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Characterization and Toxicological Evaluation of Combretum glutinosum (Habil) Gum of Sudanese Origin.

Amani A. Elmozamil[*1], Elfatih A. Hassan[2] and Yousif M. A. Idris[3]

[1] Sudanese Standards and Metrology Organization, Khartoum,. Sudan. amanimozamil@hotmail.com.

[2]Department of Chemistry College of Science, Sudan University of Science and Technology, P.O. Box 407, Khartoum, Sudan.

[3]College of Agricultural Studies, Sudan University of Science and Technology, Khartoum North, Sudan.

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ABSTRACT

Twenty samples of *Combretum glutinosum* gum, collected from Blue Nile state in Sudan during the seasons 2007 and 2008, were characterized using different physicochemical methods. Results obtained showed that the mean value for moisture content was 7.96%, ash content was 4.51%, pH value was 4.5, nitrogen content was 0.37%, protein content was 2.43%, acid equivalent weight was 1524.15, total uronic acid was 15.53%, intrinsic viscosity was 11.2 ml g^{-1} where as tannin and starch or dextrin were not detected in any samples. It is also found that *Combretum glutinosum* gum had low solubility in water, but it dissolved perfectly in basic media. Atomic Absorption spectrophotomtric analysis showed the most abundant cations present are calcium, potassium and magnesium with mean values in ppm 46.73, 35.97 and 17.36 respectively. Acid hydrolysis showed that the gum content of monosccharides were arabinose 56.1%, galactose 33% and rhamnose 10.9%. Toxicological study using *in vitro* cytotoxic methods on different types of normal and cancer human cell lines, showed that the IC_{50} was less than 100 µg/ml in the majority of gum samples studied. Prediction of LD_{50} starting doses was estimated from the values of IC_{50} using Halle's RC prediction model and was found to be in the range of 0.03 to 0.02 g kg^{-1} body weight on both normal and cancer cell lines.

المستخلص

جُمعت عشرون عينة من صمغ الهبيل من ولايه النيل الازرق بالسودان خلال موسمى 2008 و2007. فى هذه العينات تم توصيف صمغ الهبيل بإستخدام الطرق الفيزوكيميائية و أظهرت النتائج أن متوسط قيم كل من محتوى الرطوبة ومحتوى الرماد وقيمة الأس الهيدروجيني ومحتوى النيتروجين ومحتوى البروتين والوزن المكافئ الحمضي وحمض اليورونيك الكلي واللزوجة الضمنية كانت 7.96%، 4.51%، 4.54، 0.37%، 2.43%، 1524.15، 15.53% و11.2 مل/جرام بالترتيب كما بينت الدراسة خلو عينات صمغ الهبيل التى تم دراستها من النشا والدكسترين والتانين. أظهرت الدراسه أن صمغ الهبيل شحيح الذوبانيه فى الماء ولكنه يذوب بسهوله فى الأوساط القلويه. بينت نتائج تقدير الشقوق الموجبة بتقنية الإمتصاص الذري أن الكالسيوم والبوتاسيوم والمغنيزيوم

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هى الأكثر وفره من بين الشقوق الموجبة التي تم قياس تراكيزها فى الصمغ حيث كانت مقاسه (كجزء من المليون) كالآتى 46.73، 35.97 و 17.36 بالترتيب. أوضحت دراسة التحلل الحمضى لصمغ الهبيل إحتوائه على السكريات الآحاديه المتمثله فى الأرابينوز والجالاكتوز والرامنوز بتركيز 56.1%، 33% و 10.9% بالترتيب . تمت دراسة سمية صمغ الهبيل باستخدام الخلايا المعزولة داخل المختبر التى طبقت على نوعين من الخلايا السليمة و نوعين من الخلايا الُسرطنة ومن ثم اُستنبطت قيمة IC_{50} وكانت أقل من 100 ميكرجرام/مل فى أغلب عينات الصمغ التى شملتها الدراسه. تم حساب الجرعه البادئه المتوقعه للجرعه القاتله من قيمة IC_{50} للخلايا وتراوحت بين 0.02 و0.03 جم/كجم من وزن الجسم فى الخلايا السليمه والُسرطنه.

