International Journal of Research in Pharmacy and Pharmaceutical Sciences
ISSN: 2455-698X; Impact Factor: RJIF 5.22
www.pharmacyjournal.in
Volume 2; Issue 2; March 2017; Page No. 11-15

Quantitative phytochemical analysis of various hexane extracts of *Gnaphalium polycaulon*

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Abstract
Medicinal plants have been reported as richest bioresource of drugs in modern medicines, folk medicines, food supplements, pharmaceuticals intermediates, nutraceuticals, chemical entities for synthetic drugs and traditional medicine system. The aim of our present study was designed to quantify the presence of phytochemicals constituents in the different fresh and dried parts of *G. polycaulon* in hexane solvents. The phytochemical analysis was carried out quantitatively in the fresh and dried hexane extracts of *Gnaphalium polycaulon* and confirmed by TLC analysis with standard procedure. All dried hexane extracts showed significant results than fresh hexane extracts due to the presence of major phytoconstituents in *Gnaphalium polycaulon*. These findings confirmed that *Gnaphalium polycaulon* plant have medicinal property for the treatment of various diseases.

Keywords: phytochemicals, TLC, phenols, flavonoids, alkaloids, steroids, tannins

Introduction
Medicinal plants have been reported as richest bioresource of drugs in modern medicines, folk medicines, food supplements, pharmaceuticals intermediates, nutraceuticals, chemical entities for synthetic drugs and traditional medicine system [1, 2]. Medicinal plant products contain complex mixture of many medicinal plant metabolites [3]. Medicinal plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack by predators such as insects, fungi and herbivorous mammals, which is being used throughout human history [4].
Medicinal plants constitute the backbone of traditional medicine practices. Medicinal plants have the ability to synthesize a wide variety of phytoconstituents, to perform variety of significant biological functions [5]. The importance of traditional medicine (TM) as a source of primary healthcare was first officially recognized by the World Health Organization [6] by Traditional Medicine Program. Medicinal plants plays an essential role in the development of human health. Most of the traditional medicinal plants are used by the tribal communities of the Nilgiri district, South India. The traditional practitioners and healers are well known about plants used to cure various ailments without side effects till date. Specifically, *G. polycaulon* is used as natural remedy for quick heal of wounds, nerve disorder and other respiratory problems but a proper document is needed for evidence of *G. polycaulon* in present scenario.
Phytochemicals are natural and non-nutritive bioactive compounds produced by variety of plants which acts as protective agents against external stress and pathogenic attack [7]. Secondary metabolite is crucial for plant defenses as an antioxidant or antimicrobial agent that has enabled plants to survive [8]. The plant-derived photochemical with therapeutic properties could be used as single therapeutic agent or as combined formulations in drug development [9]. Hence medicinal plants are important substances for the study of their traditional uses through the verification of pharmacological effects and can be natural sources that act as a disease curing agents [10].
The complete phytochemical profile of a given plant species can be investigated, fractionation of a crude extract is desirable to separate the main classes of phytoconstituents from each other through chromatographic analysis. The separation and purification of plant constituents is mainly carried out one or more chromatographic techniques [11]. The choice of technique depends largely on the solubility properties and volatilities of the compounds to be separated [12].
Medicinal plants in the Western Ghats contains rich mega biodiversity due to numerous plants species with significant biological properties that acts as a potential source of phytoconstituents. Many reports stated that Asteraceae family is considered as abundant flowering plants with many therapeutic properties and reported as folkloric medicinal plants [13]. The genus, *Gnaphalium* was reported to have antioxidant, antibacterial, antifungal, insect anti-feedant, cytotoxic, anti-inflammatory, anti-diabetic and anti-hypouricemic properties [14] due to the presence of flavonoids, phenolic glycosides, phytosterols and other major phytocompounds. The genus *Gnaphalium*, an herb distributed worldwide, comprises approximately 200 species of the Compositae (Asteraceae) family that belongs to the tribe Gnaphalieae. *G. polycaulon* is an annual widespread weed in tropical and subtropical Africa, Asia, Australia, and America [15]. *G. polycaulon* are grown in moist climatic areas due to its taxonomic characteristics [16]. The literature survey reported that there are limited research works on *Gnaphalium* sp. The main objectives of the study was to investigate the phytochemicals constituents quantitatively by TLC analysis.

