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Research Article

Therapeutic Efficacy of *Moringa oleifera* and *Camellia sinensis* Extracts in Combination Against Peritonitis Induced Rat Model

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ABSTRACT

Objective: The present study evaluates the efficacy of *Moringa oleifera* and *Camellia sinensis* in combination in order to investigate anti-peritonitic activity in rat model. Material and methods: Methanol extracts in combination were assayed using varying concentration of extracts (25 $mgml^{-1}$, 50 $mgml^{-1}$, 75 $mgml^{-1}$and 100 $mgml^{-1}$) by well diffusion and minimum inhibitory concentration method against *E. coli, K. pneumonia, P. aeruginosa, B. subtius* and *S. aureus*. *E. coli*induced peritonitic rat model was givencombinative extracts orally at the dose of 200 $mgkg^{-1}$ bw $day^{-1}$and 400 $mgkg^{-1}$ bw$day^{-1}$for seven days. Results:Well diffusion assay had shown variation in zone of inhibition (ZOI) depending on type of bacteria and concentration of extract used. *P. aeruginosa, B. subtius, K. pneumonia, S. aureus* and *E. coli* had shown maximum significant ZOIat the dose of 100mg/ml which corresponded to 21.37±1.19mm, 23.00±2.74mm, 20.75±0.65mm, 20.13±0.85 mm and 20.25±1.19 mm respectively. Minimum inhibitory concentrations (MIC) were ranged between 1 $mgml^{-1}$ to 6$mgml^{-1}$. *E. coli, P. aeruginosa* and *B. subtitles* had shown least value followed by *K. pneumonia* with highest MIC for *S. aureus*. Rat models shown increase in survival percentage, decrease bacterial count in blood keeping RBC and WBC count normal. Survival percentage was found to be reduced to 83.33% when treated with combinative extracts for seven days. Discussion: *In vitro* model revealed anti-bacterial properties whereas peritonitis induced *in vivo* model had shown recovery by using combinative extracts almost comparable to potent antibiotics with no signs of toxicity, mortality and anemia. *Moringa oleifera* and *Camellia sinensis* extract contain possible source of bioactive compounds which can be useful in prevention from infectious diseases. Conclusion: Such combinative extract is safe, non-toxic and can be supplemented orally with other known drug to combat emergence of multiple drug resistance which is a threatening to human health. Further, it is warranted for further investigation with different regimens to explore new kind of medications against bacterial induced peritonitis.

Keywords: Peritonitis, *Moringa oleifera, Camellia sinensi, Escherichia coli,* Antibacterial activity, Minimum Inhibitory Concentrations, *In vitro* and *in vivo* model

INTRODUCTION

Peritonitis is an inflammation of peritoneum, characterized by abdominal pain, inability to pass stool, nausea, fever and shock[1].It leads to high mortality by damaging liver, heart and kidney. It is associated with transfer of intestinal bacteria and its product into systemic circulation through mesenteric lymph nodes as well as portal vein circulation[2-3]. *E. coli* is a very common pathogen that causes peritonitis followed by *K. pneumoniae, S. pneumoniae* and few *Streptococcal* species including *Enterococci*[4-5]. Mono-microbial infection caused by *Staphylococci, Enterococci*and *Candida* species has shown lower pathogenicity in human subjects[6]. In this aspect, a number of other microorganisms like *N. gonorrhoeae, C. trachomatis, M. tuberculosis, C. immitis, Lactobacilli, B. Fragilis* and *Candida* species have also been isolated from gastrointestinal perforation in human subjects[5]. *P. aeruginosa* has been investigated as a primary pathogen in children having bacterial peritonitis[7]. Peritonitic animal model has already been created to treat both facultative and anaerobic Gram-negative bacilli[8]. Antimicrobial therapies were found to be more effective against peritonitis causing organisms using clindamycin along with aminoglycoside which was further replaced by combination of cephalosporin and metronidazole because of their nephrotoxicity effect[9-10]. Emergence of resistance with third generation cephalosporin among certain Gram negative bacilli have been evolved as β-lactamases producers whereas microaerophilic Gram-positive cocci had also shown resistant to metronidazole[11]. Animal model study revealed that *E. coli* is primarily responsible for sepsis of peritoneum and mortality. Late peritonitis occurs due to mixed infection through anaerobes and facultative microorganisms mainly by *B. fragilis, E. coli* and *Enterococci*[5, 12, 13]. Similar studies have also been carried out using polymicrobial intra-abdominal infection with *Enterococci, B. fragilis* and *E. coli.* These microorganisms have been found to enhance abscess formation, bacteraemia, weight loss with high mortality in animal models[14-15].

