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Full Length Research Paper

Nitric oxide producing effect of six extracts from *Harungana madagascariensis* Lam. ex Poiret (Guttiferae) stem bark

E. O. Iwalewa[1,2]*, V. Naidoo[3], V. Bagla[2] and J. N. Eloff[2]

[1]Department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.
[2]Phytomedicine Programme, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.
[3]Section of Pharmacology and Toxicology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa.

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Preliminary pharmacological screening of the aqueous extract from the leaves, stem and root of *Harungana madagascariensis* Lam. ex Poiret (Guttiferae) are reported to have effect on some cardiovascular diseases like bleeding, cadiopathy, hematuria, and other complications. However, the mechanism underlying its therapeutic effect is not known. The effects of six extracts from the stem bark of *H. madagascariensis* on equine platelets and chicken hepatocyte cells were explored to examine the involvement of nitric oxide (NO) in platelet aggregation and cell viability capacity. Extracts exhibited concentration dependent platelet aggregation, and synergistically increased NO synthesis in platelets pre-treated with NO and prostaglandin (PG) inhibitors (L-NAME and ASA). The ED_{50} values observed in the extracts of *H. madagascariensis* extracts in this study were potentiated and the graphs were shifted to the left in the presence of both inhibitors. However, a contrary effect was observed in berberine, in which its ED_{50} was shifted to the right non-competitively by the inhibitors. These results demonstrate that extracts from the stem of *H. madagascariensis* stimulates NO release and this may be a mechanism whereby the constituents of the plant elicit its therapeutic effects in herbal medicine. This study may have relevance in hemostasis, thrombosis and cancer chemotherapy.

Key words: *Harungana madagascariensis*; nitric oxide; equnine platelets, chicken hepatocyte cells, cytotoxicity

INTRODUCTION

Platelets play a prominent role in homeostasis and thrombosis and considerable evidence implicates platelets as inflammatory cellular elements (Weksler, 1983; Metzger and Page, 1998). Several pro-inflammatory mediators are derived from platelets. These include thromboxane A_2, serotonin, nitric oxide (NO), ATP, Platelet activating factor (PAF) and lipooxygenase (LOX) metabolites. These mediators have been implicated in the pathogennesis of chronic diseases (Metzger and Page, 1998). NO, a potent vasodilator and inhibitor of platelet activation produced by the vascular endothelium, it is sequestered in red blood cells (RBCs) (Pawloski and Stamler, 2002). The frequent occurrence of NO in the living cells suggests hat NO plays an important role in the maintenance of health. As free radical species with vasodilator property, NO exerts dual effects on tissues and cells in various biological systems. At low concentrations NO can dilate the blood vessels and improve the circulation, but at high concentrations it can cause circulatory shock and induce cell death. Thus, inflammatory diseases can arise in the presence of the large amount of the physiological concentrations of NO (Wang et al., 2003). However, in some infectious diseases (parasitic, microbial and cancers) extreme concentrations could be beneficial (MacMicking et al., 1997; Wang et al., 2003).

Constituents from medicinal plants as remedy can serve as an alternative to synthetic drugs in cardiovascular diseases. *Harungana madagariensis* has been shown to be valuable in humans and ethnoveterinary medicinal uses as an antiparasitic (antitrypanosomal and antiplas-

*Corresponding author. E-mail: eoiwalewa@yahoo.com.

modial), anti-anaemic, spasmolytic and antibacterial in skin diseases, wounds, natural source of dermatological agents and cosmetics (Tona et al., 1998, 2000; EMEA, 1999; Lukwa et al., 2001; Okoli et al., 2002; Erah et al., 2003; Kamanzi Atindehou et al., 2004). Traditionally, the leaves and stem bark are used for the treatment of anaemia, while the stem bark is indicated for nephrosis, malaria, gastro-intestinal disorders and fever. All these disorders are a result of inflammatory processes. From our previous report, we suggested the possible mechanism of *H. madagascariensis* on RBC to release NO (Iwalewa et al., 2007). Here in this study, the hypothesis was tested, that one potential pharmacological mode of *H. madagascariensis* action could be via the release of NO. As far as we know, there is no evidence that *H. madagascariensis* induces secretion of NO in any biological system. After the administration of the extracts, the production of NO and the expression of inducible NO synthetase could be possible compared with the control. We therefore examined the cytotoxicity of the extracts on chicken hepatocyte cells indicating its effect on cell viability and effects of the extracts, acetylsalicyclic acid (ASA), N-nitro-L-arginine methyl ester (L-NAME) on platelet aggregation and to explore the involvement of NO induction in the activity of *H. madagascariensis* stem bark in platelet aggregation.

