Original Article

Protective Effect of Ethanolic Extract of *Lycopodium Clavatum* Against Pathogenic *Escherichia Coli* and *Proteus Vulgaris*

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Dr. Jagadeesan J, MSc, PhD worked as a Post-Doctoral fellow at CARE from 2016-2018, his work was mainly focused on Lycopodium clavatum, a plant extract used for treating alimentary canal related indigestion and inflammations of urinary organs and dermal problems. His work aims to explore the anticancer activity for therapeutic use and the present study was, therefore undertaken with an aim to explore the possible anti-microbial activity and toxic studies of the mother tincture of Lycopodium clavatum. He has published 10 international research paper related to his work. He did his PhD from University of Madras, in 2012 and Master Degree in Biomedical Genetics from University of Madras in 2008.

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Chettinad Health City Medical Journal 2021; 10(1): 4 - 9

DOI: https://doi.org/10.36503/chcmj10(1)-02

Abstract

Background and aim: This study aimed to assess the antiproliferative and antioxidant activities of Lycopodium clavatum against the pathogenic strains Escherichia coli and Proteus vulgaris.

Methods: Effects of Lycopodium clavatum plant extract was assessed in Escherichia coli and Proteus vulgaris by the analysis of cell viability and disc diffusion assays. Status of the toxicity biomarkers like lipid peroxidation (LPO), superoxide dismutase (SOD), catalase and reduced glutathione (GSH) were analyzed in Escherichia coli and Proteus vulgaris before and after administration of the plant extract.

Results: The growth curves of both Escherichia coli and Proteus vulgaris revealed the inhibitory effects of Lycopodium clavatum, as compared to the control after treatment. The Lycopodium clavatum extract shows susceptibility in higher concentrations (from 30 µl to 100 µl) in both the test organisms. Increased levels of catalase activity and decreased levels of LPO, SOD and GSH content were observed for L. clavatum plant extract thus clearly exhibiting their functional ability to reduce cytotoxicity.

Conclusions: This study revealed that Lycopodium clavatum extract has inhibitory effects against the growth of pathogenic strains Escherichia coli and Proteus vulgaris.

Keywords: Antioxidant, Cytotoxicity, E. coli L. clavatum

Introduction

Escherichia coli is a rod-shaped, gram-negative coliform bacterium that is commonly found in the small intestine of warm-blooded organisms.[1] Most E. coli strains are non-pathogenic which form the part of the symbiotic normal flora of the gut and help the hosts by producing vitamin K2, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination. E.coli serotypes are the causative agents of colibacillosis. Lycopodium clavatum (L. clavatum) plant extract is playing a major role in naturally derived plant biomolecules, since it's used in the treatment against pathogenic bacterial cells.[2] Similarly, Proteus vulgaris is a member of other gram-negative rod- shaped bacilli that causes serious infections in humans, including urinary tract infections and wound infections, burn infections, blood-stream infections, and respiratory tract infections along with Escherichia, Klebsiella, Enterobacter, and Serratia species.[3] P. vulgaris is also responsible for bacteremia and brain abscesses, with the suspected point of entry through the digestive tract.[4] The strains like E. coli (ATCC25922) and P. vulgaris (NCTC8313) are well maintained ones that make excellent microbial models for experimental studies in laboratory conditions.[5] Recently, a world trend to reduce the use of antibiotics in animal food due to the contamination of meat products with antibiotic residues and concern that makes some plant extracts are used for the treatments for human diseases.[6] For the past decade, the use of antimicrobial drugs especially in L. clavatum plant extract for therapeutic uses has received much attention.

