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ISOLATION, GC-MS ANALYSIS AND ANTIOXIDANT ACTIVITY OF THE ETHYL ACETATE LEAF EXTRACT OF *ANNONA SQUAMOSA L*

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ABSTRACT

The study was undertaken to investigate antioxidant activity of *annona squamosa* linn using Ferric reducing assay on different fractions. Ethyl acetate fraction of Annona squamosa leaf was found to exhibit the strongest antioxidant activity *in vitro*. Column chromatography and thin-layer column chromatography of the ethyl acetate fraction lead to an isolated compound. The structure of the isolated compound was examined using 1-D and 2-D NMR which revealed very intense benzene proton and carbon, an amide alongside a sugar glycone while Gas Chromatography Mass Spectrometer showed significant abundance of a benzolated compound which belong to the aporphine class of alkaloids.

KEYWORDS

Annona squamosa, Antioxidants activity, Reducing power assay and Aporphine alkaloids.

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INTRODUCTON

The identification of anti-oxidants from natural products has triggered great interests in the pharmaceutical field for an outstanding role in nullifying the destructive effects of ROS (Ma *et al*, 2016)[1]. Oxidative stress occurs when the effect of reactive oxygen (ROS) and nitrogen (RNS) species overcomes the physiological antioxidant defense mechanisms. Oxidative stress plays a significant role in many pathological conditions such as: cancer (Sosu *et al*, 2012)[2], cardiovascular diseases (Lee *et al*, 2012)[3] including atherosclerosis (Peluro *et al*, 2012)[4], hypertension (Ahmed and Tang, 2012)[5] and heart failure (HF) (Montezeno and Touye, 2012)[6];

metabolic disorders and diabetes (Stadler, 2012[7], Styskal, *et al*, 2012[8]).

Free radicals are fundamentals to any biochemical process and represent an essential part of aerobic life and metabolism (Velavan *et al*, 2012)[9].

Apart from the critical role of photosynthesis, plants can also be manufactured as natural products. Natural products have been used to help human sustain its health since the start of medicine. Over the past centenary, the phytochemicals and active constituents in plants have played a pivotal role in pharmaceutical discovery. The importance of the bioactive materials of plants in medicine and agriculture has stimulated significant interest in the bioactivities of substances (Moghadamtousi *et al*, 2013)[10].

Annona squamosa L, an ever-green tree reaching 3-8m in height is a member of the Annonaceae family which is commonly known as sugar apple, custard apple, sweet sop, sweet apres and sitaphal, is, comprising approximately 135 genera and 2300 species (Raj *et al*, 2009)[11]. The leaf is oblong lanceolate or lanceolate, 6-17cm long and 3-5cm wide, alternately arranged on short petioles; the bark is thin, gray; flower is greenish, fleshy, drooping, extra-axillary, more on leafy shoot than on the older wood and tending to open as the shoot elongates; the fruit can be round, heart-shaped,ovate or conical, 5-10cm in diameter, with many round protuberance; the seeds are 1.3-1.6cm long, oblong, smooth, shiny, blackish or dark brown (Chen *et al*, 2011)[12]. Nowadays, it is cultivated in tropical and sub-tropical regions worldwide (Yang *et al*, 2009)[13].

All portions of *Annona squamosa* tree, are widely used as medicine against various ailments and human diseases, especially for cancer and parasitism (Gajalakshmi *et al*, 2011)[14].

An alkaloid, *p*-hydroxy benzyl-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisoquinoline, isolated from the leaves is reported to be active as a cardiotonic. A fraction of root alkaloids is reported to be antihypertensive, antispasmodic, antihistaminic and a bronchodilator (Thang *et al*, 2013)[15].

Despite investigations in a range of plant species, all established wisdoms are relatively inadequate concerning their underlying role in nature. Therefore, the present study was prompted by this need.

