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Evaluation of effect of selective plants on diarrhea in experimental animal model representing chronic diarrhoea in malnourished children

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Abstract
There is a marked negative relationship between diarrhoea and physical growth and development of a child. Ayurveda literature indicates that the decoction of processed *Zingiber officinale* (sunthi), *Cyperus rotundus* (musta), *Cuminum cyminum* (jeera) can be used as an anti-diarrhoeal home remedy. In order to find out their activity, a systemic study of these medicinal plants is very important. We have made an attempt to prepare suitable condition in animal model representing chronic diarrhoea in malnourished children and to study *in vivo* effect of above mentioned plant drugs considering various parameters like colony count of intestinal organisms and organisms of mesenteric lymph nodes, barrier mucosal content of the intestine and histopathological examination of jejunum and ileum that is change in villous morphology and villous height and incidence of cryptitis. Plant extract was prepared by Hot water decoction method. Total eight groups of experimental animals were made. Test groups received high and low doses of plant extracts. In all three plant drugs, there was definitely less translocation of intestinal flora from small intestine to mesenteric lymph nodes in test group animals than in disease control group animals. High dose of all three plant drugs was more effective towards protection of translocation of intestinal flora of small intestine to mesenteric lymph nodes. Maximum effective protection was observed by musta, followed by sunthi and jeera on comparison. There was reduction in gastrointestinal mucosal damage. In colony count of mesenteric lymph nodes shows no bacterial growth, which indicates that translocation of bacteria from intestine to mesenteric lymph nodes, has not occurred. Thus translocation of indigenous intestinal flora into the systemic circulation is prevented. Test animals with High dose showed more mucus content values in all three drugs tested, than disease control and low dose test animals. Mucus content values in low dose test animals was less than disease control animals in jeera and sunthi, where as musta values were more in test animals than disease control animals. Hence, in comparison, musta was observed more effective than jeera and sunthi to control mucus content level in Ileum and Jejunum. Histopathological examination of small intestine tissue test animals showed slight change in shape of villi, anatomy of villi, altered Crypt : villi, and incidence of cryptitis on comparison with Healthy control animals. These results shows usefulness of musta, sunthi, and jeera in antidiarrhoeal model and Musta is found to be a potent antidiarrhoeal drug.

Keywords: chronic diarrhoea, malnourished children, medicinal plants, musta, sunthi and jeera

1. Introduction
Since ancient times, diarrhoea has been recognized as one of the most important health issue world widely, particularly afflicting those populations of socio-economically backward classes and from tribal areas to third-world countries [1-2]. Globally, about 2.2 million people have been killed annually by diarrhoea, majority of them are infants and children below the age of 5 years [3-4]. Epidemiological studies have demonstrated a marked negative relationship between diarrhoea and physical growth and development of a child. Malnutrition, particularly wasting, is a strong predictor of diarrhoeal duration and the prolonged illness could exacerbate nutritional faltering, thereby increasing the subsequent risk of death. A child may lose almost as much water and electrolytes from the body during an episode of diarrhoea.

Among the various risk factors, the risk factors which may predispose to diarrhoea are delayed repair of intestinal damage [5], decreased host immunity [5], nutritional problems [5] reduced food intake [5], malabsorption [5]. Diarrhoea is characterized by rapid movement of

semisolid or watery feacal matter through intestine, three or more times in a day with severe or light abdominal pain and bowel sounds [6-8]. Such type of symptoms in diarrhoea is treated by the administration of oral rehydration salts (ORS) in children or adults to maintain the body fluids osmolality [9]. Alternatively, some drugs are available in the market for treating diarrhoea, but all of the existing drugs suffer from adverse effects [10]. In order to overcome these menace of anti-diarrhoeal drugs in world market, the World Health Organization has introduced a programme, which encourages towards traditional herbal medicines [11]. Therefore, there has been great interest in herbal remedies for the treatment of such ailments.

