ssInternational Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 21-11-2020; Accepted: 06-12-2020: Published: 15-12-2020
www.botanyjournals.com
Volume 5; Issue 6; 2020; Page No. 564-571

Effect of lunar cycle based seasonal variation in physiochemical content of seeds and floral part of *Calotropis gigantea* (L.) R BR

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Abstract
Calotropis gigantea (L.) R. BR. (Asclepediaceae) is an evergreen, perennial shrub commonly known as "Sweta arka". Plant exhibited medicinal and agricultural characteristics as they contain a rich source of bioactive metabolites. Ayurvedic and Indian medicinal systems (*Charak* and S*ushruta Sanhita)* have recommended the collection of the drug on time and place. The objective of the present work was to collect the plant on the lunar cycle and seasons to attain a good requisite therapeutic drug without additional exploitation of medicinal plant resources from the planet. All plant parts were collected from Maliba college campus on every Full moon and No moon days in winter, summer, and rainy season in the year 2016. An aqueous extract was obtained by sample-solvent ratio (0.5:10) of *C. gigantea*. Quantitative primary and secondary metabolite content were determined. Statistical analysis was expressed with principle component analysis that interprets all possible related variables. Results revealed that summer Full moon and No moon was a favorable season for total carbohydrate, tannin, total phenolic content and total flavanoid content respectively while total protein, and total cardiac glycoside content were pronounced in winter season No moon and Full moon respectively for Floral part. Rainy Full moon was favorable season for total protein, flavanoids, and total tannin content while rainy No moon was favorable for carbohydrate while winter Full moon and No moon was favorable for tannin and cardiac glycoside respectively for seeds. The observation of experimental studies validates the concept of seasons and lunar collection of *C. gigantea* for superior quality and maximum therapeutic yield of active principles.

Keywords: new moon, full moon, *C. gigantea*, climate, ethno medicine drug, Allelo chemicals

Introduction

The Indian classic literature of *Acharya Charaka* and traditional medicine, advocates *evam rutu* (seasons) plays an important role in the collection of an optimum drug in the field of plant drug research. Ethnomedicinal drugs reported from different plant parts in different season have immense potential in the Indian traditional system [1, 2, 3] and for that active phytoconstituents are act as a "signatures" in the interaction of plant and environmental climatic condition. Phytoconstituents are influenced by variety of factors like temperature, rainfall, duration of the daylight, an effect of the lunar cycle, and soil condition as well as methods of collected [4, 5].

From an ancient to a modern era, *Calotropis gigantea* (Asclepiadaceae) is a wildly growing common plant and all the plant parts used as medicinal and agricultural purposes. In ancient Ayurvedic medicine, it is an important and valuable medicinal plant popularly known as "Sweta Arka" with a significant composition of phytoconstituents like flavanoids, cardiac glycoside, phenolics, proteins, triterpenes, etc. which are playing a vital role in curing various diseases [6]. Cardiac glycosides (steroids) are enhancing the force and myocardial contraction and inhibit the Na^+/K^+ ATPase [7]. Flavanoids are class of polyphenol which acts as a coloring and antioxidant agent with donating the electrons to stabilize the free radicals. Phenolic compounds are aromatic metabolites synthesized by plants which confer unique taste and flavor to plant-derived product [8]. Due to its high medicinal uses and easy availability, the plant is seeking more attention for Ayurvedic, Vedic, and modern research [9].

It was reported that root and Floral exhibited anticancer activity, stem bark exhibited anticonvulsant activity, hepatoprotective activity. leaves, whole plant, root, stem, and leaves exhibited rheumatism and, Floral exhibited antioxidant activity, leaves and Floral exhibited anti-inflammatory activity, wound healing and antidiabetic activity [10, 11, 12].

According to the world health organization (WHO), 4 billion people use herbal remedies for their primary healthcare as, it is safe, easily available, cheap, and with little or no side effects [13]. However, a natural and artificial disaster leads to the loss of natural resources. Hence to conserve the flora, the focus is on the highest therapeutic quality and lowest dosage of drugs. The lunar cycle along with seasons play a significant role in the production of phytoconstituents as Moonlight is about 15% as strong as sunlight [2]. Moreover, during the lunar cycle (New moon(NM)/*Amavasya* and Full moon (FM))/*Purnima* forces of the gravitational pull of the moon and water content of the soil are affected, these lead to drastic changes in the production of active primary and secondary metabolites. Hence, this study aimed at the Effect of lunar cycle based seasonal variation in phytochemical content of Floral and seeds part of *Calotropis gigantea* with an objective was to get a high - quality drug based on the lunar cycle and seasonal variation.

Materials and Methods

Materials

Anthrone, glucose, cuprous sulphate, sodium hydroxide, Na-K tarterate, sodium carbonate, FC(Folin Ciocalteu) reagent, sodium carbonate, sodium nitrite, tannic acid, giucose, aluminium chloride, 3,5-dinitrobenzoic acid, Folin's phenol reagent, were purchased from sd fine, Gujarat, India. Bovine serum albumin, gallic acid, rutin was purchased from Himedia Ltd., digoxin was purchased from SRL, ethanol was purchased from chalthan sugar factory, Gujarat, india

Collection, Authentication, extraction of plant

C. gigantea plant parts leaves and Floral were collected between 9:00 am-11:00 am to kept the "time" variable constant; during the seasons: winter (November-December), summer (April-May), and monsoon (July–August) of the year 2016 considered as the peak months for three seasons during the day after New moon(NM) and Full moon (FM) from Uka Tarsadia University, Maliba Campus, Bardoli (was Latitude - 21˚12", Longitude - 73˚11" 30 m above the sea level). The collected plant was identified at Navsari Agriculture University, Navsari, India. The voucher specimen (UTU/CGBIBT/17-18/04) was deposited in CGBIBT, UTU, India. The collected plant parts (floral and seeds) were washed thoroughly under tap water and dried at room temperature (37˚C). The dried plant parts were ground to a coarse powder and stored at 4˚C for further usage.

