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Study of antioxidant potential in leaves, stems, nuts of *Juglans regia* L.

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Abstract: Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods. The role of antioxidants has protected effect against free radical damage that may cause many diseases including cancer. Primary sources of naturally occurring antioxidants are known as whole grains, fruits, and vegetables. Several studies suggest that regular consumption of nuts, mostly walnuts, may have beneficial effects against oxidative stress mediated diseases such as cardiovascular disease and cancer. The role of antioxidants has attracted much interest with respect to their protective effect against free radical damage that may cause many diseases including cancer. *Juglans regia L.* (walnut) contains antioxidant compounds, which are thought to contribute to their biological properties. Polyphenols, flavonoids and flavonols concentrations and antioxidant activity of Leaves, Stems and Nuts extract of *Juglans regia L.* as evaluated using DPPH, ABTS, Nitric acid, hydroxyl and superoxide radical scavenging activity, lipid peroxidation and total oxidation activity were determined. The antioxidant activities of Leaves, Stems and Nuts extract of *Juglans regia L.* were concentration dependent in different experimental models and it was observed that free radicals were scavenged by the test compounds in all the models.

Key words: *Juglans regia L.;* Phytochemicals; Free Radicals; Lipid Peroxidation; Antioxidants

Introduction

The term antioxidant refers to the activity of numerous vitamins, minerals and other phytochemicals to protect the damage caused by reactive oxygen species (ROS). These are various forms of activated oxygen, which include free radicals such as superoxide ions ($O^{2-\cdot}$) and hydroxyl radicals ($OH^{\cdot}$), as well as non-free radical species such as hydrogen peroxide (H2 O2) [1]. In living organism various ROSs are formed in different ways, including normal aerobic respiration, stimulated polymorphonuclear leukocytes, macrophages and perioxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides [2]. Consequently, accumulation of potentially harmful ROSs causes increase in stress, disease and aging periods and there is loss of haemostatic control and organ functions [3]. In living cells, ROS are continuously produced during normal physiologic events and removed by antioxidant defense mechanism [4]. Synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxytoluene (BHT) have restricted use in food as they are under great consideration for toxicological reasons [5].

In consequence, recently interest has considerably increased in finding naturally occurring antioxidant to replace synthetic antioxidants, which were restricted due to their side effects such as carcinogenesis [6].

The vast biodiversity of Indian forests provides several plants, which are mentioned in Ayurveda for medicinal care. *Juglans regia* L. the royal species from family Juglandaceae has been used in traditional medicines from ancient times. It is a frost tender deciduous tree growing up to 40-60m. It is found in the Himalayan regions in India. The walnuts consume extensively as a food, which are rich unsaturated fatty acids and its leaves has been widely used in traditional medicine for the treatment of skin inflammations, hyperhidrosis and ulcers and for its antidiarriec, antihelmintic, antiseptic and astringent properties [7]. All parts of the plant; stem, bark, leaves, fruits, seeds, seed oil are used in folk medicines to treat variety of health disorders including cancer [8]. Walnut has been widely used as herbal medicine in the treatment of diabetes [9] and in folk medicine to treat prostate and vascular disturbance [10]. Antiradicalar and antibacterial activities have also described for different *J. regia* cultivars [11]. In addition, walnuts have other components that may be beneficial for health including plant protein, dietary fiber,

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melatonin [12], plant sterols [13], folate, tannins and polyphenols [14]. Walnuts possess a high content of α-tocopherol, a vitamin E family compound, which has antioxidant activity, mainly in the prevention of lipid oxidation process [15]. Plant-derived products can also be used as antimicrobial agents, with phenolics and polyphenolic having major interest. In the present study ethanolic extract of Juglans regia L. Leaves, Stems and Nuts are evaluated for the *in vitro* antioxidant properties using various experimental models.

Materials and Methods

Collection and Preparation of sample extracts: *J.regia L.* Leaves Stems and Nuts with shell were obtained from the local market. The Plant materials were identified by the Professor of Department of Botany and Sample specimen was voucher. The shells were peeled-off and broken kernels, leaf and stems were shade dried for 4-6 weeks, and minced and powdered in mixer grinder in a dry manner and sieved twice to obtain fine powdered. 100 gm of dried powder of nut of *Juglans regia L.* were extracted with Soxhlet extractor using ethanol till solvent was colorless. The extract was dried till constant weight was obtained. This residue was boiled in water bath for 5 minutes, cooled and centrifuged at 4000 rpm for 10 minutes. The clear supernatant was used for evaluating antioxidant properties in various assays.

