

## Antimicrobial activity of crude extracts from plant parts and corresponding calli of *Bixa orellana* L.

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Ethanol extracts from the different parts of *B. orellana* showed differential antimicrobial activity. It was found that the extracts of *in vitro* leaves showed maximum activity against *Bacillus pumilus* followed by the extracts from the roots and hypocotyls. The callus derived from different explants too showed antimicrobial activity. The leaf callus showed maximum activity. The zone of inhibition for the diluted extracts of *in vitro* hypocotyls and roots and their corresponding calli showed minimum zone of inhibition at concentration 24mg/ml, whereas the diluted extract of *in vitro* leaves and leaf derived callus showed minimum zone of inhibition at 16 mg/ml.

A special feature of higher plants is their capacity to produce a large number of organic chemicals of high structural diversity, the so-called secondary metabolites. Some of these secondary metabolites are produced for self-defense<sup>1</sup>. Such metabolites are divided into three different categories based on their mechanism of function i.e. chemotherapeutic, bacteriostatic, bactericidal and antimicrobial<sup>2</sup>.

Over the last 20 years a large number of plant species have been evaluated for their antimicrobial activity. One of the plants known for having many medicinal uses in traditional system of medicine is *Bixa orellana* L. Both roots and leaves are used as medicine for treating sore throats, jaundice, dysentery, gonorrhoea, liver diseases<sup>3</sup> and as antipyretic agents<sup>4</sup>. The present work is an initial attempt to study the antimicrobial activity of leaves, hypocotyls and root extracts of *B. orellana*.

The accumulation of phyto-chemicals in plant cell cultures has been studied for more than thirty years and the generated knowledge has helped in realization of using cell culture for production of desired phyto-chemicals. Hence an attempt has been made to check whether *in vitro* cultured callus from the leaf, hypocotyls and root explants of *B. orellana* too have the ability to accumulate the compounds with antimicrobial activity? In present study attempts were made to

find out the minimal inhibitory concentration (MIC.) of all the extracts against the test microorganism *Bacillus pumilus*.

### Materials and Methods

**Callus induction**—Two-month-old *in vitro* germinated seedlings of the red variety capsule of *Bixa orellana* L. were used for the present work. The leaf, root and hypocotyl were separated, wrapped in perforated aluminum foil and then dried in an oven at 40°C for 72 hr and stored till further use.

Leaf, hypocotyl and root explants were inoculated on MS medium<sup>5</sup> supplemented with 2.0ppm 2-iso Pentenyladenine (2-iP) and 0.5ppm NAA at 25°C. Callus was cultured at 3000 lux light intensity provided for 16 hr per day followed by a dark period of 8 hr.

Two months old callus derived from the three explants, were separately weighed and dried by wrapping them in perforated aluminum foils and keeping at 40°C for 72hr in an oven. The dry weight was recorded.

**Extraction**—To 10g of dried callus of each explant and the three above mentioned explants, 100ml absolute ethanol was added and refluxed at 78°-80°C for 6 hr using three necked round bottom flask having a solid liquid reflux unit with an attached stirrer and a coil type condenser.

Refluxed ethanol extract was cooled to room temp. and filtered through Whatmann No.1 filter paper. Filtrate was used as antimicrobial compounds (AMC).

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Pure ethanol was used as negative control for assessing the antimicrobial activity. Roxythromycin and Harida extracts were used as positive controls. The extracts were stored in amber bottles at 4°C till further use.

**Antibiotic Assay**—Prior to antimicrobial assay the extracts were concentrated by evaporation and diluted with ethanol to prepare various concentrations (8, 16, 24, 32, 64, 200 and 400mg/ml) of the extracts.

*Bacillus pumilus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were used as the test micro organisms to check their sensitivity towards the various plant extracts of *B. orellana* L.

Culture (48 hr old) of *Candida albicans*, which was cultured on Sabouraud Dextrose Agar (Hi media-M063) and 24 hr old culture of *Bacillus pumilus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, which were cultured on Soybean Casein Digest media (Hi media- M290) respectively were used.

