

IN VITRO ANALYSIS OF ORNITHINE DECARBOXYLASE INHIBITORY ACTIVITY OF THE EXTRACTS OF *EXCOECARIA AGALLOCHA* L.

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Received: 15 October 2016, Revised and Accepted: 02 November 2016

ABSTRACT

Objective: Ornithine decarboxylase (ODC) is one of the key enzymes involved in polyamine synthesis that has a prominent regulatory role in cell growth in prokaryotes and eukaryotes. Its regulation/inhibition is a potential means of controlling cell growth. The purpose of the study was to check the effect of the methanol extracts of leaves and seeds of the mangrove plant *Excoecaria agallocha* in controlling/inhibiting ODC enzyme produced by *Escherichia coli*.

Methods: The methanol extracts of leaves and seeds of the mangrove plant *E. agallocha* were made. The inhibitory effect of the extracts on ODC enzyme produced by *E. coli* was tested by a modified spectrophotometric assay (measured in terms of μM of putrescine produced), and the antibacterial effect of the extract was determined by well diffusion assay.

Results: It was found that both the leaf and seed extracts inhibited ODC activity thereby preventing the conversion of ornithine to putrescine (the prominent by-product of ODC activity) in a concentration-dependent manner. The leaf extract exhibited 50% inhibition of ODC activity at a concentration of 17.5 $\mu\text{g/ml}$ whereas seed extract inhibited 50% of the activity at a concentration of 16.1 $\mu\text{g/ml}$. In the growth inhibition assay by well diffusion method, the effect of the extracts on the growth of *E. coli* was studied and a concentration-dependent growth inhibition was found - 100 μl of leaf extract showed a zone of inhibition of 12.31 mm (diameter) and seed extract showed 12.64 mm, confirming the antibacterial activity of the extracts.

Conclusion: The ODC inhibition-mediated growth inhibition of *E. coli* is a novel finding as far as the extracts of *E. agallocha* are concerned. The extract was potent enough to be suggested as an antibacterial agent.

Keywords: Ornithine decarboxylase, *Excoecaria agallocha*, *Escherichia coli*, Ornithine decarboxylase inhibition, Putrescine, Ornithine, Antibacterial effect.

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INTRODUCTION

Polyamines are low molecular weight aliphatic polycations. They are highly charged and are ubiquitously produced by/in all living cells. Although formed in wee amounts, they are involved in a large number of cellular processes such as cell differentiation and growth, cell division, senescence, growth regulation, and tumor progression. They have a decisive role to play in cell proliferation [1].

Ornithine decarboxylase (ODC) is one of the main rate-limiting enzymes in polyamine biosynthesis that is under strict regulatory control. Decarboxylation of L-ornithine results in the formation of alkaline by-products like putrescine. Further metabolic processes will result in the formation of standard polyamines [2]. Various microorganisms are capable of producing decarboxylase enzymes and each enzyme produced by the organism is specific to the amino acid on which it acts. These enzymes are highly inducible and are capable of removing the carboxyl group from amino acids. Inhibition of these enzymes can prove to be an effective strategy to control many of the diseases especially those where polyamines tend to assist the growth of cells.

Many molecules are capable of inhibiting the activity of this enzyme [3]. Plant secondary metabolites are capable of depleting the available pool of polyamines through inhibition and degradation of ODC. Apigenin, a plant flavonoid, inhibited TPA-induced epidermal ODC in a dose-dependent manner [4]. Although a wide range of ODC

inhibitors is available, most of them exhibited a very narrow range of action in clinical and therapeutic trials. The urge to find more typical inhibitors of ODC is even sturdier. Recent researches attempt at finding a more suitable inhibitor of ODC. With more and more plant products being tested, it is expected that one or the others will yield promising result. Thus, this study was initiated with the objective to check the inhibitory effects of the methanol extract from the leaves and seeds of *Excoecaria agallocha* L. against the production of ODC enzyme by *Escherichia coli*.

E. agallocha is a mangrove plant commonly known as "blind your eyes" tree. It is the only mangrove plant with milky latex. It is understood that this plant is rich in a variety of chemical compounds as exclusively reviewed [5]. Although studied extensively for phytochemical and antimicrobial activity, the ODC inhibitory activity of the extracts from *E. agallocha* was not exploited previously. Thus, the purpose of this study is to find if the extracts are capable of inhibiting the activity of the enzyme, and consequently, serve as a novel source of ODC inhibitor.

