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SEASONAL DISCREPANCY IN PHENOLIC CONTENT AND ANTIOXIDANT PROPERTIES FROM BARK OF *NOTHAPODYTES NIMMONIANA* (GRAH.) MABB.

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ABSTRACT

Seasonal discrepancy in the antioxidant potential from bark of *Nothapodytes nimmoniana* was evaluated using two different methods (DPPH and FRAP) and were related to total phenolic content as well as flavonoid content. Higher yield of phenolic and flavonoid were observed during summer (May) similarly, the antioxidant activities (DPPH & FRAP) also were high. The antioxidant activities were strongly correlated with total phenolics (DPPH, FRAP) in all seasons, the maximum being during the summer (May) and minimum during monsoon (August). Total phenolic content showed highest correlation with DPPH and FRAP antioxidant assays during the month of winter (December). A specific pattern of increase from August-December-May was observed in phenolic content and antioxidant activities (FRAP and DPPH) tested for respective standards.

KEY WORDS

Nothapodytes foetida; HPLC; Phenolics; Flavonoids; DPPH; FRAP

INTRODUCTION

Nothapodytes nimmoniana (Grah.) Mabb. (also known as *Mappia foetida* Meirs or *Nothapodytes foetida* Sleumer) belonging to family Icacinaceae is a shrubby small tree, with broad dark green leaves and flowers distributed naturally in Western Ghats of India. The plant occupies important position in the plant based anticancer drugs because of the alkaloid camptothecin (CPT).

Plant polyphenols are the aromatic hydroxylated compounds, which are among most potent and therapeutically useful bioactive substances. They represent the largest group synthesized by higher plants. Phenolic compounds present in all plants are considered to have a great deal of biologically active constituents and therefore have been studied extensively. One of the more prominent properties of the phenolics is their excellent radical scavenging ability. Interest in these compounds has intensified in recent years

because of their possible health benefits including anticancer and antiviral activities[1] and reduced risk of coronary heart disease and stroke[2]. More than 8,000 naturally occurring phenolic compounds have been identified from various sources[3].

Plant polyphenols in general are multifunctional and can act as reducing agents, hydrogen donor and singlet O_2 quenchers exhibiting their antioxidant activity, via hydrogen atom transfer, electron donation, through metal chelation, interaction with other antioxidants (co-operative actions) or by localization and mobility of the antioxidant[4]. Many plant-derived substances, collectively termed "phytonutrients" or "phytochemicals" are becoming increasingly known for their antioxidant activity.

Besides many traditional natural antioxidants viz. tea, wine, fruits, vegetables and spices are commercially exploited as nutritional supplements[5] and many other species have been investigated in the search for novel antioxidants[6 - 8], yet there is an increasing need to find more information regarding the antioxidant potential of plant species.

Seasonal variation in plant secondary metabolites has been a subject of considerable interest. Researchers over the years are trying to understand effects of seasonal constraints on variation in the secondary metabolites. Generally it is suggested that environment alone has diminutive effect on production of secondary metabolites. However, synthesis is strongly activated by various biotic (animal grazing, cutting, fungal, microbe attack etc.) and abiotic (light, temperature, soil etc.) factors.

Owing to the different abiotic dynamics and variations in them, found across the globe, it is assumed that the patterns of seasonality would change in different parts of the world but the effect in production will remain the same.

The present work describes a comparative account on the antioxidant potential of *Nothapodytes nimmoniana* with total phenolic content (TPC), total flavonoids measured seasonally and also with their geographically isolated populations. The work herein also verifies TPC measured by UV-VIS and HPLC methods.

EXPERIMENTAL PROCEDURE

SAMPLING

Field surveys were carried out in the Southern parts of Maharashtra State, India (part of Western Ghats) locating populations of *N. nimmoniana* and a voucher specimen was deposited in the Herbarium, Laboratory of Angiosperm Taxonomy, Department of Botany, Shivaji University, Kolhapur (MS) India (voch. no. SRP 1/09).

The collection work was carried out during the year 2007-08 (Aug. 2007; Dec.2007 and May 2008). Populations were identified based on their size, flowering, and were marked using Global Positioning System (GPS). The data for its distribution, altitude was collected. Plants above 2 m height and more than 15 cm girth at breast height (gbh) were selected from each population. Based on population size five such sites were sampled with different geographical and cliamtological settings (Table 1). Western Ghats is one of the global biodiversity hotspots with discrete tropical climate and distinct forest types. The peak periods of these seasons i.e. August, December and May were selected as representatives for monsoon, winter and summer seasons, respectively. The samples collected were oven dried at 55 ± 2 ^{o}C for 48 h and were powdered in a grinding mill.

Table 1.
Localities and their attributes selected for collection and study of *N. nimmoniana*

Sr. No.	Localities	Altitude M	Longitude	Latitude	Forest type
1.	Amba	860	E73º 47’	N16º 56’	Semi Evergreen
2.	Amboli	781	E73º 59’	N15º 57’	Semi Evergreen
3.	Chandoli	906	E73º 50’	N17º 03’	Semi Evergreen
4.	Naikwadi	617	E73º 56’	N16º 09’	Deciduous
5.	Panhala	932	E74º 06’	N16º 48’	Deciduous

SAMPLE PROCESSING:

Extracts for *N. nimmoniana* samples (Stem bark) were prepared according to the method previously described by Fulzele and Satdive (2005)[9] with minor modifications. Powdered plant material (Stem bark) 1g obtained from the localities during different seasons were put individually in to 150 ml Erlenmeyer flask and 20 ml 90% Aq. MeOH (v/v) was added to it. The flasks with suspension were exposed sporadically for 12 min in a microwave oven, during which the suspension was cooled after every minute of exposure and excess boiling was avoided. The extracts were then cooled and filtered through filter paper (Whatmann No. 1) followed by centrifugation at 10,000 X g for 10 min, diluted 1:15 with same solvent and used for all experiments except for HPLC analysis, where the extract was injected directly without dilution after filtering it through a 0.2 µ nylon filter.

