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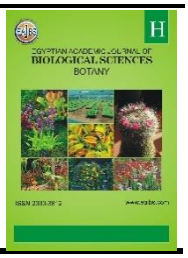

Genotoxic Activities of Polysaccharides from Cotyledon and Coat of Fermented and Unfermented *Annona squamosa* L. Seed

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ABSTRACT

This study extracted polysaccharides from cotyledon and coats of fermented and unfermented *A. squamosa* seed and determined the genotoxic potential of the polysaccharides. Fresh and ripe sugar apple fruits were collected from an orchard at Ota-Efun, Osogbo, Nigeria and authenticated at IFE Herbarium, Obafemi Awolowo University, Ile-Ife, Nigeria. The seeds were divided into two: a portion was fermented traditionally and the other unfermented. The cotyledon and the coat of both the fermented and unfermented seeds were separated and defatted. Polysaccharides were isolated to yield fermented cotyledon polysaccharide (FCP), fermented seed coat polysaccharide (FSCP), unfermented cotyledon polysaccharide (UCP), and unfermented seed coat polysaccharide (UFSCP). The estimation of hexoses, total hexosamines, and total uronic acid concentrations in the polysaccharide was carried out using HPLC. Genotoxic activities of the polysaccharides were investigated using standard methods.

The analyses of the polysaccharide composition revealed that the polysaccharides were all heteropolysaccharides. Genotoxic investigations of the polysaccharides revealed very low mitotic indices, chromosomal aberration, and very low LC_{50}.

The study concluded that the polysaccharides from fermented and unfermented cotyledon and coat of *A. squamosa* seeds have excellent genotoxic activities and potential compounds in the search for drugs for combating cancer and other diseases related to oxidative stress.

INTRODUCTION

One way to evaluate the toxicity of natural extracts of medicinal plants is the *Allium cepa* assay. This *in vitro* test is very useful as a first-tier analysis of cytotoxicity and genotoxicity, because of the simplicity, low relative cost, versatility and minimum laboratory

facilities required for its performance. Moreover, the results obtained using the *A. cepa* root model show a high degree of conformity with the results obtained from mammalian assays (Çavuşoğlu, 2019). The *Allium cepa* test has been used by many researchers mainly as a bioindicator of environmental pollution (Cabuga *et al.*, 2017), testing crude extracts of cyanobacteria (Bonciu *et al.,* 2018), as well as evaluating the genotoxic potential of medicinal plants (Singh *et al.,* 2017), because this test uses a model that is adequately sensitive to detect in numerous substances that cause chromosomal alterations. High sensitivity and good correlation with mammal tests and the same sensitivity as test systems of algae and human lymphocytes exist when compared with *Allium cepa* (Hammann *et al.,* 2020).

Studies have revealed that plant-derived compounds dramatically improve hard-to-treat illnesses, such as cancer, while a number of plant components are characterised by their ability to prevent the development of certain diseases (Shukrullo, 2020). Plant polysaccharides have been proven to possess excellent antioxidant and anti-inflammatory activities which confer on them their therapeutic potentials (Mzoughi, 2018). Presently, there is no scientific information on the genotoxic activities of polysaccharides from *Annona squamosa* seed, hence this study.

MATERIALS AND METHODS

Collection and Identification of *Annona squamosa* Fruits:

Fresh and ripe sugar apple fruits were collected from an orchard at Ota-Efun, Olorunda Local Government, Osogbo, Osun State, Nigeria (07º 32′ 30.2496″ N, 04º 31′ 41.7036″ E) between July and August. The fruits were identified and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria, and voucher number (IFE-17805) was obtained.

***Allium cepa* Bulbs:**

Healthy and fresh bulbs of *Allium cepa* (onion) were purchased from Ota-Efun Market, Osogbo, Osun State, Nigeria and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria and voucher number (IFE-17944) was obtained.

Reagents and Chemicals:

All chemicals and reagents used for this study were of analytical grade and purchased from various sources. All buffers, reagents and solutions were prepared with distilled water except otherwise stated.

Methods

Fermentation And Processing of Sugar Apple Seeds:

The *A. squamosa* seeds were removed from the matured, ripe, soft fruits into clean containers and divided into two portions. A portion was fermented traditionally by wrapping the seeds in banana leaves, kept inside a dark cupboard for 7 days, and oven-dried at 40 ºC (Dare *et al*., 2013). The second portion was washed with clean water to remove the pulp and also oven-dried at 40 ºC as unfermented seeds. The cotyledons of both fermented and unfermented seeds were separated from the seed coat and powdered separately in a Warring Blender. The powdered materials were termed fermented cotyledon (FC), unfermented cotyledon (UC), fermented seed coat (FSC), and unfermented seed coat (USC) respectively. The powdered cotyledon samples were defatted with n-hexane, extracted with 80% (v/v) ethanol for 24 hr to remove organic compounds, and the residues were air-dried.

Preparation of Polysaccharides:

The preparation of polysaccharides of FC, UC, FSC and USC was carried out using a procedure that was based on the earlier methods (Liu *et al.,* 2012) as illustrated in Fig. 1.

The precipitates were labelled as fermented cotyledon polysaccharide (FCP) and unfermented cotyledon polysaccharide (UCP) respectively and kept in the refrigerator for further analyses.

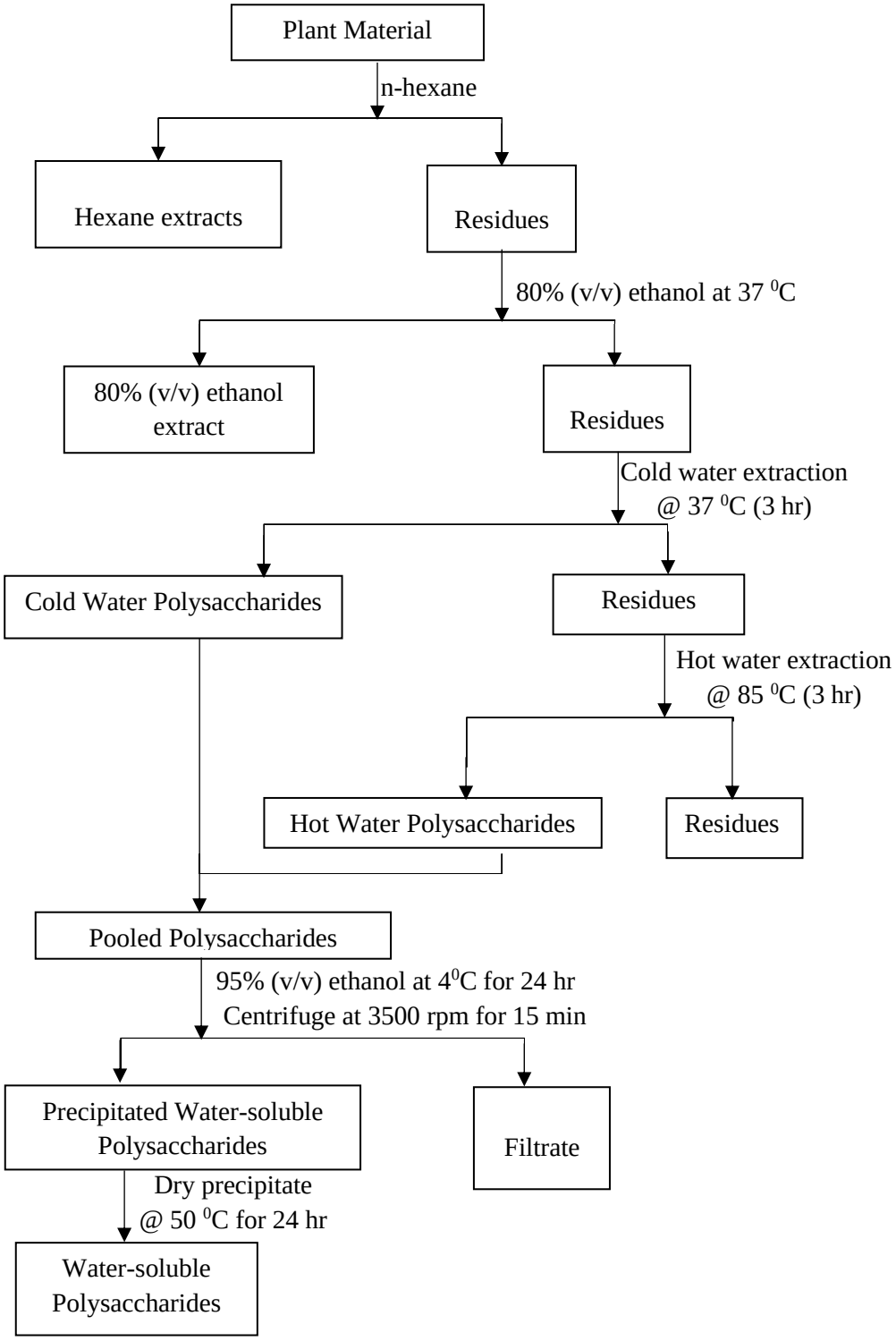

Fig. 1: Fractionation Scheme of *Annona squamosa* Fermented and Unfermented Cotyledon and Seed Coat

HPLC Analysis of the Polysaccharides:

The composition of the extracted polysaccharides was analysed by HPLC method (Yan *et al*., 2016).

