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EFFECT OF GEOGRAPHICAL DISTRIBUTIONS ON THE PHYTOCHEMICAL PROFILE OF METHANOLIC EXTRACT OF DRIED LEAVES OF *ROSMARINUS OFFICINALIS* L.

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ABSTRACT

Rosmarinus officinalis L. (Rosemary) is a medicinal herb belongs to Lamiaceae family have shown a variety of pharmacological activities as cancer chemoprevention and therapy using *in vitro* and *in vivo* models. The study was conducted to the herb that was chosen from different locations (named as RO1, RO2, RO3, RO4 and RO5) with respect to qualitative and quantitative phytochemical analysis. Preliminary phytochemical screening reveals that the presence of phytoconstituents including alkaloids, phenols, cardiac glycosides, saponins, flavonoids, tannins, terpenoids, steroids, carbohydrates, proteins, coumarins and quinines, while anthraquinones was absent in all the samples collected from different locations of kodaikanal hills. RO3 sample was found to contain high level of phenolic content (62.39 mg/g) followed by RO2 (55.53 mg/g) and RO1samples (53.51 mg/g), while RO4 (44.36 mg/g) and RO5 samples (40.75 mg/g) contain low level of phenolics. The total flavonoid content was found to be maximum (35.18 mg/g) in RO3 followed by RO2 (26.17mg/g) and RO1 (23.28 mg/g) samples and minimum in RO4 (18.94 mg/g) and RO5 (17.75 mg/g) samples. The total tannin content was found to be maximum (29.70 mg/g) in RO3 followed by RO2 (22.28 mg/g) and RO1 (21.48 mg/g) and minimum in RO4 (18.16 mg/g) and RO5 (15.86 mg/g). The total carbohydrate content was maximum (31.85 mg/g) in RO3 followed by RO2 (24.88 mg/g), RO1 (23.75 mg/g), RO4 (19.92 mg/g) and RO5 (17.64 mg/g). The protein content was maximum (25.20 mg/g) in RO3 followed by RO2 (18.16 mg/g), RO1 (16.00 mg/g), RO4 (13.18 mg/g) and RO5 (11.59 mg/g).

KEY WORDS: *Rosmarinus officinalis* L., Lamiaceae, Phytoconstituents, Medicinal herb.

INTRODUCION

Medicinal plants are major bio resources of folk medicine, food supplement, nutraceutical, pharmaceutical and chemical entities of synthetic drugs.[1] One fourth of the World population is dependent on traditional medicines, since time immemorial. Plant drug for curing various ailments due to safe and without any adverse side effect when compared to synthetic drugs. Phytomedicine can be derived from barks, leaves, flowers, roots, fruits and seeds.[2] Rosemary (*Rosmarinus officinalis* L.) is a spice and medicinal herb belongs to the Lamiaceae family and receives an increasing attention due to its antimicrobial, anti-inflammatory and antioxidative constituents.[3] It is native to the Mediterranean region; however, it has been cultivated throughout the world and accepted as one of the spices with highest antioxidant activity.[4] *R. officinalis* is used for the treatment of various human diseases such as coronary atherosclerosis, Alzheimers disease, cancer, as well as in ageing processes. Many studies have shown that antioxidant activity of rosemary extract is mainly due to the presence of rosmarinic acid (RA) and carnosic acid (CA), which are both responsible for the anti inflammatory and antioxidant properties.[5]

Recent advances in plant sciences have led to great interest in increasing the production of plant secondary metabolites for their medicinal and aromatic uses.[6] and its production is enhanced by the genetics and cultivation conditions such as climate, plant density and use of fertilizers.[7] Plants respond to an adverse ecosystem by altering their morphology, physiology, and biochemistry.[8] Some of the adaptations to stress may include the changes in both the nature and levels of primary and secondary metabolites.[9]

To the best of our knowledge, there is no comparative work has been published on chemical composition of leaves of Indian *R. officinalis* collected at five different locations of Kodaikanal hills with respect to the impact of geographic variation. The aim of this study was to determine the phytochemicals present in the rosemary leaves were collected from five different locations of Kodaikanal hills, Dindigul district, Tamil Nadu, India.

MATERIALS AND METHODS

Collection of Plant material

R. officinalis leaves were collected from five different locations of western ghats of Kodaikanal hills namely, Shenpaganur (2100m above the sea level), Kodaikanal town (2200m above the sea level), Observatory (2343m above the sea level), Attuvampatti (2000m above the sea level) and Adesarai (1900m above the sea level). The taxonomic identity of the plant was confirmed by Botanical Survey of India, Southern circle, Coimbatore, Tamil Nadu. The collected leaves samples were rinsed with tap water dried and powdered and then stored at 4 °C.

