SIMULTANEOUS DETERMINATION OF BIOACTIVE CONSTITUENTS IN *GARCINIA MORELLA* USING UPLC-Q-TOF-MS

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ABSTRACT

A simple, precise and robust UPLC-Q-TOF-MS method was developed for the quantification of four marker compounds, namely citric acid derivative, iso garcinol, garcinol and garcimultiflorone-A in *Garcinia morella,* in order to study the fragmentation pathways of four marker compounds**.** The developed method was validated according to ICH guidelines in terms of linearity, LOD and LOQ, precision and accuracy. Optimized chromatographic conditions were Waters Acquity BEH C18 (100 X 2.1mm, 1.9 μ) column, flow rate of 0.6 mL min^{-1}, 8.0 minutes run time and mobile phase being 0.1 % formic acid in water / acetonitrile. The gradient elution programme 0 (95/5), 0.5 (95/5), 2.50 (5/95), 5.0 (5/95), 6.5 (95/5) and 8.0 (95/5) was carried out on Waters instrument UPLC-Q-TOF-MS. The obtained retention times of citric acid derivative, iso garcinol, garcinol and garcimultiflorone-A were 2.14, 3.86, 4.31 and 5.05 minutes, respectively. LOD and LOQ data was found to be in 0.32-0.56 and 0.99-1.69 μg mL^{-1}, respectively.

Keywords: *G. morella,* UPLC-Q-TOF-MS, fragmentation pathway of citric acid derivative, Iso Garcinol, Garcinol and Garcimultiflorone-A

INTRODUCTION

The tree *Garcinia morella* (Clusiaceae) has a lot of culinary, pharmaceutical and industrial applications. The genus *Garcinia* includes some 200 species found in the tropics, especially Asia and Africa. Out of the 35 species found in India, 17 are endemic. Of these, seven are endemic to the region of Western Ghats as well as the state of Goa, six in the Andaman and Nicobar Islands and four in the North-Eastern region of India. The fruit, locally known as 'Kokum', is steeped in sugar syrup to make a drink which is used to avoid skin damages and allergies from the sun and tropical climate. The plant grows extensively on the western coast of India and is known by various names across India including Biran, Bhirand, Bhinda, Katambi, Panarpuli, Ratamba or Amsool[1]. The extract has been used as a pink and purple food coloring agent and as a spice to give a sour and sweet taste to curry. In addition to food usage, it has also been used as a cosmetic ingredient, as well as in traditional medicine for the management of inflammation and other disorders. The structure of garcinol includes a phenolic hydroxyl group and a β-diketone moiety, which is structurally related to curcumin, a well-studied naturally occurring anti-oxidant.

Polyisoprenyl phloroglucinols occur widely in nature and are known to exhibit diverse range of pharmacological activities. Garcinol (1) and iso-garcinol (2) are two major poly-iso-prenylated phloroglucinol compounds present in *G. indica* and also in *G. cambogia* (Guttiferae)[2-5]. Garcinia species has been reported to exhibit multiple pharmacological activities, including anticancer, anti-obesity, diuretic, anti-inflammatory, antibacterial, antiviral, antifungal, anti-HIV, antidepressant and antioxidant activities[6-9]. Garcinol, gambogic acid and α-mangostin isolated from Garcinia were found to possess antioxidant, antibiotic, antitumor, anti-inflammatory and anticarcinogenic properties[10]. Hydroxy citric acid (HCA), a potential anti-obesity and hypo-cholesterol-emic agent, is used in fruits and leaves of Garcinia species and used found as an ingredient in popular dietary supplements that are used for weight loss[11-15]. Bioflavonoids, triterpenoids, flavonoids and phenolic acids found in Garcinia are also responsible for a range of pharmacological activities[16-17]. As the genus Garcinia is in enormous demand due to its use in many marketed formulations, simultaneous quantitative assessment of the multi-class bioactive constituents is essential for effective quality control.

