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Research Article

IN VITRO ANTIBACTERIAL, MEMBRANE DAMAGE, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF *BARLERIA PRIONITIS* L EXTRACT ON UTI CAUSING MULTIDRUG RESISTANT *E.COLI*

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ABSTRACT

Resistance to antibiotics is a ubiquitous clinical problem and is compounded by a dearth of new therapeutic agents. A variety of compounds from natural sources that modify membrane permeability are employed in management of multidrug resistant organisms. In the present investigation total phenol, flavonoid contents (0.33±0.1 mg of Gallic acid and 0.9±0.5mg of Quercetin equivalent per gram of dry extract respectively), anti-oxidant (IC_{50} 0.3±0.02 mg/ml compared to standard Ascorbic acid 0.5±0.01 mg/ml) and anti-inflammatory activity (in terms of albumin percent inhibition was 85.77% as compared to standard Ibuprofen 90.0% at 50µg/ml) of the extract were evaluated along with the effect of extract on membrane potential of *E.coli* by fluorescence spectroscopy method. Extract of *B.prionitis* L effectively disrupted *E.coli* cell membrane by depolarization peak at 516.62 nm with intensity 210.91 a.u. suggesting decrease in membrane potential after the treatment with extract. The presence of reactive oxygen scavenging agents in the extract effectively exerted their antibacterial action through membrane perturbations. Thus *B.prionitis* L extract containing bioactive compounds can be studied as future alternative to treat UTI infections caused by *E. coli*.

Keywords: *E.coli*, UTI, *Barleria prionitis* L, Membrane potential, Antioxidant and Anti-inflammatory activity

INTRODUCTION

Urinary tract infections (UTIs) caused due to multidrug resistant *E.coli* has constituted serious global healthcare problems by affecting millions of people each year. *E.coli* is a major nosocomial pathogen of infectious diseases in human and proved to be the most frequent etiological agent of 80% UTIs[1,2]. Now a days, the ample exploit of common and novel antibiotics used to treat UTI provides a selective pressure for amplification of *E.coli* resistance gene and as a result of this outer membrane permeability of *E.coli* reduces the antibiotic uptake by loss or change in porin structure. Entry of hydrophobic antimicrobials such as β-lactams into MDR *E.coli* occurs via outer membrane porins channels[3]. Hence by monitoring cells membrane potential and permeability one can get important information about its state and changes regarding to diseases including Parkinson's ,Epilepsy and Bartter's Syndrome etc[4]. It is reported that the membrane potential plays a critical role in the cells antibiotic uptake and cells bactericidal action[5]. Towards the end of this MDR *E.coli* growing and worrisome problem plant derived natural compounds could be an alternate approach[6]. Nature's large arsenals of bioactive phytochemicals are capable of disrupting cell membrane and are found in plants. Plant extracts containing these bioactive compounds modulate ion channel activity of bacterial cell membrane by increasing permeability and making difficult microbial adaptability[7]. Although plants are great source of many medications but still large number of plant species need to be analyzed for their antimicrobial (drug) activity against multi-drug resistant species including *E.coli*. To identify compounds that modulate membrane ion channel activity, it is imperative that rapid and economical evaluation of biological activities in a high throughput manner be available and according to this, optical read outs using membrane impermeable fluorescent probes to detect trans membrane ion-flux mediated changes in membrane potential or intracellular ionic levels such as Ca^{+}, porins channels have emerged as an surrogate activity[8,9]. Changes in membrane potential is an early indication of injury and ability of *E.coli* cell to maintain stable membrane potential and can be determined by probe uptake or exclusion[10]. Probes are sensitive to change in membrane potential by passively diffusing through the cell wall of bacteria and assessing viability of a cell[11-13]. In our quest to assess criteria defining the death of MDR *E.coli*, we searched the fluorescent probes ($DiBAC_4$ (3)) to find out changes in bacterial membrane potential as markers for the detection of early injury in bacteria. In addition the permeability properties of the membrane influence the size of the membrane potential. Thus only those metabolically active bacteria whose membrane has not been injured are able to generate and maintain a sizable membrane potential[14].The Negatively charged oxonol dye i.e. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)) has a high voltage sensitivity and[15]enters depolarized cells depending on the cytoplasmic membrane potential, where it binds to lipid-rich intracellular components, becomes concentrated and self-quenches fluorescence spectra indicating increased membrane permeability.

