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Phytochemical Screening and Antibacterial Properties of Organic Solvent Fractions of *Psidium guajava* Aqueous Leaf Extracts

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Abstract: Resistance of some bacteria, especially some stains of *E. coli* to common antimicrobial agents has created an urgent need to develop alternative antimicrobial drugs from herbs that are safe, cheap and may overcome the resistance of the pathogens. The crude aqueous extract of *Psidium guajava* leaf which is known to possess some antibacterial properties was further subjected to sequential fractionation with organic solvents (chloroform, ethyl acetate, normal butanol) of different polarity. This was done until the organic layer was visibly clear to obtain chloroform, ethyl acetate and n-butanol soluble fractions and residual aqueous fraction. Phytochemical screening and antibacterial activity of organic solvents soluble fractions and residual fraction of the extract on some gram positive and gram negative microbes were carried out. The different fractions showed variation in phytochemical constituency and thus in their antibacterial properties. The ethyl acetate soluble fraction of the extract showed broad spectrum antibacterial properties against all the organisms tested. The fraction also showed a good activity against *E. coli* at a relatively lower concentration and hence could possibly be use against *E. coli* infections.

Key words: Phytochemistry, antibacterial, *Psidium guajava*, organic solvents fractions

INTRODUCTION

Infections with Avian Pathogenic *Escherichia coli* (APEC) cause colibacillosis, an acute and mostly systemic disease resulting in significant economic losses in poultry industry worldwide (Ewers *et al*., 2003). Antimicrobials are valuable tools in colibacillosis control, however, a substantial proportion of avian pathogenic *E. coli* strains have developed resistance to antimicrobial drugs commonly used in poultry production (Yang *et al*., 2004). Of particular concern is the emergence of resistance to frontline antimicrobials, such as the fluoroquinolones, which because of their low toxicity and relatively broad-spectrum coverage are extremely valuable for treating infections (Angulo *et al*., 2000; Livermore *et al*., 2002). The emergence of these resistant bacteria has created a major concern and an urgent need for new antibacterial agents (Emori and Gaynes, 1993; Davis, 1994; American Society of Microbiology, 1995). Furthermore, using antibiotics to subside infection produces adverse toxicity to host organs, tissues and cells (Prakash, 2006). Herbal molecules are safe, will overcome the resistance produced by the pathogens since they are in combined form or in pooled form of more than one molecule in the protoplasm of the plant cell (Prakash, 2006).

Psidium guajava leaves are employed in the treatments of a cascade of diseases including diarrhea, dysentery and vertigo. It is also used in regulating menstrual periods and dressing wounds in many parts of Africa (Iwu, 1993). The crude extract of *Psidium guajava* has been reported to possess some antibacterial properties (Guan and Damello, 1999; Geidam *et al*., 2007). Majority of medicinal plants used for herbal treatments are flowering plants (angiosperms) and are readily available in the rural areas (Farnsworth and Morries, 1976) and this has made traditional medicine relatively cheaper than orthodox medicine in the third world countries. Since over 80% of the world's population use plant as their primary source of medication (Farnsworth *et al*., 1985; Cordell, 2000) and antibiotics are sometimes associated with adverse side effects to the host including hypersensitivity, immuno-suppressive and allergic reactions (Ahmed *et al*., 1998; Prakash, 2006), it is important to develop alternative antimicrobial drugs that are herbal based, for the treatment of infectious diseases (Clark, 1996; Cordell, 2000). Some herbs have been recommended for the treatment of some diseases like tuberculosis (Mata *et al*., 2004) and ischemic heart disease (Gauthaman *et al*., 2005). McLaughlin (1991) reported that using bioassay guided screening and fractionation of

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plant extracts ensures that the compounds will have better biological activity and therefore, stands a good chance for drug discovery through subsequent structure-activity relationship studies to obtain a compound with improved activity and least toxicity.

The present study is therefore to further determine the phytochemical constituents and the antibacterial activity of the organic solvent fractions of *Psidium guajava* on some bacterial organisms. The result of the antimicrobial study would identify the most active organic solvent fraction of *Psidium guajava* leaf and give validity to its possible use as an antibacterial agent against *E. coli* infections.

MATERIALS AND METHODS

Sample collection, identification and preparation of extract: Fresh samples of the leaves without stalk were collected in February, 2006 from the University of Maiduguri campus, Maiduguri, Nigeria. The plant was identified and authenticated by Dr. S.S. Sanusi of the Department of Biological Sciences, University of Maiduguri, Nigeria. A voucher specimen (Chemistry 242 B) was deposited in the Department of Chemistry, University of Maiduguri, Maiduguri.

