Brazilian Journal of Botany (2021) 44:587–599
https://doi.org/10.1007/s40415-021-00738-4

BIOCHEMISTRY & PHYSIOLOGY - ORIGINAL ARTICLE

Dynamic alterations of metabolites in *Plectranthus amboinicus* (Lour.) Spreng. to encounter drought and Zn toxicity

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Received: 1 April 2021 / Revised: 27 June 2021 / Accepted: 2 August 2021 / Published online: 25 August 2021

Abstract
Heavy metal toxicity and drought stresses are two growing concerns of the global population as these have a disastrous effect on the agriculture sector, creating oxidative stress in plants and leads to deleterious effects, which end up causing a major decline in yield. Hence, carving out the best candidate for combating both these stresses have become the prime objective of researchers. *Plectranthus amboinicus* (Lour.) Spreng., a medicinal plant, is regarded as one such candidate that can tolerate both drought and zinc stressors by the elicitation of metabolic changes. Variation in the composition of primary and secondary metabolites of *P. amboinicus* was evaluated in the leaf tissues of the plants subjected to drought and $ZnSO_4$ (4 mM) treatments. Drought stress resulted in the accumulation of amino acids and sugars in the leaves of plants exposed to this stress. Similarly, zinc stress exhibited a remarkable impact on the synthesis of secondary metabolites like alkaloids, phenols, and flavonoids. Further, on carrying out GC–MS profiling, the compositional variation of secondary metabolites produced in *P. amboinicus* implicated its inherent potential to survive environments of these two stresses. Compounds like 2-methoxy-4-ethyl-6-methylphenol, gamma-sitosterol, hexadecanoic acid, alpha-amyrin, and ethyl linalool were some of the major secondary metabolites developed in leaves of plants under drought, whereas during $ZnSO_4$ treatment, the major compounds developed were trans-alpha-bergamotene, squalene, 1,5-dimethyl-1-vinyl-4-hexenyl isovalerate, and spathulenol. This in turn makes the plant more compatible with the stressful environmental conditions, aiding it with better survival and protection. At the same time, the enhancement in the content of these metabolites in this medicinal plant under the influence of these stressors may have applications in the pharmaceutical industry, necessary for the development of novel drugs.

Keywords Drought · GC–MS · *Plectranthus amboinicus* · Stress · Zinc

1 Introduction

According to the estimation by the World Health Organization, about 80% of people on the globe are still dependent on traditional herb-based medications due to their low cost, easy accessibility, and likely negligible side effects in comparison to allopathic medicines (Arumugam et al. 2016). Medicinal plants have curative properties due to the presence of various complex chemical substances of different chemical nature, which are found as secondary plant metabolites in one or more parts of these plants (Soni et al. 2015). These secondary metabolites also have an important role in the defensive mechanism of the plant, which enables the survival of the plant under different environmental stresses. Moreover, the medicinal and aromatic plants induce qualitative and quantitative modifications in the biosynthesis process of different metabolites when encountered with a stressor (Pradhan et al. 2017).

Drought is generally characterized by stunted growth, lower stomatal conductance, and reduced plant biomass, which results in lesser carbon assimilation via photosynthesis (Farooq et al. 2009; Jaleel et al. 2009). But, it has got a positive effect on the synthesis of secondary metabolites such as anthocyanins and flavonoids content in the plant. Water deficit affects the composition of essential oils of medicinal and aromatic plants. Flavonoids, which get accumulated in plants under various stresses, aid to mitigate the oxidative stress by the ROS-scavenging property (Pradhan et al. 2017).

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Heavy metals get released into our environment from major anthropogenic activities like industrial emissions, mining activity, fertilizer and pesticide use, and automobile exhausts. Exposure to heavy metal (chromium) stress has caused an increase in the secondary metabolite accumulation of medicinal plant like *Ocimum tenuiflorum* L. (Rai et al. 2004). Zinc (Zn) is one of the essential micronutrients that a plant requires in a specific amount for the proper functioning of its physiological processes. But, higher concentrations of Zn become toxic to plants. According to the phytotoxicity studies on metals, it was revealed that Zn is a major environmental pollutant (Orcutt and Nilsen 2000; Vaillant et al. 2005; Alloway 2008; Azzi et al. 2015; Saha 2015). In *Matricaria chamomilla* L., zinc treatment enhanced its essential oil content (Nasiri et al. 2010), while for *Lepidium sativum* L. and *Beta vulgaris* L., the production of secondary metabolites (lepidine, betalain) was enhanced (Saba et al. 2000; Savitha et al. 2006).

Heavy metal-induced stimulation of secondary metabolites accumulation in medicinal plants is strongly influenced by several aspects including plant growth stage, concentration and duration of treatment, and composition of growth medium (Nasim and Dhir 2010; Street 2012). It has been reported that certain medicinal plants when grown in polluted soils enhance the secondary metabolite yield (Rai et al. 2004). Abiotic stresses can elevate the phytomedicine production and essential oil yield of medicinal/aromatic plants by increasing its secondary metabolite accumulation potential.

Plectranthus amboinicus (Lour.) Spreng. is a potential medicinal herb belonging to the family Lamiaceae. It is found in tropical and warm regions of Asia, Africa, and Australia (Arumugam et al. 2016) and is known by a bunch of vernacular names ranging from Indian borage to "Panikoorka". An overview of several literature reveals that this plant contains 76 volatile and 30 non-volatile compounds under different classes of phytocompounds (Arumugam et al. 2016). *Plectranthus amboinicus* is a large fleshy succulent perennial herb with aromatic pubescence and inherent medicinal power due to the presence of phytochemicals like flavonoids, esters, phenolics, monoterprenoids, diterpernoids, triterprenoids, and sesquiterprenoids (Arumugam et al. 2016). These phytochemicals attributes antibacterial, antihelminthic, allelopathic, antifungal, antiepileptic, larvicidal, antioxidant, anti-inflammatory, and analgesic properties to the plant (Arumugam et al. 2016). It is used to treat malarial fever, hepatopathy, renal and vesical calculi, cough, chronic asthma, hiccough, bronchitis, colic convulsions, and epilepsy (Chopra and Nayar 1956; Nadkarni 1996; Kirtikar and Basu 2005).

The impact of drought and NaCl stresses on the metabolomics of *P. amboinicus* was investigated by different scientists (Abdelrazik et al. 2016; Sabra et al. 2018). Abdelrazik et al. (2016) studied the changes of essential oil constituents and proline content of *P. amboinicus* plants under the exposure of NaCl stress. At the same time, the effect of water stress on physiological and biochemical functions of *P. amboinicus* was evaluated by Prathyusha et al. (2019). Both these studies gave an insight into the role of different metabolites in the stress tolerance potential of the plant.