Preparation of extract

Powdered samples (5 gm of each) of the plant parts were extracted with 100 ml of sterile distilled water and macerate for 2 hrs at room temperature with continuous shaking. The resulting extracts were filtered with what-man filter paper no. 1(0.11µm) and concentrated to dryness by evaporation on a water bath. The yielded extracts were stored for further analysis.

Quantitative phytochemical analysis

Total Carbohydrate Content (TTC) and Total Protein Content (TPRC)

Carbohydrate content was estimated by modifying the anthrone method [10].1 ml of extract sample mixed with 4 ml of acidic anthrone reagent. Boiled in a boiling water bath for 8 min. Cooled rapidly and read the green to dark green color at 630 nm on a spectrophotometer (Shimadzu, UV-1800). The amount of total carbohydrate expressed in the mg g^{-1} of glucose (GLU).

Total protein content estimated by Lowry's method, based on under alkaline condition the reactivity of the peptide nitrogen(s) with the cu^{++} ion and subsequent reduction of the Folin-Ciocalteu(FC) phosphomolybdic phosphotungstic acid to hetero poly molybdenum blue[10]. 100µl of extract mixed with 500µl of alkaline copper sulphate reagent and incubated for 10 min at RT. 50µL of FC reagent added and incubated for 30 min at room temperature in dark. The blue color developed was read at 660 nm on a spectrophotometer. Protein content was expressed in mg g^{-1} of the dry mass of Bovine Serum Albumin (BSA).

Determination of Total Phenolic Content (TPC) and Total Flavanoid Content (TFC)

The method used for the determination of total phenol was used by Folin Ciocalteu reagent [14]. It works by measuring the amount of substance needed to inhibit the oxidation of the reagent. 100 µl extract mixed with 500 µl FC reagent (1:1 diluted) including the blank after 5 minutes add 2.5 ml of aqueous Na_2CO_3(20%), vortexed well and incubated in dark for 40 minutes. The presences of phenolics were read at 725 nm. Total phenolic values were expressed as Gallic acid equivalents per gm material (mg g^{-1} dry mass).

The method used for the determination of total flavanoids using aluminum chloride method in which it forms acid stable complexes with the C-4 keto group and either the C-3-C-5 hydroxyl group of flavones and flavonols was adapted from S. Karthikumaran, T. Sajeesh (2013), S. McDonald, Paul D (2000) [15] with some modification. It is based on 100 µl of extract added in 100 µl 5% $NaNO_2$, after 6 min. of incubation add 100 µl 10% $Alcl_3$. Incubate it for 6 minutes, and then add 500 µl of 4% NaOH and 2500 µl D/W. The presence of flavanoid was read at 510 nm. Total flavanoid value was expressed as Rutin equivalents (mg/ g^{-1} dry mass).

Determination of Total Tannin Content (TTC)

The Total Tannin Content was determined by the Phenol-Denis method [16]. 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 allowed to stand 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 the calibration curve of Gallic acid and the results were expressed as Gallic acid equivalent (mg g^{-1}).

Determination of Total Cardiac Glycoside Content (TCGC)

Total Cardiac Glycoside content was determined by DNBA (3, 5-dinitrobenzoic acid) method [16]. 1 ml of DNBA was added in extract and tubes were mixed well with vortex, 0.1 ml of sodium hydroxide solution was added to each test tube and mixed well. The resulting color was read at 565 nm to give optical densities proportional to test concentrations. Total cardiac glycoside value was expressed as Digoxin equivalent (mg g^{-1} dry weight).

Results and Discussion

The phytochemical analysis of *C. gigantea* Floral and seeds parts indicates significant variation in bioactive compound content with seasonal and lunar cycle. Table 1 shows the meteorological data for collection of Floral and seeds of *C. gigantea* according to the lunar and season cycle. Sunrise, sunset, moonrise, and moonset times were noted according to www.Timeanddate.com (Table: 1).

Table 1: Meterological report for *Calotropis gigantea*

Season	Date	Lunar Phase	Sun-Rise (am)	Sun-Set (pm)	Moon rise	Moon set	Moon rise	DN(h)	NL(h)	Illumination	Rainfall (mm)
Winter	14-11-2016	FM	06:47	17:57		06:14	18:04	11:09:54	12:51	99.30%	0
	29-11-2016	NM	06:56	17:55	06:34	18:03		10:58:45	13:02	0.20%	
Summer	22-05-2015	FM	05:58	19:11		06:20	19:38	13:13:28	10:47	99.80%	±0.6
	05-06-2016	NM	05:56	19:17	06:06	19:33		13:21:14	10:39	0.20%	
Rainy	03-08-2016	NM	06:12	19:15	06:28	19:36		13:03:05	10:57	0.30%	±303.9
	18-08-2016	FM	06:17	19:06		06:00	19:06	12:48:17	11:12	99.50%	

FM: full moon, NM: no moon, DL: Day length, NL: Night length Courtesy: Irrigation Division, Government of Gujarat, Navsari

Since Treta Yug, plants are globally valued, as they hold medicinal, agriculture, spiritual qualities and the presence of a natural product. *Calotropis gigantea* is growing everywhere such as roadside so that all the common people are used as primary healthcare, as they have a high medicinal value with richness in phytoconstituents.

