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Are the white and black morphs of *Sida alnifolia* phytochemically different?

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Abstract
We compared the phytochemical constituents of the white and black morphotypes of the medicinal plant *Sida alnifolia* the latter which is believed to have better medicinal properties than the former as per traditional knowledge prevailing in Kerala, India. Alkaloids, Flavonoids, Phenols, Starch and Ephedrine and fourteen plant minerals were quantified in plant samples collected from all 5 agro-geographical zones in Kerala. Soil was collected from two depths from the locations of the plants and nineteen soil parameters were analyzed. The study unequivocally showed that there exists no significant variation in the phytochemical profile of the white and black morphotypes of *S. alnifolia*. However, the phytochemical contents of plants collected from different agro-ecological zones showed prominent variations. Edaphic factors did not possess any correspondent correlation with both the colour morphs. This probably indicate that either the genotype or abiotic environmental effects have led to genetic drift of *S.alnifolia* across zones.

Keywords: *Sida alnifolia,* white and black morphs, medicinal value, phytochemicals, plant minerals, soil properties

Introduction
Sida alnifolia is a sturdily branched biennial subshrub or woody herb which belongs to the family Malvaceae. It is widely distributed at higher and lower altitudes in the plains and hills of southern Peninsular India, occurring in moist deciduous forests, secondary growth in forest clearings, lateritic hill slopes and occasionally as a weed in upland cultivation. Bala is an important plant drug in Ayurvedic system of medicine and it is equated with *S. alnifolia* in Kerala, while in North India *S. cordifolia* is used as the source plant (Shylaja and Nishitha, 2009) [1]. In Ayurveda, Bala is attributed the function of balancing all the doshas – *vata*, *pitta* and *kapha*, however, it has more effect on *vata* dosha. The rejuvenating action of this herb extends to the nervous, circulatory, and urinary systems (Chopra, *et al*., 1958) [2]. *S. alnifolia* is chiefly used to treat rheumatism by imparting strength. It forms a chief component of '*Chyavanprash'*, which is a popular ayurvedic preparation placed under '*Rasayana'* group of drugs and is widely used as tonic in improving health and in prevention of the diseases. *S. alnifolia* is one of the vital constituents in formulations of more than 70% of the products manufactured in Ayurveda, some of which are *Mahanarayana taila*, *Balati taila*, *Prabhanjana Vimardhana taila* and *Ksheera-bala taila*. Though the whole plant is used in various medicine formulations in Ayurveda, the chief therapeutic agent - Ephedrine (Anon, 1972) [3], is found high in roots. The root of this plant is extensively used in the treatment of rheumatism in Ayurveda and is also used as astringent, diuretic and tonic. Its infusion is useful in cystitis, strangury, haematuria, chronic dysentery, leucorrhoea, and gonorrhoea (Chopra *et al*., 1956; Kirtikar and Basu, 2001) [4, 5]. Thus, it is evident that *S. alnifolia* is a pertinent plant species with immense medicinal properties and wide application. Sida is one among the plants showing extent of phenotypic variations within the species. *S. alnifolia* exhibit prominent variations in the phenotypic characters such as the stem and leaf colour, leaf shape, branch architecture etc. The species is known to have two major morphometric forms termed as *Vellakurunthotti* (white sida) and *Karimkurunthotti* (black sida) in the vernacular. As per the traditional knowledge, the black morphotypes is considered to be more medicinal than the white. This study tested this traditional knowledge by comparing the phytochemical profiles of both the white and black morphs of *S. alnifolia*.

Material and Methods
Sample collection and processing
Both White and Black Sida samples were collected from all agro-ecological zones prevailing in Kerala: (i) Coastal Plains, (ii) Midland Laterites, (iii) Foothills, (iv) High Hills and

(v) Palakkad Plains (Nair and Rajase kharan, 2013) [6] in which the elevation ranged from 0-30, 30-300, 300-600, 600-1800 and 70-240 meters above sea level respectively. Samples were also collected from commercially cultivated site at Pookkode, Thrissur.

Five to ten plants of both the black and white morphs of *S. alnifolia* were collected randomly from the same locality in order to represent the whole region and their phenotypic characteristics were recorded. Further analysis was carried out in triplicates by dividing the whole root mass in to three. The altitude of collection sites was recorded. Electric conductivity, pH, organic carbon, mineral content and heavy metal content of the soil was estimated. Soil samples were collected in two consecutive depths, soil pits of 0-60 cm were dug in every grid and samples collected from 0-30 and 30-60 cm depths to represent the surface and subsurface horizons, to understand the variation in soil parameters at different layers of root growth. Collected soil samples were air dried in shade, powdered, sieved and stored for analysis. After uprooting the plant specimen, preliminary phenotypic evaluation was carried out, washed to remove mud and other extraneous materials and blotted dry till the excess moisture was removed and roots cut in to small pieces and dried under controlled (shade) condition. After complete drying, samples were cut in to smaller pieces, pulverized into homogeneous powder, sealed and stored in vacuum desiccators until chemical characterization.

