UNIVERSITY OF PORT HARCOURT

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JOURNAL OF MALARIA AND PHYTOMEDICINE
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EDITORIAL

MALARIA CONTROL AND INSECTICIDE RESISTANCE: A REVIEW

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ABSTRACT

Introduction: Malaria has been referred to as a global health concern and it's the main mosquito-borne disease that results to global human mortality. The primary vector control interventions that include the use of insecticides are responsible for the decrease in malaria burden worldwide. Malaria control programmes were reported to have challenges due to insecticide resistance. Numerous studies have shown that the strength and distribution of this resistance has increased in recent years.

Methods: Improved knowledge of current resistance levels and underlying mechanisms are important in designing proper management strategies and to avoid future selection for resistance. Vector control is regarded as one of the major methods that can be used to decrease the transmission of malaria at local level, it can bring down malaria transmission from extremely high levels to about zero level.

Results: Malaria is a disease that can be prevented and treated with proper implementation of recent recommended interventions. Currently, there are four classes of chemical insecticides used for vector control programmes. Insecticide is known as a substance that is toxic which eliminates or kills insects or vectors/pests that transmit diseases. Resistance in mosquito can be in form of target-site insensitivity that occur as result of mutations in the target proteins, metabolic resistance which is due to an increased breakdown of the insecticide by enhanced detoxification activities, or a decrease penetration or sequestration of the chemical insecticide.

Conclusion: Malaria can be controlled by effective vector control measures that can be achieved by proper use of insecticides as means of eliminating the disease vector. Knowledge and early identification of insecticide resistance can provide assurance for the eradication and elimination of malaria through vector control strategy.

Key Words: Malaria, Insecticide resistance, Insecticides, Vector control, Mosquito.

INTRODUCTION

Malaria is the main mosquito- borne disease well known among other major causes of human deaths globally[1]. Malaria is a global public health concern, approximately 3.2 billion people are at risk of malaria infection worldwide. World Health Organization (WHO) database recorded about 92% of the global mortalities in Africa, most were recorded in children under the age of five [2,3]. About 228 million malaria cases were recorded globally in 2018. Most of these cases (93%) were in the African region, 3.4% from the South East Asia Region and 2.1% from Eastern Mediterranean Region. Nineteen countries in sub- Saharan Africa and India take about 85% of the malaria burden worldwide. Six countries are responsible for more than half of all the global malaria cases, these countries include Nigeria, The Democratic Republic of Congo and Uganda with 25%, 12% and 5% of cases respectively[4]. Malaria contributes to poverty and annually cost the economies of the African continent an estimated range of 0.5% to 9%

of GDP[5], it also results in loss of productivity. Malaria, together with tuberculosis and HIV, are important causes of morbidity and mortality, especially in children[6]. It also has a strong negative impact on developmental stages in children leaving many that survive the disease disabled for life[7].

In Nigeria, about 97% of its population are at risk of malaria[8], about 51 million cases and 207,000 deaths are recorded annually. Nigeria had the highest malaria cases than any other country across the globe with an estimated 25% of the total malaria burden within Africa[9]and up to 27% of the total African malaria burden in 2016[10]. Malaria cases in pregnancy is an important public health issue in Nigeria due to their implication in maternal mortality. The major complications of malaria in pregnant women include high placental plasmodia burden, foetal complications, low birth weight in new born babies, and sometime new born death[11,12.]

Malaria Burden

Malaria burden worldwide is reduced mostly due to the improved core vector- control interventions, that include: indoor residual spray (IRS) with insecticides and long-lasting insecticidal nets (LLINs)[13, 14]. However, insecticide resistance and climate change threaten the achievement made by IRS in malaria control. Insecticide resistance has been observed in all the four classes of insecticides approved by the WHO for vector control intervention namely Organophosphates, organochlorines, pyrethroids and carbamates[15] Change in environmental temperature among other causes of resistance is implicated, worsening the resistance condition through alteration in the genetic structure, protein profiles and enzymes of mosquitoes[3]. Malaria control programmes are having a challenge of rising insecticide resistance in the main anopheline vectors in Africa and beyond. This affects the main malaria vector control interventions[7].

Since the first established relationship between malaria vectors and transmission of pathogens to humans and other vertebrates in the late nineteenth century[16], a key control strategy against major mosquito-borne diseases such as malaria, dengue, yellow fever, Zika virus infection and chikungunya fever, is targeting mosquito vectors to interrupt the transmission of diseases[17]. Preventing infection, prompt diagnosis and effective treatment are other measures used to control malaria and its complications[18].

Insecticides are significant in malaria control through vector control strategies. As the efforts to control the disease intensifies, so the selection pressure on mosquitoes to develop resistance to the available insecticides. The strength and distribution of this resistance has increased in present years and presently threatens the success of malaria control programmes[19]. Improved knowledge of current resistance levels and underlying mechanisms is important in designing proper management strategies and to curtail future selection for resistance[20].

MALARIA CONTROL

Malaria is a disease that can be prevented and treated with proper implementation of the present recommended interventions. WHO recommendations include vector control measures, diagnostic testing, preventive therapies, intensive malaria surveillance, artemisinin-based therapies. These are part of measures required to control and eradicate malaria[21]. The choice of malaria control measure in a particular region is being affected by numerous factors that include seasonality, vector specie, vector behaviour, health facilities, disease pattern, endemicity among others[3].

Vector control is a primary method that can be used to decrease the transmission of malaria at local level, it can reduce malaria transmission from extremely high levels to about a zero level[3]. Malaria prevention by vector control leads to decrease human-vector contact and also reduce the average lifespan of the mosquito population at local level. Residual insecticides are applied to the inner surfaces of living areas with the aim of targeting Anopheles mosquitoes that rest indoors[21]. ITMs (Insecticide Treated Materials), when used at large scale are expected to have major effect on

vector populations and this help the whole community including those areas without nets. Populations not making use of ITMs, but within areas with increased ITMs coverage, have been shown to be at lower risk of infection due to decrease in overall malaria transmission in such areas[22,23]. Many studies have reported that malaria-related morbidity and mortality were reduced by the use of ITMs[24,25]. Antimalarial drugs can be used in malaria prevention especially for individuals travelling to malaria endemic regions. Also, intermittent preventive treatment with sulfadoxine-pyrimethamine is recommended by WHO for pregnant women and infants living in high malaria transmission regions[3].

Insecticides use in vector control

Use of insecticides for vector control is a significant component of numerous vector-borne disease control measures[9]. Currently, there are four classes of chemical insecticides used in vector control programmes consisting of the organochlorines (DDT exclusively), the pyrethroids, the carbamates and the organophosphates. However, only pyrethroids are recommended insecticide by WHO for treating bed nets due to their relatively low toxicity to humans and fast knock-down effect[26].

Dichlorodiphenyltrichloroethane (DDT)'s insecticidal efficacy was identified around 1940 and was used worldwide for eradication of malaria between the years 1950 and 1978 to eliminate malaria in 37 of the 143 malaria endemic countries[9].Other insecticide groups that include benzyl phenyl urea and biological control agents like *Bacillus thuringiensis* are used less often against mosquitoes. Pyrethroids have better advantage of multiple modes of action on the vector. They affect sodium channels by opening the channels resulting in continuous nerve excitation, paralysis and death of the vector. They also cause irritant effect, rapid knockdown, that lead to hyperactivity, shorter landing times, undirected flight, and feeding inhibition, all of which reduce the biting ability of the vector[27].

Organochlorines (DDT) are used for Indoor Residual Spray (IRS) in vector control. The persistent use of DDT for vector control measures is approved based on condition under the Stockholm Convention on Persistent Organic Pollutants in line with WHO guidelines and recommendations[18]. Pyrethroids and DDT have identical modes of action, and hence cross resistance to these two classes of insecticides mayoccur[28]. Carbamates and organophosphates are also used for IRS, they are very effectivebut with relatively short residual activity when compared to DDT and pyrethroids. Encapsulation (CS) technology is now applied to increase the residual performance of some carbamate and organophosphates insecticides[29].

Carbamates and organophosphates inhibit cholinesterase, this prevents the breakdown of neurotransmitter acetylcholine, and this leads to neuromuscular overstimulation and death of the vector[27].

INSECTICIDE RESISTANCE

Insecticide is regarded as a substance that is toxic, it eliminates or kills insects or vectors/pests that transmit diseases. Insecticides are regarded as silver bullets or strong agents used in both agricultural practices and public health sectors in the developing countries[30].

Insecticides are strongly efficient when used appropriately[31], however, numerous factors affect its efficacy including operational capability and inadequate resources[32], insecticide resistance[33], and the use of poor-quality or adulterated insecticides[27]. These factors need to be properly taken care of by Governments, and all the stake holders concern. Insecticide resistance can be genetic, or natural ability of an organism to withstand exposure to a chemical substance that would ordinarily kill a member of that specie[34].

Resistance of mosquitoes to the four classes of insecticide primarily used in Insecticide Treated Nets or Indoor Residual Spray pose threats to efforts in malaria prevention and control. Insecticide resistance to at least one insecticide in a study site was recorded in 61 countries, while in 50

countries the resistance was to two or more insecticide classes in a site.

Insecticide resistance to the four classes of insecticides was observed in malaria vectors from all WHO regions with the exception of Europe, but the level of monitoring differed from one region to another. Most of the data (about 70%) were recorded in countries of the WHO African Region[10].

Different types of resistance include cross resistance, multiple resistance and metabolic resistance. Cross resistance is when a pest population have resistance to more than one member of a chemical family. Multiple resistance include multiple, independent resistance mechanisms, and can result into resistance to pesticides/chemicals from different chemical families. Metabolic resistance involves detoxification enzymes that can function in the biotransformation of xenobiotics to less or non-toxic substances[34].

