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Research Article

Phytochemical, Antimicrobial and GC-MS Analysis of *Juglans regia* LINN.

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Abstract

Juglans regia L. is a monoecious, heterodichogamous, deciduous tree species valued for its high quality timber as well as its nuts. It belongs to the family Juglandaceae. i It is native to central Asia, India, Nepal, China and some parts of Europe. Qualitative analysis of seed extracts of *Juglas regia* indicates the presence of alkaloids, flavonoids and tannin and its aqueous solution indicates the presence of alkaloids, carbohydrates, flavonoids, tannin and phenolic content as well. The seed extract of *Juglans regia* shows high potential of antimicrobial activity for both gram positive and gram negative control. The GC-MS of the seed extract of *Juglans regia* showed the presence of different compounds at different retention time. The mass spectrum of the compound reported at retention time 18.157 min, 18.785 min, 19.851 min and 19.977 min can be isopropyl myristate, hexadecanoic acid, 9,12,15-Octadecatrienoic acid and Octadecanoic acid respectively.

Keywords: Phytochemicals, Antimicrobial, GC-MS analysis, ethanol extract, *Juglans regia*

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Introduction

The high protein and oil content of the kernels of *Juglans regia* L. makes this fruit indispensable for human nutrition. Therefore, the walnut is classified as a strategic species for human nutrition and is included in the FAO list of priority plants [1]. Walnut has been widely used as herbal medicine in the treatment of diabetes [2] and in folk medicine to treat prostate and vascular disturbance [3].

The most important product from the *Juglans regia* is, of course, its fruit. The bark and leaves have medicinal uses. The wood is even-grained, grayish brown with dark brown streaks and is highly valued for furniture and gunstocks [4]. The leaves are used internally the treatment of constipation, chronic coughs, asthma, diarrhea, dyspepsia etc and also used to treat skin ailments and purify the blood [5, 6]. The seeds are ant lithic, diuretic and stimulant. They are used internally in the treatment of low back pain, frequent urination, weakness of legs, chronic cough and asthma, constipation due to dryness or anemia and stones in the urinary tract [7].

The oil from the seed is anthelmintic [6]. It is also used in the treatment of menstrual problems and dry skin conditions and the cotyledons are used in the treatment of cancer [6].

Materials and Methods

Collection of plant materials

Around 10 kg of fruits of *Juglans regia* were collected from Dhorpatan Hunting Reserve which is situated in Daulagiri Himal of western Nepal. The collected fruit is identified by Department of Botany Trichandra Multiple Campus, Tribhuvan University,Kathmandu,Nepal.

Preparation of extract

Ethanol extract of seed of *Juglans regia* were prepared by using soxhlet extraction method. About 150 grams of seed of *Juglans regia* was extracted with ethanol (400 ml) for 34 hours in soxhlet. The collected ethanol extract of seed of *Juglans regia* was concentrated dried to solid on water bath.

Phytochemical Analyses

Qualitative Phytochemical Analysis

The ethanolic root extracts of *Juglans regia* was tested for the presence of different phytoconstituents like alkaloids, flavonoids, steroids, phenols and so on by method described by Tiwari P. et al.[8] and Bhatt S. & Dr. Dhyani S. [9] with slight modification.

Test for Alkaloids

Wagner's Test: About 2-3 ml of plant extracts was acidified with hydrochloric acid in separate test tube, few drops of Wagner's reagent was added to each. A reddish brown precipitate indicated the presence of alkaloids.

Test for Flavonoids

Pew's Test: About 2-3 ml of plant, added zinc powder in a test tube, followed by drop-wise addition of conc. HCl. Formation of purple red or cherry color indicates the presence of flavonoids.

Test for Tannins

Lead Acetate Test: To 5 ml of extract, few drop of 10% of lead acetate solution were added, formation of yellow or red precipitate indicated the presence of tannins.

Test for Sterols

Salkowski's Test: About 2ml extracts were treated with about 2ml chloroform and 2ml of conc. H_2SO_4 and well shaken. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence indicated the presence of sterols.

Test for Glycosides

Molisch's Test: About 1 ml of extract, 2 drops of Molisch's reagent was added in a test tube and 2 ml of conc. H_2SO_4 was added carefully keeping the test tube slightly curved. Formation of violet ring at the junction indicated the presence of glycosides.

Test for phenols

Phenolic test: About 2 ml of plant extracts was taken in test tube and few drops of $FeCl_3$ was added to it. The formation of deep violet color indicated the presence of phenols.

Antimicrobial Screening

The antimicrobial activity of ethanol extract of seeds of *Juglans regia* is tested by disk diffusion method using Mueller Hinton agar commonly known as agar diffusion method. This method of Antimicrobial susceptible test is proposed by Kirby and Bauer. [10]

Collection of test organism

The identified microbial strains that were available at Department of Microbiology, Tri-Chandra Multiple Campus, Kathmandu, Nepal were taken as the test organism. The strains includes two different types of bacteria i.e. Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*).

Preparation of working solution

An amount of 25% of working solution was made by transferring 25 mg of ethanol extract of seed of *Juglans regia* to sterile vile ascetically containing 1 ml ethanol solvent, After making working solution, the test-tube were capped sealed and stored in refrigerator until use.

Preparation of media Muller Hinton agar (MHA)

About 0.5 gm of media was suspended in 250 ml of distilled water. It was dissolved by boiling and sterilized by autoclaving of 15 lb pressure and 14^{o}C for 15 minutes. It was then allowed to cool to about 50^{o}C and poured to Petridish in 20 ml per plate quantities. The plates were left as such for solidification.

