									
	RCE	RPF	RDF	RMF	SCE	SPF	SDF	SMF	Gent	Fluc
B. subtillis	0.1562	0.0195	0.0195	0.0781	0.7812	0.7812	0.7812	1.5625	0.00033	NT
S. faecalis	0.0781	0.0391	0.0781	0.0781	0.3906	0.7812	0.3906	0.3906	0.00063	NT
B. cerius	0.6248	0.0781	0.1562	0.3124	0.7812	0.7812	0.7812	0.3906	0.00125	NT
S. aureus	0.0781	0.0391	0.0391	0.0781	1.2500	0.7812	0.7812	2.5000	0.00078	NT
B. anthracis	0.0391	0.0195	0.0391	0.0391	2.5000	2.5000	1.2500	1.2500	0.00033	NT
E. coli	5.0000	2.5000	2.5000	NA	NA	NA	NA	NA	0.00063	NT
P.aeruginosa	5.0000	2.5000	2.5000	NA	1.2500	1.2500	NA	NA	0.00125	NT
S. flexineri	NA	2.5000	2.5000	NA	NA	NA	2.5000	2.5000	0.00125	NT
S. typhi	NA	2.5000	NA	NA	NA	NA	NA	NA	0.00125	NT
K.pneumoniae	NA	2.5000	2.5000	2.5000	2.5000	2.5000	1.2500	1.2500	0.00033	NT
C. albicans	NA	5.0000	5.0000	NA	NA	5.0000	2.5000	NA	NT	0.00625
C. neformans	NA	5.0000	2.5000	NA	NA	2.5000	2.5000	NA	NT	0.00313

NB: NT = Not tested, NA = No activity, RCE= Root Crude Extract, RPF= Roots P/ether Fraction, RDF= Roots Dichloromethane Fraction, RMF= Roots Methanol Fraction, SCE= Stem bark Crude Extract, SPE= Stem bark P/ether Fraction, SDF= Stem bark Dichloromethane Fraction, SMF= Stem bark Methanol Fraction

fication of plant samples was done by Mr. Haji Selemani, a botanist from the Department of Botany, University of Dar Es Salaam, and voucher specimen No.HOS 974 is deposited at the Herbarium of the Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, Tanzania. Plant materials were separately dried under the shade, finely powdered using a milling machine, and then extracted using ethanol for 48 hours and thereafter concentrated under reduced pressure using a rotary evaporator. Three grams of each crude extract were reserved for bioassays while the remaining material (82 g of stem barks and 39 g of root barks) were separately fractioned by extracting with petroleum ether, dichloromethane and methanol for 24 hours each, respectively.

Test organisms

Twelve pathogenic microbes were used which included five Gram positive bacteria; *Streptococcus faecalis* (clinical isolate), *Staphylococcus aureus* (ATCC25923), *Bacillus cerius* (clinical isolate), *Bacillus subtilis* (clinical isolate), *Bacillus anthracis* (NCTC10073), five gram negative bacteria; *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 29953), *Salmonella typhi* (NCTC 8385), and *Shigella flexneri* (clinical isolate) and two fungi; *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (clinical isolate). All the microbes were obtained from the Department of Microbiology and Immunology, Muhimbili University of Health and Allied Sciences and were maintained on Tryptone soya agar slants except for C. albicans and C. neoformans which were maintained on Sabouraud's dextrose agar slants.