B.prionitis L (Acanthaceae) is a perennial shrub widely distributed throughout Africa, India, Srilanka and Tropical Asia[16].Crude extract of this plant is commonly used in folk medicine to treat whooping cough and many infectious diseases. Previous studies have reported that the plant exhibit anti-respiratory syncytial virus[17], anti-arthritic and anti-fertility activities[18,19]. Extraction of aerial parts shows phenylethanoid glycosides, barlerinoside, iridonoid glycosides, flavonoids, alkaloids, saponin etc as a phytochemical constituents.

In the present investigation, by using fluorogenic technique we studied changes in membrane integrity of *E.coli* cells after exposure to methanolic extract of *B.prionitis* L as well as anti-microbial, antioxidant and anti-inflammatory activities of the study plant.

MATERIALS AND METHODS

Isolation and identification of *E.coli*

Urine samples (n=50) of clinically diagnosed UTI infected patients were collected in wide mouthed sterile plastic container (Hi-Media) from the Local Medical College with the help of Clinicians. Loopful of each sample was streaked on Mac Conkey's agar media (Hi-Media) plates for selective isolation of *E.coli* and incubated at 37⁰C for 24 h. After incubation colonies were selected and characterized on the basis of morphological, cultural, biochemical characteristics[20] and were identified using Enterobacteriaceae identification Test Kit (Hi-MViC™ Biochemical Test kit, KB001, Hi-Media)

E.coli screening for multi-drug resistance

All identified *E.coli* strains were further subjected to antimicrobial sensitivity test (AST) by Kirby-Bauer Disc Diffusion Technique[21] for determining their multi-drug resistance pattern (antibiogram) against 18 different antibiotics specified for Enterobacteriaceae. A Mac Farland turbidity (0.5) adjusted overnight grown *E.coli* culture was swab inoculated on Muller-Hinton agar plates. Different antibiotic discs of varying concentrations were applied aseptically on above inoculated agar plates. After 24 h of incubation at 37⁰C, isolates were evaluated for multidrug resistance by comparing the zone of inhibition with standard interpretative chart recommended by NCCLS standards[22].

Collection and Authentication of plant specimen

Barleria prionitis L belonging to Acanthaceae family has been selected for the study. Leaves of this plant were collected from different natural habitats of local forest. Taxonomic identification was done by the experts.

Preperation of extract

Leaves were shade dried and powered into coarse form. The resulting powder (100g) was exhaustively extracted with methanol in the ratio of 1:5(w/v) for 24 h using Soxhlet apparatus. The extract was concentrated under reduced pressure using rotary vacuum flash evaporator to get a constant volume and total yield was determined. Extracts were preserved at low temperature until its use.

Phytochemical prospecting

The phytochemical tests to detect the presence of saponins, tannins, flavonoids, steroids, triterpenes, coumarins, organic acids and alkaloids were performed according to the standard methods[23]. The tests were based on visual observation of a change in color or precipitate formation after addition of specific reagents.

Sensitivity testing of crude plant extract

Methanolic extract of *B.prionitis* L was tested against multi-drug resistant *E.coli* for preliminary antimicrobial activity using Agar Well Diffusion Bioassay[24]. Tubes containing 7 ml of nutrient broth was inoculated with an overnight grown test culture (*E.coli*) until the density reaches up to 0.5 Mac Farland turbidity standards (Approximately 10^5 CFU/ml) and swab inoculated on sterile Muller-Hinton agar plates. By using 5 mm diameter cork boarer, wells were prepared in the agar and 100 µl of methanolic extract was added in to the well. Methanol was employed as a control. Plates were incubated at 37^0C for 24 h. Presence of clear inhibitory zone surrounding the well indicates antimicrobial activity of the extract towards MDR pathogen. Diameter of this zone was measured and recorded.

Determination of MIC

Minimum inhibitory concentration was determined by tube dilution method[25]. Varying concentrations of plant extracts were added in nutrient broth test tubes. Active aliquot of 0.5 Mac Farland turbidity adjusted (0.1 ml) was added to each tube. Tubes were incubated at 37^0 C for 24 h. Two control tubes were maintained for each test batch that included as antimicrobial control (Tube containing extract and the growth medium without inoculums) and organism control(the tube containing growth medium and inoculums).The lowest concentration of extract that do not show any turbidity (complete growth inhibition) in comparison with control was considered as MIC.

