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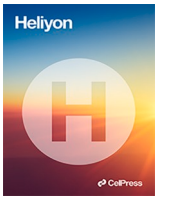

Research article

Untargeted metabolomics of *Aloe* volatiles: Implications in pathway enrichments for improved bioactivities

Nehal Batra, Priyankar Dey *

Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, 147004, Punjab, India

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ABSTRACT

Enhancing the production of economically and medically important plant metabolites by genetic and metabolic manipulation is a lucrative approach for enhancing crop quality. Nevertheless, the task of identifying suitable biosynthetic pathways related to certain bioactivities has proven to be challenging due to the intricate interconnections of the major metabolic and biochemical processes in commercially important plants. The commercial significance of plants belonging to the genus *Aloe* stems from their extensive utilization across several industries, such as cosmetics, pharmaceuticals, and wellness items, due to their medicinal properties. In the present study, we have utilized a reverse association approach to identify potential target metabolic pathways for enhancing the production of commercially important metabolites of *Aloe* spp., based on their metabolic pathway enrichment profile. The leaves of five highly utilized *Aloe* sp. were subjected to untargeted gas chromatography-mass spectrometry analysis followed by testing of free-radical scavenging effects against components of the Fenton and Haber-Weiss reaction. Through the application of appropriate bioinformatics tools, we identified distinct phytochemical classes and determined the enrichment of their corresponding biosynthetic pathways, associated the pathways with bioactivities, and also identified the inter-relation between the commonly enriched pathways. The strong association between metabolic pathways and antioxidant potentials suggested the necessity to enhance distinct but closely related metabolic pathways in order to enhance the quality of *Aloe* spp. and maximize their antioxidant effects for commercial exploitation in cosmetic industries.

1. Introduction

The genus *Aloe* comprise of the largest group of pants (over 650 spp.) under the family *Xanthorrhoeaceae* [1]. The *Aloe* spp. contains over 75 micronutrient components and over 250 different phytochemicals, including flavonoids, saponins, anthraquinones, and lignin, that exhibit various bioactivities, including antiviral, antibacterial, antifungal, anticancer, anti-inflammatory, moisturizing, anti-aging, immunostimulatory, anti-radiation, and wound healing properties [2,3]. Currently, these plants are considered to be highly significant in terms of their economic value in the field of medicine and pharmacology. They are frequently utilized in primary healthcare and traditional medicinal practice to effectively cure a wide range of disorders by modifying biochemical and molecular pathways [4,5]. In recent years, there has been a considerable increase in the use of *A. vera* in the cosmetic industry, in addition to a widespread acceptance of *Aloe*-based products by consumers due to its low dermatological sensitivity, and skin smoothening and

* Corresponding author.
E-mail addresses: priyankar.dey@thapar.edu, priyankardey28@gmail.com (P. Dey).

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moisturizing properties [2,6]. Especially due to potent antioxidant effects, an essential pre-requisite for cosmetic products, *Aloe*-based formulations are often included in commercial products [7,8]. For instance, various *Aloe* compositions have been demonstrated to possess anti-aging effects [9], UV-protective effects [10], and anti-proliferative effects [11] through potent free-radical scavenging and antioxidant properties. The amounts of *Aloe* plant components in cosmetic products can vary significantly, ranging from less than 1 % to as high as 20 % [12]. Due to the presence of a large variety of non-polar constituents, components of *Aloe* gel, upon topical application, are claimed to profoundly enter the deeper layers of the skin, facilitating the restoration of lost moisture and replenishment of the fatty layer, while neutralizing the free-radicals [13].

The composition of non-polar secondary metabolites, such as essential oils, found in medicinal plants are closely linked to their therapeutic effectiveness and can be targeted for enhancing crop quality. One can gain an understanding of how these substances are regulated metabolically by identifying the genes and enzymes involved in their biosynthesis pathways. Research on economically significant phytometabolites is accelerating as researchers aim to understand the pathways responsible for the manufacture of key metabolites and manipulate the phytochemical composition to match commercial demands [14]. Plant species belonging to the genus *Aloe* are commonly considered safe and serve as valuable resources for traditional remedies, pharmaceuticals, nutraceuticals, aromatherapy, preservatives, beverages, scents, cosmetics, and botanical pesticides [1]. Quantity and diversity of the metabolites are influenced by their biosynthetic pathways. To improve the production of industrially relevant phytochemicals, metabolic and genetic engineering techniques have been employed. For instance, by exploring full-scale functional genomics, one study generated a whole transcriptome sequence database with a focus on the metabolic specificity of *A. vera,* to identify the pathways related to secondary metabolite formation, metabolic regulation, and signal transduction, that contributes to the growth and physiology of the plant [15]. However, for the practical application of these strategies critical for crop improvement, it is necessary to first identify the specific biochemical pathways associated with the secondary metabolite formation. However, it is challenging since many of the plant's biosynthetic pathways are interconnected through a shared carbon pool [16,17]. Instead of focusing on individual genes responsible for a particular industrially significant phytochemical, it is more effective to enhance the complete biosynthetic route or multiple co-occurring biosynthetic pathways. This approach is more efficient and smarter strategy for developing economically valuable phytochemicals [14,18]. To achieve this objective, it is necessary to first identify the co-occurring plant biosynthetic pathways responsible for producing specific metabolites associated with distinct bioactivities.

Therefore, in the present study, we intended to identify the predominant and inter-correlated metabolic and biochemical pathways that commonly dictate the formation of the volatile secondary metabolites in the *Aloe* spp. This was based on the hypothesis that metabolome-based reverse association strategies for identification of the predominant biosynthetic pathways could lead to crop improvement through metabolic engineering approaches. For this purpose, we selected *Aloe vera* (L.) Burm.f., *AristAloe aristata* (Haw.) Boatwr. & J.C.Manning (aka, *Aloe aristata*), *Aloe jucunda* Reynolds, *Aloe* aspera Haw., and *Aloe* albiflora Guillaumin, which are among the highly utilized plants in the cosmetic industry that belong to the genus *Aloe* [19]. The volatile and non-polar metabolome of these plants were fingerprinted in an untargeted manner, followed by the study of the chemical-class enrichments and pathways analysis. The bioactivity potential was evaluated by testing the free-radical scavenging effects against the Fenton and Haber-Wess reaction-associated intracellular free-radical formation. Collectively, several clusters of enriched co-occurring metabolic pathways were detected, which could be potentially targeted using metabolic engineering strategies to enhance the antioxidant properties of plants belonging to the genus *Aloe*.

2. Methodologies

2.1. Plant material collection

The plant materials were collected from the medicinal garden of North Bengal University and processed as described previously [5]. In brief, disease-free leaves of *A. vera*, *A. aristata*, *A. jucunda*, *A. aspera, and A. albiflora* were hand-picked. The plant materials were identified and authenticated with accession numbers 09781, 09766, 09767, 09753, and 09779. The voucher specimens of the leaves were stored in the herbarium of North Bengal University.

