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Purification of Hydroxychavicol from *Piper betle* Linn and Evaluation of Antimicrobial Activity against Some Food Poison Causing Bacteria

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The aim of the present study is carried out to purify the medicinally potent Hydroxychavicol from methanolic extract of Betel leaf (*Piper betle* L.) and evaluation of its antimicrobial activity against some of the selected food poison causing pathogens. Hydroxychavicol was successfully purified by silica gel assisted column chromatography. Authenticity of purified Hydroxychavicol was confirmed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). Minimal Inhibitory Concentration (MIC) and Minimal Bacterial Concentration (MBC) of *E.coli and Salmonella* were found to be in the range between 20-50 µg. Furthermore, hydroxychavicol was also tested for Time-kill curve and its potential to inhibit the formation of biofilm against *E.coli and Salmonella* respectively. In summary, antimicrobial study strongly suggests that the hydroxychavicol possess potent antimicrobial activity against *E.coli and Salmonella*.

Keywords: *Piper betle*, Hydroxychavicol, Antimicrobial suspitability, Food Poison.

Multi drug resistance in human, animal and plant pathogens have developed due to misuse of antibiotics commonly applied in the treatment of infectious diseases[1]. In recent years, common oral and food poison infections are having more health problems in human beings. Among the common oral diseases, Food Poison infections having more infective pathogens. The prevalence of antimicrobial resistance towards Food Poison infection has increased during the recent decades.

However, many of the available antibiotic drugs for Food Poison infection have undesirable side effects or secondary effect like hepatoxicity, hypertension, show drug-drug interactions or lead to the development of resistance and some shows mutagenic effect[2]. This alarming situation has led microbiologists to discover new antimicrobial compounds from various sources, including medicinal plants. According to World Health Organization (WHO), 80% of world population depends on traditional herbal medicine for the treatment of fungal and bacterial infections[1]. It has now been established that the plants which naturally synthesize and accumulate secondary metabolites like phenolic acids, alkaloids, flavonoids, glycosides, tannins, and volatile oils possess antimicrobial properties[3].

Piper betle Linn, is a handsome evergreen and perennial creeper, with glossy heart-shaped leaves commonly known as the Betel leaf. It belongs to the family of piperaceae growing to a height of 150 to 180 cm. The plant is originated from India, Philippines, Bangladesh and Sri Lanka. In Indian system of medicine and health has adopted the use of betle leaves in various ways. Especially in folkloric medicine, Betle leaf is popular used as an antiseptic and is commonly

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applied on wounds and lesions for its healing effects. It contains biologically[5]active phyto molecules like Beta-carotene, Hydroxychavicol, Vitamin E, Urosolic acid, Sisterols, Eugenol etc., and it possesses potent anti-oxidant[4], anti-inflammatory, anti-bacterial, anti-fungal, anti-cancer, anti, anti-diabetic, hepatoprotective, anti-ulcer, and immunomodulatory effects.

Generally, phenolic compounds are considered as potential therapeutic agents against a wide range of ailments including neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunction, inflammatory diseases and in ageing. One such abundant and medicinally important phenolic compound in the betle leaf is hydroxychavicol. Epidemiological studies suggest that Hydroxychavicol possesses various biological activities[6] like antioxidant, anti mutagenic, antimicrobial, anti-inflammatory and cytotoxic action. The antibacterial and antifungal studies of Hydroxychavicol and various extract of Betel leaves are already established against some of the common human pathogens[7]. However, there are no scientific reports dealing the antimicrobial suspitability of oral cavity pathogens against purified Hydroxychavicol of methanolic extract of betel leaves. In this connection, an attempt has been made to evaluate the antimicrobial activity of purified hydroxychavicol against some selected Food Poison pathogens.