Major methods in preventing malaria by vector control are still based on chemicals, these methods include larviciding, personal protection, environmental management, biological control among others. Indoor Residual Spray (IRS) is an important measure to control malaria vector in regions with significant number of housing facilities having enough sprayable areas and most of the vectors rest indoors (endophilic). Insecticide Treated Nets (ITNs) can be used in places where coverage rates are much and a high proportion of vector human-biting occurs at late night when people are asleep. These methods require susceptibility of vectors to the chemical insecticides. The use of ITNs and IRS has shown promising effect in reduction of mortality and severe disease as a result of malaria in endemic regions[24,35].

Insecticide resistance evolution in insect population can arise due to a rise in the frequency of one or more resistance genes in the insect population as a result of exposure to insecticides. Genetic drift and natural selection act on genetic differentiation in the population that is created by genetic recombination, mutation and gene flow[36].

Insecticide Resistance Management

Insecticide resistance management is an effort to reduce or eliminate the development of resistance[34]. Resistance management can easily be basedprimarily on insecticides, this can take various forms such as combinations, rotation, mosaic, or mixtures. Reducing the selection pressure posed by a certain insecticide or a particular mode of action is of major significance in order to attain success in resistance management[37]. Insecticide resistance of mosquitoes is a growing threat in the African region with few insecticides recommended for public health purposes, and drawbacks in the development of new chemical molecules in years to come. Therefore, application of resistance management strategies is important to maintain the efficacy of control programmes[38].

Insecticide Treated Nets with mixture of pyrethroid and carbamate were endorsed as tools for insecticide resistance management[39]. It has been reported that synergists have the ability to delay control failure, as a result of insecticide resistance in agricultural practices. Synergists are substances that improve the toxicity of some insecticides, although having limited toxicity when used alone. These substances include S,S,S-tributylphosphoro-trithioate (DEF), piperonylbutoxide (PBO), and N-Octylbicycloheptenedicarboximide (MGK-264), etc. Synergists improve the efficacy of various classes of insecticide, including the pyrethroids, carbamates and organophosphates, they achieve their actions by inhibiting the insecticide metabolizing enzymes like Cytochrome P450s, Glutathione S Transferases and esterases in the insects' system. Now, there are Insecticide Treated Materials (ITMs) like Permanet® 3.0 that contain a mixture of a pyrethroid (insecticide) and PBO (synergist) to defeat pyrethroid resistance in mosquitoes. This technique has been reported to be more effective against mosquitoes with multiple resistance mechanisms when compared to other ITMs treated with only pyrethroid insecticide[40].

Insecticide Resistance Mechanisms

To develop resistance management strategies, knowledge of factors that influence insecticide resistance in addition to characterizing the insecticide resistance mechanisms are of primary importance. Factors that are likely to cause insecticide resistance in mosquitoes include: agriculture, industrialisation and urbanization. These factors are related to some environmental parameters; use of insecticides/pesticides in agriculture, natural or anthropogenic xenobiotic, and interactions of vectors with other organisms[38].

Insecticides impair the role of molecular targets that are important for survival. Mutation of any kind that changes the physiology or the behaviour of the insect in a way by which the target becomes less functional will be able to induce resistance. As such, insecticide resistance may be caused by one or more than one of these mechanisms: increased detoxification or excretion, target insensitivity, decreased penetration, or behavioural avoidance of the insecticide by the insect species[41]. Insecticide resistance by insects can be as a result of numerous physiological changes that include mutations of the targeted proteins termed as target-site insensitivity[42], or an increased breakdown of the insecticide due to enhanced detoxification activities termed as metabolic resistance, or a decreased penetration or sequestration[43]. Resistance to pyrethroids has been reported to occur due to target-site mutations termed as the 'knock down resistance' (kdr) and metabolic resistance mechanisms, however, other mechanisms like cuticle alteration have also been reported[44]. Knock down resistance (kdr) mutations and increased levels of detoxification enzymes also result in resistance to DDT while resistance to carbamate can be due to ace1 mutation and detoxification[45].

Metabolic Resistance

Metabolic resistance is a more diverse phenomenon that involves active regulation of detoxification system of the mosquito as such counteracting the effect of chemical insecticide. Metabolic resistance include elevated levels or enhanced activities of insecticide-detoxifying enzymes. This results in development of resistance in insects due to sufficient amount of insecticide substances being degraded before getting to their target in the nervous system of the insect[46].

Detoxification enzymes initially linked to insecticide resistance involve three main gene families: the cytochrome P450 (monooxygenases), glutathione S transferases and the carboxyl/choline esterases, but other enzyme families such as UDP glucosyl-transferase(UGTs) may also be included[47].

Insecticide degradation can be as a result of the increased production or structural modification of one or more of these enzymes either from common or distinct family. These enzymes can work sequentially or simultaneously to bring resistance. Many studies are aimed at the increased production of detoxification enzymes while few are on the selection of particular detoxification enzyme alleles which results in significant insecticide degradation in mosquito vectors[48]. High throughput sequencing approaches like RNAseq can be used to fill this knowledge gap, RNAseqprovides information on gene expression and nucleotide variations over the whole transcriptome from an experiment[49,50].

These enzymes metabolise insecticides to less toxic or non-toxic chemicals or, sequester them. Enhanced detoxification is a known resistance mechanism that stops inhibition of the targets[51].

Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases are Phase I detoxification enzymes. They catalyse many reactions, but are mostly identified for their role as monooxygenases by adding polar or reactive groups into endogenous compounds or xenobiotics[52]. CYP enzymes' catalytic cycle require electron supply to the heme iron in presence of oxygen. Most of CYP enzymes only accepts electrons fromNAD(P)H. But for some other CYPs another pathway has been identified with peroxides as donors of activated oxygen[53]. This pathway is called “peroxide shunt” and provide significant features for not only the preparative but also

analytical applications[54]. The problem of this pathway results from the faster inactivation of the enzyme by the peroxide. To avoid the inactivation of the protein, continuous photochemical or electrochemical generation of peroxide has been applied for preparative substrate conversion[55].

The Cytochrome P450 gene family was identified to have large expansion of more than 100 CYPs identified in *An. gambiae* according genomes of mosquito[56], more than 200 genes in *Culex quinquefasciatus*[57]and 160 in *Ae. Aegypti*[58]. Increased levels of P450 activity have mostly been identified in mosquitoes with pyrethroid-resistant[43]. These same enzymes can also be induced by most xenobiotics[52]. After the development of the 'Anopheles Detox Chip' microarray[59], most over-transcribed mosquito CYPs were observed in either laboratory colonies or resistant field mosquito populations by microarray analysis[60, 61, 62]. Numerous CYP genes were also associated to pyrethroid resistance by QTL approaches and positional cloning[63, 64]. *An. gambiae* CYP6P3 and CYP6M2 among others were identified as pyrethroid metabolizers[65, 66], *Ae. aegypti* CYP9J32, J24, J26 and J28 and *Anopheles minimus* CYP6P7 and CYP6AA3[67,68].

Carboxyl/choline esterases

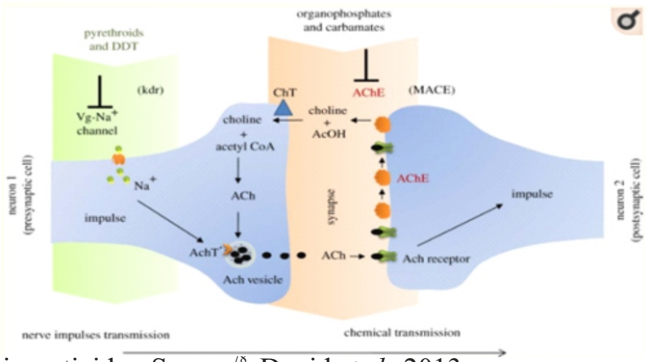
Esterases (E.C. 3.1.1.1) are carboxyl ester hydrolases that catalyse; the hydrolysis of esters having short-chain fatty acids, transesterification reactions and ester synthesis[69]. Carboxyl esterases can be defined as those enzymes catalysing the hydrolysis of acylglycerols with less than 10 carbon atoms (short chains)[70]. Tributyrine is known as the standard substrate for carboxylesterase activity. The enzyme exhibit activity in organic solvents[71]. Carboxyl esterases' active site consist of three residues: an acidic residue (aspartic acid or glutamic acid), a nucleophilic serine residue in a GXSXG motif, and a histidine. These residues work together to catalyse ester hydrolysis. The catalytic triad consists of aspartic acid, serine, and histidine with the serine enveloped in the sequence Gly-X-Ser-X-Gly (where X stands for any amino acid) at the active site[72]. The enzymes have also been reported to show a common α/β hydrolase fold[73] which is common to other hydrolases.

Glutathione S-transferases

The glutathione S-transferases (GSTs) are significant parts of the detoxification pathway in most living organisms. These enzymes work in phase II of the detoxification pathway, they act by conjugating glutathione to products of xenobiotics or metabolism, and these enzymes enhance the excretion of xenobiotics by making them more water soluble. GSTs are currently identified to play major roles in cells' protection against the harmful effects of cell signalingpathways, oxidative stress, intracellular transport, and numerous biosynthetic pathways[74]. About thirty GST genes from various subfamilies have been recognised in mosquitoes[56, 58]. Increased levels of GSTs have been identified mainly in insecticide resistance, they are being over expressed in pyrethroid resistance mosquito populations [58,59,75].

