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Changes in Antioxidants and Minerals in Noni (*Morinda citrifolia* L.) Fruits during Development Process

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Authors' contributions

This work was carried out in collaboration between all authors. Author DRS conceptualized the study. Author SS designed, wrote the protocol and first draft of the manuscript. Author VSB managed the literature searches and managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Noni (*Morinda citrifolia* L.) fruits of different maturity stages are used in traditional medicine and modern beverage or herbal industry for its rich profile of more than 200 bioactive compounds. The present study was aimed to evaluate the changes in metabolites during Noni fruit development stages by spectrophotometeric methods and identification of individual compounds in phenolics and carotenoids groups. During fruit development polyphenol content was increased by 41.4%, tannin by 81.1%, flavonoids by 166.1%, carotenoids by 404.1%, anthocyanin by 40.8%. Micronutrients showed variable concentration during fruit development and highest Zn was observed at 10 days after fruit initiation (DAFI; 70.3 ppm), Cu at 50 DAFI (34.7 ppm), Mn at 130 DAFI (34.9 ppm), Na at

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20 DAFI (0.37%), K at 90 DAFI (1.5%). Free amino acids, total sugar, reducing sugar and non-reducing sugar increased upto 120 DAFI while nitrate, oxalate and phytate contents were decreased significantly ($p<0.05$). HPLC analysis identified lutein, zeaxanthin, beta- carotene and beta- cryptoxanthin while epicatechin gallate, syringic acid, epigallocatechin gallate, vanillic Acid, naringin and cinnamic acid in Noni fruits. The information is useful for determining the fruit harvest stage of Noni fruit and showed the changes in predominant metabolites which can be used for investigating the synthesis pathways for bioactives in Noni.

Keywords: Morinda citrifolia L; fruit maturity stage; phytochemicals; micronutrients; phenolics; carotenoids.

1. INTRODUCTION

The Noni (*Morinda citrifolia* L.) belongs to Rubiaceae family and native of tropical islands in South-east Asia, particularly Andaman & Nicobar Islands (India) and Indonesia [1,2]. Its fruits are used as key source of traditional medicine in Nicobari tribes [3], Polynesian and Australian tribes [4], Indian [5] and Chinese region [6]. Noni is reported to have antibacterial, antifungal, analgesic, hypotensive, anti-inflammatory and immune enhancing effects [3,4,6]. Though, Noni has been in use for more than 2000 years in tribal and traditional health system [7], but it got major interest of researchers and industry in past 30 years only. Since then, more than 200 bioactive compounds have been identified in Noni plant parts which are presumed to be involved in health benefits from Noni juice [3].

The Noni is reported as rich source of metabolites which are organic compounds synthesized by enzyme-mediated chemical reactions called 'metabolic pathways'. The primary metabolites have major role in growth and development while secondary metabolites are known for specific functions in reproduction and defence mechanism [8,9]. Many of them are reported as 'natural antioxidants' and gained wider attention, particularly due to 'feel good factor' of consumers for fruits and vegetables which are rich in such compounds [10] (Halliwell, 1996). The scientific community also observed that the frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer [11,12]. However, the metabolites are influenced by the environmental, genetic and tissue factors [13]. Among them, the stage of fruits influence synthesis of certain compounds drastically [14] and very meagre information is available on Noni for changes in metabolites during fruit development and ripening processes.

Though, ripe fruits are used extensively by herbal and pharmaceutical industry for juice and pulp extract [15] but its fruits are also being used at different stages in traditional health systems for medicine or sometimes as 'stress food' [16]. Researchers reported changes in phenolic compounds during fruit maturation in apple [17], grape [18], peach [19] and loquat [20], but such information was not reported in Noni. Although, bioactive compounds were investigated in ripe fruits of Noni [21], but the change in kind and concentration in such compounds with maturity stages was not available to prioritize the harvesting stage for industrial use. High-performance liquid chromatography (HPLC), provide opportunity to identify and observed the changes in individual specific phenolic compounds. Such studies will help in finding the role of individual phenolic compounds has been shown to vary in their health and pharmaceutical industry. Therefore, the paucity in the information on stage associated changes in Noni, the present study was aimed to observe changes in morphology and metabolites in fruits and identification of carotenoid and phenolics in different stages of Noni fruits.

