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Anti-inflammatory and analgesic activity of a cucurbitacin isolated from *Lagenaria breviflora* Roberty fruit

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Abstract

Background: In this study, the bioactive compound in *Lagenaria breviflora* Roberty responsible for its anti-inflammatory and analgesic activities was isolated and chemically characterized.

Method: Compounds in the whole fruit, bark, pulp and seed of *L. breviflora* were partitioned utilizing their various polarity in n-hexane, ethyl acetate, chloroform and ethanol. The fractions of the extract obtained were tested for their bioactivities. The fraction with the most consistent anti-inflammatory and analgesic activities was further purified using accelerated gradient chromatography (AGC) and open column chromatography. Elution of compounds in this fraction was monitored through the different chromatography methods using thin layer chromatography (TLC). The pure compound isolated from the chromatography methods was taken for chemical characterization and elucidation of the structure.

Results: Ethyl acetate fraction of the whole fruit exhibited the most consistent anti-inflammatory and analgesic activities out of the 16 fractions obtained. Purification of this fraction with AGC yielded 7 sub-fractions composing of eluents with similar R_f values on the TLC plate. One of the sub-fractions yielded a compound which was further purified using the open column chromatography method. Eluent obtained from this sub-fraction was renamed YO1.

Conclusion: From the result of mass spectroscopy and nuclear magnetic resonance spectroscopy of the compound, the structure of YO1 was determined as a cucurbitacin with 10á-cucurbit-5-ene skeleton (9â-methyl-19-norlanosta 5-ene) backbone structure, with six carbon atoms attached to double bonds and one hydroxyl group.

Keywords: *Lagenaria breviflora, bioactivity, isolation, characterization*

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Résumé

Contexte: Dans cette étude, les composés bioactifs Lagenaria dans breviflora Roberty responsable de son anti-inflammatoire et analgésique activités était isolé et chimiquement caractérisé.

Méthode: composés dans les fruits entiers, de l'écorce, les pâtes et les semences de L. Breviflora ont été partitionnées en utilisant leurs divers la polarité du n-hexane, l'acétate d'éthyle, de chloroforme et de l'éthanol. Les fractions de l'extrait obtenu ont été testés pour leur bioactivities. La fraction de la façon la plus cohérente anti-inflammatoire et analgésique activités il a purifié l'utilisation accélérée chromatographie gradient (AGC) et ouvrez chromatographie sur colonne. L'élution des composés de cette fraction a été surveillé par les différentes méthodes de chromatographie par chromatographie sur couche mince (TLC). Le composé pur isolé des méthodes de chromatographie n'a été prise pour caractérisation chimique et à l'élucidation de la structure.

Résultats: acétate d'éthyle fraction de l'ensemble des fruits présentaient la plus cohérente anti-inflammatoire et analgésique activités des 16 fractions obtenues. Purification de cette fraction avec AGC cédé 7 sous-fractions qui composent des éluants similaires avec valeurs Rf sur la plaque pour chromatographie en couche mince. L'un des sous-fractions cédé une enceinte qui a été purifiée en utilisant la chromatographie sur colonne méthode. Éluant obtenus à partir de cette sous-fraction a été renommé YO1.

Conclusion : à partir du résultat de la spectroscopie de masse et la résonance magnétique nucléaire spectroscopie du composé, la structure de YO1 a été déterminé comme un cucurbitacin avec 10á-cucurbitacées-5-ene squelette (9â-méthyl-19-norlanosta 5-ene) structure de base, avec six atomes de carbone fixé à doubles liaisons et un groupe hydroxyle

Introduction

Lagenaria breviflora Roberty (family Cucurbitaceae) is a medicinal plant with several traditional uses in West Africa. These include treatment of diseases of inflammatory origin,

digestive disorders and wound sepsis including umbilical incision wounds [1, 2]. *L. breviflora* has been reported to possess several bioactivities including anti-implantation, miracicidal, cercaricidal, antibacterial, anti-ulcerogenic, antioxidant, hepatoprotective, anti-inflammatory and analgesic activities [2-8]. The fruit extract was also found to have a wide safety margin [9, 10]. *L. breviflora* is known locally as *tagiri* in Yoruba and *egwoli* in Igbo.