For antibiotic assay, medium No 11 (Hi media-M004) was used. 25ml media was poured into sterilized petridishes (O.D.9.0cm). The agar was allowed to solidify and a well of 12 mm diam. was punched in the center of the plate. 200µL of extract was added to the well. Loop full of all the 5 microbial cultures were streaked along the radii of a pentagon of antibiotic assay plate. The extract was allowed to diffuse at 25°C for 120 min. The plates were then incubated at 37°C for 18 hr.

Five ml of 0.9% saline was used to wash the culture of *Bacillus pumilus* (NCTC 8241). 0.2ml of this stock solution was added to 100ml of autoclaved antibiotic assay medium (No 11) at 45°-50°C. 25ml of this medium, which contained the bacterial stock solution, was poured into petridishes of 9.0 cm diameter. It was allowed to solidify at room temperature. Four wells of 12mm size were punched in the petridishes and 50µL of plant extract of varying concentrations was added to the wells. The extract was allowed to diffuse at 25°C for 120 min. The plates were incubated at 37°C for 18hr. The inhibition zones were recorded using an antibiotic zone reader (TAB machine).

## Results and Discussions

Initial recording of fresh weight of callus generated from all the three explants showed that leaf and root had high callus producing capacity (Table 1). However, the dry weight was 11% of the fresh weight for

callus derived from all the three explants. Whereas dry weight of plant parts was percent in case of roots, percent for leaves and percent for hypocotyls.

Extracts from all the three parts of the plant and callus derived from them showed antimicrobial activity, though the effective concentration varied and so did the sensitivity of microbes to the extracts. *Bacillus pumilus* was found to be the most sensitive as compared to *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* (Fig. 1). *Candida albicans* appeared to be resistant to all the 6 extracts.

Antimicrobial activity was confirmed to be because of the plant and callus extracts; because Roxythromycin and Haridra extracts both inhibited the growth of test microorganisms. Roxythromycin was more inhibitory than Haridra extract.

There was an increased gradient inhibitory response starting from 16 to 64mg/ml extract, after that

Table 1—Fresh and dry weight of various plant parts (leaf, root and hypocotyl taken from 2 months old seedlings and 2 months old callus (cultured on MS medium supplemented with 2mg/l 2iP + 0.5 mg/l NAA) from leaf, root and hypocotyl explants.

Plant Part	Fresh wt. (g)	Dry wt. (g)	Dry wt. (%)
Leaf	0.3	0.027	9.0
Leaf callus	0.75	0.0825	11.0
Root	0.28	0.0238	8.5
Root callus	0.70	0.0735	10.5
Hypocotyl	0.15	0.012	8.0
Hypocotyl callus	0.69	0.069	10.0

Table 2—Effect of different concentrations of extracts of *Bixa orellana* leaf, root, hypocotyl and callus derived from them on the growth of *Bacillus pumilus* as recorded by inhibition in the zone of bacterial growth using agar well technique

[Values are mean ± SD of three determinants]

Extracts from	Inhibited zone (mm) of microbial colonies grown in different concentrations (mg/ml) of extracts				
	8	16	24	48	64
Leaf	No Zone	17.1 ± 0.15	21.6 ± 0.16	25.6 ± 0.13	26.1 ± 0.26
Leaf callus	No zone	16.5 ± 0.09	20.7 ± 0.19	24.8 ± 0.17	26.9 ± 0.09
Root	No zone	No zone	15.8 ± 0.25	19.8 ± 0.28	22.3 ± 0.20
Root callus	No zone	No zone	15.3 ± 0.05	19.4 ± 0.16	21.8 ± 0.15
Hypocotyl	No Zone	No zone	15.2 ± 0.12	19.3 ± 0.11	20.6 ± 0.12
Hypo-callus	No Zone	No zone	14.9 ± 0.25	18.6 ± 0.12	20.2 ± 0.22

it plateaued, as it can be seen from Table 2. Leaf and leaf callus extract was effective in causing inhibition in growth of *Bacillus pumilus* (Fig. 2) at and above 16mg/ml. Whereas all the other extracts were effective at concentrations higher than 24 mg/ml, 64mg/ml showed maximum effectiveness for all the extracts

from all the samples. Since the inhibitory effect plateaued after that, the results are not presented. Extracts from leaf and leaf-derived callus had highest antimicrobial activity. Such antimicrobial activity in leaf extracts have been reported earlier<sup>6,7</sup>.

