



Antibacterial and Hemagglutinating Activity of the Fruit Pulp of *Trilepisium madagascariense* DC

Oludele O. Odekanyin^{1*}, Temitope T. Aibinu¹ and Olutayo Abioye²

¹Department of Biochemistry and Molecular Biology, Obafemi Awolowo University, Ile-Ife, Nigeria.

²Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria.

Authors' contributions

The entire study was carried out in collaboration between all authors. Author OOO designed the study and wrote the protocol. Author TTA carried out the experiments. Author OA supervised the antibacterial study. Author OOO wrote the manuscript and all the authors read and approved the final manuscript.

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ABSTRACT

Aim: This study aimed at exploring the hemagglutinating and antibacterial activities of crude lectin from the fruit pulp of *Trilepisium madagascariense* DC.

Methods: Crude lectin was obtained by maceration of the fruit and ammonium sulphate precipitation. Hemagglutinating activity assay was carried out by determining the ability of the crude extract to agglutinate red blood cells, serial dilution of the crude lectin was done followed by addition of erythrocytes. The crude lectin was subjected to evaluation for inhibition of bacterial growth by the agar well diffusion method against fourteen human pathogenic gram-positive and gram-negative bacteria.

Results: The extract agglutinated trypsinized and glutaraldehyde-fixed rabbit and human erythrocytes with higher specificity for rabbit erythrocytes and better agglutination with trypsinized erythrocytes. Among the various sugar tested, the hemagglutination was best inhibited by galactose. The crude lectin demonstrated mild spectrum antibacterial activity against four pathogenic gram-

*Corresponding author: E-mail: dekanyin@gmail.com, dekanyin@oauife.edu.ng;

positive and gram-negative bacteria. The minimum inhibitory concentration ranged from 1.56 mg/ml to 6.25 mg/ml for *Streptococcus faecalis*, *Bacillus anthracis*, *Staphylococcus aureus* and *Pseudomonas fluorescens*.

Conclusion: The study showed that there is presence of hemagglutinins in the extract and the extract possess antibacterial potential against some pathogenic bacteria tested and can be developed as alternative antibacterial drug that could be employed for the treatment of infectious diseases caused by these pathogens.

Keywords: Antibacterial; lectin; pathogen; *Trilepisium madagascariense*; hemagglutination; infectious disease.

1. INTRODUCTION

Globally, the infectious diseases that cause mortality and morbidity are on the increase due to the emergence of multidrug resistant bacterial strains which is caused by incomplete course of antibiotic dose, overuse of antibiotic and horizontal transfer of resistant gene among bacterial species [1-3]. The indiscriminate uses of unbranded antibiotics drugs in developing countries are due to the high cost of conventional drugs that have been reported to have toxic side effects.

In recent years, the traditional usage of natural compounds of plant origin has been receiving a lot of attention as an alternative source of remedy for the treatment of infectious disease coupled with the belief of their better safety nature, affordability and of less or non-toxicity [4]. Over the years, plants have served as the starting material for the development of a novel drug compounds, as plant-derived medicines have made large contribution to human health and well being [5]. Majority of the plant-based drugs discovered till date have come from whole plant, root, stem, stem-bark, leaves and few of them from seeds. Yet a large number of phytochemicals with great medicinal values are present in seeds, seed coat or fruit pulp of some plants. These chemical includes alkaloids, saponin, lectins, and phenolic compounds among others.

Lectins are known to be proteins or glycoproteins binding to specific sugar moieties on the cell surfaces [6]. They are considered strong candidates for therapeutic use, for they are macromolecules with noticeable resistance to unfavourable conditions like pH and temperature variations and isotonicity, with no significant alterations to their biological function [7]. Extensive studies discovered that some plant hemagglutinins have useful application in biotechnology [8] and can also be used for

prevention and/or treatment of some pathological disorders such as cancer [9], malarial [10] among others. Another application reported in the literature involving lectins is their antimicrobial activity where lectins may act against microorganisms by interfering with their growth and playing a role in defense systems [11,12]

In antibacterial defense, lectins are correlated with pore-forming activity, which causes bacterial membrane permeabilization [13]. Also, lectins serve significant role in the host's defense against pathogens by activating antibacterial autophagy [14] and vacuole lysis [15]. Lectins with antibacterial activity have been reported by many researchers [10, 16-21]. Their ability to reversibly bind to a specific saccharide has attracted the attention of scientists. Each lectin has a characteristic sugar-binding specificity profile indicating that they are able to recognize different saccharides [22]. The capacity for identifying and binding glycoconjugates from the microorganism's surface is exclusive to lectins. Consequently, they are capable of inhibiting the motility and multiplication of microorganisms [12]. The interaction between carbohydrates and lectins act in many biological processes and in a wide range of activities, including bacterial and fungal growth inhibition [23].