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Detail review on MO and CS

Moringa oleifera (family: Moringaceae) is often found in the Indo-Bangla subcontinent. Different parts of MO have versatile uses. MO leaves are highly nutritious which carries important vitamins (beta-carotene and Vitamin C), protein, amino acids, iron, potassium and many other health benefits [16]. Dried and powdered form of its leaves has been used commonly in soups and sauces. Leaves of MO have been found to be used in various therapeutic methods as an antibiofilm[17], antibacterial[18], antifungal[19], hypoglycemic[20-21], hypocholesterolemic, hypotensive[22] and hypolipidemicagent[23]. MO has also been reported as a potential source for biodiesel production and possess antioxidant activities [24]. Green Tea (*Camellia sinensis*) has been used as beverage since ancient times but in recent times, a huge curiosity has been given among scientific community because of its antioxidant properties[25-30]. Some epidemiological studies have suggested that tea consumption lowers the risk of several types of cancer[31-33]. Its polyphenols have an effective chemo preventive agent[27, 34]. It has many other health benefits properties such as enhancing the activity of insulin[35], antimicrobial[36-37], imunostimulatory[38], anti-inflammatory[8], protective effect against cardiovascular diseases[39] and cerebral ischemic damage[40]. Recently, scientists have found that Epigallocatechingallate (EGCG), a green tea catechin, could have anti-HIV effects when bound to CD4 receptor [41]. Different factors can influence on the composition of tea like species of the tea, season and climate of the tea, age of the tea leaf and horticultural area[42-43]. Its contains different chemical components such as some proteins, carbohydrate, caffeine, alkaloids, saponins, tannins, catechin and some poly phenol compounds like Epigallocatechingallate (EGCG), Epicatechingallate (ECG), Epigallocatechin (EGC),Epicatechin (EC), Myricetin, Querection, Kaempferol. Some of the chemicals have already been proved for antioxidant activity as well as antimicrobial activity[44-45]. Peritonitis is becoming more prevalent due to emergence of multiple drug resistance (MDR) in pathogens. So there is an urgent surge to explore neutraceutical products to combat with peritonitis and associated infections. Methanol extracts of *Moringa oleifera* (MO) and *Camellia sinensis* (CS) revealed antibacterial activity individually in many studies[46-47]. This is the first report of its kind that showed MO and CS in combination increases its efficacy and work significantly.In the present investigation, it has been tried to evaluate the efficacy of MOextract in combination with CSagainst peritonitis in rat model.

MATERIALS AND METHODS

Plant Material

Leaves of MO (*Moringaceae*) and CS (*Theaceae*) were obtained from the local surroundings at Bhopal, Madhya Pradesh, India and Guwahati, Assam, India during the month of January to March 2014 respectively. The plant was acknowledged by a Senior Botanist Dr. Zia Ul Hasan (Professor and Head) Department of Botany, Govt. Safia College of Science, Bhopal, India. The leaves were shed dried for further extraction and experimentation.

Extraction and determination of extractive value

75gm of CS and 25gm of MO leaves (dried) were taken. After that, the leaves were powdered (coarse) and subjected to Soxhlet thimble using 70% methanol. The mixture was filtered using Whatman filter no. 1then evaporated using rotary evaporator to get a powdery mass. The percentage yield of extracts was calculated by using the formula: [(weight of extract/total of weight of powder) ×100] and stored at 4°C till further use[48-49].

Phytochemical screening

The phytochemical screening was analyzed for various important metabolites such as carbohydrates, proteins, amino acids, alkaloids, saponins, terpenoids, steroid, flavonoids, tannin and phenolic compounds according to the standard procedures[50-51].

In vitro antibacterial assay

In vitro antibacterial activity of the extract was determined using well diffusion method[52] against *E. coli, K. pneumonia, P. aeruginosa, B. subtius* and *S. aureus* on Mueller Hinton agar media which were procured from Pinnacle Biomedical Research Institute (PBRI), Bhopal, India. ZOI was observed after 24 hours of incubation at 37 °C. Double dilution method was used for determination of minimum inhibitory concentration (MIC)[53].

