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Anti-asthmatic effect of *Juglans regia* Linn. in mice

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Abstract: Plants are considered as an essential source to treat different diseases. In traditional system of medicine, *Juglans regia* (*J. regia*) has been used in curing sinusitis and cough. The aim of the present study was to evaluate the anti-asthmatic activity of *J. regia* in ovalbumin-induced allergic asthmatic BALB/c mice. The mice were sensitized intraperitoneally and subsequently challenged with ovalbumin (intranasal) to induce allergic asthma. Mice were treated with methanolic, n-hexane and ethyl acetate extracts of *J. regia* and methylprednisolone for 7 consecutive days, along with intranasal challenge. The total and differential leukocyte counts in blood, bronchoalveolar lavage fluid (balf) and lung wet/dry ratio were determined. GC-MS analysis was also performed. The results showed that ethyl acetate extract of *J. regia* significantly reduced inflammatory cells count in both blood and balf more significantly. Lung wet/dry weight ratio was reduced in asthmatic mice treated with the different extracts of *J. regia*. Serum IgE antibodies level was also significantly decreased in extracts treated groups. GC-MS analysis of all three extracts of *J. regia* showed the presence of various phytochemicals responsible for its anti-inflammatory and anti-asthmatic activity. The results of the present study validated the traditional use of *J. regia* in respiratory disorders like asthma and sinusitis.

Keywords: Asthma, inflammation, *Juglans regia,* total leukocyte count.

INTRODUCTION

Asthma is one of the respiratory disorder that affects more than 300 million populations worldwide (Stern *et al*., 2020). The main features of asthma are airway inflammation, hypersecretion of mucus, leukocyte invasion, remodeling of the airway wall and bronchial smooth muscle spasm. Various kinds of inflammatory cells involved in asthma are mast cells, macrophages, monocytes, neutrophils and basophils. Recent allergic asthma management methods include usage of glucocorticosteroids which inhibit inflammation and reduce swelling of the airway linings (Kandil *et al*., 2017). The most effective anti-inflammatory treatment for asthma is use of glucocorticoids which are non-specific in their mode of action and their use also produce side effects such as adrenal suppression, reduce bone metabolism and retard growth in children (Hathout *et al*., 2016). Alternative medicines especially herbal extracts have acquired greater acceptance both by the patients and medical practitioners. Plants have been a part of the curative practice both in conventional and modern period. Herbs contain many phyto-constituents which contribute to their vast array of pharmacological activities. Herbal medicines have got popularity over conventional medicines owing to reduce risk of side effects; enhance effectiveness, lower cost and widespread availability (Jaiswal *et al*., 2016).

J. regia Linn commonly known as English or Persian walnut is a beneficial therapeutic plant with the ability to treat several diseases. It has been used as an antimicrobial, anthelmintic, astringent, keratolytic, antidiarrhoeal, hypoglycaemic, depurative, carminative and for the treatment of sinusitis, cold and stomach ache. The fresh leaves of *J. regia* have been used in Turkish folk medicine on the naked body to reduce fever or on swollen joints to alleviate rheumatic pain. In Iranian folk medicine, the kernel (fruit) of *J. regia* has been used for the treatment of inflammatory bowel disease. It is used to treat diabetes, asthma, prostate and vascular disorders in Palestine (Panth *et al*., 2016).

The objective of the current study was to evaluate the anti-asthmatic activity of *J. regia* by using BALB/c mice model of ovalbumin-induced allergic asthma. In this study, total leukocyte count (TLC), differential leukocyte count (DLC), lung wet/dry ratio, enzyme linked immunosorbent assay (ELISA) and GC-MS analysis was performed.

MATERIALS AND METHODS

Plant collection

The kernels of *J. regia* were collected from Mansehra, Khyber Pakhtunkhwa Province, Pakistan. The plant was identified by Department of Botany, Government College University, Lahore (GC. Herb. Bot. 3740).

Preparation of extracts

The kernels of *J. regia* were grinded to fine powder. The powder (500 g) was soaked in methanol (2 L) and was set down for 10 days with daily random shaking. All the material was filtered through muslin cloth and then through filter paper (Whatman number 1). The filtrate was obtained and evaporated to a concentrated extract

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under reduced pressure on a rotary evaporator. The percentage yield was calculated (10.39%). The methanolic extract was dissolved in distilled water (200 ml). The solution was subjected to liquid-liquid extraction with n-hexane (organic solvent) by using separating funnel. The n-hexane fraction was collected and the solvent was evaporated by using rotary evaporator under reduced pressure. After separation of n-hexane layer, aqueous layer was again subjected to fractionation with ethyl acetate. The same method was used as described for the preparation of n-hexane fraction.

