International Journal of Botany Studies
ISSN: 2455-541X
Impact Factor: RJIF 5.12
www.botanyjournals.com
Volume 3; Issue 2; March 2018; Page No. 42-47

Qualitative and quantitative analysis of scopoletin in the morphotypes of *Morinda citrifolia* from South India

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Abstract
Scopoletin is considered as a marker compound of *Morinda citrifolia.* Hence, its presence in the fruits of nine different morphotypes of *M. citrifolia* collected from different locations of South India was investigated. Six different growth stages of the fruit were taken into consideration and qualitative analysis by TLC profiling revealed that scopoletin was present even in the inflorescence stage of the five morphotypes namely, *M. citrifolia* var. *citrifolia* BCMML10005, *M. citrifolia* var. *citrifolia* BCMML10006, *M. citrifolia* var. *citrifolia* BCMML10007, *M. citrifolia var. citrifolia* BCMML10008 and *M. citrifolia var. citrifolia* BCMML10009. Interestingly, scopoletin was present in all other fruit stages in all the nine morphotypes. However, quantitative analysis using spectrophotometric assay revealed that scopoletin was present in all the fruit stages of all the nine morphotypes of *M. citrifolia*.

Keywords: *Morinda citrifolia*, morphotypes, scopoletin, fruit stages

1. Introduction
Morinda citrifolia L. popularly known as noni or Indian Mulberry belongs to the family Rubiaceae is a tropical ever green tree growing in the coastal regions at the sea level. The plant grows up to 10 m height, and bears cauliflorous compound fruit throughout the year with a typical characteristic rancid cheese odour and taste when ripe [1, 2, 3, 4, 5, 6, 7]. *Morinda* has been used since ancient times by people for various ailments in Ayurveda, Sidda and Unani, which are amongst the oldest systems of Indian traditional medicines [8]. The plant has been used as folk remedies in the Asian subcontinent, Hawaiian and Tahitian Islands for centuries. *M. citrifolia* is reported to have a wide range of nutritional and therapeutic value. The fruit juice is used all over the world for a host of chronic conditions such as arthritis, diabetes, hypertension and cancer [9].

Amino acids, anthraquinones, coumarins, fatty acids, flavonoids, iridoids, lignans, polysaccharides, sterols, sugars, sulphur containing compounds and terpenoids have been isolated and identified from parts such as roots, leaves and fruits of *M. citrifolia* plant [10]. More than 160 compounds have been isolated from *M. citrifolia* and the major phytoconstituents are being phenolics, organic acids and alkaloids [11]. Plants exhibit different chemical profiles according to the groups they are categorized, in addition to their individual chemical profiles [12, 13, 14, 15]. This amount of discrepancy among the level of chemical variation within species in comparision to the variation among species to a great extent remains unexploited. The chemical components of a plant are also dependent on ecological factors such as the soil, climate and various geographical factors [16]. The chemical composition of the fruit at different growing stages differs significantly [17, 18, 19]. With this background, the aim of this study was to evaluate the major components of the mature fruit and quantify the compound scopoletin starting from the inflorescence stage to the mature fruit stage of nine different morphotypes of *M. citrifolia* from Andaman, Calicut, Mangalore, Chengalpattu (Tamil Nadu) and also from the Herbal Garden of the University of Madras, Chennai. This experiment also helps in recommending the better morphotype for cultivation.

2. Materials and Method
2.1 Sample collection
The fruits of M.citrifolia for the present study were collected from the National Research Centre for Noni (NRCN), Salavakkam, Chennai, Tamil Nadu, India and the Herbal Garden, Centre for Advanced Studies in Botany, University of Madras, Chennai, Tamil Nadu. Different types of *M. citrifolia* collected from different locations of India were planted in separate plots and maintained in the field. The fruit development was studied for a period of one year from to understand the changes in colour and firmness. It was found that the period between August and December was the best time for fruiting. Triplicate sampling was carried out to ensure the reliability of the data and also the samples were collected from different parts of the well grown healthy trees starting from the inflorescence stage to the fully ripened stage which made totally six different stages (Table 1).