Ayurveda, the ancient medical science of India, is not merely a doctrine of medical treatment, but a way of healthy long life. Propounded by the seers of ancient India, Ayurveda still stands on its own, despite the oddities it faced in the past. Ayurveda offers solutions to many human maladies, which the modern medicine fails to heal. The purificatory and rejuvenative processes of Ayurveda are globally accepted today. More over the increasing use of plant extracts in the food, cosmetics and pharmaceutical industries suggest that, in order to find active components, a systemic study of medicinal plant is very important Mamatha *et al*. [12] documented that extracts of Tea (leaves), coffee (beans), *Ocimum sanctum* (leaves), *H. antidysentrica* (bark), *Centella asiatica* (leaves) and *Mystrica fragrans* (seeds) showed significant inhibitory activity against enteric pathogens. The antibacterial activities of ethanolic extracts of Ginger were investigated on selected pathogens. The extracts exhibited antibacterial activity against the pathogens, which indicated that, Ginger root contains compounds with therapeutic activity.

Above records suggests there is availability of various plant drugs against diarrhoeal condition, but accurate scientific support and data is necessary to confirm their properties to use them for treatment in such conditions. Ayurveda literature also indicates that the decoction of processed *Zingiber officinale* (sunthi), *Cyperus rotundus* (musta), *Cuminum cyminum* (jeera) can be used as an anti-diarrhoeal home remedy [13].

Keeping above facts in mind we have selected these plants, to be more specific, part of the plant as antidiarrhoeal remedy, which will with ORS, may be able to use as adjuvant. In order to find out their activity, a systemic study of these medicinal plants is very important. Scientific study towards various Ayurvedic plant drugs and its effect on various pathogens *in vitro* as well as *in vivo* are not well documented [14]. Hence, it is need of today to establish such documents for its regular use as remedy, which will be useful for the control and cure of the various infectious conditions like diarrhoea. Keeping in mind the above facts, we have made an attempt to prepare suitable condition in animal model representing chronic diarrhoea in malnourished children and to study *in vivo* effect of selective plant drugs in suitable animal model considering various parameters like Colony count of intestinal organisms, Colony count of organisms of mesenteric lymph nodes, Barrier mucosal content of the intestine and Histopathological examination of jejunum and ileum that is change in villous morphology and villous height and incidence of cryptitis.

2. Material and Methods

This study was conducted at animal house and department of Microbiology of T.N. Medical College and B.Y.L. Nair Charitable Hospital, Mumbai, India. An animal model preparation, similar to the condition of malnourished children, suffering from chronic infective diarrhoea was attained by feeding animals selected for the study with non autoclaved red kidney beans for a period of fifteen days. Parallel to these specimens, Control group were fed autoclaved red kidney beans for fifteen days. These ground raw red kidney beans were mixed in normal chow in 40:60 proportions and given as diet to the animals.

2.1. Plant material

All plants / Plant parts (sunthi-roots, Jeera-seeds, musta-roots) were obtained from authentic sources and their identity was confirmed using standard pharmacognostic method.

Plant extract was prepared by Hot water decoction method [14]. Ten grams dry powder was taken in a clean glass stoppered flask-containing 100ml of distilled water. The solution was boiled in a water bath for two minutes. The extract was cooled and filtered to get a clear solution. The filtrate was evaporated on a water bath and dried in a vacuum oven at 100 ^{o}C. Dried residue was collected in a mortar and ground to get a fine powder. The powder was then passed through #80 mesh to get a uniform powder. For every 10 gms of finely ground dry approx 5 gms of residue was obtained.

The plant extract powder was reconstituted in distilled water at the time of feeding the experimental animals. Freshly prepared doses were fed to animals. The doses were obtained by extrapolation from recommended doses ranged for humans.

2.2. Experimental animals

Wistar weanling rats of either sex, weighing between 60-100gms were procured from animal house of Haffkine Bio-pharmaceuticals corporation Ltd, Mumbai, India. The study protocol was approved from the Institutional Animal Ethics Committee under the reference number 407/01/a//CPCSEA of 2001. Guidelines were adhered during the maintenance and experiment. All animals were maintained under standard husbandry conditions with food and water *ad libitum.*

