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ORIGINAL ARTICLE

High performance liquid chromatographic determination of marmelosin and psoralen in bael (*Aegle marmelos* (L.) Correa) fruit

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Abstract Marmelosin and psoralen, two bioactive coumarins present in bael fruit, are reported to have antihelminthic, antibacterial, antispasmodic, artemicide and cytotoxic activities. A high performance liquid chromatographic (HPLC) method using diode array detector was standardized to estimate marmelosin and psoralen in bael pulp. Both marmelosin and psoralen were extracted from the pulp in benzene and benzene was filtered. The benzene free residue was dissolved in 10 ml of HPLC grade methanol. The average recovery of marmelosin from bael pulp ranged between 98.7 % and 110.3 % after adding 4 or 10 μg ml^{-1} concentrations with a minimum limit of detection of 0.02 μg ml^{-1}. The corresponding recovery for psoralen at same concentrations ranged from 94.9 % to 97.6 % with the limit of detection being 0.01 μg ml^{-1}. The limit of quantification for both the molecules was 0.1 μg ml^{-1}. HPLC was found an easy, quick, effective and sensitive method to estimate marmelosin and psoralen in bael fruits.

Keywords Marmelosin · Psoralen · Bael fruit · HPLC analysis

Bael or stone apple (*Aegle marmelos* (L.) Correa) is a prominent member of Rutaceae family and an indigenous fruit tree of India. All parts of the tree i.e., leaves, fruits, stem, bark and roots are found effective as ethnomedicines against various human ailments (Badam et al. 2002). Bael pulp can be processed into nectar or squash, marmalade, jelly, powder, toffee, etc. for both food and therapeutic use (Rakesh et al. 2005). The fresh ripe pulp of good quality cultivars and the "sherbat" made from it are used for their mild laxative, tonic, digestive and restorative properties as well as in biliousness (Kirtikar and Basu 1995). Green immature bael fruits are used for preparing "murabba" or preserve which is generally used for stomach ailments (Singh and Roy 1984). A popular drink, called "sherbat" in India, is made by beating the seeded pulp together with milk / water and sugar. The bioactive compounds identified in bael fruits are marmelosin, psoralen, luvangetin, aurapten, marmelide and tannins (Goel et al. 1997; Maity et al. 2009). Among these, marmelosin ($C_{16}H_{14}O_4$) and psoralen ($C_{11}H_6O_3$) are furanocoumarin type of polyphenols found generally in fruits. Marmelosin is believed to be the therapeutically active principle of bael fruit. It has shown antihelminthic and antibacterial activities (Ghosh and Playford 2003; Shoba and Thomas 2001). It can also be used as a laxative and for diuretic treatment. Psoralen is used for antispasmodic (Hansel et al. 1994), artemicide (LD_{50}= 5.93 μg ml^{-1}) and cytotoxic (Saqib et al. 1990) activities. It can increase skin's tolerance to sunlight and aids in the maintenance of normal skin colour. It is employed in the treatment of leucoderma (Bag et al. 2009). Despite their proven medicinal properties very few studies have been conducted earlier to quantify them in fruit pulp. High performance thin layer chromatography (HPTLC) was recently employed to quantify the amount of marmelosin in pulp, seed, leaves, rind and powder (Shailajan et al. 2011). There is a need to standardize a simple, easy, quick and effective method for quantitative estimation of marmelosin and psoralen in bael pulp. Keeping this in view, the present investigation was aimed to use high performance liquid chromatography (HPLC) for the determination and quantification of marmelosin and psoralen in bael pulp.