The fruits were cut into small pieces with clean, sterilized secateurs, dried under shade and finely ground to powder. The fruit extract was prepared from the method of Eloff [20]. In this method, ground powder was extracted with methanol in the ratio of 1:20 in a conical flask under shaking condition for overnight and fresh solvent was added every 24 h after filtering the extract through the Whatmann No. 1 filter paper

in a separate container. The solvent was removed from the extract using a rotary evaporator. The extracted residue was further evaporated by keeping it for air dry, the final crude was weighed and stored for further analysis.

2.2 Identification and separation of scopoletin through thin layer chromatography (TLC)

The sample was loaded on pre-coated silica gel plates of grade F_{254} (E-Merck, Germany) and then, 1.5 µL of the sample was spotted at 1 cm from the bottom of the silica gel plates with the help of capillary tube along with the pure standard compound. Different solvents at various combinations were used. Development of the chromatograms was done in closed tanks, in which the atmosphere has been saturated with eluent vapour by wetting a filter paper lining. The chromatograms were visualized under UV light at 365 nm and 254 nm and the R_f value of the compound was calculated.

2.3 Quantification of scopoletin in *M. citrifolia* fruit extracts

A preliminary analysis was carried out to determine the wavelength at which the maximum absorbance takes place by the authentic scopoletin. The standard curve of scopoletin was prepared using the standard stock solution of scopoletin (10 mg/10 mL) in glass distilled (GD) water. From this, different concentrations (1 - 10 µg/mL) were prepared in GD water and the intensity of absorbance was recorded in a Hitachi U-2900 UV-Vis Double beam spectrophotometer. The standard graph was constructed by plotting concentration versus intensity of absorbance. The solution was scanned using the above spectrophotometer between the wavelength region 200 and 700 nm to determine the wavelength of maximum absorbance. The methanolic crude extracts of nine morphotypes of *M. citrifolia* were screened following a modified method of Nahata and Dixit [21] and Malik *et al* [22]. Accurate weight of 10 mg of the fruit crude extracts of all the 9 samples from stage I to Stage VI were weighed and each extract was well dissolved in 1 mL of methanol. From this, 50 µL (500 µg) of the sample was pipetted and made up to a volume of 2.5 mL with distilled water and assayed. All the analyses were carried out in triplicates and the absorbance was recorded. The experiment was carried out in triplicates and the results are expressed as mean of ±Standard deviation (SD).

3. Results

3.1 Developmental stages of *M. citrifolia* fruit morphotypes

The colour, firmness and surface morphology of fruits were considered to categorize the fruits into different stages, which were studied for a year and all the seasons. The flowering and ripening of the fruits totally depended on the amount of sunlight, the different branches received (Table 2). The upper branches received more light and the development of the fruit was much earlier than the fruits in the lower branches (Table. 1 and Table. 2). The color evolves from dark green to translucent grayish and the texture too changes from very hard to soft. It was also found that the process of ripening of the fruit caused permeability which was confirmed by the moist surface of the VI stage in all the morphotypes. The total number of days for fruit to ripen was 128±29, which depended upon the amount of sun light, water, humidity and severity of pests. The details of all the six different fruit stages are presented in Figure. 1; Table 1 and Table 2.

Table 1: Fruit developmental stages in *M. citrifolia* morphotypes

Maturity stage	Degree of maturity	No. of days	Colour	Firmness	Surface /Protrusions
Stage I	Inflorescence	26±7	Dark green	Hard	With buds and flowers
Stage II	Immature	23±8	Dark green	Hard	Big scars, highly tubercled
Stage III	Middle	30±9	Dark green	Very hard	Triangular projections, smaller scars
Stage IV	Sub mature	25±15	Green yellowish	Very hard	More or less hexagonal flattened areas
Stage V	Mature	15±10	Pale yellow	Fairly hard	Clear hexagonal areas flattened scars, leathery skin
Stage VI	Fully ripened	9±4	Translucent	Grayish soft	Clear hexagonal areas flattened scars, slimy coat

Table 2: Time taken for fruit to ripen in the different branches of *M. citrifolia*.

