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Research Article

Seasonal impact on the yield, chemoprofiling, antibacterial and antioxidant activities of the essential oil of *Juglans Regia*

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ABSTRACT

The essential oil from the leaves of *Juglans regia L*. (Juglandaceae) growing wild in Kashmir (India) was obtained by hydro-distillation and analysed during different stages of growth ranging from April to October. The analysis was carried out by a combination of capillary GC-FID and GC-MS analytical techniques. Both qualitative and quantitative differences were observed in the essential oil collected at different developmental stages in which the sample Jr-3 exhibited the presence of thirty eight (38) molecules, followed by Jr-4 (32), Jr-6 (30), Jr-2 (29), Jr-7 (26), Jr-5 (25) and Jr-1 (18) constituents. The essential oil yield varied from 0.020 % to 0.035 % in fresh leaves of the different samples of *J. regia*. The Jr-1, Jr-2, Jr-3, Jr-4 and Jr-6 oil samples exhibited the dominance of monoterpene hydrocarbons constituting 61.0 %, 56.4%, 39.8%, 55.9% and 29.0% of the total oil composition respectively, where as sesquiterpene hydrocarbons form the major portion of oil samples of Jr-5 (40.4%) & Jr-7(38.9%). Major constituents like α-Pinene, β -Pinene, Germacrane-D, β -Caryophyllene, Methylsalicylate, α-Humulene though present in all samples exhibit significant quantitative variation. The various oil samples possess varying degrees of antioxidant and antimicrobial properties which shows decreasing order with the decreasing number of constituents. The current study is the first attempt that reports the seasonal impact on the essential oil composition, antioxidant and antimicrobial activities of *J. regia* growing in Kashmir valley.

Key words: *Juglans regia*, GCMS, α-Pinene, Caryophyllene

INTRODUCTION

Essential oils are natural extracts of aromatic plants used in many fields like agriculture, aromatherapy and nutrition. The constituents of essential oil contribute the overall activity of each essential oil against target organisms, some of them have been used in cancer treatment and some have been used as antimicrobial agents. Therefore, there is an urgent need for novel treatment options with improved features. Many plant-derived compounds, e.g., Paclitaxel, ACH-1, Vinblastine, or Vincristine, and semi-synthetic derivatives of natural products, such as Etoposide and Teniposide are used as anti-cancer drugs. As pointed out recently, natural products from medicinal plants represent a fertile ground for the development of novel antimicrobial, antioxidant and anticancer agents. Plants from tropical regions are considered to be one of the potential sources for the screening of anticancer agents [1]. *Juglans regia* L. (Juglandaceae) commonly known as Persian or common walnut "Akhrot" in Kashmir is an important deciduous tree found primarily in the temperate areas and commercially cultivated in the United States, Western America, Asia and Central and Southern Europe. Green walnuts, shells, kernels and seeds, bark and leaves have been used in the pharmaceutical and cosmetic industries [2]. Walnut leaves are considered a source of healthcare compounds and have been intensely used in traditional medicine for treatment of venous insufficiency and haemorrhoidal symptomatology and for its antidiarrheic antihelmintic, depuratine and astringent properties [3]-[5] Keratolytic, antifungal, hypoglycaemic, hypotensive, anti-scrofulous and sedative activities have also been described [6],[7]. In walnut leaves, napthaquinones and flavonoids are considered as major phenolic compounds. Juglone (5-hydroxy-1,4-napthoquinone) is known as being the characteristic compound of *Juglans* spp. and is reported to occur in fresh walnut leaves and roots.[8]. Literature survey revealed that a lot of

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research has been carried out on non-volatile phytochemicals of *J. regia* [9]-[13]. However, information regarding volatile oil composition *J.regia* is meagre. Volatile chemical constituents of the mature nuts and green husks of *J. regia* have been investigated [14]-[16]. The essential oil from the leaves of the plant is found to posses potent antioxidant and antimicrobial properties [17]. However, to the best of our knowledge seasonal variability of essential oil of *J. regia* have not been investigated from any part of the world. Therefore objective of the present research work was to determine the seasonal impact on the yield and chemical composition, antibacterial and antioxidant activities of the leaf essential oil of *Juglans regia* at different stages of growth and development.