KEYWORD: *Combretum glutinosum;* habil gum; characterization; cytotoxicity; MITT assay

INTRODUCTION

Combretum glutinosum, locally known as Habil gum, is an exudate obtained by incision of the bark and branches of *C. glutinosum* in a form of large whole spheroidal nodules and tears that break with glassy fractures with colours ranging from dark to pale brown. *C. glutinosum* type genus of the family *Combretaceae* [(1)], is a small savanna tree, durable for about years. It's a fast growing species, particularly resistant to arid condition [(2)] and widely distributed in Sudan in Kordofan state, Nuba mountains and Jebel Mara in Darfur [(1)]. Habil trees are mainly used in Sudan as a wood source (charcoal, tool handles, general carpentry). Extracts from the bark, leaves and roots are used in traditional medicine (treating influenza, rheumatism, urinary, liver and kidney complaints). In West Africa it is mainly used as an important source of yellow to brownish yellow dyes for cotton textiles [(2)]. Little is known about the chemistry of *C. glutinosum* gum derived from Sudanese botanical sources and no toxicological study, what so ever, have been undertaken to evaluate its safety as food or pharmaceutical additive or ingredient, particularly in comparison with the *Acacia Senegal*, where extensive work has been carried out in the field of its chemistry, taxonomy, toxicity and technological applications[(3)]. It has neither been commercialized in Sudan, where there is a large potential for its commercial production. This study aims to physicochemically characterize *C. glutinosum* gum of Sudanese origin and toxicologically evaluate it by cytogenetic technique as a preliminary study to pave the way for its commercialization and industrial applications.

MATERIALS and METHODS

Twenty Samples of habil gum were collected during seasons 2007 and 2008 from Blue Nile state in Sudan. The samples of habil gum were cleaned by hand from any extraneous materials, and prepared according to procedures cited in AOAC [(4)]. The prepared gum samples were kept in sterile screw capped polyethylene containers at room temperature till further use.

Physicochemical characterization of *C. glutinosum* gum

Moisture content, ash content, tannin and starch or dextrin were determined according to the methods of FAO [(5)]. Nitrogen content and protein content were determined by Kjeldahl method AOAC [(4)]. Determinations of metals was done using Atomic Absorption Spectrophotometric Technique ,using a GBC, Avanta, Austuralia

spectrophotometer. Determination of Sugars was done by High Performance Liquid Chromatography (HPLC) technique after acid hydrolysis of the gum following Randall *et al* procedure [6]. Acid equivalent weight and uronic acid were determined according to the method reported in Encyclopedia of Chemical Technology [7].The solubility of *C. glutinosum* gum was tested in three solvent, namely water, EDTA and Na_2CO_3 according to Jefferies *et al*. [8,9]. Osmotic pressure was determined using Osmomat 050 Colloidal Osmometer at 21˚C and modified Van't Hoff's equation. Intrinsic viscosity was determined using Ubbelohde viscometer and extrapolation of the plot of reduced viscosity against concentration.

Toxicity evaluation method

The effect of *C. glutinosum* aqueous gum solution on proliferation and apoptosis of baby hamster normal kidney fibroblast cell line (BHK), human normal melanocytes cell line (HFB4), human hepatocellular carcinoma cell line (HEPG2) and human colon carcinoma cell line (HCT116) was investigated using 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay [10]. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm using micro plate Enzyme Linked Immunosorbent Assay (ELISA) reader. The results were represented as half maximal inhibitory concentration (IC_{50}) of the gum [11]. Data were expressed as the percentage of relative viability compared with a control gum sample.

