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Sulfated Cellulose from Agriculture Wastes, Anticoagulant, Fibrinolytic and Toxicological Studies

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ABSTRACT

This study was aimed to the optimum conditions for cellulose extraction from different agriculture wastes (rice straw, rice husk, wheat straw and sugar-cane bagasse) and the biological activity of sulfated cellulose. Sulfation of the isolated cellulose was achieved through adopting the method by using sulfuric acid as sulfation agent in presence of catalyst (D.C.C or 4-(Dimethylamino) pyridine. *In vitro* anticoagulation and fibrinolytic activities for sulfated cellulose were determined. The lysis percentages of the plasma clots at 37°C were recorded with each sample and compared to that of standard hemoclar. The fibrinolytic and anti-coagulation activities of different sulfated cellulose with and without catalyst were measured in order to verify the traditional uses of this modified cellulose. The FT-IR analysis was done to elucidating the product cellulose and sulfated cellulose. The highest substitution was in sulfated cellulose bagasse, rice straw, rice husk and wheat straw, respectively. It was concluded that addition of sulphate group into extracted cellulose enhance anticoagulation activities. The results indicated that the highest clotting time was found by using D.C.C catalyst at concentration of 500 μg mL^{-1}. The lowest clotting time was reported without catalyst sulfated celluloses at same concentration. Determination of fibrinolytic effects of the modified sulfated cellulose revealed that they have good fibrinolytic activities. Sulfated bagasse cellulose in presence of catalyst whether D.C.C or 4-(Dimethylamino) pyridine exhibited fibrinolytic activities equivalent to the same amount of standard "Hemoclar" preparation at concentration of 2000 μg mL^{-1}. No cytotoxic effect of sulfated cellulose was observed on VERO cells. Results indicated that the LD_{50} of sulfated cellulose is more than 5000 mg kg^{-1} b.wt. and these compounds are practically non-toxic.

Key words: Agriculture waste, cellulose, sulfation, anticoagulant, fibrinolytic, toxicity

INTRODUCTION

Agricultural crop residues such as straws of rice, wheat, husk and bagasse, represent an enormous underutilized energy resource which has a great potential as feed for ruminants and also as raw materials for paper, chemicals and other technical products (Theander, 1985). Chemical

methods have been used to improve the digestibility of straws (Theander and Aman, 1980; Sahoo *et al*., 2002). Alkaline treatment of lignocellulosic substances disrupts the cell wall by dissolving hemicelluloses, lignin and silica by hydrolyzing uronic and acetic acid esters and by swelling cellulose (Jackson, 1977). This increase of the degradability of the cell walls is also due to the cleavage of the bonds between lignin and hemicelluloses or lignin and phenolic acids. In general, ammonium hydroxide (NH_4OH) usually produces a positive response (Todorov, 1974; Fadel Elseed *et al*., 2003) but is generally less effective than NaOH (Klopfenstein, 1978; Stacey, 1976). Chemical studies of straw and its components may provide decisive factors not only for its applicability but also for the economic feasibility of many industrial processes involving straw. Cellulose, hemicellulose and lignin are the main organic compounds which make up the biomass of agricultural by-products Lignocellulosic Materials (LM). Therefore, the present study intended to determination the chemical compositions of agriculture wastes (rice straw and husk, wheat straw and sugar-cane bagasse) and then qualitative and quantitative total carbohydrates after acid hydrolysis. Cellulose was isolated by different alkaline concentration sodium hydroxide and temperature to determine the optimum conditions. The α-cellulose was isolated from four hollocellulose samples by purification. For isolation and utilization of cellulose from straws in a pure and undergirded form, its polymer properties need to be studied; however, no reasonably simple methods for elucidation of polymer properties are available. There are many technologies to characterize the physicochemical properties of cellulose, one of them is FTIR.

The blood compatibility of cellulosic materials is limited (Cordonnier and Foret, 1989; Colton, 1987). Among the related processes, thrombosis is a major problem (Cheung and Henderson, 1986). Thrombus formation is a life-threatening event resulting from a series of enzymatic hydrolysis steps known as the blood coagulation cascade. The serpin Anti-Thrombin (AT) is the most important regulator of the coagulation enzymes under physiological conditions. Its activity is drastically enhanced after complexation with polyanionic polysaccharides such as heparin (Linhardt and Toida, 1997). Although, chemically bound heparin shows a reduced biological activity compared to free heparin (Byun *et al*., 1996), the blood compatibility of cellulos was found to be improved upon heparinization (Cheung *et al*., 1992). Beyond that, partially sulfated cellulose was considered promising for biomaterials (Baumann *et al*., 2003). Due to their action on AT, such sulfated cellulose were demonstrated to reduce the coagulation processes at the blood/material interface (Baumann *et al*., 2000; Baumann, 2001). However, neither the Degree of Polymerization (DP) of the cellulose chain nor the Degree of Sulfation (DS) were so far systematically studied with respect to the bioactivity of the obtained coatings. Since, these characteristics influence the structure and AT affinity of the cellulose layers knowledge about the relevance of these parameters appeared important for the intended application of this approach in the surface modification of biomaterials, we performed a series of experiments to explore the impact of these features.

MATERIALS AND METHODS

Agriculture wastes collection: Rice straw, husk and wheat straw were collected from Belbes, Sharkya while sugar-cane bagasse from Upper Egypt. The collected agriculture straw wastes were dried and then milled into powder in a grinding machine.

Chemicals: Heparin sodium salt was purchased from Sigma-Aldrich Chemical Co., USA; Hemoclar from Clin-Midy, Paris, France. Human plasma was purchased from the "Egyptian Organization for Biological and Vaccine Production", Egypt. All other chemicals were of reagent grades and obtained from the local scientific distributors in Egypt.

