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GC-MS, FT-IR and ^{1}H NMR Profiling of Bio-Active Phytoconstituents of The TLC Fraction of Ethyl Acetate Leaf Extract of Medicinal Plant, *Annona reticulata L.*

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Abstract

The aim of the present study is to identify the phytocompounds of the TLC fraction (R_f value 0.43 using solvent system of mixture of petroleum ether and ethyl acetate in 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* L. through GC-MS analysis. Functional groups of the phytocompounds of the tested sample were detected by FT-IR analysis and types of protons in the phytocompounds have been confirmed through ^{1}H NMR spectra analysis. Totally ten different bio active phytochemical compounds have been identified from mentioned TLC fraction of ethyl acetate leaf extract of *Annona reticulata* L. at different retention time (RT) with different percent area, and percent height through GC-MS. Several identified phytocompounds were 2-hydroxy-N,3,3-trimethylbutanamide (RT=2.5 min; % area=0.45), 2-hexanol (RT=2.85 min; % area=0.360), N-benzyl-4-hydroxypiperidine (RT=24.15 min; % area=2.17), 6,7-Dimethyl[1,2,4] triazolo [4,3-b][1,2,4] triazine (RT=29.14 min; % area=2.78), Methyl formyl (methyl) dithiocarbamate, (RT=30.95 min; % area=5.69), Butylamine N-methyl-(RT=33.11min; % area=3.03), 2-hydroxyisobutyric acid (RT=36.65 min; % area=0.61), Oxalic acid dipropylester (RT=38.25 min; % area=0.16),1,4,7,10,13 pentaoxacyclo-pentadecane (RT=38.85 min; % area=0.88), and 3,6,9,12,15-pentaonaheptadeoane-1,17-diol(RT=40.84 min; % area=2.61). The FT-IR analysis of the tested sample confirmed the presence of alcoholic OH, amine N-H, Carboxylic acid O-H, asymmetric C-H stretching, symmetric C-H stretching, CH_3, ketone C=O stretching, amide C=O stretching, NH (deformation) and ring (stretching). ^{1}H NMR spectra confirmed the presence of RCH_3, ROH, R_2NH, RCH_2R, $ArCH_3$, $RC{\equiv}CH$, $R_2C{=}CRCHR_2$, $RCOCH_2R$, and RCH_2OR types of protons. Identified several phytochemical compounds have the activities in pharmaceutical, agrochemical, perfume, and cosmetic industries. The presence of such phytochemical compounds may be used in traditional medicine and in agrochemical, perfume, and cosmetic industries. Some isolated pure bio active phytochemical compounds may be used for the preparation of drugs in pharmaceutical industries.

Keywords

Annona reticulata, phytocompounds, GC-MS, FT-IR, ^{1}H NMR

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INTRODUCTION

Plants are chemical factories as they produce a large number of chemical compounds known as secondary metabolites (SMs), derived from metabolic pathways [1, 2]. There are many ancient communities who use the medicinal plants in traditional medicine to cure different diseases. Secondary metabolites provide protection to the plants from the attack of pathogens. Some secondary metabolites can absorb UV rays and prevent severe leaf damage caused by UV rays [3]. Several secondary metabolites of medicinal plants possess many biological activities responsible for health benefits through pharmaceutical and food industries and many of SMs have great value in perfume, agrochemical, and cosmetic industries [2]. Daily use of synthetic drugs may cause addiction, but plant-based medicines are comparatively safer to use than synthetic drugs. Pharmaceutical industries use commercially important plants as a source for the production of synthetic compounds also [4]. Exploration of plant secondary metabolites may provide new leads to develop new drug [5].

Annona reticulata (family: Annonaceae) is evergreen and small tree. It is also named as custard apple, ramphal, sitaphal, sarifa, and bullock's heart. In traditional medicine, the plant is used to cure several diseases like, epilepsy, constipation, cardiac problem, worm infection, and cancer [6]. About 119 several species under genus *Annona* have been identified and most of them are trees and shrubs [7]. Leaf decoction of the plant is used traditionally as vermifuge and stem bark decoction is used for the treatment of dysentery, diarrhea and acts as tonic. Ethanol root extracts of the plant has anticancer potential against human cancer cell lines [8]. Crude and ethyl acetate root extracts of the plant have mosquito larvicidal activity [9].

