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In vitro salt tolerance induced secondary metabolites production in *Abrus precatorius* L.

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ABSTRACT

The white seeded *Abrus precatorius* L. is an important herbaceous medicinal plant with broad range of therapeutic effects. In the present study, the internode selected as explant for *in vitro* salt tolerance analysis. For callus induction, MS media with different concentrations and combinations of BAP, Kinetin and IBA were used. Better callus fresh weight and dry weight observed on MS medium supplemented with BAP 0.5 mg/L and Kinetin 1 mg/L. To detect the *in vitro* salt tolerance potential of callus, NaCl at different concentrations (0, 20, 40, 60, 80 and 100 mM) were supplemented on MS + BAP 0.5 mg/L + Kinetin 1 mg/L of which 40 mM NaCl induced better callus proliferation. The callus grown without NaCl stress showed the presence of eight phytochemical compounds in GC-MS analysis. While the NaCl stress tolerant callus exhibited the presence of seventeen phytochemical compounds. All these analyzed compounds are with antimicrobial/anti-oxidant properties. The present work will be very much helpful to ameliorate the production of medicinally significant compounds in pharmaceutical industry.

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KEYWORDS: Callus, plant growth regulators, salt tolerance, secondary metabolites, GC-MS analysis, retention time.

INTRODUCTION

The genus *Abrus Adans.* includes about 18 species which belongs to the family Leguminosae and native to Africa, Madagascar, India and Indo-China (Swanepoel and Kolberg, 2011). The generic name, *Abrus* is derived from the Greek word *habro* which means delicate, elegant, pretty or soft in reference to the leaflets (Lewis *et al*., 2015). Among the species, white seeded *Abrus precatorius* L. is a garden ornamental plant characterized by climbing, twining or trailing vine with slender branches. It is commonly known as 'white kunni' in Malayalam and 'kunch' in Bengali. The plant is best known for its white seeds which are used as beads and in percussion instruments. The species contain various kinds of alkaloids such as glycerrhizin, precol, abrol, abrasion, abrin A and abrin B (Joshi and Joshi, 2000). Moreover, the presence of abrin indicates the toxicity of white seeds. In addition to the toxic effect, the plant parts have many medicinal properties due to the presence of different secondary metabolites that including antimicrobial, anti-inflammatory, immunomodulatory and antitumor activities (Roy *et al*., 2012).

Plants need balanced abiotic factors for optimum biomass productivity. Occasionally, the proper plant development is arrested by different abiotic stresses. Salinity is one of the abiotic stresses that negatively influences the plant growth and development. Nowadays, soil salinity is a major issue in much crop plant cultivation, especially in case of rice cultivation. It can be negatively influenced on growth, development and yield of rice plants. In medicinal plants, salt stress induces the production of a number of secondary metabolites which are medicinally important (Jaleel *et al*., 2008). Currently, this method of production of medicinal compounds has a significant role in the pharmaceutical industry. The tissue culture technique is a better opportunity to implement salt stress in *in vitro* cultured plants. This *in vitro* approach can be produced a lot of progenies from a single explant by direct or indirect organogenesis. The explant may be any tissue of the mother plant with desired characters including leaf, node, internode, seed, rhizome, apical meristem etc. Application of plant growth regulators like auxins and cytokinins in an optimum ratio causes the regeneration of *in vitro* plantlets directly from explants (Loberant and Altman, 2009). In contrast, somatic embryogenesis on callus tissue in turn results micropropagation of plantlets by the regeneration of embryos from somatic cells of the callus. The potential of a single plant cell to regenerate an entire plant is the totipotency which is the basic reason behind the micropropagation (Marton and Czako, 2011).

In vitro salt stress has been researched in many medicinal plants like *Centella asiatica* (L.) Urb., *Catharanthus roseus* (L.) G. Don, Trigonella foenum-graecum L., *Carthamus tinctorius* L. etc. (Jaleel et al., 2008; Gengmao *et al*., 2015; Sahari *et al*., 2016; Ibrahim *et al*., 2018). To adapt unfavorable stress conditions, the salt tolerant plants produce different secondary metabolites to serve cellular functions essential for physiological processes. Secondary metabolites have no role in the growth and development of the plants, but they required to survive in the environment (Yang *et al*., 2018). The *in vitro* produced metabolites can be separated using GC-MS (Gas Chromatography-Mass Spectrometry) analysis, a technology for secondary metabolite profiling in extracts of plant and non-plant species. Gas chromatography separates the components of the mixture and mass spectroscopy analyzes each of the components separately (Sermakkani and Thangapandian, 2012). These stress induced metabolites frequently show medicinal properties to suppress different microbial diseases (Patra and Mohanta, 2014). Due to the present relevance, the study was focused on *in vitro* salt tolerance and related metabolite production in white seeded *A. precatorius*.

