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Simultaneous determination and quantification of three pentacyclic triterpenoids—betulinic acid, oleanolic acid, and ursolic acid—in cell cultures of *Lantana camara* L.

Priyanka Srivastava · Rakhi Chaturvedi

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Abstract A simple procedure has been described for simultaneous determination and improved yield of three pentacyclic triterpenoids—betulinic, oleanolic, and ursolic acids—from callus cultures of *Lantana camara*. Cell biomass was obtained from leaf disk explants cultured on Murashige and Skoog (Physiol Plant 15:473–497, 1962) medium supplemented with 5 μM 6-benzylaminopurine, 1 μM 2,4-dichlorophenoxyacetic acid, and 1 μM α-naphthaleneacetic acid. Optimum separation of the three compounds was achieved by reverse-phase high-pressure liquid chromatography on a C_{18} column with 80:20 (*v*/*v*) acetonitrile/water as mobile phase. With this route, a yield of 3.1% betulinic acid, 1.88% oleanolic acid, and 4.12% ursolic acid per gram dry weight was obtained from cultures. Leaves from the parent plant, used as control, showed total absence of betulinic acid, and the quantities of oleanolic and ursolic acids present in them were only marginally higher than that found in *in vitro*-raised cultures. Presence of the three compounds was further confirmed by electrospray ionization mass spectrometry.

Keywords Betulinic acid · Leaf cultures · Mass spectrometry · Oleanolic acid · RP-HPLC · Ursolic acid

Introduction

Lantana camara L., or Caturang, an aromatic, evergreen shrub belonging to family Verbenaceae, is a reservoir of several important bioactive molecules. It has been listed as one of the important medicinal plants of the world (Sharma *et al.* 2000). Several classes of compounds such as mono- and sesquiterpenes, triterpenes, iridoid glycosides, furanonaphthoquinones, flavonoids, and phenylethanoid glycosides have been reported to be present in this genus (Ghisalberti 2000). For many years, natural products from *Lantana* have been used in the prevention and cure of many serious diseases, including cancers, all over the world.

Although a few reports have described chemical constituents of *Lantana* plants growing in the wild, the biotechnological utilization of its foliage, somehow, is still unnoticed. For utilization of valuable pharmacological compounds at a commercial level, it is vital to obtain stable *in vitro* lines for uniform qualitative and quantitative production of secondary metabolites. Betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA) are highly sought-after pentacyclic triterpenoids because of their wide spectrum of biological activities. BA is most highly regarded for its anti-HIV-1 activity and specific cytotoxicity against a variety of tumor cell lines (Cichewicz and Kouzi 2004). Also, their anti-inflammatory, hepatoprotective, antitumor, anti-HIV, antimicrobial, antifungal, anti-ulcer, gastroprotective, hypoglycemic, and antihyperlipidemic activity are reported in the literature (Liu 2005). There are a few incidences in which co-occurrence of OA and UA have been observed in plant cell cultures, but no *in vitro* source has been identified for BA so far.

This work reports establishment of cell cultures from leaf disks of *L. camara*, as they are the principal reservoir of metabolites in this genus. Furthermore, simultaneous determination and quantification of BA, OA, and UA from the cell cultures have been accomplished for the first time in this report. The extraction procedure of the cells and

P. Srivastava · R. Chaturvedi (✉)
Department of Biotechnology,
Indian Institute of Technology—Guwahati,
Guwahati 781039 Assam, India
e-mail: rakhi_chaturvedi@iitg.ernet.in
e-mail: rakhi_chaturvedi@yahoo.co.uk

