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Facile formulation of Cr (III) organo-metallic adduct from natural pomegranate juice: An effective alternative for Cr (III) supplementation

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Abstract
Trivalent chromium is known for attenuating insulin resistance, lowering plasma cholesterol levels in human body and is used as nutritional supplements. In contrast, hexavalent chromium Cr (VI) is a known hemotoxin, genotoxin and carcinogen. Therefore, oxidation of Cr (III) to Cr (VI) is a serious concern for human health. Unfortunately, the natural oxidation of Cr (III) to Cr (VI) is thermodynamically feasible in both aerobic and mildly anoxic environment. Thus, to get the optimum benefit from Cr (III), it has to be prevented from auto-oxidation to harmful Cr (VI). This study dwells on the preparation of organometallic adduct between Cr(III) with natural pomegranate juice which is known to contain diverse phenolic compounds like punicalagin isomers, ellagic acid derivatives and anthocyanins which are known to be potent antioxidants. These antioxidants prevent the oxidation of Cr (III) to Cr (VI) which is evaluated by oxidative challenges posed by various known oxidants. The prepared adduct is also characterised by various physicochemical techniques *viz*. chromatography, spectroscopic studies etc. The present study shows that the adduct can successfully inhibit the oxidation of Cr (III) to Cr (VI) in compare to bare Cr (III) compounds which in turn can be used as an effective alternative for Cr (III) supplementation like chromium picolinate, chromium polynicotinate etc.

Keywords: Chromium, pomegranate juice, antioxidant, supplementation.

Introduction
Chromium, one of the most common elements found on the surface of the Earth, exists in several oxidation states, principally as metallic Cr (0), trivalent Cr (III), and hexavalent Cr (VI) [1]. The interest in trivalent chromium as a nutritional supplement to metabolism of glucose started way back to 1950, when, it was suggested the brewer's yeast contained a glucose tolerance factor, which is biologically active form of trivalent chromium, which showed to lower plasma glucose levels in diabetic mice [2]. Later, several studies suggested that, suboptimal intake of Cr (III) is a major contributing factor for chronic diseases such as type 2 diabetes and cardiovascular diseases [3]. Over the last two decades, supplementation with Cr (III) has increasingly gained popularity. Currently, Cr (III) supplements such as Chromium picolinate and Chromium polynicotinate have been ranked as the second largest selling supplement in the United States [4, 5]. Cr (III), unlike its oxidised counterpart Cr (VI), has minimal toxicity with low mutagenic potential [6, 7].
On the other hand, Cr (VI), is a known potent carcinogen and respiratory irritant causing mutagenesis and lipid peroxidation leading to cell death [8, 9]. Thus, the main challenge in formulation of Cr (III) supplement is to keep it in its non-toxic trivalent state which is the case with strong antioxidant (picolinic acid and polynicotinic acid) bounded Cr (III). Studies showed Cr (III) can also be successfully adducted with natural Polyphenolics of tea and amalaki fruits [10]. Another study also suggested that the beneficial effects of Cr (III) occur independent to the anionic ligand bound to Cr (III) [11]. Hence, it may be assumed that Cr (III) supplements can also be formulated with other Polyphenolics bearing agents which can successfully inhibit the oxidation of Cr (III) to Cr (VI). Fresh pomegranate juice may be ideal for such case.
Pomegranates are reported to contain a wide range of polyphenolic compounds that include flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallic acid and ellagic acid esters of glucose) [12].

Due to its high polyphenolic content and excellent antioxidant activity, pomegranate juice adducted with Cr (III) may be an excellent alternative to other chromium supplement available in the market.

The present study is designed to formulate an adduct between pomegranate fresh juice and Cr (III), check the capacity to prevent the oxidation of Cr (III) to Cr (VI) of the adduct, which is evaluated by oxidative challenges posed by various known oxidants. Also, an attempt has been made to characterise the possible adduct between Polyphenolics of pomegranate juice and Cr (III) by chromatographic and spectroscopic methods.

Materials and Methods

Samples, Chemicals and reagents

Samples of pomegranate was procured from local market of Kolkata, West Bengal, India. All the chemicals and reagents are of AR grade and were obtained from Sigma-Aldrich Chemicals.

Preparation of pomegranate juice and Cr (III) adduct

1.4 gm of Cr $(NO_3)_3.9H_2O$ was taken in 500 ml beaker. Distilled water (50 ml) was added and the solution was stirred for 15 minutes to completely dissolve Cr $(NO_3)_3.9H_2O$ in water.