Table 1: Location details and GPS coordinates of wild and cultivated sites

Sl No.	Locations in each Zones	Morphs	Elevation	Longitude	Latitude
Coastal Plains					
1	Kanjikkuzhi	White	19	76°20'9.00"E	9°32'59.82"N
2		Black	19	76°20'28.68"E	9°32'58.44"N
I. Midland Laterites					
3	Chengannur	White	38	76°43'4.20"E	9°23'34.86"N
4		Black	38	76°43'2.82"E	9°23'28.56"N
II. Foothills					
5	Pattazhi	White	318	76°58'39.78"E	9° 6'12.96"N
6		Black	320	76°59'4.86"E	9° 5'53.88"N
III. High Hills					
7	Kalpatta	White	1157	76° 7'7.38"E	11°36'38.22"N
8		Black	1152	76° 7'6.90"E	11°36'50.04"N
IV. Palakkad Plains					
9	Pothundy	White	114	76°38'36.24"E	10°37'13.26"N
10		Black	115	76°38'28.08"E	10°36'52.14"N
V. Commercial cultivation					
11	Pookkod	White	11	76°18'4.95"E	10°26'20.04"N
12		Black	11	76°17'8.83"E	10°26'26.14"N

Phytochemical analysis

Gravimetric method was followed to analyse total Alkaloid content in plants (Haborne, 1973) [7]. Total flavonoid content was estimated by Aluminium chloride colorimetric method (Akbay, *et al*., 2003; Kaufman, *et al*., 1999) [8, 9] with the help of UV-Visible Spectrophotometer (Shimadzu, Japan) and read the absorbance at 415 nm. Phenol content in both the plants were analysed following the Folin – Ciocalteu method with the help of UV-Visible Spectrophotometer and read the absorbance at 725 nm and Starch content at 630 nm (Sadasivam and Manickam, 2008) [10]. The chief active ingredient, Ephedrine content of both the white and black morphed plants were analysed with the help of High-Performance Thin-Layer Chromatography (HPTLC) (Gupta *et al*., 2005 and Khatoon *et al*., 2005) [11, 12].

HPTLC conditions

HPTLC plates consisted of 20×10 cm, precoated with silica gel 60 F_{254} HPTLC plates (E. Merck) (0.2 mm thickness) with aluminum sheet support. The spotting device was CAMAG Linomat V Automatic Sample Spotter (Camag, Switzerland); syringe, 100 µL (Hamilton); developing chamber was CAMAG glass twin trough chamber (20×10 cm); the densitometer consisted of CAMAG TLC Scanner linked to win CATS software; experimental condition temperature 25±2 °C, relative humidity 65±15%.

Sample preparation

Ephedrine is soluble in methanol, methanolic extract was prepared by accurately weighing 10 g of the powdered drug and extracted with methanol (100 ml) under reflux on a water bath. The methanolic extract was filtered through Whatman I filter paper, filtrates were combined, concentrated and added 2 ml methanol filtered through syringe filter (0.2 µm) volume was made up to 2 ml. This extract was used for the quantification of Ephedrine

Estimation of ephedrine

10 µL of sample solutions of methanolic extract along with the standard was applied on HPTLC plate and the plate was developed in n-Butanol: Acetic acid: water (4:3:1v/v) solvent system to a distance of 8 cm. The plates were dried at room temperature in air and derivatized with Ninhydrine reagent and heated at 105 °C for 10 min. and scanned densitometrically at 480 nm in absorbance mode using tungsten lamp. The area of the resolved peaks was recorded.

Mineral analysis

Minerals in plant and soil samples were analysed by categorizing into macro, micro and heavy metal contents. In plants micro and macro nutrients and heavy metal content were analysed by plant digestion method (Black, *et al*., 1965) [13]. Total Nitrogen content was analysed with the help of Kel

plus Nitrogen Analyzer, Phosphorous by using Spectrophotometer, Potassium and Sodium with the help of Flame Photometer. Other nutrients such as Calcium, Magnesium, Iron, Copper, Zinc, Manganese and heavy metals such as Cadmium, Lead, Nickel, and Chromium were determined (Carbonell, *et al*., 2009b) [14] by feeding the samples to Atomic Absorption Spectrometer (Varian, 240).

In addition to the minerals mentioned, available Sulphur (Chesin and Yien, 1951) [15] and Boron were also analyzed to find the available mineral content of soil and soil physical properties – pH, electrical conductivity, organic carbon (Jackson,1958) [16] were also analyzed. Available Nitrogen (N) was estimated by alkaline permanganate method (Subbiah and Asija, 1956) [17]. Available Phosphorous (P) (Watanbe and Olsen, 1965) [18]. Potassium (K), Calcium (Ca) and Magnesium (Mg) were estimated from neutral normal ammonium acetate extract of the soil (Piper, 1966) [19]. Available Iron (Fe), Manganese (Mn), Zinc (Zn) and Copper (Cu) and available heavy metals such as Lead (Pb), Nickel (Ni), Cadmium (Cd) and Chromium (Cr) were determined using 0.1 N Hydrochloric acid (Lindsay and Norvell, 1978) [20] by atomic absorption spectrophotometer (Varian 240). Sodium (Na) content was estimated from neutral normal ammonium acetate extract of the soil using digital type Elico (CL-360) Flame Photometer (Jackson,1958) [16] and Boron (B) content was determined by hot water extraction method (Gupta, 1967) [21] and estimated using UV spectrophotometer.

Statistical analysis

Independent samples Kruskal-Wallis test and Mann–Whitney U test (Mann–Whitney–Wilcoxon (MWW)) were done using IBM.SPPS. Statistics.v21Win. Since the sample size of commercially cultivated plants were less than the wild collected ones, Wilcoxon test was carried out.

