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Phytochemical Screening, Phytotoxic and Antimicrobial Prospective of Rangoon Creeper (*Combretum indicum* L.) Against Known Plants Bacterial and Fungal Pathogens

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Abstract. Currently, the world is facing a very serious issue of antibiotic resistance, the scientist is trying to develop new techniques and advance drug to compete with them. For this purpose, drug production from medicinal plants is a top trending act around the world. Keeping in view this idea the crude ethanolic extract and fractions of ethyl acetate and chloroform were screened to assess the preliminary phytochemical, phytotoxic and antimicrobial potential of leaves of *Combretum indicum* and also the same activities were carried out for the essential oil of *Combretum indicum*. The presence of various secondary metabolites in terms of alkaloids, proteins, carbohydrates, phenols, saponins, flavonoids, tannins and steroids determined through phytochemical screening. A comparison of the results were made with standard nystatin against *Alternaria alternate, Aspergillus flavus, Polysphondylium pallidum* and *Fusarium oxysporum* and tetracycline against *Staphylococcus aureus, Streptococcus mutans* and *Methicillin-resistant* and *Staphylococcus aureus* (*MRSA*) and *Serratia marcescens*. The highest antibacterial activity among leaves extracts was shown by ethyl acetate with an 18.33 mm zone of inhibition at a concentration of 15 µg/mL of inhibition and the highest activity by of essential oil was obtained with 14.67 mm zone of inhibition at a concentration of 20 µg/mL, while among the ethanolic leaves extracts and fraction of essential oil the highest antifungal activity was shown at 80 µg/mL each, with inhibition zones 12.67 mm and 13.33 mm, respectively. An efficient phytotoxic effect was shown by extracts of both leaves and essential oil against *Lemna minor* at 2000 µg/mL, while their activity was low at 20 µg/mL. Ethyl acetate leaves extracts showed the highest activity with 70.21% inhibition. A fraction of essential oil showed significant activity with 57.44% inhibition. The tested medicinal plant thus proved by the current research can be used to cure many diseases.

Keywords: antibacterial, antifungal, ethanolic extract, phytotoxic, phytochemical

Introduction

Medicinal plants can be defined as plants that possess therapeutic properties or extract beneficial pharmacological effects on the human or animal body. These medicinal plants play a very basic role in the development of various formulations. According to one report by WHO, 80% of the world's population is using medicinal plants for curing various ailments (Hu *et al*., 2020, Uzun and Koca, 2020). Throughout the world, many death cases are reported because of infectious diseases (Vincent and Taccone, 2020). Antibiotics are powerful medicines that fight certain infections and can save lives when used properly. They either stop bacteria and fungi from reproducing or destroy them (Hutchings *et al*., 2019). Scientists are trying to develop new and strong medicines but antibiotic resistant becomes a very critical and global issue nowadays. New and surprising types of multidrug resistant pathogens are developing day by day because of various activities due to which the clinical efficiency of many antibiotics is being threatened (Shahrour *et al*., 2021; Pilmis *et al*., 2020). From a very old age, human beings are using herbal remedies for the treatment of various infectious diseases (Ghany *et al*., 2020). Infectious diseases are worrying because we need to discover the new, novel mechanism of action and having diverse chemical structures antimicrobial compounds continuously (Álvarez-Martínez *et al*., 2020). Diverse chemical compounds produced by higher plants have a biological and therapeutical role (Aamir *et al*., 2020). Different plants and human pathogens can be treated by those antimicrobial compounds which are obtained

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from different plant extracts (Manandhar *et al*., 2019; Wolde *et al*., 2018; Subramani *et al*., 2017). In many countries all over the world especially in developing countries the mortality and morbidity are caused by infectious diseases in a population. There are certain bacteria reported in research that can resist the routinely used drugs available in markets (Ikwap *et al*., 2021; Tariq *et al*., 2019). The development of multi-resistance by micro-organisms against antimicrobial drugs has striving pharmaceutical companies to develop new techniques and prepare solid antimicrobial drugs in recent years by altering the chemical molecular skeleton of prevailing prescriptions to make them more competent. Generally, most skin diseases are caused by bacterial and fungal infections in developing countries reported by Bandara and Samaranayake (2019) and Orchard and van Vuuren (2017). To control these skin diseases most synthetic chemicals are used which are not eco-friendly and are also causing many environmental issues. Therefore, to overcome such dis-advantages it is important to develop more effective and less toxic novel, antimicrobial agents. Similarly, the reduction of crop yield due to observation of weeds is common in all countries including Pakistan(Hussain *et al*., 2017). Instead of other insects and diseases, more losses occurred because of weeds. Along with the decrease in productivity, weeds may also provide habitat for all those insects causing crop destruction.

