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Research Article

## Effect of *Phaseolus vulgaris* on *E. coli* induced peritonitis and bacteraemia in mice

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### ABSTRACT

Infectious or non-infectious peritonitis leads to systemic inflammation due to violation of the peritoneum which is often fatal. Evidences suggest that common bean (*Phaseolus vulgaris* L.) is a source of nutrients and contains phenolic compounds having antioxidant activity and its consumption has been linked with improved health benefits. The aim of the present investigation was evaluate the in vitro antibacterial, antioxidant activity and protective potential of the methanolic extract of *P. vulgaris* in *E. coli* induced model of peritonitis in albino wistar rats. Rats were pre-treated with 200 mg/kg and 400 mg/kg/bwt dose for 3 days and fourth day with *E. coli* (1×10<sup>8</sup> CFU/ml) strain and consecutively 3 days treatment. Mortality was monitored for 14 days. After the death of rats or completion of the experiment rats were sacrifice and kidney were used for our protocol. Colonies were count and statically analysis was done. Results showed dose dependent antibacterial activity. Thus the methanolic extract of *P. vulgaris* exhibited significant protection against *E. coli* induced peritonitis in normal rats. It significantly reduced the viable cells of *E. coli* when inoculated in rats. Activity is attributed to flavonoids and phenolic compounds. The present study thus suggests that methanolic extract of *P. vulgaris* significantly reverses peritoneal infection by *E. coli* in rats. It can be suggest that this medicinal formulation will be used as herbal medicine with no side effects. The high content of phenolic compounds, antioxidant activity and antibacterial activity of *P. vulgaris* indicate that they may impart health benefits when consumed and should be regarded as a valuable source of antioxidants. Thus, consumption of *P. vulgaris* seed along with coats might be recommended to gaining better nutritive benefits.

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### INTRODUCTION

*Phaseolus vulgaris* (Linn) belong to the family of Fabaceae are widely consumed throughout the world. They play a significant role in human nutrition, being an important source of plant proteins, minerals and certain vitamins and exhibit, for this reason, high nutritional value. In last years, bioactive effects, associated with the fibres, polyphenols, and other beans components related to human health have gained attention<sup>1,2</sup>. Beans contain substantial amount of phenolic acids and flavonoids, some cultivars (red, black, and blue-violet coloured beans) show also anthocyanins, such as delphinidin and cyanidin, that overall attribute them a very strong antioxidant and antiradical activities<sup>3</sup>. Polyphenols are essentially present in the seed coats and in minor amount in cotyledons. The beneficial effects of polyphenols on human health are expressed primarily through the reduction of the oxidative stress<sup>4</sup>. Some polyphenols are also

able to exert antiapoptotic, antiaging, as well as anticarcinogenic activity, overall inhibiting the cell proliferation processes<sup>5,6</sup>. Epidemiological studies have shown a strong link between the consumption of legumes and prevention of cancer risk, as well as the reduction of diabetes and cardiovascular risk through a normalization of the blood lipid and glucose profile<sup>7</sup>. Wedick et al. (2012) demonstrated that, among flavonoids, higher intakes of anthocyanins could be significantly associated with a lower risk of type 2 diabetes (risk: 0.85)<sup>8</sup>. Peritonitis is a serious disease, due to the inflammatory response in the serous membrane lining the abdominal cavity and viscera. The immediate answers to peritonitis are hyperthermia, bowel distension, hyperaemia, accumulation of gases and liquids, hypovolemia and pain. At the same time, there are cardiac, respiratory, renal and metabolic responses. It is also high contribution of fibroblasts that produce fibrin, responsible for the formation of intra-abdominal adhesions<sup>9-12</sup>. Although

often the treatment of peritonitis include mechanical removal of contaminants through peritoneal washings with saline, antibiotics and abdominal integrity restoration associated with modern intensive and surgical care units, currently peritonitis still accounts for approximately 50% of deaths consequent to sepsis<sup>13-16</sup>. Given popular use of *P. vulgaris* in treating diseases and previous research demonstrating antibacterial and antioxidant activity. The objective of this study was to estimate the total phenolic content, flavonoid content, antioxidant activity, antibacterial and protective potential of the methanolic extract of *P. vulgaris* in *E. coli* induced model of peritonitis in albino wistar rats.

