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Original Article

Identification of Phorbol Ester and its Degradation by Traditional Purification Method in the Root of *Baliospermum montanum* (Willd.) Muell. Arg

Jayshree Vishnupant Changade[1], Ashwini Shete[1], Asha Thomas[2,*], Rabindra K Nanda[2]

[1]Department of Dravyaguna, Dr. D. Y. Patil College of Ayurved and Research Centre, Dr. D. Y. Patil Vidyapeeth (Deemed to be University), Pune, Maharashtra, INDIA.
[2]Department of Pharmaceutical Chemistry, Dr D.Y. Patil Institute of Pharmaceutical Sciences and Research (Affiliated to Savitribai Phule Pune University), Pimpri, Maharashtra, INDIA.

ABSTRACT

Background: Currently five types of phorbol esters are reported in the root of *Balliospermum montanum* Muell. Here we observed the presence of 12-myristate 13-acetate ($C_{36}H_{56}O_8$) in the root, an additional phorbol ester which restricted its use in food or medicine due to its carcinogenic property. No specific method is reported that can remove or disintegrate the phorbol esters in *Balliospermum montanum* Muell. root. **Aim:** Identification of 12 myristate13 acetate phorbol ester ($C_{36}H_{56}O_8$) in *B. montanum* Muell. root and its purification and degradation by traditional method recommended by Acharya *Charak*. **Materials and Methods:** The traditional '*putpak*' (similar to traditional oven method) method was employed to detoxify the raw sample of *B. montanum* root. Ethanolic extract of root of *B. montanum*. unprocessed and processed samples were analysed on the Camag HPTLC system. **Results:** The HPTLC analysis gave a band at R_f 0.49 corresponding to the standard 12 myristate 13acetate ($C_{36}H_{56}O_8$). Further the band corresponding to the identified phorbol ester (biomarker standard, 12 myristate 13acetate, Sigma Aldrich, USA) was scraped out, the phorbol ester was separated by vortexing with methanol. The resulting sample was then subjected to LC-MS analysis using Impact HD system employing Electrospary Ionization (ESI) in positive ion mode to identify the isolated phorbol ester. **Conclusion:** The LC-MS analysis indicated the presence of this phorbol ester. The present *'putpak* (similar to traditional oven method)' method of detoxification was able to reduce the identified new phorbol ester by 75% when compared to the raw/unprocessed sample.

Keywords: Phorbol esters, 12 myristate13 acetate, *Baliospermum montanum*, *putpak*, Traditional, Detoxification, Purification.

Correspondence:
Dr. Asha Thomas
Department of Pharmaceutical Chemistry, Dr D.Y. Patil Institute of Pharmaceutical Sciences and Research (Affiliated to Savitribai Phule Pune University), Pimpri, Maharashtra, INDIA.
Email: asha.thomas@dypvp.edu.in

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INTRODUCTION

Maharishi Charak has mentioned that the root of *Balliospermum montanum* is laxative in nature. The processed or detoxified root is useful in the treatment of several disorders including jaundice, piles, splenomegaly, anaemia, oedema, skin diseases, urine stones.[1] Recently a lot of work has been conducted on *Baliospermum montanum*. It is effective in cervical cancer,[2] lymphocytic leukaemia,[3] prostate cancer[4] and hepato-protective[5] in nature. This drug is included in schedule E1, Drug and Cosmetics act 1940.[6] To remove hazardous content in the root, purification or detoxification process is suggested by Maharshi Charka. This plant belongs to Euphorbiaceae family, habitual at tropical, sub-tropical Himalayas and South India. This plant shows tap root system and is brown in colour and internally cream coloured and bitter in taste (Figure 1). This plant is called as 'Danti' in Sanskrit and 'Red Physic Nut' in English. Five types of Phorbol esters are montanin ($C_{32}H_{48}O_8$), baliospermin,12-deoxyphorbol-13-palmitate,12-deoxy16-hydroxyphorbol-13-palmitate and 12-deoxy5β-hydroxyphorbol-13-myristate.

