Original Article

Taxus wallichiana as a Potential *in vitro* Antioxidant with Good Lethal Effect on Pathogenic Bacterial Strains

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ABSTRACT

Objective: *Taxus wallichiana* as a potential *in vitro* antioxidant with good lethal effect on pathogenic bacterial strains.
Methods: Antioxidant activity of the different crude extracts of *Taxus wallichiana* were evaluated using total phenolics, DPPH, Reducing power, DNA sugar damage and lipid peroxidation assays and antibacterial activity against some food borne pathogens were evaluated by using agar dilution method.
Results: The maximum percentage inhibition for DPPH standard solution was recorded (72.78%) for methanolic extract, 72.45%, 68.3% and 94.81% for ethyl acetate, chloroform and petroleum ether extracts respectively. The antioxidant activities of the extracts were also determined using the microsomal lipid peroxidation system and the highest antioxidant activity was again observed in petroleum ether extract (97.63%) followed by the methanolic extract (97.56%). The minimum inhibitory concentrations (MICs) of the extracts determined by the agar dilution method were ranging from 50 to 400 mg/ml. However, the extracts like ethyl acetate, chloroform and petroleum ether exhibited marked inhibitory effect against all the test organisms and were more effective than that of methanol extract of *Taxus wallichiana*.
Conclusion: These findings indicate promising antioxidant and antibacterial activity of crude extracts of the *Taxus wallichiana* and the need for further exploration of their effective use in both modern and traditional systems of medicine.

Keywords: Antioxidant, Antimicrobial activity, Lipid peroxidation, *Taxus wallichiana*.

INTRODUCTION

Taxus wallichiana Zucc. (Himalayan Yew) is a small to medium sized evergreen tree, growing 10–20 m tall. In exceptional cases, it can grow up to 28 m tall. Its leaves are flat, dark green, arranged spirally on the stem.[1] Depending on taxonomic treatment, *T. wallichiana* are found to have a wide growth range in Asia, stretching from Afghanistan through the Himalayas to Philippines. It is found growing in Afghanistan, Bhutan, China, India, Indonesia, Malaysia, Myanmar, Nepal, Pakistan, Philippines and Vietnam. This plant is used traditionally for the treatment of high fever and painful inflammatory conditions. The leaves of this plant are used to make herbal tea for indigestion and epilepsy. Previously published literatures on *T. wallichiana* have reported immune-modulatory, anti-bacterial, anti-fungal, analgesic, anti-pyretic and anti-convulsant activities.[2,3] In India, extracts from its bark and leaves are used in Unani medicine as a source of the drug Zarnab, prescribed as a sedative, aphrodisiac and as a treatment for bronchitis, asthma, epilepsy, snake bites and scorpion stings.[4] In Ayurvedic medicine, young shoots are used to prepare a medicinal tincture for the treatment of headache, diarrhea and biliousness. The leaves are also used for the treatment of hysteria, epilepsy and nervousness. Its bark and leaves are considered to possess anti-fertility properties. It has also been used in steam baths to treat rheumatism.[5] A paste made from the bark is used to treat fractures and headaches. The inhabitants of the buffer zone villages of the Nanda Devi Biosphere Reserve in India collect the Taxus bark and leaves, mainly for traditional teas and for curing colds and coughs, a practice also commonly seen in other rural areas. Extracts from this tree are also used in medicinal hair oils. In Pakistan, a decoction of the stem is used as a treatment against tuberculosis. The objective of the present study was to evaluate the protective effects of methanolic, ethyl acetate, chloroform and petroleum ether extracts of *T. wallichiana* against free radical mediated damages under *in vitro* conditions. The *in vitro* antioxidant activity was evaluated by the ability of the extracts to interact with DPPH, Hydroxyl (OH), PMS and microsomal lipid peroxidations. Reducing power ability and total phenolic content of the extracts were also evaluated. In addition to *in vitro* antioxidant potential the different extracts of this plant was also evaluated to determine its antimicrobial activities against different strains.

