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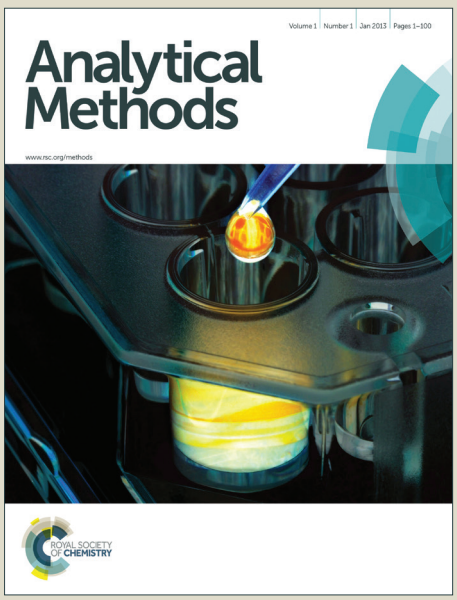

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Purification of Flavonoid Metal Complexes from *Alhagi camelorum* with Calix[4]arene Based Impregnated Resin

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Calixarenes are well known of their molecular recognition properties. Thus, the present study was carried out to explore their usage in the isolation of flavonoids (i.e. Quercetin (**I**) and Catechin (**II**) from plant extracts, e.g. *Alhagi camelorum* through metal complexation. The synthesis of flavonoid metal complexes from plant extracts through direct complexation and their purification by using calix[4]arene based resin (**R-1**) was carried out simultaneously to ensure the separation efficiency of **R-1**. It was observed that direct complexation of flavonoids present in plant extracts and purification by using column loaded with **R-1** could be the best choice for industries working and searching for isolation and purification of flavonoids or their complexes from plants.

Introduction

Flavonoids are secondary metabolites, which have shown a variety of actions against different ailments actively [1]. However, attempt to derivatize flavonoids to increase activity or reduce un-necessary action is done time to time [2]. Complexation of flavonoids is one of those attempts, which have been made by different groups [6-7] that considerably increased their antioxidant activity.

Like other natural products, flavonoids (phenolic antioxidants) are mostly found in plant kingdom that is a leading option for obtaining bio-molecules. Altogether with other bioactive constituents, Quercetin (**I**) and Catechin (**II**) are widely found in different medicinal plants such as *A. camelorum* which has been focused in current study [8-10]. Nevertheless, it is believed that plants are capable of curing diseases but simultaneously they have some substances, which might be harmful in certain cases. Hence, there has been felt a great need for separation and isolation of their biomolecules for making drug formulations [11]. On the contrary, to isolate a biomolecules from plants in pure form, one has to go through a very long, tedious, time and money consuming process.

Thus, in order to overcome such problems, various techniques have been reported to identify and determine the flavonoids [12]. Therefore, efforts were made to purify flavonoids from plants by complexation. The chelating activity of the flavonoids was used to separate them from plants by making direct complexation with metals [12]. It could be worthwhile following the direct complexation with decomplexation providing pure flavonoids from cheapest source i.e. plants. However, in the study, carried out by J. Zhang [12], the final purification step remained un-attempted. In said work, instead of doing preparative chromatographic approach to purify metal complexes, direct complexation of flavavonoids with metal was carried out and confirmed on HPLC only. Due to different pharmological abilities and other factors like that, make it necessary to have such compounds in pure and isolated form rather than mixed in extract which has been done as stated above. Keeping in the view that isolation of plants constituents in pure form is not an easy job, in our previous study [13] as well as in present study; the focus is to minimize laborious work with simple approach to purify the compound of interest in pure stat. Since many other compounds may also be present in plant extracts which do not form complexes in the proposed method and easy to separate flavonoids from those un-wanted constituents. However, these constituents co elute with flavonoids in traditional speration methods and make it difficult to isolate flavonoids.

Among a large variety of biomedicinal plants in Sindh region of Pakistan, *A. camelorum* (previously called *A. maurorum*) has largely been used as folk medicine as purgative, laxative, diaphoretic, expectorant and diuretic. Besides this, its flowers are used to treat piles, migraine and wart; as well as oil

from the leaves is used in the treatment of rheumatism [14, 15]. Water extracts of roots have also been used to enlarge the ureter and to remove kidney stones [16]. Isolation of bioactive compounds, such as flavonoids has been carried out form genus Alhagi by various groups of scientists [17-18]. Thus it has been conceived that if flavonoid rich fraction in methanol solvent is treated with metal (e.g. copper sulphate) it will result the formation of flavonoid metal complex thereby leading to easier separation from other plant constituents or unreacted phenolics. In addition, a preparative chromatography may lead to obtain pure flavonoid metal complexes with increased bioactivity such as antioxidant activity. Not only this but easy to decomplex these flavonoid metal complexes makes this approach more appealing as it can provide pure flavonoids from plant via an easy method. Accordingly, herein we report the isolation of **I** and **II** as their copper complexes from *A. camelorum* through complexation with copper and purification using **R-1** loaded column.

