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Research Article
In vitro Study of Phytochemical Composition and Antifungal Activity of *Dicerocaryum senecioides* Leaf Extract

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Abstract

Background and Objective: Drug resistance causes fungal infections such as dandruff, athlete`s foot and ring worm to become difficult to treat. The study was undertaken to investigate the antifungal activity and phytochemical composition of *Dicerocaryum senecioides* extracts as an alternative remedy in primary health care. **Materials and Methods:** Agar well diffusion test were used to assess the antifungal activity. Thin Layer Chromatography (TLC) was used to screen for the phytochemical composition of the crude extracts by visualizing the developed plates under the Ultra Violet light and staining with standard revealing agents. **Results:** Ethyl acetate extract showed the highest antifungal activity followed by dichloromethane, ethanol, aqueous and hexane extracts in respective order of decreasing activity. The Minimum Inhibition Concentrations (MIC) of ethyl acetate were 1.25, 2.50, 1.25 and 5.00 mg mL^{-1} while those for dichloromethane extract were 2.50, 1.25, 0.625 and 2.50 mg mL^{-1} for fungi strains *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxisporum* and *Penicillium italicum*, respectively. The TLC phytochemical profile of the ethyl acetate extract revealed the presence of 8 compounds while dichloromethane extract revealed 5 compounds. The phytocompounds observed include tannins, phenolics, flavonoids, carbohydrates, glycosides, saponins, alkaloids, terpenes and steroids. **Conclusion:** The present study shows that *Dicerocaryum senecioides* moderately polar and polar extracts consist of remarkable antifungal activity and significant amount of health benefiting phytocompounds therefore it is a good candidate for searching for lead compounds to develop new antifungal therapies to deal with drug resistant fungal strains.

Key words: *Dicerocaryum senecioides*, antifungal activity, ringworm, drug resistance fungal, antifungal therapies, antidandruff, fungal resistance

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fungal infections including dandruff, athlete's foot and ring worm remain an important problem to the human populace[1-3]. Clinical failure and relapses have been observed in some patients treated with current antifungal agents[4,5]. In some patients the drugs have several side effects which include, renal toxicity, infusion reactions, electrolyte abnormalities and hepatotoxicity[6]. Thus the search and development of new, safer and more efficient antifungal drugs is triggered by the prolonged duration of treatment, drug toxicity, fungal resistance and high costs exhibited by most known antifungal drugs used clinically for topical treatment. The need to develop antidandruff agents with powerful fungicidal activity led us to investigate the herb *Dicerocaryum senecioides* for potential antifungal compounds. The selection of this plant was based on its ethnopharmacological use. In this application which is popular in rural communities in southern Africa, the leaves are rubbed into moist hair to form a creamy lather[7]. The cream lather is then washed off with water. *Dicerocaryum senecioides* subsp. *Transvaalense* (Klotzsch) J. Abels [family: Pedaliaceae], is a creeping perennial herb that is also used both as a traditional medicinal plant and a nutritional source in many parts of southern Africa and China[8]. The plant is found throughout the year and grows more vigorously in the summer as compared to the winter season. The seeds of the plant have two sharp distinct cones which helps it stick to the body of animals for easy dispersal. The plant grows mostly in sandy soils and abandoned lands. Previous study by Madiga *et al.*[9] revealed that dichloromethane crude extracts from the herb exhibit anti-inflammatory and anti-proliferative activities against cancer cells. The herb is also reported to exhibit strong anti-oxidant properties[10] which enables it to be used as a hair permanent[11]. Its lathering properties facilitates its shampoo properties[12-14]. The local Zimbabwean people prefer to use the water extract of the leaves as a hair shampoo because it's perceived to have antidandruff and hair rejuvenation properties. Although, the use of the mucilage as a shampoo that is believed to cure dandruff is widely practiced, scientific studies to support this claim are still limited. Thus the present study was designed to screen for phytocompounds in *Dicerocaryum senecioides* leaf extracts for antifungal agents.

