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A tale of two medicinal plants: A comparative study of medicinal bioactivity of homoeopathic tinctures of *Coleus aromaticus* and *Coleus zeylanicus*

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Abstract
A comparative study of medically useful biological activites of homoeopathic tinctures of two closely related medicinal plants, namely *Coleus aromaticus* and *Coleus zeylanicus* was conducted. It was revealed that the tincture of *C. zeylanicus* exhibited potent anti-bacterial activity against gram negative *Klebsiella pneumoniae, E.coli and Enterococcus faecalis* compared to *C aromaticus* which exhibited activity against *S. aureus* alone. Both plant tinctures exhibited similar anti-oxidant activity. LOX inhibition assay proved that both plant tinctures inhibited the enzyme and *C. zeylanicus* was more inhibitory. *C. zeylanicus* showed 54.7% inhibition and *C aromaticus*, 13.5% when used in the same concentration. The results of inhibition of denaturation of protein indicated that *C. zeylanicus* was more effective as an anti-inflammatory agent compared to *C. aromaticus*. The extent of inhibition was 68% and 55% for the two plant tinctures respectively. It was therefore concluded that *C. zeylanicus* was more useful medicinally out of the two plants.

Keywords: *Coleus aromaticus*, *Coleus zeylanicus*, anti-bacterial, anti-inflammatory, antioxidant

1. Introduction
Herbal medicine is a prominent component in all popular traditional systems of medicine and is a common factor in ayurvedic, homoeopathic, traditional Chinese and American medicine. Ayurvedic system of medicine is the major traditional health care discipline evolved in India, literally meaning 'the science of living'. The primary focus of ayurvedic system of medicine is the assumption that health and well being of a person depends on a delicate balance between the human mind and body. It uses herbal preparations, special diets and some other unique health practices to bring about cure of human diseases. Hundreds of medicinal plants are used in ayurveda in various forms, such as decoctions, medicated oils, powders and tablets. Some of the medicinal preparations are produced by processes involving fermentation which involves the biotransformation of phytochemicals responsible for the curative action. Some of the phytochemicals such as berberine undergo demethylation reactions and produce derivatives having better pharmacological potential than parent molecule [1].

Two medicinal plants of the same genus and belonging to Lamiaceae family namely *Coleus zeylanicus* Benth. and *Coleus aromaticus* Benth. were used in this study. The present study was aimed to understand the similarities and differences in biological activities of these very closely related and morphologically similar herbaceous plants. They are both used in ayurvedic and homeopathic systems of medicine. They are quite similar in external appearance (Figure 1) and have distinctly aromatic aerial parts. Such a study was aimed to understand whether the two plants were mutually substitutable in their use in medicine. The term *Coleus* is derived from the word 'koleos', which literally implies sheath around the style [2]. *Coleus aromaticus* Benth. is a perennial herb having highly aromatic leaves and possesses anti microbial, anti epileptic, antioxidant and anti leshmanial activities. The leaf extract is used in bronchitis, asthma, cold cough etc [3]. *Coleus aromaticus* exhibits effective anti fungal activity. This was revealed in a study by using its leaves as disc [4]. In another study, different solvent extracts of the dried powder of this plant were prepared and antibacterial activity was determined. The extracts showed significant activity against *Bacillus* and *Staphylococcus aureus* [5] *Coleus zeylanicus* Benth. is the chief ingredient in many ayurvedic medical preparations [6]. It is a small profusely branched succulent aromatic herb with blue flowers in terminal racemes. It has antipyretic, antibacterial, anti diabetic and hepatoprotective activities. It is commonly used in disease conditions such as vomiting diarrhoea, leucoderma etc. [7]

The present research work is a comparative study some of the medically useful biological activities of the alcoholic tinctures of these two plants prepared according to homeopathic pharmacopoeia [8] and is therefore distinct from the previous studies conducted, such as dried powder extract and using leaf disc etc.