Results and discussion

*Synthesis and Characterization of metal complexes of **I** and **II***

The synthesis of metal complex is mostly preferred in slightly acidic or neutral pH, rarely in basic media, so reaction mixture pH was checked at initial and final step and found almost neutral or slightly acidic but not less than 6.5. The observation proved the fact that flavonoids are weak polybasic acids that tend to protonate.

Synthesis of metal complex and their characterization has already been carried out and described in details. Briefly some evidencing things are discussed. Taking a fix molar ratios (i.e. 2:1 and 1:1 for **I** and **II** respectively) of flavonoids and copper sulphate and stirring is quite enough to make complex. Colour change is first clue to assure the complexation. The yield of complex of **I** was 75 % that is in agreement with our previous report [6], whereas complex **II** was formed with 81 % yield. However, after evaporation of solvent and washing with butanol, UV-vis was first technique to apply on and confirm for, the desired product. Actually two maximum as major absorptions are observed in UV-vis spectrum of flavonoids. The absorption maxima observed in the range 240-285 nm may be considered as originating from $\pi \rightarrow \pi^*$ transition in the A ring, a benzene system. The other between 300-400 nm is attributed to transitions in the B ring, a cinnamoyl system.

Metal-flavonoid chelates are usually coloured. In the presence of metal ions, a bathochromic shift is typically observed in the absorption spectra of flavonoids. In **I** it was observed as 256 nm to 370 nm and 350 nm to 460 nm whereas, for **II**, it was 280 to 380 nm.

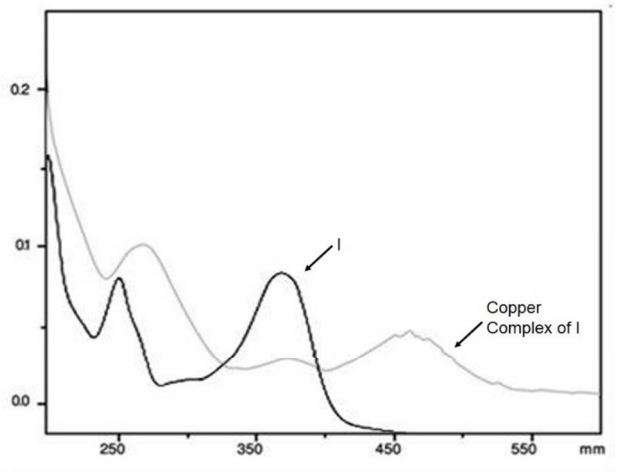

Figure-1. UV-vis spectra of **I** and its complex.

In our previous study as well as in the other reports [6, 19-21], the confirmation of metal complexation of Cu and **I** is through observation of some diagnostic wavelength (cm^{-1}) shifts in FT-IR. Some of the prominent are described here. The shifting of diagnostic peak of C=O for **I** appears at 1661 (cm^{-1}). It's shifting from 1661 cm^{-1} to lower wavelength (i.e. 1600 cm^{-1}) confirms the metal complexation that was observed in during present study also and shown in figures where two curves are given corresponding to standard **I** and **II** and their metal complexes. In Figure-3 the coloured curves (green) are of **I** & in Figure-4 the colour curves (blue) are for metal complex of **II**.

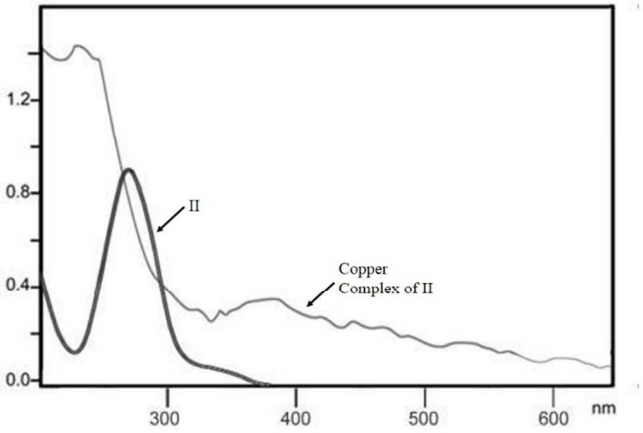

Figure-2. UV-vis spectra of **II** and its Complex.

