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Biochemical Profiling of Primary and Secondary Metabolites of *Annona reticulata* Leaf with their Seasonal Fluctuations

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Published Online: Jul 07, 2020

eBook: Recent Trends in Biochemistry

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: *Annona reticulate*; Medicinal plant; Primary biochemical; Secondary biochemical; Seasonal fluctuations

Abstract

The qualitative phytochemical analyses of ethanol and aqueous dried dust leaf powder of *Annona reticulata* (*A. reticulata*) revealed the presence of different phytochemicals. Quantitative estimations of some primary and secondary biochemicals of dried leaves of *A. reticulata* in winter, summer and monsoon seasons were done. Quantitative estimation of some primary and secondary metabolites of dried leaves of *A. reticulata* in difference seasons (winter, summer and monsoon) was done. Amount of sugar contents were highest in winter (3.21 ± 0.005 mg/g dry weight) and lowest in monsoon (0.74 ± 0.003 mg/g). Like sugars phenolics contents were found to be highest in winter (1.10 ± 0.06 mg/g) and lowest in rainy season (00.52 ± 0.01 mg/g). Amount of starch, and protein content were highest in summer and were 45.00 ± 0.05, 25.83 ± 0.01 mg/g respectively and lowest in monsoon (34.36 ± 0.31 and 19.68 ± 0.22 mg/g respectively). Tannin contents were highest in winter (10.10 ± 0.06 mg/g) and lowest in monsoon (6.10 ± 0.06 mg/g). Flavanoids and alkaloids concentrations were highest in summer (25.22 ± 0.04 and 95.18 ± 0.56 mg/g respectively) and lowest in winter (22.43 ± 0.03 and 40.93 ± 0.54 mg/g respectively). Amount of steroid content was highest in winter (0.018 ± 0.0005 mg/g and lowest in monsoon (0.005 ± 0.0005 mg/g). Estimated amount of moisture content was highest in monsoon (751.67 ± 0.88 mg/g fresh weight) and lowest in winter season (661 ± 0.58 mg/g). Total ash content was highest in winter (152.43 mg/g) and lowest in monsoon (101.00 ± 0.58 mg/g).

Two ways ANOVA analysis was done using the amount (mg/g) of different primary biochemicals in different seasons as dependent variables and different seasons and different primary biochemical as independent variables and the amount of different primary biochemicals showed significant values in terms of seasons and primary biochemicals ($p<0.05$) and it also showed significant values when the interaction of primary biochemicals and seasons were also considered.

Two ways ANOVA analysis was also done in case of amount of secondary biochemical production. The amount of different secondary biochemical production showed significant values in terms of seasons and secondary biochemicals ($p<0.05$) and it was also showed significant values when the interaction of secondary biochemicals and seasons were also considered.

Multiple comparisons by Tukey HSD test was done on difference in amount of primary biochemicals as well as secondary biochemicals between different seasons and revealed significant values ($p<0.05$) in all cases, except between winter and monsoon, and between monsoon and winter seasons in case of secondary biochemicals.

Introduction

Near about 60-85% of the world population has to rely on traditional medicine [1]. Natural products are the sources of novel drugs. During the last 30 years, approximate 50% approved drugs have been prepared from natural products and mainly in the area of treatment of cancer. Upto recent time 35,000 to 70,000 medicinal plants have been identified for their medicinal values. In early drug discovery, plants having ethno pharmacological uses have been the main sources for medicines [2]. Screening of natural product to a new isolate needs experience and well expertise. Recent years, new technologies revolutionized the isolation of natural products and discovery of pharmacotherapeutic agents [3]. Medicinal plants are the most important for human beings because of presence of several secondary metabolites, viz., alkaloids, terpenoids, tannins, flavonoids, saponins, phenolic compounds etc. [4]. Analyses of different phytochemical constituents in medicinal plants are needed from crude plant extracts and investigation their seasonal fluctuation is also important in the context to their pharmacological potential [5].

Annona reticulata L. (*A reticulata* L) belongs to the family Annonaceae. It is also known as Custard Apple, Bullock's- heart, Ramphalam, Sitaphala, Nona etc. Its native land is South America and West Indies. It is widely distributed in India, Bangladesh, Pakistan and Thailand [6,7]. It is a small deciduous and semi evergreen tree. Leaves are narrow-lanceolate, alternating, in nature. The flowers are yellow green in colour, with 3 outer narrow, fleshy petals. 2-3 cm long which are never fully opened. The fruits vary in structure, like, spherical, heart-shaped, irregular or oblong [8,9].