Total eight groups of experimental animal were made as follows. Group 1, Control group was received the diet containing autoclaved red kidney beans autoclaved at 121^{o} C at 15 atmospheric pressure for 20 minutes, to abolish lectin activity, every day for 15 days. Group 2, Diseased control groups received lectin containing diet, non-autoclaved red kidney beans. In group 3, Rats were maintained on lectin containing diet and received 67.5 mg/kg dose (low dose) of *Zingiber officinale* orally every day for 15 days and served as test group. In group 4, rats were maintained on lectin containing diet and received 135 mg/kg dose (high dose) of *Zingiber officinale* orally every day for 15 days. In group 5, rats were maintained on lectin containing diet and received 270 mg/kg dose (low dose) of *Cuminum cyminum* orally every day for 15 days. In group 6, rats were maintained on lectin containing diet and received 540 mg/kg dose (high dose) of *Cuminum cyminum* orally every day for 15 days. In group 7, rats were maintained on lectin containing diet and received 90 mg/kg dose (low

dose) of *Cyperus rotundus* orally every day for 15 days. In group 8, rats were maintained on lectin containing diet and received 270 mg/kg dose (high dose) of *Cyperus rotundus* orally every day for 15 days.

2.3. Estimation of microbial count of mesenteric lymph nodes and intestine [15]

On 16th day, over night fasting animals were scarified by ether anesthesia. A midline incision on the abdominal wall was made using aseptic precautions. Three mesenteric lymph nodes were isolated. A 10 cm segment of jejunum below the ligament of Treitz and 10 cm segment of ileum proximal to ileocaeal sphincter were isolated from each rat. The lumen of each segment was rinsed gently with 20ml sterile distilled water. These intestinal segments and mesenteric lymph nodes were homogenized separately. The homogenates were collected in sterile McConkey's broth and plated after serial 10 fold dilutions on McConkey's agar. Colony count of intestinal flora and of mesenteric lymph nodes was carried out using plate dilution frequency technique in test group, control group and disease control group animals.

Further, McConkey's agar plates were prepared and then partially dried. Eight approximately equidistant circular areas were marked lightly with a sterile cork borer. Ten-fold dilution of homogenized tissue was done. Total 6 dilutions were made by using sterile MacConkey's broth as 10^{-1} to 10^{-6}. From each dilution 0.01ml of sample was dispensed to the center of prescribed circle in the eight replicates on McConkey's agar plate. These plates were incubated at 37ºC for 24hours. Culture growth from each dilution was noted. Colony count was calculated by referring the reference table to estimate the number of organisms present.

2.4. Estimation of Barrier mucous content by Alcian blue dye extraction [16]

On the 16th day over night fasting animals were scarified by ether anesthesia. The representative twenty centimeter segments of jejunum and ileum were selected and cut by keeping ten centimeter from the ligaments of treitz and ileo-caecal junction respectively. The intestinal segment of the jejunum and ileum were opened longitudinally and blotted dry. The entire length of the intestine was examined grossly for any abnormal changes. Two centimeter piece from proximal side of jejunum and ileum were dissected for histopathology. The rest of the segment was opened longitudinally, washed, weighed and immediately transferred to 10 ml of 0.1 % W/V buffered Alcian blue solution and stained for 2 hours at room temperature. The dye binds to the mucosa and excess uncomplexed dye was removed by two successive washing in 0.25 M sucrose solution. Dye complexed with intestinal wall mucus was extracted with 10 ml of 0.5 M Magnesium chloride solution, which was intermittently shaken for 1 min after 30 min intervals for 2 hours. Four ml Alcian blue extract was then vigorously shaken with an equal volume of diethyl ether. The amount of Alcian blue in the aqueous layer was measured spectrophotometrically at 540 nm. The quantity of Alcian blue was extracted per gram weight of the tissue was extrapolated using standard curve obtained by using various dilutions of 0.1 % Alcian blue. The intestinal barrier mucus (mcg/gm) was calculated by taking ratio of optical density multiplied by standard alcain blue in microgram divided by weight of intestinal segment in grams.

2.5. Histopathological examination [17]

After sacrificing and dissecting the animals, two centimeter piece from test group, control group and disease control group from the proximal end of the jejunum and the distal end of the ileum was cut and preserved in 10 % formalin and used for histopathological examination. Stained histopathological slides of the intestinal sections were studied for change in villous morphology, villous height and incidence of cryptitis was noted by microscopical examination. The villi observed in the section were classified as it appears like Finger, Ridge, Leaf and thumb.

