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Biological Activities of Stem, Leaves and Essential Oil of *Cedrus deodara* from District Poonch, Rawalakot Azad Kashmir, Pakistan

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ABSTRACT

Cedrus deodara (Roxb. ex D. Don) Loudon, is a high value medicinal plant found in flora of Poonch part of Himalayan region. The present study was conducted to determine and compare the antioxidant activity, quantification of phenolics and flavonoids, chelating ability, biofilm inhibition, thrombolytic activity and cytotoxicity of the stem, leaves of crudes extracts and essential oil. It can be claimed that that all parts of *C. deodara* including its essential oil is a rich source of phytochemicals that exhibited high quantity of phenolics ranged from (49.76±0.22 GAE to 60.36±0.44GAE mg/g) and flavonoids ranged from (4.78±0.61mg/g to 6.62±0.45 mg/g)but also exhibited antioxidant, metal chelating agent ability, antibacterial potential ranged from (35.59±0.50 to 61.61±0.61%)and thrombolytic activities ranged from (22.86±0.7 to 32. 64±0.5 %)with minimal toxicity ranged from (0.40±0.35 to 3.73±0.23%). Further studies are required to determine the bioactive compounds and bioactivity of plant extracts and fractions.

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Introduction

Plants are important to humans' beings not only environmentally, economically and industrially but also spiritually, historically and aesthetically. They sustain human life through numerable tangible and intangible benefits. Now a day at least 120 different types of important drugs containing chemical substances are identified. These chemical substances are derived from plants. These important drugs are used in one or more countries. Some of the drugs are copies of naturally obtained substance. Most of them are simple synthetic modification (Taylor, 2000).

Medicines including high anti biotic and normal syrups were never found a century ago. However, people have been taking different medicines for illness and other health problems. Herbal medicines power cannot be overlooked as prior to all the modern medicines. Even deadly diseases are cured by these herbal medicines. A definite physiologic action on human body is produced by some chemical substances in plants. Alkaloids, flavonoids, tannins and phenolic compounds are most important bioactive compounds (Duraipandiyan et al., 2006). Several authors have reported the *in vitro* antioxidant activities of plants (Selamoglu et al., 2017; Maria et al., 2014; Dusgun et al., 2015; Selamoglu et al., 2016). Different dietary approaches have been used for the management of free radicals and for the treatment of different health disorders (Badr et al., 2017; Sevindik et al., 2017). Salmas et al. (2017) has reported the protective effect of propolis, caffeic acid and pollen on renal injury in hypertensive rats.

In last fifteen years various aspects of *C. deodara* were explored. In the treatment of remittent and intermittent fevers; diarrhea and dysentery the bark of cedar wood proves to be a good remedy (Nadkarni, 1984). In ulcers treatment the powder of *C. deodara* is used. Cedar wood possesses anti-fungal properties (Nadkarni, 1984). The outer bark and stem of the tree is used to astringent, carminative and antispasmodic and is found in biomedical action of the plant. The digestive function promoted by the Ayurvedic functions of the plant it also removes toxins from the bowel and alleviates coughing. This plant also cures skin disorders, such as eczema. Essential oil of *C. deodara* wood chip was investigated against the diamond back moth which emphasized the study larvicidal activity of *C. deodara*. Ayurvedic medical practice has been carried out since ancient days it was carried out by wood of *C. deodara* (Nadkarni, 1984). *C. deodara* is also known as Himalayan Cedar.Some activities against house flies and stored pests are found in its essential oil. Flavonoid such as taxifolin, quercetin and

saponins are the major components of *C. deodara*. Phytosterols of the plant are very effective on hyperlipidemia (Singh et al., 1984). *C. deodara* also showed a significant anti-spasmodic activity when a 50% ethanol extract of wood was taken (Vikas et al., 2013). Literature did not quote any studies carried out on the properties of *C. deodara* from Azad Kashmir, Pakistan. Therefore, the objective of present research was to uncover the biological and antioxidant properties of *C. deodara* species for their possible use in food and pharmaceuticals.

Material and Methods

Collection of Plant Material

The selection of plant has been on the basis of ethno pharmacologic information and intensive review. Leaves and stem of *C. deodara* have been collected from the high altitude of the Azad Jammu and Kashmir (AJK). It is located at Latitude 33°51'32.18" N, Longitude 73°45'34.93" E and an Elevation of 5374 feet. Different parts of the plant were collected and identified by a Taxonomist at University of Poonch, Rawalakot AK Pakistan.

