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Isolation And Purification of Hyaluronic Acid Like Components From *Manihot esculenta*(tapioca)

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Abstract: *Hyaluronic acid is a substance that is present in the human body. It is found in the most elevated fixations in liquids in the eyes and joints. Individuals take hyaluronic acid for different joint issue problems, including osteoarthritis. Hyaluronic acid is utilized in certain eye surgeries like, corneal transplantation, and repair of a disengaged retina and other eye wounds. Hyaluronic acid is additionally utilized as a lip filler in plastic surgery. A few people apply hyaluronic acid to the skin for mending wounds, cosmetics, skin ulcers, and as a lotion. Hyaluronic acid has been advanced as a wellspring of youth. In the present investigation Manihot esculenta (tapioca) was chosen for the study. The sample was extracted with methanol and chloroform. Goat brain and Visceral fluid were chosen as standard for the study. Estimation was carried out to check the presence of total carbohydrates, proteins and reducing sugars. Qualitative and quantitative studies were carried out which indicated the presence of hyaluronic acid like components in tapioca in comparison to visceral fluid and brain. Further ion exchange chromatography was carried using DEAE-Sepharose. The elution obtained from ion exchange showed a peak at 230nm which indicates the presence of hyaluronic acid like components. The peak eluents were further purified by gel permeation chromatography using Sephadex G-100. The result confirmed the presence of hyaluronic acid like components. The results of the present work suggested that hyaluronic acid like components could be present in Tapioca.*

Keywords: *Tapioca, Hyaluronic acid, Chromatography, Visceral fluid.*

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I. Introduction

Hyaluronic acid (HA) is a carbohydrate, more specifically a mucopolysaccharides, occurring naturally in all living organisms. It can be several thousands of sugars (carbohydrates) long. When not bound to other molecules, it binds to water giving it a stiff viscous quality similar to "Jello". The polysaccharide hyaluronan (HA) is a linear polyanion, with a poly repeating disaccharide structure [(1→3)-β-dGlcNAc-(1→4)-β-d-GlcA-]. HA is found primarily in the extracellular matrix and peri-cellular matrix, but has also been shown to occur intracellularly. The biological functions of HA include maintenance of the elastoviscosity of liquid connective tissues such as joint synovial and eye vitreous fluid, control of tissue hydration and water transport, supramolecular assembly of proteoglycans in the extracellular matrix, and numerous receptor-mediated roles in cell detachment, mitosis, migration, tumor development and metastasis, and inflammation (Balazs et al., 1986; Toole et al., 2002; Turley et al., 2002; Hascall et al., 2004). Although the predominant mechanism of HA is unknown, in vivo, in vitro, and clinical studies demonstrate various physiological effects of exogenous HA. Hyaluronic acid possesses a number of protective physiochemical functions that may provide some additional chondroprotective effects in vivo and may explain its longer term effects on articular cartilage. Hyaluronic acid can reduce nerve impulses and nerve sensitivity associated with pain. In experimental osteoarthritis, this glycosaminoglycan has protective effects on cartilage (Akmal et al., 2005); exogenous hyaluronic acid is known to be incorporated into cartilage (Antonas et al., 1973). Exogenous HA enhances chondrocyte HA and proteoglycan synthesis, reduces the production and activity of proinflammatory mediators and matrix metalloproteinases, and alters the behavior of immune cells. These functions are manifested in the scavenging of reactive oxygen-derived free radicals, the inhibition of immune complex adherence to polymorphonuclear cells, the inhibition of leukocyte and macrophage migration and aggregation (Balazs and Denlinger, 1984) and the regulation of fibroblast proliferation. Many of the physiological effects of exogenous HA may be functions of its molecular weight (Noble, 2002; Uthman et al., 2003; Medina et al., 2006). Hyaluronan is highly hygroscopic and this property is believed to be important for modulating tissue hydration and osmotic balance. In addition to its function as a passive structural molecule, hyaluronan also acts as a signaling molecule by interacting with cell surface receptors and regulating cell proliferation, migration, and differentiation. Hyaluronan is essential for embryogenesis and is likely also important in tumorigenesis (Kosaki et al., 1999; Camenisch et al., 2000).

