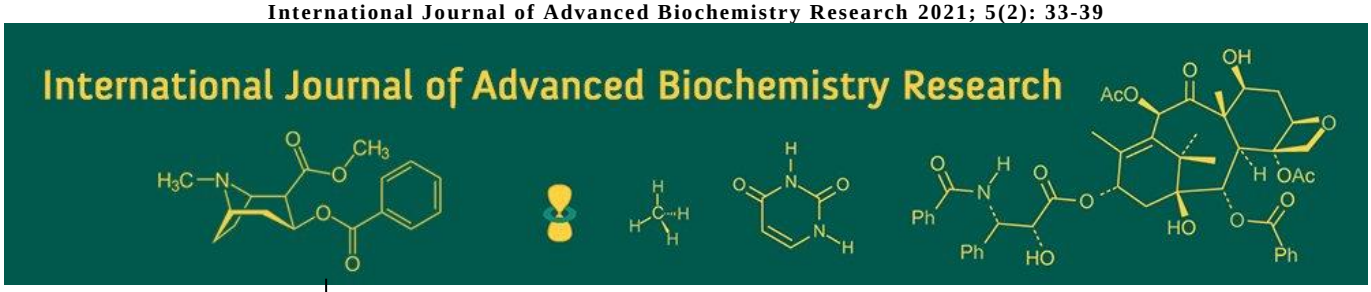

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2021; 5(2): 33-39
www.biochemjournal.com
Received: 25-05-2021
Accepted: 27-06-2021

Ako Alwell Chinonso
Biochemistry Programme, Department of Chemical Sciences, Faculty of Science, Clifford University, Owerrinta, Abia State, Nigeria

Nweje-Anyalowu Paul Chukwuemeka
Biochemistry Programme, Department of Chemical Sciences, Faculty of Science, Clifford University, Owerrinta, Abia State, Nigeria

Ejiofor Emmanuel Uchenna
Biochemistry Programme, Department of Chemical Sciences, Faculty of Science, Clifford University, Owerrinta, Abia State, Nigeria

Chigbu Charles Chukwuemeka
Microbiology Programme, Department of Biological Sciences, Faculty of Science, Clifford University, Owerrinta, Abia State, Nigeria

Ebere Wisdom Onyedikachi
Biochemistry Programme, Department of Chemical Sciences, Faculty of Science, Clifford University, Owerrinta, Abia State, Nigeria

Corresponding Author:
Ako Alwell Chinonso
Biochemistry Programme, Department of Chemical Sciences, Faculty of Science, Clifford University, Owerrinta, Abia State, Nigeria

Evaluation of qualitative phytochemical constituents, antimicrobial activity and *in vitro* anti-inflammatory activities of *Mappianthus iodoides* methanol leaf extracts

Ako Alwell Chinonso, Nweje-Anyalowu Paul Chukwuemeka, Ejiofor Emmanuel Uchenna, Chigbu Charles Chukwuemeka and Ebere Wisdom Onyedikachi

DOI: https://doi.org/10.33545/26174693.2021.v5.i2a.74

Abstract
Mappianthus iodoides is a plant remedy exploited in the management of various microbial infections and it is also useful in reducing pain. The present study screened the leaf extracts of *Mappianthus iodoides* for its phytochemical constituents, anti-microbial and *in vitro* anti-inflammatory activities. Heat- and hypotonic induced haemolysis of red blood cells were used to access the anti-inflammatory activities and the anti-microbial activity was determined by the agar well diffusion method. The plant extracts were screened for qualitative phytochemical constituents and the presence of alkaloids, flavonoids, tannins, cardiac glycosides, phenols, steroids, amino acids, anthraquine and soluble carbohydrates were observed. The results showed that the extracts had significant inhibition of microbial growth against all the organisms tested. The minimum inhibitory concentration (MIC) of the extracts showed that almost all the organisms tested were responsive to the extracts at a given concentration. Also, the extracts significantly ($p<0.05$) and dose dependently decreased the haemolysis of erythrocyte membrane induced by heat and water. The activities recorded substantiate the ethno-medicinal claims of the plant.