Extraction of Essential Oils

For the extraction the volatile compound from medicinal plant material many analytical methods were used. To extract essential oils hydro distillation method was used. This method is cost effective and simple (Hashemi *et al.,* 2007).

Hydro distillation method

C. deodara stem and leaves were cut into small pieces. Then these small pieces are air dried in a shady place. These pieces are then immersed in water to carry out hydro distillation (3-5 hours). Clevenger type hydro distillation apparatus was used. Along with the water vapors essential oils were evaporated and condensed in condenser. From aqueous layer the distillate was separated and dried over anhydrous sodium sulphate (Irshad et al., 2012).

DPPH Radical Scavenging Assay

Scavenging of the stable DPPH radical (ethanolic solution of 0.25 mM) was assayed *in vitro* (Hatano et al., 1988) and the absorbance was measured at 517 nm. Percent inhibition was calculated from the control. Ascorbic acid was used as a standard in DPPH assay.

Determination of Phenolics

The total phenolic content of each plant extract was determined by using the Folin Ciocalteau method (Singleton et al., 1999). Aqueous extracts (0.5 mL) were added to 2.5 mL of 10% Folin ciocalteau's reagent (v/v) and the content was mixed. After 5 minutes 2 mL of 7.5% sodium carbonate was added in the mixture. The mixture in the test tube was mixed and allowed for incubation for 40 minutes at 45°C. The absorbance was measured at 765 nm using a spectrophotometer. Gallic acid will be used as a standard phenol. The concentration of total phenolic is expressed as milligram of Gallic acid/g of mixture. All the determinations were carried out in triplicate.

Determination of Flavonoids

Total flavonoid content was determined using aluminium chloride. The total flavonoid content as quercetin equivalents was determined (Kosalecet al., 2004). Quercetin was used to construct calibration curves. Standard solutions or extracts (0.5 mL) was mixed with 1.5 mL of 95% ethanol (v/v), 0.1 mL of 10% aluminium chloride (w/v), 0.1 mL of 1 mol/L sodium acetate and 2.8 mL of water. The volume of 10% aluminium chloride will be replaced using the same volume of distilled water for use as a blank.

Metal Chelating Activity

The iron chelating ability of the extractwas determined using a modified method (Puntel et al., 2005).

Biofilm inhibition assay (By microtitre-plate method): Bacterial strain *Escherichia coli* was obtained and cultivated at 37°C and maintained on nutrient agar slant at 4°C.The wells of a sterile 96-well flat bottomed plastic tissue culture plate were filled with 100 uL of nutrient broth (Oxford, UK), 100 uL of testing sample and 20 uL of bacterial suspension inoculated. Nutrient broth was taken as negative control only, while a standard chemical synthetic drug Rifampicin was taken as positive control. At 37°C for 24 hours these plates were covered and incubated aerobically. With 220 uL of sterile phosphate buffer the content of each well was washed thrice. In order to remove all non-adherent bacteria, the plates were vigorously shaken. With 220 microlitres of 99% methanol per well, the remaining attached bacteria were fixed and after 15 min plates were emptied and left to dry. Then, plates were stained for 5 min with 220 mL of 50% crystal violet per well. By placing the plates under running tap water, the excess stain was rinsed off. After the plates were dried after that with 220µLof 33% (v/v) glacial acetic acid per well the bounded adherent than air dried dye was re solubilized. At 630 nm using microplate reader, the OD of each well was measured (BioTek, USA). All the tests were carried thrice times against both selected bacterial strain and the results were averaged (Shahid et al., 2015).

The bacterial growth inhibition (INH%) was calculated as follows:

$$INH\% = 100 - (OD_{630\ sample} \times 100) / OD_{630\ control}$$

Thrombolytic Activity

Five different pre-weighed sterile micro centrifuge tube were used to take 5 mL of venous blood from each volunteer and permitted to incubate at 37°C for 40 min. From each centrifuge tube, the fluid was completely released after clot formation and the clot weight was determined by subtracting weight of clot containing tube from weight of tube alone. To the centrifuge tube s 100uL of each sample were added separately, streptokinase and distilled water (100uL) were taken as positive and negative control respectively. For almost an hour at room temperature these test tubes were incubated and observed for clot lysis. The released fluid was discarded after incubation and tubes were again weighed to assess the differences in weight after clot disruption ((Prasad et al., 2007)). By following formula, the percentage of clot lysis was determined.

