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DETERMINATION AND PHYTOCHEMICAL ANALYSIS OF ANTI-FUNGAL/RINGWORM/ ACTIVITY OF NEWLY GROWING STEM EXTRACT OF *Croton macrostachyus* (BISANA)

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ABSTRACT. Phytochemical analysis and antifungal activity of the newly growing bud of *Croton macrostachyus* were determined in this study. The study employed the maceration method for the extraction of bioactive compounds from the study plant material. The solvents used were methanol, ethyl acetate, chloroform, and petroleum ether. Phytochemical investigations were carried out to determine the presence of secondary metabolites by using standard procedures. The antifungal activity was tested against *Epidermophyton floccosum*. Among the solvents used, the chloroform extract showed prominent antifungal activity, with a zone of inhibition of 11.00 ± 0.17 mm at a 0.15g/0.5 mL concentration. This was slightly higher than the activity of the standard antifungal drug clotrimazole, which showed a zone of inhibition of 10.00 ± 0.10 mm, while the methanol and petroleum ether extracts remained completely inactive. This study contributes significantly to the current scientific understanding of *Croton macrostachyus* medicinal potential by offering a new perspective on the phytochemical makeup and possible therapeutic qualities of the plant. By identifying the active components responsible for its antifungal activity, it provides a scientific basis for the traditional use of this plant in Ethiopian medicine.

KEY WORDS: Antifungal activity, *Croton macrostachyus*, *Epidermophyton floccosum*, Traditional medicine, Phytochemical

INTRODUCTION

Traditional medicine is a conventional medical practice used in cultures before the development of current health sciences [1]. The evolution of modern health sciences depends on these ancient medicinal practices. It differs from scientific medicine and is largely transmitted orally by communities of different cultures. The information about traditional medicine comes from indigenous theories, beliefs, and experiences that are conserved from generation to generation [2]. Still now, the use of medicinal plants plays an important role in the daily lives of people living in developing and developed countries due to the great contribution of traditional practitioners [2-4]. It not only serves as a complement or substitute for modern medical treatments, which are often inadequately available, but also enhances the health and security of local people [3]. In addition to the many benefits, there are also risks associated with the different types of traditional medicine (TM). Although consumers today have widespread access to various TM treatments and therapies, they often do not have enough information on what to check when using TM in order to avoid unnecessary harm [5].

Over one-third of the population in developing countries lack access to essential medicines. Safe and effective traditional medicine (TM) therapies could thus become a critical tool for increasing access to health care [6]. Ethiopia has a long history of traditional medicine and has advanced methods to combat disease through it [3, 4].

Like other developing countries, in Ethiopia medicinal plants have been used as remedies, with around 95% derived from plants, and they have been used for many years and still represent

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the main therapeutic tool in traditional medicines due to the cultural acceptability of healers and local pharmacopeias, their low cost relative to modern medicines, and difficult access to modern health facilities [4, 7- 9].

Despite their existence and continued use in many countries, and their popularity and extensive use during the last decades, medicinal plants were not officially recognized in most countries [10].

Furthermore, the importance of medicinal plants and their derivatives has become enormous at a time when antibiotic resistance has become a global concern. In recent years, there has been an increasing incidence of multiple resistance in human pathogenic microorganisms, largely due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases [11].

Due to the fast spread of drug-resistant bacterial and fungal pathogens, the need for new drugs with less toxic effects is a highly demanding public interest. Therefore, intensive research activities are required to find alternative solutions against drug-resistant microorganisms. This needs evaluation of crude extracts and active constituents of traditionally used medicinal plants against human pathogens [5].

The plant *Croton macrostachyus* Hochst. (Euphorbiaceae) is a flowering plant, with nearly 1300 species of herbs, shrubs, and medium-sized deciduous trees [12]. The plant is widely used to treat many infectious diseases all over the world. Different parts of the plant were used for the treatment of malaria, rabies, evil eye, wounds, liver problems, ascariasis, tapeworm, gonorrhea, heart disease, ringworm, constipation, abdominal problems, and abortion [13]. In Benishanigul Gumiz, southwest of Ethiopia, we mostly observed that the Shinasha and Gumiz peoples (nations) use the juices or sap of fresh, newly growing stems for the treatment of ringworm (tinea corporis), an infectious fungal disease that appears on the face, neck, and any part of human skin. However, several hydroalcoholic *Croton macrostachyus* stem bark extracts against a clinical strain of Neisseria gonorrhoeae were reported with a minimum inhibitory concentration (MIC) of 125-250 mg/mL [14].