Statistical Analysis:

The data obtained were analysed using One-way ANOVA followed by Tukey multiple comparison test. Differences were considered to be significant if $p < 0.05$.

Genotoxic Activity Assessment: *Allium cepa* Assay:

The *Allium cepa* test is used for screening and monitoring environmental chemicals with mutagenic and carcinogenic potential (Cabaravdic, 2010; Sharma and Vig, 2012).

Pre-treatment of *Allium cepa* Bulbs with Polysaccharides:

The *Allium cepa* bulbs (63) were purchased from Ota-Efun market, Osogbo. The scally outer leaves of *A. cepa* were carefully removed and old roots were carefully scraped off to promote the emergence of new roots. The bulbs were grown in distilled water (25 ml) inside cups at room temperature for 24 hr to initiate root growth. The onion bulbs were then grown in 25 ml of different concentrations of the polysaccharides (0, 100, 200, 300, 400, and 500 µg/ml) in triplicates for 48 hr, while another set of *A. cepa* bulbs was grown in distilled water to serve as the negative control. The solutions were changed daily. After 48 hr, the roots (10) from each bulb were harvested and the length was measured with a ruler (in cm).

Percentage root growth inhibition was calculated from the expression:

$$\text{Percentage growth inhibition} = \frac{\text{Root length of control} - \text{Root length treated with extract}}{\text{Root length of control}} \text{x } 100$$

The harvested roots were fixed in Carnoy's fixative solution (1:3 acetic acid: ethanol) for 24 hr and used for the preparation of slides.

Preparation of Microscope Slides:

The root tips were rinsed with distilled water and hydrolysed with 1 M HCl solution at room temperature for 10 min to soften them. The meristems (tips) of the roots were cut with a clean blade onto a microscope slide [MEDI-SCAN clear glass ground edges 25.4 x 76.2mm (1" x 3") THICK]. A drop of the fixative solution was added and the root was mashed with a clean crushing pin. A drop of 2% (w/v) FLP (formic-lactic-propionic acid)-orcein stain was added and a clean coverslip was gently placed on the root-stain mixture and allowed to stand for 15 min for the cells to take up the stain. The excess stain was removed by blotting the slide with a Whatman filter paper. The prepared slides were allowed to dry at room temperature and viewed under a microscope at a magnification of x 100. Five slides were prepared for each concentration.

Analyses of the Slides:

The cells in different cell division stages (prophase, metaphase, anaphase, and telophase) and interphase were counted and scored in each slide. The numbers of dividing cells, non-dividing cells and cells with chromosomal aberrations (such as chromosomal breakages, simple or multiple anaphasic bridges, micronucleus, laggard, or lost chromosomes) were also counted and recorded.

Photomicrography:

Photomicrographs of the slides showing chromosomal aberrations were taken using an Accu-scope trinocular microscope (ACCU-scope 33001 LED Trinocular microscope with 3.2 MP CMOS digital camera) at the Department of Botany, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

The cytotoxic potential was calculated through observation of the mitotic index (MI). The MI was calculated for each treatment using the expression:

$$\text{Mitotic Index (MI)} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \text{ x } 100$$

RESULTS AND DISCUSSION

Percentage Yield of Water-Soluble Polysaccharides:

The polysaccharides from the fermented cotyledon and seed coat of *A. squamosa* had lower yields than the unfermented cotyledon and seed coat. The increase in the unfermented cotyledon and seed coat could be a result of some metabolic activities which took place during fermentation (Table 1).

Table 1: Percentage Yield of Purified Polysaccharides

Sample	Percentage Yield (%)
FCP	1.27 ± 0.10
FSCP	0.67 ± 0.01
UCP	3.72 ± 0.71
USCP	2.82 ± 0.25

Each value represented Mean ± SEM of n = 4 replicates
FCP = fermented cotyledon polysaccharides, FSCP = fermented seed coat polysaccharides, UCP = unfermented cotyledon polysaccharides, USCP = unfermented seed coat polysaccharides

Polysaccharides Components from HPLC Analysis:

The HPLC analyses of the polysaccharides revealed the presence of certain monosaccharides and derivatives of monosaccharides (Appendices 1 to 12) like rhamnose, mannose, fructose, fucose, glucose, galactose, arabinose, xylose, galactosamine, fructosamine, glucosamine, mannuronic acid and glucuronic acids, alluronic acid, galacturonic acid. All the polysaccharides could also be noted as pectic polysaccharides due to the high content of uronic acids in them except for FCP.

Genotoxic activities of *A. squamosa* polysaccharide:

a. Allium cepa root growth inhibition assay:

The *Allium cepa* root growth inhibition assay showed the effects of the polysaccharides on the growth of the root length of *Allium cepa* (onion bulbs). The root growth inhibition was observed to be concentration-dependent i.e. the higher the concentration of the polysaccharides, the higher the root growth inhibition. Table 2 is the summary of *A. cepa* root growth inhibition. The results showed that as the concentration of polysaccharides increased, the root lengths decreased. At the highest concentration used (500 µg/ml), FCP elicited the highest percentage inhibition in root length growth (48.61%) with reference to the control and USCP had the lowest percentage inhibition in root length growth (8.33%) with reference to the control.

The statistical analyses of the concentration-dependent reduction in root length (RL) of *Allium cepa* treated with different concentrations (100, 200, 300, 400 and 500 µg/ml) of FCP, FSCP, UCP and USCP for 48 hours showed the difference was significant in Pearson's correlation analysis where correlation coefficient (r) was -0.9839 with significance level $p < 0.0001$ and 95 % confidence interval (CI) for r -0.9990 to -0.7706 for FCP, correlation coefficient (r) was -0.9728 with significance level $p < 0.0001$ and 95 % confidence interval (CI) for r -0.9983 to -0.6383 for FSCP, correlation coefficient (r) was -0.9816 with significance level $p < 0.0001$ and 95 % confidence interval (CI) for r -0.9988 to -0.7413 for UCP, and correlation coefficient (r) was -0.9377 with significance level $p < 0.0001$ and 95 % confidence interval (CI) for r -0.9960 to -0.3206 for USCP.

Table 2: Root Growth Inhibition *Allium cepa* Mean Root Length and Percentage Change in Length**.**

Concentration (µg/ml)	FCP		FSCP		UCP		USCP	
	Root Length (cm)	% Inhibition	Root Length (cm)	% Inhibition	Root Length (cm)	% Inhibition	Root Length (cm)	% Inhibition
0.00 (Control)	3.60 ± 0.10	0.00	3.60 ± 0.10	0.00	3.60 ± 0.10	0.00	3.60 ± 0.10	0.00
100	3.0 ± 0.50	16.67	3.40 ± 0.70	9.56	4.05 ± 0.95	-12.5	4.70 ± 0.40	-30.56
200	2.70 ± 0.10	25	3.15 ± 0.55	12.5	3.35 ± 0.25	6.94	4.35 ± 0.15	-20.83
300	2.55 ± 0.05	29.17	2.80 ± 0.30	22.22	2.85 ± 0.25	20.83	3.50 ± 0.80	2.78
400	2.30 ± 0.10	36.11	2.00 ± 0.20	44.44	2.70 ± 0.50	25.00	3.45 ± 0.25	4.17
500	1.85 ± 0.15	48.61	1.90 ± 0.60	47.22	1.90 ± 0.30	47.22	3.30 ± 0.80	8.33