2.2. Sample preparation and derivatization

The collected whole leaves were rinsed with double distilled water three times, chopped into 3–5 mm pieces with a sterile scalpel, and placed in a dehydrating incubator (37 °C) for 7-d until completely dried. Dried leaves (100 mg) were separately mixed with 3 mL n-hexane in recti vials and incubated in the dark for 24 h at 25 °C and 120 rpm. Next, 40 μL N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA + TMS) was added into the mixture and incubated under the same condition for 6 h. The mixture was then passed through activated charcoal and Na_2SO_4 (1:2, w/w) in a mini-column. The filtrate was spun at 15,000 rpm for 20 min at 25 °C, filtered through a 0.2 μm membrane syringe filter, and the resultant was used for GCMS analysis.

2.3. Gas chromatography-mass spectrometry

The processed and derivatized samples were analyzed in a Trace 1300 Gas Chromatography instrument and ISQ QD single quadrupole mass spectrophotometer (Thermo-Scientific) as per prior standardized protocol [14,20]. The separation column used was TG-5MS with a dimension of 30 m × 0.25 mm × 0.25 μm. Samples were injected (1 μL) in splitless mode using AI-1310 auto-sampler (Thermo-Scientific). The inlet port temperature was set at 250 °C, the initial column temperature was 60 °C with a solvent delay of 5

min (4 min hold), and the final temperature was 290 °C with 4 min hold at the end. The temperature ramp was set at 5 °C/min, achieving a total run time of 54 min (1 mL/min flow of 99.99 % helium passed through hydrocarbon and dehydrating columns). The transfer line for the mass spectrometer was set at 290 °C and an ion source temperature was kept at 230 °C (electron ionization mode). MS analyzer range was 50–650 amu and the samples were analyzed at electron energy 70 eV (vacuum pressure of 2.21 x 10^{-5} torr). MS data for identified peaks was analyzed using AMDIS (V2.7) where the major peaks were identified based on the base peak and molecular peak patterns of the library references using MS Interpreter (V2.0). All peak identification was done based on the integrated MS library of National Institute Standards and Technology. An abundance filter cutoff of 0.1 % and a probability filter cutoff of 75 % were used for the compound identification.

2.4. Free-radical scavenging bioassays

The Fenton and Haber-Weiss reaction serves as one of the key intracellular free-radical forming reactions generating the highly reactive radical ($OH^{\bullet}$), superoxide radical ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) from oxygen. Therefore, previously standardized *in vitro* assays regarding the formation and scavenging of $OH^{\bullet}$, $O_2^{\bullet-}$ and H_2O_2 were performed [21,22]. Additionally, iron chelation assay was performed to evaluate the Fe^{2+} to Fe^{3+} transition potentials of the *Aloe* leaf extracts [23]. This was important since transition metals (e.g., iron) can accelerate the Fenton reaction by triggering the formation of $OH^{\bullet}$ from H_2O_2. The overall reducing efficacy of the extracts was measured by monitoring the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which serves as a surrogate indicator of the total antioxidant assay of the extracts [24]. Since intracellular free-radical formation triggers the vicious cycle of membrane lipid peroxidation, the potential of the extracts to prevent $OH^{\bullet}$ radical mediate lipid peroxidation was measured using fresh chick brain samples. All assays were performed in 96-well plates as indicated previously [14].

3. Data analysis

Data were statistically analyzed using GraphPad V8 and quantitative data was represented as mean ± SEM. The inhibitory percentage (%) for the antioxidant assays was calculated by the formula $((X_0 - X_1) \div X0)*100$, where X_0 represents the absorbance of the control and X1 represents the absorbance of the sample and standard treated wells. The half maximal inhibitory concentration (IC_{50})

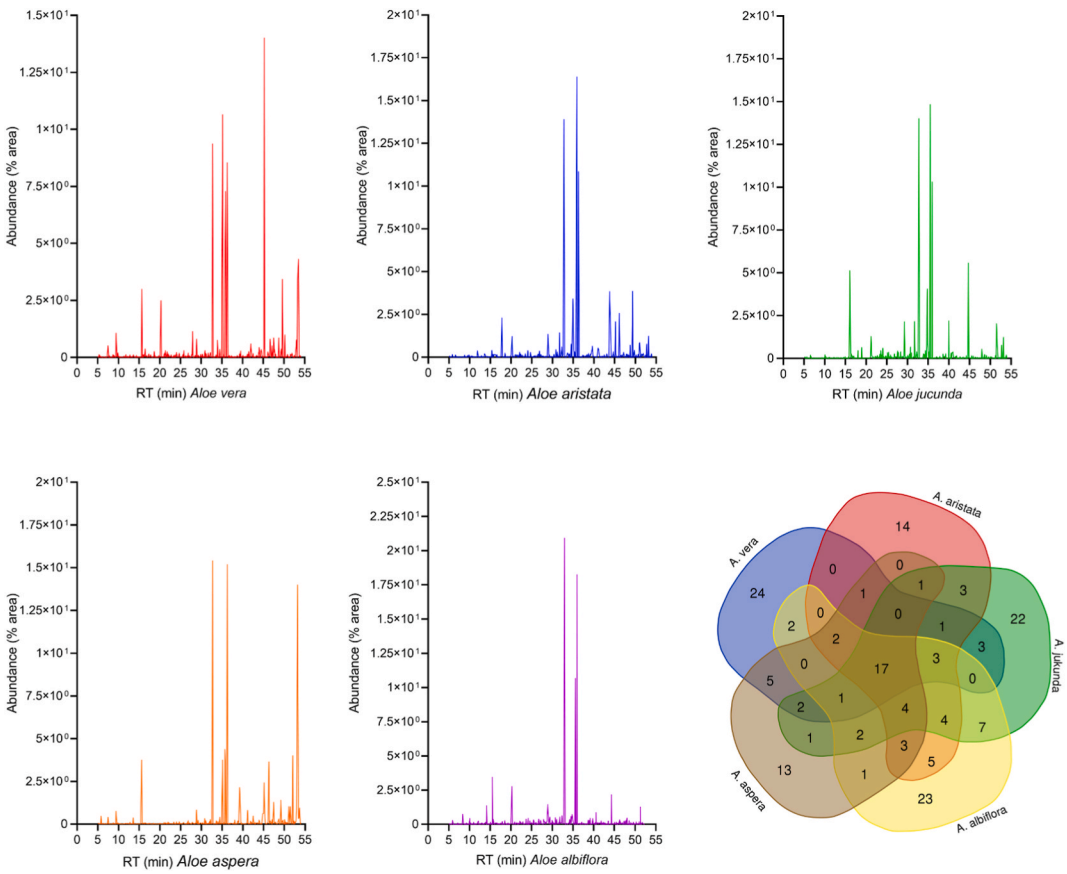

Fig. 1. (A–E) Gas chromatograms of *Aloe* spp. Data corresponds to Supplementary Tables 1–5. (F) Vein diagram demonstrating number of common and unique metabolites identified. Data corresponds to Supplementary Table 6.

values were calculated by formula $Y = A_1 \div (X + A_1)*100$, where $A_1 = IC_{50}$, Y = response (Y = 100 % when X = 0), X = inhibitory concentration. The observed hit/expected hit values were used to compute the enrichment ratio (ER) for metabolites and pathways. For each enrichment analysis entry, Holm-Bonferroni correction for P-value and false discovery rate (FDR) was computed [25]. Fisher's Exact Test was used to perform the pathway analysis. Multivariate analysis was performed using a dimension reduction tool using SPSS V23. KMO and Bartley's tests were performed to study the underlying dimensions of the variables. $P < 0.05$ was considered significant.