MATERIALS AND METHODS

Plant materials

Freshly peeled stem bark of *H. madagascariensis* were collected from the main University campus, Ile-Ife, in September 2006. The plant was identified by Mr. O.A Oladele of the department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife. The specimen prepared was also identified at the Forestry Research Institute of Nigeria, Ibadan and a voucher specimen with a number FHI 107392 was kept at the herbarium. An ethanolic extract of the plant was used in these experiments.

Extraction and preparation of plant extracts

Separate samples of finely ground plant material (1.0 g) were extracted each with 10 ml of hexane (H), dichloromethane (D), chloroform (C), ethylacetate (E), acetone (A), and methanol (M) in a centrifuge tube shaken for 30 min in a shaking machine. The extract was decanted after centrifuging at 300-xg for 5 min and the process repeated three times on the marc. The combined supernatant was placed in pre-weighed glass vials, the solvent was then removed by a stream of air at room temperature and the residues weighed (Eloff, 1998).

Cell culture and drug treatment

Chicken hepatocyte cells were prepared and maintained in MEM supplemented with 100 U/ml of penicillin (AB Biodisk) and 100 mg/ml of streptomycin (Oxoid), and 10% fetal bovine serum (GIBCO BRL (USA). Cells were grown at 37℃ and 5% CO_2 in humidified air.

Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP)

Fresh Equine (*Equus caballus*) horse blood was collected from a representative breed in the Equine Research Centre, Faculty of Veterinary Sciences, University of Pretoria by Dr V. Naidoo into sterile 5 ml glass tubes containing 3.8% trisodium citrate solution at a concentration of 1 to 9 vol of blood. PRP was prepared when the blood was centrifuged at l60 g for 10 min to give (PRP) or at 1,600 for 15 - 30 min to prepare (PPP) using a Beckman(R) GS-15R centrifuge. The platelet count of the PRP was adjusted to 300,000/pl by adding PPP. PRP and PPP were then stored at room temperature.

Anti-platelet assay procedures

Effect of extract using platelets aggregation

The activity was done according to the procedure of El-Tahir and William (1980) modified by us using spectrophotometry method as adapted from Fratantoni and Poindexter (1994). Distribution into 96 wells flat microtitre culture plates (total volume 0.020 mL = 200 µL) was done in the following step wise procedures:

(i). 50 µL adrenaline (ADR) (1:1000) as agonist were first distributed into wells.
(ii). 100 µL of PBS were put into wells in column 3A – 12A as control.
(iii). 200 µL of PBS as blank into wells in column 1 A-H, while 150 µL PBS was aliquot into wells in column 2 A-H.
(iv).100 µL PBS + tested agents and positive controls (L-NAME and ASA) in increasing concentrations.
(v). Take the baseline OD at 560 nm.
(vi). Add 50 µL PPP to wells in column 2 A-H and 50 µL of PRP to all other wells in column 3(B-H) -12 (B-H).
(vii). Repeat the OD at 560 nm after 30 min incubation time using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 37℃.
(viii). Calculation of % light transmission (% LT) or the % membrane stability will be estimated from the expression:

$$100 - \frac{(\text{absorbance drug test} - \text{absorbance drug control})}{\text{absorbance drug control}} \times 100$$

and the % inhibition of platelet aggregation is calculated as :

100 - % LT

Determination of NO release and inhibitory effect

NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Inhibitory effect on NO production was evaluated using a modified method from that previously reported (Banskota et al., 2003). Nitrite in culture supernatants were measured, as previously described by Green et al. (1982), by adding 100 µl of Griess reagent 1% sulfanilamide and 0.1% *N*-w1- naphthylx-ethylenediamine in 5% phosphoric acid to 100 µl supernatant from samples of medium, for 10 min at room temperature. The OD at 550 nm was measured as 550 using a Spectramax 250 microplate reader Molecular Devices, Sunnyvale, CA, USA. The nitrite concentration in µM was calculated from sodium nitrite standard curve.