Hyaluronan functions are diverse. Because of its hygroscopic properties, hyaluronan significantly influences hydration and the physical properties of the extracellular matrix. Hyaluronan is also capable of interacting with a number of receptors resulting in the activation of signaling cascades that influence cell migration, proliferation, and gene expression. To discover and establish plant source as a cheaper substitute for extraction of hyaluronic acid like components, the present study is an attempt to isolate and purify Hyaluronic Acid like components from plant source such as tapioca.

II. Materials And Methods

2.1 Chemicals

Acetone, chloroform, methanol, sodium acetate, cysteine, disodium ethylenediaminetetraaceticacid, sodium chloride, absolute ethanol, dinitrosalicyclicacid, glucose, maltose, anthrone, sodium carbonate, sodium hydroxide, sodium potassium tartarate, copper sulphate were purchased from Himedia Pvt. Ltd. Folin-ciocalteu reagent, bovine serum albumin, hydrochloric acid purchased from Fisher Scientific. DEAE-Sepharose matrix, CM- Sepharose matrix, Sephadex G-100 purchased from Sigma.

2.2 Equipment

Weighing balance (Auy 220 Shimadzu and Elb 300), Homogenizer, Rocker, Vacuum pump, REMI R-8C centrifuge, Elico LI-120 pHmeter, Systronics UV-VIS Spectrophotometer 117.

2.3 Plant Source

Manihot esculenta(tapioca) was taken from near by market in H.B.R Layout, Bangalore , Karnataka. Fresh tubers of these samples were used for the study.

2.4 Animal Source

Fresh Goat brain was collected from Dhodi slaughter house, Frazer town , Bangalore, Karnataka. Visceral fluid was collected from common fish's eye from Thanisandra, Bangalore, Karnataka. The brain and the visceral fluid was used as a standard.

2.5 Extraction of The Sample

Extraction was carried out using protocol in paper "Purification and characterization of hyaluronic acid from chicken combs" (Claudia Severo da Rosa, 2012).The samples were washed thoroughly and weighed separately. Brain (100 g), sweetpotato and tapioca (50 g each) was taken. The samples were crushed and immersed in volume of acetone to get 50% extract. These samples were kept in rocker for 1 hour. Chloroform:Methanol (2:1) ratio was prepared for the three samples of 100 ml. The samples were kept in the chloroform:methanol solution and incubated for 24 hours at 25 °C. After the incubation period, Digestion buffer (100Mm sodium acetate pH 5.0, 5.0mM cysteine and 5.0mM disodium EDTA) was prepared in ratio approximately 2ml buffer to 100 mg of tissue. The samples were hydrated in the digestion buffer for 44 hours at 5°C. After hydration the mixture was centrifuged at 3200 rpm for 30 minutes. Supernatant was discarded and the pellet was washed with 3 ml of 2.0M NaCl and absolute ethanol. Absolute ethanol was added in 2:1 ratio and incubated for 24 hours at -16°C. After the incubation period, the sample was centrifuged again at 3200 rpm for 30 minutes. Supernatant was discarded and the pellet was washed with 80% ethanol. The solution was centrifuged again (3200 rpm / 30 min), the supernatant was discarded and the pellet was dried for 24 hours at 25°C. The final solid was re-suspended in 5 ml of distilled water and further assays were carried out.

2.6 Estimation of Total carbohydrates by anthrone method

Total sugars were estimated by Anthrone method in the samples (Yemm. EW and Willis AJ.1954). The readings were noted and a standard curve plotted to determine the total carbohydrate content in the samples. The results were expressed as mg/ml of total carbohydrate.

2.7 Estimation of Reducing sugars by DNS method

Reducing sugars were estimated by DNS method in the samples. (Nelson.1994).The readings was noted and a standard curve plotted to determine the reducing sugars content in the samples. The results were expressed as micromoles/ml of reducing sugars.

2.8 Estimation of Proteins by Lowry's Method

The protein content in the samples was estimated by Lowry's method (Oliver H Lowry et al.1951). The readings were noted and a standard curve plotted to determine the protein content in the samples. The results were expressed as micrograms/ml.