Animals used

Mice (BALB/c) of either sex, aging 6-8 weeks weighing 25-35 grams were used. All mice were kept in the animal house of Faculty of Pharmacy, The University of Lahore, Lahore for one week in order to acclimatize with environment. Animals were given tap water and standard diet and were kept at 12 hrs dark/light cycles. Standard conditions of humidity (40-60%) and temperature (24-26 ^{o}C) were maintained (Shabbir *et al*., 2014). The study was approved by the Institutional Research Ethics Committee, The University of Lahore, IREC-2017-39.

Ovalbumin Sensitization

Mice of all groups except negative (normal) control were sensitized on days 0 and 14 intraperitoneally with ovalbumin (20µg in a vehicle which contained 2mg $Al_2(SO_4)_3$ in a volume of 0.1 ml phosphate buffer solution). Next day after the second sensitization, the mice were subjected to intranasal challenge of ovalbumin once daily for 7 days (days 15-21). Animals of the negative (normal) control group were sensitized and treated with PBS only (Yang *et al*., 2010).

Study design

Total 36 mice were divided into six groups. Each group had 6 mice. The schedule for dosing and grouping was as follows, Group I; Negative (Normal) Control; mice were treated with PBS for seven consecutive days, Group II; (Diseased control) asthma was induced in mice with ovalbumin and treated with vehicle for 7 consecutive days, Group III; methanol extract treated group, Group IV; n-hexane extract treated group, Group V; ethyl acetate extract treated group.

Asthmatic mice were treated with the methanolic, n-hexane and ethyl acetate extracts of *J. regia* (500 mg/kg) orally for 7 consecutive days (from day 15 to day 21 for group III to V) (Shahzad *et al*., 2009). Group VI; Reference drug treated (Methylprednisolone), asthmatic mice were intraperitoneally injected with methylprednisolone (15 mg/kg). The duration of treatment was similar to other extracts treated groups (Bassett *et al*., 2010)

Inflammatory cells count in blood and balf

All the mice were dissected under ether anesthesia and blood was collected using cardiac puncture at day 22. The trachea with whole lung was dissected out for the collection of balf after euthanization. Then, lungs were lavaged with PBS (0.5 ml) through trachea by slow distillation and taken out with direct needle and collected in sterile eppendorf tubes. Total leukocyte count and differential leukocyte count in blood and balf was measured by automated hemocytometer (Carvalho *et al*., 2010).

Lung wet/dry weight ratio

The wet/dry weight ratio was used as an indication of lung tissue edema. The wet weight of fresh dissected right upper lobe of lung was measured on a weighing balance instantly. Then the weight of the same lobe was taken by drying it in an oven for 15 min at 56°C (Rana *et al*., 2016).

ELISA

IgE level was measured by ELISA according to Kit Manufacturer's Protocols. Briefly, samples were added in pre-coated wells of a 96 well micro titer plate. Then IgE antibody was added, followed by streptavidin–horseradish peroxidase. Plate was slowly shaked, sealed, and incubated at 37ºC for 1 hour. Plate was washed for 3 times with washing solution and chromogen solution A and B were added eventually. An incubation period of 10 min was permitted at 37ºC in the absence of light. After this, stop solution was added and optical density was measured at 450 nm (Shabbir *et al*., 2014)

GC-MS Analysis

GC-MS analysis was performed by using capillary column (0.25µm, 30m x 0.25mm). Helium gas was used as the carrier to enumerate different components. Column velocity flow was adjusted to 1ml/min using the split less mode and 0.5 µL injection volume. The oven temperature was set at 110˚C at first for 2 min., then steadily increased at the rate of 10˚C per min. until the temperature reached to 200˚C. The rate was then decreased to 5˚C per min. to 280˚C per min and final temperature was maintained at 280˚C. 70 eV ionizing voltage was used for MS and 200˚C was set for the quadrupole analyzer. The mass-to-charge range was set at 20 to 800 (Uroosa *et al*., 2017).