MTT cytotoxicity assay for cell viability

To evaluate the cell viability effect of the extracts, appropriate cells cultures compatible for the growth to be used were cultivated in 96-

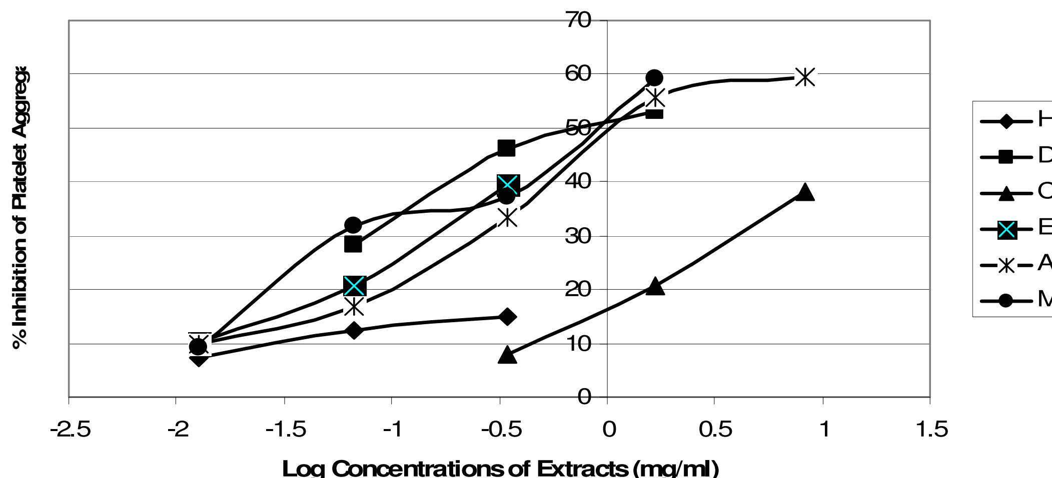

Figure 1. Graphical representation of % platelet inhibition of different six extracts of *H. madagascariensis* stem bark separately against log concentrations used to calculate the ED_{50} values.

well culture plates. Cultured cells were incubated for 24 h of incubation at 37°C in a humidified CO_2 atmosphere (5% CO_2) to allow for 80% confluence. Thereafter, cells were exposed to increasing concentrations of the extract, using four wells for each concentration. Cytotoxicity was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric method. Chicken hepatocyte cells were seeded in 96 well plates at a density of $1x10^6$ cells/ml in MEM supplemented with 100 IU/ml of penicillin and 100 mg/ml of streptomycin, and 10% fetal bovine serum. After pre-incubation for 24 h, the various concentrations of *Harungana* extracts, or positive control drug berberine were incubated for 24 h under the same conditions. After 24 h incubation with test samples, 30 µl MTT solutions (5 mg/ml in PBS) were added to the wells. After 4 h incubation, the medium was removed, and 50 µl DMSO was then added to dissolve the formazan in the cells. The optical density of formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. The stock solution of each test sample was dissolved in DMSO. % inhibitions were determined graphically (n = 4) and IC_{50} calculated.

Statistical analysis

Data are expressed as Mean ± S.E.M and analyzed using Student - t-test for comparing pairs of data. The significant level was set at $p < 0.05$. The EC_{50} and IC_{50} values were calculated using the Microsoft excel program.