3. Results

3.1. Colony count of intestinal organisms and mesenteric lymph nodes to find out bacterial translocation

Table 1: Effect of *Zingiber officinale* (ZO) on bacterial translocation (log values)

Tissue	control group	Disease control group	Zo (low concentration)	Zo (high concentration
Small intestine	63200	1364089	204189.5	66378
mesenteric lymph nodes	0	68740	894.5	96.5

Bacterial translocation from small intestine to mesenteric lymph nodes was observed less in test group animals than disease control group animals. Zo prevented bacterial translocation form intestine to mesenteric lymph nodes more in high dose but it was not statistically significant.

Table 2: Effect of *Cuminum cyminum* (Cc) on bacterial translocation (log values)

Tissue	control group	Disease control group	Cc (low concentration)	Cc (high concentration)
Small intestine	63000	1364089	127817.7	111823.2
mesenteric lymph nodes	0	68740	11816.83	1912.667

Bacterial translocation from small intestine to mesenteric lymph nodes was observed less in test group animals than disease control group animals. Cc prevents bacterial translocation form intestine to mesenteric lymph nodes more in high dose but not statistically significant

Table 3: Effect of *Cyperus rotundus* (Cr) on bacterial translocation (log values)

Tissue	control group	Disease control group	Cr (low concentration)	Cr (high concentration
Small intestine	63000	1364089	118765.4	64440
mesenteric lymph nodes	0	68740	0	0

Bacterial translocation from small intestine to mesenteric lymph nodes was observed only in disease control group animals where as all test group animals from Low and High dose of Cr were showed total protection against translocation of intestinal indigenous flora from small intestine to mesenteric lymph nodes.

Table 4: Comparison of Effect of all three plant drugs on bacterial translocation (log values)

Tissue	control group	Disease control group	Zo (low concentration)	Zo (high concentration)	Cc (low concentration)	Cc (high concentration)	Cr (low concentration)	Cr (high concentration)
Small intestine	63000	1364089	204189.5	66378	127817.7	111823.2	118765.4	64440
mesenteric lymph nodes	0	68740	894.5	96.5	11816.83	1912.667	0	0.0

In comparison of all three plant drugs, there was definitely less translocation of intestinal flora from small intestine to mesenteric lymph nodes in test group animals than in disease control group animals. High dose of all three plant drugs was more effective towards protection of translocation of intestinal flora of small intestine to mesenteric lymph nodes. Maximum effective protection was observed by Cr, followed by Zo and Cc on comparison.

The colony count of intestinal segments revealed that reduction in the bacterial colonization, which shows less translocation of intestinal bacteria. This indicates reduction in gastrointestinal mucosal damage. In colony count of mesenteric lymph nodes shows no bacterial growth, which indicates that translocation of bacteria from intestine to mesenteric lymph nodes, has not occurred. Thus translocation of indigenous intestinal flora into the systemic circulation is prevented.

3.2 Barrier Mucus content of small Intestine in mcg/ gms.

Table 5: Effect of *Zingiber officinale* on small intestine mucous damage

Tissue	Control group	Disease control group	Low Dose	High Dose
Ileum	573.1571	234.819	196.39	247.122
Jejunum	501.6729	257.511	213.425	244.664

Mucus content in Ileum and Jejunum was more in healthy Control animals than disease control animals and Test animals studied. Test animals with High dose showed more mucus content values than disease control and low dose test animals

Table 6: Effect of *Cuminum cyminum* on small intestine mucous damage

Tissue	Control group	Disease control group	Low Dose	High Dose
Ileum	573.1571	234.819	192.66	303.133
Jejunum	501.6729	257.511	157.36	295.376

Mucus content in Ileum and Jejunum was more in healthy Control animals than disease control animals and Test animals studied. Test animals with High dose showed more mucus content values than disease control and low dose test animals

Table 7: Effect of *Cyperus rotundus* on small intestine mucous damage

Tissue	Control group	Disease control group	Low Dose	High Dose
Ileum	573.1571	234.819	249.39	401.68
Jejunum	501.6729	257.511	223.24	399.64

Mucus content in Ileum and Jejunum was more in healthy Control animals than disease control animals and Test animals studied. Test animals with High dose showed more mucus content values than disease control and low dose test animals

Table 8: Comparison of Effect of all three plant drugs Zo, Cc and Cr on small intestine mucous damage

