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Evaluation of terrestrial plants extracts for uranium sorption and characterization of potent phytoconstituents

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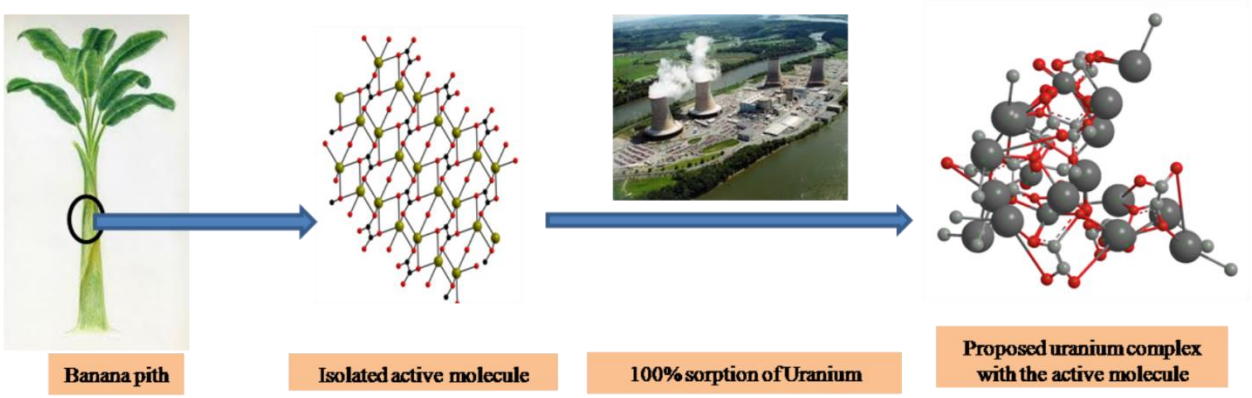
Graphical Abstract



Abstract

Sorption capacity of four plants (*Funaria hygrometrica*, *Musa acuminata*, *Brassica juncea* and *Helianthus annuus*) extracts/fractions for uranium, a radionuclide was investigated by EDXRF and tracer studies. The maximum sorption capacity i.e. 100% (complete sorption) was observed in case of *Musa acuminata* extract and fractions. Carbohydrate, proteins, phenolics and flavonoids contents in the active fraction (having maximum sorption capacity) were also determined. Further purification of the most active fraction provided three pure molecules, mannitol, sorbitol and oxo-linked potassium oxalate. The characterization of isolated molecules was achieved by using FTIR, NMR, GC-MS, MS-MS, and by single crystal-XRD analysis. Of three molecules, oxo-linked potassium oxalate was observed to have 100% sorption activity. Possible binding mechanism of active molecule with the uranyl cation has been purposed.

Keywords: Terrestrial plants, uranium, sorption capacity, potassium oxalate, mannitol, sorbitol

1.1 Introduction

World over, nuclear power stations are principally fuelled by natural and enriched uranium. Nuclear fuel cycle processes for example milling, purification, fabrication of fuel in the front end and storage, reprocessing and waste management in the back end are expected to contaminate the environment. Alongwith the nuclear industrial activities, military operations, and soil fertilizers also contribute for the soil and ground water contamination. Over the last fifty year, the mentioned activities have increased considerably and amplified the uranium contamination. Uranium contamination is a challenge for the scientists due to its severe consequences on living beings as well as to the environment. Complexation of uranium with biological constituents regulates its contamination to the human beings. Radioactive toxicity observed in humans has been studied at many places including the Bhatinda region of Punjab state, India (Busch, 2010).

Chemical techniques, such as leaching of waste with carbonate based reagents, use of anion exchanger and green techniques such as phytoremediation and biosorption for cleaning of nuclear waste are frequently applied for efficient elimination of uranium from polluted areas (Santos and Ladeira 2011). Using plants, either living or its biomass for remediation is an economically promising and environmentally safe approach as compare to the conventional techniques. Various plants show good potential of the removal of radionuclides. For instance, *Pteris vittata* L., a Chinese brake fern have immense power for the accumulation of uranium from mining soil (Chen, Zhu, and Smith 2006). Water imbibed seeds of *Ocimum basilicum* proved to be a good sorption material for Cs-137 and Sr-90 (Chakraborty *et al.* 2007). Aquatic plants such as *Apium nodiflorum, Callitriche stagnalis, Lemna minor, Fontinalis antipyretica*

and *Hydrilla verticillata* efficiently transfer uranium from contaminated sites to their tissues (Srivastava, Bhainsa, and D'Souza 2010). In most of the cases, plants were allowed to grow on polluted sites and the concentrations of radionuclides in plants or residual concentration of polluted site were evaluated. In other cases plant biomass was taken as adsorbent for the contaminants (Ashraf, Mahmood, and Wajid 2011).