2. MATERIALS AND METHODS

2.1 Chemical Reagents

The analytical grade chemical reagents used in the study and 1,1-diphenyl 2 picrylhydrazyl (DPPH), Gallic acid, Anthrone reagent, Aluminium chloride and Formic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tannic acid, Ascorbic acid, conc. HCl, Sodium acetate buffer (pH= 1:4.5 ratio) and Sulphuric acid were purchased from Himedia Laboratories Pvt. Ltd., Mumbai. Rutin, Folin-Ciocaulteu reagent, Potassium Chloride, Copper sulphate, Sodium hydroxide were purchased from Merck (Darmstadt, Germany) and Sodium acetate, Orthophosphoric acid, Chloroform,

Petroleum ether, Acetone and Sodium carbonate solution were obtained from Rankem (RFCL Ltd. New Delhi). Methanol, acetic acid and Acetonitrile of HPLC grade purchased from Merck, all the standards for carotenoid, and phenolic acids of HPLC grade purchased from Sigma-Aldrich.

2.2 Sample Collection and Preparation

Noni fruits from five random plants of cv. CARI-TRA-1 were collected at 10 days interval from 10th day of fruit setting (Fig. 1). Fruit weight, fruit volume, fruit diameter and fruit length were recorded from each of the sample fruits using standard procedures. Three fruits from each plant was taken randomly and prepared three sets representing replications for laboratory analysis. Samples were prepared using the procedure described by Singh et al. [16] with minor modifications. In brief, the sample fruits were washed with distilled water and cut into small pieces. 2 g pieces of fruits were ground using mortar in 10 ml of methanol, pestle and filtered through Whatman No. 1 filter paper. The processes were repeated four times till colourless fruit samples. The collected sample-solvent mixtures were centrifuged (Heraeus Biofuge, Taylor Scientific Pvt. Ltd., Missouri) at 8000 rpm for 10 min and filtered through Whatman No. 1 filter paper and concentrated by rotary evaporator (Cyper Lab Corp., Millburg, USA). For anthocyanin estimation, the solvent was MFW solution (Methanol: Formic acid: Water: 70:2:28) while for carotenoids estimation, acetone was used as extraction solvent. The samples were kept at -20℃ for further analysis.

Fig. 1. Different stages of Noni fruits during maturity stages

2.3 Phytochemical Compounds

The major phytochemical compounds in different stages of fruit setting were estimated in the present study. The total polyphenol content was analysed by Folin-Ciocalteau reagent (10%, v/v) [22] with some modifications, gallic acid was used as reference standard and results were expressed as mg of gallic acid equivalent (mg/100 g sample) in fresh weight (FW). Total anthocyanin content was analysed using pH differential method [23], cynidin-3-glucoside was used as the reference standard and results were expressed as mg of cynidin-3-glucoside equivalent (C_3GE)/100 g sample in fresh weight (FW). Total carotenoids content was estimated by protocol described by Sadasivan and Manikam [24] and formula given by Lichtenthaler and Buschmann [25]. Total tannin content was estimated using AOAC method [26] (1990) with tannic acid as standard. Anti-nutritional factors viz. phytate, nitrate and oxalate were estimated by the standard protocols described by Hassan et al. [27] while saponin content by AOAC method [28].

