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PHYTOCHEMICAL PROFILES, ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF LEAVES FROM THE *Psydrax dicoccos* (Gaertn)

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Abstract

The phytochemical analyses and antimicrobial activity of *Psydrax dicoccos* leaves were extracted successively with different solvents *viz*., petroleum ether, chloroform, ethyl acetate and methanol and screened for their antimicrobial activity against *Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecalis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Vibrio cholerae*, *Candida albicans, C. parapsilosis* and *C. tropicalis* by using Disc diffusion method. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were also determined in the present research. The methanol extract of *Psydrax dicoccos* showed the highest antimicrobial activity against all the bacterial and fungal strains tested than the other extracts. The mean zones of inhibition produced by the extracts in Agar diffusion assays against the tested bacterial strains was ranged from 7.0 to 22.5 mm. The MIC values were between 125 and 250 µg/ml, MBC values 250 and 1000 µg/ml and MFC 500 and 1000 µg/ml values were recorded. Phytochemical analyses of different extracts of *Psydrax dicoccos* leaves was analyzed. The methanol extract of *Psydrax dicoccos* leaves showed the presence of strong phytochemicals *viz*., steroids, tannins, phenolic compounds and terpenoids compared to other extracts. The highest mean of zone inhibition (22.5 mm) was observed in the methanol extract of *Psydrax dicoccos* against the bacteria *Staphylococcus aureus*. From the above findings, we concluded that the methanol extract of *Psydrax dicoccos* will be needed to isolate and characterize the compounds.

Key words: Antimicrobial activity, *Psydrax dicoccos*, MIC, MBC and MFC.

1. Introduction

Psydrax dicoccos belongs to the family Rubiaceae and commonly called Ceylon boxwood and Nazhuvai, Irambarathaan in Tamil and distributed in all parts of tropical and sub-tropical region of India. All parts of the plant have been recognized to have medicinal properties. Plant possesses antipyretic activity. In India, bark was used as febrifuge and also applied as plasters. The decoction of roots was used internally for treating diarrhoea. Bark powder boiled with sesame oil was used externally for rheumatic pains (Neelima *et al.*, 2011).

Microbial infections are major public health problems in the developed countries. Antibiotics are used to treat these infections. Due to indiscriminate use of commercial antibiotics, the incidence of multiple antibiotic resistances in human pathogens is increasing. This has forced the scientists to search for new antimicrobial substances from various sources like medicinal plants. Medicinal plants constitute the main source of new pharmaceuticals and health care products (Ivanona *et al.*, 2005). The use of traditional medicines was widespread in India (Jeyachandran, *et al.*, 2007).

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Infectious disease caused by bacteria, viruses, fungi and parasites are still a major threat to public health, despite the tremendous progress in human medicine (Cosa, 2006). The past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents (Chopra, 1996). Such situation stimulates the development of new anti-microbial agents in order to treat the infectious disease in an effective manner. So, this matter continued to an era to identify the potential antimicrobial agent from the natural resources. The edible plants that used for traditional medicine contain a wide range of substance that can be used to treat abundant of infectious disease with reduced side effects (Duraipandiyan, 2006). In Asia, the use of medicinal plants to cure specific illness has been use for many years (Bhattacharjee, 1998). Furthermore, Malaysia is rich in various edible plants with diverse biological & pharmacological properties (Yoga, 2005).

The numbers of resistant strains of microbial pathogens are growing since penicillin resistant and multiresistant *Pneumococci* caused a major problem in South African hospitals in 1977. (Berkowitz, 1995) calls the emerging of drug resistant bacteria a medical catastrophe. Leggiadro (1995) stated that effective regimens may not be available to treat some *Enterococcal* isolates and that it is critically important to develop new antimicrobial compounds for these and other organisms before we enter the post-antibiotic era. New compounds inhibiting microorganisms such as benzoin and emetine have been isolated from plants (Cox, 1994). The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains. A number of traditional natural products have been increased and much work has been done on selected ethno medicinal plants for antibacterial activity against pathogenic strains of Gram negative and Gram positive bacteria (Singh, 2002).