MATERIALS AND METHODS

Plant Material and Sterilization

The shoots of *A. precatorius* collected from Botanical Garden of Korambayil Ahammed Haji Memorial Unity Women's College, Manjeri, Kerala, India and immediately transferred to sterile polythene bag and tightly tied. Using a sterile blade, the internodes of the plants were separated and used as explants for *in vitro* studies. The explants incubated in 0.1 % Bavistine for 3 hours followed by 1% Teepol treatment for 1 hour. Then, the explants washed thoroughly with double distilled water to remove the sterilizing agents. For further sterilization process, the explants treated with 0.1% mercuric chloride ($HgCl_2$) for 3-5 minutes that followed by washing with sterile double distilled water and 70 % ethanol.

Callogenesis

The sterile explants inoculated on selected culture media supplemented with proper concentrations of plant growth regulators. The MS media supplemented with different plant growth regulators including MS + BAP 0.5 mg/L+ IBA 0.5 mg/L, MS + BAP 1 mg/L+ IBA 1 mg/L, MS + BAP 0.5 mg/L + IBA 1 mg/L, MS + BAP 1 mg/L+ IBA 0.5 mg/L, MS + BAP 0.5 mg/L+ Kinetin 0.5 mg/L, MS + BAP 1 mg/L+ Kinetin 1 mg/L, MS + BAP 0.5 mg/L+ Kinetin 1 mg/L and MS + BAP 1 mg/L+ Kinetin 0.5 mg/L, were used to induce callus from the internodes. The prepared media sterilized at 121 °C for 30 minutes in an autoclave at the pressure of 15 psi. The pH of the medium maintained to 5.8 for optimum growth. The cultures kept under controlled conditions of light intensity, temperature and photoperiod which maintained in the incubation room.

In vitro Salinity Stress

The different concentrations of NaCl (0, 20, 40, 60, 80 and 100 mM) used to provide salt stress in *in vitro* culture. The best callus inducing and proliferating medium, MS + BAP 0.5 mg/L+ Kinetin 1 mg/L, with pH of 5.8 selected to supply the salinity stress. The cultures were maintained at 24 ± 2 °C with light intensity of 1500 $\mu Em^{-2}S^{-1}$ and photoperiod of 8 hours. The growth changes were observed and photographed weekly. The data analyzed statistically using single factor ANOVA.

Secondary Metabolite Analysis

The secondary metabolites in the hexane extract of the calli which were grown with and without NaCl stress analyzed using GC-MS method. The analysis carried out using the instrument method - C:/Xcaliber/RCE KKD/DRUGS//METHOD/NAT. PRODUCTS. Meth - from Department of Applied Chemistry, KFRI (Kerala Forest Research Institute), Peechi, Trissur, Kerala, India.

RESULTS AND DISCUSSION

Callus Induction

The valuable medicinal plant, *A. precatorius*, is using to treat different types of human diseases due to the presence of different secondary metabolites. While the hard seed coat and difficulty in vegetative propagation is the major problem to produce the healthy seedlings in bulk. To overcome the issue, the tissue culture technique is highly useful nowadays. The present study reveals the callus regeneration from inter nodal explant within 14 days when cultured on MS medium fortified with BAP 0.5 mg/L + Kinetin 1 mg/L and BAP 1 mg/L + IBA 0.5 mg/L. The remaining media induced callus regeneration on explants within 21 days (Table 1). The specific concentration and combination of BAP, IBA and Kinetin is the basic reason for callogenesis. Each plant needs an optimum concentration of cytokinine and/or auxin for

Table 1: Fresh weight (gm), dry weight (gm) and biomass (gm) of calli that grown on different media. The values are mean ± SE, N = 5, significant at 5 % level using single factor ANOVA.

