

Total phenolic and flavonoid content, antioxidant effects and antidiarrheal activity of balacaturbhadrika churna – an Ayurvedic preparation

Rahul Pratap^{1,#}, HN Aswatha Ram^{*1,+}, KS Chandrashekar^{1,\$}, Vasudev Pai^{1,^}, Saleemulla Khan^{3,!} & Venkatesh Kamath^{2,%}

¹Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal 576 104, Karnataka, India

²Department of Pharmaceutical Biotechnology, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal 576 104, Karnataka, India

³P A College of Pharmacy, Nadupadav, Montepadav Post, Kairangala, Mangalore 574 153, Karnataka, India

E-mail: [#]ayilyath.rahul@gmail.com; ⁺aswatharam@gmail.com; ^{\$}cksbhat@yahoo.co.in; [^]vasupharmacist@gmail.com; [!]saleemulla.khan@gmail.com; [%]venkatesh.kamath@manipal.edu

Received 15 January 2019; revised 30 April 2019

Balacaturbhadrika churna (BC) is an important pediatric preparation in Ayurveda. It is used to treat various diseases of gastro-intestinal tract particularly diarrhea in children. Free radicals cause oxidative damage in the intestine and are correlated with its diseases. In the current study, total phenolic and flavonoid contents of BC extracts were determined and their antioxidant evaluation in various *in vitro* models was carried out. Procedures mentioned in The Ayurvedic Formulary of India were followed to prepare in-house BC; alcoholic (BCAL) and aqueous (BCAQ) extracts were prepared and tested by standard procedures. BCAL was tested for antidiarrheal activity in castor oil induced model. The ethanolic extract showed better antioxidant activity in many of the models as compared to aqueous extract. The present study showed substantial amounts of flavonoids and phenolics in churna extracts. BCAL showed significant antidiarrheal activity against the tested model. The potential antioxidant and antidiarrheal activities displayed by Balacaturbhadrika churna extracts could be attributed to these contents.

Keywords: ABTS, Antidiarrheal, Antioxidant, Balacaturbhadrika churna, DPPH, Flavonoid, Phenolic

IPC Code: Int. Cl.¹⁹: A61K 39/395, A61K 9/00, A61P 17/18, A61K 36/00, A61K 8/97, C12G 3/05, A61K 31/05

Free radicals are highly reactive oxygen species *viz* generation of superoxide ion, singlet oxygen, hydroxyl ion and hydrogen peroxide occur in the cells during metabolism. They cause tissue injury by producing oxidative damage to lipids, enzymes, proteins as well as DNA through covalent binding. Naturally occurring antioxidant agents have gained much attention due to their ability to scavenge free radicals¹. Antioxidant properties from Plant based phenolics as well as flavonoids have been reported regularly². Several gastrointestinal diseases have been correlated to free radical species and their damage due to oxidation in the intestine³. These free radicals directly or indirectly play a role in asthma also⁴.

Ayurveda is gaining importance globally under traditional medicines and are accepted for their clinical efficacy. BC is a reputed classical Ayurvedic pediatric preparation used in the management of gastrointestinal disorders like as diarrhea, emesis and cough. It is also

*Corresponding author

used to treat asthma, fever and emaciation in children⁵. The literature survey has revealed that the various ingredients of churna have been reported to have shown either antidiarrheal activity or antibacterial activity against the diarrhea causing organisms. The first ingredient of churna *viz* *Cyperus rotundus* decoction exhibited commendable effect on diarrhea causing pathogens in *in vitro* models⁶. The second, third and fourth ingredients of the churna namely *Pistacia integerrima* galls extract, *Piper longum* essential oils and *Aconitum heterophyllum* have reported to possess antibacterial activity against diarrhea causing *E. coli*^{7,8,9}. However, no *in vivo* study has been conducted on churna or its extracts directly for its antidiarrheal activity in experimental animals. It was therefore, thought worthwhile to test extract of churna for its claimed activity. Our study was thus, envisaged to estimate the total phenolic as well as flavonoid contents. The study also was to investigate the antioxidant capacity and antidiarrheal activity of the extracts of BC.

Methodology

Plant materials and chemicals

Western ghats form a major habitat for flora and the crude drugs that was obtained from the local market of Ayurvedic shop in Udupi district, Karnataka, India. The crude drugs were authenticated by the botanist Usharani K Suvarna, Department of Botany, MGM College, Udupi, Karnataka, India and the voucher specimens of each crude drug (PP575A, PP606, PP604 and PP605) has been deposited in the museum of the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India. ABTS and DPPH were procured from Sigma Chemicals, USA. Analytical grade chemicals were used for experimental purpose. Castor oil was procured from Suvindhinath Laboratories, Baroda. Propylene glycol was obtained from Universal Laboratories Private Limited, Mumbai and Loperamide capsule (standard) used is from Janssen-Cilag Pharmaceuticals, Mumbai.