2.4 DPPH Free Radical Scavenging Activity

The antioxidant activity of the extracts, on the basis of the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined as per protocol described by Braca et al. [29]. Samples extracts (0.1 ml) was added to 3 ml of a 0.001 M DPPH solution in methanol. Absorbance at 517 nm was taken with UV spectrophotometer after 30 min of incubation and antioxidant activity was determined using formula; Antioxidant activity (%)= [(Ao-Ae)/Ao] x 100 (Ao= absorbance without extract; Ae = absorbance with extract). The IC_{50} value was calculated using standard calibration curve from the 517 nm OD values of samples.

2.5 Micronutrient Analysis

The micronutrients *viz*. iron, cobalt, copper, manganese and zinc were analysed in fruit samples using Atomic Absorption Spectrophotometer (AAS; Shimandzu AA 6200). In brief, the samples were prepared using Muffle Furnace at 500℃ for 2 hrs and dissolved in 5 ml of 20 percent HCl and filtered the solution with Whatman filter paper No. 42 into 50 ml volumetric flask. The standards were prepared by making up 100 ml volume from 2, 4, 6, 8, 10 µl of standard solutions. Standard curve for different elements were prepared from element concentration and their corresponding absorbance.

2.6 HPLC Analysis for Carotenoids

The carotenoids were extracted from Noni fruits using the method of Olives Barba et al. [30], with minor modifications. In brief, 2 g fruit sample ground in 10 ml of 100% methanol, centrifuged at 5000 rpm for 20 min to separate the supernatant, and these operations were repeated until the samples were completely colourless. The extracts were filtered through Whatman No. 1 filter paper and than through 0.45 µm membrane filter and 20 µl sample was injected for HPLC analysis. Analysis was performed using Reversed phase chromatography RP-HPLC; DIONEX, Ultimate 3000 series), comprising a solvent rack (SRD-3200), a pump (HPG-3200SD), a column oven (TCC-3000SD), and a diode array detector (variable wavelength detectors VWD-3100 and VWD-3400). The mobile phase consisted of methanol (solvent A) and acetonitrile (solvent B) in 90:10 ratio at a flow rate of 1.0 ml/min. The column temperature was 22℃ and the absorbance was read at 450 nm. Retention time from available information was used as basis to identify the individual carotenoids.

2.7 HPLC Analysis for Phenolics

Phenolics in different stages fruits of Noni were extracted by modifying the procedure described by Ahmad et al. [31]. Fruit sample (5 g) ground well in 25 ml methanol and kept for overnight incubation. Mixture filtered through 0.45 µm membrane filters and kept in -20℃ for further analysis. The phenolic acid separation was done using RP-HPLC in the flow rate of 0.8 ml/min and the injection volume was 20 µl. The mobile phase was a binary solvent system consisting of (solvent A) dilute acetic acid (0.9%; pH-2.7) and (solvent B) 100% acetonitrile and the gradient used was 9% (0- 5 min), 11% (5-15 min), 18% (15-22 min), 23% (22-38 min), 90% (38-43 min), 80% (43-44 min), 80% (44-45 min), 5% (45-60 min) at 38℃ temperature and 280 nm. Individual phenolics were identified on the basis of retention time.

2.8 Statistical Analysis

For morphological traits, data from five replications and observations of three replications in laboratory analysis of fruits of

different stages were analysed for mean, range and ANOVA using OPSTAT software (www.hau.ernet.in).

3. RESULTS AND DISCUSSION

3.1 Morphological Observations

The observations from fresh Noni fruits showed slow increase in fruit weight at the early growth period of fruit (within 50 DAFI), then increased sharply reaching the peak (53.7 g) until 130 DAFI (Fig. 2). The relative change is shown in Fig. 3. No significant ($p<0.05$) change was seen after 110 DAFI (48.6 g) to 130 DAFI (53.7 g) in fruit weight. The growth rate (GR) in fresh weight increased speedily after fruit setting and reached the summit (78.0%) than showed moderate growth phase (25.4-36.1%) during 40th to 110th DAFI and finally decreased to 7.6 and 2.7% at 120th and 130th DAFI. The fruit length and fruit diameter showed slight increased during fruit development stages. Growth of Noni fruit can be divided into four stages: early fruit stage (upto 30 DAFI), lag phase (30 to 60 DAFI), fruit maturation (60 to 90 DAFI) and fruit ripening (90 to 130 DAFI). The morphological observations suggests for harvesting of Noni fruit after 110 to 130 DAFI, depending on distance of processing centre and end use facility. The findings are in conformity with reports of Kondo et al. [32] and Aydin and Kadioglu [33].