Each value represented Mean ± SEM of n = 10 replicates. The control group change rate (%) was taken as 100 %, and the other concentrations were compared to this group.

$$\% \text{ Inhibition} = \left(\frac{Root\ length_{control} - Root\ length_{sample}}{Root\ length_{control}} \text{ x}100\right)$$

FCP = fermented cotyledon polysaccharides, FSCP = fermented seed coat polysaccharides, UCP = unfermented cotyledon polysaccharides, USCP = unfermented seed coat polysaccharides.

b. Mitotic Indices and Chromosomal Aberrations:

Tables 3-6 are the summaries of the mitotic indices (the proportion of cells in a population that are in mitosis at a given time) and chromosomal aberrations exhibited by varying concentrations of the purified polysaccharides. The mitotic indices (MI), and chromosomal aberration (CA) analyses showed that MI decreased with increase in the concentration of the polysaccharides while chromosomal aberration increases with increase in the polysaccharides. This implied that cell division reduced as the concentrations of the polysaccharides increased and there was a rise in the frequency of abnormal chromosome (CA) with an increase in the polysaccharides concentration. However, FCP elicited the lowest mitotic index and highest chromosomal aberration and USCP with the highest mitotic index and lowest chromosomal aberration (Table 7). Table 8 presented the change in MI with respect to the control, providing summarised evidence of a decrease in MI with an increase in the polysaccharides concentrations.

Table 3: Mitotic Indices in Root Cells of *Allium cepa* Treated with FCP

Concentration (µg/ml)	Total Number of Cells (TC)	Total Dividing Cells (TDC)	Total Normal Cells (TNC)	Total Abnormal Cells (TAC)	Mitotic Index (MI)	Chromosomal Aberration (CA)
0.00 (Control)	2207.56 ± 253.24	418.99 ± 56.77	418.99 ± 56.77	0.00 ± 0.00	18.98 ± 0.54	0.00 ± 0.00
100	1110.31 ± 98.56	118.58 ± 34.17	112.65 ± 22.18	5.93 ± 0.53	10.68 ± 0.43	5.00 ± 1.35
200	2253.81 ±197.29	198.11 ± 42.75	176.65 ± 32.13	21.46 ± 2.18	8.79 ± 0.23	10.83 ± 2.50
300	2001.19 ± 214.65	119.27 ± 25.83	89.43 ± 15.27	29.84 ± 3.92	5.96 ± 0.70	25.52 ± 1.27
400	2000.23 ± 242.91	79.21 ± 21.12	53.28 ± 9.36	25.93 ± 2.76	3.96 ± 0.14	32.73 ± 2.16
500	2358.38 ± 345.62	24.76 ± 3.15	14.79 ± 1.76	9.97 ± 1.51	1.05 ± 0.01	40.26 ± 1.28

Each value represented Mean ± SEM of n = 10 replicates.

MI = $\left(\frac{\text{TDC}}{\text{TC}} \text{ x}100\right)$, CA = $\left(\frac{\text{TAC}}{\text{TDC}} \text{ x}100\right)$

FCP = fermented cotyledon polysaccharides, FSCP = fermented seed coat polysaccharides, UCP = unfermented cotyledon polysaccharides, USCP = unfermented seed coat polysaccharides

Table 4: Mitotic Indices in Root Cells of *Allium cepa* Treated with FSCP

Concentration (µg/ml)	Total Number of Cells (TC)	Total Dividing Cells (TDC)	Total Normal Cells (TNC)	Total Abnormal Cells (TAC)	Mitotic Index (MI)	Chromosomal Aberration (CA)
0.00 (Control)	2207.56 ± 253.24	418.99 ± 56.77	418.99 ± 56.77	0.00 ± 0.00	18.98 ± 0.54	00.00 ± 0.00
100	1920.56 ± 323.71	336.87 ± 35.68	307.26 ± 28.42	29.61 ± 4.23	17.54 ± 0.17	8.79 ± 0.92
200	1728.23 ± 198.35	199.26 ± 42.19	174.41 ± 33.59	24.83 ± 5.83	11.53 ± 1.11	12.46 ± 1.47
300	1661.89 ± 226.89	134.95 ± 33.45	109.89 ± 25.68	25.06 ± 3.49	8.12 ± 0.29	18.57 ± 0.88
400	1557.19 ± 127.45	105.42 ± 29.63	81.68 ± 22.53	23.74 ± 4.22	6.77 ± 0.92	22.52 ± 1.11
500	1348.47 ± 201.61	52.19 ± 9.12	36.57 ± 5.94	15.62 ± 2.78	3.87 ± 0.84[a]	29.93 ± 0.96

See footnote of Table (3)

Table 5: Mitotic Indices in Root Cells of *Allium cepa* Treated with UCP

Concentration (µg/ml)	Total Number of Cells (TC)	Total Dividing Cells (TDC)	Total Normal Cells (TNC)	Total Abnormal Cells (TAC)	Mitotic Index (MI)	Chromosomal Aberration (CA)
0.00 (Control)	2207.56 ± 253.24	418.99 ± 56.77	418.99 ± 56.77	0.00 ± 0.00	18.98 ± 0.54	00.00 ± 0.00
100	2072.51 ± 356.72	583.20 ± 65.17	572.76 ± 51.03	10.44 ± 2.34	28.14 ± 2.20	1.79 ± 0.01
200	1960.66 ± 217.52	442.52 ± 61.82	430.62 ± 55.60	11.90 ± 1.06	22. 57 ± 1.46	2.69 ± 0.34
300	1915.24 ± 245.14	324.06 ± 48.76	306.04 ± 38.79	18.02 ± 2.57	16.92 ± 1.58	5. 56 ± 0.26
400	1642.11 ± 156.39	166.35 ± 36.75	153.16 ± 32.82	13.19 ± 1.94	10.13 ± 0.57	7.93 ± 1.02
500	1495.37 ± 176.23	99.89 ± 23.61	89.33 ± 21.84	10.56 ± 1.11	6.68 ± 0.58[a]	10.57 ± 0.53

See footnote of Table (3)

Table 6: Mitotic Indices in Root Cells of *Allium cepa* Treated with USCP

Concentration (µg/ml)	Total Number of Cells (TC)	Total Dividing Cells (TDC)	Total Normal Cells (TNC)	Total Abnormal Cells (TAC)	Mitotic Index (MI)	Chromosomal Aberration (CA)
0.00 (Control)	2207.56 ± 253.24	418.99 ± 56.77	418.99 ± 56.77	0.00 ± 0.00	18.98 ± 0.54	00.00 ± 0.00
100	1779.77 ± 261.41	631.28 ± 178.34	626.55 ± 56.81	4.73 ± 0.03	35.47 ± 1.01	0.75 ± 0.01
200	1501.34 ± 181.56	422.78 ± 99.23	418.72 ± 76.55	4.06 ± 0.11	28.16 ± 1.48	0.96 ± 0.06
300	1472.19 ± 214.58	297.68 ± 57.38	291.55 ± 32.47	6.13 ± 0.32	20.22 ± 0.26	2.06 ± 0.04
400	1548.42 ± 201.23	196.80 ± 42.17	188.93 ± 26.71	7.87 ± 0.27	12.71 ± 0.25	4.00 ± 0.29
500	1484.69 ± 222.16	121.30 ± 27.62	117.45 ± 23.41	3.85 ± 0.02	8.17 ± 0.92[a]	3.17 ± 0.08

Each value represented Mean ± SEM of n = 3 replicates.