Churna preparation

Procedures mentioned in The Ayurvedic Formulary of India were followed to prepare in-house BC. Equal proportions of ingredients from the rhizomes of *Musta* (*Cyperus rotundus*), galls of *Karkatasringi* (*Pistacia integerrima*), fruits of *Pippali* (*Piper longum*) and root tubers of *Ativisa* (*Aconitum heterophyllum*) were powdered individually using cyclone mill and sieved through #80. The powdered ingredients were then mixed to get uniformly blended churna.

Preparation of alcoholic extract (BCAL)

Continuous hot extraction process was carried out to extract 30 g of BC using ethanol (90%). A desiccator was used to store the dried extract (28.93%) until its use.

Preparation of aqueous extract (BCAQ)

Chloroform water (2.5 mL of chloroform in 1000 mL of purified water) was used to macerate 30 g of BC. The process was carried out for seven days following which the contents were filtered and evaporated to dryness. A desiccator was used to store the dried extract (14.9%) until its use.

Determination of total phenolic content

Extract samples were taken and estimated their total phenolic content by Folin-Ciocalteu reagent method. In the process, 2.5 mL and 2 mL of Folin-Ciocalteu reagent (1/10 dilution) and 7.5% sodium carbonate (w/v) respectively were added to 0.5 mL of each extract in concentrations of 125, 250, 500,

1000 µg. The mixture was kept aside for 0.5 h with intermittent shaking during which a blue color was formed. The intensity of the color developed was measured at 765 nm using UV-Vis Spectrophotometer (UV-1650PC, Shimadzu Corp., Kyoto, Japan). A control tube with sodium carbonate (2.55 mL of distilled water+2 mL of 7.5% sodium carbonate) along with 2 mL of Folin-Ciocalteu reagent was used as blank. A standard Gallic acid curve plotted using different concentrations of gallic acid (50, 100, 150, 200, 250, 300, 350, 400 and 450 µg/mL) in 5.0 mL of Folin Ciocalteu reagent (diluted tenfold) and 4.0 mL of sodium carbonate solution (75 g/L) was used for comparative assessment. Thus, the content obtained was mentioned as gallic acid equivalent (GAE) in µg/mL of the dry extracts¹⁰.

Total flavonoid content determination

Sample solution (1 mg/mL; 5 mL) of the extract was mixed with equal volume of aluminium trichloride (2%). Contents were kept aside for about 10 min and using 415 nm, the absorbance was measured¹¹. Five mL of methanol extract solution without aluminium trichloride was used as control. Standard curve of quercetin (10–100 µg/mL) was employed to determine total flavonoid content and the average of 3 readings was taken and expressed as QE, that is, Quercetin equivalent, on dry weight basis.

Antioxidant assays

DPPH radical scavenging assay

Spectrophotometric method was employed to measure DPPH scavenging activity¹². To 0.05 mL of alcoholic, aqueous extracts (10-100 µg/mL) of BC and standard ascorbic acid in ethanol, added an 0.05 mL of ethanolic solution of DPPH (200 µM). Equal volume of ethanol was used as blank. DPPH free radicals quenching was observed and measured at 517 nm after 20 min following which inhibition percentage was calculated as given below;

$$\% \text{ inhibition} = (\text{control} - \text{test}) / \text{control} \times 100$$

ABTS radical cation depolarization assay

ABTS solution (7 mM) in presence of ammonium persulfate (2.45 mM) generates ABTS⁺ radical cations. The reaction mixture was kept aside at room temperature overnight. 0.5 mL of alcoholic, aqueous extract (10-140 µg/mL) and standard ascorbic acid were then added to ABTS solution (0.3 mL). One mL ethanol was used to make up the final volume. Percentage inhibition was calculated after measuring the absorbance at 745 nm¹³.