QUANTITATION OF TOTAL PHENOLIC CONTENT (TPC):

Total phenolic content was quantified using modified Folin – Ciocalteu method described by Wolfe *et al*., (2003)[10]. The absorbance of blue colour was read at 760 nm on double beam spectrophotometer. The results were compared to the standard curve and were expressed as gram per Tannic and/or Caffeic acid equivalent per 100 gram dry bark powder.

QUANTITATION OF TOTAL FLAVONOIDS:

Total flavonoid contents were quantified using method given by Luxmon-Ramma *et al*., (2002)[11]. Absorbance was measured at 368 nm on double beam spectrophotometer. The results were expressed as gram per Rutin and/or Quercetin equivalent per gram dry bark powder.

ANTIOXIDANT ACTIVITY:

2, 2 – DIPHENYL – 1-PICRYLHYDRAZYL (DPPH) ASSAY:

The antioxidant activities were determined as the measure of radical scavenging using DPPH assay as determined by Brand-Williams *et al*., (1995)[12]. The absorbance at 515 nm was measured using methanol as blank. The results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC) and Trolox equivalent antioxidant capacity (TEAC) as determined by Gil *et al*., (2000)[13].

FERRIC REDUCING ANTIOXIDANT POWER (FRAP):

The ferric reducing / antioxidant power (FRAP) assay was used to measure the total antioxidant power. Antioxidant activity assay was preformed as previously described and absorbance was taken at 593 nm[14]. The results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC) and Trolox equivalent antioxidant capacity (TEAC) [13].

QUANTITATION OF TOTAL PHENOLICS BY HPLC TECHNIQUE:

Instrumentation: The HPLC apparatus consisted of a water 6785 multi solvent delivery system, equipped with a UV dual detector. Data were processed using Waters Empower software. Chromatographic separation was achieved on a Waters C 18 column (Symmetry, 5 µm, 3.9 x 150 mm).

Chromatographic Conditions: Chromatography was carried out using two solvents: A) 25% methanol in 1% acetic acid; B) 30% methanol in 1% acetic acid in a linear gradient programme in a volumetric ratios as follows: 0-30 min, 100 A/0 B; 30-45 min, 82 A/18 B; 45-60 min, 72 A/28 B. The flow rate of the mobile phase was 0.75 ml/min. Peaks were detected at 280 and 360 nm absorption spectrum. Sample volume is 20 µl. The analysis time was 60 min for both, standards and samples used for analysis.

Calculations, Calibration Curves and Linearity: The stock solutions of standards were serially diluted with methanol to prepare working solutions for the calibration curves at three concentration levels (25, 50 and 100 ppm). The calibration curves for standards with the column were established by the peak areas (Area Under Curve AUC) and correlating it with concentrations of working solutions.

Recovery and validation: The recovery studies were performed with powdered bark of *N. nimmoniana*. Known amount of stock solutions of standards were added to a bark extract (100 µl each), and the mixture was analyzed using the proposed method. The peak area of the standard in the bark extract of *N. nimmoniana* with and without prior addition of standard compound was acquired, and the extraction recoveries of standards were calculated by the percentages of amount found divided by the amount added.

System Suitability: The system suitability test was assessed by three replicate injections of the standard solutions at a particular concentration. The peak areas of which were used to evaluate repeatability of the proposed method, and their peaks were analyzed for resolution.

DATA ANALYSIS:

Data are presented as means ± standard deviations (STDEV) of the three values and the results were processed for Student-Newman-Keuls Multiple comparison test. Differences up to $P < 0.05$ were considered significant. In addition, simple regression analysis was performed to seek relationships within and among the phenolic, flavonoids contents, antioxidant DPPH and FRAP assays during the months under study (August, December and May). The average content was calculated as a mean of 3 different readings obtained at different seasons.

RESULTS AND DISCUSSIONS:

SAMPLING:

Frequent field visits made in the selected localities for sampling reveled that there were patches of population of *N. nimmoniana* distributed in semi-evergreen and deciduous forest types. Cultivation of *N. nimmoniana* from seeds and cuttings have been successfully achieved in greenhouses of Department of Botany, Shivaji University, Kolhapur (MS) India, however growth is very slow and will certainly take years to attain maturity, similar observations are quoted by Patwardhan and Vasudeva (2006)[15].

QUANTITATION OF TOTAL PHENOLIC CONTENT (TPC):

Extraction yields of TPC for 5 localities and 3 seasons under study are depicted in Table 2. Amboli averaged lowest 11.25 ±4.71 g Tannic acid equivalent (TAE)/100g dry stem bark powder and 0.89 ±0.37 g Caffeic acid equivalent (CAE)/100 g dry stem bark powder and Panhala averaged highest (20.75 ±1.85 g TAE/100g dry stem bark powder and 1.64 ±0.15 g CAE/100 g dry stem bark powder) among all localities irrespective of the seasons (Table 2). There was >45% difference in average phenolic content between highest and lowest ranked localities against both the equivalents tested. The localities of sample collected may be arranged on basis of phenolic content from lowest as in Amboli < Naikwadi < Chandoli < Amba < Panhala to highest.