## MATERIALS AND METHODS

### Plant material

The leaves of *P. vulgaris* were collected from Pinnacle Biomedical Research Institute (PBRI), Bhopal, (M.P). The sample was identified by senior Botanist, Professor and head department of Botany, Safia College of Arts and Science, peer gate Bhopal. A herbarium of plants was submitted to the specimen library of Safia College of Arts and Science, peer gate Bhopal and The specimen voucher no. of *P. vulgaris* is 388/Bot/Saf.

### Chemicals and reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All the chemicals used in this study were of analytical grade.

### Bacterial strain

The test organisms *Escherichia coli* (MTCC 2075) were obtained from the stocks of the Pinnacle Biomedical Research Institute, Bharat Scout and Guide Campus, Shyamla hills, Bhopal, (M.P.).

### Extraction of plant material

#### Cold maceration

Leaves of *P. vulgaris* were collected, washed and rinsed properly. They were dried in shade and powdered mechanically. About 1kg of the powder leaves was extracted with methanol and allow to standing for 2-3 days. At the end of the third day extract was filtered using whatmann No. 1 filter paper to remove all un-extractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts<sup>17</sup>.

### Qualitative analysis of phytochemicals

The extracts prepared for the study were subjected to preliminary phytochemical screening by using different reagents for identifying the presence or absence of various phytoconstituents viz., carbohydrates, proteins, alkaloids, tannins, steroid, flavonoids, polyphenols and terpenoids in various extracts of apple. The above phytoconstituents were tested as per the standard method<sup>18</sup>.

### Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in extracts. For this TPC and TFC are determined. Methanolic

extracts of *P. vulgaris* are subjected to estimate the presence of TPC and TFC by standard procedure.

### Total phenolic content estimation (TPC)

The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Concentration of (10-50µg/ml) of gallic acid was prepared in methanol. Concentration of 1mg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced in to test and mixed with 2 ml of a 10 fold dilute folin ciocalteu reagent and 4 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and it was then incubated at room temperature for 30 min with intermittent shaking and the absorbance were taken at 765 nm against using methanol as blank. Total phenolic content was calculated by the standard regression curve of gallic acid and the results were expressed as gallic acid equivalent (mg/g)<sup>19</sup>.

### Total flavonoid content estimation (TFC)

Different concentration of rutin (10-50µg/ml) was prepared in methanol. Test sample of near about same polarity (1mg/ml) were prepared. An aliquot 0.5ml of diluted sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% NaNO<sub>2</sub> solution. After 6 min, 0.15 ml of a 10% AlCl<sub>3</sub> solution was added and allowed to stand for 5min and then 2 ml of 4% NaOH solution was added to the mixture. The final volume was adjusted to 5ml with distilled water and allowed to stand for another 15 min. Absorbance was determined at 510 nm against water as blank. Total flavonoid content was calculated by the standard regression curve of rutin/ quercetin<sup>20</sup>.

### In vitro Antioxidant activity

#### DPPH radical scavenging activity

For DPPH assay, the method of Gulçin *et al.*, 2006<sup>21</sup> was adopted. A solution of 0.1mM DPPH (4mg/100ml) in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of *P. vulgaris* extracts. The change in colour was measured at 517 nm wavelength using methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. The percentage inhibition of free radical DPPH was calculated from the following equation:

% inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC<sub>50</sub>), IC<sub>50</sub> was calculated based on the percentage of DPPH radicals scavenged. The lower the IC<sub>50</sub> value, the higher is the antioxidant activity.

#### Reducing power assay

A spectrophotometric method was used for the measurement of reducing power. For this 0.5 ml of each of the extracts was mixed with 0.5ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% potassium ferricyanide (10 mg/ml). The reaction mixture was incubated at 50 °C for 20 min separately, and then rapidly cooled, mixed with 1.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (0.5ml) of the supernatant was diluted with distilled water (0.5ml) and then ferric chloride (0.5ml, 0.1%) was added and allowed to stand for 10 min. the absorbance was read spectrophotometrically at 700 nm. Ascorbic acid

(AA) was used as standard for construction of calibration curve<sup>22</sup>.

$$\text{Reducing Power (\%)} = (\text{As} / \text{Ac}) \times 100$$

Here, Ac is the absorbance of control and (As) as is the absorbance of samples (extracts) or standards.