Assay of Nitric oxide radical scavenging activity

Nitric oxide generated through sodium nitroprusside was measured using Griess' reaction at the temperature conditions maintained at 25°C for 5 h. Sodium nitroprusside (5 mM) (in standard phosphate buffer solution) was incubated with various concentrations (25-400 µg/mL) of ethanolic, aqueous extracts and standard ascorbic acid in phosphate buffer of 0.025 M and pH: 7.4. Similarly, control experiment was carried out by omitting the test compound which was replaced with equal volume of buffer. Then, 0.5 mL of incubated solution was mixed with 0.5 mL of Griess' reagent (0.1% naphthyl-ethylene-diamine-dihydrochloride and 1% sulphanilamide and 2% O-phosphoric acid). The color developed through diazotization and coupling of nitrite with sulphanilamide and naphthyl-ethylene-diamine respectively, was read at 546 nm¹⁴.

Superoxide radical scavenging assay

Alkaline DMSO method was used to perform the potassium superoxide scavenging activity. Dry DMSO and Potassium superoxide mixture were kept in contact for a day. Filtration of the particular solution was ensured before it was used. Two hundred µL of the filtrate was pipetted into 2.8 mL aqueous solution having 56 µM NBT, potassium phosphate buffer (10 mM) and EDTA (10 µM). At 560 nm, absorbance was recorded for extracts and standard (ascorbic acid) at different concentrations (20-140 µg/mL) by using pure control DMSO¹⁵.

Inhibition (%) = [(Control - Test) / Control] x 100

Hydrogen peroxide scavenging assay

To 40 mM of H₂O₂ solution (0.6 mL) in phosphate buffer with pH 7.4 extract samples (100 µg/mL in distilled water) was added. Ten min later using 230 nm the absorbance of above mixture was read. A blank without hydrogen peroxide in the buffer solution was prepared. The percentage scavenging capacity of H₂O₂ by both BC extracts and the standard (ascorbic acid) were obtained using the below formula¹⁶.

% Scavenged [H₂O₂] = [(A₀ - A₁) / A₀ x 100]

Animals

Healthy Wistar albino rats weighing between 200–250 g for the study were obtained from Central Animal Research Facility of MAHE, Manipal, Karnataka. Polypropylene cages were used to house the animals and standard conditions were maintained

throughout (12 h light: 12 h dark cycle, temperature range of 25±3°C and humidity ranging between 35–60%). Animal feed included standard rat pellet diet which was procured from VRK Nutritional Laboratory Animal Feed, Maharashtra, India and water *ad libitum*. Approval for conducting the present work was obtained from KMC, Manipal, India and the reference No. is IAEC/KMC/112/2013.

Antidiarrheal activity**Diarrhea-induced by administering castor oil in rats**

Orally administered castor oil in the dose of 3 mL/kg, *po* has induced diarrhea in rats. The experimental animals were not fed for 16 h. During this fasting time, rats were allowed to drink sufficient water. Before the commencement of the experiment, arbitrarily animals were distributed into six groups and each group having 6 rats (36 animals). Following this, each rat was then placed separately in polypropylene cages having transparent sheet at its bottom. All the rats of Group I were given 10 mL/kg oral administration of propylene glycol which served as vehicle control. Group II served as the disease control and received Castor (3 mL) oil alone. Rats from the Groups III, IV and V were given 10 mL/kg oral administration of BCAL 100, 150 and 200 mg/kg as test doses respectively. Group VI rats were orally administered with the standard drug Loperamide in the dose of 10 mg/kg. After 30 min of pretreatment, all the rats were given castor oil in the dose of 3 mL/kg orally. Rats were monitored for their diarrheal droppings for 6 h. Factors like total number of fecal output, time elapsed between castor oil administration and the first instance of diarrheic fecal excretion were noted. The other factors including total diarrheic fecal weight and its weight particularly in that period were also recorded. Each animal's excreted diarrhea stool was numbered and calculated in the said period. The diarrhea induced after castor oil administration and its severity in rats were also noted and scored. Dicarolo *et al.* (1994) method of numerical score which on the basis of stool consistency was given as normal stool (or without diarrhea) = 1, semisolid = 2, and 3 for watery stools/ feces respectively. Correspondingly, the percentage and purging indices (through comparison with control group) were calculated. Interval of time (min) between castor oil administration and the first diarrheic stool appearance was considered for the measurement of onset of diarrhea. All the fecal matter that was collected during 6 h period was weighed. Body

weight differences at pre as well as post treatment periods were noted down. Calculation of percentage score defecation inhibition and weight of the feces was done by¹⁷ the following formula.

$$\frac{[\text{control mean} - \text{treated (test) mean}]}{\text{control mean}} \times 100$$

Enteropooling, intestinal transit time and fluid accumulation in the intestine induced by castor oil administration