Reports on the antibacterial activity of methanol extracts from *Croton macrostachyus* leaf are mixed, with both negative and positive findings [15]. These contrasting results may be attributed to the plant's locality, the parts used, the time of collection, storage conditions, and analytical methods. Even though the majority of the Ethiopian population uses *Croton macrostachyus* to treat different diseases traditionally, only one study has been conducted regarding the antibacterial activity of Croton macrostachyus, and no study has been conducted on the antifungal activity of any part of *Croton macrostachyus*. With the above background information, the present study aimed at determining phytochemical analysis of various parts of the plant (leaf, newly growing stem, stem bark and root bark) and determination of antifungal activities of newly growing stem extract of *Croton macrostachyus* on selected test pathogenic fungal species, namely: *Epidermophyton floccosum* with respects to synthetic antifungal drugs (clotrimazole).

MATERIALS AND METHODS

Chemicals and reagents

Analytical grade of the following solvents was used for this study. Distilled water, organic solvents (hexane 98%, petroleum ether 80%, chloroform 99.5%, ethyl acetate 97%, acetone 99.5%, ethanol 97% and methanol 99%), appropriate media for fungi (Potato Dextrose Agar, PDA), 0.14 M DMSO (Dimethyl Sulfoxides), sulfuric acid 98%, clotrimazole, hydrochloric acid 37%, 1% of aqueous Iron (III) chloride ($FeCl_3$), 10 % ferric chloride solution ($FeCl_3$), potassium hydroxide pellet, sodium hydroxide, benzene, 10% ammonia solution, ammonium hydroxide (NH_4OH), Wagner's reagent (Iodine in potassium iodide). 2% copper sulfate and glacial acetic acid.

Instruments and apparatus

Grinding machine, electronic (analytical) balance, rotary evaporator, autoclave, fume hood, Thermostat incubator (DNP-9052-1A), micropipette, cutter, Petri dish, cork borer, forceps, funnel, and Whatman filter paper.

Collection of plant materials

Healthy aerial parts of the leaf, stem bark, and newly growing stem/bud/ and roots of *Croton macrostachyus* were collected in December 2023 on the hills of Assosa district, southwest of Ethiopia. The collected plant material was identified by Desalegn Ayele, MSc in Botany, from the Department of Biology, Assosa University.

Processing of plant materials

The collected plant materials were washed thoroughly with distilled water to remove any dust from the study plant materials. The washed plant material was shade-dried at room temperature immediately after collection for three weeks. This slow shade drying technique is a conventional approach used to reduce moisture content and prevent microbial growth. However, it may lead to partial loss or degradation of highly volatile organic compounds and certain heat- or light-sensitive phytochemicals. The overall effect is a complex balance between the preservation and degradation of volatile compounds. Then, the shaded, dried healthy plant parts were powdered using an electrical grinder (200 μm) and kept in a sealed container for extraction purposes.

Extraction of the plant materials

The most important components of the medicinal plants were isolated by extraction methods using the right solvents [3]. Therefore, the extraction of the plant material was carried out as follows. 25 g of each pulverized plant material was macerated in 250 mL of analytical grade methanol, ethyl acetate, chloroform, and petroleum ether in separate conical flasks for 48 hours with minimal shaking. Each extract was then filtered through Whatman filter paper. Filtrates of each solvent were separately concentrated using a vacuum rotary evaporator until they became completely dry. After the evaporation of solvents, the crude extracts were weighed and recorded to determine the percentage yield of each crude extract and stored for further chemical tests to identify their phytochemicals. This type of extraction method (non-exhaustive approach) was intentionally chosen because it is simple and easily reproducible and allows a relative comparison of extraction efficiency and bioactivity of different solvents under identical experimental conditions, especially when compared to prolonged exhaustive extraction methods used for preliminary phytochemical screening studies [16]. The percentage yield of the crude extracts was determined using the following formula [17].