The main aim of the present study was to investigate the variation in the composition of primary and secondary metabolites in *P. amboinicus* grown under different abiotic stresses (drought and heavy metal stress). We hypothesize that the difference in the metabolite composition as influenced by the abiotic stresses can increase the abiotic stress tolerance as well as the pharmaceutical value of the plant.

2 Materials and methods

Plant material and growth conditions – *Plectranthus amboinicus,* an aromatic medicinal succulent plant belonging to the family Lamiaceae, was selected for the present study, and the plant propagules were collected from Kallai, Kozhikode District (longitude of 11°13′57.72″ N, 75°47′35.8692″ E) of Kerala, India. Stem cuttings of length 15–30 cm with uniform size and healthy appearance were treated with indole butyric acid (15 μM) for 1 h to induce root initiation.

For drought stress analysis, the cuttings were placed in poly bags of size (18 × 13 cm) filled with 1 kg solarized soil. After 30 d of growth, the plants were imparted with drought stress by withholding irrigation for 21 d and the well-watered plants were chosen as the control.

For imparting Zn stress, the cuttings were transferred to half-strength, modified Hoagland solution (Epstein 1972) taken in glass tubes (32 × 200 mm). After 15 d of growth, the plants were treated with 4 mM $ZnSO_4$ which was added to the nutrient solution (Shackira et al. 2017). The plants grown in half-strength Hoagland solution were considered as control.

Plants were maintained in the greenhouse at Department of botany, University of Calicut, Kerala, India, under controlled conditions of temperature (28 ± 2 °C), relative humidity (65 ± 5 °C) and natural daylight (800 ± 400 μmol^{-2} s^{-1}). The leaves collected from 21-d-old plants after treatment were used for different biochemical assays.

Reactive oxygen species – *Hydrogen peroxide.* Hydrogen peroxide was estimated as described by Junglee et al. (2014). Two hundred milligrams of leaf tissue of *P. amboinicus* was homogenized in 5 mL of 0.1% ice-cold trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min. Potassium phosphate buffer (pH 7) and 1 M potassium iodide solutions were used for the estimation of hydrogen peroxide. The absorbance of the mixture was measured at 390 nm.

Superoxide content. Superoxide content was estimated as described by Sruthi and Puthur (2020). Two hundred milligrams of leaf tissue was cut into 1×1 mm size and then immersed in 0.01 M potassium phosphate buffer (pH 7.8) containing 0.05% nitro blue tetrazolium (NBT) and 10 mM sodium azide. The mixture was kept in water bath at 85 °C for 15 min. After incubation, the mixture was quickly transferred to an ice bath for effecting a sudden decrease in the temperature of the solution. After cooling, the absorbance of the mixture was measured at 580 nm and sodium nitrate was used as the standard.

Lipid peroxidation. The MDA content was estimated according to the procedure of Wahsha et al. (2012). Two hundred milligrams of leaf tissue was homogenized in 5 mL of 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min at room temperature. Two milliliters of the supernatant was mixed with an equal aliquot of 0.5% of thiobarbituric acid (TBA) in 20% TCA. The solution was heated at 95 °C for 24 min, cooled, and then centrifuged at 3000 rpm for 2 min. The absorbance of the supernatant was measured at 532 and 600 nm, and the MDA content was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}.

Primary metabolites – The total soluble sugar was estimated according to the method of Kafi et al. (2011). Two hundred milligrams of fresh samples was homogenized in 10 mL of 80% ethanol (v/v) and centrifuged at 8,000 rpm for 10 min at 4 °C, and the supernatant was collected. For the estimation of sugar content, 5% (v/v) phenol was added to 100 µL of supernatant. Five milliliters of concentrated sulfuric acid was added to the tube quickly from a burette. After cooling, the absorbance was measured at 490 nm, and *D*- glucose was used as the standard.

Total free amino acids were estimated according to the method of Vardharajula et al. (2011). Five hundred milligrams of tissue was homogenized with 80% (v/v) ethanol. The extract was centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatant was made up to 10 mL with 80% ethanol. One milliliter of the sample was mixed with 1 mL of ninhydrin reagent and was kept in a boiling water bath for 20 min and further 5 mL of diluent (equal volume of water and *n*-propanol) was added to it. This mixture was incubated at room temperature for 15 min, and absorbance was read at 570 nm. Free amino acids were calculated from a standard curve prepared with glycine.

Secondary metabolites – Total phenolic was estimated using Folin-Denis reagent according to the method of Mane et al. (2011). One gram of fresh tissue was weighed and homogenized in 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min, and the supernatant was collected. Two milliliters of Folin-Denis reagent and 1 N sodium carbonate was used to estimate phenolics content, and the optical density was measured at 650 nm. *L*- catechol was used as the standard.

Anthocyanin content was determined according to the method of Thabti et al. (2011) with some modifications. Fresh leaf samples (0.2 g) were homogenized and extracted in 5 mL of acidified methanol (1: 99, HCl/methanol, v/v). The extract was kept at 4 °C for 24 h and was made up to 10 ml. The absorbance was read at 530 nm.

Total flavonoid content was measured by aluminum chloride colorimetric assay as per the method of Tambe and Bhambar (2014). Methanolic extract of *P. amboinicus* was used to estimate the flavonoid content. An aliquot (1 mL) of the extract was added to a 10 mL volumetric flask containing 4 mL distilled water. To this 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 0.3 mL 10% $AlCl_3$ was added. Again, after 5 min, 2 mL 1 M NaOH was added and the total volume was made up to 10 mL with distilled water. The solution was mixed well, and absorbance was measured against reagent blank at 510 nm.

Total alkaloid content was measured by the bromocresol green method according to Shamsa et al. (2008). One milliliter of the methanolic extract of the leaves of *P. amboinicus* was added into dimethyl sulfoxide (DMSO) and the extract gets dissolved in it. To this mixture, 1 mL of 2 N HCl was added and filtered. Then, 5 mL each of bromocresol green and phosphate buffer was added to the filtrate. These mixtures were transferred to volumetric flasks (10 mL) and shaken well after adding 4 mL of chloroform. The absorbance of solutions was recorded against blank at 470 nm. Atropine was used as the standard.

GC–MS analysis – GC–MS analysis of the crude extract prepared from shade-dried leaves of *P. amboinicus* was done according to the modified protocol of Kozlowska et al. (2015). Initially, fresh leaves were collected, shade-dried, and made into fine powder using a blender. Later on, extraction was performed by mixing and heating of 2 g of the powdered sample with 20 mL of methanol (90%) on a water bath for 10 h at 45 °C. On cooling, the resultant solution was filtered and the solvent was removed to dryness in a rotary evaporator at 40^0C. Further, the obtained extracts were stored at – 26 °C. Extracts were analyzed by GC–MS (QP2010S, Shimadzu, Italy). A Rxi-5Sil MS column (30 m length $\times$ 0.25 mm ID $\times$ 0.25 µm thickness) was used for gas chromatographic separation. Initially, the column oven temperature was maintained at 80 °C for 4 min, then increased to 260 °C and held for 6 min. Compounds were identified in the samples by comparing with the mass spectra of NIST 11 and WILEY 8 library. The important bioactive compounds induced during drought and Zn stresses are illustrated with the software ChemDraw pro 12.