Animal handling

Albino Wistar rats (n=36) of either sex were obtained from PBRI, Bhopal, India. Animals were kept under controlled conditions of humidity (50±15%), temperature (23±1°C) and 12-12 hours light-dark cycles in polypropylene cage on rice husk as bedding. Animals were fed with standard dry pellets (Golden feeds, New Delhi, India) and water *adlibitum*[12]. All animal experiments were approved by Intuitional Animal Ethics Committee (IAEC) PBRI, Bhopal (CPCSEA Reg. No. 1283/C/09/CPCSEA, Protocol Approval No. PBRI/10/IAEC//PN-185).

Acute toxicity tests (LD_{50})

The acute oral toxicity study was carried out according to OECD (Organization for Economic Co-operation and Development) 423 guidelines which is based on a stepwise procedure with the use of a minimum number of animals per step. Healthy, young, adult albino Wistar rats (200 ± 30 gm) were used for this study and kept for 5 days prior to dosing for acclimatization to the laboratory conditions. Animals were fasted prior to dosing. On the next day, dose was calculated according to the body weight[54]. Effects on their behaviour had been observed for 72 hours.

Experimental design

The animals were divided into six groups consisting of six animals in each group. Peritonitis was induced using *E. coli* (1 x 10^8 viable) at the dose of 10 $mlkg^{-1}$ bw ip and considered as first day of experimentation. Total protocol of experiment was maintained for 7 days.

Group 1: Control rats

Group 2: Peritonitic control (PC)

Group 3: PC + 1% Carboxymethyl-cellulose (CMC)

Group 4: PC + Gentamycin 10 $mlkg^{-1}$ bw ip

Group 5: PC + Methanol extracts 200 $mgkg^{-1}$ bw day^{-1} orally + 1% CMC

Group 6: PC + Methanol extracts 400 mgkg-1 bw day-1 orally + 1% CMC

Table 1: Average zone of inhibition (mm) and minimum inhibitory concentration (mg/dl) against specific microbes

Microorganism	Gram reactions	Average MIC (mg/ml)	Extract concentration (mg/ml)			
			100	75	50	25
			Zone of inhibition in diameter (mm)			
E. coli	-ve	1.45	20.25±1.19*	18.00±1.96	16.25±3.01	14.00±4.92
K. pneumonia	-ve	3.25	20.75 ± 0.65*	18.13±1.11	17.38±1.49	16.63±1.44
P. aeruginosa	-ve	1.50	21.37± 1.19*	20.88±0.75	19.13±1.32	16.13±1.75
B. subtius	+ve	1.50	23.00± 2.74*	20.88±0.63	18.75±1.32	15.88±3.17
S. aureus	+ve	6.25	20.13± 0.85*	17.75±1.26	16.75±2.10	12.75±1.19

N=6; values are expressed as mean ±SD; Superscripts * indicates values which are significantly different at $p<0.05$ when compared to minimum extract concentration; -ve=Gram negative, +ve = Gram positive

Table 2: Survival percentage, average bacterial count, RBCs and WBCs count on 7th day of treatment in different group (n=6).

Group	% Survival	Average bacterial colony count per ml blood*	RBCs (million cells/ µL)	WBCs (thousands cells/ µL)
1	100	Sterile	5.88±0.22	6.08±0.09
2	33.33	283.3±10.50*	4.04±1.44*	15.63±1.99*
3	33.33	292.3±10.50	4.04±1.44	16.53±1.99
4	100	064.2±9.22	5.12±0.75	9.06±2.08
5	66.66	125.5±9.95	6.96±0.46	12.45±1.88
6	83.33	96.2±8.87	5.10±1.15	11.81±1.78

N=6; values are expressed as mean ±SD; Superscripts * indicates values which are significantly different at $p<0.05$ when compared to minimum extract concentration

Bacterial clearance studies

E. coli induced bacteraemia was determined in each group by serial dilution of blood sample and inoculation on McConkey agar in duplicate. Bacterial colonies were counted after 24 hours of incubation[55].

Blood cells count

Blood sample was collected in tubes containing EDTA. The total RBC and Leucocytes (WBC) were counted using Thoma Zeiss haemocytometer[56-58].

Statistical analysis

All the data were expressed as Mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni multiple comparison tests. Values were considered statistically significant at $p<0.05$.

