Anti-proliferative, Cytotoxicity and Anti-oxidant Activity of *Juglans regia* Extract

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Abstract

**Objective:** *Juglans regia* (walnuts), the royal species from *Junglandaceae* family, well-known for its valuable medicinal uses, their regular consumption may have beneficial effects against oxidative stress mediated diseases including cancer. The present study was aimed to explore the total phenolic content, anti-proliferative and anti-oxidant activity of *Juglans regia* leaves. **Methods:** The leaf powder was extracted using different solvents and subjected for phytochemical investigation. The total phenolic contents were determined by the Folin-Ciocalteu method. The extracts comprising a good amount of secondary metabolites especially polyphenols were used to evaluate their antioxidant activity using Fenton’s reaction and DPPH scavenging assay, while cytotoxic and anti-proliferative activity against B16F10 mice melanoma and A375 human melanoma cell were screened using MTT Assay. **Observation:** Methanolic extract presented the highest total phenolic content (94.39 ± 5.63 mg of GAE/g of extract) as compared to aqueous extract (27.92 ± 1.40 mg of GAE/g of extract). Similarly, methanolic extract presented the highest antioxidant activity (EC 50 of 0.250mg/ml) followed by water extract (EC 50 of 0.325mg/ml) in Fenton’s reaction and 0.199 ± 0.023 and 2.991 ± 0.740, respectively in DPPH assay. The extracts showed concentration dependent growth inhibition activity (IC 50 0.234 and 0.304mg/ml) against B16F10 mice melanoma and A375 human melanoma cell line (IC 50 0.298 and 0.350mg/ml) respectively. The extracts proved least toxic when treated with normal lymphocytes. The results indicate that walnut leaves are an excellent source of antioxidant and anti-cancerous agents and may prove fruitful herbal remedy in near future. However, the extracts proved effective against mice melanoma and human melanoma cells. Despite, more study is required before coming to any conclusion.

**Keywords:** *J. regia*, anti-oxidant, anti-proliferative, B16F10, A375, Lymphocytes, MTT


1. Introduction

Epidemiological studies have consistently shown that there is a clear significant positive association between regular consumption of fruits, nuts and vegetables, and a reduced incidence of ischemic heart disease and some types of cancer, particularly stomach, esophagus, lung, oral cavity and pharynx, endometrial, pancreas and colon cancers [1,2,3,4,5]. These antioxidant and chemopreventive properties have been attributed to their high content of natural antioxidants, especially ascorbic acid (vitamin C), tocopherols (vitamin E), b-carotene (provitamin A), anthocyanins and other polyphenols [6,7,8,9]. Several studies showed that phenolic compounds are the major bioactive phytochemicals with human health benefits [10,11,12]. In fact, many authors have reported a direct relationship between total phenolic content and antioxidant activity in numerous seeds, fruits and vegetables [9,10,11,12,13].

The *Juglans* genus (family *Juglandaceae*) comprises several species and is widely distributed throughout the world. The walnut tree (*Juglans regia* L.) is its well-known member, constituting an important species of deciduous trees found primarily in temperate areas and cultivated commercially throughout southern Europe, northern Africa, eastern Asia, United States and western South America. In Portugal, walnut trees can be found all over the country, being the nuts very popular and largely consumed as part of the Mediterranean diet. Nevertheless, not only dry seeds (nuts) are used but also green walnuts, shells, bark, green husks (epicarps) and leaves, which have been used in the cosmetic and pharmaceutical industries [14].

Walnut leaves are considered a source of healthcare compounds, and have been widely used in traditional medicine for treatment of skin inflammations, hyperhidrosis and ulcers and for its antidiarrheic, anti-helmintic, anti-septic and astringent prop-erties [15]. In Portugal, as in some other European countries, especially in rural areas, dry walnut leaves are fre- quently used to...
prepare infusions [16]. Antiradical and antibacterial activities have also been recently described for different J. regia cultivars [14,15,16,17].

Interest in natural antioxidant sources prompted us to continue investigating the biological activity of J. regia phytochemicals. The present study was aimed to explore the anti-proliferative and anti-oxidant activity of Juglans regia leaves. For these purposes, methanolic and aqueous extracts were prepared. The anti-oxidant activity was performed by Fenton’s reagent. In addition, walnut extracts were tested for their potential anti-proliferative properties with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) bioassay on mice melanoma (B16F10) and human melanoma cell lines (A375). However, their cytotoxicity was screened against normal lymphocytes.

2. Materials and Methods

2.1. Chemicals and Reagents

Solvents were purchased from Merk, Fenton’s reagent and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from sigma. Lymphocytes were obtained from human blood sample. All other chemicals were obtained from sigma.