Enzymatic antioxidants – *Superoxide dismutase (SOD).* Five hundred milligrams of fresh leaf tissue was homogenized in 5 mL of phosphate buffer (50 mM, pH 7.8), and 100 mg of polyvinyl polypyrolidone was added to it as a phenolic binder. The homogenate was centrifuged at 16,000 rpm for 15 min in centrifuge at 4 °C. For the estimation of SOD activity, the protocol of Kubiś (2008) was adopted and the measurements were taken using a UV–visible double-beam spectrophotometer (Systronics 2201, India).

*Catalast (CAT).*Five hundred milligrams of leaf tissue was homogenized in 5 mL of 50 mM phosphate buffer (pH 7.0). The homogenate was filtered through two-layered muslin cloth and was made up to 10 mL using phosphate buffer. The filtrate was then centrifuged at 16,000 rpm for 15 min at 4 °C in refrigerated centrifuge. The supernatant was used for the enzyme assay. The activity of CAT in the fresh samples was determined, following the method of Radhakrishnan and Lee (2013).

Guaiacol peroxidase (GPOX). Guaiacol peroxidase activity in leaves of *P. amboinicus* was measured according to Jisha and Puthur (2016). Three milliliters of assay mixture consisted of 2.77 mL of 100 mM phosphate buffer (pH 7.8), 30 µL 1% guaiacol, and 200 µL enzyme extract. For the initiation of the enzyme activity, 12 µL of hydrogen peroxide was added. Immediately after the addition of hydrogen peroxide, the increase in absorbance due to oxidation of guaiacol was measured at 420 nm for 3 min at intervals of 30 s.

Ascorbate peroxidase (APX). Five hundred milligrams of fresh leaf tissue was homogenized in 5 mL of extraction medium (100 mM phosphate buffer, pH 7.8). APX activity in the fresh samples was assayed by the method of Weisany et al. (2012).

Statistical analysis – To evaluate the significant difference in the traits among control and stress affected plants, SPSS software (SPSS 16.0, Chicago, USA) was used. Pearson's correlation analysis was performed to evaluate the relationships between the most important variable measured in *P. amboinicus* during drought and Zn stresses. The data represent mean ± standard error (SE), and the values are the average of recordings from three independent experiments, each with three replicates (i.e. $n = 9$).

3 Results

In this study, the concentration of Zn chosen to impart heavy metal stress in *P. amboinicus* was 4 mM, which was based on the potential of this concentration to develop visible stress symptoms. For imparting of drought stress to *P. amboinicus*, irrigation was withheld for a period of 21 d until visible drought symptoms were observed in the leaves.

Reactive oxygen species – Superoxide content showed an increasing trend in both drought and $ZnSO_4$ treated plants when compared to their respective controls. Superoxide content was increased by 35% in *P. amboinicus* grown under drought stress, while it increased by 28% in plants grown under $ZnSO_4$ treatment over their respective controls (Fig. 1a). Significant accumulation of hydrogen peroxide content was observed in plants subjected to drought stress on 21 d (37%) than its respective control. Hydrogen peroxide content was decreased by 23% in *P. amboinicus* grown under $ZnSO_4$ treatment as compared to control plants (Fig. 1b).

Lipid peroxidation – Treatment of *P. amboinicus* with $ZnSO_4$ exhibited an increase (30%) in the MDA content on 21 d of treatment as compared to its respective control plant, while for plants subjected to drought, the accumulation of MDA was increased by 405% as compared to the respective control (Fig. 1c).

Primary metabolites – Primary metabolites like total sugars and total free amino acids exhibited significant variation in *P. amboinicus* upon imparting of drought as well as zinc stresses (Fig. 2).

Treatment with $ZnSO_4$ caused a gradual increase (24%) of total sugar content in *P. ambionicus* grown under hydroponics conditions, while for drought-stressed plants, the total sugar content was found to increase by 83% over its respective control plants on 21 d of treatment (Fig. 2).

On imparting of drought stress, *P. amboinicus* showed an exponential increase (19-fold) in total free amino acids content on 21 d of treatment. But the total free amino acids content in $ZnSO_4$-treated ones exhibited a reduction (42%) as compared to the respective control (Fig. 2).

Secondary metabolites – In drought-affected plants, total phenolics content was found to increase by 116% on 21 d of treatment, while in plants subjected to $ZnSO_4$ treatment, the phenolic content increased by 71% (Fig. 3a).

During drought stress, a sharp increase in the accumulation of flavonoid content (347%) was recorded in *P. amboinicus,* whereas in plants subjected to $ZnSO_4$ treatment only a lesser increase (58%) was induced (Fig. 3b). Flavonoid content showed a strong significant positive correlation with amino acids ($r = 0.943$, $P \leq 0.01$), sugars ($r = 0.853$, $P \leq 0.01$), phenolics ($r = 0.737$, $P \leq 0.01$), and MDA ($r = 0.777$, $P \leq 0.01$) content in the leaves of *P. amboinicus* exposed to drought and Zn stresses (Table 1).

A significant increase in anthocyanin content was recorded in plants subjected to $ZnSO_4$ treatment on 21 d (36%) over its respective controls, while in those subjected