Animals: Male albino rats, weighing 200 g±10 were obtained from the Animal Breeding House of the National Research Centre (NRC), Dokki, Cairo, Egypt and housed in clean plastic cages. Rats were acclimatized under laboratory conditions at room temperature of 20±2.0°C for one week. Food and water were provided *ad-libitum*.

Chemical composition: Chemical composition (e.g., moisture, ash, crude protein, crude lipids, wax and Low-Molecular Weight Carbohydrates (LMWC) was determined according to AOAC (1970).

Total carbohydrates content: Total carbohydrates were determined as glucose, using the phenol-sulphuric acid method (Dubois *et al.*, 1956). After suitable dilution, 1 mL 5% phenol solution was added to 1 mL of the resulted diluted solution and then 5 mL conc. H_2SO_4 were added rapidly to the mixture, shaked and set aside for 10 min at room temperature, then at 20-30°C (in water bath) for 20 min. Thereafter, the color density was measured at 480 nm for pentoses and 490 nm for hexoses.

Acid hydrolysis by sulphuric acid (H_2SO_4): Complete acid hydrolysis of the plant material residues polysaccharides was carried out according to the modified method by Fischer and Dorfel (1955). The 0.5 mg of the (rice straw, rice husk, wheat straw and/or bagasse) material were carefully stirred with 0.5 mL ice cold 80% H_2SO_4 to give a paste and was kept at room temperature for 15 h. Then diluted with a mixture of ice and distilled water (upto 605 mL) until the strength of sulphuric acid reached 2N. The solution was further hydrolyzed by heating in a sealed tube in a boiled water bath for 6 h. The resulted hydrolyzate was neutralized by addition of a calculated amount of $BaCO_3$. The precipitate was filtered and thoroughly washed with water. The filtrate and washings were treated by a cation exchange resin, Amberlit IR-120(H^+).

Acid hydrolysis by trifuloroacetic acid: The 0.1 mg of the (rice straw, rice husk, wheat straw and/or Bagasse) materials were dissolved in 10 mL 1N and/or 6N Trifuloroacetic acid. The solution was further hydrolyzed by heating in a sealed tube in a boiled water bath for 8 h. The resulted hydrolyzate was filtered and thoroughly washed with water. The resulted solution in two cases (sulphuric and TFA) were concentrated and then subjected to qualitative and quantitative paper chromatography.

Qualitative examination of hydrolysis sugars: Qualitative examination of hydrolysis sugars was performed by chromatography of the resulted hydrolyzate on Whatman No.1 filter paper, using the solvent system: n-butanol-acetone-water (4:5:1) (Jayme and Knolle, 1956). Authentic samples of D-glucuronic acid, D-glucose, L-arabinose and D-xylose were co-chromatographed as reference substances. Detection of spots was achieved by spraying with aniline-phthalate reagent (Partridge, 1949).

Quantitative determination of the hydrolysis sugars: Quantitative determination of the hydrolysis sugars was done according to the modified method of Wilson (1959). After chromatographic separation, the chromatogram was air dried and dipped in 40-50 mL of the color reagent (1.66 g of O-phthalic acid and 0.91 mL aniline were dissolved in a mixture of 48 mL n-butanol, 48 mL diethyl ether and 4 mL water), air dried and then heated at 105°C for 10 min in an oven for developing the colored spots. The individual spots were cut off, divided into small strips

and dropped into 4 mL eluting agent (0.7 N HCl in 80% ethanol (v/v)) in test tubes and shaked for complete elution. The absorbances of the resulting colored solutions were determined at 390 nm for hexoses and 360 nm for pentoses. The quantities of sugars were determined by comparison to appropriate standard curves constructed under the same conditions.

Cellulose extraction and purification: Alkaline treatment of lignocellulosic substances such as rice straw, rice husk, wheat straw and bagasse disrupts the cell wall by dissolving hemicelluloses, lignin and silica and by swelling cellulose and by decreasing the crystallinity of cellulose. Different sodium hydroxide concentrations have been used for the extraction to determine which one is the best, depending up on the yield and total carbohydrate content. In 75 mL sodium hydroxide (Ph 12, 1, 4 and 18%) 5 g of rice straw, rice husk, wheat straw and/or bagasse separately were treated at (90, 55, 90 and 22°C), respectively for 1 h (liquid to solid ratio, 15 mL g^{-1}) for three times. The reaction mixture was filtrated, the residue (cellulose) washed more times by water till neutralization (Fig. 1). The α-cellulose was isolated from four crude cellulose samples (rice straw, husk, wheat and bagasse). The extracted cellulose was dissolved in 30% potassium hydroxide at