In Ayurveda *A. reticulata* is used for the treatment of cancer, dysentery, epilepsy, cardiac problem, worm infestation, constipation, haemorrhage and also has antifertility, antitumour and aborfacient properties [10]. Ripe fruits are good tonic and sedative. It increases the blood, increases muscular strength, reduces burning sensation, lessens, tendency to biliousness and vomiting [11]. *A. reticulata* leaves are used in the treatment of colic. Decoction of the leaves is used to cure malaria and syphilis [9]. A decoction of leaves is used as a vermifuge and decoction of bark is used as tonic, treatment of diarrhea and dysentery. Fragments of root bark are used to relieve toothache and root's decoction is used as febrifuge [12]. Ethanol extract of its roots has an inhibitory effect against Hela, A- 549, K-562, and MDA- MB human cancer cell lines [13]. Study on *A. reticulata* leaves resulted in the identification of nine compounds, a new triterpenoid, annonaretin which have significance effect on NO inhibition in most of LPS-activated mouse peritoneal macrophages [14]. Bhalke and Chavan, 2011 [15] investigated that analgesic and CNS depressant activity increases from ethyl acetate, methanol and petroleum ether extracts of *A. reticulata* bark. The methanol leaf extract of *A. reticulata* L. possesses antimicrobial (antifungal and antibacterial) activity and has also remarkable antioxidant activity leaves, stem berks and roots have strong mosquito larvicidal potential [16-21].

The present study was carried out to examine biochemicals present in the mature leaves of *A. reticulata* and to obtain a preliminary data of seasonal fluctuation of some primary and secondary biochemicals of the plant for utilization of bioactive ingredient.

Materials and methods

Study area

Experiments were carried out in Mosquito, Microbiology and Nanotechnology Research Units, Parasitology Laboratory, Department of Zoology, The University of Burdwan (23°16′ N, 87°54′ E).

Collection of leaves

Mature leaves (fresh) of *Annona reticulata* L. plant (aged about 1-4 years) of winter, summer, and monsoon (November, 2014 to September, 2015) collected from Burdwan town, West Bengal, India (23°16' N, 87°54' E). Leaves were initially washed with running tap water and then with distilled water. Thereafter, soaked with paper towels. Leaves of winter, summer, and monsoon seasons were collected and dried in shade for 9-10 days and kept separately. The shade dried leaves ground into uniform powder using a Thomas-Willey milling machine.

Qualitative phytochemical analyses of leaf extracts of *Annona reticulata*

Aqueous and ethanol leaf extracts of *A. reticulata* were tested for qualitative phytochemical analyses following the standard protocols [22-24] slight modification.

For the preparation of aqueous extract, 500 mg of dried leaf powdered material was taken in a conical flask and added 100 ml of distilled water and boiled in a hot water bath at 100°C for 30 minutes and cooled. The leaf tissues were homogenized and shaken well. Then the extract was centrifuged at 2000 rpm for 20 minutes. Clear supernatant was used as aqueous leaf extract.

For the preparation of ethanol leaf extract, 500 mg of dried leaf powdered material was taken in a conical flask and added 100 ml of 80% ethanol and boiled in a hot water bath at 100°C for 30 minutes. The leaf tissues were homogenized and shaken well. The extract was charcoal filtered to remove pigments and then filtered through Whatman No. 1 filter paper. The clear supernatant was used as ethanol leaf extract.

Detection of tannin and phenolic compounds

2 ml of aqueous extract was taken in a clean test tube and was added 0.5 ml of 0.1% ferric chloride solution. The colour of solution change into blue green, indicated the presence of tannin and phenolic compounds.

Detection of flavanoids

2 ml of aqueous extract was taken in a test tube and was added few drops of NaOH solution. Intense colour formation

occured which became colourless on addition of dilute HCL and that indicated the presence of flavanoids.

Detection of alkaloids

2 ml of ethanol extract was taken in a clean test tube and added few drops of 2 N HCL. Then mixed 1 or 2 drops of Mayer's reagent [1.36 g of dissolved in 60 ml of H_2O and then this solution poured in potasium iodide solution (5 g potasium iodide dissolved in 100 ml of water)]. Appearence of cream or pale yellow colour precipitation indicated the presence of alkaloids.

Detection of terpenoids (Salkowski test)

2 ml of ethanol extract was taken in a test tube and then added 2 ml of chloroform and 3 ml of concentrated H_2SO_4 carefully by the interior wall of the test tube. A reddish brown colouration of the interface was formed which indicated the presence of terpenoids.

Detection of steroids

2 ml of ethanol extract was taken in a test tube and then 5 ml of chloroform and 5 ml of concentrated H_2SO_4 was added carefully by the interior wall of the test tube. The upper layer turned red and H_2SO_4 layer showed yellow with green fluorescence. This indicated the presence of steroids.

Detection of anthraquinones

2 ml of aqueous extract was added with 2 ml of 2 N HCL and NH_3. The appearence of pink red was turned blue violet, indicating the presence of anthrocyanines.

Test of saponins (frothing test)

10 ml of aqueous extract were taken in a test tube and was shaking vigorously. Persistence of frothing that is the indication of the presence of saponines in the sample. 3 drops of olive oil was mixed with the frothing and shaken vigorously, then observed for the formation of emulsion.

