Journal of Ecobiotechnology 2011, 3(5): 03-07
ISSN: 2077-0464
www.scholarjournals.org

Journal of Ecobiotechnology

www.journal-ecobiotechnology.com

Potential Antibacterial Efficacy of Bioactive Principles of Fruit Extracts of *Acacia arabica* Lam. Willd against Enterotoxigenic *Escherichia coli*

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Article Info

Article History

Received : 29-01-2011
Revised : 29-02-2011
Accepted : 30-02-2011

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Abstract

The present study revealed antibacterial activity of six different fruit extracts of *Acacia arabica* against ETEC Enterotoxigenic *E.coli* strains. Preliminary phytochemical analysis of plant extracts and quantitative estimation revealed presence of large amount of tannins. In addition the extract was found to be rich in cardiac glycosides. Antibacterial activity of plant extracts by well-diffusion and broth microdilution method revealed that fruit extract of *Acacia arabica* act as alternative therapeutic agent to cure diarrhea associated with ETEC more effectively.

Key Words: Enterotoxigenic *E.coli*, Well-diffusion, Broth microdilution method

Introduction

Hamburg's disease or Traveler's diarrhea is one of the most common and frequently occurring maladies faced by human throughout the world during their life time. The main etiological agent responsible for Hamburg's disease is *Escherichia coli.* Vehicle transmission of this food born etiological agent is always contaminated food and water (Ajayi and Akintola, 2010). Diarrheal diseases caused by *E.coli* not only infect human but also have been frequently reported in piglets, calves and various other poultry and domesticated animals (Nagy & Feket, 2005, Savita *et al.,* 2007). Though there are six major classes of Diarrheagenic *E.coli* but one of the most important class responsible for Hamburg's disease is Enterotoxigenic *E. coli* (ETEC).ETEC produces two different enterotoxins referred as heat stable (ST) and heat –labile enterotoxins (LT) that separates it from other six diarrheagenic *E.coli* (Prescott *et al.,* 2002). ETEC was reported to be responsible for major cause of neonatal diarrhea in Spain and isolated serotypes revealed presence of heat stable enterotoxins (Blanco *et al.,* 1991). Collibacillosis in piglets and calves was reported to be caused by Enterotoxigenic *E.coli* due to presence of plasmid mediated adhesions and enterotoxins (Nagy & Feket, 2005). Horizontal transfer of self transmissible Ent enterotoxigenic plasmid was responsible for virulence in ETEC stain (Ochi *et al.,* 2009). Yates, 2005 has reported more than 400,000 morbidity infants throughout the world due to ETEC strains according to recent survey of WHO. Implementation of control strategies is very essential to control diarrheal infections (Rahaman *et al.,* 2010). Prusti *et al.* (2008), strongly advocated the use of medicinal plants as an effective alternative healer to control bacterial infections. Medicinal plants were reported as cheap and safe alternative for treatment of infections but their activity may be bacteriostatic or bactericidal (Ngemenya *et al.,* 2006).

Acacia arabica Lam. Willd is a wild medicinal tree of family Fabaceae of 2.4 to 16 meter height found through out the world including India, Egypt, Kenya, Saudi Arabia and Tanzania. The tree is reputed for its various medicinal value use for treatment for various ailments both bacterial as well as non bacterial (Asolkar *et al*., 1992, Warrier *et al.*, 1994) but also economically useful for timber production. Leaf extract of *Acacia arabica* was reported to inhibit various fish pathogens (Muniruzzaman and Chowdhury, 2004). Ethanolic fruit extracts of *A.nilotica* showed remarkable inhibitory response against hospital isolates of *E.coli* associated with superficial abscesses & wound infections (Ali & Yagoub, 2007). The present study is aimed to investigate antibacterial activity of fruit extract of *Acacia arabica* against standard and clinical Enterotoxigenic stains of *E.coli* as well as to investigate phytochemicals by performing preliminary test of different solvent extracts.

Materials and Methods

Collection of Plant material

Plant material was collected during February March months in early stage of fruiting of *Acacia arabica* from road side of Bhilai. Collected fruits or pods were thoroughly washed under tap water, cut into small pieces and shade dried for 2-3 weeks. Dried plant material was pulverized using domestic mixer for preparation of plant extracts.