5 pieces of medium sized pomegranates were washed and peeled. Juice has been extracted from the peeled part by means of a juicer. The juice is filtered and solid content was measured. The solid content was found to be `9% and 220 ml juice was obtained.

100 ml of pomegranate juice was then added to Cr $(NO_3)_3.9H_2O$ solution slowly with constant stirring. Stirring was continued for another 15 minutes after addition of juice solution. Thus, pomegranate juice and trivalent Cr was prepared having 2% Cr (III) (w/w).

Preparation of Standard Curve of Cr (VI)

A standard stock solution of 500 mg/L was prepared by dissolving 0.05 g dried K_2CrO_4 (CRM grade) in 18.2 MΩ-cm deionized water and diluted to 100 ml. A 0.2 N sulfuric acid was prepared by adding 1 mL of concentrated sulfuric acid to deionized water and diluted to 100 ml. A 1, 5-diphenylcarbazide (DPC) solution was prepared by dissolving 250 mg of DPC in 50 ml acetone. Working standard Cr (VI) concentrations of 0.2, 0.4, 0.5, 0.8, and 1 mg/L were prepared from the stock solution. The pH of solutions was adjusted to ~2 with dilute sulfuric acid before complexation. The absorbance-concentrations calibration curves were plotted with a correlation coefficient.

Oxidation Challenges: Following oxidation challenges were carried out to pomegranate-Cr (III) adduct.

1. **Oxidation by air bubbling for 30 minutes**: 50 ml of sample solution was taken and air bubble was passed (medium speed) through the sample solutions by air pump for 30 minutes. Estimation of free Cr (VI) was done in the aerated samples by spectrophotometer
2. **Oxidation by 30% H_2O_2 and $FeSO_4$:** 5 ml of sample solutions were oxidized by 100 µl and 400 µl 30% H_2O_2 and 1 mg $FeSO_4$ by incubating 1 hr at room temperature. Sample solutions were centrifuged (8000 rpm for 2 minutes) after 1 hr incubation. The percent of Cr (III) oxidized to Cr (VI) were measured by spectrophotometer.
3. **Oxidation by Ammonium peroxodisulphate (4 times and 10 times that of sample) and $AgNO_3$:** The sample solutions were oxidized by Ammonium peroxodisulphate (4 times and 10 times that of the weight of sample) and 1 mg $AgNO_3$ by warming 10 minutes on steam bath (temperature 75±5°C). Sample solutions were centrifuged (8000 rpm for 2 minutes). The percent of Cr (III) oxidized to Cr (VI) were measured by spectrophotometer.

Characterisation of Pomegranate juice-Cr (III) adduct

The characterization was attempted using spectroscopic and chromatographic techniques. Both juice and adduct were subjected to spectrum scanning from 200-800 nm by UV-visible spectrophotometer. HPLC analyses were also carried out to obtain comparative fingerprint profile. The findings of HPLC analyses suggested that there are changes in the peak pattern in the polar region in adduct. Hence, the major compound from polar region was isolated from adduct by preparative thin layer chromatography. The Corresponding band was also isolated from juice. Both the isolated bands showed blue color when dipped in 3% $FeCl_3$ solution, indicating presence of phenol moieties. Isolated band from adduct was also analysed for total Cr content by ICP-OES. Finally, both the isolated bands from juice and adduct were analysed in UV spectroscopy and HPLC.

Spectrum Scanning in UV-Visible Spectrophotometer

The UV/Vis spectrophotometer (Shimadzu; UV 1900) was used for the analysis of juice and adduct.

Conditions for HPLC analysis

Shimadzu assembly (LC 2030), equipped with a UV detector, quaternary pump, injector, ZORBAX Eclipse-AAA [RP C18 150 x 4.6 mm; 3.5 µm] column were used, 40mM phosphate buffer having pH 7.8 (solvent A) and acetonitrile (Solvent B) were used as the mobile phase with a flow rate of 1.5 ml/min. 10µl sample was injected and detection was done at 270 nm. The gradient was maintained as follows:

Time (min)	Mobile Phase A (%v/v)	Mobile Phase B (%v/v)
0	100	0
18	55	45
25	20	80
30	100	0

Conditions for ICP-OES analysis

The total Cr determinations were performed employing a Agilent Technologies ICP-OES spectrometer, model 5110, serial number MY17060007 using the software ICP expert version 7.4.2.10790, for data acquisition. The experimental parameters used in ICP-OES are summarized below.

