Comparative metabolic profiling, isolation of alkylated phenols and antioxidant activity of roots of *Plumbago* species using GC-MS and NMR based metabolomics study

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**ABSTRACT**

In this study, NMR and GC-MS based comparative metabolomic profiling of the roots of three different species namely, *Plumbago indica*, *P. auriculata* and *P. zeylanica* were investigated followed by multivariate statistical analyses and their antioxidant activity. Also, two alkylated phenols i.e., 2,6-di-tert-butyl phenol and 2,4-di-tert-butyl phenol not reported earlier from this taxon were isolated from *P. indica*. This metabolic study resulted in the identification of 25 and quantification of 18 metabolites. Principal component analysis showed the clear distinction among the three species. The antioxidant activity in the extracts was tested by free radical scavenging method. The three *Plumbago* species revealed interesting antioxidant potential, in particular, *P. indica*, which was rich in naphthoquinones, coumarins, alkylated phenols, sterols, triterpenes, fatty acids showed lowest IC\textsubscript{50} value. The results highlighted the role of *P. indica* in the management of oxidative stress especially when they are utilized in the formation of fermented food products.

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1. Introduction

Genus *Plumbago* belongs to the Plumbaginaceae family is native to India, Africa, Australia, Indonesia and Southeast Asia (Chopra et al., 1956). The root and chemical constituents of *Plumbago indica* Linn., *P. auriculata* Linn. and *P. zeylanica* Linn. are credited to own therapeutical properties according to the Indian traditional medicine system (Jain et al. 2014). These species have been reported for their nutritional and antioxidant potential which is associated with their richness in naphthoquinones, sterols, terpenes and fatty acids content (Kaewbumrung and Panichayupakaranant 2012). Metabolomics is a powerful approach which has gained widespread applications in order to understand the variations in primary and secondary metabolism in closely related species of plants and animals (Sayed et al. 2020).

Since no attention has been paid to *Plumbago* species variation associated with secondary metabolites, this study was constructed to compare the roots of *Plumbago indica*, *P. auriculata* and *P. zeylanica* using GC-MS and NMR based metabolomics approach. Also, first time isolation of two alkylated phenols from the roots of *P. indica* was conducted. Additionally, we investigated their in vitro antioxidant capacities so that nutraceuticals and food supplement industries get benefitted from such findings.

