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Quantification of bioactive compounds in selected medicinal plants of Dakshina Kannada district

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Abstract
Introduction: Medicinal plants have been the main stay of traditional herbal medicine amongst rural dwellers worldwide since antiquity to date. The present study was conducted to quantify the bioactive compounds present in leaf methanol extract of selected medicinal plants.
Methods: Primary and secondary metabolites like carbohydrate, protein, fat, L-ascorbic acid, phenolic, alkaloids, flavonoid, tannin and saponin content was determined by following standard procedures.
Results: *C. papaya* showed highest protein (884.2±0.1 µg/ml), fat (8.616±0.104 %), ascorbic acid (162.96±0.05 mg/g), phenolic (255.82±0.01 mg/g) and tannin (32.46±0.408 mg/g) content. *K. pinnata* showed high amount of carbohydrate (45.13±0.11 mg/100ml) and saponin (99.67±0.323 %) content. *A. ficoidea* showed high alkaloid (12.11±0.01 mg/g) content and *E. perfoliatum* showed highest flavonoid (222.46±0.202 mg/g) content.
Conclusion: The bioactive compounds such as phenolics, flavonoids and tannins which will contribute to the antioxidant and antimicrobial activity of these plants. They also play important role in the growth and development, including protection from predators and environmental stress.

Keywords: Bioactive compounds, medicinal plants, primary metabolites, secondary metabolites.

Introduction
Biologically active compounds derived from plants have become an important source of drugs due to the increasing recognition of herbal medicine as an alternative form of health care [1]. Traditional medicinal plants, spices, vegetables and fruits have been found to protect human beings from acute and chronic diseases such as cancer and cardiovascular disease [2]. Different bioactive compounds and microelements from different dietary functional foods can be used as preventive and therapeutic medicines [3, 4].
The pharmacological action of crude drug is determined by the nature of its constituents. Thus the plant species may be consider as a biosynthetic and for the chemical compounds example proteins, carbohydrates, and fats that are utilized as food by the animals and humans, but also for a huge number of compounds including alkaloids, terpenoids, flavonoids, glycosides etc. which exert definite physiological effects. These chemical compounds are mostly responsible for the desired beneficial properties [5].
Pure isolated alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects [6, 7]. Flavonoids, on the other hand are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anticancer activity [8-10]. As antioxidants, flavonoids from these plants provide anti-inflammatory activity [10]. Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases. For example, alkaloids protect against chronic diseases. Saponins protect against hypercholesterolemia and antibiotic properties. Steroids and triterpenoids show the analgesic properties. The steroids and saponins were responsible for central nervous system activities. The importance of alkaloids, saponins and tannins in various antibiotics used in treating common pathogenic strains has recently been reported. Earlier records showed that bitter leaf contains an alkaloid which is capable of reducing headaches associated with hypertension [11, 12].

Polyphenolic flavonoids occurring as glycosides were found to have strong antioxidant properties, to be effective in scavenging free radicals and reactive oxygen species with their phenolics hydroxyl groups [13] and to decrease the risk of diseases such as cardiovascular and degenerative diseases by counteracting macromolecular oxidation [14, 15]. Gallic acid (3, 4, 5-trihydroxybenzoic acid) is an endogenous plant phenolic compound that is found in many phytomedicines [16]. Many gallic acid rich plants exhibit protective effects against liver injury [17-19] and they have been found to have various pharmacological properties, including neuroprotective, antioxidant [20], anti-inflammatory [21, 22], antiobesity [23-25] and anticancer [26] properties.

Several studies have reported that gallic acid and its derivatives can selectively induce cancer cell death by apoptosis without harming healthy cells [27-29]. A major class of phenolic compounds is hydroxycinnamic acids, which are found in almost every plant and the major representative of hydroxycinnamic acids is caffeic acid [30, 31]. Caffeic acid possesses both antioxidant and prooxidant properties. The prooxidant action of polyphenols may be an important mechanism in their anticancer and apoptosis-inducing properties [32, 33].