Compositions of media	Fresh weight (gm) + SE	Dry weight (gm) + SE	Callus biomass (gm) + SE
MS+BAP0.5mg/L+IBA0.5mg/L	1.84±0.01	0.75±0.02	1.43±0.02
MS+BAP1mg/L+IBA1mg/L	1.72±0.03	0.78±0.01	1.15±0.01
MS+BAP0.5mg/l+IBA1mg/L	1.88±0.01	0.73±0.01	1.57±0.03
MS+BAP1mg/L+IBA0.5mg/L	2.05±0.02	0.80±0.03	1.56±0.01
MS+BAP0.5mg/L+Kinetin0.5mg/L	1.76±0.03	0.80±0.006	1.20±0.02
MS+BAP1mg/L+Kinetin1mg/L	1.80±0.04	0.79±0.01	1.28±0.01
MS+BAP0.5mg/L+Kinetin1mg/L	2.08±0.01	0.72±0.02	1.89±0.04
MS+BAP1mg/L+Kinetin0.5mg/L	1.96±0.02	0.72±0.01	1.72±0.01
MS+BAP0.5mg/L+Kinetin1mg/L+NaCl20mM	1.24±0.02	0.59±0.02	1.10±0.03
MS+BAP0.5mg/L+Kinetin1mg/L+NaCl40mM	1.68±0.04	0.65±0.01	1.58±0.02
MS+BAP0.5mg/L+Kinetin1mg/L+NaCl60mM	1.06±0.05	0.46±0.002	1.30±0.01
MS+BAP0.5mg/L+Kinetin1mg/L+NaCl80mM	0.81±0.01	0.45±0.003	0.80±0.005
MS+BAP0.5mg/L+Kinetin1mg/L+NaCl100mM	0.47±0.04	0.29±0.001	0.62±0.01

callogenesis from the explants. In the investigation of Biswas *et al.* (2007), the callus developed from the cut surface of nodal explants that cultured on MS medium fortified with 5.0 mg/L BAP and 0.5 mg/L NAA. While the callus induction from nodal, intermodal and tendril explants of A. *precatorius* was successfully done by Ramar *et al.* (2018).

In the study, the callus was white in colour, compact and non-embryogenic initially; but gradually it turned to green (Figure 1). In contrast, the formation of yellowish and compact callus from the nodal segments of A. *precatorius* on MS + 2,4-D 1 mg/L + NAA 1 mg/L reported by Hassan *et al.* (2009) in which the different concentration of auxins induced the callus formation. The callus is the mass of undifferentiated tissue that in turn regenerates the *in vitro* plantlets. The nature of the callus, compact or friable/embryogenic or non-embryogenic, depends to the plant species. Within 63 days, the callus grown on MS medium supplemented with BAP 0.5 mg/L + Kinetin 1 mg/L and BAP 1 mg/L + IBA 0.5 mg/L showed maximum rate of fresh weight (FW) and dry weight (DW). It proves the higher callus induction and proliferation potential in presence of specific media composition. As the age of calli increased, the colour changed gradually from green to yellowish-brown. The browning of calli may be due to nutrient depletion or accumulation of phenolic compounds in cells. Sometimes, these cells get ruptured by higher plasmolysis that causes releasing of phenolics into the media (Jones and Saxena, 2013).

In vitro Salt Stress

In vitro production of salt tolerant lines has a significant role in agriculture and pharmacognosy. The regenerated plants can overcome the salinity stress by the presence of an internal genetic mechanism that controls the salt tolerant plant metabolism (Tuteja, 2007). Typically, the salt stress declines the callus induction and regeneration frequency in salt sensitive plants. But, some plants can resist the negative influence of *in vitro* salinity and regenerate the salt tolerant lines (Basu *et al*., 2002).

