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Research Article

Comparative Effects of *Datura stramonium* Leaf and Seed Extracts on Membrane Stabilization and Platelet Aggregation *In-vitro*

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ABSTRACT

Despite the progress in developing new therapies, inflammatory diseases remain one of the major causes of mortality. In this study, anti-inflammatory activity of the aqueous extracts of *Datura stramonium* leaves and seeds were evaluated. Heat and hypotonicity-induced haemolysis of human red blood cells (HRBCs) were used to determine the effect of the extracts on membrane stabilization, and the effect of the extracts on platelet aggregation was determined using calcium chloride ($CaCl_2$)-induced platelet aggregation. The concentrations of the extracts used were 0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml. Indomethacin (0.4 mg/ml) was used as the standard drug. The leaf extract significantly ($p < 0.05$) inhibited heat-induced haemolysis by 44.47% and 52.89% at 0.2 and 0.1 mg/ml respectively compared to the seed extract. On the other hand, the seed extract significantly ($p < 0.05$) inhibited heat-induced haemolysis by 29.5%, 44.88% and 50.01% at 0.4, 0.6 and 0.8 mg/ml respectively compared to the leaf extract. Effect of the leaf extract on hypotonicity-induced haemolysis showed that it significantly ($p < 0.05$) inhibited haemolysis from 27.27% - 68.67% corresponding to concentrations 0.1 – 0.8 mg/ml, compared to the seed extract. The effect of the extracts on platelet aggregation showed that the leaf extract exhibited significantly ($p < 0.05$) higher inhibition of platelet aggregation from 0 – 120 seconds, compared to the seed. Both extracts thus, have comparable effect on heat-induced haemolysis of HRBCs, though at different concentrations. The ability of the leaf extract to inhibit hypotonicity-induced haemolysis and platelet aggregation outweigh that of the seed.

Keywords: *Anti-inflammatory, heat-induced haemolysis, hypotonicity-induced haemolysis, platelet aggregation, Datura stramonium*

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INTRODUCTION

Inflammation is a complex and protective response of the immune system to stimuli such as microbes, damage-associated molecular patterns (DAMPs) or pathogens (Arman *et al*., 2015). Processes, such as extravasations of leukocytes, release of proteases and oxidants from phagocytes, activation of platelets and their aggregation are associated with inflammation (Ward, 2010). Platelets, also known as thrombocytes, are small anucleate cells circulating in the bloodstream (Mekaj, 2016). They are derived from the bone marrow, and play a major role in maintaining vascular integrity and homeostasis. Platelets also play roles in inflammation where they promote vascular permeability and recruit inflammatory cells (Dovizio *et al*., 2014; Gros *et al*., 2015). They contain alpha, dense and lysosomal granules which store many important inflammatory and immune mediators that are rapidly released after platelet activation

(Mekaj, 2016). The release of inflammatory mediators such as, cytokines, chemokines and eicosanoids enables platelets recruit white blood cells to the site of inflammation or injury (Arman *et al*., 2015). P-selectin from platelets α-granules binds to P-selectin glycosylated ligand (PSGL-1) thereby initiating platelet-endothelial and platelet-leukocytes interaction. Continued or chronic platelet interactions with white blood cells or endothelial cells can lead to adverse effects from excessive immune stimulation and inflammatory insult (Morrell *et al*., 2014). Platelet interactions with leukocytes may therefore contribute to vascular injury and tissue damage in several inflammatory diseases.

The rupture of the red blood cell membrane and the subsequent release of haemoglobin and other cellular constituents into the plasma, occurs in a process known as haemolysis. The life span of the red blood cells ranges from 110-120 days, after which it naturally breakdown and eventually removed from the circulation by the spleen. However, red blood cells could breakdown prematurely due to some conditions. Auto-immune haemolytic anaemia may be the reason for *in vivo* haemolysis of red blood cells, while *in vitro* haemolysis could be due to lack of accurate precautionary measures in specimen collection, transport and processing. Some of the factors that are believed to cause haemolysis include shear stress, intrinsic red blood cell membrane defects, osmotic and pH changes, bacterial contamination and temperature (Sowemimo-Coker, 2002). This process is evidenced by the presence of free haemoglobin in the red cell suspending media, such as plasma or additive solutions (Makroo *et al*., 2011).