Results and discussions

From the various metabolites analysed, figure (1) represents the difference in major secondary metabolites such as Alkaloids, Flavonoids and Phenols present in the white and black morphs of *S. alnifolia*, from the figure it is evident that total Alkaloid content found to be high in all the samples analysed and the mean Alkaloid content found slightly varied in commercially cultivated white (7.96 ± 0.16) and black (10.03 ± 0.16) morphs, test results did not show any significant difference between both the morphs. Similarly, the mean of wild collected plants from Coastal plains (white 9.58 ± 0.04 and black 6.18 ± 0.04) and Midland laterites (white 3.96 ± 0.04 and black 5.08 ± 0.04) were also found varied but test statistics showed no significant difference. Correspondingly, Flavonoids and Phenols (Fig.1) also showed no significant difference between the black and white morphs of *S. alnifolia* collected from different agro-ecological zones as well as commercially cultivated plants. Among the metabolites, figure (2) enlightens the Starch content present in the different morphs of the plant and Starch found to be high in all samples collected from wild and commercially cultivated black and white morphed plants and it did not show any significant difference between them. Similarly, from figure (3) it is evident that Ephedrine (chief therapeutic component) did not possess any variation in the wild collected black (0.02 ± 0.0008) and white (0.04 ± 0.0008) coloured morphs of *S. alnifolia* plants, while commercially cultivated plants showed slight variation in the mean value although there was no significant variation observed. Slight variation in the mean value may be due to the intake of fertilizer supplied or the influence of other environmental factors.

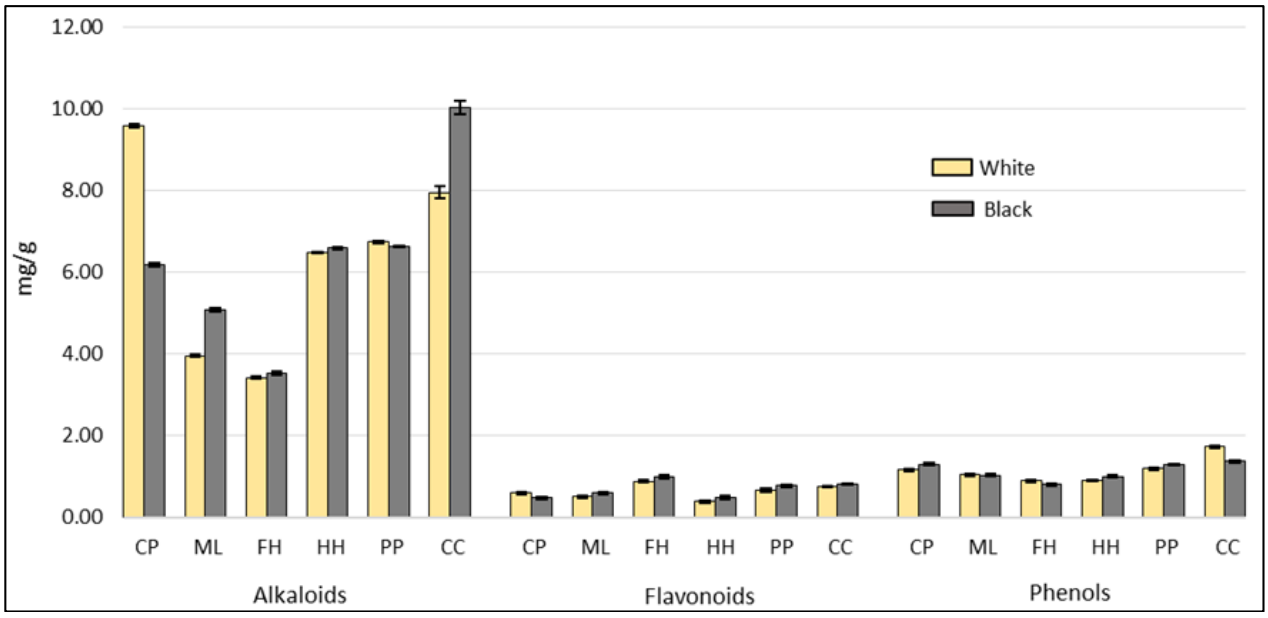
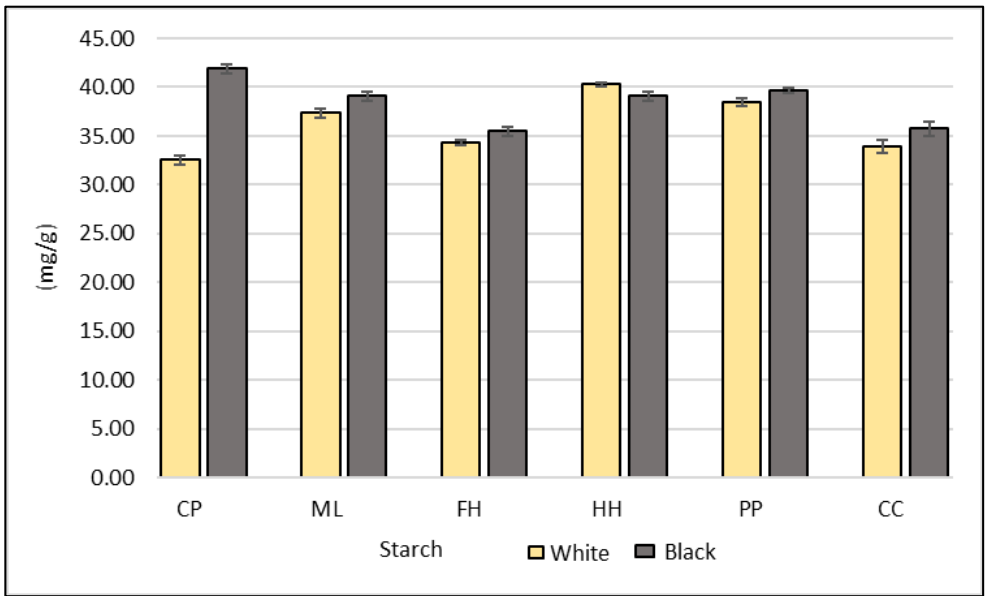


