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INVESTIGATION OF ANTI-BACTERIAL ACTIVITY OF *CUPRESSUS TORULOSA* LEAF EXTRACT

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ABSTRACT

The aim of present study was to investigate antibacterial activity of *Cupressus torulosa* leaf extract. The dried material was extracted by using 3 different solvents namely methanol, hexane and ethyl actetate. Plant extracts were subjected to phytochemical screening which showed the presence of Alkaloid, Glycoside, tannins, carbohydrate and amino acids. In test for antibacterial activity against four microorganisms (*Streptococcus pyogens, Acinetobacter calcoaceticus, Escherechia coli, Staphylococcus aureus*), methanol extracts showed highest antibacterial activity whereas hexane extracts showed less activity. This activity was assessed by measuring the diameter of ZOI of four concentrations used. ZOI was larger in 200ug/ml and least in 25ug/ml concentration of extracts. The largest zone of inhibition (15mm) was obtained with *S.pyogens in* methanol extract. When hexane extract was used, largest ZOI was 10mm against *acinetobacter calcoaceticus.* When ethyl acetate extracts was used, largest ZOI was 12mm against staphylococcus aureus. Among four pathogenic bacteria used all were found susceptible (ZOI<10) to plant extracts at different concentrations in methanol extracts. In ethyl acetate extracts, *S.aureus* and *E. coli* were found less susceptible whereas in hexane extracts, *Acinetobacter calcoaceticus* was found less susceptible.

INTRODUCTION

Plants have been used for medicinal purposes from the beginning of human civilization. Antiquities of medicinal herbs are to be traced back as far as the Vedic period, 4500 B.C to 1600 B.C. Ayurveda, the science of life in Hinduism, remains to be the main source of medicinal knowledge and skill in most part of South Asia including Nepal. Vaidhyas and Kabirajs followed Ayurveda in their pursuit of knowledge and practice in medicine.[1] After a period of decline of these traditional system "green medicine": are once again back to the center stage of our health programs.[2]

More than 35,000 plants species are being used in various human culture around the world for medicinal purposes.[3] World Health Organization has estimated that 80% of the world population relies chiefly on traditional medicine. A major part of traditional therapies involve the use of plant extracts or their active constituents.[4]

Cupressus variety is basically utilized as diuretic, stimulant, mitigating and disinfectant for basic cold and twisted recuperating in society prescriptions. The compound investigation of basic oil of Cupressus torulosa contains mono-and di-terpenes, and these fundamental oils appearing antibacterial and antifungal movement against Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Microspora, Trycophytonruberum and Trycophytonmentagrophytes was additionally detailed. The oil of C. lusitani was used in the treatment of rheumatism, whooping cough and styptic problems, and also showed antidermatophytic activity, while the ethanolic extract of C. lusitanica demonstrated cytotoxicity against cancerous cell line. Similarly, antibacterial and antifungal activity from oil of C. arizonica and C. torulosa and the larvicidal activity form oil of C.arizonica was also reported.[5]

The medicinal plants containing compounds with antibacterial activity are found all around the world and many efforts have been made to discover novel effective antibacterial compounds. Antibacterial screening is done to specify the presence of antibacterial molecule in the plant against pathogen and microbes. In present study an attempt is made for analysis of the phytochemicals and antibacterial properties of *Cupressus Torulosa* D. Don from the collected samples. Antibacterial test will be carried out by disc diffusion method.

MATERIALS AND METHODS

Collection of the plant

The medicinal plant *Cupresustorulosa* selected for the present investigation was collected from college premises, which lies in Hattiban, Lalitpur.

Identification of the sample

The plant was identified with the help of taxonomist and then official ID was obtained from Department of Herbarium and Plant Resources, Godawari, Government of Nepal.

Processing of the sample

The plants were collected and stem and leaves are separated. The samples were spread dried under the shade at room temperature until completely dried. Samples were turned up and down at least twice a day to hasten drying. Then the dried samples were subjected to grinding. Finally, the powdered sample was packed in water proof bags.