Plant extracts preparation

5g of each sample of *R. officinalis* was extracted with 100 ml of methanol using Soxhlet apparatus. The obtained extract was filtered and the methanol was evaporated by rotary evaporator and then stored at 4°C for future use.

Preliminary Phytochemical Screening

The methanolic extracts of plant samples were subjected to different chemical tests for the detection of different phytoconstituents using standard procedures.[10, 11]

Test for Phenols

To 1 ml of the extract, 3 ml of distilled water followed by few drops of 10% aqueous Ferric chloride solution was added. Formation of blue or green colour indicates the presence of phenols.

Test for Flavonoids

To 2 ml of the extract, 1 ml of 1% ammonia solution was added. Appearance of yellow colour indicates the presence of flavonoids.

Test for Tannins

To 1 ml of the extract, 1 ml of 0.008 M Potassium ferricyanide was added and then add 1 ml of 0.02 M Ferric chloride containing 0.1 N HCl. Appearance of blue-black colour indicates the presence of Tannins.

Test for Alkaloids

1 ml of crude extract was mixed with 2 ml of Wagner's reagent. Reddish brown colored precipitate indicates the presence of alkaloids.

Test for carbohydrates

a. Fehling's test

Equal volume of Fehling A and Fehling B reagents were mixed together and then add 2ml of crude extract in it and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicates the presence of reducing sugars.

b. Benedict's test

1 ml of crude extract was mixed with 2ml of Benedict's reagent and boiled. A reddish brown precipitate was formed which indicates the presence of the carbohydrates.

Test for proteins

a. Millon's test

1 ml of crude extract was mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

b. Ninhydrin test

1 ml of crude extract was mixed with 2ml of 0.2% solution of Ninhydrin and boiled. A violet colour precipitate was appeared suggesting the presence of amino acids and proteins.

Test for Cardiac glycosides (Keller-Kiliani test)

5 ml of extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Test for Saponins

2 ml of crude extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously. Add some drops of olive oil. The formation of stable foam was taken as an indication for the presence of saponins.

Test for Coumarin

10 % Sodium hydroxide was added to the extract and chloroform was added. Formation of yellow color shows the presence of Coumarin.

Test for Terpenoids (Salkowski test)

5 ml of extract was mixed with 2 ml of chloroform and 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish brown colouration of the inter face was formed which indicates the presence of terpenoids.

Test for Steroids

2 ml of acetic anhydride was added to 0.5 ml of crude extract containing 2 ml of sulphuric acid. The colour changed from violet to blue or green in samples indicates the presence of steroids.

Test for Quinones

Diluted sodium hydroxide was added to the 1 ml of crude extract. Blue green or red coloration indicates the presence of quinones.

Test for anthraquinones (Borntragers test)

0.5 g of each extract was boiled with 10% hydrochloric acid for few minutes in water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% ammonia was added to the mixture and heated. Formation of rose – pink color indicates of n-hexane, chloroform, ethyl acetate and methanol of the presence of the anthroquinones.

Quantitative Determination of Phytochemical Constituents

Total phenolic content

The total phenolic content was determined according to McDonald *et al*. (2001).[12] To 1 ml of plant extract or standard, 5 ml of Folin Ciocalteau reagent and 4 ml of sodium carbonate were added. The mixture was kept for 15 min under room temperature. The blue colour formed was read at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated by calibration curve of gallic acid and the results were expressed as gallic acid equivalent (mg/g).

Total Flavonoid content

The total flavonoid content was determined by the method of Chang *et al*. (2002).[13] To 0.5 ml of plant extract or standard was mixed with 4.5 ml of methanol. 0.1 ml of 10% aluminium chloride and 0.1 ml of 1M sodium acetate were added to the mixture. Then the reaction mixture was kept at room temperature for 30 min. Then the absorbance was read at

415 nm by UV/visible spectrophotometer. The flavonoid content was calculated by calibration curve of quercetin and the results were expressed as quercetin equivalent (mg/g).

Total Tannin content

The total tannin content was determined according to Schanderl (1970).[14] To 1 ml of the plant extract or standard was mixed with 0.5 ml Folin's phenol reagent and then added 5 ml of 35% sodium carbonate and the mixture was kept for 5 min at room temperature. The blue color produced was read at 640 nm using UV/visible spectrophotometer. The tannin content was calculated by calibration curve of gallic acid and the results were expressed as gallic acid equivalent (mg/g).

