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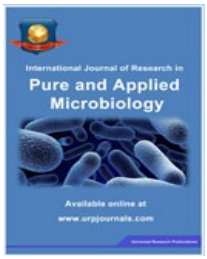
International Journal of Research in Pure and Applied Microbiology



Original Article

ISOLATION OF ANTIMICROBIAL COMPOUNDS FROM CHICORY (*Cichorium intybus* L.) ROOT

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Received 27 July 2011; accepted 18 August 2011

Abstract

Chicory root powder is routinely used in mixing with coffee because of the cost advantage compared to coffee beans. Chicory also adds to the strength or "body" of the beverage. The ethyl acetate extract of chicory root was tested for anti-bacterial and anti-fungal properties. Fractionation by column chromatography of ethyl acetate extracted root powder contains the compound, inhibiting both Gram positive and Gram negative bacteria and was found to be bacteriostatic rather than bacteriocidal. The effect of chicory root extract has more bacteriostatic effect on Gram Positive bacteria than Gram negative bacteria as MIC value is more in case of Gram negative bacteria than Gram positive bacteria. The ethyl acetate fraction of ethyl acetate root which is obtained by silica gel column chromatography has also antifungal activity as it inhibits growth of yeast and moulds.

Key words: *Cichorium intybus*, Antimicrobial activity, Zone of inhibition, Minimum Inhibitory Concentration (MIC), Column Chromatography.

1. Introduction

There is an increasing demand for biologically active substances from plant origin which is the current interest and focus of new research approach. The synthetic chemical pharmaceuticals showed various side effects on the functioning of different parts of the body, both internally and externally. Plant products have been shown to have side effect free, good therapeutic potential, due to the presence of active pharmacologically important substances, such as terpenes, alkaloids, flavonoids and glycosides [1, 2]. Screening is a tool in discovering new biologically active molecules which have been found to be most productive in the area of antibiotics. In view of this, many plants have been screened for antimicrobial activity in India and abroad [3, 4, 5, 6].

Cichorium intybus L is a perennial herb of 1.0 to 1.8 m height with a deep dandelion type root and bright blue flowers. Chicory is one of the earliest known and most widely used raw materials for manufacturing of coffee substitutes [7]. The leaves of chicory plant can be used as salad as they are rich source of vitamin A & C. and also micronutrients [8]. Chicory root is reputed to be a blood detoxifier, tonic, and decongestant of the internal organs. Chicory rhizome contains many useful compounds [9]. The boiled leaves and flowers have anti-inflammatory properties. Dried chicory roots are being extensively used by the beverage industries. It has also bifidogenic property as it is rich in inulin [10, 11].

Chicory root extract is known to inhibit the growth of *Salmonella typhi* [12]. Chicory root extract has the free radical scavenging activity and liver protecting property. It has tumor inhibitory property also [13]. Antifungal activity of chicory root extract was reported earlier [14, 15].

In our present study, chicory root extract is found to have antimicrobial effects on a number of Gram positive and

Gram negative bacteria and some fungal species. The crude and partially purified fraction of chicory root extract are tested for their antimicrobial activity on *Staphylococcus aureus, Bacillus subtilis, Pseudomonas fluorescens, Rhizobium leguminosarum, Escherichia coli, Vibrio cholerae* Yeast(*Sachharomyces cerevisiae*) and *Aspergillus niger*, by agar cup assay and growth kinetics assay. Active antimicrobial fractions from chicory root extract are obtained by refluxing in ethyl acetate. The growth inhibitory effects of chicory root extract are observed on the tested microbes.

2. Materials and Methods

2.1 Bacterial stains and Plant material

The microorganisms employed in this study consisted of three Gram Positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Rhizobium leguminosarum*) and three Gram negative (*Vibrio cholerae*, *Escherichia coli*, *Pseudomonas fluorescens*) bacterial species & two fungal (*Aspergilus niger* and *Sachharomyces cerevisiae*) species. The microorganisms were obtained from various sources (Table-1). The plant material i.e chicory root powder was purchased from commercial sources at Bangalore.