2.2. Samples

Walnut (J. regia L.) leaves were collected from Kashmir valley in the month of Sep. to Oct. The leaves were collected from the middle third of branches exposed to sunlight. Leaves were shade dried, made to coarse particles and were put in plastic bags, immediately frozen at 20°C and then freeze dried. Lyophilization was carried out to remove the excess moisture.

2.3. Extraction Procedure

The dried coarse powder (172gm) was extracted in separating funnel sequentially in 400ml of methanol and distilled water. The process was run till the decolourisation of the solvent, after which the sample was concentrated in water bath at the temperature of 45°C and further condensed to powdered form. The dried extracts were weighted and kept in labelled sterile specimen bottles.

2.4. Preliminary Phytochemical Screening

The secondary metabolite classes such as alkaloids, carbohydrates, flavonoids, steroids and tannins were screened according to the standard phytochemical methods [18].

2.5. Total Phenolic Content Measurement

Phenolic compounds concentration in the obtained extracts was determined by using the Folin–Ciocalteu’s phenol reagent, according to the previously described procedure [19] with some modifications. Briefly, 1 mL of the extract solution was mixed with 1 mL of Folin–Ciocalteu’s phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used for constructing the standard curve. The contents of total phenolics are expressed as mg of gallic acid equivalents (GAE)/g of extract.

2.6. Determination of in-vitro Anti-oxidant Activity By Fenton’s Reaction

Fenton’s reaction [20] was used for determination of in-vitro antioxidant activity. The hydroxyl radical attached deoxyribose and initiated a series of reaction that eventually resulted in the formation of thiobarbituric acid reaction substance (TBARS). The measurement of TBARS thus gives an index of free radical scavenging activity. The reaction mixture consisted of a deoxyribose (3 mM, 100µl), ferric chloride (Fe3++ 0.2 mM 50µl), EDTA (0.1mM 50 µl), ascorbic acid (0.1 mM 100 µl), stock solution of all the extracts (pet. Ether, aqueous, ethyl acetate and ethanol) at 10 mg/ml were prepared from which 100-1000 µl were added in reaction mixture, the final volume was made up to 1 ml by adding adequate quantity of phosphate buffer saline (pH, 7.4) and incubated for 1 hour at 37°C. The reaction was stopped by adding 0.5 ml of 5% TCA and 0.5 ml of 1% TBA the mixture was than incubated for 20 minutes in a boiling water bath. The absorbance was measured at 532nm. DMSO was used as control. The results are expresses in the form of % inhibition.

2.7. Determination of DPPH Radical Scavenging Activity

The capacity to scavenge the DPPH free radical was monitored according to a method reported before [21]. Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals (6 × 10⁻⁵ M). The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = [(ADPPH - AS)/ADPPH]×100.

2.8. Cell Culture

Human cancer cell lines were procured from National Centre for Cell Science (NCCS) Pune and B16F10 mice melanoma was obtained from tumour donor mice. Cells were grown in tissue culture flask in complete growth medium (RPMI-1640 medium with 2mM glutamine, pH 7.4, supplemented with 10% fetal calf serum, 100µg/ml streptomycin and 100 units/ml penicillin) in a carbon dioxide incubator (37°C, 5% CO₂, 90% RH). The cells at sub confluent stage were harvested from the flask by treatment with trypsin [0.05% in PBS (pH 7.4) containing 0.02% EDTA]. Cells with viability of more than 98% as determined by trypan blue exclusion were used for determination of cytotoxicity. The cell suspension of 1 x 10⁵ cells/ml was prepared in complete growth medium.

2.9. MTT Assay

After cell incubation with the potential extract for the desired time in a 24-cell culture plate (with 1ml of culture
medium), add 100µl of MTT stock solution (final concentration-0.5mg/ml) to each well. Incubate at 37°C for 2hrs in the humidified CO₂ incubator. At the end of the incubation period, take off the medium and add 1ml of acidic isopropanol/DMSO to solubilise the purple formazan dye. Carefully sonicate each well to solubilise completely the converted dye. Transfer 200µl of the dye solution of each well for a 96-well plate in duplicate. Read the absorbance at 570nm using 690nm as reference in a plate reader spectrophotometer. Express the results as % of sample absorbance in relation to the absorbance in the negative control.