Extraction of plant material

Maceration method

Each powdered plant samples (70g) was subjected into first maceration (simple) in 500 ml of methanol and hexane and ethyl actetate for 24 hrs followed by second maceration for another 24 hr. Then the filtered extract obtained from first and second maceration was mixed thoroughly and allowed for evaporation on water bath till the solvent fully evaporates. Thus obtained extracts was stored in glass vial and kept inside the vacuum dessicator containing silica crystals for complete drying. The weight of extract was noted on daily basis till the dry weight remains constant.

Removal of solvent

After complete extraction, the extract of round bottom flask were poured in beaker and labeled with name of respective solvent extracts. The beakers were kept in water bath and the solvents were allowed to evaporate till dryness. Then the crude extracts were transferred by using sterile spatula to weigh the yield of extracts in butter paper. After weighing, they were transferred in bottles, labeled and stored in a refrigerator.

Physical parameter and phytochemical screening

Determination of Loss on Drying

About 2.08 g of the drug was weighed in a petri plate, kept in hot air oven at 105^{o}c and dried for a period until constant weight was obtained. The weight loss on drying was noted and difference in weight gives the loss on drying of the powdered drug. The total loss on drying of powder was noted.

Determination of ash value

About 3 g of the powdered drug was weighed and placed in a silica crucible, which was previously ignited and weighed. The powdered drug was spread uniformly in a fine layer at the bottom of the tarred silica crucible. The crucible was kept inside the muffle furnace and the temperature increased to make crucible dull red hot until free from carbon. The crucible was cooled, kept in a desiccator and weighed. The same procedure was repeated to arrive at constant weight. The percentage of total ash obtained was calculated with reference to the air dried drug. The total ash value of powdered sample noted.

Determination of Extractive Values

Powdered air dried material was accurately weighed and placed in a glass-stopper conical flask, macerated with 300ml of the (methanol, hexane and ethyl acetate) for 6 hours, shaking frequently and then allowed standing for 18 hours. It was then filtered rapidly taking care not to lose any solvent, transferred 25ml of the filtrate to a tarred flat-bottomed dish and evaporated to dryness on a water bath. The extract was dried at 105°C for 6 hours, cooled in a desiccators for 30 minutes and weighed immediately. The content of extractable matter in w/w of air-dried material was calculated.

Preliminary Phytochemical screening[6,7]

Phytochemical screening was carried out by the methods referred from text book authored Pulok Mukherjee and Kokate.

Detection of carbohydrates

Small quantity of acetone, alcohol and aqueous extracts were dissolved separately in distilled water and filtered. The filtrate was subjected to various tests to detect the presence of different carbohydrates.

1. **Molisch's test**
2. **Fehling's test**
3. **Barfoed's test**

Detection of proteins and free amino acids

Small quantities of alcohol and aqueous extracts were diluted separately in water and tested for the presence of proteins and free amino acids by subjecting the extracts to various tests.

1. **Biuret's test**
2. **Millon's test**
3. **Ninhydrin test**

Table I: Detection of secondary metabolites.

SN	Tests	Procedure	Result for positive test
1	**General test for glycosides**	200 mg of extract + 5ml of dilute sulphuric acid + warm+Filter+ neutralized with 5% solution of sodium hydroxide+0.1 ml of Fehling's solution A and B and heat on a water bath for 2 minutes	Red precipitate
2	**Detection of phytosterols and triterpenoids**	extracts refluxed with ether + evaporated to dryness + residue subjected to Liebermann-Burchard's	green colour in the upper layer indicate presence of steroids and deep red colour indicates triterpenoids.
3	**Detection of tannins**	Test solution + a few drops of 5% freshly prepared ferric chloride solution indicates the presence of tannins.	a blue-black or green-black colour
4	**Detection of Flavanoids**	test solution + few magnesium turnings + conc. HCl dropwise from the sides of test column	Pink, scarlet, crimson red or occasionally green to blue color appears after 5min
5	**Detection of alkaloids**	**Mayer's test** **Wagner's test** **Hager's test**	cream colour precipitate brown precipitate yellow precipitate
6	**Saponin test**	1 ml of extracts + 20 ml water + shake	formation of froth above the surface

Anti-bacterial screening [8,9]

Preparation of plant extract

1. Stock solution of 400mg/ml was prepared by weighing 200mg of plant extract in 1.5ml eppendorf tube and the final volume of 0.5ml of DMSO was added by micropipette.
2. Extract was completely dissolved by vortexing for 5-10 minutes.