Our previous study on the anti-inflammatory and analgesic effect of the fruit of *L. breviflora*, showed that the individual parts of the fruit viz bark, pulp and seeds contained bioactive compound(s), but at varying concentrations [8]. However, the bioactive compound(s) in the fruit was not characterized.

Various studies on the bioactive compounds from some plants in the family Cucurbitaceae such as *Cucurbita andreana, Cayaponia tayuya, Wilbrandia ebracteata* and *Ecballium elaterium* showed a wide spectra of anti-oxidant, anti-inflammatory and analgesic activities. These compounds have been scientifically proven to be relevant not only for the acute forms but also for chronic type inflammatory conditions such as arthritis, ulcers, gouts, cancers and hypersensitivity reactions [11-16].

The study was aimed at conducting a bioactivity guided fractionation of each fruit part. Isolation, chemical characterization and elucidation of the structure of the bioactive compound(s) in the fruit of *Lagenaria breviflora* Roberty responsible for the potent anti-inflammatory and analgesic activity observed was carried out.

Materials and methods

Fresh fruits of *Lagenaria breviflora* Roberty were obtained from the Teaching and Research Farm of University of Ibadan and identified at the Department of Botany and Microbiology, University of Ibadan, Nigeria. Fresh fruits were washed, cut into small pieces and weighed out in small portions for the whole fruit extraction. The bark was dehulled from another batch of whole fruits, the pulp and seeds were separated. The whole fruit and the different parts were first soaked in n-hexane for 72 hours. The extract was drained and the plant residue was air dried for about 24 hours. The dried residue from the whole fruit and each part of the fruit part were soaked in ethyl acetate for 72 hours. Drying of the residue plant material and soaking were subsequently repeated using chloroform and ethanol respectively as the solvent. The extracts obtained were clarified by filtration through celite on water pump and were concentrated *in vacuo* using a rotary evaporator (Rotavapor R-210, Switzerland) at low temperatures. The remaining moisture was removed by freeze-drying. A total of 16 extracts were obtained from the fruit parts.

Experimental animals

One hundred and seventy adult Wistar rats of both sexes (150 -180g) were used for the anti-inflammatory study, while 170 adult Swiss mice (20-30g) of both sexes were used for the analgesic study. The experimental animals were housed in 12 hour light: dark condition and maintained on standard rat and mouse diet respectively. Water was provided *ad libitum*. The rats were randomly and equally divided into groups of five animals. Two groups were assigned to each fraction and were administered with extract doses of 25 and 50mg/kg body weight. One group was administered with 10mg/kg b.w of indomethacin and another group was administered with distilled water (10ml/kg b.w). The standard requirement of Experimental Animal Use and Ethical Committee of the Faculty of Veterinary Medicine, University of Ibadan was strictly adhered to for the research protocols adopted.

Anti-inflammatory study

Formalin-induced paw lick test in rats

Formalin (2.5%; 20 μL) was injected into the plantar surface of the left hind-paw of the rat [17] 60 minutes after treatment with fruit extract (25mg/kg and 50mg/kg, p.o.), indomethacin (10mg/kg b.w) or distilled water (10mls/kg b.w). The amount of time spent licking the injected paw was indicative of pain and these were recorded within 0 to 5 minutes (early phase) and 20–30 minutes (late phase) post injection of formalin. These phases represented neurogenic and inflammatory pain responses respectively.

Analgesic study

Acetic acid-induced abdominal writhing test in mice

The mice were injected intraperitonealy with 0.1ml/10g body weight of 0.6% acetic acid solution 1hour after treatment with fruit extract (25mg/kg and 50mg/kg, p.o.) or indomethacin (10mg/kg b.w) or distilled water (10mls/kg b.w) to induce the characteristic writhing. The number of writhing was observed between 5-25 minutes post injection of acetic acid.

Chromatographic separation of potent fraction(s)

Accelerated Gradient Chromatography (AGC)

The dried extract was pre-adsorbed on silica gel 60 (Merck 40-63 microns; 230-400 mesh) and loaded

to the column in a dry state. It was eluted with different solvent combinations of increasing polarity. Graded combinations of analytical grades of n-Hexane, ethyl acetate and ethanol were used for the separation. Samples were collected into labelled test tubes, with particular attention to the solvent combinations used for elution. The progress of elution was monitored with Thin Layer Chromatography (TLC).

