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Isolation and Antimicrobial Evaluation of *Bixa orellana* and *Linum usitatissimum*

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Article

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ABSTRACT

This research work was carried out to investigate the antimicrobial activities of isolated fraction of *Bixa orellana and Linum usitatissimum* against five bacterial and five fungal pathogen. Leaves Powder of bixa orellana and seed powder of *Linum usitatissimum* subjected to soxhlet extraction. The separation of various fraction was carried out by column chromatography, analyzed and purified by thin layer chromatography(TLC) until homogeneity of the fraction achieved. The homogeneity and the purity of the different fractions were checked by HPLC. Antimicrobial evaluation of isolated fractions was carried out. Two fraction Fr-1 (352mg) and Fr-2(260mg) were collected from Bixa orellana had Rf was o.99 and 0.35. fraction 3 and 4 was isolated from linum usitatissimum with Rf 0.45 and 0.25. When compare to the standard drug gentamycin, phenolic fraction of 1 and 2 had good antibacterial activity against all bacterial strain where as glycoside fraction 3 and 4 were poorest activity against all bacterial strain. When compare to standard drug tioconazole fraction-1 and 2 had very good activity against fungal strain and fraction 3 and 4 had moderate activity against fungal strain.

INTRODUCTION

In developing countries, people of native communities use *Bixa orellana* L. commonly known as 'achiote/ annatto'(Family: Bixaceae)' as folk medicine in the form of decoctions, teas & juices for the treatment of common infections. The dye obtained from the pulp of the *B.orellana* seed (called bixin) is used all over the world as a red-orange dye for coloring rice, cheeses, soft drinks, oil, butter, and soup. The dye is also used in some regions to dye textiles and the Sionas and Secoyas color fabrics and weapons with it. In the Philippines, the red pulp from the seeds is used in the as polish for russet leather) and the seeds are ground and used as a condiment [1,2,3,4]. Various indigenous groups paint their hair and bodies with the pulp to repel insects and protect from sunburn. Bixin was also the original Amerindian warpaint. The seeds are given to bulls to make them aggressive for bullfighters and are taken by indians as an aphrodisiac. A fiber may be obtained from the bark. A flagellate of the family Trypanosamatidae has been isolated from the fruit of *B.orellana*. Average price of seeds exported from Brazil in 1987 was $1.57 per kilogram, and as production increases, prices remain low. It has been found recently that particle attrition and impact rather than solvent extraction is a more cost efficient method for processing the red carotenoid bixin. Flax or linseed is among the oldest crop plants cultivated for the purpose of oil and fiber. It belongs to the genus *Linum* and family Linaceae. The botanical name, *Linum usitatissimum* was given by Linnaeus in his book "Species Plantarum". It is an annual herbaceous plant with shallow root system.[16-17] The common names flax and linseed are used in North America and Asia, respectively, for *L. usitatissimum*. Oil seed varieties and fiber varieties are specialized development of this species [5,6,7,8,9,10,11,12].

In the present invstigation phenolic compound of *bixa orellana* and *Linum usitatissimum* were isolate and subjected for its antimicrobial activity.

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MATERIAL AND METHODS

Micro organisms

Bacterial strains to be used were selected on the basis of their pathogenicity. 2 strains of gram positive and 3 strain of gram negative strain were taken for the antibacterial susceptibility tests. Bacterial strains were obtained from MTCC, Chandigarh which are summarized in Table 1

Table 1: Bacterial strains were obtained from MTCC, Chandigarh

Sr. no	Bacterial strains	MTCC no.
	Gram positive bacteria	
1.	*Staphylococcus aureus*	2079
2.	*Bacillus cereus*	2016
	Gram negative bacteria	
3.	*Escherichia coli*	2089
4.	*Klebsiella pneumonia*	2957
5.	*Salmonella typhi*	2263

Fungal strains to be used were selected on the basis of their pathogenicity. *Candidiasis* and *aspergillosis* are the main fungal infection prevailing today worldwide. These diseases are caused by Candida and Aspergillus fungal species and so, 3 strains of *Candida* and 2 of *Aspergillus* species were taken for the antifungal susceptibility tests. Fungal strains were obtained from MTCC, Chandigarh which are summarized in Table 2

Table 2: Fungal strains with their MTCC number

Sr. no	Fungal strains	MTCC no.
1.	*Aspergillus fumigatus*	2550
2.	*Aspergillus fiavus*	871
3.	*Candida albicans*	183
4.	*Candida tropicalis*	184
5.	*Candida parapsilosis*	1744

