



Chromatographic and mass spectroscopic methods for the evaluation of phytochemical variability in different accessions of *Baliospermum solanifolium* (Burm.) Suresh

*Sulaiman C.T.¹, ²Geetha S.P., ³Sadheeshna Kumari, ⁴Satheesh George & ⁵Indira Balachandran

^{1,2,3} & ⁵Phytochemistry Division, Centre for Medicinal Plants Research, Arya Vaidya Sala, Kottakkal

⁴Department of Botany, St. Joseph's College (Autonomous), Devagiri, Calicut

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Abstract

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Baliospermum solanifolium (Burm.) Suresh is a vulnerable medicinal plant belonging to the family Euphorobiaceae. In the present study, phytochemical variability has been evaluated in 14 accessions of *B. solanifolium* collected from different geographical and agro climatic regions of south India. Qualitative chemical evaluation was done using thin layer chromatography. The total polyphenolic compounds were estimated spectrophotometrically. The variation of so far reported toxic factor, Phorbol 12, 13-dibutyrate, was analysed by comparing the retention time and mass spectrum of standard compound using LC-ESI-MS analysis. The results suggest that both genotypic and geographical factors influence the chemical profiles of various accessions.

1. Introduction

Baliospermum solanifolium (Burm.) Suresh is an important medicinal plant, which is known as *Danti* In *Ayurveda*. The plant is a stout monoecious under shrub with many shoots from the base. The various parts of the plant like roots, leaves, and seeds are used traditionally for the treatment of various ailments. In *Ayurveda*, root are used to cure jaundice, leucoderma, skin diseases, wounds, and as an anthelmintic (Ravindra & Raju, 2008). Leaves are found to be useful in asthma, bronchitis (Nadkarni, 1988) and in treating abdominal tumor (Chopra *et al.*, 1994). Seeds are used as purgative and in gastric complaints

(Goel *et al.*, 1984). Decoction of stem is used to get relief from toothache (Bhatt *et al.*, 1982).

Plant species react against the environmental changes by producing some secondary metabolites. The concentrations of various secondary plant products are strongly dependent on the growing conditions and have impact on the metabolic pathways responsible for the accumulation of the related natural products (Ramakrishna *et al.*, 2011, Talei *et al.*, 2013). A wide range of environmental conditions such as rainfall, temperature, humidity etc. are known to influence the production of plant secondary metabolites



(Moroson & Lawlor, 1999). Thus, the standardization of various accessions of medicinal plants growing with different agro-climatic conditions is essential for increasing the biomass yield and secondary metabolites. Chromatographic techniques are the most often used method for the evaluation of chemical variability of plant extracts. The authentication and identification of herbal medicines can be accurately conducted using chromatographic fingerprints (Sulaiman *et al.*, 2014).

Though most of the plant drugs are safe, yet few are toxic for human health. These poisonous/toxic plants are categorized as *visha* (poison) and *upavisha* (toxic but not lethal for human health) in Ayurvedic texts. Hence, to promote and introduce their use for medicine, such plant drugs must be detoxified or purified before their use (Maurya *et al.*, 2015). The detoxification or purification process of any toxic material used for medicinal purposes is termed as "*Śodhana*". The root of *B.*

solanifolium is used for preparation of medicine only after purification process as it contains toxic phorbol derivatives.

The objective of the present work was to evaluate the phytochemical variability of different accessions of *B. solanifolium* collected from various agro climatic conditions in terms of qualitative and quantitative assessment of major secondary metabolites including toxic compounds.

2. Material and methods

2.1 Selected Plant Material

The roots of *B. solanifolium* were collected from the germplasm maintained at Centre for Medicinal Plants Research (CMPR), Arya Vaidya Sala (AVS), Kottakkal, Kerala, India which were originally collected from various geographic zones of South India (Table-1) and all the materials were authenticated by Plant Systematics and Genetic Resources Division, Centre for Medicinal Plants Research (CMPR), Arya Vaidya Sala Kottakkal, Kerala, India.