The medicinal properties of Datura genus have long been exploited. All over the world, it has been used effectively as a hallucinogen. About ten species have been found, with *Datura stramonium* and *Datura anoxia* being the most exploited for medicinal use (Soni *et al*., 2012). *Datura stramonium* is a widespread annual medicinal plant belonging to the Solanaceae family. It is commonly called jimson weed, thornapple, moon flower or angel's trumpet. It is distributed over tropical and warm temperate regions of the world and mostly found in areas with high intensity of sunlight such as roadsides, waste grounds and agricultural fields. The plant is known as "gegemu" in Western Nigeria, where it serves as sources of dyes, poison, intoxicants, and medicine (Adegoke and Alo, 2013). *Datura stramonium* poisoning has been reported in several cases (Adegoke and Alo, 2013; Devi *et al*., 2011). Studies have also shown that the seeds of *Datura stramonium* have analgesic, anti-helmintic and ant-inflammatory properties. This makes them suitable for the treatment of pains in the stomach and intestine resulting from worm infestation, toothache and fever resulting from inflammation (Soni *et al*., 2012). The leaf extract of the plant can be taken orally for treating asthma and sinus infections, while its stripped bark have been used in the treatment of swellings, burns and ulcers by applying it externally at the site of interest. *Datura stramonium* have been claimed to be used as a sedative and also believed to have membrane stabilizing potentials. The *in vivo* anti-inflammatory effect of the plant leaves has previously been demonstrated (Sonika *et al.*, 2010; Abbas, 2013). The purpose of this study was to carry out a comparative study on the *in vitro* membrane stabilizing and the anti-platelet aggregatory potentials of *Datura stramonium* leaves and seeds.

MATERIALS AND METHODS

Collection and extraction of plant material: Fresh leaves and fruits of *Datura stramonium* were obtained from Calabar, Nigeria. The plant parts were authenticated by Mr. Alfred Ozioko of International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nsukka. The fruits were cut open to expose the seeds, after which the seeds and leaves were air-dried for one week and milled. The ground seeds (28.0 g) and the ground leaves (14.18 g) were suspended in 200 ml of distilled water. The suspensions were placed in the water bath at 40 oC for four hours. The mixtures were thereafter filtered with a clean muslin cloth and the filtrates concentrated using a rotary evaporator at an optimum temperature of 40-50 oC.

Collection and preparation of blood samples: Blood sample was prepared according to the method of Shinde *et al*. (1989) and Rein *et al*. (2000). Fresh whole blood samples were collected intravenously from apparently healthy volunteers who had not taken any medication for at least 14 days. The blood samples were centrifuged at 3000 rpm for 10 minutes. The supernatant, which is rich in platelets was diluted twice its volume with normal saline and served as the platelet-rich plasma (PRP), for anti-platelet aggregation assay. The red blood pellets were resuspended with a volume of normal saline equal to the volume of the supernatant. The volume of the resuspended red blood pellets was measured and reconstituted with normal saline as a 40% v/v HRBC suspension, which was thereafter used for the assay of membrane stabilization.

Heat-induced haemolysis: The effect of *Datura stramonium* leaf and seed extracts on haemolysis of red blood cell membrane induced by heat was evaluated using modified method of Shinde *et al.* (1989. *Datura stramonium* leaf extract and standard drug (indomethacin) were dissolved in normal saline solution. A set of eleven centrifuge tubes containing 5 ml graded doses of the seed and leaf extracts (0.1, 0.2, 0.4, 0.6, and 0.8 mg/ml) and 0.4 mg/ml of indomethacin were arranged in quadruplicate (4 sets per dose). HRBC suspension (0.1 ml) was added to each of the tubes and gently mixed. A pair of the tubes was incubated at 54 oC for 20 min in a regulated water bath, while the other pair was maintained at −10 oC in a freezer for 20 min. A pair of control tube containing 5 ml of normal saline and HRBC suspension was also incubated in the water bath. The tubes were thereafter centrifuged at 1300 rpm for 3 min, followed by the determination of the haemoglobin content of the supernatants using a spectrophotometer at 540 nm. The formula below was used in calculating the percentage inhibition of haemolysis by the extract and indomethacin.

$$\% \text{ inhibition of haemolysis} = 1 - \frac{\text{Absorbance of heated test sample} - \text{Absorbance of unheated test sample}}{\text{Absorbance of heated control sample} - \text{Absorbance of unheated test sample}} \times 100$$