MATERIAL AND METHODS

Pant Material

The leaves of *Annona squamosa* plant were collected at Kwadam area of Gombe State, Nigeria. The leaves were identified by a Taxonomist in the Pharmacognosy department, Faculty of Pharmacy, University of Maiduguri, Nigeria.

Sample Preparation and Extraction

The plant material was air-dried, powdered with the use of a porceclain glass mortar and pestle and then kept in an airtight container until required for further laboratory analysis. Three kilogram (3kg) of the pulverized sample material was extracted with 90% methanol using cold marceration. The crude extract was then concentrated over a water-bath and then exposed to air at 25^{o}C to dryness. The dried extract was weighed, labelled and stored in a desiccator, subject to further analysis. Methanol extract of *Annona squamosa* were fractionated using solvents of graded polarities which include hexane, ethyl acetate, butanol and distilled water.

Reducing Power Assay

The plant extract 2.5ml (or standard gallic acid solution) was mixed with 2.5mL of 0.2M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide solution (2.5mL) and incubated at 50^{o}C for 20 minutes. Sodium phosphate buffer was prepared by dissolving 8.9g disodium hydrogen phosphate in 200mL distilled water, while the pH at 6.6 was acclimatized with monosodium dihydrogen phosphate. After incubation, 10% trichloroacetic acid solution (2.5mL) was added and centrifuged at 650rpm for 10 minutes. The supernatant, 5ml was taken mixed with 5mL distilled water and 0.1% ferric chloride solution (1mL). The control had methanol instead of the plant sample (Gupta, 2004)[16]. The absorbance was measured at 700nm.

Spectroscopic Analysis using NMR

The ^{1}H NMR and ^{13}C NMR spectroscopic analyses were carried out on Brucker AVANCE FT-NMR (850 MHz) at King Abdul Azeez University Jidda, Saudi Arabia.

RESULTS AND DISCUSSION

About 53.6g was obtained from 500g of dried plant sample using crude methanol as solvent of extraction and were later fractionated using solvents of increasing polarities as follows: hexane, ethyl acetate, *n*-butanol and aqueous with 14.00, 27.7, 3.66 and 9.80 respectively. The percentage yields were also presented in Table No.1.

The result of ferric reducing antioxidant assay of crude methanol, hexane, ethyl acetate, butanol and aqueous fractions were presented in Table No.2. The antioxidant potential were estimated from their ability to reduce Fe (III) to Fe (II). The ethyl acetae fraction has the strongest FRAP value of (87.97ug/ml) followed by the crude methanol fraction (84.62ug/ml) which may be due to the relatively high content of tannins and flavonoid. In the study of Narayanan *et al*. (2017)[17] methanol had the highest activity, followed by butanol. Zhia (2008)[18] also reported butanol being highest followed by crude methanol, ethyl acetate, hexane and aqueous fraction which is in line with this study.

Column chromatography

27 collections were obtained, 20 ml each was collected and pulled together based on TLC profile to give 9 major fractions, one of which is a clear crystal (Table No.3).

Thin layer chromatography of the ethyl acetate fraction of *Annona squamosa*

On elution with chloroform and methanol at 50:50 ratio, five collections of 20ml each were obtained, one of which was a clear crystals formed after drying. A TLC plate was prepared by spotting six different points at 2cm above the lower edge of the same plate with the extract (crystals) using a micro pipette. Using a solvent system of butanol, acetic acid and water in the ratio (4:1:5) developed in a tank and sprayed with 10 % H_2SO_4 after it dried, on heating one clear spot was observed from all the six points (Figure No.1) on a TLC plate indicating a compound for structure determination using NMR. The compound was labeled as UM with a melting point of 200^{o}C-202^{o}C.

The R_f value was measured to be 0.58 which was not soluble in hydrophobic (non polar) solvent but soluble in the hydrophilic (polar group) solvents.

NMR spectral analysis

The proton NMR spectra (Figure No.1. H^1.NMR) showed the presence of a benzene ring with peaks at 7.0 and 7.2 which appeared as doublet of a doublet which is characteristic of benzene ring having equivalent proton. (Pretsch, 2008)[19].

