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Full Length Research Paper

Phytochemical characterization, antibacterial screening and toxicity evaluation of *Dichrostachys cinerea*

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To provide scientific rationale to the traditional use of *Dichrostachys cinerea* as medicinal plant in Kenya, phytochemical analysis, antimicrobial screening and evaluation of toxic concentration levels of *D. cinerea* extracts were done. Qualitative assessment of phytochemicals, *in vitro* antimicrobial (selected bacteria and fungus) and brine shrimp toxicity assays were done. Explants (leaves, bark of stems and roots) were collected from *D. cinerea* trees growing in Jomo Kenyatta University of Agriculture and Technology (JKUAT) fallow land behind Botany laboratory. They were washed and then air dried under light exposure (27°C − 30°C for 14 days). A portion of each extracts was used for phytochemical screening. Sensitivity of different bacterial strains to various extracts was measured in terms of zone of inhibition using disc diffusion assay. Brine shrimps lethality test (BST) was used to predict the presence of bioactive compounds in the extracts. Methanol extracts contained all the tested phytochemicals while hot water extracts lacked steroids. Methanol and hot water extracts had no significant difference in terms of antibacterial screening. The LC_{50} value was found to be 2000ppm (parts per million). The results suggest that extracts of *D. cinerea* contain potential antibacterial and antifungal agents.

Keywords: Extracts; Phytochemical; microorganisms; antimicrobial activity; Toxicity bioassay.

INTRODUCTION

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years (Mahidol *et al.,* 2003).In industrialized nations at the present time, some fifty percent of all prescribed drugs are derived or synthesized from natural products, the only available sources for which are animals, marine species, plants, and microorganisms (Farnsworth and Morris, 1976). The importance of natural products is also evidenced by the fact that in 1991, nearly half of the best selling drugs were either natural products or their derivatives (O'Neill and Lewis, 1993). It is considered that because of the structural and biological diversity of their constituents, terrestrial plants offer a unique and renewable resource for the discovery of potential new drugs and biological entities (Balandrin *et al.,* 1985; Hamburger *et al.*, 1991; Cox and Balick, 1994; Cordell, 1995; Clark, 1996; Hostettmann *et al.,* 1998; Cordell, 2000). Moreover, in developing countries, medicinal plants continue to be the main source of medication (Mahidol *et al.,* 2003).

Dichrostachyscinerea is one of the very useful wild medicinal plants of semi-arid areas in Kenya, Eritrea and Somalia (Rukangira, 2004). Despite substantial efforts by ethnobotanical researchers to document majority of medicinal plants used in indigenous health systems (Rukangira, 2004), few researchers have examined and documented their safe dosages and extinction threats posed to habitat-specific species. Plants are endowed with free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites, which are rich in antioxidant activity (Zheng and Wang, 2001 and Cai *et al.,* 2003). Studies have shown that many of these antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, antitumor, antimutagenic,anticarcinogeni, antibacterial, and antiviral activities (Sala, 2002; Rice *et*

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al., 1995). Phytochemistry have been instrumental in rationalization of the use of various herbal medicines, however unscreened herbal products still find their way to markets owing to their high demand. For instance, the bark of *D. cinerea* is used to prepare concoction tradition ally used to treat dysentery, headache and elephantiasis. Its root infusions are used to treat syphilis, gonorrhea coughs and sore eye and also serve as an anthelmintic, laxative and strong diuretic. Seeds of this plant are edible and the leaves are good fodder for domesticated animals (www.worldagroforestrycentre.org/sea/products).
However, there exists no documented scientific research work to authenticate the above-mentioned remedies. To provide a scientific justification for used of these traditional remedies, the present study was planned to assess their antibacterial potential using aqueous and organic extracts against some clinically important bacteria. Phytochemical screening was carried out to identify major biologically active phyto-constituents.

MATERIAL AND METHODS

Collection of plant material

Explants were collected from *D. cinerea* trees growing in Jomo Kenyatta University of Agriculture and Technology (JKUAT) fallow land behind Botany laboratory. This research work was first done on 8th January to 31st May 2007 and repeated on 3rdFebruary to 30th November, 2011. The leaves, bark of stems and roots were picked and washed with water to remove all unwanted plant materials and sand, air dried under light exposure (27°C −30°C for 14 days), separately pulverized using a mill machine found in the department of food science, JKUAT and stored in an airtight container for further use.