Quantitative analysis of antioxidative components

Determination of total phenols

Amount of phenol in the methanolic extract of *B.prionitis* L was determined by Folin-Ciocaltue reagents by Spanes method[26]. Folin-Ciocaltue reagent (10%, 2.5 ml and 2 ml of Na_2CO_3 (2% w/v) was added to 0.5 ml sample (Methanolic extract 1mg/ml) in 3 replicates. Mixture was incubated at 45^0C with continuous shaking for 15 min. Absorbance was measured at 750 nm by using UV/VIZ Spectrophotometer (Shimadzu UV). Results (Phenolic contents) were expressed according to Calibration curve of Gallic acid (0-0.5 mg/ml).

Estimation of total flavonoids

Flavonoid content was estimated by aluminum chloride colorimetric method. One milliliter of Methanolic extract (1 mg/ml) was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of potassium acetate and 5.6 ml of Distilled water. The reaction mixture was incubated for 30 min at room temperature. Absorbance was measured at 420 nm with UV/Visible spectrophotometer. Total Flavonoid was determined by extrapolating the calibration curve which was made by preparing standard Quercetin solution (0-0.8 mg/ml).

Determination of antioxidant activity

DPPH radical quenching activity was determined by a standard method[27] with slight modification. Briefly, 1 ml of methanol solution of DPPH (0.135Mm) was added to 1ml of the sample solutions at different concentrations (0.2-0.8 mg/ml) and vortexed thoroughly. The mixtures were allowed to stand in dark at room temperature for 30 min. Reference compound; Ascorbic acid (0.2-0.8 mg/ml) was prepared in methanol. Changes in absorbance of extracts were measured at 517 nm. The scavenging activity of the extract was expressed as 50% effective concentrations (IC_{50}).The percent inhibition was calculated using following formula

DPPH Scavenging activity(%)=[(Abs control-Abs extract)/Abs control] × 100

Anti-denaturation (anti-inflammatory) activity

B.prionitis L methanolic extract was employed for anti-denaturation assay by Williams et al method with slight modification[28]. Briefly, 0.2% (w/v) of BSA solution was prepared in Tris buffer saline (pH 6.8). Stock solution of 10,000µg/ml of extract was prepared. Using the stock solution different extract concentrations (50,100,200,400,600 and 800 µg/ml) were prepared. In Eppendorf tube, 50 µl of plant extract and 5 ml of 0.2 ml of 0.2 w/v BSA solution and 50µl of methanol were added. Ibuprofen (100 µg/ml) was employed as a standard in methanol with 5 ml of 0.2% (w/v) BSA solution. Test tubes containing the sample mixture were heated at 72^0C for 5 min and cooled for 10 min. Absorbance was measured at 660 nm on spectrophotometer. The % inhibition of denaturation of protein was determined on a % basis relative to control, using the formula

% inhibition of denaturation= [(Abs of control-Abs of extract) / Abs of control] × 100

Fluorometric Membrane Potential Assay

Multidrug resistance *E.coli* membrane potential was assayed fluorometrically by Pag et al method[29] with slight modification for measuring changes in membrane integrity of *E.coli* cell after exposure to methanolic extract of *B.prionitis* L. In brief *E.coli* cells were grown in 10 ml of Muller-Hinton broth (Hi-Media) at 37^0C on rotary shaker to obtain 0.5 Mac Farland turbidity (Approximately 1×10^7 CFU/ml). About 2 ml of broth culture was dispensed in a cuvette and incubated with extract of (10× MIC) for 30 min-1 h. The membrane potential sensitive fluorescent probe Bis-(1, 3-dibutylbarbituric acid) trimetine oxonol (DiBAC4 (3), fluorescent probe, Fluka) was added for 5 min. After 5 min incubation fluorescence was measured at excitation and emission wavelength 492 and 516 respectively. Background fluorescence was determined and results were corrected.

RESULTS AND DISCUSSION

Till the date Urinary Tract infections (UTIs) are the most common encountered prevalent infections in clinical practices. To treat UTI in early stage it is necessary to identify the correct etiological agent and its susceptibility to various antibiotics (Aminoglycosides, Bêta-lactams etc). Inhibition of the target cell growth by measuring the permeability alterations of the gram negative *E.coli* outer membrane helpd us to serve this study purpose for the screening of new plant derived natural antimicrobials

In the present investigation/study we isolated total 31 clinical isolates of *E.coli* from urine samples of UTI infected patients on the selective isolation media.All the isolates were further characterized to species level by using standard microbiological techniques and finally confirmed by *Enterobacteriaceae* identification Test kit (Hi-media). Results revealed total 20 *E.coli* positive strains.