The metabolite abundance data sets from the GC-MS study were utilized to investigate the biochemical pathway enrichment using MetaboAnalyst V5 [14,26]. The Enrichment analysis was performed utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to explore the significant enrichment of functionally related metabolite groups. This analysis aimed to identify metabolites that could potentially obviate the need for preselecting compounds based on arbitrary cut-off thresholds [27]. The metabolites that were identified were cross-referenced with identifiers from PubChem and KEGG [28]. The generation of secondary metabolite pathway maps was accomplished through the utilization of the interactive pathway explorer (iPATH) version 3. These maps were constructed by aligning the biosynthesis of secondary metabolites' global pathways with the KEGG background. One factor statistical analysis was performed where data were log-transformed to achieve equal variance and mean-centered normalized and divided by the SD of independent variables. To identify common metabolic co-regulatory networks, a Debiased Sparse Partial Correlation (DSPC)-based correlative network was established (degree filter 2 and betweenness filter 1) as described previously [29,30], where nodes represented independent variables and edges represented the extent of associations. A false discovery rate (FDR)-adjusted p-value <0.2 was applied for the DSPC analysis. Partial least squares-discriminant analysis (sPLS-DA) was performed for dimension reduction. Variable Importance in Projection (VIP) scores were estimated for independent variables used in the PLS model.

4. Results

4.1. Phytochemical fingerprinting

A total of 65, 60, 76, 77, and 55 metabolites were identified in *A. vera*, *A. aristat*, *A. jucunda*, *A. albiflora,* and *A. aspera*, respectively (Fig. 1A–E; Supplementary Tables 1–5). The top three abundant metabolites were squalene (20.3 %), palmitic acid (13.5 %) and stearic acid (12.4 %) in *A. vera*; linolenic acid (22.3 % and 19.2 %), palmitic acid (18.9 % and 18.2 %), and stearic acid (14.7 % and 13.3 %) in *A. aristata* and *A. jucunda*; palmitic acid (28.7 %), stearic acid (25 %) and linolenic acid (14.4 %) in *A. albiflora* and palmitic acid (20.6 %), stearic acid (20.3 %) and β-sitosterol (18.7 %) in *A. aspera*. A majority of metabolites were commonly identified in all the *Aloe* spp.

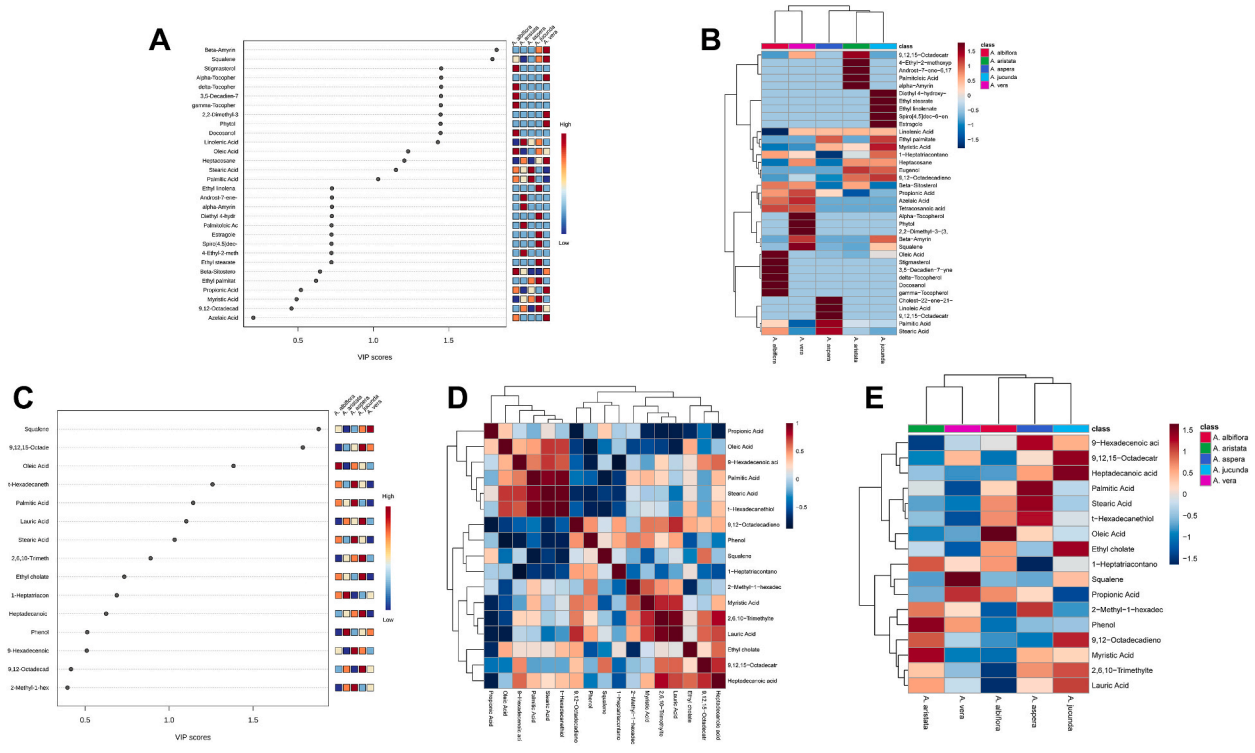

Fig. 2. (A) Variable Importance in Projection (VIP) score of top 25 abundant metabolites. (B) Correlation heatmap between top 25 metabolites and *Aloe* spp. (C) VIP scores of metabolites commonly identified in all the *Aloe* spp. (D) Heatmap depicting linear correlation between the comkon metabolites. (E) Heatmap representing association between common metabolites and different *Aloe* spp.