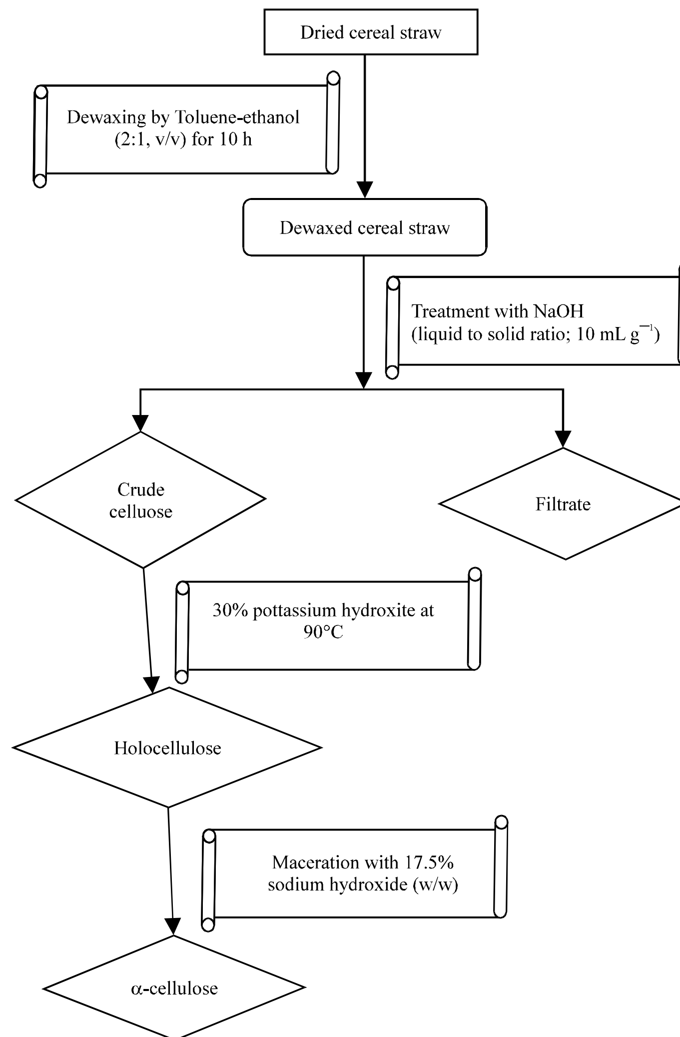

Fig. 1: Scheme for isolation of cellulose from plant material residues

90°C and then filtration. The residue washed thoroughly by distilled water till neutralization and completely deride at 50°C. The total carbohydrates were determined as glucose, using the phenol-sulphuric acid method (Dubois *et al.*, 1956) as previously described in determination of low-molecular weight carbohydrate IR spectra were collected directly from the cellulose powder on to a detector prism using a Bruker Vectra 22 FTIR spectrometer equipped with a DuraSampleIR II™ detector. All spectra were taken at a spectral resolution of 4 cm^{-1} between wave number range 4,000-400 cm^{-1}. DP was performed viscosimetrically after dissolving different α-cellulose in solvents, of which the most common are cupriethylenediamine (CED) and cadmiumethylenediamine (Cadoxen).

Sulfation of purified cellulose: This was achieved through adopting the modified method of Hussein (1994) by using sulfuric acid as sulfation agent. The 4.5 mL of H_2SO_4 was dropped in 8 mL freezed formamid in ice bath. Further, reaction mixture was poured into 0.3 g cellulose wetted by drops of formamid. The reaction mixture stirred overnight and then, diluted by water. Neutralization was occurred by 30% NaOH then dialysis and drying. The same reaction was repeated for two times using catalyst N, N'-Dicyclohexcarbodimid (D.C.C) and 4-(Dimethylamino) pyridine (D.A.P). The resulted sulfated polysaccharide (cellulose) was isolated from the reaction mixture by precipitation with methanol (3 volumes) and further purification by dissolving in water and re-precipitation with methanol.

Determination of sulfate ester groups: Firstly, cleavage the sulfate ester groups sulfated cellulose (5 mg) was hydrolyzed in a sealed tube with 1 mL HCl (1N) at 85°C for 24 h after cooling, the resulted hydrolyzate was neutralized to pH 7 with NaOH and the total volume was made up to 10 mL with distilled water (Larsen *et al.*, 1996). Secondly, Turbidimetric assay of the liberated sulfate, sulfate content (of the aforementioned hydrolyzate) was determined, adopting the turbidimetric method of Garrido (1964). To 10 mL of the analyzed solution (the hydrolyzate), 1 mL dilute HCL (0.3N) and 1 mL of barium-Tween 20 reagent was added. after mixing, standing for 15 min and remixing, the optical density of the mixture was read at 500 nm against blank containing distilled water instead of sulfate solution. The amounts of sulfate were obtained from a graph relating the optical density to the sulfate concentration.

Reaction with toluidine blue: Solutions of the investigated cellulose preparations were abilities to react with Toluidine Blue (TB) as a characteristic reagent for sulfated and other acidic polysaccharides. This was achieved according to Hussein and Helmy (2000) as follows: An aqueous solution (4 mL) containing 100 mg of the investigated polysaccharide material was titrated with an aqueous solution of TB (0.005%) until the reaction mixture reached Optical Density (OD): 1.00 at 600 nm the volume of TB solution used in this titration experiment is expressed as "Toluidine Blue Volume".

***In vitro* anti-coagulation activity of sulphated cellulose:** Adopting the method of US Pharmacopeia (1960), for the assay of sodium heparin, the anti-coagulation activities of the sulfated cellulose were used. Hard glass test tubes (31×100 mm) were cleaned by immersion overnight in chromic acid. To each tube was added either 0.8 mL of sample solution (0.01%), 0.8 mL of standard heparin sodium solution (1.4 U.S.P unite/0.8 mL) or 0.8 mL saline solution. To each of the prepared tubes, 1 mL plasma and 0.2 mL calcium chloride solution were added. The tubes were placed in water bath at 37°C. The time immediately recorded and each tube was stoppard. The time required for clotting was then determined as an average of three readings.

***In vitro* fibrinolytic activity of sulphated cellulose:** Fibrinolytic activity was determined by exposing a plasma clot to the effect of an aqueous solution (at suitable concentration) of the investigated polysaccharide sample. Preparation of the plasma clot was achieved under the same conditions mentioned previously for determination of anti-coagulation activity US Pharmacopeia (1960). Sets of three-hard glass test tubes (31×100 mm) were cleaned by immersion overnight in chromic acid. To each tube 0.8 mL saline solution (0.89% w/v), 1 mL plasma and 0.2 mL calcium chloride solution (1% w/v) were added. After mixing, the tubes were placed in water bath at 37°C and when clotting was complete, 1 mL of either the saline solution, hemoclar preparation (2 mg tube^{-1}), or the tested sample (2 mg tube^{-1}) was added individually. The lysis percentages of the plasma clots at 37°C were recorded with each sample and compared to that of standard Hemoclar.

