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IN VITRO BIOACTIVITY STUDY OF BARK EXTRACT OF *TERMINELIA ARJUNA* ON PROBIOTICS, COMMERCIALLY AVAILABLE PROBIOTIC FORMULATION

Arpita Mandal[1], Koushik Das[2], Dilip Kumar Nandi[*3]

1. Department of Microbiology, Raja N L Khans Women's college, Midnapore, Pin-721102, West Bengal.
2. Department of Human Physiology & Department of Nutrition, Raja N L Khans Women's College.
3. Dialysis Unit,Vidyasagar Institute of Health, (a 100 bedded Private Hospital) (Centre for advancement of Higher Education & Research) Rangamati, Midnapore, West Bengal.

Abstract

Probiotics are living microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community. Plant extract often shows antimicrobial effect. Thus there is always confusion about the using of herbal medicine or plant extract co administration with probiotic supplements. The aim of the study was to prove that effect different solvent extracts viz. hexane, chloroform, ethyl acetate, ethanol, methanol, hydro-methanol (40:60 *v/v*) and distilled water of *Terminelia arjuna* (TA) on growth of probiotic beneficial bacterial mixture. A modified disc-diffusion and dilution-broth method were used for antimicrobial susceptibility testing of above different solvent extracts and antibiotics. It was observed that methanol, ethanol, hydro-methanol and distilled water extract of bark of TA showed inhibitory activity on bacterial growth as antibiotics but hexane, chloroform and ethyl acetate extracts did not show any inhibitory activity. Otherwise, hexane, chloroform and ethyl acetate extracts also exhibited to increase growth of bacteria. So, hexane, chloroform and ethyl acetate extracts might be used with beneficial bacteria for future experimental study (effect of probiotics and TA on uremia) upon synergic action of both plant and bacteria.

Keywords: Probiotic; Herbal drug; *Terminelia arjuna*; Chloroform.

Introduction

The medicinal plants give us with large number of phytocompounds to cure specific disease since prehistoric times. *Terminellia arjuna* (TA) has been a part of ayurvedic medicinal system. TA is a tree that is very commonly seen in areas having warmer climatic conditions. It is a deciduous tree that attains a height of 25 meters i.e. 60 to 70 feet. The tree trunk bears a bark that is gray in color. The bark is smooth in texture. TA can be said as the most extensively used herb in heart related problems (Miller, 1998). It is one of the best cardio protective herbs that not only tones up the heart but is also helps in treating various heart problems. It is also used in pains, helps in healing up of wounds especially related to trauma and in lowering uremia (Das *et al.,* 2010). Aquoues extract of TA reduces the uremia on dehydration induced uremic rats (Das *et al.,* 2010). The aqueous extract of the bark of TA could protect the liver and kidney tissues against CCl4-induced oxidative stress probably by increasing antioxidative defense activities**.** Reduction in lipid peroxidation and increased in superoxide dismutase, catalase, glutathione peroxidase, glutathione-s-transferase glutathione reductase and glucose-6-phosphate dehydrogenase, reduced glutathione, vitamin A, vitamin C, vitamin E by the application of TA against oxidative stress (Raghavan B and Krishna Kumari S, 2006). TA ethanolic extract exhibited renal protection (Adeney *et al.,* 2008). Aquoues extract of TA protects dehydration induced uremia. The aqueous extract of the bark of TA showed optimum protective activity against NaF-induced oxidative damages. Beside this probiotic are living microorganisms which when administered in adequate amount confer health benefits to the host. The major groups are *Lactobacilli*, *Bifidobacteria* and some minor groups are *Saccharomyces*, *Streptococcus*, have been reported as potential therapeutic agents (Dunne *et al.,* 2010). Probiotic bacteria possess the ability to survive in the host depending on their metabolic activity, resistant to gastric acidity, adhesion to the mucosal surface, friendly to the

***Corresponding Address**

Dr. Dilip Kumar Nandi
Email: - dilipnandi2004@yahoo.co.in

host and protect the host against infection (Gillor *et al.,* 2008). An innovative "enteric" approach to mitigate uremia using live bacteria that, when ingested, catabolize uremic solutes in the gut has been tested recently (Ranganathan *et al.,* 2005).

But a question arise when extract of plants from 157 families have been reported to be active against microorganisms (Chitravadivu *et al.,* 2009) and there is a chance for showing antagonistic effect by plant extracts from TA bark against normally used probiotic bacteria (Ranganathan *et al.,* 2006). The worldwide interesting medicinal plants and also the use of probiotic bacteria have to think us about their combined effect. There is no previous study on the said plant extract explaining their effect on probiotic bacteria. Therefore, the present study was to find out the efficacy of TA on beneficial bacteria.

Material and methods

Collection of plant parts: The bark of *T. arjuna* was collected from Gopali, Indian Institute of Technology, Kharagpur, Paschim Medinipur district of West Bengal. Taxonomist of Botany Department, Raja N. L. Khan Women's College, Midnapore identified the material and voucher specimen (number-BVS-7) was deposited in the Department of Botany, Raja N. L. Khan Women's College.