Thin layer chromatography (TLC)

Pre-coated Thin Layer Chromatography (TLC) plates (aluminium foil) were used in this study. The samples to be analyzed were dissolved in minimum amount of solvent and spotted on the TLC plate with fine capillary tube. The plate was developed in a solvent tank which contained carefully selected solvent mixture (mobile phase). The solvent ascended the plate by capillary action and the components of the mixture were separated according to their retardation or resolution factor (R_f). Detection of the components after air-drying of developed plate was carried out by (i) visual inspection for coloured compounds, (ii) UV light (wavelengths 254 or 365nm) and (iii) use iodine vapour (in a closed chamber) which was used as a stain. Iodine forms a brown complex with unsaturated and aromatic molecules. The reaction is reversible, so that I_2 staining can be followed by another chemical stain if the plate is allowed to sit in air for about half an hour.

Elution of the compounds was monitored during and after the chromatography methods with TLC. The resolution factor and solvent combination used to elute were noted. Test tubes containing samples exhibiting similar compounds were pooled and further purified by the open column chromatography method.

Open column chromatography

The pooled samples were dried and tested on TLC plates to ascertain the compound(s) in them. Fractions with one or two compounds were selected for further purification using the open column chromatography method. The column was packed with silica gel as the dry stationary phase and the dried fraction was also loaded into the column. Solvent combinations with varying polarity were used for elution of the fraction in the column and the eluent were collected into well labelled test tubes. Each test tube was tested on TLC plate to monitor the elution of each compound. Test tubes with the same compound were pooled and dried. The pooled sub-fractions were taken for further analysis using Nuclear Magnetic Resonance (NMR) Spectroscopy and Mass Spectroscopy (MS) to elucidate the chemical structure of the compound(s) in the samples and obtain further information on the chemistry of the compounds.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The structure of the isolated compound was elucidated using NMR spectroscopy which is based on the pulse variant. The groups of pulses may be purely radiofrequency (rf) or include magnetic gradient pulses. The acquisition was carried out many times, incrementing the delay (evolution time - t1) between the two pulse groups. The evolution time was labelled t1 and the acquisition time, t2. Two dimension NMR (2D-NMR) techniques was used to save time and acquire connectivity between different types of nuclei (e.g. proton and carbon). Proton (1H) and carbon-13 (13C) NMR spectra were recorded on a Bruker Avance II 400 NMR spectrometer at SAIF Panjab University, Chandigarh, India. Deuterated chloroform was used to measure NMR spectra. The chemical shifts are indicated in parts per million (ppm) with the solvent shift as reference and coupling constants J in Hz. The pure fraction was dissolved in the chloroform after which it was poured inside the NMR tube and read. The chromatographs obtained were interpreted to determine the structure of the compound(s).

Mass spectrometry (MS)

The extract sample was loaded onto the MS instrument and undergoes vaporization. The ions formed are analyzed and their mass spectra are generated. Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio (m/z) of charged particles [18]. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. MS works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratio [18, 19].

Statistical analysis

Values of the experiments were represented as mean ± standard error of mean (SEM) of at least five independent measurements. The data for all observations were statistically analysed on GraphPad Prism 5.01 version, using one way analysis of variance (ANOVA), to compare treatment values among the groups. Test of significance were carried out by Duncan Multiple range Test. Values with $p<0.05$ were considered statistically significant.

Results

Anti-inflammatory study of fruit fractions

All the fractions significantly ($p<0.05$) inhibited inflammatory responses more than indomethacin (61.80±1.64 seconds) in both phases of inflammation. In the early phase of inflammation, rats administered with the whole fruit fractions had the least cumulative licking time, and rats administered with the chloroform fraction of the whole fruit exhibited the least licking response in the early phase (10.25±1.70 seconds and 28.75±0.48 seconds) and was followed by the ethyl acetate fraction (20.65±1.44 and 15.75±3.25 seconds). Of all the fruit extracts, rats administered with the ethanol fraction of the pulp extract at 25mg/kg bw (47.50±3.66 seconds) demonstrated the longest licking time [Table 1].

