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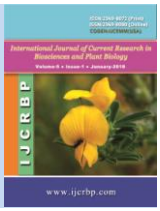

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Elicitor Application Influences the Phytochemical Profile of *Salacia reticulata* with the Formation of New Novel Metabolites of Medicinal Value

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ABSTRACT

Salacia reticulata which is a well known plant for antidiabetic properties were subject to elicitor application. The field grown plants were sprayed with aqueous extracts of 2% *Aspergillus niger* mycelium as biotic elicitor and 0.2mM salicylic acid as abiotic elicitor. Elicitation of phenolics and flavonoids were marginal in both the treatments. Formation of seven new compounds in biotic elicitor treatment and eight new compounds in abiotic elicitor treatment was evident from GCMS studies. Significantly Ala-Gly, Trimethylsilyl Ester; Cyclotrisiloxane, Hexamethyl and Eicosanoic Acid, 2,3-Bis[(Trimethylsilyl) Oxy] Propyl Ester were the new compounds found in both the elicitor treatments. Cyclotrisiloxane, Hexamethyl- is a known molecule with Antioxidant, Antiasthmatics and Antogonositic effects. Interestingly, Benzene acetonitrile, Alpha.-(.Beta.-D-Glucopyranosyloxy)-,(R)-, which is a potent anticancer agent and Antioxidant ,was found only upon biotic elicitor treatment. Thus this study has led to the finding of new molecules in *Salacia* with potent novel medicinal properties.

Introduction

Salacia reticulata belongs to the family Celastraceae. *Salacia* species are commonly known as saptacharka or saptarangi in Sanskrit, and native of Asia and Africa continent used as a herbal drug to treat many diseases (Sujana et al., 2015). *Salacia* species contain unique phytochemicals like salcinol, Kotanol (Hiromi and Sei Ozaki, 2008), Mangiferin (Jaykumar et al., 2015) which are of pharmaceutical value in the treatment of many ailments like diabetes (Jeyakodi et al., 2016), kidney disorder, anti cholesterolemia (Singh et al., 2015), rheumatism, asthma (Romero-Pérez et al., 2016) and cancer (Musini et al., 2015).

Elicitors are the signal molecules when applied to plants induce the synthesis of secondary metabolites and offers protections from pathogens (Manivannan et al., 2016). Elicitor are of two types, biotic (biological substance *viz.*, metabolites, proteins, carbohydrates obtain from microbes, for example: Yeast, Fungal spores, mycelial cell wall) and abiotic (chemicals like salicyclic acid, jasmonic acid, abscisic acid, etc) (Giridhar and Parimalan, 2010; Saini et al., 2013; Ramakrishna and Ravishankar, 2011).

Ali et al. (2016) showed that foliar spray of methyl jasmonate as abiotic elictor to potato plant enhanced the secondary metabolites like phenolics, flavonoids and anthocyanins. These molecules proved to be good antioxidants with anticancer activity. Patade et al. (2016) reported that campesterol content in the cell suspension of *Blumea lacera* enhanced when cells were treated with salicyclic acid and extracts of mycelia of *Aspergillus niger*. Thiruvengadam et al. (2016) showed isofavone enhancement in soybean plant upon elicitor treatment.

In view of the importance of *Salacia* for the treatment of various ailments, it is of interest to enhance the potency of the extracts through augmentation of the secondary metabolites of importance. Moreover the expression of the novel molecules in *Salacia* would add value to this herb for finding newer applications of medicinal value. Hence we embarked up on the study to evaluate the effect of biotic and abiotic elicitor on *Salacia* species .The study involved the application of elicitor to the plant and evaluation of phytochemical changes upon treatments, also tracking the formation of new molecules through GC MS analysis for enhanced utility of the plant for medicinal value.

Materials and methods

Plants for the studies: *Salacia reticulata* plants were obtained from Government Ayurvedic college herbal garden Bangaluru, and maintain in the DSI Campus at Bangaluru, India**.** The plants were grown in the pot in green house.