MATERIALS AND METHODS

Plant material collection and extraction

The Leaves of *Taxus wallichiana* was collected from higher reaches of Afarwat in the month of June- July, and were identified by the courtesy of centre of Plant Taxonomy, Department of Botany, University of Kashmir and authenticated by a Botanist Akhter Hussain Malik (Curator, centre for plant Taxonomy, University of Kashmir). The voucher specimen has been retained in the herbarium of Taxonomy department of Botany University of Kashmir for future reference under herbarium No: (KASH- bot/Ku/*PH*- 1943- MAS).

The authentically identified plant material (rhizome) was shade dried under room temperature at 30 ± 2°C. The dried plant material was grind into powder using mortar and pestle and sieved with a sieve of 0.3mm aperture size. The powder obtained was successively extracted in ethyl acetate, methanol, chloroform and petroleum ether by using Soxhlet extractor (60-80°C). The extracts were then concentrated with the help of rotary evaporator under reduced pressure and the solid extracts were stored in refrigerator for further use.

Total phenolics

The total phenolics in different extracts of *Taxus wallichiana* were determined by using Folin-Ciocalteau reagent according to the protocol of Chandler.[7] One hundred milligram of extracts was dissolved in 100 ml of methanolic/water (60:40, v/v, 0.3% HCl). To 1ml of sample, add 1ml of 95% ethanol, 5ml of distilled water and 0.5ml of 50% Folin-Ciocalteau reagent. The mixture was allowed to react for 5 minutes and 1ml of 5% sodium carbonate was added and mixed completely. After one hour incubation at room temperature, the absorbance of the solution at 725 nm was measured with spectrophotometer. Quantitation was based on the standard curve of gallic acid (10mg %), which was dissolved in methanol/water (60:40, v/v, 0.3% HCl). The concentration of polyphenols was expressed in terms of mg/100ml of sample.

DPPH radical scavenging activity

The DPPH assay was performed by using the method of Braca.[6] Various concentrations of plant extracts (100-1000µg/ml was added to 1ml of the 0.004% methanol solution of DPPH, and the mixture was vortexed vigorously. The tubes were then incubated at room temperature for 30 minutes in dark, and the absorbance was taken at 517nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. BHT, α-tocopherol and catchin were taken as known free radical scavengers. Percentage inhibition activity was calculated by using the formula.

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100.$$

Where A_0 was the absorbance of the control and A_1 was absorbance in the presence of *Taxus wallichiana* extracts/ known antioxidants.

Reducing power

The reducing power of *Taxus wallichiana* extracts were evaluated according to Oyaizu.[8] Different concentrations of the plant extracts were mixed with 2.5ml of 0.2 M phosphate buffer (pH 6.6), and 2.5ml of 1% potassium hexacyanoferrate. The mixture was incubated at 50°C for 20 minutes, 2.5ml of 10% TCA was added to the mixture and centrifuged at 3000rpm for 10 minutes. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%), and the absorbance was measured at 700nm. The percentage reduction of the sample was calculated by using formula:

$$\text{Reduction } (\%)=[1-(1-A_C/A_S)] \times 100.$$

Where, A_C is absorbance of standard at maximum concentration tested and A_S is absorbance of sample.

Lipid peroxidation (LPO)

LPO was induced and assayed in rat liver microsomes according to the method of Wright.[9] The reaction mixture in a total volume of 1.0ml, contained 0.58ml phosphate buffer (0.1M, pH 7.4), 0.2ml ferric nitrate (20 mM), 0.1ml of ascorbic acid (100mM) and 30mM H_2O_2, and was incubated at 37°C in a shaking water bath for 1h. The reaction was stopped by addition of 1.0ml TCA (10% w/v). Following which of 1.0ml of TBA (1%w/v) was added and all the tubes were placed in a boiling water bath for 20min. At the end, the tubes were shifted to ice bath and centrifuged at 5000rpm for 10min. The amount of malonaldehyde formed in each of the samples was assessed by measuring the optical density of the supernatant at 535nm against a reagent blank. The molar extinction coefficient for MDA was taken to be 1.56×10^5 M$^-$1 cm$^-$1.