2.9 Purification

Extraction was followed by Partial purification such as Ion exchange chromatography and Gel permeation chromatographyDEAE- Ion Exchange ChromatographyThe sample (1 ml) was loaded onto the DEAE–sepharose column and the column washed with two bed volumes of start buffer. The bound proteins were eluted with stepwise increase in the ionic strength (0 M and 1.5 M NaCl). Fractions of 5 ml were collected. The absorbance was taken for the eluents in UV spectrophotometer (200-270 nm).CM-Sepharose Ion Exchange chromatographyThe sample (1 ml) was loaded onto the CM – sepharose column and the column washed with two bed volumes of start buffer. The samples were eluted with stepwise increase in the ionic strength (0 M and 1.5 M NaCl). Fractions of 5 ml were collected. The absorbance was taken for the eluents in UV spectrophotometer (200-270 nm).The absorbance was compared between the two matrices and the results are shown. Sephadex G-100 Gel-filtration chromatographyThe DEAE –Sepharose fraction, Hyaluronic acid containing fractions were subjected to gel permeation chromatography separately using Sephadex G – 100. The samples were eluted with start buffer and fractions of 2 ml were collected. Absorbance was taken at 230 nm in UV Spectrophotometer. A single peak of glycosaminoglycan was obtained.

III. Results And Discussion

The results were obtained and the data is represented in the form of graphs which help in the comparison and understanding of the presence of hyaluronic acid. Presence of carbohydrates indicates that hyaluronic acid is present which was further purified by ion exchange and gel permeation chromatography. Results are given as follows:-

3. 1Estimation Of Total Carbohydrates By Anthrone Method

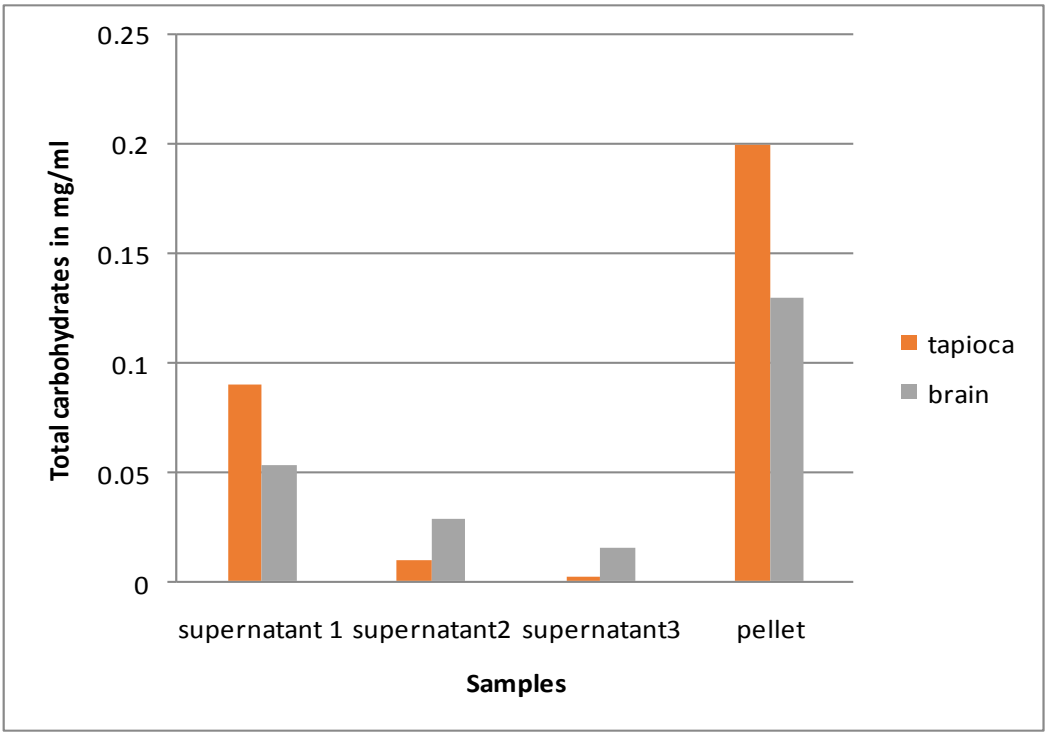

Figure 1: Bar Graph showing a comparison between total carbohydrate content in the two samples.

Estimation of total carbohydrates of the samples was carried out by anthrone method. The above figure illustrates that there is highest yield of the total carbohydrates in the pellets obtained of both the sample compared to the supernatants. 1mL aliquot of each sample were used for the assay all of which showed high yield in the pellet. The total carbohydrate concentration in the pellet was found to be 0.2mg in tapioca and 0.13mg in brain. The lowest yield of total carbohydrates was found in supernatants 3, 2 and 1.