The present study was carried out to unfold the presence of bioactive phytoconstituents in the sample of TLC bands with R_f value 0.43 (using petroleum ether and ethyl acetate solvent system with 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* plant with the help of GC-MS, FT-IR and ^{1}HNMR techniques which may provide a clear idea to its application in traditional medicine system and the plant will be the source of products for several pharmaceutical, industrial and biological application.

MATERIALS AND METHODS

Collection of leaves and identification of the Plant:

Leaves of *A. reticulata* plant (1–5-year-old) were collected during the month of August from Haldibari municipality town, Coochbehar, West Bengal, India (88^0 45′ 12.00′′ E longitude and 26^0 19′ 48.00′′ N latitude). After identification of the plant by Professor, Dr A. Mukhopadhyay, Department of Botany, The University of Burdwan, West Bengal, India, the voucher specimen of the plant (voucher no. GCZSM-4) was preserved in the Department of Zoology, The University of Burdwan, West Bengal, India.

Preparation of solvent extracts:

The present study was done in the Mosquito Microbiology and Nanotechnology Research Units, Parasitology Laboratory, Department of Zoology, The University of Burdwan, West Bengal, India ($23^0$16′ N, $87^0$54′ E). Maceration method was used to obtain the ethyl acetate leaf extract of the plant as per protocol described by Sharma *et al.*, 2016 [10] with slight modification. Collected fresh and cleaned leaves of the plant were dried in shade for a period of 15 days. Dried leaves were ground in stainless electric blender and then sieved to obtain fine powder material. Petroleum ether, hexane, and ethyl acetate solvents (from low to high polarity) were used to obtain said different solvent extracts. 50 g dried leaf powder material was kept in a brown bottle and poured 500 ml petroleum ether. Thereafter the mouth of bottle was closed tightly by lid and kept for 21 days with frequent agitation daily. The extract was then filtered through Whatman no.1 filter paper to obtain a liquid extract. Collected liquid extract was concentrated by direct evaporation. Thereafter the same plant material was soaked in hexane and finally in ethyl acetate, one after another maintaining the same procedure as described as before like petroleum ether leaf extract. Each of the semisolid leaf extract of petroleum ether, hexane, and ethyl acetate were kept separately in a refrigerator at 4°C. Only ethyl acetate leaf extract of the plant is used in the present study.

Isolation of distinct band from ethyl acetate leaf extract of *Annona reticulata*:

Thin Layer Chromatography (TLC) silica gel plates (0.25 mm thickness) were prepared using Uno plan coating apparatus (Shadon, London) and dried with heat (100°C for 30 minutes). Semisolid ethyl acetate extract (~45 mg) was taken in a test tube and added ethyl acetate solvent (~3 ml) to get liquid extract. Liquid extract was applied to the bottom of each of the prepared (silica gel coated and heated) glass plates using capillary tube as separated drops along a straight line (10 cm from extreme bottom part of silica gel plate). After drying (5-7 minutes), each of the plate was placed in a TLC glass chamber using mixture of petroleum ether and Ethyl acetate (1:1) solvent system as a mobile phase. After the movement of solvent at the top of the plate, each plate was removed from the glass chamber and air-

dried. Twenty plates were chromatogrammed. One distinct band with R_f value 0.43 was detected from ethyl acetate leaf extract. Bands of same R_f value were scrapped from twenty TLC plates and kept in a cleaned beaker and then mixed absolute alcohol to dissolve the phytoconstituents. Phytoconstituents dissolved in absolute alcohol and silica gel remained on the bottom of beaker. Thereafter, the alcohol with phytoconstituents was separated from silica gel and filtered by whatman no. 42 filter paper and filtrate was deposited on a vial. After evaporation of alcohol, semi solid fraction was obtained and used for analyses.