Medicinal plants composed of a wide variety of bioactive molecules, making them rich source of different types of treatments.[7] Many drugs today

are obtained from natural sources or semi synthetic derivatives of natural products used in the traditional systems and as well as main stream of medicine.[8] Hence, it is a reasonable process towards the drug discovery to screen all kind of traditional natural products, which have a potential medicinal value.[9] The ethanolic extract of L. clavatum demonstrates that folk medicine can be used along with main stream medicine to combat pathogenic and non-pathogenic microorganisms.[10]

The spore extract of L. clavatum is generally used successfully to ameliorate/cure ailments of the digestive system, particularly those related to liver malfunction, and the intestine often affecting the microflora, producing flatulence as one of the great indications for the use of this remedy. L. clavatum, commonly referred to as "club moss", is a creeping perennial found worldwide and the spores are the chief medicinal source.[11] Previously scientific inventions, the spore extract of L. clavatum and its various parts have been reported to have anti-oxidative and anti-proliferative effects and also had the ability to induce positive biochemical modulation in liver tissues.[12] It also showed its anticancer potential.[13] However, notwithstanding such published studies, a section of the people still remained skeptical and reluctant to give any credence to its medicinal value, believing such effects to be "placebo effects" without proper scientific evidences.

The intestinal tract protects microbiota which has an extensive influence on the health and nutrition status of the host. Balanced gut microbiota is necessary to benefit the host while the imbalances are linked with metabolic disorders.[14-15] Among all the environmental factors, diet plays a major role in alteration in gut microbial diversity which might affect the relationship with the host.[16] The alteration of the microbiota might even affect the morphology of the intestinal wall and instigate immune reactions as well. In recent years, there is a number of foods and food components that provide advantageous roles beyond normal nutrition, leading to the progressing of nutraceuticals.[17] These nutraceuticals are food components that play a role in altering and maintaining the physiological functions that support the host.[18]

Thus, in this current study, our first aim is to examine if the L. clavatum plant extract could actually retard the bacterial growth effectively, showing evidence of their bactericidal effects. Along with this, the enzymatic parameters like super oxide dismutase (SOD), catalase, reduced glutathione (GSH) and lipid peroxidation (LPO), which are considered as acceptable biomarkers for reduction of cytotoxicity were also analyzed. Finally, our prime objective is to correlate the findings obtained through these multiple sets of protocols and to understand, if possible and to evaluate the impact and suitability of the administration of these drugs.[19] Therefore, we are much focused in this study to examine the effect of L. clavatum plant extract against pathogenic bacterial strains.

Materials and Methods - Materials

The L. clavatum plant extract and chemicals, solvents (analytical grade) used in this study were purchased from HAPCO pharmaceuticals, India.

Bacteriological assays - Bacterial strains:

The studied bacterial strains comprised of E. coli (ATCC25922) and P.vulgaris (NCTC8313). Microorganisms were maintained at 4°C on nutrient agar slants. For this study the 0.5 McFarland standardized number of CFU for E. coli and P. vulgaris strains were used to ensure that a uniform number of bacteria were used for all the experiments, a set of graphs of viability assays for each strain of bacterial species was prepared and the results compared for final selection. A final concentration of 5 × 105 cfu /ml was adopted for this assay.

Methods - Bacterial cell viability:

Wells of a 96 well plate were filled with 50 µl of LB except in the first column. 100 µl of L. clavatum plant extract was filled in the first column and serially diluted. 50 µl antibiotic solutions was filled in the last second row and serially diluted. The E. coli and P. vulgaris strains were added around 10 µl to fill the entire column and also 10 µl resazurin dye (filter sterilized, 0.2 mg/ml concentration) was added in each well with bacteria. The plate was sealed with parafilm to avoid drying of the cultures, and incubated under mild shaking conditions in incubator shaker at 37°C for 4 h. After incubation, OD was measured at 600 nm.

Bacterial growth:

E. coli was cultured in 5 ml Luria Bertani (LB) broth at 37°C for 12-16 hours (h) in shaker incubator at 150 rpm. 1 ml of overnight-grown culture was again inoculated into 100 ml LB broth and allowed to grow up to early log phase at an OD600 of 0.5 ml of E.coli culture that was then treated with L. clavatum plant extract and incubated at 37°C for 24 hrs. After incubation, optical density was measured at 600 nm using UV visible spectroscopy.