Test of amino acid

4 ml of ethanol extract was taken in a cleaned test tube and added 2-3 drops of 0.1% nin-hydrin solution. There after boiled for one minutes and cooled. Appearance of violet, purple to blue colour indicated the presence of amino acids.

Seasonal fluctuation of amount of some primary and secondary metabolites of leaves of *Annona reticulata* (mg/g dry weight of leaves)

Quantitative estimations of some primary and secondary biochemicals were done in each season. In West Bengal three prevalent seasons exist. (April, May, June- **Summer,** July, August, September- **Monsoon** and November, December, January- **Winter**).

Estimation of primary biochemical of dried leaf powder of *Annona reticulata* of three seasons

Estimation of total soluble carbohydrate

Total soluble carbohydrate was measured by Anthrone method (modified) as per procedure adopted by Dubois et al. (1951) [25]. 100 mg of ground leaf powder was taken in a small conical flask and added 80% ethanol (20 ml) and the mouth of the conical flask was plugged with cotton and boiled the ethanol on hot water bath at 100°C for 30 minutes and then cooled. The leaf tissues were homogenized and shaken well. After that, leaf extract was centrifuged at 2000 rpm for 20 minutes and the supernatant was taken. To remove pigments, supernatant was passed through charcoal powder (saturated with 80% ethanol) and filtered through what man No.41 filter paper and measured the volume of clear supernatant. For the estimation of total soluble sugar 1 ml of clear supernatant was taken in a test tube and added 4 ml of anthrone reagent (freshly prepared and ice cold) and heated exactly 11 minutes at 100°C (in hot water bath) and cooled rapidly to 0°C on ice. For standard curve preparation, standard solution was made dissolving 10 mg of D-glucose on 100 ml of distilled water. From standard solution 0.2-1 ml aliquots were taken in cleaned test tubes. Each test tube was made up to 1 ml adding required volume of distilled water. Thereafter added 4 ml of anthrone reagent in each test tube and heated each test tube exactly 11 minutes at 100°C (in hot water bath) and cooled rapidly on ice. Then read at 630 nm against reagent blank in the UV visible spectrophotometer. Standard curve was prepared plotting known concentrations of standard solution on X axis and OD value on Y axis of graph. From standard curve, sample concentration was detected putting OD value of sample.

Estimation of Starch

Modified anthrone method with slight modification was adopted for the determination of the amount of starch. For the estimation of the starch components of dried leaf material, 100 mg of the ground sample material was homogenized in hot 80% ethanol (30 ml) to discard soluble sugars. The residue was washed repeatedly with hot 80% ethanol until the discarded 80% hot ethanol did not give blue color with anthrone reagent. After completion of washing, added 10 ml of 2.5 N HCL and heated in a water bath at 100°C for 30 minutes and shaken well. Volume remained fixed to 10 ml by adding distilled water. Then the extract was centrifuged at 2000 rpm for 20 minutes and the supernatant was taken. The clear supernatant was used as the sample extract. From sample extract, 1 ml was transferred in a test tube. For standard curve preparation, standard solution was made dissolving 10 mg of D-glucose on 100 ml of distilled water. From standard solution 0.2- 1 ml aliquots were taken in cleaned test tubes. Each test tube was made up to 1 ml adding required volume of distilled water. Thereafter added 4 ml of anthrone reagent in each test tube and heated each test tube exactly 11 minutes at 100°C (in hot water bath) and cooled rapidly on ice. Then read at 630 nm against reagent blank in the UV visible spectrophotometer. Standard curve was prepared. From standard curve, sample concentration was detected putting optical density value of the sample.

Estimation of total protein

Total proteins were measured as per procedure of Lowry et al. (1951) [26]. 100 mg of ground leaf powder was taken in a small conical flask and added 80% ethanol (20 ml) and the mouth of the conical flask was closed with cotton plug and boiled the ethanol on hot water bath at 100°C for 30 minutes and the leaf tissues were homogenized and shaken well, then removed the ethanol (80%) and the leaf sample was washed with 80% ethanol (20 ml) thrice to remove pigments from the sample. The leaf sample was transferred in a centrifuge-tube and added 5 ml of 5% TCA solution at 0-5°C in an ice bath for 10 minutes and then centrifuged at 5000 rpm for 45 minutes and removed the solution. Thereafter added 5 ml of 10% TCA solution at 0-5°C in an ice bath for 10 minutes and centrifuged at 5000 rpm for 45 minutes. The supernatant was discarded and the leaf tissues were re-extracted once with absolute ethanol