Isolation of bands by preparative thin layer chromatography

Silica gel G (E-Merck) was used for preparing TLC plates (20 × 20 cm). Finely powdered silica gel G (30 g) was mixed thoroughly with 60 mL distilled water. The slurry was then poured into the TLC applicator, which was adjusted to 0.5 mm thickness and slowly moved on to the clean glass plate. A fine layer of 0.5 mm thick wet silica gel was formed uniformly on the entire glass plate. The glass plate was allowed to dry in the open air for 1 h. The glass plate with silica gel coating was then heated in the oven at 110 ◦ C for 2 h. After activation of the TLC plate, the

sample (500 µL) was spotted on the plate with the aid of a capillary tube, without disturbing the silica gel layer. The Solvent system used for development is toluene, ethyl acetate and formic acid (5:4:1). The TLC chamber was allowed to get saturated with solvent (1 hr) prior to development.

ICP-OES parameter	Type or value
Spray chamber	Cyclonic
Nebulizer	Concentric
RF generator	27 MHz
RF power	1200 W
Plasma flow rate (Ar)	12.0 L/minute
Auxiliary gas flow rate (Ar)	1.0 L/minute
Nebulization flow rate (Ar)	0.7 L/minute
Wavelength for chromium	267.716 nm
Torch mode	Axial

Results and discussion

Standard Curve of Cr (VI)

Standard curve of Cr (VI) was prepared with 0.1, 0.2, 0.4, 0.6 and 0.8 mg/L standard concentrations against absorbance at 540 nm. The absorbance values in different dilutions are given in the Table 1 and figure 1.

Table 1: Standard Curve of Cr (VI) by Spectrophotometer

Concentration of Cr (VI) in mg/L	Absorbance at 540 nm
0.1	0.063
0.2	0.123
0.4	0.258
0.6	0.388
0.8	0.514

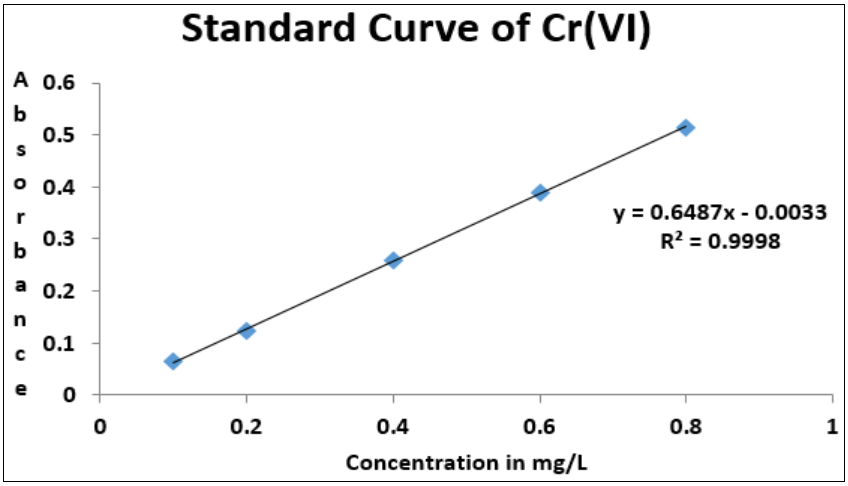


Fig 1: standard curve of Cr (VI)

The linear regression data for the calibration plot was indicative of a good linear relationship between absorbance and concentration of standard. The linear regression equation is y = 0.648x-0.003 and the regression coefficient is 0.999.

Oxidation Challenges

Following oxidation challenges were carried out to pomegranate-Cr (III) adduct was done and incorporated in Table 2 and Figure 2.