Assessment of Hydroxyl radical scavenging property

Hydroxyl radical, generated from the Fe^{3+}- Ascorbate- H_2O_2 (Fenton reaction), was evaluated by degradation of deoxyribose that produced thiobarbituric acid reactive species (TBARS).[10] The reaction mixture containing 25mM deoxyribose, 10mM Ferric chloride, 100mM ascorbic acid, 2.8mM H_2O_2 in 10mM KH_2PO4 (pH 7.4) and various concentrations of *Taxus wallichiana* extracts. The reaction mixture was incubated at 37°C for 1h. Then one ml of 1% thiobarbituric acid and 1ml of 3% trichloroacetic acid were added and heated at 100°C for 20min. The TBARS was measured spectrophotometrically at 532nm. The results were expressed as percentage inhibition of deoxyribose oxidation, as determined by the following formula.

Percentage inhibition = [(A-B)/A] x 100.

Where A was the malondialdehyde produced by Fenton reaction treated alone, and B was the malondialdehyde produced in the presence of *Taxus wallichiana* extracts/ different known antioxidants.

Anti bacterial activity

Bacterial cultures namely, *Pseudomonas aureginosa*, *Pentoea agglerans, Staphylococcus aureus* and *Escherichia coli* were grown in nutrient agar media (HiMedia India) at 37°C. Each bacterial strain was transferred from stored slants at 4-5°C to 10 ml nutrient broth and cultivated overnight at 37°C. A preculture was prepared by transferring 1 ml of this culture to 9 ml nutrient broth and cultivated for 48 h. The cells were harvested from the broth by centrifugation at 1200g for 5 min, washed and suspended in saline.

Determination of minimum inhibitory concentrations (MICs)

The MICs of tested extracts were determined against different bacteria by agar dilution method as described by Negi.[11] One hundred microlitre (about 10^6 cfu/ml) of each bacterium to be tested was inoculated into the flasks under aseptic conditions. The media was then poured into sterilized petri plates and incubated at 37°C for 20-24 h. The colonies developed after incubation were counted and expressed as colony forming units per ml of culture (cfu/ml). The inhibitory effect was calculated according to the following formula:

% Inhibition (1 - T/C) X 100).

Where T is cfu/ml of test sample and C is cfu/ml of control. Experiments are of four replications per test organism and the values are Mean SD. The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the extract capable of inhibiting the complete growth of the bacterium being tested.[12]

RESULTS

Total phenolics

Phenols are very important plant constituents and because of the presence of hydroxyl groups they have a great scavenging ability. Phenolic compounds are therefore known to be powerful chain-breaking antioxidants. Different extracts of *Taxus Wallichiana* were screened for their total phenolic content. Total phenolic concentration was found to be highest for Petroleum ether extract (106.60mg%) followed by ethyl acetate extract (105.80mg%) and then for chloroform extract it was (103mg %) and lowest was observed for methanolic extract (19.15mg%) Fig. 1.

DPPH radical scavenging activity

The total phenolic content strongly correlated with the antioxidant activity. The

DPPH radical scavenging shown by the extracts is concentration dependent. With an increase in concentration, the radical scavenging activity increases in all the extracts of *Taxus Wallichiana* as depicted in Fig 2. It was observed that petroleum ether extract showed strong radical scavenging activity with percentage inhibition of 25.17% at a concentration of 100µg/mL, followed by methanolic extract which showed 19.63% while as for chloroform extract shows 4.63 % and for ethyl acetate extract is 1.30% respectively. However, at higher concentrations (700µg/mL) it shows 94.81 for petroleum ether extract followed by methanolic extract which showed 72.78% and for ethyl acetate extract and chloroform extract the percentage inhibition of 72.45 and 68.30% was observed. While as for α-tocopherol (standard) percentage inhibitions were found to be 34.01% and 90.49% at 100 µg/mL and 700 µg/mL.

Reducing power

As illustrated in Figure 3, Fe^{3+} was transformed to Fe^{2+} in the presence of *Taxus Wallichiana* extracts. The reducing power of the extracts increased with increasing in concentration. At 50 µg/mL, the absorbance of the plant extracts were 0.075, 0.128, 0.256 and 0.084 respectively, while at the higher concentration 350 µg/mL, the absorbance of all the increased to 0.608, 0.332, 0.501 and 0.354 respectively.

