Journal of Experimental Biology and Agricultural Sciences, October - 2024; Volume – 12(5) page 705 – 729

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

Effect of salinity stress on antioxidant activity and secondary metabolites of *Piper betle*

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Received – August 06, 2024; Revision – November 04, 2024; Accepted – November 21, 2024
Available Online – November 29, 2024

DOI: http://dx.doi.org/10.18006/2024.12(5).705.729

KEYWORDS

Betel vine

Salinity stress

Antioxidant activity

GC-MS

Secondary metabolites

ABSTRACT

Salt stress is the most devastating abiotic stress that drastically limits the productivity and quality of crops. This study assessed the impact of NaCl concentrations (100, 200, and 400 mM) on betel vine's antioxidant activities and secondary metabolites (*Piper betle* L.). Results of the study suggest that the activity of antioxidative enzymes was enhanced at 100 and 200 mM NaCl levels but reduced at 400 mM NaCl. Further, the GC-MS analysis revealed the increased production of secondary metabolites such as alkane, ester, fatty acid, phenolic, and terpene compounds during salt stress. These findings would be helpful for further investigations that could lead to enhanced production of secondary metabolites in betel vine for industrial and medicinal benefits.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

Salinity affects the plant's phenotype, physiology, and metabolism. It can restrict plant development by disturbing photosynthesis, photorespiration, respiration, nutrient homeostasis, and energy generation, resulting in an imbalance of redox homeostasis, osmotic stress, and ion toxicity (Zhou et al. 2024). Additionally, salinity stress produces oxidative damage via increasing reactive oxygen species (ROS) and ROS levels above a toxicity threshold, resulting in cell death. Plants employ numerous defence strategies against salt-induced oxidative stress at morphological, physiological, and metabolic levels. For this, plants generate a diverse range of enzymatic (CAT, APX, SOD, and GPX) and non-enzymatic antioxidants (GSH, ASC, and carotenoids) to scavenge ROS (Hatami and Ghorbanpour 2024). Further, plants can synthesize key classes of volatile fatty acid derivatives, benzenoids, phenolics, terpenes, and phenylpropanoids to eliminate ROS under salinity stress.

The betel vine is a major perennial medicinal herb that encircles famous flavourings and is edible worldwide. There are over 2000 species in the genus *Piper*, and 21 of these are evergreen vines that may be recognized in India (Sen and Rengaian 2021). Betel vine contains secondary metabolites such as phenolics, alkanes, esters, essential oils (EO), fatty acids, and amino acid derivatives, which are involved in various pharmacological activities. For instance, phenolic metabolites have anti-inflammatory, anti-thrombotic, antimicrobial, antioxidant, and cardioprotective properties (Kumar et al. 2024). The constituents of betel vine EO (4-allyl-1, 2-diacetoxybenzene, chavicol, acetyleugenol, eugenol, bicycle (4.1.0), hept-3-en-camphene, germacrene B, 4-methyl-decanal, cis-ocimene, germacrene, and cyclohexene) also have anti-inflammatory, antimicrobial, and antioxidant properties capacities (Biswas et al. 2022).

The secondary metabolites, such as phenolics, alkanes, EOs, esters, terpenoids, and fatty acids, are produced in plants during exposure to heavy metals, salt stress, light intensity, and drought stress (Ait Elallem et al. 2024). Flavonoids, coumarins, phenolics, and lignins accumulate in plants during salt stress. The EO compounds (menthol, cineole, and neomenthol) also exhibit antioxidant activity in *Mentha piperita* under drought stress (Lala 2021). Bistgani et al. (2019) suggested that salinity stress enhanced the level of EO (such as oxygenated monoterpenes like α-thujone and 1,8-cineole), carotenoids and flavonoids, and volatile compounds like γ-cadinene and α-cadinol synthesis in marigold (*Calendula officinalis*).

Although the detrimental impacts of salinity on various phenotypical traits, physiological processes, and biochemical processes of betel plants have been well documented, the current investigation was carried out to evaluate the effect of salt stress on antioxidant activity and biosynthesis of secondary metabolites in betel vine.

2 Materials and Methods

2.1 Analysis of leaf disc senescence and chlorophyll content

Healthy betel vine leaves were chopped into small discs with a diameter of 1 cm and incubated in distilled water (DW) containing NaCl (200, 400, and 600 mM), $CdCl_2$ (10, 20, and 40 mM), and mannitol (200, 400, and 600 mM) for 3–4 days, respectively. Leaf discs were subjected to cold stress for 5 days at 4ºC and heat stress for 10 h at 42ºC. Leaf discs kept in DW were used as the control. After an extraction of 80% acetone, the chlorophyll concentration was measured by spectrophotometry. The equation below was used for calculating the amount of chlorophyll (Sahu et al. 2024a):

$$\text{Chl } a \text{ (mg ml}^{-1}) = -1.93A_{646} + 11.93A_{663}$$

$$\text{Chl } b \text{ (mg ml}^{-1}) = 20.36A_{646} - 5.50A_{663}$$

$$\text{Total Chl (mg ml}^{-1}) = 6.43A_{663} + 18.43A_{646}$$

2.2 Testing of betel vine plants under salt stress

The 4-week-old plants were grown under a 16/8 h light-dark period at 36±2°C and 28±2°C temperature and relative humidity of 45±5% and 63±5%, respectively. These plants were irrigated with 100, 200, and 400 mM NaCl solutions, and control plants were irrigated with Hoagland nutrient solution only. Three plants from each salt-treated and control line were taken for physio-biochemical analysis.

2.3 Histochemical detection and estimation of $O_2^{\bullet-}$, H_2O_2, and cell death

Daudi and O'Brien (2012) used the protocol for the histochemical localization of H_2O_2 in leaf tissues. Healthy leaves from each experimental setup were dipped in a DAB solution (1mg ml^{-1}, pH=4.0) in a sterilized glass beaker for 12 h under light at room temperature (RT). To bleach the leaves, they were boiled for 20 min after being immersed in 95% ethanol. After cooling, the localized H_2O_2 was observed as brown spots under a light microscope.

The $O_2^{\bullet-}$ content was also determined by following the protocol of Kumari et al. (2015), which involved immersing leaf tissues for 1 h in 3 ml of a 10 mM phosphate buffer (pH=7.8) containing 10 mM sodium azide and 0.05% nitroblue tetrazolium chloride (NBT). The tissue extracts were boiled for 15 min at 85ºC, and the optical density (OD) was measured at 580 nm by spectrophotometry and expressed in µmol g^{-1}f.w.

The protocol of Noreen et al. (2009) was utilized to estimate the H_2O_2 content. Each sample's leaf tissues weighed 0.1 g, then separately ground with 2 ml of 0.1% trichloroacetic acid. The homogenates were centrifuged for 15 min at 12,000 rpm. After that,

the reaction mixture, which consisted of 1 ml of 1 M KI, 0.5 ml of 10 mM phosphate buffer (pH=7.0), and 0.5 ml of the supernatant, was made and measured at 390 nm by spectrophotometer. The H_2O_2 content was expressed in µmol g^{-1}f.w.

Trypan blue was used to localize the dead cells following the Kerschbaum et al. (2021) protocol. For 1 min at RT, the leaves were merged in 40 ml of 0.01 g of trypan blue. In order to see the polymerized blue color as dead cells, the leaves were washed using a washing solution [ethanol and water (1:1)]. Gölge and Vardar (2020) method was used for the estimation of cell death content. Sterilized test tubes with 10 ml of 0.25 % Evans blue were used to dip the leaf tissues. The aliquot was measured at 600 nm and expressed in µg ml^{-1} using spectrophotometry.

2.4 Assay of enzymatic antioxidants

The SOD activity of each sample was determined utilizing the NBT protocol of Kumari et al. (2015). 2 ml of 50 mM phosphate buffer (containing 2% PVP and 1 mM EDTA, pH=7.0) was used to homogenize 0.5 g of leaf tissue. The homogenate was centrifuged for 20 min at 4ºC and 13,000 rpm. Next, 50 µl of the extracted enzyme was poured into sterile test tubes together with 50 mM phosphate buffer (0.3 ml of 750 µM NBT, 0.3 ml of 130 mM methionine, 0.3 ml of 20 µM riboflavin, 0.3 ml of 10 mM EDTA, and 0.25 ml of DW). For 10 min, the test tubes were kept beneath a mercury lamp. Using spectrophotometry, the OD was measured at 560 nm. The number of enzymes necessary to block the photoreduction activity of the NBT by 50% was denoted by one unit (U) of SOD activity. The SOD activity was denoted as U g^{-1}f.w. and estimated by the given formula:

% of inhibition= [1- Absorbance of each sample / Absorbance of the control] × 100

The CAT activity was determined by the method of Zhang et al. (2021). 0.5 g of leaf tissue was homogenized with 2 ml of 50 mM phosphate buffer (pH=7.0) (containing 1 mM EDTA and 2% PVP). The extracts were centrifuged at 13,000 rpm for 20 min at 4ºC. For the experiment, 2.9 ml of 50 mM enzyme extract and 50 µl of 30 mM H_2O_2 were mixed to prepare a reaction mixture in a cuvette. The decreased OD was estimated at 240 nm for 3 min by spectrophotometry and expressed in U g^{-1}f.w.

The APX activity was estimated by the protocol of Sahu et al. (2024b). 1 ml of the reaction mixture [contained 600 µl of 50 mM phosphate buffer solution (pH=7.0),100 µl of 1 mM EDTA, 100 µl of 5 mM ascorbic acid, 100 µl of H_2O_2, and 100 µl of the enzyme extracts] were taken in a cuvette. The decreased OD was recorded at 290 nm by spectrophotometry and expressed in U g^{-1} f.w. The enzyme is required to decrease 1 µmol of H_2O_2 min^{-1} under the same condition determined to be 1U of APX activity.

The GPX activity was estimated using the Techer et al. (2015) method. 0.5 g of leaf tissues was homogenized with a 50 mM PBS buffer solution (pH=7.4) that contained 2% PVP and 1 µM EDTA. The homogenates were centrifuged at 13,000 rpm for 20 min at 4ºC, and the reaction mixture (contained enzyme extract, 30 mM guaiacol, 40 mM H_2O_2, and 50 mM PBS buffer) was kept for 30 min. Then, the OD was taken at 470 nm by spectrophotometry, and the enzyme activity was expressed in U g^{-1}f.w.