Screening and evaluations of Anti-bacterial activities

The already prepared sterile MHA plates were dried to remove the moisture from the surface of media. Sterile cotton swab was dipped into the prepared inoculums and the excess of inoculums were removed by processing and rotating against the upper side wall of the tube above the liquid level then swabbed carefully all over the plates. The inoculated plates were left dry to dry for few minutes of room temperature with the lid closed. The wells were made in the plates with the help of sterile cork borer (6 mm) and labeled properly. The 20 ml of the working solution of the plate extract were loaded into the respective wells with the help of micropipette. The separate activities of the solvent and the extract were tested at a same time in the separate hole in the same plate. The plates were then left for half an hour with the lid closed so that the extract diffused to the whole media. The plates were incubated for 24 hrs at 37^{o}C. After 24 hrs, the plates were then observed for the Zone of Inhibition (ZOI) produced by the extract of *Juglans regia*. At the same time ZOI were measured with the help of ruler for the determination of the activities of the extracts as an anti-microbial.

ZOI = Total Diameter including Inhibition – Diameter of Bore

GC-MS Condition

Gas chromatography combined with mass spectroscopy is a preferable methodology for routine analysis of compounds. Ethanol extract of seeds of *Juglans regia* was injected into the Gas chromatography unit Agilent Technologies 7880A GC was the instrument used for GC-MS analysis. It is separated into various constituents with different retention time which are detected by mass spectrophotometer. The chromatogram a plot of intensity against retention time was recorded by the software attached to it. From the graph the compounds are identified comparing the data with the existing software libraries. 1 µl of the ethanol extract of seeds of *Juglans regia* was injected into GC. The injection mode was used split method with a flow of 1ml/min. The carrier gas used was Helium. The injector temperature was maintained at 300ºC. The oven temperature was set at 220ºC to 300ºC with a gradual increment of 5ºC per min.

Results and Discussions

Phytochemical Analyses

Qualitative analysis

The results of phytochemical screening of ethanolic seed extracts of *Juglans regia* is presented in **Table 1**. Qualitative tests for alkaloids, flavonoids, tannins, sterols, glycosides and phenols were carried out in order to know the presence of primary and secondary metabolites in these crude extracts of the plant. Qualitative analysis of ethanolic seed extracts of *Juglans* indicates the presence of alkaloids, flavonoids, tannins and phenols.

Table 1 Phytochemical screening of ethanolic extract of *Juglans regia*

Phytochemical constituents	**Qualitative analysis of *Juglans regia***
Alkaloids	+
Flavonoids	+
Tannins	+
Sterols	-
Glycosides	-
Phenols	+

Antimicrobial Analysis

Antibacterial activity of ethanol extract of seed of *Juglans regia* was examined against two bacterial samples like *S. aureus* and *E. coli* for antibacterial potential of the extracts. The zone of inhibition is shown in **Table 2**. The ethanol extract of seed of *Juglans regia* shows high potential of antimicrobial activity for both gram positive and gram negative control. The zone of inhibition of gram negative (14 mm) is higher than the zone of gram of positive control (11 mm).

Table 2 Antimicrobial Activity in Diameter (mm) of Inhibition Zone

Bacteria tested	**Ethanol extract (*Juglans regia*)**	**Control**	
		Streptomycin	**Erythromycin**
E. Coli (gram negative)	14	12	8
S. aureus (gram positive)	11	10	30

ZOI of ethanol extract of seed of *Juglans regia* was compared with the ZOI of standard control like Streptomycin and Erythromycin. The ethanolic extract of *Juglans regia* showed 16.67 % more ZOI as compared to Streptomycin and 75 % more ZOI as compared to Erythromycin for gram negative control. Similarly, the ethanolic extract of *Juglans regia* showed 10 % more ZOI as compared to Streptomycin and 63.33 % less ZOI as compared to Erythromycin for gram positive control.

Some other recent reports such as Deshwal [11] in 2012, Deshwal and Vig [12] in 2012 indicated that medicinal plant inhibited the growth of certain human pathogen. Deshwal and Siddiqui [13] in 2011 also mentioned that antimicrobial activity of alcohol leaf extract of in vitro raised plant part of the *Tylophora indica* which was found to be significant ($P<0.05$) against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Bacillus* species.

Present study revealed ethanolic extract of *Juglans regia* effectively inhibited the growth of *E. coli* than *S. aureus* in compared with Streptomycin and Erythromycin. This can be due to present of different phytochemical such as alkaloids, flavonoids or tannins in ethanolic extract of *Juglans regia*. This study clearly indicated that folk medicine can be as effective as antibacterial drug to control the growth of pathogen.

GC-MS Analysis

The chromatogram of ethanol extract of *Juglans regia* (**Figure 1**) shows different area peaks at different retention time. Chromatogram shows the different peak areas at retention time (t_R) 18.157 min, 18.785 min, 19.805 min, 19.851 min and 19.977 min. The chemical compounds identified in the ethanolic extract of seed of the *Juglans regia* are presented in **Table 3**.

Mass spectrum of a compound (**Figure 2**) reported at retention time 18.157min shows the different fragmentation with m/z of 102, 129, 157, 185, 207, 228.2 and 256 along with other fragmentation peak. The base peak of the compound was recorded as 102.

Analysis of chromatographic peak at t_R 18.175 min. revealed molecular ion at m/z 270 (M^+) suggesting the molecular formula $C_{17}H_{34}O_2$. Diagnostic ions are at m/z 185 ($M^+ - C_6H_{13}$), 157 ($M^+ - C_8H_{17}$) and 129 ($M^+ - C_{10}H_{21}$) are due to alkyl group fragmentation from the molecular ion. The characteristic base peak at m/z 102 was due to γ–hydrogen shift followed by α–cleavage resulted from the Mc Lafferty rearrangement [14]. The other significant fragment ions observed are m/z 207, 228, 256, etc.