However, as per the report, *Calotropis gigantea* is a common wild plant on which ample studies had been done but not a single study is accessible based on the lunar cycle along with seasonal variation. Hence, here we evaluate the proper collection of plants along with the perfect season. The lunar cycle gained attention for potency, purity, richness in quality, and highly active stage of the drug. Therefore, to prevent an environmental loss and get a high quality of drug we access the study of the effect of the lunar cycle on phytoconstitutents along with seasonal variation. Environmental conditions are not uniform for all the phytochemicals or for the entire plant organs investigated as they have different physiology and function[1]. Hence, they varied in quantity which is directly praposnal to the effect of the lunar cycle, seasons, collection time, day length and night length, soil physiology, etc.

Table 2 and Table 3 showed that according to the phytochemical composition of *C. gigantea* Floral and seeds parts were collected on winter, summer, and rainy season at New moon and Full moon.

Table 2: Phytochemical analysis of C. gigantea seed extract in winter, summer and rainy season during lunar cycle

Season	Carbohydrate (mg/g)	Protein (mg/g)	Flavanoid (mg/g)	Phenolic (mg/g)	Tannin (mg/g)	Card. y. (mg/g)	pH
GWNM	3.05±0.76	330.70±1.67	135.50±0.00	94.07±0.26	5.09±0.61	158.83±1.61	6.00
GWFM	1.49±0.37	330.93±0.94	130.50±1.00	94.15±0.32	5.08±0.87	148.17±2.02	6.50
GSNM	4.36±0.02	322.67±0.23	102.19±0.95	79.97±1.22	6.40±1.22	119.83±1.76	5.00
GSFM	2.74±0.55	325.80±1.00	103.83±2.89	83.85±1.60	7.00±1.56	129.45±0.79	5.50
GRNM	6.95±0.08	350.07±0.23	195.34±1.16	38.21±0.78	7.43±1.36	65.59±1.51	5.20
GRFM	6.19±0.73	355.60±0.35	198.99±1.01	21.07±0.90	7.83±0.76	69.56±0.50	5.80

All the values are expressed as Mean ± SD (Standard Deviation) (n=3) GWNM - *C. gigantea* Winter New moon, GWFM – Winter Full moon, GSNM - Summer New moon, GSFM - Summer Full moon, GRNM - Rainy New moon, GRFM - Rainy Full moon

Table 3: Phytochemical analysis of C. gigantea flower extract in winter, summer and rainy season during lunar cycle

Season	Carbohydrate (mg/g)	Protein (mg/g)	Flavanoid (mg/g)	Phenolic (mg/g)	Tannin (mg/g)	Card. gly. (mg/g)	pH
GWNM	5.05±0.49	180.15±1.03	12.07±0.24	13.89±0.11	6.40±0.54	15.83±0.58	6.00
GWFM	10.69±0.19	178.88±0.93	13.00±0.15	14.96±1.18	6.20±1.04	19.08±0.67	6.50
GSNM	13.32±0.21	170.45±0.39	27.45±0.48	25.44±0.45	9.47±0.28	13.67±1.04	5.50
GSFM	14.26±0.05	169.00±1.00	25.93±0.94	25.75±1.13	9.68±0.15	14.37±0.60	5.00
GRNM	04.70±0.08	70.77±1.14	21.25±2.15	23.19±0.95	7.27±0.99	9.80±0.78	4.90
GRFM	03.34±0.08	80.48±0.50	23.74±1.24	20.93±0.45	6.27±0.76	10.68±0.30	5.40

All the values are expressed as Mean ± SD (Standard Deviation) (n=3)

The results of carbohydrate content revealed that GSFM and GRNM exhibited highest TCC content in Floral (14.26±0.05mg g^{-1}) and seeds (6.95±0.08mg g^{-1}) followed by GWFM (10.69±0.19 mg g^{-1}) and GSNM (4.36±0.02mg g^{-1}) in Floral and seeds respectively as compared to other variables. It was observed that the TCC content from NM to FM, doubled in winter (GWNM-GWFT), slightly increased in summer (GSNM-GSFM) and decreased in rainy (GRNM-GRFM extract) in Floral, while in seeds exhibited half in summer (GSNM-GSFM) and fall in rainy season). Primary metabolites are playing a vital role in growth and development. Carbohydrates are plays a key role in nutrient assimilation [17]. While protein exhibit an important role throughout the plant cell of maintaining the structure and function of life [18]. Among all the samples, the highest protein content was found in GRNM and GRFM in Floral (180.15±1.03mg g^{-1}) and seeds (366.60±0.35mg g^{-1}) respectively, while lower in GRNM in Floral (70.77±1.14 mg g^{-1}) and GSNM in seeds (322.67±0.23 mg g^{-1}). TPRC was drastically raised from NM to FM in Floral in rainy and summer while raised in all the season in seeds. These results are in agreement with earlier studies in Sheth *et al*. wherein *C. procera* rich in protein content followed by carbohydrates. In present study the protein content was inversely praposnal to the carbohydrate content at the same season. It means reduction in carbohydrate content leads to rises in other constituents [19]. It was reported that the higher the protein value in plant higher the rate of increasing protein-based bioactive compound and food value [1].

moreover, in the present study the protein content in seeds are about 32-35% which was comparable with an earlier study wherein 19% protein was present in same spp [20] which was very low compare to our study and hence it indicate the role of environmental factors.