and twice with hot ethanol-ether mixture, every time discarding the supernatant. Then added 5 ml of 0.1N sodium hydroxide solution and shaken well. 1 ml of solution was taken in a test tube and added 5 ml of reagent C and mixed well and allowed to stand for 10 minutes. Then added 0.5 ml of folin ciacalteaue reagent and incubated at room temperature in the dark for 30 minutes to develop blue colour. For the preparation of standard curve, standard solution was made dissolving 100 mg of bovine serum albumin on 100 ml of distilled water. From standard solution, 0.2-1 ml were transferred into a series of test tubes and made up the volume to 1 ml in all the test tubes diluting with required volume of distilled water. 1 ml of distilled water was transferred in a test tube which served as blank. In all test tubes added 5 ml of reagent C and mixed well and allowed to stand for 10 minutes. Then added 0.5 ml of folin ciacalteaue reagent in each test tube and incubated at room temperature in the dark for 30 minutes to develop blue colour. Then read at 660 nm against reagent blank in the UV visible spectrophotometer. Standard curve was prepared. From standard curve, amount of the protein in the sample was determined.

Estimation of total lipid

Amount of lipid content of dried leaf powder of *A. reticulata* were measured as per protocol of Folch et al., 1957 [27]. 1 g of each of leaf powder of three seasons was homogenized in 20 ml of chloroform: methanol (1:2 v/v) solvent mixture for 10 minutes in a cell homogenizer separately. Then the extract was shaken vigorously and filtered (filtrate- 1). Each residue was mixed again with 25 ml of chloroform: methanol (1:2 v/v) solvent mixture and stirred for 30 minutes. The mixture was filtered through activated charcoal to remove any pigments (filtrate- 2). The Filtrate-1 and filtrate-2 was combined and shaken with 0.9% crude sodium chloride solution, and non-lipid contaminants were discarded. The lipid thus extracted were dried in desiccators and weighed.

Quantitative phytochemical estimation of some secondary metabolites of dried leaf powder of *Annona reticulata* of three seasons

Estimation of tannins

Sample preparation for tannin estimation

Estimation of tannins was carried out using Folin-Denis reagent [28]. 100 mg of dried leaf powder was boiled with 50 ml of distilled water for 30 minutes and then filtered and cooled and the extract was transferred in a volumetric flask (50 ml capacity) and made up the volume up to 50 ml, adding distilled water. 10 ml of extract from 50 ml was taken and again transferred in a 50 ml volumetric flask and made up the volume up to 50 ml adding distilled water. Then the extract was centrifuged at 2000 rpm for 20 minutes to remove any pigments and the clear supernatant was collected. 1 ml extract was taken and added 1 ml Folin- Denis reagent and 2.5 ml of saturated sodium carbonate solution.

Standard curve preparation and estimation of sample

10 mg of tannic acid dissolved in 100 ml of distilled water. From this standard solution, 10 ml was taken and added 90 ml of distilled water in a 100 ml volumetric flask. So 1 ml extract contained 0.01 mg tannic acid. From this 100 ml of solution 0.2-1 ml aliquots were taken in clear test tubes and made the volume 1ml of all the test tubes adding required volume of distilled water in each test tube. Then added 1 ml Folin- Denis reagent and 2.5 ml of sodium carbonate solution to each test tube. All the reagents in each tube including sample test tube were mixed well and kept undisturbed for about 30 minutes and read at 760 nm against reagent blank. From standard curve, sample concentration was determined.

Estimation of phenolics

Sample preparation for phenol estimation

For the estimation of phenolics, the protocol of Bray and Thorpe (1954) [29] was adopted with slight modification. 100 mg of ground leaf powder was taken in a small conical flask and 80% ethanol (30 ml) was added and the mouth of the conical flask was plugged with cotton and boiled the ethanol on hot water bath at 100°C for 30 minutes and then cooled. The leaf tissues were homogenized and shaken well. After that leaf extract was centrifuged at 2000 rpm for 20 minutes and the supernatant was taken. To remove pigments, supernatant was passed through charcoal powder (saturated with 80% ethanol) and filtered through Whattman No. 41 filter paper. The clear supernatant was used for phenol estimation. 1 ml was taken and added 1 ml folin ciocalteu reagent and 2 ml of 20% sodium-carbonate solution. Then boiled the mixture on hot water bath and cooled. Blue colour developed and diluted with 10 ml of distilled water.

Standard curve preparation and estimation of sample

10 mg of catechol dissolved in a 100 ml of distilled water. From this 100 ml of solution, 0.2- 1 ml aliquots were taken in clear test tubes and made the volume 1ml of all the test tubes adding required volume of distilled water. Then 1 ml Folin- ciocalteu reagent and 2 ml of 20% sodium carbonate solution were added to each test tube. Then the mixture was boiled on hot water bath and cooled. Blue colour developed and diluted with 10 ml of distilled water in each tube. Optical density was read at 650 nm against reagent blank. Standard curve was prepared and from this standard curve sample concentration was determined.