RESULTS and DISCUSSION

Physicochemical characterization

Table (1) shows the analytical data of physicochemical and functional properties for twenty samples of *C.*

Table 1 : Analytical data of physicochemical analysis of Combretum glutinosum gum

Physicochemical parameters	sample studied	data obtained by Awad*[1]	data for *Acacia senegal**[2]
Moisture content%	7.96	9.88	10.75
Ash%	4.51	4.31	3.77
pH	4.54	4.79	-
Nitrogen %	0.37	0.2	0.33
Protein%	2.43	1.31	2.1
Starch and dextrin	ND***	ND***	ND***
Tannin	ND***	ND ***	ND***
Acid equivalent weight %	1524.15	1291.71	1436
Uronic acid%	15.52	15.54	13.71
$[\alpha]_D$ in Water, degrees	-	-	-31.3
Intrinsic Viscosity, $[\eta]$ mlg^{-1}	11.2	9.80	16
Number Average Molecular Weight M_n	$1.8x\ 10^4$	$5.5X\ 10^5$**	$9X10^5$**
Sugar composition after hydrolysis (%)			
4-O- Methylglucuronic acid	-	-	1.5
Glucuronic acid	-	-	16
Galactose	33	-	44
Arabinose	56.1	-	25
Rhamnose	10.9	-	14

*[1] Awad, (2011). *Characterization studies on Combretum glutinosum gum*.
*[2]Karamalla *et al* (1998). Analytical data season1993/1995.
** Molecular Weight M_w

*** ND: not detected

glutinosum gum namely samples labled 1-20, collected from blue Nile area in season 2007 and 2008 compared to results published in the literature by Awad [12] and Karamalla *et al*. [13]. The moisture content of nodules samples was found to be at the range of 6.7 to 9.9 % with the mean value of 7.96%, The ash content of the gum was found to fall at the range of 1.84 to 6.39% with a mean value of 4.51 % while the pH mean value was found to be 4.5. Nitrogen content was in the range of 0.18 to 0.88% with a mean value of 0.37%. The protein content was calculated from the nitrogen content, using nitrogen conversion factor (NCF) of 6.6 Anderson [14] and it falls in the range 0.95 to 5.9% with a mean value of 2.43% .Tannin, starch and dextrin in *C. glutinosum* gums were not detected at any level. The mean value of acid equivalent weight of *C. glutinosum* was 1524.15 and the calculated mean value of uronic acid content was 15.52 %. These results compare well with the data published by Awad [12] except for nitrogen and hence protein which was almost twice his cited values. *C. glutinosum* gums showed low solubility in water and have an unclear solution that prevents measurement of its specific optical rotation. The number average molecular weight found from osmotic pressure measurements of the gum aqueous solution was1.8×10^4 Dalton. Values of limiting intrinsic viscosity number of *Combretum* gum samples examined in this study was 11.2 ml g^{-1}. Determination of monosaccharides after acid hydrolysis of the gum samples showed presence of three sugars namely rhamnose (10.9%), arbinose (56.1%) and galactose (33%). Comparing these physicochemical parameters with those of *Acacia Senegal* [13] showed that both contain almost the same types of sugars and almost equal amounts of protein which might reflect on their emulsification characteristics and functional behavior. The solubility of *Combretum glutinosum* gum in several solvents is shown in Table (2).

Table 2: Solubility of Habil gum in different solvents

	Solubility of gum Samples	
Solvent	season2007	season 2008
Water	58.6 %	30 %
EDTA	63.8 %	40 %
Na_2CO_3	96.3 %	96 %

In cold water it was as low as 30%, while it dissolved in EDTA to an extent of 40% and dissolved readily in Na_2CO_3 to an extent of 96.3%. The high solubility of the gum in Na_2CO_3 is attributed to depletion of calcium ions from the gum molecule and transferring it to a sodium salt which is obviously water soluble like any other sodium salt. From results in Table (3) it is clear that calcium, potassium and magnesium, are the most abundant element in *Combretum glutinosum* gum with the mean values 46.73, 35.97 and 17.36 ppm respectively. This explains the sparing solubility of the gum in water as Ca and Mg represent about 64% of the cations present in the gum.