Media and reagents

Iodonitrotetrazolium chloride was bought from SIGMA (Sigma- Aldrich, St Louis, USA). The Brine Shrimps eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa). Tryptone soya agar and tryptone soya broth were purchased from Himedia Laboratory pvt Ltd (Mumbai, India) while Sabouraud's dextrose agar and Sauboraud's dextrose broth were obtained from Biotec Laboratory Ltd (Ipswich, United Kingdom). Dimethylsulfoxide (DMSO) was purchased from SIGMA (Poole, Dorset, England), Cyclophosphamide and Gentamicin susceptibility test discs (10 µg) were purchased from Oxoid (Oxiod Basingstoke, Hampshire, England), while fluconazole was purchased from CADILA Pharmaceutical Limited (Dholka, India)

Screening for antimicrobial activity

Antimicrobial activities were determined by the broth microdilution technique using sterile flat bottomed 96-well polystyrene microtiter plates (Nondo et al, 2010). Bacterial suspensions equivalent to 0.5 McFarland concentrations were prepared by suspending microbes' inocula in sterile distilled water and adjusting to get the right turbidity. Test extracts were prepared by dissolving 20 mg in 0.1 ml of DMSO and diluted with 0.9 broths to make a concentration of 20 mg/ml. The stock solution (50 µl) was pipetted and added into the first well of each row of plates pre- loaded with 50 µl of broths. Then serial dilution was performed by transferring the test sample from first row wells to wells of the next rows, down to the last rows. The 50 µl from the last row wells were discarded. This was followed by addition of 50 µl of broth containing the test organisms (0.5 McFarland dilutions) to each of the wells. Wells in two columns were used as growth controls, where no drugs were added, while other two were used as the positive controls in which gentamycin and fluconazole were used for bacteria and fungi, respectively. The microtiter plates were incubated at 37oC for 24 hours for bacteria and 27 oC for 48 hours for fungi.

After the incubation period 20µl of a 0.2% p-iodonitrotetrazolium chloride (INT) were added to the wells followed by incubation at 370C for 0.5 h. Presence of microbial growth was indicated by change of INT colour to pink, while absence of growth was indicated by absence of colour change. The lowest concentration at which there was no growth was taken as the minimum inhibitory concentration (MIC).

Brine shrimp lethality test

Toxicity of the extracts was determined using the brine shrimp lethality (Artemia salina) test. The larvae were prepared by allowing eggs to hatch in saline water prepared by dissolving 3.8 gm of sea salt in 1 litre of distilled water for 24 hours. The tested extracts were prepared by dissolving 40 mg or 20 mg of the roots or stem barks extract respectively in 20% DMSO in water. Then 3, 5, 10, 15 and 30 µl of the test extract were pipetted to vials containing 10 larvae in enough salt water to make 5 ml of the final mixture and left for 24 hours after which the number of survivors in each concentration were counted and percentage mortalities were determined. The solvent was used as the negative control while cyclophosphimide was used as the positive control.

Analysis was performed using Fig P computer software in which LC50 was determined. LC84 and LC16 were used to calculate 95% confidence intervals. Inference was made on the safety and activity of the plant as previously described by Moshi et al, (2010)

Phytochemical Screening of Crude Extracts

The different extracts of A. venosum were tested for the presence of steroids, saponins, flavonoids, terpenoids, cardiac glycosides, and tannins using the standard procedures described by Trease and Evans (1996).

Test for saponins: The ability of saponins to produce frothing in aqueous solution was used as screening test for the sample. Dried extract (0.5 g) was shaken with water in a test tube; frothing which persist on warming was taken as evidence for the presence of saponins.

Test for tannins: Dried extract (5.0 g) was stirred with 10.0 ml of distilled water, filtered and ferric chloride added to the filtrate. A blue-black precipitate was taken as evidence for the presence of tannins.

Test for cardiac glycosides: The dried extract (0.5 g) was dissolved in 2.0 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under laid with 1.0 ml of concentrated H2SO4. A brown ring obtained at the interface indicated the presence of cardenolides.

Test for flavonoids: Lead acetate (10%; 1.0 ml) was added to 1.0 ml of the extract contained in a test-tube. Formation of a yellow precipitate was taken as a positive test for flavonoids.

Test for steroids: The dried extract (0.5 g) was extracted with 2.5 ml of chloroform in a test tube and 1ml of concentrated sulphuric acid added to form a lower layer. A reddish-brown interface indicated the presence of steroids.

