Original Research

Effect of elicitors on cell suspension culture of *Urginea maritima* L. towards production of proscillaridin A

Heba Shahin[1],*, Atef A. El-Hela[2], Mahmoud I. Nasr[3], Ghada M. Nasr[4]

[1] Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, 79/22857 Sadat City, Egypt

[2] Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, 11884 Nasr city, Cairo, Egypt

[3] Department Molecular biology, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, 79/22857 Sadat City, Egypt

[4] Department Molecular diagnostics, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, 79/22857 Sadat City, Egypt

*Correspondence: heba.shahin@gebri.usc.edu.eg (Heba Shahin)

Abstract

Background: *Urginea maritima* (L.f.) Baker (Hyacinthaceae) is a perennial bulbous medicinal plant that is currently at risk of extinction. Squill (white sea onion) is an analogous cardiotonic to digitalis. The purpose of the current work was to assess the optimal growth conditions for *Urginea* cells to synthesize the cardiac glycoside proscillaridin A by involving illumination, carbon source, methyl jasmonate (MJ), and culture system. **Results**: When cells were cultured for 28 days at $21 \pm 2\,^\circ$C in the dark on Murashige and Skoog (MS) media containing 1 mg/L 2, 4-D and 0.5 mg/L Kin and 30 g/L sucrose, the cell proliferation, and proscillaridin A synthesis were effectively controlled. At low concentrations, MJ stimulated the synthesis of proscillaridin A (PsA). High-performance liquid chromatography (HPLC) analysis of suspension extracts demonstrated that the callus maintained in MS media enriched with 1 mg/L 2, 4-D and 0.5 mg/L Kin yielded a greater formation of Proscillaridin A (141.31 mg/g DW) than untreated plants. **Conclusions**: The results indicate that *in vitro* cultures of *U. maritima* may be an excellent source of proscillaridin A. Moreover, it is one of the most important cardiac glycoside, which has been found to exhibit anticancer activities. Suspension cultures of *Urginea* cells could be as highly productive as a callus culture.

Keywords: *U. maritima*; Proscillaridin A; Elicitors; Suspension culture; Methyle jasmonate

1. Introduction

Urginea maritima (L.f.) Baker (Hyacinthaceae) is a critically endangered perennial bulbous medicinal plant [1]. It is commonly referred to as squills. Squill glycosides have cardioprotective properties as digitalis. *U. maritima* produces many bioactive substances, including bufadienolides, scilliglaucoside, 6α-dihydroxy-4(15)- eudesmane, 1β, plybotrin, and a polyhydroxylated furan derivative [2].

Cardiac glycosides are used to treat congestive heart failure and cardiac arrhythmias. Proscillaridin A (PsA), (Fig. 1), is one of the most important cardiac glycoside constituents of *Urginea maritime* that has been found to exhibit anticancer activities, as it inhibits proliferation and induces apoptosis in cancer cells [3]. Moreover, it has been clinically applied for treating cardiac disorders [4]. Anticancer and epigenetic effects of this compound have been investigated against various cell lines, such as human lymphoma [5], breast cancer [6,7], human fibroblasts [7], multiple myeloma [8], Embryonal rhabdomyosarcoma [9], prostate cancer [10], and advanced adrenocortical carcinoma [11]. It is an inhibitor of the Na(+)/K(+) ATPase (NKA) pump, against the proliferation and migration of glioblastoma cell lines, and as a potent candidate for drug repositioning [12].

The PsA can successfully inhibit hepatocellular carcinoma (HCC) progress and may assist as a potential therapeutic mediator for HCC treatment [13]. It has the potential to be repurposed as a gene drug in personalized oncology, especially in leukemias with MYC overexpression [14]. PsA affects hormone-regulated systems as a suppressor, and it may help predict endocrine disruption. This function may be beneficial to detect environmentally significant ERα modulators in upcoming high-throughput transcriptomic screens [15]. Novel studies have explored significant insights for improved diagnosis and therapeutic options for COVID-19 using proscillaridin A [16,17]. Analgesic, T-cell suppressive and insecticidal activities have been also reported for proscillaridin A [18,19]. The significance of these bioactive molecules has gained great interest recently.

