Development of an elicitation strategy on enhanced accumulation of oleanolic acid in suspension cultures of *Ocimum tenuiflorum* L.

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Research Article

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Abstract

Ocimum tenuiflorum Linn. is an important aromatic medicinal plant which produces several secondary metabolites responsible for diverse pharmacological activities. The present study focuses on enhanced production of biomass as well as oleanolic acid (OA), an anticancer and antioxidant compound, in suspension cell cultures of *O. tenuiflorum* upon elicitation with different elicitors. Leaf explants derived friable calli were inoculated intol iquid Murashige and Skoog (MS) media containing plant growth regulators [0.25 mg/L of α-naphthaleneacetic acid (NAA) and 0.5 mg/L of 6-benzyl amino purine (BAP)] for the establishment of suspension cultures. Influence of several factors such as, age of the suspension cultures, different concentrations, and exposure times of various elicitors such as yeast extract (YE), methyl jasmonate (MeJ) and salicylic acid (SA) respectively were analysed for cell biomass production and accumulation of OA during this study. Among the elicitors tested, YE at 50 mg/L was found to be the most efficient in terms of increased biomass production and accumulation of OA in the cultures. The highest increase in OA production (13.16-fold in elicited cultures compared to untreated cultures) was noted on 17-day-old suspension cultures when exposed to 50 mg/L of YE for four days. Enhancement of 2.72-fold in OA content was also recorded in 17-day-old cultures when treated with MeJ (60 mg/L) during two days of exposure. SA was not efficient in inducing accumulation of OA in 17- and 22-day-old suspension cultures at any concentration and exposure time. Furthermore, it was observed that the effect of different elicitors on biomass production and OA content depended on concentration and the duration of exposure times. Therefore, utilization of elicitation method could be a promising tool to enhance cell growth and OA accumulation in the suspension cell cultures of *O. tenuiflorum.*

Introduction

Medicinal plants have been utilized since millennia by different civilizations, such as those from China, India, Egypt, Rome and Greece [1]. These plants are rich sources of food, drugs, cosmetics, dyes and essential oil [2, 3, 4]. They have been exploited for traditional as well as modern medicinal purposes since these plants and their constituents exhibit an array of beneficial ethno-pharmacological activities such as, spasmolytic, antiviral, sedative, hepatoprotective, anti-inflammatory, antiseptic, antihyperglycemic, and immuno-stimulating. In addition, the consumption of these plants or plant parts or their extracts are regarded effective and safe [5, 6]. Hence these plants are considered as future of medicine [7].

Most medicinally important plants contain an array of secondary metabolites produced in response to biotic as well as abiotic stresses mainly for protecting the plants [8]. These active constituents are responsible for various pharmacological bioactivities imparted by the medicinal plants [9]. However, these ethno-botanically valued plants are getting depleted day by day due to pressure of increasing population, urbanisation, and exploitation of herbal biodiversity. Additionally, the changes in environmental factors are also affecting the content of bioactive compounds in these plants [10]. In order to get adequate desirable secondary metabolites from these plants, biotechnological approaches such as plant tissue cultures provide a platform for sustainable production of these secondary metabolites, under controlled condition throughout the year to meet the increasing demand by pharmaceutical companies.Amongst

these approaches, the application of plant cell suspension culture system has been reported to be an efficient method for the enhanced accumulation of several secondary metabolites like cosmetics, food and drugs [11]. However, the low yield of secondary metabolites as well as problems associated in scaling up of suspension cultures are the major challenges, thereby restricting accumulation of secondary metabolites in cell cultures [12]. Various strategies, such as elicitation and metabolic engineering, have been designed, optimized and applied to overcome these challenges. Moreover, several researchers reported the influence of various elicitors such as ultraviolet, and visible light, chitin, yeast extract (YE), salicylic acid (SA),and methyl jasmonate (MeJ) on the enhancement of biomass and accumulation of secondary metabolites in differentiated cells viz.hairy root, adventitious root, callus, suspension and cambial meristematic cells of various plants [11, 12, 13]. *Ocimum tenuiflorum* L. (Tulsi in Hindi and Holy Basil in English) is an aromatic annual herb belonging to the family Lamiaceae (tribe ocimeae). It is believed to have originated in north central India and now grows as native throughout the entire tropics of the eastern world. In Ayurveda, it is known as “The Queen of Herbs”, “The Incomparable One,” and “Mother Medicine of Nature”. The plant has been used as medicinal herb for curing various ailments for centuries [14]. Several studies reveal that it exhibit combination of actions including antimicrobial (antibacterial, antiviral, antifungal, antiprotozoal, antimalarial, anthelmintic), mosquito repellent, anti-diarrheal, anti-oxidant, anti-cataract, anti-inflammatory, hepato-protective, neuro-protective, cardio-protective, anti-diabetic, anti-hypercholesterolemia, anti-hypertensive, anti-carcinogenic, analgesic, anti-pyretic, anti-allergic, immunomodulatory, central nervous system depressant, memory enhancement, and anti-coagulant [15]. Some of the medicinally important bioactive metabolites reported in *O. tenuiflorum* are eugenol, linalool, oleanolic acid, linalyl, camphor, methyleugenol, citral, methyl chavicol, geraniol, methyl cinnamate, thymol, rosmarinic acid, safrol, taxol, ursolic acid etc. which impart an array of pharmacological potentials to the plant [9, 16, 17]. Amongst these metabolites, oleanolic acid (OA) is one of them in active constituents found in various parts of *O. tenuiflorum*.

OA (3β-hydroxy-olean-12-en-28-oic acid) is a pentacyclic triterpenoid compound having 30 carbon atoms in its backbone which is abundant in plants of the *Oleaceae* family such as olive [18]. OA are generally found in the form of free acid or aglycones of triterpenoid saponins in plants, the chemical structure is depicted in Fig. 1. It imparts anti-inflammatory, anti-hyperlipidemic, hepatoprotective and anticancer properties to the plants [19]. A recent study also demonstrated that OA can act as a potential inhibitor to M^{pro} protein having role in controlling viral replication during COVID-19 [20]. Since *O. tenuiflorum* is abundant and can be grown easily, it can be a cheaper source of OA and can be utilized for its extraction as an alternative source. Till now, several efforts have been applied for the establishment of cell, callus and suspension cultures of *O. tenuiflorum* for the accumulation of rosmarinic acid, eugenol and flavonoids [21, 22, 23]. Moreover, several researchers investigated the impact of important factorson augmenting biomass and accumulation of secondary metabolites like rosmarinic acid, phenolic compounds, alkaloids, terpenoids, phenylterpenoids) in suspension cultures of *O. tenuiflorum* through elicitation and culture optimization [24, 25, 26].