Target-site resistance mechanism

Synthetic insecticides target significant molecules of the nervous system. Organophosphorus (OP) and carbamate (CB) insecticides target synapse acetylcholinesterase (AChE), DDT and pyrethroids (PY) target Na^+ voltage- dependent (NaVdp) channel, while cyclodienes (CYD) targets the GABA receptor of GABA-gated chloride channels (GABAA)[76]. Insecticide resistances as a result of target proteins have been linked to decrease in the targets' binding affinity to insecticide, and not due to increase production of target molecules as was reported for drugs or herbicides[77].



insecticides. Source[78]: David *et al.,* 2013.

Behavioural Resistance

Behavioural resistance is a type of resistance restricted to insects, rodents and mites. This refers to any change in the behaviour of an organism that can help that organism to avoid the harmful effects of chemical pesticides. This mechanism of resistance has been shown for numerous classes of insecticides. The insects might simply avoid feeding after coming across some insecticides, or avoid sites that have been sprayed[79]. This type of resistance does not have same significance as the physiological resistance mechanisms but could be regarded as a factor that contributes to the avoidance of lethal doses of a chemical pesticide[79].

Sequestration

In insects there is significant amplification of metabolic enzymes to about 15% of the total body protein and these enzymes bind to the insecticide, but this binding does not permit the metabolism of the insecticide. The insecticide is sequestered by the enzymes and this renders it harmless to the target organism[79].

CONCLUSIONS

Malaria can be controlled by effective vector control measures that can be achieved by proper use of insecticides as a way of eliminating the disease vector. This should include; education/awareness programmes on the ways to effectively use insecticides in a proper manner so that insecticide resistance can be prevented or reduced. Knowledge and early identification of insecticide resistance can provide assurance for the eradication and elimination of malaria through vector control strategy since the disease is transmitted by the disease vectors. Also, knowing the mechanisms of action of the insecticide detoxifying enzymes will aid in preventing malaria.

New innovations for vector control are recommended in order to maintain the use, and efficacy of chemical insecticides. Eliminating the transmission of malaria through vector control is feasible.

DISCLOSURE OF CONFLICT OF INTEREST

Authors hereby declare that they have no competing interests.

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A STUDY OF ANTI-INFLAMMATORY ACTIVITY OF *JAUNDEA PINNATA* (P. BEAUV.) G. SCHELLENB. (CONNARACEAE) STEM BARK METHANOL EXTRACT AND FRACTIONS

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ABSTRACT

Introduction: *Jaundea pinnata* (P. Beauv.) G. Schellenb. (Connaraceae) is known for its limited medicinal uses including treatment of rheumatoid arthritis and as a repellent.

Methods: The anti-inflammatory effect of crude methanol (MeOH) extract of stem bark and aqueous (AQ) fraction was investigated by both bovine serum albumin and fresh hen's egg albumin *in vitro* models. In addition, rats dosed with MeOH extract (250 - 1000 mg/kg), and standard anti-inflammatory drug, Diclofenac sodium® (50 mg/kg) up to 14 days were investigated for effects on rat paw oedema in a formaldehyde model, as well as alteration of haematological and biochemical parameters.

Results: Oral administration with methanol extract up to Day 14 produced dose-dependent inhibition in rat paw oedema diameter comparable to the standard drug. Extract also gave non-dose dependent inhibitions of erythrocyte sedimentation rate (ESR) which was higher than the standard drug. Effect of extract on haematological parameters and liver function parameters (total protein, albumin and alanine aminotransferase) was not dose-dependent, but comparable to the Diclofenac sodium® at 50 mg/kg.

Conclusion: The results of this study justify the ethnomedicinal relevance of *J. pinnata* leaf in treating rheumatoid arthritis.

Keywords: Anti-inflammatory activity, Connaraceae, *Jaundea pinnata, in vitro, in vivo,* stem bark

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory, and systemic autoimmune disease with characteristic symptoms of pain, swelling, and destruction of cartilage and bone[1]. An estimate of 0.3-1% of the global population are victims of rheumatoid arthritis with the females being more susceptible than males[1]. Demerits of high costs and side effects such as high risks of infections often accompany orthodox treatment strategy involving use of disease modifying anti-rheumatic drugs (DMARDs) or non-steroidal anti-inflammatory drugs (NSAIDs)[1,2]. Current treatment approach through the use of medicinal plants is advantageous being more acceptable, relatively cheaper, safer, and polyherbal formulations are prepared to reduce side effects and increase the benefits[1]. In Nigeria, prevalence studies of RA have been documented in the north east[3], Rivers state[4] and Lagos[2], and preponderance of the female population to the disease were reported.

Furthermore, the increasing prevalence of RA in Nigeria[1] has resulted in the emergence of the country as a leading nation in anti-inflammatory research among developing nations globally.

Jaundea pinnata (P. Beauv.) G. Schellenb (Connaraceae) is a shrub or small tree attaining 6-7m and widely spread from Guinea to South Nigeria, to North East and East Africa[5]. Fruit is poisonous to animals in Kenya, and stem a repellent. The plant is employed in folkloric treatment of rheumatoid arthritis is south west Nigeria (personal communication). Limited published work on *J. pinnata* included the antibacterial activity against plasmid-bearing multiple antibiotics resistant bacteria[6] and recently, anti-inflammatory potential of its leaf[7]. Ethnobotanical surveys of Nigerian anti-rheumatic plants in Lagos[8], Ogun state[9], the South West[10] and North West[11] have been published without mention of *J. pinnata*.

Global reviews of anti-inflammatory activity of medicinal plants in India[12,13] and elsewhere[1, 14] is a pointer to increasing scientific research in this subject area. Furthermore, some Nigerian plants[14,15,16] and others growing elsewhere[17,18,19] have been investigated for anti-inflammatory activities. The recent publication on anti-inflammatory activity of *J. pinnata* leaf[7], and the growing interest at discovering promising anti-inflammatory medicinal plants have stimulated investigation of *J. pinnata* stem bark by *in vivo* and *in vitro* experimental models, and results are published herein.

MATERIALS AND METHODS

Chemicals and reagents

Formaldehyde (Needham Market, Suffolk, England), standard drug, Diclofenac sodium®, sodium chloride and bovine serum albumin (Sigma-Aldrich, USA), disodium hydrogen phosphate (Merck, Germany), potassium dihydrogen phosphate (Riedel-de-Haen, USA), analytical grade chemicals (Merck), analar grade organic solvents (Sigma, UK), hydrochloric acid (BDH, England) and egg albumin from fresh hen's egg were used.

Collection, preparation and extraction of plant material

Fresh stem bark peels of *J. pinnata* were collected from trees growing at the Forestry Research Institute of Nigeria, Ibadan, Oyo State, Ibadan in December 2019, and authenticated (voucher no. FHI 109517) by Mr. Odewo of the Institute. Plant material was cut into small pieces, shade dried at room temperature, ground into coarse powder using locally fabricated machine, and stored in an air tight container at ambient temperature. Ground stem bark (1100 g) was extracted to exhaustion with methanol (MeOH) in a Soxhlet apparatus. Crude MeOH extract (7.63%) (Table 1) was concentrated *in vacuo,* weighed and fractionated with dichloromethane (DCM) to yield DCM and aqueous fractions. Fractions were concentrated to dryness on steam water bath, yield determined (DCM 2.32%, AQ 26.85%) and refrigerated until needed.

Phytochemical screening

Basic phytochemical screening was carried out on the crude methanol extract of the plant according to Evans[20] and the presence of secondary metabolites recorded.

Inhibition of protein denaturation using bovine serum albumin (BSA) model

Evaluation of *in vitro* anti-inflammatory effects of the crude methanol extract and fractions followed the protocol described by Alamgeer *et al*.[18] with some modifications. The reaction mixture consisted of 1 mL of extract (aqueous and DCM fractions) at 100, 200, 500, and 1000 µg/mL) homogenized with 1mL aqueous solution of bovine serum albumin (5% BSA). Solution was adjusted to pH 6.3 by 1N HCL and incubated at 37°C for 15 minutes. The procedure was repeated with Diclofenac sodium® and distilled water which served as positive and negative controls, respectively. Each experiment was replicated thrice. Denaturation of the proteins was caused by placing the mixture in a water bath for 10 minutes at 70°C. Mixture was cooled at the ambient room temperature, then 0.5 mL of phosphate buffer saline was added and the absorbance measured spectrophotometrically (UV/VIS Spectrophotometer UV 752 (D) PEC Medical, USA) at 660nm. Percentage inhibition was calculated from the formula:

$$\frac{\text{Absorbance control - Absorbance test}}{} \times 100$$

Absorbance control

Inhibition of protein denaturation in a fresh hen's egg albumin (FHEA) model

Modified procedure of Alamgeer *et al*.[18] was employed to evaluate the *in vitro* anti-inflammatory effects of the extract and fractions. Reaction mixture (5 mL) consisted of 0.2 mL fresh hen's egg albumin, 2.8 mL phosphate buffered saline (pH 6.4), and 2 mL of different concentrations (100, 200, 500 and 1000 µg/mL) of each test agent. Diclofenac sodium® dissolved in 2 mL double distilled water served as positive control. Mixtures were incubated at 37°C for 15 min and heated on a water bath at 70°C for 5mins. Absorbance was measured spectrophotometrically (UV/VIS Spectrophotometer UV 752 (D) PEC Medical, USA) at 660nm and percentage inhibition calculated from the formula:

$$\frac{\text{Absorbance control - Absorbance test}}{\text{Absorbance control}} \times 100$$

Experimental Animals

Male Wistar rats weighing 180–200g were obtained from the central animal house of Igbinedion University, Okada, Nigeria. They were housed in 12 h light & dark cycle condition (28-35°C) and fed with pelleted chew (Bendel Foods and Flower Meal, Edo state, Nigeria) and water *ad libitum* for 2 weeks. The animals were handled in compliance with the Igbinedion University Okada Ethics Committee on Research in Animals, as well as internationally accepted principles for laboratory animal use and care.