Duration	Top Branches	Middle Branches	Lower branches
Time taken for ripening (in days)	123±7	129±6	138±12

Fig 1: Developmental stages of *M. citrifolia* fruit. a. Stage I, b. Stage II, c. Stage III, d. Stage IV, e. Stage V, f. Stage VI

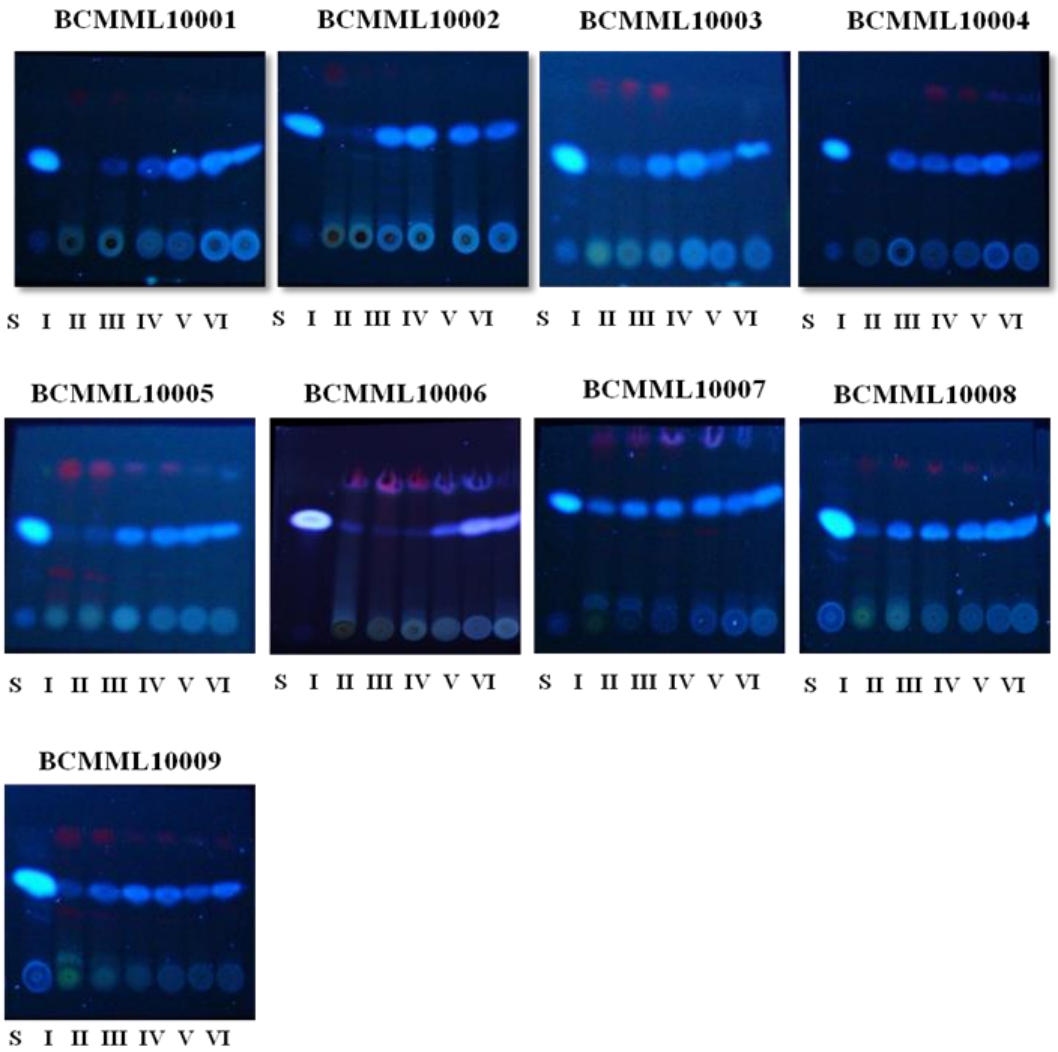
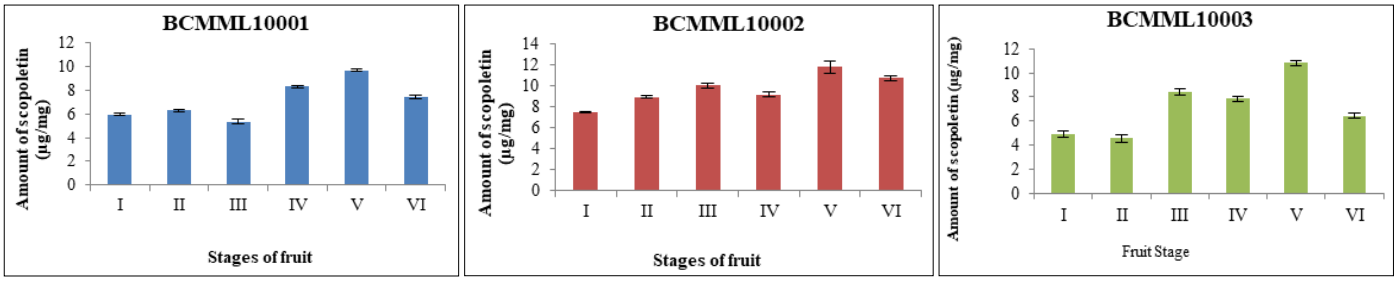