MATREIAL AND METHODS

Plant material

The leaves of *Piper Betle* linn were collected from the botanical garden at KCT, Coimbatore, India. The species was identified and confirmed at Botanical Survey of India (BSI), Southern Circle, Coimbatore, India and the Voucher specimen was retained in the laboratory for future reference. All the chemicals and reagents were used the analytical grade purchased from Sigma Chemical Co. and Merck.

Extraction and Purification of Hydroxychavicol

About 1 kg of air-dried leaves were dissolved in 3 vol of 85 % methanol and kept in an continuous stirring for 4 hr[8]. The obtained extract was filtered with Whatman no.1 filter paper and the filtrate was collected. The solvents were then removed by Rotovac Evaporator under reduced pressure at 50°C to obtain the concentrated extract. Concentrated extract was then extracted with hexane in a separating funnel. The Hexane fraction was concentrated under reduced pressure to yield a residue (10.05g) containing hydroxychavicol, again the concentrated hexane layer undergone with ethyl acetate wash to get 85% hydroxychavicol where monitored by RP-HPLC. The Hydroxychavicol-enriched residue (5.0 gm) was chromatographed on a silica gel column (200 g; 100 to 200 mesh filter; 60 cm by 3.2 cm using 1.0% ethyl acetate in chloroform (v/v) as eluting solvent. Fractions of 100 ml each were collected and subjected to RP-HPLC in methanol-water (10:90). The fractions containing pure Hydroxychavicol were pooled, crystallized at reduced pressure of MP 48°C. Purity of the hydroxychavicol and its concentration in the crude were established by RP-HPLC.

RP-HPLC analysis of Hydroxychavicol

The purity of Hydroxychavicol and its concentration in the crude extracts were established by Agilent 1220 HPLC at 30°C using C_{18} column (5 µm pore size; 250, 4.0-mm internal diameter) and UV detection at 280 nm. Sample was eluted at a flow rate of 1.5 ml/min with methanol-water containing 1.5% acetic acid (10:90) for 5 min, and the methanol concentration was increased in the gradient up to 20% over 60 min and held for 5 min, followed by a decrease in the methanol concentration up to 8% over 70 min and held for 5 min.

Bacterial strains and culture condition

The pathogenic bacterial strains were obtained from MTCC (Microbial Type Culture Collection, Chandigarh, India). *E.coli* and *Salmonella* were maintained on Luria–Bertani medium brain heart at 37°C.

Determination of MIC and MBC

The MIC of the Hydroxychavicol of (P*iper Betle* leaf) was determined by tube dilution techniques in Mueller-Hinton broth [9]. Inoculates were prepared in the same medium at a density adjusted to 0.5 McFarland turbidity standard (108 CFU/ml). The series of concentration used was 50, 100,150,200, and 250,ug/ml and were prepared by using Double Distilled water. The MIC was done at 37°C, and was recorded after 24 hours of incubation. The MIC was defined as the lowest concentration of extracts at which the microorganism tested did not demonstrate visible

growth. The minimum bactericidal concentration (MBC) was determined by spreading a 100 µl on a Mueller-Hinton agar plate from the wells showing no visible growth. The plates were incubated at 37°C for 24 h. The minimum concentration of compound that showed 99.9% reduction of the original inoculums was recorded as the MBC[10]. Minimum bactericidal concentration (MBC) was defined as the lowest concentration yielding negative subcultures or only one colony.

Reduction of hydroxychavicol against *E.coli*

Biofilm formation by *E.coli* was performed[11]. Briefly, 100 µl culture of *E.coli* (1 X 107 to 1 X 108 cells/ml) was inoculated into 10 ml of fresh Luria–Bertani medium brain heart broth containing 2% sucrose (wt/vol) in the test tubes and incubated at 37^0C for 24 h at an disposition of 30°C. The fluid containing plank tonic cell was gently removed. The water-insoluble glucan containing cells of *E.coli* were gently washed with 10 ml of sterile water and resuspended in 10 ml of citrate buffer (10 mM, pH 6.0) containing 20-50µg/ml of hydroxychavicol, followed by incubation at 37°C for 5 min. The mixture was gently washed again with sterile water containing 0.1% tween 80 (wt/v), followed by the resuspension of treated cells in 10 ml of BHI broth containing 2% sucrose (wt/v) and 0.1% tween 80 (wt/v). After incubation of cells at 37^0C for 6, 12, 18 and 24 h the acid produced by the culture was measured by using pH meter. The fluid containing free cells of *E.coli* was gently removed. The water insoluble glucan was resuspended in 10 ml of sterile water and homogenized using ultrasonic bursts, and the turbidity was measured at 595nm.