Several human health problems and soil water pollution would be caused by syntheticdrugs, use to control weeds (Raven and Wagner, 2021). This necessitates the control of weeds through harmless means to enhance the yield of many crops and to protect the environment. The present study has been conducted on *Combretum indicum* L. to check the different phytochemicals also the phytotoxic, antibacterial and anti-fungal potential of various fractions of leaf and essential oil of this plant against selected strains of bacterial and fungal pathogens to detect new sources of antimicrobial agents.

Materials and Method

Collection. The fresh leaves of *Combretum indicum* were collected from the botanical garden of Islamia College University of Peshawar in April 2020. The leaves were cleaned and washed carefully to remove all the impurities and dust particles properly and were kept in shade for complete drying. The dried leaves were ground separately in an electric grinder. Also, the essential oil of the *Combretum indicum* was taken and different pharmacological activities on essential oil and leaves powder were carried out which is shown in Fig. 1-3.

Preparation of crude extract. *Crude extract.* The grounded leaves about 100 g were mixed in ethanol and kept for two days to get the crude ethanolic extract. Further from the crude extract, some fractions were

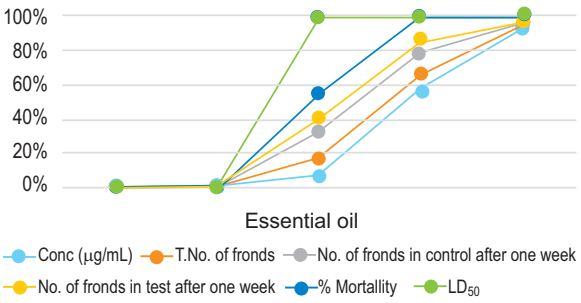

Fig. 1. Antibacterial activity of leaves extracts of *Combretum indicum* L.

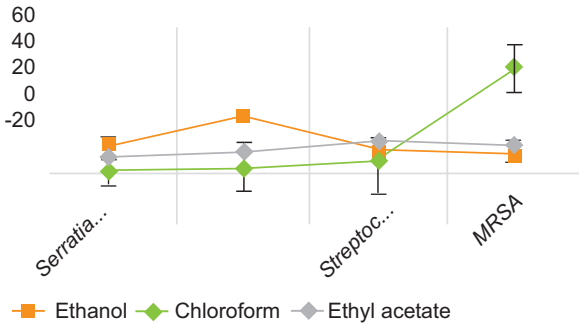

Fig. 2. LD_{50} of antibacterial activity of *Combretum indicum* L.

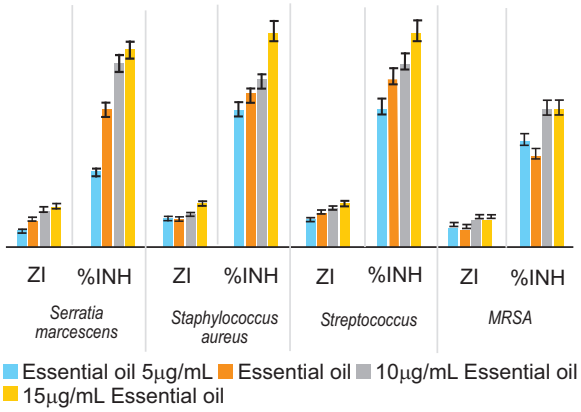

Fig. 3. Antibacterial activity of essential oil of *Combretum indicum* L.

made using solvents like chloroform, ethyl acetate and water and over a water bath, the fractions were concentrated by boiling at 60 °C. These extracts were used for different types of activities (Siddique *et al*., 2020).

Preliminary phytochemical screening. Various qualitative chemical tests are performed for the determination of the presence of various primary and secondary metabolites like carbohydrates, proteins, phenols, flavonoids, tannins, saponin and steroidal glycosides in extracts of both the leaves and for essential oil of *Combretum indicum* (Bhardwaj and Dubey, 2019).

