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Effect of Seasonal Environmental Variations on Morphological and Physicochemical Properties of *Aegle Marmelos*

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Abstract

Background and Aim-

Aegle marmelos L. is a medicinal plant used in traditional formulations for the treatment of various disease conditions. The physicochemical properties fluctuate with the season and in response to stress. To obtain the highest medicinal value it is necessary to collect the plant raw material in the correct season. The present study was aim to evaluate the physiochemical fluctuations in the leaves of *Aegle marmelos* and the aegelin, an antidiabetic constituent in these leaves.

Experimental procedure

Leaves were collected in summer, winter, and rainy seasons and subjected to authentication and standardization. The leaves were evaluated for the macroscopic and microscopic evaluation, quantitative microscopy, proximate analysis, extractive values in petroleum ether, chloroform, ethyl acetate, ethanol, water, and determination of the concentration of antidiabetic component aegelin.

Results and Conclusion

The leaves showed morphological and microscopical variations in different seasons. The phytochemical composition was the same in all seasons however the levels of extractive values fluctuated in response to seasonal variations. Maximum levels of ethanol and water extract were obtained in the winter season also the aegelin concentration was higher in this season. The suitable raw material collection season can be decided based on the present study according to the purpose of utilization of plant material.

Keywords: Secondary metabolites, Herbal medicines, alkaloids, antidiabetic, seasonal variations.

INTRODUCTION

In developed and developing countries about 80% of the population relies on herbal medicines for the treatment of various disease conditions. About 60% of these are utilizing it as medicines directly in the form of traditional medicine or its formulation whereas the 40% material is used in the pharmaceutical industry for manufacturing of formulation using modern techniques. Plants are the integral component of most of the traditional systems of medicine practiced in various countries throughout the world to treat several diseases. Many modern medicines are also obtained from the chemical constituent of herbal origin thus the plants have remained essential therapeutic aid for mankind. The World Health Organization (WHO) has defined herbal medicine as culminated labeled medicinal products that incorporate lively ingredients as aerial or underground accessories of plants. Plants are the basic source, as a natural chemical library, that serves as a basic molecule for the many semisynthetic modern medicines as well as it is continued its reputation from thousands of years as traditional systems of providing new remedies various diseases. The traditional and modern medicines are nowadays used complementary to each other providing better health care to society.

The therapeutic potential, as well as toxicity of the plant material, is dependent upon the number of phytoconstituents present in it. Many internal and external factors affect the levels of primary constituents and secondary metabolites in the plants. Genetic factors, geographical locations, and seasons are the most important factors causing variations in the levels of phytoconstituents (Sultan et al., 2018). Ayurveda has emphasized the collection of various parts of the plants in a particular season considering the highest therapeutic benefits (Tavhare et al., 2016). Various researchers reported that the essential oils polyphenol, flavonoids, glycosides, alkaloids etc are influenced significantly by seasons (Soni et al 2015).

Aegle marmelos (L.) of family Rutaceae is the sacred plant of India extensively utilized in the traditional system for the treatment of various diseases (Rahman and Parvin, 2014). Almost all parts of the plants possess some medicinal property however, most commonly used are the fruits and leaves for antidiabetic, anticancer, and immunomodulatory activity (Pathirana et al., 2020; Neeraj et al., 2017). The market of the plant and its product is increasing consistently. The Fact.MR report on Aegle Marmelos Market Forecast, Trend Analysis & Competition Tracking - Global Market Insights 2018 to 2028 suggest that the market of *Aegle marmelos* is going to increase tremendously in the future (https://www.factmr.com/report/1996/aegle-marmelos-market). To provide a quality product it is necessary to have the quality raw material. The seasonal variations affect the quality of raw plant material. It is reported that the minerals (Mandal et al., 2003), essential oil (Verma et al., 2014), and proteins and amino acids (Vasant and Nitin, 2019) levels in the *Aegle marmelos* plant material varies seasonally. However, there is a paucity of data of such seasonal variation in morphology and other phytoconstituents in *Aegle marmelos* if any.