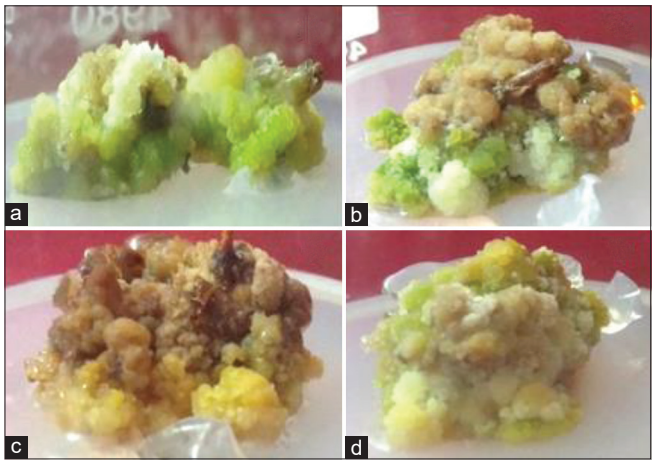

Figure 1: Callus induction from the internodes of *A. precatorius* on different culture media; a. MS + BAP 0.5 mg/L + Kinetin 1 mg/L, b. MS + BAP 1 mg/L + IBA 0.5 mg/L, c. MS + BAP 1 mg/L + Kinetin 0.5 mg/L and d. MS + BAP 0.5 mg/L + IBA 1 mg/L

In the study, the salinity brings about the morphological and biochemical changes in calli that were sub-cultured on MS medium supplemented with BAP 0.5 mg/L, Kinetin 1 mg/L and different concentrations of NaCl (20, 40, 60, 80 and 100 mM). The proliferation, fresh weight, dry weight and biomass of calli were enhanced when the calli cultured on MS medium fortified with NaCl concentration up to 40 mM (Table 1). Then the calli proliferation gradually decreased when the concentration of NaCl increased from 40 mM to 100 mM. Now, many of the recent studies have focused on the regeneration of salt tolerant rice varieties viz., Navara, IR-64, SR-26B, Chini Kanai, BRRI Dhan 38 etc. through the tissue culture techniques in which the plantlets urged the genetic potential to survive under salt stress condition (Basu *et al*., 2002; Priya *et al*., 2011; Zinnah *et al*., 2013). Furthermore, the reduction of proliferation frequency and fresh weight of *in vitro* calli under salinity stress observed in different medicinal plants (Patade *et al*., 2008). The influence of salinity leads to the colour variations in calli. The calli of A. *precatorius* grown on medium supplemented with NaCl upto 40 mM concentration showed yellow colour with compact and non-embryogenic consistency. While the yellowish callus changed to brown colour when the concentration of NaCl increased from 60 mM to 100 mM (Figure 2).

Most of the studies related to salinity stress encompass the darkening of calli. It may due to the unavailability of water for proper metabolic activities. Meanwhile, the higher concentration of NaCl has an influence on the secondary metabolism that results in the amelioration of medicinally important metabolite production compared to the calli cultured without salt stress (Hasegawa *et al*., 2000; Zhu, 2001). The presence of these metabolites also causes the browning of calli. To avoid the negative impact of soil salinity on agriculture, the regeneration of salt tolerant plantlets is significant which can be done by the application of appropriate ratio of auxins and cytokinins in the culture medium of salt tolerant calli.

Secondary Metabolite Production

Application of salt stress in *in vitro* culture is a better method to produce valuable secondary metabolites with medicinal value. In the pharmaceutical industry, there are different methods to isolate and detect secondary metabolites from plant extracts. GC-MS analysis is one of the advanced techniques to identify different organic metabolites from the extracts of medicinal plants (Safaei-Ghomi *et al*., 2009). In the study, the secondary compounds of calli which cultured on medium with and without NaCl stress were detected using the GC-MS method. The number of detected compounds was higher in callus grown under NaCl stress compared to the calli cultured without NaCl. The enhancement of secondary metabolite production under salt stress is detected in many of the *in vitro* plant cultures (Jaleel *et al*., 2008; Rishla *et al*., 2017). In *Carthamus tinctorius* L., the supplementation of NaCl below 100 mM concentration in Hoagland solution improved the production of medicinal flavonoids (Gengmao *et al*., 2015). Presently, seventeen compounds were analyzed from the salt tolerant callus of A. *precatorius*; of which the

compound, 5-ethyl-1-nonene showed the lowest retention time (RT), 4.24. While, the highest RT exhibited by 1,1,1-trifluoroheptadecen-2-one, 33.19. Retention time indicates the time taken by the organic compounds/solutes to pass through the chromatography column that influenced by many factors including column length, column degradation, gas flow rate etc. (Ezhilan and Neelamegam, 2012). Among the compounds, stigmasterol showed the highest area percentage (33.94%) and the lowest area percentage by ethyl palmitate (0.43%) Table 2, Figure 3 & 4). Interestingly, thirteen new