Tissue	Control group	Disease control group	Zo (Low Dose)	Zo (High Dose)	Cc (Low Dose)	Cc (High Dose	Cr (Low Dose)	Cr (High Dose
Ileum	573.1571	234.819	196.39	247.122	192.66	303.133	249.39	401.68
Jejunum	501.6729	257.511	213.425	244.664	157.36	295.376	223.24	399.64

Mucus content in Ileum and Jejunum was more in healthy Control animals than disease control animals and Test animals studied. Test animals with High dose showed more mucus content values in all three drugs tested, than disease control and low dose test animals. Mucus content values in low dose test animals was less than disease control animals in Cc and Zo, where as Cr values were more in test animals than disease control animals. Hence, in comparison between 3 drugs, Cr was observed more effective than Cc and Zo. Cc and Zo was more or less equally effective to control mucus content level in Ileum and Jejunum.

3.3. Histopathological study

Table 9: Histopathological examination of Ileum (Im) and Jejunum (Jm)tissue.

Groups	Type of tissue	Height/ Width	Shape	Anatomy	Crypt : Villi	Cryptitis	Other Comments
Control	Im	N/N	Finger	Tall, slender	N	No	Sloughed out epithelium
Control	Jm	N/N	Finger	Tall, slender	N	No	Submucosal Band of Lymphocytes
Dis-Ctrl	Im	N/N	Leaf	Flattening	Altered	Moderate	Submucosal lymphoid aggregate
Dis-Ctrl	Jm	N/N	Leaf	Distorted, Branching	Altered	Moderate	Lymphoid aggregate causing ulceration
Zo High Dose	Im	N/N	Leaf	Tall/slender	N	No	Mucosal lymphoid aggregate
Zo High Dose	Jm	N/N	Leaf	Tall/slender	N	No	Submucosal band of lymphoid aggregate.
Zo Low Dose	Im	N/N	Finger	Tall	Altered	Moderate	Lymphoid hyperplasia
Zo Low Dose	Jm	N/N	Finger	Tall	N	Mild	Short mucosal lymphoid aggregate
Cc High Dose	Im	N/N	Leaf	Tall, slender	N	Mild	Submucosal Band of lymphocytes
Cc High Dose	Jm	Short/N	Finger	Tapering blunt	Altered	Mild	Lymphoid hyperplasia
Cc Low Dose	Im	Short/N	Finger	Distortion of crypts	Altered	moderate	Shortening & Irregularity: Only at one end
Cc Low Dose	Jm	Short/N	Finger	Distortion	Altered	Moderate	Lymphoid Aggregate causing ulceration
Cr High Dose	Im	N/N	Leaf	Tall, slender	N	Mild	Submucosal lymphoid aggregate
Cr High Dose	Jm	N/N	Finger	Tall	N	Mid	Mucosal lymphoid aggregate
Cr Low Dose	Im	Short/N	Finger	Distortion	Altered	Moderate	Mucosal lymphoid aggregate causing ulceration of surface epithelium
Cr Low Dose	(Jm)	Short/N	Finger	Distortion	Altered	Moderate	Submucosal band of lymphocytes

Histopathological examination of small intestine tissue i.e. Ileum and Jejunum of Test animals who were fed with three plant extracts as drugs individually showed slight change in Shape of villi, Anatomy of villi, altered Crypt : Villi, and incidence of cryptitis on comparison with Healthy control animals. While comparing with Disease control animals, High dose of all three plant drugs tested showed less damage to Villi morphology (shape and anatomy) and less incidence of cryptitis than low dose of drug in Test animals.

4. Discussion

The emergence of infection caused by multiple drug resistant enteric pathogens has now necessitated the search for alternative parenteral agents and the introduction of natural plant products. This is a very important replacement for the resistance. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic values. Recently the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led us to investigate the antimicrobial activity of medicinal plants [12].

Ayurveda describes certain plant drugs, which can be used in treating or reducing severity of diarrhoea [13-14]. Diarrhoea is a condition, usually related with poor hygiene, consumption of contaminated water and food, as well as patient's immune status. Immune status is commonly associated with malnutrition, which enhances the severity and integrity of diarrhoea in patient.

In developing countries like India, malnutrition in children is very common observation, often associated with poverty and illiteracy. Hence, chronic diarrhoea is one of the major problems associated in health management. There are many treatment regimens available for diarrhoeal disease management. Always due to drug resistant factor or its side effects, none of the drug is found to be safe and promising to cure the condition. In India, traditional uses of many household remedies are seen to be useful in such condition which are found to be effective with out any side effects.