3.2 Estimation Of Reducing Sugar By DNS Method

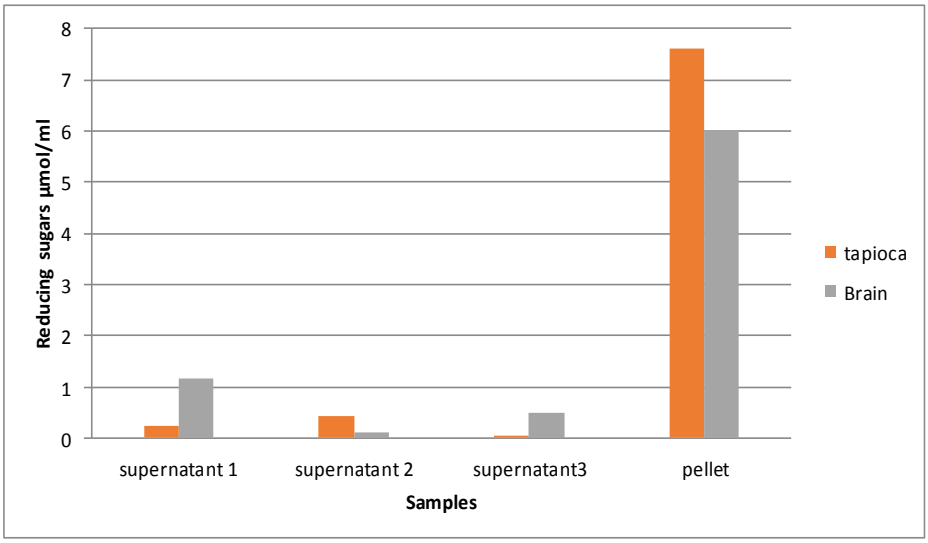

Figure 2: Bar graph showing comparison of the two samples in reducing sugars

Estimation of reducing sugars of the samples was done by dinitro salicyclic method. The above figure illustrates that there is a significant high yield in reducing sugars in the pellets of both the samples as compared to the supernatants. 1ml aliquot of each sample was used for the assay. The reducing sugar concentration in the pellet of each extract was found to be 7.6 µmol/ml in tapioca and 6 µmol/ml in brain. The lowest yield of reducing sugar content was found in supernatants. These results indicated that the pellets have highest levels of reducing sugar after extraction indicating that it can be subjected to further purification.