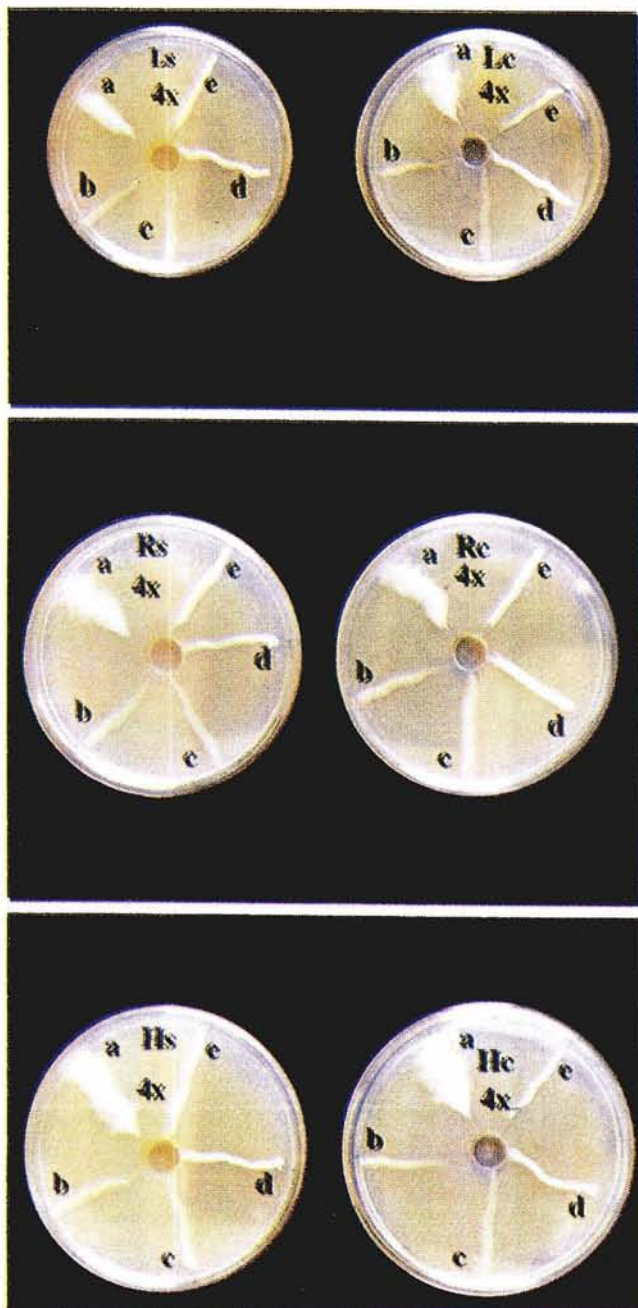


Fig. 1—Sensitivity of microbial cultures to various extracts of *B. orellana* L. a. *Bacillus pumilus*. b. *Pseudomonas aeruginosa*. c. *Staphylococcus aureus*. d. *Candida albicans*. e. *Escherichia coli*. [Extract from Ls = leaf, Lc = leaf callus, Rs = root, Rc = root callus, Hs = hypocotyls and Hc = hypocotyls callus].