2.2 Preparation of Chicory root extract

The extract was prepared by taking 5 g of chicory root powder which was mixed in 50 ml of solvents (Ethylacetate or Hexane). After 2 hrs at room temp, refluxed at 65^0C for ethyl acetate and at 56^0C for Hexane for 30 min. The filtration was done through Whatman filter paper and the filtrate was tested for their antimicrobial potential. Both the ethyl acetate and hexane extract (1ml) were taken in test tube & weight was measured .then the both extract was dried and the weight was again measured. The difference in weight is the concentration of the extracts respectively. Concentration of Ethyl acetate extract was 95mg/ml and Concentration of Hexane extract was 33mg/ml.

2.3 Antibacterial assay of extract

Antimicrobial assay of the samples against the selected bacterial cultures were carried out in vitro by the agar well diffusion method and growth kinetics assay.

2.3.1 Agar well diffusion assay

A number of colonies were picked up from the bacterial stock cultures & transferred to 5 ml Lauria Bertini medium and shaken in a water bath at 37^oC for 18 hr. Agar plates were prepared earlier and then 200 µl from that culture was added to the agar plates & spread plating was done by glass spreader. Wells of uniform diameter (9mm) were made on solidified agar plate using a sterile borer. Chicory root extracts in ethyl acetate and standard antibiotic (Tetracycline-SIGMA) as well as pure solvent, as control were pipetted in the respective wells, under aseptic condition. The plates were then maintained at room temperature for 1 hr, allowing the solution to diffuse. After incubation at 37^0 C for overnight, the zone of inhibition around each well was measured. There is no or almost nil zone of inhibition using solvent alone. All the experiment is repeated at least five times. Similar experiment was done using hexane extract of chicory root but no growth inhibition is noticed.

2.3.2 Growth kinetics assay

For determining bacterial growth inhibition by growth kinetics assay, a few test tubes, each containing 5 ml of sterile Lauria Bertini medium were taken. Then in each tube 100 µl of individual bacterial cultures were added. One test tube in each exp contained the pure solvent as control & in other test tubes different concentration of chicory root extract in ethyl acetate was added. Then all the test tubes were kept in incubator shaker at 37^0C overnight and next day the O.D was measured at 610nm. Similar experiment was done using hexane extract of chicory root but no growth inhibition is noticed.

2.4 Antifungal assay of extract

For determination of antifungal activity of chicory root extract on *Aspergillus niger*, three YEPD agar plates were prepared. One plate(A) contains 500µl of solvent ethyl acetate, another plate(B) contains chicory root extract in ethyl acetate (Conc. 95 mg /ml).Other two plates (C and D) contain 1ml (conc 33mg/ml) and 500µl of chicory root extract in hexane respectively. In each plate, 500µl of fungal spores were spread by a glass spreader. After incubation at 30ºC for 3-4 days, the growth of fungal myecellium was checked. Similar experiment was performed on growth of yeast (*Saccharomyces cerevisiae*)

2.5 Fractionation of extracts by Column Chromatography and Paper Chromatography

Ethyl acetate extract was passed through silica gel column (120 mesh size), and then step gradient elution with increasing polarity was done. The different solvents and their ratio are as follows: Petroleum ether(fraction-1), Petroleum ether:Hexane (1:1) (fraction-2), Petroleum ether:Hexane (1:3)(fraction-3), Petroleum ether:Acetone(1:2)(fraction-4), Acetone:Ethylacetate(1:1)(fraction-5),Ethyl acetate:Chloroform (1:1)(fraction-6).Column volume was 172 cc. As fraction 5 showed both anti bacterial and antifungal activity, this fraction again analyzed (5a, 5b, 5c) using acetone and ethyl acetate in different ratios. Fraction 5a consisted of Acetone:Ethyl acetate (1:2), fraction 5b consisted of Acetone:Ethyl acetate (1:3) and fraction 5c contained Acetone:Ethyl acetate (1:4).Concentration of fraction 5 was 9.2mg/ ml. Concentration of fraction 5a ,5b and 5c were 4.1mg/ml , 1.8mg/ml and 3.1 mg/ml respectively.