Toxicological studies

***In vitro* cytotoxicity:** The VERO cells incubated into culture bottle were checked using inverted microscope for its proper physical conditions, i.e., sheet and normal shape. Then, the media overlaying cell monolayer was poured off. Cells can be released from tissue culture flask by treatment with about 5 mL pre-warmed trypsin-EDTA solution. [Trypsin cleaves cell surface proteins that the cells used to adhere to the flask. EDTA chelates metal ions that are involved in cell adherence], the flask was rocked so that trypsin completely cover the cell monolayer. The trypsin was aspirated with a pipette, then 2 mL of trypsin were dispensed, the bottle rocked and was incubated at 37°C. Cells were examined from time to time to avoid trypsin over action. The bottle was struck with hand to completely dislodge the cells from the bottle surface. Cells were suspended in about 8 mL of growth media. Use 10 mL pipette to disperse cell aggregates by sucking up and expel the cells about 4 times, expel the media with the tip of pipette pressed against the bottom of the bottle to ensure that no clumps of cells are present. Cells were counted using haemocytometer and using trypan blue vital stain. About 10 mL of 2×10^5 VERO cell suspension were transferred to 50 cm^3 TC bottle (Falcon) tightly closed then was incubated at 37°C. Cells were sub-cultured once weekly. For seeding 96 well plate, 0.1 mL (2×10^5 cells) was transferred to each flat bottomed well and incubated at 37°C for 24-48 h to develop a complete monolayer sheet.

Sulfated cellulose cytotoxicity: Sulfated cellulose cytotoxicity was determined according to Van den Berghe *et al.* (1978). Growth medium was decanted from 96 micro titer plate after confluent sheet of Vero cell were formed. Ten fold serial dilution of different extracts were made in MEM medium without FCS, starting from 10^0 (1 mg mL^{-1}) till 10^{-5} dilution. The 0.2 mL of each dilution was tested in three different wells leaving two wells/row as control, receiving only maintenance medium. Plate was incubated in incubator at 37°C and examined frequently for up to 3 days. Cells were checked for any physical signs of toxicity, e.g., partial or complete loss of monolayer, rounding, shrinkage or cell granulation. The Maximum Non-Toxic Concentration [MNTC] of each extract was determined and was used for further biological studies.

Acute toxicity studies: The acute toxicity studies were performed as per economic co-operation and development (OECD) 423 guidelines (OECD, 2001). Five male albino rats (n = 5) were used for each sulfated cellulose in this study. One rat for each sulfated cellulose was fasted overnight with free access to drinking water. It was given compound at dose of 5000 mg kg^{-1} and was observed for one day for mortality. The animal was survived after 24 h and then the other four

mice were given the same dose of each compound (5000 mg kg^{-1} b.wt.). All tested rats were observed for 24 h and daily for 14 days. After 14 day, the experimental protocols and procedures were approved by the Local Ethics Committee at the National Research Centre (NRC), Dokki, Cairo, Egypt.

RESULTS AND DISCUSSION

In the present investigation, it was rather of interest to survey agriculture wastes (rice straw, rice husk, wheat straw and bagasse) constituents including moisture, ash, lipids, wax, Low-Molecular Weight Carbohydrates (LMWC) and protein (Table 1). The results revealed that bagasse has the highest moisture content 21% and the lowest was rice husk 8.5%. Rice straw, rice husk, wheat straw and bagasse ash content was 18, 15, 9 and 4%, respectively. In case of rice husk lipids, protein and wax was high (5.1 and 1.65%) and LMWC in bagasse was 26.4%. The total carbohydrates content was determined for plant material residues after hydrolysis (Table 2). Strong and mild hydrolysis by sulfuric acid, 1N and 6N Tri-Fluoro Acetic Acid (TFA) was done, respectively. The best total carbohydrates results were obtained by 1N TFA (bagasse 55.9%, rice husk 47.3%, rice straw 38.7 and wheat straw 37.8) but percent content was low, so that may be some of them lost during dewaxing processes. As we predict, total carbohydrate content for wax samples was measured and gave us 40, 17, 25 and 15%, respectively. Ratios of Glucuroinc acid, Glucose, Arabinose and Xylose were determined according to paper chromatography analysis. Strong acid hydrolysis by sulphuric acid indicated just glucose due to over hydrolysis for arabinose and xylose (Table 3).

Isolation crude cellulose was done in different alkaline concentration (NaOH) and temperature (Table 4). Cellulose percent was decreased by increasing alkalinity and temperature. Increasing alkalinity and temperature was deformed some of cellulose and hemicelluloses, so that lignin percent was increased. From quantitative cellulose, hemicelluloses and lignin 1N NaOH and 55°C

Table 1: Chemical composition for plant material residues

	Chemical composition of plant residues (% w/w)					
Plant residues	Moisture	Ash	Lipids	LMWC	Wax	Protein
Rice straw	11.0±1.56	18±0.63	2.0±0.08	7.0±0.42	6.0±0.32	1.06±0.07
Rice husk	8.5±1.88	15±0.34	5.1±0.12	4.0±0.21	13.0±0.65	1.65±0.09
Wheat straw	7.0±0.18	9±0.25	2.0±0.09	5.0±0.23	9.0±0.41	0.71±0.02
Bagasse	21.0±1.09	4±0.17	2.8±0.03	26.4±0.87	4.5±0.15	0.67±0.04

LMWC: Low molecular-weight carbohydrate, ±: SE

Table 2: Effect of Acid hydrolysis on total carbohydrates of plant material residues at different conditions