Biochemical parameters:

Lipid Peroxidation (LPO)

The LPO was determined from malonaldehyde

(MDA, an end product of LPO) content by the method from.[20] Briefly, 0.5 ml of bacterial supernatant was mixed with 1 ml of TBA–TCA–HCl reagent and the mixture was heated in boiling water bath for 15 min. The precipitate was removed by centrifugation at 4000 rpm for 10 min and absorbance of supernatant was recorded at 535 nm against a blank. The MDA concentration was calculated using a molar extinction coefficient of 1.56 × 105 M–1 cm–1 and reported in nmol/mg protein.

Catalase activity

Catalase activity was measured essentially following the standard method.[21] 3 ml reaction mixture contained 1.9 ml of 0.05 M potassium phosphate buffer (pH-7.4), 1 ml of 30 mM H2O2 and 100 µl of test sample's bacterial supernatant and absorbance at 240 nm was recorded at an interval of 30 s for 3 min. Enzyme activity was calculated using the molar extinction coefficient of H2O2 as 436 mol l–1 cm–1 at 240 nm and reported in U/mg protein.

Superoxide dismutase (SOD) activity

SOD activity in the bacterial supernatant was assayed by measuring its ability to inhibit the auto oxidation of pyrogallol according to the standard method.[22] To 50 µl of bacterial supernatant, 2.85 ml of Tris-succinate buffer (0.05 M, pH 8.2) was added and the reaction was started by adding 100 µl of 8.0 mM pyrogallol. The change in absorbance was recorded at an interval of 30 s (seconds) for 3 min at 412 nm. The activity was reported in terms of IU/mg protein.

Glutathione (GSH) estimation

GSH levels were estimated by the standard method[23] using the classical DTNB reagent. GSH activity with DTNB was read at 412 nm. The concentration was reported in nmol/mg protein.

Disc diffusion assay method

The standard bacterial strains were used for antimicrobial susceptibility testing for L. clavatum suspension. The bacterial strains were as follows: E. coli (ATCC25922) and P. vulgaris (NCTC8313). The inoculum size of the test strain standardized according to the Clinical and Laboratory Standards Institute (CLSI, 2016) guidelines. Susceptibility tests were performed by a modified agar-well diffusion method. 1 ml volume of the standard suspension of test bacterial strain was spread evenly on Mueller-Hinton Agar plate using a sterile cotton swab and the plates were allowed to dry at room temperature. Subsequently 6-mm diameter wells were bored in the agar and a 20 µl volume of sample was transferred into wells. After holding the plates at room temperature for 2 h to allow diffusion of the extract into the agar, the plates were incubated at 370C for 24 hours. Inhibition zone diameter was measured to the nearest millimeter (mm).

Statistical analysis

The statistical significance of the data was expressed as mean ± SEM (Standard Error of Mean). Student's t' test was performed between control and treatment group using the software Graph Pad prism version 7.0. P value $p \leq 0.05$ were considered as significant.

Results - Antimicrobial assay

Micro-dilution methods were widely used to determine the MIC values. This may be used to quantitatively measure the in vitro antimicrobial activity against bacteria. The results of both the strains (E. coli and P. vulgaris) depicts that when compared to control, the L. clavatum plant extract were found to have significantly increased inhibition effect with increasing concentration. (Figure 1A and 2A).

Growth curve analysis

The growth curve analysis has shown a clear decrease in the OD which corresponds to inhibition of the bacterial cells and thereby represents the bactericidal effect of L. clavatum. It can be depicted that L. clavatum plant extractinhibits the growth of E. coli and P. vulgaris when compared to control (Figure 1B and 2B).

Enzymatic parameters

Lipid peroxidation (LPO)

The specific activities of LPO in supernatant of gram-negative bacterial cells in the control and different treatments were shown. On comparison with control, the level of LPO in both E. coli and P. vulgaris strains were found to be significantly decreased with increasing concentration by addition of L. clavatum plant extract after 30 min (Figure 1C and 2C).

