International Journal of Bioassays
ISSN: 2278-778X
CODEN: IJBNHY
OPEN ACCESS

Original Research Article

IN VITRO ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT OF *PITTOSPORUM VIRIDIFLORUM* LEAVES EXTRACT AGAINST LABORATORY STRAINS OF SELECTED MICROORGANISMS

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Received for publication: August 08, 2014; **Revised:** August 15, 2014; **Accepted:** September 13, 2014

Abstract: The aim of this study was to analyse the antibacterial Activity of *Pittosporum viridiflorum* leaves extract against laboratory strains of selected microorganisms. Infusions of the bark of *P. viridiflorum* are used to treat stomach complaints, chest pain, malaria and other fever. The mean zones of inhibition of the extract against microorganisms were 12.67±0.882 mm for *Enterobacter aerogenes,* 12.50±0.281mm for *Escherichia coli,* 11.67±0.333 mm for *Proteus vulgaris,* 11.67±0.000mm for *Bacillus cereus* and 7.67±0.333mm for *Salmonella typhi.* The penicillin positive control showed large zones of inhibition and the dimethylsulfoxide negative control did not show any zone of inhibition. This report suggests that the extract was active against all the other organisms except *S. typhi* because the zone of inhibitions was less than 8 mm. Analysis of variance showed that the zones of inhibition of the extract and antibiotic control against the microorganisms were significantly different ($p<0.0001$). The Tukey's honestly test further showed both significant and non-significant comparisons between the extract and controls for various bacterial organisms. This study has shown that controlling the growth of microorganisms *in vitro* can be achieved by the ethanolic extract of *P. viridiflorum.*

Key Words: Pittosporum viridiflorum, Leaves, Antibacterial, Ethanol Extract.

INTRODUCTION

In continuation of our interest in biological activities of natural medicinal plants extracts (Anthoney *et al.,* 2013; Anthoney *et al.,* 2014 and Obey *et al.,* 2014), we report here the antibacterial activities of ethanolic extract of *Pittosporum viridiflorum* leaves used in the treatment of infectious and intestinal diseases.

Plants have been a source of medicine and a major resource for health care since ancient times, with some traditional herbal medicines having been in use for more than 2,000 years. Currently, the modern pharmaceutical industry is showing more interests to plants as scientists re-discover that plant life is an almost infinite resource for medicine development. One fourth of the modern medicines that are available on prescription today owe their origins of raw material to higher plants of tropical forests (Samy, 2005 and Graeme, 2007). Out of these, 74% are derived from plants that have some related use in traditional herbal medicine.

Natural product medicines come from various source materials including terrestrial plants, marine organisms terrestrial vertebrates and terrestrial microorganisms (Raja, 2010). The traditional medical practitioners provide the useful indigenous knowledge route employed in the search for novel drugs (Amsuan, 2007).

Medical practice has taught us to understand that ethno pharmacological data is an important source of new drugs. About 140 new drugs have originated directly or indirectly from Chinese medicinal plants by means of modern scientific methods, confirming that these plants are an important resource. Increasing emphasis on the use of medicinal plants in searching for new drugs is undoubtedly a correct strategy (Chang, 2005).

The charming *Pittosporum viridiflorum* (Cheesewood) is a really useful evergreen tree with an attractive dense, straight or rounded crown and lovely glossy deep green foliage. The Cheesewood varies in size and shape depending on where it is planted and can be maintained as a small tree of about 4 m or left to grow to its full height. These delightful trees are irresistible to insectivorous birds when the sweetly fragrant flowers appear, along with a host of insects, while a wide variety of seed eating birds such as the red eyed dove, flock to the tree when the startlingly bright red seeds appear. *P. viridiflorum* is a truly excellent all-rounder and is becoming increasingly popular as a garden and street tree throughout the country. (Treeco, 2012).

The Cheesewood has a number of medicinal properties and the bark as well as the roots has traditionally been used for a variety of ailments. Infusions of the bark are used to treat stomach

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complaints and fever, easing pain and having a generally calming effect. The powdered root is believed to have an aphrodisiac effect and is sometimes added to beer. The wood is pale and soft but is sometimes used for kitchen utensils (Treeco, 2012).