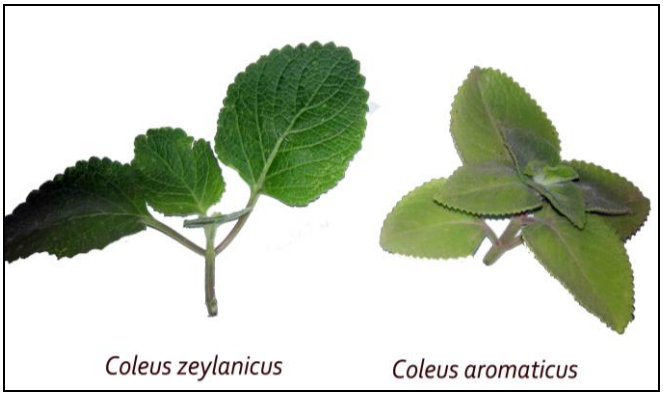

Fig 1: Arial parts of *Coleus aromaticus* and *Coleus zeylanicus*

2. Materials and Methods

Authentic samples of the two medicinal plants *Coleus aromaticus* and *Coleus* zeylanicus were collected from the medicinal plant garden of Kannur University. Voucher specimens of the two plants, C. *aromaticus* Benth. and C. *zeylanicus* Benth. were deposited in the herbarium of School of Life Sciences, Kannur University with specimen No 56/2019 and 57/2019 respectively, for future reference. All reagents used were of analytical grade, from E Merck India Ltd, Mumbai. Microbiological media were purchased from Hi Media Laboratories, Mumbai.

2.1 Preparation of alcoholic extract

Alcoholic tinctures of the two plants were prepared in accordance with the common procedure used for the preparation of medicinal tinctures in homeopathy [8]. Ten grams of the leaves of the two plants were minced finely using a surgical scalpel and were added to 50 ml rectified ethyl alcohol in reagent bottles and mixed thoroughly by vortex for a few minutes. The samples were kept for ten days for extraction with occasional vortex everyday. At the end of the period, the extract was filtered through Whatman No 1 filter paper and collected.

2.2 Determination and comparative analysis of antimicrobial activity

The homeopathic tinctures of the plants were screened for their antimicrobial activity for the following microorganisms: *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Enterococcus faecalis Klebsiella pneumoniae and Pseudomonas aeruginosa.* The concerned microorganisms were purchased from the MTCC culture collection at Institute of Microbial technology, Chandigarh, India.

2.2.1 Preparation of inoculums

Isolated colonies of same morphological type were selected from nutrient agar plate culture. The top of colony was touched with a loop and was transferred into a tube containing 5 ml of nutrient broth. The broth culture was incubated at 35ºC until a turbidity of 1-2x10^8 CFU/ml (usually 2-6 hours) was achieved. That was the turbidity of 0.5 McFarland standard, which implies the turbidity of actively growing broth. Culture was adjusted by dilution with sterile saline to obtain a turbidity which is optically comparable to that of 0.5Mc Farland standard.

2.2.2 Preparation of discs

Sterile Whatman No 1 filter paper discs with 5 mm diameter were impregnated with 20 µl, 40 µl, 60 µl, 80 µl and100 µl of the selected plant extract per disc and allowed to dry under laminar air flow cabinet.

2.2.3 Disc diffusion method

Screening of the plant extracts for antimicrobial activity was performed by disc diffusion method. It was done using an 18 hour culture (approximately 10^5 CFU/ml at 37°C in 10 ml of broth). Suspensions were spread over the petri plates containing Mueller-Hinton agar using a sterile cotton swab, so to get a uniform microbial growth on test plates. The discs which contained extract of the plants at different concentrations were placed on the inoculated agar. A standard disc containing Kanamycin (5µg/disc) was used as positive reference control whereas blank disc was used as negative control. All petri dishes were sealed with sterile parafilm to prevent evaporation of the samples. The plates were left for 30 minutes at room temperature to allow the diffusion of extract, and then incubated at 37ºC for 24 hours. The results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear zones of inhibition around the discs indicated positive antimicrobial activity. The extent of antibacterial activity was interpreted from the diameter of the clear zones surrounding the discs. After incubation, the diameter of inhibition zone obtained was measured manually in triplicate, using a standard stainless steel ruler calibrated in millimeters.

2.3. Minimum inhibitory concentration

Minimum inhibitory concentration is defined as the lowest concentration of an antimicrobial material that would inhibit the visible growth of a micro-organism after overnight incubation. Equal volumes of the extracts and nutrient broth were mixed in the test tube. Specifically 0.1ml of standardized inoculum of 1to 2 X10^7 CFU/ml was added to each tube. The tubes were incubated aerobically at 37 ºC for 18-24 hours. Two control tubes were maintained for each test batch. This is as follows: tube containing extracts and the growth medium without inoculum (antibiotic control) and the tube containing the growth medium, physiological saline and the inoculum (organism control). MIC was determined as the lowest concentration of the extracts permitting no visible growth, hence no turbidity, when compared with the control tubes.

