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Original Article

Preliminary Phytochemical Screening, Anti-oxidant activity, Multi elemental analysis by ICP- Spectroscopy and Antimicrobial activity of bulbs extracts of *Crinum woodrowii* Baker from Bhimashankar, Maharashtra, India

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ABSTRACT

Crinum woodrowii Baker is a critically endangered bulbous plant belonging to family Amaryllidaceae found only at Kates point, Mahabaleshwar is an important medicinal plant widely used in several indigenous medicinal formulations. Presently, these plants could be collected from wild habitat only. Due to indiscriminate collection from natural habitat it has become endangered. There was new location of its habitat at Bhimashankar Wild Life Sanctuary. There were only 200-250 individuals along the hilly slope and water spring. In present study the preliminary phytochemical study and antioxidant activity of the bulb extracts of *Crinum woodrowii* were evaluated. Phytochemical screening indicated that, bulbs are rich in a variety of primary and secondary metabolites such as carbohydrates, alkaloids, vitamin C, vitamin E, flavonoids, phenols, glycosides and saponins. The study highlights the biochemical and ethno pharmacological significance of a critically endangered *Crinum woodrowii.*

Keywords: *Crinum woodrowii,* critically endangered plant, phytochemicals, antioxidants, medicinal plants.

INTRODUCTION

The distribution of *Crinum woodrowii* is restricted to the North-Western Ghats of Western India, where it occurs in three areas: in the Dharmapur forest range of the Balsar district in Gujarat State at about 700 m above sea level; Mahabaleshwar; Kas Plateau and Kates point Mahabaleshwar from Satara, Khandus plateau of Bhimashankar Wild Life Sanctuary and Katraj Ghat from Pune, Vahigaon from Thane, districts of Maharashtra [1-6]. Tall herbs; bulbs 8.6–16.2 cm in dia., globose–spheroidal, outer tunics brown, membranous. Leaves contemporary with the flowers, sometimes appear after flowering, many (8–17), 45.5–80 cm x 4.5–14 cm, uniform, flat, bright green, slightly glaucous beneath, glabrous, apex acute, white waxy, scabrous along margin; leaf sheaths forming a pseudo stem. Scapes one, rarely two, arising from bulb outside the tuft of leaves, stout, compressed,53.5–82.5 cm x 1–3 cm, green at base and apex, purple in middle, faintly channeled. Flowers 10–20 in umbel, fragrant; pedicels 1–3 cm long, green with purple tinge [3]. *Crinum woodrowii* is used as ornamental as well as medicinal herb. The scented flowers may be used in perfume and pharmaceutical industry. There were 200-250 individuals of *Crinum woodrowii* Baker were identified along the water springs and hill slope of Khandus plateau of BWLS in Pune district of Maharashtra in August, 2012 [6]. It is new record of its distribution apart from Kates point Mahabaleshwar after 2004, bank of Vaitarna river at Vahigaon in Thane in 2013[4] and Katraj Ghat in Pune in 2014[5]. In the present study, we reported a new location of critically endangered distribution of *Crinum woodrowii*, a high-throughput micro-scaled method which enables digestion of small quantities of plant samples for subsequent elemental profiling by ICP-spectrometry, DPPH anti-scavenging activity, antimicrobial activity. The investigations led to the identification of phytochemical contents of bulbs of *Crinum woodrowii* of indigenous origin.

MATERIALS AND METHODS

Chemicals: All solvents were distilled prior to use. TLC was performed on silica gel 60 F254 (Merck). All reagents and solvents purchased from Merck

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Chemicals. The standard flavonoids were purchased from Sigma Aldrich. The UV–Vis spectra were recorded on a Shimadzu UV-1700 spectrophotometer. The HPLC were recorded on Agilent Technologies 1200 series Quaternary. Minerals detection was performed by using CEM Mars 6 microwave digester and Teledyne Leeman, ICP OES model Prodigy Dual View (Induction Coupled Plasma). The HPTLC were recorded on CAMAG LINOMAT-5.

Sampling: Fresh samples of bulbs of *Crinum woodrowii* were collected during monsoon (July, 2012) from Bhimashankar Wild Life Sanctuary area regions of Western Ghats of Maharashtra (Fig. 1A-D). The plants were identified and authenticated using herbarium collection at Botany research laboratory, DST-FIST School of Life Science, SRTM University, Nanded (MS) and Department of Botany Walchand College, Solapur (MS) India. Fresh bulbs were washed thoroughly under running tap water followed by sterile distilled water and dried under shade. The shade dried material was ground into coarse powder using mechanical grinder .This coarse powder was sieved by 1 mm pore size sieve. The powder was stored in airtight containers at room temperature till further phytochemical screening of secondary metabolites.