Percent of clot lysis = (wt of released clot /clot wt) × 100

Cytotoxicity (Hemolytic Activity): Fresh heparinized bovine blood (3mL) was collected from Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. For 5 min at 1000×g the blood was centrifuged, and the plasma obtained was discarded while the cells were washed thrice with 5mL chilled (40°C) sterile isotonic Phosphate-buffered saline at pH 7.4. For each assay erythrocytes were maintained 108 cells per mL. Each compound of 100 uL was mixed with human (108cells/mL) separately. At room temperature these samples were incubated and agitated satisfactorily after 10 min. These samples were placed over an ice berg immediately after incubation followed by centrifugation for 5 min at 1000×g. From each tube the supernatant of 100uL was taken and diluted 10 times with chilled Phosphate-buffered saline (PBS). PBS was taken negative control while the Triton X-100 was taken as positive control and pass through the same process at 760 nm the absorbance was noted using Quant (BIiotech, USA). The calculation was made at the end for percent RBCs lysis for each sample (Shabbir et al., 2015)

Data Analysis

The results were expressed as means± SD. The data were analyzed by one-way ANOVA and different group means were compared by applying Duncan's multiple range test (DMRT); P<0.05 was considered significant in all cases. The software package, Statistica was used for statistical analysis

Results

DPPH Radical Scavenging Activity

The entire extracts under study were analysed by free radical scavenging activity by the DPPH method. The antioxidant activity of extracts was found to dependent upon the concentration which is shown in the Table 1. There is the difference in the activity of the different parts of plants. It is cleared from the results that all the extracts have significant antioxidant potential. The strong scavenging activity was shown by the leaves of *C. deodara*. Total antioxidant activity of the leave extracts of the *C. deodara* is lower than the stem but more than other plants extracts. Leaves extract showed IC50 value of 28.16.

Phenolic and Flavonoids Content

In the present study the phenolic compounds in the different parts of the plant *C. deodara* were analysed. The total phenolic content was taken as milligram of gallic acid per gram of the extracts. The results are shown in Table 2. The highest phenolic contents were detected in the essential oils of leaves and wood of *C. deodara* (49.76±0.22 GAE mg/g) and (60.36±0.44GAE mg/g) respectively among all the tested results. The stemof these plants were found to be comparatively higher in phenolic contents than the leaves. On the other hand flavonoid content ranged from 4.78±0.61 mg/g to 6.62±0.45mg/g for stem and leaves in methanolic extract respectively, while essential oil have 6.62±0.45 mg/g of flavonoid content (Table 2). It was also concluded that antioxidant activity has the strong relation with the total phenolic (R^2=0.72) and flavonoids (R^2=0.816) contents.

Metal Chelation

The results showed that the *C. deodara* was found to show the high chelating ability (Table 3). Increasing the concentration of the extracts chelating ability also increased. Our results showed that the maximum chelating ability is shown by the essential oil than the normal extract both in stem and leaves of the *C. deodara*.

Biofilm Inhibition by Microtitre-Plate Method

C. deodara methanlic extracts of leaves and stem and their respective oil were subjected for biofilm inhibitory assay shown in Table 4. Rifampicin was taken as standard inhibitory drug which showed highest percentage inhibitory effect of 95.08±0.65. The percentage inhibition ranged from 35.59± 0.50 to 61.61 ± 0.61 for *C. deodara* leaves methanolic extract to stem essential oil, while the methanolic extract of stem and essential oil of the leaves also showed considerable percentage inhibition (42.18±0.71% and 45.74±0.54%) respectively. The ascending order of percentage inhibition by taking Rifampicin as standard is as CdL (leaves) <CdM (stem) <CdLEO (leaf oil) <CdSEO (stem oil) <Rafampicin.

Table 1 Percentage DPPH scavenging activity of the leave and stem extracts of the *Cedrus deodara*

Concentration of extracts µg/ml	Percentage scavenging by leaves extracts	Percentage scavenging by stem extracts
25	44.38±0.36	54.16±0.41
50	34.17±0.085	61.48±0.43
75	29.88±0.1	66.48±0.45
100	22.83±0.13	69.16±0.86
200	15.13±0.514	70.18± 0.52

Table 2 Total Phenolic and flavonoids contents of methanolic extracts of the plants, stem and leaves

Plants	Phenolic content (mg GAE/g extract)	Flavonoids content (mg /g extract)
Cd LME	23.95±0.48^a	5.56±0.55^a
Cd SME	28.16±0.55^b	4.78±0.61^b
Cd LEO	49.76±0.22^c	6.62±0.45^c
Cd SEO	60.36±0.44^d	6.48±,0.44^d

Values in Table which share different letters are significantly (P<0.05) different from each other by DMRT.CD= *Cedrus deodara* LME=leaves methanolic extract, SME=stem methanolic extract LEO=Leave essential oil, SEO=Stem essential oil