GC-MS analyses of bio active phytoconstituents:

GC-MS analysis of active compounds was carried out at Bose Institute Laboratory, Kolkata, West Bengal, India. GC-MS analysis of the active fraction was performed using GC (Model TRACE-GC-ULTRA) and gas chromatograph interfaced to a Mass Spectrometer (MS- Model POLARISQ) [GC-MS] equipped with capillary column (TRWAX) of 30 m length, 0.25 mm diameter and 0.25 µm thicknesses and polyethylene glycol was used as a stationary phase. For GC-MS detection, an electron ionization energy system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.0 ml/minute and an injection volume of the sample was 1 µl. The oven temperature was programmed initially at 40^0 C for 2 minutes, thereafter an increase to 130^0 C and then programmed to increase to 270^0 C and hold for 15 minutes. The MS transfer line was maintained at a temperature of 270^0 C. The injector temperature was 240^0 C. The sample was dissolved in ethyl acetate solvent and split ratio was 1:20. Identification of active compounds on the Mass spectrum was performed by using the data on National Institute Standard and Technology (NIST) library.

FT-IR analysis of bio active phytoconstituents:

1 mg of active fraction were mixed with 10 mg of potassium bromide (KBr) and pressed by the use of hydraulic press apparatus to form a pellet. Control KBr pellet was made by only with KBr but without any active fraction. FT-IR spectrometer (Model: Jasco, FT/IR-4700) was used for the detection of functional groups in the sample. Prepared pellet was loaded in said spectrometer and scanned at room temperature (25^0 C±5^0 C) with a scan range from 400-4500 cm^{-1}.

^{1}H NMR analyses of bio active phytoconstituents:

^{1}H NMR spectra of aforesaid bioactive phytoconstituents were determined on 400 MHz spectrometer as solutions in $CDCl_3$. Chemical shifts were expressed in parts per million (δ) and the signals were reported as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), m (multiplet), and coupling constants (J) were given in Hz. Chemical shifts as internal standard were referenced to $CDCl_3$ (δ = 7.26 for ^{1}H) as internal standard.

RESULTS AND DISCUSSION

Presence of various bio active phytoconstituents on TLC bands with similar R_f value 0.43 (using solvent system of mixture of petroleum ether and ethyl acetate in 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* has been evaluated through GC-MS analysis (Figure 1). The name of identified several bio active compounds (through GC-MS analyses) with their retention time (RT), molecular formula, molecular weight (MW), percent (%) area, and percent (%) height has been depicted in Table 1. Identified several bio active phytocompounds were 2-hydroxy-N,3,3-trimethylbutanamide, 2-hexanol, N-benzyl-4-hydroxypiperidine, 6,7-Dimethyl [1,2,4] triazolo [4,3-b] [1,2,4] triazine, Methyl formyl (methyl) dithiocarbamate, Butylamine, N-methyl-, 2-hydroxyisobutyric acid, Oxalic acid dipropylester, 1,4,7,10,13 pentaoxacyclo-pentadecane, and 3,6,9,12,15-pentaonaheptadeoane-1,17-diol. The structure of said several phytocompounds have been presented in Table 2. Activities of identified compounds have been presented in Table 3. Among ten total compounds, methyl formyl (methyl) dithiocarbamate ($C_7H_{15}NO_2$) showed the highest percent area (5.69) and percent height (4.19) with the retention time (RT)=30.95 minutes. Dithiocarbamate has been used in the separation of metal ions via solvent extraction and N-methyl-N-phenyl dithiocarbamate acts as antibacterial and antifungal agents [11]. 2-hydroxy-N,3,3-trimethylbutanamide ($C_7H_{15}NO_2$) (identified at the retention time=2.5 minutes, percent area of 0.45, and percent height of 0.58) has not found reported activity. 2-hexanol ($C_6H_{14}O$) has been identified at the retention time 2.85 minutes with percent area of 0.36, and percent height of 0.42. (+/-)-2-Hexanol is s an important raw material and intermediate used in organic synthesis, pharmaceuticals, agrochemicals and dyestuff field. It is used as perfuming agent [12]. (*R*)- (–)-2-hexanol and (*S*)-(+)-2-hexanol were used to prepare of some key intermediates for model studies in the total synthesis of antivirally active glycolipid cycloviracin B_1 [13]. N-benzyl-4-hydroxypiperidine ($C_{12}H_{17}NO$) (with retention time =24.15 minutes, percent area of 2.17, and percent height of 2.08) is used as an alternative molecule to study the ligand concentration attached to the epoxy-activated Sepharose 6B. It is used as reactant for synthesis of inhibitors of rho kinase, muscarinic acetylcholine receptor antagonist and beta 2 adrenoceptor