3.3 Estimation Of Proteins By Lowry's Method

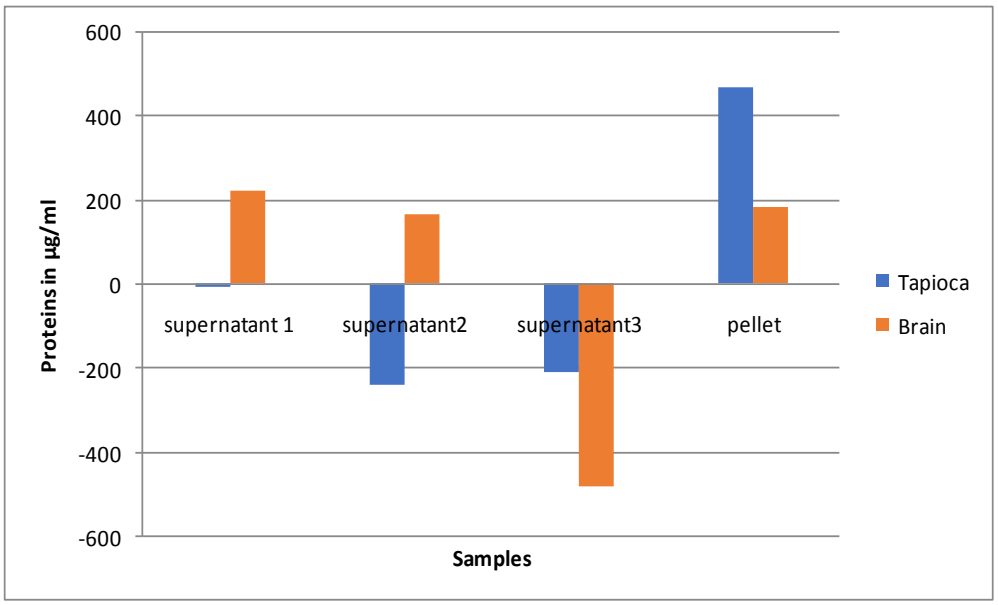

Figure 3: Bar graph showing comparison of protein content in two samples

Protein content of the samples was estimated using Lowry method. The above figure illustrates that there is very low concentration of protein content in each sample after extraction. This indicated that proteins were effectively degraded during the extraction enabling the samples to be further purified for HA like components. 1mL aliquot of each sample were used for assay and the concentrations were depicted in micrograms per mL. All the samples yielded low concentrations of protein.

3.4 Partial Purification Of Hyaluronic acid Like Components

ION EXCHANGE CHROMATOGRAPHY (DEAE-Sepharose)

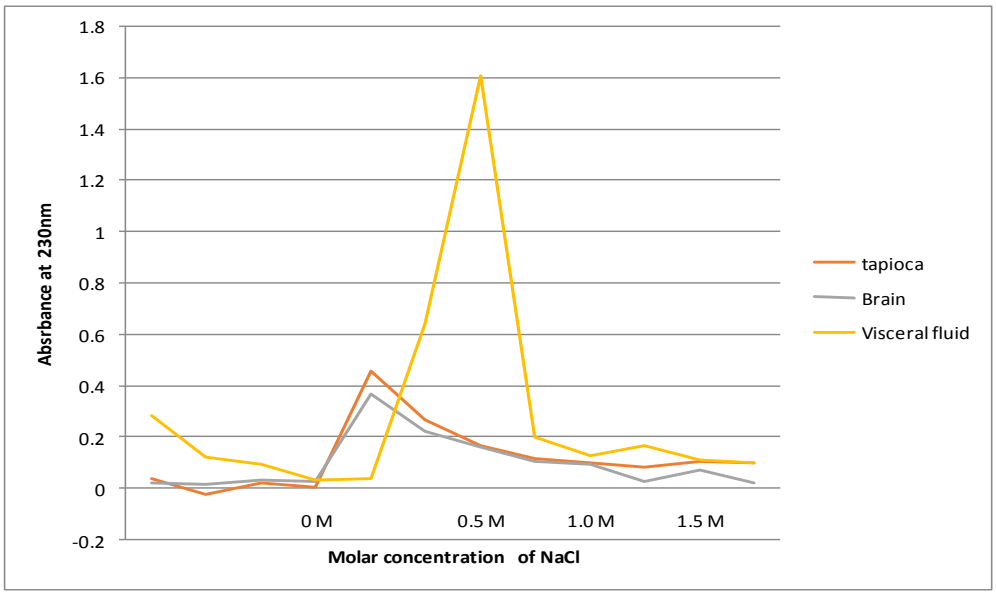

Figure 4: Line graph showing comparison of absorbance between different elutions of three samples at 230nm. Highest peak was obtained at 0.5M elution in all the three samples.

Partial purification was carried out using ion exchange chromatography. The figure depicts that elutions obtained at 0.5M concentration gave highest absorbance at 230nm, the column flow rate was maintained at 25ml/hr.The number of fractions collected where 14 and each fractions were analysed for HA like components at 200 -270nm in which 0.5M fraction showed maximum absorbance at 230nm.