2.5 Analysis of stomata

The leaf specimens were taken and processed for scanning electron microscopy (SEM) to measure the stomatal density and length of the aperture of the stomata. Leaf materials were chopped into 5-10 mm long sections, dehydrated in various amounts of ethanol (50, 70, 90, and 100%) for 30 min each, then air dried for 12 h at RT. Then the leaf sections were attached to aluminium stubs with electron-conductive carbon cement. The length of the stomatal aperture was determined by Image J software. The stomatal density (SD) was calculated by counting the number of stomas per unit of leaf surface area using the following formula (Paul et al. 2017):

$$SD = \text{Number of stomata} \times 1mm^2 / \text{Area of the field of view } (\pi r^2)$$

Leaves were chopped into discs (1 cm) and kept in DW for 1 d. The discs were dried for 4 h at 70ºC. For every experiment, 6 discs were selected. The relative water content (RWC) was also determined by the given formula (Tahjib-Ul-Arif et al. 2018):

RWC (%) = (fresh weight – dry weight) / (turgid weight – dry weight) × 100.

2.6 Identification of volatile compounds by GC-MS

The betel vine leaf extraction for GC-MS analysis was performed following the procedure of Annegowda et al. (2013). Leaf tissue (1.0 g) was homogenized with 1ml of methanol and ethyl acetate, and the homogenates were purified, evaporated at RT, and mixed again with methanol and ethyl acetate solutions. The samples were analyzed using Shimadzu GC-MS QP 2020 NX GC-MS instrument (operating conditions: SH-Rxi-5ms capillary standard non-polar column, 30 m length, ID: 0.25 mm, film thickness: 0.25 m). Helium was used as a carrier gas, and the mobile phase flow rate was set (1.0 ml/min). The injected quantity was 1 µl, and the heat setting range (Oven temperature) in the gas chromatography section was 35°C to 280°C at 5°C/min. The mass spectrometer was in EI/PCI/NCI mode, with a mass range of 35–650 m/z and a scan speed of 20,000 amu/s. The detector MS collected the data from FASST, AART, COAST, and LRI. Compared to the NIST20 library reference compounds, the non-polar compounds were identified, which used SH-Rxi-5ms capillary as a standard non-polar column.

2.7 Statistical analysis

Every assay was run separately and three times independently. All the biochemical data were statistically assessed by ANOVA, followed by a post hoc test with significance at $p \leq 0.05$ (*). Data was given as mean ± standard deviation (SD). The graph was performed using the GraphPad Prism software.

3 Results and Discussion

Development and productivity are highly affected by salinity stress. The novelty of this work is to provide new information on how salt stress modulates physiological and biochemical processes with secondary metabolite production.

3.1 Effects of abiotic stress on chlorophyll content

The abiotic stress tolerance of betel vine plants was assessed using the leaf disc senescence assay. The betel vine leaf discs were exposed to various abiotic stresses such as NaCl, mannitol, $CdCl_2$, and temperature (4ºC and 42ºC). The chlorophyll content of leaf discs from each stress was measured. Abiotic stress triggered oxidative stress, which resulted in a reduction in chlorophyll content.

One important physiological-chemical process extremely vulnerable to stress is photosynthesis, especially in salinity stress. Salt stress significantly lowers pigment concentration, stomatal conductance, and photosystem II activity. The photosynthetic mechanism uses both Chl *a* and *b* as main pigments, but their biosynthesis is inhibited under salt stress, chlorophyll breakdown increases, or both occur. The contents of Chl *a*, Chl *b*, and total chlorophyll varied during salt stress (Figure 1a). The 200 mM NaCl treatment decreased the photosynthetic pigments (Chl *a* by 49.09%, Chl *b* by 10.00%, and total chlorophyll by 48.27%), whereas 400 and 600 mM NaCl treatments showed relatively high reduction in photosynthetic pigments (Chl *a* by 79.09%, Chl *b* by 50.00%, and total chlorophyll by 69.82%) and (Chl *a* by 86.36%, Chl *b* by 83.33%, total chlorophyll by 87.06%), respectively, as compared to control. This is reliable, as previous findings show lower photosynthetic pigment levels in *Capsicum annuum* (Kusvuran et al. 2024).

Similar types of results were found in other abiotic stresses. Figure 1b shows a high reduction in content of Chl *a* by 73.31 to 91.66%, Chl *b* by 20.0 to 90.0%, and total chlorophyll by 60.00 to 90.00% during 10 to 40 mM $CdCl_2$ stress. There was a nearly complete loss of chlorophyll under 40 mM $CdCl_2$ stress. Similarly, the Cd stress reduced chlorophyll pigments in *Triticum aestivum* (Farhat et al. 2022) due to reduced size and number of chloroplasts, chlorophyll disintegration, and impaired photosystems. Figure 1c also showed high reductions in the content of Chl *a* by 26.66% to 55.83%, Chl *b* by 10.0% to 60.0%, and total chlorophyll by 25.20% to 56.91% after exposure to 200 to 600 mM mannitol stress as compared to control. In general, mannitol-induced drought stress also reduces the chlorophyll content due to inhibition of photosynthetic enzymes, disruption of the thylakoid membrane, and inhibition of PS-I and PS-II in *Apocynum pictum* and *A. venetum* (Yang et al. 2021). Furthermore, exposure to cold (4ºC) and heat (42ºC) stress significantly reduced chlorophylls in the leaves of betel vine (Chl *a* by 73.77%, Chl *b* by 60.00%, and total chlorophyll by 68.55%) and (Chl *a* by 86.88%, Chl *b* by 75.07%, and total chlorophyll by 88.24%), respectively, as compared to control (Figure 1d, e). In the current findings, a negative relationship has been found between chlorophyll content and abiotic stress; this suggests that the abiotic stress causes the breakdown of photosynthetic pigments in betel vine. Similar reports have been found in tomato (Mesa et al. 2022) as well as in rice (Li et al. 2024) plants due to disruption of photosynthetic components, carbon reduction cycles, and impaired chlorophyll molecules under both cold and heat stress (Chaudhry and Sidhu 2022). It is concluded that reductions in chlorophyll content lead to a decline in plant development and the creation of dry materials.

3.2 Effect of salt stress on ROS scavenging enzymes and stomata

Plants hold different antioxidant molecules to endure both biotic and abiotic stresses. The salt stress interferes with the homeostasis between ROS scavenging and formation, leading to oxidative bursts in cellular organelles, including chloroplasts, mitochondria, and peroxisomes. ROS occurs in various biological signaling pathways at low or high levels in the cells.

The performance of plants under prolonged exposure to salt stress was also tested. The salt-treated plants showed curling and yellowing leaves, wilting, and dropping characteristics compared to control plants (Figure 2a). The salinity caused a significant formation of reddish-brown spots (an indicator of H_2O_2) in leaves (Figure 2b), which was followed by an increase in H_2O_2 content in the leaves; equivalent outcomes in rice have been found (Mostofa et al. 2015). Moreover, the salt-treated leaves exhibited more accumulation of blue-colored spots (an indicator of cell death) than the control (Figure 2c), which was also significant with the increment in cell death content. A rise in H_2O_2 content (27.72, 45.54, and 60.41 µmol) and $O_2^{\bullet-}$ content (23.80, 36.02, and 44.96 µmol) in betel vine leaves exposed to 100 mM, 200 mM, and 400 mM NaCl stress, respectively than the control (Figure 2d, e). H_2O_2 and $O_2^{\bullet-}$ are the prime signaling molecules, taking crucial functions in various metabolic pathways during salt stress; they induce stress-responsive pathways. In extreme conditions, salt stress leads to the overproduction of ROS because the mitochondria's and chloroplasts' electron transport chains (ETCs) are damaged (Hameed et al. 2021). The findings of the betel vine were consistent with rice and *Hibiscus cannabinus* (Hatami and Ghorbanpour 2024). The betel vine leaves exhibited cell death 0.55, 1.09, and 1.76 µg in 100 mM, 200 mM, and 400 mM NaCl-induced salt stress compared to the control (Figure 2f).

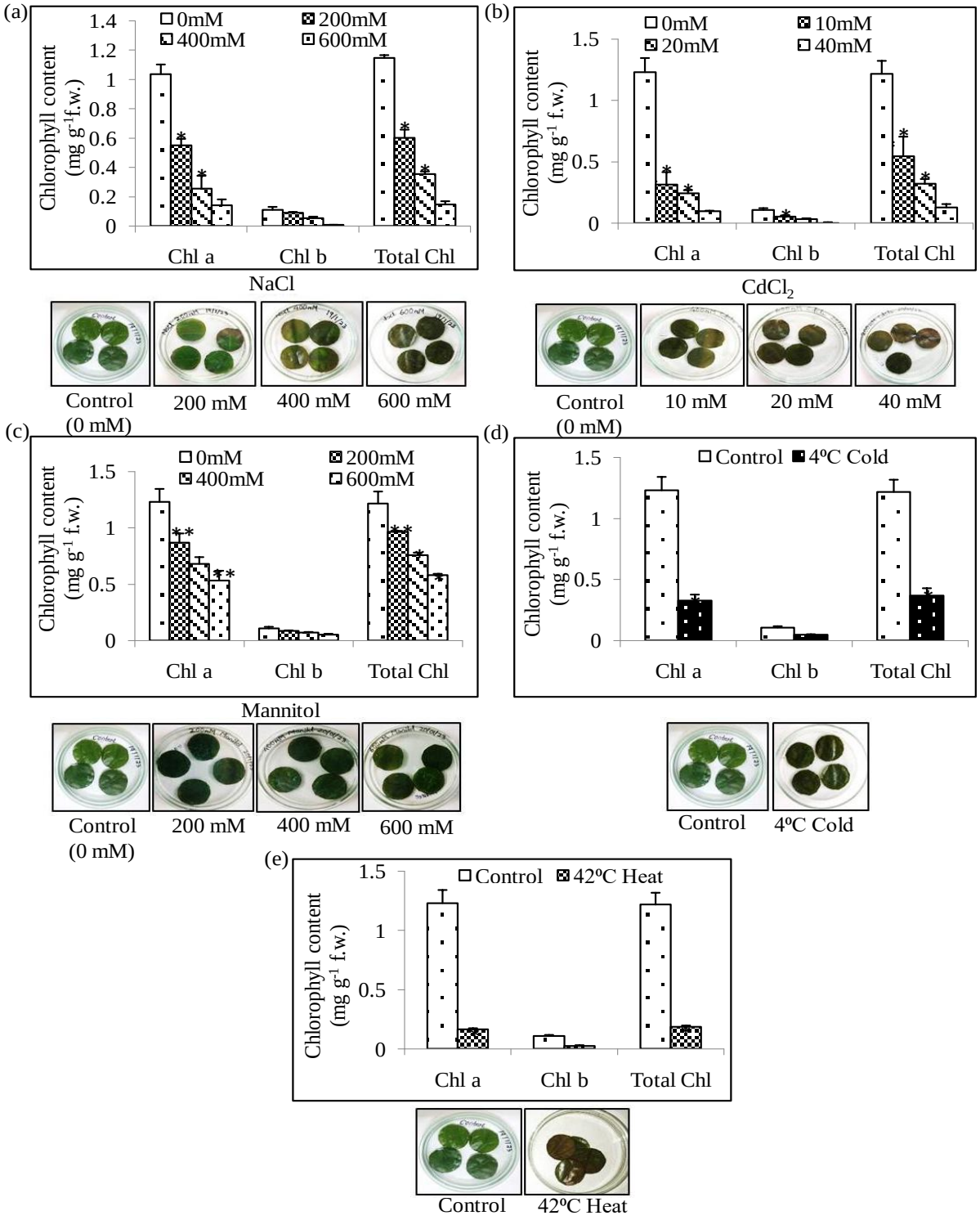


Figure 1 Assessment of betel vine plants for their tolerance towards various abiotic stresses. Leaf disc senescence assays for abiotic stress tolerance in betel vine plants. Leaf discs kept in Hoagland solution served as the experimental control. The chlorophyll content (mg g^{-1}f.w.) retained in corresponding leaf discs shown by histograms, (a) NaCl, (b) $CdCl_2$ (c) Mannitol, (d) 4°C Cold, and (e) 42°C Heat. The data represents mean ± SD of three biological replicates. *, ** denote significance at p≤0.05 and p≤0.001, respectively.