Secondary metabolites are responsible for plant growth and protection. Present study revealed that the TFC content in *C.*

gigantea Floral part was maximum (27.45±0.48 mg g^{-1}) in GSNM and minimum (12.08±0.24mg g^{-1}) in GWNM sample while moderate in GRNM extract (21.25±2.15mg g^{-1}), while in seeds, highest TFC present in GRFM (198.99±1.01 mg g^{-1}), moderate in winter (GWNM) (135.50±0.00 mg g^{-1}) and significantly low in GSNM (102.19±0.95 (mg g^{-1})). The result revealed that the TFC content was remarkably increased in all seasons from new moon to Full moon except winter (GWNM-GWFM) in seeds and summer (GSNM-GSFM) in floral part. Flavanoids are powerful antioxidants that help neutralize harmful reactive oxygen species which damage the cell and DNA, which can lead to degenerative disease. It was observed that the TPC content was pronounced in GSNM in Floral (25.44±0.45mg g^{-1}) and in GWFM in seeds (94.15±0.26mg g^{-1}) both compared to other sample. TPC was found to be significantly decreased from NM to FM in rainy and summer in floral while rainy season in seeds. Phenols might play an important role as dietary chain break antioxidants which prevent the oxidative damage in the living system [21].

Moreover, the antioxidant properties of TFC and TPC may be due to their redox potential and metal chelators. Similar studies have been reported on the same spp. wherein showed that variations were found in phytochemicals due to the different collection time. In According to observations of our study TPC and TFC content were higher than the previous study except for Floral, the flavanoids content which was maximum 27.45 mg g^{-1}. GSNM (9.68±0.28 mg g^{-1}) and GRFM (7.83±0.76mg g^{-1}) exhibited highest TTC content in Floral and seeds respectively. Tannins are known to react with proteins to provide typical effect which is important for the inflammation or altered tissue. It was found to be decreased in all the season in Floral while in seeds fall in winter only from NM to FM.

Furthermore, the cardiac glycosides are secondary metabolites and a group of steroid compounds that are traditionally used to increase the cardiac contractile force with congestive heart failure and enhance the velocity of myocardial contraction [17]. GWNM and GWFM showed highest CG content in seeds (158.83±1.61 mg g^{-1}) followed by Floral (19.08±0.67mg g^{-1}) respectively. GRFM exhibited minimum CG content in Floral and seeds. Cardiac glycoside was decreased in winter from NM to FM in seeds and increased in all the season in Floral. CG content was decreased from winter to rainy season in both the plant organs. Previous study of *C. procera* showed that CG content was very low compare to the present study. Floral and seeds parts had the same optimum pH 6.5 in winter Full moon season.

The results obtained were analyzed using Principle Component Analysis (PCA) through excel Minitab 16 software.

PCA is a statistical procedure that uses an orthogonal linear transformation that transforms the data to a new coordinate system. PCA results state the samples are well differentiated from each other as they lie in four different quadrants. Bi-plot shows a 2-D application to the original multidimensional space. *C. gigantea* seeds (Fig. 1) shows that variables are well differentiated from each other as they lie in four different quadrants. Winter samples (GWNM and GWFM) were distributed based on TCGC and pH with positive correlation. There was a negative correlation between TCGC and TFC, TPRC. Summer samples (GSNM and GSFM) were distributed based on TPC with a positive correlation with TCGC on PC1.

Rainy samples (GRFM and GRNM) were distributed based on TFC, TPRC which showed a strong positive correlation with TTC, TCC. TTC and TCC were distinguished in winter season at New moon (GRNM).