MI = $(\frac{\text{TDC}}{\text{TC}}$ x100), CA = $(\frac{\text{TAC}}{\text{TDC}}$ x100)

FCP = fermented cotyledon polysaccharides, FSCP = fermented seed coat polysaccharides, UCP = unfermented cotyledon polysaccharides, USCP = unfermented seed coat polysaccharides

Table 7: Summary of *Allium cepa* Mitotic Indices and Chromosomal Aberration for the Polysaccharides

Concentration (µg/ml)	FCP		FSCP		UCP		USCP	
	Mitotic index (MI)	Chromosomal aberration (CA)	Mitotic index (MI)	Chromosomal aberration (CA)	Mitotic index (MI)	Chromosomal aberration (CA)	Mitotic index (MI)	Chromosomal aberration (CA)
0.00 (Control)	18.98 ± 0.54	0.00 ± 0.00	18.98 ± 0.54	0.00 ± 0.00	18.98 ± 0.54	0.00 ± 0.00	18.98 ± 0.54	0.00 ± 0.00
100	10.68 ± 0.43	5.00 ± 1.35	17.54 ± 0.17	8.79 ± 0.92	28.14 ± 2.20	1.79 ± 0.01	35.47 ± 1.01	0.75 ± 0.01
200	8.79 ± 0.23	10.83 ± 2.50	11.53 ± 1.11	12.46 ± 1.47	22. 57 ± 1.46	2.69 ± 0.34	28.16 ± 1.48	0.96 ± 0.06
300	5.96 ± 0.70	25.52 ± 1.27	8.12 ± 0.29	18.57 ± 0.88	16.92 ± 1.58	5. 56 ± 0.26	20.22 ± 0.26	2.06 ± 0.04
400	3.96 ± 0.14	32.73 ± 2.16	6.77 ± 0.92	22.52 ± 1.11	10.13 ± 0.57	7.93 ± 1.02	12.71 ± 0.25	4.00 ± 0.29
500	1.05 ± 0.01[a]	40.26 ± 1.28	3.87 ± 0.84[a]	29.93 ± 0.96	6.68 ± 0.58[a]	10.57 ± 0.53	8.17 ± 0.92[a]	3.17 ± 0.08

Each value represented Mean ± SEM of n = 10 replicates. The values with alphabet superscripts are statistically significant at $p < 0.05$, a compares Control with the rest.

FCP = fermented cotyledon polysaccharides, FSCP = fermented seed coat polysaccharides, UCP = unfermented cotyledon polysaccharides, USCP = unfermented seed coat polysaccharides

Table 8: Change in Mitotic Index (MI) Relative to Control

Concentration (µg/ml)	FCP	FSCP	UCP	USCP
	Change in MI (%)	Change in MI (%)	Change in MI (%)	Change in MI (%)
100	↓56.27	↓92.44	↑148.31	↑187.86
200	↓46.31	↓60.78	↑118.92	↑148.38
300	↓31.38	↓42.80	↓89.17	↑106.52
400	↓20.85	↓35.65	↓53.40	↓66.99
500	↓5.54	↓20.41	↓35.20	↓43.07

Key: ↓ represents decrease relative to control, ↑ represents increase relative to control

FCP = fermented cotyledon polysaccharides, FSCP = fermented seed coat polysaccharides, UCP = unfermented cotyledon polysaccharides, USCP = unfermented seed coat polysaccharides.

The statistical analyses of the concentration-dependent reduction in mitotic indices (MI) of *Allium cepa* root tip meristem cells treated with different concentrations of FCP, FSCP, UCP and USCP for 48 hours showed the difference was significant in Pearson's correlation analysis where correlation coefficient (r) was -0.9976 with significance level $p <$

0.0001 and 95 % confidence interval (CI) for r -0.9999 to -0.9624 for FCP, correlation coefficient (r) was -0.9688 with significance level $p < 0.0001$ and 95 % confidence interval (CI) for r -0.9980 to -0.5957 for FSCP, correlation coefficient (r) was -0.9963 with significance level $p < 0.0001$ and 95 % confidence interval (CI) for r -0.9998 to -0.9423 for UCP, and correlation coefficient (r) was -0.9961 with significance level $p < 0.0001$ and 95 % confidence interval (CI) for r -0.9998 to -0.9391 for USCP.

Plate 1 showed some of the observed chromosomal aberrations like chromosome laggards, spindle disturbance and extended cell at metaphase, anaphase clumping, anaphasic bridge, and chromosomal breakage at anaphase.

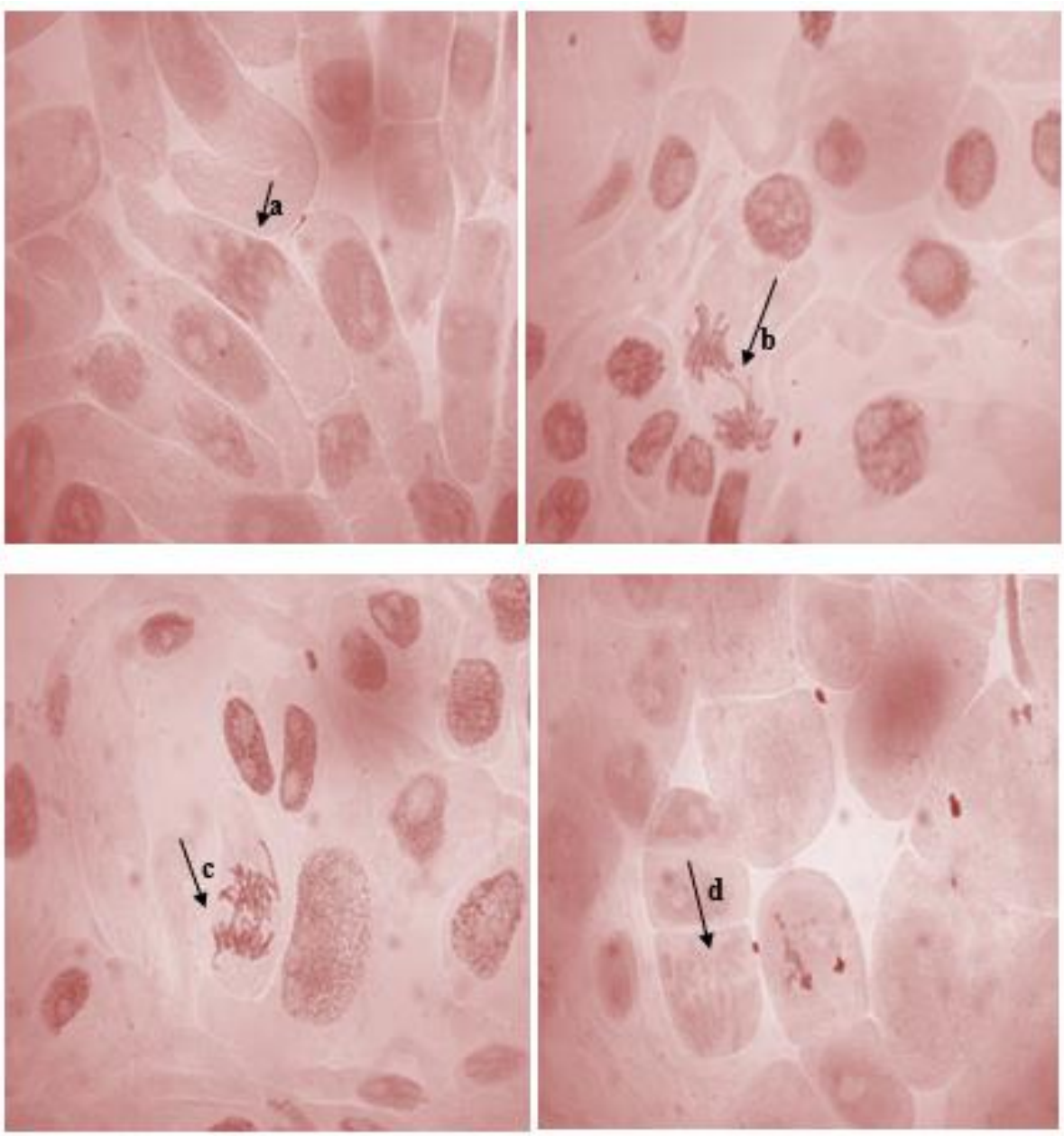

Plate 1: Some Observed Chromosomal Aberrations in *Allium cepa* Roots Treated with the Polysaccharides at x 100

a" is chromosome laggards, spindle disturbance, and extended cell at metaphase, "**b**" is the formation of anaphasic bridge "**c**" is anaphase clumping and formation of anaphasic bridge, "**d**" is chromosome breakage at anaphase.

DISCUSSION

The *Allium cepa* test is an important test, where the roots grow in direct contact with the substances of interest enabling possible damage to the DNA of humans to be predicted. The *Allium cepa* assay involved measurements of the length of the root and chromosome damage and proved to be a suitable model system for the measurement of environmental cytogenotoxic potential of pollutants (Olorunfemi *et al.*, 2015). Rubeena and Thoppil, (2020) also reported that analysis of metaphase chromosomes is an important and most widely used method to assess the mutagenic potential of a given agent.