Phytochemical Analysis: The extracts were subjected to preliminary phytochemical studies using standard procedures to detect the phytochemicals present.

Estimation of Total Phenolic Compounds: Total phenolic content was determined by the Folin Ciocalteu method [16]. To 0.5ml of 1-5 mg/ml of leaves, stems and nuts extracts individually made up with 0.5ml of distilled water, 0.5 ml of Folin Ciocalteu reagent was added and gently mixed. After 2 minutes 0.5ml of 100mg/ml sodium carbonate was added. The contents were mixed and allowed to stand for 2 hours. The optical density of the blue coloured samples was measured at 765 nm spectrophotometrically. Standard gallic acid of concentration 100-500 microgram/ ml was used. The concentration of total phenolics is expressed as milligram of gallic acid) /g of mixture. All determinations were carried out in triplicate (Table1).

Estimation of flavonoids: Flavonoids was determined by using Chang *et al* 2002 [17] with modifications. 0.5ml of concentration having 100-500 µg/ml of leaves, stems and nuts extracts were individually mixed with 1ml aluminium trichloride in ethanol (20g/l) and diluted with ethanol to 25 ml. The absorbance at 415 nm was read after 40 minutes at 37°C. Rutin (citrus flavonoids glycoside) a class of flavonoids also called as rutoside; of concentration 0.5mg/ml, 1.0mg/ml, 1.5mg/ml, 2.0mg/ml and 2.5mg/ml was used as a reference compound and absorbance was measured under the same conditions. All determinations were carried in triplicate. The amount of flavonoids calculated as milligram of rutin/g of mixture (Table.1).

Estimations of flavonols: The flavonols was determined by Yermakov *et al* 1987 [18] with slight modifications like reducing the total volume. 0.05 ml of various concentrations (100-500 µg) of leave, stems and nuts were treated with 1ml of 2% aluminium trichloride in ethanol and 1ml of 5% sodium acetate. The absorption at 400nm was read after 2.5 hours at 37°C. The same procedure was carried out for 2ml of reference compound rutin for concentration 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml and 1.0mg/ml. All determinations were carried out in triplicate. The content of flavonols was calculated in terms of milligram of rutin /g of mixture (Table.1).

Table 1: Total Phenols, Flavonoids and Flavonols of *Juglans regia L.*

Content	*Juglans regia L.*		
	Leave	Stems	Nuts
Total Phenols (mg GAE/g)	385 ± 12.25	275 ± 12.50	135 ± 2.5
Flavonoids (mg rutin/g)	310 ± 11.50	234 ± 10.5	38 ± 2.0
Flavonols (mg rutin/g)	142 ± 12.32	122 ± 11.50	12 ± 1.5

Value = Mean ±SD of 3 determinations,
*significant values and $p < 0.001$

DPPH radical scavenging activity [19]: Scavenging activity of DPPH was measured by the method of Sreejayan N *et al*, 1996 using spectrophotometric analysis. To ethanolic solution of DPPH (200µM), 0.05ml of the test compounds dissolved in ethanol were added at different concentration (100-500 µg/ml). An equal amount of ethanol was added to the control. After 20 min, the decrease in the absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition calculated by using the following formula [20].

Formula: $\text{Inhibition (\%)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$

ABTS radical cation decolorisation assay [21]: In this improved version, $ABTS^{-}$, the oxidant is generated by persulfate oxidation of 2,2,-azinobis (3-ethylbenzoline-6- sulfonic acid) – ($ABTS^{2-}$). ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate and the mixture were allowed to stand in dark at room temperature for 12 – 16 hr before use. For the study, different concentrations (100 – 500µg/ml) of ethonolic extract (0.5ml) were added to 0.3ml of ABTS

solution and the final volume was made up with ethanol to make 1 ml. The absorbance was read at 745nm and the percentage inhibition calculated.

Scavenging of nitric oxide radical [22, 23]: Nitric oxide was generated from sodium nitroprusside and measured by Griess' reaction [24,25]. Sodium nitroprusside (5m*M*) in standard phosphate buffer solution was incubated with different concentration (100 – 500 µg/ml) of the ethanol extract dissolved in phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25°C for 5 hr. Control experiments without the test compounds, but with equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5ml of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% napthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthylethylene diamine was read at 546nm. The experiment was repeated in triplicate.