2.4. Determination of antioxidant potential by DPPH radical scavenging assay

Antioxidants are those chemical substances which possess the ability to protect the body from damage caused by the free radical induced oxidative stress. A large number of plants exhibit a strong antioxidant activity and have a powerful scavenger activity against free radicals.

Radical scavenging activity of extracts was measured by a modified method [9]This method had been developed to determine the antioxidant activity of food which utilizes the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The odd electron in the DPPH free radical (purple colour) gives a maximum absorption at 517 nm. When the odd electron of DPPH radical makes pair with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H, the

colour turns from purple to yellow. The resulting colour change is stoichiometric with respect to number of electrons captured. The prepared alcoholic tinctures (200 mg/ml of crude plant) were diluted with rectified ethanol to obtain lower dilutions. The DPPH solution with a concentration of a 0.004% (w/v) solution of DPPH in methanol was prepared. Three ml of this solution was mixed with one ml extract solution in a test tube. Each mixture was then shaken vigorously by vortex and held for 30 minutes at room temperature in the dark. Discoloration was measured at 517 nm after incubation for 30 min by using a Hitachi U2900 spectrophotometer [10]. The actual decrease in absorption induced by the test was compared with the positive control. The inhibition percentage of the absorbance of DPPH solution was calculated using Eq 1.

$$\text{Inhibition\%} = [(Abs_0 - Abs_{30}) / Abs_0] \times 100 \quad (1)$$

Where:
Abs_0: the absorbance of DPPH at time zero.
$Abst_{30}$: the absorbance of DPPH after the 30 minutes of incubation.
Ascorbic acid was used for comparison as positive control. The experiment was carried out in triplicate. A plot of absorbance vs. concentration was made to establish the standard curve. [10] Absorption of blank sample containing the same amount of ethanol and DPPH solution was prepared and measured. The percentage of inhibition was determined from the extent of optical absorbance shown by DPPH solution at 517 nm, and was compared between with each concentration of the sample extract solution added.

2.5. Determination of total phenolic content
Various phenolic substances are also known to possess ability to reduce oxidative damage and act as antioxidant chemicals. They possibly react with the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes. [11] Total phenolic content was determined by the Folin- Ciocalteu's method [12] which is based on complex formation of molybdenum-tungsten blue. The samples were allowed to react with Folin-Ciocalteu's reagent and sodium carbonate solution. The phenolic contents were estimated using a standard curve of gallic acid and expressed as mg of gallic acid equivalents (GAE) per gram mass of the sample.
Aliquots of 1 ml of each of the extract were mixed with one ml Folin- Ciocalteu reagent in a test tube that contained 9 ml of distilled water. Samples were mixed using vortex mixer and added 1 ml sodium carbonate solution (20% w/v anhydrous sodium carbonate in distilled water). Samples were vortexed again and left to stand for 30 minutes to obtain maximum development of colour. The absorbance was measured at 765 nm using Hitachi U2900 UV-Vis spectrophotometer. Absolute ethanol was used as a blank. Pure gallic acid was used as the standard reference compound. A calibration curve of gallic acid (range 0–50 µg/ml) was prepared and the results determined from regression equation of the calibration curve were expressed as mg of gallic acid equivalents (GAE) per gram of the sample on dry weight basis. The contents of phenolic compounds in the sample were reported as of gallic acid equivalents per gram of the dried sample. Total phenolic content of each sample was calculated by using Eq 2

$$\text{GAE mg/g} = [(\text{Slope} \times \text{absorbance}) + C] \quad (2)$$

Where: GAE is gallic acid equivalents and C is the y intercept.