Estimation of alkaloid

For the estimation of alkaloid content of dried leaf powder, the protocol of Harborne (1973) [23] with slight modification was adopted. 1 gm of leaf powder material was weighed and kept in a 250 ml conical flask and poured 50 ml of 10% acetic acid in ethanol and covered the mouth of flask and allowed to stand for 4 hrs. This was filtered via Whatman No. 42 filter paper and the extract was concentrated to one-quarter of the original volume by evaporation. Concentrated aqueous ammonium hydroxide solution was added drop wise to the extract until the total alkaloid was precipitated. Then the precipitation was collected and washed with one percent ammonia solution and dried. The dried residue was the alkaloid content which was weighed.

Estimation of flavonoid content

The protocol of Bohm and Kocipai-Abyazan (1994) [30] was used for the estimation of flavonoid content of dried leaf powder. 10 g of the plant sample was extracted repeatedly with 100 ml of 80% crude methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42. The filtrate was later transferred into a crucible and evaporated into

dryness over a water bath and weighed to a constant weight.

Estimation of steroid content

5 g of dried leaf powder was homogenized in 100 ml MeOH: H_2O in the ratio of 4:1 and filtered by Whatman No. 1 filter paper. The filtrate was evaporated to one tenth of its original volume and acidified with 2N H_2SO_4. The filtrate was further extracted with chloroform thrice and the chloroform part was collected by removing the crude portion. Chloroform part was dried and weighed.

Terpenoid estimation

100g of plant powder were taken separately and soaked in alcohol for 24 hrs. Then filtered, the filtrate was extracted with petroleum ether; the ether extract was treated as total terpenoids [31].

Figure 1: *Annona reticulata* plant.

Figure 2: Collected leaves of *Annona reticulata.*

Ash and moisture content

Fresh mature leaf (1g) of *A. reticulata* was weighed and placed in a hot-air oven at 50°C for 72 hrs and materials that showed constant dry weight was removed from the oven and weighed. The difference between fresh and dry weight was the moisture content. A portion of each kind of oven-dried leaves was separately ashed in a muffle furnace at 450°C for 20 minutes. The ash thus produced in each crucible was soaked with 2 ml distilled water and placed in a watch glass for drying at 110°C for 2 hrs. The ash sample was weighed. The ash and ash-free weight of the sample was determined and moisture and ash content of leaves of *Annona reticulata* of said three seasons were determined.

Results

Qualitative Phytochemical analyses of dried leaf powder of A. reticulata revealed the presence of many secondary metabolites, such as terpenoids, alkaloids, steroids, tannins, flavonoids, phenols, amino acids and anthraquinones but the absence of saponins (**Table 1**)

Table 1: Result of qualitative analyses of photochemical from dried leaves of *Annona reticulata.*

Phytochemical	Presence (+)	Absence (-)
Terpenoids	+	
Alkaloids	+	
Steroids	+	
Tannins	+	
Flavonoids	+	
Phenols	+	
Amino acids	+	
Anthraquinones	+	
Saponins		_

Quantitative phytochemicals analyses of some primary and secondary biochemicals of dried leaves of A. reticulata in difference seasons were done which was presented in **Table 2**. Amount of sugar contents were highest in winter and lowest in monsoon. Like sugars phenolics contents were found to be highest in winter and lowest in monsoon. Amount of starch, and protein contents were highest in summer and lowest in monsoon respectively. Tannin contents were highest in winter and lowest in monsoon. Flavanoids and alkaloids contents were highest in summer and lowest in winter respectively. Amount of steroid was highest in winter and lowest in monsoon. Moisture content was highest in monsoon and lowest in winter. Total ash content was highest in winter and lowest in monsoon.

Two way ANOVA analysis was done using the amount (mg/g dry weight) of different primary biochemicals as dependent variable and different seasons and different primary biochemical as independent variables and was presented in **Table 3** and the amount of different primary biochemicals showed statistically significance in terms of seasons and primary biochemicals (Significance at $p<0.05$) and it also showed significant result when the interaction of primary biochemicals and seasons were also considered.

Table 2: Seasonal fluctuations in the amount of some primary and some secondary biochemicals in the leaves of *Annona reticulata* plant (amount estimated as mg/g dry weight).