Carbohydrate detection test. *Benedict test*. The addition of few drops of Benedict's reagent with extract solution and then its boiling on water bath resulted in the appearance of reddish-brown precipitates which indicated the presence of reducing sugar (Shaikh and Patil, 2020).

Fehling's test. Few drops of sample solution were added with Fehling's A and Fehling's B of equal volume and boiled. A brick-red precipitate indicated the presence of sugar.

Proteins detection test. *Millon's test*. The respective extract was treated with 2 mL of Millon's reagent which based on the appearance of whiteprecipitate the detection of the presence or absence of proteins was made and also might turn the white precipitate to red upon gentle heating or not (Koroma and Kamara, 2020).

Alkaloid detection test. *Mayer's test*. Mayer's reagent was mixed with the sample solution dropwise. The reddish-brown precipitate's appearance indicated alkaloids (Karmakar *et al*., 2020).

Phenol detection test. *Ferric chloride test*. The addition of both extract solution and ferric chloride solution ($FeCl_3$) in a test tube at a concentration of 2 mL each, brought deep bluish-green solution formation which represents the presence of phenols (Shafiq *et al*., 2021).

Flavonoid detection test. *Shinoda test (magnesium hydrochloride reduction test*). Few fragments of Mg ribbon and concentrated HCl were added to the extract solution dropwise. The indication of flavonoids presence was done by the appearance of pink scarlet, crimson red, orgreen to blue colour, after few minutes.

Tannins detection test. *Ferric chloride test*. An extract solution was treated with $FeCl_3$. The blue-green colouration gave detection of the presence of tannins (Al-Kaf *et al*., 2019).

Saponin detection test. *Frothing test*. In a test tube, the vigorously shaking of 5 mL extract with water would produce froth. The saponin's presence is detected due to the persistence of froth formation reported by (Liwata *et al*., 2020).

Steroidal glycosides test. *Killer killani test*. A glacial acetic acid was mixed with 2 mL of extract, followed by the addition of concentrated H_2SO_4 and a drop of $FeCl_3$. The presence of steroidal glycosides is detected by the appearance of bluish-green colouration in the upper layer and reddish-brown colour formation at the junction of two liquid layers (Chintamani *et al*., 2020).

Antibacterial activity. The agar disc diffusion method was followed for the analysis of the antibacterial activity of fractions of *Combretum indicum* leaves and essential oil against *Staphylococcus aureus, Streptococcus mutans* and *Methicillin-resistant, Staphylococcus aureus* (*MRSA*) and *Serratia marcescens* (Chandra *et al*., 2020).

Sample preparation. DMSO at a concentration of 2 mL was added to 6 mg of each extract for the preparation of the stock solution and four different concentrations *i.e*. 5 µL, 10 µL, 15 µL and 20 µl were then taken from this stock solution (Kosciuk *et al*., 2020).

Determination of antibacterial activity. The determination of the antibacterial activity of the extracts was carried out in Petri dishes under consideration of the disc diffusion method. The paper discs of about 6 mm in diameter were prepared and for each test microorganism, the discs were impregnated separately with 5, 10, 15 and 20 µL of the sample solution. The Petriplates were then incubated for 24 h at 37 °C. The results were compared with standard tetracycline and ampicillin (Okla *et al*., 2021).

Antifungal activity. The antifungal activity of fractions of *Combretum indicum* leaves and essential oil was carried out against *Alternaria alternate, Aspergillus flavus, Polysphondylium pallidum* and *Fusarium oxysporum* by the following agar well method.

Sample preparation. The stock solution was formed by dissolving 10 mg of each extract in 2 mL of DMSO and four different concentrations *i.e*. 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL of this stock solution were selected by taking 20 µL, 40 µL, 60 µL and 80 µL, respectively.

Determination of antifungal activity. The determination of the extract's antifungal activity was done by the

agar well method. For medium preparation, PDA (potato dextrose agar) at a concentration of 39 g was dissolved in 1000 mL of distilling water. This medium along with the selected test micro-organisms was inoculated separately in Petri dishes. Through sterile borer, wells were formed and for each test micro-organism, the wells were filled with the stock solution at different concentrations like 20 µL,40 µL, 60 µL and 80 µL. For about 48 h the Petri dishes were placed in an incubator and by inhibition zone (mm) measurement for the test organisms, the assessment of antifungal activity was done. Both the results and standard nystatin were do compared with each other (Ahmad *et al*., 2018).