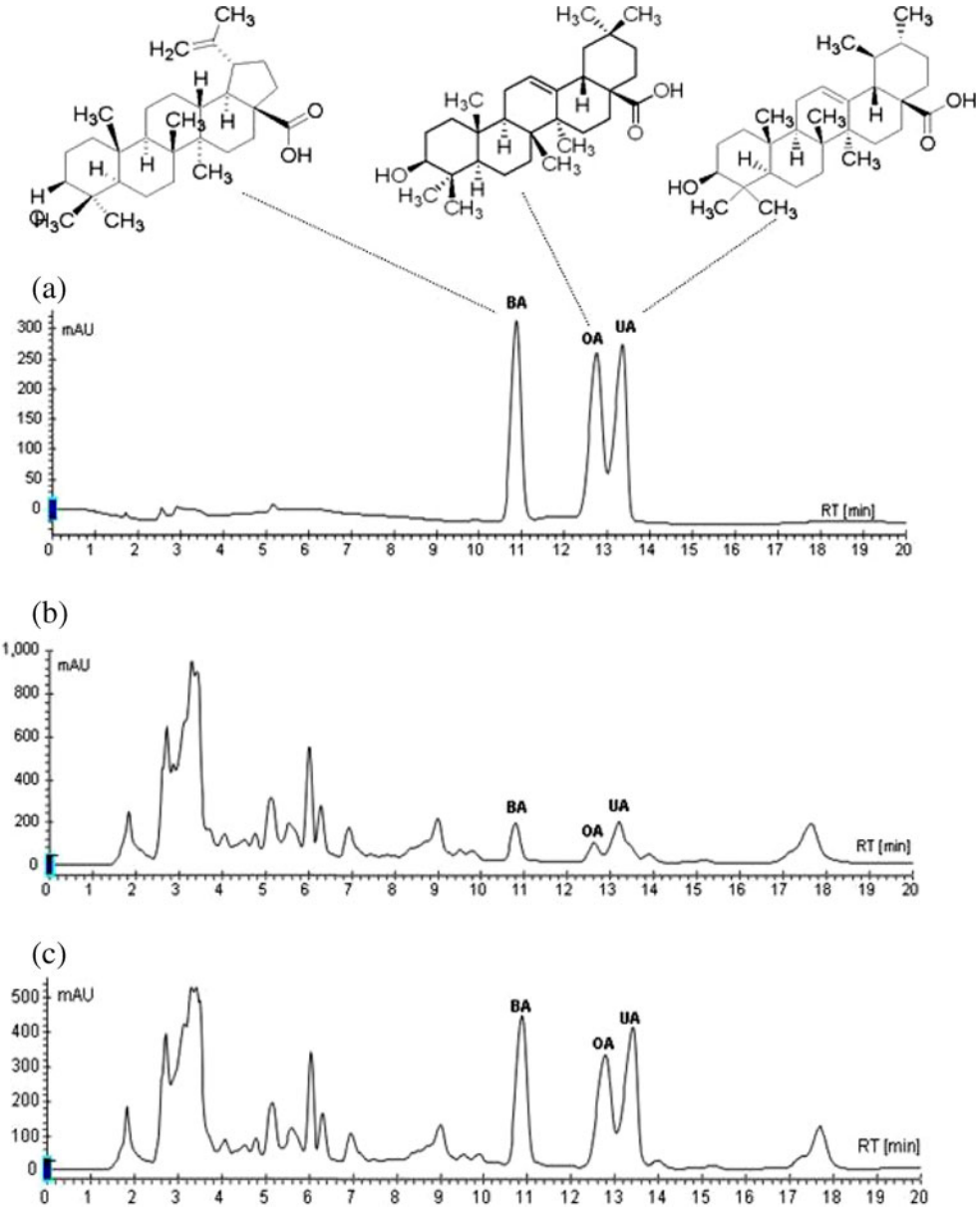

Figure 1. High-performance liquid chromatography of the organic extract from callus cultures of *L. camara*. (*a*) Chromatogram of mixture of betulinic acid (*BA*), oleanolic acid (*OA*), and ursolic acid (*UA*) standards. (*b*) Chromatogram of the crude sample. (*c*) Chromatogram of the crude sample spiked with all three standards.

HPLC method adopted for determining the compounds is efficient and reproducible. Such quantification studies have not been performed on *L. camara* plants.

Materials and Methods

Reagents and standard solutions. The constituents of MS medium, HPLC-grade acetonitrile, analytical-grade methanol, ethyl acetate, and 95% ethanol used for analysis were purchased from Merck, Mumbai, India. Water used for HPLC analysis was purified by Milli-Q system. All the plant growth regulators, standards of betulinic, oleanolic, and ursolic acids, were purchased from Sigma-Aldrich, St. Louis, MO.

Plant material and cell cultures. Studies were conducted on healthy *Lantana* plants, bearing pink-yellow variety of flowers, growing around the campus of Indian Institute of Technology—Guwahati, Assam, India. Leaves were dis-

Table 1 Standard curves and retention times of three triterpenes in cell cultures of *L. camara*

Compound	Retention time (min)	Standard equation	R^2
Betulinic acid	10.80	$Y=51x-2.4333$	0.9866
Oleanolic acid	12.61	$Y=64.465x-17.387$	0.9892
Ursolic acid	13.20	$Y=55.734x-4.5191$	0.9914