Plant cell cultures were used effectively to generate massive amounts of secondary metabolites from various plants. It was observed that *Thevetia peruviana* callus tissue culture could produce high levels of cardiac glycoside [20].

In the culture of plant cells, several methods, such as lightening and nutrient modification, can regulate the secondary metabolites and enhance the properties of cell
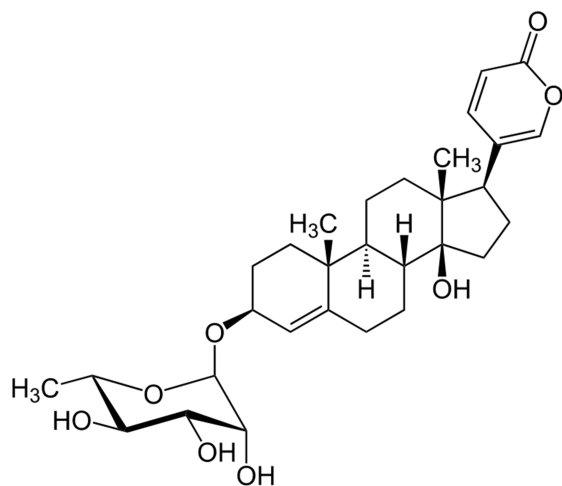
Fig. 1. Proscillaridin a structure.

biomass. Recent developments have shown that manipulating the cultural environment can enhance product accumulation. Furthermore, the application of exogenous methyl jasmonate (MJ) to *in vitro* cultures has developed as an important approach for hyperaccumulation and affecting the expression of several secondary metabolites [21].

Cells are cultured with simple sugars acting as the carbon source in the medium to provide energy. The fact is that sucrose level affects the secondary metabolites productivity in different plants [22,23]. Recently, suspending culture has been recognized as a critical step towards commercialization [24]. The liquid medium maintains intimate contact with tissue, stimulating and facilitating nutrition and hormone uptake [25], resulting in enhanced cell growth. Scholars have reconstructed several models to reach commercial scale and great production as shikonin in *Lithospermum erythrorhizon* cell line [26] and paclitaxel in *Taxus brevifolia* [27]. Hence, the chief objective of this study was to establish friable callus and cell suspension culture from H. costaricensis *in vitro* seedlings and investigate their betalain content as well as their antioxidant potential.

In this research, we assessed the cell proliferation and PsA content of calli from the leaf scale segment of *U. maritima*.

2. Materials and methods

2.1 Plant material and establishment of callus culture

Bulbs were collected from the Mediterranean coastal zone between El-Arish and Rafah (Egypt) during 2018 and 2019. The bulbs were carefully washed with detergent and rinsed with tap water. From each bulb, the two outer scales, the apical one-third and ca, 1 mm of the basal plate, were removed and discarded. The clean bulbs are cut longitudinally into quarters, and the scales were separated freely from each other to be first treated with hot water to eliminate the endogenous contamination. The bulb scales were submerged for 1 h in hot water at 50 °C for 1 h [28]. Sub-

sequently, the segments were disinfected with 80% ethanol for 1 min, immersed for 30 min with stirring in 5% sodium hypochlorite containing 0.003% (w/v) of Tween 20; and then rinsed three times with sterile water. Finally, the bulb scale segments were cut transversely into four or five segments (1.0 cm × 1.5 cm) based on the leaf size and cultured on Murashige and Skoog (MS) media [29] supplemented with 88 μM sucrose and 2.0 g/L of gelrite (Duchefa Biochemie, Haarlem, The Netherlands). The pH was adjusted to 5.7–5.8 using 0.1 N sodium hydroxide (NaOH) and 0.1 N hydrochloric acid (HCl) before autoclaving at 121 °C for 20 min. 0.5 mg/L 6-Benzylaminpurin (BA) and 3 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) were added to the media for callus induction. The callus was then subcultured after 28 days to MS media enriched with 0.5 mg/L kinetin (4-Amino-3,5,6-trichloropicolinic acid) and 1 mg/L 2, 4-D. Before autoclaving, the pH of the MS media adjusted to 5.8. Then, the media was autoclaved for 20 min at 121 °C, under a pressure of ~105 kPa. The cultures were incubated in the dark at a temperature of 21 ± 2 °C and a humidity of 80%.