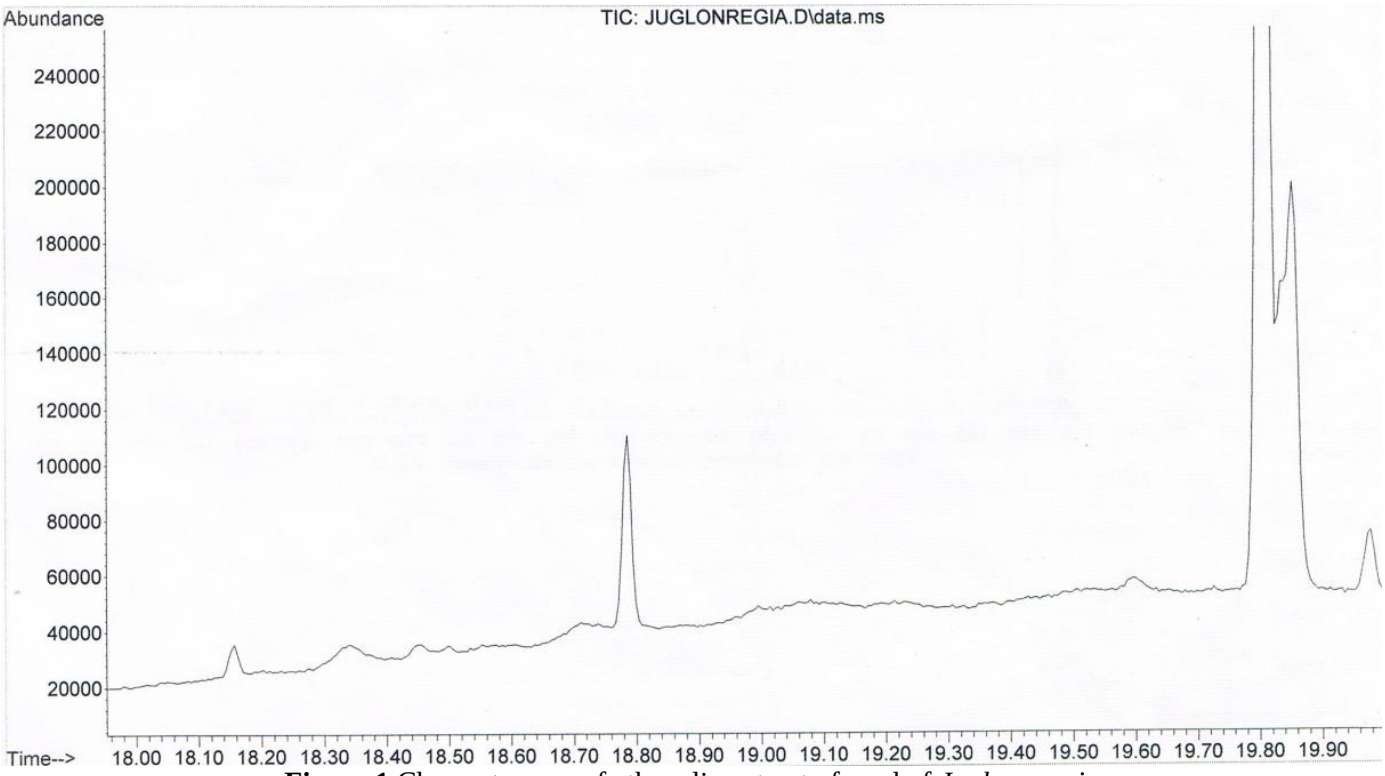

Figure 1 Chromatogram of ethanolic extract of seed of *Juglans regia*

Table 3 GC-MS Data of ethanolic extract of seed of *Juglans regia*

SN	Compound name	Mol. wt.	Mol. formula	Ret. time (min)	S. I.
1	Isopropyl Myristate	270	$C_{17}H_{34}O_2$	18.157	72
2	Hexadecanoic acid	270	$C_{17}H_{34}O_2$	18.785	90
3	9,12,15-Octadecatrienoic acid	292	$C_{19}H_{32}O_2$	19.851	81
4	Octadecanoic acid	298	$C_{19}H_{38}O_2$	19.977	70