RESULTS

Figure 1 showed the graphical representation of % platelet inhibition of different six extracts of *H. madagascariensis* stem bark separately against log concentrations used to calculate the ED_{50} values. A single highest concentration of 2.5 mg/ml of each extract was chosen here to compare their activity. The order of activity showed that methanol (M) had the greatest effect followed by dichloromethane (D), ethylacetate (E), acetone (A), chloroform (C), and hexane (H) extracts according to their EC_{50}. Table 1 showed the ED_{50} values of different extracts and berberine as positive control in inhibiting platelet aggregation. Inhibition here was not time dependent. It is possible that the major anti-platelet constituents were in the polar and intermediate polar component of the plants. Most of the extracts appeared to produce their highest activity at 1.25 mg/ml concentration (Figure. 1), the inhibition were however concentration dependent. The ED_{50} values observed by the plant extracts were potentiated and were shifted to the left in the presence of both inhibitors (L-NAME and ASA) (Figures 2, 3, 4, 5). This however, was contrary to the effect of berberine, in which its ED_{50} was shifted to the right non-competitively by the inhibitors (Figure 6).

Cytotoxic effect of all extracts (2 - 2000 µg/ml) and berberine (0.1 - 100 µg/ml) were evaluated by MTT test and were incubated with chicken hepatocyte cells for 24 h. Cell viability of polar and intermediate polar extracts, (E, A and M) were greater than 80%, and occur between 2 - 200 µg/ml concentrations and their IC_{50} values were 1.06, 1.10 and 1.13 mg/ml respectively, while non-polar extracts (C, D, H) were only viable between 2 - 20 µg/ml. The non-polar IC_{50} values were 0.89, 0.92 and 0.93 mg/ml respectively, an indication that toxicity could resides in the non-polar (especially in the chloroform extract) than the polar extracts. Berberine, however, showed cell viability between 0.1 – 1 µg/ml (Figure. 7).

DISCUSSION

Nitric oxide (NO) plays an important role in mediating many aspects of diseases. NO is an effector substance of cellular injury, and can act as either an anti-oxidant or oxidative stress which could be beneficial or detrimental depending on concentrations and site of action (Grisham, et al., 1999). It can modulate the release of various infla-

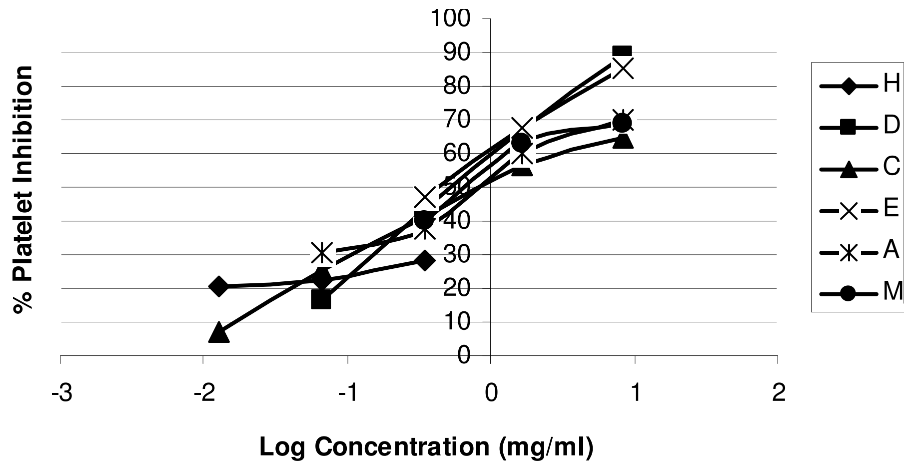

Figure 2. Graphical representation of % platelet inhibition (PI) of six different extracts of *H. madagascariensis* stems bark in the presence of L-NAME (NO inhibitor) against log concentrations.

