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Research Article

Elemental Analysis of Vitexaltissimalinn. leaves by X-ray Fluorescence and its Biological Implications and Studies

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Abstract

Elemental analysis of *V.altissima* (L) leaf was carried out by using X-ray fluorescence spectrometer and SEM-EDX. Fifteen of the detected elements are essential, which includes some heavy elements (Al, Si, P, K, Ca, Mn, Fe, Cu, Zn, Sr, Nb, Mo, Cd, Th and U) while 20 others (Mg, S, Ti, V, Cr, Co, Ni, As, Se, Rb, Y, Zr, Ag, Sn, Sb, Ba, W, Hg, Pb and Bi) were found to be below detection limit in Hand held X-ray Fluorescence (HHXRF) analysis. From SEM-EDX results, non-transition elements such as C, O, Si, S, K, Ca and Br were found to be present in the dried leaf powder of *V.altissima*. The distribution of elements was found to be in the order: crude leaf powder> Hexane> Chloroform> Methanol. From the results it is clear that *V. altissima* contain many essential elements which are all required minerals for human Biological studies such as DPPH scavenging assay and reducing power was carried out on these plant extracts to evaluate their anti-oxidant capacity. It was seen that these plant extracts do have a good anti-oxidant levels.

Introduction

Medicinal plants are rich sources of bioactive components which play important role in the prevention of variety of diseases. *Vitexaltissima* belonging to the family Verbenaceae, is a moderate to large sized tree [1]. Leaves of the plants are reported to use for the treatment of rheumatism [2]. Antioxidant and anti-inflammatory activities of the plant leaves are also reported [3]. Phytochemical screening of secondary metabolites present in dry leaves and their percentage of yield was also calculated. Phytochemical analysis revealed the presence of flavonoids, terpenoids, and spooning in dry leaf extracts. Isolation is the main step in Photochemistry, where the biologically active compounds are separated in its purest single form. This process was carried out using repeated column chromatography, whereby a single biologically active compound was separated. This isolated compound was identified with the help of its spectral data, such as IR, ^{13}CNMR, ^{1}H NMR and mass spectra.

Photochemical analysis of *V.altissima* leaf extracts reveals the presence of triterpenes, phenol acids, steroids, coumarone, flavonoids, fatty acids etc. These secondary metabolites were reported to play a major role in various biological activities, which explains its use as a traditional medicine. An increased scientific interest is noticed presently on modern medicine and herbal products. Medicinal plants have a prominent role in the pharmaceutical industry of 21st century [4, 5, 6].

Various essential and non-essential metals are also present in plants in addition to the secondary metabolites. The higher and lower concentrations of these trace metals may cause metabolic disturbances [7] These metals may include both essential micronutrients and toxic metals, the deficiency and excess of which may cause serious effects on human health [8, 9]. Hence, the safety of the herbal products and its use has recently been questioned due to the reports of illness and causalities [10]. The intake of heavy metal by human may cause disturbances of normal functions of brain, kidney, lungs, heart, liver etc. and leads to ulcers and different types of cancers [11].

Experimental

Collection of plant material and its Extraction

Vitexaltissima leaves were collected from Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram, Kerala state, India. *V. altissima* leaves were

cut into small pieces, and shade dried and powdered using a cross beater mill. *V. altissima* leaf powder (520 g) was subjected to sequential extraction on Sox let apparatus with hexane, chloroform and methanol (2.5 L each) successively (24 hr, each). The extracts were filtered and concentrated under reduced pressure using a rotary evaporator. Percent yields of each extracts were noted.

The methanol extract, obtained after sox let extraction is then subjected to liquid-liquid partition and again into two on the basis of the polarity of the solvents. The methanol extract was dissolved in minimum quantity of water, which was then made up to 200ml with rest of water. Equal amount of ethyl acetate was added to it and mixed in a separating funnel, allowing to separate into aqueous and organic layers. Water containing aqueous layer is the lower part of the separating funnel and the upper layer is ethyl acetate containing organic part. The upper organic part was collected and concentrated. The procedure was repeated several times each time with 200ml of ethyl acetate, until the organic layer has no color. After washing using ethyl acetate, same procedure as repeated with butanol. The collected ethyl acetate, butanol and aqueous water fraction were concentrated and dried with rotary evaporator for further studies.

Results and Discussion

V. altissima leaf powder (520 g) was extracted with three solvents of increasing polarity namely hexane, chloroform and methanol. The extracts were concentrated under reduced pressure using a rotary evaporator. The percentage of yield for each extract is that 2.82, 6.01 and 11.5 (weight of each extracts are 14.72, 31.333 and 60.05). The yield of methanol extract was found to be high compared to hexane and chloroform extracts.