In the late phase, rats administered with the chloroform fraction of the pulp (0.25±0.25 and 1.00±0.41 seconds) demonstrated the least cumulative licking time, and was closely followed by the ethanol fraction of the whole fruit (1.00±0.58 and 2.00±0.41 seconds) [Table 2].

Analgesic fraction study

Rats administered with the whole fruit fractions showed the least mean number of abdominal writhing with ethyl acetate fractions (7.75±1.38 and 11.25±0.95 seconds) and ethanol fractions (3.00±0.91 and 20.25±2.06 seconds) showing the longest analgesic effect. Less than half of the fractions inhibited abdominal writhing response more than indomethacin did, but rats administered

Table 1: Effect of N-Hexane, ethyl acetate, chloroform and ethanol fractions of whole fruit and fruit parts of *L. breviflora* or Indomethacin on response of rats (in seconds) to pain in early phase of inflammation of Formalin paw lick test

Dose (mg/kg)	N-Hexane	Ethyl Acetate	Chloroform	Ethanol
Bark (25)	26.75±1.65^c	37.25±2.96^c	39.75±5.54^c	32.25±3.97^c
Bark (50)	26.75±2.93^c	35.25±2.96^c	36.00±1.68^c	28.25±10.01^c
Pulp (25)	26.00±2.93^c	30.50±1.66^c	26.25±2.87^c	47.50±3.66^c
Pulp (50)	27.00±0.91^c	30.50±1.32^c	30.25±1.03^c	42.00±2.16^c
Seed (25)	28.00±2.74^c	36.00±2.04^c	33.50±2.72^c	22.50±2.02^c
Seed (50)	16.00±2.58^c	25.75±4.05^c	31.75±1.32^c	28.00±1.41^c
Whole (25)	25.00±4.49^c	20.75±1.44^c	28.75±0.48^c	19.50±1.44^c
Whole (50)	33.50±2.78^c	15.75±3.25^c	10.25±1.70^c	19.75±2.06^c
Indomethacin (10)	61.80±1.64^b			
Control	109.66±8.41^a			

Different superscripts on a column are statistically significant ($p<0.05$)

Table 2: Effect of N-Hexane, ethyl acetate, chloroform and ethanol fractions of whole fruit and fruit parts of *L. breviflora* or Indomethacin on response of rats (in seconds) to pain in late phase of inflammation of Formalin paw lick test

Dose (mg/kg)	N Hexane	Ethyl Acetate	Chloroform	Ethanol
Bark (25)	2.50±0.29^c	1.00±0.20^c	12.25±4.52^c	3.25±0.63^c
Bark (50)	4.00±0.41^c	5.01±2.04^c	4.00±1.35^c	0.75±0.48^c
Pulp (25)	12.50±1.32^c	4.00±1.78^c	0.25±0.25^c	5.75±0.75^c
Pulp (50)	6.25±1.65^c	1.00±0.41^c	1.00±0.41^c	0.00±0.00^c
Seed (25)	6.75±1.55^c	0.00±0.00^c	5.50±1.32^c	2.50±0.29^c
Seed (50)	4.50±0.29^c	2.75±0.75^c	3.00±0.75^c	1.50±0.29^c
Whole (25)	8.75±1.89^c	2.50±0.50^c	8.25±0.63^c	2.00±0.41^c
Whole (50)	10.75±0.48^c	1.25±0.63^c	8.25±0.61^c	1.00±0.58^c
Indomethacin (10)	22.88±0.89^b			
Control	73.10±6.88^a			

Different superscripts on a column are statistically significant ($p<0.05$)

with the whole fruit fractions at 50mg/kg bw consistently showed reduced response to pain demonstrated by the mean number of abdominal writhing. The least analgesic activity from the fractions was observed in rats administered with the seed fractions, but these also demonstrated significantly longer analgesic activity compared with rats in the control group [Table 3].

activity of all the fractions. It was therefore selected for further chromatographic separations and elucidation of active compound(s). Accelerated gradient chromatography separation of the ethyl acetate fraction of the whole fruit yielded ninety seven (97) sub-fractions which were collected into test tubes. The eluent in each test tube was spot-tested on Thin Layer Chromatography (TLC) to monitor

Table 3: Effect of N-Hexane, ethyl acetate, chloroform and ethanol fractions of whole fruit and fruit parts of *L. breviflora* or Indomethacin on abdominal writhing in mice injected with acetic acid intraperitonealy.