For instance, 17 common phytochemicals (Fig. 1F–Supplementary Table 6) were identified in all the plants. Contrarily, several metabolites were identified as unique in the plants. For instance, 24 metabolites were identified only in *A. vera*. Despite the metabolites commonly identified in the various *Aloe* spp., the abundance was highly variable. Because of this, the metabolite abundances were normalized on the log scale to achieve equal variance (Supplementary Fig. 1). Data accounting for all the metabolites indicated the abundance of β-amyrin and squalene to be highly variable across the plant samples (Fig. 2A). The abundance of the metabolites was also correlated with the *Aloe* spp. For instance, phytol, and α-tocopherol were highly correlated with *A. vera* whereas oleic acid and stigmasterol were closely associated with *A. albiflora* (Fig. 2B). Further, analysis using metabolites that were identified in all five *Aloe* spp. indicated that (Fig. 2C) squalene and 9,12,15-octadecatrienoic acid were the highly abundant and highly variable among all the *Aloe* spp. Among these metabolites top three positive correlation was observed between stearic acid with palmitic acid ($r_P = 0.92$) and t-hexadecanethiol ($r_P = 0.96$), and between lauric acid and 2,6,10-Trimethyltetradecane ($r_P = 0.4$); whereas top inverse correlation was observed between 9-Hexadecenoic acid and 1-heptatriacontanol ($r_P = -0.88$), oleic acid and phenol ($r_P = -0.85$), and 9,12-Octadecadienoic acid and propionic acid ($r_P = -0.82$) (Fig. 2D). Finally, among the common metabolites, a highly positive association was observed between squalene and *A. vera*, and oleic acid and *A. albiflora*, while t-hexadecanethiol was strongly inversely associated with *A. aspera* (Fig. 2E).

4.2. Chemical class enrichments

Based on the identified metabolites, chemical class enrichment analysis was performed based on MetaboAnalyst. Data showed that saturated fatty acids were most highly enriched in all the *Aloe* spp., followed by alkenes in *A. vera, A. aristata, A. albiflora*, and *A. aristata*, while unsaturated fatty acids were the second highest chemical class in *A. jucunda* (Fig. 3A–E; Supplementary table 7-11). In case of enrichment ratio (ER), the top three chemical classes entiched were non-metal phosphates (ER 4854), phenols (ER 1615) and benzenetriols (ER 1615) in *A. vera*; hydroquinones (ER 6024), benzoyl derivatives (ER 6024) and primary alcohols (ER 3012) in *A. aristata*; para cresols (ER 5681), hydroquinones (ER 5681) and benzoyl derivatives (ER 5681) in *A. jucunda*; hydroquinones (ER 5376), phenols (ER 1788) and benzenetriols (ER 1788) in *A. albiflora*; and primary alcohols (ER 3205), phenols (ER 2136), and

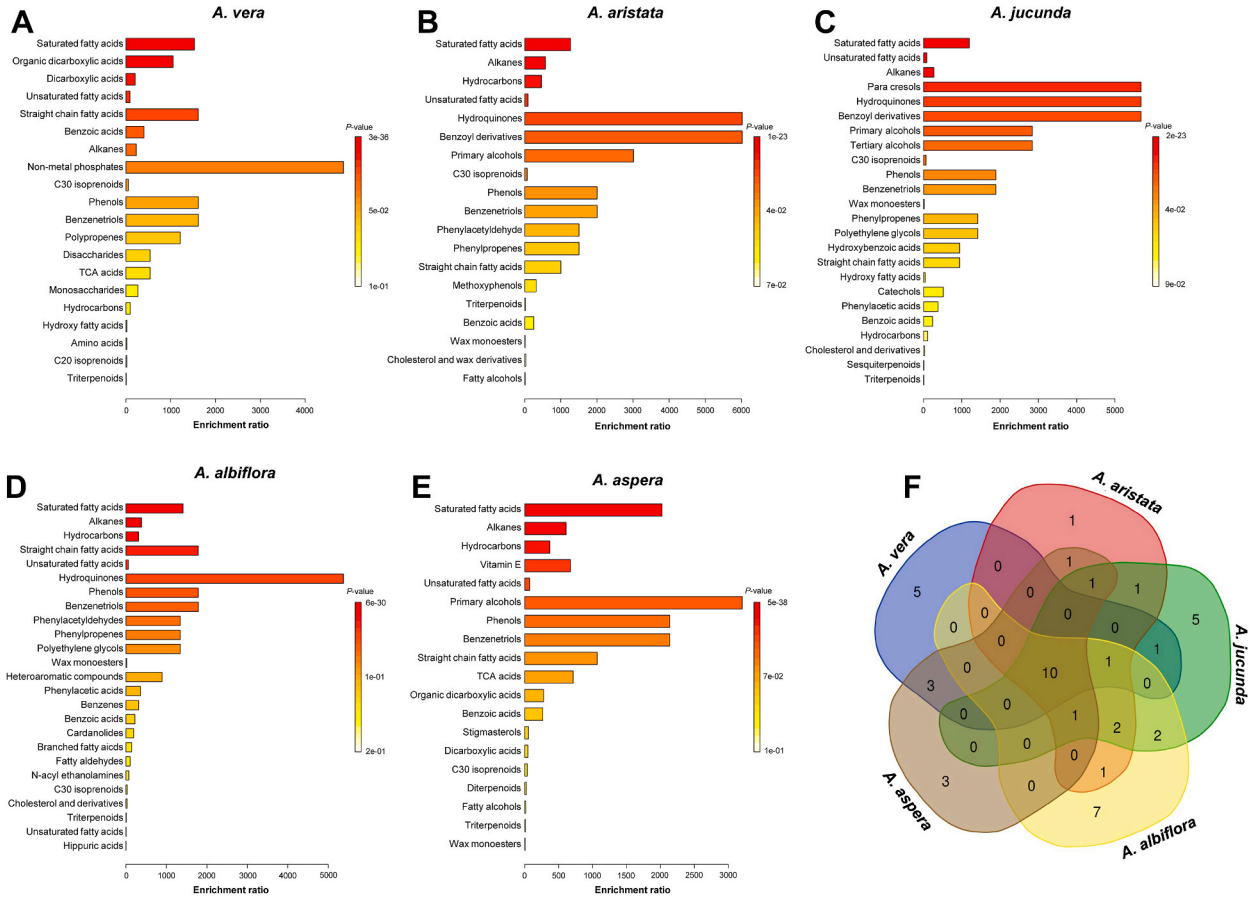

Fig. 3. Metabolite sub-class enrichment analysis of (A) *A. vera*, (B) *A. aristate*, (C) *A. jucunda*, (D) *A. albiflora*, and (E) *A. aspera*. Data corresponds to Supplementary Tables 7–11. (F) Vein diagram demonstrating number of enriched common and unique metabolite sub-classses. Data corresponds to Supplementary Table 12.

benzenetriols (ER 2136) in *A. aspera*. A total of 10 chemical class enrichments were common in all the *Aloe* spp., while 5, 1, 5, 7, and 3 unique metabolite class enrichments were observed in *A. vera*, *A. aristata*, *A. jucunda*, *A. albiflora*, and *A. aspera*, respectively (Fig. 3F–Supplementary Table 12).