agonist, inhibitors of fatty acid amide hydrolase, Urotensin-II receptor antagonists, and inhibitors of PI3 kinase-alpha [14]. 6,7-Dimethyl [1,2,4] triazolo [4,3-b][1,2,4] triazine ($C_6H_7N_5$) has its retention time =29.14 minutes, percent area of 2.78 and percent height of 2.62. [1,2,4] triazolo [4,3-b][1,2,4] triazine is used as protein tyrosine kinase inhibitor particularly as a c-Met inhibitor [15]. Derivatives of [1, 2, 4] triazolo [4,3-b][1,2,4] triazine act as antibacterial, antifungal, and anti-inflammatory activities [16]. Butylamine,N-methyl- ($C_5H_{13}N$) (retention time =33.11 minutes, percent area of 3.03 and percent height of 1.05) may be useful as an early marker for both insulin resistance and impaired glucose regulation [17]. 4-(O-benzylphenoxy)-N-methylbutylamine acts as an antidepressant and cerebral activator [18]. 2-hydroxyisobutyric acid ($C_4H_8O_3$) (retention time =36.65 minutes, percent area of 1.61 and percent height of 1.06) is used as bio marker for several metabolic diseases such as diabetes mellitus and adiposity. It acts on antibacterial degration of the fuel oxygenates methyl tert-butyl ether [19]. Oxalic acid dipropylester ($C_8H_{14}O_4$) (retention time =38.25 minutes, percent area of 0.16 and percent height of 0.54) is used as chelating agent, plasticizer, and solvent [20]. 1,4,7,10,13 pentaoxacyclo-pentadecane ($C_{10}H_{20}O_5$) (retention time =38.85 minutes, percent area of 0.88 and percent height of 1.11) is used as an efficient phase transfer catalyst and acts as a complexing agent [21]. 3, 6, 9, 12, 15-pentaonaheptadeoane-1, 17-diol ($C_{12}H_{26}O_7$) (retention time =40.84 minutes, percent area of 2.61 and percent height of 1.05) is used as eye liners or brow coloring products, Power steering fluids, fuel injector cleaners, gas treatments, or leak stoppers [22]. The result of FT-IR analyses of bio active compounds of said TLC fraction of ethyl acetate leaf extract of the plant was presented in Figure 2. FT-IR analysis was done to know the functional groups of the several bioactive compounds in the sample based on the peak values in the region of IR radiation. Peak values of FT-IR analysis of bio active phytocompounds confirmed the presence of several functional groups, such as alcoholic OH, and amine N-H (for peak value 3427.85 cm^{-1}); Carboxylic acid O-H, and asymmetric C-H stretching (for peak value 2921.63); symmetric C-H stretching and CH_3 (for peak value 2851.24 cm^{-1}) and ketone C=O stretching, amide C=O stretching, NH (deformation), ring (stretching) [for peak value 1622.8 cm^{-1}]. Result of analyses of ^1H NMR spectroscopy of bio active phytoconstituents of said TLC fraction of ethyl acetate leaf extract of *Annona reticulata* was presented in Figure 3. The chemical shift is the position on the δ scale (in ppm) where the peak occurs in ^1H NMR spectroscopy chemical shift. Chemical shifts 0.831, 0.849 and 0.865 ppm (δ) confirmed the presence of RCH_3 [alkyl (methyl)], ROH (alcohol), and R_2NH. Chemical shifts1.230 ppm (δ) confirmed the presence of RCH_2R [alkyl (methylene)], ROH (alcohol) and R_2NH. Chemical shifts 2.491, 2.495 and 2.500 ppm (δ) confirmed the presence of $ArCH_3$ [benzyl (C is next to C=O)], RC≡CH, R_2C=$CRCHR_2$, $RCOCH_2R$, ROH, R_2NH. Chemical shifts 12.504, and 2.508 ppm (δ) denoted the presence of RC≡CH, R_2C=$CRCHR_2$, $RCOCH_2R$, ROH, and R_2NH. Chemical shifts 3.369 confirmed the presence of RCH_2OR, ROH, and R2NH. So, ^1H NMR spectra confirmed the presence of RCH_3, ROH, R_2NH, RCH_2R, $ArCH_3$, RC≡CH, R_2C=$CRCHR_2$, $RCOCH_2R$, and RCH_2OR types of protons.