Total carbohydrate content

The total carbohydrate content was determined by Hedge and Hofreiter method.[15] To 0.5 ml of plant extract or standard, added 0.5 ml of distilled water and 4 ml of anthrone reagent. The mixture was heated for 8 min in boiling water bath and cooled. The green colour developed was read at 630 nm using UV/visible spectrophotometer. The carbohydrate content of the plant samples were calculated from the calibration curve of glucose and the results were expressed as mg/g of glucose equivalents.

Total protein content

The total Protein content of the plant extracts were measured by Lowry's method.[16] To 1 ml of the plant extract or standard, added 5 ml of alkaline copper sulphate reagent, mixed well and allowed to stand for 10 min and then added 0.5 ml of Folins-Ciocalteau's reagent and mixed well. The reaction mixture was allowed to stand at room temperature under dark for 30 min. The blue color developed was read at 660 nm using UV/visible spectrophotometer. The protein content of the plant extract was calculated from the calibration curve of Bovine Serum Albumin (BSA) and the results were expressed as mg/g of BSA equivalents.

Statistical analysis

All determinations were based on triplicate measurements and the results were expressed as means ± standard error. The data were evaluated by one-way ANOVA and the significance of the difference between means was determined by Duncan's multiple range test. Differences at $P < 0.05$ were considered statistically significant. The SPSS 20.0 (Chicago, Illinois, USA) was used to perform statistical analysis.

RESULTS AND DISCUSSION

Medicinal plants comprise the group of plants which is mainly used for health care. All plants contain primary and secondary metabolites. They are commonly used in the human therapy, veterinary, agriculture, scientific research and countless other areas.[17] Information of the chemical constituents of plants is desirable in order to synthesize complex chemical substances.[18,19,20] The preliminary phytochemical screening was carried out on the methanolic extract of all the samples revealed the presence of a wide range of phytoconstituents including alkaloids, phenols, cardiac glycosides, saponins, flavonoids, tannins, terpenoids, steroids, carbohydrates, proteins, coumarins and quinones, while anthraquinones was absent in all the samples of *R. officinalis* collected from Kodaikanal hills as shown in table 1.

Table1.Qualitative phytochemical analysis of methanolic extract of *Rosmarinus officinalis*

S.No	Phytoconstituents	TEST	RO1	RO2	RO3	RO4	RO5
1.	Phenol	FeCl3 test	+++	++	+++	++	++
2.	Flavonoids	Shinoda test	++	+++	+++	++	++
3.	Tannins	FeCl3 test	++	++	+++	++	++
4.	Alkaloids	Wagner's reagent	+	+	+	+	+
5.	Carbohydrates	Fehling's test Benedict's test	++	+++	+++	++	++
6.	Proteins	Millon's test Ninhydrin test	++	++	+++	++	++
7.	Glycosides	Keller-Kiliani test	+	+	+	+	+
8.	Saponins	Foam test	+	+	+	+	+
9.	Coumarins	Coumarins Test	+	+	++	++	++
10.	Terpenoids	Salkowski test	++	++	++	+	+
11.	Quinones	Quinone test	+	+	++	+	+
12.	Steroids	Salkowski test	+	+	+	+	+
13.	Anthraquinones	Borntragers test	-	-	-	-	

Key: RO1=Senphaganur; RO2=Kodaikanal Town; RO3=Observatory; RO4=Attuvampatti; RO5=Adesarai; +++= Copiously present; ++= moderately present; + =slightly present; - = absent.

During present investigation, the phytochemicals including phenols, flavonoids, tannins carbohydrates and proteins were estimated in five different samples of *R. officinalis* collected from Kodaikanal hills as shown in table 2.

Table 2. Quantitative phytochemical analysis of methanolic extract of *Rosmarinus officinalis* L.

Sample	Phenols (mg/g)	Flavonoids (mg/g)	Tannins (mg/g)	Carbohydrates (mg/g)	Proteins (mg/g)
RO1	53.51 ± 0.32^{c}	23.28 ± 0.30^{c}	21.48 ± 0.68^{c}	23.75 ± 0.16^{b}	16.00 ± 0.44^{c}
RO2	55.53 ± 0.29^{b}	26.17 ± 0.43^{b}	22.28 ± 0.23^{b}	24.88 ± 0.26^{b}	18.16 ± 0.23^{b}
RO3	62.39 ± 0.42^{a}	35.18 ± 0.37^{a}	29.70 ± 0.28^{a}	31.85 ± 0.38^{a}	25.20 ± 0.61^{a}
RO4	44.36 ± 0.23^{d}	18.94 ± 0.33^{d}	18.16 ± 0.33^{d}	19.92 ± 0.51^{c}	13.18 ± 0.42^{d}
RO5	40.75 ± 0.62^{e}	17.75 ± 0.24^{e}	15.86 ± 0.19^{e}	17.64 ± 0.23^{d}	11.59 ± 0.35^{e}

Key

Mean values ± standard error with the same letters within the same column are not significantly different at p> 0.05.