Keywords: Phytochemicals, anti-inflammatory, anti-microbial, *Mappianthus iodoides*

Introduction
The first phase of the body's defense against invading pathogens, foreign bodies and/or injury is known as the acute inflammatory phase [1]. The acute inflammatory phase involves the immune cells, blood vessels and a host of other agents that mediate infection. The purpose of these reactions is to get rid of the initial cause of cell injury, evacuate dead cells and to initiate tissue repair [1]. However, if the acute phase of the inflammatory process is not resolved, it can be a contributing factor to organ dysfunction and aggravate many widely occurring chronic inflammatory clinical manifestations which include arthritis, asthma, allergy, diabetes, inflammatory process of aging and cancers, organ fibrosis, metabolic syndrome, neurodegenerative diseases, cardiovascular and periodontal diseases [3-13]. Phytochemicals are known to contain important bioactive principles. They are present in different parts of plants in varying amounts. They are important bioresource of drugs used mainly in traditional medicine, modern medicine, pharmaceutical intermediates and this leads to the synthesis of drugs [14]. They have been reported to be responsible for the anti-microbial, antibiotic, anticancer, antihelminthic and antisickling properties of many products used in medicine [15].
Mappianthus species are a group of flowering plant belonging to the family Icacinaceae. The genus *Mappianthus* comprises of three important species which are widely spread in subtropical and tropical zone. It is used by traditional people for the treatment of traumatic injury, rheumatalgia, arthralgia etc. [16].
However, considering the myriad uses of this plant, no significant work has been done on *Mappianthus iodoides* leaf extract; especially so far, there is no scientific report on its anti-inflammatory properties, phytochemical constituents and anti-microbial properties of the

extract. Therefore, the present study seeks to evaluate the phytochemical constituents of the methanol extract of *Mappianthus iodoides* to ascertain the folkloric use of the extract in the management of pain and microbial infections.

Materials and Methods

Chemicals and materials

All chemicals used in this study were of analytical grade and were purchased from Sigma Aldrich Chemical Co. Ltd. (USA).

Plant material

Mappianthus iodoides was collected from the environment of Osusu Umuikpeghi Village in Obingwa L.G.A of Abia State. The plant material was confirmed by Mr. Alfred Ozioko of Bioresources Development and Conservation Programme (BDCP), Aku Road, Nsukka, Nigeria in November, 2020, where, the voucher specimen was kept for future reference.

Fig 1: A photograph of *Mappianthus iodoides* in its natural habitat.

Test Microorganisms

The gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *S. typhi* and gram positive bacteria (*Bacillus cerus* and *Staphylococcus aureus*) were obtained from Microbiology Laboratory, Biological Sciences Department, Clifford University, Owerrinta, Abia State.

Methods

Extraction of plant

The leaves were plucked fresh from the stalk and were dried at room temperature for about two weeks and crushed. The crushed leaf was weighed and divided into three equal parts and was soaked in 200 ml of methanol, 200 ml of cold water and 200 ml hot water respectively for 24 hours with intermittent shaking and covered to avoid the growth of mould. Filtration was carried out using Whatman No 1 filter paper and a funnel into a 100 ml volumetric flask. The filtrate was evaporated to dryness using a water bath at 45 °C. The crude extracts were kept in a refrigerator at 4 °C until further use.

The extraction yield was calculated using the relation; % yield = (weight of extract (g)/weight of plant material (g) × 100.

Preparation of media

A known amount of 38 g of the Mueller-Hinton agar was measured using weighing balance with a spatula and aluminum foil, and dissolved in 1000 ml of distilled water in a conical flask. The dissolved solution was homogenized by heating gently on a Bunsen burner, the conical flask was then covered with a non absorbent cotton wool and sealed with an aluminum foil and then autoclaved at 121 °C for 15 minutes.