Phytochemical screening of extracts

The extracts (hexane, chloroform and methanol) obtained after sox let extraction were subjected to photochemical screening using standard procedure. Results show that hexane extract is enriched with steroids while chloroform is carbohydrate rich extract. Because of higher polarity of flavonoids and spooning, methanol extract produces positive result for both of them.

Elemental studies of various extracts of *V.altissima* leaves

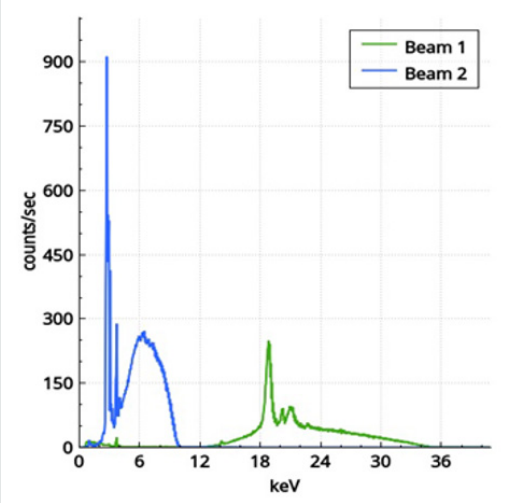
All these extracts were analyzed for trace elements using HHXRF and SEM-EDX using the crude samples. Hand held XRF (HHXRF) is the need of the hour to analyze metals, powders and alloys, as other conventional XRF techniques were found to be cumbersome and difficult to handle.

The HHXRF was directed at the samples and irradiated using X-ray tube. Rhodium tube the spectrum was obtained in 60 seconds. A Typical spectrum of leaf extract is shown in (Figure1). The prominent peaks of K (3.3 keV) and Ca (3.6KeV) are seen. The beam lines were from 12 to 36 keV (Beam 1) and from 0 to 12 KeV (Beam 2) (Figure 2, Table 1).

Table 1: Concentrations of elements in leaf extracts in ppm.

Samples	Crude	SE1	SE2	SE3	SE4	SE5
Al	5400	5700	--	4100	3300	1800
Si	1050	2310	--	1066	1700	1350
P	220	290	--	210	160	--
K	300	280	--	200	250	444
Ca	2325	1207	--	8670	1077	3950
Mn	99	59	73	--	41	56
Fe	51	45	160	137	98	118
Cu	9				132	17
Zn	20	9	21	15	31	15
Rb	--		6	7	15	12
Sr	14	17	14	16	9	23
Nb	6	7	--	6	--	--
Mo	8	9	---	--	--	--
Cd	34	41	--	35	--	--
Th	29	32	31	31	25	23
U	10	10	15	13	15	9

Figures 1: HHRF used for the analysis of *V. altissima* leaf powder.



Figures 2: Spectrum of the analyzed *V. altissima* leaf extracts.

*crude- dried leaf powder; SE1-Hexane extract; SE2- Chloroform extract; SE3- Ethyl acetate extract; SE4-Butanol extract; SE5- Water extract. (**SE- Solvent extract) Al, Si, P, K, and Ca are seen to be present significantly which are useful elements. Al which cannot be detected by many conventional instruments in XRF due to low energy X-ray absorption of detector window could be detected by HHXRF. HHXRF has SDD (Silicon drift detector) which has a grapheme window and the Al X-rays do not get absorbed unlike in the Si (Li) (Lithium drifted). Silicon X-ray detector which has Be window, absorbs low energy X-rays below Z<19. Thus, HHXRF can be a useful tool for detecting Al in samples. There are no toxic elements in the sample except Cd, which is very below MPL. The role of each of these elements needs to be investigated and the study is underway for further conclusions.

DPPH Radical Scavenging Assay

The radical scavenging activity of different extracts was determined by using DPPH assay according to Chang et al. [12]. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm.

Principle

1, 1-diphenyl-2-picryl hydroxyl is a stable free radical with pink color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

DPPH + [H-A] →DPPH-H + (A)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Reagent Preparation

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of methanol.

Procedure

Different concentrations of sample such as 12.5µg/mL- 200µg/mL from stock solution were made up to a final volume of 20µl with DMSO and 1.48ml DPPH (0.1mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

Calculation

Percentage of inhibition = $\frac{control-test}{control}X100\frac{control-test}{control}X100$

Results

(Tables 2,3)

Table 2.