MATERIALS AND METHODS

Plant material collection: *Dicerocaryum senecioides* leaves were collected around the city of Bulawayo in Zimbabwe and identified and authenticated by the Harare Botanical garden. Voucher specimens 2017/5 were deposited in the Bindura University of Science Education, chemistry laboratory for future reference.

Sample preparation: Fresh leaves of the plant were washed first with tap water and rinsed using distilled water to remove soil and debris. The leaves were then allowed to dry under the shade by spreading thinly on stainless steel sheet boards. The dried material of each sample were crushed and hand shaken vigorously to separate the leaves from the stems. The stems were then discarded and the dried leaves separately ground into a fine powder using a commercial blender.

Extraction: The powdered plant leaves of *Dicerocaryum senecioides* were successively extracted with different solvents in the increasing polarity order. Powdered leaves (80 g) were macerated separately in 800 mL of hexane with intermittent shaking for 12 h. Then they were first filtered with muslin cloth and then through Whatman number 1 filter paper. The residue was further extracted twice using the same fresh solvent and all the filtrates were pooled together. The resulting residue was air dried and further extracted with dichloromethane (DCM), ethyl acetate (EA), ethanol and lastly water using the procedure similar to the one carried out for the DCM extraction. Finally from each filtrate the solvent was removed using rotary evaporator under reduced pressure and a temperature of 40°C. The water extract was precipitated by adding 100 mL of absolute ethanol followed by concentrating on a rotary evaporator at a temperature of 40°C. When all the solvent had evaporated, the extract was lyophilized using a freeze dryer (LyoQuest-85 Telsta Laboratory Freeze Dryer). The yield of each extract was weighed and samples were stored under refrigerator until required for analysis.

Antifungal tests: The pure fungal cultures were maintained on Potato Dextrose Agar (PDA) medium. Each fungal culture was further maintained by sub culturing regularly on the same medium and stored at 4°C until required for analysis.

Agar well diffusion method: Agar well-diffusion method was set up to determine the antimicrobial activity guided by a method reported by Sen and Batra[15]. All the media prepared and the apparatus used were sterilized by auto-claving at 121°C and 15 psi for 15 min. Inoculation was done by streaking the respective fungi colonies on Potato Dextrose Agar (PDA) media plates with sterilized inoculating loop. Wells (5 mm diameter and about 2 cm a part) were made in each of the plates using sterile cork borers. Stock solution of each

plant extract were prepared at a concentration of 20 mg mL^{-1} in different plant extracts which are hexane, dichloromethane, ethyl acetate, methanol and water. A micropipette was used to dispense 100 µL of each plant extract into the prepared wells. The extracts were allowed to diffuse through the solid PDA agar for 2 h before incubation. Control experiments comprising inoculums without plant extract except the extracting solvents were set up. The plates were incubated at 28°C for 72 h for fungal pathogens to grow. The diameter of the inhibition zone (mm) were measured for each extract. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were record. Commercial standard, miconazole nitrate cream was used as a positive control (20 mg mL^{-1}). All experiments were performed in duplicate and repeated three times.

Minimum Inhibitory Concentration (MIC): Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a micro-organism. The plant extracts, which were positive for zone of inhibition in agar well diffusion test were assayed for the determination of MIC. Extracts stock solutions of concentration 20 mg mL^{-1} were prepared by dissolving 500 mg of extracts in 25 mL of the respective pure solvents. From this stock solution different concentration of 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 mg mL^{-1} were prepared by serial dilution. Agar well-diffusion method was followed to determine the antifungal activity of different concentrations of extracts. The same method of inoculating and sterilization done on the stock solutions was followed for determining the minimum inhibition concentrations. The lowest concentration (highest dilution) required to arrest the growth of fungus was regarded as minimum inhibitory concentration.

Minimum Fungicidal Concentration (MFC): This was carried out to assess the crude extract for fungicidal or fungistatic effect. The MFC was determined by sub culturing the test dilution (used in MIC) on to a freshly prepared PDA medium and incubated further to assess fungal growth. A loopful from each of these plates were sub cultured into appropriately labeled triplicates of sterilized PDA plates using sterilized wire loop and streaked uniformly on the labeled triplicates. This was incubated for 7 days at 28°C after which they were observed for growth[16]. The MFC was the triplicate with the lowest concentration of the extract without growth.