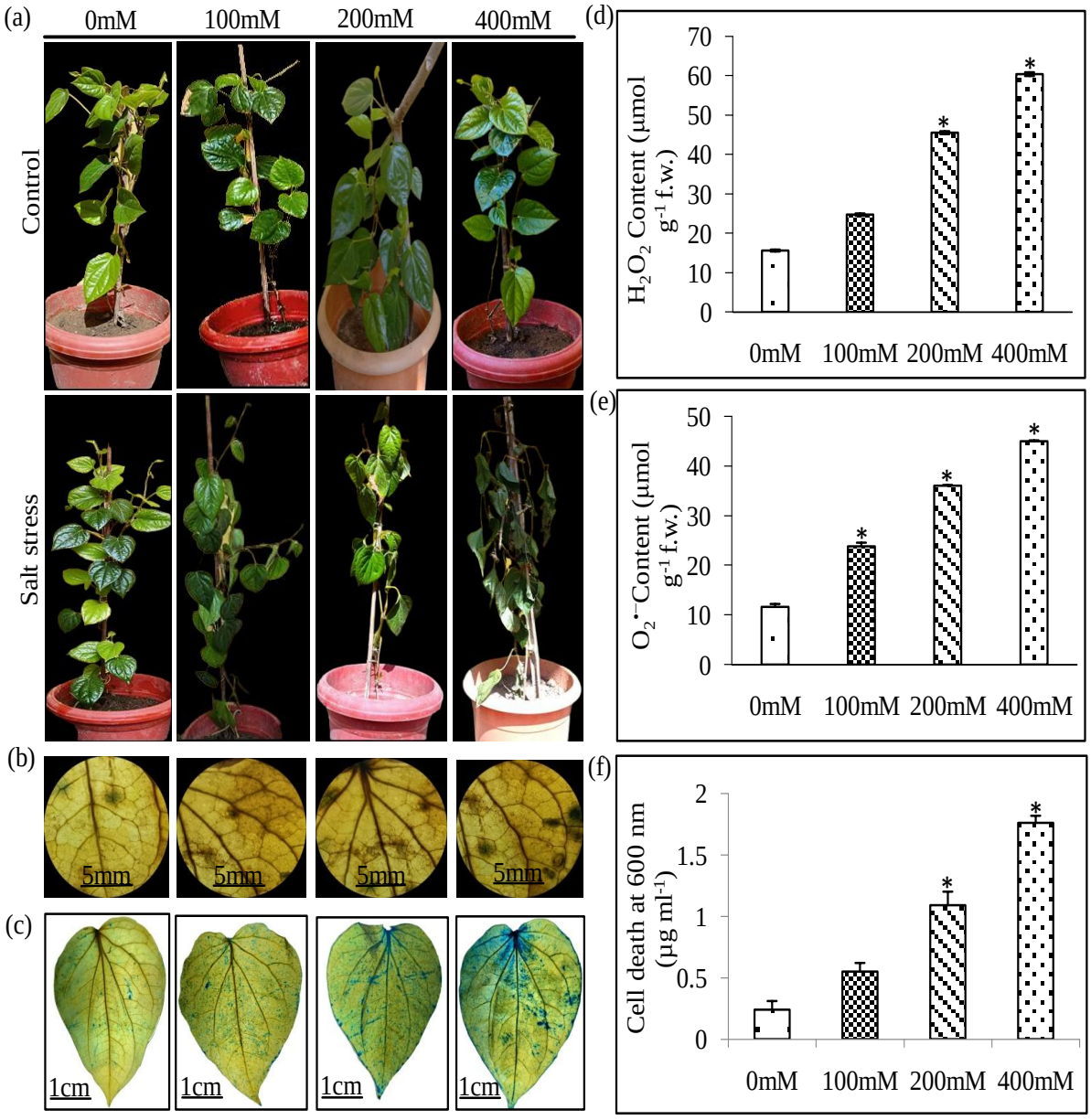


Figure 2 Effect of exposure of betel vine plants to salinity stress, (a) *Control panel* shows the phenotype of betel vine plants under non-stress condition. *Salt stress panel* shows the phenotypes of betel vine plants under salinity stress, (b) Histochemical localization of H_2O_2, (c) Histochemical localization of dead cells, (d) $O_2^{\bullet-}$ content, (e) H_2O_2 content, (f) Cell death content. The data represents mean ± SD of three biological replicates. *denote significance at $p \leq 0.05$, respectively.

To inhibit the generation of ROS, plants have a complex network that is systematically organized and comprises signalling components. Plants have dynamic enzymatic antioxidant machinery required to reduce ROS under stress. SOD is one of those enzymatic antioxidants that catalyses the transformation of $O_2^{\bullet-}$ into H_2O_2. Then, H_2O_2 is detoxified by CAT, APX, and GPX. All of these enzymes' activities, i.e., SOD, CAT, APX, and GPX, were increased by 7.49 to 16.39, 7.25 to 17.64, 0.08 to 0.15, and 1.8 to 2.68 U, respectively, in 0 mM to 200 mM of NaCl (Figure 3a, b, c, and d). These reports are concomitant with earlier reports that showed increased SOD in wheat seedlings (Esfandiari et al. 2007), *Solanum melongena* (Shumaila Ullah and Nafees 2023), CAT activity in *Cymbopogon nardus*, *Cynodond actylon*, and *Pennisetum alopecuroides* (Mane et al. 2010), APX in sweet

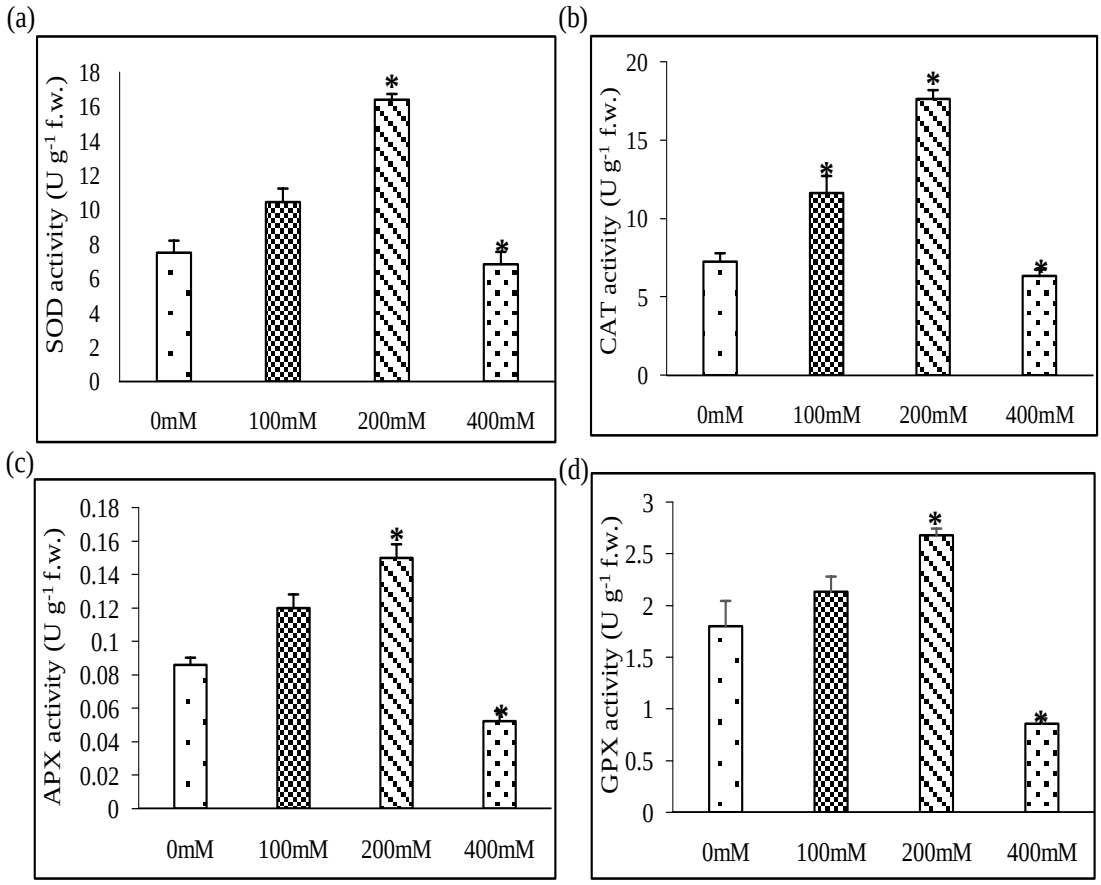


Figure 3 Antioxidant enzymes in the leaf of betel vine plant, (a) SOD activity, (b) CAT activity, (c) APX activity, and (d) GPX activity. The data represents mean± SD of three biological replicates. *denote significance at p≤0.05, respectively.

potato cultivars (Lin and Pu 2010), and GPX in foxtail millet under salt stress (Rathinapriya et al. 2020). The increased antioxidant enzymes might detoxify or prevent the detrimental effect of ROS in betel vine leaves under 100 and 200 mM NaCl. Moreover, these enzymes are prone to high oxidative stress, which affects their structural integrity and functions at the cellular level. In the current investigation, the betel vine leaves subjected to 400 mM NaCl stress showed decreased antioxidant enzyme activity. The *Sesuvium portulacastrum* (Kannan et al. 2013) and Tabarka variety *Cakile maritima* (Amor et al. 2006) showed decreased antioxidant enzyme activities. These findings suggest that under 100 and 200 mM NaCl stress, the betel vine plant preserved redox equilibrium by inhibiting enzyme denaturation and triggering the antioxidant machinery, but above that threshold, an imbalance occurred between the ROS-generating and scavenging systems.