Hypotonicity-induced haemolysis: The modified method of Shinde *et al.* (1989) was used to determine the effect of *Datura stramonium* leaf and seed extracts on haemolysis of red blood cell membrane induced by hypotonic solution. A set of eleven tubes, arranged in duplicates contained 5 ml of the seed and leaf extracts (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml) and 0.4 mg/ml indomethacin dissolved in distilled water (hypotonic solution). Another set of eleven tubes containing 5 ml of the extracts (0.1- 0.8 mg/ml) and indomethacin dissolved in an isotonic solution (normal saline) were also arranged in duplicates. The control tube, containing 5 ml of distilled water was arranged in duplicate. HRBC suspension (0.1 ml) was thereafter added to the tubes and mixed gently. The mixtures were incubated for 1 hour at room temperature (37 ^{o}C), and afterwards, centrifuged for 3 minutes at 1300 rpm. A spectrophotometer was used to determine the haemoglobin content of the supernatant at 540 nm. Percentage inhibition of haemolysis by the extract and indomethacin was calculated using the formula below, assuming the haemolysis of the control to be 100 %.

$$\% \text{ inhibition of haemolysis} = 1 - \frac{\text{Absorbance of test sample in hypotonic solution} - \text{Absorbance of test sample in isotonic solution}}{\text{Absorbance of control sample in hypotonic solution} - \text{Absorbance of test sample in isotonic solution}} \times 100$$

Anti-platelet aggregation assay: This was achieved following the method of Rein *et al*. (2000) with modifications. An aliquot of PRP (0.2 ml) was put into each of a set of ten test tubes containing 1ml each of varying concentration of the leaf and seed extract (0.1, 0.2, 0.4, 0.6 and 0.8) dissolved in normal saline. Another test tube contained an aliquot (0.2 ml) of PRP and 1ml of 0.4 mg/ml indomethacin in normal saline. The contents of the respective tubes were made up to 2.0 ml with normal saline. The control tube contained 1.8 ml of normal saline and 0.2 ml of PRP. The tubes were allowed to incubate before the induction of aggregation by the addition of 0.4 ml of 1.47% $CaCl_2$ solution. The tests were performed in duplicates. Changes in the absorbance of the solutions were taken at intervals of 30 sec for 2 min at 520 nm.

Statistical analysis

Data from this study were analyzed using statistical product and service solutions (SPSS), version 18. Tests of statistical significance were carried out using one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$. Duncan multiple test range was used to compare the group means obtained after each treatment with the control. The results were expressed as mean ± standard deviation

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RESULTS

Effect of aqueous extract of *Datura stramonium* leaves and seeds on heat-induced haemolysis of human red blood cells

As seen in Table 1, there were significant ($P < 0.05$) inhibitions of heat-induced haemolysis for both the leaves and seed extracts compared to control, with the leaf extract exhibiting the most significant ($P < 0.05$) inhibition, 52.89%.

Table 1:
Effect of the aqueous extract of *Datura stramonium* leaves and seeds on heat-induced haemolysis

Treatment	OD 540nm		Percentage inhibition of haemolysis (%)
	Heated solution	Unheated solution	
Normal saline	$0.85 \pm 0.11_{g}$	-	-
Indomethacin (0.4 mg/ml)	$0.67 \pm 0.04_{de}$	0.01 ± 0.00	21.39
Leaf extract (0.1 mg/ml)	$0.42 \pm 0.00_{a}$	0.04 ± 0.00	52.89
Seed extract (0.1 mg/ml)	$0.73 \pm 0.01_{f}$	0.02 ± 0.01	14.71
Leaf extract (0.2 mg/ml)	$0.49 \pm 0.01_{b}$	0.04 ± 0.00	44.47
Seed extract (0.2 mg/ml)	$0.74 \pm 0.01_{f}$	0.02 ± 0.00	13.29
Leaf extract (0.4 mg/ml)	$0.75 \pm 0.01_{f}$	0.03 ± 0.00	12.20
Seed extract (0.4 mg/ml)	$0.61 \pm 0.00_{c}$	0.02 ± 0.00	29.50
Leaf extract (0.6 mg/ml)	$0.64 \pm 0.01_{cd}$	0.04 ± 0.00	25.77
Seed extract (0.6 mg/ml)	$0.49 \pm 0.02_{b}$	0.04 ± 0.00	44.88
Leaf extract (0.8 mg/ml)	$0.69 \pm 0.02_{e}$	0.05 ± 0.00	20.50
Seed extract (0.8 mg/ml)	$0.45 \pm 0.00_{a}$	0.06 ± 0.00	50.01

Data are represented as means ± SD. Values with different letters as superscripts are considered significant at $p < 0.05$

Table 2:
Effect of the aqueous extract of *Datura stramonium* leaves and seeds on hypotonicity-induced haemolysis