3. Test solution of 200mg/ml, 100mg/ml, 50mg/ml, and 25mg/ml concentration was prepared.

Preparation of inoculums

1. Each culture to be tested was streaked onto nutrient agar to obtain isolated colonies.
2. Overnight incubation was done at 37°C.
3. Then isolated colonies were transferred by the help of sterile loop onto Muller Hinton Broth.
4. Overnight incubation was done at rotary shaker at 37°C.

Inoculation Procedure

1. For inoculation, a sterile cotton swab was dipped into the suspension and was pressed firmly against the inside wall of the tube just above the fluid level and the swab was rotated to remove excess liquid.
2. The cotton swab was streaked over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum.
3. Finally, swabbing was done all around the edge of the agar surface.

Antimicrobial discs and plant extract discs

1. Upon removal of the antimicrobial disks from the refrigerator (4^{o}C), the package containing the disk was left unopened at room temperature for approximately 1hour to allow the temperature to equilibrate. This reduced the amount of condensation on the disks.
2. For plant extract, disc was made from No.1 Whatman filter paper by the help of punching machine. Discs were sterilized by autoclaving. After sterilization, the discs were dipped into the desirable concentration of the plant extracts prepared initially in a sterile condition.
3. The discs were applied to the plates as soon as possible, but no longer than 15 minutes after inoculation. The discs were placed individually with sterile forceps, and then gently pressed down onto the agar (In general, place no more than 12 disks on a 150-mm plate and no more than 4 disks on a 100-mm plate. This prevents overlapping of the zones of inhibition and possible error in measurement. Diffusion of the drug in the disk begins immediately; therefore, once a disc contacts the agar surface, the disk should not be moved).

Recording and interpreting results

1. After the discs were placed on the plate, the plate was inverted and incubated at 37°C for bacteria and fungi was placed without inverting for 24 hours.
2. After incubation, the diameter of the zones of complete inhibition was measured (including the diameter of the disc) and recorded it in millimeters. The measurements were done with a ruler on the undersurface of the plate without opening the lid.

RESULTS AND DISCUSSION

Determination of LOD, Ash value and Extractive value

Various physical constants of the powdered leaf were performed like loss on drying, ash values and extractive values which are presented in tables II, III and IV respectively.

Loss on drying was 9.13%, which indicates lower quantities or absence of volatile constituents. It also shows that the drug was dried enough to control the bacterial growth.

Total Ash value was found to be 5.09%. The low Ash value represents that the drug is less adhered with dirt and sand which in turn represents the purity of the drug.

Extraction was carried out in three different solvents, methanol, hexane and ethyl acetate. Extractive value was found to be 6.20, 2.09 and 3.25 in methanol, hexane and ethyl acetate respectively. Methanol extractive value was more compared to hexane and ethyl acetate.

Table II: Loss on Drying of Leaf of *Cupressus Torulosa.*

Fresh weight (gm)	Dry weight(g)	Loss on Drying(g)	Loss on drying (%)
2.08	1.89	0.19	9.13

Table III: Ash Value of Leaf of *CupressusTorulosa*

Fresh weight(g)	Ash weight(g)	Total ash (%)
3.02	0.15	5.09

Table IV: Extraction Values.

Name of plant	Method of extraction	Solvent used	Yield (amount) in gram	Yield amount in %
Cupressus torulosa	Maceration	Methanol	4.34	6.20
		Hexane	1.46	2.09
		Ethyl acetate	2.26	3.25

Phytochemical screening

Phytochemical tests were performed from extracts of collected plant. When methanol was used as solvent, all plant extracts showed positive phytochemical results. There was

difference in the presence of phytochemical when tested for same plant extracts with different solvents. Methanolic extract showed less presence of tannin in comparison to ethyl actetae extracts. Reducing sugar was present in all plant extracts tested for phytochemical properties. Saponin was present in all three extracts which more in methanol extracts. Presence of alkaloid was comparatively more in ethyl acetate. Presence of steroids was comparatively more in methanol extracts than ethyl acetate extracts. Presence of terpenoid was comparatively more in methanol than in ethyl acetate extracts.