Pittosporum viridiflorum used as an emetic. The bark is also boiled in water, the decoction added to soup and then stirred, and finally drunk as remedy for chest complaints, malaria and other fevers. The liquid is bitter and induces violent vomiting (Kokwaro, 2008).

The genus *Pittosporum* comprises about 200 species that are distributed throughout the world and three of the species are described for Portugal: *P. coriaceum*, *P. undulatum* and *P. tobira (*Nicolau *et al.,* 2007). *P. coriaceum* is a Madeiran endemic tree with creamy fragrant flowers (Press, 1994). *P. tobira* was the first *Pittosporum* species noticed by Europeans (Cayzer, 2000). It is a tall shrub, like *P. coriaceum*, a native of China and Japan and is grown in gardens for ornamental purposes. *P. undulatum* is abundant in several hill forest areas in the Portuguese mainland and it is one of the most frequent species in the wet hill forest near Lisbon. The name *Pittosporum* is derived from two Greek words meaning 'pitch' and 'seed', referring to the stickiness of the seeds (Nicolau *et al.,* 2007).

Pittosporum is used as a source of wood for firewood, for charcoal, for engraving and, due to the large amount of nectar; it is recorded as good for honey bees. As a medicinal plant, only one record was found for Portugal: on Madeira, the whole plant crushed and applied in poultices is said to repair muscles, tendons and ligaments strained or torn by violent movement (Rivera, 1995 and Nicolau *et al.,* 2007).

The present study was carried out to evaluate the antibacterial activity of ethanolic extract of *Pittosporum viridiflorum* leaves against selected pathogenic organisms.

MATERIAL AND METHODS

Sample collection and Extraction procedure

The leaves of the *Pittosporum viridiflorum* were collected around Baraton University campus. The samples were identified by a taxonomist in the University of Eastern Africa, Baraton. The fresh leaves of the *Pittosporum viridiflorum* leaves were air – dried for three weeks; the dried leaves were ground into powder. Forty grams (40 g) of the powdered leaves were mixed with 400 ml of ethanol – water (70:30). The mixture was kept for 24 hours on a shaker for effective extraction of the plant components. The extract was filtered and the solvent was evaporated to dryness at a temperature of 40°C using rotary vacuum evaporator. The extract was brought to dryness using vacuum and pressure pump. The yield was kept at 4°C prior to use.

Bioassay Study

Preparation of the Bacterial Suspension: The turbidity of each of the bacterial suspension was prepared to match to a 0.5 McFarland standard. The McFarland standard was prepared by dissolving 0.5 g of $BaCl_2$ in 50 ml of water to obtain a 1% solution of Barium chloride (w/v). Sulphuric acid (1%) was prepared in a 100-ml volumetric flask. To prepare the 0.5 McFarland Standard, 0.5 ml of the 1% $BaCl_2$ solution was mixed with 99.5 ml of H_2SO_4 solution. Measure the turbidity of the 0.5 McFarland Standards with the aid of a spectrophotometer at a wavelength of 625nm to read an optical density of between 0.08-1.0. At this absorbance, the McFarland Standard represents a bacterial cell density of approximately 1.5×10^8 CFU/ml ($1.0 \times 10^8 - 2.0 \times 10^8$ CFU/ml. It was then transferred to a screw-capped bottle and sealed with parafilm to prevent evaporation due to exposure to air. The bacterial suspensions were then tested against the McFarland standards until they reached the absorbance of the McFarland standard and then they were ready for use.

Preparation of the Extract Concentrations and Antibiotic: Stock solutions for the extract were prepared by dissolving 500 mg in 1 ml of dimethylsulfoxide (DMSO). An antibiotic control was made by dissolving 1µg of penicillin in 1 ml of sterile distilled water. DMSO served as a negative control.