Time-kill studies against *E.coli*

E.coli was grown in Luria–Bertani medium brain heart broth at 37°C for 24 h separately[13]. The turbidity of the suspension was adjusted to 0.5McFarland standard in sterile normal saline. A total of 100ul of this suspension was used to inoculate 20 ml of Luria–Bertani medium brain heart broth containing increasing concentrations of hydroxychavicol ranging from 25 to 250µg/ml. Suspensions were incubated at 37°C, and the number of CFU was determined on Luria–Bertani medium brain heart agar using a serial dilution method at various time points.

Statistical analysis

All data were calculated as means ±S.D (n=3). The significance among different data was estimated by one way (ANOVA) and student t-test.

RESULT AND DISCUSSION

Natural products are in great demand for their extensive biological properties and bioactive components (phenols, flavonoids, saponins, glycosides, terpenoids etc.,) which have been proved to be useful against large number of causative agents of diseases[14]. Many researchers have been established isolation and purification of phytoactive compounds from natural source in recent decades. These phytoactive compounds are 100% natural, have less side effect and fight against wide range of disease alignment[15]. Extraction of Hydroxychavicol from aqueous, ethanol and chloroform extract of betel leaves were already reported[16]. However, this is the first scientific report deals with the extraction of hydroxychavicol from betel leaf by using 85% methanol as an extracting agent. Present study, Hydroxychavicol is extracted from methanolic extract of Betel leaf and purification is carried out by silica assisted column chromatography. Hydroxychavicol containing crude sample is concentrated and further extracted with hexane. Finally, Hydroxychavicol-enriched residue is chromatographed on a silica gel column (200 g; 200-mesh filter; 60 cm) for purification purpose.

Generally, hydroxychavicol having high polar nature which resulting, RP-HPLC is most common and suitable method for the separation of Hydroxychavicol in the plant material. Recently it is reported that the separation of hydroxychavicol by using TLC in Betel leaf of various extract[16]. In this study, the purity of hydroxychavicol and its concentration in the crude were established by RP- HPLC. Hydroxychavicol is eluted (Fig. 1a) at a flow rate of 1.5 ml/min with mobile phase A-acetonitrile and B-water containing 1.5% acetic acid (10:90) for 5 min, and the acetonitrile concentration is increased in the gradient up to 20% over 60 min and held for 5 min, followed by a decrease in the acetonitrile concentration up to 8% over 70 min and held for 5 min. The purity of Hydroxychavicol in the column chromatography (Fig. 1b) fractionate was checked by using RP-HPLC and it is found to be 80.23±0.25%.

In Indian scenario, many researchers have reported antimicrobial activity for various crude plant extract and few of them only have established antimicrobial activity against purified bioactive compounds from natural source[8]. It has been already proved that various extract of Betal leaves and its isolates possess antimicrobial activity against some common pathogens. Moreover, this is the first scientific report deals the antimicrobial activity of purified hydroxychavicol against Food poison causing pathogens. In this study, Antimicrobial activity of hydroxychavicol against *E.coli* is determined by MIC and MBC. Hydroxychavicol exhibites an MIC range of 20 to 50µg/ml against the selected Food poison pathogens, whereas the MBC is found to be twofold greater than the inhibitory concentration, as shown in the Table 2. MIC is determined by decreasing of turbidity of microbial growth with the increasing of drug (hydroxychavicol) dose at 595 nm. MBC is determined (Fig.2) by zone formation of organism due to the drug action. It is clearly observed that zone formation of microbial growth inhibition is increased when drug concentration increase. This antimicrobial activity is due to the inhibition of cell wall growth proteins of *E.coli* by hydroxychavicol. Recent studies have shown that many plant derives phenolic compounds and related polyphenols contribute[17]significantly to the inhibition of cell wall growth proteins of many pathogenic microbes.