Figure 1. Result of GC-MS analyses of bioactive phytoconstituents of TLC bands with R_f value 0.43 (using solvent system of mixture of petroleum ether and ethyl acetate in 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* L.

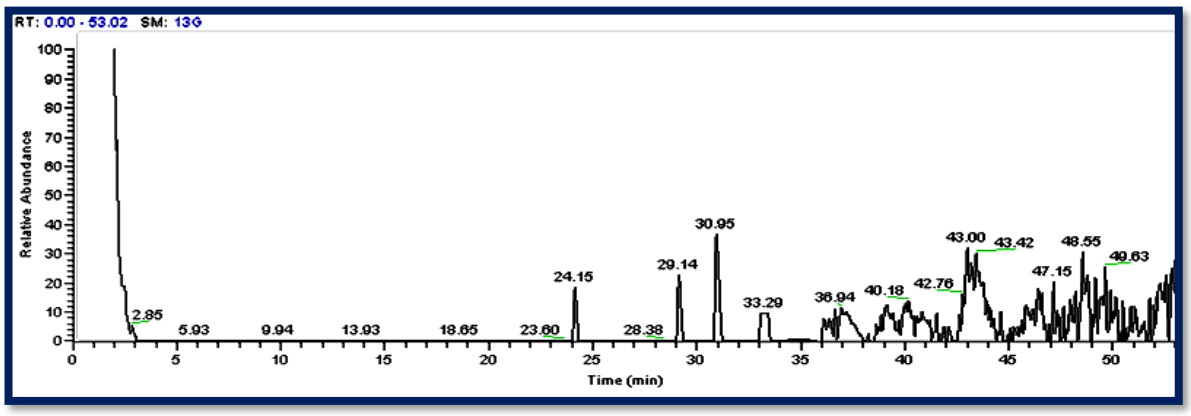


Table 1. Phytoconstituents identified through GC-MS analyses of TLC bands with R_f value 0.43 (using solvent system of mixture of petroleum ether and ethyl acetate in 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* L.

Sl. No.	Retention Time (RT)	Name of the compound	Molecular formula	Molecular Weight (MW)	% Area	% Height
1.	2.5	2-hydroxy-N,3,3-trimethylbutanamide	$C_7H_{15}NO_2$	145	0.45	0.58
2.	2.85	2-hexanol	$C_6H_{14}O$	102	0.36	0.42
3.	24.15	N-benzyl-4-hydroxypiperidine	$C_{12}H_{17}NO$	191	2.17	2.08
4.	29.14	6,7-Dimethyl[1,2,4] triazolo [4,3-b][1,2,4] triazine	$C_6H_7N_5$	149	2.78	2.62
5.	30.95	Methyl formyl (methyl) dithiocarbamate	$C_4H_7NOS_2$	149	5.69	4.19
6.	33.11	Butylamine,N-methyl-	$C_5H_{13}N$	87	3.03	1.05
7.	36.65	2-hydroxyisobutyric acid	$C_4H_8O_3$	104	0.61	1.06
8.	38.25	Oxalic acid, dipropylester	$C_8H_{14}O_4$	174	0.16	0.54
9.	38.85	1,4,7,10,13 pentaoxacyclo-pentadecane	$C_{10}H_{20}O_5$	220	0.88	1.11
10.	40.84	3,6,9,12,15-pentaonaheptadeoane-1,17-diol	$C_{12}H_{26}O_7$	282	2.61	1.05