Bacterial infections especially those caused by multi-drug resistant (MDR) bacteria have become one of the great challenges for modern healthcare. Majority of scientists define MDR as ″resistance to at least 3 classes of antimicrobial agents″ (Falagas *et al.,* 2006). Antimicrobial agents have substantially reduced the threat posed by infectious diseases over a period of time since their discovery in the 1940's (Lewis and Ausubel, 2006). However, the escalation of multidrug resistance in bacteria in recent years has seriously jeopardized these gains. This has gained worldwide attention due to the high impact on public health. Increased usage of antimicrobial agents to treat bacterial infections has lead to the emergence of MDR strains (Bonnet, 2004). Invasive fungal infections are of great concern for human beings because they are associated with unacceptable high mortality rates. More than 90 % of all reported fungal - related deaths result from species that belong to one of three genera: *Cryptococcus, Candida* and *Aspergillus*. In turn, superficial infections of the skin and nails are the most common fungal diseases in humans, affecting *C. albicans* 25 % of the population worldwide (Karan, 2009). Hence, present study was carried out to evaluate the phytochemical properties and antimicrobial activity of petroleum ether, chloroform, ethyl acetate and methanol extracts of leaves of *Psydrax dicoccos* against bacterial and fungal strains.

2. Materials and methods

Collection of Plant material and preparation of extracts

The fresh leaves of *Psydrax dicoccos* were collected from Authukurichi region (11°24′37″N 79°21′4″E), Ariyalur District, Tamil Nadu, India. During the months from March to April 2014, the specimens were deposited in the Herbarium of Department of Botany, Annamalai University, Annamalai Nagar. Collected leaves were initially washed with water, then surface sterilized with disinfectant solution of 10 % sodium hypochlorite solution and finally rinsed with sterile distilled water and shade dried under room temperature and grounded in to a coarse powder. One hundred grams of coarse powder was extracted with different organic solvents like non-polar to polar

viz., Petroleum ether, chloroform, ethyl acetate and methanol for 8 hours using Soxhlet apparatus and the solvents were evaporated under vacuum in a rotary evaporator (Heidolph, Germany) and the dried powder was stored at 4˚C for further use.

Phytochemical analysis

The solvents such as, petroleum ether, chloroform, ethyl acetate and methanol extracts of leaves of *Psydrax dicoccos* were used for qualitative phytochemical analyses. Various bioactive phytochemicals namely, flavonoids, tannins, steroids, glycosides, saponins, phenolic compounds, terpenoids and alkaloids were analyzed according to described by Harborne (1983) and Trease (1983).

Microorganisms

The seven clinical bacterial isolates and three fungal species were obtained from Raja Muthaiah Medical College Hospital, Annamalai University, Tamil Nadu. Gram positive bacteria: *Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecalis* and Gram negative bacteria: *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Vibrio cholerae* and fungal species, *Candida albicans, Candida parapsilosis* and *Candida tropicalis* were tested in the present study. The stock cultures were maintained on Muller Hinton Agar medium and Sabouraud's Dextrose Agar at 4 °C separately for both bacteria and fungus respectively. *In vitro* antibacterial activity of the isolates was determined by using Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB). *In vitro* antifungal activity was determined by using Sabouraud's Dextrose Agar (SDA) and Sabouraud's Dextrose Broth (SDB) respectively.

Antibacterial and Antifungal assays

Disc diffusion method

The agar diffusion method (Bauer *et al.*, 1966) was employed for the initial assessment of antimicrobial potential of the extracts. Sterilized mediums of 20 ml MHA (Bacteria) and 20 ml SDA (Fungi) were separately poured in each petri plate and kept untouched until it solidify. The standard inoculum of bacterial suspension containing10^8 CFU/ml and suspension of yeast containing 10^6 CFU/ml were swabbed on the top of the solidified media and the plates were dried and uniformly spread. After drying, the extracts were placed on the disc with sterile forceps and gently pressed to ensure the contact with the incubated agar surface. Ciprofloxacin (5 µg/disc) for bacteria and Amphotercin-B (100 units/disc) for yeast were used as positive control. Ten per cent DMSO was used as blind control. Finally, the inoculated plates were incubated at 37 °C for 24 hrs. The zone of inhibition was observed and measured in millimeters. Each experiment was carried out in triplicates.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations of plant different extracts were tested in MHB for bacteria and SDB for fungi described by Ericsson and Sherris (2002). The extracts were dissolved in 10 % DMSO to obtain 2 mg/ml and 0.5 ml of stock solution was incorporated into 0.5 ml of MHA to get concentrations of 1000, 500, 250, 125, 62.5, 31.2 and 15.6 µg/ml, 100 µl as standard. Bacterial suspension of the test organism was transferred into each tube and incubated at 37 °C for 24 hrs. The tube devoid of plant extracts kept as control and the MIC of the extracts were examined.