Trilepisium madagascariense DC. is a tropical rain forest tree and a member of the botanical family, *Moraceae*, formally refer to *Bosqueia angolensis*. Yoruba-speaking people of Western Nigeria called this plant "koko eran" and the Igbo-speaking people of Eastern Nigeria, where the roasted seeds served as indigenous snacking nuts called the plant "Oze". The stem bark is used traditionally to treat venereal diseases, arthritis, rheumatism and stomach troubles (diarrhoea and dysentery) and also as a pain-killer while the roots are used against cutaneous and subcutaneous parasitic infections [24]. The methanol extract from the leaves of *T. madagascariense* has been reported to inhibit

the growth of *Staphylococcus aureus* [25]. Nwosu [26] reported the presence of eleven antinutrients with alkaloids and total phenols predominating in the *B. angolensis* seeds subjected to different treatment. In 2013, Ampa et al. [27] evaluated the antidiabetic potential of the plant leaves. Prior to this, the stem bark was reported to have antidiarrheal, antimicrobial and antioxidant activities [28,29]. The nutrient, antinutrient and phytochemical compositions of roasted *B. angolensis* seeds were evaluated by Nwamarah [30]. There is, however, little or no information regarding the pharmacological activity of the fruit pulp of this plant.

There is no report of scientific and systematic investigation with regard to the pharmacological potential of the pulp of the *T. madagascariense* fruit. The current study, therefore, sought to establish the ability of the protein isolates to agglutinate red blood cells and to precipitate carbohydrate and to investigate in detail the antibacterial potential of the hemagglutinins against gram-negative and gram-positive bacteria.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

Matured fruits of *T. madagascariense* were collected from the Botanical Garden of Obafemi Awolowo University, Ile-Ife, between May and June. The fruits were identified at Ife Herbarium, Botany Department, Obafemi Awolowo University, Ile-Ife, Nigeria.

2.2 Preparation of Crude Lectin of the Fruit Pulp

Matured fruit were gently rinsed with distilled water, drained and fully ripe fruit were picked. The picked fruits were macerated in Phosphate Buffered Saline (PBS, pH 7.2) and left undisturbed for 6 hours. The resulting slurry was sieved with cheese cloth to separate the seeds. The filtrate obtained was subjected to centrifugation at 6,000 rpm for 20 minutes to remove particulate and other cellular debris. The proteins in supernatant were precipitated with 70% ammonium sulphate saturation. The mixture was stirred gently to completely dissolve the ammonium sulphate and was later left for 24

hours. The precipitate was collected by centrifugation at 6,000 rpm for 10 minutes and extensively and exhaustively dialyzed against PBS and later with double distilled water to remove all the traces of ammonium salt. The dialysate was lyophilized into powder. The lyophilized powder was termed crude lectin extract and used for the various bioassays.

2.3 Estimation of Protein Concentration

The total soluble protein content was estimated by Lowry method [31] using bovine serum albumin as a standard.

2.4 Hemagglutinating Activity Assay

The crude lectin of the fruit pulp of *T. madagascariense* was assayed for the presence of lectin by hemagglutinating activity assay performed in 96-well U-shaped microtitre plates using glutaraldehyde-fixed erythrocytes. PBS (100 μ l) was delivered sequentially into wells arranged in rows (each row contained 12 wells). The extract (100 μ l) was added into the first well and a serial dilution was done by transferring 100 μ l of the diluted sample in a particular well into the next well containing 100 μ l PBS. Aliquots (50 μ l) of the 4% glutaraldehyde-fixed erythrocyte suspension were added to each well and the microtitre plates were left undisturbed for 1 hour. The titre value was taken as the reciprocal of the highest dilution of the extract causing visible hemagglutination.

The sugar specificity of the lectin was also investigated by determining the sugars that completely inhibited the agglutination of erythrocyte by the lectin [32]. Lectin (extract) was diluted serially until the end-point dilution causing hemagglutination was obtained. The sugar solution (0.2 M) was added to each well at 50 μ l per well while the control well contained PBS instead of sugar solution. Erythrocyte suspension (50 μ l), was added to each well, and the titre of lectin activity was determined as described above. Inhibitory sugars caused a reduction in the titre of the lectin activity shown by the PBS-control experiment. The sugars tested include: D(+)-glucose, D(+)-mannose, D(+)-arabinose, D(+)-glucosamine hydrochloride, D(-)-sorbitol, mannitol, maltose, sucrose, fructose, lactose, 1-O-methyl- α -glucopyranoside, galactose, N-acetylmannosamine, α -methyl mannoside and N-acetylglucosamine.