Preparation and application of elicitors: Biotic elicitors were prepared according to Giridhar and Parimalan (2010), using fungal mycelium of *Aspergillus niger*. Fresh cultures of *A. niger* were grown on potato dextrose agar (HiMedia, Mumbai) slants and incubated for 7 days at 37 °C. The spores of the respective fungi were used to prepare a spore suspension in 0.1% sodium lauryl sulfate (w/v) and diluted with sterile distilled water under sterile conditions to obtain a spore density of ~2.5×100 spores mL^{-1}. Later the same was inoculated into 150mL Erlenmeyer conical flasks containing 40mL of potato dextrose agar and the cultures were incubated in the dark for 10 days. After incubation, fully grown mycelia cultures were autoclaved and the mycelium was separated from the culture broth by filtration using Whatmann no. 1 filter paper and its fresh weight was recorded. An aqueous extract of the mycelium was made by homogenizing with a mortar and pestle using neutralized sand. The extract was filtered through Whatmann no. 1 filter paper, and kept as the stock solution from which a working concentration of 2.0 % w/v (wet weight of fungal mycelium in 100mL of distilled water) were prepared in sterile water and used for the elicitation experiment. Abiotic elicitor, salicylic acid was dissolved in distilled water and made up to 0.2 mM for the study.

The biotic (2% w/v *A. niger*) and abiotic (0.2 mM Salicyclic acid) elicitor prepared as described above were sprayed to the plants, as a fine mist, every week up to 3 weeks in the early morning hours. Control plants received distilled water sprays. The levels were selected based on the publication of Giridhar and Parimalan (2010), who have reported effective influence of elicitors on secondary metabolite in treated plants.

Plant extract preparation: Leaves of control and treated plants of *Salacia* were dried in shade and powdered in a blender. Soxhlet extraction of the leaf powder of the plants, both control and treatments, was done using methanol.

Estimation of total phenolic content: Total phenolic content in the plant extract was

determined as described by Lim and Quah (2007). Briefly 300 µL of plant extracts were thoroughly mixed with 1.5 mL of freshly diluted Folin–Ciocalteu reagent, to which 1.2 mL of sodium carbonate solution (7.5%) was added and the mixture was incubated for 30 min in dark. The absorbance was measured at 765 nm using Spectrophotometer (UV- VIS Spectrometer SL159 ELICO). Gallic acid (Sigma Chemical Co. USA) was used as the reference standard. The concentration of phenolic content was expressed as mg of gallic acid equivalents (GAE) per g dry weight of leaf tissue.

Determination of total flavonoid content: Total flavonoid content was measured by the method of Pallab et al. (2013). One mL aliquots of standard quercetin (Sigma Chemicals Co, USA) solution (100, 200, 400, 600, 800, 1000 µg/mL) was positioned into test tubes followed by 4mL of distilled water and 0.3 mL of 5% sodium nitrite solution. After 5 minutes, 0.3 mL of 10% aluminum chloride was added immediately followed by 2 mL of 1 M sodium hydroxide. Finally, the reaction volume was made up to 10 mL with distilled water and mixed well. The absorbance of orange yellowish color product developed was measured at 510 nm in a spectrophotometer (UV- VIS Spectrometer SL159 ELICO). The blank was performed using distilled water. Quercetin was used as reference standard. The calibration curve was plotted using standard quercetin. The data of total flavonoids were expressed as mg of quercetin equivalents/ 100 g of dry mass of leaf tissue.

GC-MS analysis: The Clarus 680 GC was used in the analysis employing a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpoly siloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 mL/min. The injector temperature was set at 260°C during the chromatographic run. One µL of extract sample was injected into the instrument and the oven temperature was set as follows: 60°C (2 min) followed by 300°C at the rate of 10°C min^{-1} and 300°C, where it was held for 6 min. The mass detector conditions were as follows: transfer line temperature 240°C; ion source temperature 240°C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. column: Elite-5MS, Dimensions: 30.0m, 0.25mmID, 250µm df; initial temperature is 60°C for 2 min, ramp 10°C/min to 300°C, hold 6 min, , total time is 32.00min, carrier gas is helium, flow (mL/min) is 1.0, split flow: 1mL/min, injection volume: 1µl, Scan mass range: 30m/z-600m/z and polarity +ve. The spectrums of the components in the range of 40 to 600 Da were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library. The identification of bioactive compounds present in the extracts was performed by comparing the mass spectra with data from NIST library. The name, of the compound in the test materials were as curtained based on retention time.