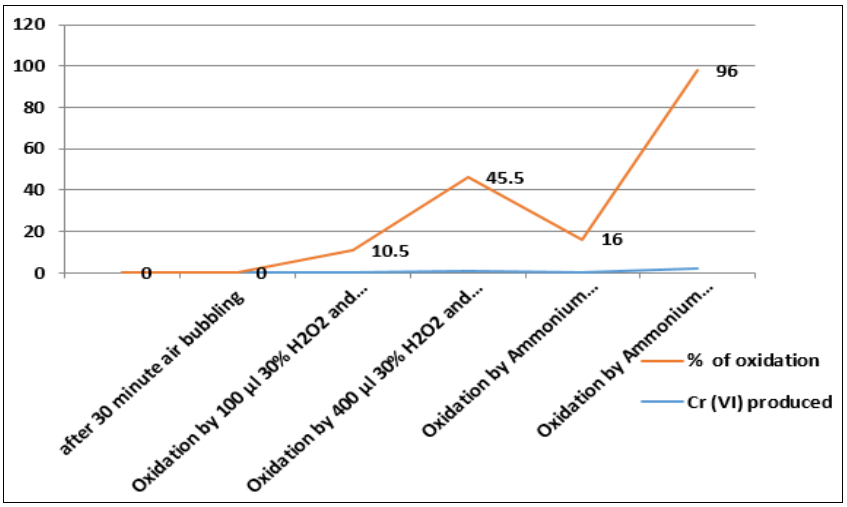


Fig 2: Oxidation of Adduct in different oxidation challenges

Table 2: Percent of total chromium as Cr (VI) of Pomegranate juice-Cr (III) adduct after oxidation challenges.

Oxidation Challenge	Cr (VI) produced (In %)	% of oxidation
after 30 minute air bubbling	Not Detected	0
Oxidation by 100 µl 30% H_2O_2 and 1 mg $FeSO_4$	0.21	10.5
Oxidation by 400 µl 30% H_2O_2 and 1 mg $FeSO_4$	0.81	45.5
Oxidation by Ammonium peroxodisulphate (4 times that of sample) and 1 mg $AgNO_3$	0.32	16.0
Oxidation by Ammonium peroxodisulphate (10 times that of sample) and 1 mg $AgNO_3$	1.92	96

Characterisation of Pomegranate juice-Cr (III) adduct: The characterisation of pomegranate juice and corresponding adduct was carried out by taking a spectrum scan in the range 200-400 nm and by HPLC.

Spectrum Scanning in UV-Visible Spectrophotometer
UV spectra scanning was carried out for both juice and adduct and it was observed that the adduct was also found to have a peak at 270 nm similar to that of pomegranate juice. This peak corresponds to the polyphenolics and the peak at 205 nm corresponds to that of Cr (III). Hence, the spectrum scan suggests the formation of adduct between any of the polyphenolics compound (s) of pomegranate juice with Cr (III). The overlaid spectra are given in Figure 3.

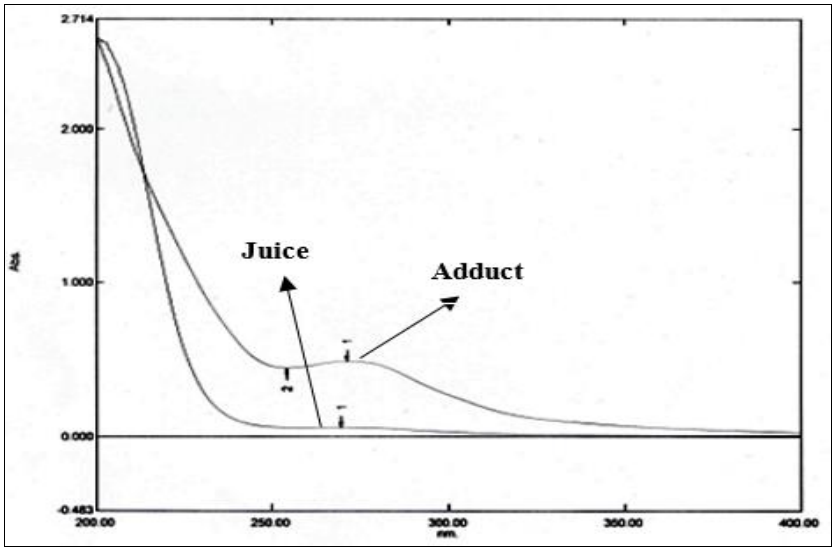


Fig 3: Overlaid UV spectra of pomegranate juice and adduct

HPLC analysis
HPLC analysis of pomegranate juice and adduct were carried out. The chromatograms are incorporated in Figures 4 and 5. It was observed that, in case of adduct, two major peaks at RT 2.8 minute and 3.6 minutes have much higher intensities in compare to juice. These intense peaks in the proposed adduct at polar region may be attributed to polymerisation of Polyphenolics and/or complexation between Polyphenolics of pomegranate juice and Cr (III).

