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ANTIMICROBIAL ACTIVITY OF DRIED AQUEOUS AND ETHANOLIC EXTRACTS OF *GNAPHALIUM POLYCAULON*

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ABSTRACT

Objective: The current study was undertaken to quantify the presence of phytochemicals constituents and to examine antimicrobial analysis of the different dried parts of *G. polycaulon* in two different solvents. **Methods:** *G.polycaulon* plants were collected from The Nilgiris District, South India and coarsely powdered for organic solvent extraction with aqueous, and ethanol solvents. Then all the extracts obtained were subjected for phytochemical screening and antimicrobial study with standard procedure. **Results:** The presence of major phytochemicals quantitatively in all solvent extracts. The aqueous leaf extract of *G. polycaulon* recorded significant antibacterial and antifungal activity against all the test bacteria and fungi than ethanolic extracts. **Conclusion:** The present study strongly supports that the crude solvent plant extracts contain medicinally important bioactive compounds due to the strongly presence of secondary metabolites which can be used to fight against resistant organisms for the treatment of different diseases.

KEYWORDS

Phytochemicals, TLC, Phenols, Flavonoids, Alkaloids, Antimicrobial Activity and MIC.

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INTRODUCTION

Medicinal plants have played an essential role in the development of human culture for medicinal purposes around before the time of man[1]. Medicinal effects of plants developed in Ancient time using direct test by physicians and lessons from animals, Ethno botanists[2]. Many of the modern herbal medicines are produced plant metabolites from medicinal plants as medicines and resources of new drugs around the world[3]. It is estimated that more than 250, 000 flower plant species helps for studying medicinal plants to understand plant

toxicity, protect human and animals from natural poisons and protect biological diversity[4]. Herbs act multi-dimensionally on the healing processes in the body[1].

Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions[5]. Approximately 25% of modern drugs derived from plants were used in the United States was reported by World Health Organization. The use of herbs to treat disease is almost universal among non-industrialized societies[6].

Phytochemicals are natural and non-nutritive bioactive compounds produced by plants that act as protective agents against external stress and pathogenic attack[7] and also to describing the isolation, purification, identification, and structure of the large number of secondary metabolic compounds found in plants using Thin layer chromatography (TLC)[8], High performance of liquid chromatography (HPLC), Gas chromatography Mass spectrometry (GC-MS) and Nuclear magnetic resonance (NMR)[4].

Asteraceae are popular garden plants due to their numerous and often brightly colored blossoms. *Gnaphalium polycaulon* is a genus of flowering plants belongs to Asteraceae family of compositae type, worldwide distribution and is mostly found in temperate regions[9]. The entire plant is harvested during flowering and is used to make herbal and homeopathic remedies[10]. Species in this genus was said to have anti-inflammatory, astringent, and antiseptic properties and are often prescribed as an herbal supplement for colds, pneumonia, and congestion[11]. It is a popular treatment for respiratory problems and neuritis among tribe may also benefit from G. *polycaulon*[12].

Pharmaceutical and scientific communities have recently concentrated their attention in the medicinal plants and various publications to validate the claims of their biological activity. There is a continuous and urgent need to discover new antimicrobial compounds for new and reemerging infectious diseases. Most recent research studies based on the species of medicinal plants revealed that the plant kingdom has not been exhausted which are yet to be discovered. So, this medicinal plant was chosen for our current study with main objectives to screen the phytochemicals constituents and antimicrobial study responsible for its medicinal property.

METHODS

Chemicals required

All chemicals used for this study were obtained commercially and were of high quality analytical grade reagents. The solvents such as ethanol, and water were purchased from S.D. Fine Chemicals Pvt. Ltd, Sigma chemicals, Lobe chemicals, Merck Chemical Supplies, Nice Chemicals and Hi media.

Collection of plant material

The fresh leaves, stem and flower of *G. polycaulon* were collected from Kotagiri in Nilgiris District, Tamil Nadu. The plant parts were selected on the basis of the knowledge on their use in different medicine system of health care and identified as *Gnaphalium polycaulon* Pers- Asteraceae family and the herbarium specimen is authenticated and incorporated in the Madras Herbarium.