Soxhlet Extraction: Exhaustive Soxhlet extraction was performed using a classical Soxhlet apparatus with accurately weighed 10 g of the drug powder for 18-40 h. Extraction was performed with water, methanol, chloroform and acetone as the extracting solvent. The extraction was conducted for 6-8 hours/day and finally all the extracts were evaporated under vacuum. The water, methanol, chloroform and acetone extracts of rhizome of these plants were prepared according to standard methods [7]. Nitrogen gas was purged through these extracts to prevent oxidation of secondary metabolites. These extracts were sealed in airtight containers and stored at -4^0C.

Phytochemical Screening: Phytochemical screening of active plant extracts was done by following the standard methods [8-9] for the qualitative analysis of various phytochemical studies such as alkaloids, carbohydrate, glycosides, saponins, flavonoids, phenols vitamin C and vitamin E which could be responsible for antioxidant activity (Table 1).

Antiscavenging activity: DPPH solution (0.1 mM) was prepared in methanol by dissolving 0.0394 gm DPPH in 1000 ml methanol. The solution was kept in darkness for 30 minutes to complete the reaction. The free radicals scavenging activity of the crude extracts was determined by the 1, 1-diphenyl-2-picryl-hydrazil (DPPH). The antioxidant activity was measured by the standard method. Where in the bleaching rate of stable free radical, DPPH was monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH absorbed at 570 nm, but upon reduction by an antioxidant or radical species its absorption decreased. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = [(ABS control-ABS sample) / (ABS control)] X 100, whereas ABS control is absorbance of negative control and ABS sample is the absorbance of the reaction mixture containing the sample extract.

Mineral analysis

Micro-scaled digestion: CEM-MARS 6 microwave oven was used for micro-scaled digestion. 0.5 gms of herbal samples were weighed and transferred to CEM- Xpress vessels. 8-10ml of conc. HNO3 was added to the samples. The samples were pre-digested for 10-15 minutes prior to capping the vessels. The CEM- Xpress vessels were assembled for microwave irradiation. The microwave program was adjusted with respect to the number of vessels and reference to the guidelines of CEM at 1000W with 100% level. 25 minutes ramping period was used to reach the digestion temperature of 1800C which there upon was maintained for 15 minutes. The CEM- Xpress vessels were kept in fume hood for cooling and to release the pressure by uncapping. The contents were transferred to 50 ml volumetric flasks and volume was made with distilled water. The solutions were filtered prior to use.

Calibration Standards: For calibration, Leeman and Thomas Baker Std. samples were used as the reference for the calibration range.

Instrument Preparation/Operation: The spray chamber, nebulizer & torch assembly was completely cleaned to eliminate any form of contamination. The plasma was stabilized for 15 minutes by flushing with distilled water. An Instrument Calibration was performed to check the wavelength shift and the same was successful with a minimum deviation of <10 % with master scan.

ICP mineral analysis: Diluted samples were used for further analysis by using Teledyne Leeman, ICP (Induction Coupled Plasma).

Vitamin E analysis by HPLC

Standard preparation: A standard dl α-tocopherol acetate (96%) (Vitamin E) manufactured by Merck was used for calibration of standard curves. 1mg of

dl α-tocopherol acetate was dissolved in 1mL in HPLC grade methanol. The dilutions of 100, 50, 25, 10 µg/ml was prepared. The pre-treated sample extracts and stock solutions were filtered through 0.45-µm syringe filters.

Reverse phase HPLC method: The concentration of α-tocopherol (Vit E) in the extracts was determined by Agilent Technologies 1200 series Quaternary system, equipped with auto sampler, quaternary pump, degasser, column oven, and a DAD detector. The spectral data was collected at UV detection 220 nm. The solvent system of acetonitrile and water (95:5)was used a gradient mobile phase on Agilent ZORBAX 300 SB column (4.6 × 150 mm × 5µm) at a flow rate of 1.0mL/min, 10 µL injection volume and detection was optimized at 220 nm with 15 min separation time.

Vitamin C: 2, 6-dichlorophenol-indophenol sodium salt (DCPI): 0.025% ethanolic solution of DCPI was prepared for the detection of Vitamin C. To the 0.5mL of sample extracts, 2 drops of DCPI indicator was added. The blue coloration changed to red confirmed the presence of vitamin C. The test was carried out for all the extracts.