Isolation of Bands by Preparative Thin Layer Chromatography
Preparative TLC was carried out and subsequent bands were scrapped from the plate and eluted with aqueous methanol. The TLC fingerprinting patterns with isolated bands are given in Figure 6.

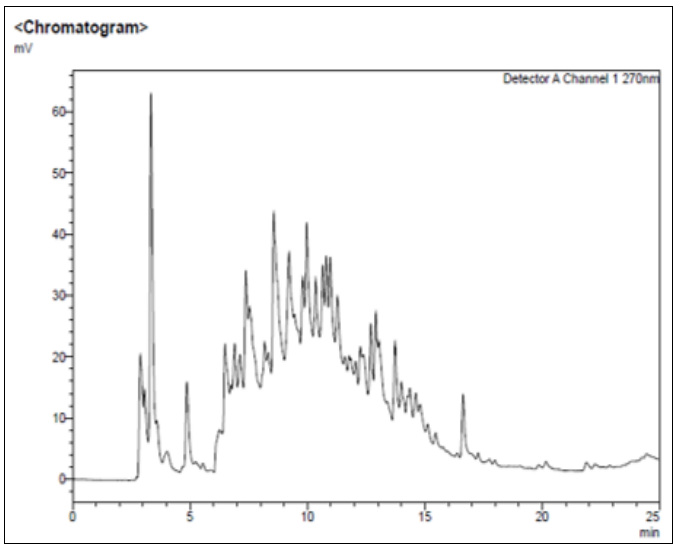


Fig 4: HPLC Chromatogram of Juice

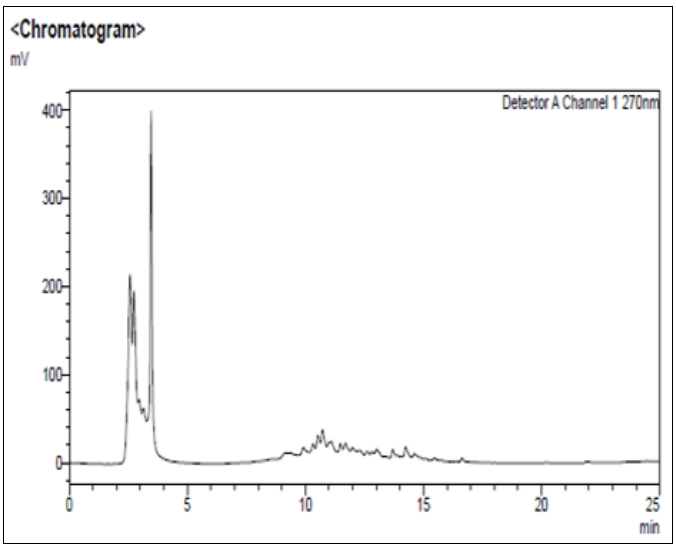


Fig 5: HPLC Chromatogram of adduct

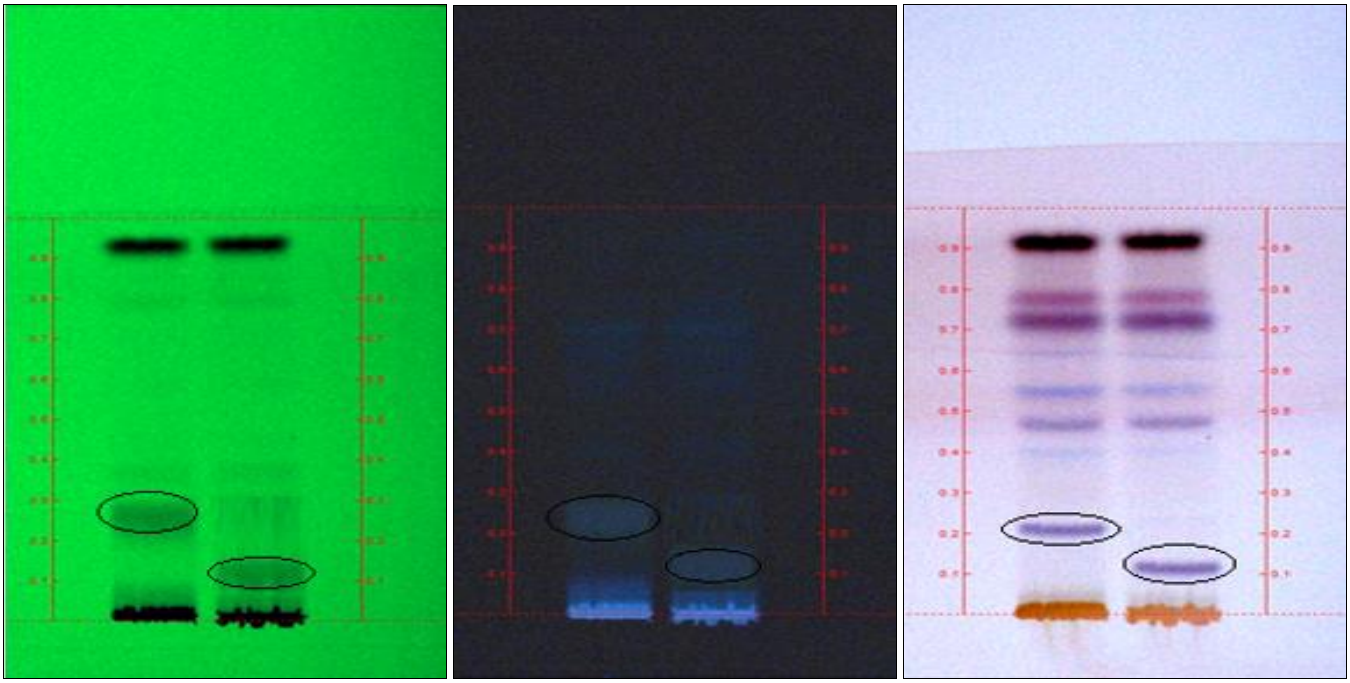


Fig 6: TLC pattern of pomegranate juice (track 1) and adduct (track 2) at 254 nm, 366 nm and after spraying with Ferric chloride solution. The circled bands were scrapped during Preparative TLC

Characterisation by Spectrum Scanning in UV-Visible Spectrophotometer: UV spectra scanning was carried out for both PTLC bands of juice and adduct and it was observed that the PTLC band of adduct was also found to have a peak at 270 nm similar to that of pomegranate juice. The overlaid spectra are given in Figure 7.