Sterilization of plates

Fifty (50) petri dishes were thoroughly washed, cleaned and allowed to dry. Thereafter, the 50 petri dishes were carefully packed in five nylon bags and sterilized using the autoclave at 121 °C for 15 mins.

Sterilization of media and plate pouring

Homogenized Mueller-hinton agar was autoclaved at 121 °C for 15 minutes. The sterilized media was poured into the 50 petri dishes, each plate containing 20 ml of agar and the plate pouring process was done close to a Bunsen burner, then allowed to cool and the gel and the plate were inverted to avoid drop of moisture on the agar which may contaminate it. The plates were incubated at 37 °C for 12 hours to ascertain its sterility and then it was ready to be used for anti-microbial screening.

Preparation of normal saline

A known amount of 0.9 g of NaCl, was dissolved in 100 ml of distilled water, 4 ml each was pipetted into 6 clean test tubes the test tubes were then sealed with non-absorbent cotton wool and then autoclaved at 121 °C for 15 minutes.

Preparation of McFarland's standard

The McFarland's standard provides the turbidity reference for checking the approximate number of cells in dilution. They are sealed tubes of Barium sulphate suspension prepared as described by McFarland for use as standard for adjusting densities of bacterial suspensions and other turbid suspension. McFarland's standard helps researcher to have an idea of the approximate microbial activity against a given approximate number of test organisms. To prepare McFarland's standard 0.05 ml of 1% Barium chloride was added to 9.95 ml of 1% Sulphuric acid to obtain a turbidity of 0.119 at (600 nm) which was compared to the turbidity of the bacterial suspensions.

Inoculant dilution

The organisms inoculated and incubated at 37 °C were removed after 24 hours. A Finn micropipette was used to prepare a dilution of the organism in sterilized normal saline to achieve turbidity equivalent to MCF 0.119. The five different organisms with the turbidity as McFarland's standard were then used to seed the well labeled Petri dishes (labeled with names of the test organisms) using the spread plate method and with the help of a Finn micropipette. Using a sterilized cork borer of 6 nm; five wells were cut round in each of the agar plates already seeded with the test organism.

Preparation of 20% dimethyl sulfoxide (DMSO)

Known amount of DMSO, 20 ml was pipette into a 100 ml volumetric flask and 80 ml of water was added to the volumetric flask to make it up to 100 ml.

Serial dilution of the extract

Known amount of the extract (0.5 g) was weighed and added into the test tube, 1 ml of 20% DMSO was added into the first test tube which is the stock (500 mg) and it was shaken to dissolve the plant extract. One (1) ml was taken

out from the first test tube into the second test tube with dilution rate (250 mg), the procedure continued until the fifth test tube to achieve concentrations of the extract equivalent to 500, 250, 125, 62.5, and 31. 25 mg/ml. This procedure was repeated for hot water and cold water extracts.

Antimicrobial studies

All tests were performed in triplicate and standard antibiotics discs were used as a positive control while 20% DMSO was used as a negative control. The six wells made round the plates with 6 mm cork borer were well labeled (using concentrations of the extract; 500, 250, 125, 62.5, and 31. 25 mg/ml), the different concentrations were introduced into appropriate well using a Finn micropipette. The set up was left to stand for 30 minutes and then the plates were incubated at 37 °C for 24 hours. At the completion of the incubation, the plates were brought out and the formation of clear zones were measured using a meter rule and recorded in millimeter (mm) and the results were recorded as mean diameter of zones of growth (with name of the test organism).

Agar well diffusion test

A loopful of bacterial culture was placed on a solidified MHA media. With a sterile cork borer, agar well of 6 mm diameter was dug on agar surface. Each well was aseptically filled with respective extracts of 20 µL. The plates were allowed to soak the extracts and incubated at 37 °C for 24 h. Zone of inhibition was measured.