3.2 Phytochemicals

The changes in phytochemicals during fruit development stages of Noni are presented in Table 1. Results showed that most of the compounds increased significantly ($p < 0.5$) till 110 DAFI. The concentration of flavonoids, tannin, carotenoids and polyphenol was maximum 110 DAFI which showed declining trend afterward. During 10 DAFI to 110 DAFI, the polyphenol content was increased by 41.4%, tannin by 81.1%, flavonoids by 166.1%, carotenoids by 404.1%, anthocyanin by 40.8% (Fig. 3a). Though, Noni fruits are poor in anthocyanin content but its fraction remained low during all the fruit development stages. Ascorbic acid content was decreased from 123.3 mg/100 g to 63.3mg/100 which again got increased to 93.3mg/100 at 120 DAFI and 86.7 mg/100g 130 DAFI. Similar observations for ascorbic acid in acerola fruit by De Assis et al. [34] and in *Vaccinium myrtillus* L by Cocetta et al. [35]. Initial increase in concentration of metabolites in Noni fruits might be due to vigorous metabolic processes occurred during fruit development stages and secondary metabolites were appeared as byproducts [9]. Therefore, harvesting of Noni fruits 110 DAFI onward is suggested for higher recovery of phytochemicals.

Excess intake of anti-nutritional factors in daily diet interferes with dietary enzymes and micronutrients [36], and present study found the Noni fruits as rich in phytate, oxalate, nitrate and saponin (Table 1). However, their concentration decreased with advancement of fruit maturation except saponin. Saponin content increased from 10DAFI (10.0 mg/100 g) to 130 DAFI (260.0 mg/100 g) but its percent change declined after 120 DAFI (Fig. 3b). Similar observations were also made by Kreuger and Potter [37] in Holly fruits. Further, the commercial Noni fruit juice is prepared through various processing steps and blending of other agents which reduce the concentration anti-nutritional compounds to safe level [5]. In Noni fruits, free amino acids, total