The stomata are the primary structure in charge of gas and water exchange; variations in stomatal density and size can help control water consumption efficiency. Salt stress reduces leaf size, cell expansion, and transpiration flux in plants. The micrographs revealed that stomatal density and the length of the stomatal aperture were drastically reduced with increasing salt concentrations (Figure 4a). The number of stomata was highly reduced in 400 mM NaCl-treated leaves. The stomatal numbers also decrease in *Populus alba* (Hasanuzzaman et al. 2009). The stomatal density was reduced by 32.3, 25, and 13.66 numbers in 100 mM, 200 mM, and 400 mM NaCl-treated betel vine leaves as compared to control, respectively (Figure 4b) and also in *Ocimum basilicum* plants (Barbieri et al. 2012). Moreover, a reduced length of stomatal aperture was also observed at 24.16, 21.66, and 13.38 µm in betel vine leaves in comparison to the control condition when exposed to 100 mM, 200 mM, and 400 mM NaCl salt stress, respectively (Figure 4c). It occurs due to disruption in starch-sugar conversion, and lower water content reduces turgor pressure in leaf tissues. Under salt stress conditions, abscisic acid (ABA) also induces stomatal closure and a reduced transpiration rate to prevent excessive water loss. Some amorphous substances were found in the salt-treated leaves compared to the control and were also reported as salt particles in *Allium cepa* during salt stress (Burkhardt 2010). In addition, the perturbation of stomatal opening

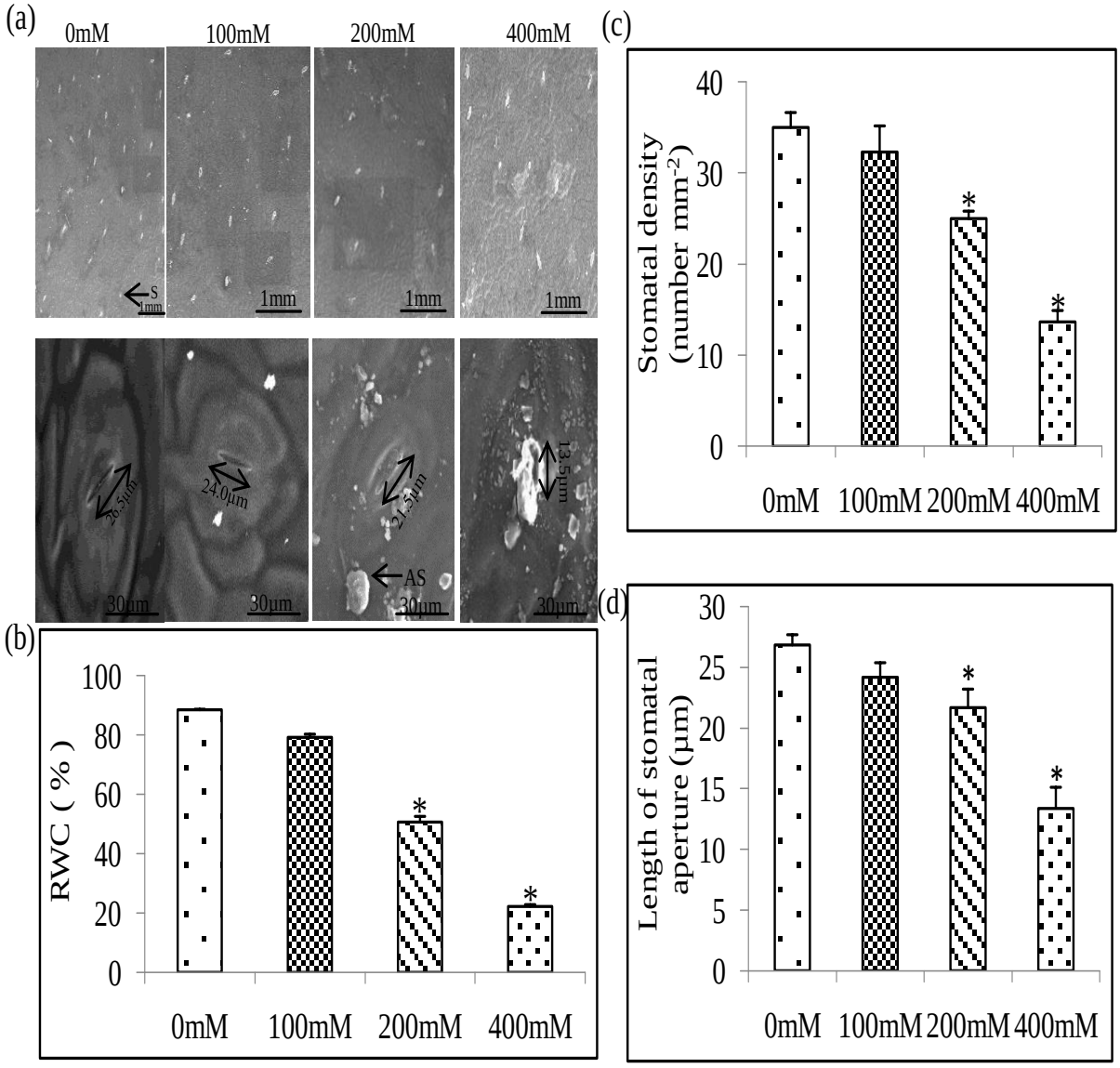


Figure 4 Effect of salt stress on stomatal anatomy, (a) Stomatal density and aperture in SEM analysis, (b) RWC, (c) Stomatal density, and (d) Length of stomatal aperture.The data represents mean± SD of three biological replicates. *denote significance at p≤0.05, respectively. S-Stomata, AS-Amorphous Substances.

causes a decrease in the absorption of CO_2 and photosynthesis, ultimately altering the synthesis of metabolites. High salt concentrations cause cellular dehydration and lead to osmotic stress, which reduces RWC in plant leaves. RWC is a crucial plant water status marker because it distinguishes between transpiration and water supply in leaf tissues. The RWC content was reduced in 100 mM, 200 mM, and 400 mM NaCl-treated plants by 79.18, 50.63, and 22.2%, respectively, compared to control plants (Figure 4d). Therefore, the uptake of water and stomatal movement is perturbed, which causes deterioration of the plant's development and yield under saline conditions.

3.3 Salinity stress effect on biosynthesis of bioactive compounds

A total of seventy-five significant compounds of methanol and ethylacetate extracts, representing five classes like alkane, fatty acid, ester, phenolic, and terpene, were identified by GC-MS analysis. Figures 5 and 6 revealed the retention time of compounds of methanol and ethyl acetate extracts of betel vine, which are also mentioned in Tables 1 and 2. The findings of this study were in close agreement with the other reports of Madhumita et al. (2020). The GC-MS analysis identified methanolic compounds like eugenol

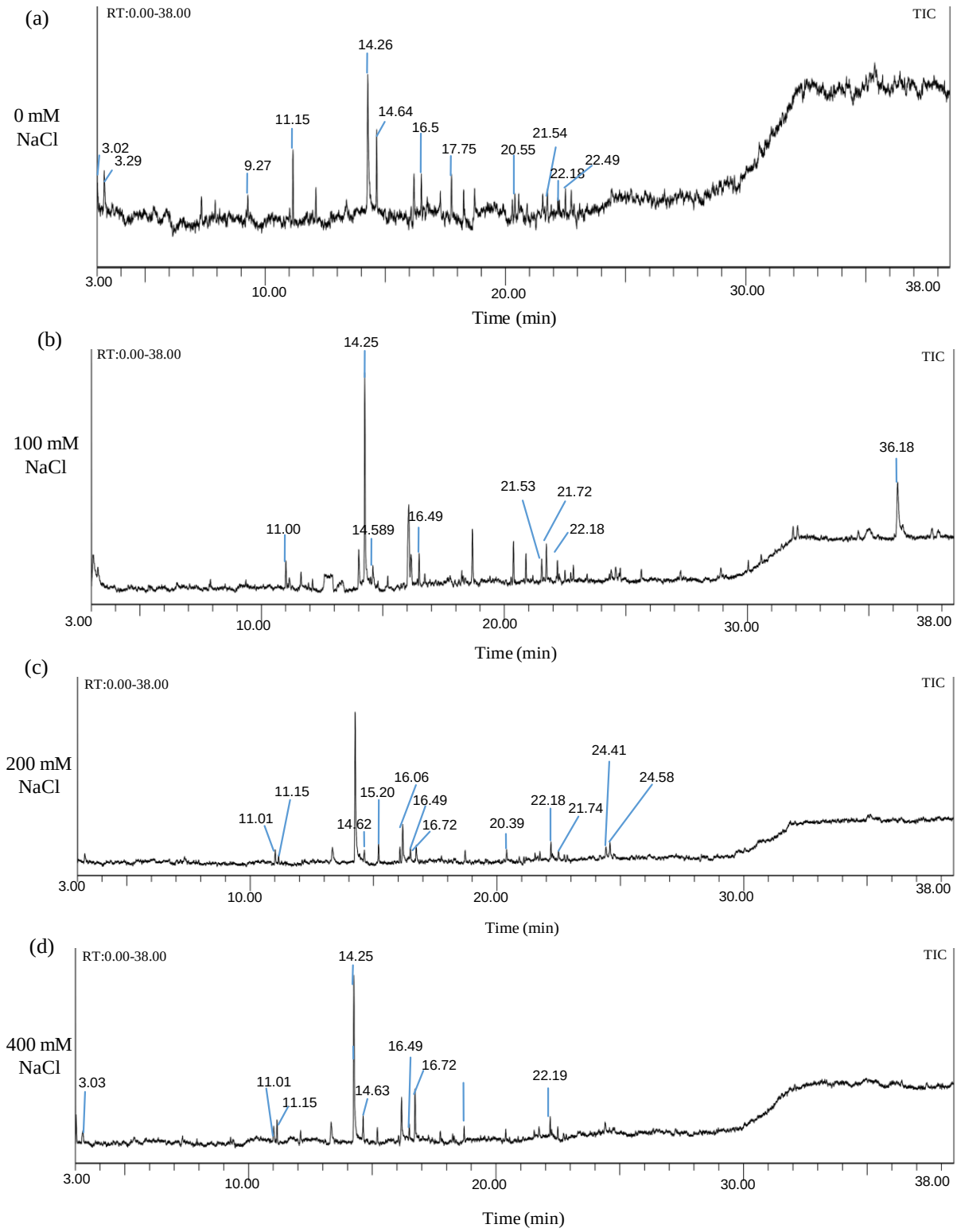


Figure 5 Chromatograph showing the retention time of volatile compounds of methanol extracts of betel vine leaves, (a) Control, (b)100 mM NaCl, (c) 200 mM NaCl, and (d) 400 mM NaCl.