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging activity was measured according to the method of Kunchandy and Rao [26] by studying the competition between deoxyribose and test extract for hydroxyl radical generated by Fenton's reaction. The reaction mixture contained deoxyribose (2.8m*M*), FeCl3 (0.1m*M*), H2O2 (1m*M),* ascorbate (0.1m*M),* KH2PO4- KOH buffer (20m*M*, *p*H 7.4) and various concentrations of the sample extracts in a final volume of 1.0ml. The reaction mixture was incubated for 1h at 37°C. Deoxyribose degration was measured as thiobarbituric acid reacting substances (TBARS) and the percentage inhition calculated.

Superoxide scavenging activity: The scavenging activity towards the superoxide radical ($O2^{-}$) was measured in terms of inhibition of generation of$O2^{-}$ [27]. The reaction mixture consisted of phosphate buffer (50m*M*, *p*H 7.6), riboflavin (20 µg/0.2ml), EDTA (12mM), NBT (0.1mg/3ml) and sodium cyanide (3µg/0.2ml). Test compounds of various concentrations of 100 – 500 µg/ml were added to make a total volume of 3ml. The absorbance was read at 530 nm before and after illumination under UV lamp for 15min against a control instead of sample. The percentage inhibitor was calculated by using the formula.

***In vitro* anti-lipid peroxidation assay:** Freshly excised goat liver was processed to get 10% homogenate in cold phosphate buffered saline *p*H 7.4 using glass teflon homogenizer and filtered to get a clear homogenate. The degree of lipid peroxidation was assayed by estimating the TBARS by using the standard method [28] with minor modifications [20]. A different concentration of the extracts (100 - 500µg/ml) in water was added to the liver homogenate. Lipid peroxidation was initiated by adding 100µl of 15m*M* ferrous sulphate solution to 3ml of the tissue homogenate. After 30min, 100µl of this reaction mixture was taken in a tube containing 1.5ml of 10% trichlro acetic acid. After 10min, tubes were centrifuged and supernatant was separated and mixed with 1.5ml of 0.67% TBA in 50% acetic acid. The mixture was heated for 30min in a boiling water bath. The intensity of the pink colored complex formed was measured at 535nm. The results were expressed as percentage inhibition. Statistical analysis-Linear regression analysis was used to calculate the IC50.

Total antioxidant activity (FRAP assay) [29]: The FRAP reagent was prepared by mixing 300mM Acetate Buffer, 10mM Tripyridyltriazine TPTZ in 40mM HCL and 20mM FeCl3. 6H2O in the ratio 10:1:1. Briefly 50µl of different concentrations of herbal preparation (200µg-1000µg) were added to 1.5ml freshly prepared and pre warmed FRAP reagent at 37°C and incubated at 37°C for 10 minutes. The absorption of blue coloured complex was read against blank using distilled water. BHT and a - tocopherol were used as standards. The absorbance was measured at 593nm. The data was expressed as mMol ferric ions reduced to ferrous form per litre FRAP. Statistical analysis: The results were expressed as % Inhibition as Mean ±SD of 3 determinations, on applying test for significance $p<0.001$ was considered as statistically significant and IC 50 values were also calculated for each assay.

Results

The phytochemical analysis of leaves stems and nut extract of *Juglans regia L.* showed that it is rich in polyphenols as shown in Table 1. The polyphenol content was 385 ± 12.25, 275 ± 12.50 and 135 ± 2.5mg/g of gallic acid equivalent of leaves, stems and nuts respectively. The amount of flavonoids in rutin equivalent was 310 ± 11.50, 234 ± 10.5 and 38 ±2.0 mg/g of leaves, stems and nuts respectively. The content of flavonols was 142 ± 12.32, 122 ± 11.50 and 12 ± 1.5 mg/g of leaves, stems and nuts respectively.

Several concentrations ranging from 100-500µg/ml of the ethanolic extract of leaves stems and nut extract of *Juglans regia L.* were tested for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner in all the manners. The ethanolic extract of leaves results showed in table 2, it was inferred that, with respect to maximum inhibition percentage of all models viz, DPPH, ABTS, Hydroxyl, nitric oxide, super oxide, and lipid peroxidation, showed value

97,92,74,73,67 and 45% of inhibition respectively at 500µg/ml concentration and their IC 50 values were 300, 310, 278, 265, 280 and 310.