METHODS

Chemicals

Putrescine dihydrochloride, sodium tetraborate decahydrate, perchloric acid, ODC broth, pyridoxal 5-phosphate hydrate, and L-ornithine monohydrochloride were from HiMedia. Picrylsulfonic acid solution and 1 - pentanol were from sigma. All other chemicals used are of analytical grade.

Plant material

The leaves and seeds of *E. agallocha* collected during the summer season of 2014, from Wadakara, near Kozhikode, Kerala, India, were used for the study. Taxonomical identification of the plant was done by Dr. P. M. Unnikrishnan, FRLHT, Bengaluru, India, by referring to the voucher specimen housed at the herbarium at FRLHT.

Sample preparation

The leaves and seeds (40 g each) were shade-dried for 7 days and ground coarsely and extracted with 100% methanol (3 times, 500 ml for 2 days) at room temperature. The extracts were combined and concentrated in vacuum at 30°C in rotary evaporator. The residue was resuspended in minimum quantity of methanol and stored at 4°C for further use.

Microbial culture

E. coli strains (#2939) were obtained from the Mycrobial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. After receiving the stock cultures, the strain was maintained as subculture and stored as per the instruction.

Preliminary assay to detect the activation of ODC enzyme by *E. coli*

The usage of ornithine by the microbe is generally achieved by ODC. The ability of *E. coli* in activating the ODC enzyme was tested in this assay. The assay was carried out according to Taylor [6], with slight modification, to find whether the test microbe was capable of using the amino acid ornithine as a source of energy and carbon for its growth and produce the diamine. In short, pure culture of test microbe (*E. coli*) was inoculated in ODC broth containing 0.5% ornithine. Enough care was taken to lock out oxygen to assist fermentation. The tubes were then incubated at 35-37°C for 24 hrs and change in color was noted. The culture was incubated for an additional 24 hrs (same conditions), and the change in color was noted again. Reverting back of the color of the media was indicative of positive ODC activity, the absence of which denotes negative ODC activity.

Inhibitory effect of the extracts on the activation of ODC enzyme

To check the effect of the extracts of *E. agallocha* on inhibiting ODC enzyme activity, the test was performed as explained above. However, once the ODC was activated and the color of the media changed to yellow (after the first incubation period of 24 hrs), the methanol extract of the leaves and seeds of *E. agallocha* was added to the respective culture tubes. The culture was incubated for an additional 24 hrs (same conditions) and the change in color was noted. Failure in purple coloration of media indicates that ODC activity is inhibited by the extract.

Preparation of crude ODC enzyme from *E. coli*

The test organism was cultured in LB broth containing 50 µg/ml kanamycin [7] and incubated for 20 hrs at 18°C. After incubation, the sample was centrifuged (3500 rpm, 10 min, 4°C) and the pellet was suspended in potassium phosphate buffer (pH 6.0) containing 1 mM DTT, 5 mM MgSO₄, and 0.04 mM pyridoxal phosphate (PLP). The sample was sonicated at 300 W for 15 s at a time for a total of 5 min using ethanol ice bath and centrifuged at 16,000 × g for 50 minutes. Further, the supernatant was dialyzed for 20 hrs using the same buffer and used directly for *in vitro* ODC assay. The amount of protein obtained was assessed by bicinchoninic acid method.

In vitro assay to detect ODC enzyme activity

Further, to test the ODC enzyme activity, an *in vitro* assay was carried out with slight modification [8]. This spectrophotometry-based assay depends on the quantitative detection of the substrate and end product of polyamine synthesis, namely, ornithine and putrescine, respectively. In short, 5 µl of various concentrations (10-100 µg/ml) of ODC enzyme (crude enzyme sample prepared as discussed under the section-preparation of crude ODC enzyme from *E. coli*) were prepared in duplicates along with proper control. 400 µl of substrate reaction mixture (β-mercaptoethanol [2.5 mM], disodium EDTA [1.5 mM], PLP [75 nM],