2.6. In vitro Lipoxygenase inhibition assay
Lipoxygenases (LOXs) are a family of non-heme iron-containing dioxygenases that catalyze the hydroperoxidation of lipids, containing a cis, cis-1, 4-pentadiene structure.The inhibition assays were performed with tinctures of plants. LOX type-1B (soybean) was purchased from Sigma Aldrich, USA. Linoleic acid (99%) was used as the substrate for the assay. The activity of LOX was monitored by measuring the increase in UV absorbance at 234 nm, which reflects the formation of hydroperoxy–octadecadienoic acid. The reaction medium (2.0 ml final volume) contained 0.2 M borate buffer of pH 8.5, 0.1g of enzyme and solution of linoleic acid prepared in tween 20 and solubilized in 0.2 M borate buffer. The assay mixture consisted of 50 µl of lipoxygenase enzyme solution (200IU/ml), 50 µl of test solution and this mixture was incubated for 1min. A definite volume of buffer was added to this mixture and reaction was initiated by adding 360 µl substrate solution and UV absorbance was recorded at 234 nm for five minutes using a Hitachi U2900 UV visible spectrophotometer. Vanillin, a known inhibitor of Soybean lipoxygenase was used as positive control [13]
Progress curve showing the time-dependent activity of the enzyme was plotted. Lipoxygenase inhibitory activity was expressed as percentage –inhibition of lipoxygenase, calculated by the following Eq 3

$$\text{Inhibition (\%)} = (1\text{-}B/A) \times 100 \quad (3)$$

Where A is the change in absorbance without test sample and B is the change in absorbance with the test solution.

2.7. Protease inhibition assay
Proteinases have been implicated in arthritic reactions. Proteinase plays an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors[14] The assay is based on hydrolysis of the substrate BAEE (N-benzoyl-L-arginine ethyl ester) at the ester linkage causing an increase in UV absorbance measured at 254 nm. The reaction mixture (3.4 ml final volume) consisted of 67 mM phosphate buffer (pH 7.6) 0.25 mM BAEE in phosphate buffer and 0.05 mM Trypsin in ice cold 0.001N HCl. The prepared homeopathic tinctures of *Coleus zeylanicus* and *Coleus aromaticus* were diluted in Dimethyl sulphoxide and used for the assay. The assay mixture consisted of 200 µl of Trypsin and 200 µl of test solution and this mixture was incubated for 10 minutes .The reaction was started by the addition of 3 ml of substrate solution and UV absorbance was measured at 254 nm for a period of 10 minutes using a Hitachi U2900 UV visible spectrophotometer. PMSF (Phenyl Methyl Sulphonyl Fluoride) which is a known Trypsin inhibitor was used as positive control. A plot showing time-dependent activity of the enzyme was drawn. Trypsin inhibitory activity was expressed as percentage inhibition of Trypsin, calculated by following Eq4.

$$\text{Inhibition (\%)} = (1\text{-}B/A) \times 100 \quad (4)$$

Where A is change in absorbance without test sample and B is change in absorbance with test solution.

2.8. Assay of inhibition of protein denaturation

Different concentrations of the two plant extracts were prepared by diluting the prepared tincture using rectified alcohol in the ratios 1:1, 1:2 and 1:3. Egg albumin was used in 0.6% concentration in 0.1M phosphate buffer saline of pH 7.40. About 50 ml of this solution was dispensed in several beakers and 0.5 ml of dilutions of plant extracts prepared were added to them. 0.5 ml rectified alcohol was added to a beaker to serve as negative control. Diclofenac sodium in different concentrations served as positive control. 0.5 ml of undiluted tinctures were also added to two of the beakers. The solutions were heated to 70º C in water bath for a period of 15 minutes and allowed to cool to room temperature. Optical density was measured at 660 nm, using a Hitachi U2900 UV visible spectrophotometer, using native protein solution as the blank. The percentage of inhibition of protein denaturation was calculated in each case. [15]

2.9. Statistical analysis

Analysis of the data for statistical significance was conducted using WASP2.0 statistical package. One way analysis of variance and triplicate values were compared and grouped by Fishers least significant difference at the significance of 5% significance level.

3. Results and Discussions

The homoeopathic tinctures of plants exhibited potential antibacterial activity against the tested microorganisms. The homoeopathic tincture of *Coleus zeylanicus* Benth.showed significant activity against gram negative *Klebsiella pneumoniae, E. coli, Enterococcus faecalis.* (Table 1).