From this study, the fermented cotyledon polysaccharides (FCP) elicited the lowest mitotic index and highest chromosomal aberration and unfermented seed coat polysaccharides (USCP) with the highest mitotic index and lowest chromosomal aberration at the highest concentration used (500 µg/ml) (Table 6). The root growth inhibition could have occurred as a result of the inhibition of cell division (indicating toxicity) and it is thus an index for estimating general toxicity. It occurs when roots are exposed to extreme pH, or to substances that prevent nutrient uptake (Owolarafe *et al.*, 2020). The inhibitory effects could also be due to cell extension, that is, cessation of root elongation which is correlated with the disappearance of mitotic figures. It has been observed that some mechanisms associated with cell division are highly sensitive to certain chemicals or metals and are permanently damaged by short exposures (Jaskowiak, *et al.*, 2018). Thus, the root growth analysis results indicated that all the polysaccharides investigated possessed and exhibited cytotoxic effects on the roots of *A. cepa*, and the polysaccharides might be inhibiting the rate of cell divisions at the meristem, thereby the root growth is either inhibited or slowed down. The cytological analyses of *A. cepa* roots exposed to different concentrations (0-500 µg/ml) of the polysaccharides examined were carried out. When compared to the negative control value of 18.98 ± 0.54%, there was a statistically significant ($p < 0.05$) decrease in mitotic index (MI) with increasing polysaccharides concentration in the root length of onions treated with the four polysaccharides investigated. The mitotic index thus gives an insight into the inhibition of cell division and it is observed microscopically by counting the number of cells in metaphase, which is an index of the meristematic cell. The mitotic index, which is defined as the ratio of the number of cells in mitosis and the total number of cells, is an indirect measurement of cell proliferation. It is considered to reliably identify the presence of cytotoxic pollutants in the environment (Wijeyaratne and Wickramasinghe, 2020).

The dose-dependent inhibition of the mitotic indices in the root of *A. cepa* could be due to intracellular stress like DNA damage, preventing cells from entering mitosis. It could also be due to a negative interference of the test compounds with DNA synthesis, microtubule formation, impaired nucleoprotein synthesis and reduced level of ATP to provide energy for spindle elongation, microtubule dynamics and chromosomal movements (Madić *et al*., 2019).

A mitotic index (MI) decrease below 22% of negative control causes lethal effects on test organisms while a decrease below 50% has sublethal effects which are called cytotoxic limit value (Sharma and Vig, 2012; Rubeena and Thoppil, 2020). Several investigators have employed MI as an endpoint for the evaluation of genotoxicity or antigenotoxicity of different chemical treatments (Sharma *et al.*, 2012; Bhat *et al.*, 2019). At the highest concentration used (500 µg/ml), MI of FCP treated *Allium cepa* root decreased by 5.54% below control, FSCP decreased 20.41% below control, UCP decreased 35.20% below control, and USCP decreased 43.07% below control (Table 7). Thus, the mitotic indices revealed that FCP was lethal at 400 and 500 µg/ml and sublethal at 200 and 300 µg/ml. FSCP was lethal at 500 µg/ml and sublethal at 300 and 400 µg/ml. Those of UCP and USCP on the other hand were sublethal at 500 µg/ml concentrations, UCP did not exhibit

any lethal effect below 300 µg/ml and USCP did not exhibit any lethal effect below 400 µg/ml. These established that the tested polysaccharides all possessed genotoxic activities at the highest concentration (500 µg/ml) used and are potential anticancer agents that can cause chromosomal damage on cancerous cells, thus induce apoptosis.

The microscopic analysis of the root tip cells of *A. cepa* exposed to the polysaccharides showed that chromosomal aberrations were induced in the root tip cells of *A. cepa* exposed to the extracted polysaccharides at different concentrations and no aberration was observed in the control group. Most aberrations were observed in *A. cepa* cells exposed to fermented cotyledon polysaccharides (FCP) indicating that it is the most toxic of the polysaccharides investigated (Table 6). The variation of chromosomal aberrations with the different polysaccharides was however dose-dependent. A possible explanation for this is that with increasing concentration, and consequently increasing toxicity, there was an inhibitory effect on cell division. This might result in prophase arrest with the attendant decline in the observation of chromosome aberration (Okereke *et al.*, 2020).

In conclusion, this study provided pieces of evidence backed up by scientific data on the genotoxic potentials of polysaccharides from fermented and unfermented *A. squamosa* cotyledon and coat and the enhancive role of fermentation to bioactivities of phytochemicals.

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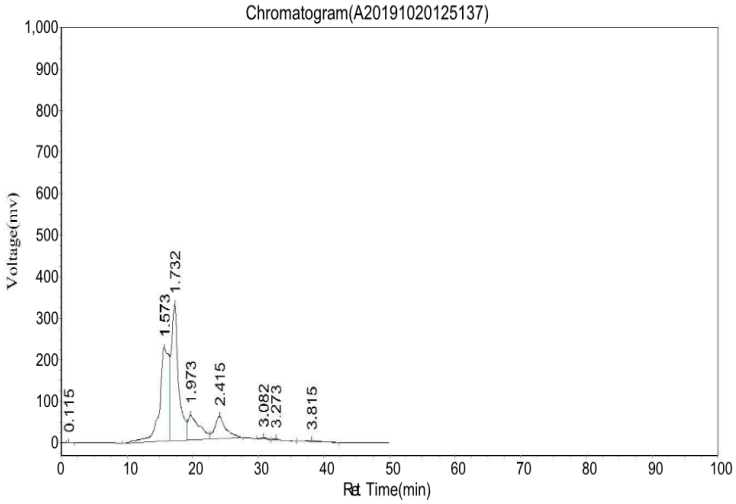
APPENDICES

Appendix 1: Monosaccharides HPLC Chromatogram for FSCP

Instrument:LC
Column Temp.(¡æ)£°40

Gradient:

Detector:UV
Wavelength(nm)£°310



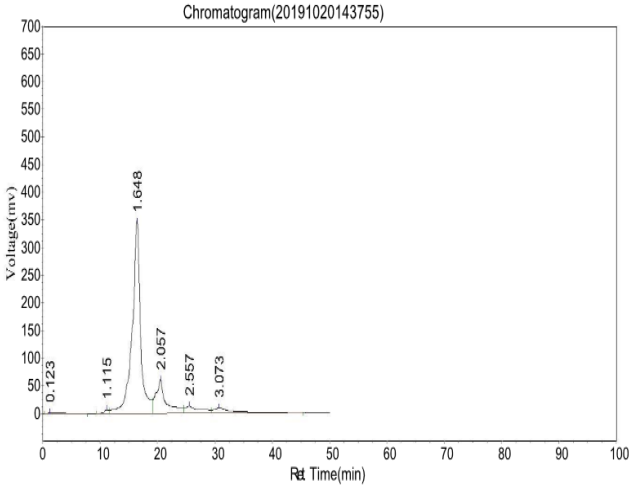
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	Unidentified	0.115	445.429	1654.400	00.027
2	Rhamnose	1.573	225427.594	2289845.750	36.801
3	Mannose	1.732	325951.594	2506970.750	40.291
4	Fructose	1.973	60302.398	737002.063	11.845
5	Fucose	2.415	53697.094	621000.000	09.981
6	Glucose	3.082	2500.507	21173.156	00.340
7	Arabinose	3.273	2500.562	16428.396	00.264
8	Xylose	3.815	2191.169	28150.199	00.452
Total			**673775.346**	**6222224.714**	**100.000**

Appendix 2: Monosaccharides HPLC Chromatogram for UCP

Instrument:LC
Column Temp.(¡æ)£°65

Gradient:

Detector:UV
Wavelength(nm)£°310



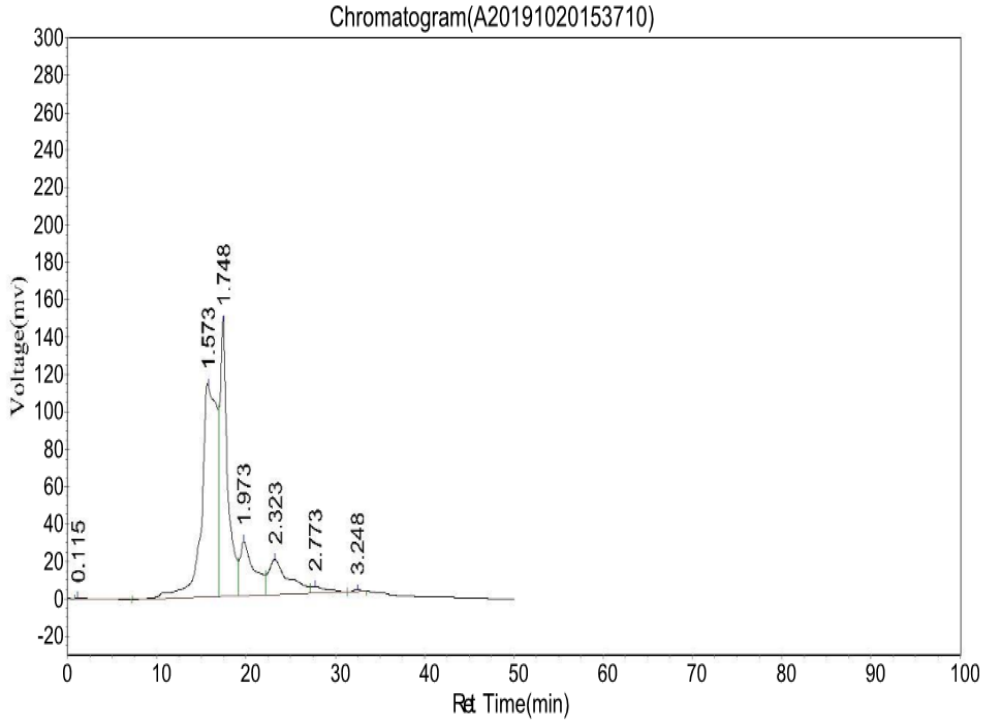
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	Unidentified	03.123	977.933	16545.400	00.341
2	Rhamnose	11.115	6305.861	37002.906	00.764
3	Fructose	15.648	346831.625	3564481.375	73.561
4	Fucose	22.057	61017.625	752821.313	15.536
5	Glucose	25.557	12638.542	233018.578	04.809
6	Xylose	31.073	8811.514	241766.469	04.989
Total			**436582.850**	**4376662.727**	**100.000**

Appendix 3: Monosaccharides HPLC Chromatogram for USCP

Instrument:LC
Column Temp.(¡æ)£º65

Gradient:

Detector:UV
Wavelength(nm)£º310



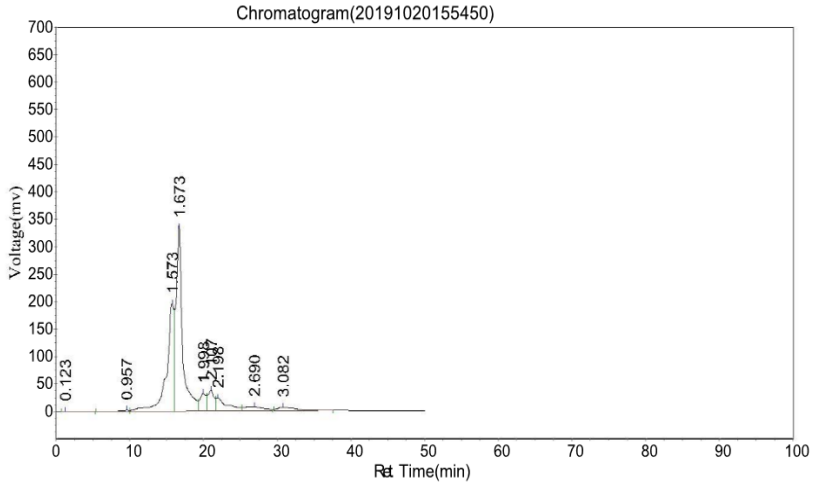
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	Rhamnose	10.115	385.474	5721.600	00.196
2	Mannose	14.573	113711.125	1340310.625	45.817
3	Fructose	19.748	148884.203	919059.313	31.417
4	Glucose	26.973	28862.158	315020.875	10.769
5	Unidentified	28.323	18727.311	292852.344	10.011
6	Arabinose	29.773	3514.221	46474.246	01.589
7	Xylose	32.248	1015.120	5934.600	00.203
Total			**315099.612**	**2925373.603**	**100.000**

Appendix 4: Monosaccharides HPLC Chromatogram for FCP

Instrument:LC
Column Temp.(¡æ)£º65

Gradient:

Detector:UV
Wavelength(nm)£º310



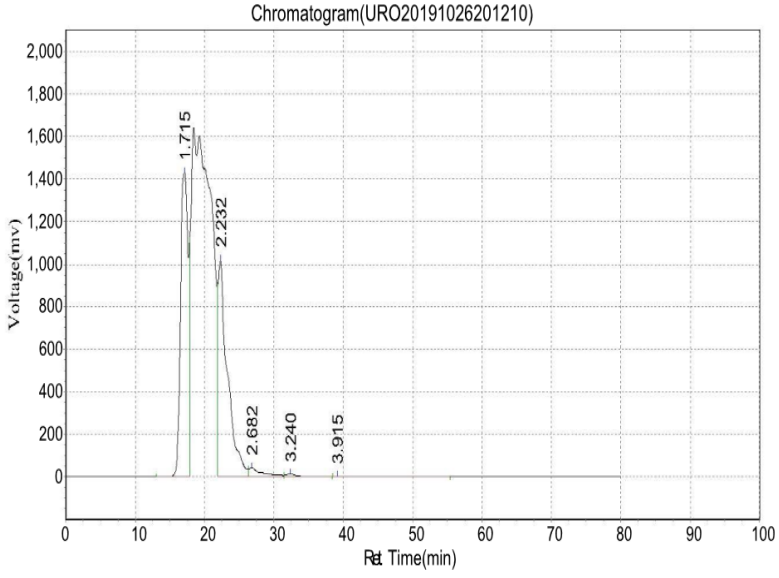
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	Unidentified	0.123	230.055	2800.450	00.064
2	Rhamnose	0.957	1583.958	12836.973	00.293
3	Mannose	1.573	195382.781	1400317.500	31.995
4	Fructose	1.673	337044.219	2031113.375	46.408
5	Fucose	1.998	32179.855	177404.250	04.053
6	Glucose	2.107	37867.402	240668.141	05.499
7	Arabinose	2.198	23428.715	248973.563	05.689
8	Galactose	2.690	7667.746	143019.547	03.268
9	Xylose	3.082	6571.347	119528.930	02.731
Total			**641956.079**	**4376662.727**	**100.000**

Appendix 5: Uronic Acids HPLC Chromatogram for UCP

Instrument:LC
Column Temp.(¡æ)£º35.1

Gradient:

Detector:UV
Wavelength(nm)£º245



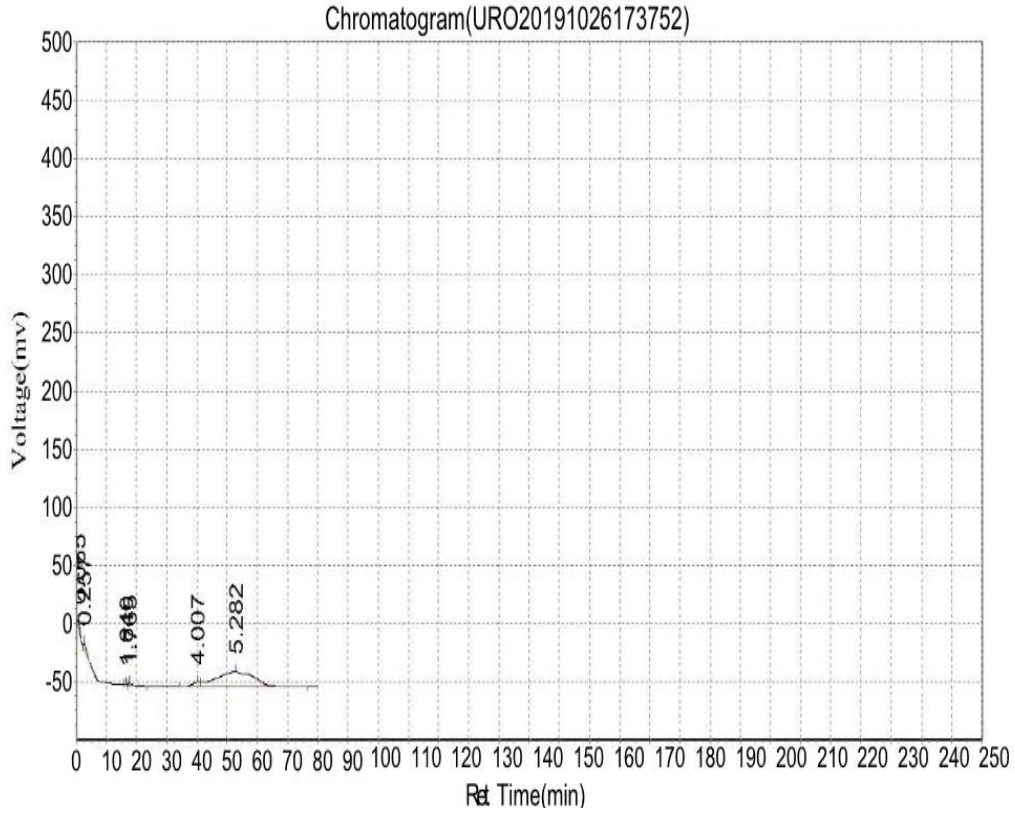
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	D-Mannuronic	1.715	1430209.125	11345963.000	51.823
2	D-glucuronic	2.232	1020558.313	9606848.000	43.880
3	Unidentified	2.682	44705.992	614597.125	02.807
4	Unidentifed	3.240	14381.701	246838.125	01.127
5	Unidentified	3.915	3092.734	79395.891	00.363
Total			**2512947.865**	**21893642.141**	**100.000**