and L-ornithine HCl [3 mM] in 150 mM phosphate buffer, pH 7.1) was added to the tubes containing the enzyme and incubated at 37°C for 30 minutes. The reaction was stopped by adding 400 µl of perchloric acid (1 M) or 400 µl of trichloroacetic acid (10%). Formation of precipitate indicates the end of enzymatic reaction. The sample was centrifuged at 5000 rpm for 5 minutes at room temperature. 200 µl of 4 N NaOH was added to 100 µl of supernatant of standards (ornithine/putrescine 0-50 µM) and/or terminated reaction mixture. 400 µl of 1-pentanol was added to the mixture and vortexed vigorously for 1 minute. The sample was centrifuged (5 minutes at 2000 rpm). 200 µl of upper (organic) phase was transferred to a fresh tube containing 200 µl of sodium borate (0.1 M, pH 8.0) and mixed properly. 200 µl of trinitrobenzenesulfonic acid (picryl sulfonic acid, 10 mM in 1-pentanol) was added and mixed properly. 400 µl of dimethyl sulfoxide was added and mixed for 1 minute and centrifuged (5 minutes at 3000 rpm). A very fine two layers will be observed with top layer containing TNP-putrescine-TNP and bottom layer containing TNP-ornithine-TNP, the absorbance of which are read at 450 nm. Based on the capacity of ODC enzyme to convert ornithine to putrescine, the amount of ornithine remaining in the solution and putrescine formed at various stages of the assay can be calculated. The amount of ornithine remaining in solution and putrescine formed was obtained by plotting a standard graph of ornithine/putrescine (0-50 µM) and calculating the absorbance of test sample in terms of µM of ornithine/putrescine produced.

Inhibition of ODC activity by the extracts and determination of growth inhibitory concentration

To check the inhibitory activity of the extracts on the enzyme activity, various concentrations of the plant extracts were added to the ODC enzyme, and the assay was repeated. The IC₅₀ concentration of the extracts was determined by plotting a graph between sample concentration and percent inhibition. Further, the percentage of enzyme inhibition was calculated using the formula:

Control difference=Mean OD of control with enzyme-mean OD of control without enzyme,

Sample difference=Mean OD of control with enzyme-mean OD of control without enzyme,

$$\text{Percent inhibition} = \frac{\text{Control difference} - \text{sample difference}}{\text{Control difference}} \times 100$$

Antibacterial activity of the extracts of *E. agallocha*

To test the antibacterial effect of the extracts, the standard agar well diffusion method was employed. Fresh cultures of *E. coli* were inoculated in agar well plates and three different concentrations (25, 50 and 100 µl/ml) of the leaf and seed extracts of *E. agallocha* were added to the agar wells and incubated overnight at 37°C with proper control. After incubation, the growth inhibition was tested by measuring the respective zones of inhibition.

Further, to test the mechanism behind the inhibition of bacterial growth by the extracts, a detailed experiment was designed to check the ODC activation by *E. coli* and its inhibition by the extracts of *E. agallocha*. The amount of ornithine and putrescine present at various stages of the assays were analyzed separately to substantiate the claim that ODC inhibition is indeed the reason behind antibacterial activity of the extracts. The various assays are as follows.

Statistical analysis

Results are expressed as the average of three independent experiments ± standard deviation.

RESULTS

Activation of ODC enzyme and its inhibition by *E. agallocha* extracts

As described in materials and methods, the media was inoculated with *E. coli* with proper control (Fig. 1a). After incubating the media for

24 hrs, a change in the color of the media was observed. The microbe used the glucose present in the media and the pH of the media dropped as indicated by change in color (purple to yellow) implying ODC activation (Fig. 1b - tubes 2-4). A further incubation of 24 hrs resulted in the reverting back of the color of media (yellow to purple) in the tube containing *E. coli* alone (Fig. 1c - tube 2), whereas media remained yellow in the tube containing *E. coli* and *E. agallocha* leaf and seed extracts (Figs. 1c, 3, and 4, respectively). This clearly demonstrated that ODC enzyme was produced by *E. coli* and was activated in the sample containing *E. coli*. However, sample tubes containing methanolic extracts of leaf and seed of *E. agallocha* (Fig. 1c - tubes 3 and 4 respectively) inhibited the activity of ODC enzyme produced by *E. coli*.