### Anti bacterial sensitivity assay

#### Well diffusion method

The agar well diffusion method technique (Bauer *et al*, 1966)<sup>23</sup> was used to determine the antibacterial activity of the plant extracts. Inoculation was done on sterile nutrient agar media plate using 18 hours old culture. A sterile 5mm cork borer was used to punch holes after solidification of media. The wells formed were filled with different concentrations of the extract which were labeled accordingly; 25mg/ml, 50mg/ml, 75mg/ml, 100mg/ml. The plates were then left on the bench for 1 hour for adequate diffusion of the extracts and incubated at 37°C for 48 hours in upright condition. The Experiment was repeated triplets and the mean values were calculated.

### In vivo study

#### Animals

All ethical and handling guidelines were followed as set by Indian Legislation and approved by Institutional Animal Ethics Committee. All animals were procured and housed in animal house maintained under standard hygienic conditions. All animals were given standard diet (Golden Feed, New Delhi) and water regularly. Animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute. Protocol approval reference no. PBRI/IAEC/PN-19013.

#### Acute oral toxicity

The acute toxic class method set out in guideline is a stepwise procedure with the use of 3 animals of a single sex per step. Depending on the mortality and/or the moribund status of the animals, on average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex. Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.; no further testing is needed, dosing of three additional animals, with the same dose and, dosing of three additional animals at the next higher or the next lower dose level. Three animals are used for each step. The dose level to be used as the starting dose is selected from one of four fixed levels, 5, 50, 300 and 2000 mg/kg body weight<sup>24</sup>.

#### Treatment

Animals were housed in a group of five in separate cages under controlled conditions of temperature (22 ± 2°C). All animals were given standard diet (Golden feed, New Delhi) and water, *ad libitum*. The environment was also regulated at 25 ± 1 °C with 12/12 h (light/dark) cycle. Animals were further divided in four groups with six animals in each group.

Group I: **Normal control** (Normal saline were administered by oral route)

Group II: **Vehicle treated with *Escherichia coli* treated group:** *Escherichia coli* (1×10<sup>8</sup> CFU/ml) were dissolved in normal saline and were administered by oral route at a dose of 10ml/kg body weight.

Group III: **Standard drug treated group:** Ofloxacin was dissolved in normal saline and was administered by oral route at a dose of 5mg/kg body weight

Group IV: **200 mg/kg extract treated group:** Extract was dissolved in normal saline and was administered by oral route at a dose of 200mg/kg body weight.

Group V: **400 mg/kg extract treated group:** Extract were dissolved in normal saline and was administered by oral route at a dose of 400mg/kg body weight.

#### Preparation for bacterial inoculums

In brief, *E. coli* strain (MTCC 2075) were grown on nutrient broth medium (3 g of beef extract, 5 g of peptone and 5 g of NaCl, pH 7, sterilized by autoclaving at 120°C for 30 min.) from a single colony and incubated at 37°C for 16-18 h at 37°C to obtain stationary growth phase cultures. The bacteria were then centrifuged (200 rpm) for 10 min at 4°C and the pellets were resuspended in PBS to an OD of 0.1 at 660 nm, with a spectrophotometer, corresponding to 10<sup>8</sup> CFU/ml<sup>25</sup>.