All rats from various groups under examination were not given food for 16 h. However, the rats had free access to sufficient drinking water during this time period. Then, the animals were arbitrarily allocated into six groups of six each. After grouping, rats were then placed separately in polypropylene cages having transparent sheet at the bottom. Group I rats were given 10 mL/kg (*po*) of propylene glycol (in which loperamide and BCAL were dissolved). Group II was administered Castor oil only. Rats from Group III, IV and V received orally 10 mL/kg of BCAL at 100, 150 and 200 mg/kg, correspondingly. Following this, castor oil (3 mL/kg) was administered orally. Loperamide, at 10 mg/kg (*p.o.*) was given to all the rats of group VI and followed by castor oil (3 mL/kg) administered orally. After thirty minutes, physiological saline containing activated charcoal (10%, 3 mL/kg) was administered orally to all the rats. This was followed up by measuring the volume of intestinal fluid and intestinal transit. Anesthetization by inhalation of ether and euthanization through cervical dislocation was carried out on all the rats after forty minutes of charcoal meal. The abdomen was cut open. Ligation through the entire intestine from the pylorus to caecum carried out followed by dissection and removal. In the small intestine, charcoal meal has traveled certain length which was determined indicating the Peristaltic index (PI) which was later expressed in terms of percentage of small intestine's total length. The whole intestine was weighed. A graduated measuring cylinder was taken in which all the intestinal contents were expelled and the volume was measured. Weight variation between full and empty intestine were noted.

For every 10 mg/kg oral dose of the standard BCAL at 100, 150 and 200 mg/kg, *p.o.* showed definite percentage inhibition of movement of charcoal meal in the intestine. It was calculated as a negative control (vehicle treated group) by the use of following formula.

$$\% \text{ transit inhibition} = \left[T_o - \frac{T_t}{T_o} \right] \times 100$$

Where,

To stands for mean length which the charcoal meal traverses in the rats of vehicle-treated group; Tt is the mean length where charcoal meal transverses in rats treated with standard loperamide and BCAL test groups¹⁷.

Acute toxicity studies

OECD 425 guidelines were followed to check for acute toxicity with a ceiling dose of 2000 mg/kg of BC. In brief, the rats were assessed every 10 min for a period of 4 h. They were also assessed for 24, 48, and 72 h to check for any kind of changes in respiration, piloerection, motor activity (spontaneous), gait and writhing once the drug was administered. The follow up process of animals went up to 30 days for mortality.

Statistical analysis

The statistical significance between the groups was determined by the one-way ANOVA (analysis of variance) followed by the post-hoc Dunnett's test using Prism 5.03 Demo Version (Graph Pad Software Inc., La Jolla, CA). The minimum level of significance was set at $p < 0.001$. All values were expressed as mean standard error of mean (SEM), $n=6$ (unless otherwise specified).

Results

The percentage yield of BCAL and BCAQ were 28.93 and 14.90 mg/g, respectively. The total phenolic content for BCAL and BCAQ was found to be 24.60 and 31.71 mg/g, respectively calculated as gallic acid equivalent (GAE). The flavonoid content in total of BCAL and BCAQ was 2.503 and 2.501 mg/g, respectively calculated as quercetin equivalent (QE).

IC₅₀ values were determined for the different models and are represented in Table 1. Ethanolic

Table 1 — IC₅₀ values of standard, BCAL and BCAQ (µg/mL)

Sample	DPPH assay	ABTS assay	Nitric oxide assay	Superoxide assay	H ₂ O ₂ assay
Standard	0.97±0.04	8.81±0.56	28.62±1.89	11.38±0.98	21.96±2.03
BCAL	5.50±0.42*	7.94±0.64 ^{ns}	25.99±3.62 ^{ns}	13.54±0.84 ^{ns}	18.10±1.08 ^{ns}
BCAQ	10.97±1.02*	9.18±0.66 ^{ns}	25.28±2.44 ^{ns}	16.45±1.24*	22.57±4.82 ^{ns}

All values are expressed as mean±S.D., $n=6$. * $p < 0.05$ vs. respective standard; ns – no significant difference vs. standard by one-way ANOVA with Dunnett's *post hoc* test.

extract of churna showed more effective results for different assays compared to aqueous extract.

Acute toxicity

Acute toxicity studies were performed for 2000 mg/kg, all animals survived and showed no abnormal behavior or signs of toxicity. For further studies, the doses selected were between 1/10th and 1/20th of the tolerated dose in rats.