Results and discussion

Phenolics and flavonoid induction by elicitors application to *Salacia*

The phenolics and flavonoids in the methanolic extract of leaves of *Salacia reticulata* plant in control and elicitor treatments were studied (Tables 1 and 2).

It was evident from the Table 1 that the phenolic compounds increased marginally in biotic elicitors whereas the abiotic elicitor resulted in the enhancement to nearly 50%. Whereas the increase in flavonoid was marginal in abiotic treatment (Table 2) and in the biotic elicitor the level of flavonoid was lower than in control. In general, the elicitor application to *Salacia* plants resulted in marginal enhancement of phenolics and flavonoids which would have positive beneficial influence on medicinal property of this plant.

GC-MS analyses of the constituents of *Salacia*

The extracts of *Salacia* were subjected to GC–MS analyses in order to carryout detailed phytochemical profiling. The results are presented in Fig. 1.

Table 1. Levels of phenolics in the elicitor treated leaf tissues of *Salacia reticulata*.

Sl. No.	Sample name	Content equivalent to Gallic acid µg/mg dry weight of leaf tissue
1	Control	38.36±0.2
2	2% *A. niger* treated plant	40.71±0.8
3	0.2mM salicylic acid treated plant	60.08±0.2

*Control: Plant sprayed with distilled water.

Table 2. Levels of Flavonoids in the elicitor treated leaf tissues of *Salacia reticulata*.

Sl. No.	Sample name	Content equivalent to Quercetin µg/mg dry weight of leaf tissue
1	Control	160.16±0.1
2	2% *A. niger* treated plant	120.24±0.2
3	0.2mM salicylic acid treated plant	180.89±0.8