Evaluation of formaldehyde-induced inflammation

The method of Alamgeer *et al*.[18] was adopted in this experiment. Animals were divided into five groups of six animals each. Oedema was induced by injection of 0.1 mL of 1% formaldehyde in normal saline, subcutaneously in the plantar side of the right hind paw of each rat in all groups. Inflammation was assessed by measurement of paw diameter with manual Vernier caliper before formaldehyde injection was given. Induction of oedema was repeated for three consecutive days (Day 1 – Day 3) and diameter of inflammation measured again on the third day of induction. At the end of this period (representing Day 1), groups A and B were orally administered with distilled water (10 mL/kg) and standard anti-inflammatory drug, Diclofenac sodium® (50 mg/kg) respectively for 14 days, while animals in groups C, D and E received 250, 500 and 1000 mg/kg extract for same period, respectively. Diameter of inflammation was measured on Days 1, 7 and 14 to determine anti-inflammatory effect. On Day 14, animals were anaesthetized in a jar containing diethyl-soaked cotton wool, and blood collected by cardiac puncture into heparinized tubes for haematological, erythrocyte sedimentation rate (ESR) and liver and renal enzymes assays.

Percentage inhibition of the inflammation was calculated from the formula:

$$\% \text{ inhibition} = (Dc - Dt)/ Dc \times 100$$

Where Dc is the average inflammation (hind paw edema) of the negative control group of rats at a given time; and Dt is the average inflammation of the drug- treated (i.e. extract or reference drug) rats at the same time.

Determination of serum liver and renal functions

Treated rats from the formaldehyde experiment were sacrificed on Day 14 by cervical decapitation after mild diethyl ether anaesthesia, a ventral longitudinal abdominal incision made and the liver and kidney were identified and immediately dissected out as described by Adeneye *et al*[21]. These dissected organs were separately rinsed and homogenized in ice-cold 0.01M Tris HCL buffer, pH 7.4 to give a 10% homogenate, and used to estimate liver and renal function parameters. Serum albumin (ALB), total protein (TP), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT) and globulin (GLOB) as liver function parameters, and serum urea and creatinine (CRT) as renal function parameters, were assayed for using Roche and Cobas commercial test kits and Roche/Hitachi 904 Automated Analyzer.

Haematological analysis
The full hematological parameters evaluated on Day 14 after the formaldehyde experiment according to Adeneye *et al*[21] include haemoglobin (Hb), white blood cell (WBC) count, neutrophil and packed cell volume (PCV) were assayed using Roche and Cobas commercial test kits and Roche/Hitachi 904 Automated Analyzer.

Estimation of erythrocyte sedimentation rate
Erythrocyte sedimentation rate (ESR) was assessed on Day 14 after the formaldehyde experiment according to Chitme and Patel[22] in Westergren pipettes having 2.5 mm internal diameter, 300 mm length, and 1 mL capacity and ESR stands. Blood was collected from all the arthritic and non-arthritic animals used in the study by cardiac puncture. Percentage (%) inhibition was calculated from the formula:
= (Dc – Dt)/ Dc × 100, where Dc is the average ESR count in the negative control group of rats at a given time; and Dt is the average count in the treated group (i.e. extract, fractions or standard drug) of rats at the same time.

Statistical analysis
Results of triplicate determinations were expressed as mean±SEM and subjected to statistical analysis using one-way analysis of variance (ANOVA, Krusta-Wallis test), and difference at $P<0.05$ were considered significant.

RESULTS AND DISCUSSION
Phytochemical screening revealed the presence of alkaloids, tannins, flavonoids, saponins and steroids in the plant (Table 2). Both MeOH extract and aqueous (AQ) fraction when tested at 100-1000 µg/mL, produced concentration-dependent inhibition of heat-induced protein denaturation in bovine serum albumin (BSA) model (Table 3), but were incomparable ($p<0.05$) to standard drug, Diclofenac sodium. The fraction (34.12% inhibition) was less active than MeOH extract (57.15% inhibition) at 1000 µg/mL. In the fresh hen's egg albumin (FHEA) model, only AQ fraction was concentration-dependent up to 500 µg/mL in inhibition of protein denaturation (Table 4). Its activity was significant ($p<0.05$) and less (38.51% inhibition at 1000 µg/mL) when compared to standard drug. In considering both protein denaturation experiments, since both active AQ fraction (38.51% inhibition at 1000 µg/mL) and standard drug (96.46% inhibition) gave higher inhibitions in FHEA model, this model may appear to be a more effective tool in determining anti-inflammatory potential of *J. pinnata* stem bark. Limited amount of DCM fraction did not permit its use for various assays. This result is a reversal of the observations recently reported for *J. pinnata* leaf which showed better activity in the BSA model[7]. Result of this present study is in agreement with the finding of Shilpa *et al*[23] who observed better inhibition of protein denaturation in the FHEA model for *Hibiscus hispidissimus*. Shilpa *et al*[23] also suggested protein denaturation as one of the main causes of rheumatoid arthritis due to the production of auto antigens. Mechanism of denaturation has been attributed to alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding[18].

Table 1: Profile of *Jaundea pinnata* stem bark and yield of extract and fractions

Plant	Voucher number	Morphological part	Aspect	Location
Jaundea pinnata	FHI 109517	Stem bark	Shrub	Forestry Research Institute of Nigeria, Ibadan

Yield		
Crude methanol extract	Aqueous fraction[+]	Dichloromethane[+]
7.63%	26.85%	2.32%

[+]Relative to crude extract

Table 2: Phytochemical screening of crude methanol extract

Phytometabolites	Result
Alkaloids	+
Tannins	+
Anthraquinone glycosides	-
Cardiac glycosides	-
Flavonoids	+
Terpenoids	-
Steroids	+
Saponins	+

Present (+), Absent (-)

Table 3: Effect of *Jaundea pinnata* stem bark extract and fraction on protein (bovine serum albumin) denaturation

Tested agent	Inhibition (%)			
	Concentration (µg/mL)			
	100	200	500	1000
Crude MeOH extract	32.45±1.92*	34.74±3.75*	48.31±0.33	57.15±0.12
AQ fraction	14.90±3.42*	19.83±3.45*	25.90±2.81*	34.12±0.27*
Diclofenac	59.45±0.12	75.36±0.95	77.85±0.51	80.31±0.92

The values above are mean of three replicates n=3, mean ± SEM, values with superscript* indicate significant difference at $p < 0.05$ compared to control (diclofenac), while values with no superscript indicate no significant difference when compared to control (diclofenac) at $p<0.05$

Table 4: Effect of *Jaundea pinnata* stem bark extract and fraction on protein (fresh hen's egg albumin) denaturation

Tested agent	Inhibition (%)			
	Concentration (µg/mL)			
	100	200	500	1000
Crude MeOH extract	39.99±2.44*	37.92±4.42*	49.35±2.21*	48.41±0.25*
AQ fraction	35.14±2.81*	38.03±0.49*	41.06±2.27*	38.51±0.28*
Diclofenac sodium®	67.36±4.22	69.44±1.39	94.86±1.25	96.46±0.55

Values are mean of three replicates (n=3), Mean ± SEM. Values with superscript* indicates significant difference at $p<0.05$ when compared with control (diclofenac), while values with no superscript

indicate no significant difference when compared with control drug (diclofenac) at $p<0.05$ using One-way ANOVA (Krustal Wallis test)

A comprehensive review of mechanisms of action of anti-inflammatory plants has been published[1]. Thus, prevention of protein denaturation by *J. pinnata* could lend credence to its anti-inflammatory effect. The results herein are in consonance with those reported by Akinnawo *et al*.[16] and Mbiantcha *et al*[24] for *Alstonia boonei* and *Piptadeniastrum africanum*, respectively. Furthermore, there appear to be no significant difference in protein denaturation effect of MeOH extract at all concentrations in both BSA and FHEA assays. Anti-inflammatory activity displayed by *J. pinnata* could be attributed to alkaloids, tannins, flavonoids, steroids and saponins[11,14] detected in this study. From this study, it may be necessary to subject AQ fraction to further phytochemical work to unravel the bioactive compounds that will serve as lead in the development of anti-inflammatory drugs.

In the haematological parameters, MeOH extract gave comparable PCV (45.20-47%), haemoglobin (15.06-15.68 g/dL), neutrophil (60.20-64 cells/mm^3) and lymphocyte (31-34.40 cells/mm^3) counts in a non-dose-dependent manner at tested doses (Table 5). However, these data were comparable to the standard drug. Extract gave higher WBC count than Diclofenac sodium® at all doses, but was not dose-dependent. PCV and haemoglobin counts can be considered to be near normal with reference to untreated control. Elsewhere, elevated levels of serum haemoglobin were observed to aid recovery from arthritis[19,22,24]. In this study, the near normalisation of PCV and haemoglobin parameters by MeOH extract (with reference to untreated arthritic control) and comparable levels of all indices (except WBC) with standard drug, is a pointer to anti-inflammatory potential of *J. pinnata* stem bark. These findings are consistent with those of Gbolade *et al* for *J. pinnata* leaf[7] and Adeneye *et al*[21].