Anomeric protons was seen around 4.8ppm, several sugar protons were seen around (4.19ppm) indicating the various hydrogen peaks on the sugar molecule. Then, a particular proton at 3.15ppm represents a CH_2 which is not part of the sugar molecule but found to establish a link between the benzene and the anomeric proton of the sugar (Bubb, 2008)[20].

The carbon NMR (Figure No.2. ^{13}C.NMR) spectra showed characteristic peaks that gave incite to determining the chemical structure. There were peaks at 130,116 which were signals on the benzene ring. The peak observed at 156ppm indicates quaternary carbonyl carbon attached to a Nitrogen (Metin, 1994[21], Pretsch, 2008[19]).

The appearance of peaks at 101ppm and 104ppm showed anomeric carbons of a sugar. There after there appeared a peak at 32ppm which was not part of the sugar molecule but indicative of a methylene group. The depth showed 3 methylene group and some methine carbons which are mostly found in the sugar region (Roslund *et al*, 2008)[22].

The HMBC shows correlation between proton to carbon up to three or 4 carbon distance. From the result there was correlation between the proton peak (7.2ppm) and the carbonyl carbon peak (156ppm) attached to a nitrogen. Also, correlation was observed between equivalent protons (7.2ppm) and the Para C at 130ppm. Also, there was correlation between carbon (32ppm) and protons on the benzene (7.0ppm) which showed that the sugar molecules were linked to the benzene via a CH_2 molecules (Pretsch, 2008[19]).

The H-H Cosy which shows interaction between proton to proton indicates that there were interaction between the methylene (CH_2) proton and the sugar protons (3.26-3.56). Also interaction was

observed between the methylene (CH_2) proton and that of the benzene proton 7.2ppm. The description of the interaction in HMBC and HSQC has been clearly shown in Table 5. Based on the information obtained from the spectral data the suggested structure was discerned to possess an N-phenylformamide aglycone nucleus and a six membered hexose sugar. The compound tested positive for alkaloid which also support the findings.

The ethylacetate portion which showed good bioactive property was subjected to GC-MS analysis and result recorded by direct inlet method (Saranya Devi *et al*, 2015).

Further structural information was obtained from the Gas chromatographic mass spectrometry analysis, as shown in Table No.4.

The compound with Molecular weight at 256 (8-Methyl-4-phenylquinoline-2-hydrazine) gave the highest relative abundance and was suggested as the most probable based on its alkaloid structure which was in agreement with the chemical test.

Table No.1: Percentage yield of crude methanol and fractions

S.No	Solvents	Weight of extract (g)	Percentage yield (%) w/w
1	Crude Methanol	53.60	10
2	**Fractions** Hexane	14	26
3	Ethyl acetate	21.70	40
4	Butanol	3.66	6.8
5	Aqueous	9.80	18.3

Table No.2: Result of Ferric Reducing Assay

S.No	Fractions	FRA value (ug/ml)
1	Crude methanol	84.62
2	Hexane	47.08
3	Ethyl acetate	87.97
4	Butanol	72.72
5	Aqueous	44.94

Key: FRA= ferric reducing assay

Table No.3: Result of Column Chromatography of ethyl acetate fraction of *annona squamosa* leaves

Fractions	Eluding solvents	No of collections	No of sports
F1	CHL 100%	1-5	Nil
F2	CHL/METH 90%-10%	5-8	Nil
F3	CHL/METH 80%-20%	8-11	3
F4	CHL/METH 70%-30%	11-13	2
F5	CHL/METH 60%-40%	13-15	3
F6	CHL/METH 50%-50%	15-20	1
F7	CHL/METH 40%-60%	20-23	3
F8	CHL/METH 30%-70%	23-25	-
F9	CHL/METH 80%-20%	25-27	-

Key: CHL (Chloroform); METH (Methanol)