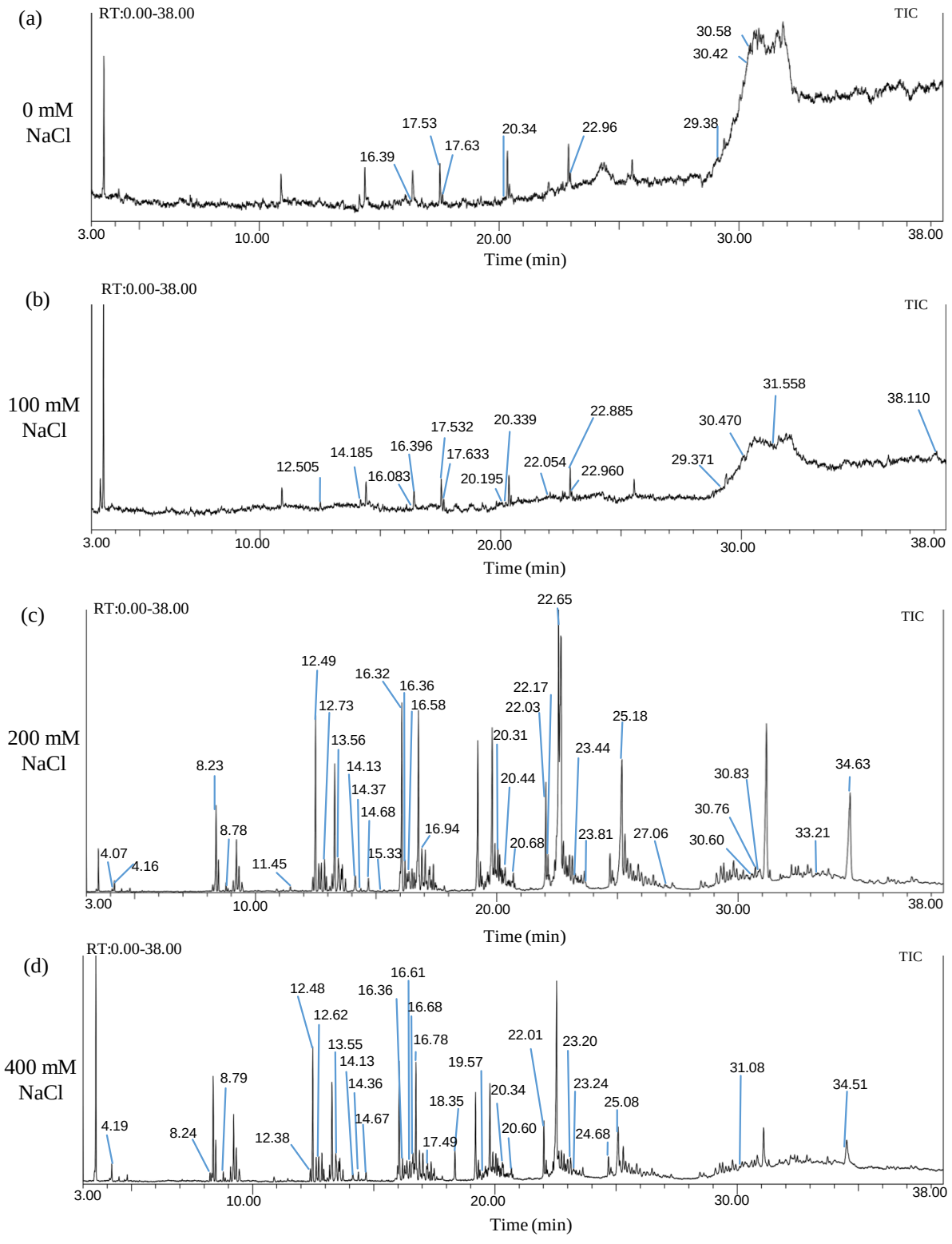


Figure 6 Chromatograph showing the retention time of volatile compounds of ethyl acetate extracts of betel vine leaves, (a) Control, (b) 100 mM NaCl, (c) 200 mM NaCl, and (d) 400 mM NaCl.

Table 1 Retention time of major volatile compounds in methanol extracts of betel vine leaves under salt stress.

Sl. No.	Components	Retention Time (min.) NaCl concentrations (mM)			
		Control	100	200	400
1	Eugenol	14.26	14.25	-	-
2	2,2-Dimethoxybutane	3.29	-	-	-
3	Hexadecane	17.75	-	14.62	-
4	2,4-Di-tert-butylphenol	16.5	16.49	16.49	16.49
5	1,2-Benzenedicarboxylic acid	21.54	21.53	-	-
6	Hexadecanoic acid	22.18	22.18	22.18	22.19
7	Undecane	9.27	-	-	-
8	Glycerin	3.02	-	-	3.03
9	Dodecane	11.15	-	11.15	11.15
10	Tetradecane	14.6	-	-	14.63
11	Octadecane	20.55	-	-	-
12	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl	22.49	-	-	-
13	13-Docosenamide, (Z)-	-	36.18	-	-
14	2,4,6-Tris(trimethyksilyl)cyclohexane-1,3,5-trione	-	21.72	21.74	-
15	Naphthalene	-	11	11.01	11.01
16	Phytol	-	-	24.58	-
17	9,12,15-Octadecatrienoic acid, methyl ester	-	-	24.41	-
18	3,5-Diisopropoxy-1,1,1,7,7,7-hexamethyl-3,5	-	14.58	-	-
19	3-Allyl-6-methoxyphenol	-	-	-	14.25
20	Caryophyllene	-	-	15.2	-
21	3-Allyl-6-methoxyphenyl acetate	-	-	16.72	16.72
22	Gamma.-Muurolene	-	-	16.06	-
23	Phthalic acid, 3,5-dimethylphenyl 4-isopropy	-	-	20.39	-

Table 2 Retention time of major volatile compounds in ethyl acetate extracts of betel vine leaves under salt stress.

Sl. No.	Components	Retention Time (min.) NaCl concentrations (mM)			
		Control	100	200	400
1	1,2,3,4-Tetrahydronaphthalen-1-yl 2,2,3,3,3-pentafluoropropanoate	-	-	30.6	-
2	11-Methylnonacosane	-	20.19	-	-
3	11-Methyltricosane	-	-	23.81	23.2
4	1-Dodecanol, 3,7,11-trimethyl-	-	-	8.78	-
5	1-Hexacosene	-	29.37	-	-
6	1-Hexadecanol	-	-	-	17.49

Sl. No.	Components	Retention Time (min.) NaCl concentrations (mM)			
		Control	100	200	400
7	1-Pentadecene	-	-	14.37	14.36
8	1-Tetracosene	-	22.88	-	-
9	2,4-Di-tert-butylphenol	-	16.39	16.36	16.36
10	2-Ethylbutyric acid, eicosyl ester	-	-	30.83	-
11	2-Undecanethiol, 2-methyl-	-	-	-	8.79
12	3,4-Dihydroxymandelic acid, 4TMS derivative	30.58	-	-	-
13	3-Allyl-6-methoxyphenol	-	-	14.13	14.13
14	3-Allyl-6-methoxyphenyl acetate	-	-	-	16.61
15	3-Ethyl-2,6,10-trimethylundecane	-	-	-	23.24
16	4-Allyl-1,2-diacetoxybenzene	-	-	-	18.35
17	5,5-Diethylpentadecane	-	-	30.76	-
18	5,5-Diethyltridecane	-	22.05	16.96	16.78
19	Benzaldehyde, 2,4-dimethyl-	-	-	11.45	-
20	Bis[di(trimethylsiloxy)phenylsiloxy]trimethylsiloxyphenylsiloxane	-	38.11	-	-
21	Carbonic acid, eicosyl vinyl ester	-	-	15.33	-
22	Cyclononasiloxane, octadecamethyl-	30.42	31.55	-	-
23	Decane, 1-iodo-	-	-	27.06	20.6
24	Decane, 2,3,5,8-tetramethyl-	-	16.08	-	-
25	Dodecanal	-	-	14.68	14.67
26	Dodecane, 2,6,10-trimethyl-	-	12.5	-	-
27	Dodecane, 2,6,11-trimethyl-	-	-	12.49	12.62
28	Dodecane, 4-methyl-	-	-	13.56	13.55
29	Dotriacontane	-	-	22.03	22.01
30	E-14-Hexadecenal	17.53	17.53	-	-
31	E-15-Heptadecenal	20.34	20.33	20.31	-
32	Heneicosane	22.96	22.96	16.32	19.96
33	Heptadecane	-	-	16.58	12.38
34	Heptadecane, 2-methyl-	-	-	-	16.68
35	Heptane, 2,4-dimethyl-	-	-	4.16	4.19
36	Hexacontane	-	-	33.21	-
37	Hexacosylnonyl ether	-	-	23.44	-
38	Hexadecane	17.63	17.63	12.73	12.48
39	Hexadecane, 2,6,10,14-tetramethyl-	-	-	-	20.34
40	Hexadecane, 2-methyl-	-	-	-	19.57
41	Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl	-	-	-	31.08

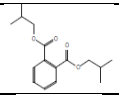
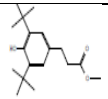
Sl. No.	Components	Retention Time (min.) NaCl concentrations (mM)			
		Control	100	200	400
42	Hexane, 2,3,5-trimethyl-	-	-	4.07	-
43	l-(+)-Ascorbic acid 2,6-dihexadecanoate	-	-	22.65	-
44	Nonane, 5-butyl-	-	-	8.23	8.24
45	Octadecane, 1-iodo-	20.42	-	20.68	-
46	Octadecanoic acid	-	-	25.18	25.08
47	Octadecanoic acid, 2,3-dihydroxypropyl ester	-	-	34.63	34.51
48	Octadecyloctyl ether	-	-	20.44	-
49	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	-	14.18	-	-
50	Phenol, 3,5-bis(1,1-dimethylethyl)-	16.39	-	-	-
51	Tetracosamethyl-cyclododecasiloxane	-	30.47	-	-
52	Tetrapentacontane	-	-	22.17	24.68

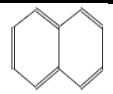
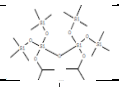
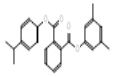
(20.85%), 2,2-Dimethoxybutane (5.51%), hexadecane (4.82%), 2,4-Di-tert-butylphenol (2,4-DTBP) (3.92%), 1,2-Benzenedicarboxylic acid (3.12%), hexadecanoic acid (1.63%), undecane (2.72%), dodecane (6.86%), tetradecane (7.87%), octadecane (1.77%), and benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)(2.57%) in control condition (Sulistyorini 2020) (Table 3).