Fig 2: TLC analysis of scopoletin



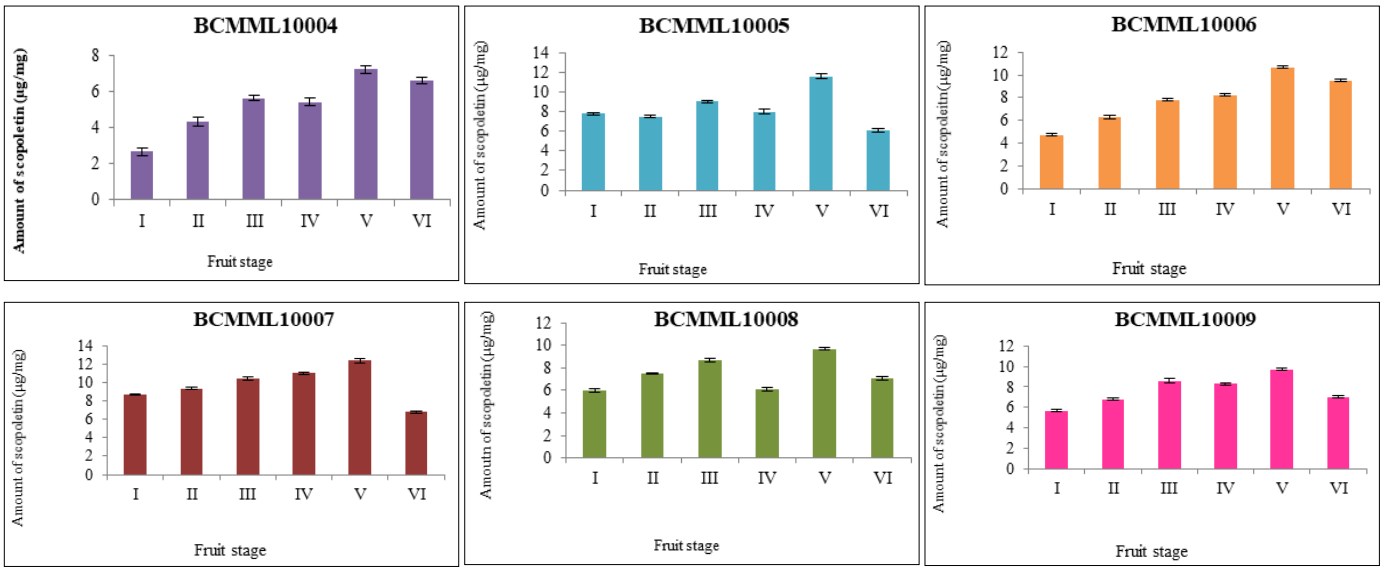


Fig 3: Quantitative analysis of scopoletin

3.2 Scopoletin in the different stages of *M. citrifolia* fruits

All the 54 methanolic extracts prepared from nine morphotypes of *M. citrifolia* have been investigated for the compound through TLC, which revealed the scopoletin presence in all the six fruit stages, except in BCMML10001, BCMML10002, BCMML10003 and BCMML10004, wherein it showed its absence in the Ist stage (Fig.2). The intensity of scopoletin in different stages of the morphotypes on the TLC also suggested that the compound was present in varying quantities in different stages of the fruit (Fig.2). The maximum absorbtion of pure scopoletin in the instrument Hitachi U-2900 UV- Vis double beam spectrophotometer was at 228nm and 344nm respectively. Further, the spectrophotometric quantification of the crude extracts confirmed the variation in all the nine morphotypes of *M.citrifolia* with the highest in the Vth stage of the fruit. Interestingly, stage VIth had lesser amount of scopletin when compared to unripen fruit of Vth and even IIIrd stage in all the morphotypes, except BCMML10006, in which a consistent increase in synthesis of the compound was noted (Fig.3). The morphotype, BCMML10007 showed highest amount of scopoletin of 12.3 µg/mg dry weight and BCMML10004 showed the least of 7.2 µg/mg dry weight in their fifth stages. The morphotype BCMML10006, showed a complete different mode of biosynthesis of compound different from the other morphotypes (Fig.3).

3. Discussion

Phytochemicals vary in quality and quantity in plants growing at different geography due to developmental and environmental variations [23]. The chemical variation within a species remains unexplored when compared to studies among the species. Among the phytochemicals, phenylpropanoids not only act as key mediators in response to plant stress, they also contribute to biotic and abiotic stimuli of plant [24]. They are also responsible for invasion of new habitats as they provide necessary biochemical resources for successful reproduction [25, 26]. Therefore, to understand this variation in phytochemicals, research was focused in quantitative approach through methanol extraction of the phytochemicals. Methanol was preferred over other solvents as it helps in extraction of maximum yield of the secondary metabolites [27, 28, 29]. However, the qualitative and quantitative nature of metabolites totally depends on the individual species.

Scopoletin is one of the important phytocomponent of the fruit of *M. citrifolia* and is referred to as the marker compound derived from the phenylpropanoid pathway [30, 31]. In the morphotype BCMML10006, scopoletin in the various stages need not be excluded on the basis that it is a human error rather than having a scientific significance. This could also probably be due to the nature of the secondary metabolites synthesized during the pathway as a response to various biotic and abiotic factors [32]. However, the information generated through this work also has given an insight on the physiological pathway involved in the synthesis of the coumarin, "scopoletin". The variation showed is due to scopoletin being synthesized as a phytoalexin in plants [33].