Materials and methods

Accurately weighed 5 mg each of reference standards of marmelosin and psoralen (Chromadex, Life Technologies India Pvt. Ltd., Mumbai) was dissolved in 25 ml HPLC grade methanol to get 200 μg ml^{-1} stock solutions. Working

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Table 1 Recovery of marmelosin and psoralen from fortified bael pulp by high performance liquid chromatography

Compound	Fortification level (μg ml^{-1})	Recovery (%)			Average recovery (%)[a]
		R_1	R_2	R_3	
Marmelosin	4	111.31	109.79	109.93	110.3±0.84
	10	101.23	97.52	97.48	98.7±2.15
Psoralen	4	100.91	96.50	95.45	97.6±2.89
	10	93.31	96.90	94.43	94.9±1.84

[a] Mean ± standard deviation (SD, n=3)

solutions of 1, 2 and 4 μg ml^{-1} concentrations were prepared by serial dilution in HPLC grade methanol. The stock solutions were quantitatively transferred to 10 ml amber colored volumetric flasks to obtain appropriate concentration range of marmelosin and psoralen (0.01 to 10.0 μg ml^{-1}). Methanol was used because both the compounds were found completely soluble in methanol.

A high performance liquid chromatograph (Model SCL 10 AVP, Shimadzu, Japan) coupled with a photodiode array (PDA) detector and a rheodyne injector (20 μl loop) was used for the analysis of these compounds. The stationary phase consisted of reverse phase μBondapak™ C-18 column (300×3.9 mm i.d., 125 Å, 10 μm film thickness) and the mobile phase consisted of methanol : water (66 : 34, v/v) with a flow rate of 1.0 ml min^{-1}. The detector wavelength was set at 254 nm. All the samples were filtered before analysis through a nylon membrane filter (Millipore, 13 mm dia and 0.45 mm thickness) held in a filter holder attached to a glass syringe. Under this condition, the retention times of marmelosin and psoralen were 10.76±0.05 and 5.62±0.04 min, respectively.

A 20 μl volume of standard solutions of marmelosin and psoralen (0.01 to 10.0 μg ml^{-1}) was injected through the injection port of HPLC in triplicate. Calibration curves were prepared by plotting peak area against concentrations of reference standards. Good linearity was obtained within the concentration range of 0.1 to 10.0 μg ml^{-1} for both the compounds. The regression equations and correlation coefficients for marmelosin and psoralen were y=7.5874×–0.5682, R^2=0.998 and y=10.095×–0.5521, R^2=0.999, respectively. The limit of detection (LOD) was determined by considering a signal-to-noise ratio (S/N) of 3:1 with reference to the background noise obtained from the blank sample. However, the limit of quantification (LOQ) of both the chemicals was determined by considering a signal-to-noise ratio of 10:1.

The recovery test for these bioactive compounds was carried out to evaluate the accuracy of the method. Accurately weighed 5 g pulp samples (in triplicate) were taken in 250 ml conical flasks and 50 ml benzene (AR grade) was added to it after spiking with 1 ml of 4 and 10 μg ml^{-1} concentrations of marmelosin and psoralen separately. After keeping this mixture overnight at room temperature, both marmelosin and psoralen were extracted from pulp by wrist action shaking for 2 min. Benzene was filtered through Whatman No. 1 filter paper. This process was repeated twice with 50 ml benzene each time. Pooled benzene extract was evaporated to dryness in a rotary vacuum evaporator at 50 °C temperature and the residue was dissolved in 10 ml HPLC grade methanol before analysis. The quantification of recovery from samples was done using the calibration curve obtained after HPLC analysis.

A bael selection CISH B-2 was chosen to evaluate the method. Fruits of this cultivar are bigger in size (fruit weight 1.8–2.7 kg) and round or oblong in shape with thin rind, less fibre and few seeds (Pandey et al. 2008). Immature and mature fruits of bael were collected from the Institute farm. The mature fruits were ripened under ambient conditions using 2000 ppm ethrel solution. Ethrel solution was prepared by dissolving 50 ml of 2-chloro ethyl phosphonic acid (40 % a.i.) in 10 L water and bael fruits were dipped in that solution for 10 min. After surface drying, the fruits were wrapped in newspapers and kept inside the cardboard boxes for ripening. Pulps of immature, mature and ripe fruit samples (5 g each) was processed and analyzed as per the optimized method described earlier for estimating marmelosin and psoralen content in them (n=3).