MATERIALS AND METHODS

Chemicals: DPPH (2,2-dipheny-1-picryl hydrazyl), L-ascorbic acid, butylated hydroxytoluene (BHT), dry ferric chloride, methanol, dimethyl sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO).

Plant material and essential oil isolation: Leaves of J. regia were collected at various stages of the plant development ranging from spring to the late autumn (April - October) 2012 from a tree growing in the campus of the Indian Institute of Integrative Medicine (CSIR) Sanatnagar Srinagar. Five kilograms of the fresh plant material were subjected to hydrodistillation every time using a Clevenger type apparatus for 3h according to the procedure described in the European Pharmacopoeia. Anhydrous Sodium sulfate was used to remove water after extraction. The essential oil was stored in air tight glass container in a refrigerator at 4 ^{o}C. The oil yield was found to be 0.035 -0.020% -(v/w) as calculated on fresh weight basis. The experiment was done in triplicate.

GC–FID and GC–MS analysis: GC-MS analysis was carried on a Varian Gas Chromatograph series 3800 fitted with a VF-5 ms fused silica capillary column (60m x 0.25mm, film thickness 0.25µm) coupled with a 4000 series mass detector under the following conditions: injection volume 0.5 µl with split ratio 1:60, helium as carrier gas at 1.0 mL/min constant flow mode, injector temperature 230 OC, oven temperature 60 0C to 2800C at 3 0C/min. Mass spectra: electron impact (EI+) mode,70 ev and ion source temperature 250 ^{0}C. Mass spectra were recorded over 50-500 a.m.u range**.** GC/FID was carried out on Perkin Elmer autosystem XL Gas Chromatograph 8500 series equipped with flame ionization detector (FID) and headspace analyzer using a fused silica capillary RTX-5 column (30m x 0.32 mm, film thickness0.25µm) coated with dimethyl polysiloxane .Oven temperature was programmed from 60 to 280^{0}C at 3^{0}C/min, with injector temperature 230 ^{0}C and detector temperature 250 ^{0}C. Injection volume1µl, nitrogen was used as a carrier gas (1.0 mL/min).Identification of the essential oil constituents was done on the basis of Retention Index (RI,determined with respect to homologous series of n-alkanes (C9-C24, Polyscience Corp., Niles IL) under the same experimental conditions), co-injection with standards (Sigma Aldrich and standard isolates),MS Library search (NIST 98 and WILEY), by comparing with the MS literature data (Jennings and Shibamoto 1980;Adams2007). The relative percentages of the individual components were calculated based on GC peak area (FID response) without using correction factors.

Determination of antimicrobial activity:

Bacterial strains: The antibacterial activity of essential oils of J. regia obtained at various stages were tested against a panel of nine bacterial strains which include four standard laboratory isolates obtained from Microbial Type Culture Collection (Chandigarh, India): Bacillus subtilis MTCC-441, Staphylococcus epidermidis MTCC-435, P.aeruginosa MTCC-1688, Proteus vulgaris MTCC-321 and five clinical isolates obtained from Department of Microbiology, Sheri-Kashmir Institute of Medical Sciences (Srinagar, India): S. aureus, Salmonella typhi, Escherichia coli, Shigella dyssenteriae and K. pneumonia. All the bacterial strains were sub-cultured on Mueller-Hinton agar (Himedia) slants every fifteen days to maintain the bacterial viability.

Antibacterial activity: Antibacterial activities of the essential oil samples were determined by agar well diffusion method. Microorganisms were grown over night at 37 ^{0}C in Mueller Hinton Broth (Himedia). 100 µl of standardized inoculum (0.5 Mac-Farland) of each strain was inoculated on molten Mueller Hinton agar, homogenized and poured into sterile 90 mm Petri-dishes and were allowed to solidify under laminar air flow. Standard cork borer of 5 mm diameter was used to made wells, into which 20 µl acetone extract and compounds dissolved in DMSO was added. 30 µg/disc of Gentamicin, streptomycin and ciprofloxacin (Sigma–Aldrich) were used as positive controls and DMSO was used as negative control. Every petri dish was sealed with laboratory film to avoid evaporation. The plates were then incubated at 37 ± 1^{0}C for 24 h. Finally zone of inhibition was measured to the nearest size in mm with the help of standard scale [18]. The experiments were carried out in triplicates and results were calculated as mean ± SD.