In vitro analysis of the effect of extracts on ODC activity

The effect of the extracts of *E. agallocha* on the inhibition of ODC and in turn, on the conversion of ornithine to putrescine was analyzed. It was

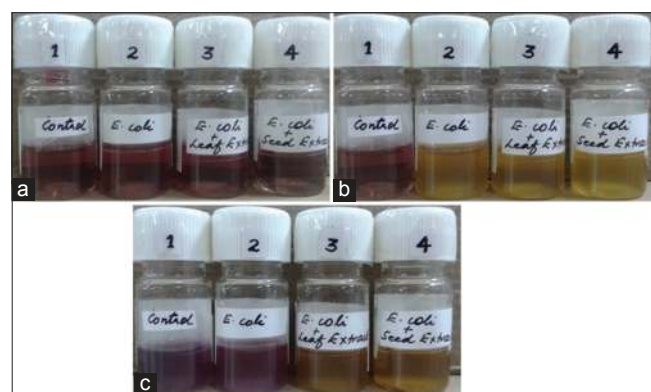


Fig. 1: Analysis of the inhibitory activity of *Excoecaria agallocha* leaf and seed extracts on ornithine decarboxylase (ODC) produced by *Escherichia coli*. (a) before inoculation, (b) 24 hrs. after inoculation (c) 24 hrs. after adding extracts. 1-control, 2-ODC broth with *E. coli* cells, 3-ODC broth with *E. coli* cells and leaf extract, 4-ODC broth with *E. coli* cells and seed extract. ODC activation is indicated by the yellow color of the media (B2, B3, B4). The reverting back of yellow color to purple is indicative of ODC activity (C2) whereas failure in purple coloration of media is indicative of inhibition of ODC activity (C3, C4)

found that both the extracts were capable of inhibiting the ODC activity as was evidenced by the *in vitro* analyses. The amount of ornithine/putrescine at various stages of incubation revealed that the extracts had potent inhibitory effect on ODC activation. Further results are discussed herewith.

Amount of putrescine/ornithine produced at various stages

The amount of putrescine/ornithine produced at various stages in the course of the experiment was analyzed to find out the activity of ODC enzyme produced by *E. coli* and also to check its inhibition by *E. agallocha* extracts. This was calculated from the standard graphs prepared using commercial putrescine/ornithine (Fig. 2a and b). The amount of ornithine and putrescine detected at various stages are represented in Fig. 2c and d.

As expected, after 24 hrs incubation, the amount of ornithine remained between 13 and 16 μ M in the samples containing *E. coli*. However, after a further incubation of 24 hrs (total incubation of 48 hrs), it was observed that the amount of ornithine decreased in samples containing *E. coli*. This was because the ornithine was being used up by the enzyme produced by *E. coli* thus producing putrescine (as evidenced by the corresponding increase in the amount of putrescine). From zero in the initial stage (0 hr), the amount of putrescine increased to 2.51 μ M in samples containing *E. coli* after 48 hrs. However, it was noteworthy that the samples containing extract had not accumulated putrescine, and the amount of ornithine was not totally diminished in these samples even after 48 hrs. This was because, the extract inhibited the activity of ODC and thereby, the conversion of ornithine to putrescine was not possible.

Extraction of ODC enzyme from *E. coli* and percent inhibition by the extracts

The ODC enzyme produced by the organism was harvested and quantified as described in the methods section. It was found that 117.5 μ g of protein was obtained from 5 ml of ODC broth inoculated with *E. coli*. It was also observed that both the extracts inhibited the enzyme activity in a concentration-dependent manner; around 100% inhibition of the enzyme activity was achieved at a concentration above 25 μ g/ml (Fig. 3a).