Table 5: Effect of *Jaundea pinnata* stem bark extract and aqueous fraction on haematological parameters on rats

Tested agent	WBC (cells /µL)	LYMP (cells/µL)	MONO (cells /µL)	GRAN (cells /µL)	RBC (10^3 cells /µL)	HGB (g/dL)
Negative Control (distilled water, 10 mL/kg)	5200±1140	3170±670	1300±380	730±90	6730±80	14.53±0.66
Positive Control (50 mg/kg)	6000±1720	4600±2510	900±150	400±32	7290±4510	16.60±3.99
Crude MeOH extract (250 mg/kg)	6100±1200	4500±1100	1030±30	500±100	6770±330	14.40±0.80
Crude MeOH extract (500 mg/kg)	10,770±920*	9000±3420*	1600±11	1000±20	6980±1110	13.80±2.81
Crude MeOH extract (1000 mg/kg)	11,600±920*	6430±150	2330±630	2000±450	6820±140	14.67±0.48
AQ fraction (100 mg/kg)	8870±2320	4530±960	1100±100	2200±1140	6530±460	13.97±1.24
AQ fraction (200 mg/kg)	6530±930	4470±570	2130±640	930±230	6070±50	13.50±0.3

WBC, white blood cells; LYMP, lymphocytes; MONO, monocytes; GRAN, granulocytes; RBC, red

blood cells; HBG, mean corpuscular haemoglobin. Values with superscript* indicate significant difference ($p<0.05$) relative to negative control.

Inhibition of rat paw oedema increased up to Day 7 with tested doses of MeOH, and fell by Day 14 (Table 6). Extract at lower doses, 250 and 500 mg/kg, were equipotent and gave maximal inhibition (56.30%) on Day 7. However, dose-dependent inhibition of inflammation was recorded with MeOH extract only on Day 14. All tested doses of extract produced insignificantly different inhibitions on Day 7 (54.76-56.30%) and Day 14 (44.80-50.40%) which were comparable to Diclofenac sodium. Results obtained in this study indicate anti-inflammatory potential of *J. pinnata* stem bark, and are in consonance with those documented for *J. pinnata* leaf[7] after 14 days, and for *Alchornea cordifolia*[21] and *Xanthium srtumarium*[17] after 28 days. Reduction of localised inflammation and biphasic pain induced by formaldehyde by *J. pinnata* extract possibly suggests[18] central and peripheral inhibition of the arachidonic pathway. Other potent anti-inflammatory plants have been previously reported[18,19,24].

Table 6: Effect of *Jaundea pinnata* stem bark methanol extract and aqueous fraction on formaldehyde-induced inflammation

Tested agent	Degree of inflammation (mm)/ % inhibition[#]			
	Day 0	Day 1	Day 7	Day 14
Negative control (distilled water, 10 mL/kg)	8.30±1.20	10.67±0.47	10.67±0.47	10.60±0.79
Positive control (Diclofenac, 50 mg/kg)	8.00±0.68 (3.61)	9.87±0.41 (7.49)	9.87±0.41 (10.13)	9.17±0.43 (13.49)
Crude MeOH extract (250 mg/kg)	6.07±0.07 (26.86)	n. d	9.60±0.45 (10.02)	7.13±0.09 (32.73)
Crude MeOH extract (500 mg/kg)	4.77±0.72* (42.53)	13.07±1.13 (-22.49)	9.40±0.31 (11.90)	6.90±0.80* (34.90)
Crude MeOH extract (1000 mg/kg)	5.67±1.58* (31.68)	12.60±1.53 (-18.08)	9.27±0.12 (13.12)	5.93±1.16* (44.05)
AQ fraction (100 mg/kg)	6.27±1.30 (24.45)	10.67±0.33 (0)	8.67±0.12 (18.74)	5.17±0.08* (51.22)
AQ fraction (200 mg/kg)	4.87±0.91* (41.32)	11.10 0.95 (-4.02)	7.87±0.17* (26.24)	5.10±0.10* (51.88)

Values above are mean of six replicates. n=6 (±SEM). Values with superscript * indicate significant difference at $P<0.05$ when compared to negative control using ordinary One –way ANOVA. [#]inhibition (%) relative to negative control, n.d = not determined.

ESR denotes haematological index for the diagnosis and prognosis of infections and inflammatory diseases[18]. From this study, crude MeOH extract at 250 - 1000 mg/kg did not give dose-dependent inhibition in ESR (39.97-42.70%) in arthritic rats, but was more active than the standard drug (21.55%) (Table 7). This is contrary to an earlier report documented for *J. pinnata* leaf[7]. Tested doses of MeOH extract gave varying responses to biochemical parameters considered in this study.

On the renal function parameters, near normalized serum levels of urea (63.36 mg/dl) and CRT (0.54 mg/dl) in extract-treated rats at 1000 mg/kg with untreated arthritic control were observed (Table 8). Extract was comparable to standard drug in serum urea level but yielded less CRT. Among the renal function indices investigated, serum ALT, ALP and TP observed in extract-treated rats (1000 mg/kg) were comparable to Diclofenac sodium, while serum GLOB was less. Moreover, dose-dependent increase in serum AST in extract-treated animals (77.30-96.18 U/I) which was higher than Diclofenac sodium was evident. Findings of some of the liver and renal function parameters investigated in this study are in agreement with an earlier report on *J. pinnata* leaf[7] and for other plants[21,23,24], and suggest potential for *J. pinnata* as an anti-inflammatory agent. Raceline *et al*.[19] have linked significant increase in the activity of liver function enzymes such as AST and ALT in this study in untreated arthritic animals to destructive effects of inflammation inducing agent on liver cells expressed by significant release of transaminases, and on kidney by renal cells necrosis that result in to glomerular filtration impairment. Therefore, *J. pinnata* stem bark may have potential as an anti-inflammatory agent since comparable activities with standard drug were observed for TP (8.36 mg/dl), urea (63.36 mg/dl), ALT (32.45 U/I) and ALP (247.49 U/I).

Table 7: Effect of *Jaundea pinnata* stem bark methanol extract administered for 14 days on erythrocyte sedimentation rate (ESR) in rats

Tested agent	Erythrocyte sedimentation rate (mm/hr)/ % inhibition[#]
Negative control (distilled water; 10 mL/kg)	7.33±3.84
Positive control (Diclofenac; 50 mg/kg)	5.75±0.85 (21.55%)
Crude methanol extract, 250 mg/kg	4.20±0.58* (42.70%)
Crude methanol extract, 500 mg/kg	4.40±0. 51* (39.97%)
Crude methanol extract, 1000 mg/kg	4.20±0.37* (42.70%)

Values above are mean of six replicates. n=6 (±SEM). Values with superscript * indicate significant difference at $P<0.05$ when compared to negative control using ordinary One –way ANOVA, [#]inhibition (%) relative to negative control.

Table 8: Effect of *Jaundea pinnata* stem bark methanol extract on some biochemical parameters

Parameters	Control		Crude methanol extract		
	Negative control (Distilled water, 10 mL/kg)	Positive control (Diclofenac sodium, 50 mg/kg)	250 mg/kg	500 mg/kg	1000 mg/kg
Liver function parameters					
TP (mg/dL)	9.60±0.63	8.35±0.91	6.75± 0.62	6.42±1.35	8.98±1.29
ALB (mg/dL)	4.49±0.72	3.74±0.18	2.80±0.19	4.82±0.25	5.27±1.28
GLOB (mg/dL)	4.21±1.10	4.49±1.20	3.97±0.98	4.29±2.11	3.29±0.91
AST (U/I)	82.89±15.51	68.26±3.77	77.40±2.12	78.39±7.39	96.18±5.94
ALT (U/I)	39.30±3.13	28.90±4.21	41.80±2.29	43.28±6.48	32.45±4.38
ALP (U/I)	222.60±79.35	259.00±34.38	249.89±75.39	352.90±45.76	247.49±66.31
Renal function parameters					
CRT (mg/dL)	0.63±0.12	1.52±0.12	0.54±0.23	0.44±0.17	0.54±0.19
Urea (mg/dL)	67.27±4.23	56.20±3.32	74.10±3.41	79.24±6.24	63.36±4.29

TP, Total protein; ALB, albumin; GLOB, globulin; CRT, Creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

CONCLUSION

This study is a follow up to that of anti-inflammatory potential of *J. pinnata* leaf, and compliments most of the earlier findings. The tested agents from *J. pinnata* stem bark inhibited protein denaturation more effectively in the BSA model, and thus may have anti-inflammatory effect which was less than that of the standard drug, Diclofenac sodium®. From this present study, MeOH extract was comparable to standard drug in most of the haematological indices, some biochemical parameters and reduction of rat paw oedema, was more active in ESR inhibition and incomparable in inhibition of protein denaturation. and hence could serve as an alternative plant remedy in the treatment of rheumatoid diseases. AQ fraction was less potent than extract in inhibition of protein denaturation and may not require further phytochemical investigation. Use of medicinal plants with anti-inflammatory effects like *J. pinnata* stem bark in this study, would be preferred to the non-steroidal anti-inflammatory drugs with a history of severe side effects. This present investigation has provided an update to the compendium of anti-inflammatory plants of Nigeria.

Conflict of interest

The authors declare no conflict of interest in this work.

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AAG was the research leader and wrote the manuscript, OPO and AOA undertook the experiments.

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ANTI-INFLAMMATORY, ALPHA-AMYLASE INHIBITION AND ANTI-OXIDANT ACTIVITIES OF THE N-HEXANE EXTRACT OF *MAESOBOTRYA DUSENII* HUTCHINSON (EUPHORBIACEAE) LEAVES

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ABSTRACT

Introduction: Sequel to earlier report on the antidiabetic activity of the N-hexane extract of *Maesobotrya dusenii*, this study investigates the anti-inflammatory, alpha-amylase inhibition, fibrinolytic and anti-oxidant activities of the n-hexane extract of *M. dusenii* leaves.