2.2 Establishment of cell suspension

The friable calli tissues were transferred to a liquid MS media after three subcultures. Cell suspension cultures were created by stirring 3 g of friable calli derived from leaf scale explants (calli was established on 3 mg/L 2, 4-D and 0.5 mg/L BA for two months until transferred to MS media enriched with 2, 4-D (1 mg/L), Kin (0.5 mg/L) and sucrose (30 g/L) in 250 mL flasks containing 100 mL of fresh liquid MS media enriched with Kin 0.5 mg/L and 2, 4-D (1 mg/L). The media pH was regulated at 5.8 prior to autoclaving. Suspension cultures incubated at 21 ± 2 ° for 16 hs light: 8 hs dark photoperiod of cool white fluorescent light (40 μmol m^{-2} s^{-1}) or in the dark with continuous shaking at 110 rpm. Every two weeks, subcellular suspensions were cultured by transferring them from an initial cell density of 3 × 10^5 cells per mL into the same liquid media. The separate flasks were sacrificed every three days for 27 days, and the proscillaridin A level was determined to validate the kinetics of glucose growth and proscillaridin A formation. For each parameter, readings were recorded from three flasks.

2.3 Eliciters application

To investigate the influence of illumination, leaf callus culture was also incubated under 600 lux light intensity with 16/8 photoperiod in MS media enriched with 2, 4-D (1 mg/L), sucrose (30 g/L), and Kin (0.5 mg/L). To investigate the impact of sucrose levels, leaf calli were grown for 28 days at 21 ± 2 °C in the dark in MS media enriched with varied sucrose levels (30, 40, or 60 g/L) and containing Kin (0.5 mg/L), and 2, 4-D (1 mg/L). For determining the PsA content of all fresh calli, they were obtained and left to be dehydrated at 40 °C for 2 days.

The elicitation effect of methyl jasmonate (MeJA) on proscillaridin A synthesis was investigated by *U. maritima* cell suspension cultures. Before introducing MeJA to the suspension cultures, it was dissolved in ethanol and filter sterilized. The feeding level of MeJA was maintained at 50 μM based on the findings of preliminary studies. As a standard, 0.3% filter-sterilized ethanol was added to the cell suspension culture.

2.4 HPLC analysis

Depending on the retention times and standard values UV spectra, HPLC was utilized to determine the PsA biosynthesized in cell cultures. A volume of 1 g left to dehydrated powder was extracted by cold maceration using chloroform for 72 h (100 mL × 3). The crude extracts were filtered through filter paper (Whatman, No. 1), concentrated to dryness, dissolved in 5 mL methanol, and again filtered through 0.45 μm membranes before being inserted into HPLC. A Breeze HPLC system from Waters Corporation (USA) was used. The mobile phase has an equal rinse with a mobile phase was a mixture of water: methanol (20:80). The flow rate was adjusted to 0.9 mL/min. An analytical column C8 (4.6 × 250 mm, 5 μm particle size; Perfectsill, MZ-Analysentechnik, Germany). HPLC chromatograms were observed utilizing a UV detector with a wavelength of 270 nm, as determined by its retention time, and spiking with standards under the same conditions described previously [30]. PsA was purchased as a standard (Sigma-Aldrich).

Statistical analysis was performed using Microsoft Excel 2010 (14.0.4734.1000 ©2010 Microsoft Corporation. All rights reserved), using the calculation of mean and standard errors, and the data were compared utilizing Duncan's many ranges method for intergroup correlations. $p < 0.05$ was selected as the criterion of significance.

3. Results and discussions

Light is a critical physical factor affecting plant development and growth, and the biosynthesis of several plant cell cultures [31]. In addition, the impact of illumination and darkness on cell growth, proscillaridin A synthesis by cell suspension culture of *U. maritima* was evaluated. At the end of the exponential phase, grown cell cultures grown in the dark had a greater cell density (14.83 × 10^5 cells per mL) than those grown in the light (11.97 × 10^5 cells per mL). The PsA level was 1.82-fold greater in cell suspension cultures grown in the dark than those grown in photoperiod conditions. PsA biosynthesis was decreased in the presence of light (Table 1). Therefore, darkness facilitated the formation of PsA in leaf callus as well as its purification by lowering the chlorophyll level in the culture. Our results are consistent with previous studies that reported that light inhibits certain secondary metabolites accumulation, such as shikonin and nicotine in cell suspension cultures and hairy root of *Lithospermum erythrohizon* [32,33], as well as phe-

nolic compounds in cell suspension of *Thevetia peruviana* [34].