The total phenolic content ranged from 5.92 ±0.28 to 23.36 ±3.33 g/100g TAE and 0.47 ±0.02 to 1.85 ±0.26 g/100g CAE of dry stem bark powder. It was high in extracts of stem bark samples collected during summer

(May) for all the localities and both the standards tested, except Amboli where monsoon (August) accumulated high percent (Table 2) whereas, low content was observed for the samples collected during monsoon (August). The difference between the highest and lowest TPC during the seasons tested in the localities was: Chandoli: 66.22%, Amboli: 60.08%, Naikwadi: 45.22%, Amba: 28.87% and Panhala: 16.23%.

A pattern of increase was observed in average TPC from monsoon (August) < winter (December) < summer (May), where monsoon averaged lowest with 13.85 ± 4.10 g TAE/100g dry stem bark powder and 1.10 ± 0.32 g CAE/100 g dry stem bark powder, winter averaged medium with 15.92 ± 5.75 g TAE/100g dry stem bark powder and 1.26 ± 0.46 g CAE/100 g dry stem bark powder and summer averaged high 20.58 ± 4.28 g TAE/100 g dry stem bark powder and 1.63 ± 0.34 g CAE/100 g dry stem powder. Yao and co-workers (2005)[16], showed higher levels of TPC during warm months and lower during cool months in Australian grown tea (*Camellis sinensis*) and similar results reported by Erturk and co-workers (2010)[17] in same species from Turkey support the findings.

The physiological and biochemical changes are synergistic effects of different biotic and abiotic parameters. In most of the plants exposure to high or low temperature than the optimum is regarded to be the main cause[18 - 22], suggesting temperature to be the regulating factor for change in TPC. There was more than quarter fold increase in the average TPC in bark during summer than observed in monsoon. Signifying seasonal changes may alter production of phenolic compounds.

QUANTITATION OF TOTAL FLAVONOID CONTENT

Extraction yields of flavonoid content for 5 localities and 3 seasons are tabulated in Table 2. Chandoli averaged lowest (0.88 ± 0.45 g Rutin equivalent (RE) /100g dry stem bark powder and 2.40 ± 1.23 g Quercetin equivalent (QE) /100g dry stem bark powder) and Amboli averaged highest (1.99 ± 0.31 g RE/100g dry stem bark powder and 5.40 ± 0.85 g QE/ 100 g dry stem bark powder) among all localities irrespective of the seasons. The difference in average flavonoid content between highest and lowest ranked localities against both the equivalents tested here was > 50%. The localities may be arranged on basis of phenolic content from lowest as in Chandoli < Panhala < Naikwadi < Amba < Amboli to be the highest.

The total flavonoid content ranged from 0.36 ±0.01 to 2.33 ±0.03 g RE/100 g dry stem bark powder and 0.98 ±0.03 to 6.34 ±0.08 g QE/100 g dry stem bark powder. There was no particular trend observed for flavonoid content during summer (May) > winter (December) > monsoon (August) as seen in case of phenolics

The difference between the highest and lowest flavonoid content during the seasons tested in the localities was: Chandoli: 69.74%, Amba: 26.24%, Amboli: 26.18%, Panhala: 18.07%, and Naikwadi: 0.59%. Unlike pattern of increase observed (summer > winter > monsoon) in TPC, flavonoid showed a mixture of patterns. Locality Amboli showed reverse pattern (monsoon > winter > summer), Chandoli and Panhala had no regular pattern wherein Chandoli, the pattern of increase was winter > summer > monsoon and that for Panhala it was summer > monsoon > winter. There was hardly any variation observed in the content at locality Naikwadi. Furthermore, monsoon averaged lowest with 1.49 ± 0.71 g RE/100 g dry stem bark powder and 4.04 ± 1.94 g QE/ 100 g dry stem bark powder, winter averaged moderate with 1.61 ± 0.32 g RE/100 g dry stem bark powder and 4.38 ±0.87 g QE/100 g dry stem bark powder and summer averaged high with 1.67 ±0.39 g RE/100 g dry stem bark powder and 4.54 ±1.07 g QE/100 g dry stem powder.

Which show, on an average warmer temperature elevating content of flavonoids. Considering grouping of flavonoids broadly under polyphenols, increase in the content during summer is sustained, nevertheless with no regular pattern observed in the localities.

The plant under study contains phenolic compounds which can serve as

natural sources to develop free radical scavengers. The phenolic compounds (TPC and flavonoid) may contribute directly to the antioxidant action. It is a known fact that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in human. Antioxidants play important role in cancer treatment, besides the already known anticancer properties of *N. nimmoniana*, present investigation adds to its radical scavenging potential of phenolic compounds.

ANTIOXIDANT POTENTIAL:

2, 2 – DIPHENYL – 1 – PICRYLHYDRAZYL (DPPH) ASSAY

Free radical scavenging potentials of bark powder of *N. nimmoniana* as tested by DPPH assay are presented in Table 3. The $DPPH_{TEAC}$ evaluated was 18.39% higher in all the seasons tested for all the localities as compared to that of $DPPH_{AEAC}$ with R^2= 1 at $P < 0.001$.

Locality Amboli averaged lowest (333.88 ±83.50 AEAC and 409.09 ±102.31 TEAC), whereas Amba averaged highest (556.31 ±86.43 AEAC and 681.63 ±105.90) among all the localites tested devoid of seasons (Table 3). In present study for antioxidant activity measured by DPPH radical scavenging assay, among the months tested, on an average samples collected during summer (May) showed the most potent radical scavenging activity than any other months. The activity was poor during monsoon (August) with an average of 398.66 ±15.86 µM AEAC and 488.47 µM TEAC, whereas summer (May) averaged more than quarter fold higher activity (576.49 ±19.29 and 706.36 ±23.64 µM AEAC and TEAC, respectively) than in monsoon.