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Table I: Linearity, LOD and LOQ characteristic parameters of Garcinia bioactive constituents

Parameter	Citric acid derivative (2.14)	Iso-Garcinol (3.85)	Garcinol (4.31)	Garcimultiflorone-A (5.05)
Calibration range (μg mL^{-1})	50-1000	50-1000	50-1000	50-1000
Detection limit (μg mL^{-1})	0.560	0.328	0.506	0.351
Quantitation limit (μg mL^{-1})	1.697	0.993	1.534	1.065
Standard error	0.419	0.056	0.104	0.083
Slope	0.091	0.090	0.207	0.222
SD of slope	0.009	0.003	0.001	0.001
% RSD of slope	1.197	3.684	0.903	0.562
Intercept	-9.668	-0.056	0.179	0.125
SD of intercept	0.168	0.026	0.017	0.039
Correlation coefficient	0.9982	0.9944	0.9970	0.9962

Currently, many analytical methods including HPLC–PDA, GC–MS and HPLC–DAD are used to evaluate the constituents of Garcinia species, focusing on the determination of only a small number of constituents in Garcinia extracts[18-19]. These reported methods have drawbacks of low sensitivity, low resolution, and long analysis time with large solvent consumption and/or derivatization. These problems could be overcome by using a more sensitive, selective and efficient technique such as LC coupled with mass spectrometry revealing (LC–MS/MS). So far, only a few constituents (camboginol,

Table II: System suitability

Name	Ret Time	Tailing	NTP	Resolution
Citric acid derivative	2.14	1.021	7000	0
Iso Garcinol	3.86	1.321	8500	2.3
Garcinol	4.31	1.521	13000	2
Garcimultiflorone -A	5.05	1.051	5000	4

Table III: Precision

Component	Concentration	Intra Day		Inter Day	
		Measured concentration	RSD (%)	Measured concentration	RSD (%)
Citric acid derivative (2.14)	50	51.25± 1.45	2.84	49.18±2.33	4.73
	200	206.29±4.02	1.95	200.21±7.76	3.87
	800	791.36± 15.80	1.99	795.66± 25.49	3.20
Isogarcinol (3.86)	50	50.51± 1.83	3.63	49.66±2.51	5.06
	200	201.03±3.98	1.98	199.03± 6.11	3.07
	800	803.66± 14.57	1.81	783.66± 42.54	5.42
Garcinol (4.31)	50	50.33± 1.52	3.03	53.00±3.60	6.80
	200	194.66±5.50	2.82	197.00± 10.58	5.37
	800	795.00± 32.78	4.12	801.66± 53.05	6.61
Garcimultiflorone-A (5.05)	50	51.21± 2.10	4.11	47.68±2.38	4.99
	200	199.63±3.94	1.97	201.66± 10.40	5.16
	800	787.00± 15.71	1.99	790.00± 26.00	3.29

Table IV: Accuracy

Analyte	Spiked concen-tration	Recovery (%) (mean±S.D)	%RSD
Citric acid derivative (2.14)	50	96.81±6.03	6.23
	200	100.66±3.05	3.03
	800	101.03±2.62	2.60
Isogarcinol (3.86)	50	98.22±4.92	5.01
	200	97.70±2.77	2.84
	800	102.70±3.77	3.67
Garcinol (4.31)	50	91.06±3.18	3.49
	200	101.69±2.48	2.44
	800	97.06±3.88	4.00
Garcimultifloron-A (5.05)	50	100.88±4.72	4.68
	200	100.27±5.36	5.34
	800	95.33±3.02	3.17