#### Systemic infection by *E. coli*

To produce infection, the rats were induced by the intra-peritoneal with suitable inoculums in a volume of 0.2 to 0.25 ml. After infection, the rats were observed twice daily and animals exhibiting profound inanition or an inability to reach food and water were sacrificed. The experimental design involved administration of each of the three test agents by daily oral dosing for a period of 14 days. dosing regimens were started on days -7,-6, -5, -4, -3, -2, -1, 0, 1, 2, 3, 4, 5, 6, 7 and relative to the day of challenge (day 0) with 1×10<sup>8</sup> CFU of *E. coli*/ ml. Before and after the challenged day animals were treated with 200 mg/kg body weight and 400mg/kg body weight *P. vulgaris* extract and 5mg/kg body weight Ofloxacin respectively. Survival was monitored for all experimental groups till 14 day. These conditions were in accordance with those of previously described method<sup>26</sup>, with slight modification. The pathological status of the rat was determined by visual examination of internal organs after their death or sacrifice at the completion of the experiment. All surviving rats were killed by cervical dislocation on 15 day determination of the numbers of CFU of *E. coli* per gram from the kidney<sup>27,28</sup>. This determination was made by aseptically removing and weighing both kidneys, homogenizing kidneys in w/v ml of saline with a high speed Homogenizer (Remi RQ-124A) and Kidney burden was determined by culturing of homogenates in physiological saline followed by plating 0.1 ml aliquots onto Nutrient agar plates. The plates were incubated at 37°C, and the number of colonies was enumerated after 48 h of growth<sup>29</sup>. All animal care procedures were supervised and approved by the Institutional Animals Ethics Committee (IAEC) of PBRI, Bhopal.

#### Bacterial clearance

*E. coli* was determined in the different groups by sampling retro blood at various intervals after 16 hrs and up to 14 days (at which time maximum mortality will be recorded). The sample was serially diluted and plated on Nutrient agar media. Incubate it at 24 hours at 37°C temperature. After incubation colony count was determine by colony counter method.

#### Biostatistical interpretation

All data are presented in Mean ±SD. Data were analyzed by One Way ANOVA followed by Bonferroni's test. P<0.05 was considered as level of significance (n=4).

## RESULT AND DISCUSSION

Phytochemical analysis of methanolic extract of *P. Vulgaris* showed the presence of carbohydrate, alkaloids, flavonoids, phenolics, tannin, saponins, triterpenoids Table 1. Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and TFC was then calculated with respect to rutin taken as standard. Results shown in Table 2 & Fig 1, 2. Antioxidant activity of the samples was calculated through DPPH assay and reducing power assay. % inhibition was calculated as an indicative of antioxidant potency. The higher the % inhibition the better the activity. Ascorbic acid was taken as standard in both the tests and the values were comparable with concentration ranging from 20µg/ml to 100µg/ml. A dose dependent activity with respect to concentration was observed (Table 3). The reducing ability of the compound usually depends on the reductants, which have been exhibited antioxidative capacity by breaking the free radical chain, donating a hydrogen atom. Reducing power assay was calculated in extracts and the values indicated a better activity (Table 4). In the acute toxicity study, no signs of toxicity were found upto the dose of 2000 mg/kg body weight. Hence 1/10th and 1/5th doses i.e. 200 mg/kg and 400 mg/kg have been fixed as ED50 for present study (Table 5). The methanolic extract was also tested for in-vitro antibacterial test using well diffusion assay (Table 6). In-vivo antibacterial activity was tested by inducing peritonitis through *E. coli*. The activity of the extract was calculated by measuring the CFU/ml of the microorganisms. The experiment was done in groups of 5 containing normal

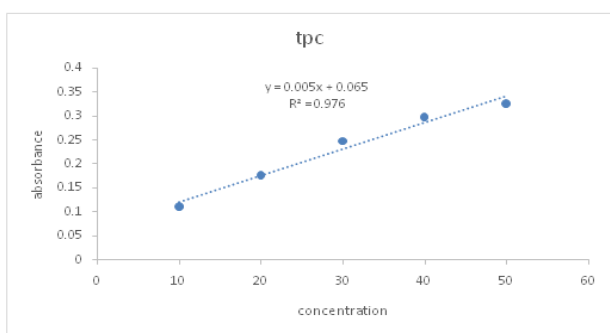
control, vehicle control, standard (5mg/kg), extract (200 mg/kg) and extract (400mg/kg). The results obtained indicated a dose dependent antibacterial activity of the extracts; the extract given at a concentration of 400 mg/kg had better activity than the one administered at 200 mg/kg (Table 7).