In many cases, *Salmonella, Campylobacter, Listeria*, are the most causative microbes for Food Poison diseases. In which, Food Poison microbes,

Table 1. Antimicrobial activity of hydroxychavicol expressed in terms of (%) growth inhibition. Chlorohexidine (0.12%) was used as a positive control. All values represent the mean (SEM of three independent experiments carried out in triplicate. All data were calculated as means ±S.D (n=3). The significance among different data was estimated by one-way (ANOVA) and student t-test;$p < 0.05$.

S. No	Concentration (µg/ml)	Inhibition (%) *E.coli*	*Salmonella*
1	20	32.8±08	27.99±11
2	25	37.99±62	30.19±07
3	30	48.23±71	41.02±77
4	35	67.55±90	50.77±19
5	40	76.11±69	65.60±90
6	45	89.15±13	74.91±78
7	50	90.1±60	89.52±56
8	Control	93.22±82	97.80±30

Table 2. Antimicrobial activity of hydroxychavicol expressed in terms of zone of diameter (mm). Chlorohexidine (0.12%) was used as a positive control (). All values represent the mean (SEM of three independent experiments carried out in triplicate. Different letters indicate significative differences between samples according to one-way ANOVA and Student test; $p < 0.05$

S. No	Concentration (µg/ml)	Zone of inhibition *E.Coli*	*Salmonella*
1	20	1±23	2±94
2	25	3±82	4±08
3	30	4±09	6±75
4	35	6±51	8±16
5	40	8±13	10±56
6	45	8±07	11±88
7	50	9±17	13±13
8	Control	12±82	13±86

Table 3. Potential of biofilm reduction of hydroxychavicol expressed in terms of inhibition (%). Chlorohexidine (0.12%)was used as a positive control (). All values represent the mean (SEM of three independent experiments carried out in triplicate. Different letters indicate significative differences between samples according to one way ANOVA and Student test; $p < 0.05$.

S. No	Concentration (µg/ml)	Inhibition (%) *E.Coli*	*Salmonella*
1	20	26.31±58	31.25±09
2	25	42.10±53	37.50±42
3	30	52.63±16	43.75±50
4	35	68.42±11	62.41±71
5	40	73.68±42	75.07±03
6	45	89.47±30	93.56±67
7	50	96.31±58	98.10±81
8	Control	85.26±39	90.06±96

in the human consuming food produce acidic environment and form biofilm on the Stomach. This may eradicate the strengthens of the food poison pathogen[12]. At this stage, it is necessary to inhibit the formation of acid production and Poison formation. In the present study, different concentration (20-50 µg/ml) of hydroxycavicol is in checked the inhibitory effect of water-soluble glucan produced by *E.coli*. Recently, it is proved that Betel leaf extract and its isolate effectively inhibits the formation against *E.coli* species[13].

The time-kill kinetics study is performed against *E.coli*. The time-kill kinetics study shows that hydroxychavicol exhibited a time and concentration dependent killing effect against *E.coli* is specifically chosen because, it has major causative agent for formation and Food Poison. Recently[13], it is reported that hydroxycavicol has effectively involved in time and concentration depended killing of *E.coli*. Present study also suggests that hydroxycavicol has potent antibacterial activity against the pathogens.