Extract Preparation

In the first experiment, crude extracts were separately extracted from ground explants (200 g) thrice using distilled water (solvent) in a shaking water-bath at 70°C for 3 hours. The extracts were filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrates were evaporated by using a rotary evaporator and stored as pure crystal dried extract (50mg). The resulting extract was reconstituted with distilled water to give desired concentrations used in this study.
In the second experiment, crude extracts were separately extracted from ground explants (200 g) using 400ml methanol (CH_3OH) as the solvent in a Soxhlet apparatus for 8 h. The obtained methanolic extracts were filtered and evaporated in a rotary evaporator and freeze dryer, respectively to give pure crystalline dried extracts. The resulting extract was reconstituted with distilled water to give desired concentrations used in this study

Test Organisms

The following microorganisms were used as test organisms: *Staphylococcus aureus,* a facultative anaerobic Gram-positive coccal bacterium frequently found as part of the normal skin flora on the skin and nasal passages (Kluytmans *et al.,* 1997), *Bacillus subtilis,* a Gram-positive obligate aerobe commonly found in soil (Madigan and Martinko, 2005)*, Escherichia coli,* a Gram-negative, rod-shaped bacterium commonly found in the lower intestine of endotherms (Feng *et al.,* 2002)*, Pseudomonas aeruginosa,* a Gram-negative, aerobic rod-shaped bacteria, (Iglewski,1996) and *Candida albicans,* a diploid fungus that grows both as yeast and filamentous cells (dEnfert and Hube, 2007).

Phytochemical screening

A small portion of the dry extract was used for the phytochemical tests for compounds which include tannins, flavonoids, alkaloids, saponins, and steroids in accordance with the methods of Trease and Evans, (1983) and Harborne, (1998) with some modifications. Exactly 1.0 g of plant extract was dissolved in10 ml of distilled water and filtered (using Whatman No 1 filter paper). A blue colouration resulting from the addition of ferric chloride reagent to the filtrate indicated the presence of tannins in the extract. Exactly 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl on steam bath. A millilitre of the filtrate was treated with few drops of Dragendorff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloid. About 0.2 g of the extract was dissolved in 2 ml of methanol and heated.

A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange colouration was indicative of the flavonoids. Saponins content was determined by boiling 1 g powdered sample in 10 ml distilled water for 15 min and after cooling, the extract was shaken vigorously to record froth formation. About 0.5 g of the extract was dissolved in 3 ml of chloroform and filtered. Concentrated H_2SO_4 was carefully added to the filtrate to form lower layer. A reddish brown colour at the interface was taken as positive for steroid ring.

Antimicrobial Assay

The susceptibility of bacterial isolates to *D. cinerea* extracts was tested by disc-diffusion assay method. Nutrient agar (OXOID LTD, Basingstoke, and Hampshire, England) was prepared by dissolving of 27 g l^{-1} in water. Sterile filter paper discs of 6mm diameter were impregnated with 2 ml of the extract solution (equivalent to 4mg of the dried extract) and also separately with serial dilutions of each extract i.e. 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. The paper discs were allowed to

Table 1: Results of phytochemical screening of extracts of various *D.cinerea* explants

		Plant extract		
Solvent	Phytochemical test	leaves	stem-barks	Roots
Hot water	Tannins	+	+	+
	Saponins	+	+	+
	Alkaloids	+	+	+
	Flavonoids	+	+	+
	Steroids	-	-	-
Methanol	Tannins	+	+	+
	Saponins	+	+	+
	Alkaloids	+	+	+
	Flavonoids	+	+	+
	Steroids	+	+	+

Key: +.....Present -.....Absent

evaporate and then placed on the surface of the inoculated agar plates. Plates were kept for 2h in refrigerator to enable prediffusion of the extracts into the agar. Then, the plates were incubated overnight (18 h) at 37°C. Sensitivity of different bacterial strains to various extracts was measured in terms of zone of inhibition using disc diffusion assay as described by Bauer *et al.,*(1966). At the end of the incubation period the antibacterial activity was evaluated by measuring the inhibition zones (diameter of inhibition zone plus diameter of the disc). An inhibition zone of 14mm or more was considered as high antibacterial activity.