$$\text{Percentage yield} = \frac{(\text{weight of final extract})}{(\text{weight of sample powder})} \times 100$$

Phytochemical screening tests

Phytochemicals present in plant material are becoming increasingly popular due to their extensive health and medicinal benefits [18]. Therefore, the crude methanol, ethyl acetate, chloroform, and petroleum ether extracts of leaf, stem bark, root, and newly growing stem bud of *Croton macrostachyus* were subjected to preliminary phytochemical testing to detect the presence/absence of different chemical groups of compounds such as flavonoids, steroids,

terpenoids, tannins, saponins, anthraquinones, alkaloids, glycosides, phlobatannins, coumarins, and emodins by using the standard methods of test.

Test for flavonoids: 2 mL of each extract was mixed with 2 mL of dilute (10%) sodium hydroxide in different test tubes. The change in color from an intense yellow color to colorless on addition of a few drops of dilute hydrochloric acid indicates the presence of flavonoids.

Test for steroids (Salkowski's test): A mass of 0.1 g of the test extract was dissolved in 5 mL of chloroform and filtered. A few drops of concentrated sulphuric acid were added to the filtrate, and the mixture was shaken and allowed to stand at room temperature (25-27 °C) for a few min. The formation of a red precipitate indicated the presence of steroids.

Test for terpenes: 2 mL of each crude extract was mixed with 2 mL of acetic anhydride. Carefully add 2-3 drops of concentrated H_2SO_4 to the mixture. The formation of a deep red coloration at the interface indicates the presence of terpenes.

Test for tannins: 2 mL of each crude extract was mixed with 2 mL of distilled water and heated in a water bath. The mixture was filtered, and 2-3 drops of 5% iron chloride ($FeCl_3$) solution were added drop by drop to the filtrate. The formation of a dark-green solution indicates the presence of tannins.

Test for saponins (Froth test): To 5 mL of each extract, 5 mL of distilled water was added in a test tube. Then, the solution was shaken vigorously for 10-15 minutes; stable froth indicates the presence of saponins.

Test for phenols: To 1 mL of the crude extract of a sample, 2 mL of distilled water followed by a few drops of 10% aqueous ferric chloride solution were added. The formation of a blue/green/blue-black color indicated the presence of phenols.

Test for phlobatannins (precipitate test): 2 mL of the crude extract was mixed with 2 mL of 1% hydrochloric acid (HCl), and the solution was warmed in a water bath for 10minutes. The formation of red precipitate indicates the presence of phlobatannins.

Test for glycosides: A mass of 500 mg of each extract or fraction was dissolved in 5 mL of distilled water. 2 mL of glacial acetic acid containing a few drops of ferric chloride was added to the filtrate. Add 1 mL of concentrated sulphuric acid along the side of the test tube. The formation of a brown ring at the interface or a violet color below the ring indicated the presence of glycosides.

Test for coumarins: 3 mL of 10% sodium hydroxide was added to 2 mL of aqueous crude extract; the appearance of a yellow color indicated the presence of coumarins.

Test for emodins: 2 mL of the crude extract was mixed with 2 mL of ammonium hydroxide, then 3 mL of benzene was added. The appearance of a red color indicates the presence of emodins.

Test for alkaloids (Wagner's Test): 5 mL of extract evaporated to dryness. The residue was heated in a boiling water bath with 2% HCl. Then filtered and treated with Mayer's reagent. The formation of a yellow precipitate indicates the presence of an alkaloid.

Test for anthraquinones: 3 mL of benzene was added to 3 mL of crude extract, followed by addition of 5 mL of 10% ammonia. The formation of violet color indicates the presence of anthraquinones.

Determination of minimum inhibitory concentration (MIC)

About 0.15 g of each crude extract from newly growing stem/bud was taken and dissolved in different volumes of 0.14 M (1% v/v) DMSO (dimethyl sulfoxide) (0.5 mL, 1 mL, and 2 mL) in separate test tubes to prepare samples with varying concentrations for the determination of the minimum inhibitory concentration of the extract. The solutions were cooled before use. The lowest extract concentration that inhibited growth of the fungal test pathogen was considered the Minimum inhibitory concentration [19]. Similarly, the minimum inhibition zone was compared with the same concentrations (0.15 g/0.5 mL, 0.15 g/1 mL and 0.15 g/2 mL) of the synthetic antifungal drug clotrimazole.