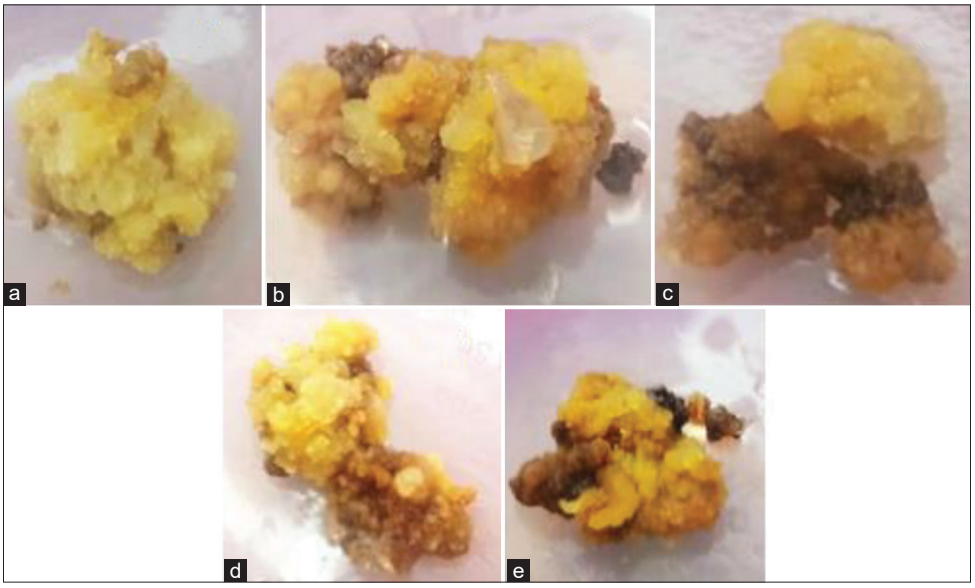

Figure 2: Callus proliferation of *A. precatorius* on MS + BAP 0.5 mg/L + Kinetin 1 mg/L supplemented with different concentrations of NaCl (20 (a), 40 (b), 60 (c), 80 (d) and 100 (e) mM)

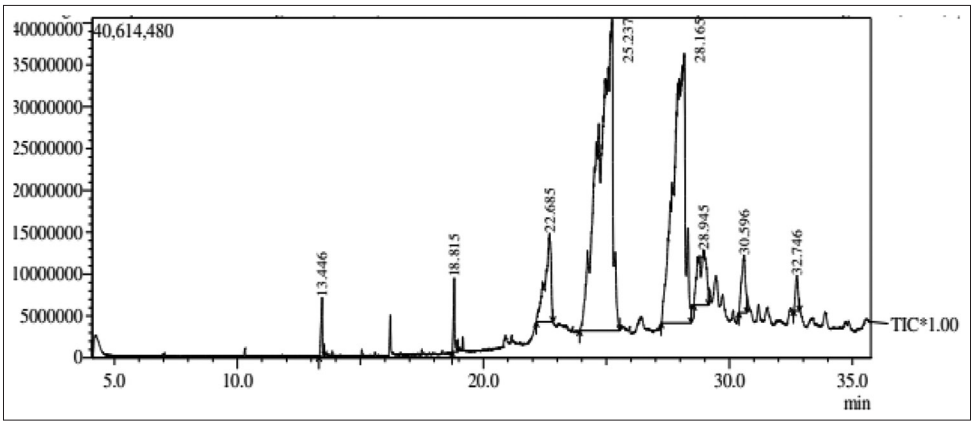

Figure 3: GC-MS chromatogram of compounds observed in callus grown without NaCl stress