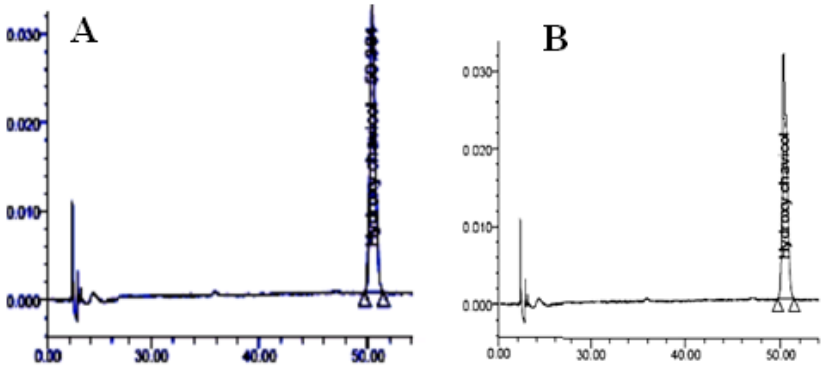

Fig. 1. RP-HPLC analysis of purified hydroxychavicol A-Standard; B-Purified sample

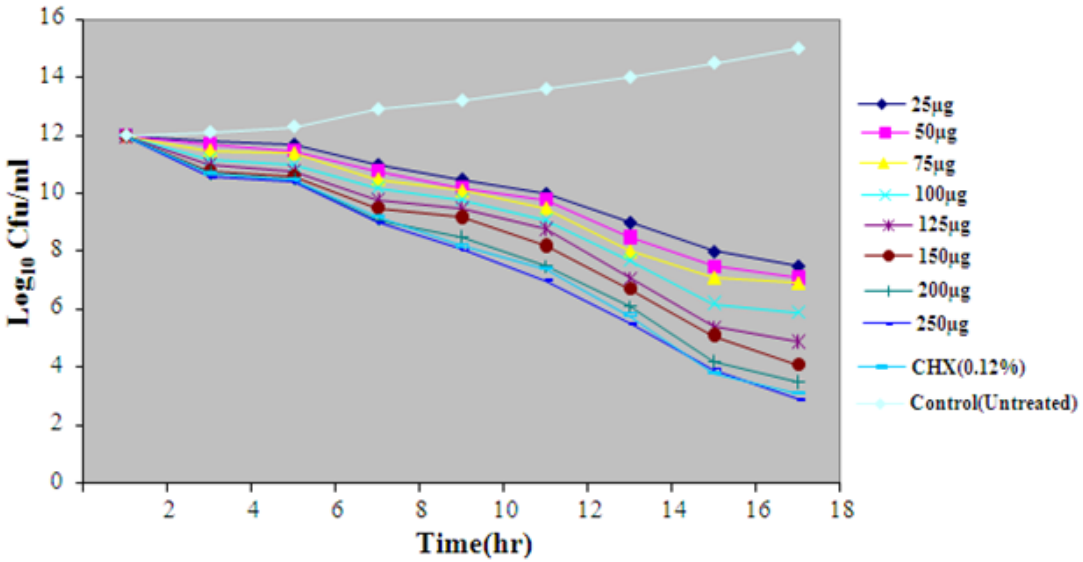

Fig. 2. Time -kill study expressed in terms of $\log_{10}$ reduction of turbidity values. Chlorohexidine (0.12%) was used as a positive control. All values represent the mean (SEM of three independent experiments carried out in triplicate. Different letters indicate significative differences between samples according to one-way ANOVA and Student test $p < 0.05$.

Similarly, many of the researches have reported that betal leaves contains phenolic compound such as hydroxycavicol, quercetin, Eugenol, carotenoids etc., effectively inhibit the growth of common food poison pathogens[4].

CONCLUSION

In summary, Hydroxychavicol showed potent antimicrobial activity against *E.coli and Salmonella*. In addition, it has effectively inhibited the formation of biofilm by against *E.coli*. The results also suggest that hydroxychavicol containing antimicrobial properties that can be useful to control Food Poison pathogens. In future, further investigations may carry out to preparation of Ready to Eat Food along with hydroxycavicol as ingredients.

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