Treatment	OD 540nm		Percentage inhibition of haemolysis (%)
	Hypotonic solution	Isotonic solution	
Normal saline	$0.91 \pm 0.02_{h}$	-	-
Indomethacin (0.4 mg/ml)	$0.52 \pm 0.01_{c}$	0.01 ± 0.00	43.08
Leaf extract (0.1 mg/ml)	$0.46 \pm 0.02_{b}$	0.03 ± 0.00	51.13
Seed extract (0.1 mg/ml)	$0.83 \pm 0.00_{g}$	0.02 ± 0.00	8.51
Leaf extract (0.2 mg/ml)	$0.67 \pm 0.07_{e}$	0.03 ± 0.00	27.27
Seed extract (0.2 mg/ml)	$0.85 \pm 0.11_{g}$	0.02 ± 0.00	7.01
Leaf extract (0.4 mg/ml)	$0.64 \pm 0.02_{e}$	0.04 ± 0.00	31.49
Seed extract (0.4 mg/ml)	$0.82 \pm 0.01_{g}$	0.03 ± 0.00	10.66
Leaf extract (0.6 mg/ml)	$0.56 \pm 0.03_{d}$	0.07 ± 0.00	41.45
Seed extract (0.6 mg/ml)	$0.76 \pm 0.01_{f}$	0.05 ± 0.00	17.33
Leaf extract (0.8 mg/ml)	$0.34 \pm 0.00_{a}$	0.08 ± 0.00	68.67
Seed extract (0.8 mg/ml)	$0.91 \pm 0.00_{h}$	0.07 ± 0.01	0.00

Data are represented in means ± SD. Values with different letters as superscripts are significantly different at $p < 0.05$

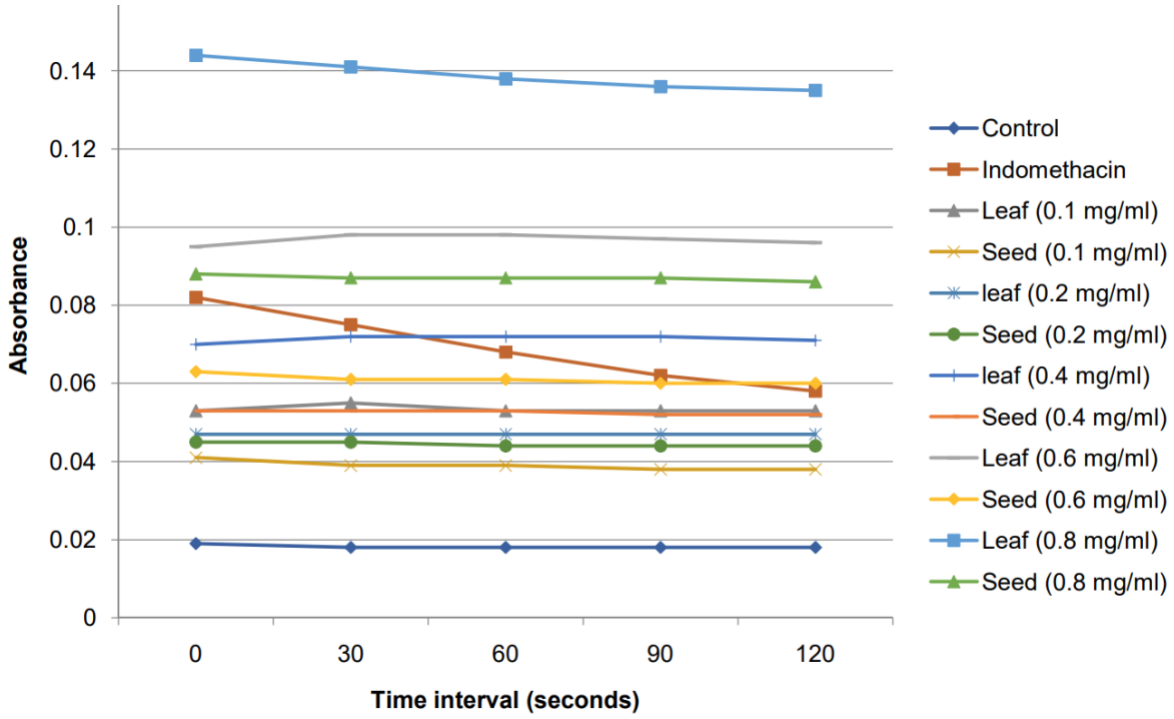

Figure 1:
Effect of the aqueous extract of *D. stramonium* leaves and seeds on $CaCl_2$-induced platelet aggregation

Effect of aqueous extract of *Datura stramonium* leaves and seeds on hypotonicity-induced haemolysis of red blood cells

Table 2 shows significant ($P < 0.05$) inhibition of hypotonicity-induced haemolysis of the extract groups compared to the control, except at 0.8 mg/ml of the seed extract. The leaf extract has the most significant ($P < 0.05$) inhibition of 68.67%.