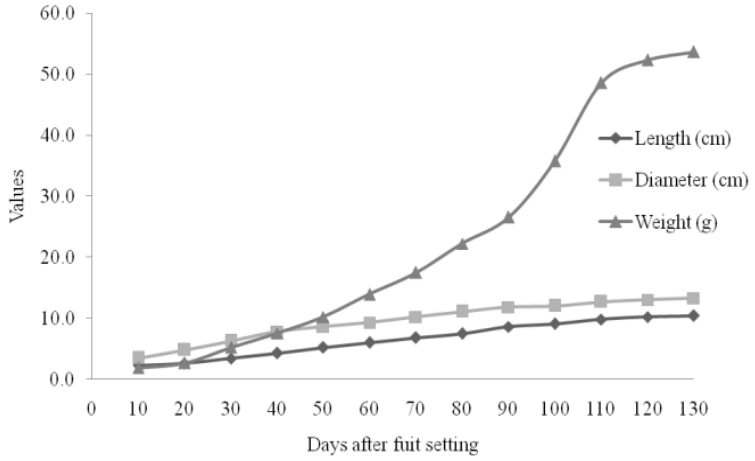

Fig. 2. Changes in fruit weight and size of Noni fruits during maturity stages

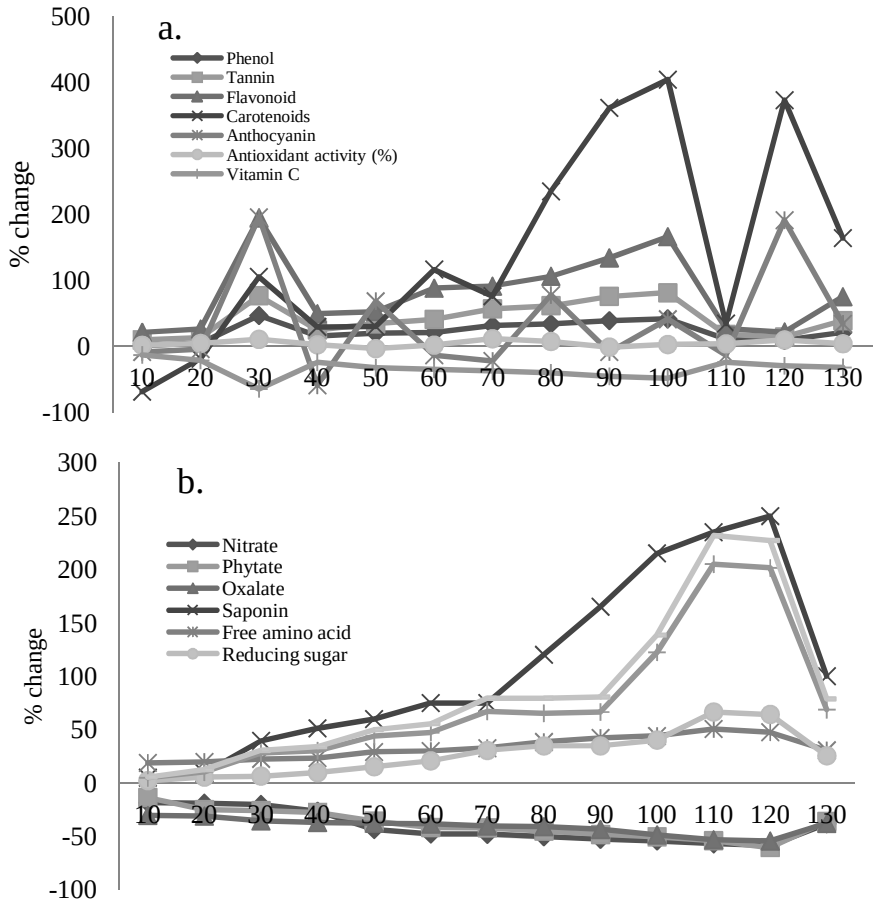

Fig. 3a-b. Change (%) in phyto-constituents in Noni fruits during maturity stages

sugar, reducing sugar and non-reducing sugar contributes in flavour and softening of texture of Noni fruit. These proximate components increased upto 120 DAFI (Table 1). Total sugar and non-reducing sugar had higher percent increase between 90 to 100 DAFI while it decreased afterwards (Fig. 3b). The percent change in free amino acids and reducing sugar also had same trend but the peaks were insignificant. Similar results were observed by Conde et al. [38] in grape.

3.3 DPPH Antioxidant Activity

Singh et al. [16] observed strong DPPH free radical scavenging activity of methanol, acetone and aqueous extracts of ripe fruits of *Morinda citrifolia*. In further investigation, the study estimated strong antioxidant activity of methanol extracts of Noni fruits of different stages which ranged from 70.3 to 81.3 percent, but zigzag trend was observed during fruit maturity stages. Although, kind and concentration of phytochemicals were changed during fruit maturity stages but percent change in antioxidant activity was not significant (Fig. 3a) which might be due to synergistic or antagonistic properties of plant matrix during fruit maturity process [16].