IC₅₀ of the extracts against ODC produced by *E. coli*

From the graph plotted between sample concentration and percent inhibition (Fig. 3a), it was found that the leaf extract showed an IC₅₀ at

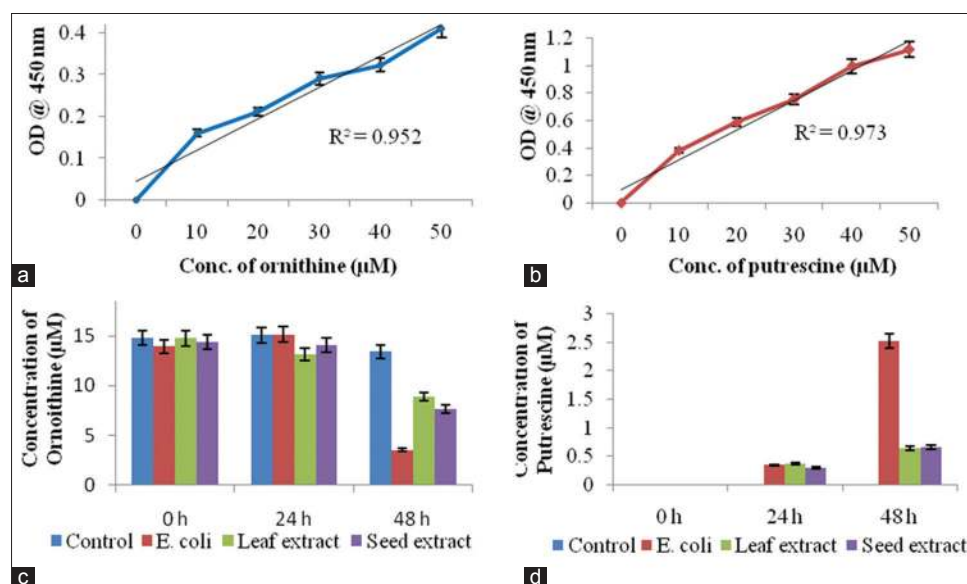


Fig. 2: Standard plots of ornithine (a) and putrescine (b). Concentration of ornithine (c) and putrescine (d) at various time intervals measured in μ M. All determinations were carried out in triplicate manner and values are expressed as the mean \pm standard deviation n=3

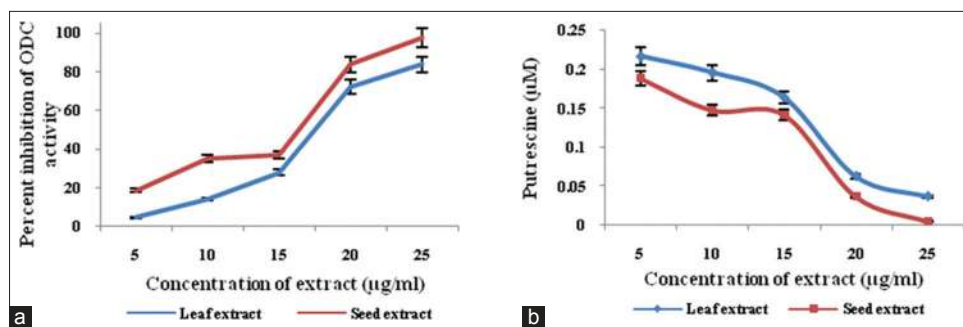


Fig. 3: (a) Percent inhibition of ornithine decarboxylase (ODC) activity. (b) Concentration-dependent inhibition of ODC activity. The diminishing gradient is approximately linear (data represents the results of experiments done in triplicates±standard deviation) n=3

a concentration of 17.5 µg/ml whereas seed extract exhibited an IC₅₀ of 16.1 µg/ml. It was understood that seed extract was slightly more potent than leaf extract. This potency was visible even in the inhibition of bacterial growth by well diffusion method.

Concentration dependent inhibition of ODC activity

Effect of various concentrations of the leaf and seed extracts on ODC activity revealed that the extracts exerted a concentration-dependent inhibitory effect on the production of putrescine (Fig. 3b). The results are indicative of the potency of the extracts toward bacterial ODC inhibition.

Effect of the presence of the substrate ornithine on ODC activity

When the substrate ornithine was externally supplied to the reagent mixture containing the extracts, there was a gradual reduction in the activity of the enzyme referring to the inhibition of putrescine production by the extracts (Fig. 4). Optimum inhibition of ODC activity was observed when the concentration of ornithine was 3 mM. Further increase in the concentration of substrate resulted in an increase in putrescine release, probably because the extracts were not potent enough to inhibit the conversion of ornithine to putrescine at higher substrate concentrations.