Minimum Bactericidal Concentration (MBC)

The MBC of the different extracts were determined by plating 100 µl of samples from each MIC assay tube with growth inhibition into freshly prepared MHB and the plates were incubated at 37 °C for 24 hrs bacteria. The MBC values were recorded as the lowest concentration of the extracts that did not permit any visible bacterial colony growth on the agar plate during the period of incubation.

Minimum Fungicidal Concentration (MFC)

The MFC of the different extracts were determined by plating 100 µl of samples from each MIC assay tube with growth inhibition into freshly prepared SDA and the plates were incubated at 37 °C for 24 hrs yeast. The MFC values were recorded as the lowest concentration of the extracts that did not permit any visible bacterial colony growth on the agar plate during the period of incubation.

Table - 1: Phytochemical analysis of the different extracts of leaves *Psydrax dicoccos*

S.No	Phytoconstituents	Petroleum ether	Chloroform	Ethyl acetate	Methanol
1	Alkaloids	+	+	+	+
2	Flavonoids	-	-	-	+
3	Phenolic compounds	-	-	+	++
4	Saponins	-	-	-	+
5	Tannins	-	-	+	++
6	Cardiac glycosides	+	+	+	+
7	Steroids	+	+	+	+ +
8	Terpenoids	-	+	-	+ +

(++) = Strong; (+) = Positive (present); (-) =Negative (absent)

3. Results

The petroleum ether, chloroform, ethyl acetate and methanol extracts of the leaf of *Psydrax dicoccos* revealed that the presence of phytochemicals such as alkaloids, flavonoids, cardiac glycosides, phenolic compounds, saponins, steroids, tannins and terpenoids. The methanol extracts of *Psydrax dicoccos* revealed the presence of strong phytochemicals, terpenoids, saponins, steroids, flavonoids and phenolic compounds. The ethyl acetate extracts were present in all phytochemicals except cardiac glycosides and saponins. The chloroform extracts were present in all the phytochemical except flavonoids, saponins, steroids, and tannins. The petroleum either extracts were absent in all the phytochemical except alkaloids, flavonoids, saponins, and cardiac glycosides.

The plant extracts were prepared from different parts (leaves) of *Psydrax dicoccos* by using the solvents with different polarity such as petroleum ether, chloroform, ethyl acetate and methanol using Soxhlet apparatus. The extracts were evaluated for antibacterial activities against Gram positive clinical isolates *viz., Staphylococcus aureus Streptococcus pyogenes* and *Enterococcus faecalis,* Gram negative clinical isolates *viz., Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris* and *Vibrio cholerae* and antifungal activities against *Candida albicans, Candida parapsilosis* and *Candida tropicalis*.

The results clearly demonstrated that the methanol extract of *Psydrax dicoccos* leaves were exhibited broad spectrum antimicrobial activity. The results of antimicrobial activity of different concentrations of petroleum ether, chloroform, ethyl acetate and methanol extracts of *Psydrax dicoccos* leaves against bacterial and fungal strains.