Preparation of extract

The air-dried powdered plants 150 g were successively extracted with solvents of increasing polarity: hexane (Prolabo, France), chloroform, ethyl acetate (Prolabo) ethanol, methanol (Prolabo) hydro-methanol (40:60) and aqueous by maceration (3 x 48 h). The extracts were then concentrated by evaporation to dryness at 45°C under reduced pressure for 15 – 30 min following solvent. The hot water extract of plants was prepared according to the standard methods with minor modification as previously reported15. Dried crude extracts (150 g) were boiled in 1000 ml of distilled water for 1h, the decoction obtained was then filtered. (Ben *et al*, 2008).

At first bark of TA was dried at 40 ± 1 ºC in incubator & the dried parts were crushed in an electric grinder machine and the powder separated. The fine 50 gm powder was dissolved in 500 ml in hexane, chloroform, ethyl acetate ethanol, methanol, hydro-methanol (40:60 *v/v*) and of distilled water airtight glass jar, separately for 48 hrs in Shaker incubator at 37 ºC. The deep reddish browns of TA extracts were collected by filtering with Whatman's filter paper in separate container. Then these crude extracts were dried in vacuum desiccators to obtain a dry mass stored in refrigerator at (0-4^{o}C) and used for the experiment. (Das *et al.,* 2010)

Collection of probiotic formulations and their composition

Four type of probiotic formulations are collected from medicine shop of Midnapur town.

These are as follows:

1. BIFILAC®: contains powdered form of *Streptococcus faecalis* T-110JPC, *Clostridium butyricum* TO-A, *Bacillus mesentericus* TO-A JPC, *Lactobacillus sporogen* [manufactured by TABLETS (INDIA) LIMITED].
2. Lactobacilli^{Plus}: contain powdered form of *Lactobacillus acidophilus, L rhamnosus, Bifidobacterium longum, B. bifidum* [manufactured by Organon (india) Limited.
3. PRO-WELL•: Contain powdered form of *Lactobacillus acidophilus, Bifidobacterium longum, B. infantis, B. bifidum* [manufactured by ALKEM LABORETORIES LTD.].
4. Folcovit™: contain powdered form of lactic acid bacilli [manufactured by ESKAG PHARMA PVT. LTD].

Preparation of the inoculums

0.1gm of each powdered form Probiotic were grown in nutrient broth medium.

Antibacterial Assay

The test bacterial cultures were poured onto solidifies nutrient agar dishes. The test strain (0.2 ml) was inoculated into the media to inoculums size (108cells/ml) when the temperature reached 40-42°C. Care was taken to ensure proper homogenization. The plant extracts were tested for antibacterial activity by paper disc diffusion method. (Bauer, 1959)

Antimicrobial activity by disc diffusion assay

A modified disc-diffusion method was used for antimicrobial susceptibility testing. The dried plant extract were dissolved in 5 percent dimethylsulphoxide (DMSO; Merck, Germany) and then in sterile water, to reach a final concentration of 20 mg/ml and sterilized by filtration by 0.22 µm Millipore filters. The media used was nutrient agar. The discs (6 mm in diameter) were impregnated with 10 µl of the extracts (200 µg/disc) at a concentration of 20 mg/ml and placed on the inoculated agar (108 Cfu/ml of bacteria). Antimicrobials (µg/disk) all from Gibco, h: tetracycline (30UI/disc), streptomycin (10UI/disc) was served as positive reference standards to determine the sensitivity of the tested microbial strains. Control tests with the solvent DMSO (5%) employed to dissolve the plant extracts were performed for all assays and showed no inhibition of microbial growth. The inoculated plates were incubated at 40-42°C for 24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the tested organisms. All inhibition assays and controls were made in triplicate. (Murray, 1995)

Dilution broth method

one mg of the extracts solubilised in methanol extract (for the methanol extract) and in the same way 1 mg of each specific extract was dissolved in that of each solvent and adjust to 10 ml with sterile distilled water the resulting mixture must have a final concentration less than 5%. The mixture is strongly agitated during 5 minutes. The obtained mother extract is diluted from 10^{-2} to10^{-3}. Three

controlled was included in the test. Each tube contains respectively sterile distilled water, the culture medium added to tubes contain 8ml of sterile medium and incubated for 24 hours. Then OD was measured calorimetrically (Bouhadjera *et al.,* 2005).