	Total carbohydrates of acid hydrolysis (% w/w)		
		TFA	
Plant residues	Sulfuric acid	1N	6N
Rice straw	35.6±0.65	38.7±0.88	36.8±0.36
Rice husk	41.5±0.46	47.3±0.58	42.2±0.68
Wheat straw	35.9±0.82	37.8±0.79	33.5±0.82
Bagasse	54.6±0.75	55.9±0.63	51.8±0.71

1N: One normal, 6N: Six normal, TFA: Tri fluoro acetic acid

Table 3: Monosaccharide constituents of acid hydrolysis for material residues (% w/w)

	Monosaccharide constituents of Acid hydrolysis for material residues (% w/w)											
	D-glucuronic acid			D-glucose			L-arabinose			D-xylose		
Plant residue	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)
Rice straw	Nil	t	t	100	28.00	56.06	t	12.50	9.38	t	59.50	34.56
Rice husk	Nil	t	t	100	51.70	59.56	t	9.11	13.95	t	39.16	26.48
Wheat straw	Nil	2.58	t	100	23.75	25.50	t	17.43	19.72	t	56.23	54.78
Bagasse	Nil	t	2.57	100	26.58	43.99	t	10.28	10.17	t	63.14	43.27

t: Traces (<1%), Nil: Nothing

Table 4: Yield of extracted cellulose from material residues (% w/w) and total carbohydrates content

Yield of extracted cellulose (% w/w)			Total carbohydrates of acid hydrolysis (% w/w)		
				Tri fluoro acetic acid	
Extraction conditions	Cereal straw	Cellulose	H_2SO_4	1N (TFA)	1N (TFA)
NaOH PH 12 (90°C)	Rice straw	52.9±0.63	39.0±0.25	58.0±0.38	53.0±0.51
	Rice husk	63.0±0.31	50.6±0.27	53.0±0.67	49.5±0.38
	Wheat straw	77.9±0.58	40.6±0.29	68.0±0.46	62.0±0.61
	Bagasse	72.7±0.91	72.0±0.82	88.9±0.67	84.8±0.29
1% NaOH (55°C)	Rice straw	51.6±0.35	43.0±0.26	69.0±0.54	64.0±0.38
	Rice husk	61.3±0.47	52.0±0.51	84.0±0.62	79.0±0.72
	Wheat straw	56.0±0.67	39.0±0.46	66.0±0.53	42.0±0.59
	Bagasse	64.3±0.45	71.0±0.25	92.0±0.18	83.0±0.91
4% NaOH (90°C)	Rice straw	44.0±0.53	31.0±0.68	47.5±0.52	41.0±0.67
	Rice husk	43.0±0.46	42.3±0.52	51.3±0.68	45.8±0.27
	Wheat straw	44.0±0.56	42.3±0.48	51.3±0.35	45.8±0.68
	Bagasse	50.0±0.61	57.0±0.54	63.0±0.68	56.3±0.43
18% NaOH (25°C)	Rice straw	57.5±0.76	28.0±0.81	39.0±0.89	31.0±0.68
	Rice husk	68.6±0.54	37.0±0.75	46.0±0.65	40.6±0.76
	Wheat straw	53.3±0.39	27.0±0.48	37.0±0.56	30.7±0.48
	Bagasse	55.1±0.61	53.0±0.52	60.9±0.62	49.0±0.38

was the optimum condition for isolation. In order to gain more information about the chemical composition of extracted crude cellulose of rice straw, rice husk, wheat straw and bagasse, they were subjected to complete strong and mild acid hydrolysis followed by qualitative separation and then quantitative determination using paper chromatography. The resulted recorded in Table 5-7 indicate that most of the investigated polysaccharide materials comprised D-glucose, L-arabinose and D-xylose. In addition to these sugar moieties, some of the studied polysaccharide products contained D-glucuronic acid unit was done. The results obtained from qualitative analysis was supported the quantitative results, 1N and 55°C is proper method for isolation (Table 5).

Ash content for the extracted cellulose was determined, the highest was cellulose husk straw 11% and the lowest was cellulose bagasse 1.5% in between rice straw 9.6% and wheat straw 2.5% (Table 8). The results obtained from acid hydrolysis and ash content indicated that crude cellulose still has impurities like hemicelluloses and lignin so that crude cellulose should be purified using

Table 5: Monosaccharide constituents of acid hydrolysis for extracted cellulose by 1N NaOH (% w/w)

	Monosaccharide constituents of acid hydrolysis for extracted cellulose (% w/w)								
	D-glucose			L-arabinose			D-xylose		
Extracted cellulose by 1N	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)
Rice straw	100	26.0	28.5	t	21.0	20.40	t	53.0	51.1
Rice husk	100	39.5	44.7	t	17.6	16.48	t	42.9	38.9
Wheat straw	100	30.1	33.9	t	14.6	13.10	t	55.3	53.0
Bagasse	100	43.9	44.6	t	18.0	17.50	t	38.1	37.9

t: Traces (<1%), traces from glucornic acid in all samples

Table 6: Monosaccharide constituents of acid hydrolysis for extracted cellulose by 4N NaOH (% w/w)

	Monosaccharide constituents of acid hydrolysis for extracted cellulose (% w/w)											
Extracted cellulose	D-glucuronic acid			D-glucose			L-arabinose			D-xylose		
by 4N	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)
Rice straw	Nil	t	t	100	43.1	59.0	t	10.6	1.2	t	46.3	39.8
Rice husk	Nil	t	t	100	51.2	56.7	t	11.3	8.8	t	37.5	34.5
Wheat straw	t	t	t	100	38.7	48.5	t	14.2	13.1	t	47.1	38.4
Bagasse	t	t	t	100	67.8	69.6	t	12.9	11.8	t	19.3	18.6

t: Traces (<1%), Nil: Nothing

Table 7: Monosaccharide constituents of Acid hydrolysis for extracted cellulose by 18N NaOH (% w/w)