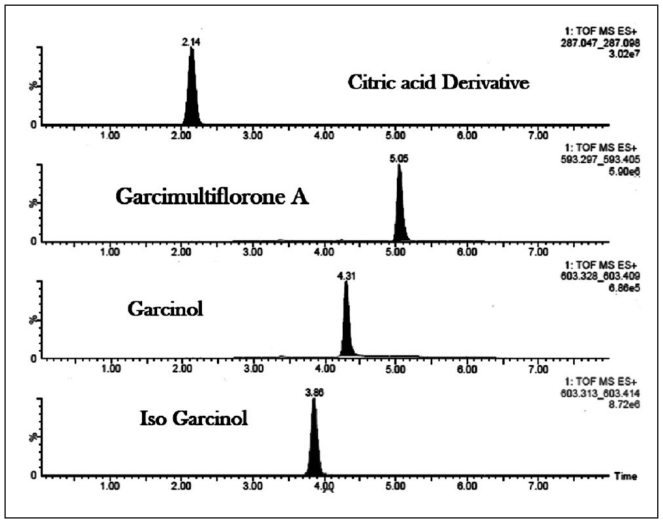

Fig. 1: Detailed chromatograms and retention times of bio active constituents

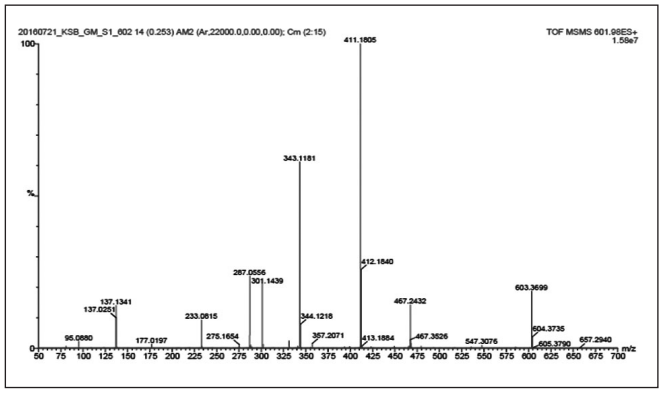

Fig. 2: LC MS/MS spectrum

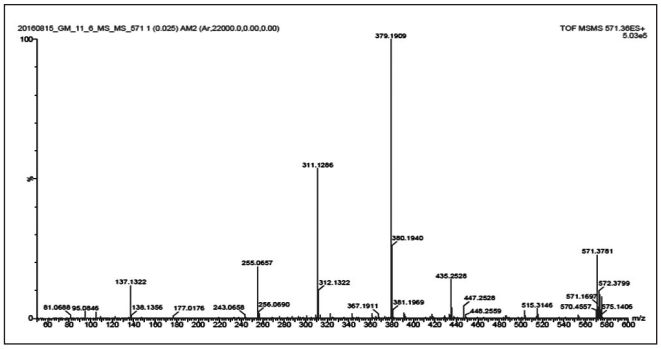

Fig. 3: LC MS/MS spectrum

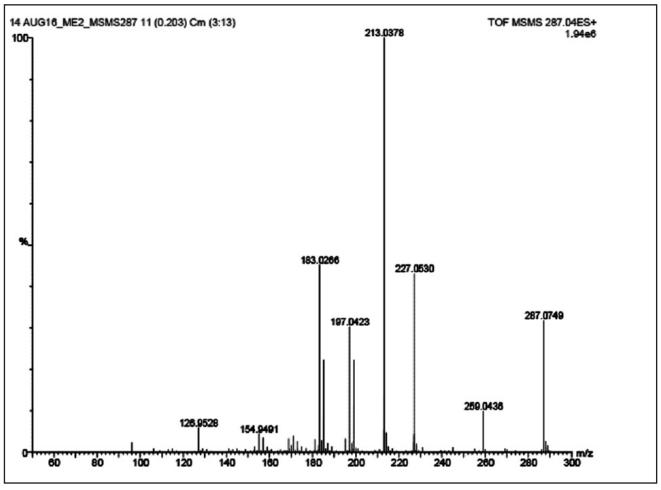

Fig. 4: LC MS/MS spectrum

garcinol, xantho-chymol and iso-xantho-chymol) have been determined by LC–MS/MS method in *G. cambogia* and *G. indica*. Therefore, there is a need to optimize a sensitive, efficient and consistent LC–MS/MS method for the simultaneous determination of multi-class bioactive constituents including organic acids, prenylated xanthones, poly-iso-prenylated benzophenones, bioflavonoids, triterpenoids, phenolic acids and flavonoids in Garcinia species.

Ultra-performance liquid chromatography (UPLC), coupled with quadrupole time-of-flight mass spectrometry (Q-TOF-MS), has been proven to be a powerful means for chemical analysis since it allows appropriate chromatographic separation and identification of individual compounds. Previously, a precursor ion discovery (PID) method on a Q-TOF-MS was reported to rapidly screen polycyclic polyprenylated acylphloroglucinols (PPAPs) in various Garcinia species. In this present study the work was extended to include characterization and the quantification or simultaneous estimation of bioactive constituents in *G. morella* like citric acid derivative, iso garcinol, garcinol and garcimultiflorone-A using UPLC-Q-TOF-MS.