*Control: Plant sprayed with distilled water

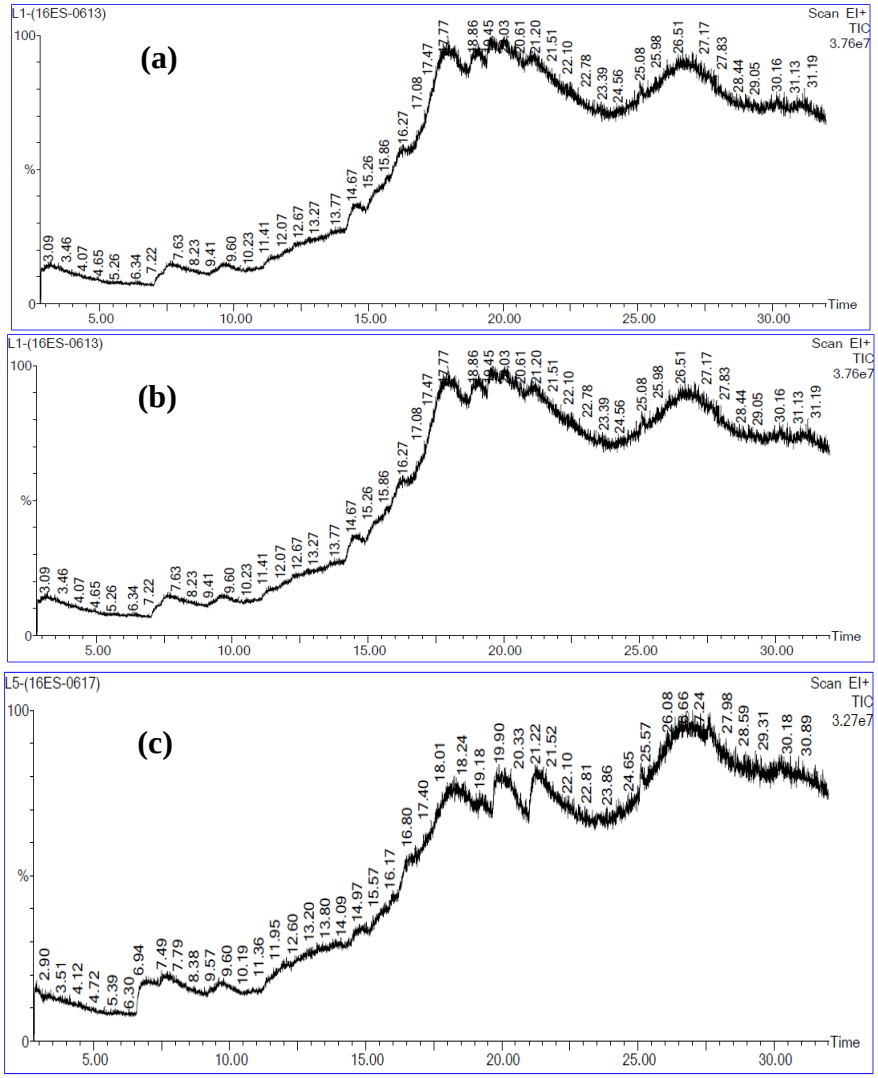

Fig. 1: Gas chromatogram of plant sprayed with distilled water (control), 2% *A. niger* (Biotic elicitor) and 0.2mM salicylic acid (abiotic elicitor) with peak. **(a)** GCMS analysis showing different phytochemicals identified based on retention time of methanol extract of control plant; **(b)** GCMS analysis showing different phytochemicals identified based on retention time of methanol extract of 2% *A. niger* (Biotic) treated plant; **(c)** GCMS analysis showing different phytochemicals identified based on retention time of methanol extract of 0.2mM salicylic acid (Abiotic) treated plant.

The control plants showed the prevalence of 6 major phytochemicals (Table 3). Interestingly the treatments showed a different scenario of new and novel metabolites which were not found in control. Control plant showed the formation of (**compound#2**) 1-(2-Decylaminoethoxy-2-[2-(2-Trimethylsilyloxyethoxy) Ethoxy] Ethan; and (**compound#3**) Butane, 1,2,3-Tris(Trimethyl siloxy)-. However these metabolites were not found in elicitor treated plants, implying that elicitor treatment also down regulated the formation of certain metabolites. Most of the publications refer to upregulation of metabolites upon elicitation (Prasad et al., 2013). However, in this study, we observed down regulation of **compound # 2** and **#3** in the elicitor treated plants. Similarly down regulation of **compound #4** (3,7,11,15,18-Pentaoxa-2,19-Disilaeicosane, 2,2,19, 19-Tetramethyl-) was evident in salicylic acid treatment; similarly, compound #5 (O-Methylisourea) and **compound #6** (Propanoic Acid, 2-Oxo-, Trimethylsilyl Ester) in *A. niger* extract treated plants (Table 3). The plausible explanation for down regulation could be attributed to *in situ* mobilization of precursors to other metabolic pathways.

It is evident from Table 3 that the treatment of *Salacia* plants with biotic elicitor resulted in the formation of (**compound#7** 1,5,9,9-Tetramethyl -2-Oxatricyclo [6.4.0.0(4,8)] Dodecane; (**compound #8**) Benzeneacetonitrile, Alpha.-(.Beta.-D-Gluco pyranosyloxy)-, (R)-; **(compound#9)** Trimethyl [4-(1,1,3,3,-Tetramethylbutyl) Phenoxy] Silane**;** (**compound#10**) Tetracosanoic Acid, Trimethyl-silyl Ester as exclusive metabolites. Hence upregulation of certain novel pathway is evident with the formation of these new compounds not found in the control plants. The biological significance of these novel molecules will be interesting to study.

In contrast, the treatment of *Salacia* plants with abiotic elicitor Table 3 resulted in formation of yet other exclusive metabolites *viz.,* C3,27-Dioxa-2,28-Disilanonacosane, 2,2,4,28,28-Pentamethyl-, (**compound#14**); Eicosanoic Acid, 2,3-Bis[(Trimethylsilyl) Oxy] Propyl Ester (**compound#15**); Undecanoic Acid, 11-Fluoro-, Trimethylsilyl Ester; (**compound#16**) Decanoic Acid, 10-Fluoro-, Trimethylsilyl Ester; **(compound#17**) Oleic Acid, Trimethylsilyl Ester; (**compound#18**) 17-Octadecynoic Acid, Trimethylsilyl Ester. Thus the exclusive influence of biotic and abiotic elicitors in the formation several novel compounds were highly evident.