Lipid peroxidation

In the present study the inhibition of lipid peroxidation induced by H_2O_2/ ferric nitrate/ascorbic acid by the extracts of *Taxus Wallichiana* was done under *in vitro* situations. It was observed that the petroleum ether shows higher percentage inhibition (97.63%) followed by methanolic extract with percentage inhibition (96.56%). However, ethyl acetate and chloroform extract shows percentage inhibition of 78.94% and 52.11% respectively Fig 4.

The antioxidant activity of *Taxus Wallichiana* extracts on Fe^{++}/ ascorbic acid model systems. This model system contained Fe^{++}/ ascorbic acid as oxidizing agent to initate lipid peroxidation in rat liver microsomes. The MDA forms a pink cromogen with TBA that absorbs at 535 nm. At concentration of 300 µg/mL around 97.73% inhibition in LPO was observed for methanolic extracts followed by petroleum ether extract 94.77%, ethyl acetate 90.10% and chloroform extract 86.75% respectively. However in presence of known antioxidant α-tocopherol, LPO was inhibited by 87.25% at the same concentration.

DNA damage

The Fenton reaction was used to induce deoxyribose sugar damage in the calf thymus DNA following 24-hour incubation and the damage was assessed by measuring the absorbance of the formation of TBARS at 535 nm. The percentage of hydroxyl radical scavenging activity increased with the increasing concentration of extracts and known antioxidants. The maximum scavenging activity was observed with petroleum ether extract 98.54% at concentration of 300 µg/mL followed by methnolic extract (93.05%), chloroform extract 89.79 % and ethyl acetate extract 84.51% respectively Fig 5. However the scavenging activity of α-tocopherol was found to be 90.67% at 300µg/mL. Thus the scavenging activity was found in following order.

Petroleum ether > methanol > chloroform > ethyl acetate.

Antibacterial activity

The methanolic extract of *Taxus wallichiana* showed variable zone of inhibition (12–16 mm) for all the bacteria tested. Ethyl acetate extract showed zone of

inhibition from ranging from (16-19 mm), chloroform showed from (18-20mm) and for petroleum ether extract zone of inhibition ranges from 16-20 mm). These extracts were tested against different bacterial strains namely, *Staphylococcus auerus, Pseudomonas aureginosa, E. coli* and *Pentoea agglerans* as shown in the Table 1. All the extracts mentioned above were compared with the standard streptomycin which showed 24 mm zone of inhibition for *Staphylococcus auerus*, 26mm for *Pseudomonas aureginosa*, 24 mm for *E. coli* and 28mm for *Pentoea agglerans*.

DISCUSSION

There is increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and therefore there is increasing interest in the protective biochemical functions of natural antioxidants contained in spices, herbs and medicinal plants.[13,14] Phenolics are the most widespread secondary metabolite in the plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidants in terms of their ability to act as both efficient radical scavengers and metal chelators. It has been reported that the antioxidant activity of phenol is mainly due to its redox properties, hydrogen donors and singlet oxygen quenchers.[15] Therefore, in the present study, total phenolic content present in the plant extract was estimated using a modified Folin Ciocalteau method.

DPPH radical scavenging activity

The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple color). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, for example a free radical scavenging antioxidant, the absorption strength is decreased and results in decolonization (yellow color) with respect to the number of electrons captured;[16] the more the decolonization the greater the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug. The percentage DPPH scavenging activities of *Taxus wallichiana* extracts are summarized in Fig 2. Dose-dependent DPPH radical-scavenging activity was exhibited at all concentrations tested. The results suggest that the medicinal property exhibited by the plant might be due to the radical scavenging activity. Our results concur with earlier investigations that found different extracts of plants such as *Pterocarpus santalinus* exhibit significant DPPH radical inhibition.[17]

Reducing power

Findings from different studies have showed that the electron donation capacity reflecting the reducing power of bioactive compounds is associated with antioxidant activity.[18] Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of Fe^{3+}/ferricyanide complex the ferrous form. Therefore Fe^{2+} can be monitored by measuring the form of Perl's Prussian blue at 700 nm.[19] Our results suggest that different extracts of *Taxus wallichiana* plays an important role in scavenging free radicals and the activity is increased with the increasing concentration of the plant extracts. Similar results were reported by Noriham, [20] who demonstrated antioxidative activity on *Pimpinella anisum* seed extracts.