2.10. Lymphocyte Isolation

For lymphocyte isolation, defibrinated or anticoagulant treated blood was taken in desired tubes. Required volume of Ficoll-Paque PLUS was added to the sample. The sample was centrifuged at 30-40minutes and the upper layer was removed leaving the lymphocyte layer undisturbed at the interface. Transfer the lymphocyte layer to a clean centrifuge tube and spin for 20-30 minutes. Add balanced salt solution and spin again. Discard the supernatant and suspend the lymphocytes in RPMI. Few drops of PHA (mitogen) were added to culture bottles containing sample. Culture bottles were incubated for 24hrs in CO₂ incubator. Cell counting was performed by using trypan blue dye.

2.11. Statistical Analysis

The experimental data were expressed as mean ± SEM. The significance of difference among the various treated groups and control group were analysed by means of one-way ANOVA. The level of significance was set at p < 0.05.

3. Results & Discussion

3.1. Phytochemical Analysis

Phytochemical screening of the crude leaf extracts of Juglans regia L. revealed the presence of carbohydrates, cardiac glycosides, phenolics, flavonoids, alkaloids, proteins, steroids and tannin [20]. Results of preliminary phytochemical screening are tabulated in (Table 1). The increasing reliance on the use of medicinal plants worldwide has been traced to the extraction and development of several drugs from these plants as well as from traditionally used herbal remedies. Further, detailed investigation needs to be underway to determine the exact phytoconstituents and isolate the active principles which are responsible for the anti-proliferative activity of the leaves of Juglans regia L. As methanolic and aqueous extracts showed presence of several secondary metabolites especially phenolic compounds, so these two extracts were subjected for further experiments.

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Benzene extract</th>
<th>Acetone extract</th>
<th>Methanolic extract</th>
<th>Ethanolic extract</th>
<th>Water extract</th>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
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<tr>
<td>Flavonoids</td>
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<td>Phenolic Compound</td>
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<td>Glycosides</td>
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<tr>
<td>Proteins</td>
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<td>Steroids</td>
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<tr>
<td>Tannins</td>
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<td>+</td>
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<tr>
<td>Carbohydrates</td>
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</table>

3.2. Total Phenolic Content

Total phenolic contents of methanolic and aqueous extracts of walnut leaf are shown in Table 2. As expected, methanolic extracts obtained presented a higher phenolic content than aqueous extract. In fact, methanol is considered as one of the best solvents for phenolics extraction[11]. Methanolic extract, presented the highest total phenolic content (94.39 ± 5.63 mg of GAE/g of extract) as compared to aqueous extract (27.92 ± 1.40 mg of GAE/g of extract). Taking into account the total phenolic concentration in walnut leaf, which is in agreement with previous studies showing walnuts as a good dietary source of phenolics [12,13,22].

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phenolic content (mg GAE/g)</th>
<th>DPPH scavenging activity EC50 (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic Extract</td>
<td>94.39 ± 5.63</td>
<td>0.199 ± 0.023</td>
</tr>
<tr>
<td>Aqueous Extract</td>
<td>27.92 ± 1.40</td>
<td>2.991 ± 0.740</td>
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</table>

3.3. Hydroxyl Radical Scavenging Activity

The leaf extracts of Juglans regia had showed potent anti-oxidant property in Fenton’s reaction model with ascorbic acid as standard. Methanolic extract presented the highest antioxidant activity (EC₅₀ of 0.250mg/ml) followed by water extract (EC₅₀ of 0.325mg/ml; Graph 1). Both extracts showed significant antioxidant activity when compared against standard ascorbic acid (EC₅₀ of 0.235mg/ml). The hydroxyl radical scavenging activity was found to increase in a concentration dependent manner.

3.4. DPPH Free Radical Scavenging Activity

The methanolic extract presented a strong concentration dependent anti-radical activity. Among both leaf extracts (methanol & aqueous extract) methanolic leaf extract exhibited the highest DPPH free radical scavenging capacity, followed by aqueous leaf extract with EC₅₀ value of 0.199 ± 0.023mg/ml and 2.991 ± 0.740, respectively (Table 2). Our results are in agreement with previous reports showing the antiradical properties of extracts from walnut seeds [23].
3.5. Anti-proliferative and Cytotoxicity Activity against Cancer Cells