Table No.4: Result of TLC analysis of compound UM

S.No	Solvent system	Spot	R_f value	Color in 10% H_2SO_4
1	Chloroform: Methanol (50:50)	1	0.58	Yellow

Table No.4: Phytoconstituents identified in ethyl acetate fraction of *Annona squamosa* Leave byGC-MS

Peak	Name	MW	RT	Formulae
1	Carbonic acid, ethyl-methyl ester	10	10.16	$C_4H_8O_3$
2	Dimethyl sulfoxide	78	12.98	$C_2H_6O_8$
3	Dimethyl sulfoxide	78	13.32	$C_2H_6O_8$
4	Dimethyl sulfoxide	78	13.40	$C_2H_6O_8$
5	8-Methyl-4-phenylquinoline-2-hydrazine	265	9.46	$C_{16}H_{15}NO_3$
6	3, 4-Hexanedione, 2, 2, 5-trimethyl	156	20.80	$C_9H_{16}O_2$

Key: MW-molecular weight RT- retention time

Table No.5: Chemical shift values of ^{13}C NMR AND ^{1}H NMR and their Correlations

S.No	C-H (ppm)	HMBC	HSQC	DEPT
1	32- 3.15 Q	3.153-3.36-3.50	7.2, 7.0, 4.8, 3.22, 3.05	CH_2
2	66- 3.65 dd 2.90 T	3.65-2.914-3.26 2.914-3.65-3.26	5.0, 4.2, 3.55, 3.26	CH_2
3	68- 3.55 dd 3.92 d	3.55-3.50-3.92 3.92-3.55-3.50	5.0, 4.2, 3.5	CH_2
4	70.77- 4.8m	4.8-3.22-3.153	5.2. 5.03, 5.0, 3.92, 3.65, 3.55, 3.5, 3.05, 2.92	CH
5	73.6- 3.22 dd	3.22-3.26		CH
6	73.8- 2.96 dd	2.96-3.06		CH
7	76.29- 3.5 Q	3.50-3.55		CH
8	76- 3.27 m	3.27-3.65-3.06-3.92		CH
9	76.84- 3.26	3.26-3.65-3.06-2.92		CH
10	76.92- 3.06m	3.06-3.65-3.26-2.92		CH
11	101- 4.8m	4.8-3.153-3.22	5.39, 3.92,3.5, 3.22	CH
12	104- 4.18 d	4.18-2.97	5.03, 3.82, 3.65, 3.55, 3.06, 2.98, 2.93	CH
13	116- 7.0 d	7.0-7.2	7.2, 7.0	CH
14	130- 7.2 d	7.2-3.5	7.2, 7.0, 4.8, 3.15	CH
15	156		7.2, 7.0, 4.8, 3.15	

Key: HMBC = Heteronuclear Multiple Bond Correlation; HSQC = Heteronuclear Single Quantum Correlation; DEPT = Distortionless Enhancement by Polarization Transfer
Q = Quartet; D = Doublet; dd = Double of doublet; m = Multiplet