Effect of the extracts on the growth of *E. coli*

The antibacterial assay clearly demonstrated the inhibitory effect of the extracts on the growth of *E. coli*. It was observed that the growth inhibition by the extracts was in a concentration-dependent manner (Fig. 5). Fig. 5a represents growth inhibition by the leaf extracts and Fig. 5b represents growth inhibition by seed extracts at three different concentrations (25 µl, 50 µl, and 100 µl - 1, 2, and 3, respectively). 4 represent control (bacteriocin disc).

DISCUSSION

Microbes are capable of producing enzymes that can specifically decarboxylate amino acids. ODC is one such enzyme produced by *E. coli*. The ODC enzyme produced by *E. coli* decarboxylates the amino acid ornithine present in the media to the diamine (putrescine) and carbon dioxide. The glucose present in the media acts as the fermentable carbohydrate which, during the early stages of incubation, is fermented by the test organisms resulting in acidification. Subsequently, the color of the media changes (from purple to yellow). After incubation, putrescine is produced which raises the pH of the media. The media becomes alkaline and the color changes from yellow to purple/violet. If the organism does not produce the appropriate enzyme, the medium remains acidic (yellow in color). Accordingly, in this study, it was observed that the media changed to purple after inoculating with *E. coli* which explained the production and activation of ODC by *E. coli*. However, the media supplied with extracts did not change to purple, but remained yellow, indicating inhibition of ODC activity by the extracts. This is a novel finding as far as the extracts of *E. agallocha* are concerned.

ODC activity has been traditionally assayed by the release of [14C] CO₂ using [14C] labeled ornithine as a substrate and expressed as nmol of CO₂ released/hrs on protein basis. The activity is generally determined

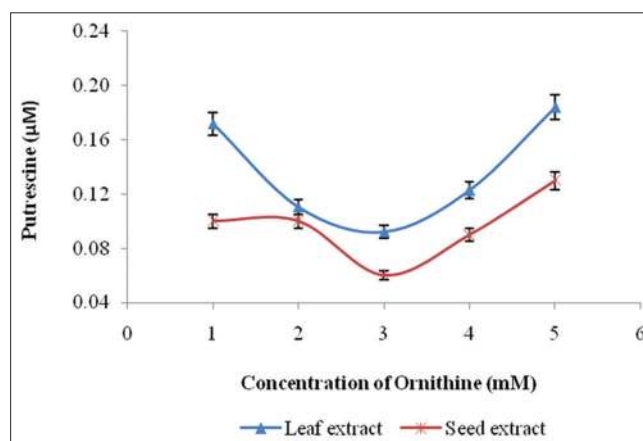


Fig. 4: Effect of the extract in the presence of the substrate (ornithine) on ornithine decarboxylase (ODC) activity. Optimum inhibition of ODC activity was observed at the substrate concentration was 3 mM when IC₅₀ concentrations of the extracts were used. Further increase in concentration of ornithine resulted in an increased yield in putrescine (data represents the results of experiments done in triplicates±standard deviation) n=3

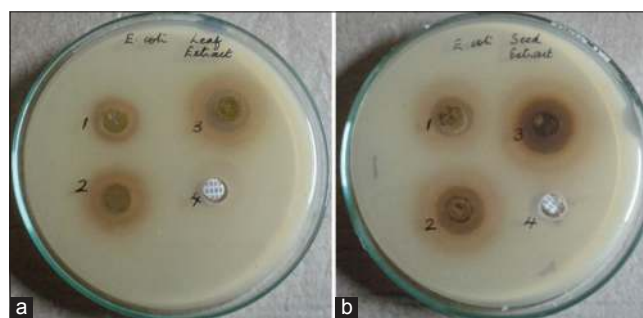


Fig. 5: Agar well diffusion assay to test the inhibitory effect of *Excoecaria agallocha* extracts on the growth of *Escherichia coli*. (a) Leaf extract, (b) seed extract. 1-25 µl, 2-50 µl, 3-100 µl, 4-control (bacteriocin disc)