Enhanced OA accumulation in response to biotic elicitors in cell suspension cultures of *Calendula officinalis* has been reported [27]. However, till date there are no reports on influence of age of the culture for biomass production as well as accumulation of OA in suspension cultures of *O. tenuiflorum* in the presence of varied types and concentrations of elicitors with different exposure times. Thus, in the present study, we established the callus and suspension culture of *O. tenuiflorum* and investigated the effect of age of culture, concentration of elicitors and their exposure time on biomass and accumulation of OA.

Material And Methods

Plant material and induction of callus

O. tenuiflorum seeds were procured from Jamia Hamdard Herbal Garden, New Delhi, India. The seeds were washed with tap water containing 0.1% detergent (Triton-X-100) for 20 min to remove dust particles and then rinsed with Milli-Q water five times to remove excess detergent. After treating seeds with 0.1% Bavistin, a fungicide to remove fungal mycelia and spores, for 20 min and rinsing with sterilized Milli-Q water four times, the seeds were brought into laminar air flow hood and rinsed once with sterilized Milli-Q water. After pretreatment with 0.1% $HgCl_2$ for 1min, seeds were again rinsed thrice with sterilized Milli-Q water. Seeds were placed on sterile Whatman filter paper no. 1 to air dry and then transferred onto autoclaved MS media [28]. medium gelled with 0.7% agar in the culture tubes/jam bottles. The tubes/bottles were placed in the culture room maintained at 23 ± 2°C under 16 h illumination of 450 µmol $m^{-2}s^{-1}$ and eight h darkness. The leaf explants from one- month-old *in vitro* germinated plantlets were cut into pieces of 3–4 mm^2 and placed onto autoclaved MS media (sucrose 3% and 0.7 % agar) supplemented with plant growth regulators [0.25 mg/L of α-naphthaleneacetic acid (NAA) and 0.5 mg/L of 6-benzyl amino purine (BAP)]. The cultures were maintained under cool white fluorescent light as mentioned above. The experiments were performed in triplicate. The callus cultures were subcultured every two weeks on fresh media of similar composition.

Development of suspension cultures

The cell suspension cultures were initiated by inoculating 2 g of friable callus into 25 ml liquid MS medium supplemented with 0.25 mg/L NAA and 0.5 mg/L BAP in 100 ml flasks. The flasks were kept on gyratory shakers at 80 rpm at 23 ± 2° C and 16 hours illumination of 450 µmol m^{-2} s^{-1} and fast-growing suspension cultures were developed as mentioned in [26]. The cultures were harvested from flasks on 5th, 10th, 15th, 20th, 25th and 30th days for estimation of biomass and OA production kinetics. The fresh weight (FW) of the cells was determined by harvesting cells from suspension cultures by filtration. The cells were dried at 45°C to get constant dry weight (DW).The pH of the medium was measured by pH meter. Cell growth was measured on the basis of FW and DW.

Elicitor treatment

For elicitor treatment, 17-and 22-day-old suspension cultured cells were treated with different concentrations of YE (0, 25, 50 and 100 mg/L), MeJ (0, 30,60 and 120 mg/L) and SA (0,30,60 and 120 mg/L) and incubated for two, four and six days. Cultures without elicitor treatments were considered as controls. The cells were harvested by filtration and DW of the cells was determined as mentioned earlier.

Solvent extraction techniques for callus

The plant extracts were prepared as mentioned in [16]. The dried cells were pulverized with liquid nitrogen to fine powder using sterile pestle and mortar. The plant extracts were prepared by dissolving the powdered cells in HPLC grade methanol followed by ultrasonic extraction for 15 mins. The extracts were kept on a shaker at 320 rpm for 2 h and centrifuged at 13,000 ×g at 4°C for 12 mins. The supernatant was collected in a fresh tube and dried in a stream of nitrogen gas. After drying, 500µl methanol was added to the extracts which was filter sterilized through 0.22 µm nylon membrane filters and stored at -20°C till further analysis.

Estimation of OA by HPLC

To estimate the amount of OA in the plant extracts, HPLC analysis was performed as described by [29] with minor modification. The separation of OA was performed on HPLC system (Agilent 1200 series equipped with a binary pump, UV detector and an auto-sampler). Separation of compounds was performed with Agilent Zorbax 300SB-C18 column (150 mm × 4.6 mm, 5 µm particle size) using mobile phase of methanol/water (95:5, v/v) with a flow rate of 0.2 ml/min. The wavelength of UV detector was set at 210 nm. The peaks of the extract were identified and compared to that of the standard. Data acquisition was performed by Agilent ChemStation software (Agilent Technologies, USA).The standard samples of OA were calibrated in 0.25, 2.5, 5, 10, and 20 µg/µl concentrations and the equation obtained was $y = 7739x - 3534$ ($R^2 = 0.99$).

Statistical analysis

The experiments were performed in a factorial manner which was completely randomized with two factors, viz. concentrations of elicitors and exposure times. All the experiments were done in three biological replicates and repeated in triplicate, data are expressed as mean values ± standard deviation (SD). The error bars in the figures represents standard deviation. All data were analyzed by two-way ANOVA Tukey's multiple comparison test. Differences at $p < 0.05$ were considered statistically significant. All statistical analyses were performed with GraphPad Prism ver. 8.0.