Figure 2. Result of FT-IR analyses of phytoconstituents of TLC bands with R_f value 0.43 (using solvent system of mixture of petroleum ether and ethyl acetate in 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* L.

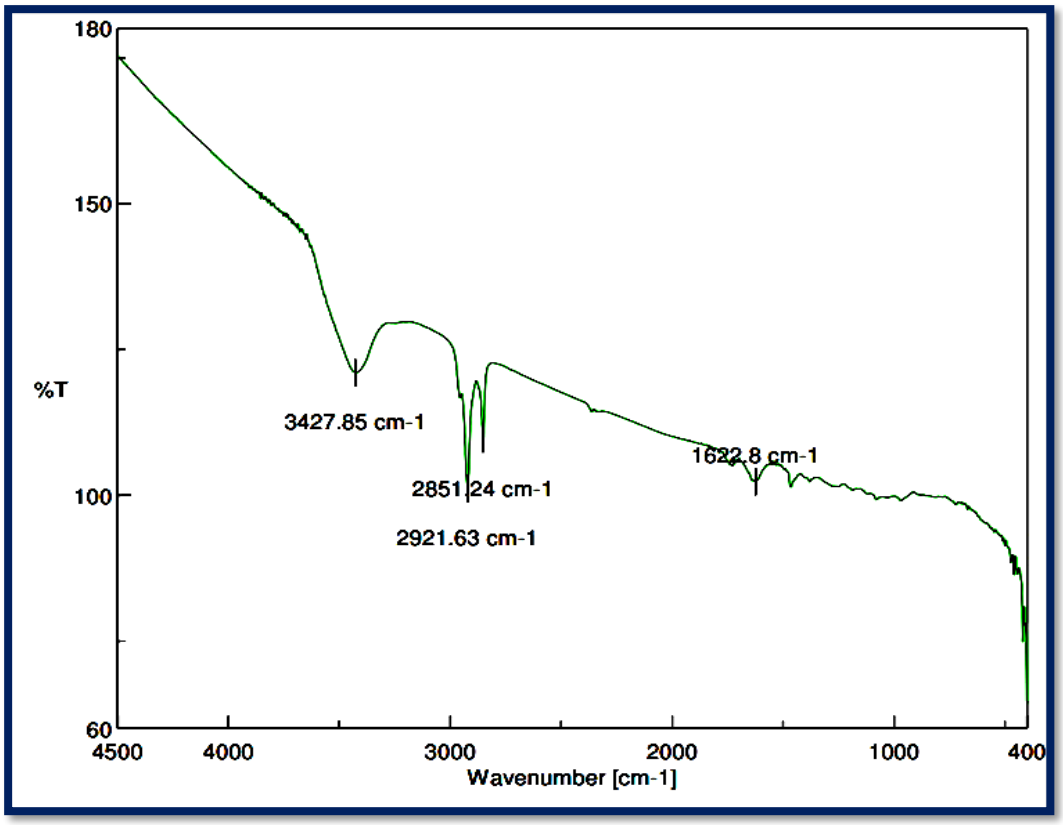
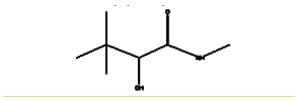
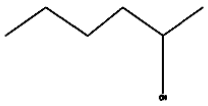


Table 2. Structure of phytocompounds identified through GC-MS from the TLC bands with R_f value 0.43 (using solvent system of mixture of petroleum ether and ethyl acetate in 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* L.