Table 2: Effect of ethanolic extract of leaves of *Juglans regia L.* on different antioxidant models

Concentration (µg/ml)	Mean% Inhibition ± SD					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	57.31 ± 0.24	55.32 ± 1.75	42.55 ± 0.85	47.35 ± 1.24	33.20 ± 1.41	8.69 ± 1.85
200	65.08 ± 0.36*	65.85 ± 1.85*	51.85 ± 0.47*	58.28 ± 1.05*	42.24 ± 1.20*	12.97 ± 1.49*
300	77.09 ± 0.21*	72.55 ± 1.90*	62.34 ± 0.62*	65.85 ± 1.58*	50.35 ± 1.50*	24.85 ± 1.52*
400	88.12 ± 0.25*	83.24 ± 1.92*	69.68 ± 0.58*	70.73 ± 1.96*	56.02 ± 1.07*	38.54 ± 1.64*
500	97.12 ± 0.32*	92.25 ± 1.90*	73.42 ± 0.81*	74.55 ± 1.62*	67.31 ± 1.48*	45.29 ± 1.81*
IC 50 (µg/ml)	300	310	278	265	280	310

Value = Mean ±SD of 3 determinations, *significant values and p< 0.001

The ethanolic extract of stems results showed in table.3, values with respect to maximum inhibition percentage of all models viz, ABTS, super oxide, nitric oxide, Hydroxyl, DPPH and lipid peroxidation, showed 81, 68, 65, 61, 55 and 32 % of inhibition respectively at 500 µg/ml concentration and their IC 50 values were 320,275,308,280,293 and 300µg/ml.

The ethanolic extract of stems results showed in table 4, showed respect to maximum inhibition percentage of all models viz, DPPH, ABTS, super oxide, nitric oxide, Hydroxyl, and lipid peroxidation, showed value of 81, 68, 65, 61, 55 and 32% of inhibition respectively at 500µg/ml concentration and their IC 50 values were 280,300,225,310,305 and 320µg/ml.

Table 3: Effect of ethanolic extract of Stems of *Juglans regia L.* on different antioxidant models

Concentration (µg/ml)	Mean% Inhibition ± SD					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	18.50 ± 0.51	45.21 ± 1.51	35.32 ± 0.25	35.15 ± 0.98	31.25 ± 1.20	6.05 ± 1.02
200	25.42 ± 0.25*	57.40 ± 1.72	42.52 ± 0.34*	43.29 ± 1.10*	40.29 ± 1.45*	11.27 ± 1.45*
300	35.31 ± 0.31*	68.23 ± 1.85	49.25 ± 0.78*	51.25 ± 1.25*	52.30 ± 1.80*	19.35 ± 1.38*
400	47.20 ± 0.30*	75.38 ± 1.65	57.35 ± 0.82*	59.58 ± 1.49*	62.45 ± 1.85*	24.68 ± 1.47*
500	55.25 ± 0.42*	81.25 ± 1.85	65.24 ± 0.65*	61.25 ± 1.39*	68.95 ± 1.52*	32.46 ± 1.65*
IC 50 (µg/ml)	320	275	308	280	293	300

Value = Mean ±SD of 3 determinations, *significant values and p< 0.001

The total antioxidant potential of *Juglans regia L,* determined by FRAP assay results showed in table 5, for this study extract of leaves showed the maximum activity of 59, following this 53 and 39 of nuts and stems respectively. It was found that *Juglans regia L.* better antioxidant potential than BHT and α-Tocopherol. IC 50 values were also determined leaves and stems value same as 600 and nuts contain 525µg/ml.

Table 4: Effect of ethanolic extract of nuts of *Juglans regia L.* on different antioxidant models

Concentration (µg/ml)	Mean% Inhibition ± SD					
	DPPH	ABTS	Nitric Oxide	Hydroxyl radical	Superoxide radical	Lipid peroxidation
100	35.51 ± 0.64	36.23 ± 2.51	33.38 ± 0.75	29.62 ± 1.32	34.64 ± 1.85	11.05 ± 1.05
200	49.08 ± 0.56*	47.52 ± 2.25*	41.25 ± 0.54*	35.28 ± 1.46*	41.86 ± 1.95*	18.25 ± 1.25*
300	65.09 ± 0.74*	59.74 ± 2.05*	47.68 ± 0.65*	42.37 ± 1.28*	52.25 ± 1.52*	27.25 ± 1.32*
400	76.12 ± 0.72*	70.25 ± 1.02*	55.25 ± 0.55*	49.45 ± 1.58*	62.54 ± 1.47*	39.85 ± 1.47*
500	92.12 ± 0.65*	85.42 ± 0.62*	63.48 ± 0.84*	58.95 ± 1.29*	71.25 ± 1.56*	52.25 ± 1.56*
IC 50 (µg/ml)	280	300	225	310	305	320