So the objective of my study to quantify the bioactive compounds present in leaf methanol extract of selected medicinal plants.

Materials and Methods

Alternanthera ficoidea L., *Carica papaya* L., *Eupatorium perfoliatum* L., *Kalanchoe pinnata* L., *Mussaenda belilla* L., and *Talinum fruticosum* L., were collected from in and around the area of Mangalore and Belthangady (Dakshina Kannada, District). The plants were identified and authenticated by the botanist of Department of Applied Botany, Mangalore University. These leaf samples were separated from each plant and were washed with running tap water, surface sterilized in distilled water, blot it dried and powdered. 150 grams of the samples were weighed and extraction was carried out using soxhlet apparatus. The extracts obtained from the solvents were concentrated using rotary vacuum evaporator and then dried. The yielded crude extracts were stored in a deep freezer at -20 ºC for the further study. Since most of bioactive compounds were qualitatively determined in methanol extract. Methanol leaf extract was used for quantification of bioactive compounds in selected plant materials.

Quantification of primary metabolites

Total carbohydrate

The total carbohydrate content was estimated by the anthrone method [34]. 100 mg of the dried powder sample was hydrolysed with 5.0 ml of 2.5 N HCl in a test tube and kept it in a boiling water bath for three hours. The cooled to room temperature and neutralized with solid sodium carbonate until the effervescence ceases out. The sample was made up to 100 ml, centrifuged and supernatant was collected. 0.2-1.0 ml of glucose served as the working standards. To 0.5 and 1.0 ml test sample was made up to 1.0 ml with distilled water. To this solution, 4.0 ml of anthrone reagent was added, heated for eight minutes in a boiling water bath and cooled rapidly. Simultaneously a blank was prepared with 1ml of distilled water and 4 ml of anthrone reagent. The dark green colour was read at 630 nm. Calibration curve was constructed by plotting the glucose concentration on x-axis and absorbance on the y-axis. From the graph the concentration of glucose in the sample was calculated.

$$\text{Amount of carbohydarte present in 100 mg of the sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

Total fat content

2 g of powdered sample was dissolved in chloroform: methanol solution (1:1) overnight and filtered. The fat content of the solution was calculated according to the method described [35].

$$\%\ \text{of sample} = \frac{\text{Weight of the extract}}{\text{Weight of the sample}} \times 100$$

Total protein

Protein content in sample was determined by using Lowry's method [36]. 500 mg of powdered sample was mixed with 10 ml of the phosphate buffer and centrifuged. Supernatant was used for protein estimation. 0.2 ml to 1ml of the working standard (Bovin Serum Albumin) was pipetted out into a series of test tubes. 0.2 ml of test sample was taken and made up to 1ml with distilled water. 1 ml of distilled water serves as the blank. 5 ml of Folin-ciocalteau reagent was added, mixed thoroughly and incubated at room temperature in the dark for 30 min. Blue colour was read at 660 nm. The amount of protein content was calculated using standard graph and expressed in mg/g.

Secondary metabolites

L-Ascorbic acid content

Ascorbic acid content in the samples was estimated by the method of Roe and Keuther [37]. 1g of powdered sample extract was homogenized in 10 ml of 4% TCA and centrifuged at 2000 rpm for 10 min. The supernatant was treated with a pinch of activated charcoal, shaken well and kept it for 10 min. Centrifugation was repeated twice to remove the charcoal residues. The volume of the clear supernatants obtained was noted. 0.5 ml and 1 ml of the supernatant was taken for the assay and the volume was made up to 2.0 ml with 4% TCA. Series of working standard ascorbic acid solution (0.2 to 1.0 ml) was made up to 2.0 ml with 4% TCA and to this 0.5 ml of Dinitrophenylhydrazine (DNPH) reagent and two drops of 10% thiourea solution to all the test tubes. The osazones formed after incubation at 37 °C for 3 hrs, were dissolved in 2.5 ml of 85% H_2SO_4 in ice cold, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea solutions were added after the addition of 85% H_2SO_4. After incubation for 30 min. at room temperature, the solutions were read at 540 nm and the levels of ascorbic acid in the samples were determined using the standard graph and expressed as mg of ascorbate /g extract.