In the present study, moss (*Funaria hygrometrica* Hedw.), banana (*Musa acuminata* Colla.*)*, Indian mustard (*Brassica juncea* L.*)* and sunflower (*Helianthus annuus* L.) were selected for investigations. Available literature revealed the use of selected plants in remediation of metals contaminated sites. *Funaria hygrometrica,* besides having antibacterial and antifungal activities, has been reported for its potential of removing ^{137}Cs and ^{90}Sr (Balaramakrishna *et al.* 2004). *Brassica juncea* is a potential crop for the remediation of redionuclides and documented for the phytoextraction of uranium from contaminated soil (Ebbs and Kochian 1998; Huang *et al.* 1998). Similarly, *Helianthus annuus* has been assessed for its multiple heavy metals hyperaccumulation potential (January *et al*. 2008). *Musa sapientum* have also been reported as bio-sorbent for heavy metals (Ashraf, Mahmood, and Wajid 2011).

Therefore, an attempt has been made to isolate and characterize the metabolites from plants those are responsible for radionuclides sorption. Besides, growing whole plant on polluted site, plants were collected from field. Extracts and fractions were prepared and evaluated for the sorption capacity. The article reports uranium remediation potential of mentioned four plants extracts and fractions for the first time and *Musa acuminata* extract and fractions showed maximum uptake potential has further been studied for characterization of active molecules.

1.2 Materials and methods

Collection, extraction, fractionation, purification and characterization of molecules was carried out at CSIR-IHBT, Palampur and uranium uptake studies was conducted at BARC, Mumbai.

1.2.1 Collection of plant materials

Plants were collected from field at different locations in India. *Funaria hygrometrica* (moss), *Musa acuminata* (banana*), Brassica juncea* (Indian mustard*)* from Himachal Pradesh (latitude/longitude-32.1200° N, 76.5300° E) and *Helianthus annuus* (sunflower*)* from Punjab (latitude/longitude-31.5300° N / 75.9200° E). Aerial parts of moss and pith (inner part of banana pseudostem) from banana were taken for studies. Different mustard plant parts (roots, stems, pods and leaves) and sunflower (roots, stems, leaves and flowers) were taken for preparation of extracts and fractions. All the plant material were washed, sun dried, oven dried and ground into powder.

1.2.2 Extraction and Fractionation

Each plant material was cold extracted separately with different solvents i.e. *n*-hexane, ethyl acetate, methanol, methanol: water (1:1) and water in a percolator. General scheme for the preparation of different plants extracts is given in Fig. 1. The process was repeated thrice with each solvent. Extracts were dried at low temperature (below 45^{o}C) and under vacuum on rotary

evaporator and lyophilized. All the lyophilized extracts were evaluated for uranium sorption capacity.

The active extracts having maximum sorption capacity, were further dissolved in water and fractionated with *n*-hexane, chloroform, ethyl acetate and finally with *n*-butanol. Fractionation was performed thrice with each solvent and fractions thus obtained were evaporated under vacuum at low temperature (below 45^{o}C).

1.2.3 Uranium sorption studies

All extracts and fractionated samples were tested for natural uranium sorption by EDXRF and for tracer ^{233}U by liquid scintillation counting in Radiochemistry Division, BARC, Mumbai. Conversion of ^{233}U from higher acidic medium to 4.5*p*H buffer was carried out by drying tracer (^{233}U) under IR lamp. 10mg of plant extract was dissolved in 10mL of 4.5*p*H buffer ($CH_3COONa + CH_3COOH$) solution to make final concentration 1mg/mL and 1mL was taken in an equilibration tube. Some suspended particles appear in organic fractions while dissolved in aqueous buffer and were removed by centrifugation. 100µl of uranium tracer and 1mL of organic layer (0.1M thenoyltrifluoroacetone (TTA) in xylene) were added to it. The tubes were kept in constant temperature (40^{o}C) shaking bath for two hours and centrifuged to separate organic and aqueous layer. Aqueous layer (100µl) was taken in a vial of known activity background, having 5mL of dioxane based liquid scintillator (cocktail W) in it. Thereafter, α count rate (no. of light flashes counted in a minute) of the sample was taken in a liquid scintillation counter. Each plant sample was measured in duplicate. For EDXRF analysis, plant extracts were dissolved in 4.5 *p*H buffer solution (1mg/mL). Uranium dissolved in 4.5 *p*H buffer was added to it and the mixture

was shaken. Uncomplexed uranium was extracted into 0.1 M TTA/xylene by equilibration for two hours followed by centrifugation of the equilibration tubes.