The present research was aim to evaluate seasonal variations in the morphology, physicochemical properties, and phytochemicals in the *Aegle marmelos.*

Material Methods

2.1 Collection and Identification of Plant material:

Leaves of *Aegle marmelos* were collected in the three different seasons i.e Summer (May), Rainy (September), and Winter (January) from the Botanical garden of JSPM's Jayawantrao Sawant College of Pharmacy and Research, Pune, Maharashtra. Authentication was done by a Taxonomist of the Botanical Survey of India, Pune. A voucher specimen (No. BSI/WRC/100-1/ Tech./ 2019/ 03) was deposited in the Herbarium of Botanical Survey of India, Pune.

2.2 Assessment of foreign matter:

Foreign matter in the collected leaves was determined following WHO guidelines for Quality control methods for herbal materials (World Health Organization, 2011). The thin layer of weighed quantity 250 g of the leaves was spread on white paper and inspected visually to sort the foreign matter. The remaining quantity of the leaves was spread in sieve No. 250, and shaken to remove and measure any mineral admixture. The sorted foreign matter was weighed to determine the foreign matter content and the percentage value of it was calculated.

2.2.1 Macroscopic evaluation:

Fresh leaves were subjected to organoleptic evaluation of the appearance, shape, color, odor, and taste and measurement of size and surface characteristics as per the WHO guidelines. (World Health Organization, 2011).

2.2.2 Microscopic evaluation:

The transverse sections of the fresh leaves containing a midrib or larger vein and some with the marginal vein were boiled with the chloral hydrate in a test tube for a few minutes till they become transparent. The selected sections were subjected to staining with a drop of hydrochloric acid and phloroglucinol and observed under a trinocular microscope under 10x or 40x. Images were captured with Trino CXR camera.

2.2.3 Quantitative microscopy:

Quantitative microscopic characters of the leaves were measured using the following method.

Stomatal number:

A fresh leaf was cleaned and upper and lower epidermis was obtained from the clean fresh leaf of the plant. The peeled-off epidermis was then mounted in glycerine water on a glass slide. The slide was placed on the mechanical stage of the microscope and observed under 40× objective and a 6× eyepiece lens. The image was sketched on paper using camera lucida. The number of stomata and the number of epidermal cells in each field were counted. The average number per square millimeter was recorded.

Stomatal index:

The stomatal index was calculated as follows:

Stomatal index =S x 100E + S

Where S = the number of stomata in a given area of the leaf; E = the number of epidermal cells (including trichomes) in the same area of the leaf. For each leaf sample, more than 10 determinations were carried out and the average index was calculated.

Palisade ratio:

The fragment of leaf, about 5 × 5 mm in size heated with 5 ml of chloral hydrate in the test tube for approximately 15 minutes or until it becomes colorless. The fragment was then observed under a microscope with the 40x objective and a 6x eyepiece lens and the image was sketched on paper using camera lucida. The palisade cells under the four epidermal cells were counted. The palisade ratio was calculated by: total number of palisade cells/ 4.

Vein islet number and vein termination number:

A small portion of the leaf was boiled in chloral hydrate. The processed portion of the leaf was then mounted on the glass slide with a drop of glycerine. The slide was observed under a microscope with a 40x objective and a 6x eyepiece. The 1mm area is drawn on the drawing board with the camera lucida and stage scale. The veins included within such four adjacent squares were traced on the paper. The number of vein-islets was counted within these squares. The average number of the veinlet islets in one square millimeter area was calculated as a vein islet number. The average number of terminations of veinlets traced in one square millimeter area was recorded as vein termination number.

2.2.4 Proximate analysis:

Loss on drying:

The air-dried leaves weighed accurately 2 gm and were placed in the thermobalance set at 100-105º C. The weight was recorded intermittently. The process was continued until a constant weight was achieved or two successive weights showing variation not more than 5 mg. The loss on drying was calculated as the final weight of material – the initial weight of the material.