Phytotoxicity. The standard procedure was employed to evaluate the phytotoxic activity of ethyl acetate, chloroform, ethanolic and aqueous extracts of leaves and essential oil of *Combretum indicum* against *Lemna minor* as shown in Fig. 4-5.

Sample preparation. The stock solution was formed by dissolving 20 mg of each extraction of 2 mL of methanol. From this stock solution three different concentrations *i.e*. 10 µg/mL, 100 µg/mL and 1000 µg/mL were prepared and in each petri dishes 5, 50 and 500 µL were taken separately from stock solutions.

Procedure. In each petri dish, 20 mL of the medium was separately added and after that 10 *Lemna minor* plants, each having 2-3 fronds were shifted to each petri dish and were then kept at room temperature for 10 days. A comparison of the results was made with control on day 10 after counting the fronds (Sharma *et al*., 2015).

Results and Discussion

During the current research, the screening of leaves and essential oil of *Combretum indicum* was carried out for bioassays, due to which its therapeutic values are determined (Fig. 6).

Qualitative phytochemical screening. Preliminary phytochemical screening is useful in finding the nature of drugs. Different phytochemicals like tannins, alkaloids, saponins, proteins, carbohydrates, flavonoids, phenols and steroids shown in (Table 1.) which have been analyzed in leaves and essential oil of *Combretum indicum* through phytochemical screening. (Agarwal *et al*., 2017; Lim, 2014) carried out research for the phytochemical screening of *Combretum indicum* L. extracts that reported similar results.

Antibacterial activity of leave extracts. The results of antibacterial activity obtained in the present research through screening of leaves and essential oil extracts are shown in Tables (2 and 3) separately, based on the presence or absence ofa zone of inhibition.

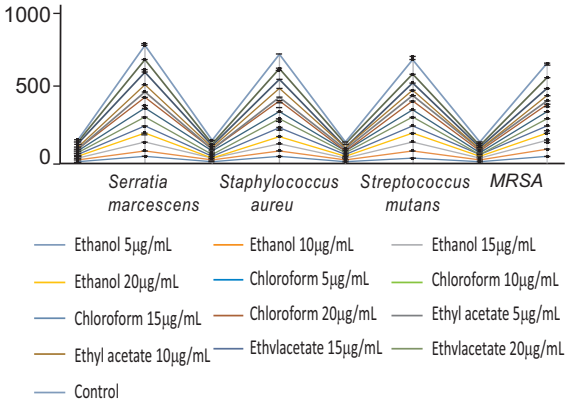

Fig. 5. Antifungal activity of leaves extracts of *Combretum indicum* L.

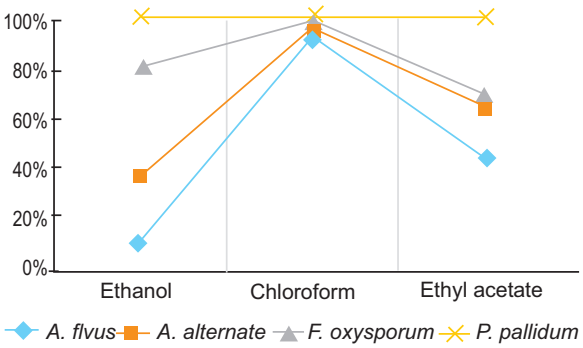

Fig. 4. LD_{50} of antifungal activity of leaves extract of *Combretum indicum* L.

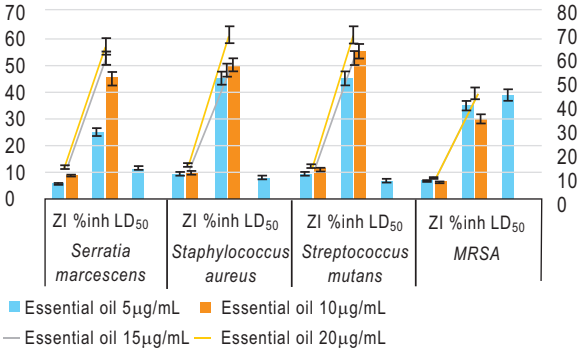

Fig. 6. Antifungal activity of essential oil of *Cambretum indicum* L.