Screening for the antibacterial potential of the plant extract: The agar well diffusion procedure used in the experiment was similar to that used by Taye *et al.*., (2011) and Jeyachandran and Mahesh (2007). The microorganisms used for this study were laboratory strains of *Proteus vulgaris, Escherichia coli, Salmonella typhi, Bacillus cereus* and *Enterobacter aerogenes*. A single colony for each of the organisms was picked from agar plate and dissolved in 5 ml of Mueller Hinton broth. The broth was incubated overnight at 37°C. Five (5) ml of plain Mueller Hinton broth was incubated alongside the organisms to ensure that the medium was not contaminated. The spectrophotometer was set to 625 nm wavelength and each of the microbial cultures was pipetted into cuvettes to measure the absorbance. A cuvette of plain Mueller Hinton broth was used a blank at 0.000 absorbance. The absorbance of the microorganisms were measured. The bacterial organisms exceeding 0.1absorbance were adjusted by adding bacterial suspension until the absorbance fell between 0.08-0.10, matching the McFarland Standard.

The organisms falling below 0.08 absorbance were also adjusted until the McFarland standard absorbance was achieved. All the organisms, therefore, reached a cell density of 1 x 10^8 cfu/ml (Ngeny *et al.,* 2013). One hundred (100) µl of each of the organisms were then inoculated onto agar plates for the bioassay (Agyare *et. al.,* 2013). Three 6 mm wells were made into each agar plate using a sterile metal cork borer. 100 µl of the standard drug penicillin was placed in one well, the extract in another well and dimethyl sulfoxide (DMSO) was placed in the third well on each plate. The experiment was run in triplicate for each extract and each organism tested. The plates were incubated for 24 to 48 hours and the zones of inhibition were measured in millimetres with the aid of a meter rule.

Statistical Analysis

A random sampling procedure was done for the entire test and the experiment was conducted in triplicate assays on Mueller Hinton agar plates (Jeyachandran and Mahesh, 2007). The mean values and standard error were calculated for the zones of inhibition. Analysis of variance was used to determine if there was significant difference among the average zones of inhibition of the bacterial organisms by the extract and controls. The Tukey's honestly significant difference test was used to determine pairwise comparisons between average zones of inhibition among the bacterial organisms by SPSS version 21.0.

RESULTS AND DISCUSSION

Table 1: Zone of inhibition (mm ± S.E.) of ethanolic extract of *Pittosporum viridiflorum* leaves against selected bacterial organisms.

Microorganism	Zone of Inhibition (mm±S.E.)	Penicillin Control	DMSO control
Proteus vulgaris	11.67±0.333	39.33±0.667	0.00±0.000
Escherichia coli	12.50±0.281	38.67±0.333	0.00±0.000
Salmonella typhi	7.67±0.333	25.33±1.202	0.00±0.000
Bacillus cereus	11.67±0.000	40.33±0.333	0.00±0.000
Enterobacter aerogenes	12.67±0.882	39.00±0.577	0.00±0.000

Key: S.E. = standard error; DMSO = dimethylsulfoxide

The main zone of inhibition for *Enterobacter aerogenes* was the highest (12.67±0.882), followed by that of *Escherichia coli, Proteus vulgaris, Bacillus cereus* and *Salmonella typhi* (table 1). The penicillin control inhibited growth at higher zones of inhibition than all the microorganisms. The DMSO negative control showed no zone of inhibition. Analysis of variance (ANOVA) showed that there was significant difference in the zones of inhibition among the microorganisms by the extract and the controls ($P < 0.001$). The extract is considered inactive against *S. typhi* because the measured zone of inhibition is less than 8mm (7.67±0.333).

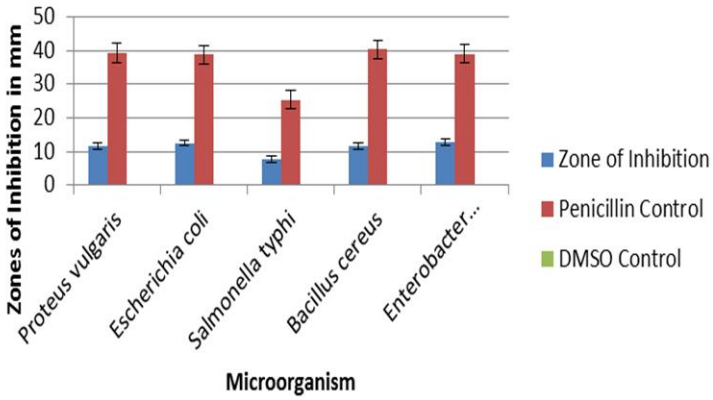

Figure 1: Antimicrobial activity (zone of inhibition ± S.E.) of *P. viridiflorum* extract against selected microorganisms