**Table 1 Result of phytochemical screening of *P. Vulgaris***

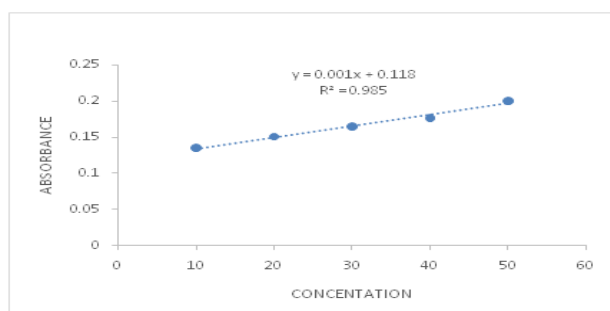
S. no.	Test's name	Result
1.	Borntrager's test	Negative
2.	Legal's test	Negative
3.	Keller killioni test	Negative
4.	Test of saponins	Negative
5.	Mayer's test	Negative
6.	Hager's test	Positive
7.	Wagner's test	Positive
8.	Molish test	Negative
9.	Fehling test	Negative
10.	Benedict's test	Positive
11.	Biuret's test	Negative
12.	Million's test	Negative
13.	Ninhydrin test	Negative
14.	Lead acetate test	Positive
15.	Alkaline reagent test	Positive
16.	Shinoda test	Positive
17.	Ferric chloride test	Positive
18.	Lead acetate test	Positive
19.	Dilute iodine test	Positive
20.	Gelatin test	Positive
21.	Solubility test	Positive

**Table 2 Total phenolic and total flavonoid content of *P. Vulgaris***

S. No.	Plant	Total Phenol (gallic acid) (mg/100mg)	Total flavonoid (Rutin) (mg/100mg)
1.	<i>P. Vulgaris</i>	5.175±0.2101	3.0833±0.7216



**Fig. 1 Graph of estimation of total phenolic content**



**Fig. 2 Graph of estimation of total flavonoid content**

**Table 3 DPPH assay of ascorbic acid, methanolic extract**

S. N.	Conc. (µg/ml)	Ascorbic acid (% Inhibition)	Methanolic Extract (% Inhibition)
1.	20	48.22	44.63
2.	40	54.98	46.44
3.	60	61.39	47.11
4.	80	69.40	50.60
5.	100	74.38	52.28

**Table 4 Result of reducing power assay**

S. N.	Concentration	Ascorbic acid	Methanolic Extract
1.	20µg/ml	0.142	3.706
2.	40 µg/ml	0.211	3.725
3.	60 µg/ml	0.269	3.747
4.	80 µg/ml	0.34	3.793
5.	100 µg/ml	0.397	3.818

**Table 5 Acute oral toxicity of Extract**

S. No.	Groups	Observations/ Mortality
1.	5 mg/kg Bodyweight	0/3
2.	50 mg/kg Bodyweight	0/3
3.	300 mg/kg Bodyweight	0/3
4.	2000 mg/kg Bodyweight	0/3

**Table 6 Antibacterial activity of extract by well diffusion assay**

Bacteria	Zone of inhibition			
	25µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
<b>E. coli</b>	12 mm	14 mm	16 mm	19 mm

**Table 7 E. coli induced peritonitis**

Groups	CFU/ml
<b>Normal Control</b>	0.004
<b>Vehical control</b>	0.0944
<b>Standard (5 mg/kg bwt)</b>	0.02
<b>Extract (200 mg/kg bwt)</b>	0.0764
<b>Extract (400 mg/kg bwt)</b>	0.0468

## CONCLUSION

Peritonitis continues to be an important problem in the health care system. *P. Vulgaris* has received some attention for its beneficial effects against several diseases. Our research study showed that the use of *P. Vulgaris* could effectively reduce the severity of acute peritonitis in our *E. coli* model. Further studies need to be done to verify the effect of phenolic compound, explore the mechanisms and promote the clinical use. An accurate biomarker for the early identification of peritonitis would be of great diagnostic value. An early finding of the correct diagnosis of peritonitis and the subsequent effective initiation of an appropriate treatment may help to lower the complication rate and to improve the prognosis

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