The most effective extracts in anti-oxidant assays were further studied for anti-proliferative activities. Therefore, walnut leaf methanolic and aqueous extracts were evaluated for their ability to inhibit the growth of mice melanoma (B16F10) and human melanoma (A375) cancer cell lines. There was a not a notable difference in the sensitivity between the two applied extracts against respective cell lines as shown in graph 2 & graph 3. The normal lymphocytes showed negligible sensitivity towards both leaf extracts of *J. regia*. However, the aqueous extract showed least sensitivity towards lymphocytes as compared to methanol extract. The cytotoxicity activity of *J. regia* extracts was screened against B16F10 Mice Melanoma, A375 human melanoma cell lines and lymphocytes for 72hrs by MTT bioassay. The methanolic extract showed potent anti-proliferative activity with IC$_{50}$ 0.234mg/ml against mice melanoma (B16F10) in comparison to human melanoma (A375) cell lines with IC$_{50}$ 0.304mg/ml. Similarly, aqueous extract of *J. regia* leaves showed notable anti-proliferative activity against B16F10 and A375 cancer cell lines with IC$_{50}$ 0.298mg/ml and 0.350mg/ml, respectively. Therefore, it was found that methanolic extract depicted considerable anti-proliferative activity when compared to aqueous extract. Despite, both the extracts (Methanolic and Aqueous) showed least sensitivity towards lymphocytes when incubated with MTT bioassay for 72hrs. Graph 2 & Graph 3 presents the concentration effectiveness of walnut extracts on viability of B16F10 and A375 cells. Nine different concentrations of both extract (0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5mg/ml of extract/ml) were applied. Both extracts inhibited mice melanoma and human melanoma cancer cell growth in a concentration-dependent manner, although there was no significant difference between the three lowest concentrations (.08, 0.1and 0.25mg/ml) of both the extracts. The cyclophosphamide was used as a standard chemotherapeutic drug. Both the extracts when applied adjuvant with standard chemotherapeutic drug showed remarkable cancer cell growth inhibition.
Graph 3. Cytotoxicity activity of Aqueous Extract against Cancer cell lines (B16F10 and A375) and Normal cell lines (Lymphocytes). Data are presented as Mean, P<0.05 was considered statistically significant

*J. regia* species has been claimed to possess anticancer activities [24]. Yang et al [12] demonstrated recently a strong anti-proliferative capacity of walnut seed extract against human HepG2, liver and Caco-2 colon cancer cells. The anticancer effects of these nuts are usually attributed to their chemical composition. Walnuts contain components that may slow or prevent cancer growth including omega-3 fatty acids, vitamin E (mainly the c-tocopherol form) [25,26] phytosterols [27], ellagic acid [28,29], gallic acid and flavonoids namely quercetin [30,31] carotenoids [32] and melatonin [33]. Recent studies conducted on cell cultures and animal models seem to indicate that polyphenols are the main phytochemicals with antioxidant and anti-proliferative properties of higher plants [7,8,9,34,35]. These molecules might act as cancer blocking agents, preventing initiation of the carcinogenic process and as cancer-suppressing agents, inhibiting cancer promotion and progression [36]. In detail, polyphenols can block cancer either through: (i) suppression of nuclear factor-kB (NF-kB) activation; NF-kB is a nuclear transcription factor that regulates the expression of various genes involved in inflammation and carcino genesis and its activation induces transcriptional upregulation of the genes involved in cell-cycle progression; (ii) suppression of activator protein-1 (AP-1) transcription factor activation; AP-1 activity is increased by several tumor promoting agents and its inhibition is a recognized molecular target in chemoprevention; (iii) suppression of mitogen activated protein kinases (MAPK); (iv) suppression of protein kinases (PK), namely PKC; (v) suppression of growth-factor receptor (GFR)-mediated pathways; (vi) cell cycle arrest and induction of apoptosis; (vii) antioxidant (protecting pre-malignant cells from oxidative breakage of cellular DNA) and anti-inflammatory effects; and (viii) suppression of angiogenesis [37,38]. In this study, the inhibition of cancer cell proliferation by walnut extracts was not correlated with the total phenolic contents in the extracts tested, suggesting that a specific phytochemical or a class of phytochemicals in extracts may be responsible for their anti-proliferative activities. Alternatively, taking into consideration the complexity of the mechanisms proposed for their chemo-preventive properties, it is likely that anti-carcinogenic effects attributed to polyphenols may be based on synergistic, additive, or antagonistic interactions of many compounds present in these foods [39]. Corroborating this, currently available data suggest that some extracts may show greater effects than an individual constituent, implying that combinations of constituents present in plant extracts may be highly important in the final biological activity.

In conclusion, the results of this study represent the first evidence that walnut leaf methanolic and aqueous extracts possess effective anti-oxidant and anti-proliferative activity against mice melanoma and human melanoma cancer cells. These properties seem more likely related to its phenolic constituents. It is therefore suggested that *J. regia* may be used as an inexpensive and easily accessible source of effective natural antioxidants and chemo-preventive agents, and future clinical investigations on this medicinal plant should be encouraged.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

References