Results and discussion

The recovery of marmelosin and psoralen from bael pulp is presented in Table 1. The average per cent recovery of marmelosin at 4 and 10 μg ml^{-1} fortification levels were found to be 110.3 and 98.7 %, respectively, while those for psoralen were 97.6 and 94.9 %, respectively. Around 95.0 % or more recovery for both the compounds proved that the extraction method was accurate and efficient. The optimized HPLC method was evaluated in screening farm samples of bael fruit to identify and quantify the contents of marmelosin and psoralen in immature, mature and ripe fruits. Marmelosin and psoralen in pulp samples were identified by their respective retention times (Fig. 1). Our study revealed that the content of marmelosin was always higher than that of psoralen at all stages of fruit. Immature fruits were found to have higher amount of marmelosin and psoralen as compared to mature and ripe fruits. The contents of marmelosin and psoralen in immature fruits were observed to be 481.11 and 15.69 μg g^{-1}, which reduced to 103.93 and 7.97 μg g^{-1}, respectively, in mature fruits. The amounts further decreased to 36.52 and 5.79 μg g^{-1} in pulp when the fruits ripened after almost one month of ethrel treatment. The findings were in contrast with the results reported by Shailajan et al. (2011) who observed that ripe fruit pulp powder contained slightly higher concentration of marmelosin than unripe pulp powder.