Total ash (Ash value):

Air-dried and pulverized leaves weighed accurately 4 g and spread in even layer in previously ignited and tarred silica crucible. The crucible was then placed in an incinerator. The temperature of the incinerated was increased gradually to achieve 500-600^0 C. the heating is continued till the formation of white ash. Crucible was kept in a desiccator and allowed to cool. And then weighed. The total ash was calculated in form of mg/gm of air-dried leaves.

Water-soluble ash:

Water, 25 ml, was mixed with total ash in the crucible and boiled for 5 min. The content was filtered through ashless filter paper. The hot water was used to wash the filter paper until the filtrate was neutral. Filter paper along with content on it was placed in the original crucible, dried, and kept in the incinerator at 450^0 C for 15 min or till obtaining constant weight. The crucible was transferred to a desiccator for 30 min and then weigh. The weight of water-soluble ash was calculated.

Water-soluble ash = weight of total ash−Weight of the residue.

Acid insoluble ash:

To the total ash in the crucible, 25 ml of hydrochloric acid (2N) was added, and the same procedure as described for water-soluble ash was followed to obtain the acid-soluble ash.

Sulphated Ash:

Accurately weighed 1gm powdered plant material was previously ignited silica crucible. The material was moistened with sulfuric acid and ignited in the incinerator at 550°C to 650°C for 30 minutes. Then the crucible was kept in a desiccator, allowed to cool, and weighed accurately.

Water, alcohol, and ether soluble extractive value

Accurately weighed 5 g air-dried and powdered plant material was placed in the flasks. Such three flasks containing plant material were added with 100 ml of individual solvent. The chloroform water, alcohol, and ether were added to flask 'A' 'B' and 'C' respectively. Flasks were closed and kept for 18 hours with intermittent shaking. After 18 hours the solution was filtered, 25 ml of filtrate was transferred to the tarred flat bottom dish and evaporated to dryness on a water bath. Content of extractable matter was calculated in mg per g of air-dried material.

2.3 Phytochemical screening:

The 100 gm air-dried powder extracted in Soxhlet apparatus with 300 ml of each solvent petroleum ether, chloroform, ethyl acetate, ethanol, successively for 6 hr each. The material was dried every time before subjecting to extraction with the next solvent to remove the traces of the previous solvent. Extracts were dried and the percentage yield was calculated. The consistency, color, appearance of the extracts was noted.

2.3.1 Establishment of qualitative phytoprofile of successive solvent extracts. (chemical tests):

The above extracts were subjected to the phytochemical test to determine the presence of alkaloids, glycosides, carbohydrates, phenolics and tannins, proteins and amino acids, saponins, and sterols using reported methods.

1. Alkaloids: The presence of alkaloids in extracts were determined by Dragondorff's test, Mayer's test, Wagner's test, Hager's test, and Tannic acid test.
2. Amino acids: The presence of amino acids in the extract was determined by Millon's test and Ninhydrin test.
3. Carbohydrates: The presence of carbohydrates in the extract was determined by Molisch's test and Barfoed's test.
4. Flavanoids: The presence of flavonoids in the extract was determined by the Shinoda test, Alkaline reagent test, and Zinc hydrochloride test.
5. Glycosides General test: The presence of glycosides in the extract was determined by fehlings test A and B. Types of glycosides present in the extract was determined by the specific tests for glycosides
i. *Anthraquinone glycosides*: Borntrager's test,
ii. *Cardiac glycosides*: Keddie's test, Killer- Killiani test, Raymond's test, Legal's test, and Baljet's test.
iii. *Coumarin glycosides*: fluorescence test.
iv. *Saponin glycosides*: Froth formation test, Haemolysis test.
6. Phenolic compounds (Tannins):
The presence of phenolic compounds in the extract was determined by the Ferric chloride test, Phenazone test, Gelatin test.
7. Proteins:
The presence of proteins in the extract was determined by the Biuret test, and Xanthoproteic test.
8. Steroids and Triterpenoids:
The presence of steroids and triterpenoids in the extract was determined Libermann-Burchard test, Salkowski test, and Sulfur powder test.

2.4 HPTLC Analysis:
***Sample preparation*:** Accurately weighed 20 mg of each extract was added to the separate volumetric flask each extract. methanol 10 ml was added to each volumetric flask and shaken complete dissolution of extract. The solution was filtered through Whatman's filter paper no. 1 and used for HPTLC analysis.