Plant material

The different parts of plants according to their use in Ayurveda and traditional systems of medicine were collected from various regions of Chhattishgarh during march to june. The botanical identify was authenticated by Dr R.K.Verma, , Department of Environmental and Herbal Science, CSVTU, Bhilai, Chhatishgarh. The plant material was dried in shade. List of plant and their ayurvedic uses mention in Table.3

Table 3: List of Plant Used and their Ayurvedic Uses

Serial No.	Botanical Name	Family	Common name	Plant part used	Ayurvedic or Traditional uses
1	*Bixa orellana*	*Bixaceae*	lipsticktree	Leaves	It is useful in jaundice, diarrhoea, leprosy treatment, kidney disease, liver trouble.
2	*Linum usitatissimum*	*Linaceae*	Flaxseed	Seed	specialized in treatment of complicated diseases like cancer, heart disease, hyperglycemia

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Preparation of powder and extracts

The different parts of plants of *Mallotus Philipinesis, Bixa orellana* and *Linum usitatissimum* were collected and dried under shade. These dried materials were mechanically powdered after keeping them in an oven at 350◦C for 24 hrs.

Hot Continuous Extraction (Soxhlet)

On the laboratory scale, the Soxhlet apparatus is used. It consists of a flask, a Soxhlet extractor and a reflux condenser. The raw material of plant was usually placed in a thimble made of filter paper and inserted into the wide central tube of the extractor. Alternatively, the drug, after imbibition with menstruum, may be packed in the extractor taking care that the bottom outlet for the extract is not blocked. 60% methanol is placed in the flask and brought to its boiling point. Its vapors pass through the larger right hand tube into the upper part of the extractor and then to the condenser where they condense and drop back onto the drug. During this period, the soluble constituents were extracted. When the level of the extract reaches the top of the syphon tube, the entire volume of extract syphons over into the flask. The process was continued until the drug was completely extracted.

Phytochemical screening

Phytochemical examinations were carried out for all the extracts as per the standard methods.

Column chromatographic separation

The methanolic extract of plant sample was adsorbed onto silica gel by triturating in a mortar and left for about 10 hours to dry. The column was packed with a solution of silica gel with n-butanol using the wet slurry method. This involves preparing a solution of silica gel, with n-butanol in this case, in a beaker and subsequently adding this unto the column till it is about three-fourths filled. The solution was stirred for dispersal and quickly added to the column before the gel settles. This method was used to prevent the trapping of air bubbles. A ball of wool was pushed into the column to settle atop the packed silica gel. A substantial for phenolic compound amount of benzene:methanol:formic acid(10:5:1) and for glycoside amount of chloroform: methanol (95:5) were poured continuously into the column and allowed to drain but prevented from reaching the cotton wool. The quantity collected was poured back into the column. Periodically, a piece of rubber tubing was used to agitate the column to allow for the escape of trapped air bubbles. The column fractions were again tested with TLC chromatogram and the Rf values were determined.

Thin layer chromatography (TLC)

Thin layer chromatographic studies of phenolic fraction of *Mallotus Philipinesis* and *Bixa orellana*, glycoside fraction of *Linum usitatissimum* were carried out. TLC plates were prepared by using silica Gel-G as adsorbent. 500 gm silica Gel-G was mixed with sufficient quantity of distilled water to make slurry. The slurry was immediately poured into a spreader and plates were prepared by spreading the slurry on the glass plates of the required size. The thickness of the layer was fixed as 1.5 mm. Plates were allowed to air dry for 1 hour and layer was fixed by drying at 105°C for 1 hour. Using a syringe and needle, about 10 µl of 1 % W/V solution of extracts were loaded gradually over the plate. The loaded plates with phenolic fraction were eluted by suitable elutent mixture containing benzene:methanol:formic acid(10:5:1) and glycoside fraction were eluted by suitable elutent mixture containing chloroform: methanol (95:5). Before the elution, the tank was allowed 30 minutes for saturation with suitable elutent mixture. The fraction showed separation into bands. The chromatograms were observed under both visible light and UV radiation (at 254 nm and 346 nm) and were photographed and Rf value were measured.

High Performance liquid chromatography(HPLC)

The homogeneity and the purity of the different fractions were checked by HPLC(pump: LC-10AD, shimadzu liquid chromatography, shimadzu Co.) using a column CC 250/4 Nucleosil 1000-7 C_{18} Cat. NO 721609.40, Ser NO. 1096117, Charge/Batch 21401011, Macherey-Nagel and elution of phenolic fraxtion with flow rate 4.0min/ml with benzene:methanol:formic acid(10:5:1) and elution of glycoside fraction with chloroform: methanol (95:5) at flow rate of 1.0min/min.