Table V: Phytochemical screening of methanolic, hexane and ethyl acetate extract.

S.No.	Secondary metabolites	Phytochemical test	Methanol	Hexane	Ethyl acetate
1.	Alkaloids	Wagner	+	+	+
		Hager	+	+	+
2	Tannin	$FeCl_3$	+	-	-
		Gelatin	+	+	+
3.	Carbohydrate	Benedict	+	-	+
		Molisch's	+	-	+
		Barford	+	-	+
		Fehling	+	-	+
4.	Protein	Biuret	+	-	+
5.	Phytosterol and Triterpenoids	Salkowaski	+	+	+
6.	Flavonoid	Shinoda	+	-	+
7.	Glycosides	Keller-killani	+	-	+
8.	Saponin	Foam	+	+	+
9.	Phenol	gelatin	+	-	+

Antimicrobial Assay

Antimicrobial assay was also performed with all plant extracts from three solvents by agar disc diffusion method. Four antibiotics were used in this process. In this study, antimicrobial assay was performed with four pathological bacteria. Among the three solvents used, methanol extracts showed highest antibacterial activity whereas hexane extracts showed less activity. This activity was assessed by measuring the diameter of ZOI of four concentrations used. ZOI was larger in 200ug/ml and least in 25ug/ml concentration of extracts. The largest zone of inhibition (15mm) was obtained with *S. pyogens in* methanol extract. Antibacterial activity was comparatively higher in methanol extract. When hexane extract was used, largest ZOI was 10mm against *acinetobactercalcoaceticus.* When ethyl acetate extracts was used, largest ZOI was 12 mm against staphylococcus aureus. Among four pathogenic bacteria used all were found susceptible (ZOI<10) to plant extracts of different at different concentrations in methanol extracts. In ethyl acetate extracts, *S. aureus*and *E. coli* were found less

susceptible whereas in hexane extracts, *Acinetobactercalcoaceticus* was found less susceptible.

The details are shown in table VI and VII and graphical representation is shown in figure I – IV.

Table VI: Zone size interpretative standards for selected antimicrobial discs (AZM[15] CIP[5], CD[2], GEN[10]) and their observed ZOI during experiment.

Antibiotics	Zone of Inhibition (mm)			
	Streptococcus pyogenes	***Acinetobacter calcoaceticus***	***Escherechia coli***	***Staphylococcus aureus***
AZM[15]	20mm	23mm	20mm	19mm
CIP[5]	29mm	35mm	18mm	17mm
CD[2]	22mm	10mm	19mm	17mm
GEN[10]	14mm	24mm	11mm	10mm

Table VII: Zone size interpretative standards for selected antimicrobial discs (AZM[15] CIP[5], CD[2], GEN[10],) and their observed ZOI during experiment using different concentration of (Methanol extract, Ethylacetate, Hexane).

Plant	Microbes	Extract Concentration (in mg/ml) and Zone of Inhibition (In MM)			
		200mg/ml	**100mg/ml**	**50mg/ml**	**25mg/ml**
CupressusTorulosa (methonal solvent)	*Staphylococcus aureus*	14mm	14mm	11mm	9mm
	Streptococcus pyogenes	15mm	14mm	12mm	10mm
	Acinetobacter calcoaceticus	12mm	8mm	8mm	0
	Escherechia coli	14.5mm	13.5mm	10mm	7mm
CupressusTorulosa (ethyl acetate solvent)	*Staphylococcus aureus*	12mm	9mm	8mm	7mm
	Streptococcus pyogenes	7.5mm	7mm	7mm	0
	Acinetobacter calcoaceticus	7mm	6.5mm	6.5mm	0
	Escherechia coli	10.5mm	7mm	6.5mm	0
CupressusTorulosa (hexane solvent)	*Staphylococcus aureus*	7mm	0	0	0
	Streptococcus pyogenes	9mm	8mm	8mm	8mm
	Acinetobacter calcoaceticus	10mm	9mm	7mm	7mm
	Escherechia coli	0	0	0	0