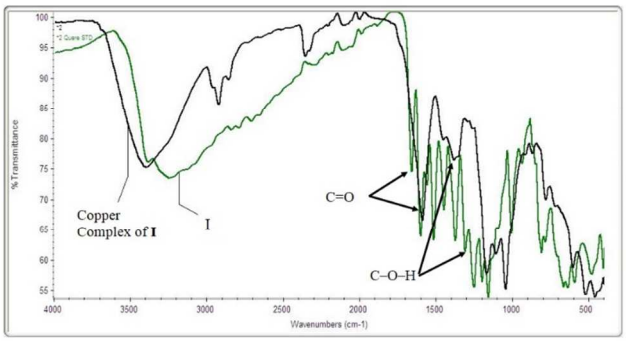

Figure-3. FT-IR spectra of **I** and its copper complex.

The C-O-H deformation was further evidence indicating an increase in bond order, which is normally observed when metal coordination involves with the ortho-phenolic (O–H) group on the B-ring of **I**. Similarly for **II** (Figure-4) there was same observation except C=O as it was not present in **I**. The deformation of C–O–H due to complexation is confirmed by shifting of its band from 1310 to 1360 cm^{-1} for **I** and 1390 cm^{-1} to 1480 cm^{-1} for **II**.

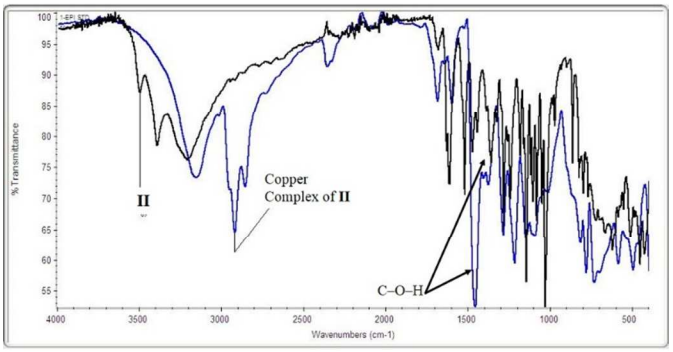

Figure-4. FT-IR spectra of Standard and Complex of **II**.

*Purification of **I** and **II** complexes from roots of A. camelorum by **R-1** loaded column*

Initially metal complexes of **I** and **II** obtained through complexation of respective reference standards were taken to proceed for trial. Three different solvents (i.e. methanol, water and hexane) were opted to optimize and select one of them as initial eluting solvent depending on efficiency. **R-1** was stirred in these three solvents individually to homogenize. After running column it was observed that water had very poor efficiency as it caused co-elution **I** and **II**, whereas, methanol was acceptable as mobile phase to separate **I** and **II** via **R-1**. However, methanol was not suitable for the plant extract as it caused early elution of **1** and **2** as well as other un-wanted plant constituents or unknown complex which might have formed during addition of $CuSO_4.5H_2O$. Nevertheless, initially homogenizing **R-1** in hexane and starting column to run by hexane and gradual increase of ethyl acetate provided optimum change of retention time and elution of **I** and **II** in pure form was achieved in plant extracts too. The only limitation of this mobile phase solvent system was relatively more retention of **I** and **II** on column that increased run time almost double of methanol (five to seven min.) but not much more to compromise on. All fractions were continuously monitored on TLC (viewed on UV-vis portable lamp) and Rf values were counted during optimization of mobile phase solvent system. The yield (considering as of dry plant material 1.0 g) was 0.031 % and 0.075 % for complex of **I** and **II** respectively. After running the column for reference standards of **I** and **II** and plant extracts, carrying same, the column was successfully washed by running 100% methanol and doing it thrice made column reusable for the similar purpose. No retention of any constituent on the coulmn loaded with **R-1** suggests a regenerate able column with maximum efficiency to purify metal complexes directly made in plant extracts.

Experimental

General

FT-IR spectra were recorded on a Thermo Nicollet AVATAR 5700 FT-IR spectrometer in the spectral range 4000-400 cm^{-1} keeping parameter as; 32 number of scans, with resolution 4cm^{-1}. Small amount of sample which could cover the ATR diamond cell were placed. All of the reagents used were purchased from Merck (Darmstadt, Germany) and or Sigma–Aldrich Chemie Gmbh. (Steinheim, Germany) and used as supplied. Thin layer chromatography (TLC) was performed on pre-coated silica gel plates (SiO2, PF254, Merck). Column chromatography was performed on R-1 loaded column that was prepared by the impregnation of Thioalkyl derivative **3** of calix[4]arene (3) (Figure-5). The synthesis of **1, 2** and **3** was carried out as reported earlier [3-5] respectively. The complexes **I** and **II** were synthesized as standards according to our previously reported methods [6-7].