Materials and Methods
Chemicals required
All chemicals used for this study were high quality analytical grade reagents. The solvents such as methanol, acetone, ethanol and hexane were purchased from S.D. Fine

Chemicals Pvt. Ltd, Sigma chemicals, Lobe chemicals, Merck Chemical Supplies, Nice Chemicals and Himedia. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

Collection and Preparation of plant samples

The presence of this plant was collected among very few locations of Western Ghats and in Kothagiri (The Nilgiri District, Tamil Nadu, India). Following the collection, the plant samples were identified and authenticated. In order to remove the debris and dust particles, collected plant materials were gently washed with sterile distilled water without damaging their parts. The standard protocols were followed to extract phytoconstituents from fresh and dried plant parts (leaves, stems and flowers) by hexane as solvents [17].

Resulting extracts were individually concentrated by applying vacuum to get dry powder using rotary evaporator and subjected to lyophilizaion. The obtained crude extracts were packed in air-tight plastic containers and stored in refrigerator at 4 ºC for further applications.

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 [18]. 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 725 nm against a reagent blank. Total phenolic contents were determined as a Gallic acid equivalent (GAE) based on Folin-Ciocalteau calibration curve 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.

Determination of Total Flavonoid Content

The flavonoid content of the plant extract was determined by a colorimetric method [19] with minor modification. Different concentration of the plant extract was prepared by diluting the stock solution (4000 µg/ml) with deionized water. Each sample (100 µl) was diluted with distilled water (200 µl). Sodium nitrite (5 %; 30 µl) was added to the samples and then at 5 min, aluminum chloride (10 %; 30 µl) and at 6 min, sodium hydroxide (1M; 200µl) were added to the mixture. Finally, 400 µl of deionized water was added. The absorbance was recorded at 510 nm. 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 Harborne method [7]. 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. Total alkaloid contents were determined using Gallic acid equivalent (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

Determination of Tannin

The Tannin was determined by the Van-Burden and Robinson method [20]. 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 5 ml of the filtered was pipetted out into a test tube and mixed with 2 ml 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 equivalent (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 Obadoni and Ochuko [21]. Twenty grams of plant sample were put into a conical flask and 100 cm^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 200 ml 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/min) for 24 h [22]. The condensed filtrate was used for chromatography. The phenols were separated using chloroform and methanol (27:3) 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, ethanol and hexane 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.

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

Results and Discussion

Quantitative analysis of phytoconstituents

The total phenolic, tannin, alkaloid, flavonoids and saponin content were found to be 0.73, 0.44, 0.23, 0.75, and 0.28 in the hexane extract of dried leaf than other extracts. The results for quantitative analysis of phytochemicals were reported in Table 1.

Table 1: Quantitative analysis of various hexane extracts of *G. polycaulon*

Plant parts	Quantitative assay of various hexane extracts of *G. polycaulon*				
	TPC (mg/g)	**TTC** (mg/g)	**TAC** (mg/g)	**TFC** (mg/g)	**TSC** (mg/g)
Fresh Leaf	0.48	0.38	0.21	0.65	0.26
Fresh Stem	0.13	0.33	0.14	0.62	0.18
Fresh Flower	0.11	0.1	0.09	0.43	0.11
Dried Leaf	0.73	0.44	0.23	0.75	0.28
Dried Stem	0.46	0.26	0.21	0.45	0.21
Dried Flower	0.24	0.29	0.2	0.38	0.16

TPC: Total Phenolic Content (mg/g); TTC: Total Tannin content (mg/g); TAC: Total Alkaloid content (mg/g); TFC: Total Flavonoid content (mg/g); TSC: Total Saponin content (mg/g)
All the values are means of three independent determinations, n=3, analyzed in triplicate.

Phytochemicals are produced for protection and repair the biological processes within the natural environment. The diverse uses of plants in the treatment of various diseases are attributable to the presence of the phytoconstituents [23]. The estimation of phytoconstituents of selected plant showed that leaf were rich in phenols, tannins, alkaloids, flavonoids, and saponins than stem and flower that are responsible for medicinal and physiological activity [24]. The differences in total phenolic, tannins, alkaloids, flavonoids, and saponins contents of each extracts were significant ($p < 0.01$) [18]. Although phytoconstituents are found to be responsible for antioxidant activities, antioxidant effects do not always correlate with the presence of large quantities of all phytoconstituents [25].