Beside this, flavonoids, glycosides, sterols, alkaloids and saponins are present.[7] Total 69 formulations of *Baliospermum montanum* are mentioned by Charaka which are useful in various diseases. Removal or disintegration of harmful constituents present in traditional drugs continues to remain a major challenge. Presently, very few methods are available for disintegration or removal of phorbol esters in the plants of Euphorbiaceae family by using various chemicals and ultra-modern devices. Presence of phorbol esters (Figure 2) has restricted its use in food or as medicine. Charaka has described '*putpak* (similar to traditional oven method)' method for detoxification. In humans, phorbol

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ester intoxication shows GI tract irritation, muscle shock, high pulse rate and even death.[8] Phorbol esters are natural derivatives causes activation of Protein Kinase (PKC) which leads to inflammation; mainly in the stomach.[8] Phorbol esters are toxic and carcinogenic in nature. It gets absorbed through the skin and intestinal tract. They may cause severe irritation of tissues like skin, eyes, mucous membranes and lungs.[9] Phorbol ester molecule is a hydrophobic oil soluble tetracyclic diterpenoid.[10] The phorbol ester 12 myristate 13 acetate which is tumour promoter induces chromosomal damage via indirect action.[11] The mechanism of action of PMA (12-myristate 13-acetate) involves release of the proteases, cytokines and NADPH oxidases which can cause tissue damage through promotion of histamine secretion and tumour production. PMA caused embryonic and larval deformities in the Zebra fish embryo.[12] A lot of work has been conducted on the degradation of PMA (12-myristate13-acetate) in Jatropha seed but very scanty work has been conducted for the degradation of phorbol ester in *Baliospermum montanum* root. The reported methods employ the combination of solvent extraction and use of high temperature which is expensive, chemical based and not environmentally friendly. The present work focuses on the *Putpak* method for detoxification of the root sample of *B. montanum* and quantification of the phorbol ester using HPTLC studies.

MATERIALS AND METHODS

Balliospermum montanum roots were collected from the field in the Pune (Maharashtra) region, washed thoroughly and cut into small pieces of 8-10 cm in length. Other required material i.e. roots of *Glycerrhiza glabra Yashtimadhu*, fruits of *Piper longum (Pippali)* and leaves of *Desmostachya bipinnata (Kush)* were procured from local market in Pune (Maharashtra, INDIA). Authentication of all samples was done at 'Sheetal', an Indian Govt. approved laboratory located in Pune, India (Report no. A.M.-135/05-2021).

Putpak method (Similar to traditional oven method)[13-15]

As per classics, pieces of wet roots were coated with paste of fruit of piper longum powder (50 g-120 mesh) root of *G. glabra* powder (50 g-120 mesh) along with water and left to dry. After this, it was wrapped by a special type of grass leaves (*Desmostachya bipinnata*) as first layer, again wrapped with fresh leaves of banyan tree as second layer. Finally, all this material was coated with clay prepared by soil which is free from fertilizers or pesticides. Again, it was left to dry in sunlight, when it was completely dry, it was kept in a medium size iron vessel in such a way that it was completely covered by all sides by fire of cow dungs cakes (nos. 10) of weight approximate-100 g each. Until the outer coating of clay became reddish in color. This process takes about 30 min. The amount of heat to be supplied is not defined for traditional Putapak method. It was taken out of the fire. When the fire was completely diminished, it was left to cool. When the outer covering of the clay came to room temperature, outer covering of clay, *banyan* leaves and leaves of *Desmostachya bipinnata* removed systematically. The sample of *Baliospermum montanum* was cleaned with tap water and dried in the shade. This sample was treated as processed (detoxified) sample (Figure 3). Then, the unprocessed and processed samples were pulverized into coarse powder. Lateron, organoleptic, physicochemical and phytochemical studies was conducted of unprocessed (Raw) and processed (detoxified) samples (Tables 1-3).

Extraction of unprocessed and processed samples

Subsequent to the testing, 50 g coarse powder of both samples, processed and unprocessed were extracted in ethanol (99.9%) by Soxhlet extraction. The solvents have been removed using rotary evaporation to yield the extracts.

(a) (b)

Figure 1: *Baliospermummontanum* (Willd.) Müll. Arg (a) and its roots (b).

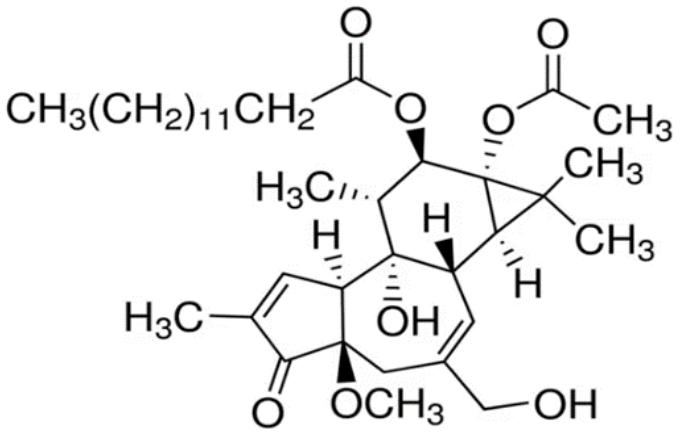

Figure 2: 12 myristate 13 acetate phorbol ester.