Antimicrobial activity determination

Antimicrobial agents

Gentamycin (10 µg/mL) and Tioconazole (0.7 mg/mL) were included as standard reference drugs in the study.

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Agar diffusion-pour plate method (bacteria)

An overnight culture of each organism was prepared by taken two wire loop of the organism from the stock and inoculated each into the sterile nutrient broth of 5ml, each incubated for 18-24hr at 37°C. From overnight culture, 0.1 mL of each organism was taken and put into the 9.9mL of sterile distilled water to obtained 10^{-2} inoculum concentration of the organism. From the diluted organism (10^{-2}), 0.2mL was taken into the prepared sterile nutrient agar cooled to about 40-45°C, then poured into sterile Petri dishes and allowed to solidify for about 45- 60min. Using a sterile cork-borer of 8mm diameter, the wells were made according to the number of the test tubes for the experiment. For this work 8 wells were made. Using a micropipette (100 µl) of each of plant sample by dissolving (125 mg) in (1 ml) distilled water, were put in the pores accordingly including the controls. The studies were done in duplicates to ascertain the results obtained. The plates were left on the bench for about 2hrs to allow the extract diffuse properly into the nutrient agar i.e. pre-diffusion. The plates were incubated for 18-24hr at 37°C.

Agar diffusion-surface plate method (fungi)

A sterile potato dextrose agar was prepared accordingly and aseptically poured into the sterile plates in triplicates and solidified properly. 0.2mL of the 10^{-2} inoculum concentration of the organism was spread on the surface of the agar using a sterile Petri-dish cover to cover all the surface of the agar. Eight wells were bored using a sterile cork-borer of 8mm diameter. Using a micropipette (100 µl) of each of plants by dissolving (125 mg) in (1 ml) distilled water, were put in the pores including the controls. All the plates were left on the bench for 2hr to allow the extract diffuse properly into the agar i.e. pre-diffusion. The plates were incubated at 25°C for 72hr [13,14,15,16,17,18].

RESULTS AND DISCUSSION

Preliminary Phytochemical Analysis

Methanolic extract of *Bixa orellana* (Extract-B) indicated the presence of tannins and phenolic compound, saponin, flavonoid, alkaloids, proteins, amino acid and steroids in plant. (Mohammed *et al.*,2010) Methanolic extract of *Linum usitatissimum* (Extract-C) indicated the presence of proteins, amino acid, carbohydrates and glycosides in plant.(Kumanan *et al.*2010). phytochemical tests and reports are shown in Table no.4

Table 4: Phytochemical analysis of plant in methanol extract

Sr.No	Phytochemicals	Leaves of *bixa orellana*	Seed *of linum usitatisimum*
1	Tannins and phenolic compound	+	-
2	Saponin	+	-
3	Flavonoid	+	-
4	Alkaloids	+	-
5	Proteins and Amino acid	+	+
6	Steroids	+	-
7	Anthraquinones	-	-
8	Carbohydrates	-	+
9	Glycosides	-	+
10	Diterpenes	-	-

Note: + : Presence, - : Absence.

Column chromatography

Methanolic extract of *Bixa orellana* (Extract-B) (1.6g) was dissolved in a 2ml methanol and carefully applied on the top of the column. The extract was eluted with methanol at a rate of 4 ml/min and two fraction Fr-1 (352mg) and Fr-2(260mg) were collected. Methanolic extract of *Linum usitatissimum* (Extract-C) (1.3g) was dissolved in a 2ml methanol and carefully applied on the top of the column. The extract was eluted with methanol at a rate of 1.0 ml/min and two fraction Fr-3 (160mg) and Fr-4(250mg) were collected.

Phytochemical test for isolated phenolic compound.

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

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Phytochemical test for isolated glycoside.

Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of glycosides.

TLC

Thin layer chromatography was performed to confirm the presence of phenolic compound in bixa orelana and presence of glycoside in linum usitatisimum. Fraction 1 was phenolic fraction of Bixa orellana was light yellow in colour with Rf value 0.99. Fraction – 2 was phenolic fraction of Bixa orellana was dark green in colour with Rf value 0.35. The fraction-3 was light yellow in colour and its Rf value was 0.45 and this equal of Rf value according to Harborre [7]. Fraction-4 was light yellow in color and its Rf value was 0.25.

HPLC

Fractions collected from column chromatography were further purified on HPLC to isolate phenolic and glycoside. Fraction-1 and 2 containing mainly phenolic compound were separated by HPLC using benzene: methanol: formic acid(10:5:1) mobile phase. All fraction shown only single peak which raveled fraction-1 and 2 were pure compound. Fraction-3 and 4 containing mainly glycoside compound were separated by HPLC using chloroform: methanol (95:5) as mobile phase. All fraction-3 and 4 shown only single peak which reveled fraction-3 and 4 were pure compound. High performance liquid chromatographic analysis of the fraction obtained from the column chromatography depicted the purity of fractions.