The fresh leaves of *P. guajava* collected were air-dried in the laboratory, ground into fine powder and stored in a glass container at 4°C. Eight hundred grams of the powdered sample was exhaustively extracted with distilled water using a reflux method. The crude aqueous extract obtained was concentrated *in vacuo*, brown in colour and yielded 33.75% (w/w). It was properly labeled and stored in the refrigerator at 4°C until used (Trease and Evans, 1989). All work was carried out in accordance with the general guidelines for methodologies on research and evaluation of traditional medicine (WHO, 2000).

Fractionation of the aqueous extract: The crude aqueous extract obtained was suspended in cool distilled water and then filtered using Whatman No. 1 filter paper. The filtrate was thereafter fractioned successively with chloroform, ethyl acetate and normal butanol (Fig. 1). The fractionation with the organic solvents which are of different polarity was done until the organic layer were visibly clear to get chloroform, ethyl acetate and n-butanol soluble fractions and residual aqueous fraction, in sequence as described by Cho *et al.* (2003) and Motohashi *et al.* (2004).

Phytochemical analysis: The *P. guajava* organic solvent fractions were subjected to qualitative chemical screening for identification of the various classes of active chemical constituents such as carbohydrates, tannins, phlabotannins, saponins, glycosides, steroids, triperpinnoids, flavanoids, anthraquinones and alkaloids. The phytochemical analysis was done by standard methods (Trease and Evans, 1989; Trease and Evans, 1997).

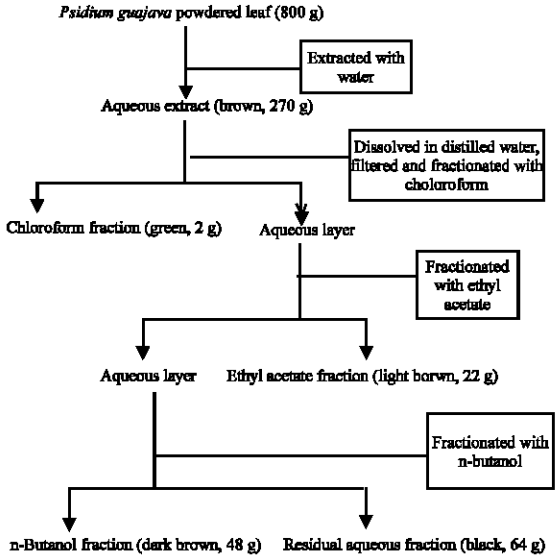

Fig. 1: Schematic diagram of extraction and fractionation of *P. guajava* leaf extract into organic solvent fractions

Microbial cultures: Laboratory isolates of the pure culture of gram-positive (*Staphylococcus aureus* and *Streptococcus fecalis*) and gram-negative (*Salmonella typhi*, *Klebsiella pneumoniae* and *Escherichia coli*) bacteria were obtained from the Veterinary Medicine Research Laboratory, University of Maiduguri, Nigeria.

The isolates were propagated and stored on nutrient agar plate. The nutrient agar medium was obtained in dehydrated powdered form (Oxoid Ltd., England) and was prepared according to the manufacturer's specification. All stock cultures were maintained in nutrient agar plate at 4°C and sub-cultured in nutrient broths (Oxoid Ltd., England) at 37°C for 8 h prior to antimicrobial testing. One milliliter of the broth culture was then used to flood the agar plates.

Extracts concentration: Stock solutions of the different organic solvents soluble fractions were prepared by dissolving 100, 200 and 400 mg of the extract in 1 mL of distilled water. The following concentrations of each fraction were prepared, 100, 200 and 400 mg mL^{-1}. Standard antibacterial agent (oxytetracycline, Cipla Ltd., Mumbai, India) at a concentration of 10 mg mL^{-1} was also used on all the bacteria organisms and their zones of inhibition were compared with those of the extract.

Antimicrobial sensitivity testing: Disc diffusion method as described by the National Committee of Clinical Laboratory Standards (1993) was used to determine the antibacterial activity of the various organic solvent fractions of *Psidium guajava* leaf. Discs containing different concentrations of dissolved extracts was prepared with sterilized filter papers (Whatman No. 1; 6 mm in diameter) soaked in different beakers containing different concentrations (100, 200 and 400 mg mL^{-1}) of the extracts. The discs were dried at 50°C.