Subsequently all the isolates (n=20) of *E.coli* were screened for their multi-drug resistance profile by Disc Diffusion method with commercially available antibiotic discs viz. Cloramphenicol (C), Cefotaxime(CE), Amoxycillin/Clavulanic Acid(AC), Nalidixic Acid(NA), Ceftazidime(CA), Fusidic acid(FC), Aztreonam(AO), Gentamicine(G), Penicilillin(P), Cephalexin (CP), Amoxicillin (AM), Cefpodoxime (CEP), Tetracycline (T), Imipenem (I), Amikacin (AK), Trimethoprim (TR), Ampicillin (A) and Vancomycine (VA) etc. Antibiogram revealed that nearly all isolates were equally multi-drug resistant except the one strain which was highly drug resistant i.e. more than 80% resistant (graph 1). The resistance to Aminoglycosides and Chloramphenicol in Gram negative multi-drug resistant *E.coli* is often mediated by B-lactamases which are unaffected by exposure to the potential drugs[30]. Hence, the strain with elevated decreased susceptibility to most of the antibiotics was continued for further work.

Extract preparation, yield and phytochemical determination

Leaves of *B.prionitis* L were extracted (dark Black color) by solvent extraction method using methanol with a final yield 24.74%. Phytochemical constituents revealed presence of Carbohydrates, Glycosides, Phytosterol, Phenolic Compounds, Tannins, Flavonoids, Saponins etc as shown in table 1. As plant is a biosynthetic laboratory, besides the chemical compounds (Carbohydrates, Proteins, Lipids) it contain many secondary metabolites having medicinal properties.The phytochemical prospecting revealed the presence of diverse classes of secondary metabolites with wide variety of biological activities like antimicrobial, anti-oxidant and anti-inflammatory[31].

Sensitivity testing of crude plant extract and MIC

The experimental results revealed that crude methanolic extract exhibited good antimicrobial activity against MDR *E.coli* with 12 mm inhibition zone. These results are of interest since they have been obtained with crude methanol extract and not a pure product which can be considered to have good potency level. It is reported that, plants are rich in wide variety of Secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids which possess *in vitro* antimicrobial properties[32] and thus in our study, susceptibility of *E.coli* to this extract may be due to presence of these compounds. We detected saponins, which are secondary metabolites of the plants containing a steroid or triterpenoid a glycan attached to one or more sugar chains and exhibited very good cell membrane permeabilizing property. It is reported that the phytochemical interaction with bacterial cytoplasmic membrane might have led to disruption of membrane structure resulting in dissipation of the transmembrane potential and eventual cell death by inserting into the cytoplasmic membrane forming channels or pores that are proposed to lead to the leakage of cell contents and cell death[33]. The methanolic extract of *B.prionitis* L containing phytochemicals may acts as permeability enhancers that decreases or removes extracellular layer resistance reversibly and allow the drug to pass through the membrane[34] and hence the activity of the extract and chemical nature of its active components suggested that it might be membrane active.

Methanolic extract of *B.prionitis* L revealed MIC at 1 mg/ml and hence indicative of the sub-lethal injury of bacterial cell membrane that alter their permeability. This finding supports the use of *B.prionitis* L in the treatment of UTI caused by MDR *E.coli.*

Estimation of Phenol and Flavonoids

Total phenol and flavonoid content of the *B.prionitis* L was 0.33±0.1 mg GAE/g and 0.9±0.5 mg of Quercetin equivalent per gram of dry extract respectively. It is well known that presence of phenolic compounds provides health-beneficial effects by counteracting reactive oxygen species (ROS)[35]. This study affirms the *in vitro* anti-oxidant potential of crude methanolic extract of the leaf *B.prionitis* L with results comparable to those of the standard compounds such as Gallic acid to clarify the *in vivo* potential of this plant in management of acute UTI infections.

Flavonoids are synthesized by the plants in response to antimicrobial infections and are effective against a broad range of microorganisms[36]. They have the capacity to form complexes with extracellular soluble proteins, which have been shown to exert their antibacterial action through membrane perturbations. This perturbation of cell membrane coupled with the action of β-lactams on the transpeptidation of the cell membrane which could lead to enhanced antimicrobial effect[37]. It is reported that some lipophilic flavonoids also rupture the plasma membrane of microorganisms[38]. Presence of phenolic compounds and flavonoids in *B.prionitis* contributed to their anti-oxidative, antimicrobial as well as cell wall permeability property hence proving the usefulness of this plant as a potent antimicrobial. It is noteworthy that no Alkaloid was detected in the extract, as these have been associated with medicinal uses for centuries but one of their common biological property is their cytotoxicity and their absence tends to lower the risk of poisoning by the plant[39].