A Jordan Valley Ex-3600 M EDXRF spectrometer equipped with rhodium source and Si(Li) detector (1-40 keV) was used, calibration for uranium determination (with its L_α x-rays at 13.6 keV) was done using solution standards in aqueous and organic media. The samples were kept inside plastic cups covered with 0.6 μ thick mylar film at the bottom and plastic lid at the top. Percentage of the uranium uptake by plant extract was calculated as per the equation mentioned hereunder:-

$$^{233}U \text{ sorption by extract (\%)} = (\alpha \text{ counts of plant extract/ initial } \alpha \text{ counts}) \times 100$$

1.2.4 Quantitative analysis of active fraction for carbohydrates, phenolics, proteins and flavonoids by UV spectrophotometer

Fraction having maximum sorption capacity was analyzed to quantify the phytochemicals present by using UV spectrophotometer (Shimadzu UV-2450, UV-Vis spectrophotometer). Total carbohydrates were quantified by common anthrone reagent method with glucose as standard and absorbance was observed at 630 nm. Phenolic content was determined by Folin-Ciocalteu method using gallic acid as standard and absorbance was measured at 725 nm. Protein content was quantified by bradford protein assay and BSA was used as standard. The absorbance was recorded at 595 nm. Total flavonoids content of active fraction was determined by earlier reported method (Meda *et al.* 2005). Standard used was quercetin and absorbance was observed at 415 nm. For every assay each sample was performed in duplicate.

1.2.5 IR spectral analysis of active fraction

FTIR spectra of the active fraction were recorded on KBr pellets using Perkin-Elmer 1760 FTIR spectrometer. Absorption spectra were in the range of 4000-400 cm^{-1} with an accumulation of 60 scans.

1.2.6 Column chromatography of active fraction

The active fraction was subjected to purify the secondary metabolites using flash column chromatography on adsorbent silica gel H (mesh size 350). Elution was carried out with 25%, 50%, 75% and 100% distilled water in methanol and four major fractions (F_1-25%, F_2-50%, F_3-75%, and F_4-100% water in methanol) were obtained. All the subfractions were monitored for activity on EDXRF. Fraction (F_4) was recorded to have maximum concentracted activity and was further taken for purification of pure molecules. Repeated column chromatography on cellulose powder as adsorbent and elution by methanol with increasing proportion of water led to the isolation of pure compounds **1.** Characerisation of this compound was carried out by FTIR, mass spectrometry, NMR (Bruker Avance^{III} 600) and single crystal XRD analysis (CCDC No.: 836369). Another fraction (F_1) was repeatedly chromatographed over silica gel (60-120 mesh) led to the separation of two pure compounds **2** and **3** which were further identified by mass spectrometry, NMR and by GC-MS after acetylation by acetic anhydride in pyridine.

1.3 Results and discussion

When ^{233}U tracer was added to aqueous phase, constituents present in plant extract bind with the tracer. Free uranium was extracted with thenoyltrifluoroacetone (TTA has the property of extracting the unbound U(VI) from aqueous solution at *p*H 4.5) so that the amount of uranium

complexed with plant metabolites can be measured. Alpha counts rate of sample having only tracer was taken as initial counts.

The plant sample with maximum ^{233}U in aqueous layer was suppose to have high uptake capacity for radionuclide and high alpha count rate close to the initial counts. In such cases, constituents of plant were able to chelate the uranium. In other cases where constituents do not interact with the tracer, the activity goes in organic layer. Analysis of both organic and aqueous layers for ^{233}U was carried out for determination of percent absorption by plant extract. In each cases alpha counts of aqueous and organic layer should be equal to the initial counts. From fifty extracts samples analyzed, only four exhibited the appreciable uptake (>75%) of radionuclide. These four samples were banana pith water extract, banana pith MeOH: H_2O (1:1) extract, mustard leaves water extract, mustard stem water extract. The result for crude extracts exhibiting high radionuclide binding capacity is shown in Fig. 2.

The results indicated that the polar constituents of extracts exhibited high absorption capacity for radionuclides. For separation of active constituents, fractionation of active extracts was carried out with five different solvents to yield twenty fractions. Results of twenty samples obtained by EDXRF analysis and tracer studies are given in Fig. 3. Results further confirm the uptake of uranium by water fraction. From the four active extracts, only one fraction (water fraction of banana pith) exhibited maximum sorption (92%). In rest of the extracts active constituents may have got percolated to other fractions and the activity got distributed. Therefore, the fraction having highest activity was further taken for detailed chemical investigations and characterization of active constituents.