Results And Discussion

Callus induction and establishment of suspension cultures of O. tenuiflorum

In our previous study, leaf and stem explants of *in vitro* germinated *O. tenuiflorum* plantlets were used to increase the frequency of callus induction. It was noticed that leaves served as better explants for callus induction [30]. Therefore, leaf derived green and friable callus were chosen to establish cell suspension cultures for further investigations on biomass and OA production. Friable calli (2g) were inoculated into

25 ml liquid MS media supplemented with NAA (0.25 mg/L) and BAP (0.5 mg/L) and kept on shaker (80 rpm). Lumps of calli were removed by filtration through 200 µm stainless steel mesh to obtain fine suspension of cells (Fig. 2a, 2b). The suspension cultured cells of *O. tenuiflorum* were characterized by fine milky yellowish dispersed cells as shown in Fig. 2b. Cells were harvested at 5 days intervals to estimate biomass and OA production.

Growth curve study

The cell growth was determined on the basis of FW and DW. The pH values of spent media and content of OA were also checked. Results showed that the *O. tenuiflorum* cells exhibited sigmoid growth curve pattern (Fig. 3). During the lag phase of 0–5days, the biomass increased slowly, and then entered the logarithmic phase on 5th day and continued up to 20th day. A rapid increase in biomass was observed with a maximum value of 5.82 g FW and 0.69 g DW on 20th day. The biomass of cells became constant during 20 to 25days depicting stationary phase. The OA accumulation increased according to the culture period. The highest OA accumulation (0.84 mg/g DW) was observed on 20th day which was 1.28-fold higher than the initial observation. During the stationary phase (20-30th day), OA content remained constant. These findings revealed that increase in OA accumulation is correlated with increase in biomass during the time course of growth of suspension cultures of *O. tenuiflorum* (Fig. 3). The value of pH of the spent medium also changed during different growth phases of suspension cell culture. The pH of the spent culture medium progressively reduced during lag period, while it increased during the exponential phase up to 20 days. A further decrease in the pH values after 20th day during stationary phase was observed. The pH of the culture medium during different growth phases probably changed due to the uptake of ammonium (NH_4^+) resulting in decrease in pH which is directly correlated to the liberation of H^+ ions. The increase in pH of the medium increase if NO^{3-} is more assimilated by the cultures rather than NH_4 nitrate (NO_3^+) [31].

The OA production in suspension cultures of *O. tenuiflorum* was detected throughout different growth phases. It was presumed that the production could be improved by applying elicitation strategy. Therefore, in the present study, the effect of elicitors was screened for the enhanced accumulation of OA in cell suspension culture of *O. tenuiflorum*.

Elicitation analysis

Elicitation techniques have been used by several researchers for enhanced accumulation of secondary metabolites in cell/tissue cultures of several plants. It is also reported that physiological state of the cells, dosage as well exposure time of elicitors are some of the important parameters which affect elicitation strategy for enhanced biomass and accumulation of secondary metabolites in several plant cell cultures [17, 32]. To investigate the effect of the elicitors on the biomass growth and OA yield, the 17- (late-exponential phase) and the 22-day-old (stationary phase) suspension cell cultures were treated with different concentrations of elicitors like YE (0,25,50 and 100 mg/L), MeJ (0,30,60 and 120 mg/L) and SA

(0,30,60 and 120 mg/L). The non-elicited cultures were treated as control. After two, four and six days of the treatment with each elicitor, the cells were harvested for DW and total OA content estimation.

Elicitation effect of YE on cell biomass and OA production

Significant differences were observed in biomass growth between YE treated 17- and 22- day-old cultures with respect to the control cultures in all concentrations of YE in all exposure times considered in the study. YE (25 mg/L) stimulated 1.23-, 1.38- and 1.42-fold increase in biomass production in comparison to their respective controls in 17-day-old suspension cultures after two, four and six days of exposure respectively. Similar trend was also observed with YE (50 mg/L) which induced 1.29-, 1.43- and 1.47-fold increase in biomass production with respect to that of their controls for two, four and six days of exposures respectively. The maximum significant biomass level in the presence of YE (100 mg/L) for all three exposure times demonstrated the biomass growth was affected by dosage of the YE (Fig. 4a). After two and four days of exposure, the biomass growth of 17-day old cultures was up to 1.73- and 1.92-foldsincrease in comparison to that of their respective controls (0.7 and 0.5 g/g DW). The biomass production increased up to 2.02-fold after six days of exposure when compared to that of the control. Significant increase in biomass production was noted at all exposure times in 22-day-old suspension cultures treated with 25 and 50 mg/L YE. However, higher concentration of YE (100 mg/L) was more effective in inducing greater biomass for all exposure times. The biomass increased 1.5 and 1.75-fold compared to their respective controls (0.62 and 0.49 g/g DW) after two and four days of treatment.YE (100 mg/L) favored biomass growth up to 0.76 g/g DW (1.9-fold) in 22-day- old cultures when incubation time was increased to six days (Fig. 4b).

This study reveals that the increase in biomass occurs under elicitor exposure-dependent manner. Similarly, the significant increase in biomass of *Ophiorrhiza mungos* was reported when incubation time was increased from 1st to 10th day with maximum biomass on 10th day at all concentrations of YE (25, 50, 100 and 200 mg/L) which declined after 15 days of incubation [33]. The increase in biomass in *Panax vietnaminis* cell suspension cultures was noticed during the first 12 days in media containing YE (0.5, 1.0, 1.5, and 2.0 g/L) with the highest increase on day 21 in presence of 1.0 g/L YE [34]. The addition of higher concentrations of this elicitor (1.5 and 2.0 g/L) inhibited the increase of biomass over the culture period.

No significant OA production was found in 17-day-old cultures treated with any concentration of YE for two days of exposure (Fig. 4c). But there was significant OA production in all concentrations of YE treated cultures compared to the control cultures after four and six days of exposure respectively. The maximum significant amount of OA content detected was11.0 mg/g DW (13.16-fold higher than the control of 0.83 mg/g DW) with YE 50 mg/L, after four days of exposure. However, when the incubation time was increased up to six days, the OA content decreased to 4.05 mg/g DW (still significantly 5.6-fold higher than the control of 0.71 mg/g DW) in 17- day-old suspension cell cultures (Fig. 4c). At YE (100 mg/L), the concentration of OA was 1.68-, 7.02-, and 3.63-fold higher than that of their respective controls of 0.83, 0.835 and 0.713 mg/g DW in 17-day-old cultures during the two, four and six days of treatment, respectively.