Fig 2: A picture showing the formation of clear zones in the plate.

Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration can be regarded as the least concentration of the anti-microbial agent found to stop the growth of a particular test organism. The MIC value explains the potency of the antimicrobial agent, it may be interpreted that the organism is ''susceptible'' intermediate'' or ''resistant'' to the antimicrobial agent [17]. The lower the MIC value, the more susceptible the organism and the more potent the antimicrobial substance. The MIC was accessed using the micro dilution method. The MIC samples were found to be active by the dilution method, the test extracts were dissolved in 20% DMSO to give the stock concentration of 500 mg/ml and this was subjected to serial dilution in a series of test tubes to a working concentration ranging from (500 to 15.63 mg/ml) and the following concentrations were obtained, (500, 250, 125, 62.5, 31.25, and 15.63 mg/ml). The broth was prepared by weighing 1.3 g of agar broth in a volumetric flask and making up to 100 ml, the broth was pipetted into 10 test tubes each for aqueous and methanol extracts respectively. The diluted extract for aqueous and methanol were transferred into the test tubes according to their concentrations respectively (500, 250, 125, 62.5, 31.25, 15.63 mg/ml). The five organisms were introduced into the test tubes and were incubated at 37 °C for 24 hrs.

Phytochemical analysis

The crude extracts were subjected to phytochemical analysis using the method described by Harbourne, 1998 [18].

Membrane Stabilization Studies

Heat and hypotonic-induced haemolysis of human erythrocyte were used to determine the stabilization of membrane and this was done using the method described by Shinde *et al.*, 1989 [19] with some modifications, Anosike *et al*., 2018 [20].

Heat-Induced haemolysis of Red Blood cell

A known quantity of the extracts was dissolved in isotonic phosphate buffer solution. Centrifuge tubes were arranged in quadruplicate containing about 5 ml doses of the extracts (100, 200, 400, 600, 800 µg/ml). Control experiment was set up by arranging two sets of centrifuge tubes containing 5 ml of isotonic phosphate buffer solution and 5 ml of 200 µg/ml of indomethacin respectively. Erythrocyte suspension (0.1 ml) was added to each tube and mixed gently. Two of the tubes from each set were incubated at 54 °C for 20 minutes in a water bath. Thereafter, the tubes were centrifuged at 1300 g for 3 minutes and the haemoglobin content of the supernatant was determined using spectronic 21D (Milton Roy) spectrophotometer at 540 nm.

The percentage inhibition of haemolysis of the extract was calculated thus

$$\text{Percentage Inhibition of haemolysis} = \frac{1 - (OD2 - OD1) \times 100}{(OD3 - OD1)}$$

Where,

OD_1 = Absorbance of test sample unheated

OD_2 = Absorbance of test sample heated

OD_3 = Absorbance of control sample heated

Hypotonic-induced Haemolysis of Red Blood Cell

A known quantity of the extracts was dissolved in hypotonic solution (distilled water). Duplicate pairs (per dose) of the centrifuge tubes contained 5 ml graded doses (100, 200, 400, 600, 800 µg/ml). However, an isotonic phosphate buffer solution containing about 5 ml graded doses of the extracts (100, 200, 400, 600, 800 µg/ml) were put into the other duplicate pairs (per dose) of the centrifuge tube. Control experiment was set up by arranging two sets of centrifuge tubes containing 5 ml of distilled water and 5 ml of 200 µg/ml of indomethacin respectively. Erythrocyte suspension (0.1 ml) was added to each of the tubes and mixed gently. The mixture was incubated for 1 hour at room temperature (25 °C) and centrifuged afterwards for 3

minutes at 1300 g. Absorbance (OD) of the haemoglobin content of the supernatant was determined at 540 nm using spectrophotometer. Percentage haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100%
The percent inhibition of haemolysis of the extract was calculated thus

$$\text{Percentage Inhibition of haemolysis} = \frac{1 - (OD2 - OD1)x\ 100}{(OD3 - OD1)}$$

Where,
OD_1 = Absorbance of test sample in isotonic solution
OD_2 = Absorbance of test sample in hypotonic solution
OD_3 = Absorbance of control sample in hypotonic solution.