Tukey's multiple comparison test showed that the zone of inhibition that did not differ significantly were those of *P. vulgaris* and *E. coli, P. vulgaris* and *B. cereus, P. vulgaris* and *E. aerogenes, E. coli* and *B. cereus, E. coli* and *E. aerogenes* and *B. cereus* and *E. aerogenes.* The microorganism *Salmonella typhi* zone was significantly lowest than the other microorganisms. The control drug showed zones significantly bigger than the extract for all microorganisms (Table 2).

Table 2: Tukey's honestly significant difference test for the zone of inhibition of ethanolic extract of *Pittosporum viridiflorum* leaves against selected bacteria organisms.

Comparison	P-Value	Significance
P. vulgaris vs E. coli	0.973	NS
P. vulgaris vs S. typhi	0.001	S
P. vulgaris vs B. cereus	1.000	NS
P. vulgaris vs E. Aerogenes	0.921	NS
P. vulgaris vs P. vulgaris control	0.000	S
E. coli vs S. typhi	0.000	S
E. coli vs B. cereus	0.973	NS
E. coli vs E. Aerogenes	1.000	NS
E. coli vs E. coli control	0.000	S
S. typhi vs. B. cereus	0.001	S
S. typhi vs E. Aerogenes	0.000	S
S. typhi vs S. typhi control	0.000	S
B. cereus vs E. Aerogenes	0.921	NS
B. cereus vs B. cereus control	0.000	S
E. aerogenes vs E. aerogenes control	0.000	S

S = significant; NS = not significant

Essential oils of leaf and fruit of *Pittosporum viridiflorum var. viridiflorum* growing in Madagascar were analyzed by GC and GC/MS. The leaf oil, which was sesquiterpene-rich, contained delta-cadinene (10.6%) and alpha-cadinol (18.3%) as major components. The fruit oil contained aliphatic as well as mono- and sesquiterpenoid compounds with sabinene (13.2%), decanal (10.3%) and beta-elemene (9.5%) as major components (Ramanandraibe *et al.,*)

The Cheesewood has a number of medicinal properties and the bark as well as the roots have traditionally been used for a variety of ailments. Infusions of the bark are used to treat stomach complaints and fever, easing pain and having a generally calming effect. The powdered root is believed to have an aphrodisiac effect and is sometimes added to beer. The wood is pale and soft but is sometimes used for kitchen utensils.

According to Ramanandraibe (2000), the antimicrobial activity of essential oil of *Pittosporum viridiflorum* leaves was done using human isolated strains of *Staphylococcus aureus, Staphylococcus epidermis Streptococcus faecali, Pseudomonas aeruginosa* and *Escherichia coli* as test organisms. The essential oils of *Pittosporum viridiflorum* leaves inhibited the growth of *Staphylococcus epidermis* with a zone of inhibition of 18.3 mm, *Staphylococcus epidermis* with a zone of 13.3 mm, *Streptococcus faecalis* with a zone *of* 13.6 mm, *Pseudomonas aeruginosa* with a zone of 11.0 mm and *Escherichia coli* with a zone of 13.0 mm.

According to Wilfred (2012), there were no significant differences in the flavonoid and proanthocyanidins contents between the leaves and bark extracts of *Gasteria bicolor* and *Pittosporum viridiflorum* respectively, while the total phenolic content of the bark extract of *P. viridiflorum* was significantly higher than that of *G. bicolor* leaf. The acetone extracts of both plants indicated strong antioxidant activities.

The leaves and stem extracts of *Gasteria bicolor* and *Pittosporum viridiflorum* respectively possess antioxidant properties and could serve as free radical inhibitors, acting possibly as primary antioxidants. Since reactive oxygen species are thought to be associated with the pathogenesis of AIDS, and HIV-infected individuals often have impaired antioxidant defenses, the inhibitory effect of the extracts on free radicals may partially justify the traditional use of these plants in the management of OFIs in HIV patients in South Africa. (Wilfred, 2012).