Toxicity test (Brine shrimps test)

Brine shrimps lethality test (BST) was used to predict the presence of bioactive compounds in the extract. The brine shrimp toxicity assay used in this study was developed by Michael *et al.* (1956) and incorporated modifications of Meyer *et al.*(1982)and Solis *et al.*(1993). Artificial seawater was prepared by dissolving 3.8g of salt in 1 litre of sterile distilled water and then diluted to obtain final concentrations of 0.5–8.0 mg/ml. Brine shrimp eggs were put into a1000ml beaker containing 80ml of artificial seawater to give a large number of shrimps (*Artemiasalina*). Brine shrimp eggs were hatched in artificial sea water after 48 h incubation at room temperature (25 − 30°C), the larvae (nauplii) were then found to be attracted to one side of the vessel (with a light source) and collected by pipette. Briefly, stock solutions (40 mg/mL) of all extracts were prepared by dissolving them in DMSO (Dimethyl sulfoxide). Different levels of concentrations (200, 400, 800, 1200, 1600, and 2000ppm) were prepared by drawing different volumes from the stock solutions and then added into vials, each containing ten brine shrimps larvae (nauplii). The negative control contained brine shrimp, artificial sea water and DMSO (0.6%) only. The number of survivor nauplii was counted after 24 h (chronic toxicity) of exposure to the various extracts. The surviving shrimps were counted and the concentration that could kill 50% of larvae (LC_{50}) was assessed (Geran *et al.,* 1972).

Statistical analysis

All values were expressed as mean ± standard deviation and comparison of the antibacterial activity of the samples with standard antibiotics was evaluated by applying t-test. $P \leq 0.05$ values were considered to indicate statistically significant difference. All data were expressed as mean ± SD. Statistical analyses were evaluated by one-way ANOVA followed by Tukey HSD test. Values with $P< 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

RESULTS

Phytochemical Screening

Of all the phytochemicals tested, steroid was absent in aqueous extracts (Table 1)

Antimicrobial Assay

E. coli and C. albican were the most and least

Table 2: Inhibition zone diameters (ID in mm) of Hot water and methanolic extracts of *D.cinerea* against Gram positive/negative bacteria species and *Candida albicans*

Microorganism	Mean					
	HTH_2O(STM)	*HT*H_2O(RT)	*HT*H_2O(LV)	*MET*(STM)	*MET*(RT)	*MET*(LV)
S.aureus	8.44±0.27^{a}	9.61±0.23^{ab}	9.78±0.48^{ab}	9.11±0.32^{a}	8.39± 0.28^{ab}	10.28±0.44^{a}
C.albican	8.94±0.31^{a}	8.83±0.34^{ab}	9.39±0.34^{a}	9.17±0.36^{a}	8.44±0.28^{ab}	9.56±0.46^{a}
P.aeruginosa	9.28±0.37^{ab}	8.72±0.29^{a}	10.27±0.39^{ab}	9.83±0.38^{ab}	9.00±0.28^{ab}	9.94±0.44^{a}
B.subtilis	9.33±0.37^{ab}	8.56±0.29^{a}	9.22±0.36^{a}	9.11±0.41^{a}	8.17±0.26^{a}	9.17±0.33^{a}
E.coli	10.50±0.38^{b}	9.94±0.31^{b}	11.28±0.38^{b}	10.78±0.46^{b}	9.44±0.38^{b}	10.61±0.51^{a}
L.S.D.$_{0.05}$	0.963	0.825	1.104	1.099	0.843	1.236

1gm of each explant extract was used in this bioassay. Mean values followed by the same letter within the column are not significantly different by Turkey's test (*P<0.05*)

Key:*HT*H_2O (STMB).....Stem-bark extract using hot water as solvent, *HT* H_2O (RT)……...Root extract using hot water as solvent, *HT* H_2O (LV)……...Leaves extract using hot water as solvent, *MET* (STM)……..Stem-bark extract using methanol as solvent, *MET* (RT)……..Root extract using methanol as solvent, *MET* (LV)……..Leaves extract using methanol as solvent