Preparation of the Erythrocyte Suspension
Fresh blood sample (3 ml) was collected from healthy volunteers and were put in heparinized bottles and centrifuged at 3000 rpm for 10 minutes. A volume of saline similar to the volume of the supernatant was used to dissolve the red blood pellets. The volume of the dissolved red blood pellet was measured and reconstituted as 40% v/v suspension with isotonic buffer solution (10 nM sodium Phosphate buffer, pH 7.4). The buffer solution contained 0.2 g of NaH_2PO_4, 1.15 g of Na_2HPO_4 and 9 g of NaCl in 1 litre of distilled water. The reconstituted red blood cell (re-suspended supernatant) was used as such.

Statistical analysis: Results were presented in tables are expressed as mean ± S.D. The level of significance was tested using one-way ANOVA. Results were regarded as significant when $p<0.05$ that is there is greater than 95% probability that the result is not by chance. All statistical analyses were performed using SPSS software.

Results

Phytochemical screening

The result of the phytochemical screening of the methanol extract of the leaf of *Mappianthus iodoides* is shown in Table 1. The result shows the presence of alkaloids, flavonoids, tannins, saponins and cardiac glycosides, anthraquinones, reducing sugar, steroids, phenol, soluble carbohydrate, proteins and amino acids by qualitative methods. Terpenoids were absent.

Table 1: Qualitative Phytochemical Composition.

	Test compound	**Leaf extract**
	Aqueous	**Methanol**
Saponins	+	+++
Amino acids	**ND**	+
Phenol	++	+++
Tannins	++	+++
Terpenoids	**ND**	**Absent**
Steroid	+	+++
Flavonoids	++	+++
Reducing sugar ND	+	
Glycosides ND	+	
Anthrquine+	+++	
Soluble carbonhydrate ++	+++	
Alkaloids +	+++	

Bioactivity key: ND –not detected
+++ = present in very high concentration
++ = present in moderate concentration
+ = present in low concentration

The qualitative phytochemical composition of *Mappianthus iodoides* presented in Table 1 shows high concentrations of some bioactive phyto-active principles such as phenols, saponins, flavonoids, tannins and steroids in the methanol extract while the concentrations were low or absent in the aqueous extract. Amino acids were in low concentration in the methanol extract and absent in the aqueous extract. Terpenoids were absent in both aqueous and methanol extract.

Antimicrobial activity

The zone diameters of inhibition of the crude extracts at different concentrations are shown in Table 2. From the result of the zones of inhibition of methanol extract of *M. iodoides*, it can be seen that it displayed credible activities on the organisms tested when compared to the standard drugs used. Table 3 shows the minimum concentration of the crude extract required to inhibit the growth of the organism. The lowest (125 mg/ml) for the methanol extract of *M. iodoides* was recorded against *S. typhi*, while the highest MIC (500 mg/ml) was recorded against *P. aeruginosa.*

Table 2: Inhibition zone diameter of methanol extract on some test organisms against different concentrations.

Conc. (Mg/ml)	**Inhibition zone diameter**				
	E. coli	***S. typhi***	***P. aeruginosa***	***Staph auerus***	***B. cerus***
500	16.66±0.34	20.33±0.47	8.76±1.0	13.85±0.77	12.0±3.46
250	12.26±2.72	15.4±0.1	8.13±1.71	1.0±5.47	11.0±3.31
125	10.0±1.15	10.3±0.83	9.66±0.61	6.3±2.5	8.00±2.82
62.5	5.6±0.31	7.83±0.67	5.73±0.14	0.00±0.00	6.00±2.44
31.25	4.0±0.57	10.60±0.67	0.00±0.00	0.00±0.00	0.00±0.00
15.63	4.0±0.57	5.46±1.03	0.00±0.00	0.00±0.00	0.00±0.00

n= 3. All values are expressed as mean zones of inhibition (mm) ± standard deviation

Table 3: Minimum inhibitory concentration (MIC) values of the test organisms against various plant extracts.