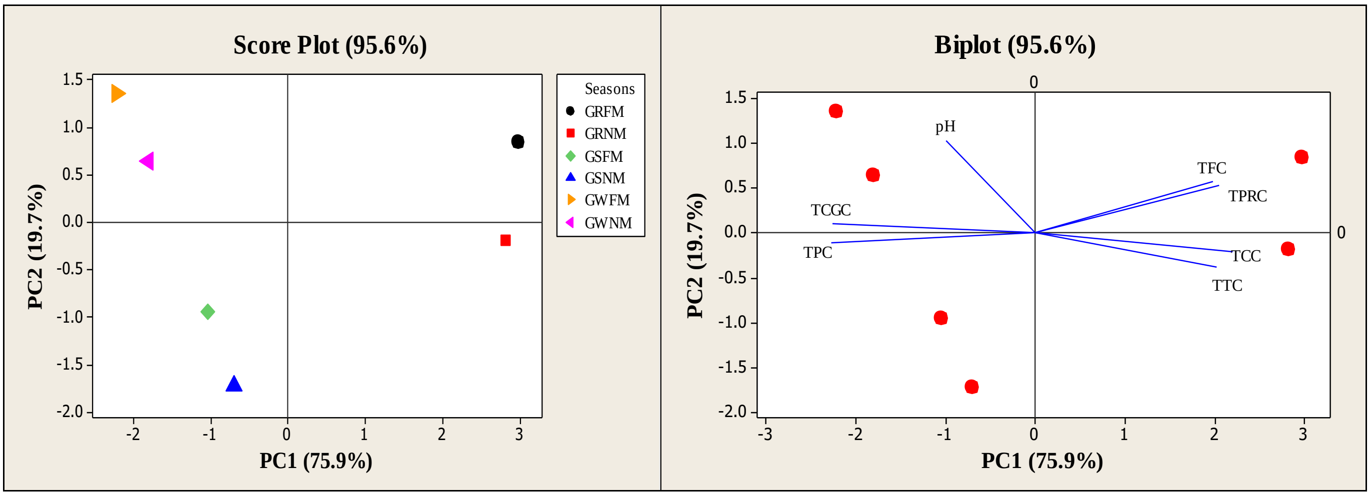

Fig 1: Phytochemical analysis of *C. gigantea* seed during lunar cycle. (A) Score plot (95.6%) (B) PCA biplot (95.6%).

C. gigantea Floral (Fig. 2) showed that GWFM separated based on TPRC, pH, and TCGC with positive correlation on PC1. GSFM and GSNM were distinguished by TPC, TFC with strong Positive correlation on PC1 and TTC was positively correlate with TCC while at the same time GWNM, GRFM, GRNM samples did not show any distribution towards variables.

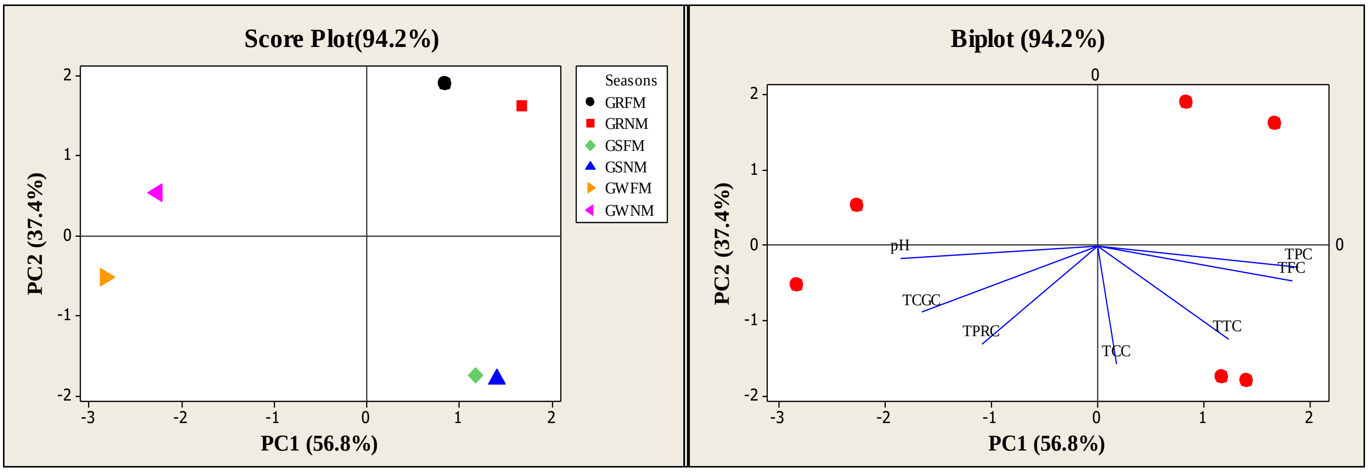

Fig 2: Phytochemical analysis of *C. gigantea* flower during lunar cycle. A) Score plot (94.2%) (B) PCA biplot (94.2%).

C. gigantea seeds and Floral New moon

Among New moon samples, seeds shows that summer (GSNM) distinguished based on TPC. GWNM was separated based on TCGC and pH with positive correlation at the same time in another quadrant GRNM sample separated because of TTC and TPRC with a strong positive correlation with each other but negative and no correlation with TCGC and pH respectively.

While TTC and TCC variables did not show any season distribution (Fig.: 1.1). While in Floral, GWNM sample distributed based on TCGC, pH, TPRC with strong positive correlation with each other. GSNM sample separated in view of TFC, TCC, TPC and TTC. For Production of TPRC and TCGC, GWNM became favorable with positive correlation. GRNM did not show any distribution for variables (Fig.: 2.1).