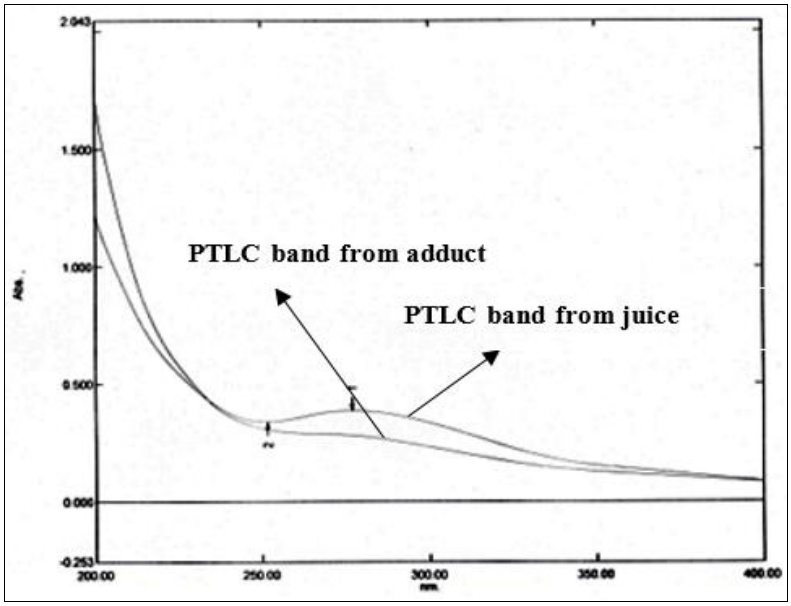


Fig 7: Overlaid UV spectra of PTLC bands isolated from pomegranate juice and adduct

Characterisation by HPLC Analysis

HPLC analyses of PTLC bands of pomegranate juice and adduct were carried out. The chromatograms are incorporated in Figures 8 and 9. It was observed that, in case of PTLC band of juice, the major peak is at RT 16.942 minute. In case of PTLC band isolated from adduct, the major peaks at RT 3.4 minutes was predominant in the adduct.

Estimation of Chromium in PTLC band of adduct: The PTLC band isolated from adduct was subjected to ICP-OES for the estimation of total chromium. The result was incorporated in Table 3.

Table 3: Total chromium content of PTLC band isolated from adduct.