Extract	**MIC values in (mg/ml)**				
	E. coli	***S. aureus***	***B. cerus***	***P. aureus***	***S. typhi***
Methanol	125	250	250	500	125
Aqueous	0.0	0.0	500	250	0.0

Table 4: Inhibitory concentrations of reference drugs

Reference drugs (30 mg)	Inhibitory zone diameter (mm)				
	E. coli	***S. aureus***	***B. cerus***	***P. aureus***	***S. typhi***
Tarind (OFX)	18	13	20	22	13
Reflacine (PEF)	23	20	13	15	22
Ciprofloxacin (CPX)	22	23	17	14	14
Augmentin (AU)	17	18	14	14	28
Gentamycin (CN)	13-14	15	15	13	13-14
Streptomycin (S)	10-14	14	15	28	15
Nalicidic Acid (NA)	29	17	10	15	16
Septrin (SXT)	16	16	19	11	10
Ampicillin (PN)	12-13	14	12	28	13

Table 5: Inhibition zone diameter of aqueous extract

Conc. (mg/ml)	Inhibition zone diameter (mm)				
	E. coli	***S. typhi***	***P. aeruginosa***	***S. aeruginosa***	***B. cerus***
500	0.00	2.06±o.28	0.00	0.00	2.00±0.53
250	0.00	2.0±0.28	1.75±0.77	0.00	1.25±0.14
125	0.00	0.00	0.00	0.00	1.73±0.03
62.5	0.00	0.00	0.00	0.00	0.00
31.25	0.00	0.00	0.00	0.00	0.00
15.63	0.00	0.00	0.00	0.00	0.00

N= 3 All values are expressed as mean zones of inhibition (mm) ± standard deviation

Membrane stabilization of human red blood cell

Effect of methanol extract of *M. iodoides* on heat – induced haemolysis of human red blood cell

Table 6 shows that the extracts significantly (p<0.05) shielded the human red blood cell membrane against breakdown induced by heat in a dose dependent manner. The result also shows that 800 µg/ml of the extract produced the highest percentage inhibition of haemolysis of 89% and this is comparable to the standard drug used.

Table 6: Effect of *Mappianthus iodoides* Methanol extracts on heat induced haemolysis of human red blood cells (HRBCs)

Treatment	Dose	Mean Absorbance ± S.D.		% Inhibition
		Heated solution	Unheated solution	
Control	_	1.08±0.06	0.49±0.15	
Extract	100	0.94±0.14	0.63±0.32	31
	200	1.04±0.04	1.00±0.10	50
	400	1.46±0.04	1.22±0.12	71
	600	1.42±0.001	1.37±0.01	83
	800	1.58±0.06	1.53±0.07	89
Indomethacin	200	1.90±0.03	1.49±0.23	100

Effect of methanol extract of *Mappianthus iodoides* on hypotonicity-induced haemolysis of human red blood cell.

Table 7 shows that *Mappianthus iodoides* extract significantly (p<0.05) prevented lysis induced by water (hypotonic solution). This is evident by the high percentage respective inhibition of 66, 31, 36, 100, and 65 obtained at doses of 100-800 µg/ml. This result is very comparable to that of indomethacin (200 µg/ml) which produced percentage inhibition of 100%.

Table 7: Effect of *Mappianthus iodoides* extract on hypotonic-induced haemolysis of HRBCs.