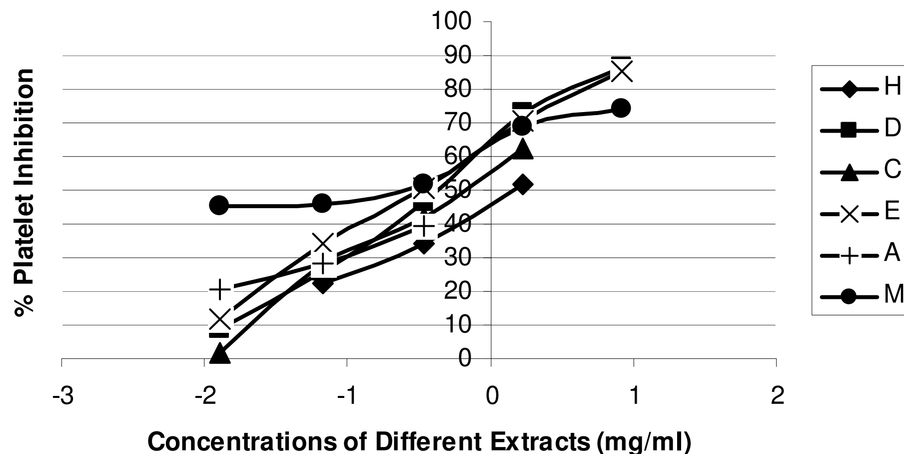

Figure 3. % Platelet inhibition (PI) of six different extracts of *H. madagascariensis* stem bark in the presence of ASA (PG inhibitors) against log concentrations

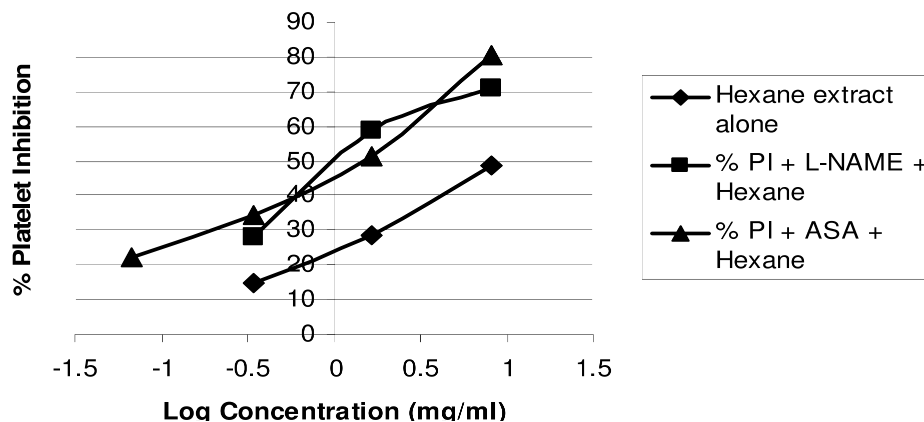

Figure 4. % Platelet inhibitions (PI) of hexane extract of *H. madagascariensis* stem bark alone and in the presence of L-NAME (NO inhibitor) and ASA (PG inhibitors) against log concentrations as a prototype of all the extracts.

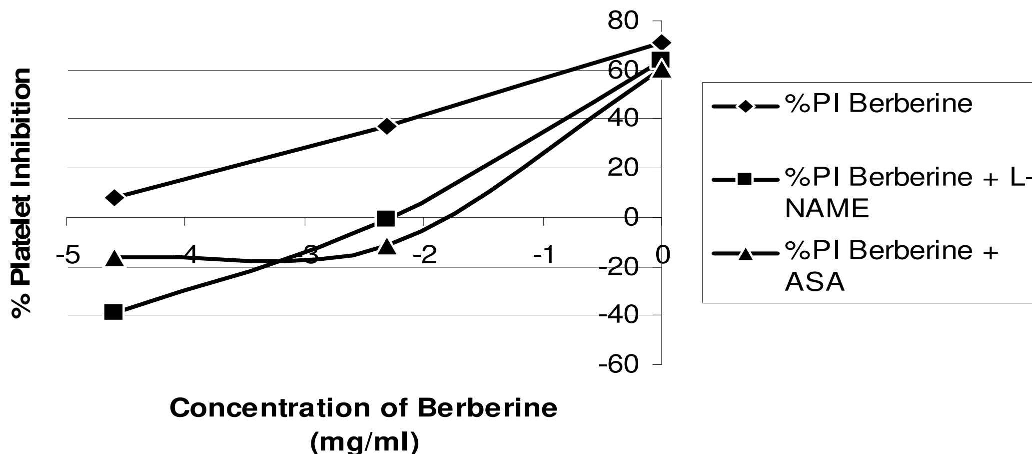

Figure 5. The % platelet inhibition (PI) of berberine alone and in the presence of both L-NAME (NO inhibitor) and ASA (PG inhibitors) against log concentrations.