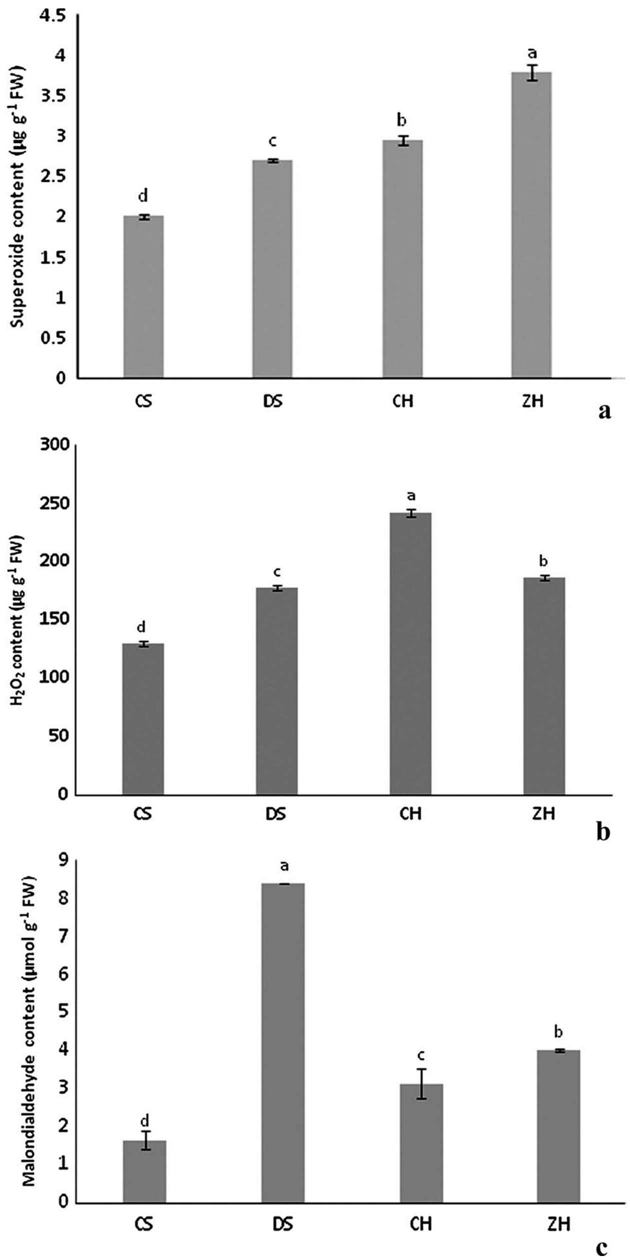

Fig. 1 ROS generation and lipid peroxidation in *P. amboinicus* exposed to drought and Zn stresses CS) control; DS) plants subjected to drought in soil; CH) control and ZH) plants subjected to Zn stress in Hoagland solution. **a** superoxide; **b** hydrogen peroxide; and **c** malondialdehyde content

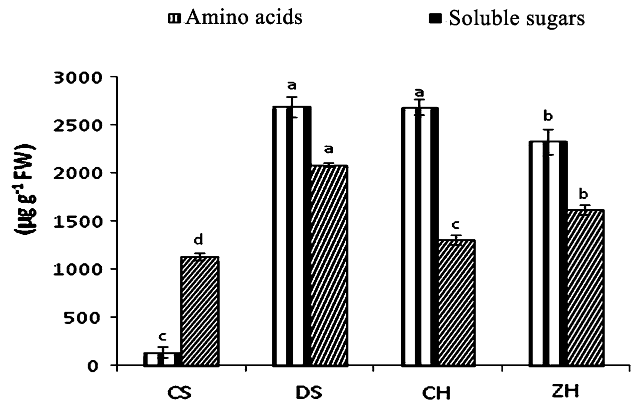

Fig. 2 Primary metabolites in *P. amboinicus* exposed to drought and Zn stresses CS) control; DS) plants subjected to drought in soil; CH) control and ZH) plants subjected to Zn stress in Hoagland solution

to drought stress, it was found to decrease by 33% (Fig. 3c). Total alkaloid content was found to be significantly higher in $ZnSO_4$-treated plants than plants subjected to drought. On $ZnSO_4$ treatment, a sharp increase in accumulation of alkaloid content (99%) was recorded in *P. amboinicus* plants, while for drought-exposed plants, the increase was only 9% (Fig. 3d).

Changes in secondary metabolites of *P. amboinicus* under drought and Zn stresses were evaluated by GC–MS analysis (Table 2). Development of different secondary metabolites was observed in drought and Zn stress-imparted plants as compared to their respective control plants. Hexadecanoic acid (9.37%), 2-methoxy-4-ethyl-6-methylphenol (5.28%), gamma-sitosterol (5.21%), alpha-amyrin (3.69%), ethyl linalool (3.55%), alpha-tocopherol (2.06%), octadecanoic acid (1.15%), isophytol, acetate (1.23%), and 9, 12, 15-octadecatrienoic acid (0.91%) were the major secondary metabolites developed under drought as compared to its respective control. But under $ZnSO_4$ treatment, squalene (3.66%), 1,5-dimethyl-1-vinyl-4-hexenyl isovalerate (3%), trans-alpha-bergamotene (2.53%), alpha-tocopherol (1.08%), gamma-tocopherol (0.80%), (-)-spathulenol (0.54%), neophytadiene (0.25%), and camphanol acetate (0.24%) were the secondary metabolites developed in *P. amboinicus* as compared to its respective control. The values in the parenthesis represent the area percentage of the bioactive compound detected in the methanolic extracts of leaves. The mass spectra of all these compounds are represented in the Supplementary Fig. 1.

More than the detection of new secondary metabolites, quantitative variations of different metabolites were found between control and both stress-affected plants. A reduction in the area percentage of phytol was observed in *P. amboinicus* exposed to drought and Zn stressors, and it was to the extent of 1.9 and 8.35%, respectively. A prominent increase in the area percentage, representing the content of 5-isopropyl 2-methylphenol, was observed under $ZnSO_4$ treatment (68%) and drought stress (38%). In plants exposed to drought and Zn stressors, the area percentage of phenol, 2-methyl-5-(1-methylethyl) decreased as compared to the control. But, the levels of few other metabolites were increased upon $ZnSO_4$ treatment. The area percentage of caryophyllene was increased in $ZnSO_4$ treated *P. amboinicus* (5.8%)