Table – 2: Antimicrobial activity of different extracts of leaves of *Psydrax dicoccos*

S. No.	Microbial strains	Mean zone of inhibition[a] (mm)[b]				MIC (µg/mL)	MBC (µg/mL)
		Concentration of the extracts (µg/disc)					
		1000	500	250	Ciprofloxacin (5 µg/disc)		
1	***Staphylococcus aureus***						
	Petroleum ether	13.5± 0.50	10.8 ±0.76	9.3 ±0.57	29.1 ± 057	250	500
	Chloroform	15.8± 0.67	13.1±0.28	10.8± 0.76	28.6± 0.76	250	500
	Ethyl acetate	20.1±0.28	16.1± 0.28	12.3± 0.57	29.0 ± 0.50	125	250
	Methanol	22.5± 0.50	17.3 ±0.57	13.0± 0.50	30.1± 0.78	125	250
2	***Streptococcus pyogenes***						
	Petroleum ether	13.1 ±0.28	9.8 ± 0.76	8.1± 0.28	30.0 ± 0.28	250	500
	Chloroform	14.6± 0.76	10.3± 0.76	8..5± 0.50	29.6 ± 0.75	250	500
	Ethyl acetate	17.8± 0.76	12.5± 0.50	10.5± 0.50	28.3± 0.50	125	250
	Methanol	18.5±0.50	14.1± 0.28	11.8 ± 0.57	28.1± 0.28	125	250
3	***Enterococcus faecalis***						
	Petroleum ether	12.0± 0.50	9.5 ± 0.50	7.8 ± 0.76	26.7± 0.76	500	1000
	Chloroform	12.6± 0.50	10.1± 0.28	9.1 ± 0.28	29.0 ± 0.50	500	1000
	Ethyl acetate	13..6 ± 0.36	10.5± 0.50	9.6± 0.76	28.1± 0.28	250	500
	Methanol	14.8± 0.76	11.1 ± 0.78	10.1 ± 0.28	29.3 ± 0.50	250	500
4	***Escherichia coli***						
	Petroleum ether	11.6 ± 0.67	9.3± 0.57	7.5 ± 0.50	30.0 ± 0.57	500	1000
	Chloroform	12.1± 0.78	9.8± 0.76	8.0 ± 0.50	29.8 ± 0.76	500	1000
	Ethyl acetate	13.1± 0.28	10.0 ± 0.70	9.1 ± 0.28	28.5± 0.50	250	500
	Methanol	13.5± 0.50	10.3 ± 0.57	9.5± 0.50	30.1± 0.28	250	500
5	***Proteus vulgaris***						
	Petroleum ether	10.3± 0.57	9.8± 0.76	7.1 ± 0.28	26.5 ± 0.28	500	1000
	Chloroform	10.6 ± 0.56	9.0 ± 0.50	7.8± 0.76	29.1 ± 0.28	500	1000
	Ethyl acetate	11.3± 0.57	9.8± 0.67	8.6± 0.76	30.5± 0.50	250	500
	Methanol	13.1 ± 0.35	10.5± 0.50	9.3 ± 0.57	27.1 ± 0.28	250	500

[a]Diameter of zone of inhibition (mm) including the disc diameter of 6 mm
[b]Mean of three assays; ± - Standard deviation ; Significant at $P<0.05$