DPPH Radical quenching activity

The DPPH assay constitutes a quick, low cost and widely used method frequently used for the evaluation of anti-oxidative potential of various natural products since it accommodate many samples in a less time and help to detect active ingredients at low concentration[40]. As, the methanolic extract of *B. prionitis* L exhibited radical scavenging activity in a concentration dependent manner (table 2) with an IC_{50} value of 0.3±0.02 mg/ml which is near about to the L-ascorbic acid (IC_{50} 0.5±0.01mg/ml).This results infers that the decrease in absorbance of DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation (a color change from purple to yellow). Thus the methanolic extract reduces the radical to the corresponding hydrazine when it reacts with the hydrogen ions released from the samples which contain antioxidant principles[41].

Determination of Anti-inflammatory activity

It is well known that, UTIs are also associated with acute inflammations. To analyze the extract for anti-inflammatory activity is one of the important objective of this study. Inflammation is normal protective response to tissue injury that is caused by physical trauma, noxious, chemical or microbiological agents[42]. Denaturation of protein is well documented cause of inflammation. Phenyl butazone, Salicyclic acid, flufenamic acid, Ibuprofen (Anti-inflammatory drugs) etc. have shown dose dependent ability to thermally induced protein denaturation[43].

As a part of investigation on the mechanism of the anti-inflammatory activity, ability of extract to inhibit protein denaturation was studied. Methanolic extract exhibited significant anti-denaturation activity. It is effective in inhibiting heat induced albumin denaturation at different concentration. Maximum inhibition (85.77%) was observed at 50 µg/ml. IC_{50} value was found to be 70 µg/ml. At the same experimental conditions Ibuprofen a standard anti-inflammatory drug showed the maximum inhibition 90.05% at the concentration of 50 µg/ml. The degree of inhibition of BSA denaturation increased with the decrease in the concentration of both the extracts showing the anti-denaturation of the drug is more at lower concentration (Table 3). The plant extract revealed to contain saponins, known to produce inhibitory effect on inflammation[44]. Literature survey also suggests that, the anti-denaturation property of BSA was due to the presence of two interesting binding sites in the aromatic tyrosine rich and aliphatic threonine and lysine residue regions of the BSA[27]It has been reported elsevere that in inflammatory diseases the excessive activation of phagocytes, production of $O_{2;}$ .OH radicles and non-free radical species(H_2O_2) which can harm surrounding tissue, initiates lipid peroxidation which results in membrane destruction and hence damaged tissue provokes inflammatory response by production of mediators and chemotactic factors[45]. Hence the reactive oxygen scavenging agents can be beneficial in the treatment of inflammatory disorders.Thus in this sturdy *in vitro* antidenaturation of Bovine serum albumin and reducing antioxidant activity was evaluated. The results revealed that the plant have moderate to significant antioxidant and anti-denaturation activity.

Fluorometric Membrane Potential Assay

The fluorescent membrane potential dye $DiBAC_4(3)$ has been used in our study for indicating the changes in membrane polarization of *E.coli* strain.The results showed that *E.coli* cells treated with methanolic extract of *B.prionits* L disrupted the cell membrane by depolarization peak at 516.62 nm with intensity 210.91 au whereas in control, the dye depolarized cell membrane showed a peak of 515.62 nm with same intensity suggesting decrease in membrane potential as an evidence of increase in flouorescence (Fig.2).Extract of *B.prionitis* L increased the fluorescence in the treated MDR *E.coli* cell due to membrane depolarization (less negative inside the cell) and as a result revealed decrease in membrane potential[46] but increase in bactericidal activity by increase in membrane permeability. The cell membrane is an active structure and acts as a barrier between cytoplasm and the extracellular medium which is essential for maintaining optimal internal conditions for metabolism and energy transduction. The dye depolarizes the cell membrane if potassium is found in the inoculum medium. Recent studies revealed that depolarization occurs primarily due to pH changes (from acidic to neutral) or by increasing movements of ions, specifically K^+ channels and affects cellular homeostasis[47].

The dye $DiBAC_4$ (3) is an oxonol which is an anionic molecule that showed enhanced fluorescence upon binding to *E.coli* membrane by depolarizing the cell membrane. It moved into the cell and subsequently bound to intracellular hydrophobic states resulting in enhanced quantum yield of DiBAC4 (3) as the dye have very low fluorescence in the extracellular aqueous environment but increase their quantum yield upon binding to the hydrophobic core of the lipid membrane. Upon depolarization of the cellular membrane, the negatively charged oxonols moves from the extracellular medium into the cell membrane and bind to cellular membranes there by results in fluorescence quantum yield and emission.