***Standard preparation*:** Accurately weighed 10 mg of each standard was added to a volumetric flask and dissolved in 10 ml methanol. The solution was filtered through Whatman's filter paper no. 1 and used for HPTLC analysis.

***Procedure*:** the sample and standards, 10 µl each, were applied on the pre-coated TLC silica gel G 60F254 (Merck) plates using Hamilton 100 µL syringe and Linomat 5 applicator (CAMAG). The plates were placed in the TLC Twin Trough Chamber containing mobile phase Toluene: Ethyl acetate (7:3) and allowed to develop to 8 cm. The plates were dried in a drying chamber, scanned by using winCATS software at variable wavelength, after spraying with detection reagent (Anisaldehyde sulphuric acid), and heated at 110 ^{0}C for 5 minutes). The Rf values and yield of the resolved bands were recorded.

3. RESULT

3.1 Assessment of quality of plant material- *A. marmelos*

3.1.1. Macroscopic evaluation:

It is green in colour, aromatic odour, bitter taste, 6 x 2.5 cm size, the shape is ovate with tapering or pointed tip and rounded base, untoothed or with shallow rounded teeth crenate margin, acuminate apex and long petiolate, smooth and shiny touch, hairy texture. (Fig-1)

Fig-1: Seasonal variation in morphology of A. marmelos L. leaf.

1a leaf Summer

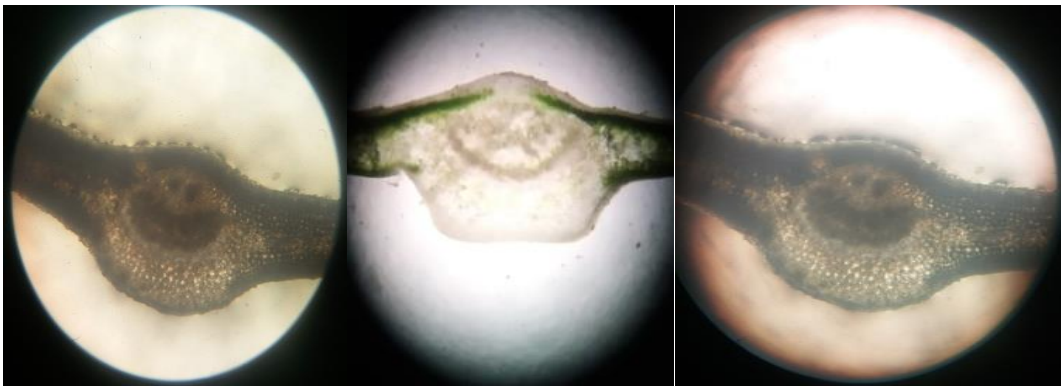
1b. Rainy season

1c. winter season

3.1.2 Microscopic evaluation:

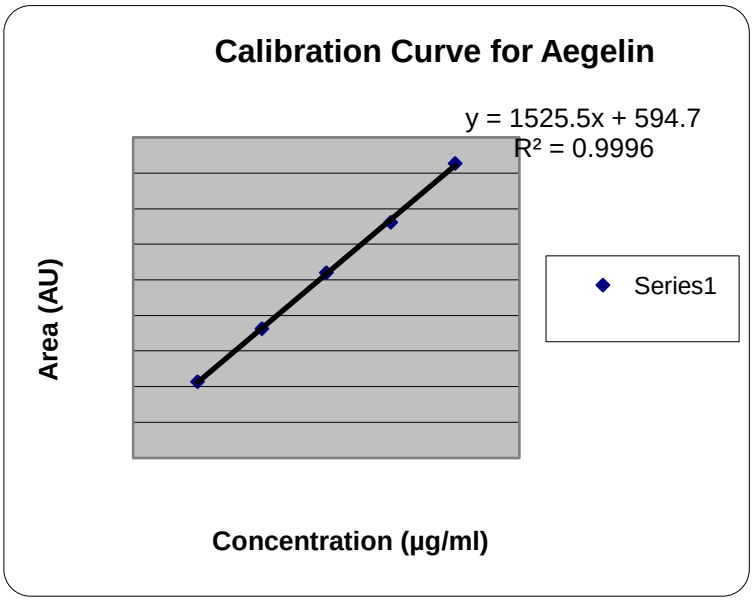
The cell wall is single-layered compactly arranged barrel-shaped with a cuticle, the vascular bundle consists planoconvex cylinder, of lower bowl-shaped part and upper flat plate, diacytic Stomata, unicellular trichomes, presence of Starch grains, and rosette Calcium oxalate crystals. (Fig-2)