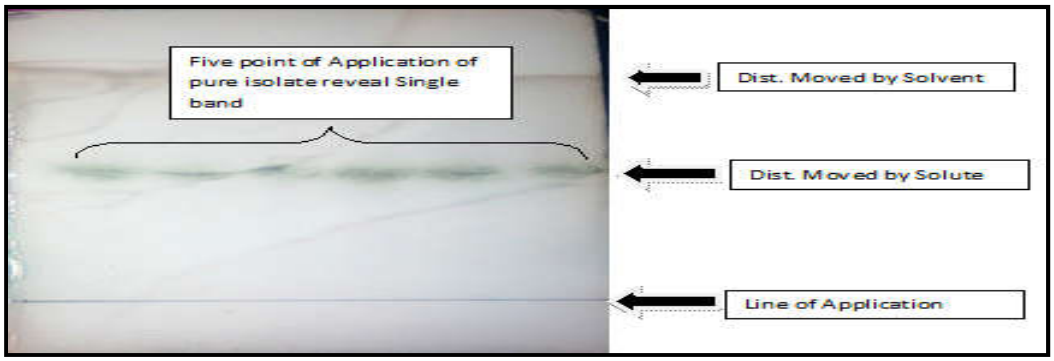
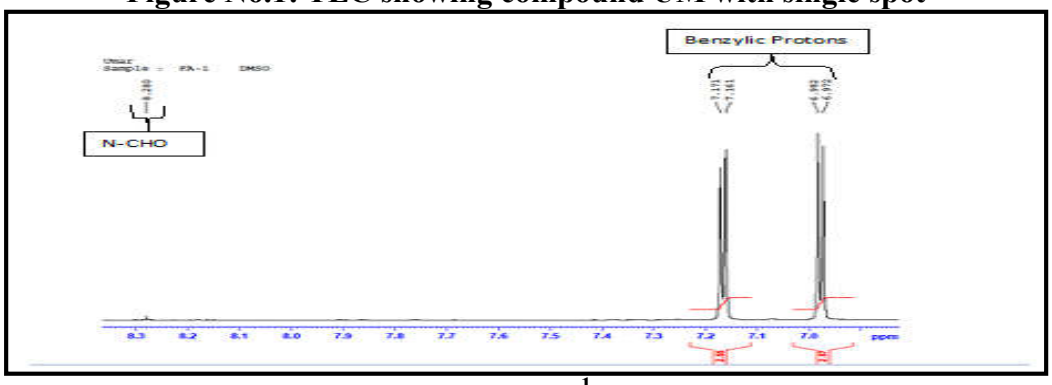
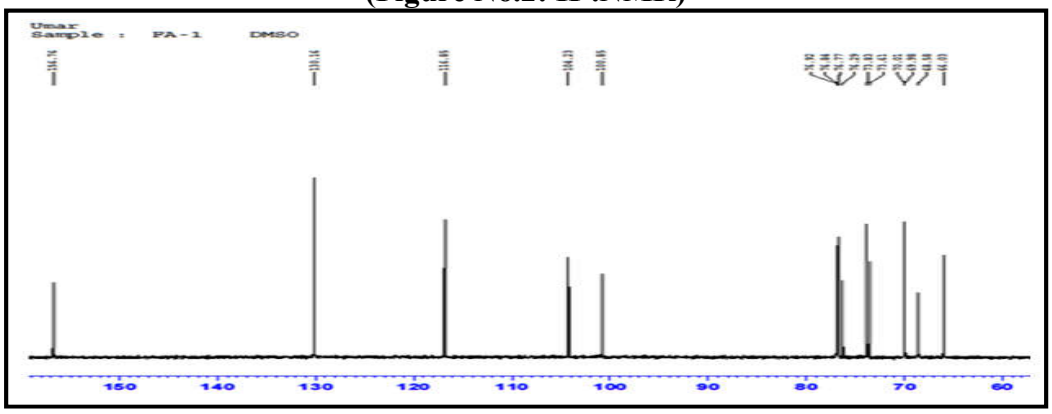
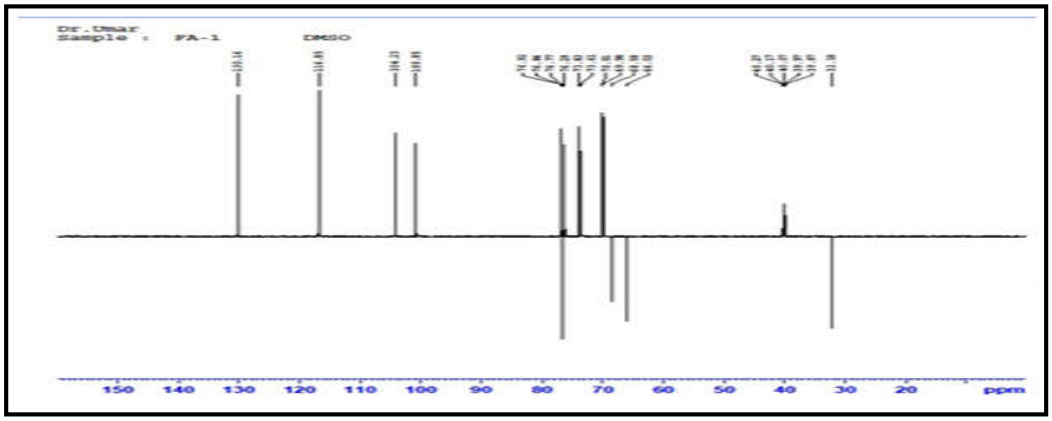