The reduced accumulation of OA (2.182-fold) was observed in 22-day-old cultures at YE (50 mg/L) for short duration of two days exposure. The highest OA accumulation of 4.3 mg/g DW was recorded with YE (50 mg/L) after four days of exposure which was significantly 5.93-fold higher compared to the control (0.72 mg/g DW; Fig. 4d). The amount of OA was 2.94-fold higher than that of the control after six days of exposure. At YE (100 mg/L), OA accumulation in 22-day-old cultures further increased to 1.23, 2.05 and 1.9 mg/g DW which were 1.54-, 2.83- and 2.72-fold greater than that of their respective controls after two, four and six days of exposure respectively (Fig. 4c and 4d). Lower OA accumulation was also observed in 22-day-old cultures when compared with the 17-day-old cultures. Therefore, it was demonstrated that with an increase in age of the cultures, concentration of YE and exposure times resulted in reduced accumulation of OA in suspension cultures of *O. tenuiflorum*.

YE has been widely employed as a complex additive for the induction of secondary metabolite accumulation in *in vitro* cultures of various plants viz. *Psoralea corylifolia* [17], *Ocimum sanctum* [22], *Agastache rugosa* [35], *Eryngium planum*[36], *Eschscholtzia californica* [37] and *Lithospermum erythrorhizon* [38].The enhanced biomass and accumulation of secondary metabolites in *in vitro* cultures might occur due to the presence of some cations like Ca^{2+}, Co^{2+} andZn^{2+}present in YE which might act as abiotic elicitors. It was also reported that YE augmented in camptothecin, plumbagin and tanshinone contentsin the suspension cell cultures of *Camptotheca acuminata* [39], embryogenic cell suspension cultures of *Plumbago rosea* [40]. and *Salvia miltiorrhiza* hairy root cultures [41] respectively.

Elicitor concentration, exposure time and growth stage of the cultures are the important parameters which influence biomass production and secondary metabolite accumulation [32]. In the present study YE at 100 mg/L was optimal for inducing higher biomass production while YE at 50 mg/L induced significantly higher accumulation of OA in 17-day-old cultures as compared to that of non-elicited cultures as well as 22-day-old cultures. It was concluded that late exponential phase was more suitable for elicitation in suspension cultures of *O. tenuiflorum* for the enhanced biomass production and accumulation of OA. This could perhaps be due to higher availability of the nutrients from the medium which the cells can better utilize for biosynthesis of secondary metabolites in a particular phase [42].

Elicitation effect of MeJ on cell biomass and OA production

The growth in biomass of 17-day-old *O. tenuiflorum* cell suspension cultures on exposure to 30, 60 and 120 mg/L of MeJ at all exposure times was less significant (Fig. 5a). However, in 22-day-old suspension cultures, significant growth of biomass was noticed at all concentrations of MeJ after two and four days of exposure times. Low dosage of 30 mg/L MeJ induced higher biomass production up to 1.24 g DW (2.0-fold higher than the control of 0.62 g DW) after two days of exposure time in 22-day-old cultures. The growth of biomass was 1.85-fold higher than that of the control (0.48 g DW) when exposure time was increased to four days. After six days of exposure, the production of biomass decreased to 1.75-fold in comparison to that of the control (0.40 g DW). Higher dosage of MeJ of 60 and 120 mg/L also favored improved biomass production but the values were lower than that of treated cultures with 30 mg/L MeJ (Fig. 5b).

Low MeJ concentration at 30 mg/L led to improved accumulation of OA of 1.59, 1.49 and 1.22 mg/g DW in 17-day-old cultures during two, four and six days of exposure respectively. The maximum level of OA production was2.27 mg/g DW (significantly2.72-fold higher compared to that of the control of 0.83 mg/g DW) for short duration of exposure of two days in the presence of MeJ at 60 mg/L. (Fig. 5c). On increasing the exposure time to four and six days, accumulation of OA reduced to 2.12 and 1.04 mg/g DW respectively. Increase in concentration of MeJ up to 120 mg/L, also decreased accumulation of OA to 0.46, 0.368 and 0.254 mg/g DW in 17-day-old suspension cultures during two, four and six days of exposure time in comparison to that of their respective controls. This result is consistent with the result obtained by Wang et al., 2015 [43] where MeJ had inhibitory effects at higher concentrations on flavonoid content in *Hypericum perforatum*. Increase in age of the cultures to 22-day-old cultures decreased the yield of OA at all MeJ concentrations (Fig. 5d). These findings suggest that MeJ at optimal concentration of 60 mg/L effectively induce enhanced accumulation of OA in 17-day-old suspension cultures of *O. tenuiflorum*. Increased age (22-day-old culture) did not favor any increase in accumulation of OA.

MeJ is a signal molecule participating in signal transduction pathway leading to accumulation of low molecular weight secondary metabolites [44]. MeJ plays crucial roles in plant growth and development as well as in protecting plants against stresses. These signal molecules had been used for the enhanced accumulation of bioactive secondary metabolites in *in vitro* cultures of various plant species [12, 45]. (Giri and Zaheer, 2016; Ramirez-Estrada et al.,2016). The treatment with MeJ has been a useful strategy to enhance bioactive compound production in *Ocimum sanctum* [23]. (Hakkim et al., 2011), *Thevetia peruviana* [46], *Hypericum perforatum* [42], *Mentha×piperata* [47], *Fagopyrum tataricum* [48] and *Taxus* × *media* [49].

Elicitation effect of SA on cell biomass and OA production

At lowest (30 mg/L) and highest dosage (120 mg/L) of SA, the biomass growth in 17- and 22-day-old cultures was not induced at any exposure time when compared to the control (Fig. 6a and 6b). However, SA (60 mg/L) favored growth of biomass upto 1.01,0.85 and 0.66g DW in 17-day-old cultures (1.49-, 1.7- and 1.57-fold higher than that of their respective controls) for two, four and six days of exposure times respectively (Fig. 6a). In 22-day-old cultures, the biomass productions were 1.62-, 1.68- and 1.7-fold higher than that of their respective controls after two, four and six days of exposure respectively (Fig. 6b). As shown in Fig. 6c and 6d, the presence of different concentrations of SA, did not enhance accumulation of OA in both 17- and 22-old-day cultures compared to control cultures of all exposure times. SA is a natural plant hormone which participates in plant defense regulation systems against biotic and abiotic stresses [50].Treatment with SA has been a useful strategy to enhance bioactive compound production in *in vitro* cell cultures of *Capsicum chinense* [51] and *Momordica dioica* Roxb Ill-Min [52].