Estimation of phorbolesters (PMA12 myristate 13 acetate) using HPTLC

The presence of phorbol esters in the extracts were analyzed using Camg HPTLC system.

Preparation of standard solutions

1 mg of standard PMA12 myristate 13 acetate was dissolved in 10 mL of methanol to obtain a stock solution of concentration of 100 ug/mL. Then working standard solutions in the concentration range of 10-100 ug/mL was prepared and used for further analysis.

Preparation of sample solution

Accurately 1 g of unprocessed and processed sample weighed separately. To each, 10 mL methanol was added. The resulting solutions were sonicated for about 30 min in an ultrasonic bath, dilution with the same solvent and then filtered through Whatman filter paper No. 42. Working sample solutions were freshly prepared.

Instrument

A Camag Linomat 5 (S/N: 080222) HPTLC system equipped with a TLC scanner 4 (S/N:170422) and win CATS 1.2.2 software (Camag, Muttens, Switzerland), visualizer (S/N: 150503), twin trough chamber (10×20 cm or 20×20 cm; Camag, Muttens,

(a) (b) (c)

Figure 3: *G. glabra* powder, *P. longum* powder treated Sample wrapped with leaves (a); Sample coated with clay (b); Sample heated by traditional method (c).

Table 1: Organoleptic study of raw and detoxified sample of *Balliospermum montanum* Muell root.

Sl. No.	Criteria	Unprocessed Sample (Raw)	Processed Sample (Detoxified)
1	Color	Outer Brown Inner Whitish	Outer-Deep Brown to Black. Inner-Yellowish White.
2	Odor	Woody	Sweet smell
3	Taste	Bitter	Slightly Sweet
4	Size Length	Length-6 to 8 cm Width-0.6 to 0.8 cm	Length-6 to 8 cm. Width-0.8-1cm.
5	Shape	Cylindrical	Cylindrical
6	Fracture	Hard to Break.	Easy to Break.

Switzerland), and saturation pad (Camag, Muttens, Switzerland) was employed in the present study.

Chromatographic condition

The chromatographic separation was carried out in triplicate. The mobile phase consisting of toluene: chloroform: ethanol (4:4:1).1 v/v/v) was used in the study. Ascending development technique was carried out in twin trough chambers. The optimized chamber saturation time for the mobile phase was 15 min at room temperature (25±2°C) that was assisted by saturation pads. Suitable aliquots of the standard/test samples were applied on a pre-coated silica gel 60°F 254 TLC plate (10×10 cm) of uniform thickness at uniform distance using the Linomat sample applicator. The plates were developed and the densitometric scanning was performed at different wavelengths i.e., at 254 and 366 nm.

Method validation

To assess the linearity range of the developed method, suitable aliquots of the standard were applied on the HPTLC plates and the peak areas were recorded in triplicate. The developed method exhibited linear response in the concentration interval of 100-1500 ng/band for the standard phorbol 12-myristate 13-acetate. Further the precision of the method was analysed by intra and inter day precision study. The accuracy of the method was also established through recovery experiments performed at 80, 100 and 120% of the test concentration.

Further to the validation studies, the band at R_f 0.49 which was matched with the reference standard was scrapped out, extracted using methanol as solvent, filtered, the solvent was than evaporated to obtain the solid residue which was further subjected to LC-MS analysis.

RESULTS AND DISCUSSION

The developed HPTLC method was suitable validated with linearity in the range of 100-1500ng/band for the standard phorbol ester (r2 0.997). The method was also found to have high degree of precision (%RDS<2%) with % mean recoveries between 98-102%. The standard densitogram of PE-PMA is shown in Figure 4.