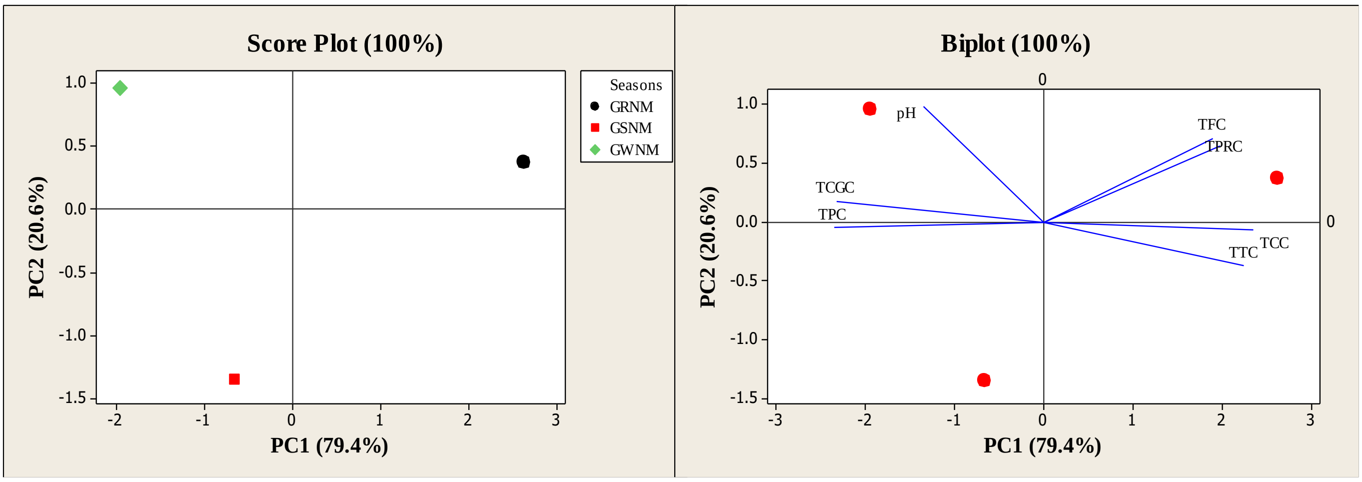

Fig 1.1: Phytochemical analysis of C. gigantea seed during New Moon. (A)Score plot (100%) (B) PCA biplot (100%).

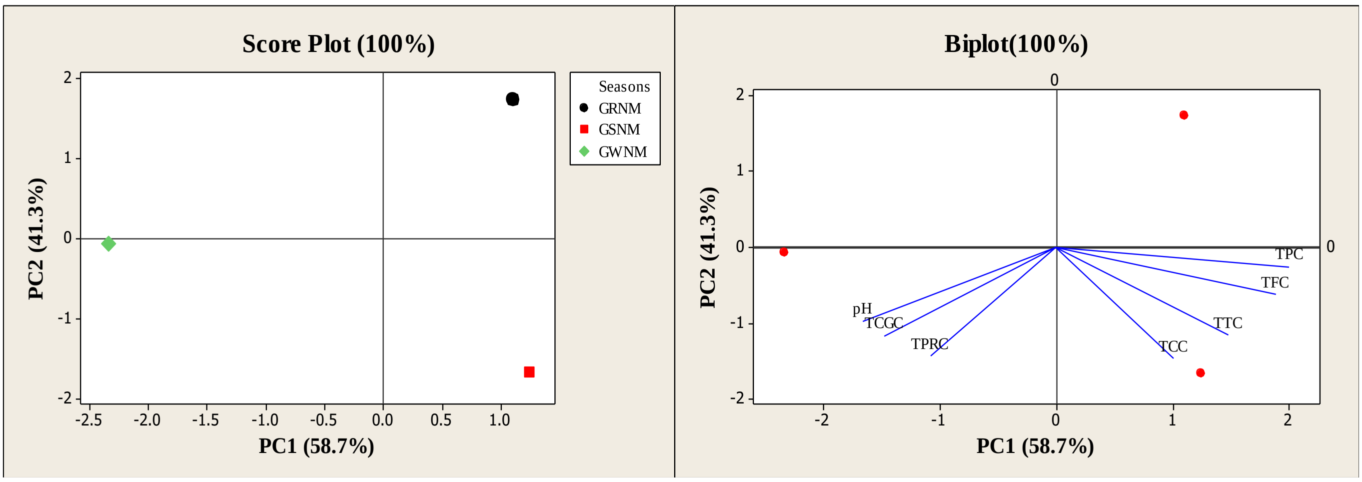

Fig 2.1: Phytochemical analysis of *C. gigantea* flower during New Moon. (A) Score plot (100%) (B) PCA biplot (100%).

C. gigantea seeds and Floral Full moon

Among Full moon samples, seeds showed that GWFM was separated in view of TCGC and pH while on the opposite side GRFM was distinguished in view of TPRC, and TFC. GSFM was separated based on TPC (Fig.: 1.2). It indicates that phenol plays an important role for C. gigantea in terms of defending stress and allelochemicals which are very useful in enzyme activity, biocontrol agent etc [22]. Floral parts showed that GSFM was separated based on TPC, TFC, and TTC variables with positive correlation on PC1. TCC, TPRC, and TCGC were not distinguished based on any season (Fig.: 2.2).