Plant cell suspension cultures have been preferred for enhanced biomass production as well as accumulation of medicinally valued secondary metabolites in a variety of plant species. Additionally, elicitation is an effective strategy which can be applied for enhanced accumulation of secondary metabolites in several *in vitro* culture systems [53]. The response of suspension cell cultures towards different elicitors differs according to types and concentration of elicitors, exposure time as well as age of

the cultures and of course the plant species [10]. In the present investigation, it was also observed that elicitors regulate critical parameters affecting cell growth and accumulation of secondary metabolites in cell suspension cultures. Additionally, the exponential phase of cell cultures is the most suitable stage for elicitation [54, 55]. Higher accumulation of OA was also found in this study in the presence of YE at 50 mg/L during four days of exposure in 17- and 22-day-old suspension cell cultures when compared to that of cultures treated with MeJ and SA.SA was found to be inhibitory to OA production in both 17- and 22-day-old cultures under the tested concentrations. The HPLC profiles of OA were depicted in Fig. 7.

Conclusion

In conclusion, elicitation in plant cell suspension cultures is a proven strategy for improving yield of secondary metabolites. In the present study, YE (50 mg/L) and MeJ (60 mg/L) were most effective for a significantly higher accumulation of OA (13.16-fold and2.72-fold for four and two days of exposure respectively) in *in vitro* suspension cultures of *O. tenuiflorum*. Our results demonstrate a direct correlation between the physiological stage of the suspension cultures of *O. tenuiflorum* with different concentrations of elicitors and their exposure times This study can help in further scale up in bioreactors for increased accumulation of secondary metabolites.

Declarations

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Figures

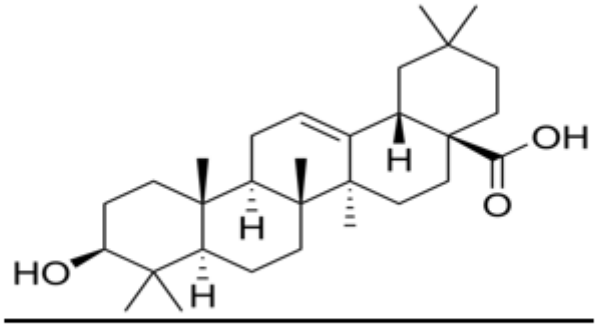

Figure 1

Chemical structure of Oleanolic Acid

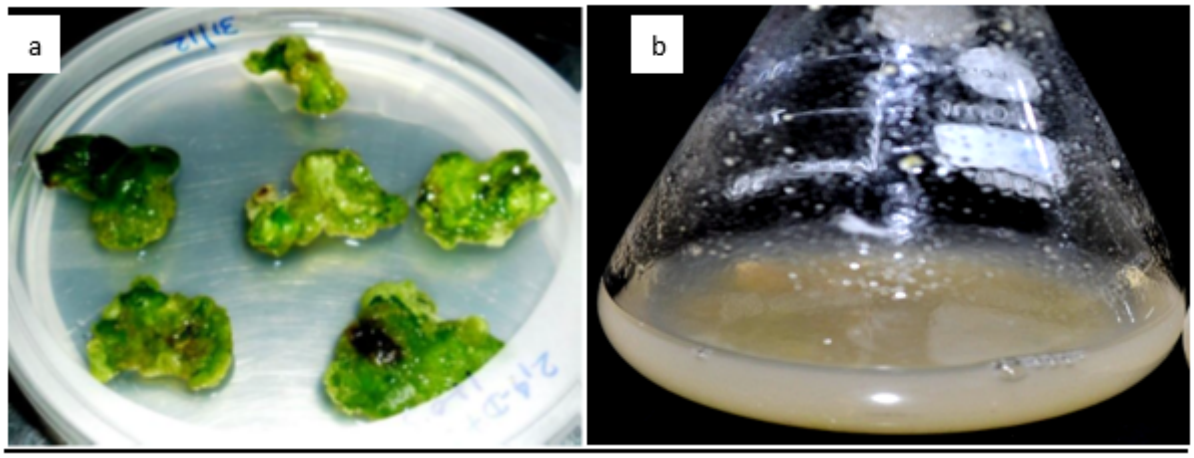

Figure 2

(a) Callus induction from leaf explants of O. tenuiflorum when inoculated into semi-MS media supplemented with NAA (0.25 mg/L) and BAP (0.5 mg/L) (b) Cell suspension cultures