2.5 Antibacterial Activity

2.5.1 Test microorganisms

The test microorganisms used in this study comprises bacterial isolates obtained from National Collection for Industrial Bacteria (NCIB) (*Staphylococcus aureus* (NCIB 8588), *Pseudomonas fluorescens* (NCIB 3756), *Escherichia coli* (NCIB 86), *Klebsiella pneumonia* (NCIB 418), *Bacillus cereus* (NCIB 6349), *B. subtilis* (NCIB 8222), *Proteus vulgaris* (NCIB 950), *B. stearothermophilus* (NCIB 8224) and *Clostridium sporogenes* (NCIB 532) and locally isolated organisms (*Micrococcus luteus*, *Streptococcus faecalis*, *Corynebacterium pyogenes*, *B. polymyxa* and *B. anthracis*) obtained from the culture collection of Prof. D. A. Akinpelu, Microbiology Department, Obafemi Awolowo University, Ile-Ife. The bacterial isolates were first subcultured in a nutrient broth (Oxoid Ltd) and incubated at 37°C for 18 hours.

2.5.2 Agar-well diffusion assay

The antibacterial activity of the crude lectin was determined in accordance with the agar-well diffusion method described by Igbinosa et al [5]. The bacterial isolates were first grown in a nutrient broth for 18 hours before use and standardized to 0.5 McFarland standards (10^6 cfu ml⁻¹). 200 µl of the standardized cell suspensions were spread on a Mueller-Hinton agar (Oxoid Ltd). Wells were then bored into the agar using a sterile 6 mm diameter cork borer. Approximately 100 µl of the crude lectin at 12.5 and 25 mg ml⁻¹ were introduced into the wells, allowed to stand at room temperature for about 2 hours and then incubated at 37°C. Controls were set up in parallel using the sterilized double distilled water that was used to reconstitute the extract. The plates were observed for zones of inhibition after 24 hours. The effects were compared with that of streptomycin at a concentration of 1 mg/ml. The experiment was carried out in triplicates.

2.5.3 Minimum Inhibitory Concentration (MIC)

The estimation of MIC of the crude lectin was carried out using the method of Akinpelu and Kolawole [33]. A two-fold dilution of the crude lectin was prepared and 2 ml aliquots of different concentrations of the solution were added to 18 ml of pre-sterilized molten nutrient agar at 40°C. The medium was then poured into sterile Petri dishes and allowed to set. The surface of the medium was allowed to dry under laminar flow

before streaking with 18 hours old bacterial cultures. The plates were later incubated at 37°C for 24 hours, after which they were examined for the presence or absence of growth. The MIC was taken as the lowest concentration that prevented the growth of the test microorganism.

2.5.4 Minimum Bactericidal Concentration (MBC)

The MBC of the plant extracts was determined by a modification of the method of Igbinosa et al [5]. Samples were taken from plates with no visible growth in the MIC assay and subcultured on freshly prepared nutrient agar plates and later incubated at 37°C for 48 hours. The MBC was taken as the concentration of the extract that did not show any growth on a new set of agar plates. The MBC was not determined for organisms that were not affected by the extract.

3. RESULTS AND DISCUSSION

Our study showed the presence of soluble proteins in the fruit pulp of *T. madagascariense* extracted with aqueous buffer (PBS). The method used in extracting the bioactive proteins may likely responsible for the good amount of protein obtained (13.47 mg of protein/ml). Extraction of protein using appropriate buffer and the use of ammonium sulphate fractionation has been used extensively in protein isolation. The same method was employed to extract the following bioactive proteins: antifungal protein from *curcuma longa* [34], haemagglutinating protein from *C. ammarissima* [35], antioxidant protein from Red gram seed coat [36] and antimicrobial protein from stem of *Tinospora tomentosa* [37].