3.4 Micronutrient Analysis

The concentration of micronutrients showed a declining trend in Noni fruits during fruit development stages (Table 2). The highest concentration of Zn was observed at 10 DAFI (70.3 ppm), Cu at 50 DAFI (34.7 ppm), Mn at 130 DAFI (34.9 ppm), NA at 20 DAFI (0.37%), K at 90 DAFI (1.5%). Ca content was highest in the 30th day fruit (7437.8 ppm) and Mg was found to be maximum in 20th day fruit (1377.5 ppm). The variation in concentration of micronutrients during fruit development might be due changes in phytochemical matrix because many of these micronutrient are co-factors for enzymes involved in biosynthetic pathways during fruit development stages [39].

Table 1. Phytochemical, anti-nutritional factors and proximate components in Noni fruits at different maturity stages

Fruit stage (DAFl)	Phytochemicals (mg/100 g)						Anti-nutritional factors (mg/100 g)				Proximate components (mg/100 g)				Antioxidant activity (%)
	Polyphenol	Tannin	Flavonoid	Carotenoids	Anthocyanin	Ascorbic acid	Nitrate	Phytate	Oxalate	Saponin	Free amino acid	Reducing sugar	Non-reducing sugar	Total sugar	
10	54.4	117.8	120.8	71.9	19.1	123.3	75.3	1186.0	63.1	10.0	62.9	0.4	95.2	95.7	73.1
20	54.8	128.9	146.1	22.3	17.4	106.7	61.5	1024.9	43.9	15.0	74.7	1.3	99.8	101.1	74.1
30	56.8	133.3	152.3	58.2	18.2	96.7	61.0	890.5	43.6	19.3	75.3	2.6	105.0	107.6	76.1
40	59.6	137.8	155.6	74.0	16.4	43.3	60.1	879.9	40.7	49.5	77.1	3.0	122.0	125.0	78.7
50	62.9	146.7	180.3	92.9	17.8	93.3	55.5	860.7	39.8	61.2	77.6	4.3	123.9	128.3	74.6
60	65.0	157.8	184.3	93.4	32.1	83.3	42.5	762.0	39.4	70.0	81.2	6.5	137.0	143.5	70.4
70	65.7	165.6	227.0	155.4	16.5	80.0	39.4	692.6	38.9	85.0	81.8	8.7	140.2	148.9	74.3
80	71.4	184.4	230.9	126.4	14.8	76.7	39.2	688.0	37.8	85.0	83.5	12.6	159.1	171.7	81.3
90	72.8	190.0	248.9	240.6	33.9	73.3	37.5	651.8	37.4	130.0	87.1	14.3	157.4	171.7	78.3
100	75.5	206.7	282.6	331.7	17.4	66.7	35.7	614.5	35.7	175.0	89.4	14.3	158.5	172.8	72.1
110	76.9	213.3	321.4	362.5	26.9	63.3	34.5	587.8	32.4	225.0	90.6	16.5	211.7	228.3	74.9
120	60.2	138.9	152.8	396.6	16.5	93.3	32.5	546.2	29.6	245.0	94.7	27.0	290.4	317.4	75.6
130	59.2	134.4	146.8	340.1	55.6	86.7	31.3	469.8	28.8	260.0	92.9	26.1	287.0	313.0	80.0
CD (*P*=0.05)	5.06	3.38	4.22	4.73	2.53	2.03	3.4	20.3	3.7	2.5	4.22	0.02	4.22	5.91	2.19

Table 2. Changes in micronutrients in Noni fruits during fruit maturity stages

Stages of fruits (DAFI)	Zn (ppm)	Cu (ppm)	Mn (ppm)	Na (%)	K (%)	Ca (ppm)	Mg (ppm)
10	70.3	32.0	3.9	0.3	1.4	7090.2	1324.2
20	66.4	37.0	3.5	0.4	1.4	7305.5	1377.5
30	65.7	33.3	2.7	0.3	1.4	7437.9	1322.5
40	35.3	30.7	3.5	0.3	1.1	4321.3	1166.9
50	38.6	34.7	2.9	0.3	1.3	3097.1	1190.1
60	20.4	30.0	1.9	0.3	0.9	3607.5	1078.6
70	19.4	27.3	1.4	0.3	1.4	1803.4	1035.7
80	15.9	24.7	6.7	0.4	1.3	1654.6	1042.4
90	27.7	18.0	4.9	0.4	1.5	3305.3	1043.8
100	22.1	16.0	4.0	0.3	1.4	2713.4	1099.9
110	17.6	15.7	4.0	0.3	1.3	1393.2	947.8
120	12.7	13.7	6.9	0.3	1.1	1334.9	930.6
130	9.8	11.0	34.9	0.3	1.2	1366.5	935.2
CD (*P*=0.05)	3.38	2.53	0.84	0.02	0.17	16.84	20.25