Antidiarrheal activity

Diarrhea in rats induced by treatment with castor oil

The control group rats exhibited copious diarrhea around two-and-half hours post oral castor oil administration. The onset of diarrhea was significantly delayed in rats pretreated with BCAL (100, 150 and 200 mg/kg, *po*). It also had an impact on the frequency and reduced the number of times of defecation as well as the fecal wetness (decrease in the number of stools which are wet and in total). Loperamide, the standard drug (10 mg/kg, *po*), exhibited a greater inhibition on diarrheal parameters under examination compared to BCAL (Table 2).

Intestinal transit in rats induced by castor oil administration

In control rats, 95% transit of charcoal along the length of the intestine was observed (Table 3) after

Table 2 — Effects of BCAL (100-200 mg/kg, *po*) on castor oil-induced diarrhea in rats

Treatment group	Dose (mL/kg) or (mg/kg)	Total no. of stools	No. of wet stools	% protected
Normal	10	0.00±0.00	0.00±0.00	100
Control	10	7.33±0.33	6.50±0.42	0
Extract	100	6.50±0.22***	0.16±0.16***	11
	150	5.50±0.34***	0.33±0.21***	25
	200	4.83±0.30***	0.16±0.16***	34
Loperamide	10	0.00±0.00***	0.00±0.00***	100

All values are expressed as mean ± S.E.M., n = 6. ****p*<0.001 vs. control by one-way ANOVA with Dunnett's *post hoc* test.

Table 3 — Effects of BCAL (100-200 mg/kg, *po*) on castor oil-induced intestinal transit in rats

Treatment group	Dose (mL/kg) or (mg/kg)	Peristaltic index (%)	Inhibition of transit (%)
Normal	10	0.00±0.00	100
Control	10	95.10±0.88	0
Extract	100	59.22±3.14***	42
	150	31.75±1.10***	69
	200	0.00±0.00***	100
Loperamide	10	0.00±0.00***	100

All values are expressed as mean ± S.E.M., n = 6. ****p*<0.001 vs. Control by one-way ANOVA with Dunnett's *post hoc* test.

0.5 h of gastric administration. In the BCAL administered group of rats, (100, 150, 200 mg/kg, *po*) a significant (*p*<0.001) decrease in propulsive motion and charcoal meal transit was observed. Loperamide (10 mg/kg, *po*) exhibited higher effect of anti-motility in comparison to maximum dose of BCAL.

Enteropooling in rats induced by castor oil

A statistically significant (*p*<0.001) and marked surge in the volume of intestinal fluid was observed on oral administration of castor oil 3 mL/kg to rats in comparison to vehicle control group which was treated with propylene glycol (10 mL/kg, *po*) alone. Prior treatment of the test group rats with BCAL (100, 150 and 200 mg/kg, *po*) inhibited fluid accumulation that was induced by castor oil and the effect was significant (*p*<0.001) in comparison with the control group (Table 4). It was also observed that loperamide (10 mg/kg, *po*) demonstrated a higher significant inhibitory effect (*p*<0.001) on fluid accumulation induced by castor oil as compared to the maximum dose of BCAL (100, 150 and 200 mg/kg, *po*).

Discussion

Balacaturbhadrika churna is an Ayurvedic pediatric preparation used for various GIT diseases since ages. The botanical ingredients of the churna prompted us to validate the claimed activity biologically and relate the activity to possible mechanistic frame. It was therefore aimed at determination of phenolic and flavonoid content of the churna; estimation of antioxidant activity and evaluation of Antidiarrheal activity in the animals. Oxidative damage is assumed to be the root cause of many diseases, we tested various extracts for possible antioxidant potential of the product.

Redox property of phenolic compounds and their activity is facilitated by hydroxyl groups, and thus act

Table 4 Effects of BCAL (100-200 mg/kg *po*) on castor oil induced intestinal enteropooling and fluid accumulation in rats

Treatment group	Dose (mL/kg) or (mg/kg)	Intestinal fluid (mL)
Normal	10	0.00±0.00
Control	10	1.05±0.04
Extract	100	0.71±0.03***
	150	0.56±0.03***
	200	0.23±0.04***
Loperamide	10	0.45±0.06***

All values are expressed as mean ± S.E.M., n = 6. ****p*<0.001 vs. Control by one-way ANOVA with Dunnett's *post hoc* test.