Total alkaloids

Total alkaloids were measured by the method of Harborne [38]. 10 mg of plant material was homogenized in 20 ml of methanol: ammonia (68:2), decanted the ammonical solution after 24 hrs and added fresh methanolic ammonia. The procedure was repeated thrice and pooled the extracts, evaporated the extracts using a flash evaporator, treated the residue with 1N HCl and kept it overnight. Acidic solution

was extracted thrice with 20 ml of $CHCl_3$, pooled the organic layers and evaporated to dryness. The basic fraction basified the acidic layer with Conc. NaOH to pH-12 and extracted with $CHCl_3$ (20 ml) thrice, pooled the $CHCl_3$ layers, dry over absorbent cotton and evaporated to dryness, weighed the fraction that contains ajmalicine and serpentine expressed as mg/100g of extract.

Total phenolic

The total phenolic content was determined using Folin–Ciocalteu (FC) method [39]. 0.1 ml of sample extract was made up to 0.25 ml with distilled water and mixed with 0.25 ml of FC phenol reagent. After 3 min, 0.5 ml of 20% sodium carbonate solution was added to the mixture and made up to 5 ml by adding distilled water. The resultant mixture was kept in the dark for 30 min, after which its absorbance was read at 760 nm. The results were expressed as µg of gallic acid equivalents/mg of the sample extract.

Flavonoid content

0.5 ml of the sample extract was mixed with 1.5 ml of methanol, 0.1 ml of 10% $AlCl_3$ followed by 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The amount of flavonoid content was expressed as µg of quercetin/mg of extract [40].

Tannin content

The tannin content was estimated by the method of Price and Butler [41] with slight modifications. 20 µl of the sample was aliquoted into a test tube containing 980 µl of distilled water. To this, 500 µl of 1% $K_3Fe(CN)_6$ and 100 µl of 1% ferric chloride ($FeCl_3$) were added and made up to 3 ml with distilled water. After 10 min, the reaction mixture was measured using a UV spectrophotometer at 720 nm. The tannin content was expressed as µg of tannic acid equivalents/ mg of the sample extract.

Saponin content

Saponin content of sample was determined by the method of Nahapetian and Bassiri [42] with slight modification. 20 g of plant sample extract was dispersed in 200 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 hrs with continuous stirring at about 55 °C. The mixture was filtered and the residue was re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 °C. The concentrated extract was transferred into a 250 ml separating 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 to aqueous layer and washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample was dried in the oven into a constant weight. The saponin content was calculated in percentage.

Statistical analysis

The experimental results were expressed as mean ± Standard Error Means (SEM) of triplicates. Analysis of data was carried out by applying alkaline copper solution was added to all the test tubes, mixed well and allowed to stand for 10 min. Then 0.5 ml of independent‘t’ test and one-way analysis of variance (ANOVA) using SPSS. P-value lesser than 0.05 (p<0.05) was considered as statistically significant.

Result and Discussion

Results of the quantitative analysis of primary and secondary metabolites of selected medicinal plants were represented in the table 1.