Effect of aqueous extract of *Datura stramonium* leaves and seeds $CaCl_2$-induced platelet aggregation of red blood cells

From Figure 1, there were significant ($p < 0.05$) increases in the absorbance values of the treated groups compared to the normal control. Increase in absorbance was found to be concentration dependent for the seed extract.

DISCUSSION

Inflammation is possibly the fastest growing metabolic disease in the world, making it imperative to seek for more appropriate therapies of controlling the disease, aided by the increasing knowledge of its heterogeneous nature (Sumathi and Anuradha, 2016). This study therefore seeks to understand the anti-inflammatory action of *Datura stramonium* leaves and seeds.

During inflammation, the stabilization of the membrane is important so as to maintain the integrity of the cell and prevent serum proteins and fluids from leaking into the tissues, during amplification in vascular permeability (Chaitanya *et al*., 2011). Furthermore, stabilization of the lysosomal membrane during inflammation is necessary to prevent activated leukocytes from causing increased inflammation and damage in tissues through the release of their constituents such as proteases and bactericidal enzymes (Oyekachukwu *et al*., 2017; Kosala *et al*., 2018). Since HRBC membranes are similar to lysosomal membrane components (Zohra and Fawzia, 2014), this study determined the ability of *Datura stramonium* leaves and seeds to stabilize the HRBC membrane. The membrane was destabilized with heat and hypotonic solution, which subsequently led to the release of haemoglobin into the medium. *Datura stramonium* exhibited significant ($P < 0.05$) membrane stabilizing activity compared to the standard anti-inflammatory drug, indomethacin. The leaf and seed extracts inhibited heat-induced lyses of the red blood cell by 52.89% and 50.01% respectively, while the leaf extract inhibited hypotonicity-induced haemolysis by 68.67% compared to indomethacin. This stabilization of erythrocyte membranes by the extracts implies that they may as well stabilize lysosomal membranes. The ability of the extracts to inhibit the release of haemoglobin therefore corresponds to its effectiveness in preventing the release of inflammatory mediators and the lysosomal contents of activated leukocytes during inflammatory response. The significant inhibition of hypotonicity-induced haemolysis correlated with the results obtained by (Rajeshkanna *et al*., 2016) for *D. stramonium* flowers. Plants contain active substances, such as flavonoids, saponins and tannins that possess ability to facilitate the stability of biological membranes when exposed to induced lyses, which could be achieved by binding to the membrane, thereby altering the surface charge of cells (Oyedapo *et al*., 2010). The membrane stabilizing activity of *D. stramonium* could be due to the presence of these substances in the leaves and seeds as previously demonstrated (Waza *et al*., 2015; Adam and Idris, 2017).

Platelet aggregation is the process by which certain stimuli trigger platelet-platelet interactions to form aggregate (Azizuddin *et al*., 2017). The present study also investigated the efficacy of aqueous extract of *Datura stramonium* leaves and seeds to inhibit the aggregation of platelets induced by calcium chloride during inflammatory response. Aggregation of platelets leads to increase in transmittance (Muthu and Durairaj, 2015), which corresponds to decreased absorbance values as shown in the control group. The inhibition of platelet aggregation by the extract is shown by the significant ($p < 0.05$) increase in the absorbance values relative to the control. During inflammation, activated platelets synthesize thromboxane A_2 (TXA_2) which induces platelet aggregation by increasing intracellular Ca^{2+}, which in turn promotes fusion of platelet granules with the membrane (Ekwueme *et al*., 2011). Platelet granules release their contents, such as adenosine diphosphate (ADP) which also promotes the aggregation of platelets. Since the metal-chelating activity of *D. stramonium* has been demonstrated (Olabinri *et al*., 2014; Ademiluyi *et al*., 2016), the extracts could possibly have chelated the Ca^{2+} ions, thereby inhibiting platelet aggregation and binding of platelets to leukocytes (Ferrer-Acosta *et al*., 2014). This inhibition of platelet aggregation is important so as reduce the susceptibility of the cell to oxidative damage, and prevent tissue damage during sustained inflammatory response. This result also complements the ability of the extract to stabilize lysosomal membrane, as platelets lysosomal granules are hindered from releasing their pro-inflammatory contents.

This study consolidates the anti-inflammatory activity of the extracts of *Datura stramonium* leaves and seeds as it established their membrane stabilizing and anti-platelet aggregatory effect. The plant can therefore serve as a source of new anti-inflammatory therapies. However, the leaf extract has a higher anti-inflammatory potential compared to the seed extract.

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