From UV spectrophotometer, carbohydrate and protein contents of active fraction were found to be 47-51% and 18-22% respectively. Protein have potential donor sites as amino acids side chains and peptide backbone which can behave as binding sites for the radionuclides (Gooding, Hibbert, and Yang 2001). Uranium exists in different oxidation states (III, IV, V and VI). However, U(VI) dominates in aqueous solutions under aerobic conditions. Sulphur containing amino acids peptides like metallothionein also have great affinity for the uranyl cation (Michon *et al.* 2010). Hydroxyl groups in carbohydrates become negatively charged at low *p*H and are responsible for the chelation of metal (Davism, Volesky, and Mucci 2003). Various polyol compounds like mannitol are also documented for the removal of metal from water (Geffen *et al.* 2006). Phenolics and flavonoids contents were found to be negligible in the active fraction.

IR spectra showed prominent band at 3391 cm^{-1}, 1684 cm^{-1} and 1320 cm^{-1} representing O-H C=O , and C-O stretching of hydroxyls, ketones and acids respectively. Besides these peaks, C-H stretching of alkanes at 2928 cm^{-1}, C-O stretching of primary hydroxyl group at 1081 cm^{-1} and methyl symmetrical C-H bending at 1384 cm^{-1} were also recorded. Prominent peaks revealed that these functional groups are involved in trapping or binding of the metal.

Using various adsorbents in column chromatography, three compounds (**1-3** (Fig. 4.)) were isolated from the active fraction. Compound **1** was isolated as white powder having a single peak at δ173.0 in ^{13}C NMR , prominent bands at ν_{max} 1595, 1311 in FTIR for C=O stretching, C-O stretching respectively, a sodiated peak at m/z: 205 $[M+Na]^+$ was observed in mass spectrometery. The compound was characterized as oxo-linked potassium oxalates having empirical formula $C_2K_2O_5$. Crystal data and structure refinement obtained from single crystal

XRD are given in Table 1. Bond lengths and bond angles of the molecule were consistent with the literature values of potassium oxalate (Dinnebier *et al.* 2003). Simplest carboxylic acid that abundantly found in plants is oxalic acid and a major precursor of oxalate is glyoxylate. In several oxalate-accumulating plants, L-ascorbic acid serves as a precursor for the biosynthesis of oxalic acid (Nuss and Loewus 1978). Oxalate content in plants depends on nitrogen source, inorganic ion availability and on the environmental conditions. It involves in the several metabolic processes and also plays a defending role to protect plant against the insects. Oxalate may cause poisoning or even death in animals after consumption of foliage containing high concentration (Libert and Franceschi 1987; Doege, 2003). In many plants oxalic acid is found in salt form commonly with the alkali metals (Dinnebier *et al.* 2003). Earlier reports have revealed the presence of monopotassium oxalate in the banana pith (Benitez *et al.* 1991). Compound **1** was active for uranium sorption and exhibited 100% uranium concentration in aqueous layer. Studies revealed that the high capacity of banana pith for uranium sorption was due to compound **1**. Easy ionization of alkali oxalates promotes their function as chelating agent for various metals (Tsushima, Brendler, and Fahmy 2010).

Compound **2** and **3** were characterized as mannitol and sorbitol and their spectral data was consistent with the literature values (Lee *et al.* 2010). Compound **2** and **3** were further identified by GC-MS after synthesizing their acetate derivatives. These two molecules showed less interaction (10-14%) with the uranyl cation.

Proposed binding of uranyl cation with the active molecule

Uranium is present as uranyl cation [$(UO_2)^{+2}$] in aqueous medium at 4.5 *p*H. The cation has highest binding capacity with oxygen donor ligands and molecule showing highest sorption has potential oxygen binding sites for the uranium. These studies suggest oxygen as possible chelating sites responsible for binding of uranium with the molecule.