MATERIALS AND METHODS

Plant material, chemicals and standards

G. morella fruits were bought from a local drug store and authenticated by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateshwara University, Tirupati, India. They were shade dried, finely powdered and used for analysis. HPLC grade acetonitrile (ACN) and Methanol (MeOH) used for the HPLC analysis were obtained from Merck India. Ultra-pure water for chromatographic use was procured from a Milli-Q system (Millipore Corp., Bedford, MA, USA). All the samples were filtered through 0.45 µm membrane filter prior to injecting into HPLC. Bioactive compounds, namely, citric acid derivative, iso garcinol, garcinol and garcimultiflorone-A, were isolated from hexane extract in our laboratory as described earlier.

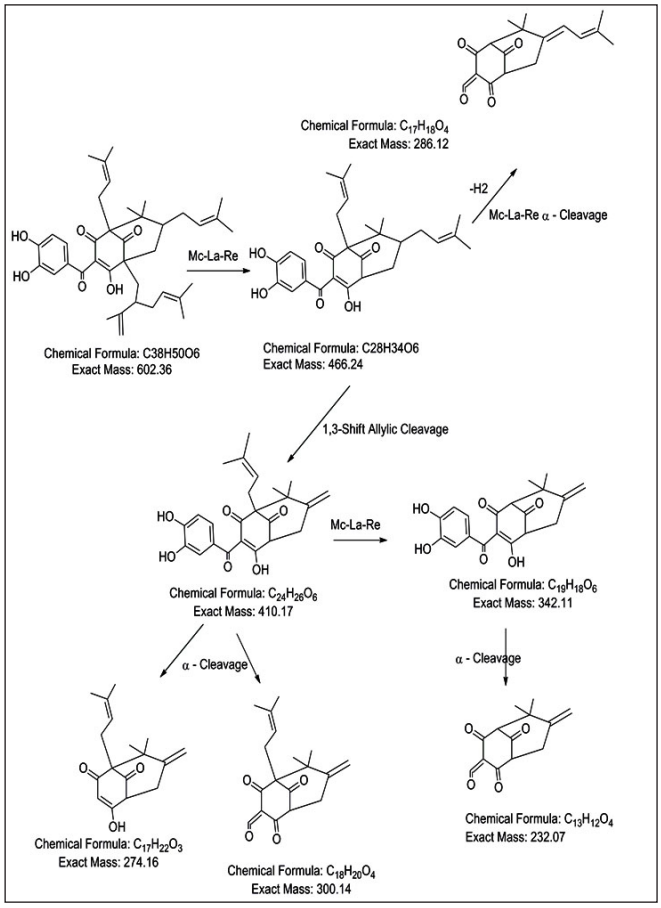

Fig. 5: Scheme of Fig. 2 LC MS/MS fragmentation pathways

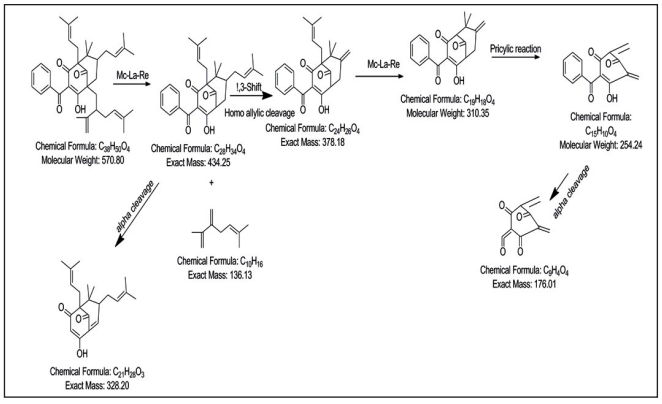

Fig. 6: Scheme of Fig. 3 LC MS/MS fragmentation pathways

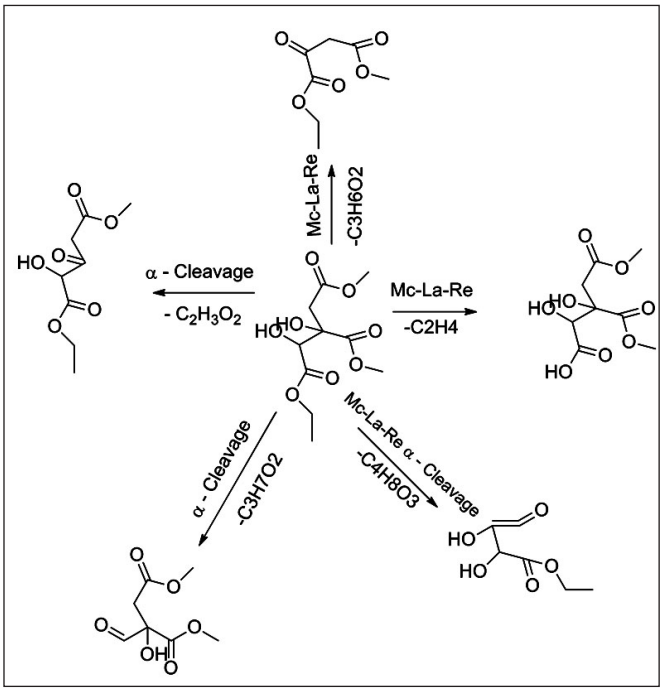

Fig. 7: Scheme of Fig. 4 LC MS/MS fragmentation pathways

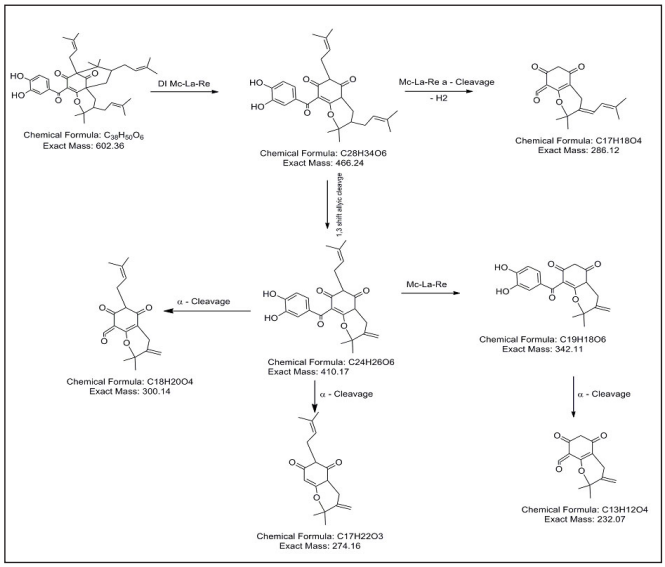

Fig. 8: Final LC MS/MS fragmentation pathway

Their structures were identified by comparison of their spectral data (UV, IR, MS, ^{1}H NMR, ^{13}C NMR and 2D NMR) with those published in earlier references. The purity of each compound was determined to be more than 98 % by normalization of the peak area detected by HPLC.

Extraction and sample preparation