Value = Mean ±SD of 3 determinations, *significant values and p< 0.001

Table 5: Total Antioxidation activity of *Juglans regia L.*

Concentration (µg/ml)	mM ferric ions reduced to ferrous ions per liter FRAP reagent				
	Juglans regia .L			BHT	α -Tocopherol
	Leaves	Stems	Nuts		
100	29.12 ± 0.185	20.36 ± 0.185	25.94 ± 0.193	50.22 ± 0.153	24.78 ± 0.145
200	40.55 ± 0.123*	24.48 ± 0.112	38.25 ± 0.168*	51.54 ± 0.165*	30.15 ± 0.173*
300	45.78 ± 0.168*	29.91 ± 0.158	41.98 ± 0.152*	78.37 ± 0.147*	33.58 ± 0.192*
400	52.26 ± 0.182*	34.47 ± 0.175	47.38 ± 0.118*	85.65 ± 0.189*	37.67 ± 0.129*
500	59.64 ± 0.195*	39.25 ± 0.127	53.49 ± 0.149*	91.75 ± 0.158*	42.35 ± 0.125*

Value = Mean ±SD of 3 determinations, *significant values and p< 0.001

Discussion

DPPH is a relatively stable radical; the assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH, which react with suitable reducing agent. The electrons become paired off and solution loses color stochiometrically depending on the number of electrons taken up [30]. DPPH was

used to determine the proton radical scavenging action of extracts of Leave stems and nuts of *Juglans regia L.* because it possesses a proton free radical and shows a characteristics absorbance at 517nm. From the results stems of *Juglans regia* L. showed the maximum reduced the radical activity [27].

The ethanolic extracts of the leaves, stems and nuts of *Juglans regia L.* were fast and effective scavengers of the ABTS radical and this activity. From the results, the extracts of leaves exhibited higher activity than stems and nuts. Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals [31]. The results imply that the extract of leaves inhibit or scavenge the $ABTS^{\cdot+}$ radical since both inhibition and scavenging properties of antioxidant towards $ABTS^{\cdot+}$ radical have been reported earlier[21].

Nitric oxide is a free radical generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological processes [32]. Excess concentration of NO is associated with several diseases [33, 34]. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite anions, which act as free radicals [35]. In the present study, the ethanolic extract of stems of *Juglans regia L.* showed better activity in competing with oxygen to react with nitric oxide and thus the inhibition of generation of anions.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation, damage to DNA and proteins [36]. The Fenton reaction generates hydroxyl radicals (OH -) which degrade the DNA deoxyribose, using Fe ++ions as catalyst. This indicates that *Juglans regia L* was capable of reducing oxidative deoxyribose damage in dose dependent manner. From the results nut extracts of *Juglans regia L.* showed the maximum activity.

Superoxide anion is the first reduction product of oxygen which is measured in terms of inhibition of generation of O2 [37]. Superoxide dismutase catalyses the dismutation of reactive superoxide anion to oxygen and hydrogen peroxide [30]. The effect of Juglans regia L of leave stems and nuts in scavenging anions may be due to inhibition of generation of superoxide.

Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and involves formation of lipid radicals leading to membrane damage. Free radical induces lipid peroxidation in polyunsaturated lipid rich areas like brain and liver [38]. In this study, in vitro lipid peroxidation was induced in goat liver by using FeSO4 and ascorbic acid. The present results show that extract of nuts of *Juglan regia L.* shows better dose-dependent prevention towards generation of lipid peroxides.

The reducing ability of the extracts was in the range of 407.60-695.98µm Fe (II)/g le 2). The antioxidant potentials of the ethanolic extracts of the leaves stems and nuts of *Juglan regia L.* were estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). The FRAP values for the ethanolic extracts of the leaves and stem of *Juglan regia L.* were significantly higher than that of BHT, but almost similar of α-Tocopherol. The ferric reducing/antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant component in dietary polyphenols [39]. Antioxidant activity increased proportionally to the polyphenol content. According to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species [40].

Conclusion

The results of the present study apparently indicated that *Juglans regia L.* leave, stems and nuts may constitute a suitable source of phenolic and could be used as alternative natural antioxidants in food industries. This further showed the capability of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathological damage. Ethanolic extracts from *Juglans regia L.* leave stems and nuts possess antioxidant and antiradical activity, which may be helpful in preventing or stopping the progress of various oxidative stress-related diseases.

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