It was revealed from the data analysis that the ethyl acetate fraction of leaves showed the highest activity against *Serratia marcescens* with 80% inhibition, 70% against *Staphylococcus aureus* and *Streptococcus mutans*. Chloroform fraction showed 65% inhibition against *Serratia marcescens* and *Staphylococcus aureus*. Ethanol fraction showed inhibition of 65% against *MRSA* (Table 2). The present result agreed with (Bhuiya, 2020) reported that *Combretum indicum* is highly antimicrobial and shows high antibacterial activity. The antibacterial results were compared with the phytochemicals of the plant that showed the antifungal activity because the plant might have secondary metabolites (alkaloids, flavonoids, tannins and phenols) which are responsible for the activity.

Table 1. Phytochemical screening of *Cambretum indicum* L.

Constituents	Tests name	Results	
		Leave	Essential oil
Carbohydrates	Benedict' test	+	+
	Fehling's test	+	+
Proteins	Millon's test	+	+
Phenols	Ferric chloride test	+	+
Flavanoids	Shinoda test	-	+
Tannins	Ferric chloride test	+	+
Alkaloids	Mayer's test	+	+
Saponins	Frothing test	+	+
Steroids	Killaerkilani test	-	+

Effect of essential oil. Essential oils are concentrated plant extracts that retain the natural smell and flavour, or "essence," of their source. Essential oils are most commonly used in the practice of aromatherapy, in which they are inhaled through various methods (Chouhan *et al*., 2017).

Table 2. Antibacterial activity of extract of *Combretum indicum* L.

Treatment	Conc.	Test species											
		Serratia marcescens			*Staphylococcus aureus*			*Streptococcus mutans*			*MRSA*		
		ZI	% inh	LD_{50}	ZI	% inh	LD_{50}	ZI	% inh	LD_{50}	ZI	% inh	LD_{50}
Ethanol	5µg/mL	8.67	40	11.08	8.66	40	24.08	7.6	35	10.48	9.66	45	8.72
	10µg/mL	9.33	45		9.33	45		9	45		10.66	50	
	15µg/mL	11.33	55		9.33	45		12	60		11.66	55	
	20µg/mL	12.33	60		10.66	50		13.66	65		12.33	60	
Chloroform	5µg/mL	11	55	2.68	11.66	55	2.37	10	50	5.07	9	45	43.56
	10µg/mL	12	60		12.66	60		11	55		9.33	45	
	15µg/mL	13	65		12.3	60		11	55		9.33	45	
	20µg/mL	13	65		13	65		12	60		10.66	50	
Ethyl acetate	5µg/mL	8.23	40	7.13	6.66	30	9.23	7.66	35	13.55	6.66	30	2.91
	0µg/mL	10.66	50		12.33	60		8.03	40		8.66	40	
	15µg/mL	18.33	90		12.33	60		9.33	45		9.33	45	
	20µg/mL	16.33	80		14.24	70		13.33	65		14.06	70	

ZI = (Zone of inhibition); LD_{50} = (Lethal dose 50).

Table 3. Antibacterial activity of essential oil *Combretum indicum* L.

Treatment	Conc.	Test species											
		Serratia marcescens			*Staphylococcus aureus*			*Streptococcus mutans*			*MRSA*		
		ZI	% inh	LD_{50}	ZI	% inh	LD_{50}	ZI	% inh	LD_{50}	ZI	% inh	LD_{50}
Essential oil	5µg/mL	5.67	25	11.64	9.67	45	8.08	9.67	45	7.09	7	35	38.79
	10µg/mL	9	45		10	50		11.33	55		6.33	30	
	15µg/mL	12.33	60		11	55		12.67	60		9	45	
	20µg/mL	13.67	65		14.67	70		14.67	70		9	45	

ZI = (Zone of inhibition); LD_{50} = (Lethal dose 50).

It was revealed from the data analysis that essential oil showed the best antibacterial activity against *Staphylococcus aureus* and *Streptococcus mutans* with inhibition of 70%, followed by *Serratia marcescens* with inhibition of 65 and *MRSA* with inhibition of 45 (Table 3). The present results were agreed with the findings of (Bhuiya, 2020) reported comparable results to that of essential oil.

Antifungal activity. The results of the antifungal activity of both leaves and essential oil are presented in Tables (4 and5) respectively.