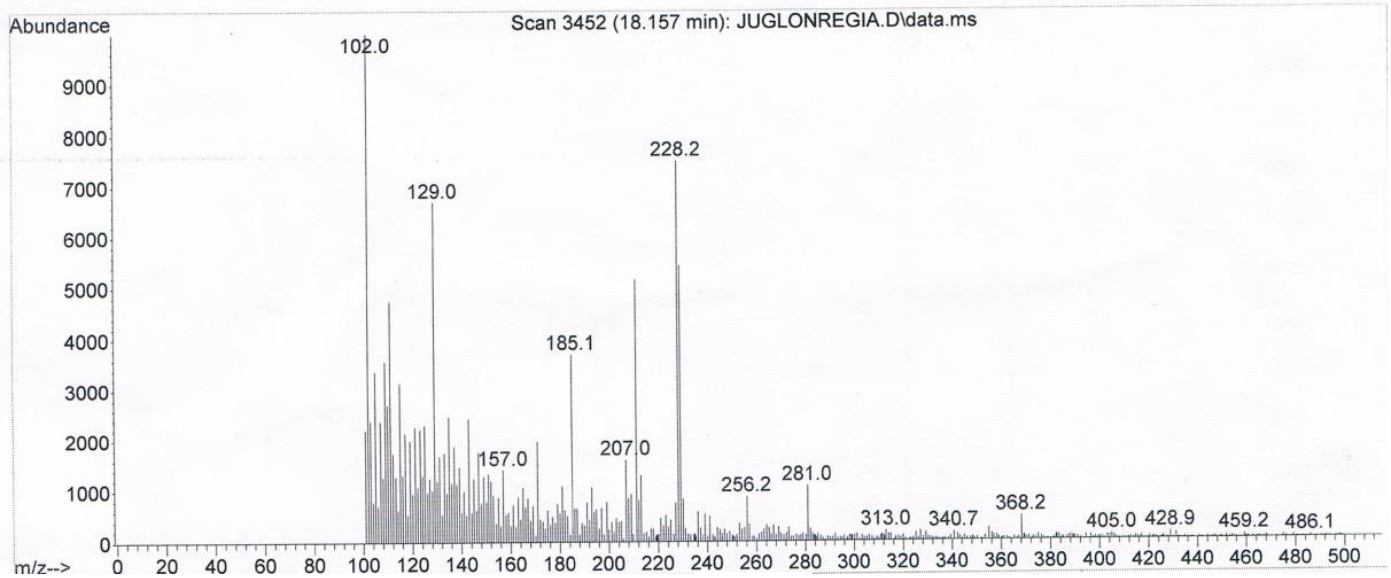

Figure 2 Continues

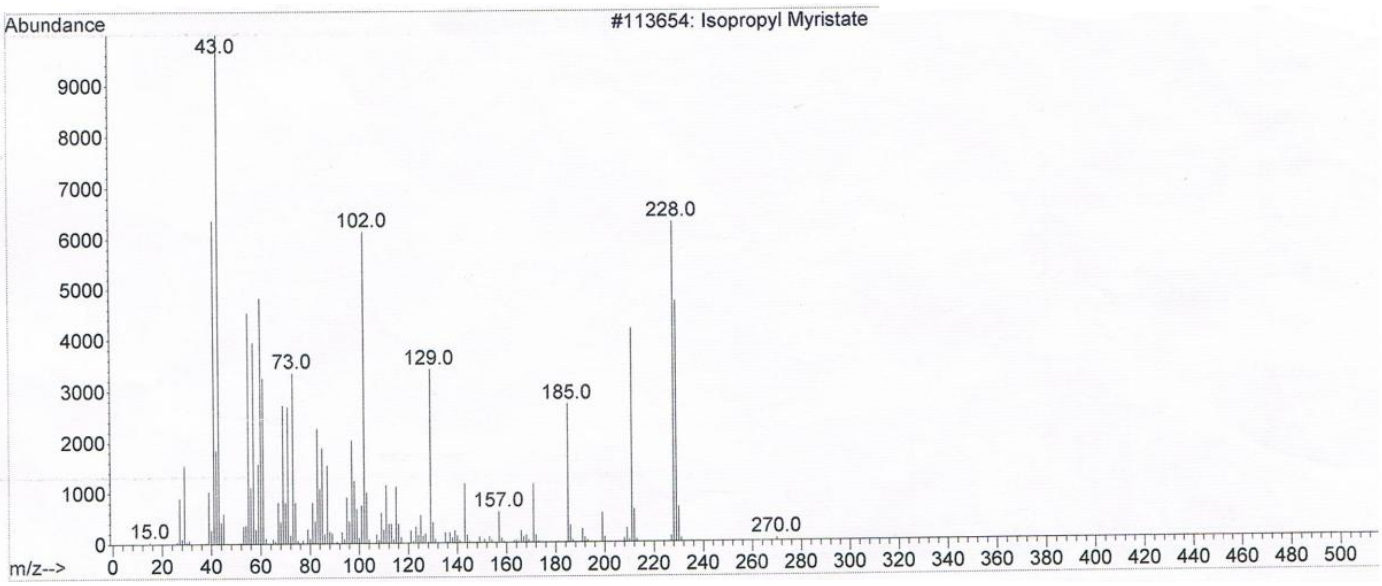

Figure 2 GC-MS spectrum of Isopropyl Myristate

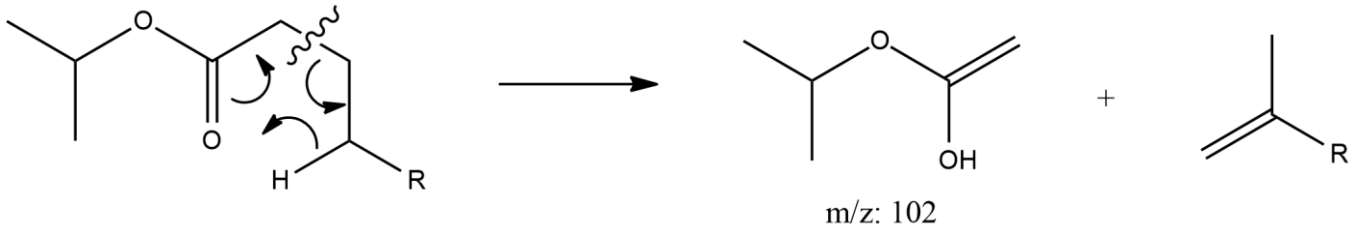

Figure 3 Mc Lafferty rearrangement [14]

With this we can conclude that the mass spectrum of a compound at retention time 18.157 min can be Isopropyl Myristate. The structure of Isopropyl Myristate is:

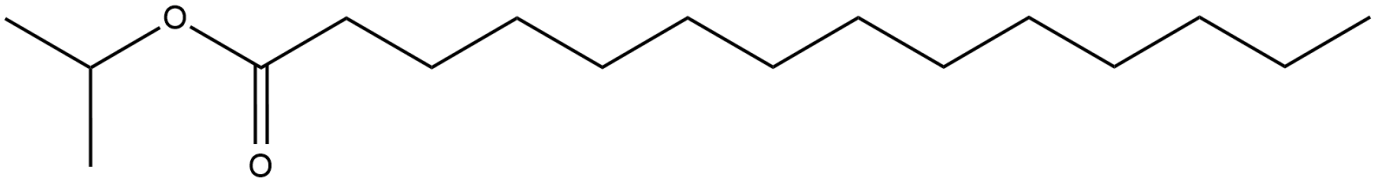

Figure 4 Isopropyl Myristate [16]

Mass spectrum of a compound (**Figure 5**) reported at retention time 18.785min shows the different fragmentation with m/z of 101.1, 122.1, 143.1, 164, 185.1, 206.2 227.2 249 and 270.2 along with other fragmentation peak. The base peak of the compound was recorded as 143.1.

Analysis of chromatographic peak at t_R 18.785 min., revealed molecular ion at m/z 270 (M^+) suggesting the molecular formula $C_{17}H_{34}O_2$. Diagnostic ions are at m/z 227 ($M^+ - C_3H_7$), 185 ($M^+ - C_6H_{13}$), 101 ($M^+ - C_{12}H_{25}$) and base peak 143 ($M^+ - C_{11}H_{23}$). In all diagnostic ions, alkyl group are fragmented from the molecular ion. The characteristic peak at m/z 74 which is present in library spectrum but not recorded in scan spectrum is due to γ–hydrogen shift followed by α–cleavage resulted from the Mc Lafferty rearrangement [14]. We can hope that such peak was present in scan spectrum of sample. However, there are significant fragment ions observed at m/z 122, 164, 206, 249, etc. More than 90% fragment peaks are matched with the mass spectrum of Hexadecanoic acid.

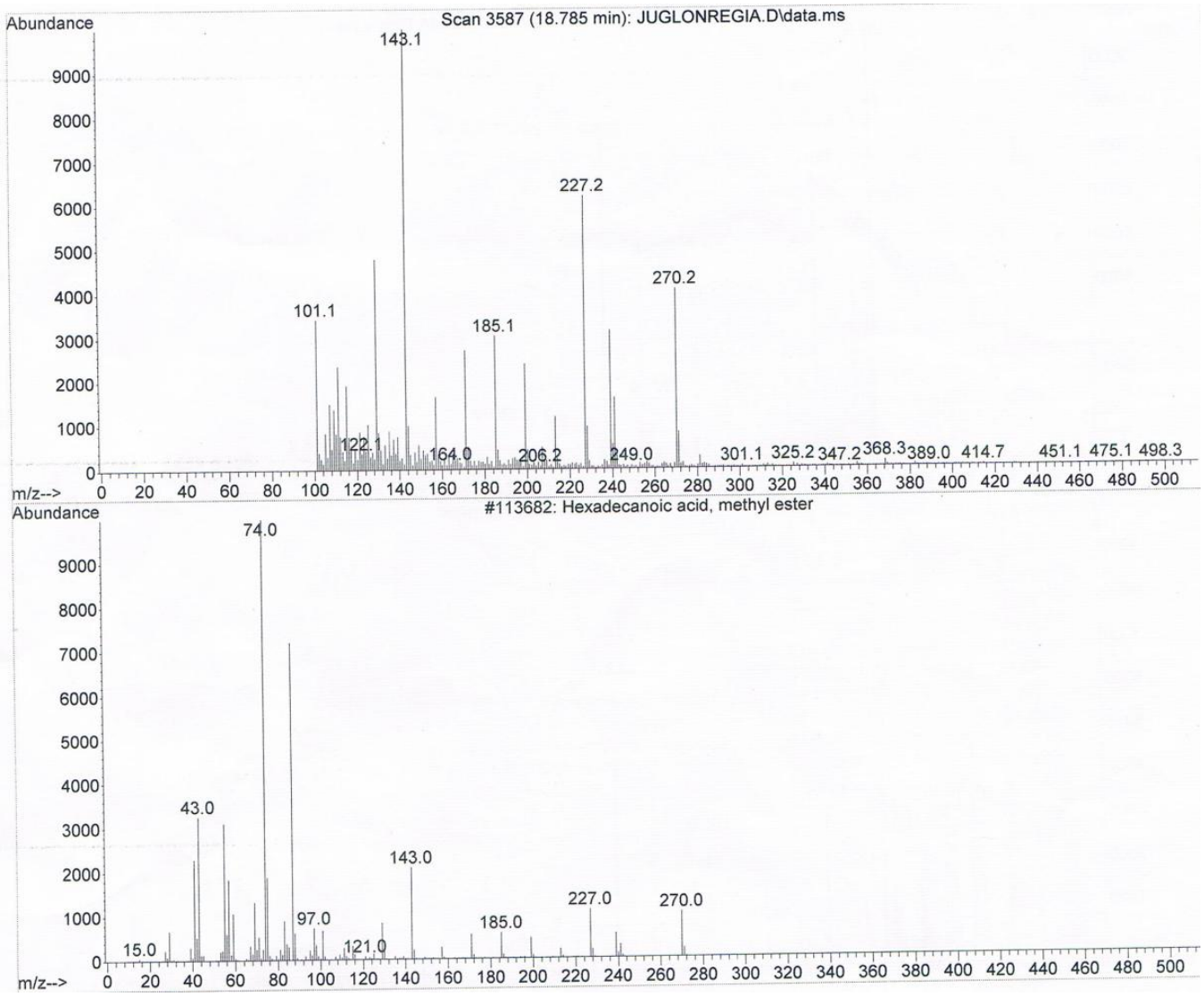

Figure 5 GC-MS spectrum of Hexadecanoic acid

With this we can conclude that the mass spectrum of a compound at retention time 18.785 min can be Hexadecanoic acid. The structure of Hexadecanoic acid is:

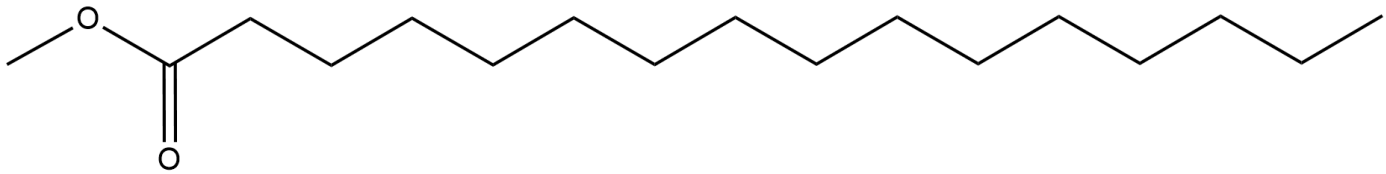

Figure 6 Hexadecanoic acid [17]

Mass spectrum of a compound (**Figure 7**) reported at retention time 19.851min shows the different fragmentation with m/z of 108.1, 135.1, 163.1, 185, 207, 236.2 261.2 and 292.3 along with other fragmentation peak. The base peak of the compound was recorded as 108.1.

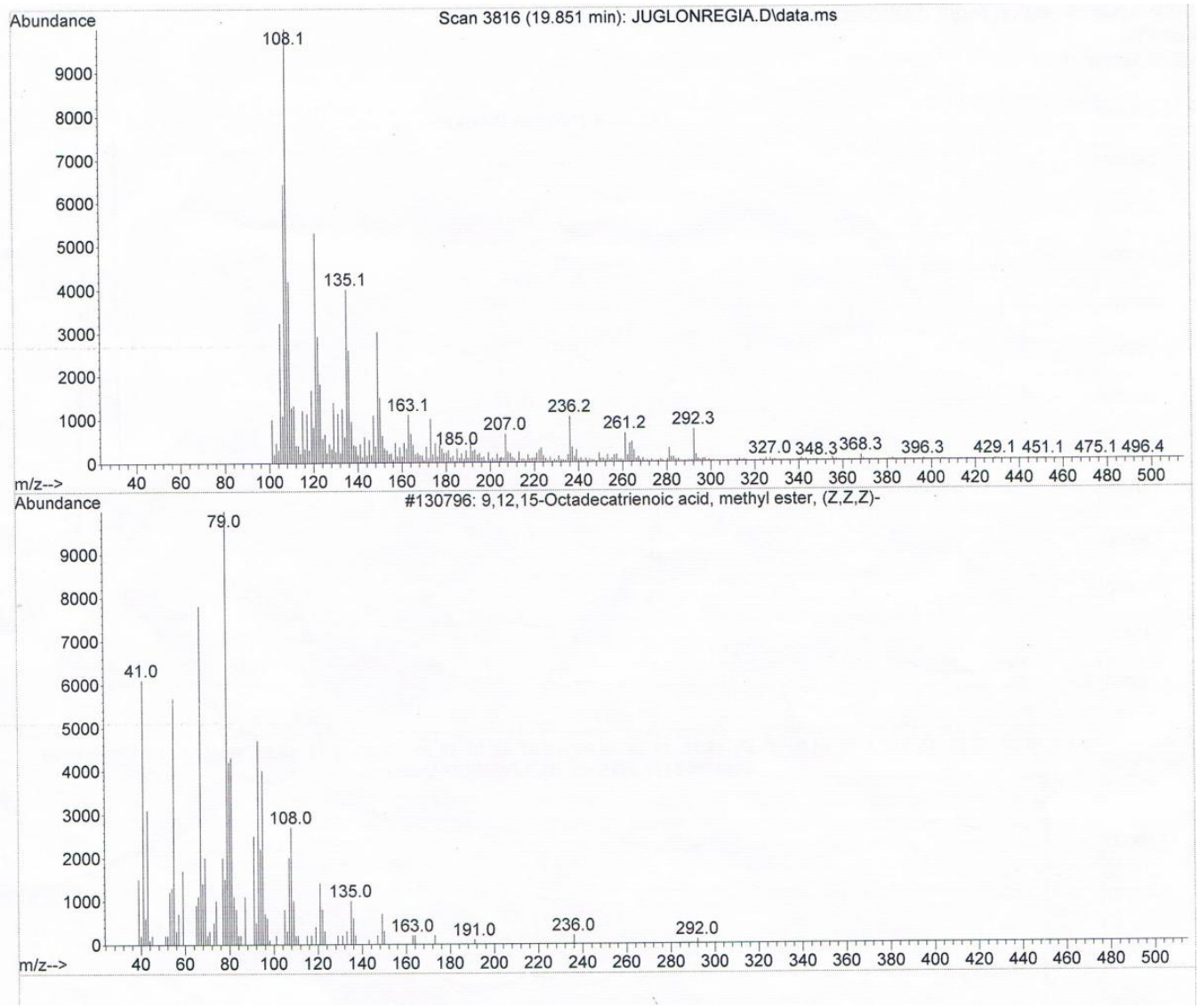

Figure 7 GC-MS spectrum of 9,12,15-Octadecatrienoic acid

Analysis of chromatographic peak at t_R 19.851 min., revealed molecular ion at m/z 292 (M^+) suggesting the molecular formula $C_{19}H_{32}O_2$. Loss of six hydrogen from parent molecular formula i.e. $C_{19}H_{32}O_2$ indicates the compound has 3 double bond. The characteristic peak at m/z 108 represents an *n*-3 terminal group [15]. The smallest peak at m/z 236 may represent the cleavage at the carboxyl end of the molecule giving a fragment containing the first two double bonds and the second methylene group. This ion was also first noted by Holman and Rahm in 1971 [15]. Another smallest peak at m/z 261 ([M-31]$^+$) representing loss of methoxyl group, and confirming that it is indeed a methyl ester. The m/z 135 was due to cleavage between C8 and C9 whereas the m/z 163 was due to cleavage between C6 and C7 and both peaks represent the polyalkene fractions.