Table 3. Identified carotenoids and phenolics in Noni fruits at development stages by RP-HPLC

Phytochemical group	Compounds	Day after fruit initiation (DAFI)											
		10	20	30	40	50	60	70	80	90	100	110	120	130
Carotenoids	Lutein	+	+	-	+	+	+	-	-	++	-	-	-	-
	Zeaxanthin	+++	++	++	+	+	+	++	++	+	++	++	+	+
	α-Crtyptoxanthin	++	++	++	+	+	+	+	+	+	+	+	-	-
	Echinenone	+	-	+	+	++	-	-	-	-	-	-	-	-
	α-Carotene	-	-	-	-	+	++	++	+++	+	+	+	-	-
	β-Carotene	-	+	-	-	-	-	+	+	-	-	-	-	-
Phenolic compounds	Epicatechin gallate	+	+	+	+	+	+	+	+	+	++	++	+	+
	Syringic Acid	-	-	-	-	+	+	+	-	-	-	-	-	-
	Epigallocate chin gallate	-	-	-	-	-	+	-	-	-	-	-	-	-
	Vanillic Acid	-	-	-	-	-	-	++	+	+	++	++	++	+
	Naringin	-	-	-	-	+	-	+	-	-	-	-	-	++
	Cinnamic Acid	-	-	-	-	-	-	-	-	-	-	-	+	-

- absent; + present; No. of + indicates peak size

3.5 HPLC Analysis

The HPLC analysis with diode array detector identified five carotenoids compounds such as lutein, zeaxanthin, β- carotene and β- cryptoxanthin in methanol extract of Noni fruit of different developmental stages (Table 3). The relative height of the peak for lutein was maximum at 90 DAFI, for zeaxanthin at 10 DAFI, β- cryptoxanthin at 120 DAFI, α- carotene at 80 DAFI, β- carotene at 70 DAFI. Lutein is not synthesized in human body and thus intake of Noni fruit can contribute in lutein uptake through dietary means [40]. The role of Noni in refreshing health, alleviate chronic diseases such as cancers and coronary heart diseases might be due its richness in carotenoids.

The HPLC chromatograph for phenolics revealed that the syringic acid, vanillic acid and cinnamic acid are the major phenolic acids while, epicatechin gallate and epigallocatechin gallate are the most abundant tannins subclass (Tabe 3). Naringin was new observed phenolics belongs to flavonoids subclass founded in Noni fruits. These phenolics compounds might be the major contributor in free radical scavenging capacity of Noni fruit extracts. Similarly, changes in phenolics were also reported by Ding et al. [20].

4. CONCLUSION

The fruit development stages significantly influence concentration of metabolites and micronutrients in Noni fruits. Antioxidants contents and total sugar content increased significantly during fruit maturation (upto 120 DAFI) while anti-nutritional factors (except saponin) were decreased significantly. The study

suggested harvesting of Noni fruits between 110 to 130 DAFI for processing purpose because of higher contents of phytochemicals and low content of anti-nutritional factors. The firmness of the fruits remains satisfactory and there is no further change in fruit weight. The HPLC analysis identified individual carotenoids and phenolics in Noni fruits but further investigation are suggested for unscrambling the mystery of other bioactive compounds and their synthesis pathways in Noni.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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