Phytochemical screening: The sequential extracts from *D. senecioides* were subjected to thin-layer chromatography (TLC) assays in order to perform a qualitative detection of their functional phytocompounds. The working solutions 10 mg mL^{-1} were prepared by dissolving 10 mg of each dried extract in 1 mL of original solvent before spotting on 10×10 cm ALUGRAM® SIL G/UV254 TLC plates[17]. The plates were then developed in different solvent systems for proper separation. The ethyl acetate fraction was successfully separated using the mobile phase, ethyl acetate; methanol; water (EMW, 10:2:1.5, v/v/v) system. The dichloromethane extract was separated using solvent system hexane; ethyl acetate; acetic acid (HEA, 50:40:10, v/v/v). The components were visualized under visible and UV light of wavelength 254 and 365 nm to detect quenching and fluorescing compounds respectively[18]. The detected sports were marked and Retention factors (R_f) values calculated using the following Eq.:

$$\text{Retention factor} = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

Phytochemicals were detected using the methods developed by Oleszek *et al.*[19] with slight modifications were necessary. Saponins were tested by taking 10 mL of 10 mg mL^{-1} extract and mixing with 5 mL of distilled water followed by shaking vigorously for a stable persistent froth. The frothing was mixed with 3 drops of cooking oil and shaken vigorously, then observed for the formation of emulsion. Detection of phenolics was achieved by spraying the plates with aqueous ferric chloride solution. Flavonoids and alkaloids were revealed by spraying with ethanolic $AlCl_3$ and modified Dragendorff's reagent respectively[20]. Steroids and terpenes were detected by spraying with antimony (III) chloride reagent as determined by Waksmundzka-Hajnos *et al.*[21]. Carbohydrates and sugars were detected by spraying with 3% p-Anisidine hydrochloride in n-butanol followed by heating the plate at 100°C for 2-10 min. Aldohexoses are seen as green-brown spots, ketohexoses as yellow spots, aldopentoses as green spots and uronic acids as red spots[22].

Statistical analysis: The results are expressed as Means±Standard deviations of three replicate analysis. Differences between the group means for antifungal assays were analyzed using the IBM SPSS version 20 software by applying a one-way ANOVA with the Turkey-karner *post hoc* test to identify significance among groups. A p-value<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Extraction solvents: Yields for the different solvents obtained from a powered mass of 80 g are shown in Table 1. The yield

(%) ranged from 1.55-2.67. The best yield was achieved with ethanol followed by dichloromethane and ethyl acetate. Hexane as an extracting solvent produced the least yield (%) of 1.55.

Antifungal activity: The results for the antifungal activities of sequential extracts are shown in Fig. 1. Results indicated that the ethyl acetate extract exhibited the highest zone of inhibition ranging from 10.58-13.92 mm against all the four fungi strains followed by dichloromethane with zone of inhibition ranging from 9.52-12.5 mm $p<0.05$. The zone of inhibition for the ethyl acetate and dichloromethane extract was significantly higher than the zone of inhibition of the miconazole commercial standard drug, 5.17-8.15 mm for all the strains ($p<0.05$). The ethanol extract also showed significantly higher inhibition effect on *Aspergillus flavus*, *Fusarium oxisporum* and *Penicilium italicum* than the standard drug miconazole ($p<0.05$), however for *Aspergillus niger* miconazole showed a significantly higher potency than the extract ($p<0.05$). The water extract showed an inhibition zone that was significantly similar to that of the standard drug miconazole ranging from 5.28-6.52 mm for all the strains ($p>0.05$). The hexane extract exhibited the least inhibitory activity ranging from 4.95-5.13 mm and this was significantly lower than that for the standard drug ($p<0.05$). Solvents used for extraction showed negligible zone of inhibitions for all the tested fungal species.