as antioxidants¹⁸. Free hydroxyl groups particularly 3-OH, plant flavonoids possess antioxidant properties¹⁹. BC is a well-known traditional Ayurvedic churna preparation containing four herbal ingredients viz, Musta, Karkatasringi, Pippali and Ativisa as mentioned earlier. At least three of them are known antioxidants and have been validated for their activity by various researchers. Phenolic and flavonoid compounds from the rhizomes of *Cyperus rotundus*-an important ingredient of BC, were found to be methyl 3,4-dihydroxy benzoate and rutin²⁰. Rutin is a common antioxidant used as standard for antioxidant activity. The galls of *Pistacia integerrima* (Karkatasringi), are celebrated antioxidant agents. 14'-phenoxytetradecanyl 3,5-dihydroxy benzoate (pistiphloro-gluciny ester) 2, 4'-phenoxy-n-butyl-1'-(3oxy-5-hydroxy) benzoic acid (pistaciaphenyl ether) 3 and 3'-(1,3-dihydroxy-5-phenoxy-1',5'-dimethoxybenzene (pisticphloro-gluciny ester) have been reported from the galls and all of them are known antioxidants²¹. Several studies have been undertaken to justify the use of Pippali as an antioxidant^{22,23}. Thus, the antioxidant activity possessed by BC extracts can be attributed to its flavonoid and the total phenolic contents.

Antioxidants have been advised in many diseases. The implication of antioxidants had also been reported in diarrhea. Hu *et al.*²⁴ have reported the application of phenols and flavonoids in diarrhea and have carried out their antioxidant activity.

The DPPH is a stable free radical that is widely used in assessing free radical scavenging activity of the plants extracts rich in secondary metabolites, like phenolics and flavonoids²⁵. In our study, the alcoholic extract of the churna (BCAL) showed better antioxidant activity when compared to aqueous extract BCAQ in DPPH free radical scavenging assay.

Inhibition of absorbance of ABTS radical cation takes place in ABTS assay and is characterized by long wavelength absorption spectrum. From the result obtained, it can be indicated that the activity of churna extracts is either by inhibiting or by scavenging property as an antioxidant activity towards ABTS free radical²⁶.

The unstable radical nitric oxide will react with oxygen to produce the intermediates NO₂, N₂O₄ and N₃O₄ and eventually stable product nitrate and nitrite. Griess reagent is used to estimate this. In our investigation, the extracts reduced the nitrite that was formed by incubating sodium nitroprusside in

standard phosphate saline buffer at 25°C. It may be due to extract constituents which show antioxidant activity competing with oxygen in order to react with nitric oxide and thus not allowing the formation of nitrite²⁷.

Superoxide dismutase acts as the catalyst for dismutation of highly reactive superoxide anion to H₂O₂ as well as oxygen. The first reduction product of oxygen is superoxide anion which is measured in terms of inhibited generation of O₂²⁸. In the present study, on *in vitro* reaction mixture, extracts showed dose dependent inhibition of generation of superoxide.

Hydrogen peroxide plays a vital role in the pathogenesis of various diseases. Under physiological pH and temperature, it is very stable and has the capacity to penetrate the membrane. Because of its weak oxidizing ability and relatively lesser toxicity, it is still regarded as less reactive²⁹. Furthermore, cellular damages are mediated by the hydroxyl radical which is the conversion product in presence of transition metal. Singlet oxygen may also be formed by reacting with hypochlorous acid (HOCl) or superoxide anion or chloramines in living system³⁰. Thus, while assessing the anti-oxidant activity, measurement of H₂O₂ scavenging activity becomes an important tool. In our study, BCAL produced better protection against H₂O₂ compared to BCAQ. The antioxidant results from different assay methods demonstrated coherent results suggesting better activity of BCAL when compared to BCAQ. Therefore, in the present study, BCAL was selected for the *in vivo* animal model.

Castor oil induced diarrhea being most widely used animal model for antidiarrheal activity and therefore, it was selected for the present investigation. Castor oil stimulates diarrhea by three different mechanisms namely-(1) releasing nitric oxide and thereby increasing permeability of gastrointestinal membrane for calcium; (2) stimulating prostaglandin synthesis and thereby increasing fluid and electrolytes into the lumen of the bowel; and (3) increasing peristalsis³¹. Our results showed sharp increase in number of stools after administration of Castor oil indicating diarrhea. An appreciably high peristaltic index was also observed in the untreated control. High intestinal enteropooling was also noticed. From the above observations, induction of diarrhea was confirmed. BCAL Extracts having shown good activity were given at three different doses to study the effect of the extract on diarrhea. At a lower dose (100 mg/kg) the

extract showed very mild activity and lower protection against castor oil induced diarrhea. At the middle and higher doses (150 and 200 mg/kg respectively) the extract exhibited significant protection against castor oil assault ($p < 0.001$ in both the cases). The elevated total number of stools due to castor oil was reduced to 4.83 from 7.33, indicating 34% protection, though, the complete reduction in wet stools indicated complete reduction in diarrhea. However, the churna extract did not stop the stools completely further leading to forced constipation. Thus, Balacaturbhadraka churna is a better option when compared to loperamide. Both Churna extracts and loperamide have successfully prevented water loss and improved peristaltic index.