Appendix 6: Uronic Acids HPLC Chromatogram for FCP

Instrument:LC
Column Temp.(¡æ)£º35

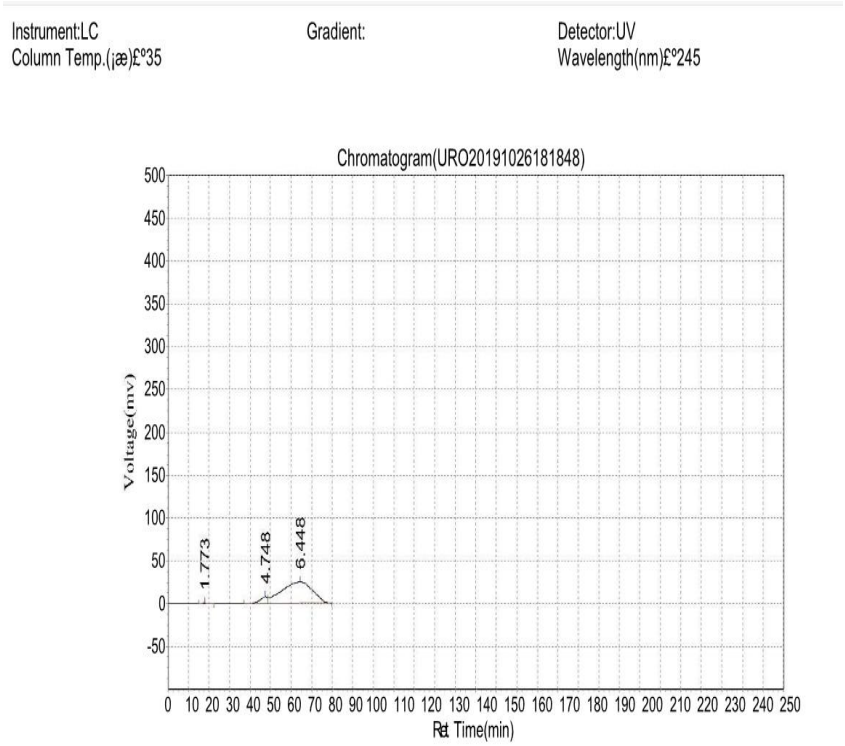
Gradient:

Detector:UV
Wavelength(nm)£º245



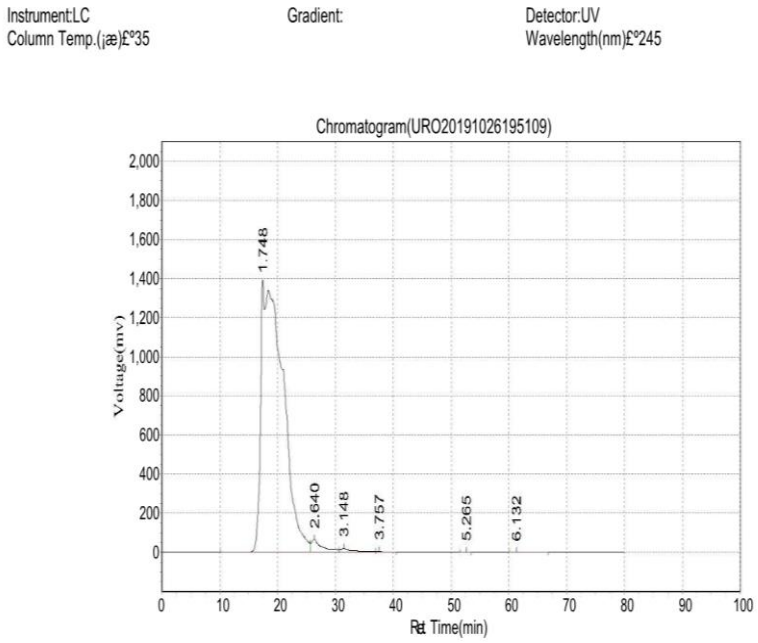
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	D-mannuronic	0.065	5595.521	27473.350	02.234
2	Unidentified	0.257	5059.222	32850.051	02.671
3	D-alluronic	1.640	401.756	1603.499	00.130
4	D-galacturonic	1.765	1332.922	5612.274	00.456
5	Unidentified	4.007	3442.980	62583.391	05.088
6	D-glucuronic	5.282	12778.194	1099945.625	89.422
Total			**28610.596**	**1230068.189**	**100.000**

Appendix 7: Uronic Acids HPLC Chromatogram for USCP



Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	D-mannuronic	1.773	781.557	10407.100	00.369
2	D-galacturonic	4.748	8329.355	205449.688	07.275
3	D-glucuronic	6.448	24846.596	2608055.500	92.356
Total			**33957.510**	**282391.287**	**100.000**

Appendix 8: Uronic Acids HPLC Chromatogram for FSCP



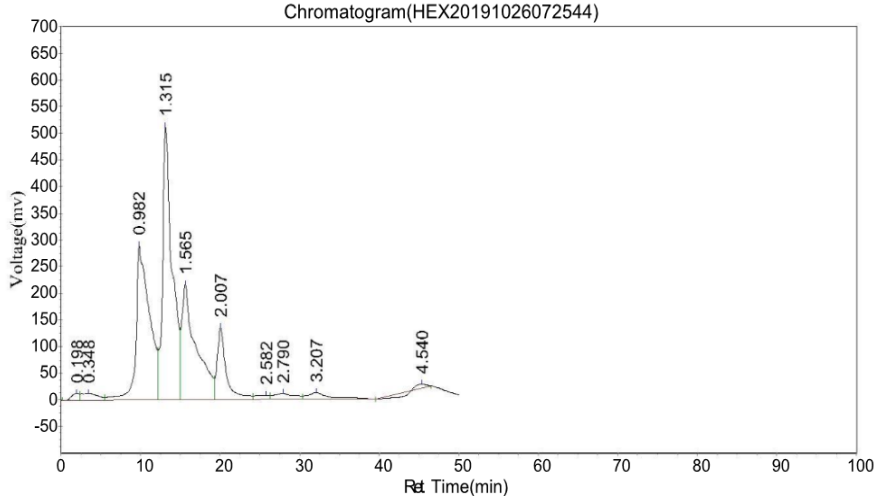
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	D-mannuronic	1.748	1381587.250	37059924.000	97.004
2	Unidentified	2.640	65173.234	833747.750	02.182
3	D-alluronic	3.148	18921.449	281595.719	00.737
4	D-galacturonic	3.757	3116.249	27401.258	00.072
5	Unidentified	5.265	68.043	425.100	00.001
6	D-glucuronic	6.132	121.037	1515.950	00.004
Total			**1468987.263**	**38204609.777**	**100.000**

Appendix 9: Hexosamines HPLC Chromatogram for UCP

Instrument:LC
Column Temp.(¡æ)£º40

Gradient:

Detector:UV
Wavelength(nm)£º265



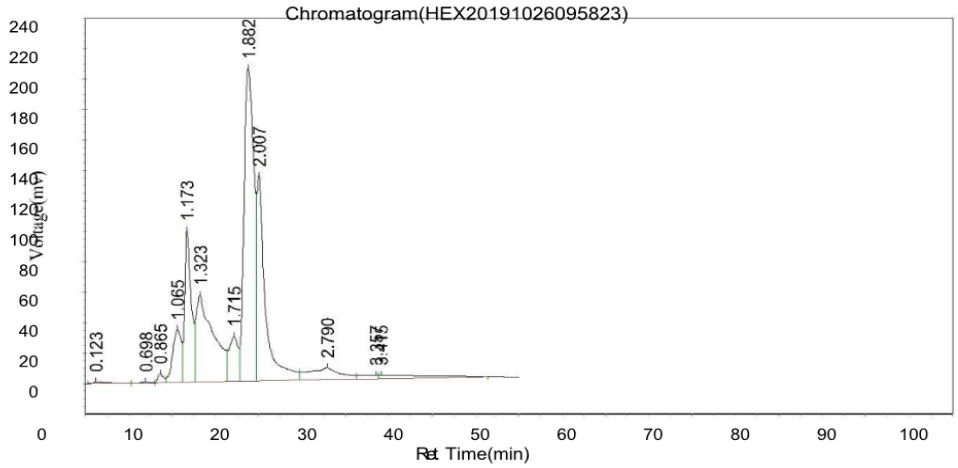
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	Unidentified	0.198	12007.768	83391.133	00.677
2	Unidentified	0.348	12449.396	176936.688	01.436
3	Galactosamine	0.982	287108.031	3424799.500	27.801
4	Fructosamine	1.315	511051.438	428691.500	34.814
5	Glucosamine	1.565	215593.484	2704858.250	21.957
6	Unidentified	2.007	134740.844	1060281.625	08.607
7	Unidentified	2.582	8090.744	96191.602	00.781
8	Unidentified	2.790	10784.860	199505.875	01.619
9	Unidentified	3.207	11796.860	221161.578	01.795
10	Unidentified	4.540	6406.831	63189.801	00.513
Total			**1209993.512**	**12319007.551**	**100.000**

Appendix 10: Hexosamines HPLC Chromatogram for FCP

Instrument:LC
Column Temp.(¡æ)£°40

Gradient:

Detector:UV
Wavelength(nm)£°265



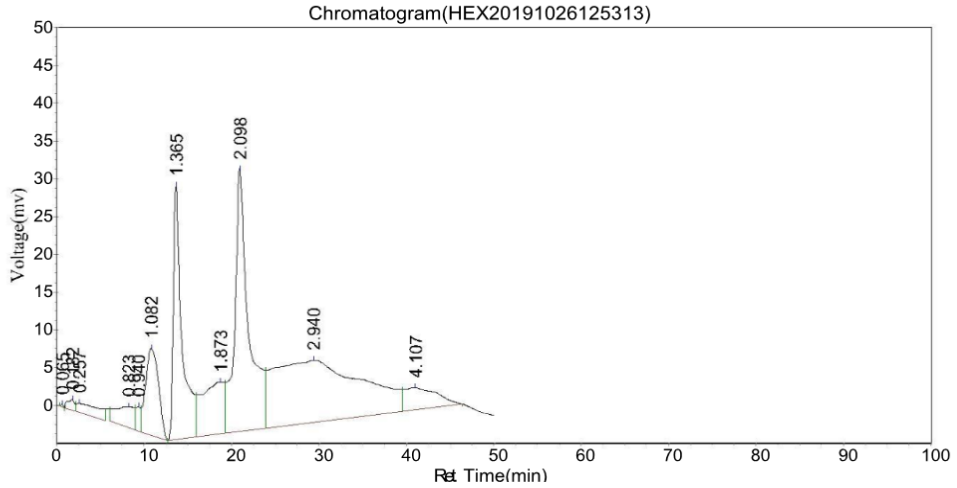
Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	Unidentified	0.123	1015.033	11422.500	00.249
2	Unidentified	0.698	568.517	4205.920	00.092
3	Galactosamine	0.865	5953.035	27144.375	00.592
4	Fructosamine	1.065	34357.656	241039.109	05.254
5	Unidentified	1.173	99339.242	517947.375	11.290
6	Unidentified	1.323	56745.207	797657.000	17.387
7	Unidentified	1.715	29422.172	211373.938	04.607
8	Glucosamine	1.882	205381.688	1614282.250	35.188
9	Unidentified	2.007	134867.828	930800.000	20.289
10	Unidentified	2.790	7731.759	196599.656	04.285
11	Unidentified	3.357	2325.517	35173.602	00.767
Total			**577707.654**	**4587645.725**	**100**

Appendix 11: Hexosamines HPLC Chromatogram for USCP

Instrument:LC
Column Temp.(¡æ)£°40

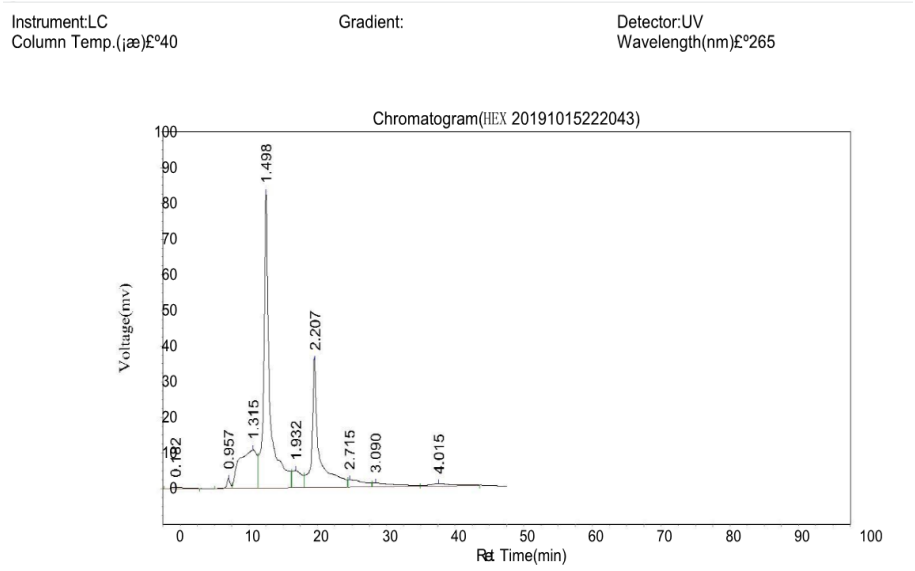
Gradient:

Detector:UV
Wavelength(nm)£°265



Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	Unidentified	0.065	283.571	465.600	00.29
2	Unidentified	0.182	1458.493	8118.241	00.508
3	Galactosamine	0.257	1196.169	26674.523	01.670
4	Fructosamine	0.823	2772.944	41847.660	02.620
5	Unidentified	0.940	3217.662	12524.015	00.784
6	Unidentified	1.082	11426.605	117298.609	07.344
7	Unidentified	1.365	33129.625	231398.844	14.488
8	Unidentified	1.873	6822.099	121149.445	07.585
9	Glucosamine	2.098	34816.637	386439.469	24.195
10	Unidentified	2.940	8250.386	576490.438	36.094
11	Unidentified	4.107	2907.356	74803.492	04.422
Total			**106280.548**	**1597210.336**	**100**

Appendix 12: Hexosamines HPLC Chromatogram for FSCP



Peak No	Peak Name	Ret Time	Height	Area	Concentration (%)
1	Galactosamine	0.182	381.290	3964.700	00.364
2	Unidentified	0.957	2691.186	8888.315	00.817
3	Fructosamine	1.315	10788.665	187414.594	17.227
4	Unidentified	1.498	81077.469	511650.938	47.030
5	Glucosamine	1.932	4832.095	46473.852	04.272
6	Unidentified	2.207	36148.301	262820.250	24.158
7	Unidentified	2.715	2098.073	31394.320	02.886
8	Unidentified	3.090	1044.808	21367.150	01.964
9	Unidentified	4.015	654.952	13958.023	01.282
Total			**139716.840**	**1087932.142**	**100.000**