Y peak area, *x* concentration (milligrams), R^2 correlation coefficient

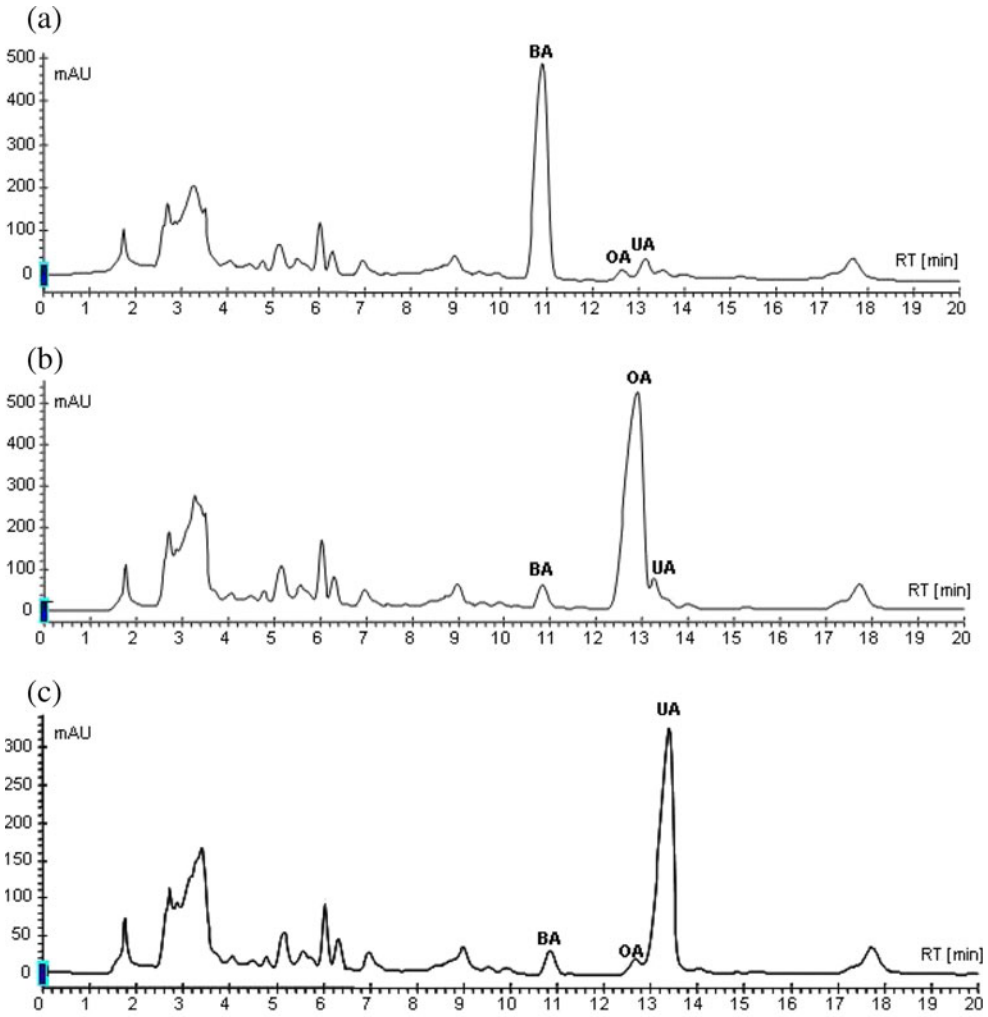

Figure 2. High-performance liquid chromatography of the organic extract from callus cultures of *L. camara*, spiked individually with three standard compounds. (*a*) Spiking of crude with betulinic acid. (*b*) Spiking of crude with oleanolic acid. (*c*) Spiking of crude with ursolic acid.

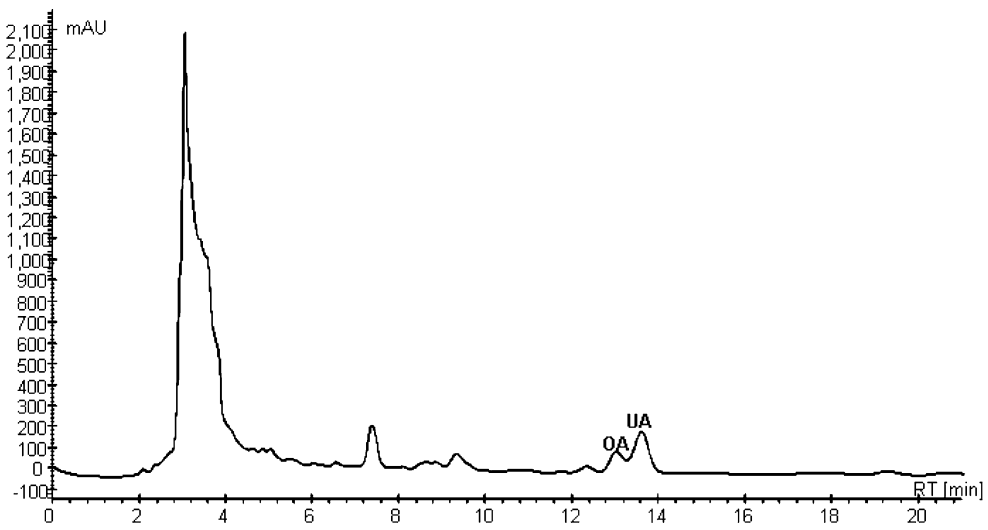

Figure 3. High-performance liquid chromatography of the leaves of *L. camara* showing the presence of oleanolic and ursolic acids; absence of betulinic acid can be clearly noted.

Table 2 Precision, content, and recovery percentages of three triterpenes in cell cultures of *L. camara*

Compound	RSD (%)		Content (% per grams dry weight)	Recovery (%)
	Intraday	Interday		
Betulinic acid	1.19	1.37	3.1	96.90
Oleanolic acid	0.60	0.55	1.88	95.07
Ursolic acid	0.80	0.65	4.12	97.60

infected using 1% (*v*/*v*) Tween-20 for 15 min and 0.1% (*w*/*v*) mercuric chloride for 10 min, followed by three rinses in sterile distilled water after each step. The leaf disk explants were prepared using a cork borer of 5 mm diameter. The basal media used in all the experiments related to callus induction and proliferation consisted of MS (Murashige and Skoog 1962) macro- and microsalts, vitamins, 100 mgl^{-1} myoinositol, enriched with 30 gl^{-1} sucrose, and solidified with 0.8% agar (HiMedia Laboratories, Mumbai, India). MS medium supplemented with 6-benzylaminopurine (BAP; 1.0, 5.0 μM) either alone or in combination with 2,4-dichlorophenoxyacetic acid (2,4-D; 1.0, 5.0 μM), α-naphthalene acetic acid (NAA; 5.0, 10.0 μM), and/or 3-indole acetic acid (IAA; 5.0, 10.0 μM) was used.