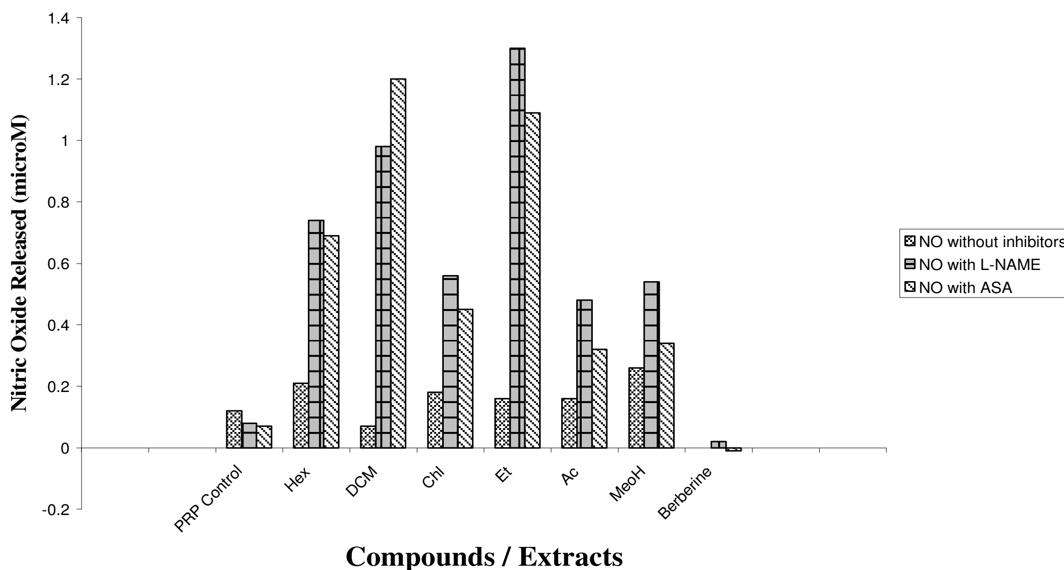

Figure 6. The concentration of nitric oxide released during the platelet aggregation reaction with extracts and berberine in the absence and presence of L-NAME and ASA.

mmatory mediators from a wide range of cells participating in inflammatory responses (e.g., leukocytes, macrophages, mast cells, endothelial cells, and platelets). It is also involved in blood flow, adhesion of leukocytes to the vascular endothelium and the activity of numerous enzymes, all of which can have an impact on inflammatory diseases and disorders (Wallace, 2005).

From this platelet aggregation study, we were able to deduce that the constituents in *H. madagascariensis* stem bark has a dual role of activities depending on the amount and types of mediators released. At low NO concentrations NO can exhibit or induce platelet aggregation, but at high concentrations, NO being free radical oxygen species it can decrease platelet aggregation and in another dimension can induce cell death and produce inflammation. Thus, inflammatory diseases can arise in the presence of the large amount of the physiological concentrations of NO (Wang et al., 2003). However, in some infectious diseases (parasitic, microbial and cancers) extreme concentrations could be beneficial (MacMicking et al., 1997; Wang et al., 2003). Ideally, if the constituents of *H. madagascariensis* are behaving as