The pattern in the activity observed for samples collected during different seasons from all the localities resembled to the patterns observed in total phenolic content in this plant. Although locality Amba deserves a detail attention as no perfect relation between them was established. However, decline in the DPPH radical scavenging activity during winter for the samples collected from Amba and Amboli would be explained from the concept that antioxidants are potent reducing agents and as like in majority of cases even here the antioxidant activity is due to phenolic compounds. The localities Amba and Amboli fall in semi-evergreen patch of the tropical forests of Western Ghats and are represented by average low temperature and high rainfall among the study sites. Low rate of dehydration due to low temperature and high rainfall forms to be the probable reason for this drop.

Also the disturbance in the regular pattern of increase in the activity of DPPH assay from monsoon to summer in Amba and Amboli may perhaps be due to the presence of other reducing agents that are not reactive towards DPPH. Antioxidant compounds like polyphenols may be good reducing agents for iron but may not scavenge DPPH due to steric reasons. Similar results for low or no result is observed by Spanou *et al*. (2007)[23]. There might also be reducing agents which are present in the extracts those are produced during winter (December) in these localities which show low or less response to DPPH antioxidant assay.

Table 2

Total phenolic and flavonoid content of bark sample of Nothapodytes nimmoniana collected from different localities during different seasons.

Locality	Month	Total Phenolics				Total Flavonoids			
		Tannic acid	Average	Caffeic acid	Average	Rutin	Average	Quercetin	Average
Amba	August	15.25 ±3.60	17.96 ±3.17	1.21 ±0.28	1.42 ±0.25	1.63 ±0.04	1.91 ±0.29	4.43 ±0.11	5.19 ±0.78
	December	17.17 ±1.36		1.36 ±0.11		1.89 ±0.07		5.15 ±0.19	
	May	21.44 ±0.96		1.70 ±0.08		2.21 ±0.06		5.99 ±0.16	
Amboli	August	14.83 ±0.56	11.25 ±4.71	1.17 ±0.04	0.89 ±0.37	2.33 ±0.03	1.99 ±0.31	6.34 ±0.08	5.40 ±0.85
	December	05.92 ±0.28		0.47 ±0.02		1.91 ±0.05		5.18 ±0.13	
	May	13.01 ±0.51		1.03 ±0.04		1.72 ±0.09		4.67 ±0.25	
Chandoli	August	07.89 ±1.36	16.60 ±7.92	0.63 ±0.11	1.32 ±0.63	0.36 ±0.01	0.88 ±0.45	0.98 ±0.03	2.40 ±1.23
	December	18.56 ±1.58		1.47 ±0.12		1.19 ±0.01		3.24 ±0.02	
	May	23.36 ±3.33		1.85 ±0.26		1.09 ±0.09		2.97 ±0.25	
Panhala	August	18.99 ±0.82	20.75 ±1.85	1.50 ±0.07	1.64 ±0.15	1.69 ±0.04	1.69 ±0.01	4.58 ±0.10	4.59 ±0.01
	December	20.59 ±1.30		1.63 ±0.10		1.70 ±0.09		4.61 ±0.25	
	May	22.67 ±2.24		1.80 ±0.18		1.69 ±0.02		4.59 ±0.04	
Naikwadi	August	12.27 ±1.09	17.35 ±5.07	0.97 ±0.09	1.37 ±0.40	1.43 ±0.03	1.48 ±0.15	3.89 ±0.08	4.03 ±0.42
	December	17.39 ±2.17		1.38 ±0.17		1.36 ±0.11		3.71 ±0.29	
	May	22.40 ±0.42		1.77 ±0.03		1.66 ±0.11		4.50 ±0.31	

Table 3 .

Antioxidant activity as measured by DPPH and FRAP assay for bark sample of Notahpodytes nimmoniana collected from different localities during different seasons.

Locality	Month	DPPH (µM)						FRAP (µM)					
		AEAC ±SD		Average	TEAC ±SD		Average	AEAC ±SD		Average	TEAC ±SD		Average
Amba	August	572.36	±18.58	556.31 ±86.43	701.30	±22.77	681.63 ±105.90	383.72	±28.42	565.32 ±233.73	193.76	±14.35	285.46 ±118.03
	December	462.98	±55.23		567.28	±67.67		483.20	±14.21		244.00	±07.18	
	May	633.58	±22.82		776.32	±27.96		829.03	±41.03		418.62	±20.72	
Amboli	August	368.04	±16.20	333.88 ±83.50	450.96	±19.84	409.09 ±102.31	326.87	±14.21	334.77 ±163.58	165.06	±07.18	169.04 ±82.60
	December	238.71	±03.15		292.49	±03.86		175.28	±08.21		88.51	±04.14	
	May	394.87	±06.05		483.83	±07.41		502.15	±21.71		253.57	±10.96	
Chandoli	August	271.04	±12.40	490.26 ±199.37	332.11	±15.19	600.72 ±244.29	170.54	±00.00	500.57 ±308.48	86.12	±00.00	252.77 ±155.77
	December	538.99	±10.94		660.42	±13.40		549.53	±32.82		277.49	±16.57	
	May	660.76	±07.46		809.62	±09.15		781.65	±61.95		394.70	±31.28	
Panhala	August	410.01	±21.18	534.75 ±108.90	502.38	±25.95	655.22 ±133.44	397.93	±14.21	581.11 ±177.91	200.94	±07.18	293.44 ±89.84
	December	583.36	±02.15		714.79	±02.63		592.16	±35.77		299.02	±18.06	
	May	610.88	±15.62		748.51	±19.13		753.23	±37.60		380.35	±18.99	
Naikwadi	August	371.83	±10.94	476.96 ±105.25	455.59	±13.40	584.42 ±128.97	307.92	±16.41	554.26 ±294.97	155.49	±08.29	279.88 ±148.95
	December	476.74	±18.83		584.14	±23.07		473.73	±21.71		239.21	±10.96	
	May	582.33	±44.51		713.52	±54.53		881.14	±37.60		444.94	±18.99	