Hydroxyl radical scavenging activity

The activities of the extracts on the hydroxyl radical are shown in Fig. 5. The hydroxyl radical is a highly reactive oxygen-centered radical that is formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecules and is known to be capable of abstracting hydrogen atoms from membrane lipids and can bring about the peroxidic reaction of lipids.[21,22,] *Taxus wallichiana* exhibited concentration-dependent scavenging activity against hydroxyl radicals generated in a Fenton reaction system.

Lipid peroxidation

In addition to free radical scavenging activity, the extracts were evaluated for their ability to protect biomembrane from oxidative damage. Initiation of the lipid peroxidation by ferric nitrate, ascorbic acid and H_2O_2 takes place through the formation of OH radical by Fenton's reaction. *Taxus wallichiana* extracts inhibited Fe^{3+}/ ascorbate-induced lipid peroxidation in a dose-dependent manner as given in Fig 4. The inhibition could be caused by the absence of ferryl-perferryl complex, or by scavenging the OH radical or the superoxide radicals, or by changing the Fe^{3+}/Fe^{2+}, or by reducing the rate of conversion of ferrous to ferric iron or by chelating the iron itself. The result shows that *Taxus wallichiana* extracts have the capacity to prevent oxidative deterioration of microsomal lipid peroxidation in a dose-dependent manner. The beneficial effect of *Taxus wallichiana* on lipid peroxidation is attributed to its phenolic content. Similar results were observed by Ganie *et al*.[23]

Antibacterial activity

Four bacterial strains *E. coli, pseudomonas Aureginosa, pentoea agglomerans* and *Staphylococcus aureus* were tested for their susceptibility to *Taxus Wallichiana* methanol extract, ethyl acetate extract, chloroform extract, petroleum ether extract respectively. The standard antibacterial compound was streptomycin and was used at concentration of 30 µg/disc. The extracts were tested at concentration of 50 µg/ml and the maximum zone of inhibition was observed with chloroform and petroleum ether extracts. Similar results were observed by Sanjana Safi *et al*.[24].

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Table 1. Anti-bacterial activity of different extracts of *Taxus wallichiana*

Extracts	Bacterial Strains (Zone of Inhibition)			
	S_a	P_a	E_c	P_a
Methanolic extract	14 ± 1.01	12 ± 0.32	12 ± 1.34	13 ± 0.65
Ethyl acetate extract	18 ± 0.94	19 ± 1.01	16 ± 1.15	19 ± 0.87
Chloroform extract	18 ± 0.89	18 ± 0.15	19 ± 0.72	20 ± 0.59
Petroleum ether extract	16 ± 1.03	20 ± 0.23	18 ± 0.56	19 ± 1.01
Streptomycin	24 ± 0.96	26 ± 0.79	24 ± 1.05	28 ± 1.56

S_a: *Staphylococcus aureus*; **P_a:** *Pseudomonas aeruginosa;* **E_c:** *E. coli*; **P_a:** *Pentoea Agglomerans*.