Table 3: Calcium, potassium and magnesium as determined by AAS, in Habil gum

Element				
	Ca	K	Mg	
Cationic content	46.73	35.97	17.36	(ppm)

Table (4) shows the quantity of iron, nickel, cadmium, lead, cobalt, copper, zinc, chromium and aluminium recorded in ppm, with the mean values 0.55, 0.07, 0.2, 0.16, 0.07, 0.02, 0.06, 2.07and 1.272 respectively. These heavy metals cations represent 4.5% of the total cations present, a value that falls within the range of the limits specified for heavy metal in Food Grade Gums Additives.

Table 4: Heavy Metals contents of Habil gum

	Elements								
	Fe	**Ni**	**Cd**	**Pb**	**Co**	**Cu**	**Zn**	**Cr**	**Al**
Metal content (ppm)	0.55	0.07	0.2	0.16	0.07	0.02	0.06	2.07	1.27

Cytotoxicity Evaluation

Cytotoxicity assay results showed that *Combretum glutinosum* gum had profound effect on four cells line used in this study namely, Human normal baby hamster kidney fibroblast cell line (BHK), Human normal melanocytes cell line (HFB4), Human hepatocellular carcinoma cell line (HEPG2) and Human colon carcinoma cell line (HCT_{116}), The cytotoxicity of the gum is expressed in terms of relative viability of normal and cancer cells treated with different doses of the gum in comparison to control cells. The percentage of cytotoxicity was calculated considering the control to have 100% cell-viability. The results represented as Median Growth Inhibitory Concentration (IC_{50}) which is the gum's dose required to produce 50% cytotoxic effect i.e. 50% cell viability. Cytotoxicity of *Combretum glutinosum* gum was compared to drugs cytotoxicity clasification cited in Shirazi *et al*. [15]. The relationship between cell survival fraction and gum doses used for treating the different cell lines used in this study are depicted in the Figures 1, 2, 3 and 4, while the values of IC_{50} obtained in each case are shown in Table (5) The value of the IC_{50} for normal human cell line HBK were 41.5 and 175 µg ml^{-1} for samples collected in the seasons 2007 and 2008 respectively. While for the normal human skin cells HFB4 the survival fraction was never less than 70% within the whole range of gum doses used, which reflects the extremely low toxic effect of the gum on the human skin cells. This is an indication of the possible application of the gum in the body and skin care pharmaceutical formularies. The IC_{50} obtained for carcinoma cell line HEPG2 were 54 and 49.5 µg ml^{-1} and those for HCT116 were 50 and 39 µg ml^{-1}. These low values indicate an appreciable level of toxicity according to Shirazi *et al*. [15].

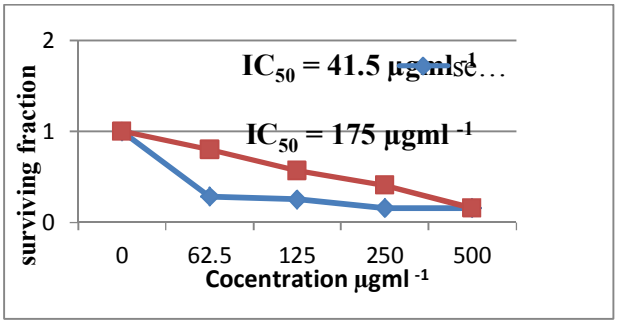

Figure 1: Variation of surviving fraction with gum dose on (BHK) cell line.

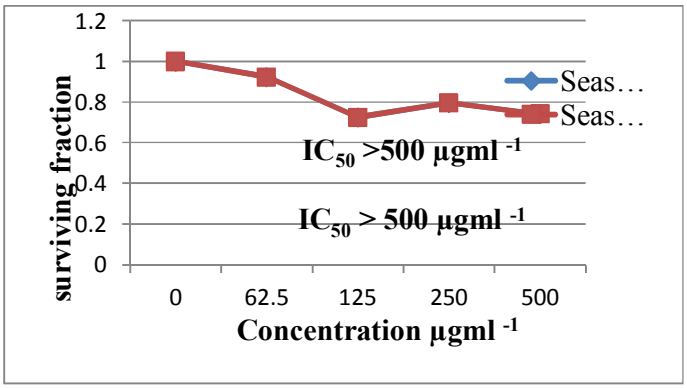

Figure 2: Variation of survival fraction with gum dose on HFB4 cell line.