FERRIC REDUCING ANTIOXIDANT POTENTIAL (FRAP)

Trolox Equivalent Antioxidant Capacity (TEAC) and Ascorbic acid Equivalent Antioxidant Capacity (AEAC) of dry stem bark powder of *N. nimmoniana* as measured by FRAP is shown in Table 3.

The $FRAP_{AEAC}$ evaluated was > 50% higher in all the seasons tested for all the localities as compared to that of $FRAP_{TEAC}$ with R^2= 1 at $P < 0.001$. An average higher level of antioxidant activity was observed for May (706.36 ± 129.32 µM TEAC and 576.49 ±105.55 µM AEAC) and average lowest was for August (488.47 ±134.56 µM TEAC and 398.66 ±109.82 µM AEAC) devoid of localities. Summer (May) yielded highest level of ferric reducing antioxidant potential than winter (December) and monsoon (August) which was lowest. Although the plants are different, similar results of $FRAP_{AEAC}$ to be higher than $DPPH_{AEAC}$ and $DPPH_{TEAC}$ higher than $FRAP_{TEAC}$ is observed.

The antioxidant capacity evaluated by the FRAP method for AEAC was higher than that evaluated by DPPH assay and the reverse was true for TEAC. These results are in accordance with previous reports on antioxidant capacities of fruits, vegetables and medicinal plants which exhibit polyphenols to play important role in the activity [24, 25]. Moreover, the antioxidants tested by FRAP assay are limited to water-soluble (i.e. soluble in aqueous alcoholic solution) components compared to DPPH assay (organic solvents especially alcohols) [26, 27].

The inhibition of accumulated colored radical reagents (DPPH) in the presence of antioxidants is expressed in the units of lag time, which is unstable for this antioxidant assay, as the time is not always linearly correlated to antioxidant concentration. Whereas in case of FRAP assay it is based on Fe^{+++} to Fe^{++} reduction in presence of Fe^{++} stabillizing agent tripyridyltriazine (TPTZ) [14]. Although there is a wealth of data on importance of antioxidants in health care and its oxidation ability, the correlation between antioxidant activity and chemical structure is not so clear. However, it is known that they interrupt lipid oxidation, either in the propagation phase or by protecting the oxidation substrates against the first formed radicals in the initiation phase. Accordingly, evaluation of plant material for antioxidative activity should not depend only on a single method, but it should include measurement by various methods compared to possible standards [28]. Hence it may be clear from the available literature that there is no 'total antioxidant' for the labeling of biological samples because of the lack of standard quantitaiton methods. However it may also be understood that the assay measure the potency of biological samples under a certain defined conditions given by the method using different oxidants.

ANALYSIS OF TOTAL PHENOLICS BY HPLC TECHNIQUE

HPLC of highest (Chandoli, May) and lowest (Amboli, December) content of total phenolics containing samples along with 3 other: highest TPC in the month of December (Panhala), lowest in the month of August (Chandoli) and moderate in May (Naikwadi) was performed.