Fig: 1 Variation in major secondary metabolites of white and black morphed *S. alnifolia*

CP – Coastal plains, ML – Midland laterites, FH – Foothills, HH – High hills, PP – Palakkad plains and CC – Commercial cultivation. The values in the figure given in Mean ± SE for each samples.



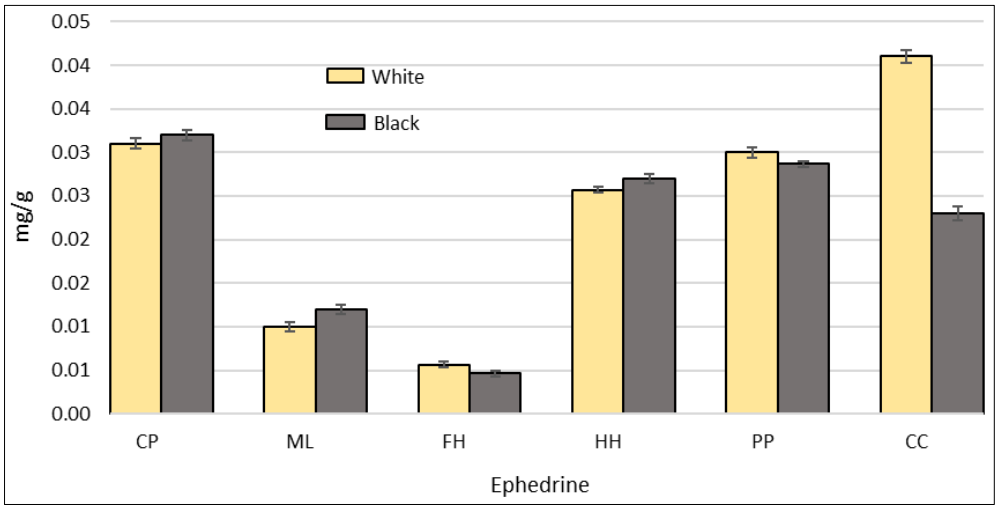


Fig 2 & 3: Variation in Starch and Ephedrine content – white and black morphs of *S. alnifolia*

From the results, the analyses indicate no significant variations in the metabolic contents between black and white morphs of *S. alnifolia* collected from different agro-ecological zones as well as commercially cultivated was observed.

Plant mineral analysis

Plant minerals were analyzed by categorizing into macro, micro and heavy metal contents present in plants (Fig 4 to 8).

From the figures it is evident that, one among the essential element, Phosphorous (Fig.6) and trace elements such as Iron (Fig.6) and Sodium (Fig.7) contents found to be high in all the samples collected from wild as well as commercially cultivated. Among the all, Sodium (Fig.7) content found to be significantly too high in all the plants collected from wild and commercially cultivated, but there was no significant difference found in the black and white morphed plants.

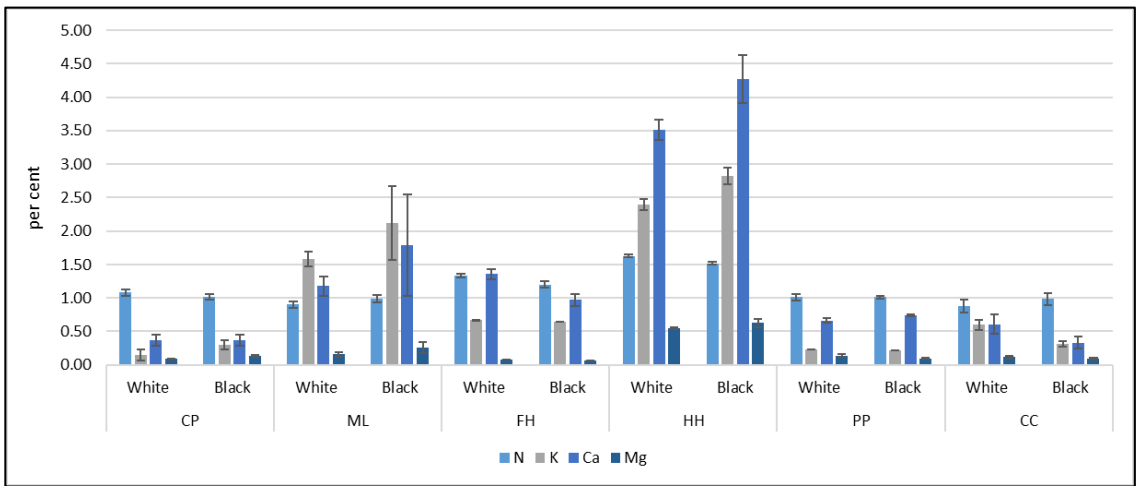


Fig 4: Variation in essential elements of white and black morphed *S. alnifolia*

When the essential elements considered, a slight to negligible variation in the mean values were evident from figure (4) and the hypothesis test summary also revealed that the distribution of essential elements found same across both the categories and the test statistics showed no significant difference between the morphs. All the essential elements found to be high in their content but there was no significant difference between them. Variation in mean value of Potassium and Calcium present in the white and black morphed plants collected from Midland laterites were observed but no significant difference found between them. Similar results were observed in commercially cultivated plants too.