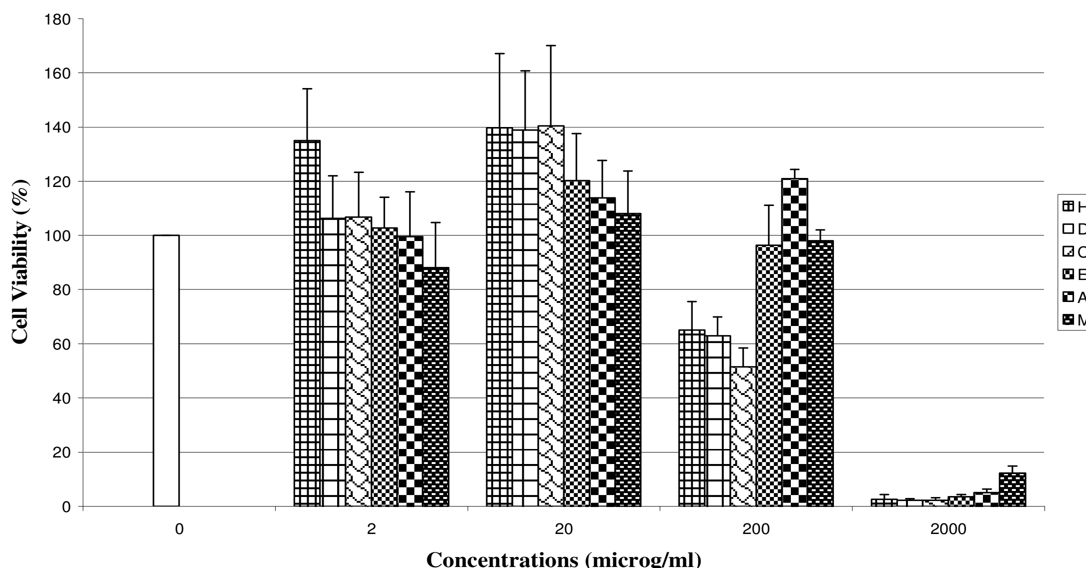

Figure 7. The cell viability test of *H. madagascariensis* six extracts on chicken hepatocyte using MTT assay.

an anti-inflammatory agent, the effect on the platelet membrane is thought to be through inhibition or stabilization of cell membrane preventing the agonist adrenaline to stimulate the thrombin/collagen receptor-sites and therefore prevent aggregation. However, in this present study, the reverse is the case. Using only the 2.5 mg/ml concentration of each extract as an example, it is evident that *H. madagascariensis* induced platelet aggregation with little amount of NO production (0.07 - 0.28 μM). The % inhibition were H = 45%, D = 32%, C = 35%, E = 38%, Ac = 58%, and M = 60%, and berberine (100 μg/ml) = 62% as compared to PPP with 0%.

In the presence of L-NAME and ASA, there was a reduction in % inhibition in platelet aggregation with berberine to 16.4% which is the normal pattern for an anti-inflammatory agent, however, for all the extracts, there was an increase in % inhibition of platelet aggregation: H = 62%, D = 86%, C = 68%, E = 82%, Ac = 72%, M = 70%, and with increasing amount of NO production (0.32 - 1.3 μM). The extracts from this plant could therefore be a source of NO generation. The mechanism through which more NO was generated/released in the presence of L-NAME and ASA (non selective inhibitors of NO and COX respectively) is not clear. However, it could be that the extracts overcome competitively the inhibitory effects of L-NAME and ASA and therefore stimulates the NO and COX receptor site to increase the synthesis of NO. There has being a link between the synthesis of NO and PG. Both mediators can influence each other's release from cells (Salvemini et al., 1993; Mollace et al., 2005). There are studies that showed lower concentrations of agent increases the platelet aggregation, while high concentrations of the same agent decrease the platelet aggregation (Hirakata, et al., 1999). Thus, the present study indicates that extracts of *H. madagascariensis* have different effects on platelet aggregation depending on concentration; relatively high concentrations of extracts could suppress platelet aggregation, whereas relatively low concentrations enhance aggregation. These complex effects of extracts have probably caused the inconsistency in the estimation of its effects on platelet aggregation. Extracts or isolated com-pounds have been shown to alter secondary aggregation without altering primary aggregation when aggregation was induced by weak agonists such as epinephrine (Hirakata et al., 1999).