There could be two probable mechanisms for binding of active molecule with the uranyl cation. First, uranyl cation may get attached with the complete moiety without exchange of potassium cation as mentioned in the reaction hereunder and in Fig. 6 A. The oxygen atoms of oxalate may form ionic interactions with the uranium cation and prevent the binding of uranyl cation with TTA. Similar types of complexes have also been reported in the literature (Morss *et al.* 1986).

$$[K_2C_2O_5]_n + [UO_2]_n^{+2} \longrightarrow [K_4(UO_2)(C_2O_4)_2O_2]_n$$

In second case, active compound may get ionized in the mildly acidic conditions and the oxalate ions are formed. The complex formation between dioxouranium(VI) or uranyl cation and oxalate ion has been investigated by various researchers. Oxalate forms stable complexes with the uranyl cation (Fig. 6 B, C) and stabilized by large entropies of the complexation (Di Bernardo *et al.* 2009).

$$[K_2C_2O_5]_n \xrightarrow{H^+} n[2K^+] + [(C_2O_4)^{2-}]_n$$

$$[(C_2O_4)^{2-}]_n + [UO_2]^{+2} \longrightarrow UO_2(C_2O_4)_2 \quad \text{or} \quad UO_2(C_2O_4)_3$$

The possible complexes of uranyl cation with the oxo- linked potassium oxalate is given in Fig. 6.

In the present study the dried powdered plant material was extracted and fractionated with organic solvents and was further subjected to different chromatographic technique using various adsorbents. There may be chances for the formation of certain artifacts. However, in nature live plants come in contact with radionuclides. These need further investigations with the living system.

1.4 Conclusions

Activity guided isolation and characterization of active molecule led to the identification of an oxalate as responsible moiety for binding of the metal cation. In the proposed binding mechanism, active molecule may bind with uranium resulting in the potassium uranyl oxalate complexes and the uranyl oxalate complexes. The present study has illustrated the uranium remediation potential of constituents of *Musa acuminata* for the first time.

Acknowledgement

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Table 1. Crystal data and structure refinement.

Empirical formula	$C_2K_2O_5$
Formula weight	182.22
Temperature	150(2) K
Wavelength	0.71073 A
Crystal system, space group	Monoclinic, C 2/c
Unit cell dimensions	a = 9.0886(4) A alpha = 90 deg.
	b = 6.2095(2) A beta = 110.683(5) deg.
	c = 10.6035(5) A gamma = 90 deg.
Volume	559.85(4) A^3
Z, Calculated density	4, 2.162 Mg/m^3
Absorption coefficient	1.633 mm^{-1}
F(000)	360
Crystal size	0.23 x 0.18 x 0.13 mm
Theta range for data collection	4.06 to 24.99 deg.
Reflections collected / unique	1357 / 498 [R(int) = 0.0121]
Completeness to theta = 24.99	99.6 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.8158 and 0.7052
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	498 / 0 / 43
Goodness-of-fit on F^2	1.187
Final R indices [I>2sigma(I)]	R1 = 0.0200, wR2 = 0.0610
R indices (all data)	R1 = 0.0205, wR2 = 0.0612
Extinction coefficient	0.048(4)