When exposed to salt stress, these compounds' proportional area (%) varied drastically. The different concentrations of salt treatment induced various types of metabolites such as phenolic (eugenol, 2,4-DTBP, 2,4,6-tris (trimethyksilyl) cyclohexane-1,3,5-trione, 3-Allyl-6-methoxyphenol, 3-Allyl-6-methoxyphenyl acetate), terpenes (naphthalene, phytol, caryophyllene), alkane (hexadecane, dodecane, tetradecane), and ester (1,2-Benzenedicarboxylic acid, 9,12,15-Octadecatrienoic acid, methyl ester, phthalic acid, 3,5-dimethylphenyl 4-isopropy, hexadecanoic acid) (Table 3). Similarly, the salt stress also induces the formation of metabolites such as phenolics, carotenoids, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), flavonoids, anthocyanins, 2,2-diphenyl-1-picrylhydrazyl, as well as vitamin C in wheat microgreen extract under organic cultivation conditions (Islam et al. 2019). Moreover, salinity causes the modification of secondary metabolites in various plant species like *Coriander sativum*, peppermint, and *Origanum majoram* (Mahalakshmi et al. 2013). The 100 mM NaCl stimulated the eugenol compound by 15.97% compared to the control, but it was reduced under 200 and 400 mM NaCl stress. Eugenol is identified in betel vine at 0.32% concentration and is an antioxidant with anticancer, antibacterial, anti-inflammatory, analgesic, and neuroprotective properties (Chaudhary et al. 2023). The 2, 4-DTBP was reduced by 9.43 to 34.94% in 100 mM to 400 mM NaCl-treated plants compared to control plants. In addition, 2,4-DTBP is also found in endophytic *Streptomyces* KCA-1, which promotes antioxidant activity at plant roots (Ayswarya et al. 2022). Another phenolic compound, like 3-Allyl-6-methoxyphenol synthesis, was higher in 200 and 400 mM NaCl and emerged as an abundant compound. The 3-Allyl-6-methoxyphenol showed antioxidant activity in the EO of *Cinnamomum verum* and antibacterial activity against two *Escherichia coli* strains (Al Ahadeb 2022). The 400 mM NaCl-treated plants also induced a phenolic compound such as 3-Allyl-6-methoxyphenyl acetate by 164.38% compared to 200 mM NaCl-treated plants. In addition, the naphthalene compound (as 4.05 and 3.17%) was obtained from plants exposed to 200 and 400 mM NaCl, which functioned as cytotoxicity and anti-tubulin activity in betel vine plants leading to senescence, along with biosurfactant and anti-inflammatory activity (Mohapatra and Phale, 2021). Caryophyllene (CP) is a natural sesquiterpene observed by 4.56% in 200 mM NaCl-treated plants, possessing numerous pharmaceutical activities such as antimicrobial, anticarcinogenic, anxiolytic, anti-inflammatory, antioxidant, and local anesthetic effects (Sharma et al. 2016).

Further, alkane compounds like hexadecane, a straight-chain 16-C compound, were reduced by 28.83% compared to the control in plants exposed to 200 mM NaCl. It is also a component of the EO of long pepper. This non-polar plant metabolite acts as a pheromone of the moth (*Acrolepiopsis assectella*) (Morris et al. 2005) and has antibacterial and antioxidant activities (Yogeswari et al.2012). In addition, dodecane is a 12-C compound and shows a 71.72 and 32.65% reduction in plants exposed with 200 and 400 mM NaCl compared to the control. It is also isolated from *Zingiber officinale*, acts as a pheromone of *Glossina morsitans*, and attracts gravid females to its larvae (Adden et al. 2023).

Table 3 GC/MS analysis of volatile compounds in methanol extracts of betel vine leaves under salt stress.

Sl. No.	Compounds	KRI literature (Column)	Molecular formula	Structure of compounds	Types of compounds	Area (%) NaCl concentrations (mM)			
						Control	100	200	400
1.	Eugenol	1356 (DB-5)	$C_{10}H_2O_2$		Phenolic	20.85±0.50	24.18±0.28*	Nd	Nd
2.	2,2-Dimethoxybutane	685	$C_6H_{14}O_2$		Ether	5.51±0.34	Nd	Nd	Nd
3.	Hexadecane	1600(DB-5)	$C_{16}H_{34}$		Alkane	4.82±0.15	Nd	3.43±0.16*	Nd
4.	2,4-Di-tert-butylphenol	1502(CP-Sil-5CB)	$C_{14}H_{22}O$		Phenolic	3.92±0.06	3.55±0.25*	2.19±0.22*	2.55±0.28*
5.	1,2-Benzenedicarboxylic acid	1917 (HP-Ultra-2)	$C_{24}H_{38}O_4$		Ester	3.12±0.10	Nd	Nd	Nd
6.	Hexadecanoic acid	1984 (DB-5)	$C_7H_4O_2$		Ester	1.63±0.11	Nd	3.48±0.13*	4.26±0.33*
7.	Undecane	1099 (DB-5)	$C_{11}H_{24}$		Terpene	2.72±0.23	Nd	Nd	Nd
8.	Glycerin	940 (9DB-1)	$C_3H_8O_3$		Triol	2.14±0.31	Nd	Nd	5.32±0.21*
9.	Dodecane	1199(DB-5)	$C_{12}H_{26}$		Alkane	6.86±0.15	Nd	1.94±0.15*	4.62±0.16*
10.	Tetradecane	1399(DB-5)	$C_{14}H_{30}$		Alkane	7.87±0.13	Nd	Nd	4.74±0.15*
11.	Octadecane	1800 (DB-500)	$C_{18}H_{38}$		Alkene	1.77±0.18	Nd	Nd	Nd
12.	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl	3823	$C_{18}H_{28}O$		Ester	2.57±0.11	Nd	Nd	Nd
13.	13-Docosenamide, (Z)-	2625 (HP-5MS)	$C_{22}H_{43}NO$		Fatty acid	Nd	12.48±0.37*	Nd	Nd
14.	2,4,6-Tris(trimethyksilyl)cyclohexane-1,3,5-trione	1904	$C_6N_6O_3$		Phenolic	Nd	4.24±0.19*	1.21±0.09*	Nd

Sl. No.	Compounds	KRI literature (Column)	Molecular formula	Structure of compounds	Types of compounds	Area (%) NaCl concentrations (mM) Control	100	200	400
15.	Naphthalene	266 (SE-52)	$C_{10}H_8$		Terpene	Nd	2.71±0.18*	4.05±0.04*	3.17±0.16*
16.	Phytol	[1949] (DB-5)	$C_{20}H_{40}O$		Terpene	Nd	Nd	2.87±0.24	Nd
17.	9,12,15-Octadecatrienoic acid, methyl ester	2058 (capillary)	$C_{19}H_{32}O_2$		Ester	Nd	Nd	3.62±0.18	Nd
18.	3,5-Diisopropoxy-1,1,1,7,7,7-hexamethyl-3,5	1648	$C_{18}H_{50}O7Si_6$		Hexamethylated cycloheptatriene	Nd	1.51±0.39*	Nd	Nd
19.	3-Allyl-6-methoxyphenol	1322 (DB-5MS)	$C_{10}H_{12}O$		Phenolic	Nd	Nd	48.8±0.58*	51.36±0.80*
20.	Caryophyllene	1428 (DB-5)	$C_{15}H$		EO	Nd	Nd	4.56±0.07*	
21.	3-Allyl-6-methoxyphenyl acetate	1513 (DB-5MS)	$C_{12}H_{14}O_3$		Phenolic	Nd	Nd	4.24±0.07*	11.21±0.21*
22.	Gamma.-Muurolene	1477(DB-5)	$C_{15}H_{24}$		Elaidolinolenic acid	Nd	Nd	3.7±0.17*	Nd
23.	Phthalic acid, 3,5-dimethylphenyl 4-isopropy	Nm	$C_{24}H_{22}O_4$		Ester	Nd	Nd	1.78±0.10*	Nd
Most identified classes									
			Alkane			19.55±1.64	0	5.37±0.34	9.36±1.22
			Ester			7.32±0.78	0	8.88±0.63	4.26±0.67
			Phenolic			24.77±1.04	31.97±1.00*	56.44±1.02*	65.12±0.52*
			Terpene			2.72±0.12	2.71±0.32	6.92±0.78	3.17±0.92

Nd: not detected

* means of three replicates ± SD with significant differences at 5% (post hoc test).

KRI: Kovats retention index from literature

Nm: Not mentioned

The esteric component phthalic acid, 3,5-dimethylphenyl 4-isopropy, was 1.78% in a 200 mM NaCl-treated plant. This compound is extracted from *Bacillus subtilis* SR22 and exhibited defence responses in tomatoes against *Rhizoctonia* root rot (Rashad et al. 2022). The hexadecanoic acid compound was synthesized by 113.49% and 161.34% in plants exposed to 200 and 400 mM NaCl, respectively, which acts as antioxidants to reduce DPPH, ABTS, and NO in the leaves of *Ipomoea eriocarpa*. It can also be antibacterial against *Klebsiella pneumonia*, *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus* (Ganesan et al. 2024). Plants have synthesized secondary metabolites, including phenolic, terpene, ester, and alkane compounds, to scavenge ROS during salt stress (Akbari et al. 2022), especially alkane compounds, which are utilized in industries and aligned with our present results. The two phenolic compounds 3-Allyl-6-methoxyphenol (51.36±0.80) and 3-Allyl-6-methoxyphenyl acetate (11.21±0.21) are found to exhibit antioxidant activity by reducing ROS or by stopping hydroperoxides from becoming free radicals (Alam et al. 2023).