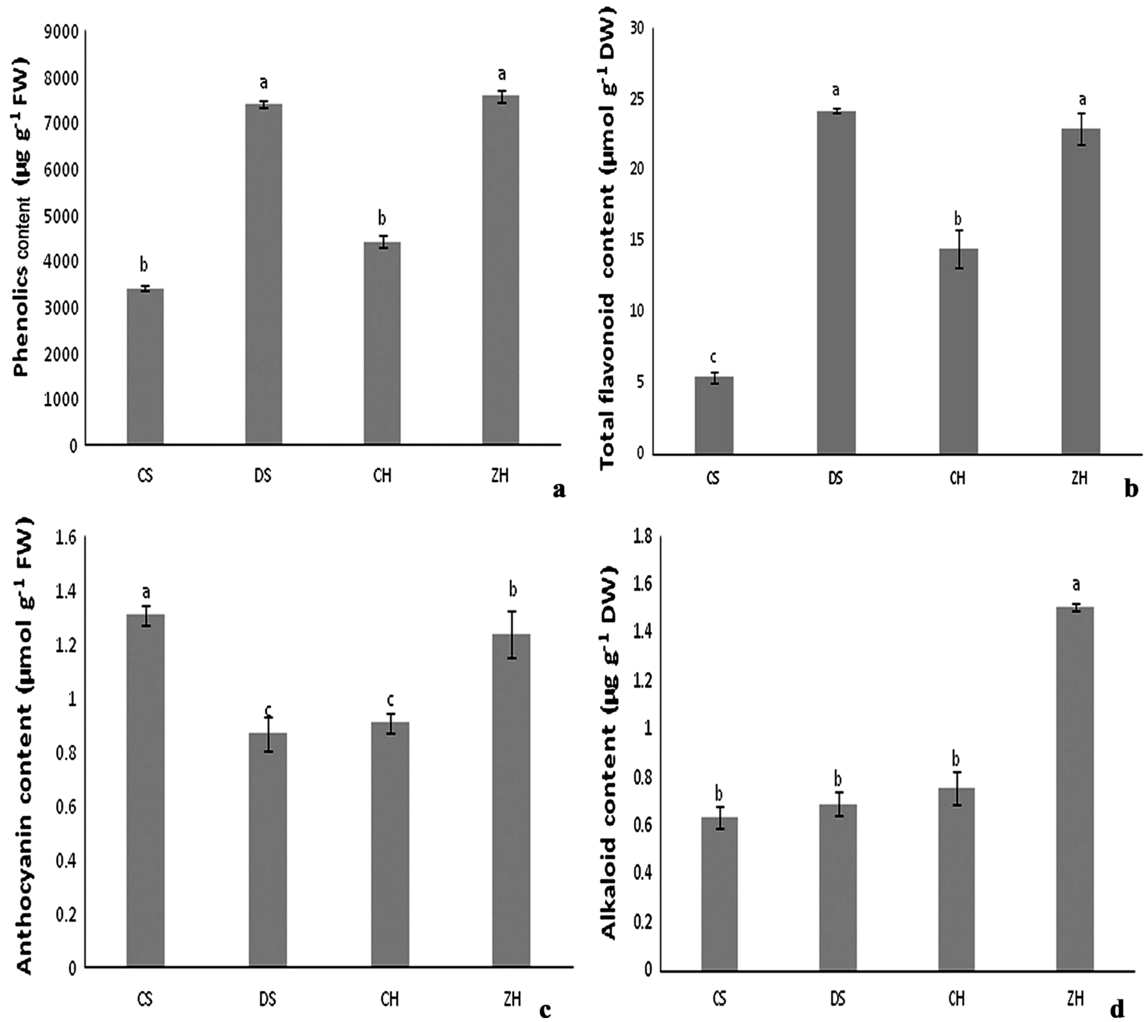

Fig. 3 Secondary metabolites in *P. amboinicus* exposed to drought and Zn stresses CS) control; DS) plants subjected to drought in soil; CH) control and ZH) plants subjected to Zn stress in Hoagland solution. **a** total phenolics content; **b** total flavonoids; **c** anthocyanin content; and **d** alkaloid content

as compared to its respective control (0.96%). The important bioactive compounds accumulated during Zn toxicity and drought are represented in Fig. 4.

Enzymatic antioxidants – Drought-exposed plants and $ZnSO_4$-treated plants exhibited differential responses with respect to the activities of enzymatic antioxidants like SOD, APX, GPOX, and CAT. The activity of SOD was found to be negligibly enhanced in both $ZnSO_4$-treated and drought-exposed plants as compared to the respective control plants. The activity of GPOX in drought-exposed and $ZnSO_4$-treated plants exhibited a similar pattern of reduction, and it was to the extent of 64–67% as compared to their respective control plants. The APX activity of drought-exposed plants was twofold higher than $ZnSO_4$-treated plants. The CAT activity of $ZnSO_4$-treated plants was found to be 4.2 fold higher than that of drought-stressed plants (Fig. 5).

4 Discussion

In this study, the concentration of $ZnSO_4$ chosen to impart heavy metal stress in *P. amboinicus* was 4 mM, which was based on the potential of this concentration to impart immediate, observable stress symptoms. The same concentration of $ZnSO_4$ to imparted toxicity in *Acanthus ilicifolius* L., causing significant metabolic alterations in the plant (Shackira et al. 2017). For imparting drought stress to *P. amboinicus*, irrigation was withheld for a period of 21 d until visible drought symptoms were observed in the leaves. The drought-induced morphological and physiological modifications were observed in *Abelmoschus esculentus* (L.) Moench on 7 d of drought (Pravisya et al. 2019), but in maize plants, these modifications were observed on 12 d of stress (Efeoğlu et al. 2009). Delayed expression of drought-induced phenological modifications

Table 1 The correlation observed between different biochemical parameters of *P. amboinicus* exposed to drought and zinc stress

	Amino acids	Sugars	Phenolics	MDA	Flavonoids	Alkaloids	Anthocyanin	Superoxide	Hydrogen peroxide	SOD	APX	GPOX	CAT
Amino acids	1												
Sugars	0.662*	1											
Phenolics	0.588*	0.754**	1										
MDA	0.631*	0.913**	0.565	1									
Flavonoids	0.943**	0.853**	0.737**	0.777**	1								
Alkaloids	0.233	0.244	0.632*	− 0.106	0.346	1							
Anthocyanin	− 0.555	− 0.361	− 0.254	− 0.461	− 0.472	0.355	1						
Superoxides	0.401	0.304	0.174	0.137	0.481	0.572	0.297	1					
Hydrogen peroxide	0.797**	0.123	0.118	0.116	0.584*	0.094	− 0.496	0.360	1				
SOD	0.384	0.001	0.452	− 0.247	0.330	0.815**	0.189	0.536	0.444	1			
APX	0.457	0.162	0.714**	− 0.074	0.425	0.786**	− 0.065	0.178	0.376	0.826**	1		
GPOX	− 0.290	− 0.844**	− 0.783**	− 0.712**	− 0.577*	− 0.446	− 0.021	− 0.280	0.314	− 0.063	− 0.219	1	
CAT	0.328	0.598*	0.836**	0.318	0.549	0.877**	0.208	0.525	− 0.072	0.575	0.640*	− 0.809**	1

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

in *P. amboinicus* indicated its high tolerance toward drought stress.

ROS production and lipid peroxidation – Drought stress disturbs the balance between the generation of reactive oxygen species (ROS) and the antioxidant defense, resulting in the accumulation of ROS that induces oxidative stress to biomolecules like proteins, membrane lipids, and other cellular components (Waraich et al. 2011). ROS generation, known as oxidative burst, is one of the major responses of plants exposed to drought stress, which triggers serious threat to plants by increasing lipid peroxidation, protein degradation, and DNA degradation which finally causes cell death (Anjum et al. 2011). Enhanced metal uptake and accumulation in roots of *P. amboinicus* induce higher levels of free radical generation, which was indicated by high MDA content in roots. A similar case of enhanced MDA content was reported in *A. ilicifolius* when subjected to zinc stress (Shackira et al. 2017).

Malondialdehyde is the end product of lipid peroxidation, which can be related to oxidative damage, and thus can be considered as a sensitive index of oxidative stress (Yan and Tam 2013; Hashem et al. 2016). As a result of lipid peroxidation, several products are formed from polyunsaturated precursors which comprise small hydrocarbon fragments such as ketones and malondialdehydes (Garg and Manchanda 2009). Once polysaturated fatty acids undergo peroxidation by ROS attack, it may lead to membrane fluidity and increased permeability (Sharma et al. 2012). According to Djebali et al. (2005), when plants are exposed to stressful environmental conditions, the rate of lipid peroxidation gets enhanced which ultimately results in loss of membrane integrity; this, in turn, results in leakage of essential elements. MDA accumulation was increased in *P. amboinicus* exposed to drought stress and Zn toxicity and the rate of increase was much higher on exposure to drought stress indicating that drought stress was more severe causing cell membrane degradation and death of cells.