Antifungal effect of leaves extracts. High antifungal activity was shown by an ethanolic fraction with inhibition of 97.72% against *A. alternate*, 92.30% against *F. oxysporum*, 85.71% against *A.flavus* and 66.66% against *P. pallidum*. Chloroform fraction showed 84.61% inhibition against *F. oxysporum*, 80% against *A.flavus*, 66.66% against *P. pallidum* and 62.50% against *A. alternate*. Ethyl acetate fraction showed the highest inhibition against *A. flavus* with 92.30% inhibition, 91.66% against *P. pallidum*, 90% against *F. oxysporum* and 64.28% against *A. alternate* (Table 4). The present results were similar to the finding of (Shah *et al*., 2017) reported antimicrobial activity of *Combretum indicum*. The antifungal results were compared with the phytochemicals of the plant that showed the antifungal activity because the plant might have secondary metabolites (alkaloids, flavonoids, tannins and phenols) which are responsible for the activity.

Effect of essential oil. It was revealed from the data analysis that a fraction of essential oil showed best antifungal activity against *A. flavus* with inhibition of 92.85% which is followed by *F. oxysporum* with inhibition of 90.90, *A. alternate* with inhibition of 67.66 and *P. pallidum* with inhibition with 64.28 (Table 5). The present results were similar to the finding of (Ramírez *et al.*, 2018; Javaid and Amin, 2009) reported antifungal activity of some species of the Combretaceae family.

Phytotoxic activity. Using natural compounds to control weeds has long been accepted as an environmentally friendly approach but during the last four decades, farmers have mostly relied on toxic synthetic agrichemicals. However, in recent years, demand for organic farming has markedly increased all over the world. In the present study, phytotoxic activity was observed by counting the number of fronds. The potential of the plant is shown in Table 6 and Fig. 7.

Effect of leaves extracts. Ethyl acetate extract of *Combretum indicum* showed significant, good and moderate phytotoxic activity at concentrations of 2000

Table 4. Antifungal activity of leaves extracts *Combretum indicum* L.

Treatment	Conc.	Test species											
		A. flavus			*A. alternate*			*F. oxysporum*			*P. pallidum*		
		ZI	% inh	LD_{50}	ZI	% inh	LD_{50}	ZI	% inh	LD_{50}	ZI	% inh	LD_{50}
Ethanol	20µg/mL	10.6	71.42	4.36	8	72.72	9.58	08.3	61.53	16.78	7	58.33	7.56
	40µg/mL	10	71.42		9	81.81		08.3	61.53		7.33	58.33	
	60µg/mL	11	78.57		9	81.81		11.33	84.61		8	66.66	
	80µg/mL	12.3	85.71		11	97.72		12.67	92.30		8.67	66.66	
Chloroform	20µg/mL	10.67	66.66	5.28	8.33	50.00	20.30	08.33	61.53	13.89	7	58.33	7.56
	40µg/mL	11	73.33		9.33	56.25		8.33	61.53		7.67	58.33	
	60µg/mL	12	80.00		9.67	56.25		10.33	76.92		8.33	66.66	
	80µg/mL	12	80.00		10	62.50		11.33	84.61		8.33	66.66	
Ethyl acetate	20µg/mL	7.67	53.84	18.09	8.33	57.14	7.67	08.67	81.00	2.17	8	66.66	12.36
	40µg/mL	10.33	76.92		8.67	57.14		8.33	80.00		8.67	66.66	
	60µg/mL	11	84.61		9.33	64.28		9.33	90.00		10.67	83.33	
	80µg/mL	12	92.30		9.33	64.28		9.67	91.00		11.33	91.66	
Control		13.67			14.67			10.33			12.33		

ZI = (Zone of inhibition); LD_{50} = (Lethal dose 50).

µg/mL, 200 µg/mL and 20 µg/mL, respectively. Ethanolic and chloroform extract showed good activity at 2000 µg/mL and 200 µg/mL. The overall % inhibition was high at 2000 µg/mL. The aqueous extract showed moderate activity at 2000 µg/mL (Table 6). The present

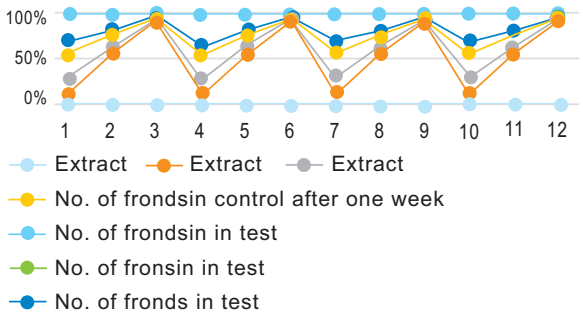

Fig. 7. Phytotoxic activity of different fractions of the crude extract.

results were similar to the finding of (Jahan *et al*., 2008) reported phytotoxic activity of *Combretum indicum*. The antifungal results were compared with the phytochemicals of the plant that showed the antifungal activity because the plant might have secondary metabolites (phenolics and terpenoids) which are responsible for the activity.