Minimum inhibitory concentrations (MIC): Minimum inhibitory concentrations were determined by broth dilution method [19]. Each sample was serially diluted in Muller Hinton broth to obtain desired concentrations ranging from 600 to 0.50_g/ml. Each bacterial strain was adjusted to 0.5 McFarland standard turbidity (equivalent to 1.5 $\times 10^8$ CFU/ml). Each sample was stirred and inoculated with 50 µl of tested bacterial suspension containing 2×10^5 CFU/ml and incubated at 37 ±1 C for 24 h. The MIC value was considered as the lowest concentration of the sample at which the tested bacteria does not show any visible growth after incubation.

Antioxidant activity: The essential oil samples of *J. regia* were subjected to screening for the possible antioxidant activity by DPPH free radical scavenging and hydroxyl radical (OH^-) scavenging methods.

DPPH free radical-scavenging activity: DPPH free radical scavenging activity was evaluated by measuring the scavenging activity of the essential oil samples on stable 2,2-diphenyl-1-picryl hydrazyl radical (DPPH). A 0.5 mM solution of DPPH in methanol was prepared. A stock solution of sample (1 mg/ml) in methanol was prepared. Different concentrations (10–100 µg/ml) were added to 1 ml (0.5 mM DPPH) and final volume was made to 3 ml with methanol. The mixture shaken was vigorously and kept standing at room temperature for 10 min. The absorbance of the mixture was measured at 517 nm on spectrophotometer. The decrease in the absorbance indicates an increase in DPPH-radical scavenging activity. The percentage inhibition was calculated by the following equation.

$$\text{DPPH radical scavenging (\%)} = \frac{Ac-As}{Ac} \times 100$$

Where Ac is the absorbance of control and As is absorbance of sample. L-ascorbic acid and BHT (Sigma) served as positive control. The experiment was done in triplicate and mean values were recorded. IC_{50}value were calculated as the concentration of sample, required to scavenge 50% of DPPH free radicals

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging was carried out by measuring the competition between deoxyribose and the essential oil samples for hydroxyl radicals generated in Fenton reaction. Hydroxyl radicals degrade deoxyribose leading to the formation of thiobarbituric acid reactive substances (TBARS) that could be measured spectrophotometrically at 532 nm. The reaction mixture containing 25 mM deoxyribose, 10 mM ferric chloride, 100mM ascorbic acid, 2.8 mM H_2O_2 in 10 mM KH_2PO_4 (pH 7.4) and different concentrations of various essential oil samples of *J. regia* were incubated at 37^0C for 1h. Then 1 ml of 1.6% thiobarbituric acid and 1ml of 3% trichloroacetic acid was added.the whole mixture was heated at 100 ^{0}C for 20 min. The TBARS was measured spectrophotometrically at 532 nm. The results were expressed as percentage inhibition of deoxyribose oxidation, as determined by the following formula.

$$\text{Percent inhibition} = \frac{A-B}{A} \times 100$$

Where A was the malonaldehyde produced by Fenton reaction treated alone and B was the malonaldehyde produced in the presence of essential oil of J. regia and known antioxidant.

Statistical analysis: All of the experiments were done in triplicate. The data was recorded as mean ± standard deviations. Analysis of variance for individual parameters was performed by Duncan's test on the basis of mean values to find out the significance at $p < 0.05$.

RESULTS AND DISCUSSION

Volatile oil yield of the fresh leaves of *J. regia* is summarised in **table 1**. The oil yield varied from 0.02 to 0.035 % in different samples obtained at different periods of growth. Maximum oil yield was noticed in Jr-3 (0.035 %) followed by Jr-4 (0.032), Jr-2, Jr-5 (0.030), Jr-6 (0.025 %) and Jr-7 (0.02 %). Low essential oil yield during hot summer might be attributed to the high temperature and partial evaporation of some constituents of an oil. Our results were in agreement to those of [20]. who investigated that growing season has a major effect on the essential oil yield in *Origanum majorana*.