Flavonoids analysis by HPTLC

Standard preparation: The standards Quercetin hydrate, Kaempferol, Catechin gallate, Rutin hydrate and Hesperidin were procured from Sigma Aldrich USA All the solutions of standards were prepared in ethanol whereas hesperidin in water.

High-Performance Thin-Layer Chromatography method: Chromatography was performed on silica gel 60F254 (10cmX10cm; 25 mm layer thickness; Merk) with aqueous, methanolic, chloroform and acetone bulb extracts of *Crinum woodrowii.* The fraction residues were collected and (10µl) subjected for HPTLC (CAMAG, Switzerland) analysis. The fractions were impregnated on silica gel $60F_{254}$ TLC plate .The plate was air dried and then inserted in CAMAG- twin through lass chamber containing solvent system of composition with ethyl acetate, acetic acid, formic acid and water (100:11:11:27) as a gradient mobile phase for 20 minutes. The well eluted TLC plate was then dried at 105^0C for 15 min and then it was scanned using Scanner 3 (CAMAG, Switzerland) at 254 and 366 nm using Win Cat 4 software.

RESULT AND DISCUSSION:

Optimisation of extraction method: In order to extract the phytochemicals from herbal samples efficiently, variables involved in this procedure were optimised, including extraction solvent (Water, Methanol, Chloroform, Acetone, 100%), extraction method (Soxhlet, reflux, percolation), and extraction time (18-40 hr). The extraction time in water was 40 hr. The biomass was refluxed for 40hours, and then it was dried naturally for 2-3 days. To the dried biomass, 100% methanol was added and the reaction was percolated for phytochemicals. The methanolic fraction was collected in amber coloured bottle under nitrogen atmosphere. The material was dried for 5-6 hrs. The procedure was repeated for chloroform and acetone. The extraction time was optimized for all the samples. All the extracts were preserved under nitrogen atmosphere in amber coloured bottle.

Phytochemical Screening: It is known that plants are rich in a variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides, saponins and volatile oils. It is necessary to identify the phytochemical components of local medicinal plants usually employed by herbalists in the treatment of diseases[10]. The presence or absence of certain phytochemicals could be used to explain some of the biological activity of certain plant extracts. For example, saponins are a special class of glycosides which have soapy characteristics and have been reported to be active antifungal agents. Antimicrobial properties of a number of tannins, flavonoids, alkaloids have been reported.

Phytochemical screening of the bulb extracts of *Crinum woodrowii* revealed the presence of different phytochemicals. Indeed phytochemical investigations of this plant have resulted in occurrences of carbohydrates, alkaloids, glycosides, saponins, flavanoids, phenols, Vitamin E and Vitamin C. Table 1 illustrates the results of phytochemical screening of all the extracts of *Crinum woodrowii*. The qualitative analysis of carbohydrates (Benedict's reagent test) and glycosides (Borntranger's Reagent) were carried out in all extracts i.e. aqueous (S1), methanol (S2), acetone (S3) and chloroform (S4) extracts. The solutions turning red and pink confirmed the presence of carbohydrates and glycosides respectively. The hydrophilic carbohydrates and glycosides were present in water (S1) whereas hydrophobic carbohydrates and glycosides were detected in rest of the organic solvents (S2-S4). The Mayer's test of extract S2 displayed appearance of white turbidity for alkaloids. The alkaloids were absent in S1, S3, S4 extracts. The dark brown coloration test for phenols was observed in S2-S4 extracts. The water soluble phenols were absent in all the extracts. The extracts S1-S4 were shaken with distilled water. The persistence of froth in S1, S2 was observed, indicated the presence of saponins. The hydrophilic flavonoids were detected in extract S1. The water soluble vitamin C was found in S1 and the vitamin

E was qualitatively analyzed by HPLC method in extracts S3 of *Crinum woodrowii.*

Antiscavenging activity: The phytochemical screening of the crude rhizome extracts showed the positive reactions for alkaloids, flavonoids, phenolic compounds, saponins, glycosides, carbohydrates, Vitamin C, Vitamin E and Minerals. The scavenging ability assayed is the ability of extracts to react rapidly with DPPH radicals and reduce most DPPH radical molecules. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule[11]. Plant derived antioxidants such as tannins, lignans, stilbenes, coumarins, quinones, xanthones, phenolic acids, flavones, flavonols, catechins, anthocyanins and proanthocyanins could delay or provide protection for living organisms from damage caused by uncontrolled production of reactive oxygen species (ROS) and the concomitant lipid peroxidation, protein damage, and DNA strand breaking[12], because of their redox properties, which allow them to act as hydrogen donors, reducing agents, free radical scavengers [13-14].

