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Evaluation of antimicrobial and toxic activity of aqueous, ethanolic and methanolic extracts of leaves and roots of *Dichrostachys cinerea* (Fabaceae)

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Abstract
Dichrostachys cinerea is a plant used in traditional Mozambican medicine to treat diarrhea, toothache, ear pain and abdominal pain, however its therapeutic and toxic potentials have not been sufficiently studied. Therefore, this study aimed to carry out preliminary qualitative phytochemistry and to evaluate antimicrobial and toxicological activity of the aqueous, methanolic and ethanolic extracts of the leaves and roots of *Dichrostachys cinerea*. Antimicrobial activity was assessed using the Muller Hinton Agar diffusion method, while the acute toxicity study was performed on rats (*Mus musculus*) using the protocol approved by the Eduardo Mondlane University Bioethics Committee, described according to the guidelines of the Organization for Cooperation and Development (OECD) for Chemical Testing. The methanolic extract of the leaves showed activity on *Staphylococcus aureus* with MIC = 125 mg / mL while the ethanolic extract of the roots showed activity for *Staphylococcus aureus* with MIC = 306 µg/ mL and *Streptococcus* sp, with MIC of 977 µg/mL. All extracts showed less activity than the drug ciprofloxacin used as a positive control. When assessing toxicity, the methanolic extract showed the highest mortality in rats, both acute and sub chronic toxicity. Although signs of systemic toxicity were observed in all groups, these were accentuated in animals that received the methanol extract. The conclusion of the study is that, the extracts of *D. cinerea* evaluated in this study show toxicity to *Mus musculus*, although most of them are very common even with commercially available medicines. Further evaluation studies are needed to better assess the risk of toxicity and its therapeutic benefit.

Keywords: Medicinal plants; *Dichrostachys cinerea;* antimicrobial activity; toxicity

Introduction
Dichrostachys cinerea is a plant widely used in traditional medicine. According to the Institute for traditional Medicine in Mozambique, roots of the plant are used, in traditional medicine, with roots of other plants to treat parasitic diseases, asthma and other diseases, the bark is used to alleviate toothaches and stomach problems, and the leaves can be applied to bits and stings. These findings are supported by studies by different authors who produced evidence about the use of this plant in medicine. Wyk (2011) report the use of *D. cinerea* leaves to treat gonorrhea and diarrhea; Aworet-Samseny et al. (2011), Sousa et al. (2011) and Geneviève et al. (2018) studied its use in the treatment of asthma; El-Sharawy et al. (2017) reported the antitrypanosomal and antiviral effect of aqueous-alcoholic extracts of the leaves. Neondo et al. (2012) studied the antimicrobial activity of aqueous and methanolic extracts of roots, leaves and bark of the plant on microorganisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*, and observed that methanolic leaf extracts and aqueous root extracts were most effective against the above bacteria. Mishra et al. (2009) reported the potential antibacterial and analgesic activity of *D. cinerea* leaf extracts. Swetha and Devareddy (2013) studied the antimicrobial activity of methanolic, petroleum ether, methanolic and ethyl acetate leaf extracts, whose results showed activity against *Staphylococcus aureus* and *Shigella Soneii*, while Shankarmurthy (2011) demonstrated hepatoprotective activity of *D. cinerea* at a dose of

3,500 mg / kg.

Banso and Adeyemo (2007) showed that tannins isolated from *D. cinerea* roots exhibited antibacterial activities against *Staphylococcus aureus*, *Shigella boydii*, *Shigella flexneri*, *Escherichia coli* and *Pseudomonas aeruginosa*. The MIC of tannins ranged from 4.0 to 5.5 mg / mL, while the minimum bactericidal concentration ranged from 4.5 to 6.0 mg / mL. Adikay et al. (2009) have shown that *D. cinera* ethanolic root extract had nephroprotective activity and Jayakumari (2011), using ethanolic root extracts at doses of 200 mg/kg and 400 mg/kg in Wistar albino rats, demonstrated the potential antiurolithic effect of *D. cinerea*.