Table 3: Means of inhibitory concentrations (IC in mg/ml) of hot water and methanolic extracts of *D.cinerea* against Gram positive bacteria species and fungal species

concentration	Mean					
(mg/ml)	*HT*H_2O(STM)	*HT*H_2O(RT)	*HT*H_2O(LV)	*MET*(STM)	*MET*(RT)	*MET*(LV)
0.00001	7.33±0.19^{a}	7.60±0.19^{a}	8.07±0.23^{a}	7.60±0 .16^{a}	7.40± 0.13^{a}	7.60± 0.19^{a}
0.0001	8.33±0.25^{ab}	8.33±0.30^{ab}	8.67±0.32^{ab}	8.80±0 .29^{b}	7.80± 0.18^{a}	8.47±0.19^{ab}
0.001	9.27±0.27^{bc}	8.80±0.18^{bc}	9.73±0.30^{bc}	8.87±0.30^{b}	8.07±0.16^{ab}	9.33±0.19^{bc}
0.01	9.60±0.32^{c}	9.40±0.25^{cd}	10.27±.38^{cd}	10.13±0.24^{c}	8.80±0.20^{bc}	10.07±0.18^{c}
0.1	10.07±0.27^{c}	9.93±0.21^{de}	11.00±0.31^{de}	10.20±0.39^{c}	9.47±0.24^{c}	11.47±0.27^{d}
1	11.20±0.24^{d}	10.73±0.18^{e}	12.20±0.22^{e}	12.00±0.24^{d}	10.60±0.27^{d}	12.53±0.29^{e}
L.S.D.$_{0.05}$	0.728	0.628	0.840	0.784	0.564	0.629

Mean values followed by the same letter within the column are not significantly different by Turkey's test (*P<0.05*)

Key: *HT* H_2O (STMB).....Stem-bark extract using hot water as solvent, *HT* H_2O (RT)……...Root extract using hot water as solvent, *HT* H_2O (LV)……...Leaves extract using hot water as solvent, *MET* (STM)……..Stem-bark extract using methanol as solvent, *MET* (RT)……..Root extract using methanol as solvent, *MET* (LV)……..Leaves extract using methanol as solvent

susceptible microorganisms to *D. cinerea*extracts respectively (Table 2 and Table 3). Leaf methanol extract and root water extract were the most and least effective inhibitory extracts of *D. cinerea*.

Toxicity test (Brine shrimps test)

(Fig 1) Methanol extracts of *D. cinerea* exhibited varied lethal concentration potential. Leaf extract had the highest lethal potential followed by root and stem-bark respectively. Of all the extracts concentrations tested, concentration 2000ppm
Had ≤50% lethal potential. Concentrations above 2000ppm had high lethal potential.

DISCUSSION

The phytochemical analysis conducted on *D. cinerea* extracts revealed the presence of tannins, flavonoids,