Finely powdered fruits of *G. morella* approximately (500 mg) were extracted using methanol. An aliquot of 2.5 mL of extraction solvent was added in the direction of 500 mg *G. morella* fruits in 15 mL centrifuge tube, sonicated for 30 min followed by centrifugation for 20 min at 2500 rpm. The supernatant liquid was collected separately in a 10 mL volumetric flask. To the residue, 2.5 mL of solvent was added and same sequence was repeated for 3 more times. The volume was made up to the mark with extraction solvent. The same procedure was followed for all the solvents used in this study. The solution was filtered through 0.45 μm syringe filter. The filtered aliquot was used for HPLC analysis. UPLC-Q-TOF-MS Conditions: The chromatographic column used was a Waters Acquity BEH C18 (100 X 2.1 mm, 1.9 μ) with flow rate of 0.6 mL min^{-1}, gradient elution was carried out by using mobile phase: 0.1 % formic acid in water / acetonitrile 0 (95/5), 0.5 (95/5), 2.50 (5/95), 5.0 (5/95), 6.5 (95/5) and 8.0 (95/5). TOF-ESI-MS experiments were carried on a Waters Xevo G2-XS time of flight (TOF) mass spectrometer (Waters, Milford, USA) connected to Waters H-Class inlet system. Spectra were acquired in ESI positive mode from m/z 50 to 1000. The capillary voltage was set at 3 kV, sampling cone at 40 and source temperature set at 120 °C. TOF-MS/MS experiments were performed at various energy collisions by varying the charge from 10 V to 55 V where argon gas used for CID-MS/MS experiments. For TOF-MS and MS/MS analysis, cone gas and desolation gas (N_2) was set at 40 L h^{-1} and 650 L h^{-1}, respectively, with desolvation temperature adjusted at 300 °C. The mobile phase composition of water and acetonitrile was mixed with 0.1 % formic acid at a ratio of 15:85, during this time adjusting the inlet flow rate at 0. 1 mL min^{-1}. Data integration and evaluation for MS experiments was performed with Mass-Lynx v4.1 software SCN949 platform. The obtained chromatograph is shown in Fig.1.