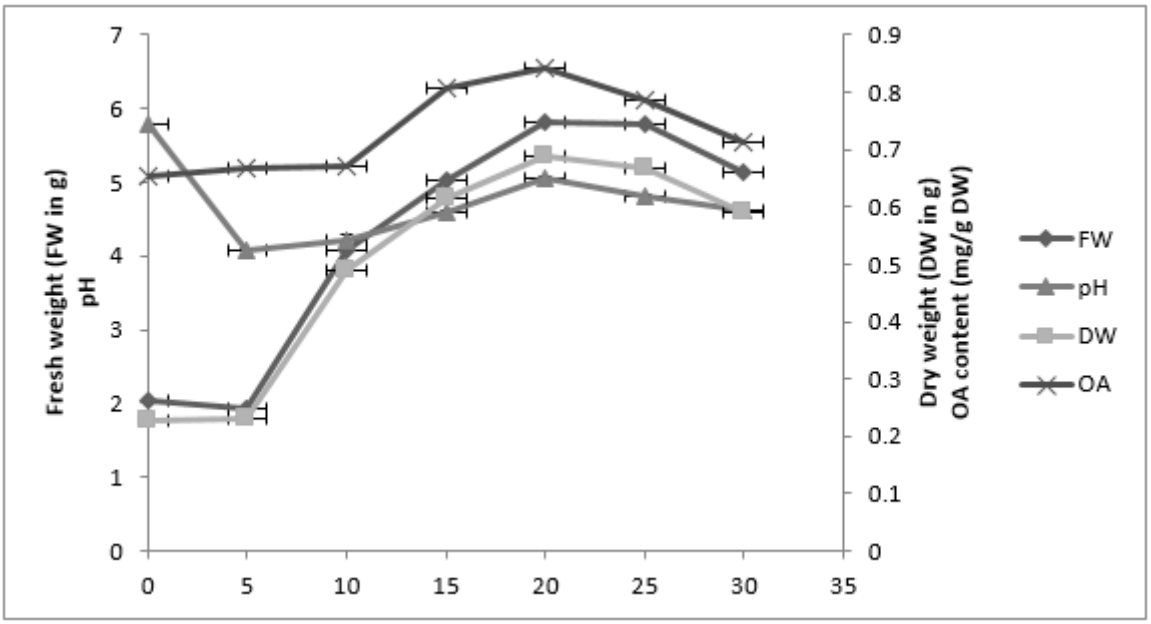

Figure 3

Time course analysis of biomass production of suspension cultures (FW and DW), pH and OA production in O. tenuiflorum suspension cultures over a period of 30 days. Data represent mean ± SD from three replicates

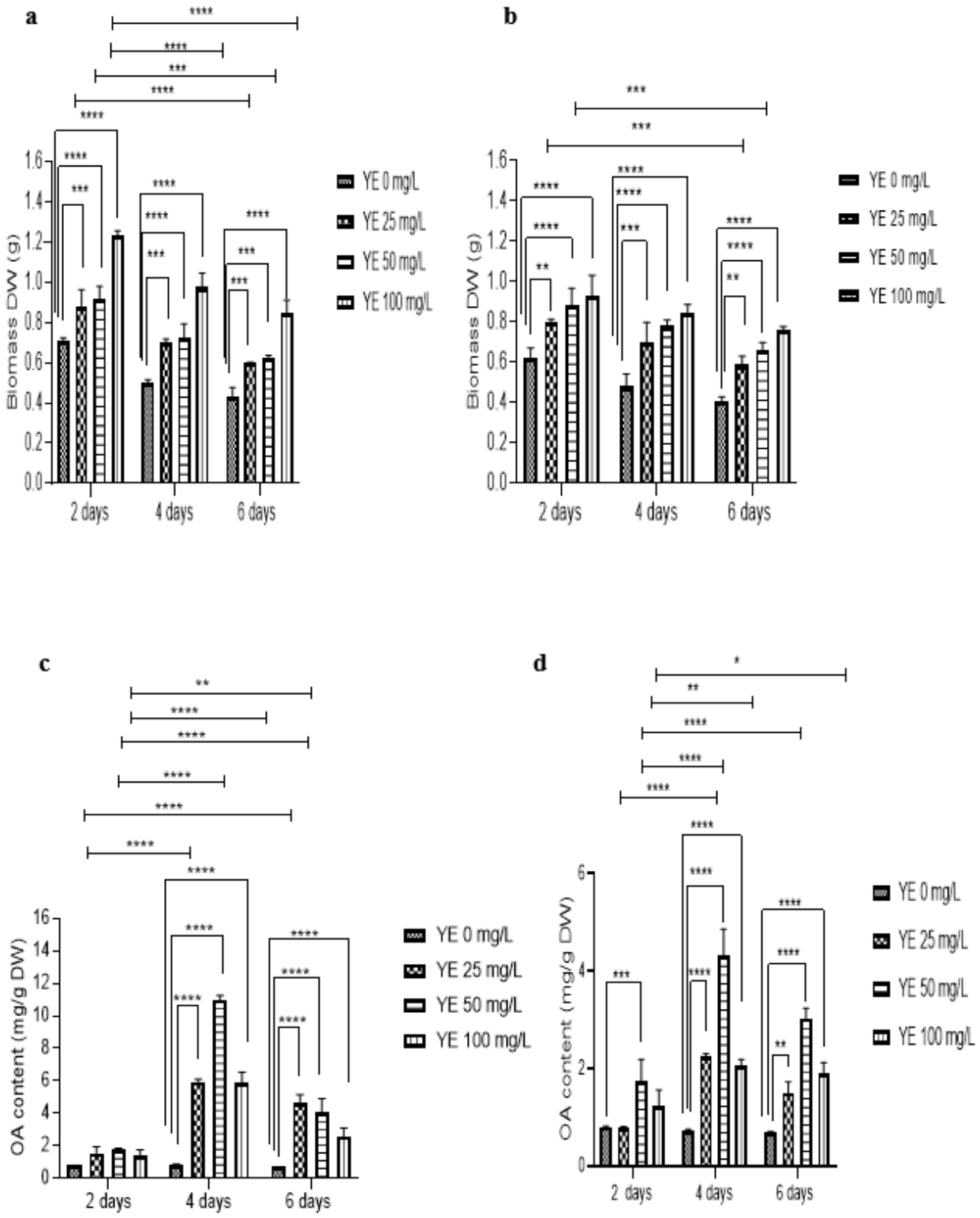

Figure 4

Influence of YE (0, 25, 50 and 100 mg/L) on (a) Biomass production and (b) Accumulation of OA in 17-day-old cultures, on (c) Biomass production (d) Accumulation of OA in 22-day-old suspension cultures of O. tenuiflorum. Data are the mean ± SD from three replicates. Means with asterisk show statistically different from the control (ANOVA two way with post hoc Tukey's test with multiple comparison P< 0.05*, P < 0.01**, P < 0.001***, P < 0.0001****)