Primary metabolites – Amino acids are considered to a play major role in plant stress metabolism. In drought-exposed and $ZnSO_4$-treated plants of *P. amboinicus*, a decreasing trend of total free amino acids content was observed, which can be correlated with the accumulation of secondary metabolites. Amino acids form precursors of alkaloids, phenols, and lignin and are often channeled to secondary metabolism for the synthesis of secondary metabolites. It may occur by shifting of amino acid biosynthesis pathway to these synthesizing secondary metabolites. Aromatic amino acids like phenylalanine, tyrosine, and tryptophan are synthesized via shikimate pathway and turn to be precursors of several secondary metabolites with chorismate as branch point (Tzin and Galili 2010). The metabolites like phenylpropanoids are

Table 2 Bioactive compounds in *P. amboinicus* after exposure to drought and zinc stresses

Sl. No	Bioactive compounds	Area percentage (%)				Retention time (t_R, min)
		Control (soil)	Drought stressed (soil)	Control (Hogland)	Zinc stressed (Hogland)	
1	(-)-Spathulenol	0	0	0	0.54	23.09
2	(-)-Beta-caryophyllene	0	0	0	3.28	17.00
3	Alpha-amyrin	0	3.69	0	0	44.04
4	Alpha-bergamotene	1.55	0.5	0	0	17.34
5	Alpha-caryophyllene	0	0	0	1.1	17.93
6	Gamma-sitosterol	0	5.21	0	0	40.83
7	Gamma-tocopherol	1.39	0.66	0	0.8	46.90
8	1,5-dimethyl-1-vinyl-4-hexenyl isovalerate	0	0	0	3.02	40.54
9	10–12-Pentacosadiynoic acid	0	0	1	0	23.08
10	1-pentadecene	0	0	0	0.25	21.26
11	2,4-ditert-butylphenol	1.55	1.62	3.63	0.39	19.30
12	2-camphanol acetate	0	0	0	0.24	39.22
13	2-methoxy-4-ethyl-6-methylphenol	0	5.28	0	0	20.70
14	5-isopropyl-2-methylphenol	0	38.64	0	68.62	13.97
15	8-octadecanone	0	0.41	0	0	27.41
16	8-pentadecanone	0.79	0.6	0	0	23.09
17	9,12,15-octadecatrienoic acid, methyl ester	0	0	0.55	0	31.76
18	9,12,15-octadecatrienoic acid, methyl ester, (z,z,z)-	1.29	0.91	0	1.87	31.77
19	9,12-octadecadienoic acid, methyl ester, (e,e)-	0	1.75	0	0	32.57
20	Caryophyllene	1.8	1.1	0.96	0	17.02
21	Caryophyllene oxide	0	0	0	0.99	21.09
22	Cis,cis,cis-7,10,13-Hexadecatrienal	0	6.8	0	0	32.74
23	Dichloroacetic acid, tridec-2-ynyl ester	2.42	0	0	0	40.56
24	Dl-alpha-tocopherol	0	2.06	0	1.08	48.41
25	Ethanone, 1-(2-hydroxy-5-methoxyphenyl)-	0	0	1.01	0	20.87
26	Ethyl linalool	0	3.55	0	0	40.55
27	Hexadecanoic acid	0	9.37	0	0	29.45
28	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	1.34	0	0	0	38.88
29	Hexadecanoic acid, methyl ester	0	0.39	0	0	28.45
30	Isophytol, acetate	0	1.23	0	0	33.84
31	Lupeol	3.66	0	0	0	44.13
32	Methyl octadeca-9,12-dienoate	0	0	0	0.47	31.65
33	N,n-bis(2-hydroxyethyl)dodecanamide	0	2.52	0	0	20.80
34	Neophytadiene	1	0.52	0	0.25	26.63
35	Octadecanoic acid	0	1.15	0	0	33.13
36	Pentadecanoic acid, 14-methyl-, methyl ester	0	0	0.33	0.55	28.44
37	Phenol, 2-methyl-5-(1-methylethyl)-	32.23	0	79.96	0	13.96
38	Phytol	37.11	1.9	5.2	8.35	31.99
39	Phytol, acetate	0	0.32	0	0	27.52
40	Propanoic acid, anhydride	0.78	0	0	0	44.68
41	Squalene	4.52	2.01	0	3.66	43.15
42	Trans-alpha-bergamotene	0	0	0	2.53	17.31
	Total percentage	100	100	100	100	

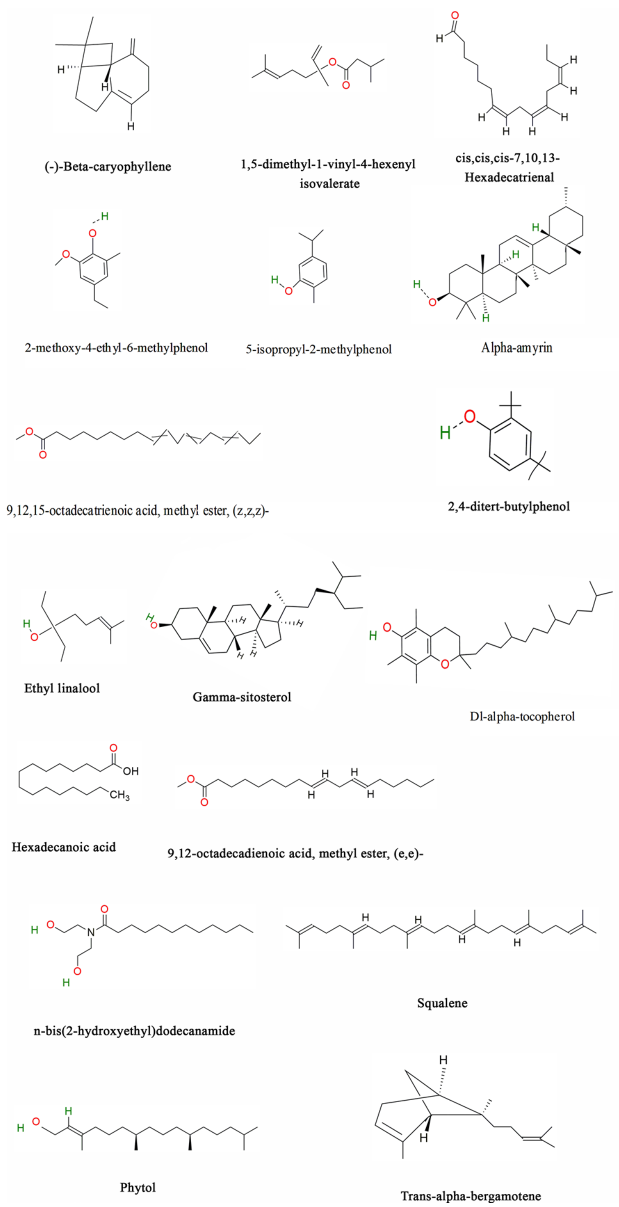

Fig. 4 Important biomolecules induced in the leaves of *P. amboinicus* exposed to drought and Zn stresses