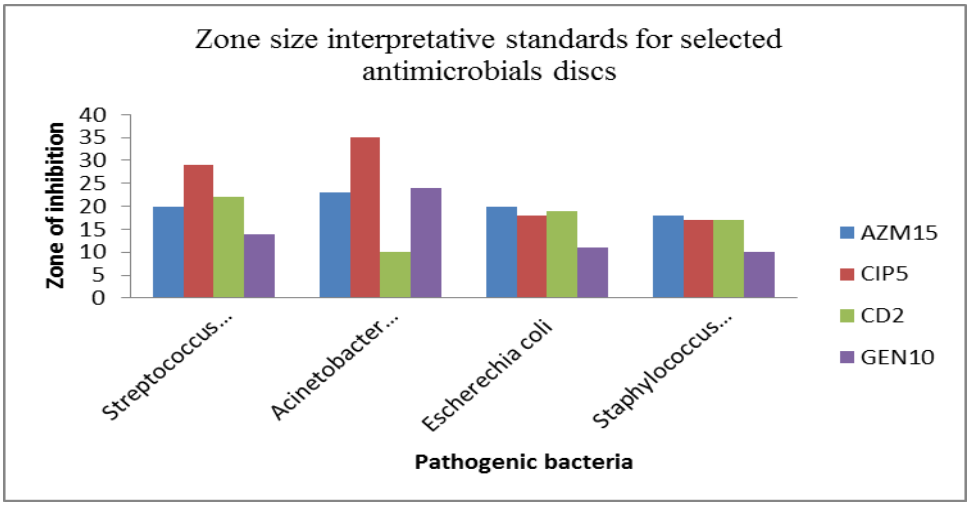

Figure I: ZOI of standard antibiotics extract against different microbes.

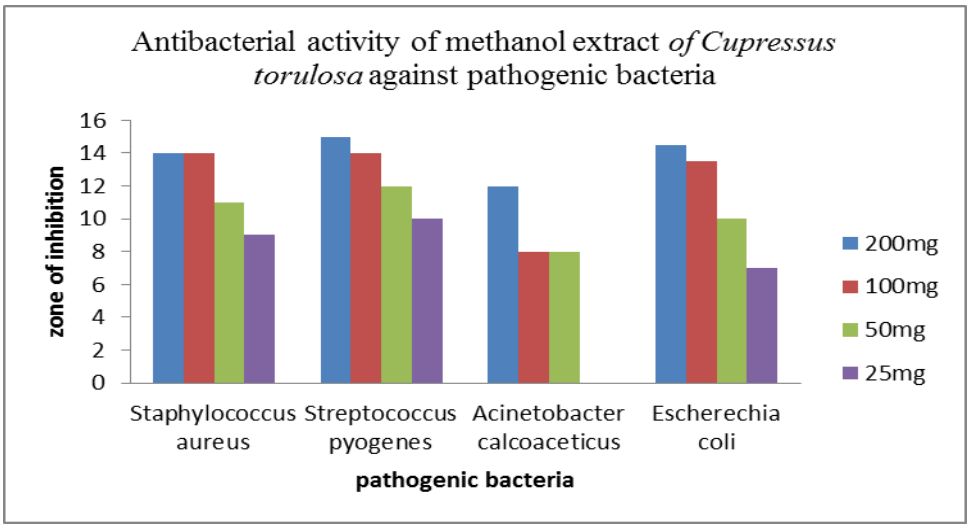

Figure II: ZOI of *Cupressus torulosa* of methanol extract against different microbes.