Preparation of extracts

The plant materials were washed, air dried and then coarsely powdered. Forty grams of the powdered leaf, stem and flower samples were extracted sequentially using Soxhlet's method for 72 h at a temperature not exceeding the boiling point of the solvent into 250ml of aqueous, and ethanol for extract preparation. Resulting extracts was concentrated in vacuum to dryness using a Rotary evaporator. Each powder was weighed and dissolved in the appropriate solvents used for extraction separately and stored at 4ºC. These extracts were subjected to screening antimicrobial study.

Quantitative Analysis of Phytochemicals

Determination of Total Phenolic Content

The total phenolic content of all extracts of the plant material was estimated according to the method of[13]. Different aliquots of the extracts were taken in a test tube and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-

Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20 %) were added sequentially in each tube in triplicate manner. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725nm against a reagent blank. Total phenolic contents were determined as a Gallic acid equivalent (GAE) based on Folin-Ciocalteau calibration curve using Gallic acid as the standard and expressed as mg Gallic acid equivalent per gram of dry sample. All tests were carried out in triplicate.

Determination of Total Flavonoid Content

The flavonoid content of the plant extract was determined by a colorimetric method as described by[14] with minor modification. Different concentration of the plant extract was prepared by diluting the stock solution (4000µg/ml) with deionized water. Each sample (100µl) was diluted with distilled water (200µl). Sodium nitrite (5 %; 30µl) was added to the samples and then at 5 min, aluminum chloride (10 %; 30µl) and at 6 min, sodium hydroxide (1M; 200µl) were added to the mixture. Finally, 400µl of deionized water was added. The absorbance was recorded at 510nm. Quercetin was used as the standard to calculate the concentrations of flavonoid content and the values were expressed as mg Quercetin equivalents/g of sample. The analysis was performed in triplicates.

Determination of Alkaloid

The Alkaloid was determined by the methods of[15]. 5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and allowed to stand for 4 h. Then filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid was dried and weighed. All tests were carried out in triplicate

Determination of Tannin

The Tannin was determined by the methods of[16]. 500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker and filtered into a 50 ml volumetric flask and made up to the mark. Then 5ml of the filtered was pipetted out into a test tube and mixed with 2ml of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide. Total tannin contents were determined using Gallic acid (ranging from 50 to 1000 mg/ml) as the standard and expressed as mg Gallic acid equivalent per gram of dry sample. All tests were carried out in triplicate. The absorbance was measured at 120 nm within 10 min.

Determination of Saponin

The Saponin was determined by the methods of[17]. 20 g of plant sample were put into a conical flask and 100cm^3 of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The saponins content was calculated as percentage.

Thin Layer Chromatography

The powdered plant sample was lixiviated in ethanol on rotary shaker (180 thaws/mins) for 24 h[18]. The condensed filtrate was used for chromatography. The phenols were separated using chloroform and methanol (27:0:3) solvent mixture. The alkaloids spots were separated using the solvent mixture chloroform and ethanol (15:1). The flavonoid spots were separated using chloroform

and ethanol (19:1) solvent mixture. The saponins were separated using chloroform, glacial acetic acid, methanol, water (3:1.5:0.6:0.2) solvent mixture. The glycosides were separated using chloroform, methanol, conc. ammonia (40:10:2) solvent mixture. The colour of spots was identified for aqueous, and ethanol extracts in leaf, stem and flower of *G. polycaulon*. The retardation factors (R*f*) of all components are reported. R*f* value is calculated by using the formula.

$$\text{Retardation factors}(R_f \text{ value}) = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent from the origin}}$$

Antimicrobial activity

Test organisms

The bacterial cultures of Gram positive (*Aeromonas hydrophila, Escherichia coli* MTCC739, *Pseudomonas aeruginosa* MTCC424) and Gram negative (*Bacillus cereus* MTCC430, *Staphylococcus aureus* MTCC3381) bacteria; the fungal cultures of *Aspergillus fumigatus* MTCC343, *Aspergillus oryzae,* and *Candida albicans* MTCC227 were used to test the antimicrobial activity.