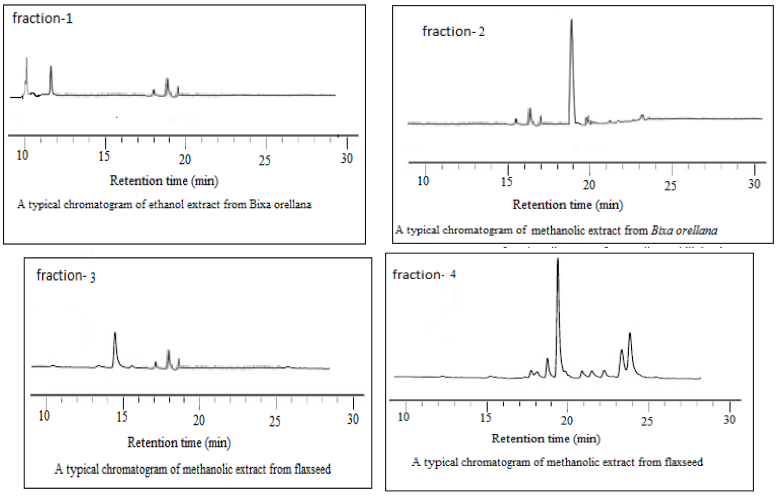

Figure1:HPLC chromatogram of different fraction.

Antibacterial Activity

MIC values for different fractions against bacterial strain are shown in Table.5 Fraction-1 shown antibacterial activity in the range of 18 to 28mm. Fraction-2 had highest activity against *pseudomonas aeruginosa.* Fraction-2 showed antibacterial activity in the range of 16-26mm. it shown highest antibacterial activity against *pseudomonas aeruginosa.* Fraction 1 and 2 Shown good antibacterial activity when compare with standard drug gentamycin. Fraction-3 had antibacterial activity In the range of 02 to 0.4mm. Fraction 3 shown poorest antibacterial activity against gram positive bacteria ineffective on gram negative bacteria. Fraction-4 had poorest antibacterial activity against all bacterial strain. When compare to the stander drug gentamycin phenolic fraction of 1 and 2 had good antibacterial activity against all bacterial strain where as glycoside fraction 3 and 4 were poorest activity against all bacterial strain.

Antifungal activity

MIC values for different fractions against fungal strain are shown in Table 6 Fraction-1 had antifungal activity in the range of 16 to 20mm. Fraction-2 had good antifungal activity in range of 10 to 22mm. Fraction-2 was more effective on candida strain and moderate active on aspergillus strain. Fraction-3 had good activity on fungal strainin the range of 06 to 16mm. fraction-4 shown moderate antifungal activity in the range of 10-18mm. Standard drug Tioconazole shown antifungal activity in range of 24-26mm. when compare to standard drug fraction-1and 2 had very good activity against fungal strain and fraction 3 and 4 had moderate activity against fungal strain.

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Table. 5 Zone of inhibition of plant sample against the bacteria by Agar diffusion-pour plate method

Isolated sample	Diameter of zone of inhibition of bacteria(mm)				
	Gram Positive Bacteria			Gram Negative Bacteria	
	S.a	B.c	S.F	K.Pne	Psea
Fraction-1	24	22	22	18	28
Fraction-2	20	16	24	22	26
Fraction-3	02	04	02	-	-
Fraction -4	02	-	02		-
Gentamycin	36	34	34	36	34

S.a= *Staphylococcus aureus*, B.c= *Bacillus cereus*, S.f= *Streptococcus faecalis*, K.Pne= *Klebsiella pneumoniae*, Pse.a= *Pseudomonas aeruginosa*

Table 6: Zone of inhibition of plant sample against the Fungal by Agar diffusion-pour plate method

Plant extract	Diameter of zone of inhibition of Fungi(mm)				
	Fungal strain				
	A.f	A.fl	C.a	C.t	C.p
Fraction-1	20	16	18	20	18
Fraction-2	12	10	22	20	22
Fraction-3	12	16	08	10	06
Fraction -4	14	18	10	12	14
Tioconazole	24	26	22	23	24

A.f= *Aspergillus fumigatus* MTCC-2550, A.Fl= *Aspergillus flavus* MTCC-871, C.a= *Candida albicans* MTCC-227, C.t= *Candida tropicalis* MTCC-184, C.p=*Candida parapsilosis* MTCC-1744

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