Concentrations (µg/mL)	Absorbance I	Absorbance II	Absorbance III
Control	1.105	1.096	1.077
Sample code: VAH			
12.5	0.906	0.917	0.891
25	0.872	0.879	0.864
50	0.812	0.835	0.801
100	0.779	0.798	0.769
200	0.747	0.749	0.747
Sample code: VAC			
12.5	0.664	0.660	0.648
25	0.623	0.623	0.633
50	0.521	0.586	0.552
100	0.495	0.498	0.491
200	0.402	0.373	0.384
Sample code: VAE			
12.5	0.675	0.575	0.641
25	0.599	0.550	0.526
50	0.442	0.401	0.489
100	0.328	0.320	0.328
200	0.183	0.131	0.147
Sample code: VAB			
12.5	0.657	0.693	0.641
25	0.464	0.511	0.438
50	0.307	0.277	0.232
100	0.160	0.126	0.149
200	0.147	0.115	0.104

Table 3: IC_{50} Value- VAH-440.173µg/mL (Calculated using ED 50 PLUS V1.0 Software). VAC-75.522µg/mL; VAE- 28.7433µg/mL; VAB-17.8732µg/mL.

Concentrations (µg/mL)	Percentage Inhibition I	Percentage Inhibition II	Percentage Inhibition III	Average Percentage Inhibition
Control	0.00	0.00	0.00	0.00
Sample code: VAH				
12.5	18.05	16.38	17.32	17.25
25	21.13	19.80	19.78	20.24
50	26.52	23.86	25.63	25.33
100	29.50	27.19	28.60	28.43
200	32.40	31.71	30.69	31.60

Sample code: VAC				
12.5	39.91	39.78	39.83	39.84
25	43.62	43.16	41.23	42.67
50	52.85	46.53	48.75	49.38
100	55.20	54.56	54.41	54.73
200	63.62	65.97	64.35	64.64
Sample code: VAE				
12.5	38.91	47.54	40.48	42.31
25	45.79	49.82	51.16	48.92
50	60.00	63.41	54.60	59.34
100	70.32	70.80	69.55	70.22
200	83.44	88.05	86.35	85.95
Sample code: VAB				
12.5	40.54	36.77	40.48	39.27
25	58.01	53.38	59.33	56.91
50	72.22	74.73	78.46	75.13
100	85.52	88.50	86.17	86.73
200	86.70	89.51	90.34	88.85

Reducing Power Activity

The reducing power of extract was determined by the method of YEN and DUH (1993).

Procedure

Different concentrations of sample such as 125µg/mL-2000µg/mL from a stock concentration of 10mg/mL were mixed with 2.5ml of phosphate buffer (200mM) (pH 6.6) and 2.5ml of 1% potassium ferric cyanide was added and boiled for 20 minutes at 50°C. A control without the test compound, but an equivalent amount of distilled water was taken. After incubation, 2.5 ml of 10% TCA were added to the mixtures followed by centrifugation at 650g it for 10 minutes. The upper layer (5ml) was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride was added and absorbance was read at 700nm.

Results

(Tables 4)

Table 4.

Concentrations (µg/mL)	Absorbance I	Absorbance II	Absorbance III	Average Absorbance
Control	0.0898	0.089	0.0866	0.0885
Sample code: VAH				
125	0.0922	0.0980	0.0109	0.0670
250	0.1065	0.1016	0.1194	0.1092
500	0.1423	0.1387	0.1482	0.1431
1000	0.1684	0.1690	0.1580	0.1651
2000	0.1794	0.1721	0.1783	0.1766
Sample code: VAC				
125	0.1078	0.0997	0.1045	0.1040
250	0.1160	0.1059	0.1162	0.1127
500	0.1663	0.1501	0.1571	0.1578
1000	0.2028	0.1998	0.2020	0.2015
2000	0.3568	0.3518	0.3572	0.3553
Sample code: VAE				
125	0.1627	0.1576	0.1688	0.1630
250	0.1986	0.1827	0.1978	0.1930
500	0.2466	0.2394	0.2344	0.2401
1000	0.3392	0.3300	0.3468	0.3387
2000	0.4016	0.3911	0.3983	0.3970

Sample code: VAB				
125	0.1774	0.1621	0.1692	0.1696
250	0.2071	0.1918	0.2011	0.2000
500	0.2950	0.2851	0.2890	0.2897
1000	0.4225	0.4097	0.4169	0.4164
2000	0.6338	0.6193	0.6020	0.6184

Conclusions

A vast amount of research is on way to explore various herbal products. In addition to various primary and secondary metabolites, photochemical include a variety of essential and non-essential elements. Thus, the safety use of herbal products lies on its content of trace elements also. Photochemical analysis of *V.altissima* leaf extracts reveals the presence of triterpenes, phenol acids, steroids, coumarone, flavonoids, fatty acids etc. In the present study, the leaf extract was estimated to contain several essential elements. This knowledge about the elemental composition will be useful while formulating drugs for specific purposes.

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