4.3. Pathway enrichment analysis

The identified metabolites were mapped against the KEGG database to identify the enriched pathways associated with each *Aloe*r spp. A close overlapping of the enriched pathways was identified based on the PCA analysis (Supplementary Fig. 2). The top three impacted pathways in all the *Aloe* spp. were common viz., linoleic acid metabolism, sesquiterpenoid and triterpenoid biosynthesis, and α-linolenic acid metabolism; while fatty acid biosynthesis was the most significantly enriched pathway in all the samples (Fig. 4A–F; Supplementary table 13-18). Interestingly, only in *A. vera*, uniquely enriched pathways were identified viz., Starch and sucrose metabolism, Aminoacyl-tRNA biosynthesis, Arginine and proline metabolism, and Galactose metabolism (Supplementary Table 13). Further, Debiased Sparse Partial Correlation (DPSC) based analysis was performed to identify the co-regulatory metabolic networks in the *Aloe* spp., providing partial correlations and significance values through nodes representing pathways and edges representing the degree of associations (Fig. 5). The DPSC algorithm created a correlation network, providing partial correlation coefficients (C_P) and *P*-values for each pair of target networks and this determine the interconnection between all the pathways. Data showed that sesquiterpenoids and terpenoid biosynthesis had the highest betweenness index (49.3), indicating its greatest contribution to the interrelated pathway networks. This was followed by steroid biosynthesis (29.8) and fatty acid elongation (20.4). Other than starch and sucrose metabolism vs. arginine and proline metabolism ($P = 0.06$; $C_P = -0.49$), all other edges represented positive association. A significant positive association was identified between glyoxylate and dicarboxylate metabolism vs. fatty acid degradation ($P = 0.036$; $C_p = 0.98$), galactose metabolism vs. purine metabolism ($P = 0.037$; $C_P = 0.98$), Starch and sucrose metabolism vs. purine metabolism ($P = 0.037$; $C_p = 0.985$), steroid biosynthesis vs. purine metabolism ($P = 0.038$; $C_P = 0.977$), phenylalanine metabolism vs. sesquiterpenoids and terpenoid biosynthesis ($P = 0.038$; $C_P = 0.980$), and one carbon pool by folate vs. fatty acid elongation ($P = 0.038$; $C_P = 0.978$).

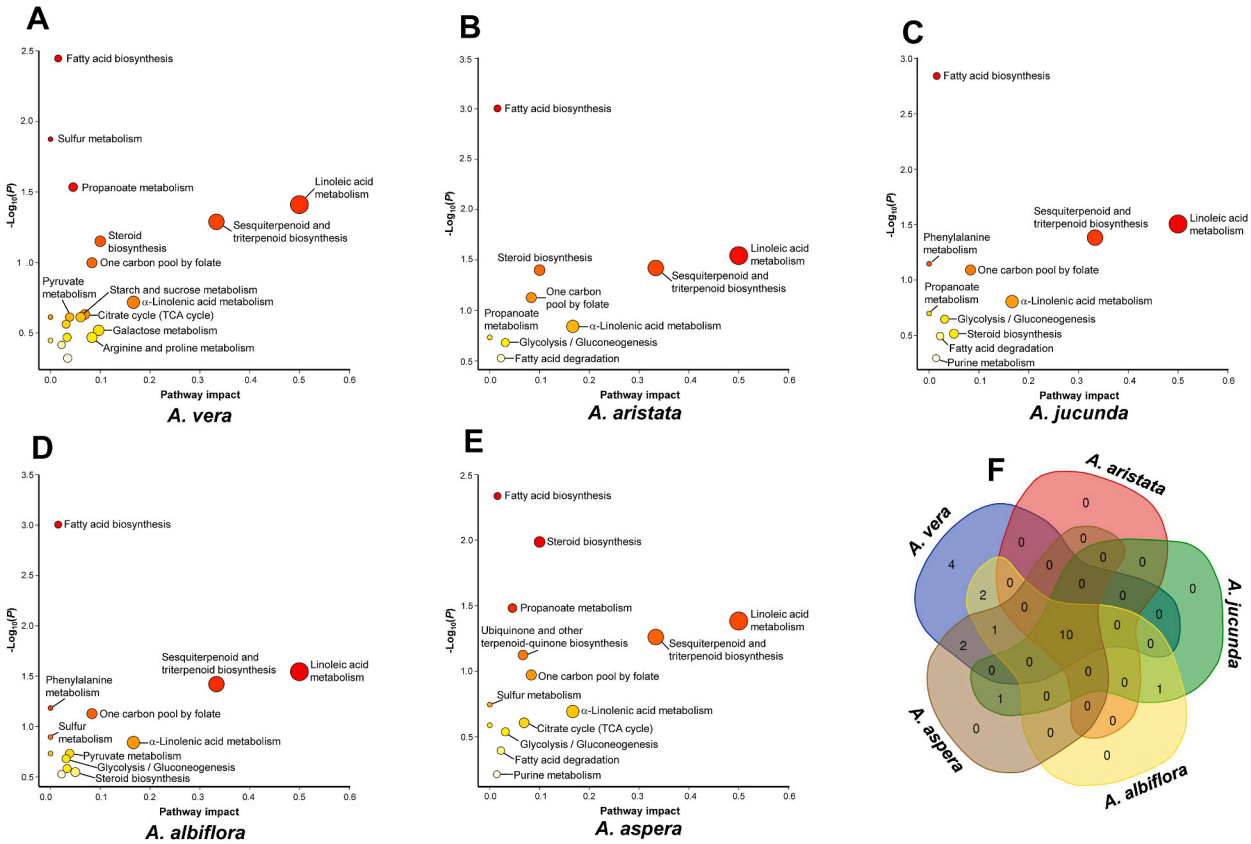

Fig. 4. Metabolic pathway enrichments of (A) *A. vera*, (B) *A. aristate*, (C) *A. jucunda*, (D) *A. albiflora*, and (E) *A. aspera*. Data corresponds to Supplementary Tables 13–17. (F) Vein diagram demonstrating number of enriched common and unique pathways. Data corresponds to Supplementary Table 18.