Volatile oil composition variability: The volatile oils of all the seven samples were analysed by GC/FID and GC/MS. A total of 43 constituents representing 82.26 to 96.20% of the total oil composition was identified **(Table 2).** Major components of the essential oil were α-pinene (2.74-15.81 %), β -pinene (2.56 to 35.63 %), Germacrene-D (6.73-20.78 %), β-caryophyllene (12.64-23.80 %), Methylsalicylate (0.085-21.75) and α–humulene (0.10-3.06). Among the terpenoids, a prevalence of monoterpene hydrocarbons compared to sesquiterpene hydrocarbons was noted in samples Jr-1, Jr-2, Jr-3, Jr-4 & Jr-6 (61.00-29.00 %) while its reverse trend could be seen in samples Jr-5 & Jr-7 which were dominated by sesquiterpene hydrocarbons (38.90-40.40). Oxygenated monoterpenes and oxygenated sesquiterpenes **(Table 3)** were also found in good amounts in different samples (0.60-22.69 % and

0.60-5.10 %). The components belonging to other classes were detected in trace amounts. As far the chemical composition of *J. regia* is concerned, both the qualitative and quantitative variations were observed (fig 1a) with Jr-3 showing maximum number of phytomolecules (38) followed by Jr-4 (32), Jr-6 (30), Jr-2 (29), Jr-7 (26), Jr-5 (25) and Jr-1 (18) constituents. Major constituents like α-Pinene, β -Pinene, Germacrane-D, β -Caryophyllene, Methylsalicylate, α-Humulene showed their presence in all samples with variation in quantities. Minor constituents showed both qualitative as well as quantitative variation. Some phytomolecules are greatly effected by seasonal variation and are present only at particular stage of development like Sbinene, Terpinolene, (E)-Sabinol, Myrtenol, Cyclosatinene, α-Mucrolene and (Z)-3-Hexenyl benzoate in Jr-3. Some persist for shorter duration like p-Cymene in Jr-2 & Jr-3; 1,8-Cineole in Jr-3 & Jr-5; Camphor in Jr-4 & Jr-6. Linalool in Jr-2, Jr-3 & Jr-4, Humulene epoxide-II in Jr-4, Jr-6 & Jr-7 and Laurenene in Jr-2, Jr-3 & Jr-4 samples. A single report on volatile compounds of *J. regia* leaves from Kashmir valley showed the presence of 38 constituents [17]. Thus, comparison of our results with earlier report clearly showed the difference in the leaf essential oil composition of *J. regia* as an impact of seasonal variation. Further, the present study identified several new compounds for the first time in the essential oil of *J. regia* leaves in significant amounts. These are β- Eudesmol, α-Cadinol, Z-Thujone, Camphor and Humulene epoxide-II . Therefore it can be concluded that seasonal variation effects chemical composition of an essential oil of *J. regia.* These differences can be attributed to the seasonal changes in temperature and humidity accompanied by different stages of plant metabolism.

Antibacterial activity: Gentamicin, streptomycin and ciprofloxacin were used as standard antimicrobial agents. The antibacterial activity of the *J. regia* essential oil obtained during different seasons was evaluated against a set of significant bacterial strains including both Gram-positive and Gram-negative, and their potency was assessed qualitatively and quantitatively by the presence or absence of inhibition zones, zone diameters (ZDS) and MIC values. The results (MICs) are given in **Table 4** which indicate that these essential oils obtained at different developmental stages displayed a broad spectrum and variable degree of antibacterial activity against the different tested strains. B. subtilis MTCC-441, S. epidermidis MTCC-435 and S. aureus were the most sensitive bacterial strains to the essential oils while as S. typhi, E. coli and K. pneumonia were the most resistant. The observed variation in the antibacterial activity of the agents against each culture may be due to difference in the number and type of the components present in essential oils obtained at different stages. The antibacterial activity of the essential oils was compared with that of standard antibiotics, gentamicin, streptomycin and ciprofloxacin. From the data it is clear that the sample with maximum number of components shows greater activity (lower MIC values) and the sample with fewer number of components shows little activity against the tested strains.