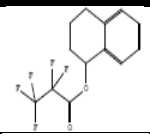
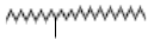
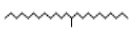
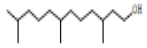
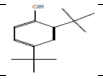
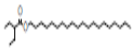
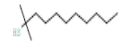
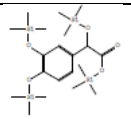
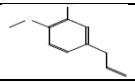
The secondary metabolites of ethyl acetate extracts of control plants included 1-Hexacosene (4.9%), 3,4-Dihydroxymandelic acid, 4TMS derivative (5.59%), cyclononasiloxane, octadecamethyl (11.58%), E-14-Hexadecenal (5.39%), E-15-heptadecenal (6.08%), heneicosane (1.5%), hexadecane (1.5%), and phenol, 3,5-bis(1,1-dimethylethyl)-phenolic (4.97%) (Madhumita et al. 2019; Biswas et al. 2022) (Table 4). The different classes of metabolites such as phenolic (phenol, 2-methoxy-4-(2-propenyl)-, acetate, 3-Allyl-6-methoxyphenol,2, 4-DTBP, 4-Allyl-1,2-diacetoxybenzene), alkane (11-Methyltricosane, 1-Pentadecene, 3-Ethyl-2,6,10-trimethylundecane, 5,5-Diethyltridecane, decane, 1-iodo-, decane, 2,3,5,8-tetramethyl-, dodecane, 2,6,10-trimethyl-, dodecane, heneicosane, 4-methyl-, dotriacontane, 2,6,11-trimethyl-, dodecane,hexadecane), EO (1-Dodecanol, 3,7,11-trimethyl-), terpene (dodecane, 1-tetracosene, 2,6,10-trimethyl-), ester (1,2,3,4-Tetrahydronaphthalen-1-yl 2,2,3,3,3-pentafluoropropanoate, 2-Ethylbutyric acid, eicosyl ester, 3-Allyl-6-methoxyphenyl acetate, 2-hydroxy-1 (hydroxymethyl) ethyl, carbonic acid, eicosyl vinyl ester, hexadecanoic acid, tetrapentacontane) and fatty acid (Octadecanoic acid, 11-Methylnonacosane, 2,3-dihydroxypropyl ester) were identified in 100 mM to 400 mM NaCl-treated plants, that have pharmacological properties, such as anti-inflammatory, wound-healing, anthelmintic, antiparasitic, antifungal, antihemolytic, antibacterial, antiseptic, antioxidant, anticancer, and antidiabetic, activities (Mahalakshmi et al. 2013) (Table 4). Similarly, the other phenolic compounds also have various properties, including 4-Allyl-1,2-diacetoxybenzene, phenol, 2-methoxy-4-(2-propenyl)-, acetate (as antibacterial and anti-biofilm activity), and 2, 4-DTBP, a toxic phenol compound (exhibiting considerable antioxidant, antibacterial, and antitumor activity) found in NaCl-treated plants, which inhibits the plant immune system (Zhao et al. 2020). The high salinity reduced 2,4-DTBP (88.8 to 78.57%) compared to plants exposed to 100 mM NaCl. In addition, a low amount of 3-Allyl-6-methoxyphenol was detected in plants exposed to 200 and 400 mM NaCl, which possess potent antioxidant, anti-inflammatory, antibacterial, nematicide, antimicrobial, and pesticide properties (Sosa et al. 2016).

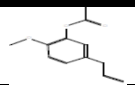
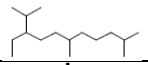
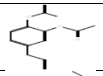
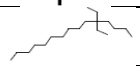
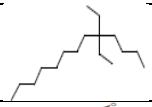
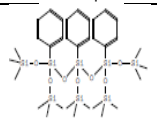
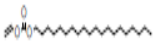
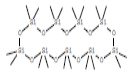
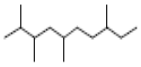
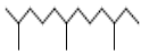
The alkylated phenol dodecane 2,6,10-trimethyl was 0.76% in 400 mM NaCl-treated plants. This compound also acts as an anti-proliferative and antioxidant agent in *Ficus pumila* (Torkornoo et al. 2019). 5,5-Diethyltridecane was induced as 0.55, 0.37, and 0.23% in 100, 200, and 400 mM NaCl-treated plants but absent in the control. This compound possesses the acaricidal activity of EO from *Satureja hortensis* (L.) and *Teucrium polium* (L.) against two-spotted spider mites (Ebadollahi et al. 2015). Another compound, heneicosane, was enhanced by 5.33% in 100 mM NaCl but reduced by 78 and 70% in plants exposed to 200 and 400 mM NaCl, respectively, compared to control. It is reported as a novel antimicrobial compound in *Plumbago zeylanica* leaf extracts, a reducing and stabilizing agent for preparing nanoparticles (ZnO, AgO), and utilized as a pheromone by the queen or king termites in the species *Reticulitermes flavipes* (Eyer et al. 2021).

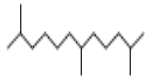
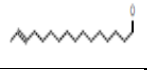
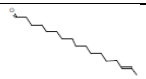
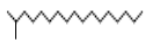
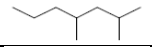
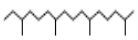
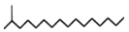
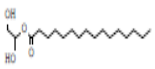
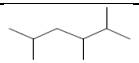
1-Dodecanol, 3,7,11-trimethyl was observed at 0.17% in 200 mM NaCl-treated plants. This component possesses anti-inflammatory, antioxidant, and antimicrobial properties and also, in drug delivery and skin protection applications, acts as a surfactant in detergents, lubricants, and cosmetics (Nazarudin et al. 2022).100 mM NaCl stimulated the 1-tetracosene terpene compound (by 4.9%) as compared to the control but not in 200 and 400 mM NaCl stress. This compound also exhibited cytotoxic and antioxidant activity in *Spiraea hypericifolia* (Kudaibergen et al. 2020).

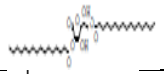
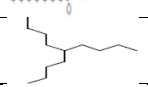
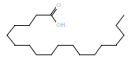
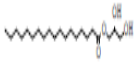
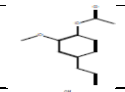
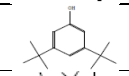
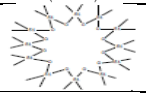
The ester compounds like hexadecanoic acid and 2-hydroxy-1 (hydroxymethyl) ethyl were 5.7% in 200 mM NaCl but decreased by 95.26% in plants exposed to 400 mM NaCl-treated plants. This compound also exhibits antifungal activity against the *Macrophomina phaseolina* pathogen and is found at 10.99% in *Chenopodium quinoa* plants (Khan and Javaid 2020). In addition, hexadecanoic acid, an ester component, possessed anticancer, antimicrobial, antioxidant, and antihemolytic activity (Dubey et al. 2014). Octadecanoic acid is a fatty acid molecule found at 2.95% in 400 mM NaCl-treated betel vine plants, which is utilized as softening plastics and in making candles, cosmetics, and hardening soaps. Compared to control, the antibiotic compound E-15-Heptadecenal was reduced by 16.61 and 95.88% in plants exposed to 100 and 200 mM NaCl. The E-15-Heptadecenal compound is also synthesized in *Jatropha curcas* under salt stress (Mahalakshmi et al. 2013). Similarly, a vitamin-type compound, l-(+)-Ascorbic acid 2,6-dihexadecanoate, was observed at 8.21% in plants exposed to

Table 4 GC/MS analysis of major volatile compounds in ethyl acetate extracts of betel vine leaves under salt stress.

Sl. No.	Compounds	KRI literature (Column)	Molecular formula	Structure of compounds	Types of compounds	Area (%) NaCl concentrations (mM)			
						Control	100	200	400
1	1,2,3,4-Tetrahydronaphthalen-1-yl 2,2,3,3,3-pentafluoropropanoate	Nm	$C_{11}H_{16}C_lN$		Ester	Nd	Nd	0.3±0.2	Nd
2	11-Methylnonacosane	2933(DB-5MS)	$C_{30}H$		Fatty acid	Nd	1.59±0.6*	Nd	Nd
3	11-Methyltricosane	2335 (DB-1)	$C_{24}H_{50}$		Alkane	Nd	Nd	0.06±0.1*	0.06±0.12*
4	1-Dodecanol, 3,7,11-trimethyl-	1563	$C_{15}H_{32}O$		EO	Nd	Nd	0.17±0.08	Nd
5	1-Hexacosene	2593 (CP-Sil-8CB)	$C_{26}H_{52}$		Aliphatic hydrocarbon	4.9±0.08	Nd	Nd	Nd
6	1-Hexadecanol	1854	$C_{16}H_{34}O$		Fatty alcohol	Nd	Nd	Nd	0.35±0.02
7	1-Pentadecene	1502	$C_{15}H_{30}$		Alkane	Nd	Nd	Nd	0.24±0.01*
8	1-Tetracosene	2395	$C_{24}H$		Terpene	Nd	4.9±0.04*	Nd	Nd
9	2,4-Di-tert-butylphenol	1502 (CP-Sil-5CB)	$C_{14}H_{22}O$		Phenolic	Nd	4.2±0.4	0.47±0.01	0.9±0.01
10	2-Ethylbutyric acid, eicosyl ester	2709	$C_{27}H_{54}O_2$		Ester	Nd	0.5±0.02	Nd	Nd
11	2-Undecanethiol, 2-methyl-	1433	$C_{12}H_{26}S$		Thiol	Nd	Nd	Nd	0.16±0.09
12	3,4-Dihydroxymandelic acid, 4TMS derivative	1936 (DB- 1)	$C_{20}H_{40}O_5Si$		Catechol	5.59±0.08	Nd	Nd	Nd
13	3-Allyl-6-methoxyphenol	1322 (DB-5MS)	$C_{10}H_{12}O$		Phenolic	Nd	Nd	0.36±0.03	0.23±0.01