RESULTS

Phytochemical screening:

Phytochemicals present in the extracts had shown selective reactivity with some reagents which form the basis of chemical tests for identification of many compounds. The biochemical qualitative test of crude extracts had shown positive reaction for glycosides, flavonoids, tripterpenoid, tannins and phenolic compounds.

In vitro antibacterial assay

In-vitro antibacterial activity of MO and CS in combination was tested against selected bacterial strains and found to be highly sensitivity. ZOI and MIC were observed after 24 hours of incubation with various concentration of the extract (Table 1).

Acute toxicity tests (LD_{50})

Oral administration of different dose of extracts in animal showed no signs of toxicity and mortality in animal model within 24hours even at 2000mgKg^{-1}bw day^{-1}extract. No changes in skin, fur, eyes, mucous membranes along with respiration, convulsions, salivation, diarrhea, lethargy, sleep, autonomic and central nervous systems functions and coma had been observed.

Bacterial clearance studies

Average bacterial count in peritonitis induced positive control was 283.3±10.50 which decreased to 064.2±9.22 when treated with antibiotics. Similarly, treatment with combinative extract (CE) had decreased bacterial count to 96.2±8.87. Bacterial colonies were found to decrease by 55.07 % and 66.04% when treated with CE at the dose of 200mgKg^{-1}bw day^{-1} and 400mgKg^{-1}bw day^{-1} respectively in compared to antibiotic treated group (77.34%).

Animal survival studies

Percentage survival was found to be increased when peritonitis induced animals were treated with CE. Untreated group (II and III) showed 33.33% survival percentage which increased to 66.66% and83.33% when treated with CE at the dose of 200mgKg^{-1}bw day^{-1} and 400mgKg^{-1}bw day^{-1} respectively for seven days. Gentamycin treated group had shown 100% survival rate (table-2).

RBCs and WBCs count

RBC and WBC count in control mice showed 5.88±0.22 million cells/ mm^3 and 6.08±0.09 thousand cells/ mm^3 respectively. A little decrease in red cells count was recorded in group II and III on 7thdays ($p<0.05$) when compared with control but leukocyte count was

significantly increased in group II and III at the end of incubation period. Peritonitic mice when fed with the extracts of MO and CS in combination, showed significant improvements in red cells and leukocytes count. Control mice when fed with the extracts showed insignificant change in red cells and leukocyte count.

DISCUSSION

MO is food supplements with high nutritional and therapeutic values which contain good source of various amino acids protein, vitamins, β-carotene, β-sitosterol, kaempferol, phenolics and caffeoylquinic acid. MO leaves, flowers seed, fruit, immature pods, bark and roots has been found to act as cardiac stimulants, antipyretic, antiinflammatory, antiulcer, diuretic, antihypertensive, antiepileptic, antidiabetic, cholesterol lowering, antispasmodic, antioxidant, hepatoprotective, antitumor, antibacterial and antifungal activities[59]. CS has been used since ancient times as a source of beverage. It had been proven antioxidant properties, lowers the risk of several types of cancer, enhance the activity of insulin, antimicrobial, anti-viral, immuno-stimulatory, anti-inflammatory, protective effect against cardiovascular diseases and cerebral ischemic damage.

The efficacies of traditional therapies have been declined due to emerging problem of multi-resistance in majority of isolated organisms[3]. So use of broad spectrum antibiotic has been suggested as an alternative in suchcondition. The susceptibility of some bacterial strains to the extract of MO and CS has been successfully demonstrated in individual experiments and has been suggested that they can be used against these susceptible bacterial strains. In this respect, chloroform leaf extract of *Azadirachta indica* and *Moringa oleifera* in combination has been already found to exhibit better antimicrobial potential against some human pathogens[48].Its extracts acted as an antibacterial agent in both *in vivo* and *in vitro* models satisfactorily. We compared the present results with gentamicin and placebo rats and found the similar properties related to antimicrobial activity. It has nutritive value and medicinal properties with no toxicity.

CONCLUSION

Leaf extracts of MOand CS showed varying antibacterial activity which suggest that MOand CS can serve as potential source of bioactive healthy compounds in the diet. Their consumption could be useful in the prevention of diseases. The use of these plants symbolizes an economic and safe alternative to treat infectious diseases. Further research is needed to isolate and identify active principle compounds present in the extracts and their combinative action could possibly be utilized for pharmaceutical use.

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