Effect of essential oil. The essential oil of *Combretum indicum* showed significant phytotoxic activity at 2000 µg/mL, good at 200 µg/mL and moderate at 20 µg/mL, essential oil showed good activity at 2000 µg/mL (57.44%) and 200 µg/mL (51.06%), while it showed moderate activity at 20 µg/mL (44.68%) (Table 7). The present results were agreed with the findings of (Wei *et al*., 2020) reported comparable results to that of essential oil of *Onopordum acanthium*. The essential oil phytotoxicity was reported first time from the flowers of *Combretum indicum* which is shown in Fig. 8.

Table 5. Antifungal activity of essential oil of *Cambretum indicum* L.

Treatment	Conc.	Test species											
		A. flavus			*A. alternate*			*F. oxysporum*			*P. pallidum*		
		ZI	% inh	LD_{50}	ZI	% inh	LD_{50}	ZI	% inh	LD_{50}	ZI	% inh	LD_{50}
Essential oil	20µg/mL	11	78.57	2.83	6.2	0.5	19.98	9	81.81	0.32	7	0.5	19.94
	40µg/mL	12.33	85.71		7.33	58.33		9.67	81.81		8.67	57.14	
	60µg/mL	12.33	85.71		8	66.66		9.33	81.87		9	64.28	
	80µg/mL	13.33	92.85		8.67	67.66		10.33	90.90		9.67	64.28	
Control		14.33			12.33			11.33			14.33		

ZI = (Zone of inhibition); LD_{50} = (Lethal dose 50).

Table 6. Phytotoxic activity of leaves extract

Extract	Concern. (µg/mL)	No. of fronds	No. of Frondsin control after one week	No. of Fronds intest after one week	Mortality (%)	LD_{50}
Aqueous	20	30	47	23	51.06	4.38
	200	30	47	20	57.45	
	2000	30	47	20	57.45	
Ethyl acetate	20	30	47	18	61.70	0.06
	200	30	47	16	65.96	
	2000	30	47	14	70.21	
Chloroform	20	30	47	23	51.06	11.02
	200	30	47	20	57.45	
	2000	30	47	18	61.70	
Ethanol	20	30	47	22	53.19	4.53
	200	30	47	19	59.57	
	2000	30	47	17	63.83	

LD_{50} = concentration required to cause 50% mortality.

Table 7. Phytotoxic activity of essential oil extract

Extract	Concern. (µg/mL)	No. of fronds	No. of fronds in control after one week	No. of fronds in test after one week	Mortality (%)	LD_{50}
Essential oil	20	30	47	26	44.68	136.04
	200	30	47	23	51.06	
	2000	30	47	20	57.44	

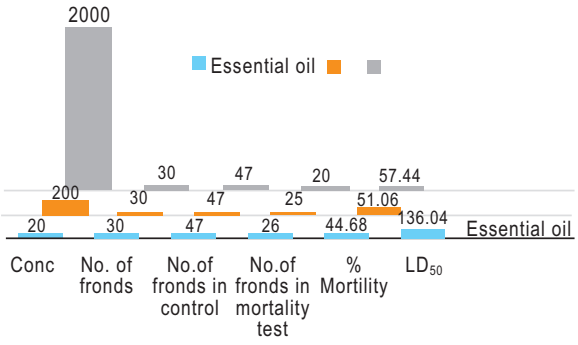

Fig. 8. Phytotoxic activity of essential oil of flowers.

Conclusion

From the current study, it is proved that *Combretum indicum* is a highly medicinal plant containing different types of secondary metabolites in different concentrations. Phytochemical screening showed that the plant holds important secondary metabolites. Some of these secondary metabolites have high antibacterial and antifungal activities also the plants show high phytotoxicity. It is recommended that advanced activity must be carried out on these plants so that we can obtain new and most operational secondary metabolites in the future to cure risky ailments.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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