Table 1: Quantitative analysis of primary and secondary metabolites in leaf extract of selected medicinal plants

Bioactive compounds	*A. ficoidea*	*C. papaya*	*E. perfoliatum*	*K. pinnata*	*M. belilla*	*T. fruticosum*
Carbohydrate (mg/100ml)	29.1±0.1	45.1±0.1	35.66±0.11	45.13±0.11	24.46±0.41	18.8±0.17
Protein (µg/ml)	692.1±0.1	884.2±0.26	548.03±0.15	292.3±0.3	356.1±0.15	224.4±0.41
Fat (%)	6.51±0.010	8.616±0.104	2.93±0.060	5.33±0.293	4.66±0.030	3.53±0.026
L-Ascorbic acid (mg/g)	37.06±0.11	162.96±0.05	48±1	87.8±0.72	11.2±0.2	69.1±0.26
Total Alkaloid (mg/g)	12.11±0.01	10.31±0.02	11.76±0.033	10.38±0.012	10.42±0.002	9.27±0.030
Total Phenolic (mg/g)	189.44±0.411	255.82±0.01	173.17±0.124	157.38±0.364	112.98±0.522	226.20±0.105
Total Flavonoid (mg/g)	70.56±0.011	76.88±0.07	222.46±0.202	42.49±0.052	52.49±0.005	91.42±0.085
Total Tannin (mg/g)	32.23±0.195	21.13±0.157	31.10±0.105	28.47±0.066	32.46±0.408	31.14±0.026
Total Saponin (%)	92.46±0.007	55.51±0.040	52.62±0.248	99.67±0.323	9.54±0.037	99.39±0.081

Values are expressed as mean ± SEM, n=3, p< 0.05 considered as significant.

Data are expressed as mean±SEM. Independent‘t’ test was used to compare the carbohydrate, fat and proteins in the selected parts of medicinal plants. p=0.001, α=0.05, p<0.05 there was a significant difference between the tests. Since ‘p’ value was equal to 0.001 was < than our chosen significant level α=0.05. We concluded that the mean of the different tests i.e., anthrone method for carbohydrate, fat content, Lowry’s content method for protein content was significantly different. One-way ANOVA statistics were used to compare the samples between all secondary metabolites. p=0.001, α=0.05, p< 0.05 was considered as significant.

Organic compounds produced in the plant kingdom have metabolic functions essential for plant growth and development produced in every plant [43]. Carbohydrate is one such group of carbon compounds, which are essential to life. Almost all organisms use carbohydrates as building blocks of cells and as a matter of fact, exploit their rich supply of potential energy to maintain life [44]. Proteins are essential to maintaining the structure and function of all life and vital for growth and development. The presence of higher protein level in the plants parts points towards their possible increase in food value or that a protein based bioactive compound could also be isolated in future [45]. Plant sugars can be used as artificial sweetener and they can even help in diabetes by supporting the body in its rebuilding [46].

Vitamins are essential to maintain normal metabolic processes and homeostasis within the body. The amount of a specific vitamin required by an individual varies

considerably and it is influenced by such factors as body size, growth rate, physical activity, and pregnancy [47]. Ascorbic acid (vitamin C) has been gaining attention as a potential treatment for human malignancies. Various experimental studies have shown the ability of pharmacological doses of vitamin C alone or in combinations with clinically used drugs to exert beneficial effects in various models of human cancers [48].

The alarming increase in the rate of infection by antibiotic-resistant microorganisms has urged scientists to search for compounds which have potential antimicrobial activity [49]. The ability to synthesize compounds by secondary metabolism possessing antimicrobial potential makes plants an invaluable source of pharmaceutical and therapeutic products [50]. More than 100 phytochemical compounds have been isolated from various parts of the plant, namely phenols, flavonoids, alkaloids, cardiac glycosides, saponins, terpenoids, steroids, and tannins. These compounds are well known to possess biological and pharmacological activity against various chronic diseases such as cancer and cardiovascular and gastrointestinal disorders [51-54]. Due to the presence of various compounds that are essential for good health, it can also be used to improve the health status of society [55].

Conclusions

Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples. If the plant was selected on the basis of traditional uses [56], then it is needed to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as TLC, column chromatography, flash chromatography, sephadex chromatography and HPLC, should be used to obtain pure compounds. The pure compounds are then used for the determination of structure and biological activity [57].

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