	Monosaccharide constituents of Acid hydrolysis for extracted cellulose (% w/w)											
Extracted cellulose	D-glucuronic acid			D-glucose			L-arabinose			D-xylose		
by 18N	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)	H_2SO_4	1N (TFA)	6N (TFA)
Rice straw	Nil	t	t	100	53.1	100.0	t	9.0	t	t	37.9	t
Rice husk	Nil	t	t	100	62.4	67.7	t	9.3	6.6	t	28.3	25.7
Wheat straw	t	t	t	100	53.8	55.0	t	1.7	0.8	t	44.5	44.3
Bagasse	t	t	t	100	87.9	85.7	t	1.5	2.4	t	10.6	11.9

t: Traces (<1%), Nil: Nothing

Table 8: Determination of ash, α-cellulose content for the crude cellulose, total carbohydrate and degree of polymerization (D.P) for α-cellulose

Extracted cellulose	Ash content (% w/w)	α-cellulose content (% w/w)	Total carbohydrate (% w/w)	D.P of α-cellulose
Rice straw	9.6±0.54	83.0±0.56	97±0.32	418
Rice husk	11.0±0.96	82.5±0.23	98±0.92	521
Wheat straw	2.5±0.12	84.0±0.89	97±0.56	406
Bagasse	1.5±0.09	76.0±0.74	98±0.67	550

alkaline system (under publication) to gain nearly pure cellulose (α-cellulose). The α-cellulose content for the extracted crude cellulose was investigated (Table 8), wheat straw 84%, rice straw 83%, rice husk 82.5% and bagasse 76%. The results were obtained from acid hydrolysis of α-cellulose indicate that total carbohydrates more than 97% in all agriculture residues, this means purification process was succeeded to obtain mainly pure cellulose (as glucose). Degree of

polymerization four α-cellulose samples were in the range of 400-550. The polydisperity of native straw cellulose is probably quite low which means that DP_n and DP_w do not deviate much from each other. The molecular weight of cellulose can be defined by its average DP. Measurements of its polymer properties indicate that cellulose in solution belongs to the groups of randomly coiling polymers.

The sulfation was optimized to gain a maximal concentration of sulfate groups. Based on that, we report a straightforward modification of cellulose layers by *in situ* sulfation of the cellulosic hydroxyl groups. Several reaction parameters (concentration of the sulfation reagent, cellulose source and catalyst) were varied to investigate their influences on the sulfation process. Table 9-11 show sulfated celluloses yield in Formamid by sulfuric acid N, N'Dicyclohexcarbodimid (D.C.C) and 4-(Dimethylamino) pyridine. The yield was increased in presence of catalyst especially in D.C.C (increased from 5-10%). The results in Table 9 reveal that the highest total carbohydrate content was recorded for sulfated husk cellulose 74.5%. On the other hand, the three sulfated cellulose (rice straw 67.5%, wheat straw 71.6% and bagasse 58.5%). Table 10 has the same sequence of total carbohydrate in four sulfated samples by 4-(Dimethylamino) pyridine with decrease 5% in all samples. The lowest total carbohydrate was recorded in presence of N, N'Dicyclohexcarbodimid (Table 11). Sulfate content of sulfated cellulose was measured to indicate the degree of substitution. In contrast the lowest sulfate content was obtained for sulfated cellulose rice husk 8.3% and the highest for sulfated cellulose bagasse 12.1% (Table 9). Generally sulfate content was increased for all sulfated samples by using 4-(Dimethylamino) pyridine (Table 10). Furthermore, the highest sulfate content was obtained in presence of N, N'Dicyclohexcarbodimid, sulfated cellulose bagasse

Table 9: Chemical and biological characteristics of sulfated cellulose

H_2SO_4	Yield (% w/w)	T.C (w/w %)	SO_4^- (w/w %)	T.B (mL)	Clotting time (min)*	Fibrinolytic activity**
Rice straw	30.0±0.47	67.5±0.46	9.4±0.13	6.0±0.11	15.30	2 (+)
Rice husk	24.0±0.85	74.5±0.84	8.3±0.20	5.1±0.10	9.30	1 (+)
Wheat straw	27.0±0.23	71.6±0.42	9.0±0.16	5.2±0.14	9.30	2 (+)
Bagasse	37.0±0.36	58.5±0.56	12.1±0.34	8.4±0.12	31.30	3 (+)

T.C: Total carbohydrates, T.B: Toluidine blue, *Clotting time in minutes (standard heparin sodium preparation "1 mg": 1.4 IU-90 min) **Lysis of plasma clot using standard Hemoclar ((2000 μg mL^{-1}): 4(+), 7(+): Lysis of more than 85% of plasma clot, 6(+): Lysis of more than 80% of plasma clot, 5(+): Lysis of more than 70% of plasma clot, 4(+): Lysis of more than 60% of plasma clot, 3(+): Lysis of more than 50% of plasma clot, 2(+): Lysis of more than 40% of plasma clot and 1(+): Lysis of less than 40% of plasma clot

Table 10: Chemical and biological characteristics of sulfated cellulose in presence of 4-(Dimethylamino) pyridine (D.A.P)

Sulfated cellulose	Yield (% w/w)	T.C (w/w %)	SO_4^- (w/w %)	T.B (mL)	Clotting time (min)*	Fibrinolyticactivity**
Rice straw	32.0±0.55	63.2±0.73	9.9±0.13	6.1±0.12	43.0	2(+)
Rice husk	25.0±0.64	71.5±0.62	9.1±0.20	5.4±0.13	11.0	3(+)
Wheat straw	30.0±0.35	69.1±0.82	10.5±0.16	6.4±0.10	16.0	2(+)
Bagasse	39.0±0.71	56.5±0.65	14.3±0.41	9.8±0.15	51.3	4(+)