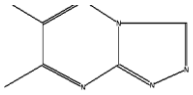


(1) 2-hydroxy-N, 3, 3-trimethylbutanamide

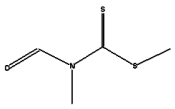


(2) 2-hexanol

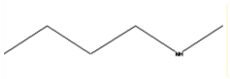
(3) 1 N-benzyl-4-hydroxypiperidine



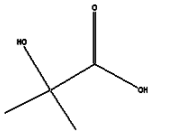
(4) 6, 7-Dimethyl [1, 2, 4] triazolo [4, 3-b][1,2,4] triazine



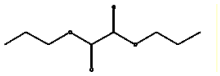
(5) Methyl formyl (methyl) dithiocarbamate



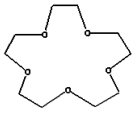
(6) Butylamine, N-methyl-



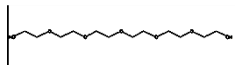
(7) 2-hydroxyisobutyric acid



(8) Oxalic acid, dipropylester



(9) 1,4,7,10,13 pentaoxacyclo-pentadecane



(10) 3,6,9,12,15-pentaonaheptadeoane-1,17-diol

Figure 3. Result of chemical shift ^{1}H NMR of TLC bands with R_f value 0.43(using solvent system of mixture of petroleum ether and ethyl acetate in 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* L.

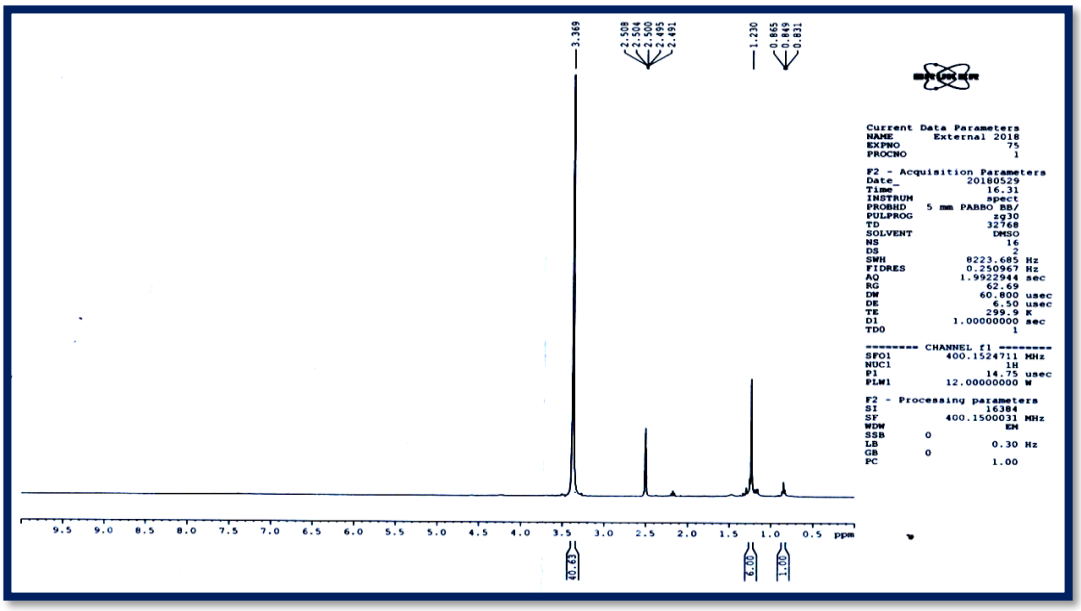


Table 3. Activity of phytoconstituents identified by GC-MS from TLC fraction having R_f value 0.43(using solvent system of mixture of petroleum ether and ethyl acetate in 1:1 ratio) of ethyl acetate leaf extract of *Annona reticulata* L.