The aim of the present study was to determine the in vitro antimicrobial activity of aqueous, ethanolic and methanolic extracts of *D. cinerea* against *Streptococcus* sp; *Escherichia coli*; *Staphylococcus aureus*; *Pseudomonas aeruginosa*, *Salmonella* sp and *Candida albicans*, including the determination of the MIC of the extracts which present an antimicrobial activity; and (2) assess weight, hippocratic, hematological, biochemical and pathological changes of *D. cinerea* in mus musculus (mice rats) submitted to acute and subchronic toxicity tests.

Materials and methods

Sample preparation

The leaves and roots of *D. cinerea* were collected by hand in February 2017 from Moma – Nampula Province, north of Mozambique. The samples were identified by the Herbarium of the Institute for Traditional Medicine – Ministry of Health. The leaves and roots of *D. cinerea* were cleaned with tap water and dried.

Sample extraction

The samples were extracted using 96 % ethanol, methanol and distilled water. Each sample of 100 g was weighed into conical flask that was wrapped with parafilm and 1000 mL 96% ethanol, methanol or water was added. The mixture was stirred and left to stand for seventy-two hours and the extracts obtained were filtered and concentrated to dryness. After that, the residues were weighed and stored in the fridge until use.

Preliminary phytochemical analysis

A preliminary phytochemical screening was carried out using the ethanolic, methanolic and aqueous extract of the leaves and roots by employing the standard procedures (Joshi et al., 2013).

Antimicrobial activity

All the extracts were submitted to evaluation of antimicrobial activity, against *Staphylococcus aureus*, *Streptococcus* sp, *Salmonella* sp, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*, using the disk diffusion test (Balouiri, 2016; NCCLS, 2003). Extracts presenting antimicrobial activity were then diluted to 250 mg/mL, 125 mg/mL 62.5 mg/mL and 31.25 mg/mL and used in the determination of the MIC using a norm from the Clinical and Laboratory Standards Institute (Queen et al., 1994).

Toxicity tests

120 Rats of both sexes, four and five months old, weighing 23–37 grams, were purchased from the Bioterium of the Mozambique Agricultural Research Institute (Maputo, Mozambique). The animals were fasted for 24 hrs before the extracts were administered orally, using a blunt needle. All experimental protocols with animals were approved by the Bioethics Commission of Eduardo Mondlane University. All efforts were made to minimize the suffering and anguish of the rats. For the acute toxicity a single dose of the different extracts was administered to a group of 10 animals (5 male and 5 female) and animals were monitored during 14 days, while for the subchronic toxicity extracts were administered orally, on a daily basis, for a period of 28 days.

Monitoring of the animals during toxicological tests included weekly determination of the body weight, determination of the percentage of erythrocytes from blood samples, observation of behavioral changes (state of consciousness, motor activity and coordination, reflexes, autonomic stimulation and other toxicity indicators) and physical changes (monitoring was performed 5, 10, 30 and 60 minutes and 2, 4, 6, 12 and 24 hours after administration and on the first day. After the 24 hours monitoring was performed once in a day until end of the test). At the end of the tests leaving animals were submitted to hematological parameters using a BC-2800 VET/Mindray analyzer and determination of biochemical parameters. Death and leaving animals were further submitted to macroscopic evaluation of organs damage (Al-Taee et al., 2019).

Statistical analysis

The experimental results were expressed as mean ± standard error of mean (SEM) of three replicates for antimicrobial activity or mean ± standard error from the weight of ten animals for toxic activity. Comparative analysis and ANOVA ($p<0.05$) were done using the STATA 14 package.

Results and Discussion

Results of the phytochemical analysis of the roots show for methanol extracts only the presence of saponins while the ethanolic extract tested positive for all metabolites, except heterosides (Table 1). In the leaf extracts (Table 2) saponins were the only metabolite detected in the three extracts; heterosides were present only in the aqueous extract, while tannins and alkaloids were found only in the methanolic extracts.

Table 1: Results of the phytochemical analysis of *Dichrostachys cinerea* roots, obtained with ethanolic and methanolic extracts.

Components	Solvent	
	Ethanol	**Methanol**
Alkaloids	+	-
Tannins	+	-
Flavonoids	+	-
Heterosides	-	-
Saponins	+	+

Table 2: Results of the phytochemical analysis of *Dichrostachys cinerea* leaves, obtained with ethanolic, methanolic and aqueous extracts.