Antifungal activity test for the extracts

Test pathogens

The test fungal pathogens were obtained from Alert Hospital, Addis Ababa. The fungal cultures were subcultured and maintained using Potato Dextrose Agar (PDA) and incubated at 27 ^{o}C.

Microorganisms and inoculum preparation

This study used *Epidermophyton floccosum*, the causative agent of the fungal disease tinea corporis. Potato dextrose agar (PDA) medium was prepared by dissolving a given amount of PDA powder in distilled water according to the manufacturer's instructions. The mixture was heated by stirring until all components were dissolved. To ensure sterility, all necessary materials (Petri dish, cork borer, forceps, cotton swab, micropipette) and the agar mixture were autoclaved at 121 °C and 15 psi for 15 minutes before use. After autoclaving, the media were cooled to an appropriate temperature before use in a fume hood. Then approximately 45-50 mL of the solution was carefully transferred into each of the eleven (11, for positive control and untreated) sterile Petri dishes and left to solidify.

Determination of antifungal activity

The antifungal properties of the crude extracts against *Epidermophyton floccosum* were tested using the disc diffusion method. A small portion of the newly growing *Epidermophyton floccosum* culture was carefully cut and transferred onto the PDA plate with sterile forceps. It was then evenly spread across the surface with a cotton swab to ensure uniform distribution, then gently pressed into the agar with a cork borer. We pipetted 20 μL of each crude extract sample at different concentrations, along with the positive control, clotrimazole, at the same concentrations as the extracts, onto discs and allowed them to dry for 15 minutes in a fume hood. The plates were carefully inverted and incubated at 27 °C for 5-14 days in a thermostat incubator (DNP-9052-1A) to determine zones of inhibition of the tested fungal pathogen. We prepared all plates in triplicate and reported data as means ± SD using R. Significance levels were also defined as $p < 0.001$. Statistical analyses were performed using appropriate software. Graphical representations were generated using the ggplot2 package in [20].

We monitored the development of *Epidermophyton floccosum* on the PDA plate over time. This involved examining colony morphology and measuring the inhibition zone against the tested pathogen and the positive control. We measured the zone of inhibition with a ruler on the underside of the plate without opening the lid, collected statistical data in triplicate for each treatment, and recorded the average inhibition zone. The extracts with the largest zones of inhibition were compared with the standard positive control, clotrimazole.

RESULTS AND DISCUSSION

Plants play a vital role in Ethiopian traditional medicine. Research on medicinal plants has been increasingly recognized both in Ethiopia and internationally. This study conducted qualitative phytochemical analysis and assessed the antifungal properties of four extracts (methanol, ethyl acetate, chloroform, and petroleum ether) from the leaves, new growth, buds, bark, and roots of *Croton macrostachyus*.

Percentage yield of plant extracts

The results clearly show that solvent polarity significantly affects the amount of crude extract obtained and the percentage yield. Polar solvents produce more product than less polar or non-polar solvents when used with plant materials, as shown in Table 1. The highest extraction yield was obtained with methanol solvent, which yielded 15.90% for leaf, 12.18% for newly growing buds, 10.50% for bark, and 10.60% for roots, and the less polar solvent ethyl acetate, which resulted in 13.43% for leaf, 9.00% for newly growing buds, 5.27 % for bark, and 5.50 % for roots. However, chloroform solvent extracts yield 10.30% for leaf, 8.94% for newly growing buds, 6.60% for bark, and 6.10% for roots. Finally, the lowest extraction yield was obtained with the non-polar solvent petroleum ether, with percentage yields of 8.92%, 7.65%, 4.71%, and 4.80% from leaf, newly growing bud, bark, and root, respectively. The results of our study clearly indicate that using methanol for extraction yields more product than other solvents.

Table 1. Percentage yield of crude extract from each plant material with different solvents.