Standard solutions

1 mg mL^{-1} stock solutions were prepared by dissolving 1 mg of citric acid derivative, iso garcinol, garcinol and garcimultiflorone a individually in 1 mL of MeOH. Stock solutions was diluted with methanol to obtain concentration in the range of 50-1000 μg mL^{-1}, respectively.

RESULTS AND DISCUSSIONS

Identification of bio active constituents

Identification of proposed bio active compounds by using LC MS/MS and possible fragmentation pathways are shown in citric acid derivative, iso garcinol, garcinol and garcimultiflorone a. The obtained mass spectra are shown in Figs. 2 - 4 and fragmentation pathways are shown in Figs. 5-8.

Method validation

The optimized UPLC-Q-TOF MS method was validated according to ICH guidelines for *G. morella* bioactive constituents[19]. All the parameters such as assay, linearity, accuracy, precision (intra and inter day), detection limits, quantification limits, robustness and system suitability were evaluated.

Linearity

The linearity was evaluated by analyzing at different concentration levels of each compound. Six series of concentration levels ranging 50-1000 μg mL^{-1} were analyzed. The linearity data is given in Table I.

LOD and LOQ

Detection and quantitation limits were determined by calculating S. D of the response and slope. The results are provided in Table I.

Selectivity

Selectivity of the method was evaluated by injecting blank, standard mixture containing *G. morella* constituents placebo individually. No peak was observed in UPLC chromatogram for blank and placebo indicating no interference.

System suitability

The system suitability parameters, which include resolution (Rs), asymmetry (A), sensitivity (S), theoretical plates (N), skewness, relative retention time (RRT) and % relative standard deviation (% R.S. D), were assessed and the results were represented in Table II.

Precision

Intraday and inter day injections were performed in triplicate for citric acid derivative, iso garcinol, garcinol and garcimultiflorone-A. Method which is calculated as % R.S.D. The % R.S.D values for citric acid derivative, iso garcinol, garcinol and garcimultiflorone-A were found to be in the range of 50-1000 for intraday and

50-1000 for inter day, which is within acceptable limits in Table III.

Accuracy

Accuracy study was performed by the standard addition method. Results indicate that the accuracy was within the acceptable limits Table IV.

CONCLUSION

Developed UPLC-Q-TOF-MS method for the quantification of four marker compounds from *G. morella* was validated and found to be rapid sensitive and precise. The developed method was subsequently applied to determine the concentration of four markers in methanolic extract of *G. morella*. The developed UPLC-Q-TOF-MS procedure can easily be utilized as a fast and sensitive analytical method for quality control of crude extracts or herbal drug formulations of *G. morella.*