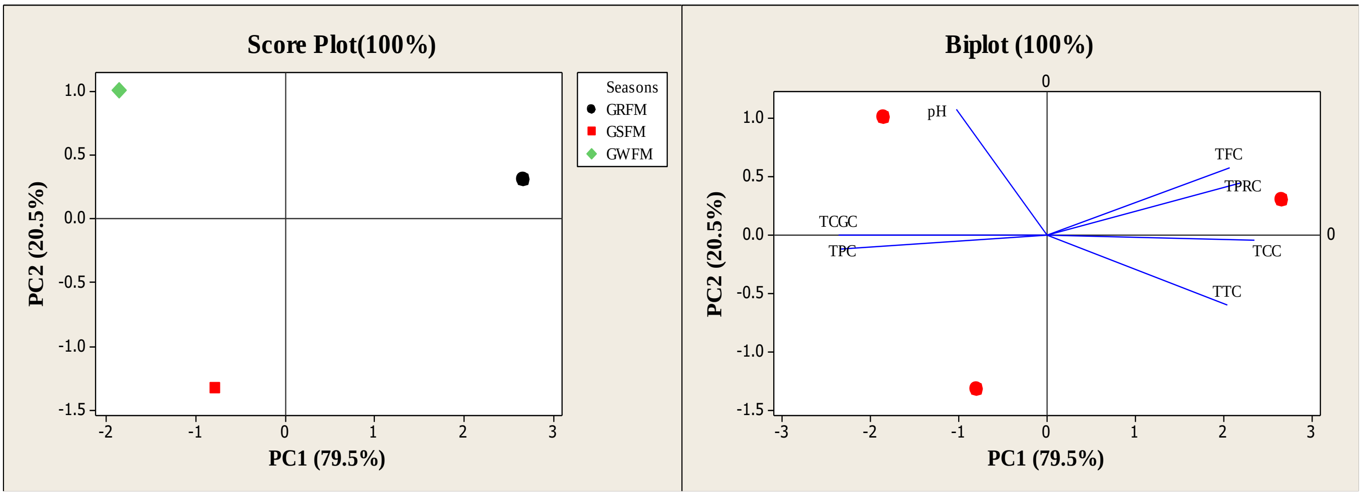

Fig 1.2: Phytochemical analysis of *C. gigantea* seed during Full Moon. (A)Score plot (100%) (B) PCA biplot (100%).

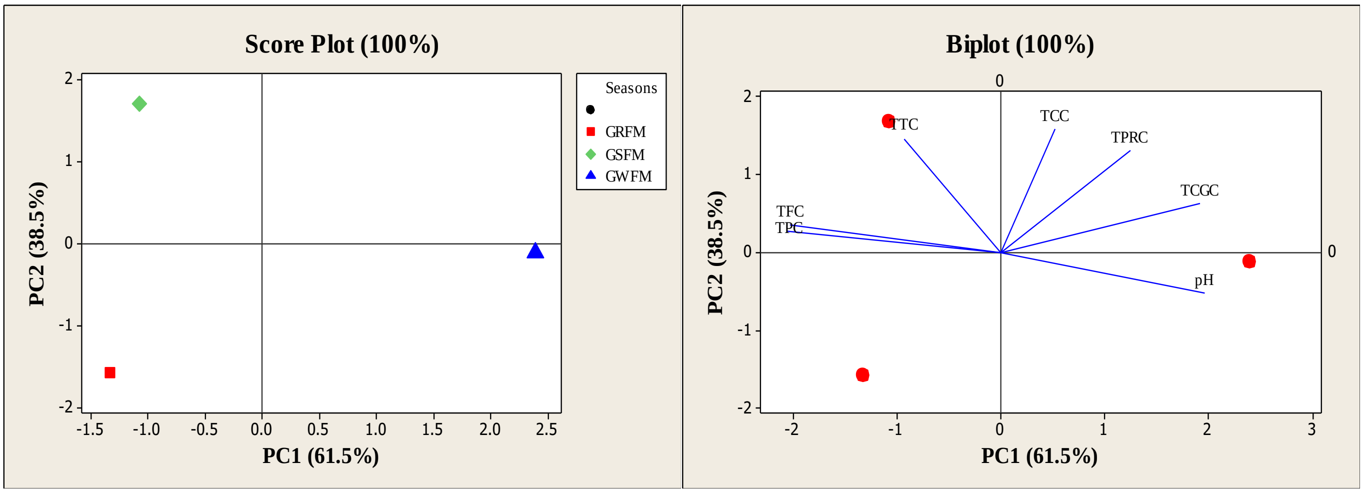

Fig 2.2: Phytochemical analysis of *C. gigantea* flower during Full Moon. (A) Score plot (100%) (B) PCA biplot (100%).

Seasons

Phenolic and Cardiac glycoside content were higher in seeds while protein and CG content were higher in flower at winter season.

This may be due to high humidity, low temperature stress and maturity of plants which might elevate the metabolites content [22].

TCC, TFC, TPC and TTC content were highest in floral at summer season indicate that sunlight might be associated with higher amount of polyphenols. TCC, TPRC, TFC TTC content were elevated at rainy season in seeds. These results might be due to maximum humidity, low intensity of sunlight and availability of large amount of water during rainy season which are favourable condition for the biosynthesis of these metabolites in seeds.

Moreover, Fig. 3 showed the comparison of seeds and Floral extracts with seasonal and lunar cycle along with phytochemicals.

Total tannin (Fig.: 3(F)) and total carbohydrates content (Fig.: 3(A)) were higher in Floral than seeds while rest were higher in seeds then Floral.

Total cardiac glycoside content was extensively higher in seeds than Floral as different organ pronounced in different phytochemical content.