Catalase assay

L. clavatum plant extract showed significantly increased catalase activity for both E. coli and P. vulgaris with increasing concentration. The enhanced expression of the antioxidant enzyme catalase in humans can protect them against reactive oxygen species (Figure 1D and 2D).

Superoxide dismutase (SOD)

L. clavatum plant extract exhibits a decreased activity for both E. coli and P. vulgaris. Moreover, the L. clavatum plants extract was having more antioxidant as well as free radical scavenging activity. Some bacteria produce superoxide which

	Target Organism	
Concentrations (µl)	*E. coli*	*P. vulgaris*
1	R	R
10	R	R
20	S	R
30	S	S
40	S	S
50	S	S
100	S	S

Table 1: : (S) Susceptibility (inhibition zone ≥ 7 mm) (R) Absence of susceptibility

acts as a defense mechanism against the elimination of foreign invaders. Upon treatment, this L. clavatum extract inhibits the bacteria to produce superoxide which in turn breaks down the defense mechanism (Figure 1E and 2E).

Reduced glutathione (GSH)

Glutathione is an important antioxidant and it is also capable of preventing damage to the important cellular components that are caused by reactive oxygen species including free radicals, peroxides, lipid peroxides and heavy metals. The effect of L. clavatum plant extract on reduced glutathione in the supernatant of gram-negative bacterial cells is represented in Figures 1F and 2F. However, when compared to control, the L. clavatum plant extract showed a significant decrease in GSH level. Since L. clavatum extract inhibits the action of bacterial strains which in turn may decreases the production of glutathione.

Disc diffusion assay

L. clavatum extract shows susceptibility in higher concentrations (from 30 µl to 100 µl) in both the test organisms (Table 1) (Figure 3).

Discussion

Research in the field of natural herbal medicine is often subjected to criticism. In the present study, the results of several widely accepted protocols would suggest that there were positive modulations in the parameters of study in the L. clavatum treated bacterial cells, which were not observed in the control group. Therefore, the concentration that bacterial cells show response to all the most effective being the L. clavatum plant extract indicates diffusely, that successes alcohol/ placebo-treated cells has an adverse effect on bacterial cells.

L. clavatum plant extracts showing a trend of uniformly efficacious functions for both the strains of bacteria. This greater resistance was probably manifested in the higher level of survivability of E. coli and P. vulgaris in the control group, as compared to plant extract treated groups.

Commonly, ethanol treatment- induced oxidative stress that generated intracellular ROS and free radicals and altered the levels of antioxidants in bacteria, which is in line with previously published report.[24] Intracellular ROS, cross-links to other molecules that block replication.[25] It is important to reduce the level of superoxide anion for the survival of cells. SOD, as part of the defense systems against oxidative damage, catalyzes O2- to oxygen (O2) and hydrogen peroxide (H2O2), which then gets reduced to water (H2O) by H2O2 -scavenging enzyme, catalase.[26] Some bacteria produce superoxide which acts as a defense mechanism against the elimination of foreign particles. After the treatment, probably, L. clavatum extract inhibits the bacteria to produce superoxide which in turn breaks down the defense mechanism.

Some molecules like ascorbic acid and GSH are constitutively present and help to maintain an intracellular reducing environment or to scavenge reactive oxygen and protect from the attack of ROS. GSH also plays an important role in the regulation of cellular proliferation and cellular defense.

Catalase is an antioxidant enzyme that quenches reactive oxygen species and protects from oxidative damage [27]. These enzymes can also breakdown mutagenic and toxic chemicals like H2O2. The L. clavatum plant extract showed significantly increased catalase activity for both E. coli and P. vulgaris with increasing concentration. Thus, the enhanced expression of the antioxidant enzyme catalase in humans can protect them against reactive oxygen species. L. clavatum plant extracts significantly reduced intracellular ROS as was supported by the decrease in SOD, LPO, growth after treatment as compared to control.