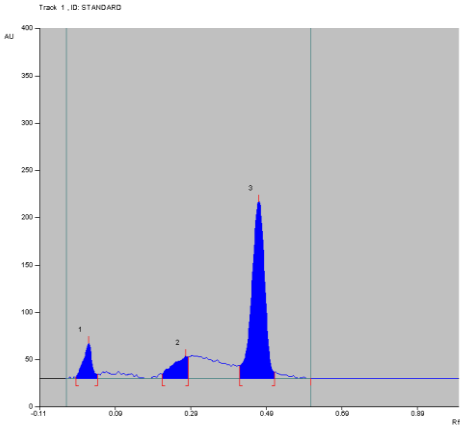
Fig-2: Seasonal variation in the microscopic characters of *A. marmelos L.* leaf



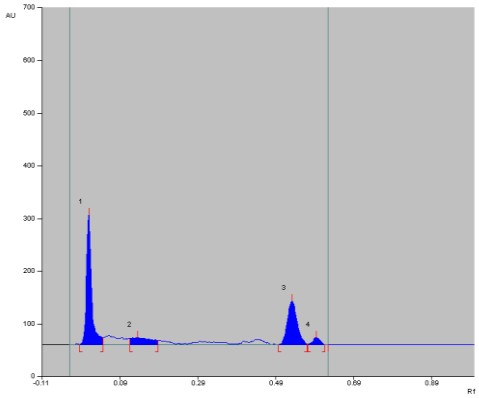
2a Summer season *Rainy season* *Winter season*

Fig-3: HPTLC analysis of ethanol extract.

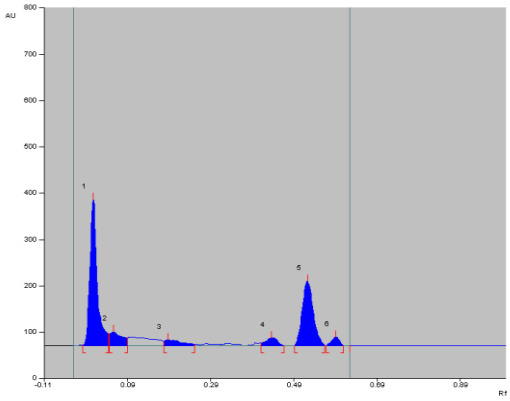




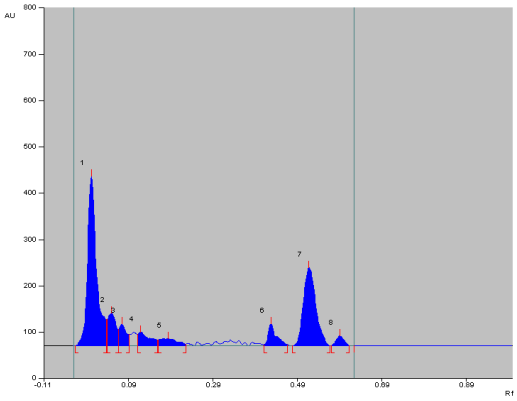
Standard aegelin



Ethanolic extract (Summer)



Ethanolic extract (Rainy)



Ethanolic extract (Winter)

3.1.3 Quantitative microscopy:

The stomatal number was found to be highest (11) in the rainy season and lowest in the winter (6). The stomatal index was constant all season. Whereas palisade ratio was 6, 8, and 9 in winter, summer, and rainy seasons respectively. Vein islet number was 8, 9, and 8 in winter, summer, and rainy season respectively; similarly vein termination number was 8, 9, 8 during these seasons. (Table-1)