Table 1. Impact of light on cell growth and PsA formation in the leaf cell suspension of *U. maritima*.

Light	Dehydrated weight/flask (g)	PsA content (mg g^{-1} dehydrated weight)
Dark	1.35 ± 0.09	61.6 ± 13.40
Light	1.11 ± 0.15	39.17 ± 12.41

Values are means ± SD of 5 replicate analyses.

Carbon sources play a role in the synthetic pathway of numerous substances, performing as building blocks for macromolecules, and may affect many cellular growth pathways [35,36]. Koch [37] noted that sugars affect the expression of several plant genes, and their relationship to growth and metabolic processes is unequivocal. Therefore, carbohydrates are of fundamental relevance for *in vitro* morphogenesis, an elevated energy needing procedure [38,39]. Sucrose must be included for growth and development through osmoregulation, as well as to increase the nutritional value of the culture. Sucrose also acts as a signaling molecule, influencing growth, development, and metabolic processes, such as carbon and nitrogen uptake and transport [40].

The ideal sucrose content for PsA synthesis was determined in the suspension culture of *U. maritima*. The sucrose level influences the development and formation of PsA of the leaf callus at sucrose levels of 20, 30, 40, and 60 g/L. As presented in Table 2, the weight of the dehydrated callus was raised with higher sucrose levels. When the sucrose amount was 40 g/L, the dehydrated weight of the callus was 2.48 ± 0.14 g per flask. This demonstrates that sucrose is a necessary nutrient for callus formation. When calli were cultured in media containing 20 g/L sucrose, the dehydrated weight of the callus was reduced by 2.8-fold compared to when cultured in a medium containing 30 g/L sucrose. However, there was no positive correlation between the total amount of PsA in the leaf callus and sucrose level. The medium supplemented with 3% sucrose was found to be the optimum concentration, resulting in the maximum rate of PsA content. The PsA peak level (151.54 ± 12.58 mg/g of cultured cells) (Table 2).

Additionally, when the level of sucrose in the leaf callus reached 30 g/L, the PsA level decreased. Hence, 30 g/L sucrose must be applied to the culture media to maximize total PsA formation. According to several investigations, increased sugar levels lead to decreased growth [41,42].

Furthermore, when sucrose levels in the culture media elevated, phenolic levels and tissue necrosis elevated, and shoot regeneration reduced significantly in the three sugar beet lines [43]. These findings agree with those of Kadota *et al.* [44], who discovered that elevated sucrose levels were

Table 2. Impact of sucrose levels on cell growth and PsA formation in the leaf cell suspension of *U. maritima*.

Sucrose concentration	Dehydrated weight per flask (g)	PsA content (mg/g of cultured cell)
20 g L^{-1}	0.79 ± 0.2c	49.86 ± 8.56bc
30 g L^{-1}	1.27 ± 0.19b	62.70 ± 12.60a
40 g L^{-1}	1.21 ± 0.13b	31.46 ± 9.19c
60 g L^{-1}	1.81 ± 0.15a	45.23 ± 11.43c

Values are means ± SD of 5 replicate analyses. Duncan's test indicates that means within a column preceded by the same letters are not significantly different at the 5% level.

deleterious to pear (*Pyrus communis*), possibly due to a reduction in osmotic potential associated with increased carbohydrate levels [45]. *In vitro*, cells recognized sugars as chemical signals, with extremely elevated levels acting as stressors [46,47].

Comparing the medium that produced higher cell densities to the medium that contained lower sucrose levels, the latter collected the highest betalain pigment concentration in *Hylocereus costaricensis* cell suspension [48]. The uptake and storage of carbon sources in the cells was the fundamental reason for increasing dry cell mass [49]. Sucrose concentrations of 2–3% are commonly used in cell suspension cultures [48]. In a study on *B. vulgaris* cell suspension cultures, dry cell weight increased with increasing sucrose concentration up to 50 g/L [50].

Increased sucrose concentrations in the culture media generate a hypertonic environment that allows water from within the cells to diffuse out while inhibiting nutrient uptake. Cell death decreases the cell number and, resulting in decreased secondary metabolite synthesis [51].