Ayurveda literature search indicates that the decoction of processed Zingiber officinale (sunthi), Cyperus rotundus (musta), Cuminum cyminum (jeera) can be used as an anti-diarrhoeal home remedy [15]. Sahoo *et al*. [18] study in 2014 showed usefulness of jeera in animal model. They showed significant ($P < 0.001$) inhibition in frequency of diarrhoea, defecation time delaying, secretion of intestinal fluid as well as intestinal propulsion as compared to control and the graded doses of tested extract followed dose dependent protection against diarrhoea.

In order to find out activity, a systemic study of medicinal plants is very important [12]. Scientific study towards various Ayurvedic plant drugs and its effect on various pathogens *in vitro* as well as *in vivo* are not well documented. Hence, it is need of today to establish such documents for its regular use as remedy, which will be useful for the control and cure of the various infectious conditions like diarrhoea.

Keeping in mind the above facts, we have made an attempt to prepare suitable condition in animal model representing chronic diarrhoea in malnourished children and to study *in vivo* effect of selective plant drugs in suitable animal model considering various parameters like Colony count of intestinal organisms, Colony count of organisms of mesenteric lymph nodes, Barrier mucosal content of the intestine and Histopathological examination of jejunum and ileum that is change in villous morphology and villous height and incidence of cryptitis.

In vivo effect of Sunthi, Jeera and Musta was studied by using Wistar rats as animal model. We have used weanling Wistar rats as an experimental model of diarrhoea as it is very close to human anatomy and body mechanisms. Lectin was used as damaging agent to develop intestinal condition similar as chronic diarrhoea in malnourished child. Mamatha *et al*. [12] in 2005 (India) reported use of animal model to study effect of plant drugs. Banwell *et al*. [19] reported that intestinal condition similar as diarrhoea can be created in rats by using red kidney beans as a source of lectin. Shoda *et al*. [20] documented in 1995 from India, that intestinal condition created by feeding red kidney beans in rats is similar as chronic diarrhoea in malnourished children.

In the present study effect of Sunthi, Jeera, Musta were studied in rat animal model prepared for chronic diarrhoea in malnourished children in comparison with Healthy control and Disease control animals. Different study parameters like bacterial overgrowth, translocation of intestinal flora into mesenteric lymph nodes, mucus content of small intestine (ileum and jejunum), and disruption of villi were compared between Healthy control, Disease control and Test group of animals. In Test group animals,

protective mechanisms were seen as, prevention of translocation of indigenous intestinal flora to systemic circulation, reduced the intestinal bacterial colonization and prevention of loss of mucus content from small intestine.

In Test group animals, protective mechanisms were observed more or less at par to Disease control animals. This effect was predominantly observed with Musta followed by Sunthi and Jeera respectively. The study suggests that use of plant drugs with ORS can be useful for strengthening of the intestinal mucosal layer. This protects mucus content of intestine and prevents absorption of bacterial toxin, and commensals to become pathogens by increasing immunity of the child. *In vivo* effect of plant drugs on experimental animal model, revealed that Musta was most effective (protective) followed by Sunthi and Jeera. High dose of each plant drugs were found more effective than low dose on comparison.

In animal study it has observed that, there was an over growth of commensals in small intestine and translocation of indigenous intestinal flora into mesenteric lymph nodes in Disease control and Test group animals. Intestinal condition of study animals were developed similar to intestinal condition of malnourished children with chronic diarrhoea cases.It suggest that normal commensal flora can be responsible to reduce mucus content, mucosal damage, overgrowth of organisms and translocation of intestinal flora into mesenteric lymph nodes. This can cause persistent diarrhoea as well as systemic infection, mainly in lowered immunity children due to malnutrition, which can turn out to be fatal in untreated cases.

5. Conclusion

These results of this study shows the usefulness of musta, sunthi, and jeera in antidiarrhoeal model. Musta was found to be a potent antidiarrhoeal drug which supports the traditional claim.

6. Recommendation

More research work on medicinal plants will be useful to treat various gastrointestinal problems in children in developing countries like India.

7. Acknowledgement

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