Sl No.	Compounds	Activity
1.	2-hydroxy-N,3,3-trimethylbutanamide	No reported activity
2.	2-hexanol	(+/-)-2-Hexanol are important raw material and intermediate used in pharmaceuticals, organic synthesis, agrochemicals and dyestuff field. It is used as perfuming agent.
3.	N-benzyl-4-hydroxypiperidine	N-Benzyl-4-hydroxypiperidine is used as an alternative molecule to study the ligand concentration attached to the epoxy-activated Sepharose 6B. It is used as reactant for synthesis of inhibitors of rho kinase, muscarinic acetylcholine receptor antagonist and beta 2 adrenoceptor agonist, inhitors of fatty acid amide hydrolase, Urotensin-II receptor antagonists, and inhibitors of PI3 kinase-alpha.
4.	6,7-Dimethyl [1,2,4] triazolo [4,3-b][1,2,4] triazine	It acts as protein tyrosine kinase inhibitor particularly as a c-Met inhibitor. Derivatives of [1,2,4] triazolo [4,3-b][1,2,4] triazine act as antibacterial, antifungal, and anti-inflammatory activities
5.	Methyl formyl (methyl) dithiocarbamate	Dithiocarbamate has been used in the separation of metal ions via solvent extraction. N-methyl-N-phenyl dithiocarbamate acts as antibacterial and antifungal agents.
6.	Butylamine,N-methyl-	May be useful as an early marker for both insulin resistance and impaired glucose regulation. 4-(O-benzylphenoxy)-N-methylbutylamine acts as an antidepressant and cerebral activator.
7.	2-hydroxyisobutyric acid	Bio marker for several metabolic diseases such as diabetes mellitus and adiposity, antibacterial degration
8.	Oxalic acid, dipropylester	It acts as chelating agent, plasticizer, and solvent
9.	1,4,7,10,13 pentaoxacyclo-pentadecane	It is used as a ligand, acts as phase transfer catalyst
10.	3,6,9,12,15-pentaonaheptadeoane-1,17-diol	It is used as eye liners or brow coloring products, Power steering fluids, fuel injector cleaners, gas treatments, or leak stoppers

Plants produce various phytoconstituents known as secondary metabolites and many of them have great values in pharmaceutical, food industry, agrochemical, perfume, and cosmetic industries. Many authors reported GC-MS, and FT-IR profiling of different solvent extracts of several plants. Sunil *et al.*, 2018 [23] worked with methanol leaf extract of *Coriandrum sativum* to observe its phytoconstituents through GC-MS and FT-IR analyses. 40 bio active compounds have been identified through GC-MS and functional groups of several compounds have been confirmed by FT-IR analysis. Paul and Devi, 2021 [24] worked with methanol fruit extracts of *Ficus racemosa* and *Ficus auriculata* to investigate their phytoconstituents through GC-MS and FT-IR analyses. Result of GC-MS and FT-IR analyses confirmed the presence of several bio active phytoconstituents with medicinal as well as pharmacological activities. Khan *et al.*, 2018 [25] reported the chemical profiling of methanol and hexane leaves and stems of *Alternanthera sessilis* red from Sabah, Malaysia through GC-MS analyses and also observed antioxidant potential. Pakkirisamy *et al.*, 2017 [26] worked with methanolic extract of *Curcuma caesia* Roxb (black turmeric) to study its chemical profiling through GC-MS and FT-IR analyses. Total 15 bio active compounds have been identified by their study.

CONCLUSION

The various phytocompounds have been identified through GC-MS analysis of TLC fraction of ethyl acetate leaf extract of *Annona reticulata* L. FT-IR analysis of the phytoconstituents of the tested sample confirmed the presence of several functional groups. ^{1}H NMR spectra of the sample confirmed the

presence of types of protons by their chemical shift (ppm). Identified several phytocompounds have their activities in pharmaceutical, agrochemical, perfume and cosmetic industries. The presence of such phytocompounds may be used in traditional medicine and research and isolated some pure bio active phytocompounds may be used for the preparation of drugs in pharmaceutical industries.

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