The antioxidant capacity *Crinum woodrowii* bulb extracts was measured by standard method and the results were expressed in Fig.2. The antiscavenging activity of aqueous extract was 84.33% higher than those of Methanolic (46.9%), Chloroform (10.41%) and Acetone (17.82%) extracts. However, the antiscavenging value of aqueous extract is three time higher than any other extracts. These results might suggest higher medicinal suitability of alcoholic extracts in various antioxidant applications.

Mineral analysis

Optimization and calibration for of *Crinum woodrowii* bulb extracts: After optimization, a new calibration method was by using Cu324.754 nm, Mn 257.610, Se 196.090, Fe 259.940 and Zn 213.856 (Table 3) wavelengths for calibration. Calibration STD solutions were measured 3 times one by one with an RSD < 1%. Once all the calibration standards were finished, a necessary back ground correction was applied for each wavelength. The samples were measured with 3 reproductions. The average sum of the 3 measurements is tabulated in the analysis report.

Quantitative multi-elemental analysis by inductively coupled plasma (ICP) spectrometry depends on a complete digestion of solid samples. However, fast and thorough sample digestion is a challenging analytical task which constitutes a bottleneck in modern multi elemental analysis. Additional obstacles may be that sample quantities are limited and elemental concentrations low. In such cases, digestion in small volumes with minimum dilution and contamination is required in order to obtain high accuracy data.

We have developed a micro-scaled microwave digestion procedure and optimized it for accurate elemental profiling of plant materials. A commercially available 40- position rotor with 5 ml Poly tetra flouro ethylene (PTFE) vials, originally designed for microwave-based parallel organic synthesis, was used as a platform for the digestion. The novel micro-scaled method was successfully validated by the use of various certified reference materials (CRM). The micro-scaled digestion procedure was applied on crude powder of dried plant material in small batches. The contents were transferred to 50 mL volumetric flasks and volume was made with distilled water. The solutions were filtered prior to use. Teledyne Leeman, ICP spectrometer was calibrated by using Leeman standard, National Institute of Standards and Technology (NIST), USA. Diluted samples were used for further analysis.

Iron and copper are of great importance for life. As redox-active metal they are involved in photosynthesis, mitochondrial respiration, nitrogen assimilation, hormone biosynthesis.

Manganese is essential for plant metabolism and development and occurs in oxidation states II, III, and IV in approximately 35 enzymes of a plant cell. Zinc is important as a component of enzymes for protein synthesis and energy production and maintains the structural integrity of biomembranes. Most of the zinc enzymes are involved in regulation of DNA-transcription, RNA-processing, and translation. Selenium (Se) is an essential trace element for animals and humans that is obtained from dietary sources including cereals, grains and vegetables. The Se content of plants varies considerably according to its concentration in soil. The Se is a component of enzymes, such as, thioredoxin reductase and glutathione peroxidase. The glutathione peroxidase is one of the antioxidant for body, which catalyzes some reactions and also inhibits the toxicity of some metals, such as, lead, mercury and cadmium [15]. There are at least 30 selenoproteins that have been identified in mammals, and it has been estimated that humans have about 25 selenoproteins[16]. We have determined the 5 elements in coarse powder of bulbs of *Crinum woodrowii* are given in Fig.3 Thereby, the concentration of minerals in bulbs extracts had the different profiles and quantitative differences had been detected.

The most abundant microelement was Fe in *Crinum woodrowii*; whereas copper was found at

the lowest concentration followed by Selenium, Manganese and Zinc. The content of Iron was especially high followed by Mn, Zn and Cu. Selenium uptake is remarkable.

Qualitative analysis of vitamin E by HPLC

A .Optimisation of HPLC method: To meet the requirements for quantitative analysis, the following HPLC parameters were examined, including different columns (Agilent SB-C18 length 250mm and 150mm, width 4.6, particle size 5um), column temperature (25^0C), and UV wavelength(220nm). The best chromatographic resolution was obtained on Agilent SB-C18 length 4.6 X 150mm, 5um column at 25^0C. The UV detector was monitored at 200-380 nm for finger printing analysis because the peaks were observed under this wavelength. The high intense peak was observed at 220nm

B. Method validation and calibration: A calibration curve is simply a graph where concentration is plotted along the x-axis and area is plotted along the y-axis (Response, absorbance, intensity, peak height, etc.). The line represents the calibration curve. Figure 3 showed a calibration curve of vitamin E. We have constructed a calibration curve for vitamin E. It was created by running 4 different calibration standards (10, 25, 50 and 100 µg/mL). Each concentration gave a peak area (287.717, 761.253, 1594, 3023.3) respectively Fig.5, 6. Peak area was then plotted against the concentrations. The linear trend line has been drawn and linear regression equation has been calculated as y = mx + C. whereas, y = Area under the peak or Response, m = Slope of the linear line (Constant), x = Concentration in µmL and C = intercept (Constant).