According to Patricia (2012), *Salmonellosis* causes more disease burden than any other foodborne pathogen. An estimated 93.8 million cases of gastroenteritis caused by Salmonella species occur globally each year and of these, nearly 80.3 million cases are foodborne (Majowicz *et al.,* 2010). In the United States, an estimated 1 million incident cases of human salmonellosis occur annually (Scallan *et al.,* 2011); however, only a small portion of these cases are recognized clinically. In industrialized countries as few as 1% of clinical cases are actually reported (Heymann, 2008). Collectively, Salmonella infections in the United States account for roughly 19,336 hospitalizations, 17,000 quality adjusted life years lost (QALYs), and $3.3 billion in total medical expenditures and lost productivity each year (Batz *et al.,* 2011).

Salmonella gastroenteritis is usually a self-limited disease in which the symptom of fever typically resolves within 48 to 72 hours and diarrhoea within three to seven days. Complications from the infection may include severe dehydration, shock, collapse, and/or septicemia. Symptoms are usually more severe among infants, young children, elderly, and those who are immune-compromised (Scallan *et al.,* 2011). Although there are many serotypes of Salmonella that are pathogenic to both humans and animals (i.e., approximately 2,500 serotypes have been identified), the vast majority of human Salmonella isolates are serotype S. enterica subsp. enterica (Heymann, 2008). Serovars Typhi and Paratyphi of this serotype, S. enterica subsp. enterica, are the etiologic agents that cause typhoid and paratyphoid fevers. These types are also common, but are generally found in developing countries, such as those in South America, Africa, and parts of Asia (Heymann, 2008). In developed countries where there is active, coordinated foodborne disease surveillance, other serovars such as Typhimurium and Enteritidis are frequently reported.

Enterohaemorrhagic E. coli (EHEC) causes bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhea and haemolytic uremic syndrome (HUS). The principal reservoir of EHEC is the bovine intestinal tract and initial outbreaks were associated with consumption of undercooked hamburgers. Subsequently, a wide variety of food items have been associated with disease, including sausages, unpasteurized milk, lettuce, cantaloupe melon, apple juice and radish sprouts (James *et al.,* 2004).

Enterotoxigenic E. coli (ETEC) causes watery diarrhea, which can range from mild, self-limiting disease to severe purging disease. The organism is an important cause of childhood diarrhoea in the developing world and is the main cause of diarrhoea in travellers to developing countries (Nataro, 1998).

Enteroaggregative E. coli (EAEC), are increasingly recognized as a cause of often persistent diarrhea in children and adults in both developing and developed countries, and have been identified as the cause of several outbreaks worldwide (James *et al.,* 2004).

The evolution of pathogenic *E. coli* that has resulted in formation of distinct pathotypes capable of colonizing the gastrointestinal tract, urinary tract or meninges illustrates how key genetic elements can

adapt a strain to distinct host environments. Using *E. coli* K-12 as a base-model', several features can be added (PAIs, plasmids, transposons or phage) or subtracted (black holes or pseudogenes) to modify the base model to adapt to specific environments and to enable these modified strains to cause disease in an immuno competent human or animal host (James *et al.,* 2004).

Proteus vulgaris meningitis is relatively uncommon. It may occur by direct extension from an adjacent otitis media or mastoiditis and occasionally as a complication of septicemia from a focus of infection, usually in the genito-urinary tract. The causative organism is a gram-negative, aerobic, non-sporulating, actively motile bacterium, usually occurring as a saprophytic non-pathogen in the upper respiratory, gastrointestinal, or genito-urinary tracts. Occasionally it produces severe infection and death (Leon, 1950).

Proteus species are the major cause of diseases acquired outside the hospital, where many of these diseases eventually require hospitalization (De Champs *et al.,* 2000). *P. mirabilis* causes 90% of Proteus infections. *Proteus* species, particularly *P. Mirabilis*, is believed to be the most common cause of infection-related kidney stone, one of the most serious complications of unresolved or recurrent bacteruria (Coker *et al.,* 2000). *P. mirabilis* has been implicated in meningitis, empyema, osteomyelitis and gastroenteritis. Also, it frequently causes nosocomial infections of the urinary tract (46%), surgical wounds (24%) and lower respiratory tract (30%). Less frequently, proteus species cause bacteraemia (17%), most often in elderly patients (Mansy, 2001).