Table.1 Collection locality of various germplasm of *B. solanifolium*

Accession	Place of collection	Latitude	Longitude
B1	AVP, Kanjikode, Kerala	10.799315	76.752098
B2	ARI, Poojappura, Kerala	8.467817	77.018904
B3	PDS, Idukki, Kerala	9.578293	76.972947
B4	Mattannur, Kannur, Kerala	11.938436	75.506973
B5	Kalpetta, Wayandu, kerala	11.619845	76.084334
B6	Nilambur, kerala	11.279432	76.239785
B7	Vellarimala, Thrissur, Kerala	10.527642	76.214435



B8	Vithura, Thiruvananthapuram	8.675349	77.085226
B9	Odakkali, Ernakulum, Kerala	10.094460	76.557567
B10	BIRD-K, Tiptur, Karnataka	13.263781	76.470295
B11	Thiruchirappalli, Tamil Nadu	10.790483	78.704673
B12	Sevayur, Tamil Nadu	11.127123	78.656894
B13	Top slip, Anamalai, Tamil Nadu	10.470405	76.843196
B14	Nedumkayam, Nilambur, Kerala	11.286666	76.314318

2.2 Chemicals

Folin-Ciocalteu reagent was procured from Sisco Research Laboratory (SRL), Mumbai, India, Gallic acid and LCMS solvents were procured from Sigma Chemicals Co. (Bangalore, India). All other chemicals employed were of standard analytical grade from Merck India.

2.3 Extraction

Dried root powder (5 g) of each accession was extracted in 80% methanol for 6 hrs by reflux method. After filtration, the filtrate was concentrated to dryness by rotary evaporator at 40°C. 10 mg of this extract was dissolved in methanol and used for thin layer chromatographic analysis and total phenolic and total flavonoid estimations. The remaining residue was kept in refrigerator until LC MS analysis.

2.4 Poly phenolic analysis

Polyphenols such as phenolics and flavonoids were estimated spectrophotometrically. The total phenolic content (TPC) was determined using Folin-Ciocalteu reagent (Singleton *et al.*, 1965). TPC was expressed as gallic acid equivalents (GAE) in mg / g of

sample. Total flavonoid content (TFC) was measured by aluminium chloride colorimetric assay (Zhishen *et al.*, 1999) and expressed as mg quercetin equivalents (mg EQ).

2.5 Thin Layer Chromatography

The thin layer chromatographic profile was developed for methanolic extract of different accessions on a precoated silica plate (F₂₅₄ Merck, India) using toluene: ethyl: acetate and methanol as mobile phase in the ratio of 7:2:1. 10 µl of the extracts was applied using Agilent HPLC syringe. The chromatogram was developed in a twin trough chromatographic chamber (CAMAG, Switzerland). The developed plates were visualized and documented under 254 nm and 366 nm.

2.6 HPLC Analysis

HPLC analysis was conducted on Agilent LC 1200 equipped with Agilent Eclipse XDB -C18 column of 5 µm, 4.6 x 150 mm. Gradient elution was performed with water/0.1% formic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.8 ml/ min. Column temperature was maintained at



30^o C. The DAD signal was recorded at 270 nm.

2.7 LC-MS analysis

The extracts stored in the refrigerator were dissolved in LC MS grade acetonitrile and filtered through PVDF membrane (0.45 µm) and these filtrates were subjected to LC MS analysis. LC-ESI-MS analysis was conducted on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 equipped with Agilent Eclipse XDB -C18 column of 5 µm, 4.6 x 150 mm. Gradient elution was performed with water/0.1% formic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.8 ml/ min. Column temperature was maintained at 30^o C. The MS analysis was performed using ESI in the negative mode. The conditions for mass spectrometry were: drying gas (nitrogen) flow 5 L/min; nebulizer pressure 40 psig; drying gas temperature 325^oC; capillary voltage -3000 V; fragmentor volt 125V; Oct RF Vpp 750 V.