From Table 1, it is observed that after purification by traditional method, the colour of *B. montanum* root changes due to heating. The woody (unpleasant) odour of unprocessed sample changes to a sweet (pleasant) odour due to application of Glycyrrhiza glabra, which on heating synthesizes isoliquirtigenin in presence of piperine/piperedine as a catalyst.[16] The isoliquirtigenin and flavone liquiritigenin are biosynthetically similar.[17] *In vitro*, isoliquirtigenin suppresses phorbol ester induced (PMA) COX-2 expression by modulating ERK-1/2 signalling. It detoxifies the effect of the phorbol esters,[18] helping to detoxify and disintegrate the phorbol esters in the sample. Acid, alkali, elevated temperature, light and atmospheric oxygen are responsible for degradation of phorbol ester.[19] The PH values recorded and are represented as: *Piper longum* fruits-5.2, root of *G. glabra*- 6.5, leaves of *Desmostachya bipinnata*-6.5, leaves of *Ficus religiosa*-5.2.

It was observed that all the drugs used in purification process of root of *B. montanum* are acidic in nature which helps in the degradation of PMA (12 myristate13 acetate) present in the sample. The processed root can be easily broken as it becomes smooth after heating. It is also observed that foreign matter in the processed material was significantly reduced. Total ash content and acid insoluble ash content decreased due to removal of impurities. The water-soluble extract increased while the alcohol soluble extract decreased. The density of root of *Baliospermum montanum* decreases due to loss of impurities. This is due to the breakdown of the ester bond of phorbol ester generating the fatty acids (Table 2).

Table 2: Physicochemical Study of raw and detoxified sample of *Balliospermum montanum* Muell root.

Sl. No.	Parameter	Unprocessed (Raw) sample	Processed (Detoxified) sample
1	Description	Brownish color, Odor pungent	Brownish color, Odor Sweet
2	Foreign matter	0.058%	0.009%
3	PH	6.38%	6.93%
4	Moisture	2.33%	8.14%
5	Total Ash	10.37%	3.32%
6	Acid Insoluble Ash	0.1%	Nil
7	Water Soluble Extract	5.47%	10.53%
8	Alcohol Soluble Extract	19.02%	4.12%
9	Density	0.3651gm/cc	0.2690%

Table 2 demonstrated that the unprocessed sample contain alkaloids, carbohydrates, amino acids, tannins and terpenoids. In the processed sample of *B. montanum* roots, the alkaloids and glycosides were totally absent. The proportion of carbohydrates, amino acids, proteins were unaltered while the amounts of terpenoids were reduced.

The HPTLC assessment indicated that the amount of the P.E (PMA) in the processed sample (Figure 4c) decreased by 75% (Table 4) when compared to the unprocessed sample (Figure 4b) with %RSD<2%. In the LC-MS study, it was observed that as the phorbol ester PMA contains a hydroxyl group in its structure, the molecule loses a mole of H_2O instantly. As a result, it was difficult to capture the molecular ion of phorbol ester at its molecular mass of 616.398 m/z. Hence, the highest mass obtained in the LC-MS (LC-ESI) recording in positive ion mode using electrospray ionization is 599.3558 *m/z* which represents M+H-H_2O ion as

Table 3: Phytochemical screening of unprocessed sample (Ethanolic extract).

Sl. No.	Test	Saponin	Alkaloid	Glycosides	Sterol	Tannins	Flavonoids	Aminooacids	Gum	Mucilage	Carbohydrates	Proteins	Terpenoids
1	Mayer's		++										
2	Wagner's		++										
3	Hager's		++										
4	Molisch's										++		
5	Fehling's										+		
6	Liebermanris				+								
7	Borntrange's			+		+							
8	$FeCl_3sol^n$											+	
9	Biuret												
10	Millon's												
11	Fehlings reagent								++				
12	Ruthenium red sol^n									+			
13	Alkalinereagent						++						
14	Ninhydrin							+					
15	Salkowski												++
16	Frothing test	_											

+ Present, ++-strongly present, - absent.