To determine the number of compounds present in both extract, paper chromatography was carried out. Solvent system was Ethyl acetate:Methanol:Water(40:6:4) and the detection was with vanillin(0.1gm vanillin in 28 ml methanol+ 1ml H_2SO_4). The chromatographic spots obtained with fraction 5 were three of which the highest mobility coincides with fraction 5c.Antibacterial and anti fungal activities were studied with these fractions using agar diffusion assay.

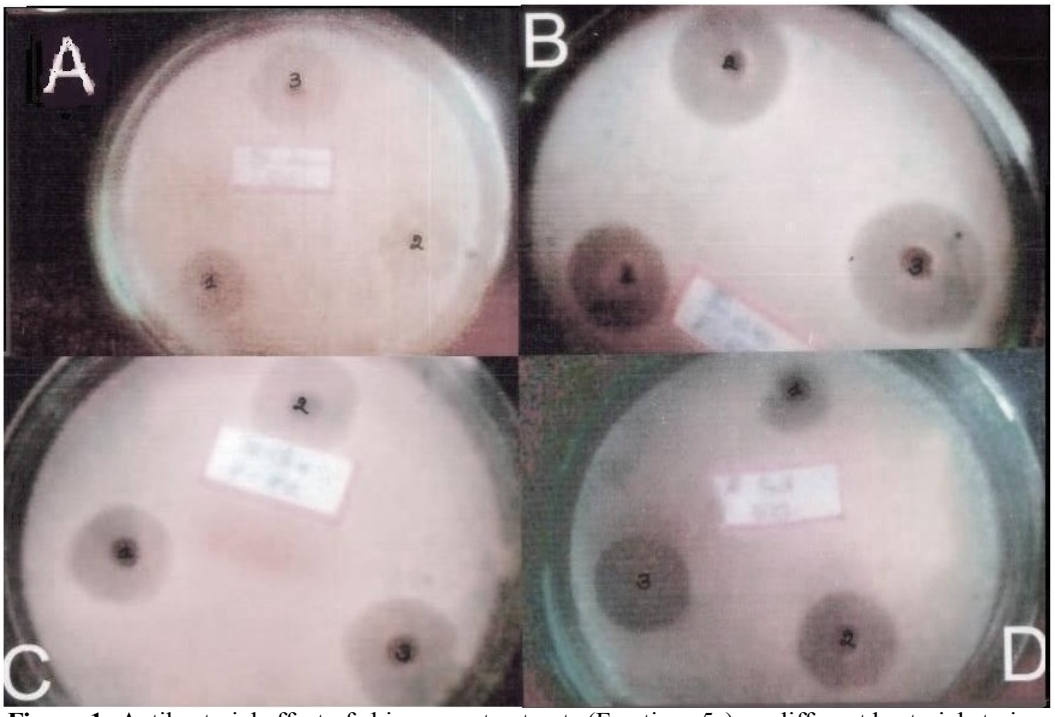

Figure 1: Antibacterial effect of chicory root extracts (Fraction- 5c) on different bacterial strain
A. Inhibitory effect of chicory root extract (5c) on the growth of *Staphylococcus aureus*
B. Inhibitory effect of chicory root extract (5c) on the growth of of *Bacillus subtilis*
C. Inhibitory effect of chicory root extract (5c) on the growth of *Vibrio cholerae*
D. Inhibitory effect of chicory root extract (5a) on the growth of *Pseudomonas fluorescens*

Lane-1 Lane-2
(Fraction-5) (Fraction-5C)