REFERENCES

1. Singh I.P. and Bharate S. B.: Phloroglucinol compounds of natural origin, **Nat. Prod. Rep.,** 2006, 23(4), 558-560.
2. Krishnamurthy N., Lewis Y.S. and Ravindranath.: On the structures of garcinol, isogarcinol and camboginol, **Tetrahedron Lett.,** 1981, 22(8), 793-796.
3. Das D., Bandyopadhyay D. and Banerjee R.K.: Oxidative Inactivation of Gastric Peroxidase by Site-Specific Generation of Hydroxyl Radical and Its Role in Stress-Induced Gastric Ulceration, **Free Rad. Biol. Med.,** 1998, 24(3), 460-469.
4. Tosa H., Iinuma M., Tanaka T., Nozaki H., Ikeda S., Tsutsui K. and Yamada M.: Inhibitory activity of xanthone derivatives isolated from some guttiferaeous plants against DNA toposiomerases i and ii, **Chem. Pharm. Bull.,** 1997, 45, 418-420.
5. Bharate J. B., Vishwakarma R. A., Bharate S. B., Kushwaha M. and Gupta A. P.: Quantification of the polyisoprenylated benzophenones garcinol and isogarcinol using multiple reaction monitoring LC/electrospray ionization-MS/MS analysis of ultrasound-assisted extracts of *G. indica* fruits, **J. AOAC. Int.,** 2014, 97(5), 1317-1322.
6. Baliga M.S., Bhat H.P., Pai R.J., Boloor R., and Palatty P.L: The chemistry and medicinal uses of the under utilized Indian fruit tree *G. indica* Choisy (kokum): A review, **Food Res. Int.,** 2011, 44(7), 1790-1799.
7. Ritthiwigrom T., Laphookhieo S., Pyne and Maejo S.G.: Chemical constituents and biological activities of *Garcinia cowa Roxb*, **Int. J. Sci. Techno.**, 2013, 7, 212-215.
8. Hemshekhar M.., Sunitha K., Sebastin M. S., Devaraja S., Kemparaju K., Vishwanath B. S., Niranjana S. R. and Girish K. S.: An overview on genus Garcinia: phytochemical and therapeutical aspect, **Phytochem. Rev.,** 2011, 10, 325-351.
9. Xu G., Kan W.L.T., Zhou Y., Song J.Z., Han Q.B., Qiao C.F., Cho C.H., Rudd J.A., Lin G. and Xu H.X.: Cytotoxic Acylphloroglucinol Derivatives from the Twigs of *Garcinia cowa*, **J. Nat. Prod.,** 2010, 73(2), 104-108.
10. Han Q.B., Yang L., Wang Y.L., Qiao C.F., Song J.Z., Sun H.D. and Xu H.X.: A Pair of Novel Cytotoxic Polyprenylated Xanthone Epimers from Gamboges, **Chem. Biodivers.**, 2006, 3, 101-103.
11. Padhye S., Ahmad A., Oswal N. and Sarkar F. H.: Emerging role of garcinol, the antioxidant chalcone from *G. indica* (choisy) and its synthetic analogs, **J. Hematol. Oncol.,** 2009, 2, 38–42.
12. Jayaprakasha, G.K., and Sakariah, K.K.: Determination of (−) hydroxy citric acid in commercial samples of garcinia cambogia extract by liquid chromatography with ultraviolet detection, **Liq. Chromatogr. Relat. Technol.,** 2000, 23(6), 915-923.
13. Acuna U.M., Dastmalchi K., Basile M.J. and Kennelly E.J.: Quantitative high-performance liquid chromatography photo-diode array (HPLC-PDA) analysis of benzophenones and bioflavonoids in eight *Garcinia* species, **J. Food Compos. Anal.,** 2012, 25(2), 215-220.
14. Zadernowski R., Czaplicki S., and Naczk M.: Phenolic acid profiles of mangosteen fruits (*Garcinia mangostana*), **Food Chem.,** 2009, 112(3), 685-689.
15. Ji X., Avula, B. and Khan I.A.: Quantitative and qualitative determination of six xanthones in *Garcinia mangostana* L. by LC–PDA and LC–ESI-MS, **J. Pharm. Biomed. Anal.**, 2007, 43(4), 1270-1276.
16. Wittenauer J., Falk S., Weisz U.S. and Carle R.: Characterization and quantification of xanthones from the aril and pericarp of mangosteens (*Garcinia mangostana* L.) and a mangosteen containing functional beverage by HPLC–DAD–MS, **Food Chem.,** 2012, 134(1), 445-452.
17. Chattopadhyay and Kumar S.K.: Identification and quantification of two biologically active polyisoprenylated benzophenones xanthochymol and Iso-xanthochymol in *Garcinia* species using liquid chromatography–tandem mass spectrometry, **J. Chromatogr. B,** 2006, 844(1), 67-83.
18. Bharate J.B., Vishwakarma R.A., Bharate S.B., Kushwaha M. and Ajai P.: Quantification of the Polyisoprenylated Benzophenones Garcinol and Isogarcinol Using Multiple Reaction Monitoring LC/Electrospray Ionization-MS/MS Analysis of Ultrasound-Assisted Extracts of Garcinia indica Fruit, **J. AOAC Int.,** 2014, 97(5), 1317-1322.
19. ICH-Q2B Validation of Analytical Procedure: Text and methodology, International Conference of Harmonization, Geneva, (1996).