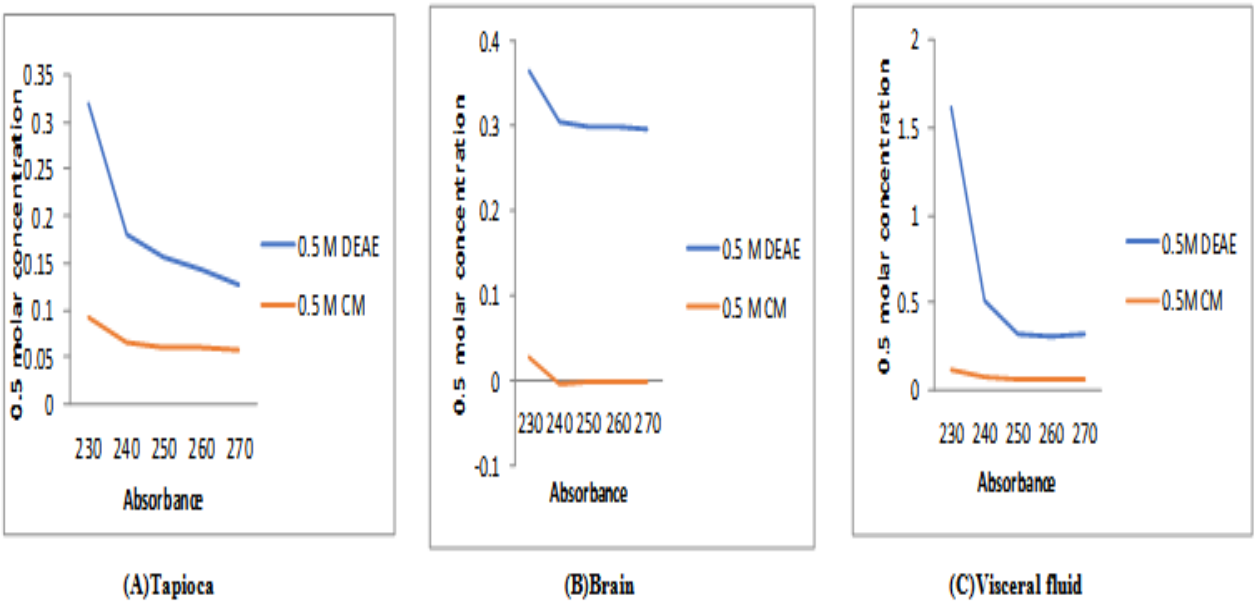

Figure 5: **A comparison of absorption spectra at different wavelengths of ion exchange chromatography using anion exchanger (DEAE) and cation exchanger (CM) in A B and C.**

The above figures depict the absorbance obtained after elutions in DEAE and CM matrix. It indicates that 0.5 M elutions of each sample showed highest peak at 230nm in DEAE matrix, showing that HA like components are present in the sample

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GEL PERMEATION CHROMATOGRAPHY (Sephadex G-100 matrix)