The pH of the media was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2} and 121°C for 15 min. Thermolabile growth regulator like IAA was filter-sterilized and added to the autoclaved medium cooled to 50°C; 20 ml of the medium was then dispensed into each 150×25 mm Borosil rimless glass tube. All the cultures were maintained in diffuse light (1,000–2,000 lx) and 16-h photoperiod at 25±2°C and 50–60% relative humidity. Calluses were subcultured at an interval of 4 wk, and each time, 500 mg of the callus was used as an inoculum to transfer to the fresh medium.

Determination of dry cell weight. Callus maintained on the responding medium was harvested at the end of the growth period of 4 wk, washed with distilled water, and filtered under vacuum. Subsequently, its fresh weight was taken on high precision analytical balance (Sartorius, Goettingen, Germany). Thereafter, the cells were dried in oven at 30±2°C until a constant weight was achieved. The drying temperature was kept low to avert decomposition of thermolabile compounds. For biochemical studies, 50 g of this dried cell mass was utilized.

Preparation of standard solutions. Individual stock solutions of BA, OA, and UA were prepared in 95% ethanol at a concentration of 5 mg/ml. The stock solutions were stored at −20°C. The quantification was performed using five levels of external standards obtained by serial dilutions of stock solutions at a concentration range of 2.5 to 0.16 mg/ml. Each concentration of standards was run at least thrice to check the repeatability and precision of results. Both external and internal standards were employed for confirmation of the compounds.

Preparation of sample solutions. To prepare samples, 50 g of dried cell mass and powdered leaf samples were drenched separately in methanol (200 ml) for 48 h and, thereafter, sonicated at 30% amplitude (pulser 5 s on/off) for 40 min, then again for 20 min at pulser 3 s on/off cycle. The methanolic mixture thus obtained was filtered, and the filtrate was centrifuged at 10,000 rpm for 10 min. Supernatant was pooled, filtered, and dried in a rotary evaporator at 40°C. This methanolic extract was further fractionated into an organic (ethyl acetate) and aqueous fraction. The ethyl acetate fraction was further dried under reduced pressure in a rotary evaporator at 40°C (Buchi Rotavapor R-200, Tokyo, Japan) and used for further study.

Chromatographic conditions. Quantitative estimation of BA, OA, and UA was carried out on Varian Prostar HPLC system (Varian, San Diego, CA) equipped with a binary pump, UV detector, and a 20-μl injection loop. Hypersil BDS RP-C_{18} column (Thermo, Waltham, MA) of dimensions 250×4.6 mm was used with acetonitrile/water (80:20, *v*/*v*) as the mobile phase at a flow rate of 1 ml/min. The eluted samples were detected at 209 nm wavelength. The identification of all three acid triterpenes was done by comparing their retention times with those of authentic standards. The crude and standard samples were filtered through 0.22 μm cellulose nitrate membrane filters prior to analysis, and aliquots of 20 μl of clean solution were injected into the HPLC system. System suitability tests were performed by checking linearity, precision, and recovery of compounds in the developed assay. Each of the three peaks, corresponding to the three compounds, were isolated from the crude extract and used for mass spectrometry.

Linearity of developed method was checked by running the standard compounds at five different concentrations. A calibration curve was generated by plotting concentration against peak area in Microsoft Office (Excel) Professional Edition 2003. The standard equation obtained from the