Test for terpenoids: A chloroform extract of the dried extracts (0.5 ml) was evaporated to dryness on a water bath and heated with 3ml of concentrated sulphuric acid for 10 minutes on a water bath. A grey colour indicated the presence of terpenoids

RESULTS

Antimicrobial activity

Both the root and stem bark crude ethanol extracts showed activity against all the 5 Gram positive bacteria that were tested (Table 1). The root crude extract was more active than the stem crude extract, with the lowest MIC of 0.0391 mg/ml against Bacillus anthracis. The stem crude extract was less active than the root crude extract for all the Gram positive bacteria tested. Likewise, the two extracts had either very weak or no activity against all the Gram negative bacteria tested. The highest activity was by the stem bark extract against *Pseudomonas aeruginosa* which had an MIC of 1.25 mg/ml. Both the root and stem bark crude extracts were inactive against *Candida albicans* and *Cryptococcus neoformans* giving MICs □ 5.0 mg/ml. Fractionation of the root ethanol extract significantly increased activity against Gram positive bacteria for all the three fractions made; dichloromethane, petroleum ether and methanol fractions, The lowest MIC was 0.0195 mg/ml against *Bacillus subtilis* and Bacillus anthracis. The pet ether and dichloromethane fractions of the root showed activity against almost all the Gram negative bacteria, although the MICs were high (Table 1). The dichloromethane fraction also exhibited low activity against all the Gram negative bacteria tested (MIC 2.5 mg/ml), except Salmonella typhi. Fractionation of the stem ethanol extract did not significantly increase antibacterial activity as compared to the crude extract. However, antifungal activity against C. albicans and C. neoformans was significantly higher for both the root and stem bark dichloromethane and petroleum ether fractions, although the MICs were between 2.5 and 5.0 mg/ml.

Table 2: Brine shrimp activity

Extract/fraction	LC50 µg/ml (95% CI)
SCR	40.93 (22.24-63.03)
SMO	31.91(19.94-51.06)
SDC	25.51 (42.35-15.38)
SPE	25.56 (15.12- 43.20)
RCR	62.97 (42.26- 93.82)
RMO	80.26 (50.48-127.61)
RDC	66.00 (41.77-104.28)
RPE	63.12 (38.73-102.89)

RCE= Root Crude Extract, RPF= Roots P/ether Fraction, RDF= Roots Dichloromethane Fraction, RMF= Roots Methanol Fraction, SCE= Stem bark Crude Extract, SPE= Stem bark P/ether Fraction, SDF= Stem bark Dichloromethane Fraction, SMF= Stem bark Methanol Fraction

Brine shrimp lethality test

The results of the brine shrimp lethality test (Table 2) indicate that the stem bark ethanol extract and fractions were all slightly more toxic (LC50 25.51- 40.93 µg/ml) than the root ethanol extract and its fractions (LC50 62.97- 80.26 µg/ml). Generally, the extracts and fractions of both the roots and stem bark were only moderately toxic, which may suggest potential for some useful biological activities.

Phytochemical screening

Phytochemical screening indicate the presence of terpenoids, tannins and steroids in both the root and stem bark crude ethanol extracts and their petroleum ether, dichloromethane and methanol fractions (Table 3). Cardiac glycosidse were detected in both the root and stem bark crude ethanol extracts and their methanol fractions only. Flavonoids and saponins were not detected in either crude extracts or fractions for both the root and stem barks.