Figure 2: Paper chromatogram of fraction 5 (Lane1) and fraction 5c (Lane2) of chicory root

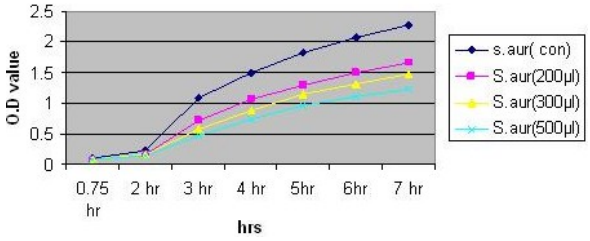

Figure 3: Bacteriostatic effect of ethyl acetate extract of chicory root (Fraction-5c) on growth of *Staphylococcus aureus* at different concentration.

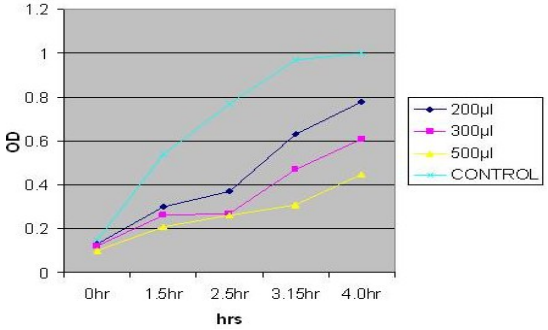

Figure 4: Bacteriostatic effect of ethyl acetate extract of chicory root (Fraction-5c) on growth of *Vibrio cholerae* at different concentration.

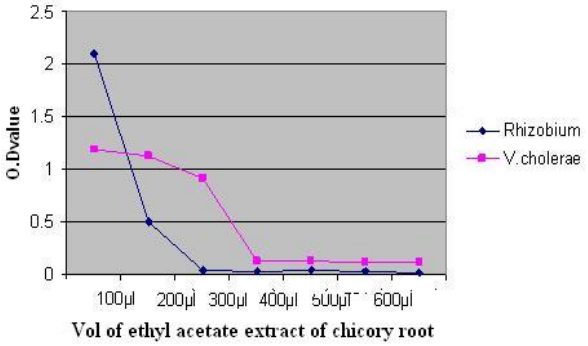

Figure 5: Determination of MIC of *Rhizobium leguminosarum* & *Vibrio cholerae* by chicory root extract (Fraction-5c) in Ethyl acetate.

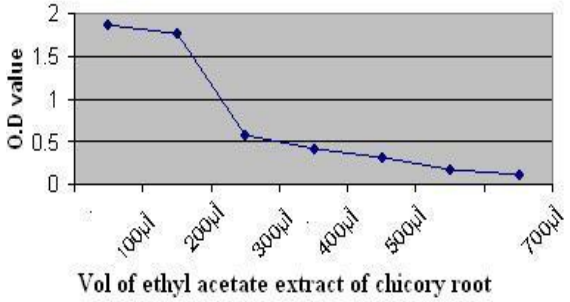

Figure 6: Inhibitory effect of ethyl acetate extract of chicory root (Fraction-5c) on growth of *Sachharomyces cerevisiae* at different concentration.

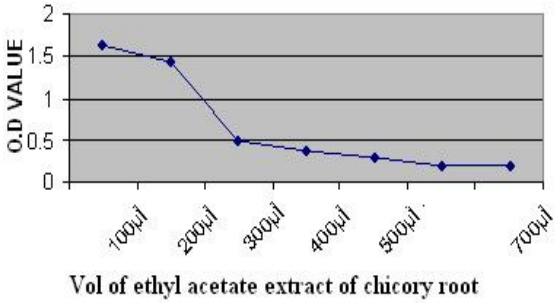

Figure 7: Inhibitory effect of ethyl acetate extract of chicory root (Fraction-5c) on growth of *Aspergillus niger* at different concentration.