Berberine, an isoquinolines derivative purified from *Coptis chinesis* (a vasodilatory, anti-inflammatory and antimicrobial agent) has been shown in this study to inhibit platelet aggregation and the inhibition was blocked by the two inhibitors (L-NAME and ASA). The inhibition was shifted to the right non-competitively. This however, was contrary to the effects produced by the extracts in this study, their effects were potentiated and the graphs were shifted to the left also in the presence of both inhibitors (L-NAME and ASA). This therefore means that the anti-inflammatory property of *Harungana* is definitely not through the same mechanism exhibited by berberine as standard anti-inflammatory agent. According to this results, synergism and potentiation occur between the *Harungana* extracts and the inhibitors, while, there was antagonism between berberine and the inhibitors. Pharmacologically, this type of antagonism between *Harungana* extracts and the inhibitors is referred to as allosteric

inhibition whereby an inhibitor can bind to a site on the receptor distinct from that of a primary agonist and thereby changes the response of the agonist (Gilman et al., 2006).

Recently, NO-releasing drugs have been developed, which exhibit very powerful anti-inflammatory effects (Muscara and Wallace, 1999). The potentiation observed between the extracts and the inhibitors could also be explained from the concept of the possibility that nitric oxide-releasing donating drugs (NODD) may be of therapeutic benefit in a wide variety of disease states including thrombosis, pain, inflammation, cancer, microbial and protozoa infections. The use of aspirin (ASA) for the prevention and treatment of cardiovascular disease, in particular thrombosis, is well established (Harter et al., 1979; Hirsh, 1979). Aspirin exerts its anti-thrombotic effects by irreversible inhibition of COX-1 thereby reducing formation of pro-aggregatory TXA_2 by platelets and anti-aggregatory PGI_2 by vascular endothelial cells] (Schror, 1997). However, NO is a potent inhibitor of platelet activation and adhesion to the vessel wall (Ignarro, 1999). Thus, NO-aspirin may, at least in theory, be expected to exhibit a greater degree of inhibition of platelet function. Increased anti-thrombotic potency of NO-aspirin, relative to aspirin, has been observed both *in vitro* and *in vivo*. Thus, NO-aspirin inhibited platelet aggregation in rat and man platelets *in vitro* with greater potency than aspirin (Lechi et al., 1996; Wallace et al., 1995b). Therefore, NO generated by the extracts with ASA, or L-NAME could possibly be the pattern through which potentiation occur between the extracts and the inhibitors as more NO were released in the course of the interactions.

Some of the herbal extracts or purified active substances whose actions have been linked to the NO pathway have been identified by Achike and Kwan (2003). These are herbal extracts/drugs that enhance the production of NO via iNOS. These drugs are generally used as anticancer, anti-parasitic and antibacterial folk remedies (Kim et al., 1998, 1999a, 1999b, 1999c, 1999d; Tanner, et al., 1999). This could possibly be the mechanism of action of *Harungana* in its ethnomedical uses as antimalarials / antiparasitics, antimicrobial, anti-anaemic, and in GIT disorders by increasing NO production.

In our previous study (Iwalewa et al., 2007) where we examined the cytotoxicity of these various six extracts on the haemagglutination potentials on equinine RBC, it was shown that the release of NO from the RBCs were evidence by the extracts and the release was blocked/reduced tremendously by the pre-treatment of L-NAME and ASA. This finding therefore buttressed the releasing potentials of *H. madagascariensis* stem bark constituents. Beneficial effects of this plant constituents can therefore be envisaged in cardiovascular diseases, as seen also in microbial, protozoan and cancer (MacMicking et al., 1997, Wang et al., 2003), since the etiology of all these diseases has to do with reduction in NO. The same constituents could be detrimental with inflammatory reactions that are involved with increase in NO generation. We therefore postulate here that *H. madagascariensis* stem is or might be producing its activities by the release of nitric oxide and related com-pounds in this study as one of its mechanisms of action.

This is the first report that describes the production of NO by the various extracts of the stem of *H. madagascariensis* in platelets. By the administration of extracts, the production of NO was also augmented in presence of nitric oxide and prostaglanding inhibitors (L-NAME and ASA) pre-treated platelets. These results suggest that the secretion of NO by the extracts is not regulated by the same mechanism exhibited by berberine.

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