(**Compound#11**) A la-Gly, Trimethylsilyl Ester; (**compound#12**) Cyclotrisiloxane, Hexamethyl- and **(compound #13)** Eicosanoic Acid, 2,3-Bis [(Trimethylsilyl) Oxy] Propyl Ester**,** were the three chemicals commonly found in biotic and abiotic treated plants of *Salacia* and not found in control plants (Table 4). Whereas, **(compound#14)** 3,27-Dioxa-2,28-Disilanonacosane, 2,2,4,28,28-Penta methyl-**; (compound#15)**. Undecanoic Acid, 11-Fluoro-, Trimethylsilyl Ester; **(compound#16)**. Decanoic Acid, 10-Fluoro-, Trimethylsilyl Ester; **(compound#17)** Oleic Acid, Trimethylsilyl Ester, were found in abiotic elicitor treatment only and not in biotic elicitor treatments or in control.

In general the GC MS profile showed 6 common compounds in control, whereas biotic elicitor showed 7 new compounds and abiotic elicitor showed 8 new compounds respectively (Table 3).
The PubMed website based analyses of biological activities of elicited metabolites of *Salacia* plants showed interesting properties (Table 4).

Evidently, (**compound#12**) Cyclotrisiloxane, Hexamethyl- was a new novel compound found in both the elicitor treatment- biotic and abiotic which is supposed to be a potent Antioxidant, Antiasthmatics, Antogonositic effects, and also it is a signal molecule. Moreover, (**compound#17**) Oleic Acid, Trimethylsilyl Ester found in abiotic elicitor treatment is a known antitumour compound. Benzeneacetonitrile, .Alpha.-(.Beta.-D-Gluco pyranosyloxy)-, (R)-(**compound#8**) found in biotic elicitor treatment is known for anticancer and antioxidant property. Further analyses through *in vitro* and *in vivo* models would throw light on the biological activities of these molecules with respect to *Salacia*.

Table 3. Comparative account of plant sprayed with distilled water (Control), 2% *A. niger* (Biotic) and 0.2mM salicylic acid (Abiotic) with GCMS chemical profiling.

Compound number	Molecular formula	MW	Retention time	Control	2% *A. niger*	0.2mM salicylic acid
1	$C_{13}H_{26}O_6NSiB$	331	22.506, 18.005, 18.240, 18.600	+	+	+
2	$C_{21}H4_7O_4NSi$	405	16.269	+	- *	-*
3	$C_{13}H_{34}O_3Si_3$	320	20.120	+	- *	-*
4	$C_{17}H_40O_5Si_2$	380	17.949, 18.345	+	+	-*
5	$C_2H_6ON_2$	74	3.204, 7.715	+	-*	+
6	$C_6H_{12}O_3Si$	160	19.090, 20.000, 19.725	+	-*	+
7	$C_{15}H_{26}O$	222	24.922	-	+	-
8	$C_{14}H_{17}NO_6$	295	19.485, 19.600, 19.980	-	+	-
9	$C_{17}H_{30}OSI$	278	27.438	-	+	-
10	$C_{27}H_{56}O_2SI$	440	20.080	-	+	-
11	$C_8H_{18}N_2O_3SI$	218	2.993, 2.903	-	+	+
12	$C_6H_{18}O_3SI_3$	222	26.768, 27.578	-	+	+
13	$C_{29}H_{62}O_4SI_2$	530	20.006, 19.895, 20.095, 21.191	-	+	+
14	$C_{30}H_{66}O_2SI_2$	514	19.815	-	-	+
15	$C_{14}H_{29}O_2FSI$	276	20.135	-	-	+
16	$C_{13}H_{27}O_2FSI$	262	20.326	-	-	+
17	$C_{21}H_{42}O_2SI$	354	21.221	-	-	+
18	$C_{21}H_{40}O_2SI$	352	21.296	-	-	+

+ Denotes Presence; - Denotes Absence of the compound; * Down regulated in elicitor treatments only.