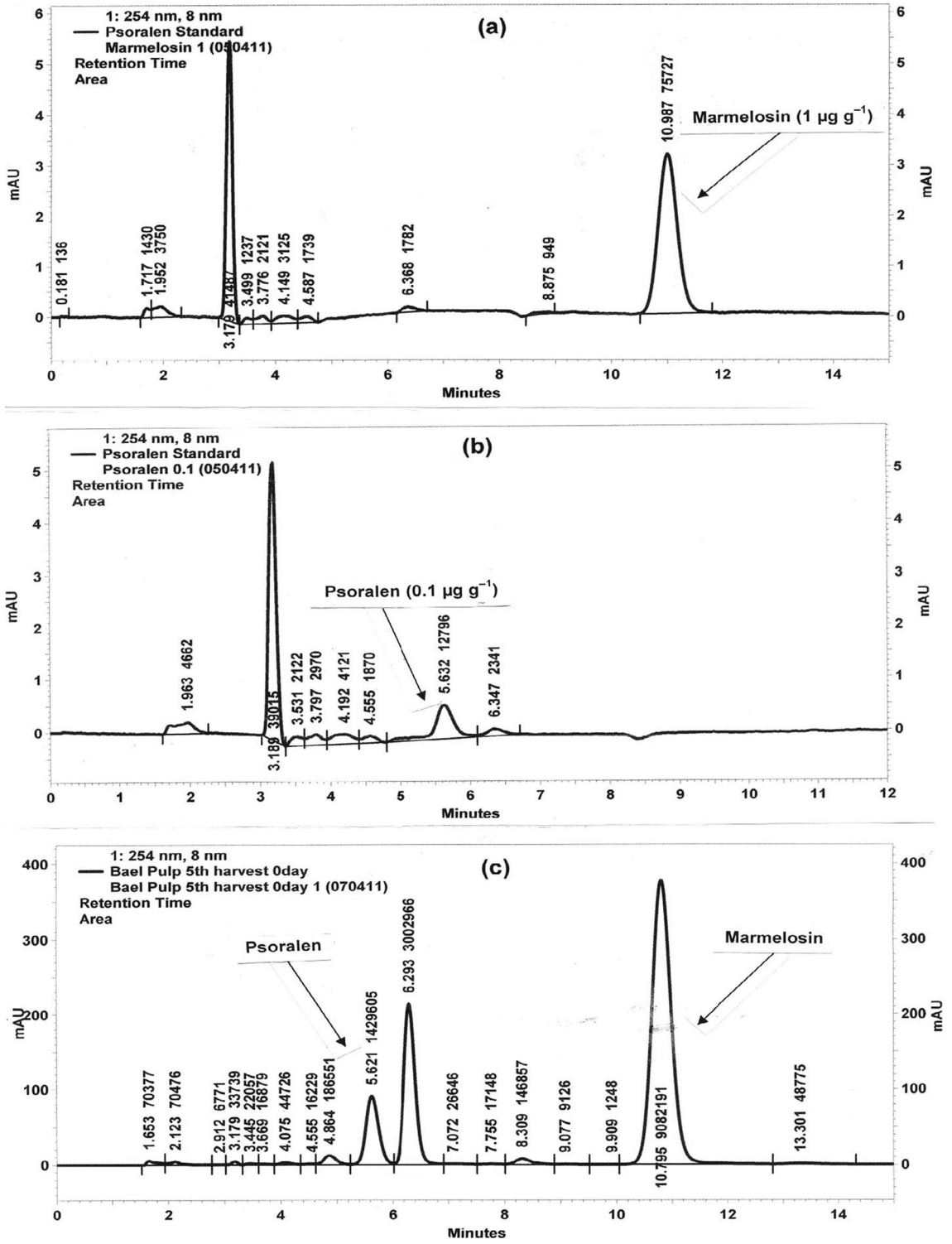

Fig. 1 HPLC chromatograms of **a** Marmelosin standard (1 µg g^{-1}), **b** Psoralen standard (0.1 µg g^{-1}) and **c** Bael pulp sample

The linearity of the HPLC method from lowest to highest concentrations for marmelosin and psoralen is presented in Fig. 2. Good linearity was established within the concentration range of 0.1 to 10.0 µg ml^{-1} with the correlation coefficient (R^2) being>0.99 for both the compounds. The LOD was observed to be 0.02 µg ml^{-1} for marmelosin and 0.01 µg ml^{-1} for psoralen by considering a signal-to-noise ratio of 3:1, whereas the LOQ for both the chemicals was found to be 0.1 µg ml^{-1} after considering a signal-to-noise ratio of 10:1. The LOD and LOQ of marmelosin by HPLC were found better than those reported by Shailajan et al. (2011) using HPTLC (LOD 0.1 µg ml^{-1} and LOQ 0.25 µg ml^{-1}).

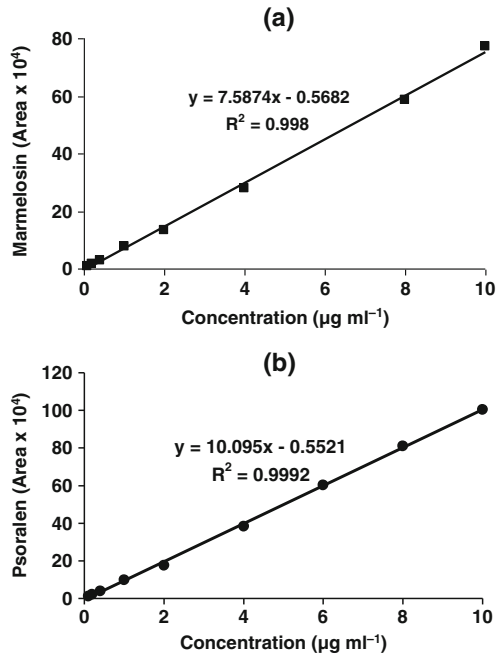

Fig. 2 Calibration curves for **a** marmelosin and **b** psoralen by high performance liquid chromatography

Linearity along with LOD and LOQ proved the efficiency and sensitivity of the HPLC method. Most of the workers now-a-days preferred HPLC for the analysis and characterization of bioactive plant molecules like vitamins and polyphenols because of its wide range of applicability, good sensitivity, accurate determination and easy to use approach (Aslam et al. 2008; Sellappan et al. 2002; Bhattacherjee et al. 2011). Sample preparation took minimum time with minimum effort and maximum efficiency because marmelosin and psoralen were extracted directly from pulp and not from powder after drying of pulp as earlier reported by Shailajan et al. (2011). CISH B-2 fruits have less number of seeds, thereby reducing the hindrance in extraction from pulp. Thus this method was found easy and quick. Also by using less number of easily available solvents this one was a cheaper method which can easily be replicated in any laboratory.