No	Plant material	Sample weight (g)	Type of solvent	Amount of solvent (mL)	Amount of crude extract obtained (g)	Percentage yield (%)
1	Leaf	25	Methanol	250	3.975	15.90
	Leaf	25	Ethyl acetate	250	3.358	13.43
	Leaf	25	Chloroform	250	2.575	10.30
	Leaf	25	Petroleum ether	250	2.231	8.92
2	Newly growing bud	25	Methanol	250	3.045	12.18
	Newly growing bud	25	Ethyl acetate	250	2.251	9.00
	Newly growing bud	25	Chloroform	250	2.234	8.94
	Newly growing bud	25	Petroleum ether	250	1.912	7.65
3	Bark	25	Methanol	250	2.625	10.50
	Bark	25	Ethyl acetate	250	1.327	5.27
	Bark	25	Chloroform	250	1.651	6.60
	Bark	25	Petroleum ether	250	1.178	4.71
4	Root	25	Methanol	250	2.650	10.60
	Root	25	Ethyl acetate	250	1.375	5.50
	Root	25	Chloroform	250	1.525	6.10
	Root	25	Petroleum ether	250	1.201	4.80

Phytochemical screening test

The result of the preliminary phytochemical screening test was conducted to analyze the presence of different secondary bioactive compounds in different parts of C*roton macrostachyus* (leaf, bark, root, and bud) using different solvents (PE = petroleum ether, CF = Chloroform, EA = ethyl acetate, and Me = Methanol). The results are represented using symbols where "+++" indicates the highest amount, "++" indicates a high amount, "+" indicates a moderate amount, and "-" indicates absence as shown in the Table 2.

Table 2. Preliminary phytochemical analysis of leaf, bark, root and newly growing bud of *Croton macrostachyus.*

No	Type of Tests	Leaf				Bark				Root				Bud			
		PE	CF	EA	Me	PE	CF	EA	Me	PE	CF	EA	Me	PE	CF	EA	Me
1	Flavonoid	-	+	-	-	+++	++	-	-	+++	+++	+	-	-	+	-	-
2	Steroids	-	-	-	-	+++	++	+++	-	+++	++	+++	-	+++	++	+++	-
3	Terpenoids	-	-	-	-	++	+	-	+	++	++	+++	+++	+	-	-	+
4	Tannins	++	+++	++	+++	-	+		++	-	-	-	-	-	+	++	-
5	Saponins	-	-	-	+	++	-	+	+	+	-	+	-	+	+	++	-
6	Phenols	-	-	+	++	-	-	-	+	-	-	-	-	-	+++	++	-
7	Phlobatanins	-	-	-	-	-	-	-	-	-	-	+	-	-	+	++	-
8	Glycosides	-	+++	++	++	-	+	+	-	-	-	-	-	+	++	+++	+++
9	Coumarins	+++	-	-	+	+	-	++	+	-	-	+	-	++	-	+	+++
10	Emodins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	Alkaloids	++	-	++	-	++	-	++	-	+++	-	-	-	-	-	+	-
12	Anthraquinones	-	-	-	-	-	+	+	++	-	+	-	++	-	+	-	-

(+++) present in highest amount; (++) present in high amount; (+) present in moderate amount and (-) absent.