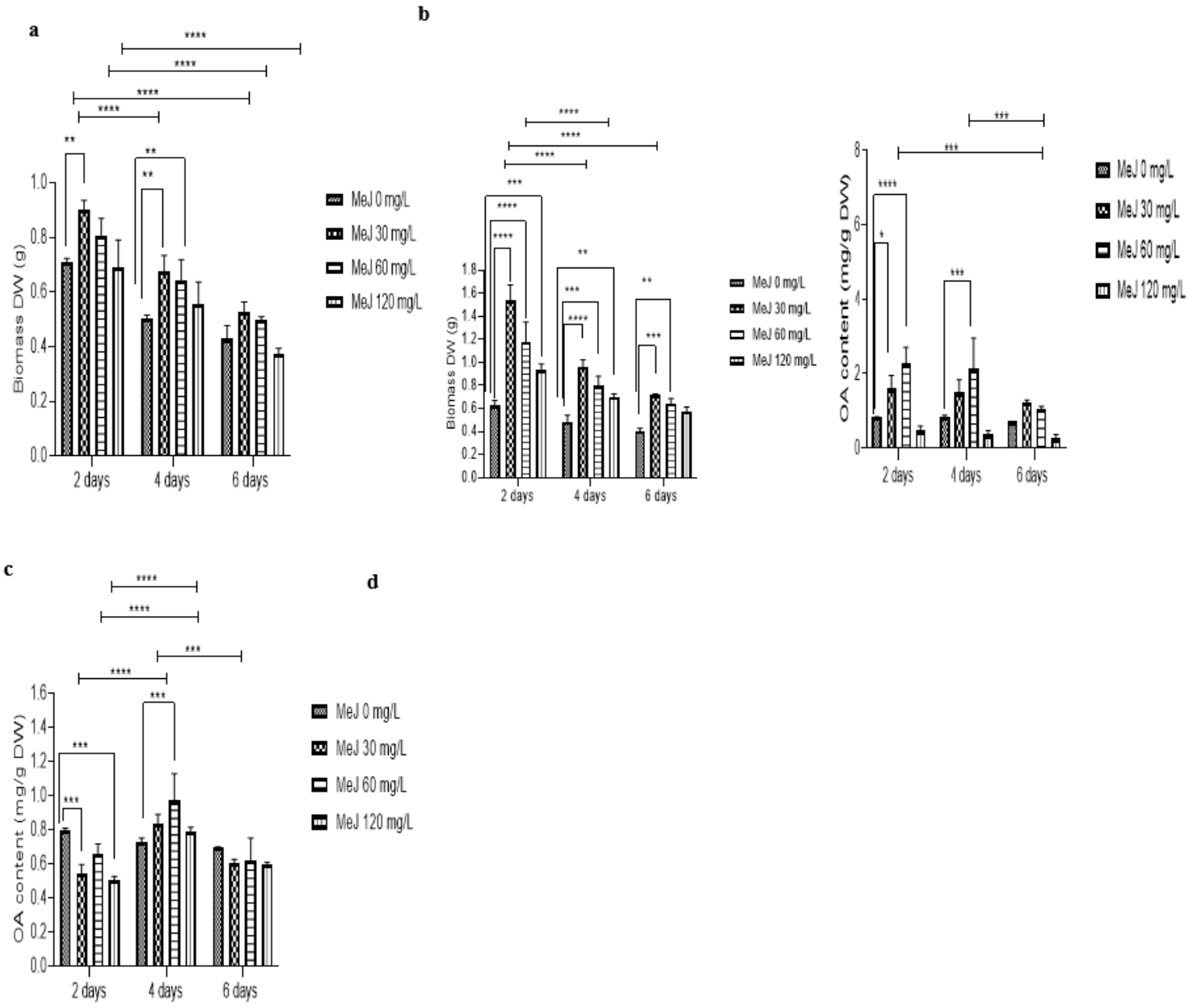

Figure 5

Influence of MeJ (0, 30,60 and 120 mg/L) on (a) Biomass production and (b) Accumulation of OA in 17-day-old cultures, on (c) Biomass production (d) Accumulation of OA in 22-day-old suspension cultures of O. tenuiflorum. Data are the mean ± SD from three replicates. Means with asterisk show statistically different from the control (ANOVA two way with post hoc Tukey's test with multiple comparison $P < 0.05$*, $P < 0.01$**, $P < 0.001$***, $P < 0.0001$****)