Vitamins are a diverse group of organic compounds essential in trace amounts for the normal growth and maintenance of life. To ensure the adequate intake of vitamins, the human diet can be completed with a high range of multivitamin tablets and food products enriched with vitamins, in other words, these compounds are usually administered as nutraceutical or functional ingredient. They are classified as either water-soluble or fat soluble. Vitamin E is fat-soluble whereas Vitamin C is water-soluble. Vitamin E is a generic term for tocopherols and tocotrienols, and it is fat-soluble antioxidant that block the production of reactive oxygen species formed when lipids undergoes oxidation. We employed reverse phase HPLC-analytical tool for qualitative estimation of vitamin E, in which HPLC has been coupled with UV detector. HPLC is most widely used technique to analyze tocols and both normal-phase (NP) and reversed-phase (RP) chromatography are applied [17-19]. Vitamin E functions as a chain-breaking antioxidant, neutralizing free radicals and preventing oxidation of lipids within membranes [20]. The lipophilic vitamin E has been detected in methanolic and chloroform extracts of *Crinum woodrowii.* Organic extracts of *Crinum woodrowii* displayed significant antioxidant activity, proposed that the concentration of vitamin antioxidants. The quantitative estimation of lipophilic vitamin E in tuber extracts of *Crinum woodrowii* is depicted in Table 2. Chloroform extract showed highest concentration (0.9163µg/mL) as compared to methanolic extract (0.3501µg/mL). The occurrence of vitamin E in both extracts suggested that the antioxidant activity Fig.7, 8. The organic extracts displayed significant antioxidant activity proposed that the concentration of vitamin E might be higher along with the other natural antioxidants.

Vitamin C: Vitamin C (L-Ascorbic acid or L ascorbate) is an essential nutrient for humans and other animal species. Deficiency in this vitamin causes the disease known as scurvy in humans. This compound is also widely used as a food additive because of its antioxidant activity. The hydrophilic vitamin C has been detected in aqueous extract.

Antimicrobial activity: All extracts of bulb of *Crinum woodrowii* were screened *in-vitro* for their anti-microbial activities against clinically isolated bacterial and fungal strains, such as *Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Aspergillus terreus.* In our results. methanolic extract showed 6mm zone of inhibition against *Salmonella typhi* , 4mm against *Staphylococcus aureus and 8mm* against *Escherichia coli* (Table-3). There were 4mm zone of inhibition was seen against *Aspergillus terreus.* There were no zone of inhibition in Acetone, Chloroform and aqueous extracts against all the microorganisms.

Conclusion:

Crinum woodrowii have an ancient history of the multiple indigenous uses and indigenous traditional medicines from India. Investigation of the preliminary phytochemical study and their biological activity has provided scientific support for many of its traditional uses. An improved RP-HPLC-UV-method has successfully applied for determination of dl α-tocopherol acetate in organic extracts. Similarly the results obtained from phytochemical analysis illustrates the occurrences of various micronutrients i.e. carbohydrates, vitamin C, vitamin E, flavonoids, phenols, glycosides, saponins, alkaloids and minerals i.e. Zn, Cu, Mn, Se, Fe. The present findings for microelements and minerals suggested that their contents are responsible for significant antioxidant

activity in all extracts. The significant antiscavenging activity in all the extracts showed its importance as natural antioxidants. All the results showed the importance of bulbs in ethno pharmacological drug formulations. Due to indiscriminate use of bulbs of *Crinum woodrowii* by locals it becomes a critically endangered. The conservation of such medicinally important plant should be the immediate need.