Antioxidant activity:

DPPH and hydroxyl radical scavenging activities: Fig. 1 shows the scavenging effect of the leaf essential oils of *J. regia* on the DPPH radical. Ascorbic acid and BHT were used as the reference standards. The essential oils obtained at different developmental stages exhibited a concentration dependant scavenging of DPPH radicals which was comparable to the reference standards (ascorbic acid and BHT) at the same doses. **Fig. 2** shows the scavenging effect of the leaf essential oil of *J. regia* on hydroxyl radical. The essential oil exhibited a concentration dependent scavenging of hydroxyl radicals which was comparable to the reference standards (ascorbic acid and BHT) at the same doses. The IC_{50} values of the essential oils were found to be 56.34, 40.15, 38.74, 39.12, 44.16, 40.08 and 42.44 µg/ml respectively for JR-1, JR-2, JR-3, JR-4, JR-5, JR-6 and JR-7as calculated by DPPH and 100.54, 78.00, 57.00, 59.05, 66.00, 59.70 and 64.00 by hydroxyl radical scavenging assays. From the values of IC_{50} and inhibition, it is clear that the antioxidant activity shows a decreasing order with decreasing number of chemical constituents present in different oil samples. No data are available in the literature regarding the antioxidant activity of *J. regia* essential oil with respect to seasonal variations with which the present activity can be compared. The changes in antimicrobial activity of the investigated *J. regia* essential oils, with regard to seasonal variations, might be attributed to the different chemical composition of the oils. Earlier reports showed that the changes in chemical composition of essential oil directly affects their biological activities [21],[22]). In general, growing season effects chemical composition, antioxidant and antimicrobial activities of the essential oils of *J. regia*. These differences can be attributed to the seasonal changes in temperature and humidity, also to different stage of plant metabolism. As discussed above, essential oils from *J. regia* showed good antioxidant and free radical-scavenging activities, and broad activity against bacteria. The production of essential oils and their utilization as potential natural food preservatives could be of economical value.

Table 1: Period of collection and essential oil yield of Kashmir Walnut (*Juglans regia*) samples.

Sample code	Period of collection	Essential oil yield[%age]
Jr-1	20-04-2012	0.028
Jr-2	10-05-2012	0.030
Jr-3	05-06-2012	0.035
Jr-4	05-07-2012	0.032
Jr-5	10-08-2012	0.030
Jr-6	05-09-2012	0.025
Jr-7	01-10-2012	0.020

Table 2: Chemical composition variability of various essential oil samples .

S.No	RI	Compound	% Composition						
			JR-1	JR-2	JR-3	JR-4	JR-5	JR-6	JR-7
1	936	Alpha pinene	15.81	14.64	15.10	11.16	8.61	5.51	2.74
2	947	Camphene	---	0.20	0.217	0.91	0.54	0.87	0.43
3	970	Sabinene	---	---	1.734	----	---	---	---
4	977	Beta pinene	35.63	33.90	30.56	12.73	11.13	5.98	2.56
5	988	Myrcene	1.34	1.24	1.111	1.09	0.90	0.77	0.54
6	1004	Delta 3-carene	---	---	0.032	0.61	0.45	0.82	0.46
7	1015	Alphaterpinene	4.48	3.79	0.28	5.33	4.31	5.64	3.94
8	1025	p-cymene	---	0.16	0.03	---	---	---	---
9	1027	Limonene	3.92	3.76	3.693	3.25	1.56	2.29	2.57
10	1035	1,8-cineole	---	---	0.028	---	0.68	---	---
11	1038	(Z)-Beta ocimene	---	0.10	1.746	---	0.80	1.14	0.79
12	1045	(E)-Beta ocimene	---	---	1.697	0.82	---	---	---
13	1057	Gamaterpinene	---	0.16	0.158	4.23	3.57	6.47	4.39
14	1084	Terpinolene	---	---	0.110	---	---	---	---
15	1101	Linalool	---	0.07	0.118	0.19	---	---	---
16	1140	(E)-Sabinol	---	---	0-179	----	---	---	---
17	1146	(z)- Thujone	---	---	---	0.82	0.54	1.14	1.63
18	1165	Camphor	---	---	---	0.19	---	0.69	---
19	1169	Borneol	---	---	0.077	0.52	0.30	0.98	1.31
20	1179	Terpine-4-ol	0.46	0.34	0.255	0.44	0.87	1.00	1.56
21	1184	Methyl salicylate	3.22	0.14	0.085	12.5	17.30	17.19	21.75
22	1191	Myrtenol	---	---	0.938	---	---	---	---
23	1196	Myrtenal	---	---	0.207	0.26	---	1.17	---
24	1291	Bornyl acetate	---	0.21	0.154	0.15	---	0.21	0.34
25	1362	Eugenol	---	0.08	0.096	0.12	---	0.25	---
26	1370	Cyclosatinene	---	---	0.074	---	---	---	---
27	1377	Alpha copaene	0.44	0.43	0.570	0.42	---	0.89	1.83
28	1381	Geranylacetate	0.35	0.38	1.077	0.11	0.63	---	---
29	1417	(E)-Caryophyllene	12.64	13.26	15.576	13.77	19.46	14.50	23.80
30	1450	Alpha humulene	1.58	2.06	0.108	2.31	2.31	1.100	3.06
31	1482	Germacrene-D	14.69	20.78	14.422	9.80	14.77	6.73	6.99
32	1496	Alpha mucrolene	---	---	0.2510	---	---	---	---
33	1503	Bicyclogermacrene	---	0.25	0.2483	0.85	0.53	0.78	0.77
34	1507	Beta bisabolene	0.38	0.14	0.0944	1.43	0.84	0.43	0.45
35	1510	Gamma cadinene	---	0.11	0.1461	0.24	---	0.23	---
36	1519	Delta cadinene	0.37	0.37	0.6610	2.16	2.12	2.00	2.30