S. No.	Microbial strains	Mean zone of inhibition[a] (mm)[b]				MIC (µg/mL)	MBC/MFC (µg/mL)
		Concentration of the extracts (µg/disc)					
		1000	500	250	Ciprofloxacin (10µg/disc)		
6	***Pseudomonas aeruginosa***						
	Petroleum ether	10.8± 0.76	9.1± 0.28	7.3 ± 0.57	29.8 ± 0.76	500	1000
	Chloroform	11.3± 0.55	9.8± 0.76	7.8± 0.76	26.7± 0.58	500	1000
	Ethyl acetate	12.3 ± 0.57	10.1 ± 0.28	8.6 ± 0.76	28.0 ± 0.50	250	500
	Methanol	13.0± 0.50	10.3± 0.75	9.5 ± 0.50	30.1± 0.28	250	500
7	***Vibrio cholera***						
	Petroleum ether	11.0 ± 0.50	8.5 ± 0.50	7.3 ± 0.57	21.1 ± 0.28	500	1000
	Chloroform	12.8± 0.76	9.8± 0.76	8..1± 0.28	26.3 ± 0.57	500	1000
	Ethyl acetate	12.1 ± 0.28	10.8 ± 0.76	9.3± 0.57	26.0 ± 0.50	250	500
	Methanol	13.8 ± 0.76	11.1± 0.28	9.6± 0.76	30.0 ± 0.50	250	500
8	***Candida albicans***						
	Petroleum ether	10.5± 0.50	9.1 ± 0.50	7.1 ± 0.28	15.1 ± 0.28	500	1000
	Chloroform	11..0± 0.50	9.1± 0.50	7.8± 0.76	13.5± 0.50	500	1000
	Ethyl acetate	12.8 ± 0.76	11.0 ± 0.50	8.5± 0.57	11.0± 0.50	250	500
	Methanol	13.8 ± 0.50	11.8 ± 072	9.1± 0.28	15.3± 0.57	250	500
9	***Candida parapsilosis***						
	Petroleum ether	10.0 ± 0.50	8.1 ± 0.78	7.0± 0.50	16.3 ± 0.57	500	1000
	Chloroform	11..5 ±0.50	8.3±0.50	7.5± 0.50	14.1± 0.28	500	1000
	Ethyl acetate	13.0 ± 0.50	11.1± 0.28	7.8 ± 0.76	15.3± 0.57	250	500
	Methanol	14.5± 0.50	1!.8± 0.28	9.3 ± 0.57	14.0± 0.50	250	500
10	***Candida tropicalis***						
	Petroleum ether	9.0± 0.50	8.1 ± 0.28	7.0 ± 0.50	16.0 ± 0.50	500	1000
	Chloroform	10.1± 0.28	8.1± 0.28	7.5± 0.50	14.7 ± 0.58	500	1000
	Ethyl acetate	11.5 ± 0.50	9.5± 0.50	8.1 ± 0.28	16.3 ± 0.57	250	500
	Methanol	12.8 ± 0.76	10.1± 0.78	9.0± 0.50	15.0± 0.50	250	500

[a]Diameter of zone of inhibition (mm) including the disc diameter of 6 mm
[b]Mean of three assays; ± - Standard deviation ; Significant at $P<0.05$

The results revealed that the highest mean zone of inhibition produced by the leaf extracts of *Psydrax dicoccos* for bacteria ranged between 7.1 ± 0.28 and 22.5 ± 0.50 mm. For fungi, the mean zone of inhibition produced by the leaf extracts ranged between 7.0 ± 0.50 and 14.5 ± 0.50 mm. The MIC and MBC values of crude extracts of leaves of *Psydrax dicoccos* for bacteria were between 62.5 and 500 µg/mL and 125 and 1000 µg/mL respectively.

The MIC and MFC values of crude extracts of leaves of *Psydrax dicoccos* for fungi were between 250 and 500 µg/mL and 500 and 1000 µg/mL respectively. The highest mean zone of inhibition for bacteria (22.5 ± 0.50 mm at 1000 µg/disc), lowest MIC (62.5 µg/mL) and MFC (250 µg/mL) values were recorded in methanol extract of leaves of *Psydrax dicoccos* against *Staphylococcus aureus.* The highest mean zone of inhibition for fungi (14.5 ± 0.50 mm at 1000 µg/disc), lowest MIC (250 µg/mL) and MFC (500 µg/mL) values were recorded in methanol extracts of leaves of *Psydrax dicoccos* against *Candida parapsilosis* and *Candida albicans.*

Ciprofloxacin (5 µg/disc) was used as the positive control for bacteria and the mean zones of inhibitions were between 26.7 ± 0.76 mm and 31.5 ± 0.50 mm. Amphotericin-B (100 units/disc) was used as the positive controls for *Candida* species and the mean zones of inhibition were between 11.0 ± 0.50 mm and 16.3 ± 0.57 mm. For bacteria, the lowest activity (7.1 ± 0.57 mm at 250 µg/disc) was recorded with petroleum ether extract against *Proteus vulgaris* and for fungi, the least zone of inhibition (7.0 ± 0.50 mm) was observed against *Candida tropicalis.*