Lectins are known for their hemagglutination properties. Hence, the crude protein precipitated with 70% ammonium sulphate were tested for hemagglutinating activity to determine the ability of the extract to agglutinate the various blood erythrocytes. The extract contains a measurable amount of hemagglutinating activity. It was found to agglutinate two human blood group erythrocytes (A and B) while no hemagglutinating activity was observed against human blood group O erythrocytes (Table 1). This is in contrast to the reported hemagglutinating activity of *B. angolensis* seed lectin which shows no human blood group specificity [38]. The crude lectin also proved capable of agglutinating rabbit erythrocytes with higher specificity. All trypsin-treated erythrocytes were better agglutinated than untreated one. Similar observation was

reported for the *B. angolensis* seeds lectin which was able to agglutinate trypsin-treated and non-treated human blood group and rabbit erythrocytes [38].

Table 1. Hemagglutinating activity of crude lectin of *T. madagascariense* fruit pulp against human and animal erythrocytes

Erythrocyte sources	Hemagglutination titre*	
	Trypsinized	Non-trypsinized
Human blood group		
A	2 ¹²	2 ⁹
B	2 ¹¹	2 ⁹
O	2 ⁴	2 ⁰
Rabbit	2 ¹⁶	2 ¹³

*Hemagglutination titre is the reciprocal of the highest dilution of the extract causing visible agglutination of the erythrocytes

The ability of lectins to agglutinate cells is a recognized physiological effect that depends on their specificity and high binding affinity for a particular carbohydrate moiety on the cell surface [6]. The sugar specificity of the crude lectin was investigated by competitive inhibition of various carbohydrates. Among all the carbohydrates tested only galactose inhibited the lectin activity of the protein extract with minimum concentration of 3.125 mM (Table 2).

Lectin-containing protein extract of fruit pulp of *T. madagascariense* exhibited a strong antibacterial activity against some human pathogenic gram-positive bacteria such as *B. anthracis*, *S. aureus*, and *S. faecalis* and also a gram-negative bacterium, *P. fluorescens* (Table 3). The result shows that gram-positive bacterial strains were more susceptible to the protein extract than gram-negative bacterial strains. It may be suggested because of the presence of lectin in the extract that the bacterial growth inhibitory activity of this extract is due to the presence of lectin. Lectins have been shown to possess good antimicrobial activities [16,19-20]. This is in line with the most researchers' reports that most plant extracts tend to have more activity against gram-positive bacteria [39,40] but in contrast to the report of Oliveira et al [20] on nonselectivity of lectin from *Eugenia uniflora* seeds against all bacteria tested. *Archidendron jiringa* seed lectin showed strong inhibitory activity against *Candida albicans* and *S. aureus* with very low MIC (0.0567mg/ml) but did not show any activity against *E.coli* and *P. aeruginosa* [41]. The growth inhibition exhibited by the crude lectin was

concentration dependent as higher concentration gave a higher zone of inhibition (Table 3). The extract was inactive against all other bacterial strains used. The zone of inhibition revealed that the extract show stronger inhibitory effect toward *S. faecalis* than the standard drug used.

Table 2. Sugar inhibition of hemagglutinating activity of the crude lectin of *T. madagascariense* fruit pulp

Sugars	Hemagglutination Titre*
Lactose	2 ¹²
Sucrose	2 ¹³
Maltose	2 ¹²
Galactose	2 ²
Mannose	2 ¹¹
Sorbose	2 ¹³
Glucose	2 ¹²
Fructose	2 ¹¹
Arabinose	2 ¹²
Mannitol	2 ¹³
N-acetylmannosamine	2 ¹²
α-methylmannoside	2 ¹¹
O-methylglucopyranose	2 ¹²
N-acetylglucosamine	2 ¹¹
Glucosamine	2 ¹²
Hydrochloride	
*Positive control	2 ¹²
*Negative control	2 ⁰

* Each experiment contained of 100 µl of serially diluted crude lectin in a U-shaped microtitre well. 50 µl 0.2 M sugar solution and 50 µl of 4 % suspension of erythrocytes (Rabbit erythrocyte) were added to each well. Positive control is without sugar while negative control does not contain extract and sugar

The susceptibility of some bacteria to the crude lectin of fruit pulp of *T. madagascariense* may be a step toward establishing its potential as a drug that can be used against these vulnerable bacterial strains especially *S. faecalis* and *P. fluorescens*. The unexpected strong susceptibility of a gram-negative bacterium, *P. fluorescens*, to this probably galactose-specific lectin tends to be a sign of relief to the current antibacterial resistance shown especially among gram-negative bacteria. The variation in bacterial sensitivity may be due to the nature of each species of bacteria tested [41]. It has been reported that aqueous extract tends to have no or low activity against microbes [39,42]. The usage of buffer with good ionic strength can improve the extraction of bioactive components like proteins/peptides which have been shown to possess good antimicrobial activity [19-21]. This