Figure No.1: TLC showing compound UM with single spot



(Figure No.2: H^1.NMR)





(Figure No.3: DEPT Spectra)

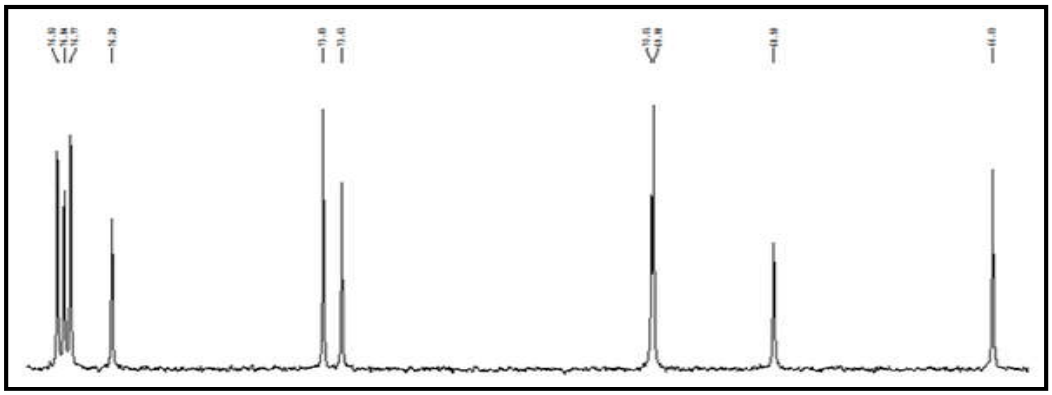


Figure No.4: 13C-NMR for Sugars

CONCLUSION

The *in-vitro* reducing antioxidant assay indicated that the five fractions have varying antioxidant potential. However, the ethyl acetate fraction of *Annona squamosa* leaves exhibited the highest antioxidant activity. Thereafter, the ethyl acetate fraction was subjected to isolation protocol and the compound obtained elucidated using 1D and 2D Proton and Carbon NMR. GC-MS analysis of the ethylacetate portion gave a prominent peak characteristic of an alkaloid compound owing to its chemical formula.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