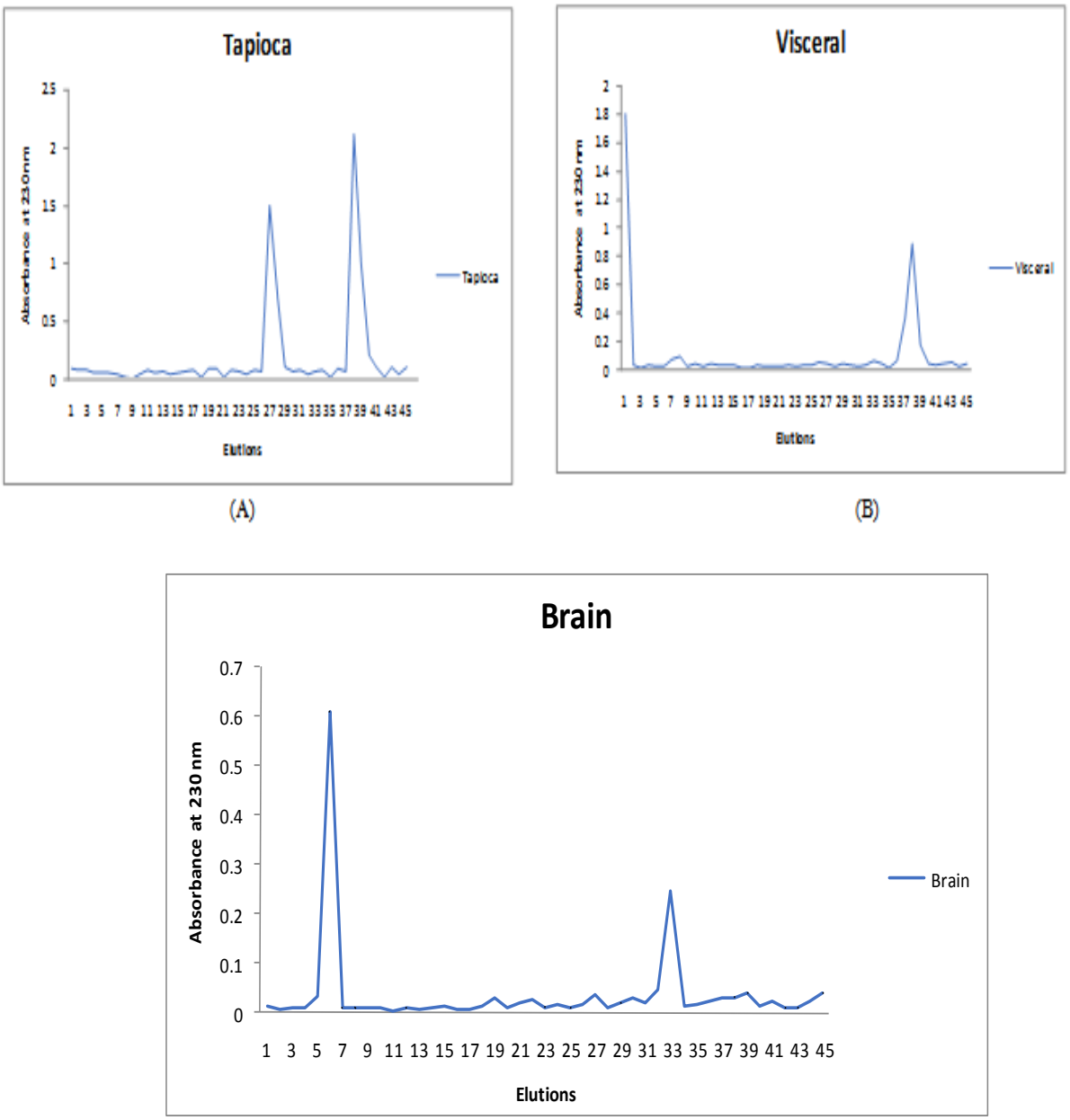

Figure 6: Peaks obtained at 230 nm for 1ml of 45 elutions in gel permeation chromatography in A, B, C

Gel permeation chromatography was performed with each sample which was eluted at 0.5M concentrations in Ion exchange chromatography. Sephadex G-100 matrix was used for separation where 100µL of each sample was loaded into the column. The flow rate was maintained at 12mL per hour and 45 elutions of 1mL was collected and then subjected to UV-vis spectra. The figure above shows peak obtained in each sample. The low molecular weight hyaluronic acids showed a peak at 230 nm and showed that there was no modification of chemical structure (Wu Yue, 2012). The peak indicating in this graph shows that there is presence of hyaluronic acid.When compared to the reference papers we get to know that the peaks obtained in the gel permeation chromatography is hyaluronic acid like components where peaks were obtained in different fractions. In tapioca, there is a peak in the 27th and 38th eluent indicating that hyaluronic acid like component was present in the sample. If we compared to the standards (brain and visceral) the peaks obtained are almost similar. The peak indicates the presence of HA like components. In Gel permeation chromatography, the calculated total volume was 32.5ml (125mm X15mm). The sephadex G-100 matrix can elute molecular weight

ranging from 10kDa to 100kDa. As G-100 is used , the fractions can have molecular weight between 10 – 90kDa. Hyaluronic acid like components having higher molecular weight would have broken into smaller fragments which can have molecular weight between 10 – 90 kDa which is obtained in the graphs showing peaks, whose absorbance is read at 230nm. Based on this assumption the molecular weight of hyaluronic acid like components from tapioca can be more than 100kDa, since it is coming within the void volume. Also, existence of one more peak assures the presence of smaller molecule mass of HA of molecular weight 22kDa and 18kDa which would be fragments of the polymer.

IV. Conclusion

Manihot esculenta is a tuber is an outstanding source of nutrients, and it is the best plant source which possess hyaluronic acid like components. The biochemical analysis was carried out to detect carbohydrates, reducing sugars, protein. The presence of carbohydrates and proteins was detected using DNS , Anthrone and Lowry's method. Further ion exchange and gel permeation techniques were carried out to purify the hyaluronic acid like components. A peak was obtained at 230nm with 0.5M NaCl linear gradient elution in ion exchange chromatography. The elution from ion exchange was further purified through Sephadex G-100 gel permeation chromatography. The results of gel permeation indicates that hyaluronic acid being a high molecular weight component would have broken into smaller fragments having a molecular weight of 18kDa. Pure Hyaluronic acid is expensive as it is manufactured by recombinant technology. These methods can further be simplified or used to isolate hyaluronic acid which can be used in the industries.

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