Preparation of the inoculum

To prepare the bacterial and fungal inoculums from each of the microorganisms, a loop ful of each test organisms was taken and subsequently sub-cultured into separate test tubes containing nutrient agar broth. Then the tubes were subjected to incubation for 24 h at 37ºC, the obtained broth with microorganisms was standardized to have a uniform population density of microorganisms in microbial culture laboratory.

Screening for antibacterial activity

The antibacterial activity of *G. polycaulon* was assayed by a modification of agar well diffusion method[19,20]. The test organisms were maintained on agar slants were recovered for testing by inoculating into nutrient broth and incubated at 37°C in a shaker at 180 rpm. The culture of each microorganism was inoculated in plates in nutrient agar and spread evenly using sterile glass spreader. Test extracts were incorporated into the wells made by sterile 5 mm size borer in media and different concentration of plant extracts were added and water alone as a control. Plates were incubated at 37ºC and after 24 h, the zone of inhibition of plant extract, standard control were measured by screened in triplicates manner.

Screening for antifungal activity

Antifungal activity of all various extracts was studied against two fungal strains by the agar well diffusion method[21]. The fungal isolates were allowed to grow on a potato dextrose agar (PDA) at 25 ºC until they sporulated. The fungal spores were harvested after sporulation by pouring a mixture of sterile distilled water. The fungal spores suspension was evenly spread on plate using sterile glass spreader. Wells were then bored into the agar media using sterile 5 mm cork borer and the wells filled with the solution of the extract and water alone as a control. The plates were allowed to stand on a laboratory bench for 1 h to allow for proper diffusion of the extract into the media. Plates were incubated at 25 ºC for 96 h and later observed for zones of inhibition of all extracts, standard control and measured in triplicates manner.

Minimum Inhibitory Concentration (MIC)

Organisms were subcultured on nutrient agar, followed by incubation for 24 h at 37ºC. Inoculum was prepared by transferring several colonies of microorganisms to sterile nutrient broth[22]. The suspensions were mixed for 15 sec and incubated for 24h at 37ºC. Required volume of suspension culture was diluted to match the turbidity of 0.5 Mc Farland standards ($1.5x10^8$ CFU/mL). MIC was considered the lowest concentration of the sample that prevented visible growth. All samples were examined in triplicates manner. Samples were prepared in dimethyl sulphoxide at the concentration of 2 mg/ml.

A series of 15 tubes were filled with 0.5 ml of sterilized nutrient broth. Sequentially, test tubes 2-14 received an additional 0.5 ml of the sample serially diluted to create a concentration sequence from 500-0.06 µg. The first tube served as the control. All the tubes received 0.5ml of inoculum. The tubes were vortexes well and incubated for 24 h at 37ºC. The resulting turbidity was observed, and after 24 h MIC was determined to be where growth

was no longer visible by assessment of turbidity by optical density readings at 600 nm.

RESULTS AND DISCUSSION

Secondary metabolites in Medicinal plants products are responsible for several biological activities in living systems and used throughout human history. In recent times, ethno medical and traditional pharmacological approaches are achieving great appreciation in modern medicine, because the search for new potential medicinal plants is often based on an ethno medicinal origin[23].

Quantitative phytochemical screening

The quantitative estimation of phytochemical screening in *G. polycaulon* showed that the aqueous leaf, stem and flower extracts were rich in phenolics, tannins, alkaloids, flavonoids and saponins due to high medicinal and physiological activities[24]. The quantitative analysis of dry extracts of *G. polycaulon* was reported in Table No.1. Phenolics are one of the major groups of phytochemical that can be found ubiquitously in certain plants. The presence of phenolic compounds contributed to their antioxidative properties in herbal medicament[25]. Phenols have been found to be useful in the preparation of some antimicrobial compounds[26]. Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable activity in cancer prevention and anticancer[27]. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity[15]. Flavonoids are capable of treating certain physiological disorder and diseases[28]. Saponin is glycosides occurring widely in plants and medicinally as antibiotic[29], antiviral[30], anti-inflammatory[31] and anti-ulcer. Thus, *G. polycaulon* plant may serve as a potential source of bioactive compounds in the treatment of cancer and related diseases.