Table 3. Antibacterial activity of crude lectin of *T. madagascariense* fruit pulp against some human pathogenic bacteria

Organisms	Diameter of zone of inhibition (mm) (Mean \pm SD)		
	Crude lectin (mg/ml)		Streptomycin (mg/ml)
	12.5	25	1
Gram Positive			
<i>Bacillus anthracis</i>	14 \pm 1.6	18 \pm 1.6	20 \pm 0.6
<i>Bacillus cereus</i>	0 \pm 0.0	0 \pm 0.0	28 \pm 1.4
<i>Bacillus subtilis</i>	0 \pm 0.0	0 \pm 0.0	20 \pm 2.9
<i>Bacillus polymyxa</i>	0 \pm 0.0	0 \pm 0.0	15 \pm 0.4
<i>Bacillus stearothermophilus</i>	0 \pm 0.0	0 \pm 0.0	20 \pm 0.8
<i>Staphylococcus aureus</i>	17 \pm 1.2	20 \pm 1.4	21 \pm 0.8
<i>Streptococcus faecalis</i>	24 \pm 1.4	32 \pm 1.7	23 \pm 2.1
<i>Clostridium sporogene</i>	0 \pm 0.0	0 \pm 0.0	25 \pm 1.4
<i>Micrococcus luteus</i>	0 \pm 0.0	0 \pm 0.0	0 \pm 0.0
<i>Corynebacterium pyogene</i>	0 \pm 0.0	0 \pm 0.0	20 \pm 1.4
Gram negative			
<i>Escherichia coli</i>	0 \pm 0.0	0 \pm 0.0	0 \pm 0.0
<i>Klebsiella pneumonia</i>	0 \pm 0.0	0 \pm 0.0	0 \pm 0.0
<i>Proteus vulgaris</i>	0 \pm 0.0	0 \pm 0.0	0 \pm 0.0
<i>Pseudomonas fluorescens</i>	18 \pm 1.0	24 \pm 1.2	30 \pm 0.5

prompts the use of the PBS for the extraction in this study. The minimum inhibitory concentration and minimum bactericides concentration of the extract against *P. fluorescens* were the lowest (1.56 and 3.125 mg/ml) followed by *S. aureus*, and *S. faecalis* 3.125 and 6.25 mg/ml respectively for each isolate (Table 4). This suggests that the crude lectin contain a component that possess the ability to totally kill the bacteria at this or lower concentration and also that the component if purify could be used as an antibiotic agent against diseases that are caused by the organisms.

Table 4. The MIC and MBC regimes of crude lectin of *T. madagascariense* fruit pulp

Test bacteria	MIC (mg/ml)	MBC (mg/ml)
<i>Bacillus anthracis</i>	6.25	12.5
<i>Staphylococcus aureus</i>	3.125	6.25
<i>Streptococcus faecalis</i>	3.125	6.25
<i>Pseudomonas fluorescens</i>	1.56	3.125

The data obtained in this study proposed that lectin possibly play an important part in plants and animal defense mechanism especially against pathogenic bacteria. The sugar-binding site may likely play a significant role in this activity, being responsible for the recognition of bacteria. Almost all microbes have surface-exposed carbohydrates. These biomolecules

may be covalently bound, as the glycosylated teichoic acid to peptidoglycan, or non-covalently bound, as capsular polysaccharides [41,43].

It has been established that proteins/peptides termed antimicrobial peptide are involved in a defense mechanism against pathogenic bacteria by inhibiting the growth of microbes through different molecular means, like binding to chitin or increasing permeability of the bacteria membranes. Koczulla and Bals [44] reported that antimicrobial peptides probably interact with membrane in two possible ways. The first is the interaction between cationic amino acid and negatively charged phospholipid head groups on the surfaces and the second is that the hydrophobic and positively charged areas of the peptide interact with the aliphatic fatty acid and anionic component. These interaction leads to destabilization of the membrane integrity and eventually kill the bacteria. It has also been proposed that the protein with antibacterial activity for a channel on cell membrane and cell dies as a result of the out flowing of the cellular contents, this mechanism being different from that of antibiotics [16,20].

4. CONCLUSION

The present study established the presence of lectin in the aqueous buffer extract of *T. madagascariense* fruit pulp and revealed the *in vitro* antibacterial potential of the crude lectin

extract against pathogenic bacteria. Further purification of this lectin may lead to discovery of more therapeutic potential of lectin(s) that may be present in the crude that could make it play a significant role in clinical microbiology or as biotechnological tools.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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