Phenolic content act as antioxidants as free radical-scavenging, oxygen radical absorbance, and chelating of metal ions. Several phenolic studies from plant sources have reported the antioxidant and antimicrobial properties [26] in medicinal plants due to rich in phenolic compounds for herbal medicament. Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and also in cancer prevention [20]. Thus, estimation of tannins contents of *G. polycaulon* may serve as a potential source of bioactive compounds in the treatment of cancer, anti-diarrhoeal activity and prevent oxidative stress related disorders [14].

Alkaloids are recognized for their biological activity that are associated with medicinal uses for centuries [7] and tend to lower the risk of poisoning by the plant. The presence of alkaloids, and flavonoids in plant parts are the main constituents responsible for the therapeutic value of the medicinal plants.

Phytosterols, anthraquinones, and chalcone derivatives [27], Diterpenoids, terpenes and Diterpenes, flavonoids [28], acetylenic compounds and carotenoids [29] derivatives were previously isolated constituents reported in *Gnaphalium* species. This previous report strongly supported the presence of phytoconstituents in *G. polycaulon* extracts. Flavonoid content showed its effective antioxidant, anti-inflammatory and anticancer activities due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall.

Saponins are glycosides occurring widely in plants [21] and used as antibiotic, antiviral, anti-inflammatory [30] and anti-ulcer. The results obtained in preliminary phytochemical screening of the different extracts showed the presence of various phytoconstituents that acts as bioactive agents to cure the wounds, antioxidant and antimicrobial agents, and pharmacological activities in the present study. Further research work is required to investigate all extracts of plant parts of *G. polycaulon* for various phytoconstituents and pharmacological activities.

Thin layer chromatography (TLC) analysis

The appearance of green and yellow colored spots indicated the presence of phenols, alkaloids, flavonoids, steroids, saponins and glycosides in fresh and dried hexane extracts of *G. polycaulon* and its retention factors, R_f values were tabulated in Table 2.

Table 2: R_f values of TLC in various hexane extracts of *G. polycaulon*

Plant parts	R_f values (mm)					
	Alkaloids	**Flavonoids**	**Saponins**	**Glycosides**	**Phenol**	**Steroids**
Fresh Leaf	0.39	0.41	0.44	0.8	0.72	0.51
Fresh Stem	0.31	0.37	0.43	0.61	0.69	0.36
Fresh Flower	0.28	0.3	0.21	0.58	0.65	0.24
Dried Leaf	0.42	0.45	0.49	0.87	0.73	0.54
Dried Stem	0.33	0.31	0.47	0.66	0.71	0.44
Dried Flower	0.31	0.28	0.42	0.61	0.67	0.33

All the values are means of three independent determinations, n=3, analyzed in triplicate.

The dried hexane leaf extract of *G. polycaulon* exhibited maximum R_f values with green coloured spots indicated the presence of major phytoconstituents than other extracts.

The appearance of colored spots on the TLC plate indicated the presence of phenols, alkaloids, flavonoids, steroids, saponins and glycosides in different fresh and dried parts of

G. polycaulon due to the presence of major phytoconstituents. The complete phytochemical profile is subjected to separate the main classes of phytoconstituents through TLC chromatographic analysis [11]. Various chromatographic systems are also useful for the identification separation and quantification of phytoconstituents [31]. TLC studies was used to analysis, identify, and elucidate the structure of the bioactive compounds of medicinal plants which are responsible for the antimicrobial activity and other medicinal values [32]. Thin layer chromatography is a globally accepted practical solution to characterize raw herbs, active constituent-enriched extracts and their formulations [33].

Conclusion

Based on the wide medicinal applications of *G. polycaulon*, various parts (both fresh and dried leaf, stem and flower) have been selected to quantify the phytoconstituents. The hexane extract of dried leaf showed significant major phytoconstituents followed by the aqueous extract of fresh leaf. Such kind of plants should be given with lot of importance and their bioactive properties should be studied in an elaborative manner and documented.

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