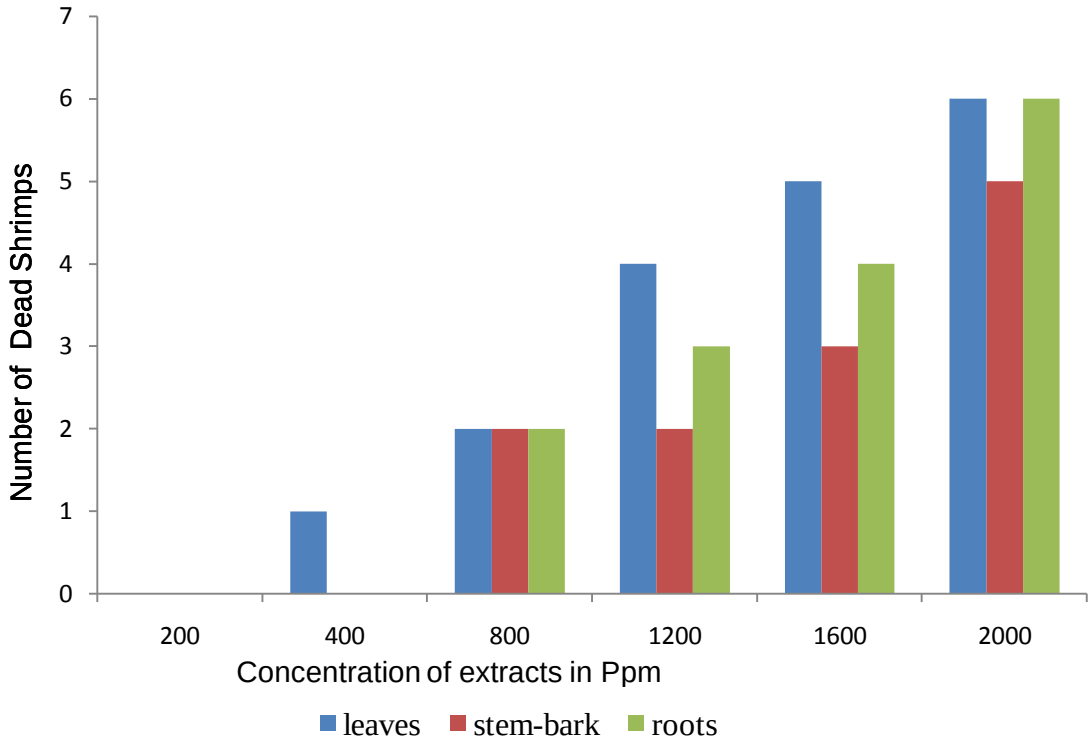

Figure 1: The mortality rate of Brimps (nauplii) at different concentrations of *Dchrostachyscinerea* leaf extract (methanol extract)

steroids and saponins.

Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2 (Li *et al*., 2003) and this property may explain the mechanisms of anti-oxidative action of *D. cinerea*. Flavonoids serve as health promoting compound as a results of its anion radicals (Hausteen, 1983; Wanda *et al.,* 2010).

D. cinerea was also found to contain saponins known to produce inhibitory effect on inflammation (Just *et al.,* 1998). Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and, they have remarkable activity in cancer prevention and anticancer (Ruch *et al.,* 1989; Motar *et al.,* 1985). Thus, *D. cinerea* containing this compound may serve as a potential source of bioactive compounds in the treatment of cancer.

Alkaloid was also detected in *D. Cinerea* extracts. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity (Nobori *et al.,* 1994). Success of *D. cinerea* extracts against both Gram positive and Gram negative bacteria are likely dependent on their content in alkaloids able to intercalate between DNA strands (Phillipson and O'Neill, 1987). The presence of these phenolic compounds in *D. cinerea* extracts contribute to its antioxidative properties and thus the usefulness of this plant in herbal medicament. Phenols have been found to be useful in the preparation of some antimicrobial compounds such as dettol and cresol.

All the evaluated extracts of various *D. cinerea* explants exhibited antibacterial and anticandidal activities i.e. all the extracts were effective against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*and *Candida albicans.* Of the two extraction solvents, methanol extracts gave better inhibition zones as compared to hot water extracts; which might be attributed to the incomplete leaching of the antibacterial substances. *E. coli* was found to be the most sensitive microorganism while *B.subtilis* was the least sensitive microorganism to the extracts.

The brine shrimp is widely accepted and used as a primary test for biological evaluation. In this procedure, assumption was made that any brine shrimp that survived to a given dose would also had survived to any lower dose, and also any cell that died at a certain dose would also die at any higher dose (Chavez *et al.,* 1997). The test period was taken as the exposure of the brine shrimps to various concentrations of *D. cinerea* extracts for a period of 24 h for chronic toxicity. This test was convenient since the brine shrimp is sensitive to a variety of chemical compounds. The assay is considered a useful tool for assessment of toxicity (Solis *et al.,*1993) and is widely used. The live brine shrimp showed internal

and external movement. The LC_{50} values obtained from *in vitro* cytotoxicity assay of the *D. cinerea* extracts against brine shrimp was 2000ppm. According to Venugopal *et al.*(2002), bioactive compounds which exhibit an LC_{50} value more than 1.0 mg/ml are considered not toxic to the nauplii of *Artemiasalina*. Therefore, the cytotoxicity result obtained in this study indicated that the *D. cinerea* extracts showed no toxicity against brine shrimp. The brine shrimp assay is a useful tool for testing lethality of bioactive compounds from plant extracts (Sam, 1993). Thus, the results suggested that the extracts could be potential candidates to be used as antibacterial and antifungal agents.

CONCLUSION

This study assent to the rational usage of this plant in Kenyan medicinal traditions. Further studies however are needed to quantify the identified phenols and also clarify the *in vivo* potential of this plant in the management of human diseases resulting from oxidative stress.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

'Author [1*]', (corresponding author) designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. 'Author [1]' and 'Author [2]' provided technical advice, assisted in literature searches and approved the final manuscript."

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