BIBLIOGRAPHY

1. Ma Q G, Xu L, Sang Z P, Wei R R, Liu W M, Su Y L, Yang J B, Wang A G. Ji T F, Li L J. Alkenes with antioxidative activities from Murraya koenigii (L.) Spreng, *Bioorg. Med.Chem. Lett,* 26(3), 2016, 799-803.
2. Sosa V, Moline T and Somoza R. Oxidative stress and cancer: An overview, *Ageing Res Rev,* 12(1), 2012, 376-390.
3. Lee R, Margaritis M, Channon K, Antoniades C. Evaluating oxidative stress in human cardiovascular disease: Methodological aspects and considerations, *Curr Med Chem,* 19(16), 2012, 2504-2520.
4. Peluso I, Morabito G, Urban L, Ioannone F, Serafi M. Oxidative stress in atherosclerosis development: The central role of LDL and oxidative burst, *Endocr. Metab. Immune Disord. Drug Targets,* 12(4), 2012, 351-360.
5. Ahmed Z, Tang W W. Pharmacologic strategies to target oxidative stress in heart failure, *Curr Hea Fail Rep,* 9(1), 2012, 14-22.
6. Montezano A C, Touyz R M. Oxidative stress, Noxs and hypertension: Experimental evidence and clinical controversies, *Ann Med,* 44(1), 2012, 2-16.
7. Stadler K. Oxidative stress in diabetes, *Adv Exp Med Biol,* 771, 2012, 272-287.
8. Styskal J, Van Remmen H, Richardson A, Salmon A B. Oxidative stress and diabetes: what can we learn about insulin resistance from antioxidant mutant mouse models? *Free Radic Biol Med,* 52(1), 2012, 46-58.
9. Velavan S, Nagulendran K, Mahesh R. *In vitro* antioxidant activity of Asparagus racemosus root, *Phar Mag,* 3(9), 2013, 26-33.
10. Moghadamtousi S Z. Biological activities and phytochemicals of swietenia macrophylla king, *Molecules,* 18(9), 2013, 10465-10483.
11. Raj D S. The hepatoprotective effect of alcoholic extract of *Annona squamosa* leaves on experimentally induced liver injury in

swiss albino mice, *Int Jo Bio,* 5(3), 2009, 182-186.
12. Chen J W, Chen Y, Li X. Beneficial aspects of custard apple (*Annona squamosa* L.) seeds. In: V.R. Preedy, R.R. Watson and V.B. Patel (eds.), *Nuts and Seeds in Health and Disease Prevention, Academic Press, London,* 10(1), 2011, 439-445.
13. Yang H J, Li X, Chen J W, Cai B C. Supercritical fluid CO_2 extraction and simultaneous determination of eight annonaceous acetogenins in Annona genus plant seeds by HPLC-DAD method, *Journal of Pharmacy and Biomedical Analysis,* 49(1), 2009, 140-144.
14. Gajalakshmi S, Divya R, Deepika V D, Mythili S, Sathiavelu A. Pharmacological activities of *Annona squamosa*: A review, *International Journal of Pharmaceutical Science,* 10(2), 2011, 24-29.
15. Thang T D, Dai D N, Hoi T M, Ogunwande I A. Study on the volatile oil contents of *Annona glabra* L, *Annona squamosa* L, *Annona muricata* L and *Annona reticulate* L. from Vietnam, *Natural Product Research,* 27(13), 2013, 1232-1236.
16. Gupta M, Mazumder U K, Gomatthi P, Ramathan S K, Kokate C K, Purohit A P, Gokhale S B. Antioxidant and Free radical, *Textbook of Pharmacognosy,* 29, 2004, 317-320.
17. Narayanan V, Raju R, Leva V, Jeya A I D, Jeya A. Phytochemical and *in vitro* antioxidant activity of various bark extracts of *Annona squamosa* (L), *Oriental Journal of Chemistry,* 33(5), 2017, 2-6.
18. Zhia P R. Evaluation of the antioxidant activity of *Annona squamosa* leaves, *Molecules,* 13(10), 2008, 2545-2566.
19. Pretsch E, Bùhlmann P, Badertscher M. Structure determination of organic compounds; Tables of spectral data, *Springer-Verlag Berlin Heidelberg,* 4th Edition, 2009, 19-23.
20. Bubb W A. NMR Spectroscopy in the study of carbohydrates; Characterizing the structural complexity, *Wiley Periodicals Inc, Concept Mag. Reson,* 19A(1), 2003, 1-14.
21. Metin B. Basic 1H and 13C NMR spectroscopy, *Saunder Elsevier books, Philadelphia,* 2nd Edition, 1994, 297-360.
22. Roslund M U, Petri T, Mattias N, Rainer S. Complete Assignment of the 1H and 13C Chemical Shifts and JH, H Coupling Constants in NMR Spectra of D-glucopyranose and all D-glucopyranonosyl-D-gucopyranosides, *Carbohydrate Research,* 343(1), 2008, 101-112.

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