Upon analyzing the data, several observations can be made. The abovTable 2 shows that (**1**) Flavonoids were present in the highest amount in the stem bark and root when using petroleum ether (PE) and chloroform (CF), and in moderate amounts in the bud when using chloroform and in the root with ethyl acetate. However, the phytochemical screening test shows that flavonoids are absent in the leaf, bark, root, and newly growing bud when using methanol (Me) solvent. (**2**) Steroids are present in high amounts in the bark, root, and bud when using petroleum ether (PE), chloroform (CF), and ethyl acetate (EA), but are absent in all parts of the plant when using methanol. In addition, the phytochemical screening test shows the absence of steroids in the leaf with all the used solvents. (**3**) Terpenoids are absent in the leaf and bud when using chloroform (CF), ethyl acetate (EA), and methanol (Me) as solvents. However, they are present in moderate to high amounts in the bark and root when using different solvents, with the highest presence observed in the root with methanol (Me) and ethyl acetate (EA). (**4**) Tannins are present in high amounts in the leaf and absent in the root when using all types of solvents. However, tannins are present in moderate amounts in the bud with ethyl acetate (EA) and chloroform (CF). (**5**) Saponins are present in moderate amounts in the bark and bud when using petroleum ether (PE) and Ethyl acetate (EA), respectively. (**6**) Phenols are absent in the leaf and bark when using petroleum ether (PE) and chloroform (CF) as solvents. On the other hand, the preliminary phytochemical screening test shows phenolic compounds are present in high amounts in the bud when using ethyl acetate (EA) and chloroform (CF) as solvents. (**7**) Phlobatanins are absent in all parts of the plant when using petroleum ether (PE) and methanol (Me) as solvents. However, they are present in high amounts in the bud when using ethyl acetate (EA) and in moderate amounts when using Chloroform (CF) as a solvent. (**8**) Glycosides are absent in the root when using all solvents, but present in high amounts in the leaf and bud when using chloroform (CF), ethyl acetate (EA), and methanol (Me) as solvents. (**9**) Coumarins are present in high amounts in the leaf and bud when using petroleum ether (PE) solvent. They are also present in moderate to high amounts in the bark and bud when using ethyl acetate (EA) and methanol (Me) as solvents. (**10**) Emodins are absent in all parts of the plant when using petroleum ether (PE), chloroform (CF), ethyl acetate (EA), and methanol (Me) as solvents. (**11**) Alkaloids are present in varying amounts in the leaf, root, and bud when using petroleum ether (PE) and ethyl acetate (EA) as solvents, while they are absent in the bark. Additionally, Alkaloids are present in moderate amounts in the bark and bud when using chloroform (CF) as a solvent, and are absent in all parts when using methanol (Me) as a solvent. (**12**) Anthraquinones are present in high amounts in stem bark and root when using

methanol (Me) as a solvent, while in moderate amounts in bark, root, and bud when using chloroform (CF) as a solvent. However, they are absent in the leaf when using petroleum ether (PE), chloroform (CF), ethyl acetate (EA), and methanol (Me) as solvents.

Minimum inhibitory concentration (MIC) of the crude extracts

The effectiveness of the antifungal activity of the extracts was assessed using the minimum inhibitory concentration (MIC) assay. Figure 1 below shows the antifungal zone of inhibition against *Epidermophyton floccosum*.