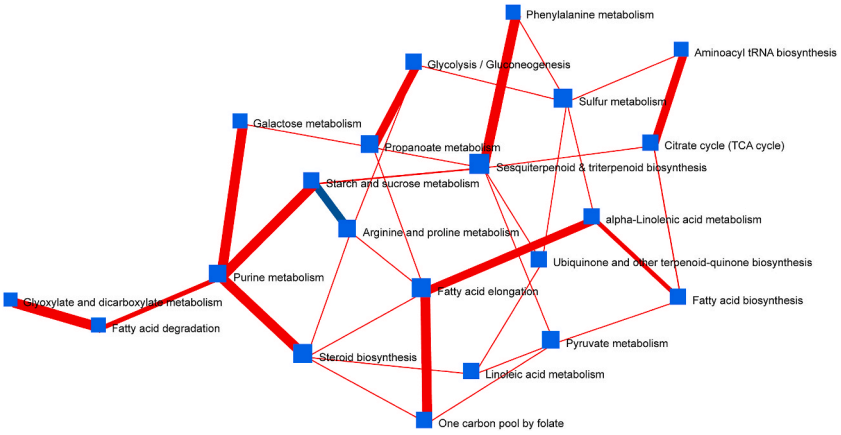

Fig. 5. Debiased Sparse Partial Correlation (DSPC)-based correlative network of commonly enriched pathways in the *Aloe* spp. Size of nodes represents direction of change and edge thickness indicates extent of significance ($P < 0.05$). Red edge and blue edge represented positive and negative correlation, respectively.

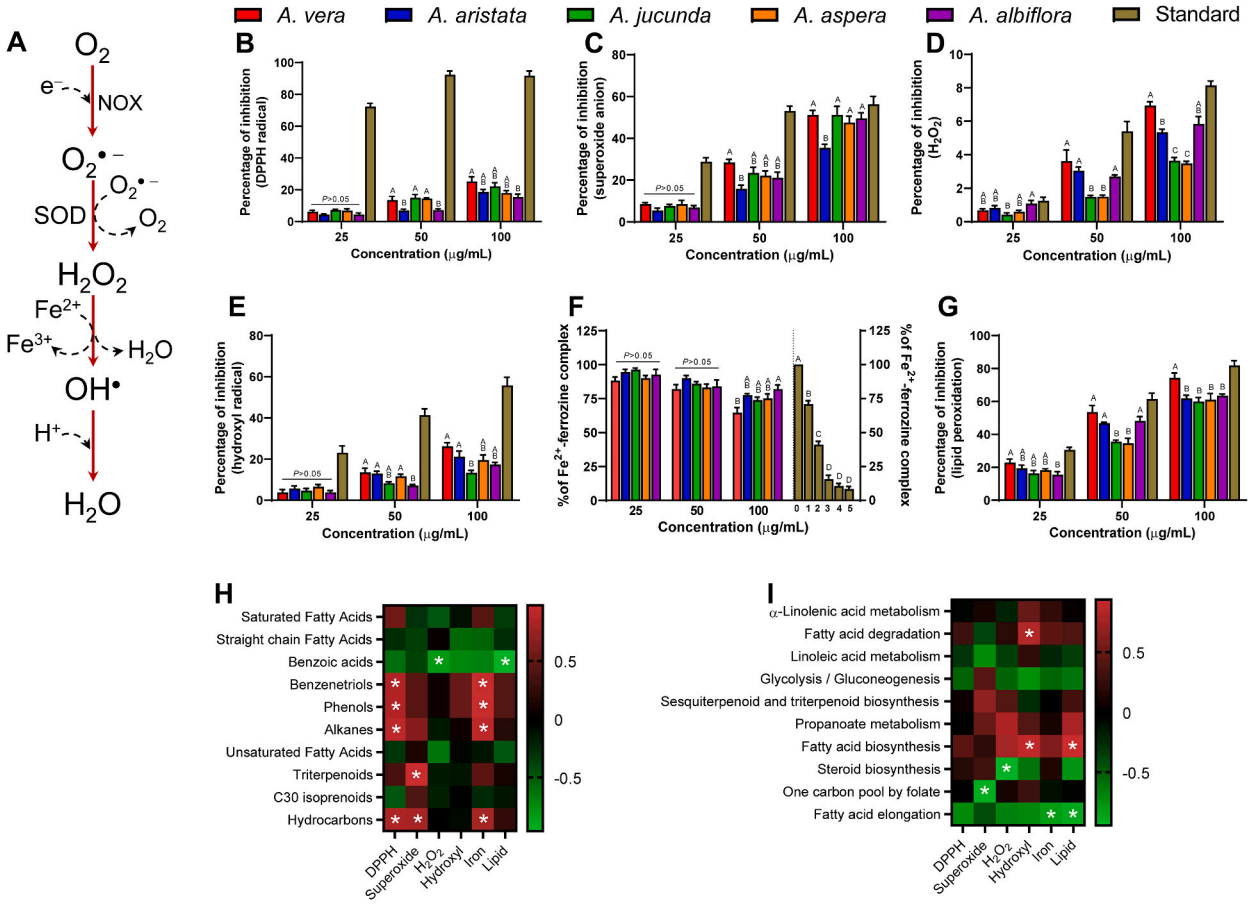

Fig. 6. (A) Fenton and Haber-Weiss reaction of intracellular free-radical formation. (B) DPPH scavenging activity; (C) Superoxide radical scavenging activity; (D) Hydrogen peroxide neutralizing activity; (E) Hydroxyl-radical scavenging activity; (F) Inhibition of iron chelation activity; (G) Lipid peroxidation inhibitory activity. Linear correlation analysis between antioxidant assays vs (H) chemical sub-class and (I) enriched pathways. Abbreviation: NOX, NADPH oxidase; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; $OH^{\bullet}$, hydroxyl radical; $O_2^{\bullet-}$ superoxide radical.

4.4. Antioxidant analysis

Based on the fact that the Fenton and Haber-Weiss reaction (Fig. 6A) serves as the classical intracellular free-radical forming mechanism, the *Aloe* spp. was evaluated for their potential to neutralize free-radicals related to these pathways, in addition to evaluation of their overall antioxidant capacity and potentials to neutralize $OH^{\bullet}$ mediated lipid peroxidation. Data showed that except for $O_2^{\bullet-}$ assay standards had superior bioactivities compared to that of the *Aloe* spp., while for each assay, the *Aloe* spp. demonstrated a dose-response (Fig. 6B–G). Although at 100 μg/mL all the *Aloe* spp. demonstrated comparable effects, overall antioxidant effects of *A. vera* were superior based on the calculated IC50 values (Supplementary Table 19). Further, linear correlation analysis was performed between the antioxidant capacities of the *Aloe* spp. with common chemical sub-classes (Fig. 6H) and the enriched pathways (Fig. 6I). The top three highest positive correlation was identified between $O_2^{\bullet-}$ scavenging activity and triterpenoids ($r_P = 0.98$; $P < 0.01$); and iron chelation activity Vs benzenetriols and phenols ($r_P = 0.97$; $P < 0.01$). Only benzoic acids demonstrated an inverse correlation with lipid peroxidation ($r_P = -0.96$; $P < 0.01$) and H_2O_2 ($r_P = -0.81$; $P < 0.05$) scavenging.