using labeled ornithine: [14C] labeled ornithine releases putrescine and [14C]O₂ which is assayed with a scintillator [9] or High-performance liquid chromatography (HPLC) assays [10]. However, other enzymes such as transaminases [11] can also release CO₂. Therefore, in this study, an alternative procedure by a modified spectrophotometric method [8] was followed for determining ODC activity. This method relied on quantitative determination of the substrate (ornithine) and specific reaction product of decarboxylation of ornithine, putrescine [12]. Since the precise reaction product is quantified, the assay is expected to be

more specific and valid. The effectiveness of the spectrophotometric analysis in the determination of ODC activity was already reported and it was understood that this method was comparable to HPLC method [13]. Furthermore, the *in vitro* spectrophotometric method to assay ODC activity requires no initial purification of polyamines from the samples [3].

The amount of ornithine remained almost equal in the samples before inoculating the media with *E. coli* (Fig. 2c). However, 24 hrs after *E. coli* inoculation, it was observed that the amount of ornithine started depleting steadily in all *E. coli* inoculated samples. This implied that the enzyme (ODC) was activated and ornithine was used up by the enzyme produced by *E. coli*. Further, the presence of putrescine detected in the media at this stage (Fig. 2d) is evidence for the decarboxylation process happening in the media containing *E. coli*. Extracts of *E. agallocha* were added to samples 3 and 4 at this stage and inoculated for a further 24 hrs time period. It was observed that at 48 hrs, i.e. 24 hrs after adding leaf and seed extracts of *E. agallocha* to the respective tubes, the amount of ornithine changed in these samples whereas the amount of putrescine remained the same (Figs. 1, 2c, and d). This is a clear suggestion that the extracts were capable of inhibiting the decarboxylation of ornithine and in turn, the production of the diamine and putrescine. In the cases where the sample was inoculated with *E. coli* (as there was no extract to inhibit the activity of the enzyme), the amount of putrescine was found to be higher than those found in samples with extract. Here, decarboxylation of ornithine continued unhindered, yielding more and more putrescine.

As the biosynthetic pathway of polyamine synthesis is heavily dependent on the activity of the enzyme ODC, it is understood that a decrease in the concentrations of polyamine levels could result in reduced cell growth. This could prove to be a rational approach to successfully control the growth of potent pathologic bacterial strains. The effect of polyamine inhibition on the growth pattern of *E. coli* was tested. This study revealed that the methanolic extracts of leaf and seed of *E. agallocha* were capable of inhibiting the growth of *E. coli*. The growth inhibition was concentration dependent. This result is in concurrence with that of the activity of plant extracts against *E. coli* as reported by [14]. A concentration of the extract above 25 µg/ml was found to inhibit the ODC activity to almost 100%. It was also observed that the concentration of ornithine as substrate was as low as 3 mM. Good inhibitor of ODC activity requires less substrate [15] and this result point toward the effectiveness of the extracts of *E. agallocha* as a good ODC inhibitor. Curcumin from turmeric is found to be inhibitory activity toward ODC [16].

Accordingly, it is understood that the prominent inhibition of *E. coli* growth, as observed in this study, is due to the depletion of polyamines by the extracts, the evidence of which was revealed by the lower concentration of putrescine in samples treated with the extracts as compared to untreated sample. It is clear from the assays that the extracts of *E. agallocha* offered fairly good inhibition of ODC activity. The physiological role of polyamines in cell proliferation [17] is undoubtedly emphasized by the results of this study.

CONCLUSION

To conclude, as postulated in the study, it was found that the extracts of *E. agallocha* were capable of inhibiting the activity of ODC enzyme produced by *E. coli* thus, serving as a novel source of ODC inhibitor. It was also found that the inhibition was concentration-dependent which

resulted in significant growth inhibition of *E. coli*. This ODC inhibition-mediated growth inhibition of *E. coli* is a novel finding as far as the extracts of *E. agallocha* are concerned. Consequently, these results suggest and support the use of plant extracts as potential, alternate sources of antibacterial agents. This could lead to the development of new strategies to control the crucial growth of pathologic bacteria and thereby bacterial infections. However, further studies are warranted to that effect. Currently, experiments are underway to check and authenticate the molecular mechanisms behind the inhibition of ODC induced by the extracts in mammalian cells.

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