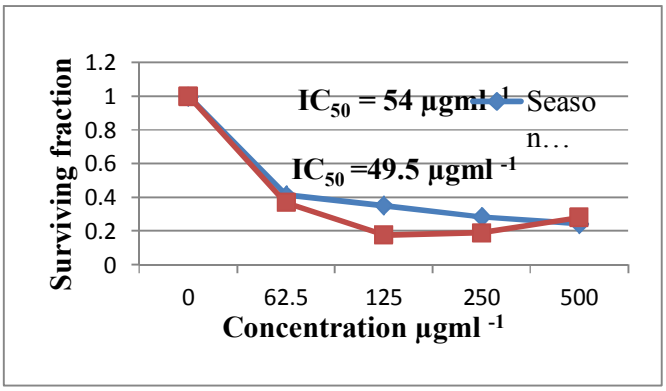

Figure 3: Variation of the survival fraction with gum dose on HEPG2 cell line.

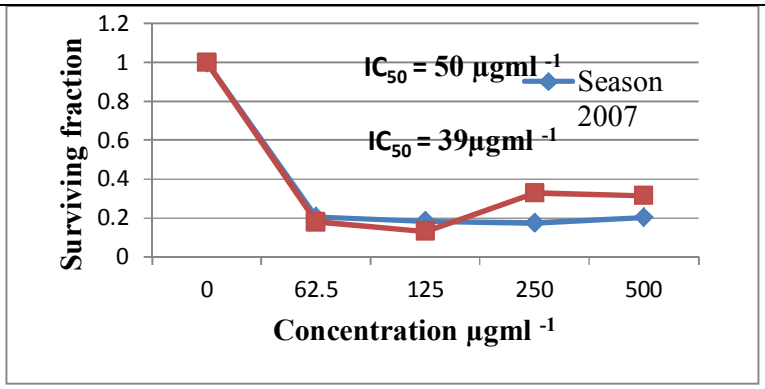

Figure 4: Variation of survival fraction with gum dose on (HCT116) cell line.

Table 5: IC_{50} values for normal and cancer cell line for samples collected at the seasons 2007 and 2008.

	IC_{50} µg ml^{-1}	
Cell line type	sample from season 2007	sample from season 2008
BHK	41.5	175
HFB4	-	-
HEPG2	54	49.5
HCT116	50	39

Correlation between LD_{50} and IC_{50}

MTT method is recommended by Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a protocol for basal cytotoxicity for predicting starting doses for acute oral systemic toxicity tests, LD_{50}. Results of IC_{50} obtained in this study were subjected to Halle's Registry of Cytotoxicity (RC) prediction model. Halle; Spielmann *et al.* [16, 17].

Table (6) shows predicted starting doses of LD_{50} for *Combretum glutinosum* samples estimated from their IC_{50} values on the normal and cancer cell lines studied. LD_{50} starting doses was found to be in the range of 0.03 to 0.02 g Kg -1 body weight on both normal and cancer cell lines respectively.

Table 6: Prediction of LD_{50} gum's starting doses for the four cell line used.

	LD_{50} starting doses gkg^{-1}	
Cell line	gum sample of season 2007	gum sample of season 2008
BHK	0.015	0.03
HFB4	-	-
HEPG2	0.015	0.015
HCT116	0.015	0.015

CONCLUSION

The solubility of habil gum can enhance greatly by calcium ions depletion, through precipitation of the ion as $CaCO_3$.

In vitro method (MTT) could be used for predication of LD50 Starting dose of other gum species, considering the rapidness and low cost of In vitro compare to in vivo method.

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