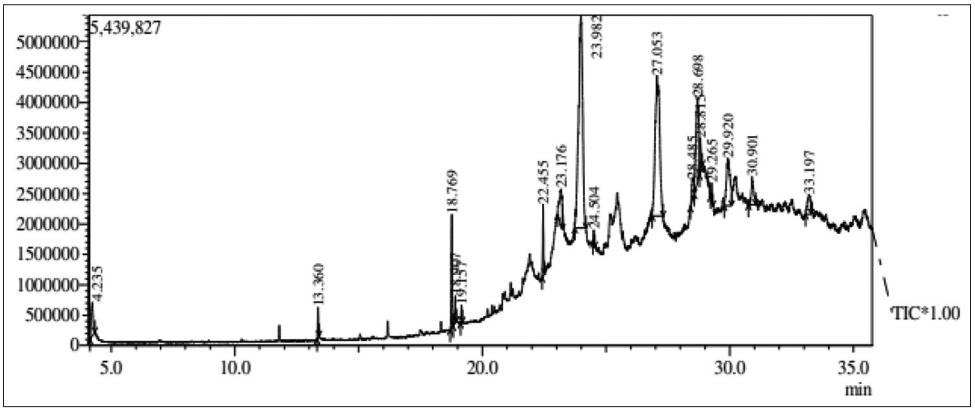

Figure 4: GC-MS chromatogram of compounds observed in callus grown with NaCl stress

Table 2: Compounds analyzed from callus grown with and without NaCl stress using GC-MS.

Sl. No.	Retention Time	Area %	Compounds	Nature of callus
1	13.45	0.89	Dodecanoic acid	
2	18.82	0.98	Pentadecanoic acid	
3	22.69	5.79	Campesterol	
4	25.24	49.18	Stigmasterol	Callus grown without NaCl
5	28.17	34.85	beta-Sitosterol	
6	28.95	4.93	beta-Amyrin	
7	30.60	2.39	Spinasterone	
8	32.75	0.99	Sitostenone	
1	4.24	2.72	5-Ethyl-1-nonene	
2	13.36	0.79	Dodecanoic acid	
3	18.77	3.90	Pentadecanoic acid	
4	18.91	1.14	Dibutyl phthalate	
5	19.16	0.43	Ethyl palmitate	
6	22.46	1.92	trans-Pinosylvin dimethyl ether	
7	23.18	3.83	Stigmasterol methyl ether	
8	23.98	33.94	Stigmasterol	Callus grown with NaCl
9	24.50	0.46	Pinostrobin chalcone	
10	27.05	24.81	Gamma-Sitosterol	
11	28.49	1.76	Beta-Amyrin	
12	28.70	10.00	5 Alpha-Stigmastane-3,6-dione	
13	28.82	1.77	Illudol	
14	29.27	0.69	Squalene	
15	29.92	6.82	Citrost-7-en-3-ol	
16	30.90	2.52	Beta-Saccharostenone	
17	33.19	2.49	1,1,1-Trifluoroheptadecen-2-one	

compounds were detected from the salt tolerant callus that was not observed in the callus grown without NaCl stress. The type and concentration of secondary metabolites produced by an *in vitro* plant is determined by its genotype, physiology, developmental stage and environmental factors during growth (Isah, 2019). Moreover, the different environmental factors viz. temperature, light intensity, the supply of water and minerals etc., have a considerable role in secondary metabolite production (Ramakrishna and Ravishankar, 2011). Though, eight different secondary metabolites were analyzed in calli cultured on medium without NaCl to which dodecanoic acid showed the lesser RT, 13.45 and sitostenone exhibited higher value. In the analysis, stigmasterol and dodecanoic acid were with higher and lower area percentage respectively (Table 2). As reported by many researchers, the detected metabolites in calli of A. *precatorius* show antimicrobial, antioxidant and anti-inflammatory activities (Agboke, 2015; Parasuraman *et al*., 2016; Patel *et al*., 2016). Remarkably, the present study will be very much helpful in pharmacology to produce medicinally significant compounds in future.

CONCLUSION

The white seeded A. *precatorius* is an important source of organic compounds with therapeutic effects. *In vitro* culture plays as the best method to multiply the species. The MS medium supplemented with BAP 0.5 mg/L + Kinetin 1 mg/L and BAP 1 mg/L + IBA 0.5 mg/L showed better callus induction within a short period. These green compact non-embryogenic calli were with highest fresh weight, dry weight and biomass. The presence of NaCl negatively influenced on callus proliferation of which calli cultured with 40 mM NaCl exhibited a greater degree of callus biomass. This salt tolerant calli is the source of 17 different medicinally important metabolic compounds that may be a new breakthrough in the field of drug discovery.

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