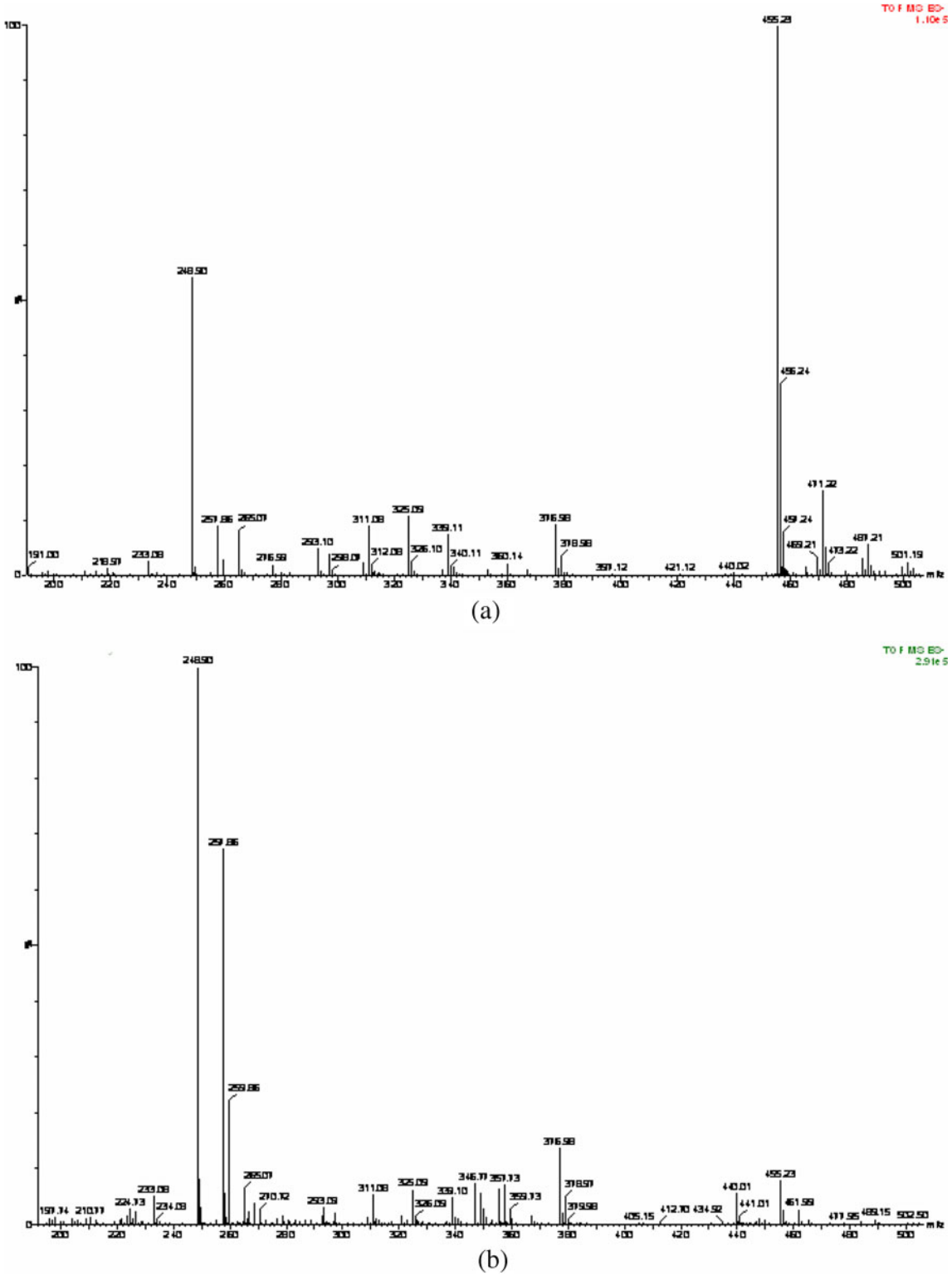

Figure 4. Comparative negative mode electrospray ionization mass spectra of standard compounds and the purified samples from cell biomass. (*a*) BA standard, (*b*) BA sample, (*c*) OA standard, (*d*) OA sample, (*e*) UA standard, (*f*) UA sample.