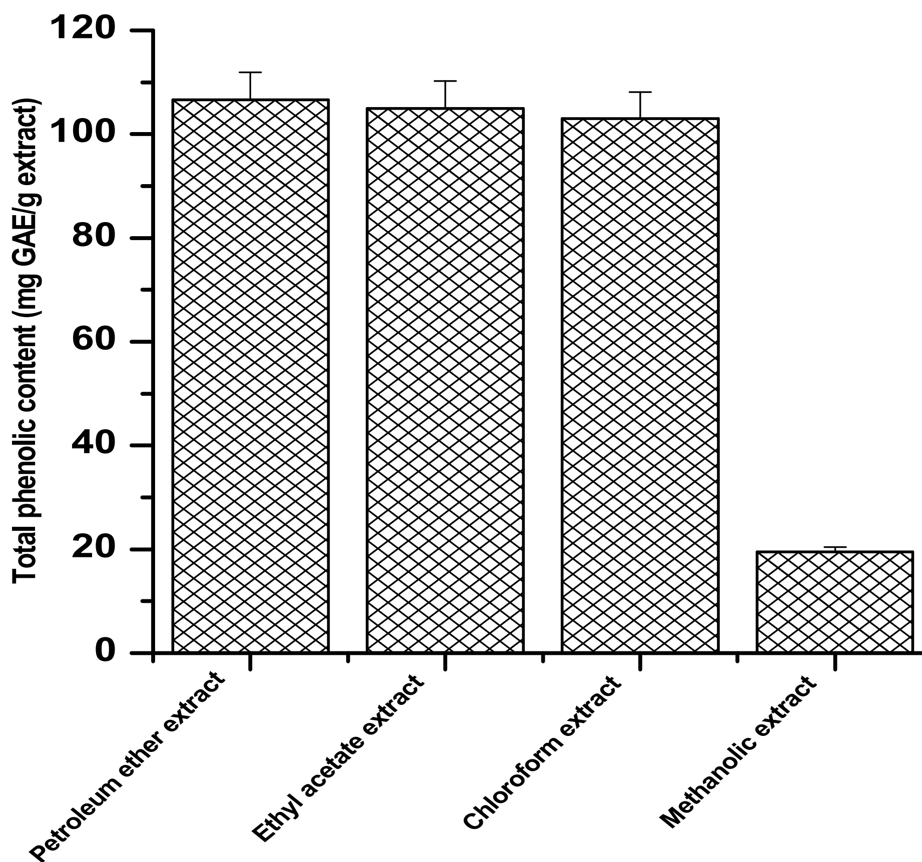

Figure 1. Total phenolic content of different extracts of *Taxus wallichiana*. The results represent mean ± SD of 3 separate experiments

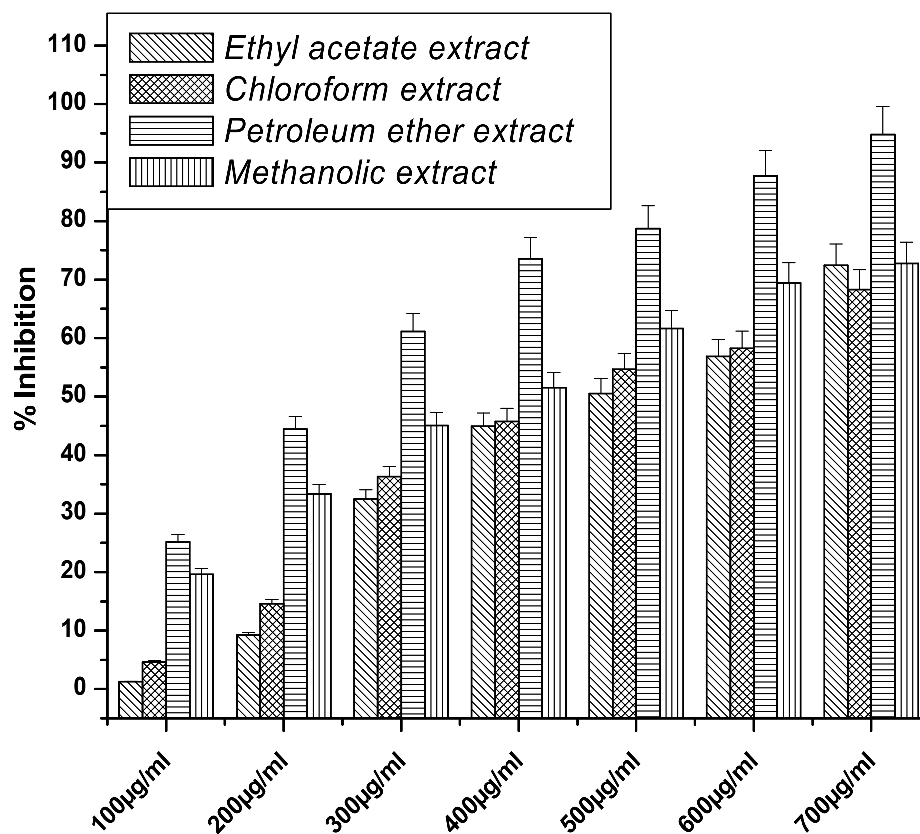

Figure 2. The effect of different extracts of *Taxus wallichiana* on DPPH radical scavenging activity. The results represent mean ± SD of 3 separate experiments. Absorbance at 517 nm