In many plant pathogen systems, the resistance of plants usually depends on the development stage at which the plant is infected. Many plants like *Arabidopsis*, tobacco, rice are generally more susceptible to diseases in an early phase or at a later stages or phase to specific pathogens [33, 34, 35, 36]. In this work the amount of scopoletin starting from the inflorescence to fully ripened stage varied in each stage due to the biological changes in the course of development. In tobacco, the young leaves were highly resistant to the activity of *Alternaria alternata* and the brown spot disease was seen only in the mature leaves [37, 38]. However, it was noted by the works of Sun *et al.* [39] that the reason for resistance in the young leaves of tobacco was due to the presence of scopoletin, a strong blue fluorescence compound seen in the infection zone. This is also a reason behind the high amount of scopoletin in the immature or unripe fruit stage. The high amount of scopoletin in the first three fruit stages can also be related to a phenomenon called as 'Ontogenic resistance' [40, 41]. In tobacco the scopoletin increased to 2-3 folds in the source-sink transition leaves than in mature leaves and the same source and sink concept is applicable for the developing fruits as well which is confirmed

by the UV-Vis spectroscopic study [42, 43]. The resistance stage at which the plant develops, persists throughout the rest of the life period. In the present study, high amount of scopletin was reported by spectrophotometric quantification in the unripen stages compared to the mature fruit, which is also correlating with the discovery of Sun *et al*. [41]. This clearly defined the differences in accumulation of scopoletin in different stages of fruit development. The dramatic changes of scopoletin from the unripen stage to the mature stage of *M. citrifolia* fruit can also be compared to that of lycopine of tomato [19]. The pathway of scopoletin synthesis is totally based on the pathogen-host interaction signaling mechanism and dependent on the F6´H´ gene regulation [30, 44].

4. Conclusion

The molecular analysis on the synthesis of scopoletin in *M. citrifolia* has to be exploited greatly, which would give a deep understanding in knowing the expression, regulation and controlling mechanism of scopoletin synthesis at different stages of the fruit. Hence, this may not be treated as a conclusive research as the follow up can be intensified by looking into the biosynthetic pathways and signal transduction mechanisms.

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