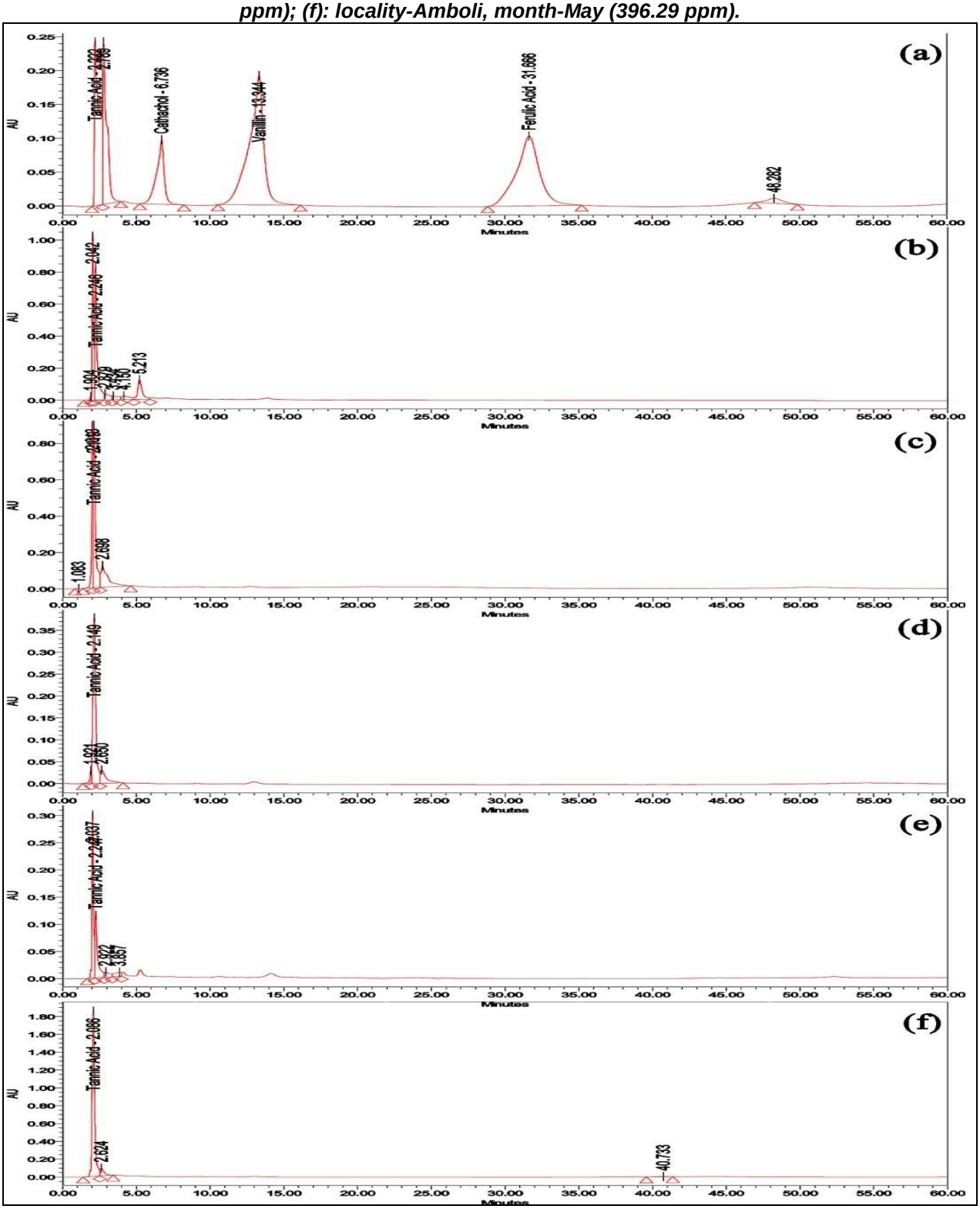
There are significant number of compounds in different phenolic classes, each of which has a different absorption maximum, most of all phenolics such as hydroxybenzoic acid derivatives absorb light at 280nm [29]. According to the HPLC analysis, the phenolic profile of each sample was expressed in ppm of tannic acid for hydroxybenzoic acid derivative. HPLC analysis of different concentrations of standards (25, 50 and 100 ppm) yielded profiles with a retention time of 2.223 (±0.026) min for Tannic acid, 6.772 (±0.035) min for Catechol, 13.370 (±0.033) min for Vanillin and 31.634 (±0.036) for Ferulic acid. Calibration curves were linear with R^2 = 0.999 for all the four standards.

The HPLC profiles showed several other peaks between RT 0 – 5 min at 280 nm for samples collected during May and August from Naikwadi and Chandoli respectively (Fig 1). One (tannic acid) out of 4 phenolic compounds was well separated by HPLC; rest three of them catechol, vanillin and ferulic acid were not detectable in any of the tested samples (Fig 1). Methanolic extract of sample collected from Chandoli during May showed highest tannic acid content (396.29 ppm) and lowest in Amboli for the sample collected during December (022.34 ppm).

The Folin – Ciocalteu based estimates were substantially higher than the HPLC – derived measurements of phenolic compounds. The highest phenolic yielding sample (Chandoli, May) among all the sampling also yielded high tannic acid in HPLC analysis similarly the lowest yielding and other selected samples also did follow the same course.

Figure 1.

HPLC profile of phenolic compounds. (a): 4 phenolic compound standards (100 ppm); locality of bark collection and month (b): locality-Naikwadi, month-May (150.52 ppm); (c): locality-Panhala, month-December (162.04 ppm); (d): locality-Chandoli, month-August (89.21 ppm); (e): locality-Amboli, month-December (22.34 ppm); (f): locality-Amboli, month-May (396.29 ppm).



DATA ANALYSIS

Statistically linear, significant and strong correlations were observed between the standards for the same tests for all the seasons (Aug, Dec and May). Correlation coefficients (r^2) and significance (P value) between TPC, flavonoid content and antioxidant activity (DPPH and FRAP) tested for bark samples of *N. nimmoniana* collected during the months of August, December and May as determined by Student-Newman-Keuls Multiple comparison test are presented in Table 4. It also shows the color coding to indicate extent of correlation. For each experiment two standards were considered to determine TPC, flavonoid content and antioxidant activity (DPPH and FRAP). The correlation coefficients between the two standards (tannic acid and caffeic acid; rutin and quercetin; trolox and ascorbic acid) used in respective experiments (TPC; flavonoid content, DPPH and FRAP) used for all the three seasons (Aug, Dec and May) is same i.e. r^2=1.000 with < p=0.01 (Table 4), hence single standard (i.e. tannic acid, rutin and TEAC) is considered for statistically representation.

August (monsoon) showed highest correlation between TPC and antioxidant FRAP $_{TEAC\ and\ AEAC}$ followed by TPC and DPPH and poor correlation was found between TPC and flavonoid, flavonoid and DPPH $_{TEAC\ and\ AEAC}$, flavonoid and FRAP $_{TEAC\ and\ AEAC}$ antioxidant assay, for both the equivalents tested (Table 4). Whereas, fair correlation was found between both the antioxidants (DPPH and FRAP) tested (Table 4). Similarly, December (winter) showed highest correlation between TPC and antioxidant DPPH$_{TEAC\ and\ AEAC}$, TPC and antioxidant FRAP$_{TEAC\ and\ AEAC}$ activity and equally good correlation was found between DPPH$_{TEAC\ and\ AEAC}$ and FRAP$_{TEAC\ and\ AEAC}$ activity (Table 4). This was followed by poor correlation between TPC with flavonoid content, flavonoid with DPPH$_{TEAC\ and\ AEAC}$ and FRAP$_{TEAC\ and\ AEAC}$ antioxidant assays (Table 4).