Still, this is scientifically unclear and would invite further studies to establish beyond any doubt about the actual mechanism involved in the action of the L. clavatum plant extract in bacterial model. However, in the present study showed some light on the biological responsiveness of the plant extract in bacterial model system. Works in this direction in the future would hopefully resolve the minutes of natural medicine science and pave the way for utilization and beneficial use of alternative mode of treatment, either alone or in combination with other mainstream treatment in the alleviation of microbial disease of intestine.

Acknowledgments

The authors are thankful to Chettinad Academy of Research and Education (CARE), Chennai, India for providing research fellowship to Jagadeesan AJ.

Conflicts of Interest

We declare no conflict of interest. All co-authors have agreed to transfer the copyright to the publisher if it is accepted for publication.

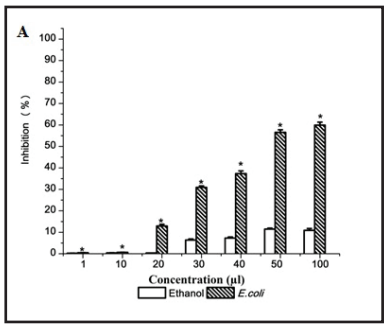

Figure 1: A. Micro-dilution method of E.coli for Lycopodium clavatum

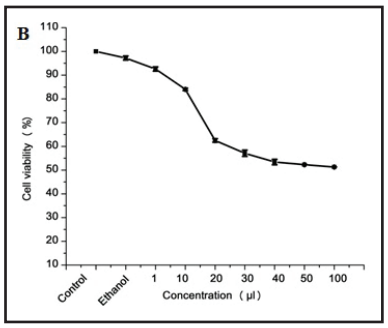

Figure 1: B. Growth curve of E.coli

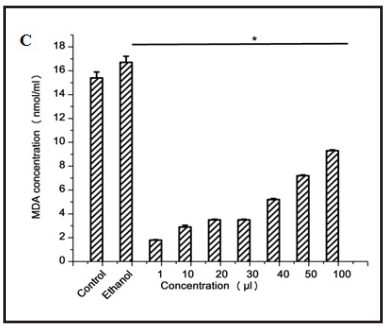

Figure 1: C. Lipid peroxidation (LPO) assay of E.coli

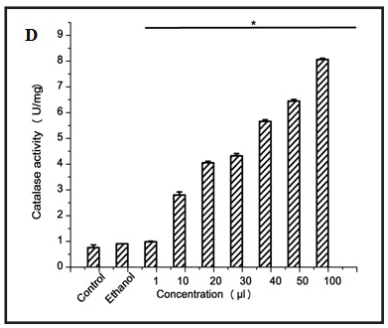

Figure 1: D. Catalase assay of E.coli

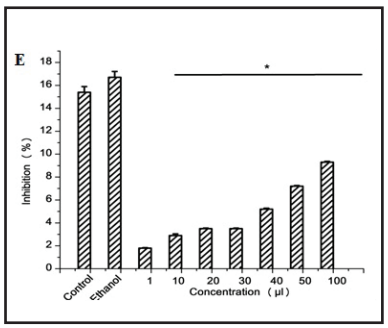

Figure 1: E. SOD assay for E.coli treated

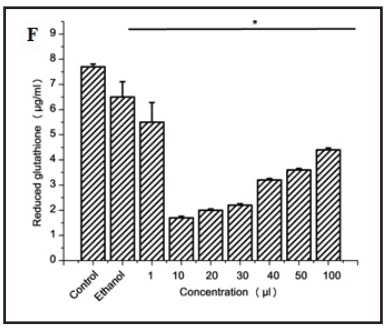

Figure 1: F. GSH assay of E.coli treated. Values are expressed as mean ± SEM, * represents p<0.05