Overnight cultures of each bacterial isolate were diluted using sterile normal saline to give an inoculums size of about 10^6 Cfu mL^{-1}. The inocula were spread on the surface of dried nutrient agar plates with cotton wool swabs, which have been dipped in the diluted suspension of the organisms. The plates were incubated at 37°C for 30 min before the discs were applied aseptically. The treated plates were incubated at 37°C for 48 h. The same procedure was carried out using oxytetracyclin (10 mg mL^{-1}) as the positive control. Plates without the antibiotic or extract discs were set up as the negative control experiment. The zone of inhibition above 6 mm diameter of each isolate was used as a measure of susceptibility to the extracts and this was compared to that of the standard antibiotic.

Minimal inhibitory concentration: The Minimum Inhibitory Concentrations (MIC) of the ethyl acetate soluble fraction of aqueous extract of *Psidium guajava* leaf were determined using the method described by Greenwood (1989). Six sterile test tubes were arranged in five rows in a test tube rack, each row for one of the five microorganisms used for the test. Half a milliliter of sterile nutrient broth was pipetted into all the tubes. In addition, 0.5 mL of the ethyl acetate soluble fraction of the extract containing 200 mg mL^{-1} was pipette into the first tubes of the 5 rows to obtain a concentration of 100 mg mL^{-1}. Thereafter there was a serial dilution of the extract in each row to obtain concentrations of 50, 25, 12.5, 6.25 and 3.13 mg mL^{-1}, respectively. The test organisms (0.5 mL) were pipetted into each of the test tubes and incubated at 37°C for 24 h. The MIC was recorded as the least concentration of the extract that completely inhibited the growth of the test organisms. The content of the tubes were further sub-cultured for 24 h to determine bactericidal or bacteriostatic activity. Bactericidal effect was demonstrated when no growth occurred on the sub-cultured medium after MIC determination.

RESULTS

The result of extraction and fractionation of the crude extract of *Psidium guajava* leaf is presented in Fig. 1. The n-butanol soluble fraction of the extract is more in quantity followed by ethyl acetate and chloroform soluble fractions respectively. However, the quantity of the residual fraction is more than any of the organic solvent fractions. The phytochemical investigation of the various solvent fractions of *P. guajava* showed the chloroform fraction of the extract to contained only steroids, which occurred in high concentration. The residual aqueous and n-butanol fractions contained similar phytochemical constituents in which the n-butanol fraction appears to contain higher concentrations of carbohydrate and flavanoid. The ethyl acetate fraction contained only tannins, steroids, glycosides and high concentrations of flavanoids. The ethyl acetate fraction did not contain any carbohydrate (Table 1).

Table 1: Phytochemistry of organic solvent fractions of aqueous extract of *P. guajava* leaf

Phytochemical constituent	Type of test	Inference			
		Chloroform	Ethyl acetate	n-butanol	Residual aqueous
Tannin	Ferric chloride	-	++	+++	+++
	Lead acetate	-	++	+++	+++
	Formaldehyde	-	+	++	+++
Saponin	Frothing	-	-	++	+
Carbohydrate	Molish's	-	-	+++	+++
	Free reducing sugar	-	-	+++	++
	Combined reducing sugar	-	-	+++	+++
	Barfoed's	-	-	-	-
Flavanoid	NaOH	-	+++	+++	-
	Ferric chloride	-	+++	+++	+++
	Lead acetate	-	+++	+++	++
	Shinoda's	-	+++	+	-
Alkaloid	Dragendorff's	-	-	-	-
	Mayer	-	-	-	-
	Wagner	-	-	-	-
Phlobatanin	1.HCl	-	-	-	-
Steroid	Lieberman's	+++	++	-	++
	Salkowski's	+++	+	+	-
	Keller-kiliani	+++	+++	+++	++
Glycoside	General test	-	++	+	+
Anthraquinone	Free anthraquinone	-	-	-	-
	Combined anthraquiunone	-	-	-	-

+ = Low concentration; ++ = Moderate concentration; +++ = High concentration; - = Absent

Table 2: Antibacterial efficacy of organic solvent fractions of aqueous extract of *P. guajava* leaf

Extract	Concentration (mg mL^{-1})	Antibacterial activity (mm)				
		Staphylococcus aureus	*Streptococcus fecalis*	*E. coli*	*Salmonella typhi*	*Klebsiella pneumoniae*
Chloroform	100	R	R	R	R	9.0
	200	R	R	R	R	10.0
	400	R	R	R	R	11.0
Ethyl acetate	100	7.0	7.0	14.0	8.0	11.0
	200	8.0	9.0	16.0	9.0	12.0
	400	10.0	10.0	17.0	10.0	13.0
n-Butanol	100	R	R	R	8.0	10.0
	200	R	R	R	9.0	11.0
	400	R	R	R	10.0	13.0
Residue	100	R	R	R	R	R
	200	R	R	R	R	R
	400	R	R	R	R	R
Oxytetracycline	10	20	20	22	20	10