5. Discussion

Plants belonging to the genus *Aloe* are extensively used in the pharmacological and cosmetic industry, and extensive photochemical fingerprinting has been performed to identify the bioactive constituents of the *Aloe* gel. While several polar secondary metabolites have been identified, non-polar volatile metabolites are mostly associated with utility in commercial cosmetic products [31]. In the current study, we have identified common metabolic and biosynthetic pathways related to plants belonging to the genus *Aloe*, which can be potentially enriched to improve the production of target metabolites to be exploited for commercial purposes.

The inner layer of *Aloe* leaves is 99 % H_2O and contains glucomannans, amino acids, lipids, sterols, and vitamins, whereas the remaining fleshy part also contains anthraquinones and glycosides [32]. Earlier studies have identified high content of antioxidant phytochemicals including vitamins, carotenoids, and phenolic compounds [3], while cholesterol, campesterol, β-sitosterol, and lupeol were reported as part of bioactive non-polar sterol compounds in the *Aloe* leaves [33]. We have identified several common metabolites in all the *Aloe* spp., while certain metabolites were commonly identified only in selected spp. (Fig. 1F). Although variation in metabolite identification could result due to environmental and experimental variables, plant aromatic fingerprints can vary due to biotic factors such as variations in gene expression patterns, modifications in enzyme activity, and alterations in plant development parameters [34]. For instance, recent studies have identified extensive genetic diversity in plants belonging to the *Aloe* spp., associated with the drought tolerance phenotype [35], whereas others have shown that differential expression of genes related to secondary metabolites saponin, lignin, anthraquinone, and carotenoid biosynthesis contributes to the extent of defense mechanism during stress [15]. We identified high enrichment of straight-chain fatty acids, the universal backbone of plant fatty acid formation [36], among which the saturated pool was predominantly enriched. This was predictable not only due to a non-polar extraction procedure but also because saturated and unsaturated fatty acids serve as the main biosynthetic source for plant volatiles [37]. Earlier studies have identified diverse fatty acid components in various *Aloe* spp. while emphasizing the need for utilizing *Aloe* metabolites for commercial exploitation [38]. Common to several other plants belonging to the family *Xanthorrhoeaceae*, palmitic acid, palmitoleic acid, oleic acid, and myristic acid [38] are abundant in all the five *Aloe* spp. Indeed, plants under diverse families, that are rich in fatty acids, have demonstrated potent antioxidant capacities [39], while composition prepared from plant-based fatty acids for cosmetic use, have demonstrated superior antioxidant activities [40]. Among other commonly identified chemical classes, benzoic acid and its derivatives, and terpenoids are well-established for their antioxidant properties in the cosmetic industries [41]. Highest abundance of the antioxidant metabolite β-amyrin, squalene, alpha tocopherol, phytol, and azelaic acid were observed in *A. vera*. Although all of these metabolites are extensively used in cosmetic products, especially squalene [42], tocopherol [43], and azelaic acid [44] are used frequently in dermatological and skin-care products.

The identified metabolites were further mapped against KEGG identifiers for identification of the predominantly enriched metabolic and biochemical pathways. Based on the identification of several fatty and lipophilic metabolites, different pathways related to fatty acid metabolism were identified as enriched. Earlier studies have identified fatty acid metabolism-related pathways as one of the most crucial and predominant biosynthetic pathways in *A. vera* based on transcriptomics data [15]. Especially in aromatic plants, various secondary metabolites are produced from fatty precursors through α- and β-oxidation, and lipooxygenase pathways [45]. Linoleic acid metabolism was highly enhanced in all the *Aloe* spp. It belongs to the polyunsaturated omega-6 fatty acid class, serves as a precursor for short-chain fatty acids, and is utilized for the preparation of industrially-relevant hydrocarbons through chemical synthesis [37]. A plant-based diet rich in linoleic acid has been recommended by the American Heart Association due to its inverse association with the risk of cardiovascular disease and antioxidant effects [46]. To date, several metabolic engineering strategies have been employed to enhance the production of linoleic acids. For instance, enhanced linoleic was produced using engineered oleaginous yeast having favorable properties such as lack of β-oxidation, inability of triglyceride accumulation and overexpression of the Δ12-desaturase gene, responsible for oleic acid to linoleic acid bioconversion [47]. Next, pathways related to sesquiterpenoid and triterpenoid biosynthesis were also commonly enriched in all the *Aloe* spp. Terpenoids are commonly biosynthesized from isopentenyl diphosphate and dimethylallyl diphosphate, where C10 monoterpenoids, C20 diterpenoids, and C40 teteraterpenoids are produced in plastids, whereas C15 sesquiterpenoids and C30 triterpenoids are formed in the cytosol [14]. Previous studies have isolated various commercially important terpenoids from *Aloe* spp. (e.g., lupeol) [48], while others have identified the presence of up to 23 different terpenes in *A. arborescens* (e.g., limonene, citronellol, eugenol, thymol) [49]. Elicitors are commonly employed to stimulate the activation of secondary metabolic pathways and enhance the production of specific terpenoids. This can be achieved by overexpressing genes involved in terpenoid biosynthesis in both related and unrelated plants, inhibiting the expression of competing metabolic

pathways, or regulating broader gene regulatory pathways such as key transcription factors, endogenous phytohormones, and primary metabolism. As a result of these approaches, the synthesis of terpenoids with desirable pharmacological properties has been successfully enhanced [50]. Our observation regarding the co-enhancement of fatty acid and terpenoid biosynthesis suggests that the augmentation of fatty acid β-oxidation can assist the co-enhancement of terpenoid and fatty acid metabolic pathways by enabling the recovery of acetyl-CoA from lipids for terpenoid production. Indeed, acetyl-CoA, generated through glycolysis, serves as a key precursor for terpenoid biosynthesis in the plastic through methylerythritol 4-phosphate and in the mevalonate pathway in the cytosol [51]. Glycolysis-associated pathways were also enhanced in all the *Aloe* spp. Among the other commonly enhanced pathways predominant were linoleic acid metabolism, α-linolenic acid metabolism, and steroid biosynthesis. Earlier studies identified linoleic acid (C18:2 n−6) and α-linolenic acid (C18:2 n−3) as the primary polyunsaturated fatty acid in *A. vera* [52]. Both are catabolized by the lipoxygenase enzyme to generate a wide variety of plant volatiles, while also being used for the chemical synthesis of industrially-relevant phytochemicals from linolenic acid [37]. Indeed, extensive genetic engineering strategies have been employed to enhance the production of commercially relevant products by enhancing the lipoxygenase expression in plants [53,54]. Steroidal compounds in plants are branched out from terpenoid backbone biosynthesis [55]. Similar to our observation, various steroidal compounds (e.g., campesterol, cholesterol, β-sitosterol) were identified in *A. vera* leaf [56]. Squalene, one of the major precursors of sterol biosynthesis which is formed by the catalytic effects of farnesyl-diphosphate farnesyltransferase [57], was also detected in the leaves of all five *Aloe* spp.