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Table 1: Preliminary phyto-chemical screening of bulb extracts of *Crinum woodrowii*

Species	Ext.	Car	Sta	Prot	Glyc	Alk	Sap	Tan	Flav	Phe	Vit. C	Vit. E
Crinum woodrowii	WE	+++	-	-	++	-	++	-	++	+	++	-
	ME	++	+	+	+	++	+	GT +	+++	++	-	+
	CE	++	-	-	+	-	-	-	+	-	-	++
	AE	++	-	-	+	-	-	-	+	-	-	-

Ext = Extract, Car = Carbohydrate, Sta = Starch, Prot = Protien, Glyc = Glycoside, Alk = Alkaloids, Sap = Saponins, Tan = Tannin, Flav = Flavonoids, Phe = Phenols,
Vit C = Vitamin C, Vit E = Vitamin E, WE = Water Extract, ME = Methanol Extract, CE = Chloroform Extract, AE = Acetone Extract, GT = Gallotannins, PT = Pseudotannins.
+ = Significant, ++ = Moderate, +++ = Very good

Table 2 : Quantitative analysis of vitamin E in bulb extracts of *Crinum woodrowii* extracts using y = mx + C

Sr. No.	Sample name	Extract	Retention time in minutes	Response	Concentration in µg/mL
STD	Standard Sample α-tocopherol acetate	Methanol	10.575	287.717	10
STD	Standard Sample α-tocopherol acetate	Methanol	10.64	761.253	25
STD	Standard Sample α-tocopherol acetate	Methanol	10.738	1594.99	50
STD	Standard Sample α-tocopherol acetate	Methanol	10.719	3023.3	100
1	*Crinum woodrowii*	Chloroform	10.397	34.4156	0.9163
		Methanol	10.369	17.1737	0.3501

Table 3: Anti-microbial activity of bulb extracts of *Crinum woodrowii*

Sample	Extract	Diameter of zone of inhibition in mm			
		Salmonella typhi	***Staphylococcus aureus***	***Escherichia coli***	***Aspergillus terreus***
Crinum woodrowii	Acetone	-	-	-	4.0
	Chloroform	-	-	-	-
	Methanol	6.0	4.0	8.0	-
	Water	-	-	-	-

Fig.1: *Crinum woodrowii* Baker, A: Natural habit on hill slope, B: Single individuals with bulb C: Young plant and D: Bulbs