Thin layer Chromatography

The appearance of coloured spots on the TLC plate indicated the presence of alkaloids, flavonoids, saponins and phenol and its retention factors, *Rf* values were determined in leaf, stem and flower of aqueous, and ethanol extracts (Figure No.1). Further studies are needed with the selected herb to identify, isolate, characterize and elucidate the structure of the bioactive compounds of the herbs which are responsible for the antimicrobial activity and other medicinal value[32].

Antimicrobial activity

Antimicrobial properties of several plant extracts have been attributed due to the secondary metabolites[19]. In this study, antibacterial activity in aqueous, and ethanol solvents of leaf, stem and flower extracts of *G. polycaulon* were screened and evaluated against bacteria and fungal cultures. Results were compared with the standard drugs such as gentamycin for bacterial cultures and Nystatin for fungal cultures. The zone of inhibition was seen in all extract against all cultures but the maximum inhibition shown in dry aqueous leaf extracts than others extracts. All extracts showed good activity against the fungal isolates with zones of inhibition ranging from 8 to 18 mm. The zone of inhibition of all various dry extracts of *G. polycaulon* was measured and tabulated in Table No.2.

Fungi can cause damage to the structures, decoration of buildings and are also responsible for their indoor air quality[21]. In conclusion, the results showed that the all various extract of *G. polycaulon* is a broad spectrum agent which can be used against both Gram positive and Gram negative bacteria[20] and also fungi[21]. The antibacterial activity of the crude extracts may be attributed to the high phenolic and flavonoidcontent[26,28]. The antibacterial activity of the extracts could be attributed to the high content of flavonoids which have been reported to be involved in the inhibition of nucleic acid biosynthesis and metabolic processes[28].

Minimum Inhibitory Concentrations (MIC)

Minimum Inhibitory Concentrations of all crude extracts was determined. The MIC values were evaluated and showed no growth at 125 µg/ml in aqueous leaf extracts. The results showing high resistant activity in aqueous leaf extract in the order of test bacterial cultures such as *S. aureus*, *A. hydrophila*, *E. coli*, *P. aeruginosa*, *B. cereus* and *L.*

monocytogenes and the fungal cultures are *C. albicans, P. notatum, A. fumigates* and *A. oryzae.* MICs of active extracts ranged from 500-0.06µg/mL against test bacterial and fungal cultures were tabulated in Table No.2. The reason for higher sensitivity of the Gram-positive bacteria than Gram negative bacteria could be attributed to their differences in cell membrane constituents. Gram-positive bacteria contain an outer peptidoglycan layer, which is an ineffective permeability barrier[33]. In conclusion, G. *polycaulon* extracts tested in present study had specific potential antimicrobial activity against the reference strains. To date, many plants have been claimed to pose beneficial health effects with the emergence of multiple strains of antibiotic resistance microorganism, great interest has been generated in search for potential compounds from plants for therapeutic, medicinal, aromatic and aesthetic uses.

Table No.1: Quantitative analysis of of various extracts of dried *G. polycaulon*

S.No	Dried Plant extracts		Quantitative assay (mg/g of extract)				
			Total Phenolic content	**Total Tannin content**	**Total Alkaloid content**	**Total Flavonoid content**	**Total Saponin content**
1	Aqueous	Leaf	0.75	0.48	0.26	0.78	0.34
		Stem	0.48	0.32	0.23	0.51	0.22
		Flower	0.27	0.31	0.22	0.44	0.19
2	Ethanol	Leaf	0.74	0.46	0.25	0.76	0.29
		Stem	0.47	0.27	0.22	0.49	0.21
		Flower	0.26	0.3	0.21	0.42	0.18

Table No.2: Antimicrobial activity and MIC determination of various extracts of dried *G. polycaulon*