4. Discussion

In the present study, different solvent *viz.*, petroleum ether, chloroform, ethyl acetate and methanol extracts of *Psydrax dicoccos* leaves were showed varied level of inhibitory activities against the bacterial and fungal strains tested. All the extracts of *Psydrax dicoccos* possessed significant antibacterial and antifungal activity against all the bacterial and fungal strains. The methanol leaf extract of *Psydrax dicoccos* showed the highest antibacterial activity against *S. aureus* followed by other strains. The highest mean zone of inhibition (22.5 mm) and the lowest MIC value (125 µg/ml) and MBC value (250 µg/ml) were observed in methanol leaf extract. In antifungal activity, the methanol leaf extract of *Psydrax dicoccos* showed highest antifungal activity and it was followed by petroleum ether, chloroform and ethyl acetate.

Similar results were also observed in previous studies, methanol extracts have been shown to result in high extraction yields with strong antibacterial activities (Jo *et al.,* 2012). Prashanth *et al.* (2001) proved that methanolic extracts of pomegranate peels were more active than water extracts against *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*. Ashik Mosaddik *et al.* (2000) reported that the methanol extracts of *Alangium salviifolium* flowers showed a wide spectrum of antibacterial activity against *Bacillus subtilis, Bacillus megaterium* and *Staphylococcus aureus* and Gram negative bacteria *viz., Escherichia coli, Shigella sonnei, Shigella shiga, Shigella boydii, Salmonella typhi* and *Klebsiella* sp.

The methanol extract of *Psydrax dicoccos* leaves showed the presence of strong phytochemicals *viz.*, steroids, tannins, phenolic compounds and terpenoids compared to other extracts.

Methanol extracts of *Psydrax dicoccos* showed potential inhibitory action on tested fungal strains. The solvent methanol is known for its ability to isolate more antimicrobials from plants including tannins, polyphenols, terpenoids, saponins, xanthoxyllines, totarol, quassinoids, lactones, flavones and phenones, while the water extracts could isolate only anthocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectins. In general, phenolic compounds possess specific physical, chemical and biological activities that make them useful as drugs. Phenolics were also responsible for the antimicrobial, anti-inflammatory, anti-feedant, anti-viral, anticancer and vasodilatory actions (Rievere, 2009). Phenolic compounds may

affect growth and metabolism of bacteria. They could have an activating or inhibiting effect on microbial growth according to their constitution and concentration (Reguant 2000). Tannins were used therapeutically as antiviral, antibacterial, antiulcer and antioxidant agents. Many tannin containing drugs are used in the treatment of piles, inflammation, burns and as astringent (Kolodziej, 2005).

The MIC and MBC values of methanol extract were lower than that of other extracts of the *Psydrax dicoccos* showed potential antibacterial effect against bacterial strains tested. Similar observations were made while studying the antimicrobial activity of seeds of *Syzygium jambolanum* (Chandrasekaran, 2004), FAME extracts of certain marine macro algae (Ananatharaj 2004) and leaves of *Ipomoea pes-caprae* (Chandrasekaran, 2005). The majority of plant derived antimicrobial compounds generally have higher MICs than bacterial or fungal produced antibiotics, thus limiting their therapeutic potential (Gibbons, 2004).

In this study, the methanol extract of leaves of *Psydrax dicoccos* showed the highest antibacterial activity against Gram positive bacteria than Gram negative bacteria. The reason for different sensitivity between Gram positive and Gram negative bacteria could be ascribed to the morphological differences between these microorganisms (Avato *et al.*, 1997). The Gram positive bacteria should be more susceptible since they have only an outer peptidoglycan layer, which is not an effective permeability barrier (Scherrer and Gerhardt, 1971). The resistance of Gram negative bacteria towards antibacterial substances was related to lipopolysaccharides in their outer membrane (Gao *et al.*, 1999).

5. Conclusion

The antimicrobial activity of the *Psydrax dicoccos* methanol leaf extract, probably due to the recognized phytoconstituents, further confirms its use as a health cure in traditional medicine. Bioactive substances from this plant as a result be employed in the formulation of antifungal agents for the healing of a variety of microbial infections. Finally, it can conclude that the methanol extract of *Psydrax dicoccos* can be used as a antimicrobial substance for the treatment of microbial infections.

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