Drugs and chemicals used

For the present study, Solu-Medrol 500mg/7.8 ml injection (reference drug) manufactured by Pfizer Belgium and the solvents methanol, n-hexane and ethyl acetate (Sigma Aldrich) were used.

STATISTICAL ANALYSIS

The data were presented as mean ± standard error of mean. One-way analysis of variance (ANOVA) followed by post hoc Tuckey's test was used to compare blood and balf inflammatory cell counts, lung wet/dry ratio and serum IgE level among groups (Graph Pad Prism version 5.0.1). The value of $P \leq 0.05$ was considered as significant.

RESULTS

TLC in blood and balf

TLC in blood and balf was found remarkably increased in diseased group (12.633±0.122, 44.5±0.763, respectively) as compared with normal control (3.991±0.094, 8.166± 0.703, respectively) group. Pre-treatment with different extracts (methanolic, n-hexane and ethyl acetate) of *J. regia* significantly reduced TLC in blood (4.471±0.319, 3.191±0.385 and 3.428±0.112) and balf (18.166±0.703, 8.5±0.991 and 9.666±0.843) as compared with diseased group. Methylprednisolone also significantly reduced TLC in blood (4.731±0.131) and balf (10.5±0.763) as compared with diseased group (figs.1 A and B).

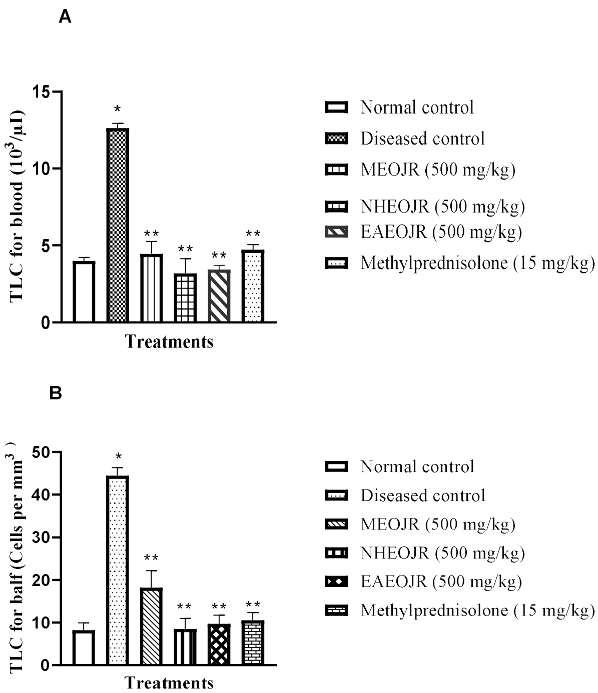

Fig. 1: Data have been presented as mean ± SEM. Effects of different extracts of *J. regia* on TLC in both blood (A) and balf (B) (**P*<0.05; compared to normal control group, ***P*<0.05 compared to diseased group; n=6) MEOJR; methanolic extract of *J. regia*, NHEOJR; n-hexane extract of *J. regia*, EAEOJR; ethyl acetate extract of *J. regia*.

Neutrophil count (DLC) in blood and balf

Neutrophil count in blood and balf was found significantly raised in diseased group (1.608±0.0465 and 108.5±5.213, respectively) as compared with normal control (0.321±0.017, 21.66±2.472). Treatment with different extracts (methanolic, n-hexane and ethyl acetate) of *J. regia* significantly lowered neutrophil count in blood (0.34±0.022, 0.308±0.024 and 0.39±0.015) and balf (62.333±4.402, 72±5.403 and 60±4.187, respectively) as compared with diseased group. Methylprednisolone also reduced neutrophil count in blood (1.361±0.067) and balf (84.5±1.688) as compared with diseased group (figs. 2 A and B).