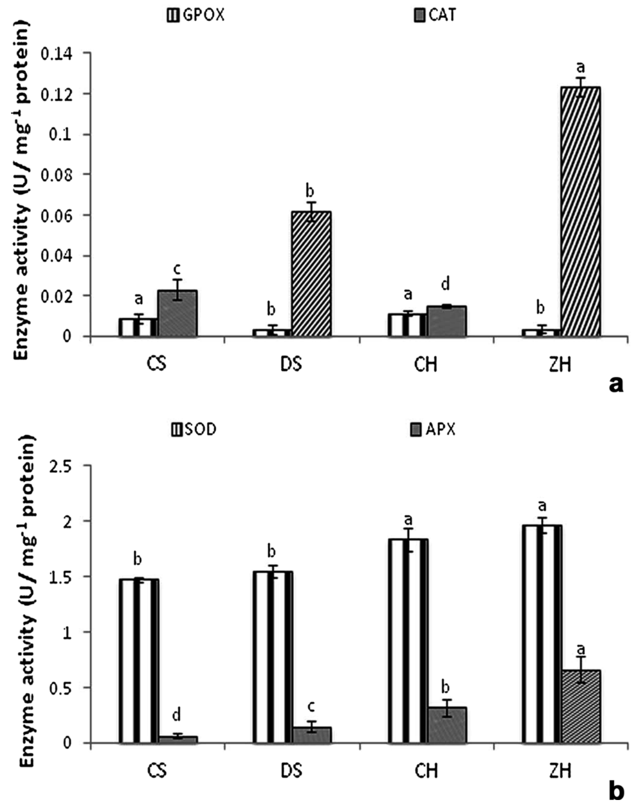

Fig. 5 Enzymatic antioxidants in the *P. amboinicus* exposed to drought and Zn stresses CS) control; DS) plants subjected to drought in soil; CH) control and ZH) plants subjected to Zn stress in Hoagland solution. **a** GPOX and CAT activity; **b** SOD and APX activity

derived from the amino acid phenylalanine (Vogt 2010). The biosynthetic units of terpenes, isopentenyl diphosphate, and dimethylallyl diphosphate that originates from mevalonate, and alternatively from methyl erythritol phosphate, are derived from the precursors of glycolytic pathway (Kirby and Keasling 2009). Nitrogen-containing alkaloids are derived from a variety of primary metabolites, including amino acids and purine nucleosides (Facchini 2001).

In drought-exposed and $ZnSO_4$-treated plants of *P. amboinicus*, the accumulation of soluble sugars was increased which accounts for its enhanced antioxidant potential and osmotic adjustment. Increased accumulation of sugar content in plants occurs due to reduced soil water content (Arabzadeh et al. 2013). Heavy metal toxicity leads to the blockage of the xylem vessels that reduces the availability of water to the roots of plants, leading to an osmotic stress situation (Shackira and Puthur 2017). Accumulation of soluble sugars in rice plants was considered as a tolerance mechanism to overcome the osmotic stress developed due to Zn toxicity (Sinisha and Puthur 2018). The accumulation of soluble sugars as a result of exposure to drought stress and Zn can be positively correlated with the increase of relative leaf water content (Irigoyen et al. 1992; Parida et al. 2007) and plays the main role in the osmotic adjustment of the plants (Sanchez et al. 2004; Parida et al. 2007; Hessini et al. 2009; Zhou and Yu 2009). Moreover, soluble sugars act as defense signal which can simultaneously sense and control both photosynthetic activity and ROS balance, by regulating defense against increasing ROS levels (Couée et al. 2006).

Secondary metabolites – Secondary metabolites like phenolics, flavonoids, anthocyanin, and alkaloids were detected in different plants exposed different abiotic stresses like drought, salinity, and heavy metal toxicity (Sen et al. 2020;

Janeeshma and Puthur 2020; Sameena and Puthur 2020). Parallel to these results, *P. amboinicus* increased the biosynthesis of all these metabolites on exposure of drought and Zn stresses. Total phenolics content was found to increase in *P. amboinicus* exposed to both stress treatments and can be correlated with the accumulation of secondary metabolites like flavonoids, anthocyanin, and alkaloids, respectively. Flavonoids are phenylpropane derivatives which comprise flavonones, flavonols, and anthocyanidins. Flavonoid accumulation was found to be higher in *P. amboinicus* when subjected to drought stress, and this was similar to the increase in flavonoid, reported in *Pisum sativum* L. (Nogués et al.1998) and also in *Labisia pumila* (Blume) Fern. by Jaafar et al. (2012) subjected to drought stress. In Zn treated plants, the increase of phenol content was lower as compared to drought-affected plants of *P. amboinicus,* indicating that latter stress could induce higher accumulation of phenolics. The phenolic acids, flavonoids, and antioxidants in *Amaranthus tricolor* L. exposed to drought stress exhibited a quantitative and qualitative improvement (Sarker and Oba 2018). And the case was similar in *Hypericum brasiliense* Choisy wherein increased production of various phenols and betulinic acid was recorded under temperature stress (Abreu and Mazzafera 2005). According to Selmar (2008), plants under stress produce more phenolic compounds than those grown under normal conditions, which plays a significant role in free radical scavenging and protects plant cells from harmful effects of oxidative stress.

Higher alkaloid accumulation in $ZnSO_4$-treated plants of *P. amboinicus* can be correlated with that of *Bacopa monneria* (L.) Pennell subjected to copper and cadmium stresses (De Backer-Royet et al. 1990). It can also be corroborated with the increasing accumulation of alkaloids in roots of *Catharanthus roseus* (L.) G. Don plants subjected to cadmium, manganese, and nickel (5 mM) treatments. Cd and Ni treatment resulted in twofold, whereas Pb treatment resulted in threefold increase in serpentine content of roots (Srivastava and Srivastava 2010). Similarly anthocyanin was also found to be higher in $ZnSO_4$-treated plants, which was similar to the consistent increase in anthocyanin content in *Arabidopsis thaliana* (L.) Heynh. subjected to treatments with higher concentrations of essential (Cu, Zn, and Mn) and non-essential (Pb and Hg) metals. The strong positive correlation observed between the flavonoid content and other parameters such as MDA, amino acids, sugars, and phenolics indicates the potential of *P. amboinicus* to tolerate the oxidative stress by the over-accumulation of these compounds, well known in combating abiotic stresses.