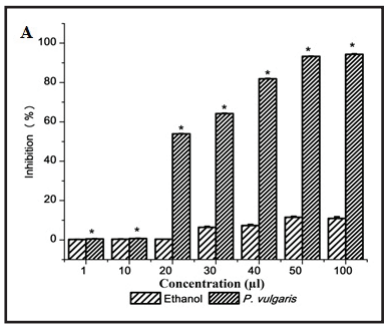

Figure 2: A. Micro-dilution method of P.vulgaris for Ethanol and Lycopodium clavatum

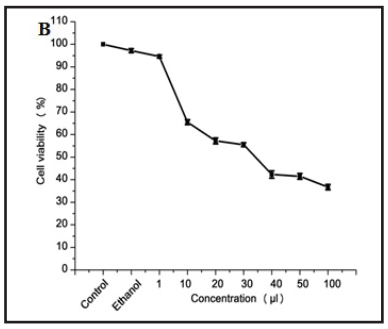

Figure 2: B. Growth curve of P.vulgaris

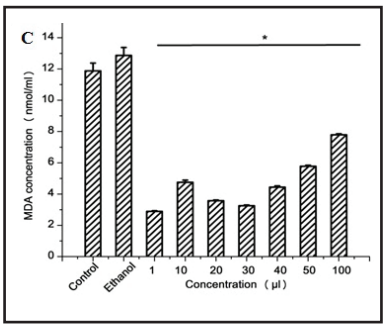

Figure 2: Lipid peroxidation (LPO) assay of P.vulgaris

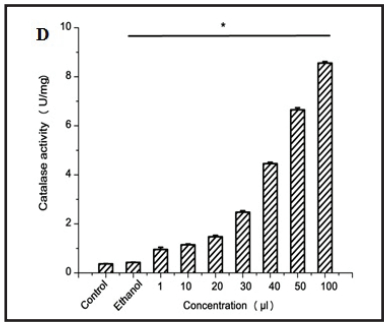

Figure 2: D. Catalase assay of P.vulgaris, E. SOD assay for P.vulgaris treated

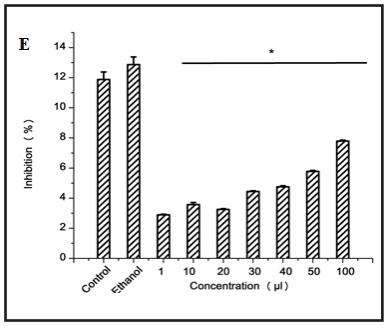

Figure 2: E. SOD assay for P.vulgaris treated

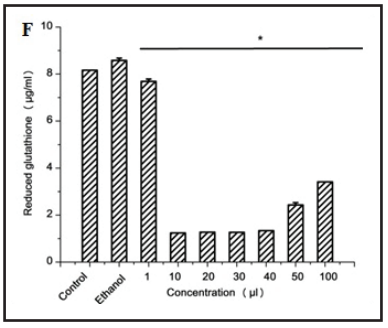

Figure 2: GSH assay of P.vulgaris treated. Values are expressed as mean ± SEM, * represents p<0.05

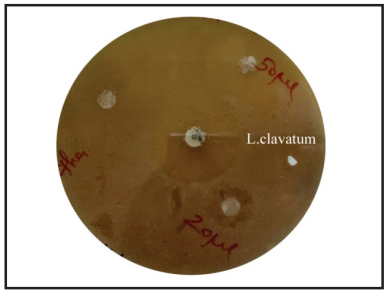

Figure 3: A. Disc diffusion assay for Escherichia coli

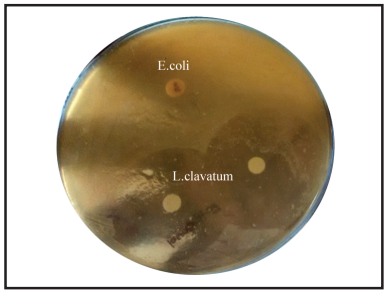

Figure 3: B. Disc diffusion assay for Proteus vulgaris

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