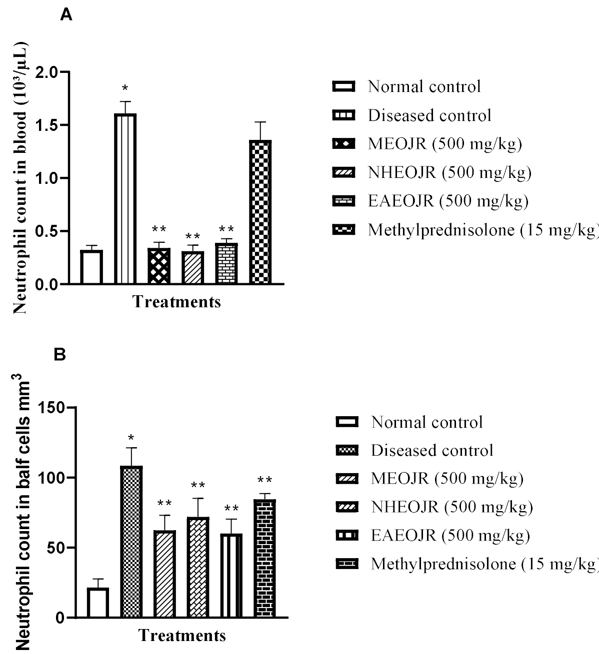

Fig. 2: Data have been presented as mean ± SEM. Effect of different extracts of *J. regia* on neutrophil count in both blood (A) and balf (B) (**P*<0.05; compared to normal control group, ***P*<0.05 compared to diseased group; n=6) MEOJR; methanolic extract of *J. regia*, NHEOJR; n-hexane extract of *J. regia*, EAEOJR; ethyl acetate extract of *J. regia*.

Effect of J. regia on lungs wet/dry ratio

There was an increase in lung wet/dry weight ratio of diseased group (0.626±0.016) as compared with normal control group (0.306±0.013). Pretreatment with different extracts of *J. regia* (methanolic, n-hexane and ethyl acetate) decreased lung wet/dry weight ratio (0.321± 0.030, 0.477±0.0198 and 0.2741±0.024, respectively) in oval albumin induced asthmatic mice as shown in fig. 3.

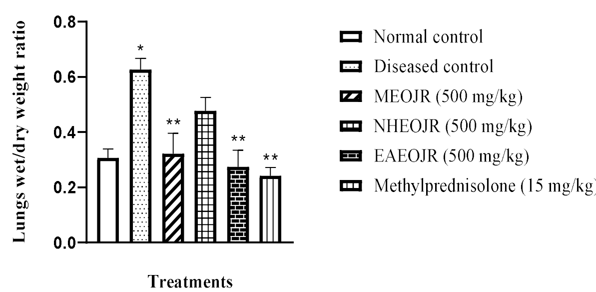

Fig. 3: Data have been presented as mean ± SEM. Effect of different extracts of *J. regia* on lungs wet/dry ratio (**P*<0.05; compared to normal control group, ***P*<0.05 compared to diseased group; n=6) MEOJR; methanolic

extract of *J. regia*, NHEOJR; n-hexane extract of *J. regia*, EAEOJR; ethyl acetate extract of *J. regia*.

Effect of J. regia on serum IgE level

There was a significant increase in the IgE level of mice exposed to ovalbumin (287.666±5.213) as compared with normal control group (122.333±2.375). After administration of different extracts of *J. regia* (methanolic, n-hexane and ethyl acetate), the serum level of IgE were restored (192.666±5.213, 200.8333±7.354, 197.666±6.581) as compared with diseased group (fig. 4).

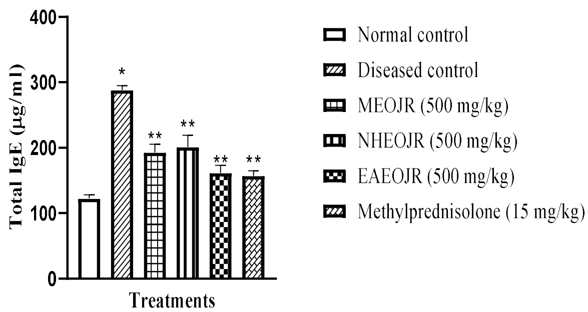

Fig. 4: Data have been presented as mean ± SEM. Effects of different extracts of *J. regia* on serum IgE (**P*<0.05; compared to normal control group, ***P*<0.05 compared to diseased group; n=6) MEOJR; methanolic extract of *J. regia*, NHEOJR; n-hexane extract of *J. regia*, EAEOJR; ethyl acetate extract of *J. regia.*

GC-MS analysis of different extracts of J. regia

GC-MS analysis of methanolic, n-hexane and ethyl acetate extracts of *J. regia* revealed the presence of a number of different compounds including octadecadienoic acid, benzenedicarboxylic acid, linoleic acid ethyl ester, sitosterol and vitamin E as shown in tables 1, 2 and 3 (fig. 5).