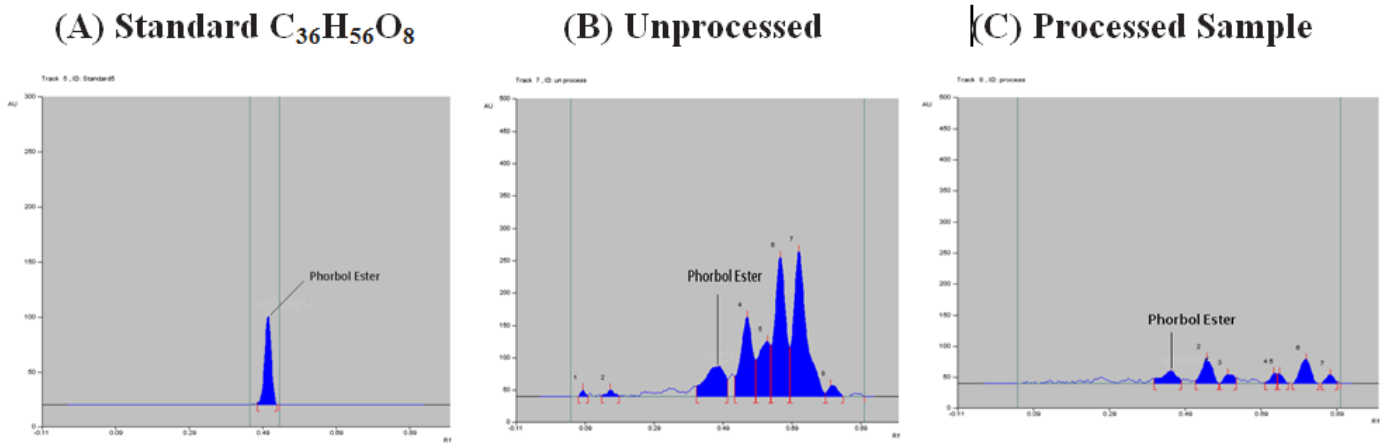

Figure 4: Densitogram (a) standard (b) unprocessed (c) processed sample.

Table 4: HPTLC analysis of the processed and unprocessed samples.

Sample	R_f	AUC	Concentration * (%RSD)	% Degradation
Raw sample	0.48	2247	973.20 ng±2.12 (0.465)	-
Detoxified sample	0.45	665	243.3 ng±2.41(0.990)	75%

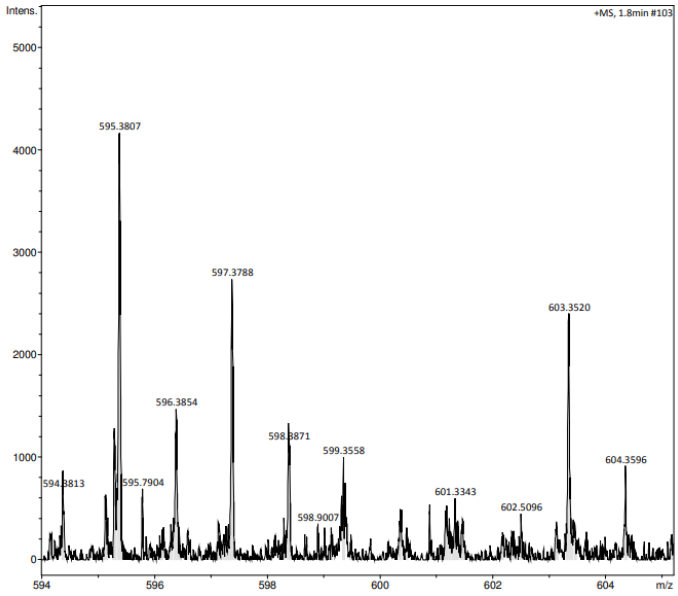

Figure 5: LCMS analysis showing presence of phorbol 12-myristate 13-acetate at 599.3558m/z.

shown in Figure 5, indicating the presence of phorbol ester PMA in the sample.

CONCLUSION

The present study using HPTLC and LC-MS analysis helped to identify and suggest the presence of 12myristate13acetate ($C_{36}H_{56}O_8$) in the root of *B. montanum* Muell. Also, there is significant decrease in the percentage of this phorbol ester ($C_{36}H_{56}O_8$) using the traditional '*Putpak* (similar to traditional oven method)' method. However, the modification in the heating pattern of '*Putpak*' method by using electric oven or repetition of this traditional procedure can help to further reduce the amount of phorbol ester. It also needs adaptation to other advanced tools for the detoxification and its comparison to the traditional method can help to choose the best tool for the purification of the root of *B. montanum* Muell. This will help to bridge the gap between traditional Ayurvedic practises and modern science, thereby promoting the effective utilization of our traditional preparation for furthering human health.

Further studies are needed to elucidate interaction of the drugs, *G. glabra, Piper longum, Desmostachya bipinnata* on *B. montanum* Muell at the molecular level during the heating procedure of '*Putpak*' method. Further the presence or formation of any other isomers needs to be confirmed using HR-MS and NMR studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPTLC: High-performance thin-layer chromatography; **P.E:** Phorbol ester; **RSD:** Relative standard deviation; **LCMS:** Liquid chromatography-mass spectrometry; **LC-ESI:** Liquid chromatograpy electrospray ionization; **HRMS:** High resolution mass spectrophotometry; **NMR:** Nuclear magnetic resonance.

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