Components	Solvent		
	Ethanol	**Methanol**	**Water**
Alkaloids	-	+	-
Tannins	-	+	-
Flavonoids *	+	-	+

	-	+	+
Heterosides	-	-	+
Saponins	+	+	+

* Shinoda reaction (upper); Reaction with NaOH (lower)

Antimicrobial tests

Ethanolic and aqueous extracts of leaves and methanolic extracts of the roots at the maximum concentration (500 mg/mL) were not active against the bacterial under study, while the methanolic extract of the leaves was effective against *Staphylococcus aureus* and the ethanolic extract of the roots showed activity against *Staphylococcus aureus* and *Streptococcus spp*. No extracts showed activity against *Candida albicans*, *Pseudomonas aeruginosa* and *Escherichia coli*. Methanolic extract of the leaves showed activity only against *Staphylococcus aureus*, with a MIC of 125 mg/mL. Ethanolic extract of the roots presented a MIC of 977 µg/mL for *Staphylococcus aureus* and 31.5 mg/mL for *Streptococcus spp*.

Assessment of acute toxicity

Table 3 present results of the body weight of the animals during the acute toxicity tests, obtained with ethanolic, methanolic and aqueous extracts, while table 4 presents results obtained with different doses of the aqueous extracts. Statistical analysis of weight variation was performed considering gender, treatment groups and the interaction of the two variables, using ANOVA. A statistically significant difference was observed for sex of the animals and extract factor, but the interaction of the two variables showed no significant differences. Tukey's test for comparing the extracts showed that: (1) the control group, the methanolic leaf extract and the aqueous leaf extract did not differ significantly from each other; (2) the control group and methanolic extract were statistically different from the ethanolic root extract, and (3) the ethanolic root extract and the aqueous leaf extract were not statistically different in weight variation. The statistical analysis of the results obtained the aqueous extract of the leaves with different concentrations (Table 4) showed that the different doses of the aqueous extract and the interaction dose and sex did not have significant differences ($p > 0.05$), while sex factor was the only one that showed significant differences between the means ($p < 0.05$).

Statistical analysis of the hematocrit means, before and after administration, of the mice rats exposed to aqueous, methanolic and ethanolic extracts of the roots (1250 mg/kg) showed no significant differences. Concerning aqueous leaf extracts (1250 mg/kg and 3250 mg/kg), (1) factor sex show no statistical differences, (2) but dose and the interaction of sex and dose show a significant difference of obtained results ($P<0.05$).

Hippocratic screening present a different behavior for the different extracts (see tables 5-7). Mortality rate ranged from 0% to 60%, with 60% for the methanolic leaf extract, 20% for the ethanolic root extract and 10% for both groups exposed to aqueous leaf extract with 1250 mg/kg and 3250 mg/kg doses, compared to the 0% for the control group. In terms of macroscopic damages, animals exposed to different extracts show (1) methanolic leaf extracts (1250 mg/kg): a red lung and liver; (2) aqueous leaf extract (1250 and 3250 mg/kg doses): reddish lung and pallor in the periphery and (3) ethanolic root extracts (1250 mg/kg): red lung. Histopathological analysis showed different damages, more pronounced on animals exposed to methanolic an ethanolic extracts than on animals exposed to aqueous extracts.

Table 3: Variation of the body weight of the animals exposed to ethanolic, methanolic and aqueous extracts, with a dose of 1250 mg/kg, during the acute toxicity tests.

Group	Body weight in grams		
	Before administration	First week	Second week
Methanolic extract	27.48±3.8	27.725±1.83	29.3±2.27
Ethanolic extract	31.47±2.6	28.71±4.13	28.125±12.6
Aqueous extract	26.01±1.9	26.17±6.22	25.7±2.97
Control group	30.63±4.1	32.26±2.9	33.13±2.81

Table 4: Variation of the body weight of the animals exposed to different doses of the leaves aqueous extracts, during the acute toxicity tests.

Group (Dose)	Body weight in grams		
	Before administration	First week	Second week
Group 1 (1250 mg/kg)	26.01±1.9	26.17±6.22	25.7±2.97
Group 2 (3250 mg/kg)	32.5±2.23	31.34±2.08	35.52±1.31
Control group	30.63±4.1	32.26±2.9	33.13±2.81

Table 5: Results of the hippocratic screening of animals exposed to methanolic leaf extracts.