Preparation of Plant Extract

Preparation of Cold Extract: About 7.5 g of finely pulverized fruit was soaked in exactly 100 ml of three different solvents Methanol, Acetone and Chloroform and shaken for 24 hrs in rotary shaker at 120 r.p.m. (Nair *et al.,* 2005, Dhanabalan *et al*., 2008, Sharma & Patel, 2009). After filtration extract was concentrated by slow evaporation of solvents at 37^0C for 3-4 days to obtain final plant extract and yield was recorded. The process was repeated to recover large quantity of extract and

was stored under refrigeration at -4^{0}C for further use in antibacterial sensitivity test. The yield was recovered as % of quantity of initial fruit powder used (7.5 g) in 100 ml of solvents taken.

Yield (%) = yield × 100/7.5

Preparation of Hot Extract: Acacia arabica fruit extract was extracted by soxhlet apparatus at 60^{0}C for 2-3 days and evaporated till dryness. About 37.5 g of plant powder was taken and filled in thimble and successively extracted using 500 ml of three different solvents (Methanol, Acetone and Chloroform) separately and slowly evaporated at room temperature for 3-4 days and yield was recorded (Johnson *et al.,* 2008, Ali and Yagoub, 2007).

Yield (%) = yield × 100/37.5

Preliminary Phytochemical Screening of Plant extracts:

Cold and Hot methanolic, acetone and chloroform fruit extracts of *Acacia arabica* were subjected to certain phytochemical tests in order to detect presence of alkaloids, flavonoids, saponin, tannins and glycosides according to standard methods (Aderotimi, & Samuel , 2006).

Test for Alkaloids: To 0.5 g of extract ,5ml of 1% of HCl was added and filtered. To the filtrate Mayer's reagent was added drop wise. Formation of white precipitate indicates presence of alkaloids.

Test for Flavonoids: To 0.5 mg of plant extract, 5 ml solvent from which the extract belongs was added excluding chloroform extract. Chloroform extract was dissolved in 5 ml of DMSO for best solubility of compound. After filtration of extract about 2ml of extract was treated with conc. HCl and magnesium ribbon added to the filtrate. Change of color from pink- tomato red to magenta indicates presence of flavonoids.

Test for Saponin: To 0.5 mg of plant extract, 5 ml distilled water was added and shaken thoroughly and slightly heated in water bath for 5 min. Frothing indicates presence of saponin. However, in case of Chloroform extract 5ml of DMSO was taken and subjected to water bath and shaken to detect froth formation.

Test for Tannins: To 0.5 mg of plant extract, 5 ml extracted solvent was added excluding chloroform extract to which 5ml of DMSO was added. After filtration 2 ml of filtrate of various extract was treated with 5% of FeCl3 solution. Blue-black precipitate indicated the presence of Tannins.

Test for Cardiac Glycosides: To 0.5 g of extract, 2 ml acetate anhydrate and conc.H2SO4 was added. Green-blue color indicated the presence of cardiac glycosides.

Determination of total polyphenolic concentration of plant extracts

Estimation of total polyphenolic concentration of plant extract was performed using Folin -ciocalteaue assay. About 0-400 mg/l of gallic acid dilutions were prepared and exactly same amount of dilutions of plant extract were prepared. About 1 ml of four different concentration of standard gallic acid solution and plant extract was taken and treated with 1 ml of 10%of Folin -ciocalteaue solution and 2ml of 7.5% of Na2Co3 solution and kept in darkness for 1 hr at room temperature for color development. After 1 hr absorbance was recorded at 755 nm using UV-Vis spectrophotometer and concentration was recorded using gallic acid standard calibration curve (Ghasemzadeh *et al*., 2010).

Collection of Bacteria and Preparation of Inoculums

Enterotoxigenic *E.coli* strain *E.coli* O78:K80:H11 was obtained from IMTECH, Chandigarh popularly known to carry heat -stable and heat -label enterotoxins. In addition Enterotoxigenic *E.coli* from patient stool suffering from severe diarrhea was also obtained from pathology laboratory. The media used for the drug sensitivity purpose were Muller-Hinton's agar (Hi-media) and Muller- Hinton's broth (Hi-media). Cultures were adjusted according to Mc Farland turbidity 0.5 standards in MH- Broth.

Antibacterial Sensitivity Test

Agar-well diffusion Assay Method: Antibacterial activity of six different fruit extracts was performed using agar-well diffusion method (Johnson *et al.,* 2008). About 100 µl of Muller-Hinton's broth carrying 24 hrs old inoculum of ETEC was evenly swabbed over MH-agar plates and 6 mm diameter well was punched using a sterile cork borer. About 100 µl of five different concentration of plant extracts 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml was introduced into the well and incubated for 24 hrs at 37^{0}C. All experiments were performed in triplicates and average of inhibition zone diameter (IZD) was recorded.