R = Indicates the resistance to the test organisms

Table 3: The minimum inhibitory concentrations of ethyl acetate soluble fraction of aqueous extract of *P. guajava* leaf on some bacteria

Organisms	Concentration of extract (mg mL^{-1})					
	100	50	25	12.5	6.25	3.13
Staphylococcus aureus	-	-	-	-	+	+
Streptococcus fecalis	-	-	-	-	+	+
Escherichia coli	-	-	-	-	-	+
Salmonella typhi	-	-	-	-	+	+
Klebsiella pneumoniae	-	-	+	+	+	+

+ = Indicates bacterial growth; - = Indicates no bacterial growth

Inhibition of bacterial growth with the chloroform soluble fraction was observed only on *Klebsiella pneumoniae*, whereas the ethyl acetate fraction showed significant inhibitory effects on the growth of the entire gram-positive and gram-negative bacteria used in this study. The inhibition of the organisms by the ethyl acetate fraction appears to be concentration and organism dependent. The *E. coli* was inhibited more by this fraction compared to the other organisms used in this study. The n-butanol fraction inhibited the growth of *Salmonella typhi* and *Klebsiella pneumoniae* both of which are gram negative organisms. No effect on the growth of *Staphylococcus aureus*, *Streptococcus fecalis* and *E. coli* was observed from the n-butanol fraction. The residual extract has no effect on any of the organisms used in this study. Oxytetracycline, the standard antibacterial agent used inhibited the growth of all the organisms used in the study. The zones of inhibition produced by the oxytetracycline on the organisms were far greater than those produced by the different concentrations of *Psidium guajava* leaf organic solvent fractions except that of *Klebsiella pneumoniae* organism where the ethyl acetate fraction had more inhibitory effect (Table 2).

The MIC of ethyl acetate soluble fraction of *Psidium guajava* aqueous leaf extract for the different micro organisms tested is presented in Table 3. *E. coli* was found to be most sensitive to the fraction since growth was inhibited at a relatively low concentration. This is followed by *Staphylococcus aureus*, *Streptococcus fecalis* and *Salmonella typhi*. *Klebsiella pneumoniae* was found to be least sensitive to the inhibitory effect of ethyl acetate fraction. There was no growth of the bacteria tested following sub-culture of contents of the tubes above the MIC.

DISCUSSION

The present study further strengthens earlier reports by Guan and Damello (1999) and Geidam *et al.* (2007) that *Psidium guajava* leaf has antibacterial activities. This may also be in agreement with the claim of herbal healers. They claim to use the plant to treat diarrhea, dysentery and wounds (Iwu, 1993). The organisms tested have been implicated in diarrhea and/or dysentery (*Salmonella* sp., *E. coli* and *Klebsiella* sp.) and wound infection (staphylococcus and streptococcus). The ethyl acetate soluble fraction showed broad spectrum of activity against all the gram positive and gram negative bacteria used in the study. This indicates that the ethyl acetate fraction has better antibacterial properties as compared to the narrow spectrum of activity of the crude extract as reported by Gnan and Demello (1999) and Geidam *et al.* (2007). This is in agreement with the findings of McLaughlin (1991) who reported that fractionation ensures better biological activity. *E. coli* was found to be most susceptible to ethyl acetate fraction of the extract while *Staphylococcus aureus* was the least susceptible. The activity of this fraction against *E. coli* is interesting since *E. coli* strains have developed resistance to antimicrobial drugs commonly used in poultry production (Yang *et al.*, 2004) and even to frontline antimicrobials, such as the fluoroquinolones (Angulo *et al.*, 2000; Livermore *et al.*, 2002).

The antibacterial properties of ethyl acetate fraction could be due to flavanoids and tannins contents of the

fraction, both of which are known to possess appreciable antimicrobial activities (Narayana *et al*., 2001). The crude extract has been reported to contain carbohydrate, saponin, steroid and cardiac glycosides (Geidam *et al*., 2007). Carbohydrates could facilitate the growth of bacterial organisms and therefore may antagonize the antibacterial activity of the active principles. This may account for the narrow antibacterial activity of the crude extract and other fractions of the leaf extract of *Psidium guajava* that contain carbohydrates.

In conclusion, this study has shown that the ethyl acetate soluble fraction of *Psidium guajava* aqueous leaf extract possesses broad spectrum antibacterial properties. This fraction also showed a good activity against *E. coli* at a relatively lower concentration and thus could possibly be use against *E. coli* infections. However, further studies need to be carried out.

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