Using DSPC network analysis, we were further able to identify the co-related metabolic and biosynthetic pathways. Sesquiterpenoids and terpenoid biosynthesis pathways emerged as the key regulatory node that was associated with other metabolic pathways. Indeed extensive metabolic and genetic engineering strategies have been employed to enhance the biosynthesis of related phytometabolites with commercial importance [58]. Interestingly, starch and sucrose metabolism (representing carbohydrate metabolism) and arginine and proline metabolism (representing protein metabolism) were inversely associated when all five *Aloe* spp. were considered. This is likely plausible due to the utilization of the shared carbon pool by both the pathways, as well as due to common metabolic intermediates of glycolysis, pentose phosphate pathway, and the citric acid cycle (e.g., pyruvate) [59]. Modulation of these pathways could be a lucrative strategy to improve the quality of agricultural produce since a vast array of plant secondary metabolites remains under the control of amino acid metabolism [60]. Arginine and proline are derived from α-ketoglutarate as part of the citrate (TCA) cycle, which is in turn tightly regulated by the availability of pyruvate in the glycolysis. Nucleotide metabolism, especially the purine metabolism pathway, was closely associated with galactose metabolism, starch, and sucrose metabolism, and steroid biosynthesis. Indeed, among the plant biosynthetic pathways, nucleotide metabolism plays an important regulatory role since not only that they form the building blocks of life (i.e., DNA), but also several common metabolic intermediates (e.g., ATP, NADH, Co-A, and Uridine diphosphate glucose) are either nucleotide themselves or structurally contain nucleotide fragments [61]. An earlier study has shown that in the potato (Solanum tuberosum), a decrease in AMK (adenylate kinase) activity in the plastid resulted in an elevation of the adenylate pool, which includes AMP, ADP, ATP, and ADP-Glc (adenosine diphosphate glucose). This increase in the adenylate pool led to enhanced starch production in the tubers [62]. Additionally, the Leloir pathway of galactose metabolism involves the conversion of galactose into glucose-1-phosphate to produce UDP-glucose, an essential perquisite for purine metabolism [63]. However, the rationale behind the close correlation between purine metabolism and steroid biosynthesis remained unexplored in the present study since there are no common intermediates through which both pathways are connected. Nevertheless, such associations were also reported previously, where the predominance of transcripts of purine metabolism was reported associated with the steroid alkaloid biosynthesis in *Fritillaria imperialis* [64].

Beyond identifying the enriched biochemical pathways, we also intended to link the antioxidant and free-radical scavenging activities of the *Aloe* spp. with that of the commonly identified phytochemicals and metabolic pathways. For this purpose, we evaluated the bioactivities against free radicals related to the Fenton and Haber-Weiss reaction. Both of these interrelated reactions serve as a major gateway to intracellular reactive O_2 and N_2 species formation, ultimately resulting in damage to cellular components. Commercial cosmetics and cosmeceuticals are often fortified with natural antioxidants to prevent the signs of aging by neutralizing free radicals [7]. Similar to our findings, others have shown that terpenoid-rich herbal extracts can neutralize $O_2^{\bullet-}$ and elevate the levels of superoxide dismutase under *in vitro* experimental conditions [65]. Benzenetriols, also known as trihydroxybenzenes, are plant polyphenols with potent free-radical scavenging activities. A strong positive correlation observed in the present study between the inhibition of iron chelation and the benzenetriols and phenolics indicates the possible use of *Aloe* volatiles for preventing the formation of the highly reactive $OH^{\bullet}$ by inhibiting Fe^{2+} to Fe^{3+} transition. Interestingly, benzoic acid, despite being an $OH^{\bullet}$ scavenger, showed an inverse correlation with lipid peroxidation inhibitory effects, which remains unexplored. We also showed that *Aloe* metabolic pathways related to fatty acids were associated with antioxidant activities. This is relevant since polyunsaturated hydrocarbons (e.g., α-linolenic acid, linoleic acid, squalene) are considered natural antioxidants, bioactivities of which depend on the chain length which is dictated by the metabolic pathways associated with their biosynthetic, elongation and degradation processes [66,67]. Finally, the close association between both the fatty acid and terpenoid biosynthetic pathways with the antioxidant effects could be explained by the fact that the augmentation of fatty acid β-oxidation can assist the co-enhancement of terpenoid and fatty acid metabolic pathways by enabling the recovery of acetyl-CoA from lipids, which is necessary for terpenoid production [68]. Nevertheless, both of these components of the *Aloe* gel are extensively used in cosmetic products.

6. Conclusion

In the present study, we identified shared metabolites that possess antioxidant capabilities that can be beneficial for cosmetic applications (Supplementary Fig. 3). *Aloe* spp. exhibited the highest abundance of cosmetic antioxidants such as β-amyrin, squalene,

vitamin E, phytol, and azelaic acid. The variations in metabolites are most likely attributable to the differential expression of genes, variations in enzyme activity, and the developmental stages of the plants. *Aloe* spp. exhibit improved metabolic pathways for fatty acids, terpenoids, and steroids. These metabolites generate beneficial antioxidants, volatile metabolites, and commercially valuable phytochemicals. By overexpressing crucial biosynthetic genes and enzymes, strategies such as genetic and metabolic engineering can significantly enhance the production of polyunsaturated fatty acids, terpenoids, and steroids to be used in cosmetic products. Modulating lipoxygenase activity and enhancing acetyl-CoA retrieval from lipids can further enhance the interconnected production of fatty acids and terpenoids. The network analysis revealed that sesquiterpenoid and terpenoid production is a crucial pathway connected to other metabolic processes. Manipulating these pathways enhances the production of commercially valuable phytochemicals. The pathways involved in the metabolism of carbohydrates and amino acids, which have common metabolites, exhibited an inverse connection between different species, most likely because they compete for carbon sources. The interconnection between nucleotide metabolism and the synthesis pathways of sugar and steroids is well-established, although the underlying mechanism requires further investigation. The antioxidant activity was linked to the enhanced pathways of fatty acids and terpenoids. These mechanisms are likely to work together, as an increase in fatty acid β-oxidation results in a greater amount of acetyl-CoA available for terpenoid production. The antioxidant benefits utilized in cosmetics could be directly derived from polyunsaturated hydrocarbons produced through these routes. Additional *in vitro* investigations are necessary to assess the impact of enhancing the phytochemical quality by focusing on potential metabolic pathways using the suggested metabolomics-based reverse association technique. Although our study showed a close association between the enriched metabolic pathways and the antioxidant potentials, *in vivo* validation experiments are critically needed to establish the link between the bioactivities and the pathway enrichments.

CRediT authorship contribution statement

Nehal Batra: Software, Methodology, Investigation, Formal analysis. **Priyankar Dey:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Data curation.

Data availability statement

Data included in the supplementary material is referenced in the article.

Ethics statement

Not applicable for the current study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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