DISCUSSION

Asthma is a respiratory tract disease characterized with the production of mucus, airway contraction and gasping. These changes result in decrease lung liability, increase fibrosis, rapid multiplication of smooth muscles and thickening of basement membrane (Walsh *et al*., 2010). In the current study, BALB/c mice were used for the induction of allergic asthma. This model is well known for the production of T-helper cell 2 (Th2) arbitrated immune response (Nials and Uddin, 2008).

Neutrophils play an essential role in defense mechanisms of body. In asthma, raised white blood cells as well as neutrophils accumulation ultimately leads to airway stenosis (Rosales 2018). There was an increase in total inflammatory cells and neutrophils in both blood and balf of ovalbumin induced asthmatic mice in this study. The raised count was remarkably reduced after treatment with ethyl acetate and n-hexane extracts of *J. regia,* indicating improvement in narrowing of airways. This is consistent with previous study (Shahzad *et al*., 2010).

The immunoglobulin E (IgE) antibody is considered as a hallmark of allergic asthma (Froidure *et al*., 2016). After binding to basophils and mast cells, it initiates a series of biochemical events leading to increased level of pro-inflammatory mediators which are responsible for the allergic signs and symptoms (Khan *et al*., 2015). In the present study, there was an elevated level of IgE in diseased group that lead to an increase in mediators accountable for the allergic reactions. The IgE level was reduced remarkably after treatment with ethyl acetate extract of *J. regia* that improved signs and symptoms of asthma. These findings are in line with previous study (Shahzad *et al*., 2009).

The inflamed lung tissue edema was estimated by lung wet/dry ratio (Shahzad *et al*., 2009). There was a notable increase in lung wet/dry ratio in diseased group that was subsequently reduced after treatment with ethyl acetate extract of *J. regia* indicating reduction in lungs edema and obstruction.

The GC-MS analysis of methanolic, n-hexane and ethyl acetate extracts of *J. regia* exhibited the existence of various phytochemicals including octadecadienoic acid, benzenedicarboxylic acid and linoleic acid ethyl ester, sitosterol and vitamin E which might be responsible for the anti-asthmatic activity of this medicinal plant. It has been shown that oxygen free radicals have vital role in asthma. An imbalance between the oxidant and antioxidant systems induced by contaminants or the development of endogenous reactive oxygen species from inflammatory cells involves the pathogenesis of oxidative stress in asthma. There is an extending prove that antioxidant therapies which include vitamins have been shown to relieve this oxidative stress while improving the features of asthma (Arulselvan *et al*., 2016).

CONCLUSION

In conclusion, *Juglans regia* showed remarkable anti-asthmatic activity by decreasing TLC, DLC, serum IgE levels and lung wet/dry ratio in ovalbumin induced asthmatic mice. The anti-inflammatory and antioxidant phytochemicals found in all three different extracts of *J. regia* might be responsible for anti-asthmatic potential of this plant. However, further clinical studies become mandatory for isolation and identification of phytochemicals and elucidation of their exact mechanisms of action. The extracts of *J. regia* might be regarded as a promising agent for respiratory disorders.