Results

The effect of different extracts of TA bark on probiotic bacteria was shown in table 1.Two commonly used antibiotic, tetracycline and streptomycin gave significant antibacterial result against probiotic bacteria (Prescott *et al.,* 1999). The result of table 1 clearly shows that methanol extract was effective against all used probiotic bacterial combination suggesting that methanol soluble polar compounds show antimicrobial activities on probiotic bacteria. Among the probiotic bacteria, *Lactobacilli* sp with *Bifidobacterium* sp (Lactobacilli^{Plus}) were less susceptible to methanol extract than other. Probiotic mixture-Folcovit™ was most susceptible to and the solvent. 1.5ml of each dilution and 0.5ml of fresh bacterial culture (4 type probiotics combination) were methanol extract. Hydro methanol extract (table 1) gave inhibition zone, but it was lower than used antibiotic (effective against both gram positive and gram negative bacteria) zones. Ethanol (table 1) and aqueous (table 1) also gave inhibition zone lower than susceptible zone giving by the used antibiotic. This result suggests that polar extracts contain lesser amount of polar compounds with antimicrobial effect. Chloroform, ethyl acetate and hexane extract did not give any inhibition zone suggesting that nonpolar extract with nonpolar compounds posses no antimicrobial effect on probiotic bacteria. Methanol extract also show growth inhibition in broth culture. Hydro methanol extract, Ethanol (table-2) and aqueous extracts show bacterial growth similar to control (without extract). Hexane, Chloroform and ethyl acetate extracts influence bacterial growth.

Table 1 : Effect of Different Solvent extract of TA on growth of different probiotic formulations (disc diffusion method).

Different solvent Extract and used antibiotics	Diameter of Inhibition zone (mm)			
	Lactobacill ^{Plus}	PRO-WELL	Folcovit™	BIFILAC®
Methanol extract	20	25	28	21
Ethanol extract	-	6	-	-
Hydro-methanol extract	-	5	5	-
Aqueous extract	-	10	-	-
Ethyl-acetate extract	-	-	-	-
Chloroform extract	-	-	-	-
Hexane extract	-	-	-	-
Tetracycline	19	20	21	28
Streptomycin	17	18	15	15

Table-2 : Effect of Different Solvent extract of TA on growth of different probiotic formulations (dilution broth method).

Different Solvent Extract and antibiotics	Bacterial growth			
	Lactobacill ^{Plus}	PRO-WELL	Folcovit™	BIFILAC®
Methanol extract	-(a)	-	-	-
Ethanol extract	+(b)	+	+	+
Hydro-methanol extract	+	+	+	+
Aqueous extract	+	+	+	+
Ethyl-acetate extract	++(d)	++	++	++
Chloroform extract	++	++	++	++
Hexane extract	++(c)	++	++	++
Tetracycline	-	-	-	-
Streptomycin	-	-	-	-

-= (a) Growth inhibition
+= (b) Normal growth
++= (d) heavy growth

Discussion

Present study was to find out inhibitory and stimulatory effect of *Terminalia arjuna* (TA) on probiotics. A modified disc-diffusion and dilution-broth method were used for antimicrobial susceptibility testing of different solvent extracts and antibiotics on commercially available probiotics. From the results, it was observed that methanol, ethanol, hydro-methanol and distilled water extract of bark of TA showed inhibitory activity on bacterial growth as antibiotics but hexane, chloroform and ethyl acetate extracts did not show any inhibitory activity. Otherwise, hexane, chloroform and ethyl acetate extracts also exhibited to increase growth of bacteria. The results obtained in this study indicated a considerable difference in antibacterial activity among the 7 solvent extracts. Maximum antibacterial effect of methanol extract was supposed to because methanol is an organic solvent and dissolve organic compounds mainly polar compounds better than aqueous extract and also other polar and nonpolar solvents (Srinivasan *et al.,* 2001). It was confirmed that extract of polar solvents mainly methanol extract from bark of TA contain antimicrobial substances those have inhibitory effect on applied probiotic formulations. In our previous work was to reduce uremia of renal failure rats (Das *et al.,* 2009). Aquoues extract of *Terminalia arjuna* reduces the uremia on dehydration induced uremic rats (Raghavan *et al*., 2006). *Terminalia arjuna* ethanolic extract exhibited renal protection (Adeneye *et al.*, 2008). Otherwise, *Streptococus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium longum*, are safe and effective and in management of renal failure in cats and probiotic therapy reduce uremia (Saunders *et al.,* 2003). So, it is clear that probiotics and extract of TA are effective to reduce uremia. Thus, we can conclude that TA bark extract (solvent system should be chloroform, or hexane or ethyl acetate) can be administered with probiotic bacteria for better improvement to reduce uremia of renal failure rats which will be further study of our laboratory. Current therapies to remove uremic solutes for the ESRD patient include peritoneal dialysis, hemodialysis, and kidney transplantation. Each of these costly and time-consuming regimens is associated with high patient morbidly. In the recent years, efforts have been undertaken to mitigate uremia in animals and humans by administration of live cultures of naturally existing microbes. So, hexane, chloroform and ethyl acetate extracts of TA might be used with beneficial bacteria for future experimental study (effect of probiotics and TA on uremia) upon synergic action of both plant and bacteria.

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