Conclusion

The current method is found simple, easy and quick for the extraction of two bioactive furanocoumarins, marmelosin and psoralen, from bael pulp. HPLC has been established as an accurate and sensitive method for the analysis of marmelosin and psoralen with good linearity and can be used effectively for quantifying these two compounds in different bael cultivars at different developmental stages.

References

Aslam J, Mohajir MS, Khan SA, Khan AQ (2008) HPLC analysis of water-soluble vitamins (B1, B2, B3, B5, B6) in *in vitro* and *ex vitro* germinated chickpea (*Cicer arietinum* L.). Afr J Biotechnol 7:2310–2314

Badam L, Bedekar SS, Sonawane KB, Joshi SP (2002) *In vitro* antiviral activity of bael (*Aegle marmelos* Corr.) upon human coxsackieviruses B1-B6. J Commun Dis 34:88–99

Bag SK, Srivastav PP, Mishra HN (2009) Desorption and adsorption characteristics of bael (*Aegle marmelos*) pulp and powder. Internl Food Res J 16:561–569

Bhattacherjee AK, Tandon DK, Dikshit A, Kumar S (2011) Effect of pasteurization temperature on quality of aonla juice during storage. J Food Sci Technol 48:269–273

Ghosh S, Playford RJ (2003) Bioactive natural compounds for the treatment of gastrointestinal disorders. Clin Sci (London) 104:547–556

Goel RK, Maiti RN, Manickam M, Ray AB (1997) Antiulcer activity of naturally occurring pyrano-coumarin and isocoumarins and their effect on prostanoid synthesis using human colonic mucosa. Indian J Exp Biol 35:1080–1083

Hansel R, Keller K, Rimpler H, Schneider G (1994) Hager's Handbuch der Pharmazeutischen Praxis. Springer, Berlin, p 1196

Kirtikar KR and Basu BD (1995) Indian medicinal plants, vol. 1, 2nd edn., In: Blatter E, Caius JF, Mhaskar KS (ed.), Lalit Mohan Basu, Allahabad. pp 499–502

Maity P, Hansda D, Bandyopadhyay U, Mishra DK (2009) Biologial activities of crude extracts and chemical constituents of bael, *Aegle marmelos* (L.) Corr. Indian J Exp Biol 47:849–861

Pandey D, Shukla SK, Pandey G (2008) Bael (*Aegle marmelos* Correa), in Underutilized Horticultural Crops, vol 3, In: Peter KV (ed.), New India Publishing Agency, New Delhi. pp 201–224

Rakesh, Dhawan SS, Arya SS (2005) Processed products of bael. Processed Food Industry 8:25–27

Saqib QN, Hui YH, Anderson JE, McLaughlin JL (1990) Bioactive furanocoumarins from the berries of *Zanthoxylum americanum*. Phytotherapy Res 4:216–219

Sellappan S, Akoh CC, Krewer G (2002) Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. J Agric Food Chem 50:2432–2438

Shailajan S, Menon S, Hande H (2011) Method validation of marmelosin from fruit pulp of *Aegle marmelos* (L.) Correa using HPTLC technique. J Pharma Res 4:1353–1355

Shoba FG, Thomas M (2001) Study of antidiarrhoeal activity of four medicinal plants in castor-oil induced diarrhoea. J Ethnopharmacol 76:73–76

Singh RN and Roy SK (1984) The Bael: Cultivation and processing, Indian council of agricultural research, New Delhi. pp 1–5