37	1566	(z)-3-hexenylbenzoate	---	---	0.0615	---	---	---	---
38	1581	Caryophyllene oxide	0.27	0.31	1.7280	0.44	0.78	1.70	1.06
39	1608	Humuleneepoxide-II	---	---	---	1.70	---	0.20	0.37
40	1636	Epi-α-cadinol	0.28	0.24	0.9791	1.77	0.88	1.02	0.54
41	1649	Beta- eudesmol	0.46	0.07	---	---	1.11	---	0.37
42	1652	Alpha cadinol	0.28	0.22	---	0.32	2.03	---	1.46
43	1877	Laurenene	---	0.17	0.0242	0.12	---	---	---

Table 3: Class compositional variability of various essential oil samples

	% age Content						
Class composition	Jr-1	Jr-2	Jr-3	Jr-4	Jr-5	Jr-6	Jr-7
Monoterpene hydrocarbons	61.00	56.40	39.80	55.90	27.34	29.00	17.90
Oxygenated monoterpenes	3.60	0.60	14.79	1.60	19.50	22.69	22.40
Sesquiterpene hydrocarbons	30.00	37.47	30.90	32.60	40.40	25.47	38.90
Oxygenated sesquiterpenes	0.60	0.70	4.10	2.60	3.50	5.1	3.20
Total identified (%)	96.20	95.17	89.59	92.70	90.74	82.26	82.40

Table 4: Antibacterial activities depicting MIC (µg/ml) of *J. regia* essential oil collected during different seasons

Sample code	B. subtillis	S. epidermidis	P. vulgaris	P. aeruginosa	S. aureus	S typhi	E. coli	S. dyssenteriae	K. pneumonia
JR-1	20.50	20.50	36.60	36.60	20.50	73.20	72.30	36.40	73.60
JR-2	17.42	17.44	35.30	35.40	17.40	67.20	67.20	35.60	67.25
JR-3	15.62	15.62	31.25	31.25	15.62	62.50	62.50	31.25	62.50
JR-4	15.80	15.80	32.40	32.44	16.00	63.40	63.44	32.40	63.40
JR-5	17.30	17.30	35.80	35.80	17.30	68.80	68.80	35.80	68.80
JR-6	16.40	16.40	33.54	33.54	16.42	65.35	65.35	33.53	65.50
JR-7	17.20	17.20	35.60	36.60	17.20	68.10	68.10	35.54	68.10
Gentamicin	5.95	5.90	7.9 0	7.81	3.90	7.81	7.81	3.90	7.81
Streptomycin	7.60	12.8 0	13.51	11.52	11.20	11.61	11.61	8.71	11.61
Ciprofloxacin	8.88	13.51	6.70	6.41	6.44	6.60	5 .11	7.75	6.65