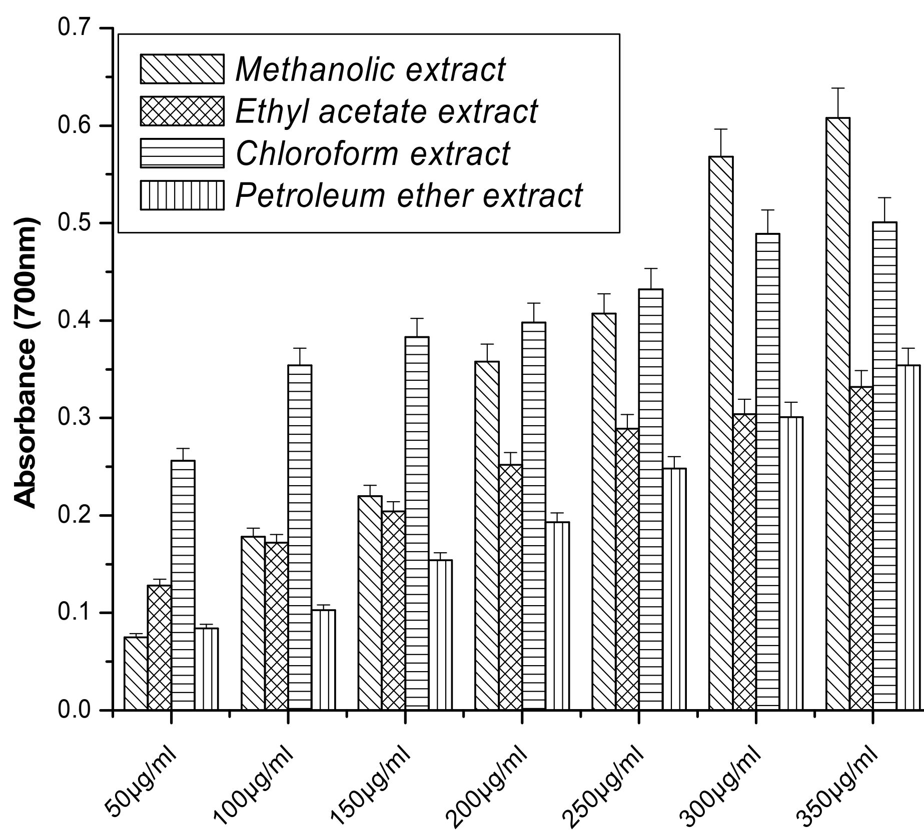

Figure 3. The effect of different extracts of *Taxus wallichiana* on reducing power. The absorbance at 700nm was measured in triplicate. The results represent mean ± SD of 3 separate experiments