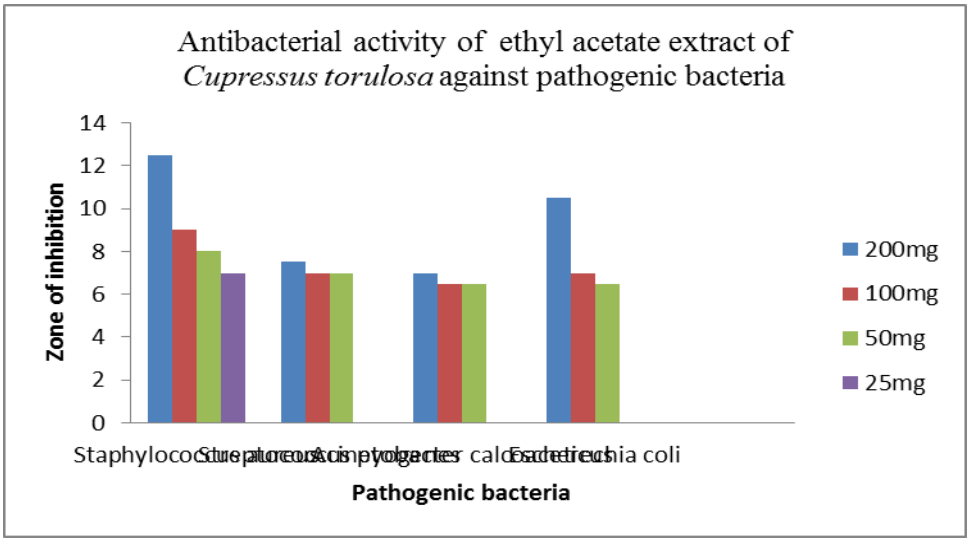

Figure III: ZOI of *Cupressus torulosa* of ethyl acetate extract against different microbes.

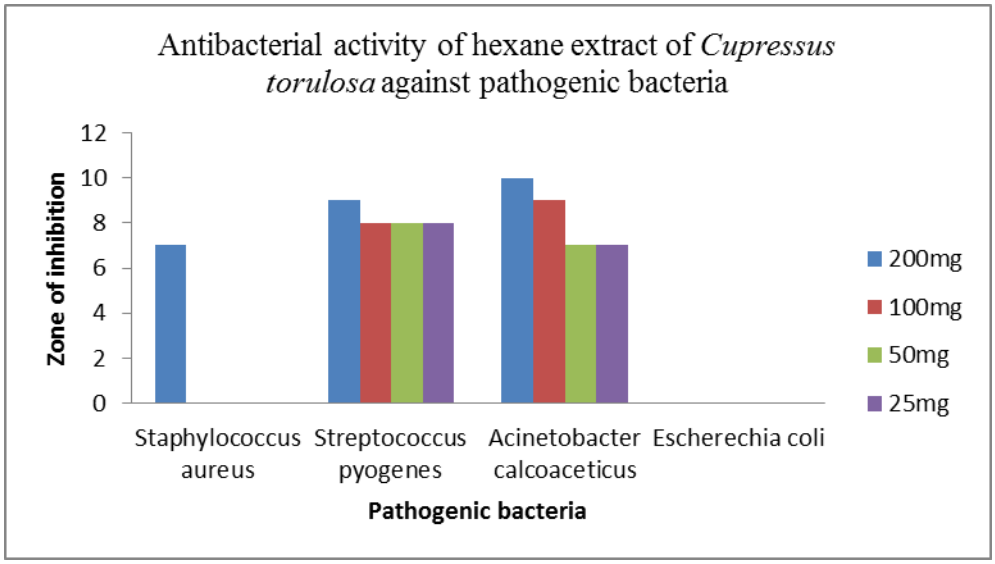

Figure IV: ZOI of *Cupressus torulosa* of hexane extract against different microbes.

CONCLUSION

The result supports the fact that the organic solvent extracts exhibited greater antimicrobial activity as the antimicrobial principle were either polar or non-polar and they were extracted only through the organic solvent medium. The present study was also successful in identifying medicinal plants with different antimicrobial activity and this can further be exploited for isolation and characterization of the novel phytochemical in the treatment of infectious disease when drug resistant microorganisms are being developed in today's world. In Nepal, there are various medicinal plants with effective antimicrobial activities that can serve in developing drugs to various diseases so effective study and analysis of these plants need to be considered.

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