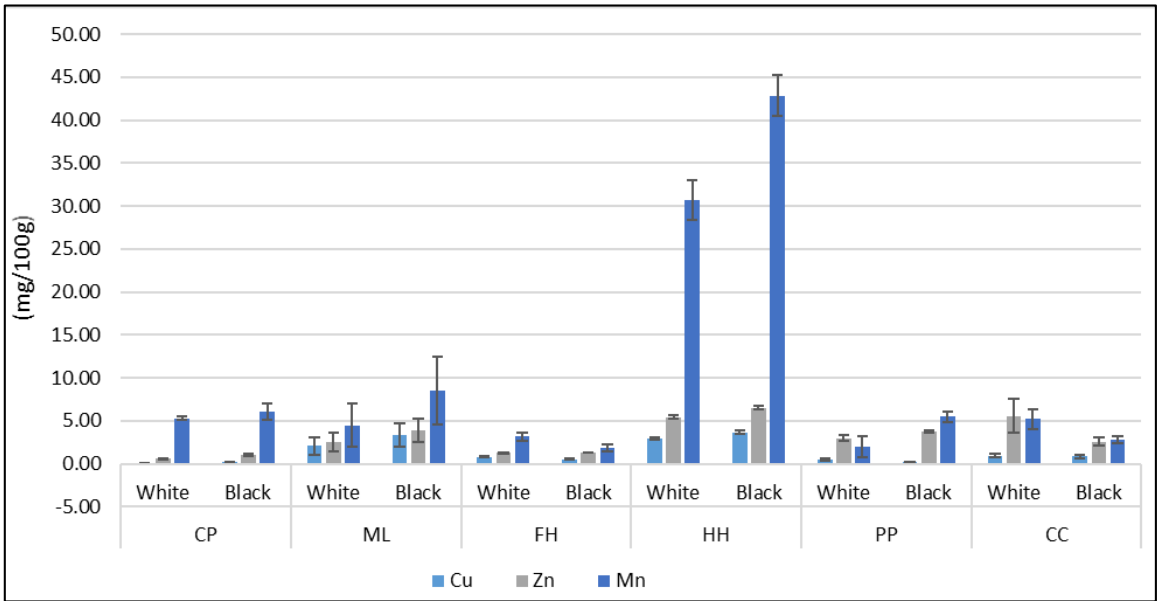
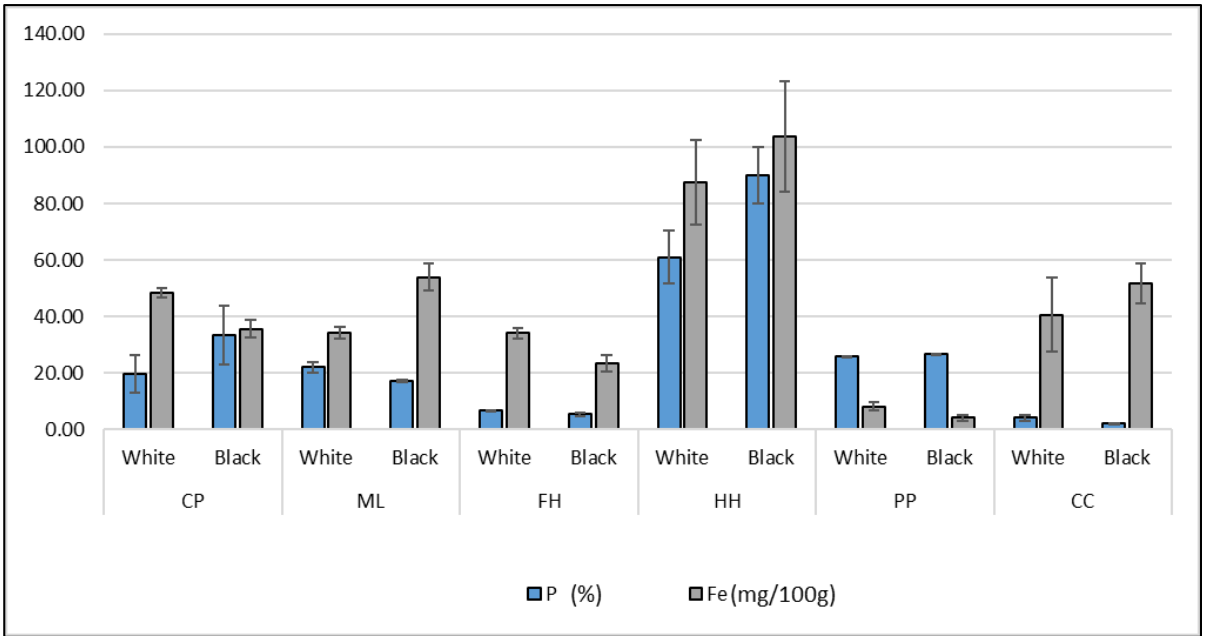


Fig 5: Variation in trace elements of white and black morphed *S. alnifolia*

From the figure (5) it is evident that trace elements found to be high in the samples collected from High hill regions and content of Copper found lesser in content throughout wild as well as commercially cultivated plants and Sodium (Fig.7), Iron (Fig.6) and Manganese content also found high in all the samples collected. However, there was no significant variation in the contents of trace elements were observed in the white and black morphs of *S. alnifolia* plants collected from wild and cultivated. Although, a slight variation in Zinc and Manganese content were observed in the commercially cultivated white and black *S. alnifolia* plants, it may be due to the variation in intake of the additionally supplied higher inputs for the growth and development of the plant but there was no significant difference found and test statistics showed non-significance.