Table 1: The antimicrobial activity exhibited by *Coleus zeylanicus* against various microorganisms. The zone of inhibition is recorded against volume of plant tincture used. Treatments found Significant at 5% level of significance CD(0.05) = 1.653

No	Vol (µL)	*E. fecalis*	*K. pneumoniae*	*E. coli*	*P. aerugenosa*	*S aureus*	*Bacillus spp*
1	20	8±0.3mm	10±1.4mm	6±0.2mm	NIL	NIL	NIL
2	40	11±0.2mm	12±1.3mm	8±0.2mm	NIL	6±0.1mm	NIL
3	60	11±1.2mm	12±1.5mm	10±1.0mm	NIL	7±0.8mm	NIL
4	80	11±1.0mm	12±1.2mm	11±1.2mm	NIL	7±0.3mm	NIL
5	100	11±1.2mm	12±1.3mm	12±1.3mm	NIL	8±1.2mm	NIL

The highest antibacterial activity of 12 mm against *Klebsiella pneumoniae* at a concentration of 12mg/ml and followed by 12 mm against *Enterococcus faecalis* and least recorded in *E. coli* measured around 10 mm. No significant activity was found against *Pseudomonas aerugenosa* and *Bacillus spp.* With *Staphylococcus aureus*, activity is feeble. *C.aromaticus* Benth. exhibited no significant antibacterial activity against the tested bacterial strains except *S. aureus* with which it showed antibacterial activity comparable to C. *zeylanicus* Benth. (Table 2).

Table 2: The antimicrobial activity exhibited by *Coleus aromaticus* against various microorganisms. The zone of inhibition is recorded against volume of plant tincture used. Treatments found Significant at 5% level of Significance CD(0.05)= 1.819

No	Vol (µL)	*E. fecalis*	*K. pneumoniae*	*E. coli*	*P. aerugenosa*	*S aureus*	*Bacillus spp*
1	20	NIL	NIL	NIL	NIL	6±0.1mm	NIL
2	40	NIL	NIL	NIL	NIL	9±0.5mm	NIL
3	60	NIL	NIL	NIL	NIL	7±0.2mm	NIL
4	80	NIL	NIL	NIL	NIL	7±0.2mm	NIL
5	100	NIL	NIL	NIL	NIL	7±0.3mm	NIL

This is in contrast to earlier study done by using dried powder extracts of *C aromaticus*, in which significant anti-bacterial activity was observed [5]. The MIC values of the ethanolic extracts of *C. aromaticus* Benth. and *C. zeylanicus* Benth. were calculated against *Klebsiella pneumoniae* using micro dilution method. MIC of *C.aromaticus* Benth. extract was 60mg/ml and *C. zeylanicus* Benth. was found to be 40mg/ml. This proves that *C. zeylanicus* Benth. is more potent antibacterial agent than *C. aromaticus* Benth. With regard to antioxidant capacity by DPPH radical scavenging assay, both tinctures exhibited a significant dose dependent inhibition of DPPH activity (Table 3).

Table 3: Results of free radical scavenging assay. The percentage of scavenging is shown against concentration of each plant. Treatments found Significant at 5% level of significanceCD (0.05) = 1.602

Plant	Concentrations			
	50 mg/ml	100 mg/ml	150 mg/ml	200 mg/ml
C. aromaticus	3.6±0.14%	7.09±0.40%	67.30±0.37%	91.55±0.14%
C. zeylanicus	41.25±0.07%	60.74±0.24%	76.98±0.19%	86.10±0.29%

C. zeylanicus Benth. exhibited high percentage of antioxidant capacity at a lower concentration itself. At higher concentrations, the percentages are comparable with each other. LOX inhibition assay proved that both plant tinctures inhibited the LOX enzyme and *C.* zeylanicus Benth. was more inhibitory (Figure 2).