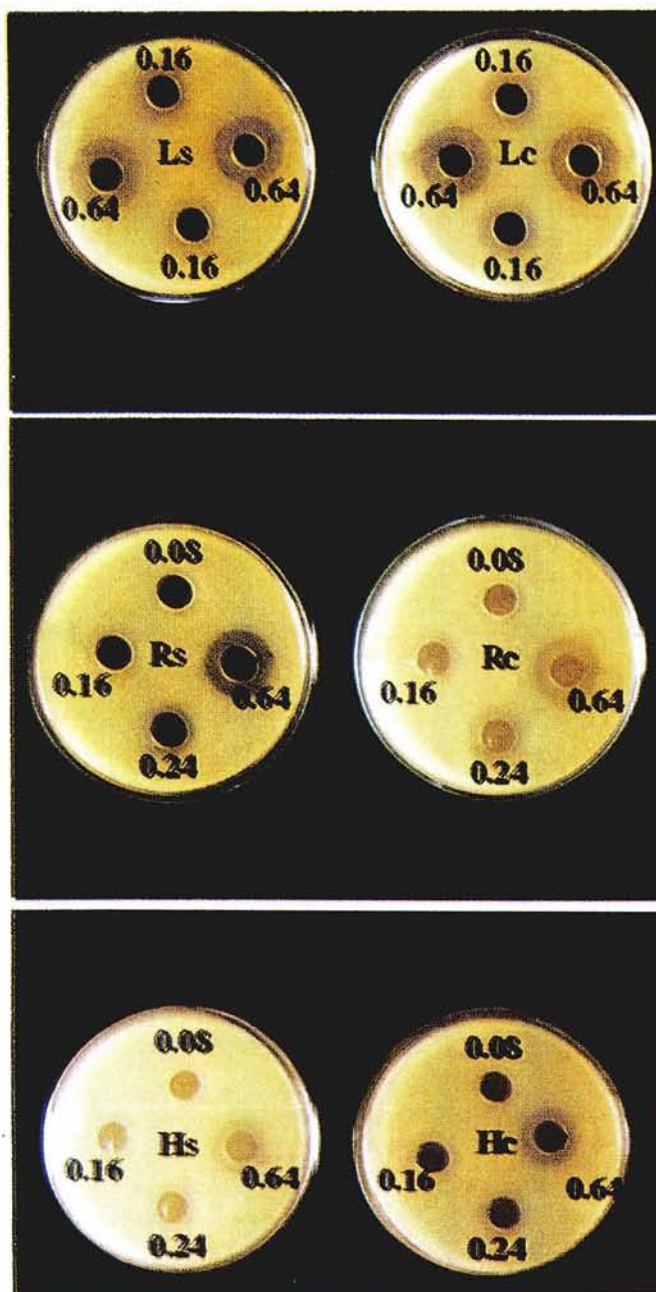


Fig. 2—Minimal inhibition concentration of the various extracts of *Bixa orellana* L. exhibited against *Bacillus pumilus*. a. *Bacillus pumilus*. b. *Pseudomonas aeruginosa*. c. *Staphylococcus aureus*. d. *Candida albicans*. e. *Escherichia coli*. [Extract from Ls = leaf, Lc = leaf callus, Rs = root, Rc = root callus, Hs = hypocotyls and Hc = hypocotyls callus]



Ontengco *et al.*<sup>7</sup> while studying the antibacterial effect of essential oil obtained from the flowers and leaves of *Bixa orellana* also found that 16-64 mg/ml extract showed antimicrobial activity.

Root and hypocotyl and their callus had almost similar antimicrobial activity.

It was interesting to note that callus of all the explants had almost same or slightly higher anti-microbial activity. The analysis of dried leaves<sup>3</sup> showed that it contains essential oils along with a sesquiterpene (bixagane). It also contains ellagic acid, 7-bisulphate luteolin, 8-bisulphate hypoluteolin, 7-glucoside apigenin, 7-glucoside luteolin, bixorellin, an unknown glycoside and a waxy material, which may account for it as antimicrobial property<sup>3</sup>. Whereas Ontengco *et al.*<sup>6</sup> have reported that it is the essential oil content in the leaves and flowers that have anti-microbial activity. Callus culture extracts with high anti-microbial activity makes it a good system for further expanding this work at commercial level, and using it for production of desired molecule.

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