Primary and secondary chemicals	Winter (mg/g dry weight) [Mean±SE]	Summer (mg/g dry weight) [Mean±SE]	Moonsoon (mg/g dry weight) [Mean±SE]
Sugars	3.21 ± 0.005	0.85 ± 0.003	0.74 ± 0.003
Starch	35.10 ± 0.05	45.00 ± 0.05	34.36 ± 0.31
Protein	22.79 ± 0.10	25.83 ± 0.01	19.68 ± 0.22
Lipids	70.33 ± 0.88	85.7 ± 0.65	84.00 ± 0.58
Phenolics	1.10 ± 0.06	0.54 ± 0.003	00.52 ± 0.01
Tannins	10.10 ± 0.06	10.06 ± 0.03	6.10 ± 0.06
Flavanoids	22.43 ± 0.03	25.22 ± 0.04	23.01 ± 0.01
Alkaloids	40.93 ± 0.54	95.18 ± 0.56	44.53 ± 0.28
Terpenoids	9.87 ± 0.19	16.23 ± 0.18	10.10 ± 0.06
Steroids	0.018 ± 0.0005	0.010 ± 0.0008	0.005 ± 0.0005
Moisture and ash contents	**Winter** (mg/g fresh weight) [Mean±SE]	**Summer** (mg/g fresh weight) [Mean±SE]	**Monsoon** (mg/g fresh weight) [Mean±SE]
Moisture content	661 ± 0.58	721.5 ± 0.76	751.67 ± 0.88
	Winter (mg/g dry weight) [Mean±SE]	**Summer** (mg/g dry weight) [Mean±SE]	**Monsoon** (mg/g dry weight) [Mean±SE]
Ash content	152.43 ± 0.23	106.75 ± 0.32	101.00 ± 0.58

Table 3: Results of two way ANOVA using the production of the amount (mg/g dry weight) of different primary biochemicals (dependent variable), and seasons and primary biochemicals as independent variables.

Source of variation	Sum of Squares (SS)	Degree of Freedom (DF)	Mean of Squares (MS)	F value	p-level
Seasons (S)	163.920	2	819599	192.453	0.000*
Primary biochemicals PB)	29,694.490	3	9898.163	23,242.248	0.000*
S×PB	541.762	6	90.294	212.022	0.000*
Within groups	102209	24	0.426	-	-
Total	30,410.393	35	868.868	-	-

*: Significance at $p<0.05$, PB: Primary Biochemical

Multiple comparisons by Tukey HSD test was done on difference in amount of primary biochemicals between different seasons and revealed significant values (at the 0.05 level of significance) in all cases

Table 4: Multiple comparisons (Tukey HSD) on differences in amount (mg/g dry weight) of primary biochemicals of leaf of *Annona reticulata* between different seasons.

Multiple comparisons	Season (I)	Season (J)	Mean diff. (I-J)	Std. Error	Significance
Tukey HSD	Winter	Summer	-4.969	0.266	0.000*
		Monsoon	-1.081	0.266	0.001*
	Summer	Winter	4.969	0.266	0.000*
		Monsoon	3.888	0.266	0.000*
	Monsoon	Winter	1.081	0.266	0.001*
		Summer	-3.888	0.266	0.000*

*: significance

Based on observed means, the mean difference is significance at the 0.05 level

Two ways ANOVA analyses was done using the amount (mg/g dry weight) of different secondary biochemicals as dependent variable and different seasons and different secondary biochemicals as independent variables and was presented in Table 5 and the amount of different secondary biochemicals showed statistically significance in terms of seasons and secondary biochemicals (Significance at $p<0.05$) and was also showed significant result when the interaction of secondary biochemicals and seasons were also considered.

Table 5: Results of two way ANOVA on the production of the amount of different secondary biochemical (dependent variable) and seasons and secondary biochemicals as independent variables.

Source of variation	Sum of Squares(SS)	Degree of Freedom(DF)	Mean of Squares(MS)	F value	p-level
Seasons (S)	1319.608	2	659.804	5288.213	0.000*
Secondary biochemicals (SB)	22,992.891	5	4598.578	36,856.808	0.000*
S×SB	4,326.240	10	432.624	3,467.406	0.000*
Within groups	4.492	36	0.125	-	-
Total	28,643.230	53	540.438	-	-

*: Significance at $p<0.05$

Multiple comparisons by Turkey HSD test was done on difference in amount of secondary biochemicals between different seasons and the significance values showed except between winter and monsoon, and monsoon and winter seasons (**Table 6**).

Table 6: Multiple comparisons (Turkey HSD) on differences in amount (mg/g dry weight) of secondary biochemicals of leaf of *Annona reticulata* between different seasons .

Multiple comparisons	Season (I)	Season (J)	Mean diff. (I-J)	Std. error	Significance
Turkey HSD	Winter	Summer	-10.467	0.118	0.000*
		Monsoon	0.040	0.118	0.939(N.S.)
	Summer	Winter	10.467	0.118	0.000*
		Monsoon	10.506	0.118	0.000*
	Monsoon	Winter	-0.040	0.118	0.939(N.S.)
		Summer	-10.506	0.118	0.000*

*: Significance at $p<0.05$

Based on observed means, the mean difference is significance at the 0.05 level

Discussion

Plants cells produce primary and secondary metabolites. Primary metabolites are directly related to plant growth, development and metabolisms. They are carbohydrates, proteins and lipids. From primary metabolites secondary metabolites are produced. These are alkaloids, flavanoids, tannins, phenolics, terpenoids, steroids etc. They basically act as defence chemicals and their absence do not affect the plants. These secondary metabolites have medicinal values as well as exhibiting physiological activity [24].