Fig.1. Antioxidant activity measured by DPPH radical assay of J.regia essential oil samples

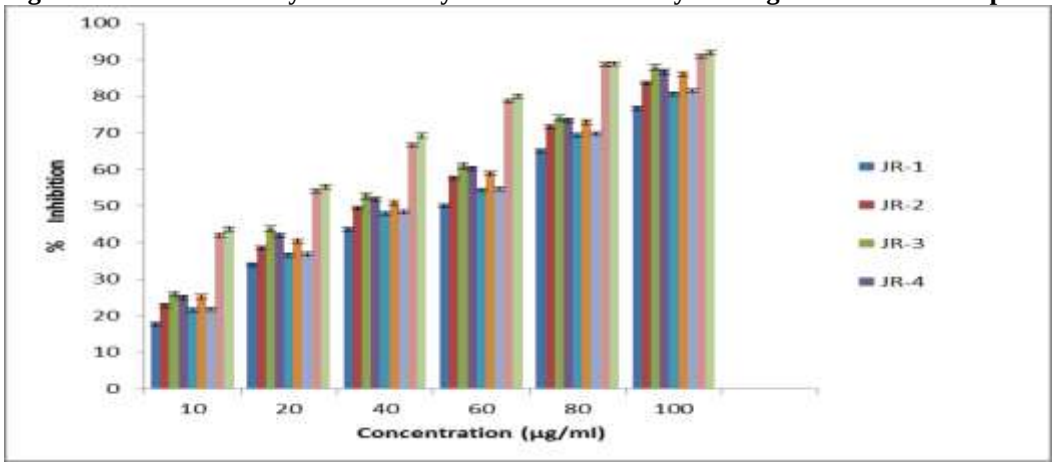
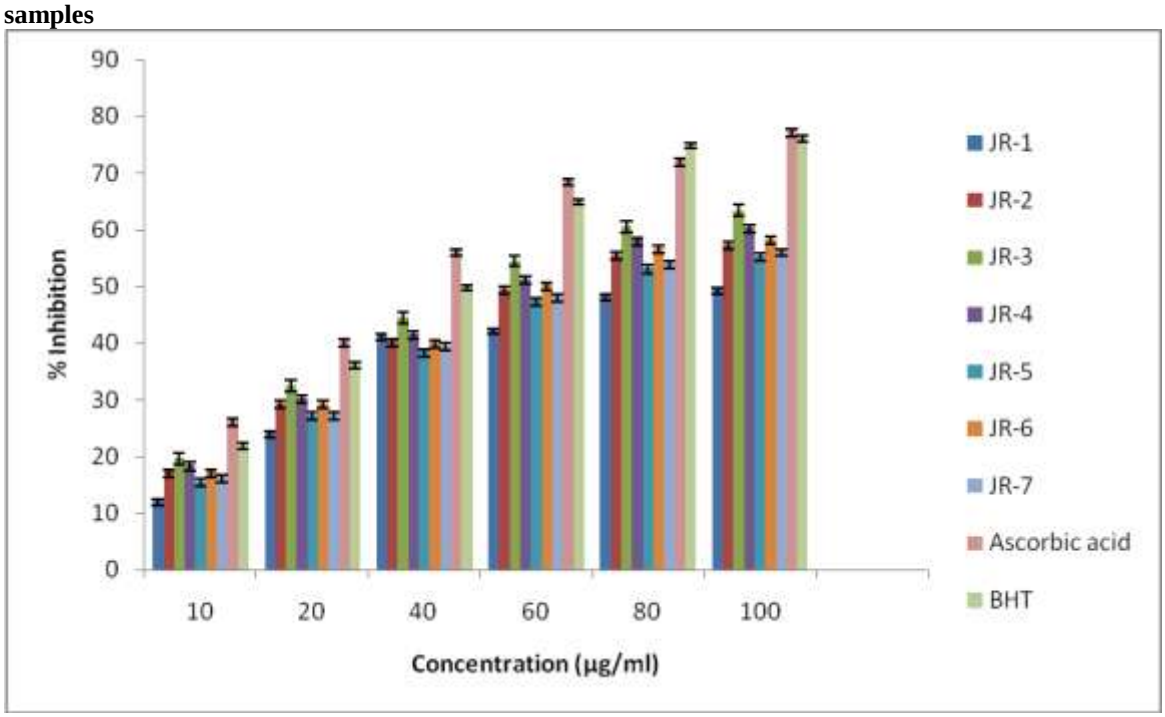