Determination of Minimum Inhibitory Concentration: MIC of different fruit extracts were performed using broth microdilution method (Kashikar & George, 2006). About 10-fold dilution of plant extracts was prepared from 10 mg/ml to 0.019 mg/ml. About 10 µl of double strength Muller- Hinton's broth was introduced into each well. Exactly same amount of different concentration of plant extracts and ETEC broth was introduced into each well and incubated at 37^{0}C for 24 hrs. On next day 10 µl of 0.2 mg/ml of 2,3,4-triphenyl tetrazolium chloride INT dye was added to each well and incubated for 3 hrs for color change to determine break point inhibition of plant extract by reaction with surviving bacteria since the dye act as electron acceptor and turns pink whereas if inhibition takes place clear well was observed.

Results

Yield of plant extracts

On the basis of two different types of extraction process cold extraction gives more amount of extract in comparison to hot extraction method. In addition, type and nature of solvents also plays a very important role in extraction. Out of three different solvents, acetone has highest yielding ability in comparison to methanol and chloroform.

Table: 1 Yield of various Fruit Extracts in gm and %

Fruit Extracts	Yield (in gm)	Yield (in %)
Cold Methanol	1.08 (From 7.5 g in 100 ml)	14.4
Hot Methanol	4.36 (From 37.5 g in 500 ml)	11.6
Cold Acetone	2.06 (From 7.5 g in 100 ml)	27.5
Hot Acetone	9.25 (From 37.5 g in 500 ml)	24.6
Cold Chloroform	1.38 (From 7.5 g in 100 ml)	18.4
Hot Chloroform	0.08 (From 37.5 g in 500 ml)	0.21

Preliminary Phytochemical Screening

Preliminary phytochemical analysis of six different fruit extracts of *Acacia arabica* revealed complete absence of alkaloids and saponins. Tannins and glycosides are present abundantly and flavonoids in moderate amount. Quantitative analysis of Gallic acid revealed that it is present in large amount in acetone extract (33.5 mg/g), methanolic extract (31.2 mg/g) and chloroform extract (28.5 mg/g).

Table: 2 Preliminary Phytochemical Screening of Fruit Extract

	Cold Methanolic fruit extract	Hot Methanolic fruit extract	Cold Acetone Fruit extract	Hot Acetone Fruit Extract	Cold Chloroform Fruit Extract	Hot Chloroform Fruit Extract
Alkaloid	-	-	-	-	-	-
Flavonoid	++	++	++	++	-	-
Saponin	-	-	-	-	-	-
Tannins	++++	++++	++++	++++	++++	++++
Glycosides	+++	+++	++++	++++	++	++

++++=Abundantly present, +++-present, ++= moderately present, - =absent

Antibacterial Assessment of Different Fruit Extracts by Well Diffusion method

All the extracts excluding hot Chloroform showed effective Inhibition zone diameter against two different Enterotoxigenic strains of *E. coli*. However, cold methanolic, acetone and chloroform extract showed strongest inhibition against Enterotoxigenic standard and Clinical isolates in comparison to hot methanolic and acetone extract. Hot chloroform extract showed no zone of inhibition against tested organisms.

Table: 3 Determination of Inhibition Zone Diameter (IZD) ± S.E (in mm)

	Enterotoxigenic *E.coli* (Standard)					Enterotoxigenic *E.coli* (clinical isolate)				
	500 mg/ml	250 mg/ml	125 mg/ml	62.25 mg/ml	31.32 mg/ml	500 mg/ml	250 mg/ml	125 mg/ml	62.25 mg/ml	31.32 mg/ml
Cold Methanolic fruit extract	11.3±0.6	6± 0	6±1.1	5.2±1.3	3.3±1.7	16.6± 0.6	9.3±0.6	6.6± 1.1	3.3±0.6	2± 0
Hot Methanolic fruit extract	12±1.1	0.6±0.6	0±0	0±0	0±0	11.3±0.6	10±0	8±0	0±0	0±0
Cold Acetone Fruit extract	18±0	12.6±0.6	12±0	8±0	6±0	21.3± 0.6	16± 0	14± 0	10.6± 1.3	9.3±0.6
Hot Acetone Fruit Extract	4±0	2.6±0.6	1.13±0.6	0±0	0±0	11.3±0.6	10.6±1.3	9.3±1.3	8.6±0.6	8±0
Cold Chloroform Fruit Extract	8.6±0.6	7.3±0.6	6±0	0±0	0±0	16.6±1.3	14±0	8±0	0±0	0±0
Hot Chloroform Fruit Extract	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0

Determination of Minimum Inhibitory Concentration by Broth Microdilution Method

Broth microdilution method revealed that all extracts showed growth inhibitory results in comparison to well diffusion method, therefore revealing that microdilution method is more sensitive and effective in comparison to well –diffusion method since very limited amount of extract showed effective inhibition against tested microorganism.