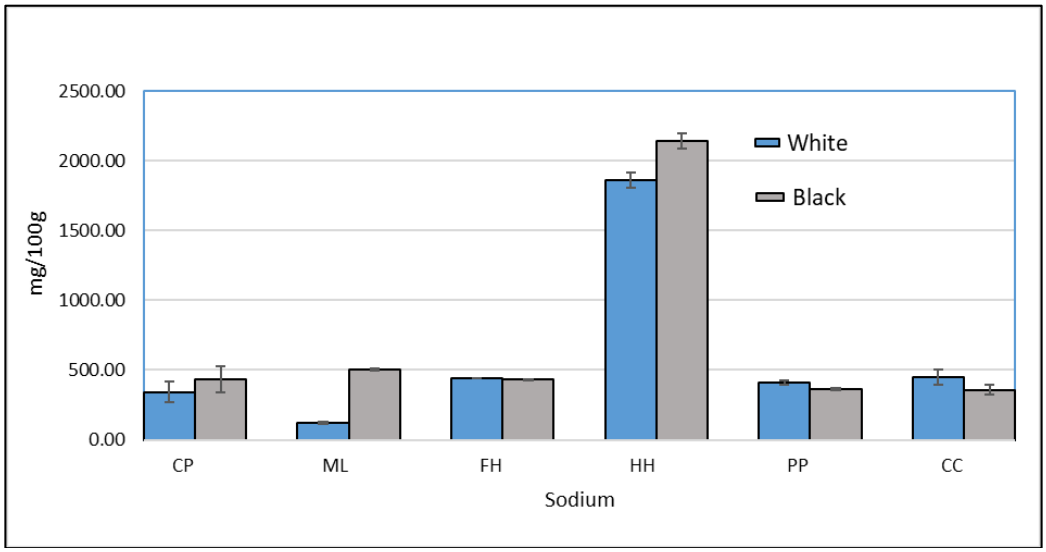


Fig 6 & 7: Variation in Phosphorous, Iron and Sodium contents of white and black morphed *S. alnifolia*

Heavy metal contents observed were Lead, Nickel, Cadmium and Chromium. From the figure (8) it is evident that, among the four, Cadmium found to be almost nil or non-detectable quantity in samples collected from Coastal plains, High hills and Palakkad plains. Similarly, Lead content was also found nil the samples of Palakkad plains. Variation in mean values are evident from the figure (8) but there was no significant difference found between the morphs. Plants never absorbs the heavy metal contents from the atmosphere it used to get absorbed through the soil, so the presence of heavy metal content present in plants were captured from the region where it grown. Heavy metal content was found to be high in plants collected from Coastal plains and similarly, high content of Lead was detected in both the white and black morphed plants collected from commercially cultivated plot, incidentally the commercially cultivated site also come under the zone, Coastal plains.

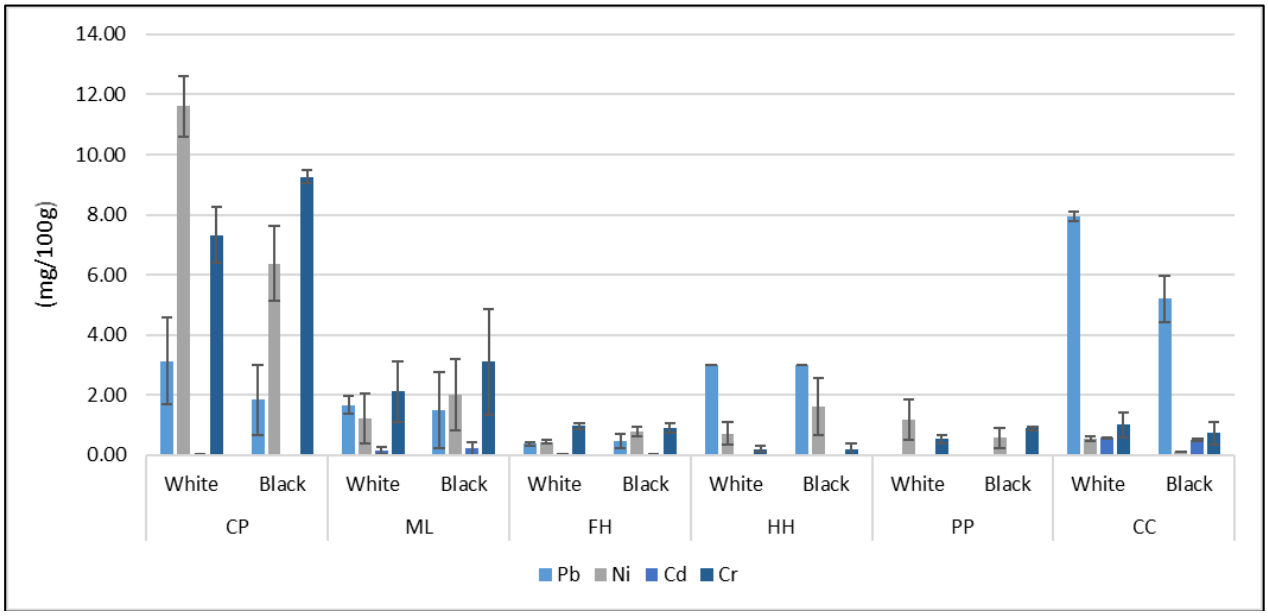


Fig 8: Variation in heavy metal contents of white and black morphed *S. alnifolia*