Table-1: seasonal variation in quantitative microscopy of A. marmelos *L. leaf*

S. No	Parameter	Summer	Rainy	Winter
1	St. number	9	11	6
2	St. Index	16	16	16
3	Palisade ratio	8	9	8
4	Vein islet no	9	8	8
5	Vein termination no.	8.5	9	8

3.1.4 Proximate Analysis

The foreign matter was within the standard limits during all seasons. The loss of drying was observed to be more 2.25% during the rainy season than the winter 1.70% and 1.55% in winter and summer respectively. Total ash and sulfated ash were highest in summer whereas, water-soluble ash and acid-soluble ash were more in the winter season. The extractive values of water extract, alcohol extract, and ether extract were higher in the winter season.(Table-2)

Table-2: Seasonal variation in proximate analysis- of A. marmelos *L. leaf*

S. No	Parameter (%)	Summer	Rainy	Winter
1	F.O.M.	1.2	1.1	1
2	L.O.D.	1.55	2.25	1.70
3	Total ash	6.75	5.80	6.30
4	water soluble ash	1.70	2.30	2.70
5	Acid Insoluble Ash	1.25	1.20	1.55
6	Sulphated Ash	1.70	1.50	1.20
7	Water S. Ext. V.	10.10	12	13.10
8	Alcohol S. Ext. V	8.1	8.0	8.3
9	Ether S. Ext. V	4	3	5

Table-3: seasonal veriations in preliminary phytoprofle of A. Marmelos *leaf extract.*

Extract	Season	Color	Consistency	Yield %w/w
Pet. ether	Summer	Green	Viscous	3.50
	Rainy	Green	Viscous	3.45
	Winter	Green	Viscous	3.40
Chloroform	Summer	Yellowish Green	Viscous and Sticky	2.80
	Rainy	Yellowish Green	Viscous and Sticky	2.90
	Winter	Yellowish Green	Viscous and Sticky	2.85
Ethyl acetate	Summer	Brown	Viscous and Sticky	2.30
	Rainy	Brown	Viscous and Sticky	2.30
	Winter	Brown	Viscous and Sticky	2.32
Ethanol	Summer	Chocolate Brown	Viscous and Sticky	8.12
	Rainy	Chocolate Brown	Viscous and Sticky	8.03
	Winter	Chocolate Brown	Viscous and Sticky	8.20

Table 4: Seasonall variation in priliminary qualitative phytochemical in A. marmelos *L. leaf*

S No.	Type of Phytoconstituent	Season		
		Summer	Rainy	Winter
1	Alkaloids	+	+	+
2	amino- acids	-	-	-
3	Carbohydrates	+	+	+
4	Flavonoids	+	+	+
5	Glycosides	+	+	+
6	Phenolic compounds	+	+	+
7	Proteins	-	-	-
8	Steroids	+	+	+
9	Saponins	+	+	+

Table-5: Result of HPTLC analysis of *A. marmelos* Leaf extract

Rf Value	Season	Area (AU)	Yield (mg/g)
0.56	Summer	1726.2	1.13
	Rainy	3078.1	2.01
	Winter	4118.0	2.70

The extracts obtained with different solvents showed seasonal variation in the extractive values. The extraction with petroleum ether gave the highest yield in the summer season, chloroform in the rainy season whereas, ethyl acetate and ethanol gave the highest yield in the winter season.

3.1.5 Phytochemical analysis (Preliminary and HPTLC):

In all seasons (winter, summer and rainy) all the phytoconstituent i.e. alkaloids, amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, proteins, steroids, and saponins were present in the extracts in qualitative analysis. However, the quantitative evaluation of the aegelin showed seasonal variation. The highest quantity (2.70 mg/g) was present in the winter whereas, the lowest (1.13 mg/g) was in summer.