Wang *et al*. [52] discovered several factors affecting cell growth and the formation of secondary metabolites in cell suspension cultures, including the elicitor concentration, culture age, cell line, growth regulators, and nutritional material composition.

After 15 days of cultivation, *U. maritima* cell suspension cultures were treated with various doses of MeJA (50–200 μM). on day 20, the dehydrated weight and proscillaridin A content are shown in Fig. 2. MeJA at all tested doses inhibited cell proliferation. Induction with 50 or 100 μM MeJA reduced DW by 13.7 to 27.51%, whereas an elevated level of MeJA (150 μM) reduced DW significantly from 8.0 ± 0.35 (control cultures) to 4.0 ± 0.48 g/L. The addition of a low concentration (<150 μM) of MeJA elicitor to cultured *U. maritima* cells enhanced proscillaridin A accumulation. As described in Fig. 2, the PsA content was elevated approximately 1.1-fold with a 50 μM dose and approximately 2.3-fold with a 100 μM dose compared to control cultures. At a 100 μM elicitation dose, the proscillaridin A content increased up to 62.8 mg/g DW. Suspension cell cultures are more sensitive to elicitor concentrations than other plant species [53,54]. At higher doses (200 μM), MeJA was also found to inhibit cell growth and metabolite synthesis in *Gymnema sylvestre* cell suspension cultures [55]. As shown in Fig. 2, the optimal dose of the elicitor for peak formation (279.5 mg/L) of proscillaridin A was 100 μM, which was 2.7-fold higher than the value for control cultures. The previous results demonstrate that the optimum technique for eliciting MeJA was to provide a 100 μM dose on day 15. Fig. 3 Assessment of optimal culture time in *U. maritima* cells. Content of proscillaridin A in *U. maritima* cell suspension cultures.
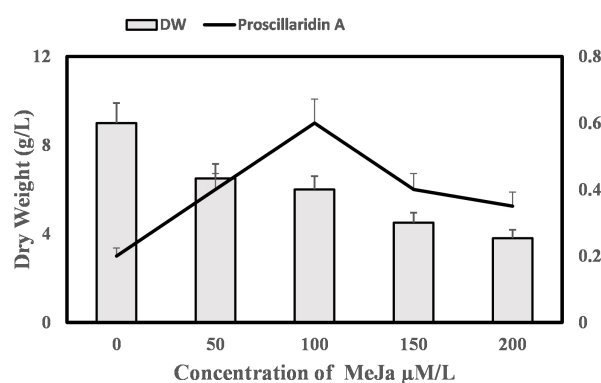
Fig. 2. Impacts of MeJA level on cell growth and proscillaridin a content (mg/g of the cultured cells).
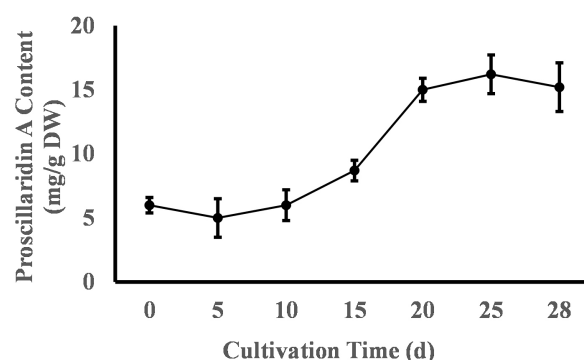
Fig. 3. Assessment of optimal culture time in *U. maritima* cells. Content of proscillaridin A in *U. maritima* cell suspension cultures.

MeJA is a good abiotic elicitor as it activates a key signal in plant defensive responses and enhances the for-

mation of secondary metabolites in plant cell cultures. Furthermore, MeJA is involved in the activation of phenolic chemical metabolism in plants as a whole, as well as in cell suspension and callus cultures from various plant families [56].