Sl. No.	Compounds	KRI literature (Column)	Molecular formula	Structure of compounds	Types of compounds	Area (%) NaCl concentrations (mM)			
						Control	100	200	400
14	3-Allyl-6-methoxyphenyl acetate	1513 (DB-5MS)	$C_{12}H_{14}O_3$		Ester	Nd	Nd	Nd	0.98±0.01
15	3-Ethyl-2,6,10-trimethylundecane	Nm	$C_{16}H_{34}$		Alkane	Nd	Nd	Nd	2.94±0.004*
16	4-Allyl-1,2-diacetoxybenzene	1647 (ZB-5)	$C_{13}H_{14}O_4$		Phenol and benzoate ester	Nd	Nd	Nd	0.96±0.004
17	5,5-Diethylpentadecane	1825	$C_{15}H_{32}$		Hydrocarbon	Nd	Nd	0.16±0.04	Nd
18	5,5-Diethyltridecane	1205	$C_{15}H_{32}$		Alkane	Nd	0.55±0.009	0.37±0.02	0.23±0.01
19	Benzaldehyde, 2,4-dimethyl-	1208	$C_9H_{10}O$		Benzaldehyde	Nd	Nd	0.22±0.1	Nd
20	Bis[di(trimethylsiloxy)phenylsiloxy]trimethyls iloxyphenylsiloxane	3343	$C_{33}H_{60}O_7Si_8$		Siloxane	Nd	0.74±0.01	Nd	Nd
21	Carbonic acid, eicosyl vinyl ester	2497	$C_{28}H_{54}O_2$		Ester	Nd	Nd	0.14±0.11	Nd
22	Cyclononasiloxane, octadecamethyl-	1860	$C_{18}H_{54}O_9Si_9$		Siloxane	11.58±0.06	1.73±0.02*	Nd	Nd
23	Decane, 1-iodo-	1430	$C_{10}H_{21}I$		Alkane	Nd	Nd	0.06±0.02	0.33±0.12
24	Decane, 2,3,5,8-tetramethyl-	4714	$C_{14}H_{30}$		Alkane	Nd	0.56±0.01	Nd	Nd
25	Dodecanal	1407 (DB-5)	$C_{12}H_{24}O$		Lauraldehyde	Nd	Nd	0.26±0.04	0.05±0.01
26	Dodecane, 2,6,10-trimethyl-	1320	$C_{15}H_{32}$		Alkane	Nd	0.76±0.01	Nd	Nd

Sl. No.	Compounds	KRI literature (Column)	Molecular formula	Structure of compounds	Types of compounds	Area (%) NaCl concentrations (mM)			
						Control	100	200	400
27	Dodecane, 2,6,11-trimethyl-	1320	$C_{15}H_{32}$		Alkane	Nd	Nd	2.77±0.2	2.87±0.02
28	Dodecane, 4-methyl-	1249	$C_{13}H_{28}$		Alkane	Nd	Nd	0.37±0.02	0.54±0.002
29	Dotriacontane	1600 (DB-5)	$C_{32}H_{66}$		Alkane	Nd	Nd	7.41±0.14	1.64±0.1
30	E-14-Hexadecenal	1824 (DB-50)	$C_{16}H_{30}O$		Palmitol aldehyde	5.39±0.08	5.07±0.08*	Nd	Nd
31	E-15-Heptadecenal	2083 (HP-5MS)	$C_{17}H_{32}O$		Antibiotic	6.08±0.08	5.07±0.08	0.25±0.02*	Nd
32	Heneicosane	2100(DB-5)	$C_{21}H$		Alkane	1.5±0.05	1.58±0.05*	0.33±00.2*	0.45±0.01
33	Heptadecane	1700 (DB-5)	$C_{17}H_{36}$		Alkane	Nd	Nd	0.27±0.05	Nd
34	Heptadecane, 2-methyl-	1746	$C_{19}H_{40}$		Alkane	Nd	Nd	Nd	0.46±0.01
35	Heptane, 2,4-dimethyl-	788	C_7H_{16}		Alkane	Nd	Nd	0.16±0.02	0.32±0.02
36	Hexacontane	1600 (DB-5)	$C_{60}H_{122}$		Alkane	Nd	Nd	0.17±0.01	Nd
37	Hexacosyl nonyl ether	3550	$C_{36}H_{74}O$		Fatty alcohol	Nd	Nd	0.63±0.12	Nd
38	Hexadecane	1600(DB-5)	$C_{16}H_{34}$		Alkane	1.56±0.09	0.24±0.04	0.45±0.03*	3.45±0.01*
39	Hexadecane, 2,6,10,14-tetramethyl-	1753	$C_{20}H_{42}$		Alkane	Nd	Nd	Nd	0.38±0.008
40	Hexadecane, 2-methyl-	1647	$C_{17}H_{36}$		Alkane	Nd	Nd	Nd	0.28±0.01
41	Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl	2498	$C_{20}H_{40}O_3$		Ester	Nd	Nd	5.7±0.21	0.27±0.01
42	Hexane, 2,3,5-trimethyl-	724	C_9H_{20}		Alkane	Nd	Nd	0.04±0.04	Nd

Sl. No.	Compounds	KRI literature (Column)	Molecular formula	Structure of compounds	Types of compounds	Area (%) NaCl concentrations (mM) Control	100	200	400
43	l-(+)-Ascorbic acid 2,6-dihexadecanoate	Nm	$C_{38}H_{68}O_8$		Vitamin C	Nd	Nd	8.21±0.06	Nd
44	Nonane, 5-butyl-	1204	$C_{14}H_{30}$		Alkane	Nd	Nd	0.22±0.1	Nd
45	Octadecane, 1-iodo-	2272 (DB-1)	$C_{18}H_{37}I$		Alkyl halide	Nd	Nd	0.25±0.03	Nd
46	Octadecanoic acid	2124 (DB-5)	$C_{18}H_{36}O_2$		Fatty acid	Nd	Nd	Nd	2.95±0.01
47	Octadecanoic acid, 2,3-dihydroxypropyl ester	2992	$C_{21}H_{42}O$		Fatty acid	Nd	Nd	4.45±0.12	1.98±0.02
48	Octadecyl octyl ether	1862	$C_{26}H_{54}O$		Ether	Nd	Nd	0.17±0.02	Nd
49	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	1451(HP-5)	$C_{12}H_{14}O$		Phenolic	Nd	1.57±0.06	Nd	Nd
50	Phenol, 3,5-bis(1,1-dimethylethyl)-	1555	$C_{27}H_{50}OP_2$		Phenolic	4.97±0.08	Nd	Nd	Nd
51	Tetracosamethyl-cyclododecasiloxane	2480	$C_{24}H_{72}O_{12}Si$		Siloxane	Nd	1.4±0.04	Nd	Nd
52	Tetrapentacontane	5389	$C_{54}H_{110}$		Ester	Nd	Nd	5.63±0.12	1.47±0.01
Most identified classes									
Alkane						3.06±0.05	3.69±0.12	12.37±0.4*	14.19±0.35*
Fatty acid						0	1.59±0.23	4.45±0.52	4.93±0.65
Ester						0	0.5±0.04	11.94±0.8*	2.72±0.14*
Phenolic						4.97±0.12	5.77±0.24	0.83±0.32*	1.13±0.16

Nd: not detected

* means of three replicates ± SD with significant differences at 5% (post hoc test).

KRI: Kovats retention index from literature

Nm: Not mentioned

200 mM NaCl but was absent in other NaCl treatments. This compound also exhibits antimicrobial, antioxidant, anticancer, and antiulcerogenic properties in *Sesuvium portulacastrum* (Kumar et al. 2014). Due to oxidation, total phenolic compounds were observed at 5.77% in 100 mM but decreased to 0.83, and 1.13% in 200 and 400 mM NaCl-treated plants. The total alkane compounds were induced by 3.69, 12.37, and 14.19% in plants exposed to 100, 200, and 400 mM NaCl, respectively, and ultimately emerged as the most prevalent chemical group in ethyl acetate extracts of salt-treated plants. In addition, the fatty acid and ester compounds were also stimulated more in the saline condition. These alkanes, benzoate esters, and fatty acid methyl esters were polymerized to develop a cuticle layer in the epidermal tissues of wheat and barley leaves to check water loss under saline stress conditions (Hasanuzzaman et al. 2017). Our results indicate that the biosynthesis of phenolic, alkane, fatty acids, ester compounds, and activation of antioxidants are simultaneously stimulated to safeguard the general health of the betel vine when subjected to salinity stress. Moreover, these findings support our presumption that salt-stress-tolerant plants could provide good systems for the biosynthesis of secondary metabolites needed by the manufacturing and pharmaceutical sectors.

Conclusion

Many companies are looking for more natural, eco-friendly, and alternative sources of antioxidants, antibiotics, crop protection agents, and antimicrobials. There are no published data regarding manufacturing secondary metabolites like phenolic, alkane, and ester components in betel vines under various salinity stresses. This study showed that the photosynthetic pigments, RWC, stomatal density, and length of the stomatal aperture were reduced while H_2O_2, $O_2^{\bullet-}$, and cell death contents were increased with increasing NaCl levels. Furthermore, the NaCl treatment enhanced the SOD, CAT, APX, and GPX activities at 100 and 200 mM NaCl to maintain cellular homeostasis, but the activities were declined in 400 mM NaCl. The production of bioactive compounds was also triggered by the salt stress, including eugenol, 3-Allyl-6-methoxyphenol, caryophyllene, hexadecane, 4-Allyl-1,2-diacetoxybenzene, 11-Methyltricosane,1-Dodecano etc. Thus, the synthesis of these molecules at different salt concentrations could be a benefit of salinity stress for some plants, encouraging the production of chemicals of commercial and medicinal value.

List of abbreviations

NaCl: Sodium chloride; RWC: Relative water content; ROS: Reactive oxygen species; H_2O_2: Hydrogen peroxidase; $O_2^{\bullet-}$: Superoxide anion; SOD: Superoxide dismutase; CAT: Catalase; APX: Ascorbate peroxidase; GPX: Guaicol peroxidase; ASC: Ascorbate; GSH: Glutathione; GC-MS: Gas chromatography-mass spectrometry; EO: Essential oils; SEM: Scanning electron microscopy;

Author contributions

A. K. Sahu: Writing, Reviewing and Editing, Investigation. P. Priyadarshini: Data curation, Methodology, and Investigation. B. Dash: Data curation, Methodology, and Investigation. B. S. Behera: Data curation, Methodology, and Investigation. S. K. Gochhi: Data curation. D. Pradhan: Data curation. P. Kumari: Supervision, Conceptualization, Writing, Reviewing and Editing, Investigation, Validation.

Acknowledgments

The grants from OHEPEE, COE for Bioresource Management and Energy Conservation Material Development, F.M. University and Science & Engineering Research Board (SERB), Department of Science & Technology, Government of India, to Dr. Punam Kumari are gratefully acknowledged. The laboratory facilities provided by the P.G. Department of Biosciences and Biotechnology, F.M. University, are also gratefully acknowledged.

Funding

This work was financial assisted by OHEPEE, COE for Bioresource Management and Energy Conservation Material Development, F.M. University, and partial funds from the Science & Engineering Research Board (SERB), Department of Science & Technology, Government of India (No. EEQ/2022/000273).

Conflict of interest

The authors affirm that they have no competing interests.

Ethical clearance statement

This certifies that no animal or human models were involved in the study; therefore, ethical clearance is not required.

References

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