Methanolic leaf extract		
Reaction	**% animals**	**Period of occurence**
Lethargy	100 % (10 animals)	Immediatelly after administration
Muscle spasms before death	20 % (2 animals)	Seconds before death on day of administration
Apathy	100 % (10 animals)	Occasionally during 1st and 2nd day after administration

Table 6: Results of the hippocratic screening of animals exposed to aqueous leaf extracts.

aqueous leaf extract			
Reaction	**Group**	**%**	**Period of occurence**
Muscle spasms before death	3250 mg/kg	10 % (1 animal)	Seconds before death on day of administration
Apathy	1250 mg/kg	10 % (1 animal)	Up to 30 minutes after administration
	3250 mg/kg		
Post-administration urination		10 % (1 animal)	Immediately after administration

Table 7: Results of the hippocratic screening of animals exposed to ethanolic root extracts.

Ethanolic root extract		
Reaction	**% animals**	**Period of occurence**
Lethargy	10 % (1 animal)	Immediatelly after administration
Apathy	10 % (1 animal)	During the 1st day after administration
Bristly hair	100 % (10 animals)	During the 1st day after administration

Assessment of the subchronic toxicity

Animals exposed to different concentrations of the methanolic leaf extract show a decrease of body weight along the 4 weeks of the study (Table 7), while the ones exposed to the aqueous leaf extract show, in general, a gain of weight (Table 8). Results of the analysis of variance, followed by the Tukey test, of the results in table 8 show that, a) sex, b) dose and c) interaction of sex and dose have a significant effect on variation of weight, with males showing the most predominant effect. Statistical analysis was not performed for the results in table 7, since only two animals survived to the test.

Statistical analysis of the hematocrit means, before and after administration, of the mice rats exposed to aqueous leaf extracts do not show a significant effect of sex, dose or interaction of both factors.

Hippocratic screening present a different behavior when comparing methanolic and aqueous leaf extracts. Animals exposed to methanolic leaf extracts present (1) lethargy and apathy in 100% of tested animals and permanently during the monitoring period and (2) muscle spasms before death, also in 100% of tested animals and immediately after administration, while animals exposed to methanolic leaf extracts present hyperactivity in 100% of tested animals, during the entire monitoring period. Mortality rate reached (1) 100% for 500 and 250 mg/kg dose and 80% for the 125 mg/kg dose, in animals exposed to methanolic leaf extracts; (2) 30% for 500 and 125 mg/kg dose and 20% for the 250mg/kg dose, in animals exposed to aqueous leaf extracts; and 0% for the control group.

Macroscopic pathological analysis of animals exposed to aqueous leaf extracts show (1) pale areas in liver and red areas in lung, in 100% of the animals administered with the 500 mg/kg dose; (2) pale and diffuse areas in liver in 10% of the animals administered with the 250 mg/kg dose and (3) splenomegaly in 10% of the animals administered with the 125 mg/kg dose. Animals exposed to methanolic leaf extracts show red areas in lung and liver and pale areas in the spleen, in 100% of the animals administered with the 500 and 250 mg/kg dose, and 10% damages in the animals administered with the 125 mg/kg dose.

Results of the phytochemical analysis of methanolic root extracts are different from the results obtained by Neondo *et al*. (2012), who identified further components than the saponins identified in this study. Methanolic leaf extracts present more components than the ethanolic and aqueous extracts, results which are more in agreement with those obtained by Neondo *et al*. (2012), Reis *et al*. (2015) and Samseny *et al*. (2011). Swetha and Devareddy (2013) obtained similar results for the leaf extracts, but using a different solvent (Ethyl acetate). Ethanolic root extracts and methanolic leaf extracts show similar components, including the alkaloids and tannins associated with antimicrobial activity (Oliveira *et al*., 2009; Negri and Tabach, 2013).

Although no extracts showed activity against *Candida albicans*, *Pseudomonas aeruginosa* and *Escherichia coli*, Banso and Adeyemo (2007) report antimicrobial activity of extracts of pure tannins isolated from the plant. Apparently, its effect may depend on concentration of the components, but also on the presence of some components with an inhibitory action or which combination can influence behaviors different from those of pure extracts (Fennel, 2004).