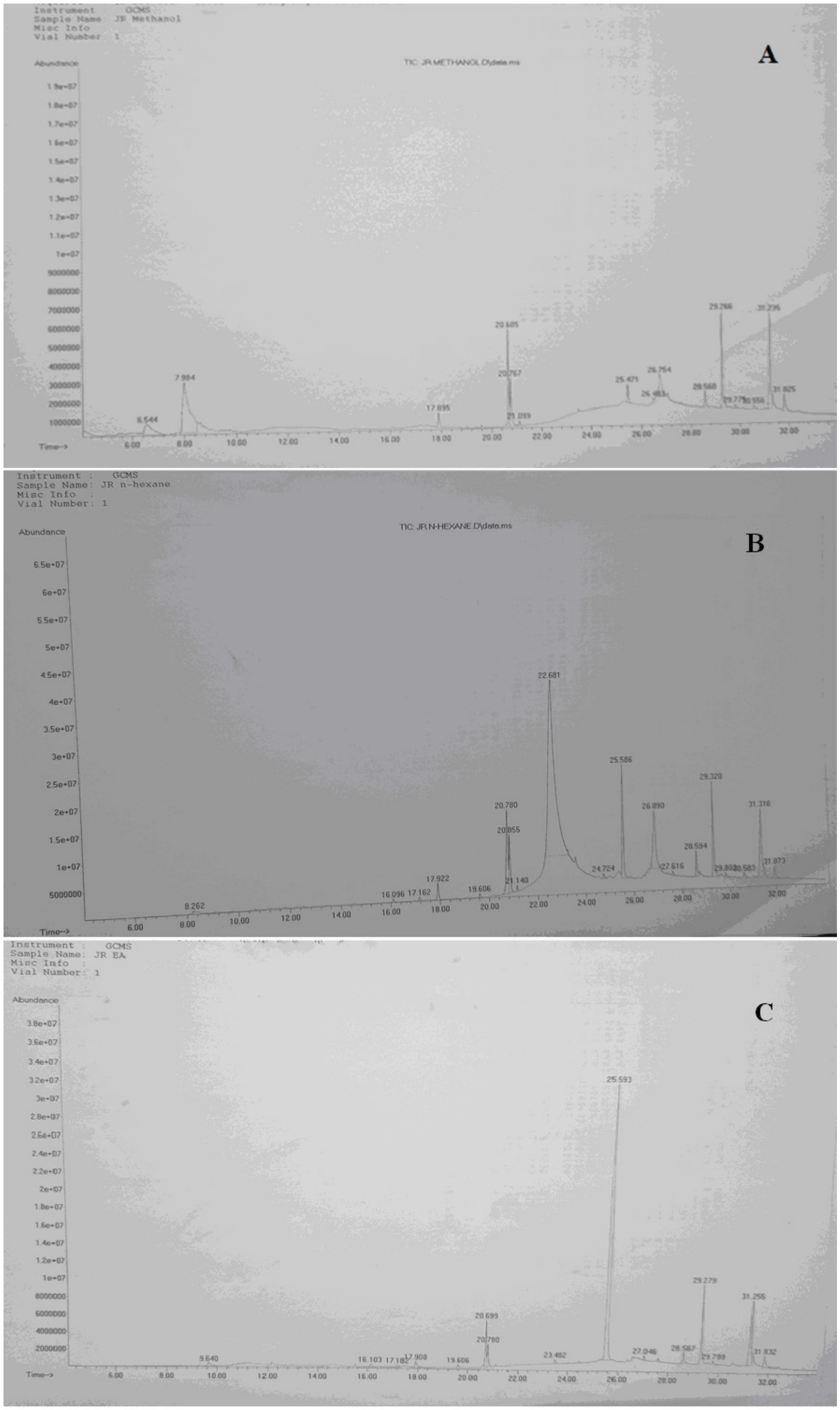

Fig. 5: GC-MS chromatogram of methanolic (A), n-hexane (B) and ethyl acetate (C) extracts of *Juglans regia*

Table 1: Phytocomponents identified in methanolic extract of *J. regia* by GC-MS

Peak #	Retention Time (mins)	Area %	Molecular wt. (g/mol)	Molecular Formula	Phytocomponents
1	6.544	27.85	144	$C_6H_8O_4$	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
2	7.984	100.00	126	$C_6H_6O_3$	2-Furancarboxaldehyde, 5-(hydroxymethyl)-
3	17.895	6.49	270	$C_{17}H_{34}O_2$	Pentadecanoic acid, 14-methy-,methyl ester
4	20.685	33.74	294	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z,Z)-,methyl ester
5	20.767	20.08	296	$C_{19}H_{36}O_2$	11-Octadecenoic acid, methyl ester
6	21.099	1.65	298	$C_{19}H_{38}O_2$	Heptadecanoic acid, 16-methyl-, methyl ester
7	25.471	5.85	278	$C_{16}H_{22}O_4$	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester
8	26.483	1.74	308	$C_{20}H_{36}O_2$	Linoleic acid ethyl ester
9	26.754	17.25	354	$C_{21}H_{38}O_4$	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropylester
10	28.560	6.94	402	$C_{27}H_{46}O_2$	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)
11	29.266	33.21	416	$C_{28}H_{48}O_2$	Beta-Tocopherol
12	29.775	1.46	430	$C_{27}H_{42}O_4$	Propanoic acid, 2-(3-acetoxy-4,4, 14-trimethylandrost-8-en-17-yl)-
13	30.556	2.48	400	$C_{28}H_{48}O$	Campesterol
14	31.235	47.56	414	$C_{29}H_{50}O$	Gamma-Sitosterol
15	31.825	10.82	426	$C_{30}H_{50}O$	9,19-Cyclolanost-7-en-3-ol