Treatment	Dose (µg/ml)	Mean Absorbance ± S.D.		% Inhibition
		Hypotonic solution	Isotonic solution	
Control	-	0.53±0.02	0.49±0.01	
Extract	100	0.39±0.002	0.74±0.002	66
	200	0.67±0.01	0.99±0.13	31
	400	0.90±0.05	1.57±0.07	36
	600	2.05±0.05	2.05±0.001	100
	800	1.52±0.09	2.06±0.02	65
Indomethacin	200	0.76±0.01	0.82±0.09	88

Discussion

In this present study, we evaluated the phytochemical constituents and its antimicrobial and anti-inflammatory activities. Though there are so many extracts with anti-microbial and anti-inflammatory activities, *Mappianthus iodoides* extracts have not been well studied. Anti-inflammatory activities can be evaluated by many methods but this study focused on heat-induced membrane stabilization and hypotonic-induced human erythrocyte membrane stabilization.

The phytochemical screening of the leaf extract of *Mappianthus iodoides* shows the presence of alkaloids,

flavonoids, tannins, terpenes, saponins, and cardiac glycosides by quantitative methods, terpenoids were found to be absent in the crude extract. These secondary metabolites possess several medicinal properties which have been documented elsewhere [21].

Five (5) of the bacterial strains tested (Gram-positive and Gram-negative) were sensitive to the methanol extracts of *Mappianthus iodoides* and this was determined by agar well diffusion method. Though agar well diffusion method is not efficient because the antimicrobial activity may be affected by various factors the type of agar used, salt concentration, incubation temperature and the molecular size of the antimicrobial agent. However, it does not distinguish between the agents that can kill the bacteria and those than can stop the growth of the bacteria [22]. The MIC values in Table 3 showed that the methanol extract had antimicrobial activity against all the bacteria tested while the aqueous extract exhibited activity against *B. cerus* and *P. aureus* only.

Table 6 shows that the extract significantly ($p<0.05$) prevented heat-induced haemolysis of HRBC. This is evidence that stabilization of the membrane is a possible method of the anti-inflammatory activity of the extract. Hypotonic solutions are known to have haemolytic effect. Haemolysis occurs when there is excess accumulation of fluid in the cell and this result in the swelling of the membrane and eventual bursting of the erythrocyte membrane. When the erythrocyte membrane gets ruptured, it will make the cell liable to secondary damage. This damage occurs by free radical-induced lipid peroxidation [23]. Membrane stabilization prevents the leakage of serum proteins into the tissue fluid. This process goes on by inflammatory mediators especially when there is an increase in membrane permeability [24]. The methanol extract of *Mappianthus iodoides* leaf could stabilize the erythrocyte membrane induced by hypotonic solution by inhibiting the discharge of enzymes that could disintegrate the cell and other active inflammatory mediators. At 600 µg/ml of the extract, it showed 100% inhibition of haemolysis and this result is similar to the standard drug, indomethacin and this result is in agreement with work of Anosike *et al*., 2018 [20].

Another possible mechanism for the membrane stabilizing activity of *Mappianthus iodoides* extract could be due to an increase in the surface area/volume ratio of the cell, which could be brought about by increase in membrane size or decrease in cell size and interaction with membrane proteins [25]. However, it has been well documented that the cell volume of red blood cells are closely related to the calcium content inside the cell [20]. This is good evidence that the ability of the extracts to protect the human red blood cell could be due to the ability of the *Mappianthus iodoides* to change the influx of calcium into the red blood cell membrane.

Conclusion

The present study reveals that the extracts possess some phyto-active principles of therapeutic importance and these possess biological activity against a number of diseases. Therefore, further studies are required to discover and isolate the active components of the pharmacological properties of the plant.

Acknowledgements

The authors are grateful to the administration of Clifford University, Owerrinta, Abia State, Nigeria led by the President/Vice-Chancellor, Prof. A.C. Omeonu for providing the materials needed for this research. We are also grateful to Mr. Ben Ikoro, the laboratory technologist in charge of the Biochemistry Laboratory for assisting us with the available materials and equipment.

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