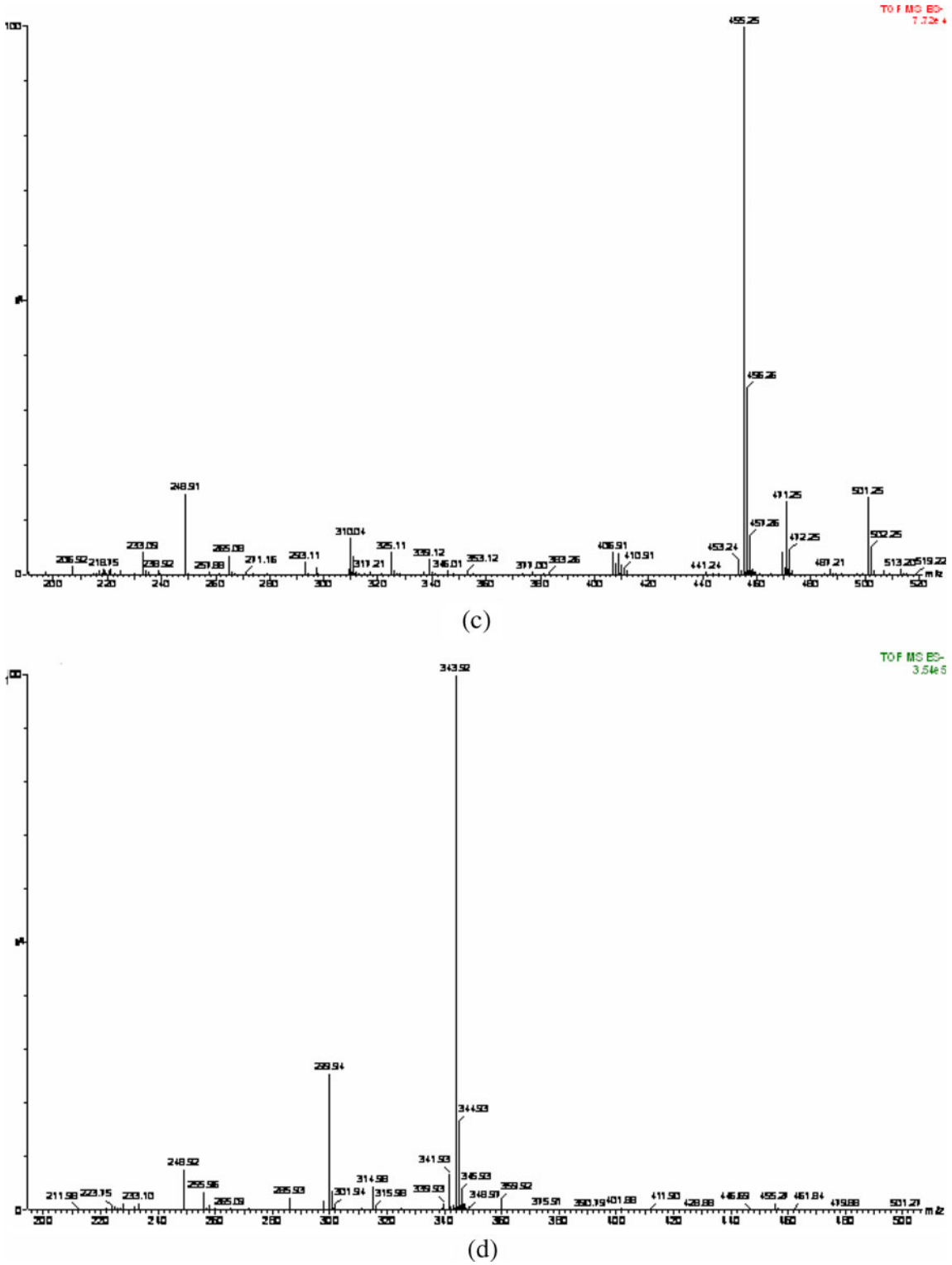

Figure 4. (continued)

curve was used for quantification of the three triterpenes in the unknown samples. The correlation coefficients (R^2) were also generated in Excel by fitting the linear trend lines to the standard curves obtained for each of the three compounds.

Precision of developed assay was evaluated by running the same concentration of standard compounds at least four times on the same day (intraday) and twice at 1-d intervals (interday). The values were calculated in terms of relative standard deviation (RSD).

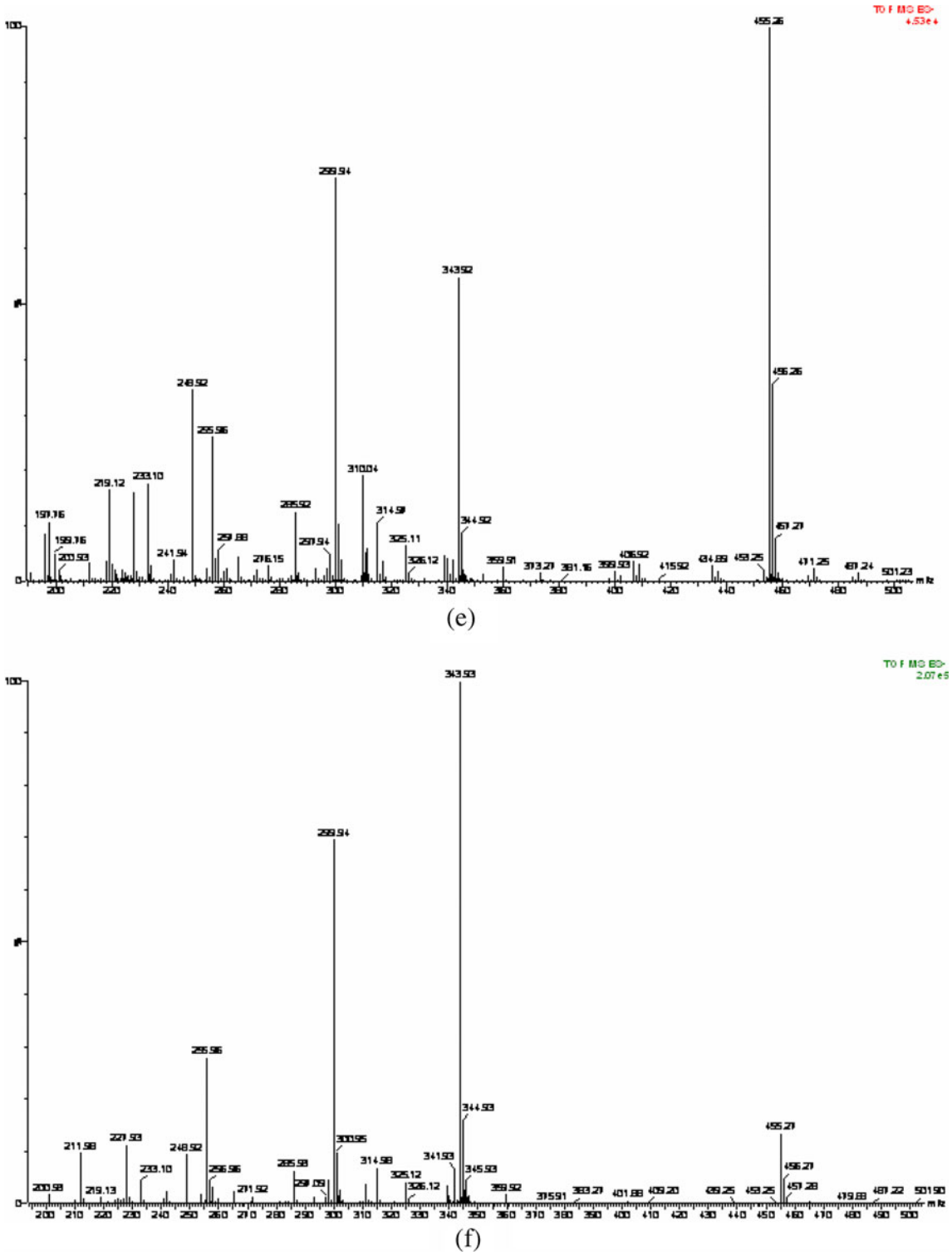

Figure 4. (continued)