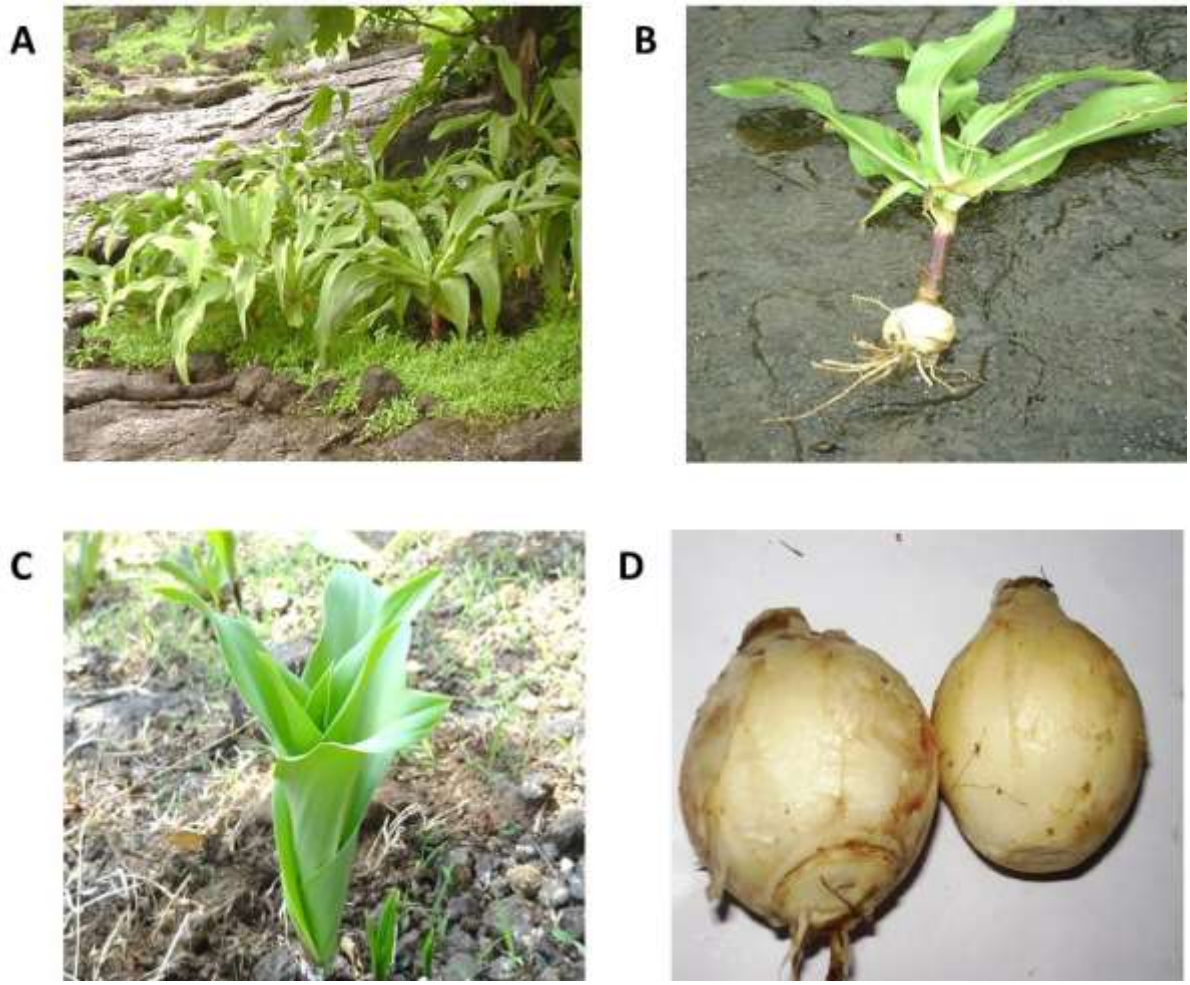


Fig. 2 : Anti-scavenging (DPPH) Activity of bulb extracts of *Crinum woodrowii*

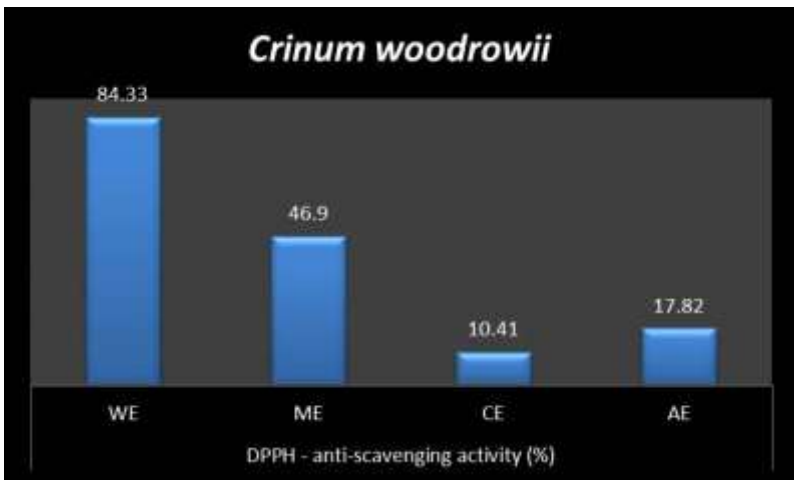


Fig. 3: Accuracy of elemental concentrations in bulbs of *Crinum woodrowii*

Fig.4: Calibration curve of α-tocopherol acetate (Vitamin E)