The underlying mechanism in the protection against castor oil induced diarrhea could be attributed to the phenolic and flavonoid contents of the churna. In some of the previous studies, Tannins and Flavonoids have been reported to possess antidiarrheal activity^{32,33}. Galvez *et al*³³ reported antidiarrheal activity of *Euphorbia hirta* extract and isolation of active flavonoid constituent. Therefore, phenolics and flavonoids present in BC are thought to be responsible for the antidiarrheal activity. The phenols and flavonoids are known to increase colonic water and electrolyte reabsorption and thus act by inhibiting intestinal motility³⁴.

Conclusion

The current experiment showed substantial amounts of flavonoids and phenolics in churna extracts. The potential antioxidant activity displayed by BC extracts could be attributed to these contents. Thus, churna extracts can be used to treat the diseases mediated by free radicals. However, BCAL showed better activity in most of the models. The experimental proof or evidence generated from the current study indicates BCAL to possess antidiarrheal activity in rats. These results could pave way towards lending pharmacological basis to or confirm the usage of BC in traditional medicine for managing and controlling diarrhea in children.

Abbreviations used

BC: Balacaturbhadraka churna

BCAL: Balacaturbhadraka churna alcoholic extract

ACAQ: Balacaturbhadraka churna aqueous extract

ABTS: 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonate)

DPPH: 1,1-Diphenyl-2-picryl-hydrazyl

OECD: The Organization for Economic Co-operation and Development

IAEC: Institutional Animal Ethics Committee

KMC: Kasturba Medical College

References

- 1 Saeed N, Khan MR, Shabbir M, Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L., *BMC Complement Altern Med*, 12 (2012) 221.
- 2 Shoib Baba A, Shahid Malik A, Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume, *J Taibah. Univ Sci*, 9 (2015) 449–54.
- 3 Kirsty Brown, Erin Molcan, Ethendhar Rajendiran, Angela Nusrat, Jessica Baker, Stephanie Ruscheinsky, Deanna Gibson L, Free Radicals and Gastrointestinal Disorders, In *Systems Biology of Free Radicals and Antioxidants*, edited by I Laher, (Springer-Verlag Berlin Heidelberg), 2014, 1692-1693.
- 4 Kanazawa H, Kurihara N, Hirata K, Takeda T, The role of free radicals in airway obstruction in asthmatic patients, *Chest*, 100 (1991) 1319-1322.
- 5 Anonymous, *The Ayurvedic Formulary of India: 2nd edn*, Part 1, (Central Council for Research for Ayurveda and Siddha, Ministry of Health and Family Welfare, Government of India), 2003, 116.
- 6 Daswani PG, Brijesh S, Tetali P, Birdi TJ, Studies on the activity of *Cyperus rotundus* Linn. tubers against infectious diarrhea, *Indian J Pharmacol*, 43(2011) 340-344.
- 7 Bharathirajan R, and Prakash M, Analysis of IR, NMR and *in vitro* antibacterial potency of *Pistacia integerrima* against 6 clinically isolated multidrug resistant bacterial, *Int J Curr Microbiol App Sci*, 4 (2015) 1174-1190.
- 8 Bhargava AK, Chauhan CS, Antibacterial activity of essential oils, *Indian J Pharma*, 30 (1968) 150.
- 9 Ahmad M, Ahmad W, Ahmad M, Zeeshan M, Obaidullah, Shaheen F, Norditerpenoid alkaloids from the roots of *Aconitum heterophyllum* Wall with antibacterial activity, *J Enzyme Inhib Med Chem*, 23 (2008) 1018-1022.
- 10 Savitree M, Isara P, Nittaya SL, Worapan S, Radical scavenging activity and total phenolic content of medicinal plants used in primary health care, *J Pharm Sci*, 9 (2004) 32-35.
- 11 Kalita Pallab, Barman Tapan K, Pal Tapas K, Kalita Ramen, Estimation of total flavonoids content (TFC) and antioxidant activities of methanolic whole plant extract of *Biophytum sensitivum* Linn, *J Drug deliv Ther*, 3 (2013) 33-37.
- 12 Sreejayan N, Rao MNA, Free radical scavenging activity of curcuminoids, *Drug Res*, 46 (1996) 169.
- 13 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice Evans C, Antioxidant activity applying an improved ABTS radical cation decolorization assay, *Free Rad Biol Med*, 26 (1999) 1231–1237.
- 14 Sreejanyan N, Rao MNA, Nitric Oxide Scavenging by Curcuminoids, *J Pharm Pharmacol*, 49 (1997) 105-107.
- 15 Henry LEA, Halliwell B, Hall DO The superoxide dismutase activity of various photosynthetic organisms measured by a