2.8 Statistical analysis

Data were presented as mean ± standard deviation (SD) of three determinations. Statistical analyses were performed using one-way analysis of variance. Results were calculated by employing the statistical software (COSTAT, Monterey, CA 93940, U.S.A)

3. Results and Discussion

3.1 Estimation of polyphenolic compounds

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants (Dong Wei *et*

al., 2010). The different accessions have shown extended variability in phenolic and flavonoid contents (Table. 2). The highest TPC and TFC has been found in accession B9 (Odakkali, Ernakulum) while least TPC was found in accessions B6 and B14. The lowest TFC was showed by accession B7. The Flavonoid/Phenolics (F/P) ratio was also calculated for each sample in order to distinguish flavonoids out of total phenolics. The highest F/P ratio has been shown by B12 (0.63). In B9, in spite of the highest TPC (21.36), the contribution of flavonoids was found to be comparatively less as F/P ratio was showed as 0.22. The F/P ratio can be used for the assessment of individual specificity of flavonoids towards the total polyphenols (Marinova *et al.*, 2005, Sulaiman & Balachandran, 2012).

Many factors such as soil composition, water stress, temperature and light intensity may affect the levels of phenolic compounds in plants (Kouki & Manetas, 2002; Turtola *et al.*, 2005). This present study indicated that accumulation of polyphenolic compounds in *B. solanifolium* in various accessions might be associated with variations caused not only by geographic areas but also by genetic diversity as the germplasm were conserved in uniform climatic conditions for more than 10 years. Similar finding was reported for many other species (Siatka & Kasparova, 2010, Santos *et al.*, 2011, Kremer *et al.*, 2012, Samec *et al.*, 2015, Ying Xua *et al.*, 2015).



Table 2 Total phenolics and Total flavonoids in different accessions of *B. solanifolium*

Sl No	Accession	TPC (mg EGa/g)	TFC(mg EQ/g)	F/P
1	B1	14.17 ±0.12	2.48±0.18	0.17
2	B2	11.25 ±0.16	2.56±0.12	0.22
3	B3	11.48±0.14	1.89±0.22	0.16
4	B4	11.49±0.12	2.35±0.17	0.20
5	B5	4.12±0.18	1.48±0.12	0.35
6	B6	2.18±0.12	1.20±0.14	0.55
7	B7	2.14±0.18	1.14±0.18	0.53
8	B8	3.18±0.16	1.58±0.16	0.49
9	B9	21.36±0.13	4.86±0.26	0.22
10	B10	15.20±0.12	2.85±0.22	0.19
11	B11	14.2±0.18	1.54±0.15	0.11
12	B12	5.18±0.24	3.25±0.18	0.63
13	B13	7.5±0.15	2.56±0.14	0.34
14	B14	2.18±0.14	1.63±0.28	0.75

3.2 Thin Layer Chromatography

Thin layer chromatography has an advantage of comparing multiple samples on a same plate (Waksmundzka-Hajnos *et al.*, 2008). The qualitative chemical variation of different extracts can be easily evaluated using chromatographic fingerprints. The TLC profile showed significant variations among the accessions both qualitatively and quantitatively (Fig. 1 &2). Accessions such as B1, B2, B9 and

B10 showed high degree of variations in terms of individual chemical constituents. The highest band intensity for separated compounds was observed in accession B9. It also showed the highest polyphenolic level. The concentrations of individual phytochemicals were found to be very less in accessions like B3, B4, B5, B6, B7 and B13.

Fig.1 TLC profile of various accessions of *B. solanifolium* (254 nm)