Largest diff. peak and hole	0.724 and -0.208 e.A^{-3}

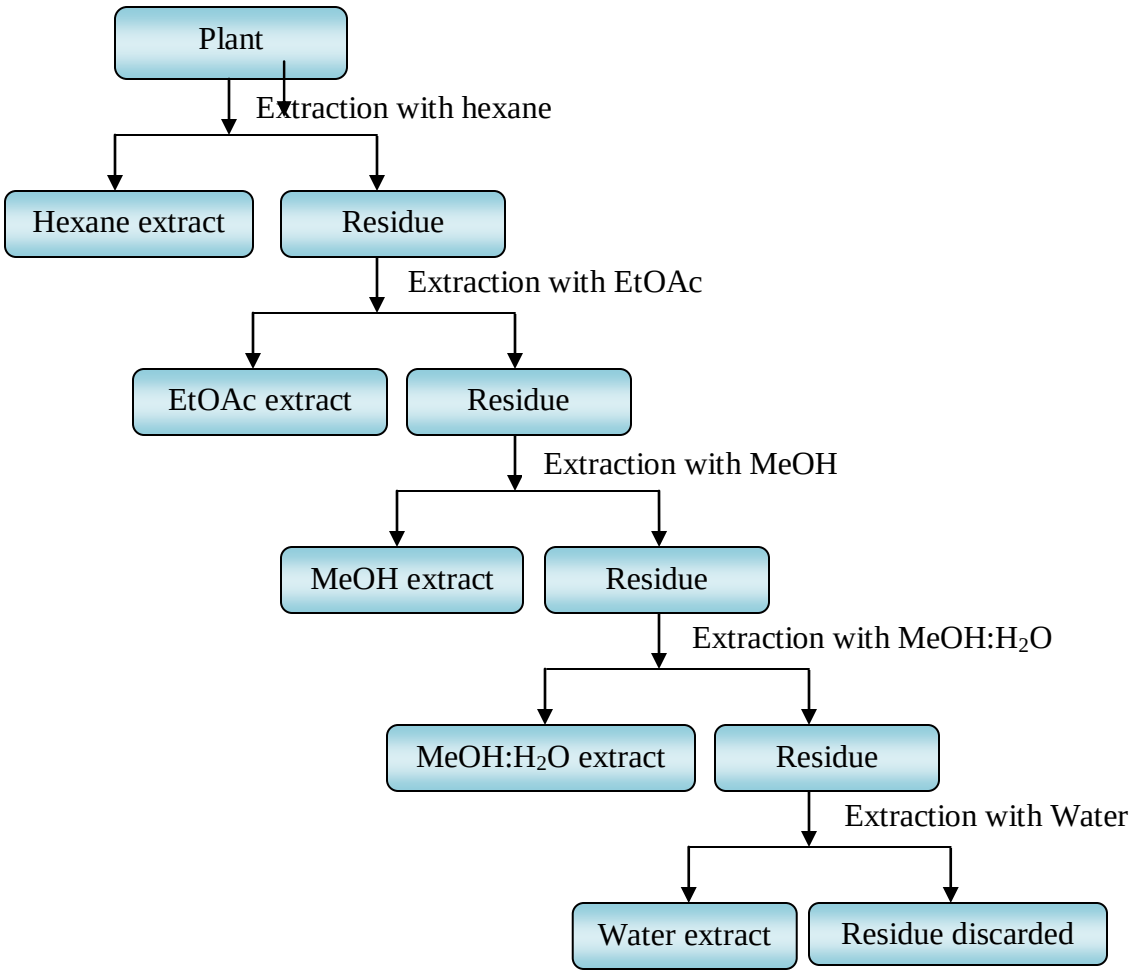


Fig. 1. General scheme followed for the preparation of different plants extracts

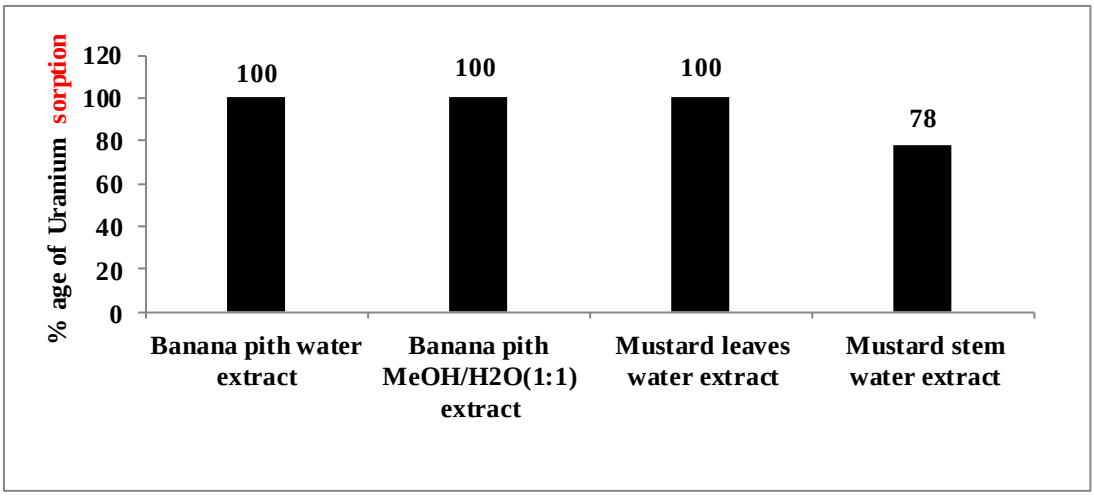


Fig. 2. Percent sorption of uranium by crude extracts

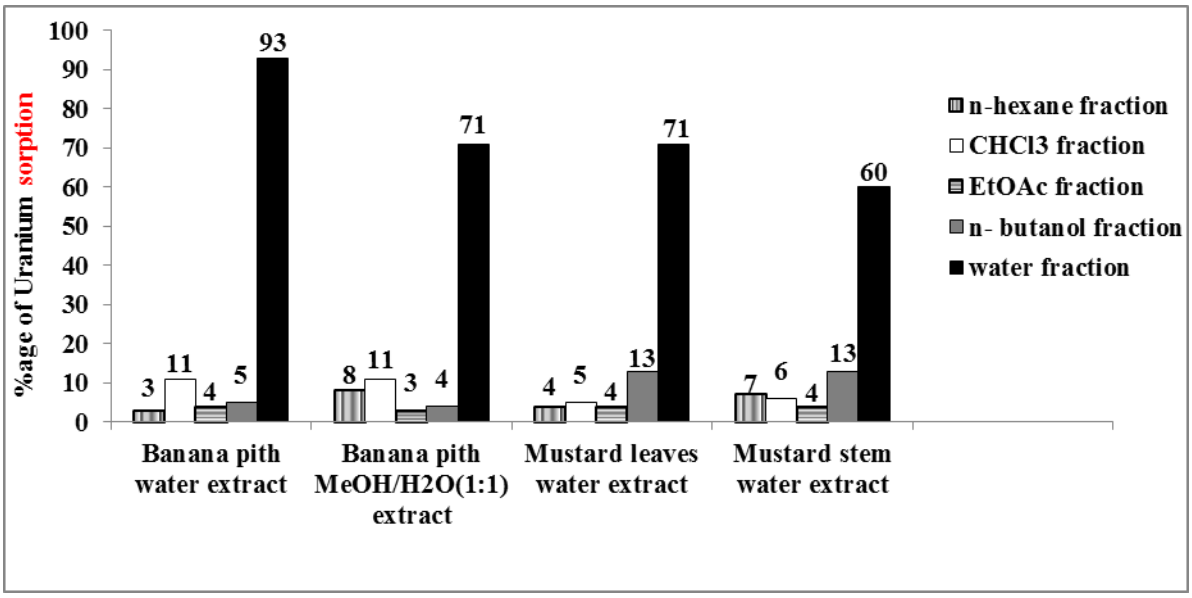