B. cereus in association with food poisoning and eye infection, recognition and appreciation for the multitude of other serious infections such as fulminant sepsis and devastating central nervous system infections are lacking. The suspicion of the association of *B. cereus* with these mounting infectious complications moves with a fatal lethargy in its recognition as a bona fide human pathogen. Clinicians and clinical microbiologists must both give serious consideration to the significance of a *B. cereus* isolate from a clinical specimen, especially if the patient is immunosuppressed (Edward, 2010).

Enterobacter aerogenes is a Gram – negative, catalase positive, indole negative, rod shaped bacterium (Sanders, 1997). *E. aerogenes* is a nosocomial and pathogenic bacterium that causes opportunistic infections including most types of infections. The majority are sensitive to most antibiotics designed for this bacteria class, but this is complicated by their inducible resistance mechanisms, particularly lactamase which means that they quickly become resistant to standard antibiotics during treatment, requiring change in antibiotic to avoid worsening of the sepsis.

According to Gabriel, some of the infections caused by E. *aerogenes* result from specific antibiotic treatments, venous catheter insertions, and/or surgical procedures. *E. aerogenes* is generally found in the human gastrointestinal tract and does not generally cause disease in healthy individuals. It has been found to live in various wastes, hygienic chemicals, and soil.

Enterobacter aerogenes causes disease in humans through inadvertent bacteria transfer in hospital settings. A selection of enteric bacteria like *E. aerogenes* are opportunistic and only infect those who already have suppressed host immunity defences. Infants, the elderly, and those who are in the terminal stages of other disease or are immunosuppressed are prime candidates for such infections (Janda, 2006).

The genus Enterobacter is more specifically a nosocomial opportunistic pathogen and is sought out to be one of the many key causes for extra intestinal infections next to *E. coli*. Infections commonly attributed to *E. aerogenes* are respiratory, gastrointestinal, and urinary tract infections, specifically cystits, in addition to wound, bloodstream, and central nervous system infections (Brooks, 2007; Lederberg, 2000 and Sankaran, 2000). Furthermore, E. cloacea and *E. aerogenes* are the species most commonly associated with adult cases of meningitis. Colonies of Enterobacter strains may be slightly mucoid.

In the clinical setting, *Enterobacter aerogenes* and *Enterobacter cloacae* are the most frequently isolated in samples of infected hospitalized patients. The majority of the infections are etiologically due to inadvertent transfer of bacteria during surgery or prolonged treatment in hospitals in patients who use venous or urethral catheters. Enterobacteriaceae may account for 80% of clinically significant isolates of gram-negative bacilli and for 50% of clinically significant bacteria in clinical microbiology laboratories. Additionally, they account for nearly 50% of septicaemia cases and more than 70% of urinary and intestinal tract infections. The severity of these infections thus create an importance to target, isolate, identify and test for susceptibility for the causes of these nosocomial infections (Sankaran, 2000).

From the data obtained in this study it is therefore worthy to mention that the plant can be used to treat against all the infections caused by

Proteus vulgaris, Escherichia coli, Salmonella typhi, Bacillus cereus and *Enterobacter aerogenes*. These results are pertinent in addressing problems like drug resistance in treating diseases caused by the selected microorganisms using conventional antibiotics.

CONCLUSION

Like other species of *Pittosporum*, *P. viridiflorum* has shown antimicrobial activity against four of the five organisms tested. These results are pertinent in addressing problems like drug resistance in treating diseases caused by the selected microorganisms using conventional antibiotics. Further analysis of the extract needs to be carried out using spectroscopic techniques for structural elucidation of the active ingredients found in the extract. The extract can also be tested against other bacterial and fungal organisms to determine if better results can be obtained for other organisms. It is recommended that purer forms of the extract be produced for testing against the same organisms used in this study.

ACKNOWLEDGEMENTS

The authors of this paper are very much thankful to the Department of Chemistry and Medical Laboratory Science, University of Eastern Africa, Baraton. Authors also thankful to Mr. Joel Ochieng Ondiek, University of Eastern Africa, Baraton.

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Source of support: Nil
Conflict of interest: None Declared