The recovery experiments for all the three compounds were performed by adding known amounts of BA, OA, and UA standards to the callus cells, which were then extracted in a similar manner as mentioned in the "Preparation of sample solutions" section. The recovery percentages were calculated as:

$$\text{Recovery}(\%) = (A - B)/C \times 100$$

where *A* is the quantity of BA, OA, and UA in the spiked powder, *B* is the quantity of BA, OA, and UA in the powder without added standards, and *C* is the quantity of added BA, OA, and UA.

Instrumentation and conditions for mass spectrometry. MS detection was carried out on Waters quadrupole time-of-flight premier mass spectrometer with microchannel plate detector (Waters, Milford, PA) and was operated in the negative ion mode, with collision energy of 5 V. The cell entrance and exit voltage was set at 2 and −10 V, respectively. The MS data were obtained in full scan mode (mass range 100–1,000 amu). A comparison of the mass spectra of the three standard compounds obtained from Sigma-Aldrich with those of the individual samples isolated from HPLC confirmed the presence of all three triterpenic acids.

Results and Discussions

Establishment of cell lines. Of all the treatments tested, callus was induced from 100% of the leaf disk explants cultured on MS medium supplemented with BAP (5 μM), 2,4-D (1 μM), and NAA (1 μM). On other growth regulator combinations, explants turned brown soon after inoculation and were unable to revive thereafter. In the responding medium, leaf disk explants first turned brown, but after a week, bright green, hard, and compact calluses were developed from the margins of leaf disk. These compact calluses were dissected out after 4 wk and subcultured on the fresh medium of same growth regulator composition. Browning of the callus cultures was the major problem in initial two to four subcultures. However, further subculturing, at every 4-wk interval, helped to reduce browning of calluses to some extent. This procedure of subculturing was repeated until the rate of proliferation and the nature of calluses improved. It took about 26 wk of regular subculturing at every 4-wk interval to improve the nature of calluses and to obtain stable, constantly growing green, and cream healthy calluses.

Naik *et al.* (1999) also observed browning of the cultures and culture media in the case of pomegranates, which he reported might be because of phenolic exudation from the explants. These authors suggested the use of antioxidants such as ascorbic acid, polyvinylpyrrolidone, or polyvinyl-polypyrrolidone to overcome browning and death of explants. To avoid any negative effect of antioxidants on triterpenoid synthesis and massive callus proliferation, repeated subculturing at regular intervals was adopted in the present study as an alternative route to overcome browning of the cultures (Rout *et al.* 1999). With this protocol, it was possible to achieve stable and profusely growing, bright green, and cream, friable cell biomass every 4 wk.

Method optimization for separation of acid triterpenes. The detection wavelength of 209 nm was selected by checking absorption maxima of the standard compounds, dissolved in ethanol, by a Cary-100 UV-Visible spectrophotometer (Varian). In our preliminary studies, methanol was used as one of the mobile phase instead of acetonitrile. As the absorption of the former was higher, high interference was observed, especially at a short wavelength of 209 nm. Moreover, as OA and UA are structural isomers, their separation needed a low absorbing solvent that does not interfere with the resolution process. Hence, acetonitrile was chosen for the purpose. Acetonitrile and water at 80:20 (*v*/*v*) ratio was found to be appropriate as the mobile phase for satisfactory separation of the three components at a flow rate of 1 ml/min. The chromatograms of BA, OA, and UA obtained by this method are represented in Fig. 1*a*. A fairly short acquisition time of 15 min was adequate for good separation of all three triterpenes (Table 1).

Linearity and precision. Calibration curves for all the three standards showed good linearity at tested concentrations (0.16 to 2.5 mg/ml) with correlation coefficients (R^2) of 0.9866, 0.9892, and 0.9914 for BA, OA, and UA, respectively. The equations generated from the curves by external standard method (Table 1) were used to calculate the amount of compounds present in crude samples. Presence of compounds was reconfirmed with the use of internal standards by co-injecting them one by one and then all three together with the extract. A very distinct and clear separation of three acids can be observed (Figs. 1 and 2). The precision of the developed method, as mentioned in "Chromatographic conditions" under "Materials and Methods" section, was evaluated by measuring intra- and interday variability in terms of relative standard deviation. The standard samples, at the same concentration, were analyzed at least four times within the same day, and the RSD values obtained were 1.19%, 0.6%, and 0.8% for BA, OA, and UA, respectively. Similarly, for interday variability, same concentration of the three standards was run at least twice at 1-d intervals, and the values for the same figured out to be 1.37%, 0.55%, and 0.65% for BA, OA, and UA, respectively (Table 2). Leaf samples from parent plants (control), on the other hand, exhibited only marginal increase in OA (ca. 2%) and UA (4.5%) content, whereas BA was altogether absent (Fig. 3).