Qualitative Phytochemical analyses of dried leaf powder of *A. reticulata* revealed the presence of many secondary metabolites such as, terpenoids, alkaloids, steroids, tannins, flavonoids, phenols, amino acids, and anthraquinones but the absence of saponins. Primary metabolites and some secondary metabolites were investigated quantitatively in winter, summer, and monsoon seasons of West Bengal.

Plants are continuously challenged by diverse phytophagous insect and to acquire their own survival fitness in nature, they produce inducible and constitutive defenses to reduce insect damage. Most plants are resistant to insect herbivores. This resistance occurs in plants due to an array of toxic and deterrent small molecules, which are responsible for prevention insects from feeding. Many plant defenses are produced constitutively, and others defences are inducible. Inducible plant defences include, nicotene in Nicotiana tabacum, protease inhibitors in *Solanum lycopersicum,* benzoxazinoids in *Zea mays*, glucosinolates in *Arabidopsis thaliana* etc. [32].

The amount of carbohydrate was found in ranged from 3.21 ± 0.005 to 0.74 ± 0.003 mg/g dry weight of mature shade dried leaf of the plant. The amount of carbohydrates was found highest in winter season and lowest in monsoon in the present study. Carbohydrates are the most important component for metabolism, due to supply the energy, needed for respiration and other metabolic processes. During summer season, photosynthesis increases because of high temperature and optimum solar radiation, but soluble sugars are mobilized in most of the growing part of the plant, so in summer season, carbohydrate content level remain lowest. During winter season

carbohydrate reserves are allocated in leaves for respiration during winter [25]. Carbohydrate concentrations in leaves increased in winter season because A. reticulata plant maintains leaves (live) throughout the winter period, because of response to environmental factors such as low temperature. Ludovici et al., (2002) [33] also observed 2-3 fold increase in glucose concentrations in winter season when compared summer production of glucose levels in needles of *Pinus taeda* L. the high levels production of glucose in leaves during the winter assessment period due to a response to the low levels of environmental temperatures. Similar types of observation was found i.e. highest levels of glucose concentrations in leaves of oak tree might increase in winter, investigated by Martinez-Trinidad et al., 2009 [34].

Starch is the reserve carbohydrates available for growth, energy and maintenance. Low starch reserve can be related with lower flowering, fruit set and yield. Trees store reserves for their survival in winter and for bud flush and leaf growth in the following spring. These reserve functions are mainly occur by starch which is degraded to soluble carbohydrates during winter season for respiration and in spring time during bud flushing [35].

Non Structural Carbohydrate (NSC) is a mixture of two fractions, starch and soluble sugars with contrasted functions. Starch is a purely storage component for future use. Soluble sugars perform a variety of functions viz., new growth, respiration, defence, and their role as intermediary metabolites, osmolytes, and substrates for transport [36]. The NSC level of a plant is an important indicator of the carbon source and sinks capacity of plant [37]. Amount of starch content of leaves of A. reticulata was highest in summer and lowest in monsoon. Hoch et al., 2003 [38] reported the highest concentrations in starch, in leaves in early summer for deciduous species. Yellow birch and sugar maple seedlings had the highest total starch by the end of the growing season [39].

Seasonal variation trends in leaf protein of *A. reticulata* were found in the present study. During summer, quantity of soluble proteins in mature leaves was higher than winter and monsoon. Protein bio molecules are the most important building block and a major determinant in controlling tree growth and reproduction, similar to other plant [40-42]. This is probably because of allocation of resource materials in fruits and flower etc., act as a sink rather than source. The results denoted here that the soluble protein is an indicator of the state (physiological) of the plant.

The amount of total lipids in the mature leaves of *A. reticulata* ranged from 70.33–85.70 mg g^{-1} dry weight during the study periods. The maximum amount of lipid content was found in summer season i.e. 85 mg g^{-1} dry weight and minimum in winter season (70.33 mg g^{-1} dry weight). The level increased in the early growing season and remained elevated throughout the summer. Momin and Kadam, 2011 [43] reported the same finding about lipid content of leaves of *Sesbania cannabina* plant, in which lipid content of leaves was higher in summer (23.6 mg/g) over than winter (22.55 mg/g). Similar finding was also observed by Pihakaski et al., 1987 [44] and Chapman et al., 1998 [45].