Table 3 Metal Chelation by methanolic extracts of *Cedrus deodara* leaves and stems and their essential oils

Concentration of extracts µg/ml	Percentage Chelation by leaves extracts	Percentage chelation by stem extracts	Percentage chelation by leaves essential oil	Percentage chelation by stem essential oil
25	28.50±0.53	34.72±0.72	37.59±0.65	34.64±0.83
50	42.95±0.6	46.77±0.75	34.88±0.6	38.91±0.85
75	46.68±0.58	52.82±0.59	48.84±0.6	46.58±0.41
100	58.76±0.5	61.13±0.51	62.86±0.8	67.68±0.89

Table 4 Biofilm inhibition assay by microtitre-plate method of different parts of *Cedrus deodara*

Sample name	Biofilm Inhibition against *E.coli* (%)
Cd leaves	35.59±0.50[a]
Cd Stem	42.18±0.71[b]
Cd LEO	45.74±0.54[c]
Cd SEO	61.61±0.61[d]
Rifampicin	95.08±0.65[e]

CD= *Cedrus deodara* LEO= Leave essential oil, SEO=Stem essential oil, Values in Table which share different letters are significantly (P<0.05) different from each other by DMRT.

Table 5 Thrombolytic activities of different parts of *Cedrus deodara*

Sample Name	Thrombolytic Activity (%)
Cd Leaves	23.79±0.5[a]
Cd Stem	22.86±0.7[b]
Cd LEO	32.64±0.5[c]
Cd STO	30.95±1.24[d]
Streptokinase	89.85±0.36[e]
Distilled Water	2.59±0.52[f]

PBS =Phosphate Buffer Saline, CD= *Cedrus deodara* LEO= Leave essential oil, STO=Stem essential oil, Values in Table which share different letters are significantly (P<0.05) different from each other by DMRT.

Table 6 Cytotoxicity by hemolytic activity of different parts of *Cedrus deodara*

Sample Name	Cytotoxicity (%) by Hemolytic activity
Cd Leaves	0.81 ±0.09[a]
Cd Stem	3.73±0.23[b]
Cd LEO	0.406±0.35[c]
Cd SEO	1.443± 0.47[d]
Trition-X 100	97.70±0.50[e]
PBS	1.61±0.51[f]

Values in Table which share different letters are significantly (P<0.05) different from each other by DMRT.

Thrombolytic Activity

The ability to breakdown the blood clot formed was detected for all the four subjects while taking streptokinase and distilled water as positive and negative control (89.85±0.36 and 2.59±0.52(%) thrombolytic activity respectively (Table 5). The percentage thrombolytic ability of *C. deodara* ranged from 22.86±0.7 to 32.64±0.5 (%) thrombolytic activity for methanolic extract of stem to essential oil of leaves. The essential oil of stem part and leaves only also showed considerable ability to lyse the blood clot which were 30.95±1.24 and 23.79±0.5% respectively. For the first time these activities were determined for *C. deodara* which may lead to isolation of some useful thrombolytic drug.

Cytotoxicity of Extracts

For positive control of experiment Triton X-100 (0.1% v/v) was taken (97.70±0.50 (%) Cytotoxicity). The phosphate buffer saline (PBS) was taken as negative control (1.61±0.51 (%). Cytotoxicity (%) by hemolytic activities for various parts were analysed (Table 6). The percentage cytotoxicity ranged from 0.406±0.35 to 3.73±0.23 (%) for essential oil of leaves of *C.deodara* to stem of the *Cedrus deodara.* Leaves and its essential oil also showed minimal cytotoxicity 0.81±0.09 and 1.443±0.47 (%) respectively. As a whole stem contains higher cytotoxicity than leaves parts.

Discussion

There is no significant correlation between the antioxidant activity and the phenolic compounds in some of the extracts. The antioxidant activity is not only related with the phenolic content but also have the relation with the other free radical scavenges (Ozgen et al., 2006). Here the antioxidant activity showed a strong correlation with phenolic and flavonoid content. Ozgen et al., (2016) have reported the antioxidant activities of flavonoids such as Quercetin. Polyphenolic compouns have several health protective properties (Selamoglu, 2017, Sevindik et al., 2017). Phenolic compounds are the important class of the antioxidants and are termed as free radical terminators. Flavonoids exhibit a broad range of activities including the anticancer activities (Selamoglu, 2017).