Table1: Name (scientific) and source of Bacterial and Fungal strains used for this study

Bacterial and Fungal strains	Source of the strains	Code No.
Staphylococcus aureus	Indian Institute of Chemical Biology, Kolkata	--
Bacillus subtillis	Institute of Microbial Technology Chandigarh	MTCC- 121
Pseudomonas fluorescens	Institute of Microbial Technology Chandigarh	MTCC-134
Rhizobium leguminosarum	Institute of Microbial Technology Chandigarh	MTCC-99
Escherichia.coli	Indian Institute of Chemical Biology, Kolkata	--
Vibrio cholerae	National Institute of Cholera & Enteric Diseases, Kolkata.	--
Aspergillus niger	Institute of Microbial Technology Chandigarh	MTCC-96
Sachharomyces cerevisiae	Institute of Microbial Technology Chandigarh	MTCC-170

Table 2: Antibacterial activity (diameter of Inhibition zone in cm) of crude ethyl acetate extract of chicory (*Cichorium intybus*) root

Bacterial strains	Vol of ethylacetate extract (µl) of chicory root	Tetracycline(µl)
	40 60 80	80
Effect on Gram+ve bacteria		
Staphylococcus aureus	2.4 2.6 2.5	3.3
Bacillus subtilis	2.3 2.6 3.0	3.4
Rhizobium leguminosarum	1.2 1.5 1.6	2.9
Effect on Gram–ve bacteria		
Pseudomonas fluorescens	1.9 2.1 2.4	3.3
Vibrio cholerae	2.1 2.3 2.4	3.1
Escherichia coli	2.6 2.8 2.8	2.8

Concentration of Tetracycline was 25µg/ml. In each case the zone of Inhibition (cm) by pure solvents (Ethyl acetate) was nil.

Table 3: Antibacterial activity of fraction 5c (Diameter of inhibition zone in cm) of chicory (*Cichorium intybus*) root

Bacterial strains	Vol of (µl) fraction 5c			Tetracycline
	25µl	50µl	80µl	80µl
Staphylococcus aureus	0.6	1.2	1.5	3.3
Bacillus subtilis	0.7	1.0	1.6	3.4
Rhizobium leguminosarum	0.6	1.1	1.7	2.8
Vibrio cholerae	0.8	1.1	1.5	3.1
Escherichia coli	0.7	1.0	1.4	3.0
Pseudomonas fluorescens	0.5	0.8	1.4	2.9

Concentration of Tetracycline was 25µg/ml. In each case the zone of Inhibition (cm) by solvent mixture was nil.

Table 4: Bacteriostatic action of Fraction 5c of chicory (*Cichorium intybus*) root extract on growth of *Satphylococcus aureus* and *Vibrio cholerae*

Tube	OD value (610nm)	OD value (610nm)
Control	1.020	0.846
Chicory 5c extract	0.125	0.212
Fresh Luria Bertani	0.925	0.831
Tetracycline	0.081	0.087

Table 5: MIC values of ethyl acetate extract of chicory root (fraction-5c) on different bacterial strains used for this study

Bacterial strains	Minimum Inhibitory Concentration (µl)	Affinity to Gram stain
Staphylococcus aureus	240	**Positive**
Bacillus subtillis	250	**Positive**
Pseudomonas fluorescens	350	**Negative**
Rhizobium leguminosarum	250	**Positive**
Escherichia.coli	350	**Negative**
Vibrio cholerae	350	**Negative**

Table 6: Effect of ethyl acetate extract of chicory (*Cichorium intybus*) root on *Aspergillus niger* and *Sachharomyces cerevisiae*

Plate number	Composition	Fungal growth
A	YEPD + 500µl Ethyl acetate.	High growth
B	YEPD+500µl Ethyl acetate extract (Fraction 5c)of chicory root.	No growth
C	YEPD + 1 ml Hexane extract.	High growth
D	YEPD + 500µl Hexane extract.	High growth