Table 4.

Correlation of determination (r^2) obtained from regression analysis of the relations between Phenolics, flavonoids, DPPH and FRAP with other during the same months. The extents of correlation as determined by Student-Newman-Keuls Multiple comparison test are shown with color coding to indicate extent of correlation.

Month	Tests	Phenolics	Flavonoids	DPPH	FRAP
August	Phenolics	1.000***	0.490^{ns}	0.312***	0.787***
	Flavonoids	0.490^{ns}	1.000***	0.217***	0.585***
	DPPH	0.312***	0.217***	1.000***	0.611***
	FRAP	0.787***	0.585***	0.611***	1.000***
December	Phenolics	1.000***	0.198^{ns}	0.936***	0.929***
	Flavonoids	0.198^{ns}	1.000***	0.245***	0.233***
	DPPH	0.936***	0.245***	1.000***	0.937***
	FRAP	0.929***	0.233***	0.937***	1.000***
May	Phenolics	1.000***	0.037^{ns}	0.765***	0.721***
	Flavonoids	0.037^{ns}	1.000***	0.021***	0.000***
	DPPH	0.765***	0.021***	1.000***	0.670***
	FRAP	0.721***	0.000***	0.670***	1.000***

Strongest	1.000	Strong	<1.000 -0.700	Fair	<0.700 – 0.400	Weak	<0.400 – 0.000

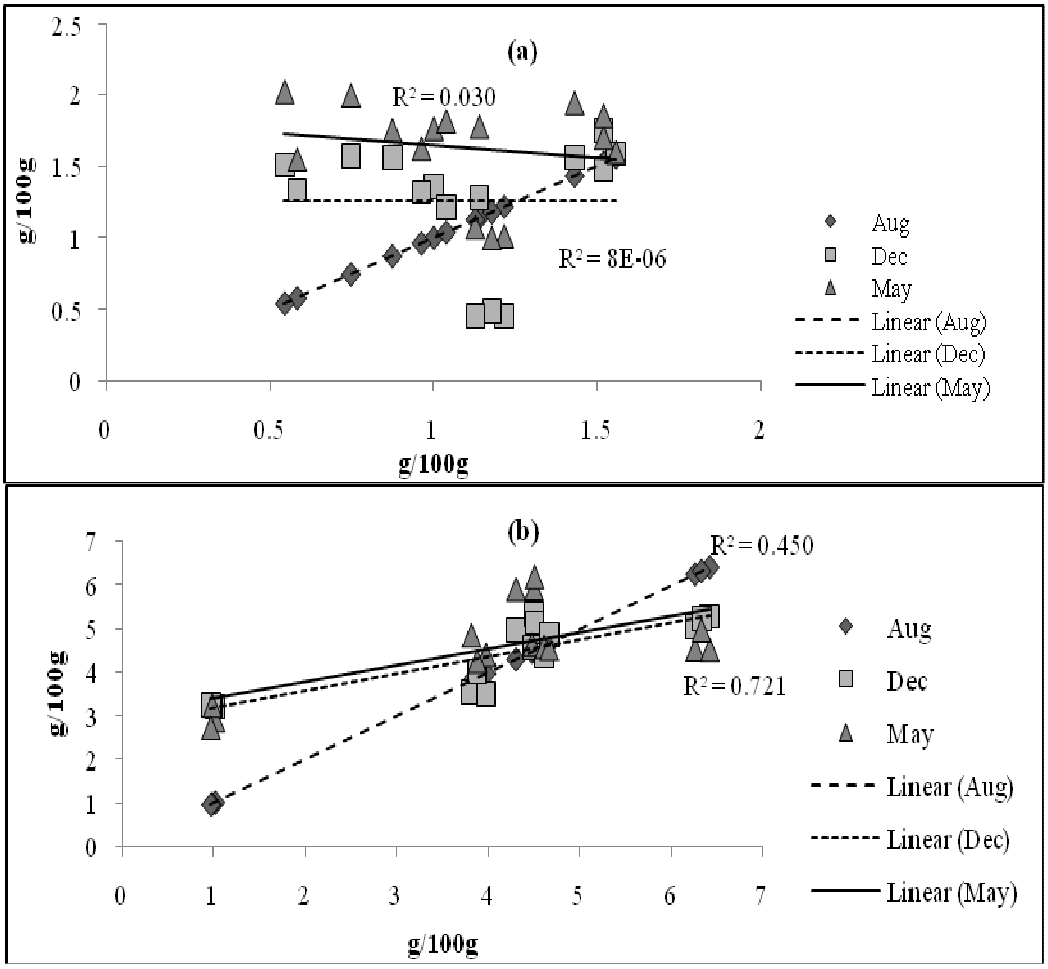
****** Extremely significant; ^{ns} not significant***