TC: Total carbohydrates, TB: Toluidine blue

Table 11: Chemical and bological characteristics of sulfated cellulose in presence of N, N'- Dicyclohexcarbodimid (D.C.C)

Sulfated cellulose	Yield (% w/w)	T.C (w/w %)	SO_4^-(w/w %)	T.B (mL)	Clotting time (min)*	Fibrinolytic activity**
Rice straw	39.0±0.13	52.2±0.73	14.9±0.25	10.0±0.19	106.0	3 (+)
Rice husk	30.0±0.42	68.5±0.62	12.1±0.37	8.9±0.72	19.3	3 (+)
Wheat straw	36.0±0.56	55.1±0.82	14.5±0.63	11.0±0.53	21.3	2 (+)
Bagasse	43.0±0.64	49.5±0.65	18.3±0.52	15.4±0.27	118.0	4 (+)

TC: Total carbohydrates, TB: Toluidine blue

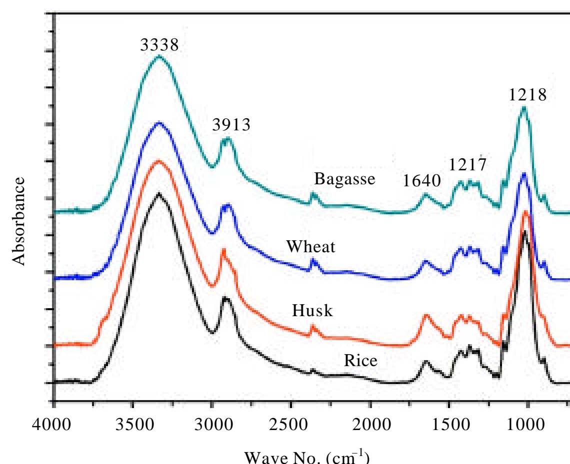

Fig. 2: FT-IR spectroscopy analysis for different crude cellulose extracted

18.3%, sulfated cellulose rice straw 14.9%, sulfated cellulose wheat straw 14.5% and sulfated cellulose rice husk 12.1% (Table 11). Similar, volume of Toluidine Blue (TB) was increase from sulfation without catalyst to presence of catalyst. The highest toluidine blue volume was in case of sulfated cellulose bagasse in presence of N, N'Dicyclohexcarbodimid 15.5 mL and then, sulfated cellulose wheat straw 11 mL, sulfated cellulose rice straw 10 mL and sulfated cellulose rice husk 8.9 mL, respectively (Table 11).

In vitro anticoagulant study, there was an important assay to evaluate the anticoagulation activity of sulfated celluloses. Heparin was used as standard anticoagulant (Fig. 2). On the other hand, the results showed the promising anticoagulation activities of the sulfated celluloses at different concentrations (2000, 1000 and 500 μg mL^{-1}) compared with corresponding native extracts. Data concerning the anticoagulation activity is shown in (Table 9-11). Briefly, the obtained results could be revealed all sulfated celluloses have anticoagulation activities more than three hours at 2000 and 1000 μg mL^{-1} concentration. Sulfated celluloses show weak anticoagulation activities comparable to that of standard preparation of heparin sodium. The results in Table 11 indicated that the highest clotting times were found by using D.C.C catalyst at concentration of 500 μg mL^{-1} (sulfated cellulose bagasse 118 min, sulfated cellulose rice straw 106 min, sulfated cellulose wheat straw 21.3 min and sulfated cellulose rice husk 19.3 min). The lowest clotting time were reported without catalyst sulfated celluloses at same concentration (500 μg mL^{-1}). Sulfated celluloses by 4-(Dimethylamino) pyridine results were intermediate. Sulfated bagasse cellulose in presence of D.C.C as catalyst has the longest clotting time (118 min) at concentration (500 μg mL^{-1}). From these data, It was concluded that addition of sulfate group into extracted cellulose enhance anticoagulation activities.

As part of our interest in this study was to evaluate various sulfated celluloses against fibrinolytic activity compared with standard fibrinolytic, Hemclar drug (Pentosan sulfuric polyester, product of Clin Midy. Paris). It is clear from the results in Table 9-11 that the Sulfated bagasse cellulose in presence of catalyst whether D.C.C or 4-(Dimethylamino) pyridine exhibited fibrinolytic activities equivalent to the same amount of standard "Hemoclar" preparation at concentration of 2000 μg mL^{-1}. On the other hand, sulfated cellulose rice straw and sulfated cellulose rice husk were showed fibrinolytic activities less than the same amount of standard "Hemoclar". In addition, the

sulfated cellulose wheat straw exhibited fibrinolytic activities equivalent to about half that of standard "Hemoclar" preparation at same concentration (2000 μg mL^{-1}). Determination of fibrinolytic effects of the modified sulfated cellulose revealed that, they have good fibrinolytic activities.

The Fourier-Transformed Infrared Spectroscopy (FT-IR) spectrum of cellulose is always similar except for the intensity of absorption bands. In the spectrum, the absorption at 2900 cm^{-1} relates to the CH and CH_2 stretching and the one at 1372 cm^{-1} to the O-H bending. The peak at 893 cm^{-1} represents the glycosidic C1-H deformation with ring vibration contribution which is characteristic of **β**-glycosidic linkages between glucose in cellulose. A peak at 1426 cm^{-1} relates to the CH_2 symmetric bending. The total crystallinity and lateral order indices can be obtained from the 1429/893 and 1372/2900 cm^{-1} absorbance ratios, respectively. Furthermore, the absorption bands at 750 and 710 cm^{-1} in FTIR spectrum of cellulose are assigned to the I_α and I_β phases, respectively. In the FTIR spectrum of cellulose extracted from the wheat straw, the absorption band at 750 cm^{-1} was not detectable, indicating that there is no cellulose I_α crystalline polymorphism in wheat straw. There is only the absorption band at 710 cm^{-1} which indicates that only cellulose I_β crystalline polymorphism exists in the wheat straw (Fig. 2). Figure 3-6 reveled that α-cellulose spectrum has more intensity OH peak than crude one and the crystallinity and lateral order has been increased and more significant. Sulfated cellulose has less OH groups due to sulfate group substitution and deformation crystallinity. The highest substitution was in sulfated cellulose bagasse, rice straw, rice husk and wheat straw, respectively.