With this we can conclude that the mass spectrum of a compound at retention time 18.785 min can be 9,12,15-Octadecatrienoic acid. The structure of 9,12,15-Octadecatrienoic acid is:

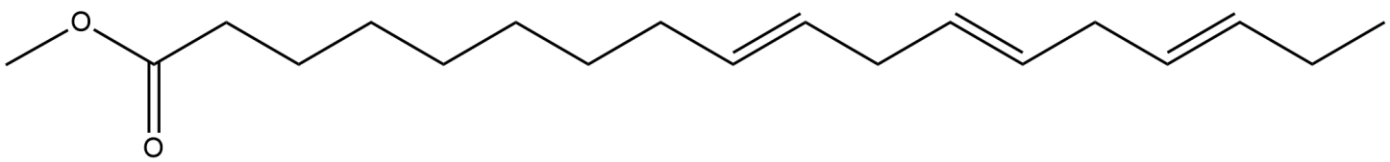

Figure 8 9,12,15-Octadecatrienoic acid [18]

Mass spectrum of a compound (**Figure 9**) reported at retention time 19.977 min shows the different fragmentation with m/z of 111.1, 143.1, 171.1, 199.1, 227.2, 255.2, 277 and 298.3 along with other fragmentation peak. The base peak of the compound was recorded as 143.1.