For the large-scale formation of secondary plant products *in vitro*, it would be preferred to combine the rapid growth rates and capacity for high biomass levels of undifferentiated cell culture systems with the genetic stability and inherent capacity for secondary metabolite formation of differentiated cells or tissues. Secondary metabolite synthesis has been demonstrated in cultures that proliferate similarly to undifferentiated cell cultures (i.e., callus or cell suspension) but are composed of differentiated cells or tissues [57]. The specialized tissue types that create and sequester vital leaf oils are increased in shoot cultures, and the strict developmental program required for shoot morphogenesis decreases genetic instability as well. In this way, tissue cultures have been shown to combine the appealing characteristics of both undifferentiated and differentiated systems. In cell suspension cultures of leaf callus, the temporal history of biomass growth and secondary metabolites was shown in Fig. 3.

MeJA, an abiotic elicitor, induced the synthesis of high concentrations of furanocoumarins in nutritional media that after various batch culture times. This is because secondary metabolites are generated intracellularly and then released into the nutritive medium in particular plant species, making it easier to collect. In some situations, cells may store secondary metabolites in vacuoles rather than secreting them into the media [58]. The simultaneous cell division and growth with the increase in biomass could be attributable to this release, which follows the growth curve through the lag phase, exponential phase, linear growth phase, and stationary phase, during which the cells stop dividing and growing [59]. Environmental factors such as nutrition depletion, oxygen deficiency, ethylene accumulation, depletion of additional growth regulators, or changes in physical and chemical elements all contribute to cell culture restriction [58]. According to Ramawat [59], the conversion of primary metabolites to secondary metabolites results in an increase in secondary metabolite synthesis at the stationary phase and by the conclusion of the exponential phase.

In the current investigation, the long-term exposure to 2.4-D was associated with the culture-age effect. Due to the homogeneity of cell suspension cultures and cells coming into close touch with a liquid medium, foreign chemicals like PGRs may have a greater effect on cells [60]. Continuous production of secondary metabolites is impeded by a loss of cell productivity, irregular production patterns, and large variability across periodical subcultures [61]. Chemical signals produced in high quantities by cells in the culture can cause cells to flip to a high secondary metabolite generating state.

It is found that the MS media enriched with 0.5 mg/L of Kin, 1 mg/L 2, 4-D, and 30 g/L of sucrose was effective in achieving high formation at 21 ± 2 °C in the dark. As a result, this finding was chosen to explore further suspending culture. The exponential growth of biomass was monitored in this suspension culture for 8–18 days. Peak biomass growth in suspension culture (15.9 ± 2.3 g/L) and the peak total PsA content (141 ± 3.27 mg/g of the cultured cells) were observed after 18 and 27 days after cultivation, respectively. Previously, the suspension system's efficacy was confirmed [62]. The plant cells growth is faster in suspension than in callus culture and is easily regulated, as the culture medium can easily be amended or altered [63].

The current research demonstrated the development of an *in vitro* technique as a useful tool for biotechnologically producing proscillaridin A molecules. Elicitation and precursor feeding will be used in future studies to improve the synthesis of these economically relevant anticancer compounds. This may be a useful tool for producing PsA sustainably and continuously for use in many treatments.

4. Conclusions

A rational approach and a very feasible technique for cell suspension studies are to follow tissue culture for the improvement of commercially important and potentially useful secondary metabolites from a phytochemical source. The authenticity as seen from the obtained data is comprehensive and supports the need of assessing all chemical activities in the current analysis of the Egyptian herbal-based formulation. The evaluation of the results led to the selection of the optimum conditions for callus induction and proscillaridin A development by *in vitro* cell suspension culture of *U. maritima*. The results obtained are accurate, optimistic and indicate that the *in vitro* cultivation of *U. maritima* can be an ideal method for generating large yields of bioactive compounds with economic importance. Further experiments are ongoing to scale up the bioreactor procedure to improve growth rates and production including enzymatic and microbial elicitors.

We found that the leaves were the best plant tissue to enhance the calli production of PsA. Furthermore, the light was not necessary for the biosynthesis of the compound under investigation in the callus, which was favorable for PsA purification by decreasing the amount of chlorophyll in the culture. However, factors stimulating calli growth appear to be different from those that induce PsA formation. Sucrose enhanced the growth of the leaf calli but did not directly affect PsA synthesis.

Author contributions

HS and GN designed the research study. HS performed the research. MN provided help and advice on performance and during writing. AH performed the chemical analysis part. HS analyzed the data. HS and GN wrote the manuscript. All authors contributed to editorial changes

in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Conflict of interest

The authors declare no conflict of interest.

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