These results support the use of A. venosum in traditional medicine for the treatment of gonorrhoea (Chhabra et al., 1993), abdominal disorders and dysentery (Hunchings et al., 1996), cut wounds (De Boer, 1989), chronic diarrhea, purulent cough, gonorrhea and tuberculosis (Kisangau et al., 2007). The antibacterial activity of A. venosum has been reported in petroleum ether, dichloromethane and 70% ethanol extracts of the leaves (Fawole et al., 2009). The leaf extracts were weakly active against Escherichia coli, *Bacillus subtilis* and *Staphylococcus aureus* with MICs ranging from 3.125 -9.375 mg/ml and the ethanol extract was most active against *Staphylococcus aureus* with an MIC of 0.650 mg/ml (Fawole et al., 2009). The current study has gone further in generating more results linking traditional medicinal uses with antibacterial activity, mainly against Gram positive bacteria. This study has also shown that the roots have the highest antibacterial activity. The study by Fawole et al., 2009 showed that the leaf extracts had weak antifungal activity against *Candida albicans* with MIC of 3.125-mg/ml and these results are similar to the stem bark and root results.

Table 3: Phytochemical screening of stem bark and root extracts of *A. venosum*

Plant extract*	Cardiac glycosides	Flavanoids	Terpenoids	Tannins	Saponins	Steroids
SCE	+	-	+	+	-	+
SMF	+	-	+	+	-	+
SDF	-	-	+	+	-	+
SPF	-	-	+	+	-	+
RCE	+	-	+	+	-	+
RMF	+	-	+	+	-	+
RDF	-	-	+	+	-	+
RPF	-	-	+	+	-	+

*RCE= Root Crude Extract, RPF= Roots P/ether Fraction, RDF= Roots Dichloromethane Fraction, RMF= Roots Methanol Fraction, SCE= Stem bark Crude Extract, SPE= Stem bark P/ether Fraction, SDF= Stem bark Dichloromethane Fraction, SMF= Stem bark Methanol Fraction; +=Present, -= Absent

DISCUSSION

Although in recent years advances in molecular biology and synthetic chemistry have made a big contribution to the development of new drugs, plants still remain a potential source of bioactive compounds (Iwu et al., 1999). In this study, A. venosum stem bark and root ethanolic extracts have shown promising activity against B. subtilis, S. aureus, B. anthracis, B. cerius and S. faecalis but were only slightly or not active against Gram negative bacteria and fungi. The root extract and its fractions were a few times more active than the stem bark extract and fractions. These results support the finding that plant extracts are usually more active against Gram positive bacteria than Gram negative (Lin et al., 1999; Palombo and Semple, 2001). Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria. The Gram-negative bacteria cell wall appears to act as a barrier to many substances including synthetic and natural antibiotics (Tortora et al., 2001).

The brine shrimp results in this study suggest that the extracts of both the roots and stem barks have a weak cytotoxic activity. These results correlate with results from a previous study which showed that A. venosum aqueous extract exhibited weak cytotoxic activity against human adenocarcinoma cells of the cervix (HeLa) and human breast cells (MCF-12A) and weak acute toxicity against porcine hepatocytes and human lymphocytes (Steenkamp et al., 2009). However, the finding that extracts of A. venosum leaves showed negative mutagenic activity against Salmonella typhimurium strain T98 (Fawole et al., 2009), which is also supported by an earlier study which tested extracts of the leaf, roots and twigs against TA98, TA100, and showed that both dichloromethane and 90% methanol extracts of these parts with and without metabolic activation did not cause genotoxic effects (Elgorashi et al., 2003) forms the basis for wanting to do further studies to explore the observed antimicrobial activity.

Certainly the phytochemical screening reported in this study remains indicative of putative compounds that may be associated with the observed antibacterial activity, but can not clearly tell which compounds are responsible. Ongoing phytochemical studies should soon show which compounds are specifically responsible for the antimicrobial activity and thus pave way for further studies to explore possible clinical application.

CONCLUSION

These results show promising activity against Gram positive bacteria, especially by the petroleum ether and dichloromethane fractions of the roots and thus support the popular use of this plant for the treatment of conditions associated with bacterial infections such as cut wounds, chest infections and some types of diarrhoea. Further studies are ongoing to identify the active compounds.

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