Minimum inhibitory and fungicidal concentration: Minimum Inhibitory Concentration (MIC) refers to the highest dilution or least concentration of the extracts that is capable of inhibiting growth of organisms while Minimum Fungicidal Concentration (MFC) is the least concentration of plant extract that completely killed the fungi. Results of MICs and MFCs are shown in Table 2. The MFC of ethyl acetate extract on both *A. flavus* and *F. oxisporum* were found to be 0.625 mg mL^{-1} while that for *A. niger* and *P. italicum* were 1.25 and 2.50 mg mL^{-1}, respectively. Thus the ethyl acetate inhibit strongly *A. flavus* and *F. oxisporum*. The dichloromethane extract was more active against *F. oxisporum* as indicated by minimum fungicidal concentration of 0.625 mg mL^{-1}. The higher MIC and MCF ranging from 10-20 mg mL^{-1} for

Table 1: Yields of crude extracts in grams and percentage

Solvent used	Mass obtained (g)	Yield (%)
Hexane	1.242±0.003	1.55
Dichloromethane	1.874±0.003	2.34
Ethyl acetate	1.842±0.003	2.30
Ethanol	2.134±0.003	2.67
Water	0.531±0.003	1.77

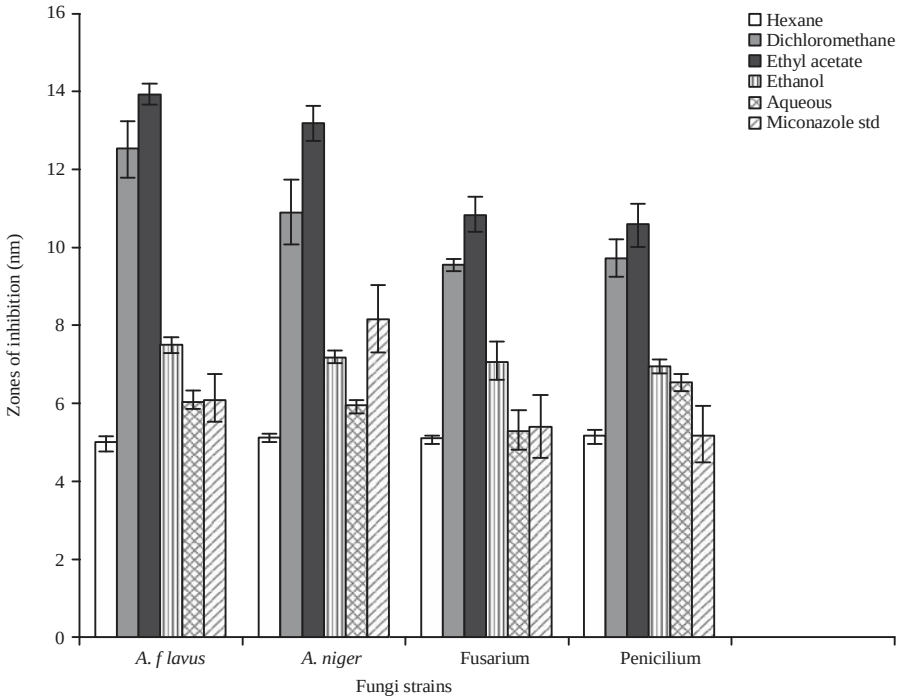

Fig. 1: Antifungal activity (zone of inhibition, mm) of sequential extracts of *Dicerocaryum senecioides* against selected fungal strains

Table 2: Minimum Inhibition Concentrations (MIC) and minimum fungicidal concentrations (MFC) of extracts on the fungi strains

	MIC and MFC values (mg mL^{-1})							
	Ethyl acetate		Dichloromethane		Methanol		Water	
Fungi strains	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus flavus	1.25	0.625	2.50	1.250	20.00	10.00	20.00	20.00
Aspergillus niger	2.50	1.25	1.25	1.250	5.00	2.50	5.00	2.50
Fusarium oxisporum	1.25	0.625	0.625	0.625	10.00	10.00	2.50	2.50
Penicilium italicum	5.00	2.50	2.50	1.250	20.00	20.00	20.00	20.00

Table 3: Phytochemical screening of sequential extracts of *Dicerocaryum senecioides*