2. Results and discussion

2.1. Comparative metabolomic profiling of hexane fraction by GC-MS

Twenty-five chemically diverse metabolites were characterized and quantified in hexane fraction of roots of *Plumbago* species using GC-MS (Table S1). Among all GC-MS detected metabolites, 2,6-di-tert-butyl phenol (2,6-DTBP), 2,4-di-tert-butyl phenol (2,4-DTBP), Ergostan-5-ol, 3,6,12-tris[(trimethylsilyl)oxy]-, (3β,5α,6β,12β) and α and β- amyrin have not been previously described from the species of *Plumbago* so far (Table S1). *P. indica* were dominated by alkylated phenols, naphthoquinones, indole derivatives,
fatty acids, sterols, triterpenes and α-asarone. Alkanes and alkenes were prevalent in *P. auriculata* and *P. zeylanica*. Among fatty acids, palmitic acid was the most prevalent metabolite in the roots of *P. indica*, *P. auriculata* and *P. zeylanica* with percent peak area of 7.89 ± 0.62%, 3.58 ± 0.31% and 4.11 ± 0.40%, respectively (Table S1). Oleic acid and linoleic acid with percent peak area of 8.95 ± 0.92% and 4.49 ± 0.41% in *P. indica* root. The high quantity of 2,4-DTBP (18.66 ± 1.54%) and 2,6-DTBP (0.82 ± 0.09%) had been detected from the hexane fraction of *P. indica* root. 2,6-DTBP had been observed in *P. indica* roots only. The high amount of alkylated phenols in *P. indica* makes it a future non-conventional source of these phenols having diverse nutraceuticals and health related benefits (Zhao et al. 2020). Triterpenes namely, α- and β- amyrin with percent peak area of 2.83 ± 0.31% and 2.49 ± 0.11% have been investigated in high amount only in *P. indica* root. These triterpenes exhibited excellent gastroprotective, hepatoprotective, antidepressive and hypolipidemic effects (Nogueira et al., 2019). Among sterols, stigmasterol, β-sitosterol and ergostan-5-ol, 3,6,12-tris[(trimethylsilyl)oxy]-, (3β,5α,6β,12β) were dominant in *P. indica* root. The percent peak areas of these metabolites were 2.56 ± 0.28%, 2.97 ± 0.33% and 1.62 ± 0.12%, respectively. In earlier studies, it had been concluded that plant sterols are taken directly by human body through diets and supplements. Moreover, daily intake of sterols can efficiently lower cholesterol level (Trautwein et al. 2018). Indazol-4-one, 3,6,6-trimethyl-1-phthalazin-1-yl-1,5,6,7-tetrahydro and 2-methyl-7 phenyl indole with peak area of 7.68 ± 0.70% and 3.46 ± 0.38% were detected in enormous amount in the roots of *P. indica*. The biological significance of indole derivatives includes antifungal, antioxidant, anti-Alzheimer’s and anti-Parkinson’s potential (Kumar et al. 2020). The peak area of 19.42 ± 2.11% of 1,4-naphthoquinone was detected in *P. indica* root which is higher in concentration than two other species of *Plumbago*. 1,4-naphthoquinone, an important antiamyloidogenic metabolite as it has the property of interacting with amyloidogenic pathway of the amyloid precursor protein (Bermejo-Bescós et al. 2010)

### 2.2. Structure elucidation of alkylated phenols and comparative metabolomic profiling of methanol fraction by NMR

Compound A (2,6-di-tert-butyl phenol) was obtained as light-yellow liquid and compound B (2,4-di-tert-butyl phenol) as yellow solid by preparative TLC. The structures of both the phenols were elucidated by NMR spectroscopic data (Figure S1) (Table S2) and the assignments were further validated with earlier reports. The 1H NMR analysis of roots of *P. indica*, *P. auriculata* and *P. zeylanica* allowed the identification of a total of 18 organic compounds belonging to different chemical classes, namely: naphthoquinones (13) and coumarins (4) with different concentrations in each species (Tables S2 and S3). Figure S2 reports, respectively, typical stack plot of 1H NMR spectra obtained from methanolic root extracts of *P. indica*, *P. auriculata* and *P. zeylanica* plants. The content of all the naphthoquinones and coumarins were higher in *P. indica* except plumbagic acid which is higher in *P. zeylanica* root (1.27 ± 0.13 mg/gm). The important biomarker naphthoquinone, namely, plumbagin was detected in *P. indica*, *P. auriculata* and *P. zeylanica* respectively with a concentration of 25.41 ± 2.02, 18.42 ± 1.5 and 16.26 ± 1.5 mg/gm, respectively. The concentration of 1.74 ± 0.10, 3.82 ± 0.42,
1.88 ± 0.15, 2.63 ± 0.25 and 2.36 ± 0.13 mg/gm of droserone, elliptinone, isoshinanolone, isozeylinone and plumbazeylanone were investigated higher in *P. indica* root. The other minor naphthoquinones were also observed higher in *P. indica* root namely, chitrtranone (0.12 ± 0.01 mg/gm), 2,3-dibromo-1,4-naphthoquinone (2.47 ± 0.21 mg/gm), 2-methyl-5-(3δ-methyl-but-2δ-enyloxy)-[1,4]naphthoquinone (1.04 ± 0.11 mg/gm), 3,3-biplumbagan (2.46 ± 0.21), 3,8-dihydroxy-6-methoxy-2-isopropyl-1,4-naphthoquinone (0.73 ± 0.07 mg/gm) and 5,7-dihydroxy-8-methoxy-2-methyl-1,4-naphthoquinone (0.86 ± 0.09 mg/gm) respectively. The concentration of elliptinone, 3,3-biplumbagin, isoshinanolone, isozeylinone were also found to be higher in *P. auriculata* root than *P. zeylanica* but lower than *P. indica* root (Table S3). Previous reports have illustrated that naphthoquinones have promising potential for the treatment of various diseases such as anti-tumor, antibacterial, antiviral and antiparasitic effects (López López et al., 2014). The identified coumarins include seselin (0.47 ± 0.05 mg/gm), suberosin (0.28 ± 0.02 mg/gm), xanthoxyletin (0.63 ± 0.06 mg/gm) and xanthyletin (0.57 ± 0.05 mg/gm) were detected in small amounts in *P. indica* than in *P. auriculata* and *P. zeylanica* root. Researchers have reported the role of coumarins that have been associated with wide range of activities such as anti-tumor, antibacterial and inhibition of symbiotic fungus (de Melo Cazal et al., 2009). A good quantity of triterpene, lupeol was also observed higher in concentration in *P. indica* (2.87 ± 0.14 mg/gm) in comparison to *P. auriculata* (0.91 ± 0.08 mg/gm) and *P. zeylanica* root (0.59 ± 0.06 mg/gm).