Results revealed that no cytotoxic effect was observed when the VERO cells were treated with all sulfated cellulose. Also, no mortality or sings of toxicity were recorded in rat received sulfated cellulose at dose 5000 mg kg^{-1} b.wt. Therefore, this results indicated that the LD_{50} of sulfated cellulose are more than 5000 mg kg^{-1} b.wt. and these compounds are practically non-toxic.

It was concluded that addition of sulphate group into extracted cellulose enhance anticoagulation activities. The results indicated that the highest clotting times were found by using D.C.C catalyst at concentration of 500 μg mL^{-1}. The lowest clotting time were reported without catalyst sulfated celluloses at same concentration. Determination of fibrinolytic effects of the modified sulfated cellulose revealed that they have good fibrinolytic activities. Sulfated bagasse cellulose in presence of catalyst whether D.C.C or 4-(Dimethylamino) pyridine exhibited fibrinolytic

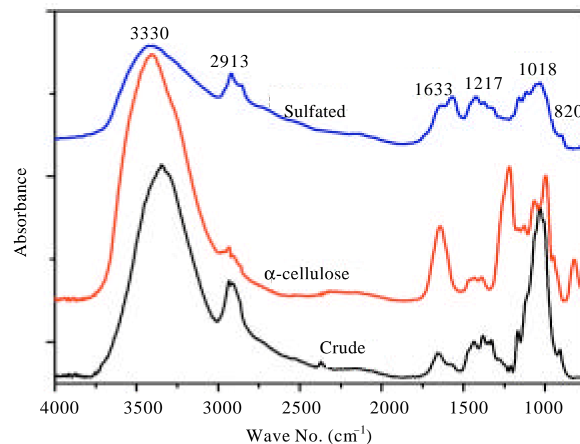

Fig. 3: FT-IR spectroscopy analysis for rice straw crude cellulose, α-cellulose and sulfated cellulose

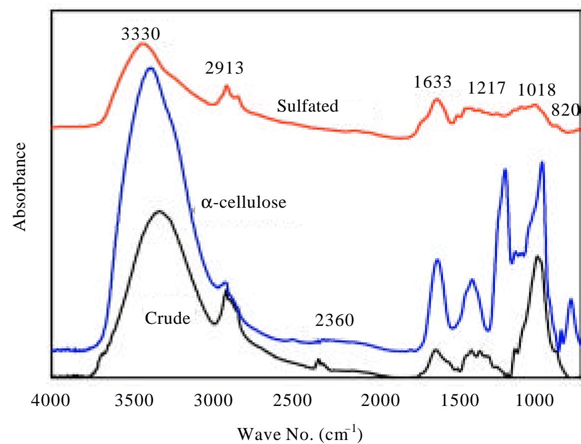

Fig. 4: FT-IR spectroscopy analysis for rice husk crude cellulose, α-cellulose and sulfated cellulose

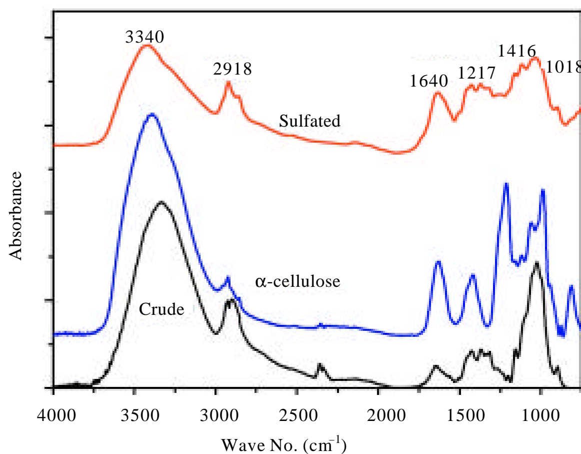

Fig. 5: FT-IR spectroscopy analysis for wheat straw crude cellulose, α-cellulose and sulfated cellulose

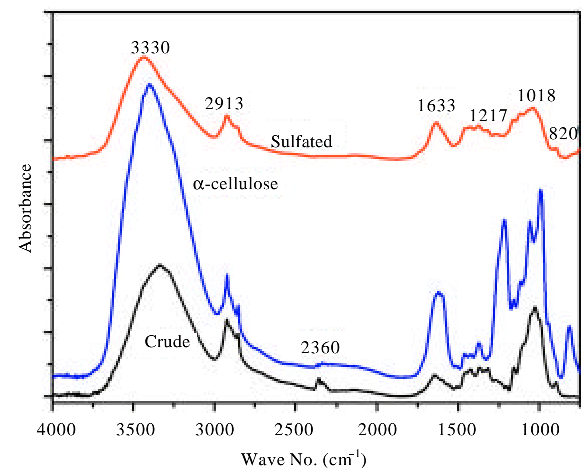

Fig. 6: FT-IR spectroscopy analysis for bagasse crude cellulose, α-cellulose and sulfated cellulose

activities equivalent to the same amount of standard "Hemoclar" preparation at concentration of 2000 μg mL^{-1}. No cytotoxic effect of sulfated cellulose was observed on VERO cells. Results indicated that the LD_{50} of sulfated cellulose is more than 5000 mg kg^{-1} b.wt and these compounds are practically non-toxic.

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