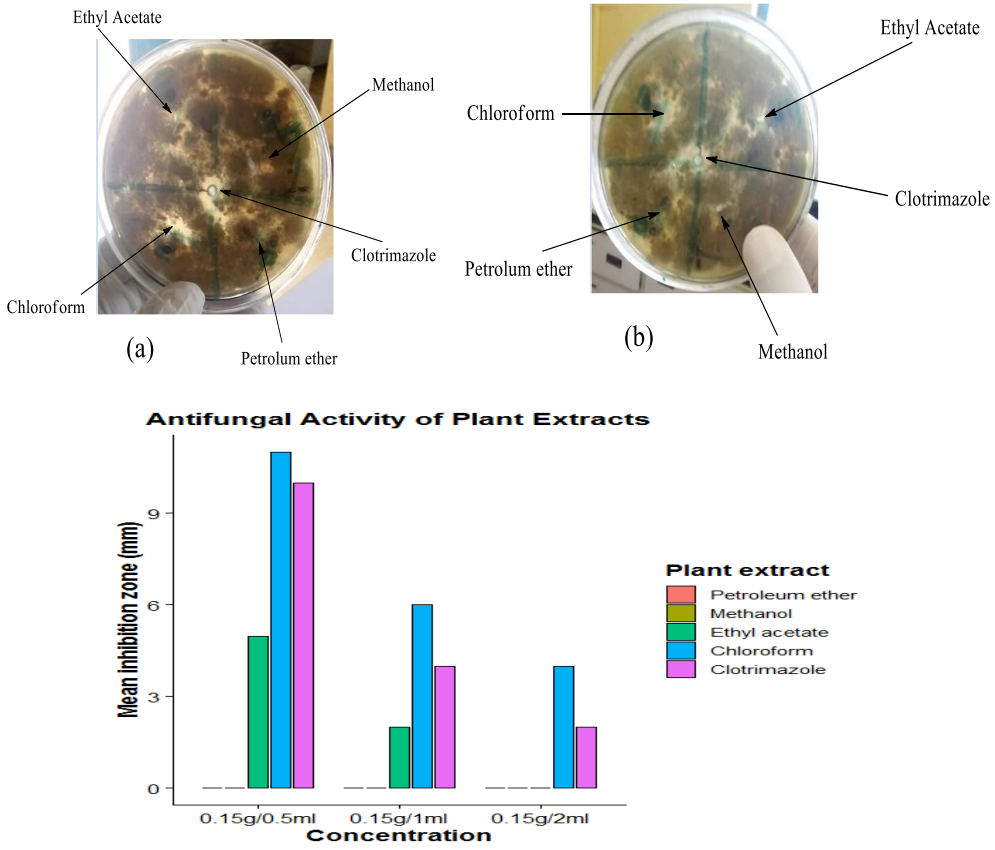


Figure 1. The average zone of inhibition (mm) for each extract of newly growing bud and the positive control clotrimazole at its higher concentration.

The crude extracts tested are methanol, ethyl acetate, chloroform, petroleum ether, and clotrimazole. The average zone of inhibition for each extract was measured in millimeters (mm) and represents the area around the extract where the growth of *Epidermophyton floccosum* is restricted.

Table 3. Mean, standard deviation (SD), and standard error (SE) of zone of inhibition.

Plant extract	Concentration	n	Mean (mm)	SD	SE
Petroleum ether	0.15 g/0.5 mL	3	0.00	0.000	0.000
Petroleum ether	0.15 g/1 mL	3	0.00	0.000	0.000
Petroleum ether	0.15 g/2 mL	3	0.00	0.000	0.000
Chloroform	0.15 g/0.5 mL	3	11.00	0.300	0.173
Chloroform	0.15 g/1 mL	3	6.00	0.100	0.058
Chloroform	0.15 g/2 mL	3	4.00	0.346	0.200
Clotrimazole	0.15 g/0.5 mL	3	10.00	0.173	0.100
Clotrimazole	0.15 g/1 mL	3	4.00	0.265	0.153
Clotrimazole	0.15 g/2 mL	3	2.00	0.100	0.058
Ethyl acetate	0.15 g/0.5 mL	3	4.97	0.115	0.067
Ethyl acetate	0.15 g/1 mL	3	2.00	0.265	0.153
Ethyl acetate	0.15 g/2 mL	3	0.00	0.000	0.000
Methanol	0.15 g/0.5 mL	3	0.00	0.000	0.000
Methanol	0.15 g/1 mL	3	0.00	0.000	0.000
Methanol	0.15 g/2 mL	3	0.00	0.000	0.000

The result obtained shows the impact of various substances on the growth of *Epidermophyton floccosum*, depending on their concentration. The data in Table 3 show a clear trend: higher concentrations mean stronger results for almost every crude extract. Chloroform extract shows a larger inhibition zone than the others and yields the largest zone of inhibition at 11.00 mm at 0.15 g/0.5 mL concentration. It even narrowly outperformed the positive control, Clotrimazole, which exhibited 10.00 mm at the same dose. The results suggest that chloroform shows a comparable average zone of inhibition against the tested pathogen as clotrimazole. This indicates that these substances are more potent against *Epidermophyton floccosum* at higher concentrations compared to other extracts. Ethyl acetate followed a similar path, though it flatlined at first and needed at least 1 mL to activate before peaking at 4.97 mm. On the other hand, petroleum ether and methanol are completely ineffective, staying at a flat 0.00 mm across the board. Because our standard deviation and error values are incredibly low, we can be confident that the three replicates ($n = 3$) yielded highly consistent, reliable, and precise data points.

Table 4. Two-way ANOVA of zone of inhibition.

Source of variation	df	Sum of squares	Mean square	F-value	p-value
Treatment	3	262.27	87.42	2538.1	< 0.001
Concentration	2	157.43	78.72	2285.3	< 0.001
Treatment × Concentration	6	62.03	10.34	300.2	< 0.001
Residual/Error	24	0.83	0.03	-	-

The two-way ANOVA data on table 4 confirms that both type of crude extract and amount of concentration along with the way they interact had a massive, highly significant impact on the results ($p < 0.001$). Treatment type was the biggest driver of differences in our data, followed closely by concentration level. Most importantly, the strong interaction effect gives us statistical proof that changing the dose doesn't affect every treatment the same way. While some extracts spiked dramatically at higher concentrations, others reacted differently or stayed flat.