Yield and recovery studies. From the standard equations, the amount of BA, OA, and UA in the cell extract were calculated and listed in Table 2. The recovery experiments for all the three compounds were performed by adding

known amount of BA, OA, and UA standards to the cells, which were extracted in a similar manner as described in the "Materials and Methods" section. The percentage recoveries for all three compounds were observed to be above 95% in all the cases (Table 2).

Razborsek *et al.* (2008) reported detection and quantification of BA, OA, and UA in five plants of Lamiaceae, growing in the wild, by GC-MS. They obtained 0.6% BA, 0.09–0.9% OA, and 0.09–1.6% UA, which is considerably less than that reported in the present study. Moreover, the extraction protocol adopted by them was too complicated, including several steps, and their studies were carried out on field-grown plants that are very much prone to climatic fluctuations and thus may result in inconsistent production of metabolites. As mentioned in the introductory section, there are only a few incidences of OA and UA occurring together in cell cultures of some plant species, but none of them have reported the simultaneous presence of BA, another very potent and selective antitumor triterpene. Skrzypek and Wysokinska (2003) identified the presence of OA and UA in cell cultures of *Hyssopus officinalis*; however, quantification studies were not done. Pasqua *et al.* (2006) could obtain 1.5% total triterpenoid content (including OA and UA) from *in vitro* callus cultures of *Camptotheca acuminata* with the best solvent (95% ethanol); Zhao *et al.* (2007) were able to achieve 1.86% of BA in white birch bark collected from different provinces of China. The method, described in the present study, provides an excellent reproducible protocol for simultaneous determination of BA, OA, and UA in cell cultures of *L. camara* with an enhanced yield of 3.1%, 1.88%, and 4.12%, respectively.

Analysis of mass spectra. In the present study, both positive and negative mode electrospray ionization conditions were applied for the three compounds, but only negative spray ionization mass spectra could be obtained during analysis. The compounds were putatively identified as betulinic acid, oleanolic acid, and ursolic acid on the basis of the HPLC analysis. Mass spectrometry analyses confirmed the presence of a peak with *m*/*z* 455 in both standards and isolated fractions of betulinic acid and ursolic acid but failed to confirm the presence of a *m*/*z* 455 ion in the fraction of oleanolic acid (Fig. 4).

Conclusions

An efficient protocol has been developed for establishment of *in vitro* cultures from *Lantana* leaves. Also, the study allows optimum separation and quantification of three pharmacologically active pentacyclic triterpenes in the cultured cells with a small acquisition time of 15 min. This identifies the merit of cell cultures as a constant source of medicinally important compounds, in high amounts, all the year round. Additionally, establishment of *in vitro* cell lines for production of compounds is justifiable in this genus, as the production profile of leaves from parent plants (control) was only marginally higher for OA and UA, while BA was entirely absent.

References

Cichewicz R. H.; Kouzi S. A. Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. *Med Res Rev* 24: 90–114; 2004.

Ghisalberti E. L. *Lantana camara* L. Verbenaceae. *Fitoterapia* 71: 467–486; 2000.

Liu J. Oleanolic acid and ursolic acid: research perspectives. *J Ethnopharmacol* 100: 92–94; 2005.

Murashige T.; Skoog F. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497; 1962.

Naik S. K.; Pattnaik S.; Chand P. K. *In vitro* propagation of pomegranate (*Punica granatum* L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. *Sci Hortic* 79: 175–183; 1999.

Pasqua G.; Silvestrini A.; Monacelli B.; Mulinacci N.; Menendez P.; Botta B. Triterpenoids and ellagic acid derivatives from *in vitro* cultures of *Camptotheca acuminata* Decaisne. *Plant Physiol Biochem* 44: 220–225; 2006.

Razborsek M. I.; Voncina D. B.; Dolecek V.; Voncina E. Determination of oleanolic, betulinic and ursolic acid in Lamiaceae and mass spectral fragmentation of their trimethylsilylated derivatives. *Chromatographia* 67: 433–440; 2008.

Rout G. R.; Saxena C.; Samantaray S.; Das P. Rapid clonal propagation of *Plumbago zeylanica* Linn. *Plant Growth Regul* 28: 1–4; 1999.

Sharma O. P.; Singh A.; Sharma S. Levels of lantadenes, bioactive pentacyclic triterpenoids in young and mature leaves of *Lantana camara* var. aculeata. *Fitoterapia* 71: 487–491; 2000.

Skrzypek Z.; Wysokinska H. Sterols and triterpenes in cell culture of *Hyssopus officinalis* L. *Z Naturforsch* 58: 308–312; 2003.

Zhao G.; Yan W.; Cao D. Simultaneous determination of betulin and betulinic acid in white birch bark using RP-HPLC. *J Pharma Biomed Anal* 43: 959–962; 2007.