Dose (mg/kg)	N Hexane	Ethyl Acetate	Chloroform	Ethanol
Bark (25)	28.50±3.59^b	20.25±3.68^b	19.00±2.80^b	28.25±2.02^b
Bark (50)	18.00±1.58^b	20.25±3.28^b	16.50±2.56^b	13.75±0.63^b
Pulp (25)	19.50±1.32^b	16.00±1.58^b	34.00±2.92^b	28.75±1.49^b
Pulp (50)	21.75±1.18^b	9.00±1.58^b	38.25±1.11^b	29.50±3.23^b
Seed (25)	28.75±0.95^b	31.00±2.04^b	30.00±2.27v	31.75±1.44^b
Seed (50)	38.75±3.07^b	31.50±1.89^b	19.00±0.71^b	23.75±0.48^b
Whole (25)	23.50±1.19^b	11.25±0.95^b	23.75±2.06^b	20.25±2.06^b
Whole (50)	16.50±2.63^b	7.75±1.38^b	9.25±1.11^b	3.00±0.91^b
Indometh (10)	19.80±5.11			
Control	48.00±3.81^a			

Different superscripts on a column are statistically significant ($p<0.05$)

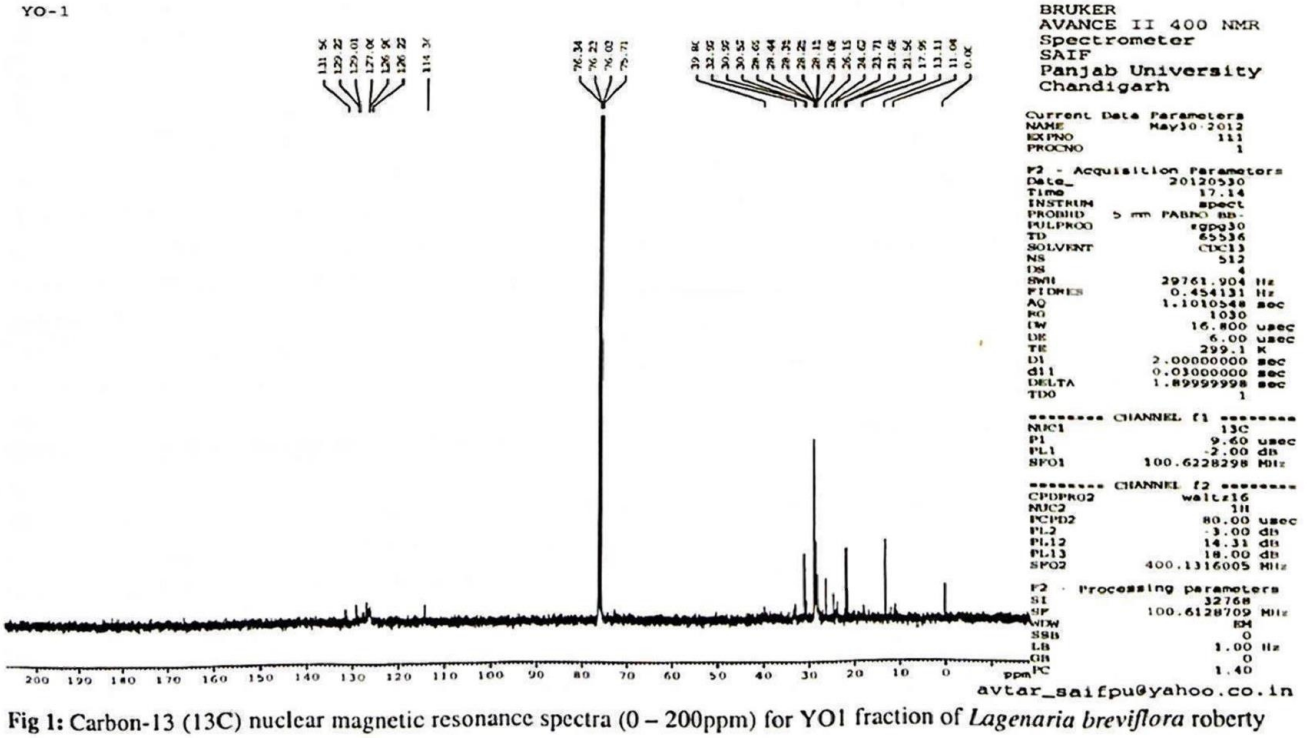

Fig 1: Carbon-13 (13C) nuclear magnetic resonance spectra (0 – 200ppm) for YO1 fraction of *Lagenaria breviflora* roberty

Accelerated gradient chromatography (AGC)

Ethyl acetate fraction of the whole fruit consistently had very potent anti-inflammatory and analgesic

the progress of elution of the compound(s) in the sub-fraction.