The total phenolic content was estimated by the Folin Ciocalteo reagent and expressed as gallic acid

equivalents. The present study revealed that the stem of the *C. deodara* have high phenolic content than the leaves. They play important role in the defence mechanism against endogenous and exogenous free radicals (Shad et al.,2013). Total antioxidant activity of the leave extracts of the *C. deodara* is lower than the stem but more than other plants extracts. One more study revealed that methanolic extracts of the *C. deodara* have more antioxidant potential than their aqueous extracts. Antioxidant potential is related with the reducing power, scavenging power and total phenolic contents (Ozgen et al., 2006). Several authors have reported the health beneficial effect of medicinal plants (Amin et al., 2018; Amin et al., 2017).

The *C. deodara* in the present study was found to show the high chelating ability against iron. Increasing the concentration of the extracts chelating ability also increased. Our results showed that the maximum chelating ability is shown by the essential oil than the normal extract both in stem and leaves of the *C. deodara*. The ability to chelate the metal ions is due to the presence of polyphenols and flavonoids. It was found that *M. ravens* extracts interfere with the most active metal iron and shown the chelation activity of 80 percent. Alzheimer's syndrome which is a neurological disorder and many other diseases caused by the ferrous ions. It is chelated and reduces the formation of the ROS (Ebrahimzadeh et al., 2008). For the toxic metals chelation many types of metal chelators are available. But it is very difficult the selection of the chelator as an ideal chelator. Metal chelators should be specific and proper administration (Flora et al., 2010). Natural plants contain the phytochemical such as phenols and flavonoid which are responsible for the chelation of the metals and also prevent the lipid peroxidation (Jayaprakasha and Patil, 2007).

On testing bases only one type of bacterium was selected to test the various methanolic extracts (stem and leaves) and its essential oil was subjected to biofilm inhibition assay. On the basis of results Table 4 showed that exhibited a significant antibacterial effect against Gram negative bacteria called *E.coli* particularly. As compare to planktonic form bacteria in biofilm have been shown to be much more resistant to antibiotics (Lewis, 2001). The success of natural compounds in inhibiting cell attachment is a promising tool for reducing microbial colonization on various surfaces (Bavington and Page, 2005). How to prevent microbial infections is a interesting approach which made the large implications of anti-adhesion agents (Ofek et al., 2003; Steinberg et al., 2004).

In order to find a natural compound able to inhibit and prevent microbial biofilm formation, we tested the effect of methanolic extracts and oil on biofilm *E. coli* strains. Crystal violet assay showed that methanolic extracts and their oil reduce the number of adherent bacteria.

Our results revealed that various extracts of *C. deodara* efficiently kills *E.coli* suspension and prevent biofilm formation. By microscopic analysis of strains grown on the surface of glass slides covers the effect on biofilm formation was confirmed. We observed a biofilm inhibition when we inoculated the strain with a particular concentration.

The result of this work showed that the methanolic extracts (leaves and stem) and their respective oil had mild to moderate to thrombolytic activity (Table 5). The results of clot lysis were indicated that test samples showed different thrombolytic activity. The significant average percent of clot lysis (32.64±0.53 and 30.95±1.24) of leaves and stem essential oil of *C. deodara* was found. Therefore, it is evident that the essential oil sample and methanolic extract were thrombolytic. As a positive control streptokinase (SK), a known thrombolytic drug is used while distilled water as negative control (Prasad et al., 2007). The comparison of positive control with negative control clearly confirmed that clot dissolution does not occur when water was added to the clot. By comparing with this positive and negative control, a significant thrombolytic activity was observed after treating the clots with essential oil and methanolic leaves and stem extracts.

Drugs either purified or crude must be screened for safe usage that's the reason why cytotoxicity assays are widely used by the pharmaceutical industries. Before investing in their development as a pharmaceutical drug, cytotoxic compounds can either look for researchers, if they are interested in developing a therapeutic that targets rapidly dividing cancer cells, for instance; or they can screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects. To kill rapidly growing cancerous cells is solely depends upon the cytotoxicity ability and also depends upon the concentration of compound(s) in the sample (Svensson et al., 2005). Cytotoxic results indicated that test samples have low toxicity and they can be recommended for human and animal consumption.

Conclusions

it can be claimed that that all parts of *C.deodara* including its essential oil is a rich source of phytochemicals that exhibited high quantity of phenolics and flavonoids but also exhibited antioxidant, metal chelating agent, antibacterial and thrombolytic activities with minimal toxicity. Hence, further studies are suggested to be undertaken to pin point the exact compounds and to better, understand its actions scientifically.

Conflict of Interest Statement

The authors declare no conflict of interest.

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