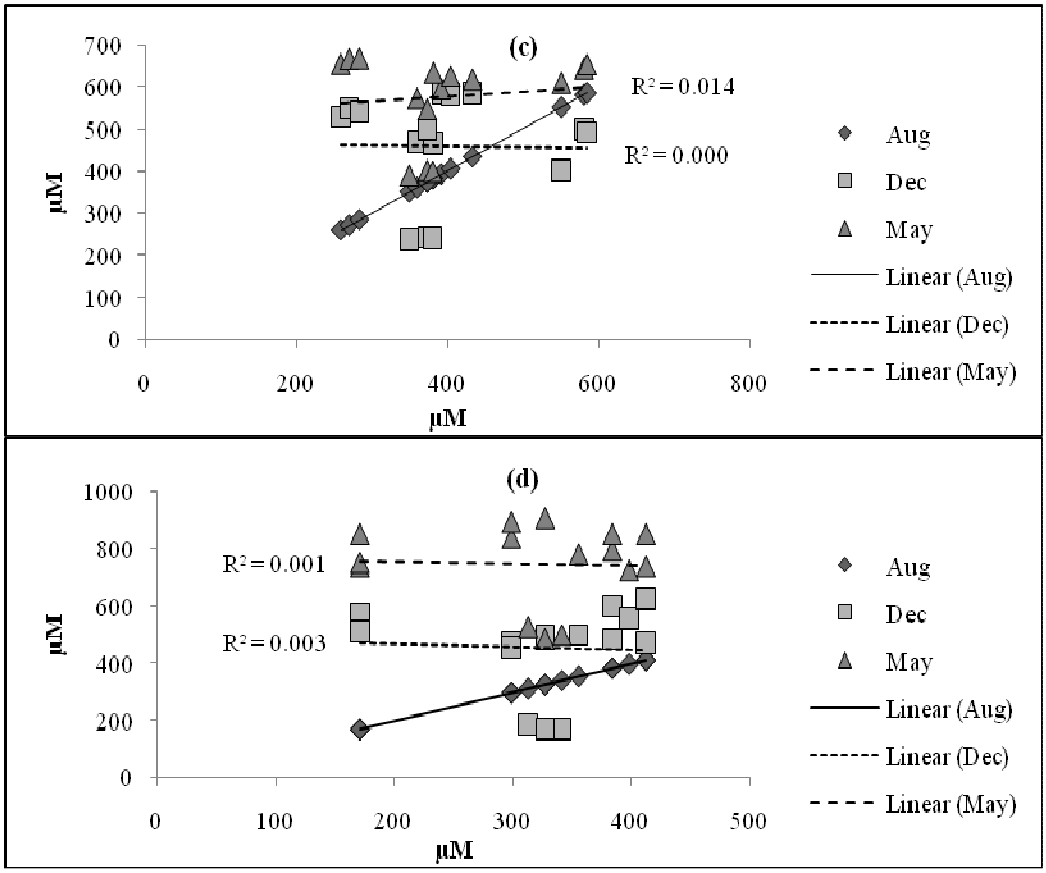
Observation made for month of May (summer) showed, TPC had highest correlation with antioxidants $DPPH_{TEAC\ and\ AEAC}$ and equally good with $FRAP_{TEAC\ and\ AEAC}$ assays, which was followed by the correlation between antioxidants $DPPH_{TEAC\ and\ AEAC}$ and $FRAP_{TEAC\ and\ AEAC}$ (Table 4). The poor correlation were observed between TPC with flavonoid content, flavonoid content with $DPPH_{TEAC\ and\ AEAC}$ and $FRAP_{TEAC\ and\ AEAC}$ antioxidant assays (Table 4).

The correlation between the three seasons for the total phenolic content showed that the yield during August had good correlation with that during May (R^2= 0.03) as compared to that yielded during December (R^2= 8E-06) which is very poor (Fig 2 a). Further, taking in to account the relation between correlation coefficients of the flavonoid contents, it was higher during the month of December than that of during August (Fig 2 b), with a significance level P<0.001. The correlation coefficients were highest during May and December where the actual increase in the content was observed.

Figure 2.

Correlations between individual assays studied during August, December and May; (a): Total Phenolic Content; (b): Total flavonoid content; (c): DPPH antioxidant activity; (d): FRAP antioxidant activity.





Correlations observed between individual assays for the three seasons tested showed a trend where flavonoids show a strong correlations followed by TPC (Fig 2 a and b) and poor correlation were seen in DPPH and FRAP antioxidant activity (Fig 2 c and d).

Statistically strong correlation was observed between TPC and $DPPH_{TEAC \text{ and } AEAC}$ radical scavenging activity during winter and summer whereas, monsoon had fairly weak correlation. This may be due to the colder environmental stress which might be elevating the content of phenolics with high antioxidant properties. These results were consistent with the findings of various research groups, who reported positive correlations between total phenolic content and antioxidant activity [30 - 32]. Furthermore TPC showed a significantly higher inhibition percent of DPPH radical compared to reference ascorbic acid and trolox. These results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the investigated plant species.

It was apparent from the regression model used here that, phenolic content has played a major role in determining the antioxidant potential in the bark of *N. nimmoniana* during the seasons tested.

CONCLUSIONS

Seasonal variation is one of the extrinsic factor which has remained sidelined by the scientists. Screening the plant wealth for antioxidant potential remains the only pin-hole to the darker side of synthetic medication, which can even ensure no or less side effects. Beyond the antineoplastic potential known, the significance of this study will add to the antioxidant prospects of this plant species. Other than that production of phenolics during summer increases antioxidant capacity (FRAP and DPPH) of the species under study. The mismatch of the total phenolic content and the extent of antioxidant activity during monsoon

(December) hints towards possible existence of certain non-phenolic compounds having DPPH radical scavenging activity.

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