### 2.3. Multivariate statistical analysis

A principal component analysis (PCA) was applied on GC-MS and NMR obtained data in order to examine the intrinsic metabolites variation among *P. indica*, *P. auriculata* and *P. zeylanica*. According to PCA score plot (Figures S3A–4A), the three species of *Plumbago* were clustered away from each other indicating different metabolic concentration in each species. 2,6-DTBP and 2,4-DTBP, fatty acids, sterols, triterpenes, indole derivatives, anthranilic acid, β-asarone, naphthoquinones and coumarins were most abundant and characteristic metabolites in *P. indica*, notably alkanes, alkenes and plumbagic acid which were significantly more abundant in *P. auriculata* and *P. zeylanica* (Figures S3B–4B).

### 2.4. Antioxidant potential

To investigate the antioxidant potency of 2,6-DTBP and 2,4-DTBP, hexane and methanol extracts of *P. indica*, *P. auriculata* and *P. zeylanica* roots towards the most common reactive oxygen species (ROS), obtained extracts were subjected to in vitro antioxidant assay, namely DPPH radical scavenging activity. As shown in Figure S5, compound 2,6-DTBP showed a remarkable radical scavenging activity (IC50 = 42.26 μg/mL) against control ascorbic acid and BHT. Pertains to this, hexane and methanol root extracts of *P. indica* partially endowed this activity (IC50 = 124.11 and 82.41 μg/mL) in comparison to *P. auriculata* (162.74 and 91.63 μg/mL) and *P. zeylanica* (188.57 and 95.28 μg/mL) These results are highly correlated to high content of alkylated phenols, naphthoquinones, coumarins, fatty acids, triterpenes and sterols in *P. indica* roots.
3. Conclusions

Results reported here clearly highlighted that the naphthoquinones and other secondary metabolites variation based on GC-MS and NMR is able to discriminate the three species of *Plumbago* viz., *P. indica*, *P. auriculata* and *P. zeylanica*. The present investigation revealed the efficiency of metabolomics in chemotaxonomical characterization of closely associated plant species. *P. indica* was characterized by its richness in naphthoquinones, coumarins, alkylated phenols, triterpenes, fatty acids and sterols and showed a broad-spectrum *in vitro* antioxidant potential. *P. indica* are currently becoming endangered threatened species because of continuous use in herbal, pharmaceutical and nutraceutical industries. Such biological specimens should be cultivated so that the roots of *P. indica* can be utilized in the production of value-added fermented food products.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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