Discussion of toxicity results has been negatively affected by the fact that the only studies available about the toxicity of *D. Cinerea* were made with the saline *arthemia* larva and evaluated cytotoxicity and average lethal concentration (Neondo *et al*., 2012) and mortality results determined through acute toxicity tests in Wistar albino rats (Shankarmurthy *et al*., 2011). Statistical analysis show that variation of body weight was, in generally, not affected by *D. Cinerea*. Significant differences hereby were found for sex, combinations of sex with other parameters or other physiological parameters.

In the acute toxicity tests, the extract type did not affect significantly the hematocrit, but the increase in the dose, in the case of the aqueous extract, affected significantly the hematocrit variation. However, tests of the subchronic toxicity registered an oscillating behavior over the exposure period, including in the control group, that was statistically not relevant.

Lethargy and apathy were a common feature in both acute and subchronic exposure to all extracts. Exception to this pattern was subchronic toxicity in aqueous extract in which the animals showed hyperactivity, a result that was also observed by Atsang et al. (2018) in *Dichrostachys glomerata*, another species of the same genera, but at higher doses (2- 5 gr/kg). Report of muscle spasms before death, occurring immediately before death, may be associated with induction, by extracts, of increased contractile force and frequency of muscle contractions, observed by Samseny *et al*. (2015), which suggest that *D. cinerea* has a varied effect on the nervous system.

The highest mortality rates in this study were observed in methanolic extracts ((1) 60% for acute toxicity and (2) 100% mortality for the 500 and 250 mg/kg doses and 80% in subchronic toxicity). Ethanolic extracts showed a mortality rate of 20% in acute toxicity tests, while aqueous extracts showed a 10% mortality rate in acute toxicity tests and 20-30% in subchronic toxicity tests.

Table 8: Variation of the body weight of the animals exposed to different doses of the methanolic leaf extracts, during the subchronic toxicity tests.

Group	Body weight in grams				
	Before	**1st week**	**2nd week**	**3rd week**	**4th week**
Group 1 (500) mg/kg)	30.76±3.5	24.4	20.7	18.4	-
Group 2 (250 mg/kg)	32.11±4.7	32.6±1.7	-	-	-
Group 3 (125 mg/kg)	31.08±5.1	26.9±6.3	28.56±4.83	22.35±3.32	22.1±1.1
Control group	29.56±4.77	29.51±4.61	28.73±4.45	29.47±4.27	30.32±3.35

Table 9: Variation of the body weight of the animals exposed to different doses of the aqueous leaf extracts, during the subchronic toxicity tests.

Group	Body weight in grams				
	Before	1st week	2nd week	3rd week	4th week
Group 1 (500 mg/kg)	28.22±2.97	28.52±2.9	28.1±4.02	30.0±2.50	30.58±2.25
Group 2 (250 mg/kg)	28.36±3.83	28.73±3.75	28.8±2.39	29.91±1.61	31.625±2.11
Group 3 (125 mg/mL)	26.97±2.66	29.09±3.53	32.04±2.33	32.7±1.96	33.54±1.64
Control group	29.56±4.77	29.51±4.61	28.73±4.45	29.47±4.27	30.32±3.35

Conclusions

The ethanolic and aqueous extracts of the leaves and methanolic extracts of the roots showed no activity against any of the bacteria under study. None of the extracts showed fungal activity against *Candida albicans*. Methanolic leaf extract showed activity against *Staphylococcus aureus* with a MIC of 125 mg/mL. The ethanolic root extract showed activity against *Staphylococcus aureus* with a MIC of 0.977 mg / mL and *Streptococcus sp* with a MIC of 3.906 mg / mL. Methanolic extract showed the highest mortality rate in both acute and subchronic exposure. Although signs of systemic toxicity were observed in all groups, they were marked in animals exposed to the methanolic extract. *D. cinerea* extracts evaluated in this study showed signs of toxicity to *mus musculus*, however they need to be better evaluated, so that more data can be obtained to allow a better evaluation between the toxicity risk and its therapeutic benefit.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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