Table 2: Phyto-components identified in n-hexane extract of *J. regia* by GC-MS

Peak #	Retention Time (mins)	Area %	Molecular wt. (g/mol)	Molecular Formula	Phytocomponents
1	8.262	0.37	150	$C_{10}H_{14}O$	Phenol, 2-methyl-5-(1-methylethyl)-
2	16.096	0.39	270	$C_{17}H_{34}O_2$	Isopropyl Myristate
3	17.162	0.50	256	$C_{17}H_{36}O$	1-Hexadecanol, 2-methyl-
4	17.922	1.92	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
5	19.606	0.40	256	$C_{16}H_{32}O_2$	n-Hexadecanoic acid
6	20.780	13.0	294	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
7	20.855	6.12	296	$C_{19}H_{36}O_2$	9- Octadecadienoic acid (Z,Z)-, methyl ester
8	21.140	0.52	298	$C_{19}H_{38}O_2$	Octadecadienoic acid, methyl ester
9	22.681	100.00	280	$C_{18}H_{32}O_2$	9,12- Octadecadienoic acid (Z,Z)-
10	24.724	0.64	280	$C_{18}H_{32}O_2$	9,12- Octadecadienoic acid (Z,Z)-
11	25.586	13.30	278	$C_{16}H_{22}O_4$	1,2-Benzenedicarboxylic acid, mono(2-ethylexyl) ester
12	26.890	13.64	354	$C_{21}H_{38}O_4$	9,12-Octadecadienoic acid (Z,Z)-,2,3-dihydroxypropyl ester
13	27.616	0.48	372	$C_{25}H_{40}O_2$	4-Hexyl-1-(7-methoxycarbonylheptyl)bicycle[4.4.0] deca-2,5,7-triene
14	28.594	2.44	402	$C_{27}H_{46}O_2$	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)
15	29.320	9.97	416	$C_{28}H_{48}O_2$	Tocopherol
16	29.802	0.59	430	$C_{29}H_{50}O_2$	Vitamin E
17	30.583	0.72	400	$C_{28}H_{48}O$	Campesterol
18	31.316	11.16	414	$C_{29}H_{50}O$	Gamma-Sitosterol
19	31.873	2.17	426	$C_{30}H_{50}O$	9,19-Cyclolanost-24-en-3ol, (3.beta.)-

Table 3: Phytocomponents identified in ethyl acetate extract of *J. regia* by GC-MS

Peak #	Retention Time (mins)	Area %	Molecular wt. (g/mol)	Molecular Formula	Phytocomponents
1	9.640	2.19	256	$C_{17}H_2O_2$	Phenol, 4,4'-(1-methylidene)bis[2-methyl-
2	16.103	0.71	270	$C_{17}H_{34}O_2$	Isopropyl Myristate
3	17.182	0.79	256	$C1_7H_{36}O$	1-Hexadecanol, 2-methyl-
4	17.908	1.92	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
5	19.606	0.79	298	$C_{19}H_{38}O_2$	Isopropyl Palmitate
6	20.699	9.92	294	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
7	20.780	5.60	296	$C_{19}H_{36}O_2$	11-Octadecenoic acid, methyl ester
8	23.482	1.05	290	$C_{18}H_{26}O_3$	2-Propenoicacid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester
9	25.593	100.00	278	$C_{16}H_{22}O_4$	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester
10	27.046	1.02	354	$C_{22}H_{42}O_3$	Isomenthone
11	28.567	2.78	402	$C_{27}H_{46}O_2$	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)
12	29.279	16.55	416	$C_{28}H_{48}O_2$	Beta-Tocopherol
13	29.789	1.16	430	$C_{29}H_{50}O_2$	Vitamin E
14	31.255	20.55	414	$C_{29}H_{50}O$	Gamma-Sitosterol
15	31.832	4.43	426	$C_{30}H_{50}O$	9,19-Cyclolanost-24-en-3-ol, (3.beta).

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