Sample Name	Intensity	Calculated concentration (mg/kg)
Standard 0.01 mg/L	364.639	0.011
Standard 0.02 mg/L	570.484	0.021
Standard 0.05 mg/L	1055.711	0.046
Standard 0.1 mg/L	2102.615	0.099
Standard 0.3 mg/L	6187.915	0.308
Standard 0.5 mg/L	10304.229	0.519
Standard 0.7 mg/L	14395.699	0.728
Blank	-	-0.001
PTLC band from Adduct	-	0.639

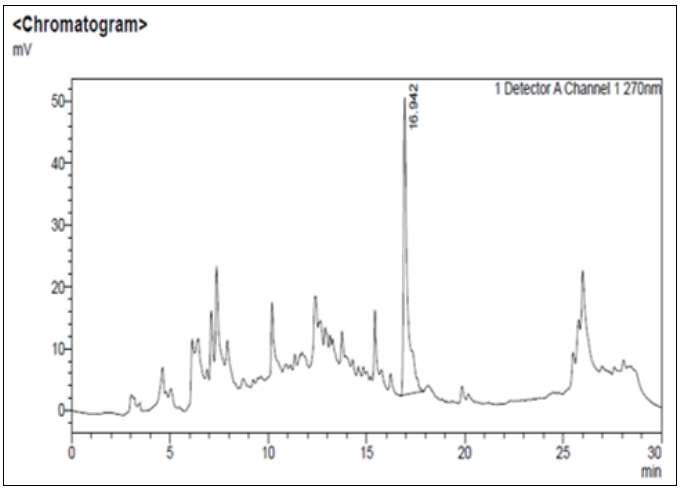


Fig 8: HPLC Chromatogram of Juice

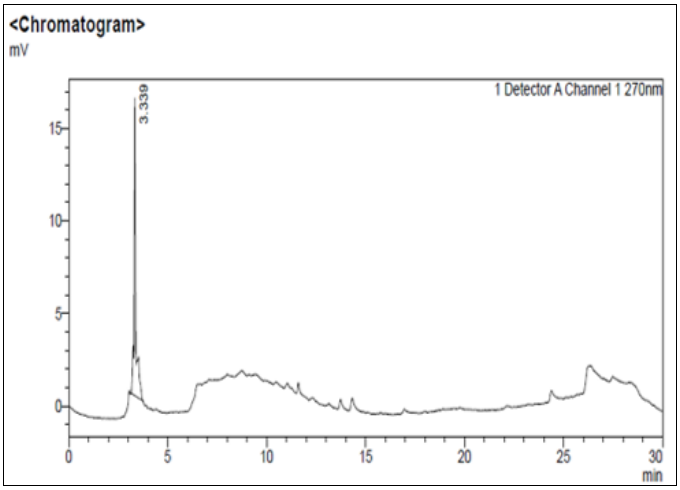
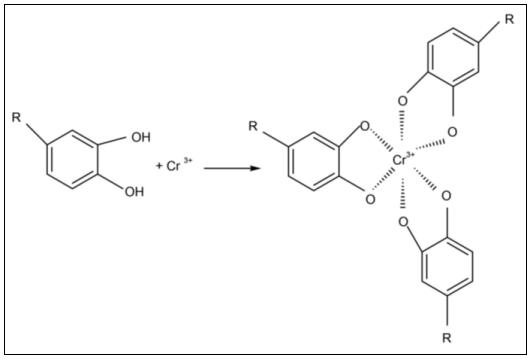


Fig 9: HPLC Chromatogram of adduct

Conclusion

The proposed adduct prepared with pomegranate juice and Cr (III) showed significant resistance to oxidation to Cr (VI) during different oxidation challenges. As the major constituent of pomegranate is Polyphenolics and complex formation between Cr (III) with other Polyphenolics was reported earlier, it may be assumed that the proposed adduct was formed between polyphenolic compound (s) and Cr (III) with the proposed structure.



The band isolated by PTLC in adduct was found to contain Polyphenolics group as it gave (i) blue color with ferric chloride, (ii) peak at 270 nm in UV spectra and (iii) major peak at 270 nm in HPLC. It was evident that the said isolated band contains Polyphenolics group. However, it was also shown to contain Cr when analysed by ICP-OES. Thus, the isolated band can be considered to contain conjugated Polyphenolics-Cr (III). The proposed adduct has shown to have great potential as an effective alternative for Cr (III) supplementation like chromium picolinate, chromium polynicotinate etc.

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