3. Result

The antibacterial property of the ethyl acetate extract of chicory root appeared to have considerable effects on studied Gram positive as well as Gram negative bacteria (Table-2). Hexane extract of chicory root on the other hand showed no such antibacterial effect. The ethyl acetate extract of chicory root had also antifungal effect on *Aspergillus niger* and *Sachharomyces cerevisiae*. Column chromatography was performed with an aim to isolate the active fraction, responsible for antimicrobial activity. The fraction 5 which was eluted by using solvent Ethyl acetate and Acetone in the ratio of 1:1 as the mobile phase, only showed antimicrobial (both antibacterial and antifungal) activity. The other fractions had hardly any antimicrobial effect. When fraction 5 was further subjected to column chromatography by changing the ratio of ethyl acetate and acetone concentration, 3 fractions (5a, 5b and 5c) were obtained. Among them the fraction 5c showed the antimicrobial effect (Fig-1) (Table-3) where fraction 5a and 5b had no such activities.

After paper chromatography analysis, it was shown that there were three spots for fraction 5 and fraction 5c had only one spot in the chromatogram (Fig-2).The fraction 5c had bacteriostatic effect rather than bacteriocidal effect on both Gram positive and Gram negative bacterial strains. *Staphylococcus aureus* (a representative of Gram positive bacteria) showed inhibition of growth which is directly proportional to increasing concentration of fraction 5c (Fig-3).Similar effect was observed in case of *Vibrio cholerae* (Fig-4) which is a Gram negative bacteria (Table-4). Minimum Inhibitory Concentration (MIC) was determined for all the studied bacteria (Table-5). In case of *Rhizobium legumiorasum* MIC was achieved when 250 µl of fraction 5c was put in the

well. In case of *Vibrio cholerae*, MIC was achieved when 350 µl of fraction 5c was put in the well (Fig-5).The concentration of fraction 5c was 3.1mg/ml.

Antifungal activity was also observed by using fraction 5c.This fraction has growth inhibitory effect on *Sachharromyces cerevisiae* (Fig-6) as well as on *Aspergillus niger* (Fig-7).Hexane extract of chicory root had no effect on the growth of *Aspergillus niger* and *Sachharomyces cerevisiae*, even with higher concentration(Table-6).

4. Discussion

Though antimicrobial activity of Chicory root was reported earlier [12, 16, 17] but the isolation of the active fraction responsible for this antimicrobial action is also very much required. From this study it is demonstrated that the action of active principle in chicory root is more intense on Gram positive bacteria than Gram negative bacteria as evident from the fact that MIC value of active fraction of chicory root is less in case of Gram positive bacteria than Gram negative bacteria. It indicates that outer envelope of Gram negative bacteria to some extent protecting the Gram negative bacteria from inhibitory action of chicory root extract. As chicory root also have fungal inhibitory actions so the mechanism of action of active principle is as applicable on both prokaryotes and eukaryotes. However hexane extract of the chicory root has neither antibacterial nor antifungal activities indicating that active principle of chicory root is certainly a polar compound. This extract can be used for the preparation of effective antimicrobial agents. The present work shows that the compounds from chicory possess potent antimicrobial activity and suggesting that the chicory root extracts contains the effective active constituents responsible for eliminating the bacterial concentration.

Finally, it can be concluded that the active chemical compounds present in chicory (*Cichorium intybus*) should certainly find place in treatment of the various bacterial infections. The results of this study are very encouraging and indicate that this herb should be more extensively studied to explore its potential in the treatment of many infectious diseases. Further investigation is needed to determine the actual chemical structure of the active compound of chicory root.

Acknowledgement

P.Roy wishes to acknowledge Mrigendranath Mandal endowment chair for professor in Biotechnology at The University of Burdwan.

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Source of support: Nil; Conflict of interest: None declared