Corresponding to the metabolic content, plant minerals were also not showed any significant difference between the black and white morphs of *S. alnifolia* between the zones and commercially cultivated. Whereas, test statistics (Kruskal wallis) showed significant difference between the localities. From the figure (3 to 8) it is evident that between the zones nutrient contents differed significantly but there was no notable or significant difference found between the white and black morphed plants.

Valuation of soil properties

Soil physical properties were signified in figure (9). From the figure it is evident that there was no notable variation in the physical characteristics were observed in 0 to 60 depth scale. Among the three soil physical properties, pH of soil found to be moderately acidic to normal in wild collected ranged from (5.17 ± 0.09 to 6.90 ± 0.17) there was no much variation in 0-60 cm depth scale. However, soils of commercially cultivated were slightly acidic in 0-30 cm depth (4.87 ± 0.19) and found normal in 30-60 depth (6.26 ± 0.03), which leads the acidic content of the top soil may be due to the atmospheric gases as also due to high input cultivation practices employing the synthetic content of bulky application of organic manure. EC ranged from (0.38 ± 0.01 to 0.69 ± 0.01 dS/m) and in commercially cultivated ranged between (0.34 to 0.35 dS/m).

Organic carbon per cent also found to be high in all region collected were ranged from 0.77 ± 0.10 to 2.26 ± 0.03 %).

Figure (10) represents the soil mineral content present at two consecutive depth scale, 0-30 and 0-60. From the figure it is evident that, among the macro nutrients considered, Calcium found to be high in all the samples analysed and the highest amount was reported in the samples collected from Midland laterites followed by Palakkad plains and Coastal plains and least content were reported at High hill region where the maximum amount of available Calcium found in the black and white morphs of *S. alnifolia*, where Calcium is considered a secondary plant nutrient. Merely Nitrogen and Potassium are required in larger amounts by plants and Calcium considered as an important constituent of cell walls and can only be supplied through the xylem sap and once fixed, the available Calcium content in plants found immobile. In general, roots absorb Phosphorus in the form of orthophosphate, but can also absorb certain forms of organic Phosphorus. In the current results Phosphorous content in plants found to be too high compared to other essential elements evaluated but the available Phosphorous content in soil found too low than the other macro nutrients. In support to the present results studies of Brady and Weil (2002) [22] reports that in comparison to other macronutrients, Phosphorous concentration in the soil solution found to be much lower in comparison to other macronutrients and was ranged from 0.001 mg/L to 1 mg/L. Nitrogen and Potassium found to be high both in plants and soil in fact which required in larger amounts by plants and followed by Magnesium. Similarly, Iron, Sodium and Manganese content were also found high in plants as well as soil in two consecutive depths. Calcium and Phosphorous content found high 30-60 depth scale than 0-30 cm in commercially cultivated plot. When compare to the wild, presence of mineral nutrients in plants as well as soil found to be lower in commercially cultivated. Sodium and Iron found comparatively higher in both plants as well as soil, and in soil both the elements present more in 30-60 depth. Copper and Zinc were present in almost equal amount in soil and was found high in commercial site. Manganese content found constantly high in soil and consecutively in plants too.

Among the heavy metal content evaluated Lead found to be high in soil compare to the other four evaluated followed by Nickel. Cadmium content found mostly low or below detectable limit in soil as well plants. Total Lead content found to be high in Foothills followed by Midland laterites and Lead content found less in soils of commercially cultivated land. Whereas available Lead content found higher in samples of commercially cultivated plants and plant samples collected from Foothills reported lower content of Lead. Nickel and Chromium found higher in samples collected from High hills, Palakkad plains and commercially cultivated area, presence of Nickel in plants found higher in High hills, Palakkad plains and Midland laterites and Chromium found higher in commercially cultivated plants.