Growth, metabolism and development of the plant are influenced directly by effect of phenolic compounds of the plant [46]. Phenols are beneficial compound for the plant, due to its roles in plant as stress protecting, feeding deterrent, and attractants. They are most important biochemicals for the plant as they act as resistance to plant pathogen [47]. Phenolic compound found in different parts of the plant, viz., leaf, shoot, root, leaf bud, flower, leaf bud, the woody tissues, phloem, styles and pollen [48]. In the current study, phenolics of leaves of *A. reticulata* ranges from 1.10 ± 0.06 to 0.52 ± 0.01 mg/g dry weight, in which highest amount was observed in winter season and lowest amount was found in monsoon. Santosh et al., 2011 [49] reported that total phenols and other secondary metabolites in *Eugenia uniflora* leaves were analysed every month for one year and the results were correlated with climatic factors such as, rainfall, humidity, mean temperature, cloudiness. These facts suggest that climatic condition changes may be one of the factors affecting phenol levels in *Eugenia uniflora*. Leaves samples of the plant collected during the dry season and were characterized by high levels of phenol content i.e. 35.43 ± 6.24 mg g^{-1}. Mikulic Petkovsek et al., 2009 [50] reported that an increase in total phenolic content found in resistant as well as in susceptible apple cultivars. They reported an increase in phenolic content due to lack of rain. The content of phenolic compounds in the leaves depends on the cultivar (genotype) and on seasonal changes.

Animals do not eat certain leaves in particular seasons due to the presence high amount of tannins. They have both beneficial and adverse effects. Shrub and tree foliage are commonly rich in tannins and their effects on animals may be beneficial to toxicity and death [51,52]. Present study revealed that tannin content range from 10.10 ± 0.06 to 6.10 ± 0.06 mg/g dry weight in the study periods. Higher amount of tannin content was found in winter leaves than leaves of monsoon and summer, under the current study. Similar type of observation was found in some leaves of medicinal plants viz., *Barleria dinteri*, *Grewia flava* and *Jatropha lagarinthoides* in which amount of tannin content was found higher in winter than summer [53]. Flavanoid content varied from 22.43 ± 0.03 to 25.22 ± 0.04 mg/g under the present study. Highest concentration was found in leaves of the plant in summer and lowest concentration was found in winter season. Similar observation was found in the amount of flavanoid in leaves of *Cistus ladanifer* L. i.e. highest amount was found in summer and lowest in winter [54]. Alkaloids are group of secondary metabolites found in plants. About 2500 alkaloids contain nitrogen in heterocyclic ring and these are basic in nature. They are usually found in plants as salts of organic acid and showed important pharmacological properties [43]. Alkaloid content ranged from 40.93 ± 0.54 to 95.18 ± 0.56 mg/g of dry weight of leaves of the plant in the current study and attained highest concentration in summer season and lowest concentration was observed in winter i.e. 40.93 ± 0.54 mg/g. Similar observation was noticed in case of leaf alkaloid of *Sesbania grandiflora* and *Sesbania bispinosa* by Momin and Kadam, 2011 [43].

Terpenoid are correlated in many plants, those responding to biotic and abiotic environmental stimulating factors. Synthesis of terpenoids should be considered as defence mechanisms of the plant against stress, like phenolics [55]. In the current study, terpenoids ranged from 16.23 ± 0.18 to 9.87 ± 0.19 mg/g dry weight of mature leaf of the plant, being highest concentration in summer and lowest concentration in winter season. Algahtani et al., 2015 [56], reported similar finding regarding amount of terpenoid contents of *Centella asiatica* (L.) like the present study.

Plants are the main source of all the elements, which is essential for human beings. There is a correlation between elemental

contents and its nutritive values of the plant. Near about, all of the mineral contents (inorganic) of plant are obtained in the ash content. Ash content acts as an indicator of the total mineral content of the plant [57,58]. Ash content of the plant ranged from 152.43 ± 0.23 mg/g to 101.00 ± 0.58 mg/g dry weight of leaves of *A. reticulata* plant during study periods, being highest its peak i.e. 152.43 ± 0.23 mg/g dry weight and lowest amount was obtained in monsoon (101.00 ± 0.58 mg/g dry weight). In summer, amount of ash content of leaves of the plant was 106.75 mg/g dry weight. Similar observation was found in *Sesbania bispinosa*, in which total ash content in leaves ranged from 5.55% to 6.2%, with higher in winter than monsoon. In Wood of *Sesbania bispinosa*, total ash content was higher in winter (12.25%) than monsoon (12.00%) and summer (11.4%) Oko et al., 2016 [59] investigated on ash content of leaves of *Aspilia Africana* which ranged from 11.67 to 18.97% during the study periods. In August, the period of onset of bloom ash content was obtained 18.97% and decreased to 11.67% at the time of flowering of the plant (September).

Conclusion

In conclusion, the present study revealed a detailed biochemical profile and variation in some primary and secondary metabolites of mature leaves of *A. reticulata*. The plant is with rich source of starch, protein, and some secondary metabolites which can be used as a drugs of several diseases like, cancer, cardiac problem, worm infestation, constipation, hemorrhage, antifertility, antitumour and aborfacient etc. Further study is needed to know the identification of active principals which is responsible for the medicinal characteristics of the plant.

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