Antifungal activity of the extract

The antifungal activities of the extract against fungal species were used to treat infections caused by fungi. The results of testing the antifungal activity of the crude extract from newly growing buds using different solvents (petroleum ether, chloroform, ethyl acetate, and methanol). The

antifungal activity of the petroleum ether, chloroform, ethyl acetate, and methanol crude extracts of *Croton macrostachyus* was determined against the ringworm species *Epidermophyton floccosum* using the disc diffusion method. The results show that a solvent extract containing a large amount of material doesn't mean it will be highly effective against fungus. Highly polar methanol yields about 12.18% from the newly growing buds, but it mostly contains inactive structural compounds that don't have any antifungal activity. On the other hand, the moderately polar chloroform extract was much more selective. It successfully concentrated the plant's active compounds, specifically phenolic compounds, flavonoids, and anthraquinones, which are the basic components behind fighting off *Epidermophyton floccosum*. In fact, this specific chloroform bud extract was so potent that it created an 11.00 ± 0.17 mm zone of inhibition, actually overtaking the standard drug, Clotrimazole.

These active phytochemicals exert their anti-dermatophytic effects through different sides: lipophilic phenolic groups alter fungal cell membrane permeability to induce cell lysis, flavonoids, on the other hand, inhibit key metabolic enzymes to prevent cell wall growth, and anthraquinones, on the other hand, create intracellular oxidative stress (ROS) through reactive oxygen species production. These powerful antifungal properties concentrated in the moderately polar (chloroform) fraction fit perfectly with what scientists see across the *Croton* family [21]. While previous *Croton macrostachyus* studies focused mostly on leaf or stem bark profiles, targeting the newly growing bud reveals it as a specialized protective metabolic bio-factory. Ultimately, this collaboration of compounds provides the exact scientific proof that validates why this plant has been trusted in traditional Ethiopian medicine for centuries. However, it is better to isolate and determine the amounts of the individual components to exactly identify the active compounds responsible for this antifungal activity.

CONCLUSION

People frequently use the fresh, newly growing buds of *Croton macrostachyus*, locally known as *Bisana*, as traditional medicine to treat ringworm infections. To evaluate the scientific basis of this practice, we conducted preliminary phytochemical screenings to assess the presence or absence of bioactive compounds across different plant parts: the newly growing buds, leaves, stem bark, and root bark. Since the extraction of these bioactive components depends mainly on solvent polarity, we treated the plant materials using four solvents: petroleum ether, chloroform, ethyl acetate, and methanol. The polarity of the solvent has a direct relation with mass recovery in *Croton macrostachyus*, where the highly polar methanol revealed the highest crude extract in all plant parts, in leaf (15.90% (3.975g) at 25 g/250 mL, followed by bud (12.18%), root (10.60%), and bark (10.50%). In contrast, the non-polar petroleum ether was the least effective, achieving only 4.71% in the bark. Intermediate solvents were moderately successful, with chloroform giving better results than ethyl acetate in the extraction of stem bark (6.60% vs. 5.27%). Across the different plant parts, these screenings revealed a rich diversity of secondary metabolites, including terpenoids, flavonoids, saponins, tannins, alkaloids, phenols, anthraquinones, and sterols, which are widely used for their antimicrobial properties. While the newly growing bud's chloroform extract didn't yield as much as the inactive methanol extract, it was relatively rich in phenolics, flavonoids, and anthraquinones. In an antimicrobial test against *Epidermophyton floccosum*, chloroform extract exhibited a clear, concentration-dependent antifungal activity that validates its traditional application. Despite the lower yield produced than the completely inactive methanol extract, chloroform overtook all variants. It achieved a potent maximum inhibition zone of 11.00 ± 0.17 mm at 0.15g/0.5 mL, remarkably exceeding the standard drug Clotrimazole (10.00 ± 0.10 mm). The extracts showed antifungal activity, but the specific bioactive compounds that are responsible for this activity were not determined in the present study. Therefore, further studies with freshly prepared extracts, quantitative phytochemical analysis of total phenolic content and total flavonoid content of the crude chloroform extract, and GC-MS/LC-MS-based

characterization are needed to confirm the compounds responsible for the revealed antifungal activities.

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