- new and a rapid assay technique, *FEBS Lett*, 66 (1976) 303-306.
- 16 Ruch RJ, Cheng SJ, Klaunig JE, Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea, *Carcinogenesis*, 10 (1989) 1003.
 - 17 Emmanuel Awe O, Simeon Kolawole O, Kamoru Wakeel O, Oyindamola Abiodun, O, Antidiarrheal activity of *Pyrenacantha staudtii* Engl. (Iccacinaceae) aqueous leaf extract in rodents, *J Ethnopharmacol*, 137 (2011) 148-153.
 - 18 Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun OT, Phenolics as potential antioxidant therapeutic agents: mechanism and actions, *Mutat, Res, Fundam, Mol*, 579(2005) 200–213.
 - 19 Geetha S, Sai-Ram M, Mongia SS, Singh V, Ilavazha-gan G, et al, Evaluation of antioxidant activity of leaf extract of sea buckthorn (*Hippophae rhamnoides* L.) on chromium (VI) induced oxidative stress in albino rats, *J Ethnopharmacol*, 87 (2003) 247–251.
 - 20 Gamal Mohamed A, Iridoids and other constituents from *Cyperus rotundus* L. rhizomes, *Bull Fac Pharm (Cairo Univ)*, 53 (2015) 5-9.
 - 21 Shamim Ahmad, Mohammed Ali, Shahid Ansari H, Phenolic constituents from the galls of *Pistacia integerrima* Stewart, *Indian J Chem*, 50B (2011) 115-118.
 - 22 Dinesha R, Chikkanna D, Antioxidant activities of Pippali (*Piper longum*) proteins, *Indian J Pharm Drug Anal*, 2 (2014) 811-814.
 - 23 Wakade AS, Shah AS, Kulkarni MP, Juvekar AR, Protective effect of *Piper longum* L. on oxidative stress induced injury and cellular abnormality in adriamycin induced cardiotoxicity in rats, *Indian J Exp Biol*, 46 (2008) 528-533.
 - 24 Hu J, Gaob W, Ling N, Liu C Antidiarrheal and intestinal modulatory activities of Wei-Chang-An-Wan extract, *J Ethnopharmacol*, 125 (2009) 450-455.
 - 25 Shoib Baba A, Shahid Malik A, Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume, *J Taibah Univ Sci*, 9 (2015) 449-454.
 - 26 Sreejayan N, Rao MNA, Free radical scavenging activity by Curcuminoids, *Drug Res*, 46 (1996) 169-171.
 - 27 Ialenti A, Moncada S, Di Rosa M, Modulation of adjuvant arthritis by endogenous nitric oxide, *Br J Pharmacol*, 110 (1993) 701–706.
 - 28 Blois MS, Antioxidant determinations by the use of a stable free radical, *Nature*, 181(1958) 1199- 1200.
 - 29 Bandyopadhyay U, Das D, Banerjee RK, Reactive oxygen species: Oxidative damage and pathogenesis, *Curr Sci*, 10 (1999) 658-666.
 - 30 Kardag A, Ozcelik B, Saner S, Review of methods to determine antioxidant capacities, *Food Anal Methods*, 13 (2009) 41-60.
 - 31 Tunaru S, Althoff TF, Nüsing RM, Diener M, Offermanns S, Castor oil induces laxation and uterus contraction via ricinoleic acid activating prostaglandin EP3 receptors, *P Natl Acad Sci*, 5 (2012) 9179-9184.
 - 32 Galvez J, Zarzuelo A, Crespo ME, Antidiarrhic activity of *Sclero aryabirrea* barks extract and its active tannin constituent in rats, *Phytother Res*, 5 (1991) 276–278.
 - 33 Galvez A, Zarzuelo ME, Crespo MD, et al, Antidiarrhoeic activity of *Euphorbia hirta* extract and isolation of active flavonoid constituent, *Plant Med*, 59 (1993) 333–336.
 - 34 Enzo Palombo A, Phytochemicals from traditional medicinal plants used in the treatment of diarrhea: Modes of action and effects on intestinal function, *Phytother Res*, 20 (2006) 717–24.