Compound #; **(1)** Alpha.-D-Glucopyranoside, Methyl 2-(Acetylamino)- 2-Deoxy-3-O-(Trime; **(2)** 1-(2-Decyl aminoethoxy-2-[2-(2-Trimethylsilyloxyethoxy)Ethoxy]Ethan**; (3)** Butane,1,2,3-Tris(Trimethylsiloxy)-; **(4)** 3,7,11, 15,18-Pentaoxa-2,19-Disilaeicosane,2,2,19,19-Tetramethyl-; **(5)** O-Methylisourea; **(6)** Propanoic Acid, 2-Oxo-, Trimethylsilyl Este**r; (7)** 1,5,9,9-Tetramethyl-2-Oxatricyclo[6.4.0.0(4,8)] Dodecane; **(8)** Benzeneacetonitrile, Alpha.-(.Beta.-D-Glucopyranosyloxy)-,(R)-; **(9)** Trimethyl [4-(1,1,3,3,-Tetramethylbutyl) Phenoxy] Silane; **(10)** Tetracosanoic Acid, Trimethylsilyl Ester; **(11)** Ala-Gly, Trimethylsilyl Ester; **(12)** Cyclotrisiloxane, Hexamethyl-; **(13)** Eicosanoic Acid, 2,3-Bis[(Trimethylsilyl) Oxy] Propyl Ester; **(14)** 3,27-Dioxa-2,28-Disilanonacosane, 2,2,4,28,28-Pentamethyl-; **(15)** Undecanoic Acid, 11-Fluoro-, Trimethylsilyl Ester; **(16)** Decanoic Acid, 10-Fluoro-, Trimethylsilyl Ester; **(17)** Oleic Acid, Trimethylsilyl Ester; **(18)** 17-Octadecynoic Acid, Trimethylsilyl Ester.

Table 4. Biological property of identified Phytochemical in methanol leaf extract of *Salacia reticulata* plants sprayed with distilled water (Control), 2% *A. niger* (Biotic) and 0.2mM salicylic acid (Abiotic) by GC-MS with molecular weight (M.W), Formula.

Compound no.	Compound name	Biological property
1	Alpha.-D-Glucopyranoside, Methyl 2-(Acetylamino)- 2-Deoxy-3-O-(Trime	NK
2	1-(2-Decylaminoethoxy-2-[2-(2-Trimethylsilyloxyethoxy) Ethoxy]Ethan	NK
3	Butane, 1,2,3-Tris(Trimethylsiloxy)-	NK
4	3,7,11,15,18-Pentaoxa-2,19-Disilaeicosane,2,2,19,19-Tetramethyl-	NK
5	O-Methylisourea	Antifungal, Antibacterial, cough suppressant, Antitussitive, Mucolytic
6	Propanoic Acid, 2-Oxo-, Trimethylsilyl Ester	NK
7	1,5,9,9-Tetramethyl-2-Oxatricyclo[6.4.0.0(4,8)]Dodecane	NK
8	Benzeneacetonitrile,.Alpha.-(.Beta.-D-Glucopyranosyloxy)-, (R)-	Antioxidant, anticancer
9	Trimethyl [4-(1,1,3,3,-Tetramethylbutyl) Phenoxy] Silane	NK
10	Tetracosanoic Acid, Trimethylsilyl Ester	Antioxidant, Antimicrobial, Insecticidal activity
11	Ala-Gly, Trimethylsilyl Ester	NK
12	Cyclotrisiloxane, Hexamethyl-	Antioxidant, Antiasthmatics, Antogonositic, Signal molecule with small MW molecule
13	3,27-Dioxa-2,28-Disilanonacosane, 2,2,4,28,28-Pentamethyl-	NK
14	Eicosanoic Acid, 2,3-Bis [(Trimethylsilyl) Oxy] Propyl Ester	NK
15	Undecanoic Acid, 11-Fluoro-, Trimethylsilyl Ester	NK
16	Decanoic Acid, 10-Fluoro-, Trimethylsilyl Ester	NK
17	Oleic Acid, Trimethylsilyl Ester	Cancer Preventive, anti-inflammmatory, hypocholesterolemic
18	17-Octadecynoic Acid, Trimethylsilyl Ester	NK

NF-Not found in website www.http//pubchem.ncbi.nlm.nih.gov/compound.
NK-Not Known.

Thus the present study has shown the potential of elicitor application in producing novel compounds with some new biological activities which are hitherto not found in the *Salaci*a plants. Hence the study has been useful in possibly enhancing the medicinal application of *Salacia* through elicitor treatment. The validation of the biological activities of *Salacia* with respect to the newly predicted pharmacological potential is in progress.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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