Fig. 2. Antioxidant activity measured by hydroxyl radical scavenging assay of J. regia essential oil samples



REFRENCES

1. Butner D et al. *Ailanthus excelsa* Roxb is really a plant of heaven. Int. J. Pharmacol 2010; 6, 535–550.
2. Stampar F et al Traditional walnut liqueur – cocktail of phenolics. Food Chemistry 2006; 95, 627–631.
3. Van Hellemont J. Compendium de phytotherapie. Association Pharmaceutique Belge, Bruxelles 1986; 214–216.
4. Bruneton, J. Pharmacognosie, Phytochimie, Plantes Medicinales. Tec. & Doc.Lavoisier,Paris, 1993; 348.
5. Wichtl M, Anton R.. Plantes Therapeutiques. Tec. & Doc, Paris 1999; 291–293.
6. Valnet J. Phytotherapie Traitement des maladies par les plantes. Maloine, Paris 1992; 476–478.
7. Girzu M et al. Sedative effect of walnut leaf extract and juglone, an isolated constituent. Pharmaceutical Biology 1998; 36, 280–286.
8. Solar A et al. Seasonal variation of selected flavonoids, phenolic acids and quinones in annual shorts of common walnut (Juglans regia L.). Plant Science 2006; 170, 461–543.
9. Fukuda T, Ito H, Yoshida Y. Antioxidative polyphenols from walnuts (*Juglans regia* L.) Phytochem 2003; 63, 795-801.
10. Ito H et al. Two novel dicarboxylic acid derivatives and new dimeric hydrolysable tannin from walnuts. Journal of Agricultural andFoodChemistry 2007; 55, 672–679.
11. Zhang J, et al. Chemical constituents in green walnut husks of *Juglans regia*. Chinese Traditional and Herbal Drugs 2009a.
12. Zhang Z et al. Antioxidant phenolic compounds from walnut kernels (*Juglans regia* L.). Food Chem 2009b; 113, 160-165.
13. Thakur A. Juglone: a therapeutic phytochemical from Juglans regia L. Journal of Medicinal Plants Research 2011; 5, 5324–5330.
14. Buttery RG et al. Volatile components of green walnut husks. Journal of Agricultural and Food Chemistry 2000; 48, 2858–2861.
15. Elmore JS, Nisyrios I, Mottram DS. Analysis of the headspace aroma compounds of walnuts (Juglans regia L.). Flavour and Fragrance Journal 2005; 20, 501–506.
16. Abbasi MA et al. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry.Allured Publishing Corp.,CarolStream,IL,USA 2007.
17. Manzoor et al. Chemical composition, antioxidant and antibacterial activities of the leaf essential oil of Juglans regia L. And its constituents. Phytomedicine 2012; 19, 1185– 1190.
18. Norrel SA, Messley KE, Microbiology Laboratory Manual Principles and Applications. Prentice Hall, Upper Saddle River NJ 1997; 85–90.
19. Barry A. The Antimicrobic Susceptibility Test: Principles and Practices. Lea and Febiger, Philadelphia. 1976.
20. Trivino M G, Johnson C B. Season has a major effect on the essential oil yield response to nutrient supply in *Origanum majorana*. The Journal of Horticultural Science and Biotechnology 2000; 75, 520-527.
21. Celiktas, OY et al. Antimicrobial activities of methanol extracts and essential oils of Rosmarinus oficinalis, depending on location and seasonal variations. Food Chemistry, 100, 2007; 553-559.
22. Van Vuuren S F et al. Seasonal and geographical variation of Heteropyxis natalensis essential oil and the effect thereof on the antimicrobial activity. South African Journal of Botany 2007; 73, 441-448.