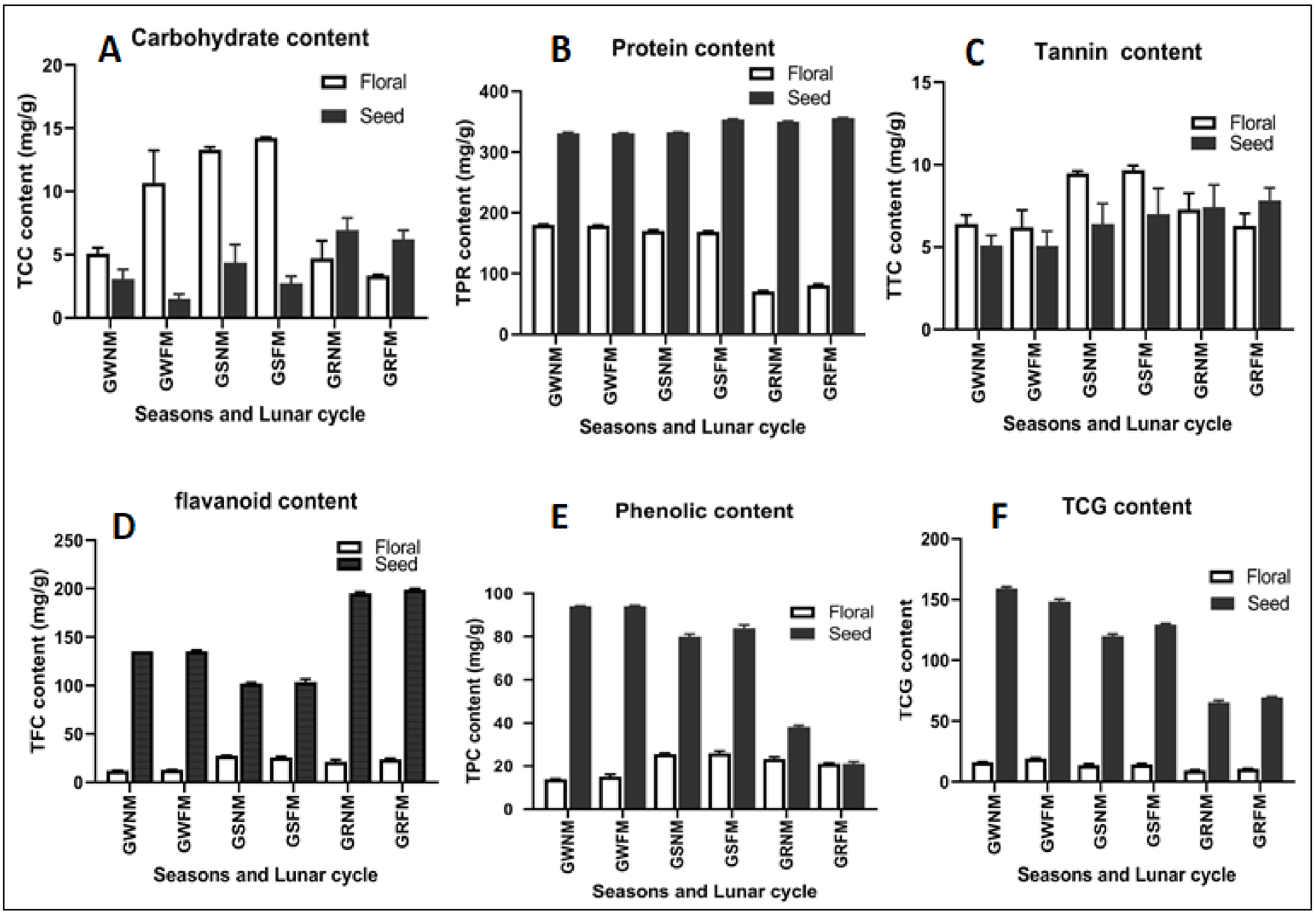

Fig 3: Physiochemical constituent's comparison of *C. gigantea* floral and seeds extract based on seasonal lunar cycle (A) Total carbohydrate content, B) Total protein content, C) Total Flavanoid content, d) Total phenolics content, E) Total cardiac glycoside

Amongst all the season, the day length of winter season days was more than the summer and the rainy season. Hours of day length of the summer season are slightly more than the rainy season. When compare with New moon *day*, Full moon days received more light which may be the key factor responsible for more photosynthetic activity depends on sunlight, anabolism activity, and moonlight (up to 99.5%).

Conclusion

The study explored the effect of lunar cycle based seasonal variation in physiochemical content of floral and seeds parts of *Calotropis gigantea*. Study showed significant variations (increase or decrease) in the bioactive constituents due to the New moon days, Full moon days, and season. *C. gigantea* is an ancient to modern medicinal plant used in treating various diseases from common cold, wound healing to rheumatism, diabetic, and cancer. To get the maximum active metabolites of high quality with minimum loss of flora, the aqueous extract of the plant parts (floral and seeds) were collected during Full Moon (FM) and New Moon (NM) of lunar cycles throughout the seasons. The finding demonstrated that the active metabolites were found more in FM days compared to NM days except for few phytochemical parameters pronounced in NM days. Summer season was favorable for TPC, TCC, and TFC from floral, winter was suitable for TPRC and CG of Floral while tannin and CG from seeds. Rainy season can yield high TPC, TFC and TCC from seeds. This confirms that floral and seeds parts extracted during the favorable lunar cycle and seasons have strong potential to increase the therapeutic efficiency by superior quality and high quantity of active phytoconstituents which in turn were influenced by the method of collection.

Financial support and sponsorship
Nil.

Acknowledgment
The authors are sincerely thankful to the C.G. Bhakta Institute of Biotechnology for providing all the necessary facilities to conduct this research.

Conflict of Interest

There are no conflicts of interest.

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