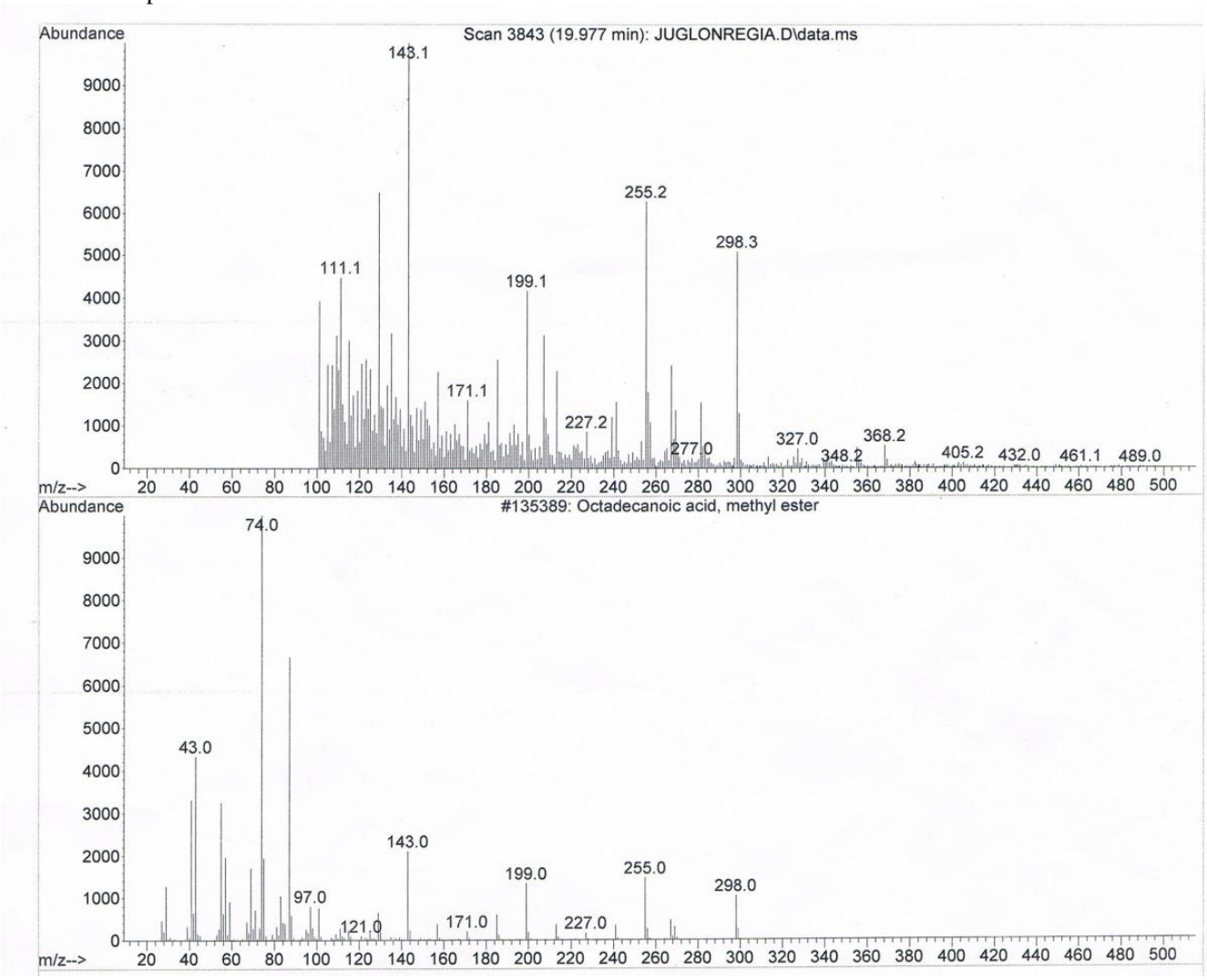

Figure 9 GC-MS spectrum of Octadecanoic acid

Analysis of chromatographic peak at t_R 19.977 min., revealed molecular ion at m/z 298 (M^+) suggesting the molecular formula $C_{19}H_{38}O_2$. Diagnostic ions are at m/z 255 ($M^+ - C_3H_7$), 227 ($M^+ - C_5H_{11}$), 199 ($M^+ - C_7H_{15}$), 171($M^+ - C_9H_{19}$) and base peak 143 ($M^+ - C_{11}H_{23}$). In all diagnostic ions, alkyl group are fragmented from the molecular ion. The characteristic peak at m/z 74 which is present in library spectrum but not recorded in scan spectrum is due to γ–hydrogen shift followed by α–cleavage resulted from the Mc Lafferty rearrangement [14]. We can hope that such peak was present in scan spectrum of sample. However, there are significant fragment ions observed at m/z 277, 111, etc.

With this we can conclude that the mass spectrum of a compound at retention time 19.977 min can be Octadecanoic acid. The structure of Octadecanoic acid is:

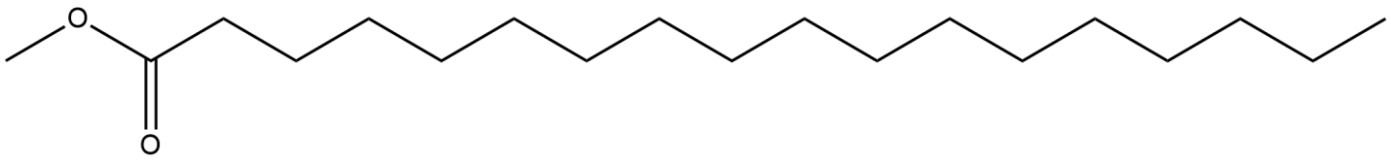

Figure 10 Octadecanoic acid [19]

Conclusions

Phytochemical screening analysis of ethanol extract indicates the presence of alkaloids, flavonoids and tannin. The ethanol extract of seed of *Juglans regia* was examined against two bacterial samples i.e. *Staphylococcus aureus* for gram positive and *Escherichia coli* for gram negative. The ethanol extract of *Juglans regia* shows high potential of antimicrobial activity for both gram positive and gram negative control. The zone of inhibition of Gram negative is higher than the zone of gram of positive control. The GC-MS of the ethanol extract of the seed of *Juglans regia* showed the presence of different compounds at different retention time. The mass spectrum of the compound reported at retention time 18.157 min, 18.785 min, 19.851 min and 19.977 min are isopropyl myristate, hexadecanoic acid, 9,12,15-Octadecatrienoic acid and octadecanoic acid respectively. The presence of various phytochemicals contributes many medicinal values of the plant. Further studies are required to confirm the compound detected by GC-MS analysis.

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References

[1] Gandev S., Bulgarian Journal of Agricultural Science, 2007, 13, 683-689.
[2] Asgary S., Parkhideh S., Solhpour A., Madani H., Mahzouni P. and Rahimi P., Journal of Medicinal Food, 2008, 11(3), 533-538.
[3] Spaccarotella K.J., Kris-Etherton P.M., Stone W.L., Bagshaw D.M., Fishell V. K., West S.G., Lawrence F.R. and Hartman T.J., Nutrition Journal, 2008, 7(13).
[4] Duke J. A., Handbook of Energy Crops, Purdue University, Center for New Crops & Plants Products, 1983.
[5] Launert E., Edible and Medicinal Plants, Hamlyn, London, 1981.
[6] Duke J. A. and Ayensu E. S., Medicinal Plants of China, Reference Publications, Inc., Algonac, 1985, Vol 1 & 2.
[7] Yeung H., Handbook of Chinese Herbal Formulas, Redwing Book Co., 1985.
[8] Tiwari P., Kumar B., Kaur M., Kaur G. and Kaur H., Internationale Pharmaceutica Sciencia, 2011, 1(1), 98-106.
[9] Bhatt S. and Dr. Dhyani S., International Journal of Current pharmaceutical research, 2011, 4(1), 87-89.
[10] Bauer A. W., Kirby W. M. M., Sherris J. C., and Turck M., American Journal of Clinical Pathology, 1966, 36, 493-496.
[11] Deshwal V.K., Global Journal of Research on Medicinal Plants & Indigenous Medicine, 2012, 1(4), 109-113.
[12] Deshwal V.K. and Vig K., International Journal of Pharmaceutical Invention, 2012, 2(3), 24-32.
[13] Deshwal V.K. and Siddiqui M.M.M., Biochemical and Cellular Archives, 2011, 11(2), 461-464.
[14] McLafferty F.W., Analytical Chemistry, 1959, 31(1), 82-87.
[15] Holman R.T. and Rahm J.J., Progress in the Chemistry of Fats and Other Lipids, 1971, 9, 15-20.
[16] http://webbook.nist.gov/cgi/cbook.cgi?ID=C110270&Units=SI, accessed on 16th Jan 2016.
[17] http://webbook.nist.gov/cgi/cbook.cgi?ID=C112390&Units=SI, accessed on 16th Jan 2016.
[18] http://webbook.nist.gov/cgi/cbook.cgi?ID=C7361800&Units=SI, accessed on 16th Jan 2016.
[19] http://webbook.nist.gov/cgi/cbook.cgi?ID=C112618&Units=SI, accessed on 16th Jan 2016.

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