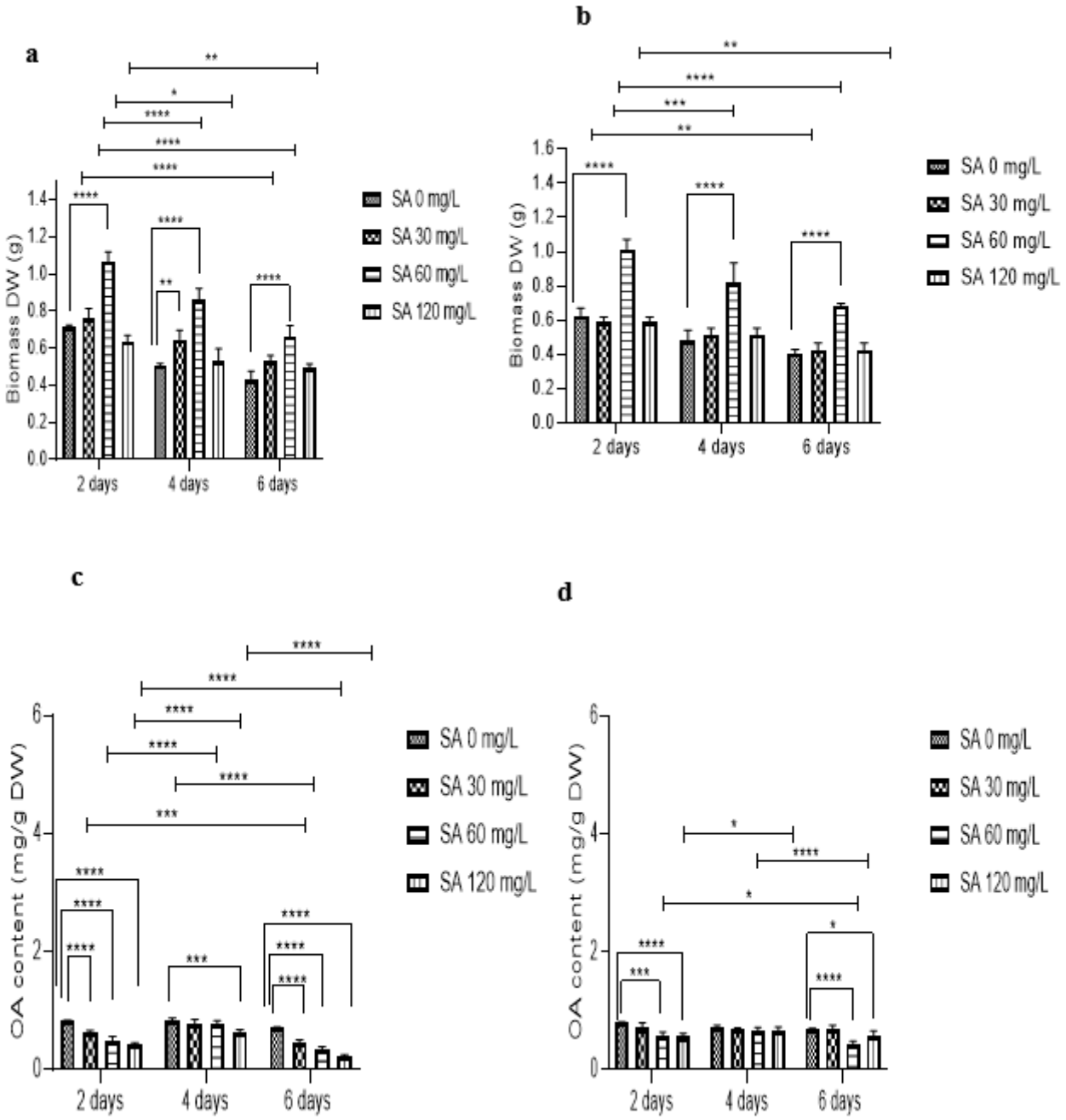

Figure 6

Influence of SA (0, 30,60 and 120 mg/L) on (a) Biomass production and (b) Accumulation of OA in 17-day-old cultures, on (c) Biomass production (d) Accumulation of OA in 22-day-old suspension cultures of O. tenuiflorum. Means with asterisk show statistically different from the control (Means with asterisk show statistically different from the control (ANOVA two way with post hoc Tukey's test with multiple comparison $P < 0.05$*, $P < 0.01$**, $P < 0.001$***, $P < 0.0001$****)

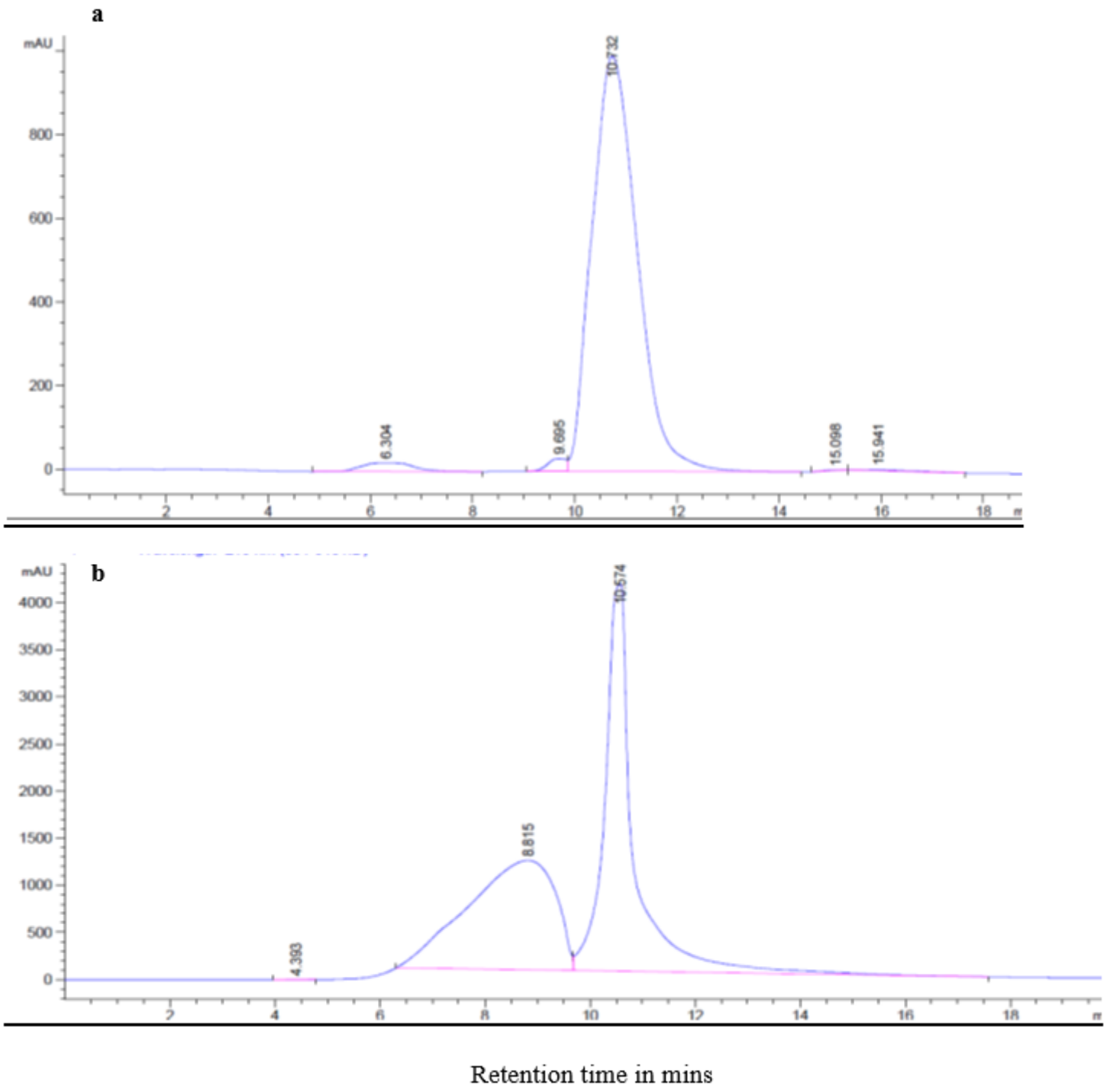

Figure 7

HPLC profiles of (a) Standard of OA (b) Sample treated with YE (50 mg/L)