Among the samples analysed, RO3 sample was found to contain high content of phenolics (62.39 mg/g) followed by RO2 (55.53 mg/g) and RO1 (53.51 mg/g), while RO4 (44.36 mg/g) and RO5 (40.75 mg/g) contain low content of phenolics when compared to RO3, RO2 and RO1. Environmental factors have been identified as responsible for changes and determination of the secondary metabolites in a plant.[21] An increase in the phenolic content with increase in altitude may be attributed as a response of plants to enhanced UV-B radiation and decreased temperatures.[22] Erkan *et al.* (2008).[23] Hernández-Hernández *et al.* (2009).[24] Kontogianni *et al.* (2013).[25] and Ana María Piedrahita *et al.* (2015).[26] reported that the total phenol content of rosemary extract was 162.00, 109.50 mg, 54.6 mg/g and 91.01 mg GAE/g extract, respectively. The content of phenolics in rosemary extract obtained is lower than previously reported in some rosemary extracts from other latitudes.[27,28] excluding Kontogianni *et al.* (2013).[25] reports. Peshev *et al.* (2011).[27] reported that difference in the concentration of rosmarinic acid and carnosic acid of rosemary extract may be due to different factors such as the region of origin and environmental conditions (altitude and latitude) and extraction method. The region of origin and environmental conditions (altitude and latitude) may also play a vital role in phenol production. Phenolics are the largest group of secondary metabolites which medicinal properties and health-promoting effects.[28, 29]

The total flavonoid content was found to be high level (35.18 mg/g) in RO3 followed by RO2 (26.17mg/g) and RO1 (23.28 mg/g) and low level in RO4 (18.94 mg/g) and RO5 (17.75 mg/g). Kontogianni *et al.* (2013).[25] reported that the total flavonoid content of rosemary

extract was 24.6 mg/g. Flavonoids and other phenolic compounds are potent water soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anti cancer activity.[30, 31] In agreement with our results, Sohair Aly Hassan *et al*. (2012).[32] are also reported that difference in antioxidants, phenolics as well as flavonoids of rosemary collected from Egypt and Malaysia may due to the effect of the polarity of the solvent, the geographical factor includes the soil composition and climate.

The total tannin content was found to be higher amount (29.70 mg/g) in RO3 followed by RO2 (22.28 mg/g) and RO1 (21.48 mg/g) and lower amount in RO4 (18.16 mg/g) and RO5 (15.86 mg/g). Mossi *et al*. (2009) suggest the existence of acorrelation between environmental factors such as average annual temperature, climate, vegetation, geomorphology, latitude and altitude and tannin production.[33] Tannins are used in the treatment of inflamed or ulcerated tissues and cancer.[34]

The total carbohydrate content was maximum (31.85 mg/g) in RO3 followed by RO2 (24.88 mg/g), RO1 (23.75 mg/g), RO4 (19.92 mg/g) and RO5 (17.64 mg/g). The protein content was high level (25.20 mg/g) in RO3 followed by RO2 (18.16 mg/g), RO1 (16.00 mg/g), RO4 (13.18 mg/g) and RO5 (11.59 mg/g). The climatic change across altitude could affect the chemical composition and ultimately the survival of some medicinal plants in high altitude regions as the stress particularly the temperature stress can affect secondary metabolites and other compounds that plants produce, which usually are the basis of their medicinal activity.[35]

CONCLUSION

In the current study, the results showed significant variations in the concentration levels of the selected phytochemicals of *Rosmarinus officinalis* with reference to the geographical area collected from kodaikanal hills. Assessing phytochemical content of plant samples at varying altitudes can help to select elite genotype and reflect the best suited altitude for commercial cultivation of the species as these phytochemicals are considered as the basis for their medicinal activity and also no major conclusion could be derived about the direct and individual effect of geographic location on the chemical constituents and biological activities of the plant. They are also affected by soil types, growing conditions, nutrient availability, fertilizer applications, plant's age, variety, climate and treatments etc. Therefore, these variations must be taken into consideration while utilizing raw plant materials for industrial applications and traditional therapies.

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