Thin layer chromatography (TLC)

The 97 sub-fractions were pooled into 7 using the polarity and R_f values of compound(s) in each test tube on TLC plates. Sub-fractions in test tubes 37 – 45 and 49 - 59 showed distinctive single spots on the TLC plate suggesting the presence of one compound each in these set of test tubes. Pooled sub-fractions 37 – 45 and 49 -59 were further purified using the open column chromatography method.

Open column chromatography

Pooled fractions 37-45 and 49-59 were eluted using solvent combinations with increasing polarity. Eluents from each sub-fraction were collected, dried and renamed fractions YO1 and YO2 respectively. Chemical characterization and structural elucidation of the compounds in both pooled sub-fractions was conducted using MS and NMR.

one had a hydroxyl group attached to it (C3). About 26 carbon atoms were observed coupled to protons only [Fig 1]. About 54 proton atoms were expressed on the Proton (1H) NMR spectra [Fig 2].

Mass Spectroscopic evaluation of fraction YO1 was done. The molecular weight of YO1 was 466.3 m/z with an electron induction weight of 467.3 m/z. About four fragments of the isolated compounds were also identified from the MS (437.2 m/z, 393.2m/z, 391.2m/z and 195.1m/z).

Discussion

In this study, a bioactivity guided fractionation of all parts of *Lagenaria breviflora* fruit was done to ensure no potential candidate was ruled out. All the fractions obtained exhibited potent anti-inflammatory and analgesic effects, but the whole