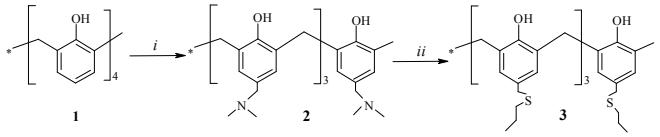

Figure 5. Synthetic scheme of calix[4]arene thioalkyl derivative **3**.

Plant material

The roots of *A. camelorum* were collected in December 2012 at Malkani, a small town of district Badin of Sindh province, Pakistan. This plant has already been identified from taxonomist of Institute of Plant Sciences, University of Sindh, Jamshoro, Pakistan; where a voucher specimen is deposited in herbarium (*Alhagi maurorum* 15460).

*Synthesis of **R-1***

The resin (**R-1**) was synthesized as reported in our previous work [22] by following the impregnation of 0.4 g (5.1×10^{-4} mol) of ligand 3 on 10 g of XAD-4 resin. The ligand content was checked after solvent evaporation through gravimetric analysis. A maximum amount of ligand **3** impregnated onto dry resin was 0.35 g (4.52×10^{-4} mol), that is, 4.52×10^{-5} mol$\times g^{-1}$.

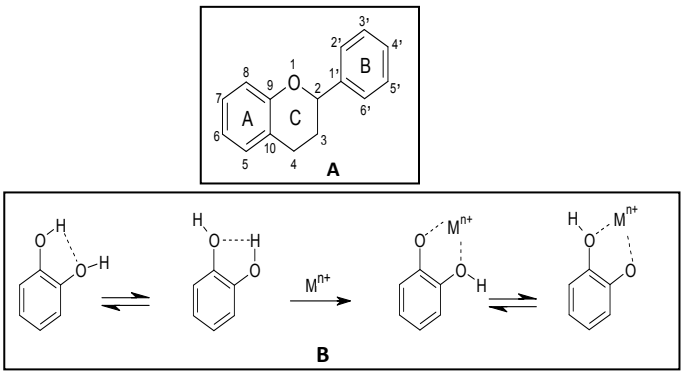

Figure 6. Basic structure of flavonoid (A), Deprotonation of 3' and 4'–OH group by complexation of Flavonoids.

*Synthesis of flavonoid complexes **I** and **II***

Flavonoid metal complexes were synthesized as previously reported methods [3-7]. Briefly 0.17g (0.01mol) of **I** or **II** stirred in MeOH (20 mL) until it was completely dissolved. Within 15 min the color of the solution was light yellow (for **I** whereas, colorless to green for **II**) and quickly solid $CuSO4._5H_2O$ (0.25g, 0.02mol) was added in the reaction mixture now the color of the solution was brownish yellow which was stirred at room temp for 1.5h. The reaction mechanism of flavonoids and complex is illustrated in Figure-6. Flavonoids are able to chelate metal ions via 3' and 4'–OH groups (if present) as their structures have an easily removable H-atom, whereas the other H-atom is intramolecularly bonded.

*Characterization of flavonoid complexes **I** and **II***

It is well known that flavonoids strongly absorb ultraviolet (UV) radiation and make pigments responsible for the color of leaves, herbs and petals. Therefore, Dušan and Vesna [23] suggest that UV–vis spectroscopy remains the main tool for structural analysis of flavonoids. UV–vis Spectra (Figure-1 & 2) were obtained by PerkinElmer Lambda 35 UV–vis spectrophotometer using standard1.00 cm quartz cells. The range was selected from 200 to 600 nm to cover the full range in which light is absorbed by both 1 (i.e. 290 nm) and 2 (i.e. 270 & 370 nm) as well as their complexes of **I** (i.e. 280 nm) and **II** where absorbance was observed as 256 nm to 370 nm and 350 nm to 460 nm for **I**, whereas, it was 280 to 380 nm for **II**.".