Table 1: Quanlitative Analysis of the Phytochemicals of *B.prionitis* L

Phytoconstituents	Methanolic extract of *B.prionitis* L
Alkaloids	-
Terpenoids	+
Steroids	+
Tannins	-
Saponins	+
Cardiac glycosides	+
Flavanoids	+
Coumarins	-
Carbohydrates	+

(+) & (-) Presence & Absence of Phytoconstituents

Table 2: *In vitro* Radicals scavenging activities*of *B.prionitis*

Concentration	Inhibition of denaturation (%)	
mg/ml	Ascorbic acid	*B. prionitis*
0.2	87.99%	75.98%
0.4	88.27%	75%
0.6	87.28%	62.85%
0.8	88.13%	63.27%

*Values represent the mean of triplicate results (Experiments)

Table 3: *In-vitro* anti-inflammatory activity*of *B.prionitis* L

Conc. Dose (μg/ml)	Inhibition of denaturation (%)	
	Standard (Ibuprofen)	*B. prionitis*
50	90.00	85.77
100	89.97	70.12
200	84.00	69.21
400	75.28	70.00
600	75.40	63.00
800	64.80	62.31

*Values represent the mean of triplicate results (Experiments)

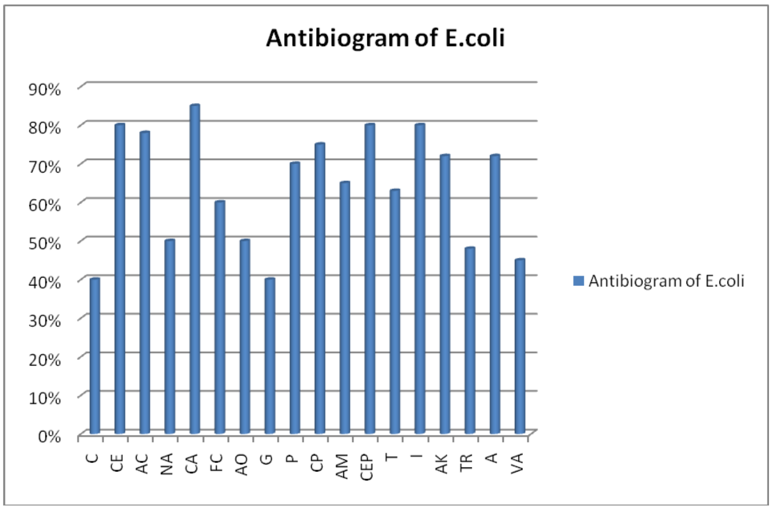

Fig. 2: Fluorometric membrane potential assay of *E.coli*

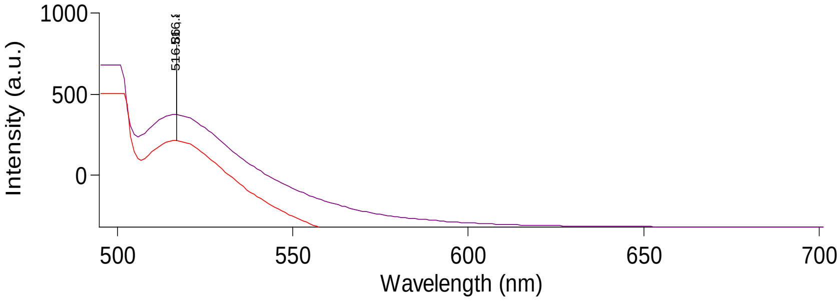

Fig. 2: Fluorometric membrane potential assay of *E.coli*

Red color: test culture, Black color: control

CONCLUSION

This study affirms that extract of *B. prionits* L can damage MDR *E.coli* cell membrane by exerting profound physiological changes that lead to bacterial death. Crude methanolic extract of *B. prionits* L revealed *in vitro* anti-oxidant, total phenol and flavonoid contents, anti-inflammatory and antimicrobial potential. The results are comparable to the standard compounds such as Ascorbic acid, Gallic acid, Quercetin and Ibuprofen to clarify the *in vivo* potential of this plant in the management of UTI infections. Thus the multi-therapeutic characteristics of this plant extract serves as a source of plant derived natural products that modify antibiotic resistance of MDR *E.coli*. Further investigations are in progress to find active component of this plant extract and to confirm its mechanism of action *in vivo.*

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