Methods: The extract was screened for phytochemicals using standard methods and then separated on column chromatography using gradient elution of hexane, dichloromethane and ethanol. Pooled column Fractions were assayed for alpha-amylase inhibition, membrane stabilizing effect for anti- inflammatory, Prasad assay for fibrinolytic activity and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) reduction for anti-oxidant activity.

Result: Phytochemical screening showed that the extract had de-oxysugars, saponins, triterpenoids and steroids. Column fractions were pooled to six fractions (F1 - F6). Apart from fraction F4, all the other fractions exhibited alpha-amylase inhibition. None of the fractions exhibited significant fibrinolytic activity. Fractions F1, F4 and F6 exhibited anti-inflammatory activities. Only fractions F4, F5 and F6 showed anti-oxidant activity. There was a significant difference in anti-inflammatory activity among the fractions ($p < 0.05$) and there was a significant and dose dependent increase in anti-oxidant activity of the different concentrations of the fractions ($p < 0.05$).

Conclusion: The study shows that the column fractions of n-hexane extract of *M. dusenii* leaves have alpha-amylase inhibitory and anti-inflammatory activity.

Key words: *Maesobotrya dusenii,* Euphorbiaceae, Alpha-amylase, Anti-inflammatory, Anti-oxidant, Membrane stabilizing assay,

INTRODUCTION

Diabetes Mellitus is a metabolic disorder characterized with chronic hyperglycemia and it is due to impaired insulin secretion with or without insulin secretion. Type 1 diabetes where the β-cells of the pancreas are destroyed and so do not produce insulin and type II diabetes where there is β-cell dysfunction leading to impaired insulin secretion and insulin resistance are the 2 main types of diabetes [1, 2]. Complications of diabetes are a major fear of patients of diabetes and their care-givers. Type 1 and type 2 diabetes, as well as diabetic complications, are linked to immunologic abnormalities. T cell abnormalities are thought to be the primary source of autoimmune illness in type 1 diabetes, which results in pancreatic islet

loss. Inflammation and monocyte activation are thought to play a role in increasing insulin resistance in type 2 diabetes and may contribute to islet cell loss of insulin secretory function [3]. Anti-inflammatory drugs are chemicals that help the body reduce inflammation (redness, swelling, and pain). Reactive oxygen species (ROS), or potentially reactive oxygen derivatives, such as O_2, H_2O_2, and OH, are continuously produced inside the human body as a result of exposure to a variety of exogenous chemicals in our environment and some endogenous metabolic processes [4]. When there is an upsurge of ROS and the antioxidants present in the body are overwhelmed, various biomolecules, such as proteins, lipids, lipoproteins, and DNA, are easily attacked and oxidised by the ROS [4]. This oxidative damage is a significant etiological element in a variety of chronic human diseases, including diabetes[4]. The creation of inflammatory mediators is also stimulated by oxidative stress, and inflammation, in turn, increases the formation of ROS [5]. An antioxidant is a chemical that prevents other molecules from being oxidised in processes that involve the transfer of electrons and hydrogen, resulting in the formation of free radicals. People prefer natural antioxidants because of concerns about the harmful and carcinogenic consequences of synthetic antioxidants – [6, 7]. Thrombotic mortality occurs in 80% of type 2 diabetes individuals [8]. Several endothelial, inflammatory, and pro-coagulant biomarkers, such as VWF (Von Willebrand Factor), inflammatory (IL-6 and TNF-– Tumor Necrosis Factor-alfa), and pro-coagulant (D-Dimer and PAI1 – Plasminogen Activator Inhibitor-1), have been found to be elevated in diabetes individuals [9]. These biomarkers could be indicators of deep venous thrombosis or other cardiovascular diseases [10]. Thrombolytic drugs convert inactive plasma plasminogen to plasmin, which then breaks down fibrin into soluble components, restoring normal blood flow (fibrinolysis) [11] Alpha-amylase (-1, 4-glucan-4-glucanohydrolases) is a key secretory product of the pancreas and salivary glands that aids in the digestion of starch and glycogen in microbes, plants, and higher animals. The alpha-amylase enzyme catalyses the first stage of starch hydrolysis, converting to maltose, maltotriose, and branching oligosaccharides of alpha-(1-6) and alpha -(1-4) oligoglucans. These are acted on by alpha-glucosidases and further reduced to glucose, which enters the bloodstream after absorption. Delaying the process of carbohydrate hydrolysis reduces glucose level after eating [2]. An ideal therapy for diabetes will be one that can reduce the level of glucose in the blood, prevent inflammation which could lead to destruction of the β-cells of the pancreas, have an anti-oxidant activity and prevent other complications of diabetes. Due to the fact that plants are made of several constituents -flavonoids, saponins, phenolics, alkaloids etc., they have a great potential to have natural multi beneficial activities compared with conventional drugs. *Maesobotrya dusenii* is of the Euphorbiaceae family.

It is a tree with a dense crown that grows up to 15 m tall in the rain-forest areas of South Nigeria, West Cameroons, and Fernando Po, as well as East Cameroon and Equatorial Guinea. The fruit has a sourish-tasting edible pulp that is used to create jam in Gabon. Its parts have been found to have anti-microbial, anti-fungal, anti- diabetic and anti-hyperlipidemic activities [12-14]. Sequel to the study carried out by Suleiman and Abo [12, 13]. The aim of this research was to investigate the alpha-amylase inhibition, the fibrinolytic activity, anti-oxidant and anti-inflammatory activities of fractions obtained from the n-hexane extract of *Maesobotrya dusenii* leaves.

MATERIALS AND METHODS

Plant Materials

The leaves of *Maesobotrya dusenii* were collected from the Etche region of River State in April 2016. Dr. N. L. Edwin-Wosu of Plant Science and Biotechnology Herbarium at the University of Port Harcourt identified and verified the fresh leaves. A herbarium specie was deposited at the Department of Pharmacognosy and Phytotherapy's Herbarium, Faculty of Pharmaceutical Sciences, Port Harcourt University with a voucher number UPHE0475. The leaves were dried at room temperature, crushed, and stored in an airtight container.

Methods

Extraction and Column Fractionation

A 5 Kg of dried, pulverized leaves of *M. dusenii* were macerated in n-hexane for 24 hours with intermittent shaking. This process was repeated for 3 days. The solvent was changed every 24 hours and the filtrate from each day was pooled. The pooled extract was filtered and concentrated *en vacuo* using the rotary evaporator (England lab science) at 40 °C. This extract was stored in a desiccator until needed. As previously reported [15, 16], the n-hexane extract was fractionated on column chromatography packed with silica gel G (60-120 mesh) and eluted with gradient mixtures of n-hexane, dichloromethane, and ethanol. On a pre-coated analytical thin layer chromatography (TLC) GF254, fractions were developed in 100 % dichloromethane, monitored and bulked into six fractions (F1 through F6). Fractions were assayed independently.

Phytochemical Screening

This was carried out on the n-hexane extract using standard methods[17-19].

In vitro anti-inflammation assay

The red blood membrane stabilization assay was used to test for anti-inflammatory activity of the n-hexane extract and fractions of the leaves of *Maesobotrya dusenii* [13]. Fresh human blood of 5 mL was transferred to an ethylene di-amine tetra-acetate (EDTA) centrifuge tube to prevent clotting. It was centrifuged at 2000 rpm for 5 min to remove the supernatant. The erythrocytes remaining were washed three times with equal volume of normal saline and centrifuged. Isotonic phosphate buffer (pH 7.4) was used to constitute the erythrocytes as a 40 % v/v suspension. A five (5) mL of buffer solution was used to make a suspension of 20, 40, 60 and 80 µg of each of the fractions F1 – F6. A set of four (4) centrifuge tubes per concentration was prepared, (0.5 µg/mL) erythrocyte suspension was added into each tube and gently mixed. A pair of the tubes from each set was incubated at 54 °C for 20 min in a regulated water bath. The other pair was maintained at 0-4 °C in ice for 20 min. The reaction mixture for each of the four tubes was centrifuged at 1000 rpm for 3 min and the absorbance of the supernatant was measured at 540 nm. A five (5) mL of diclofenac (100 µg/mL) was also prepared and tested as the reference. Negative control was buffer solution without diclofenac and extracts. Percentage inhibition of haemolysis was calculated thus:

$$100 \times \left(1 - \frac{OD_2 - OD_1}{OD_3 - OD_1}\right)$$

OD_1 = Unheated test sample, OD_2 = Heated test sample and OD_3 = Heated control sample

Alpha-amylase Inhibition Assay

The modified chromogenic method previously described using Dinitrosalicyclic acid (DNSA) reagent was used [13] . DNSA reagent and 20nM phosphate buffer were prepared as described by Kamtekar, Keer and Patil [2]. Porcine amylase (EC3.2.1.1, type VI, Sigma) was dissolved in ice cold distilled water to give a 5 unit/ml concentration. Potatoe starch (0.5% w/v) in 20 mM phosphate buffer (pH 6.9, NaCl -6.7 mM) was used as the substrate solution. Plant extracts of 1 to 5 µg/ml each were dissolved in 1-ml Tween 80 for each fraction 1 to 6. Each prepared plant fraction was mixed with 1 ml of 0.5% w/v alpha-amylase in a tube and incubated for 10 min at 25 °C. One (1) mL of potato starch solution was introduced into the tube and further incubated at 25 °C for 10 min. Then, one (1) ml of prepared DNSA was added to the tube and boiled at 90 °C in a water bath (Water bath TT-6, Techmel and Techmel, USA) for 15 min. The reaction was left to cool and diluted with 10 ml of de-ionized water. This method was repeated for all prepared plant fractions to yield the test products. By omitting alpha- amylase, a blank was prepared, while the negative control was prepared by replacing the plant extracts with their solvent, (Tween 80). Commercially available acarbose was used as a positive control. The absorbance of the test products, negative control, positive control and blanks were measured at 540 nm using the Uv-Vis spectrophotometer (LobachemiePVT Ltd, India). Percentage alpha-amylase inhibition was calculated as follows:

$$Percentage\ inhibition = \frac{Absorption\ of\ control - Absorption\ of\ test}{Absorption\ of\ control} \times 100\%$$

Fibrinolytic assay

The Prasad method for the determination of fibrinolytic activity earlier described was used[11,20].