Phytochemicals	Extract	Colour	R_f value	Spray reagent
Phenolics	EA	Green	0.80, 0.63	Iron (III) chloride in 50% methanol, UV light
	DCM	1, 3	0.75, 0.65	
Flavonoids	EA	Orange, yellow	0.73, 0.53, 0.63	Ethanolic aluminum (III) chloride, UV light
	DCM	Green, orange	0.60, 0.55	
Alkaloids	EA	Orange	0.68	Dragendorff's reagent UV light
Terpenoids and steroids	EA	Purple	0.58, 0.48, 030	Antimony (III) chloride in chloroform, UV light
	DCM	Red	0.70, 0.60	
Glycosides	EA	2, 4, 7	0.58, 0.63	Methanolic potassium hydroxide, UV light
	DC	4	0.60	

EA: Ethyl acetate, DCM: Dichloromethane

methanol and water extracts against *P. italicum* and *A. flavus* shows that the extracts are not good inhibitors of these strains.

Thin layer chromatography phytochemical screening: Dichloromethane and ethyl acetate extracts showed significant antifungal activities, therefore; the extracts were further subjected to analytical thin layer chromatography to investigate the phytocompounds responsible for the activity. Phytochemicals targeted where phenolics, flavonoids, steroids and terpenoids, alkaloids and alkaloids because these have been reported previously to exhibited antifungal activity[23]. Results for the thin layer phytochemical screening are shown in Fig. 2, 3 and Table 3. Compounds with serial numbers 1, 3 and 6 (Fig. 2) quenched the UV fluorescence light at wavelength 254 nm. This characteristic is common for aromatic compounds as explained by Ngobeni[17]. Conjugated compounds appear as dark sports because they block the fluorescence by absorbing the UV light on a green background at this wavelength. Compounds coloured yellow, blue and red under UV light are a characteristics of flavonoids. The TLC chromatogram developed for dichloromethane extract revealed the presence of 5 compounds (Fig. 3). Only two of them were active at wavelength 254 nm. Detailed retention factors and identity of compounds are as described in Table 3. The TLC profiling of dichloromethane and ethyl acetate extracts in different solvent system confirms the presence of diverse group of phytochemicals (Table 3). Revealed spots comprise of tannins, phenolics, flavonoids, carbohydrates, glycosides, saponins, alkaloids, terpenes and steroids.

In phytochemical analysis successful extraction of the active principles depends on the judicial choice of solvents. In the present study solvents of different polarities where chosen and results show that polar to intermediate polar solvents were able to extract more compounds than the non-polar solvent hexane. This shows that most of the phytocompounds in *Dicerocaryum senecioides* are polar to intermediately polar. These findings are in line to the traditional mode of extracting active principles from *Dicerocaryum senecioides*. Water infusions of the leaves are used as a hair shampoo and to treat measles and wounds[13].

Although, ethanol managed to extract the greatest amounts of phytocompounds, it did not tally with antifungal activity. Ethyl acetate and dichloromethane exhibited the best antifungal activity proving that the bioactive compounds are moderately polar. The antifungal activity surpassed that of miconazole, a commercial drug used to treat fungal infections. The order of antifungal activity was ethyl acetate extract>dichloromethane extract>ethanol extract>extract hexane for the strains *A. flavus*, *A. niger, P. italicum* and *F. oxisporum.* The MIC and MFC results showed clearly that ethyl acetate and dichloromethane extracts can be possible starting points for lead compound search for the development of antifungal agents. In their studies, Mandiga *et al.*[9] found out that dichloromethane extracts exhibited significant ant-proliferation and anti-inflammatory activity.