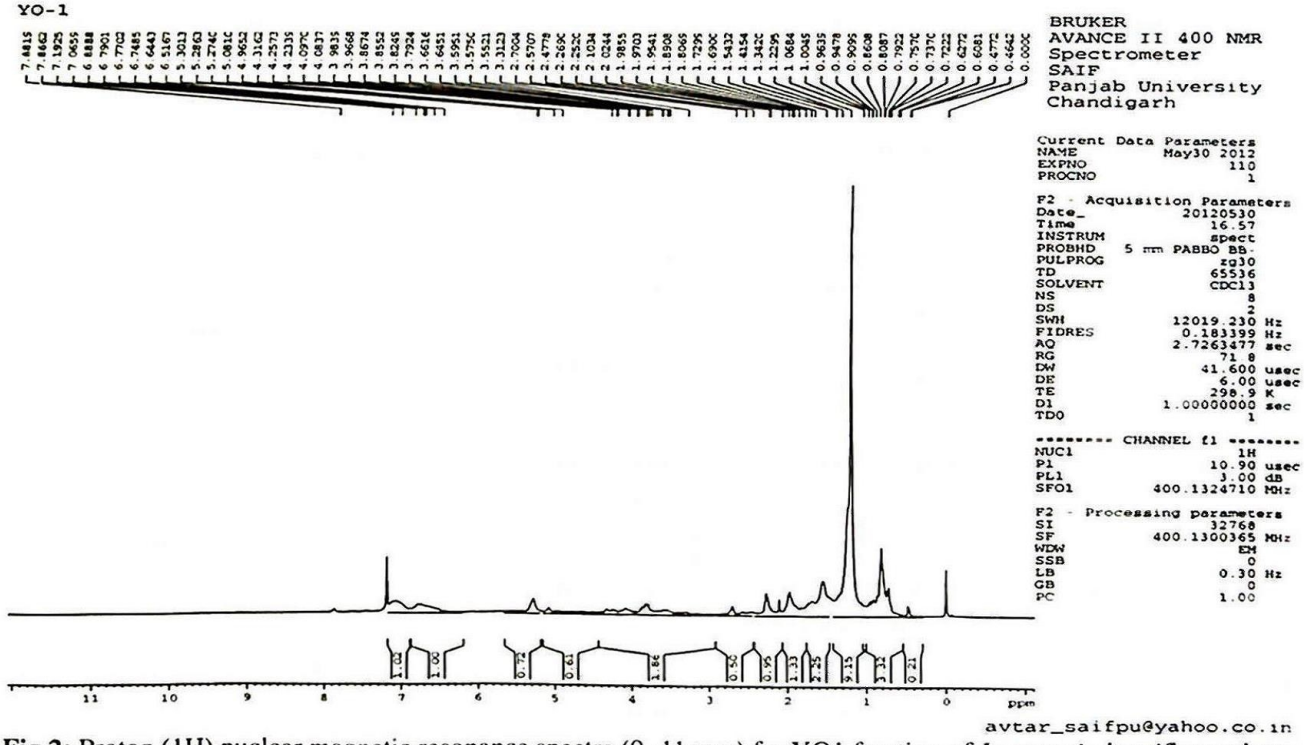

Fig 2: Proton (1H) nuclear magnetic resonance spectra (0 -11ppm) for YO1 fraction of *Lagenaria breviflora* roberty

Nuclear Magnetic Resonance Spectroscopy and Mass Spectroscopy

NMR was used to study the spectra of YO1 using deuterated chloroform as the elution solvent. The carbon 13 (13C) NMR spectra of YO1 showed that the compound has 7 carbon atoms coupled to either a double bond or an oxygen atom (hydroxyl group) as observed after the solvent peak between 75.71 – 76.39 parts per million (ppm). The spectra showed that six out of these seven carbon atoms were coupled to double bonds (C1, C2, C6, C7, C23 and C24) and

fruit showed the most consistent activity in both the anti-inflammatory and analgesic models used. Ethyl acetate fraction of the whole fruit had the most consistent anti-inflammatory/analgesic activities which was why it was selected for further chemical elucidation of the bio-active compound(s) in the fruit.

The bioactive compound isolated from *L. breviflora* is a Cucurbitacin compound with possibly a novel structure. The proposed chemical formula of the compound is $C_{33}H_{54}O$ (Molecular weight 466.3m/z). The structure of the compound has the

10á-cucurbit-5-ene (9â-methyl-19-norlanosta 5-ene) backbone with one hydroxyl group and six carbon atoms attached to double bonds. Numerous cucurbitacins such as B, C, D E, J, I with same basic or primary structures but differing functional groups have been isolated from plants and named. The Cucurbitacin identified in this study does not have same functional groups as any of the existing Cucurbitacins so far reported. The hydroxyl group in the structure explains the antioxidant capacity of this compound in *Lagenaria breviflora* (Benth.) Roberty because hydroxyl groups are known to confer antioxidant activity on a compound [20-22].

Cucurbitacins are triterpenoid steroids reported to have several biological activities and are predominantly isolated from Cucurbitaceae family. These compounds are reported to possess potent anti-inflammatory, analgesic, antioxidant, anti-tumorigenic activities [15, 23-25]. They are able to inhibit the expression of TNF in macrophages and lymphocytes and the expression of proinflammatory mediators such as nitric-oxide synthase-2, lipooxygenase and cyclooxygenase-2 [26, 27]. They can also suppress phosphotyrosine STAT3 levels which result in inhibition of STAT3 DNA binding, inhibit cell proliferation and induce significant S-phase cell cycle arrest and apoptosis [28, 29].

This finding is typical for plants in the family Cucurbitaceae which are renowned for their bioactivities. The Cucurbitacin content of *Cucurbita andreana, Cayaponia tayuya, Wilbrandia ebracteata* and *Ecballium elaterium* have been proven to be responsible for their anti-inflammatory, analgesic, antioxidant and anti-tumorigenic activities [11,13-16]. Several researches are still on-going to determine the ability of these compounds to selectively destroy existing cancer cells or inhibit growth of cancerous cells [27, 30-34].

Further study is recommended to investigate the chemistry and other pharmacological activity of the Cucurbitacin isolated from this plant. This Cucurbitacin may be developed into a new anti-inflammatory, analgesic, antioxidant or anticancer drug of plant origin which is biologically safer and more ecologically friendly.

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