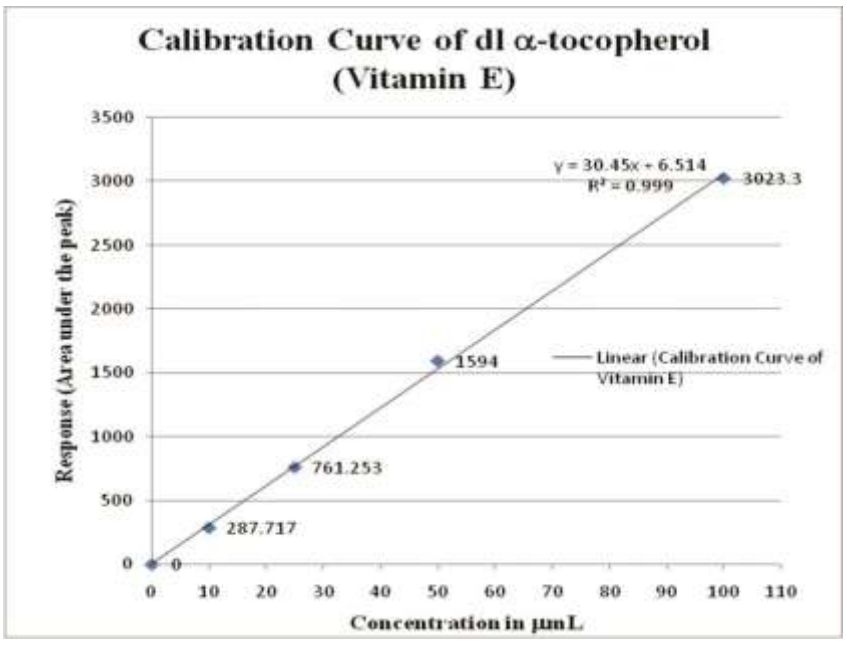


Fig:-5.Vit.E standard peak at 10µL

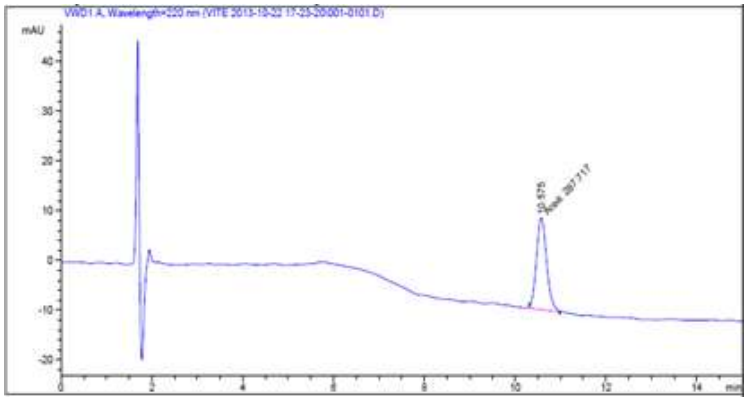


Fig:-6.Vit.E standard peak at 25µL

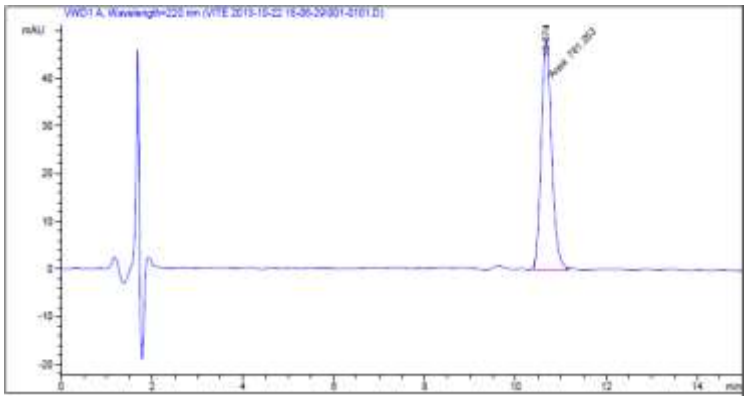


Fig:-7. Chloroform extract contains Vit.E

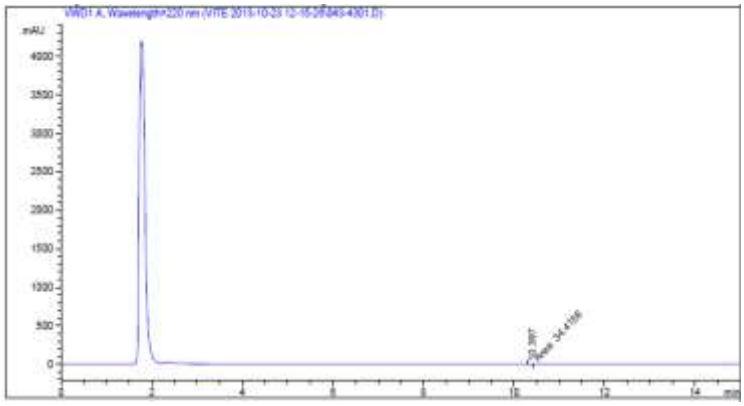
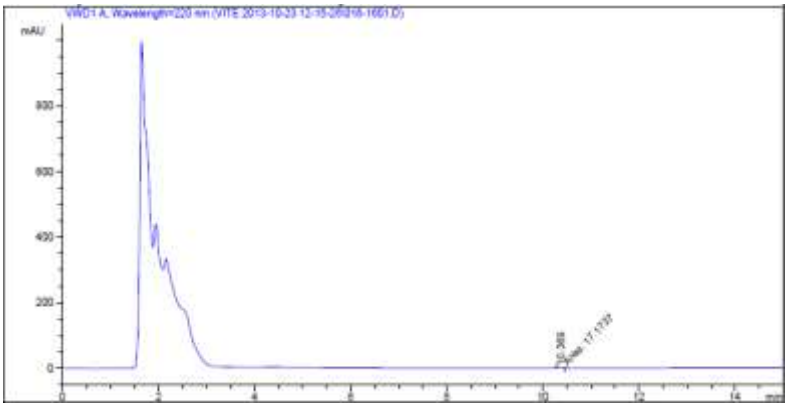


Fig:-8. Methanolic extract contains Vit.E



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