GC–MS profiling of the methanolic extracts of *P. amboinicus* leaves revealed the occurrence of different chemical compounds belonging to different classes, and most of them were reported to exhibit important biological activities. Ethyl linalool is the plant product to which receptor of neurons responds primarily and it has been identified in different pests living on angiosperms (Mustaparta and Stranden 2005). This compound is widely used in perfume industry due to its slow rate of evaporation and in *P. amboinicus* its production was induced by drought stress. Amyrins are three closely related natural chemical compounds of the triterpene class. They are designated as α-amyrin, β-amyrin, and δ-amyrin and it was over-synthesized in *P. amboinicus* during drought stress. γ-sitosterol has been reported for the first time in *Girardinia heterophylla* (Decne) and has the potential to be used as an antidiabetic owing to its remarkable medicinal properties (Tripathi et al. 2013).

Drought-induced over-accumulation of γ-sitosterol was observed in *P. amboinicus*. Detection of gamma-tocopherol and alpha-tocopherol in plants subjected to zinc stress was an interesting observation during the study. Tocopherols are methylated phenols, many of which have vitamin E activity, with antioxidation potential. It was reported that vitamin E was the chemical compound determining the metal tolerance potential of *A. thaliana* and the vitamin E deficient mutant exhibited a drop in the tolerance potential toward metal stress (Collin et al. 2008). The enhancement of this antioxidant may help to impart tolerance to *P. amboinicus* toward a higher concentration of zinc. The antioxidant, antibacterial, and other prophylactic activities of *Eupatorium odoratum* L. were attributed to the presence of different compounds; 2,4-bis (1-phenylethy)-phenol, hoslundin, 2,4,6-tris (1-phenylethyl)-phenol; dl-α-tocopherol, phytol, 1,2,4-oxadiazol-5-amine, 3- (4amino-1,2,5-oxadiazol-3-yl)-n-[2- (4-methoxyphenyl), 1-heptacosanol, stigmasterol, γ-sitosterol, tetra-O-methylscutellarein, neophytadiene, (35)-7-omethoxymethylvestitol, α-amyrin, methylcommate *D,* and 4-acetyl-3-hydroxy 2,6-dimethoxytoluene (Raman et al. 2012). Most of these compounds were detected at higher levels in *P. amboinicus* subjected to both stresses. It indicates the antioxidant, antibacterial, and prophylactic potential of *P. amboinicus*. 5-isopropyl-2-methylphenol/phenol, 2-methyl-5-(1-methylethyl) is the most prominent phytochemical detected in *P. amboinicus,* and it is a phenolic constituent of many essential oils. Commonly it is known as carvacrol and it is a monoterpenic phenol. The reduction of carvacrol in plants subjected to both drought and zinc stresses can be related to the development of other metabolites, which are formed from the intermediary compounds of the same pathway operational in plants subjected to these two stresses. Phytol, the side chain of the photosynthetic pigment chlorophyll, is the most abundant plant terpenoid (Davis and Croteau 2000). Phytol was reported in *Glycosmis pentaphylla* (Retz.) DC. leaves as a dominant compound in essential oils (Prakasia and Nair 2015). It was increased in *P. amboinicus* subjected to drought and Zn stresses, which indicates severe pigment degradation and at the same time enrichment of different essential oils by this compound.

Antioxidant enzymatic system – Enzymatic antioxidant defense system consists of several enzymes such as SOD, APX, GPOX, and CAT which mediate the scavenging of toxic ROS in cells. Quick scavenging of ROS occurs due to the enhanced activity of antioxidant enzymes, which helps the plant in encountering oxidative stress (Alqarawi et al. 2014; Hashem et al. 2016). SOD detoxifies superoxide radicals which prevent stress-induced cellular damage (Gratão et al. 2005). The SOD activity produces hydrogen peroxide which is converted to water by the action of catalase or ascorbate peroxidase (Cakmak 2000; Hegedus et al. 2001; Hashem et al. 2016). Glutathione reductase (GR) and ascorbate peroxidase are important enzymes of ascorbate–glutathione cycle, and GR catalyzes the conversion of oxidized glutathione (GSSG) to reduced form (GSH) and thus tries to maintain a higher ratio of GSH/GSSG (Noctor and Foyer 1998; Rausch et al. 2007; Hashem et al. 2016). When *P. amboinicus* was subjected to drought and zinc stresses, the activity of antioxidant enzymes was found to be activated at increased levels. Increased accumulation of Zn in plant tissue has increased the ROS levels and antioxidant enzymes play a major role in removing these destructive oxygen species (Zhu 2003; Parida and Das 2005). In plants treated with $ZnSO_4$, the H_2O_2 persisting in its cells was less as compared to its control and that was due to the high activity of CAT. CAT constitutes to be the vital one as compared to APX and GPOX, in detoxification of H_2O_2 in *P. amboinicus*, which can be correlated with the higher CAT activity in *A. ilicifolius* exposed to heavy metal stress (Shackira and Puthur 2017).

Plectranthus amboinicus showed significant variations in the accumulation rate of primary and secondary metabolites after imparting drought and zinc stresses. These two stressors elicited different primary metabolic changes in the plants, and the variation was specifically observed in the content of amino acids. In *P. amboinicus*, exposure to drought and zinc stresses has resulted in a remarkable increase in the accumulation of various secondary metabolites like flavonoids, alkaloids, phenolics, and anthocyanin, enhancing the potential of the plant to withstand both stresses. Compositional variation of secondary metabolites accumulated on exposure to drought and zinc stresses indicates the inherent potential of this medicinal plant to withstand adverse environmental conditions with the aid of over synthesized secondary metabolites. This study conclusively proved the potential of drought and Zn stresses to increase the accumulation of secondary metabolites in *P. amboinicus*, which aid in drought and zinc stress alleviation, and at the same time, these metabolites are also medicinally important.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40415-021-00738-4.

Acknowledgements The authors greatly acknowledge the financial assistance provided by University Grant Commission (UGC), India, in the form of JRF and the corresponding author expresses sincere thanks to KSCSTE for funding through research grant (KSCSTE/5179/2017-SRSLS). The authors extend their sincere thanks to the Department of Science & Technology (DST), Government of India, for granting fund under Fund for Improvement of S&T Infrastructure (FIST) program (DST-FIST/15-16/28.05.2015).

Author contributions HH performed the analysis, processed the experimental data, and interpreted the results. EJ drafted the manuscript and designed the figures. JTP helped to shape the research and analysis aided in interpreting the results and worked on the manuscript.

Funding This work was supported by the University Grant Commission (UGC), India, in the form of JRF under Grant 319492.

Availability of data and materials Data sharing is not applicable to this article as all new created data are already contained within this article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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