Extracts	Plant parts	Concentration (µg/ml)	Antibacterial activity (Zone of inhibition, mm)					Antifungal activity (Zone of inhibition, mm)		
			Ah	*Ec*	*Pa*	*Bc*	*Sa*	*Afu*	*Aor*	*Can*
Aqueous extracts	Dried leaf	50	8	9	10	10	10	9	9	10
		100	8	9	10	11	11	9	9	11
		150	10	10	11	11	11	10	10	12
		MIC	125	125	125	125	62.5	125	125	125
	Dried stem	50	6	7	9	3	4	4	4	6
		100	7	8	9	5	5	4	5	6
		150	8	8	10	7	6	5	6	8
		MIC	125	125	125	125	125	125	125	125
	Dried flower	50	7	7	9	2	4	5	5	6
		100	7	7	9	2	7	8	5	11
		150	8	8	10	5	8	8	7	11
		MIC	-	125	125	125	-	125	125	125
Ethanolic extracts	Dried leaf	50	9	10	11	11	10	10	10	11
		100	9	10	11	11	10	10	10	12
		150	10	11	11	12	11	11	11	13
		MIC	125	125	125	125	62.5	125	125	125
	Dried stem	50	7	9	10	4	5	4	5	7
		100	8	9	10	5	5	5	5	7
		150	8	10	11	8	9	6	7	9
		MIC	125	125	125	125	125	125	125	125
	Dried flower	50	8	8	9	5	5	5	6	7
		100	8	8	9	5	8	6	9	7
		150	9	9	10	7	9	6	11	9
		MIC	-	125	125	125	-	125	125	125
	Standard	100	18	17	19	28	28	8	8	14

Ah: Aeromonas hydrophila; Ec: Escherichia coli; Pa: Pseudomonas aeruginosa; Bc: Bacillus cereus; Sa: Staphylococcus aureus; Afu: Aspergillus fumigatus; Aor: Aspergillus oryzae; Can: Candida albicans;
MIC: Minimum Inhibitory Concentration

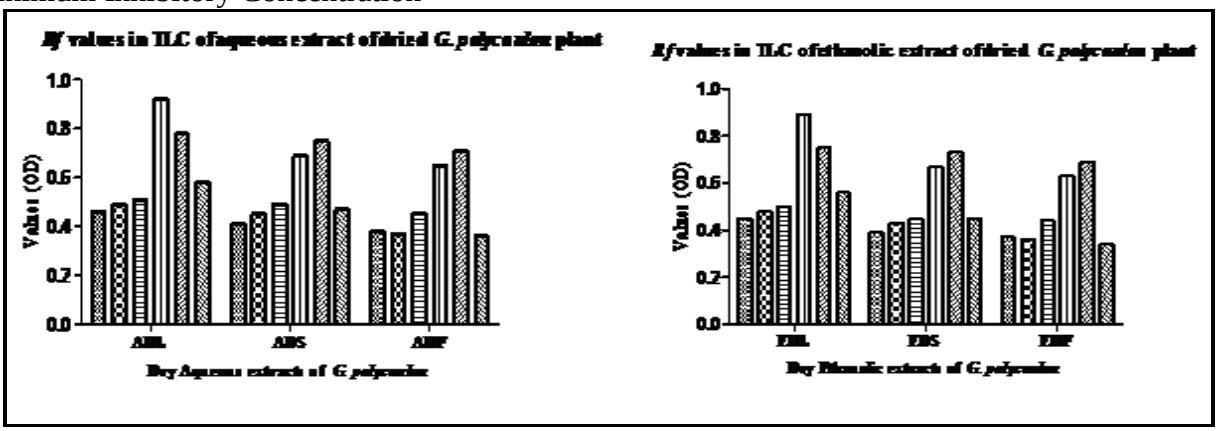

Figure No.1: The *Rf* values in Thin Layer chromatography of various extracts of dried *G. polycaulon*

CONCLUSION

Herbal medicines are not only providing traditional medicine but also promising for highly efficient novel bioactive molecules. Many medicinal plants lie unexplored or remain under explored. The results reported the presence of major phytochemicals quantitatively in all solvent extracts and showed that *G. polycaulon* plant is good source of phytochemical and antimicrobial activity. The resource base of the traditional folk medical practices is more prevalent in rural and tribal villages of India. From results finding, it strongly supports that the efficiency of crude solvent plant extracts contain medicinally important bioactive compounds due to the strongly presence of plant secondary metabolites which can be used to fight against resistant bacteria and fungi for the treatment of different diseases in traditional medicine.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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