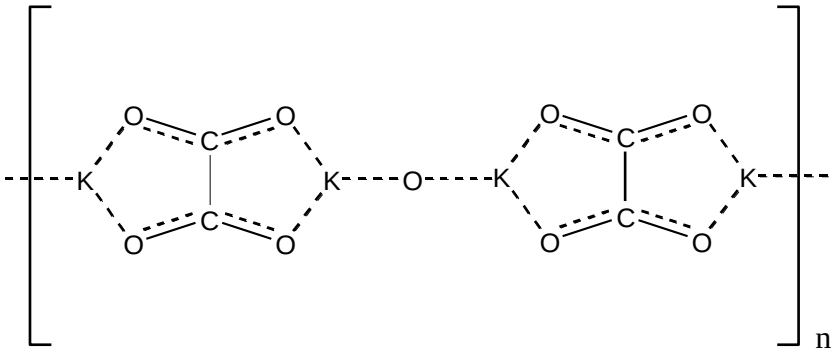


Fig. 3. Percent sorption of uranium by different fractions



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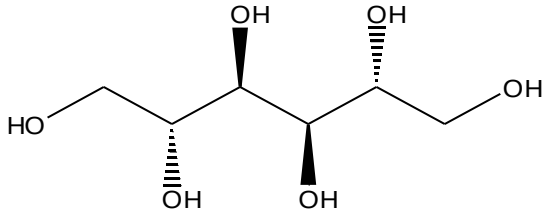

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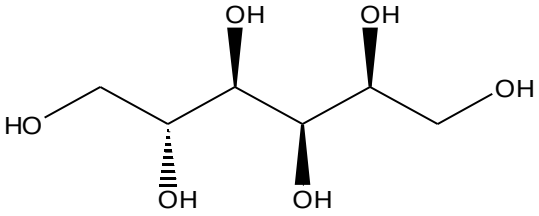