Phytochemical screening results showed that the ethyl acetate and dichloromethane consist of several phytocompounds including tannins, phenolics, flavonoids, carbohydrates, glycosides, saponins, alkaloids, terpenes and

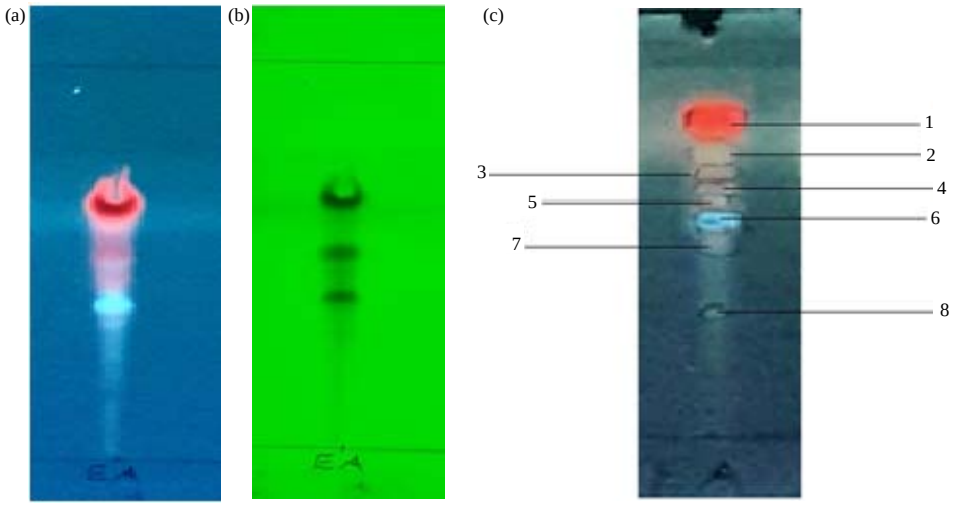

Fig. 2(a-c): Typical TLC chromatograms of the ethyl acetate extract visualized at wavelength, (a) 366 nm, (b) 254 nm and (c) 366 nm in antimony (III) chloride reagent

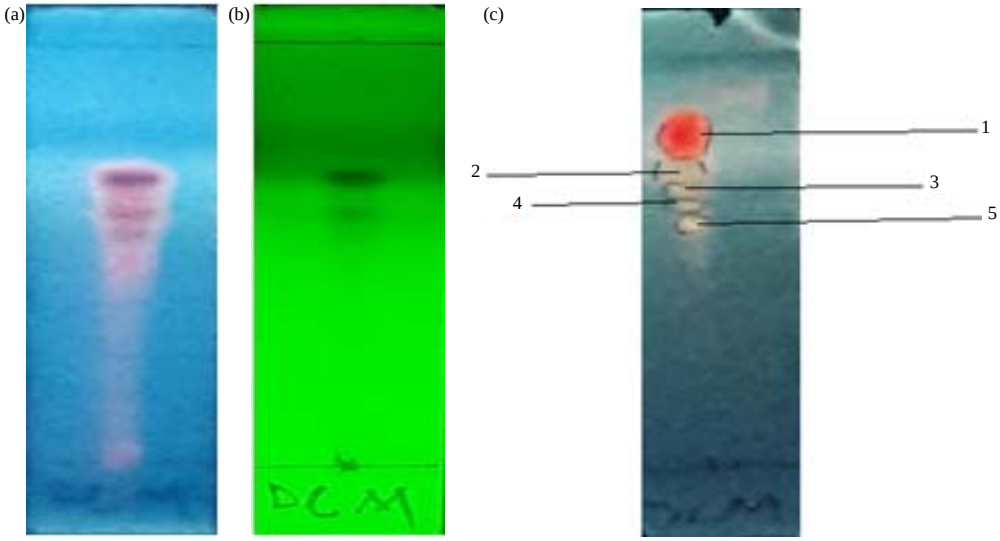

Fig. 3(a-b): Typical TLC chromatograms of dichloromethane extract visualized at wavelength, (a) 366 nm, (b) 254 nm and (c) 366 nm in antimony (III) chloride reagent

steroids. These have been reported before to have several health effects. Terpenes and steroids have been shown to consist of antifungal activities[24] while tannins exhibited wound healing capabilities[25]. Phenolics and flavonoids also exhibited antifungal and antibacterial activities[26].

CONCLUSION

This study revealed that *Dicerocaryum senecioides* extracts consist of significant antifungal activity and various phytocompound types. The bioactive phytocompounds are moderately polar. Thus the present results should give an impetus for fractionating the compounds such that the actual compounds responsible for the antifungal activity can be identified.

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