The commercially available streptokinase (1,500,000 IU) was carefully mixed with five (5) mL of sterile injection water. 100 µL (30,000 IU) of streptokinase were obtained from this stock. Venous blood (5 mL) was obtained from healthy volunteers and transferred into already weighed 500 µL – Eppendorf tubes and incubated at 37 °C for 45 min. A Pasteur pipette was used to remove serum completely from each tube after clotting had taken place. The clot remaining and tube were weighed. The plant fractions were each dissolved with dichloromethane and introduced into the different Eppendorf tubes containing clots, 100 µL of streptokinase was used as a positive control while water and dichloromethane each without streptokinase or plant fraction respectively were used as negative controls. All tubes were incubated at 37 °C for 90 min. After incubation, fluids obtained were completely removed and the tube plus clot for each sample was reweighed to check for the difference in clot weight. In all cases clot weight was obtained using the formula below:

$$Clot\ weight = Weight\ of\ clot + tube - weight\ of\ empty\ tube$$

The difference in clot weight before and after the addition of the plant fractions and streptokinase was expressed as a percentage of clot lysis using the formula below:

$$Percentage\ weight\ of\ blood\ clot\ lysed = \frac{Average\ difference\ in\ clot\ weight}{Average\ original\ weight\ of\ clot} \times 100\%$$

Antioxidant assay

The modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) model of the free radical scavenging activity method used has been earlier described by Okonkwo *et al* [21]. Weighed 5mg of DPPH was transferred to a 100ml conical flask covered with foil paper. 100ml of methanol was added to the reagent in aliquots, with intermittent shaking at intervals. The solution was kept in the dark at room temperature. Using dichloromethane, different concentrations of fractions 1 -6 obtained from column chromatography separation were produced (200, 400, 600, 800, 1000 µg/ml). After that, 2.0 mL of 50 g/mL DPPH in methanol was added to the various concentrations of the test samples and left in the dark for 30 minutes. After calibrating the instrument with a blank of 2mls methanol and 2mls dichloromethane (1:1 v/v), the absorbance of the various test samples and the standard was measured using a UV spectrophotometer (517nm). The negative control was the absorbance of DPPH: Dichloromethane (1:1v/v). Ascorbic acid was employed as a positive control and was produced at concentrations of 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 g/ml. The percentage inhibition of the various concentrations was calculated using the method below:

$$Percentage\ inhibition = \frac{Absorption\ of\ control - Absorption\ of\ test}{Absorption\ of\ control} \times 100\%$$

The anti-oxidant activity was recorded as the percentage reduction in the absorbance of DPPH by the test samples or standard.

Statistical Analysis

Values were expressed as mean ± SEM. A one-way ANOVA and two – way ANOVA tests were performed using the GraphPad Prism version 6 and Ms Excel 2019. A p-value lower than 0.05 was considered significant.

RESULTS

Percentage yield

The percentage yield of the hexane extract as shown in Table 1 falls in line with the result obtained in the study of Suleiman and Abo [12] where the yield was 1.32%. where the yield was 1.32%.

Table 1: Yield of the fractions of *M. dusenii*

Sample	Weight (g)	Yield (%)
n-hexane extract	52.804	1.06
F1	4.85	9.18
F2	2.3	4.36
F3	8.75	16.57
F4	1.95	3.69
F5	3.55	6.72
F6	9.05	17.14

F= fraction

Phytochemical Screening

The qualitative phytochemical test in Table 2 shows that de-oxysugars, saponins. triterpenoids and steroids were present in the hexane extract similar to earlier studies[12,14].

Table 2: Phytochemical components of n-hexane leaf extract of *M. dusenii*

Chemical constituent	**Result**
Alkaloid	-
Tannins	-
Saponins	+
Flavonoids	-
Anthraquinones	-
	-
Triterpenoids	+
Steroids	+
Deoxysugars	+

+ = Present - = Absent

Membrane stabilizing effect of the fractions from n-hexane extract

In Table 3, fraction F1 demonstrated dose-dependent efficacy with the greatest percentage inhibition of 97% at 80 µg/ml. F2 inhibited in a dose-dependent manner, however there was no statistical significance between the concentrations employed ($p > 0.05$). F5 and F6 showed a decrease in percentage inhibition as concentration increased. The anti-inflammatory activity of the fractions of the n-hexane extract were comparable with that of the standard, Diclofenac (100 µg/mL). Fractions exhibited IC_{50} in increasing order of F1<F6<F4<F2<F5<F3 ($p < 0.05$).

Table 3: Membrane stabilizing effect of the fractions from n-hexane extract of *M. dusenii*

Fraction	Concentration µg/mL	% Inhibition	IC_{50}
F1	20	62	8.2748
	40	90	
	60	93	
	80	97	
F2	20	5	16.4497
	40	7	
	60	12	
	80	21	
F3	20	70	86.6439
	40	81	
	60	88	
	80	94	
F4	20	91	12.9224
	40	93	
	60	95	
	80	91	
F5	20	82	20.0249
	40	49	
	60	35	
	80	20	
F6	20	99.8	8.3869
	40	89	
	60	86	
	80	82	

Alpha-amylase Inhibition effect of the fractions from n-hexane extract

Except for fraction F4, all of the fractions of the n-hexane extract showed alpha-amylase suppression, as shown in Figure 1. Figure 1, shows that fractions F3 and F5 exhibited a substantial inhibitory ability at doses of 4 µg/mL and 5 µg/mL, respectively. Figure 2 demonstrates that at their peak activity, F3 and F5 exhibited no significant difference in alpha-amylase inhibition when compared to acarbose (the standard).

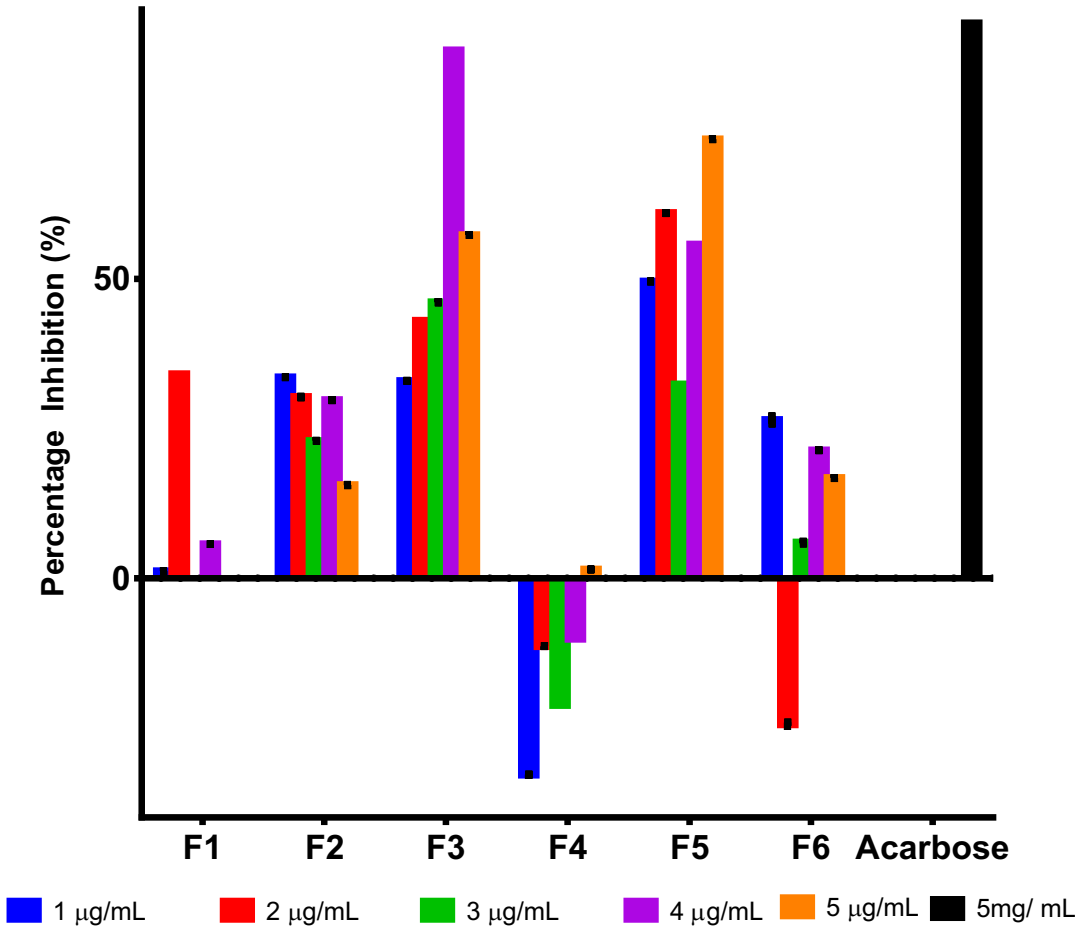

Figure 1: Alpha amylase inhibition Assay. Acarbose was used at 5mg/mL.