Table: 4 Minimum Inhibitory Concentration in mg/ml of six different Fruit Extracts of *Acacia arabica*

	Enterotoxigenic *E.coli* (Standard)						Enterotoxigenic *E.coli* (Clinical Isolated)					
Conc (in mg/ml)	Cold Metha-nolic fruit extract	Hot Methanolic fruit extract	Cold Acetone Fruit extract	Hot Acetone Fruit Extract	Cold Chloroform Fruit Extract	Hot Chloroform Fruit Extract	Cold Methanolic fruit extract	Hot Methanolic fruit extract	Cold Acetone Fruit extract	Hot Acetone Fruit Extract	Cold Chloroform Fruit Extract	Hot Chloroform Fruit Extract
10	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
2.5	-	-	-	-	-	-	-	-	-	-	-	-
1.25	-	-	-	+	-	-	-	-	-	-	-	-
0.6	-	-	-	+	-	-	-	-	-	-	-	-
0.3	-	+	+	+	-	-	-	+	+	+	-	-
0.15	-	+	+	+	+	+	-	+	+	+	+	+
0.07	+	+	+	+	+	+	+	+	+	+	+	+
0.03	+	+	+	+	+	+	+	+	+	+	+	+
0.019	+	+	+	+	+	+	+	+	+	+	+	+

●- = Absence of growth, + = presence of growth

Discussion and Conclusion

Cold acetone extract showed highest yield (27.5 %) in comparison to hot acetone extract (24.6 %). Yield of cold methanolic extract (14.4%) was found to be less in comparison to cold chloroform extracts (18.4%). Soxhlet extraction provides limited yield of plant material in comparison to cold extraction. The yield of hot chloroform extract was found to be very limited of 0.21%. Therefore it was concluded that solvent extraction and yield were highly affected the antimicrobial activity (Ekwene & Elegalam, 2005).

Preliminary phytochemical screening of six different fruit extracts revealed presence of abundantly large amount of polyphenolic compounds, tannins and glycosides. Tannins present in plants played an important role to inhibit microorganisms (Ramakrishnan *et al*., 2006, Gowri and Vasantntha, 2010). Plants rich in gallic acids and tannic acids were reported to be having antidiarrheal properties (Sharma *et al*., 2009). Fruit extract shows complete absence of alkaloids and saponins. Therefore it was concluded that therapeutic as well as antibacterial activity of fruit extracts of *Acacia arabica* depends upon presence of tannin and glycosides.

Estimation of Antibacterial activity by well diffusion method revealed that Enterotoxigenic strains were inhibited more by cold extracts since average diameter of zone of inhibition were found to be greater in comparison to hot extracts of three different solvents. However, well diffusion method is quite unreliable due to difference in dissolving nature of polar as well as nonpolar solvents in various media (Hashmi *et al*., 2008). Diameter of zone of inhibition of acetone extracts against both Enterotoxigenic Standard and Clinical isolates of *E.coli* were found to be much greater in comparison to cold and hot methanolic extracts. Cold chloroform extract showed limited zone of inhibition against tested organism where as hot chloroform extract showed no inhibitory result in well diffusion method.

Minimum Inhibitory concentration of plant extract by broth microdilution method reveals that MIC of Cold Methanolic extract of ETEC (standard) and ETEC (clinical isolated) strain was found to be 0.15 mg/ ml where as MIC of Hot Methanolic fruit extract was 0.6 mg/ml for both strains. MIC of Cold Acetone extract was 0.6 mg/ml for both strains. MIC of Hot acetone fruit extract was 2.5 mg/ml for ETEC standard strain and 0.6 mg/ml for clinical isolated strain. MIC of Cold and Hot Chloroform fruit extract against two strains were reported to be 0.3 mg/ml. Determination of MIC using microtiter plate or broth microdilution technique was reported to be one of the most useful technique to determine activity of large number of samples. Its advantage over disc diffusion method includes increased sensitivity for small amount of extract and ability to distinguish bacteriostatic and bactericidal activity (Ncube *et al*., 2008). Broth microdilution method provides accurate results with out any error as compared to disc diffusion method (Edelmann *et al.,* 2007).

Photographs of result obtained

Photo 1). Diameter of zone of inhibition of cold methanolic fruit extract at conc, of 500 mg/ml and 250 mg/ml against ETEC Clinical isolated strain. Photo 2). Diameter of zone of inhibition of cold methanolic fruit 500 mg/ml and 250 mg/ml against ETEC standard stain.

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