DISCUSSION

Medicinal plants are the base of most of the traditional systems of medicine in the world. About 80% population of the world relies on traditional medicines. World Health Organization (WHO), reported that there are 21,000 species 45,000 varieties of the plant are utilized in the traditional system of medicine, Ayurveda for treatment of various disease conditions. (Pant et al., 2021) Plants are interacting continuously with the environment and are depends on environmental factors for their growth and survival (Mohiuddin, 2019). Living organisms and plants are not independent. The changes in the environment directly or indirectly affect the physiology or phytochemistry of the plants. The environment consists of the external factors viz intensity of light, water content, temperature, wind, type of soil etc. (Ceyhan et al., 2012).

The plants respond to the prolonged environmental changes, seasonal variation, by changing their phenotype. Many plant species were reported to show changes in leaf thickness, and stomatal density as well as the density of xylem vessels during the dry weather (Guo et al., 2017). Similarly, it is reported that the phloem also shows changes in its anatomy and physiology for the transportation of carbon and signals during the seasonal variations (Ray and Savage, 2021). In the present study, the stomatal number was less in winter and more in the rainy season while the stomatal index was constant in all seasons. Earlier, Beerling and Chaloner (1993), reported the variation in stomatal density in response to environmental CO_2 conditions. The stomatal number in *Psychotria horizontalis* was reported to show a positive relationship with the rainfall (Franks et al.,2009) and in *M. lanceolata* negative relation to rainfall (Hogan et al.,1994). The environmental temperature and stomatal density are found to be positively correlated in many studies (Hill et al., 2014). The palisade ratio remains constant for the related texa and is considered a reliable taxonomical characteristic which is least affected by the seasonal variations (Simon, 2018). In the present study, the palisade ratio of the A. *marmelos* was also found to be stable and did not show seasonal variations. Similarly, the result of vein islet number and vein termination number did not show seasonal variation. Zhu et al., 2012 reported the leaf vein density is a constant parameter that does not vary with the seasonal variations, however, changes in latitude may show some variation.

Acid soluble ash represents physiological ash as well as non-physiological ash. The high value was observed in the summer season which may be due to the presence of more mineral content. Acid-insoluble ash determines the amount of earthy matter present in the sample indicative adulteration or contamination with soil. Acid insoluble is observed to be more in winter. Water-soluble ash is roughly indicative of water-soluble extractable matter present in the ash which was found to be higher in winter in *A. marmelos* L. leaf.

Seasonal changes in the environment such as availability of water, change in temperature, radiation, etc affect the synthesis of primary and secondary metabolites (Ceyhan et al., 2012; Sampaio et al., 2016). During the rainy season, the plant material is rich in primary metabolites such as sugars and nucleosides. The leaves and stem contain the maximum amount of secondary metabolites in the summer (dry) season whereas lactones, acids, and esters are more in winter

(Sampaio et al., 2016). In the present study, the ethanol and water extractive values are found to be higher in the winter season.

The alkaloid content in various plants was reported to show seasonal variation. The concentration of different alkaloids in the same plant may vary seasonally. For example, the bisindole alkaloid in leaves of Catharanthus roseus was reported to be a higher concentration in summer whereas, in the same plant, another alkaloid vindoline was at peak concentration in winter (Mall et al., 2019). Levels of phenolic compounds are reported to change in response to temperature and moisture, flavonoids in response to light and temperature whereas, alkaloids content changes according to light moisture and temperature (Ncube et al., 2012). Bakhshi and Arakawa (2006) reported that irradiation of apple with the mixture of white and UV light increases the contents of phenolic acids, anthocyanin, and flavonols in apple fruit. In the present study, the alkaloid aegelin concentration was found to be higher in the winter season.

CONCLUSION

The observations noted in the present study indicated that leaves of *A. marmelos* show physiological variations viz the stomatal number, proximate analysis and extractive values. The phytochemical composition also varies with the season showing the highest concentration of aegelin in winter. This data can help to decide the time of collection of plant raw material according to the need and purpose of the utilization. It will also help to achieve the highest extractive value for the development of formulation with maximum efficacy by deciding the correct season for collection.

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Conflict of interest:

The authors declare that the research was conducted in absence of any conflict of interest.

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