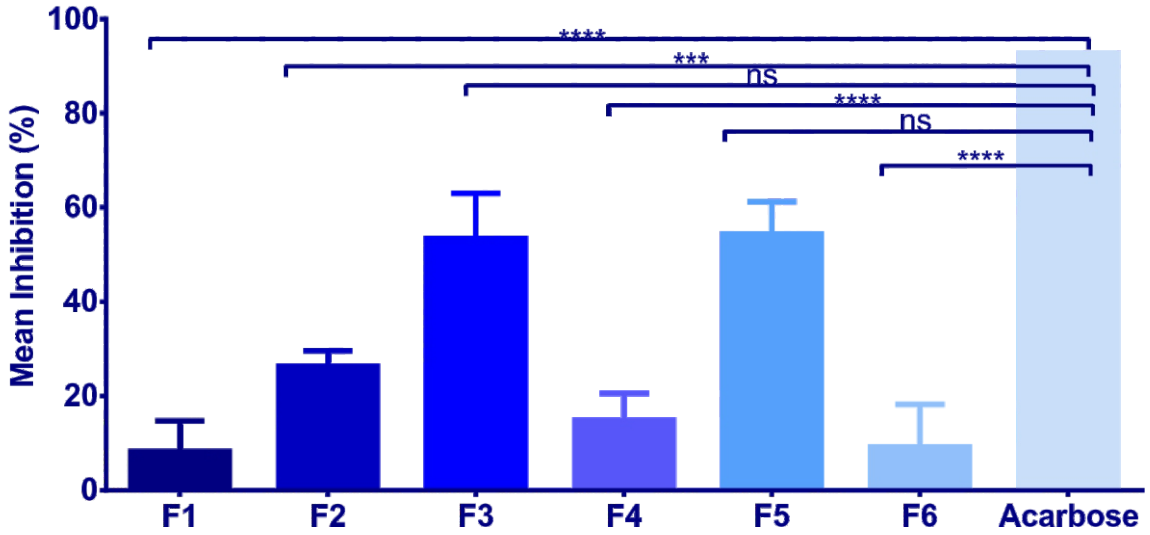

Figure 2: One- way ANOVA with Tukey post hoc analysis comparing the mean alpha amylase inhibition of each fraction with Acarbose (commercial antihyperglycemic drug) at $P = 0.05$

Key: F= fraction; The statistical difference between Acarbose and the fractions is represented by each line with * showing significant difference and 'ns' meaning not significant.

Fibrinolytic effect of the fractions from n-hexane extract

The fractions F1 to F3 exhibited no thrombolytic activity in Table 4. Although fraction F4 exhibited the highest fibrinolytic activity when compared with the other fractions, it fell short of the 30 % cut off.

Table 4: Fibrinolytic effect of the fractions from n- hexane extract of *M. dusenii*

Sample	Average difference in weight ± SEM	Average weight of clot	Percent clot lysis (%)
Streptokinase	0.331 ± 0.038	0.496	92.24
Dichloromethane	0.097 ± 0.049	0.499	19.40
Water	0.356 ± 0.021	6.386	54.65
F4	0.069 ± 0.066	0.485	14.24
F5	0.007 ± 0.009	0.379	1.79
F6	0.026 ± 0.022	0.477	5.49

Key: A percentage clot lysis of 30% and above is taken as significant. SEM = standard error of mean

DPPH reduction effect of the fractions from n-hexane extract

Figure 3 shows the result of the antioxidant assay. Fractions F1, F2, and F3 showed no anti-oxidant activity while fractions F4, F5 and F6 showed a dose - dependent anti-oxidant activity as they all reduced DPPH. The result indicated a significant difference ($p < 0.05$) in the antioxidant activity affected by varying concentrations of n-hexane fractions (within group), but no significant difference in anti-oxidant activity ($p > 0.05$) among the different fractions (between groups).

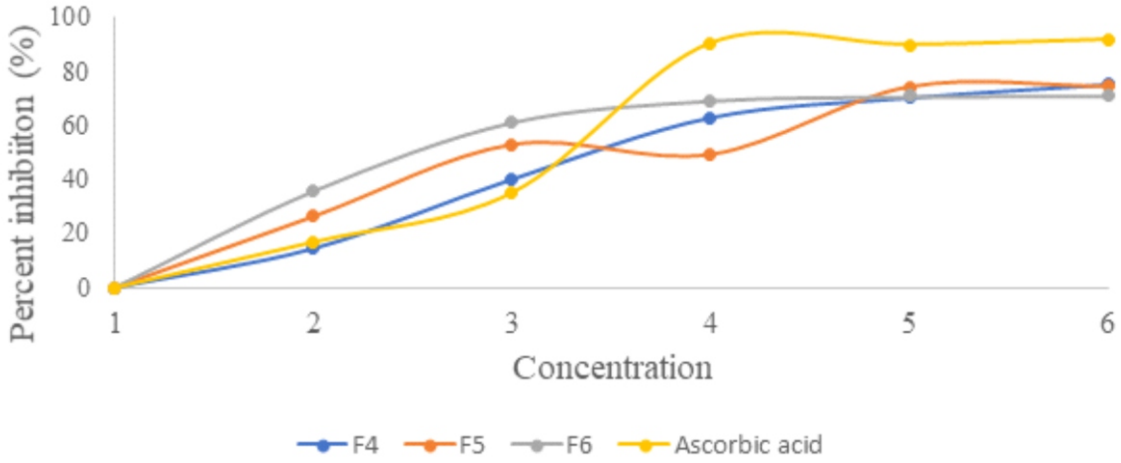

Figure 3: Anti- oxidant effect of fractions 4 to 6.
Key: Concentrations 1, 2,3, 4, 5, 6 = 0, 200, 400, 600, 800 and 1000 µg/mL respectively for the fractions. For ascorbic acid, the concentrations 1, 2, 3, 4, 5, 6 = 5, 10, 20, 40, 80 µg/mL respectively.

DISCUSSION

The control of diabetes with drugs falls in to several mechanisms of action and one of the mechanisms is the alpha-amylase inhibition. Some drugs also have complementary effects by prevention of blood clotting, reduction of free radicals and degeneration of the body cells. These were investigated on the reported antidiabetic fractions from *M. dusenii.*

Lysosomal components are released during inflammatory processes. Stabilizing the lysosomal membrane is critical for controlling inflammation because it limits the release of lysosomal components such as neutrophils, proteases, and other inflammatory mediators. The erythrocyte membrane is similar to the lysosomal membrane. The concept underlying the membrane stabilising test is that any extract that stabilises the erythrocyte membrane will likewise stabilise the lysosomal membrane – [22, 23]. The anti-inflammatory efficacy of the various concentrations of the different fractions from the n-hexane extract of *M. dusenii* leaves, as shown in Table 3, indicated that all the fractions exhibited percentage inhibition of above 80% except fraction F2. However the significant inhibition of lysosomal enzymes could be attributed to the steroids –[24] detected in the extract in Table 2. This suggests that the n-hexane extract of the *M. dusenii* leaves will be able to ameliorate the pains attributed to diabetes.

From the result in Figure 1, fraction F4 favoured the breakdown of starch into monosaccharides. In addition, when the absorbance of fraction F4 was compared to the control (Tween 80), it revealed no significant difference ($p > 0.05$). This therefore explains that alpha - amylase inhibition is not the mechanism of the F4 fraction's antidiabetic activity reported in its *in vivo* studies. [12]. The response of fractions F3 and F5, however, agrees with the antidiabetic activity reported [12]. Figure 2 shows how similar in potency fractions F3 and F5 are to the standard acarbose. From this report it can be deduced that most of the anti-diabetic action of the *M. dusenii* leaves is as a result of its alpha-amylase inhibitory effect.

In the fibrinolytic assay, using Prasad method, taking 30% of clot lysis and above as being significant, Table 4 shows that streptokinase which was the standard was able to lyse the blood clot by 92.24%, water which was the negative thrombolytic control had 54.65%. However, the fractions from the plant showed no activity as the percentage of clot lysed by each fraction was insignificant. This, therefore, shows that the

fractions do not have the potential of preventing blood clots although it has an antihyperlipidemic effect as reported.[12]

The DPPH assay was focused on determining the antioxidants potential of the fractions. By acquiring a hydrogen atom from antioxidants, the electrons of the nitrogen atom in DPPH are decreased. When DPPH interacts with a donor, it produces its reduced form, which is followed by the loss of the violet colour and the formation of a yellow colour [25, 26]. Figure 3 clearly shows that the antioxidant capacity of fractions F4, F5, and F6 is relatively poor according to the concentration of the fractions that provoked the DPPH reduction activity seen in contrast to the concentration of the standard, ascorbic acid.

CONCLUSION

The hexane extract of the leaves of *M. dusenii* contained mostly saponins, cardiac glycosides and carbohydrates. All fractions exhibited anti-inflammatory inhibition. Fraction F2 and F3 showed significant alpha-amylase inhibition. The fractions showed weak anti-oxidant potentials and no fibrinolytic activity. This suggests that the n-hexane leaf extract can be rationally developed for use in the management of type II diabetes since it has been seen in this study that its column fractions have significant alpha-amylase inhibition, anti-inflammatory inhibition and antioxidant potentials.

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Conflict of interest

The authors declare that there is no conflict of interest.

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