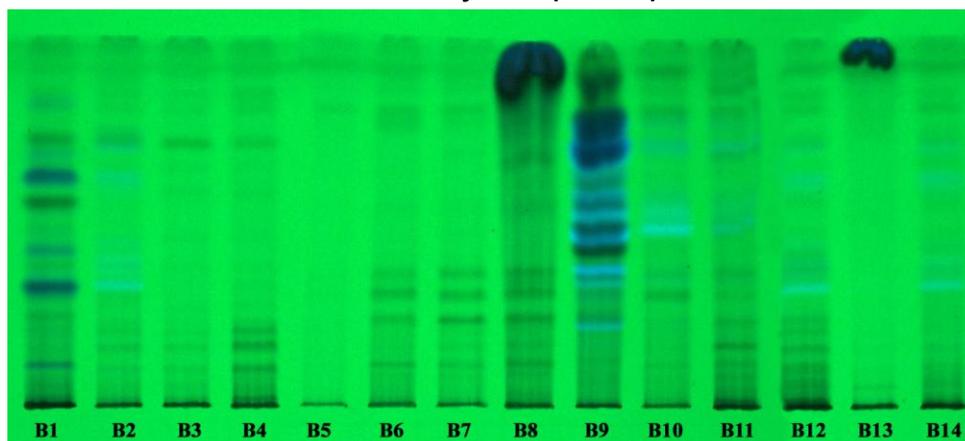
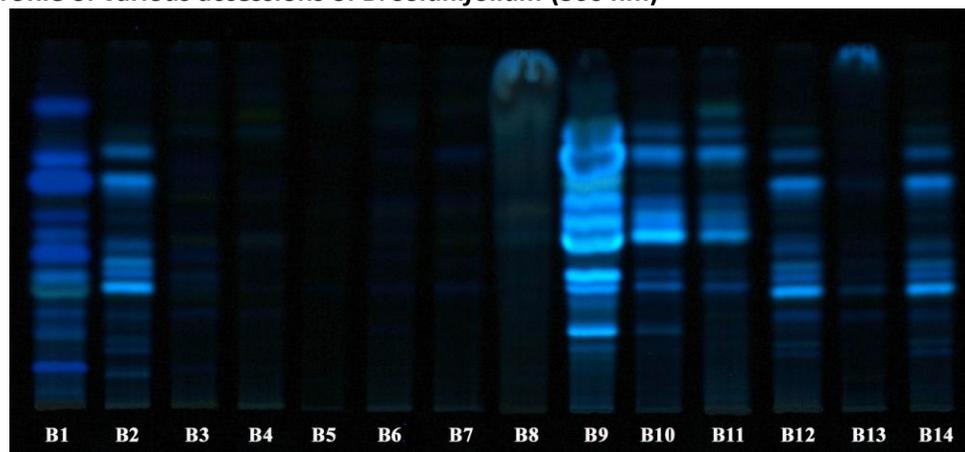


Fig.2 TLC profile of various accessions of *B. solanifolium* (366 nm)

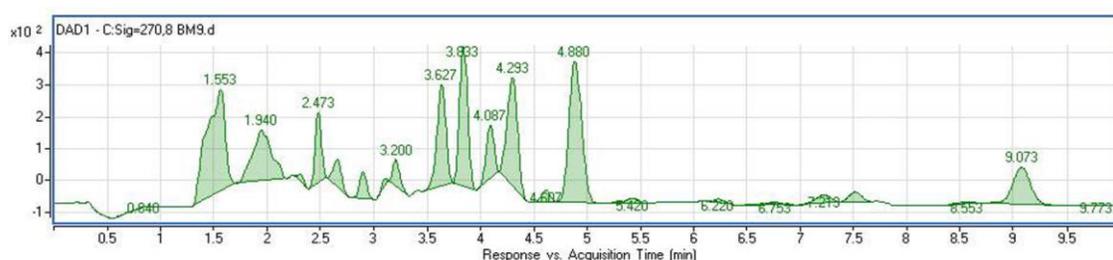


3.2 HPLC Analysis

The HPLC finger prints of various accessions also showed variation in number of peaks and peak areas which support the TLC results. The difference in the area percentage of various resolved peaks indicated the quantitative variations of

phytochemicals with respect to the accessions. The HPLC chromatogram of accession B9 showed maximum peaks (Fig. 3). The major peaks were obtained at 1.55, 1.94, 2.47, 3.20, 3.62, 3.83, 4.08, 4.29 and 4.88.