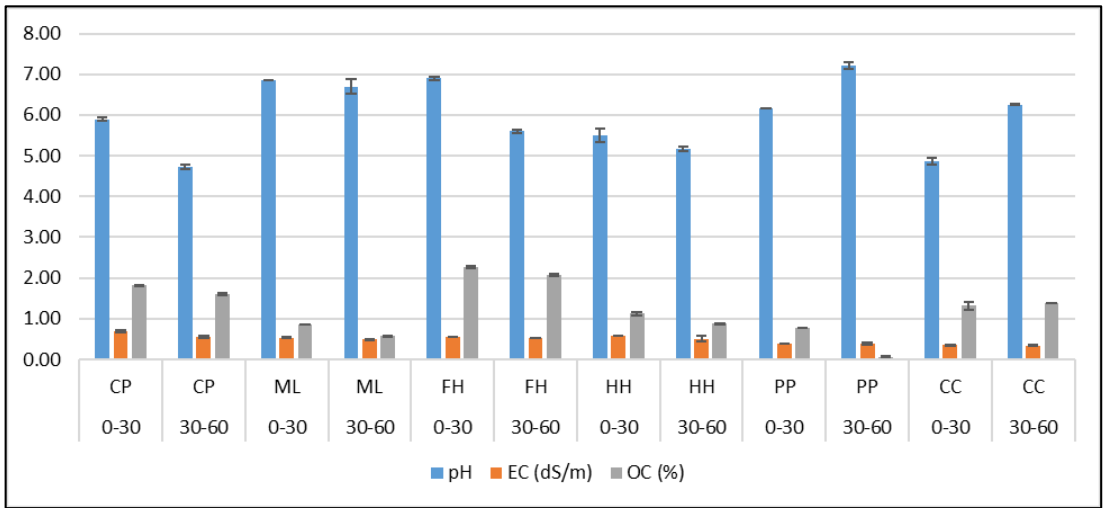


Fig 9: Soil physical properties 0-30 and 30-60 cm depth scale.

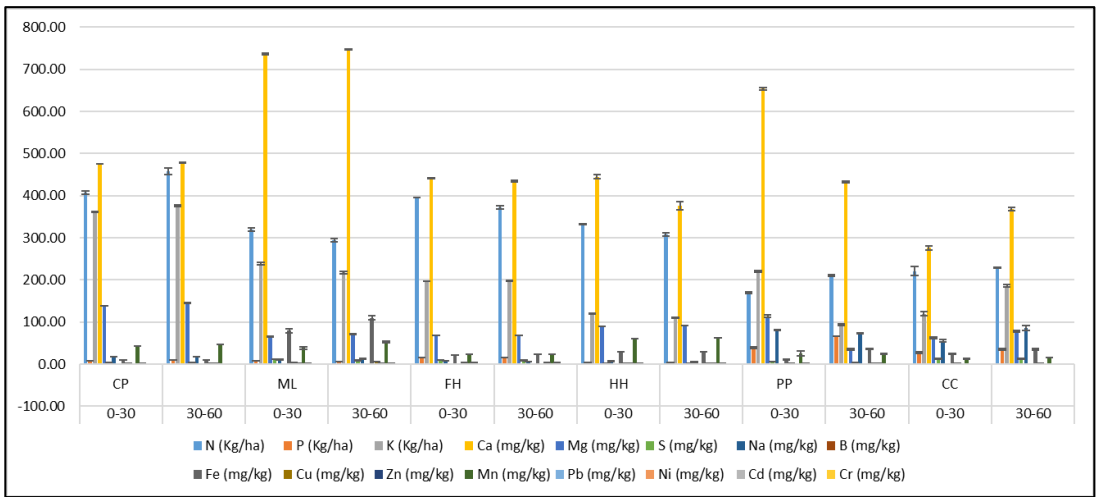


Fig 10: Soil Mineral contents 0-30 and 30-60 cm depth scale.

Analogous to the phytochemical contents of white and black morphs, edaphic factors also did not show any notable correlations between the morphs. However, the test revealed that the locations determined the significant variations in phytochemical contents rather than the colour morphs.

Precisely, from the results it was evident that regional discrepancy thoroughly impart variation in the phytochemical profiles whereas the plant colour did not make any impact on phytochemical profiles of the *S. alnifolia* plants collected from wild as well as commercially cultivated. Similar studies conducted by Weiss (1995) [23] and Bond (2007) [24], reported that in different species evaluated, they could have noticed colour polymorphisms are very prevalent in plants of different species. Slight variation in the mean value may be due to the variation in mineral content or other soil properties prevailing in the soils of specific area and other environmental factors of particular locality. Similar studies were conducted by Epling and Dobzhansky (1942) [25], Wright (1943) [26] and Rausher (2008) [27], in their studies the results imply the possibility of the co-occurrence of different colour morphs has ecological and evolutionary interest and can be due to a variety of evolutionary processes, from direct or indirect selection mediated by pollinators, frugivores, herbivores, pathogens or abiotic environmental effects, to random genetic drift. Many evolutionary biologists have been engaged in the study of the processes underlying colour polymorphism thus, last two decades onwards reports of colour polymorphism in the scientific literature have shown a sharp increase (Forsman, 2016) [28]. Here, we use polymorphism to refer to discrete variants within a population in a frequency high enough not to be the result of recurrent mutations (Ford, 1945; Huxley, 1955) [29, 30]; thus, a species is considered as colour polymorphic when possessing at least one population where two or more morphs coexist. Because of the temporally variable selective environments, morph frequencies usually vary within each population; consequently, species may be polymorphic in some populations and monomorphic in others (Wright 1978; McLean and Stuart-Fox 2014; Forsman, 2016) [31, 32, 28]. As well, most of the colour polymorphic species show variation in morph frequencies, usually related to environmental heterogeneity (McLean and Stuart-Fox 2014; Carlson and Holsinger 2015; Forsman and Wennersten 2016) [32, 33, 34].

Conclusion

White and black morphs of *S. alnifolia* did not show any significant difference in their phytochemical constitution. However, the phytochemical contents of plants collected from different agro-ecological zones and commercially cultivated showed prominent variations. This may probably indicate that either the variations in genotype of the plants collected from the different zones may have affected the consequent variations in phytochemical contents as noted in our more comprehensive study (Unpublished Ph.D. Thesis, 2019). Nevertheless, the influence of the soil features and local environment prevailing in the wild collected locations on the concentrations of the active ingredient cannot be ruled out. However, this need to be examined further by conducting genetic studies and controlled experimentation.

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