Isolation of flavonoids from plant extracts through metal complexes

Extraction of flavonoids from roots (1 g) of *A. camelorum* was carried out by previously reported method [24] with some modifications. 0.12 M solution of methanol:water:hydrochloric acid (70:29:1 v/v/v) was used as extraction solvent. Samples of root parts of *A. camelorum* were transferred to pressure controlled Teflon vessels of the Start Microwave extraction system © 2003 Mile Stone Inc. followed by the addition of 10 mL of extraction solvent. The programming of microwave extraction system was set in two steps. In the first step, gradient rise of temperature was maintained from ambient to 45 °C within five min. and in the 2nd step it was kept intact for 15 min. using an energy level of 400 W, followed by ventilation for 10 min. The obtained solution was filtered and evaporated. The residue was washed with mild cold water then dried and again washed with hexane to remove fatty materials followed by dissolving the flavonoids fraction in 10 mL of methanol. Finally, 0.5 g of powdered $CuSO_4.5H_2O$ was added in to the solution and stirred. Formation of soft solid material was observed soon.

*Purification of metal complexes by **R-1***

Metal complexes of **I** and **II** of their reference standards prepared as described previously, were mixed and loaded into glass column of small size (dia 2 mm and length 5 inch) loaded with **R-1**. **R-1** was already stirred with hexane (starting eluting solvent) for up to 1h to homogenize and then packed into a glass column. Mobile phase was consisted of hexane and ethyl acetate (1:9). Metal complex of **I** was eluted first and followed **II** after successive collection of 10 eluted fractions approximately of 2.5 mL. Whereas in case of plant extract of flavonoids complexes was subjected to same type of column chromatography but running initially hexane until a pale yellow colored fraction eluted which might be of non or less polar compounds of plant. Addition of ethyl acetate gradient to mobile phase was done gradually to assure prevention of co-elution of many unwanted complexes as well as other substances. Both complexes of **I** and **II** were eluted on same composition e.g. hexane and ethyl acetate (1:9). Some fractions are kept for future work and structure elucidation of those is planned.

Decomplexation/Decomposition of Flavonoid Complexes

Above stated work is carried out to obtain individual flavonoid metal complexes which are themselves important but if desired, these can easily be decomplexed to have pure flavonoids by proposed method and used in previous study [12]. Briefly the complex is refluxed in ethanol:water (1:1, v/v) with stirring putting EDTA in equal ratio for 120 min at 50 °C. It results the formation of Metal-EDTA complex which is filtered and dried. Filtrate can be washed with methanol to obtain free flavonoids and insoluble EDTA-Metal complex is removed.

Conclusions

The study focused on complexation of flavonoids present substantially pure fraction of *A. camelorum* flavonoids (i.e. **I** and **II**) followed by their purification through a column (loaded with **R-1**, a calixarene based resin). It has been noticed that metal complexation is not only important due to increase of certain bioactivities but it is also best at isolating flavonoids from plants extracts which is a difficult task in other ways. On the other hand, **R-1** is fine enough to get the complexes of flavonoids, formed in plants, in pure form with minimum efforts. It is therefore, optimistic proposal, to use the technique,

for industry as well as all those who are in search for flavonoids from plants. Easy process of decomplexation is also advatnge to those who do not wish to have metal complexes of flavonoids rather than pure form.

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Notes and references

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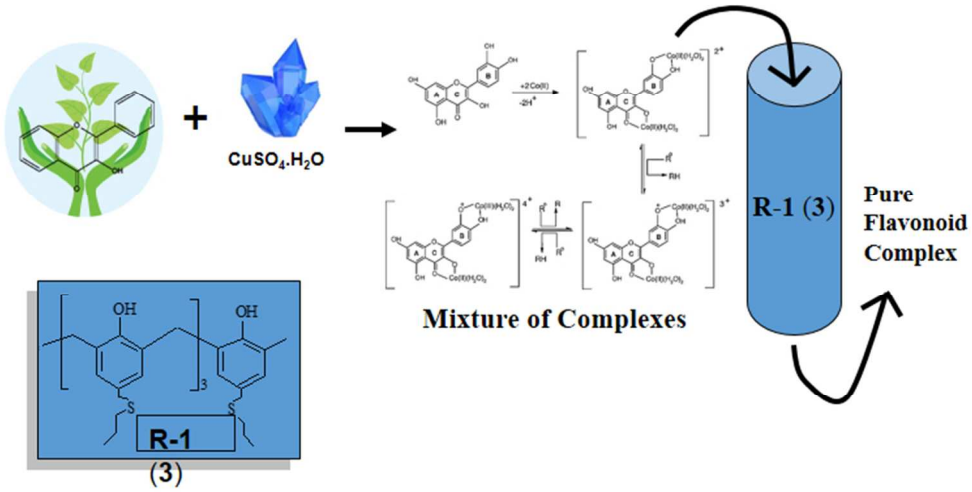

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