Fig. 3 DAD Chromatogram of accession B9 at 270 nm

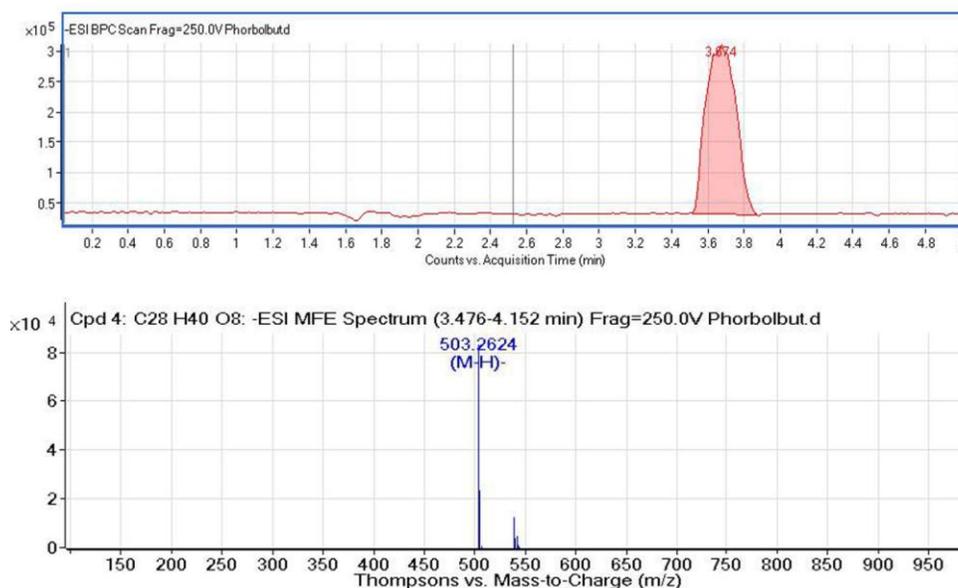


3.3 LC MS analysis

Active constituents of many plant drugs may exert severe toxic effect at high concentrations. Ayurvedic classics have emphasized various methods of detoxification (*Sodhana*) to overcome the undesired effects from various poisonous drugs. The purification processes are basically intended to reduce the toxicity level (Maurya *et al.*, 2015). Detoxification process is being employed in the case of *B. solanifolium* prior to medicine manufacture, as it contains Phorbol esters such as Phorbol 12, 13-dibutyrate. The phorbols and their different derivatives are reported to be potent tumor promoters (Chaube *et al.*, 1996, Goel *et al.*, 2007). The various accessions were screened for the detection of Phorbol 12, 13-dibutyrate using LC MS analysis to ensure the limit of detection up to ppm level.

The samples stored in refrigerator were dissolved in LC MS grade acetonitrile and was used for LC/MS analysis. The analysis was performed by LC-ESI-MS in negative polarity mode. The total Ion Chromatogram (TIC) obtained for various accessions were extracted to base peak chromatogram (BPC) using Agilent Mass Hunter algorithm. The BPC of standard Phorbol 12, 13-dibutyrate showed a mass with m/z [M-H] 503.62 at 3.6 minute (Fig. 4). All the accessions were evaluated by comparing the retention time of DAD signal at 270 nm as well as the BPC of mass spectrum with that of standard compound. The quantitative variation of phorbol ester was observed in different accessions as they showed differences in peak areas. Phorbol 12, 13-dibutyrate was found to be absent in accessions B-10 (Tiptur, Karnataka).

Fig. 4 BPC and MS spectra of standard Phorbol 12, 13-dibutyrate





On evaluating the factors like total phenolics, individual phytoconstituents separated on TLC and presence of toxic chemical, the accession B10 (Tiptur, Karnataka) found to be superior with comparatively higher concentration of secondary metabolites with absence of toxic compound.. Genotype variation of phytochemicals among the various accessions analysed indicates that it would be possible to select accession B10 for crop improvement program so that the same can be used for medicine preparation without purification process.

4. Conclusion

The phytochemical variability studies in *B. solanifolium* were successfully accomplished using chromatographic and spectroscopic analyses. The variation of polyphenolics was established spectrophotometrically. The result indicates that the genetic and environmental factors might influence the accumulation of polyphenolic compounds. The chromatographic finger prints showed variations in individual secondary metabolites in various accessions. The level of toxic compound was also examined using LC/MS analysis. In conclusion, a novel analytical methodology has been developed for the evaluation of phytochemical variability in different accessions of *B. solanifolium* for selecting superior line for large scale breeding program so as to ensure the quality of raw drug with maximum active phytochemicals at the same time with least toxic factors.

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