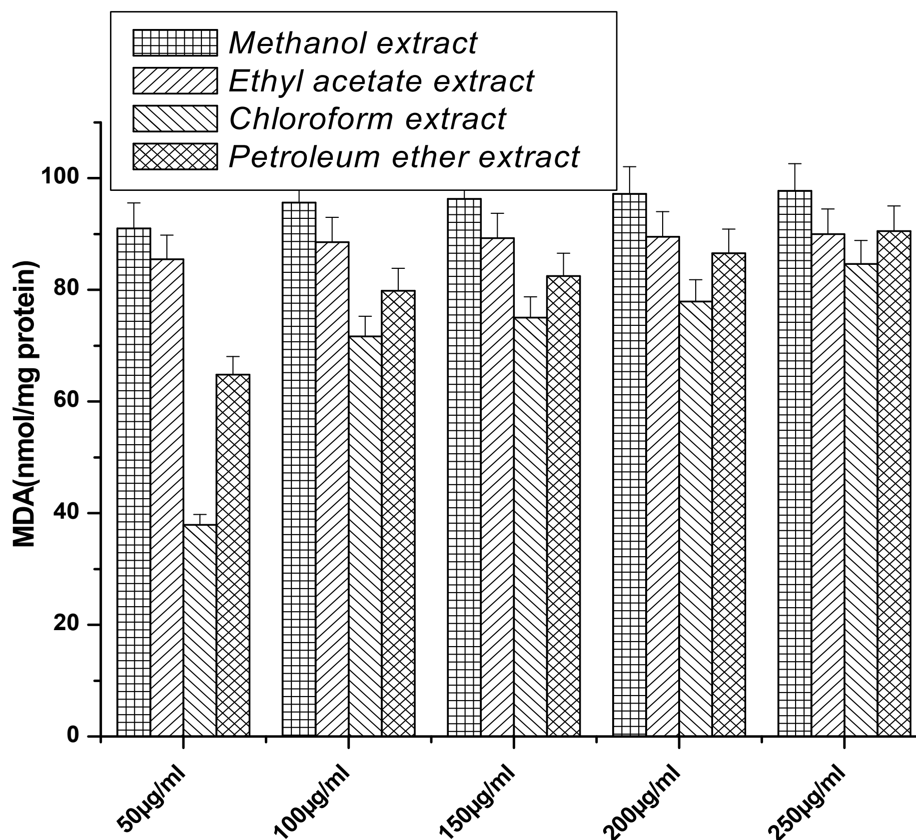

Figure 4. The effect of different extracts of *Taxus wallichiana* on microsomal lipid peroxidation radical scavenging activity. The results represent mean ± SD of 3 separate experiments. Absorbance at 532 nm

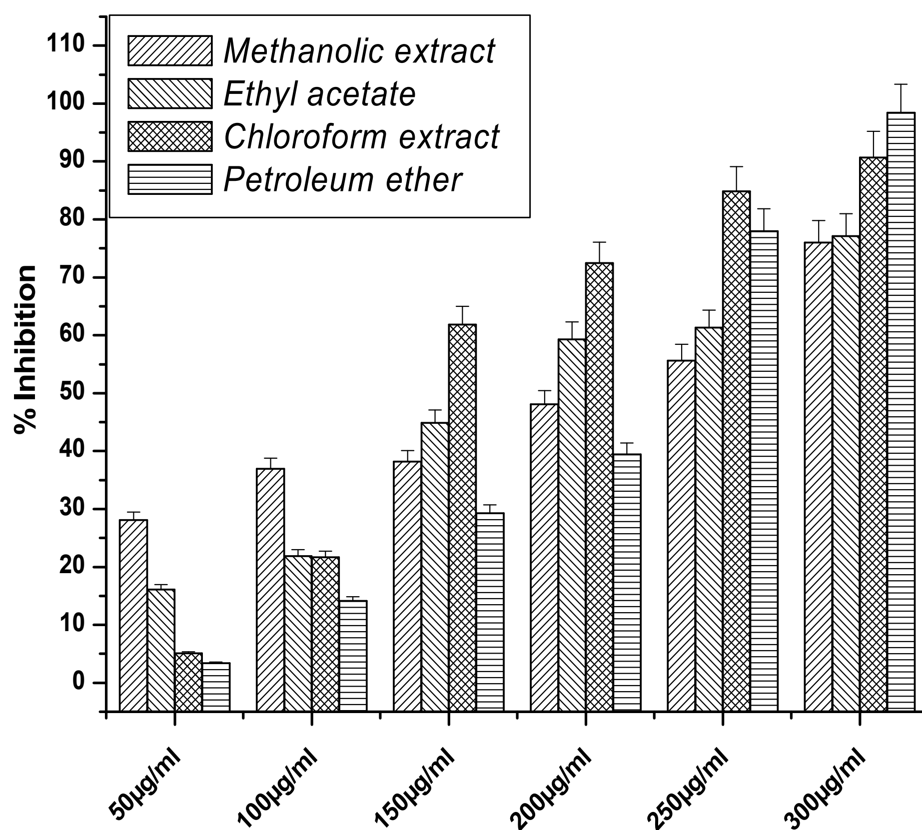

Figure 5. The effect of different extracts of *Taxus wallichiana* on hydroxyl radical scavenging activity. The results represent mean ± SD of 3 separate experiments. Absorbance at 532 nm