3

Fig. 4. Metabolites isolated from active fraction of *Musa acuminata*

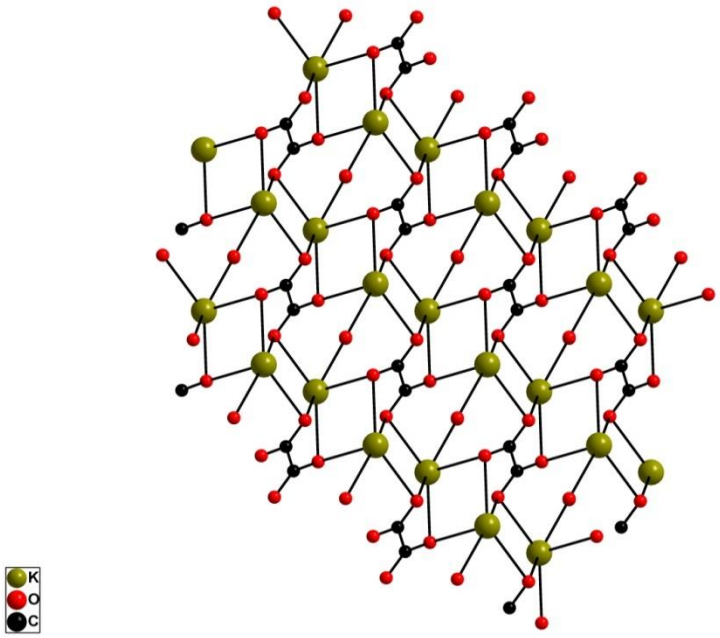
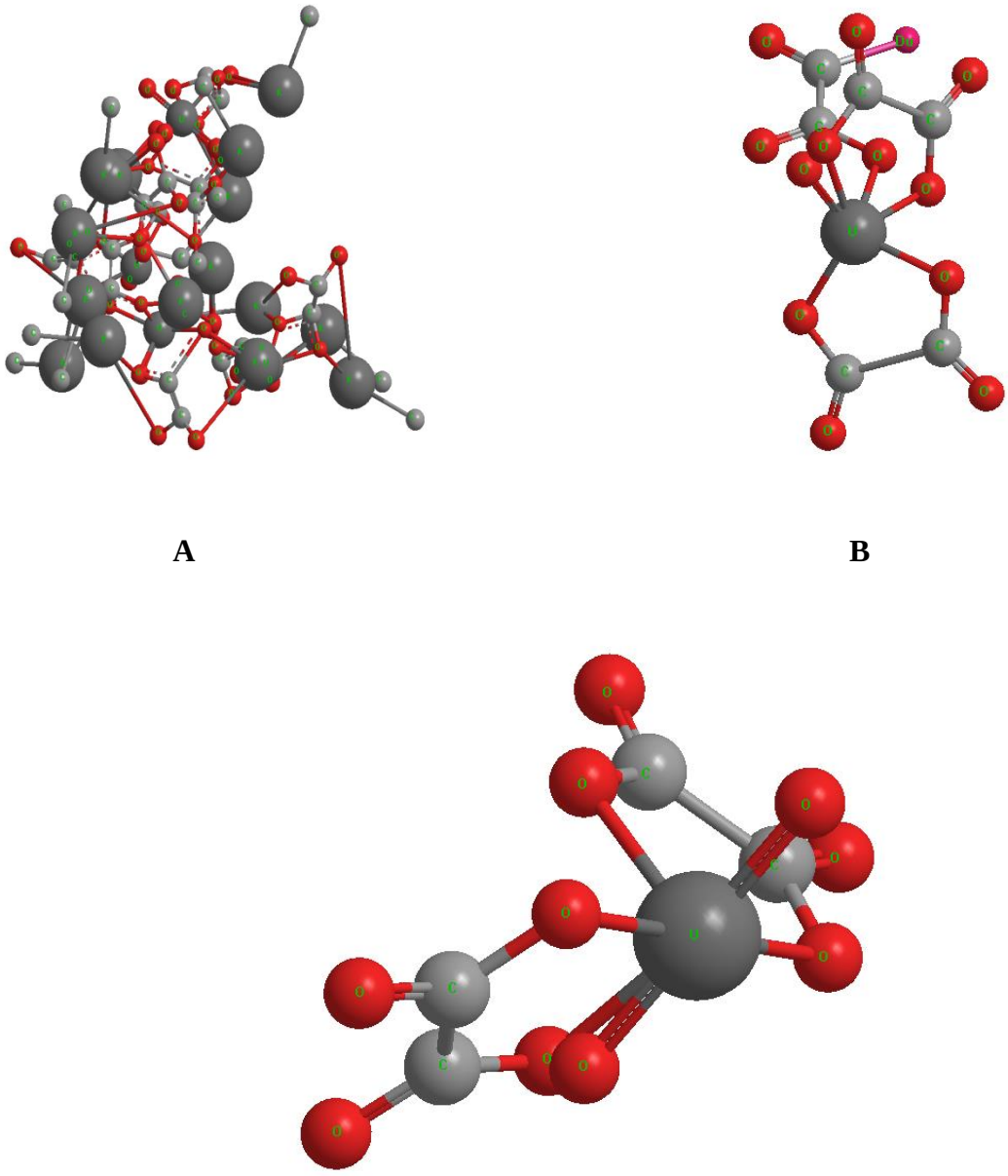


Fig. 5. Crystal structure of compound oxo-linked potassium oxalate (**1)**



A **B**

C

Fig. 6. Probable complexes of potassium oxalate with the uranyl cation, (A- without ionization and B, C- with ionization)