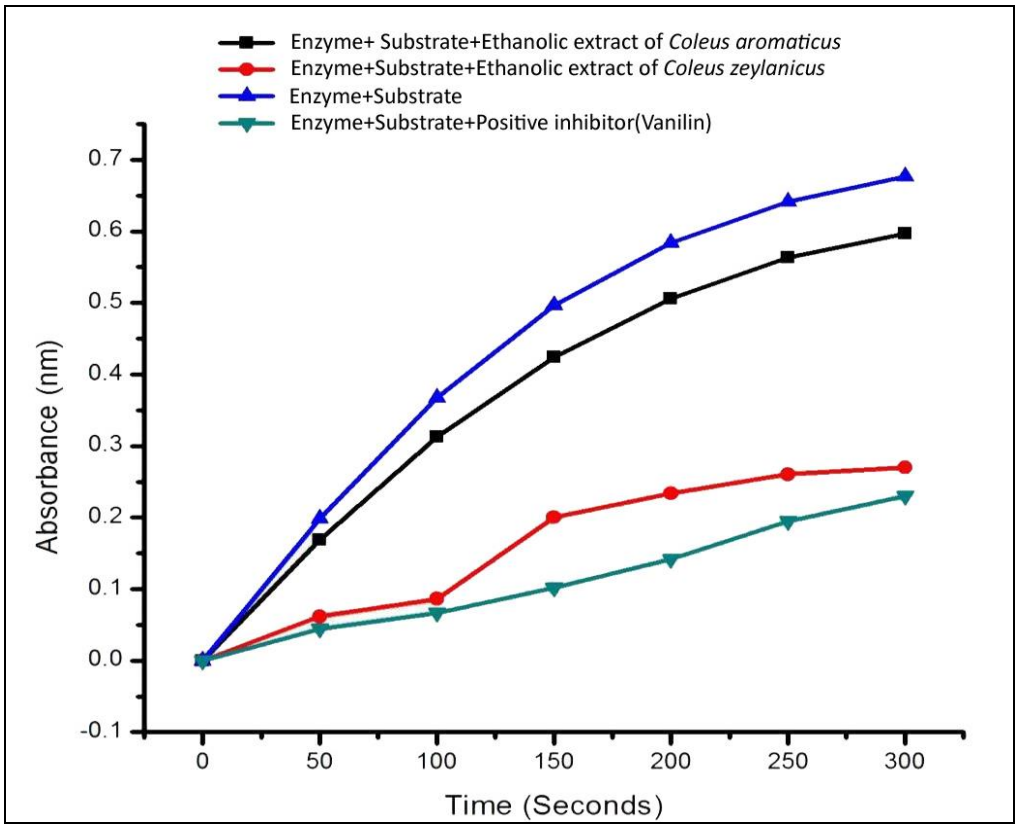

Fig 2: Progress curves of LOX inhibition by the two plant tinctures against positive control of Vanillin.

The percentage of inhibition was calculated to be 54.7% for *C. zeylanicus* Benth. and 13.5% for *C. aromaticus* Benth. when used in the same concentration. The results of protein denaturation inhibition studies indicated that *C. zeylanicus* Benth. Was found to be more effective as an anti-inflammatory agent compared to *C. aromaticus* Benth. *C. zeylanicus* Benth. shows protein denaturation inhibition activity comparable to diclofenac sodium while *C. aromaticus* Benth. exhibited less activity (Table 4).

Table 4: Percentage of protein denaturation inhibition shown by different ratios of plant extracts compared with diclofenac sodium as standard. Treatments found Significant at 5% level of significance CD(0.05) = 5.857

Material	25% extract	50% extract	75% extract	100% extract
C. zeylanicus	23.69	47.57	60.53	68.43
C. aromaticus	7.90	15.79	39.48	55.27
Diclofenac Na 10mg/ml	34.22	42.11	52.64	65.79

This is in accordance with LOX inhibition studies shown by the two plants. The total phenolic content was found to be 0.00396 mg Gallic acid equivalent/g sample for *C. zeylanicus* Benth. and of the *C. aromaticus* Benth. was 0.002315mg Gallic acid equivalent/g sample. This proved that the two plants were not much different from each other in terms of phenolic content. This was the same in the case of proteinase inhibition studies where the two plants, *C aromaticus* Benth. and *C. zeylanicus* Benth. exhibited 63.4% and 65.2% inhibition respectively.

4. Conclusions

Both plants *Coleus aromaticus* Benth and *Coleus zeylanicus* Benth. are used in ayurvedic system of medicine since several centuries. Our investigation revealed that these two plants, though of the same genus and showing good morphological similarity, exhibit marked differences in biological activity. *Coleus zeylanicus* Benth. shows pronounced anti bacterial activity against several micro organisms and possesses excellent anti inflammatory effect evidenced by LOX inhibition, protein denaturation inhibition and protease inhibition assay. The antibacterial activity of some extracts of *C. aromaticus* were investigated earlier [5]. Their work involved extracts of dried powder of this plant extracted by soxhlet apparatus. They had reported significant anti bacterial activity against several micro organisms. But our present study compares the bioactivity of homoeopathic tinctures of *C. aromaticus* with C. *zeylanicus* and proves that the latter possesses stronger antibacterial effect than the former. This study proves that *Coleus zeylanicus* Benth. is more useful as a medicinal plant compared to *Coleus aromaticus* Benth. Also it was proved that these two plants cannot be substituted mutually in medical treatment as they differ vastly in their medically important biological activity, in spite of being belonging to the same genus and showing morphological similarity.

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