Antioxidant and Anti-ulcerogenic Effect of Chloroform and Methanol partitioned leave extracts of *Harungana madagascariensis* on wistar albino rats: A Comparative analysis

**Abstract**

**Background:** This research investigated the antioxidant and anti-ulcerogenic potentials of *chloroform and* methanolic leave extracts of *Harungana Madagascariensis* leave.Antioxidants are substance that prevents or slows down the damage caused by free radicals. An ulcer is an open sore that occurs when surface cells die and are shed, creaking a break in the lining of an organ. **Method:** The *in-vitro* antioxidant activity was tested using the following models; 1,1-diphenyl-2-picrylhydrazyl (DPPH), Ferric reducing power (FRAP) and Total antioxidant capacity (TAC). **Results:** The *H. Madagascariensis* extracts exhibited a moderate *in vitro* antioxidant activity when compared with ascorbic acid. The extracts showed inhibitory potential against DPPH free radical, the inhibitory percentages are 47.5, 50.1, 54.5, 56.7, 61.9, 66.8 and 72.5µg/ml for the chloroform extract while for the methanol extract was 61.6, 70.1, 73.3, 82.6, 88.2, 91.2 and 93 µg/ml. The extracts (chloroform and methanol) also significantly (P <0.05) reduced free radical activities for Ferric reducing power and Total antioxidant capacity across all concentrations. *In-vivo* antioxidant enzymes (SOD, CAT and GPx) were evaluated for its ability to prevent indomethacin-induced gastric ulcer in rats. Pre-treatment with extracts at oral doses 100, 200 and 400 mg/kg body weight significantly (P <0.05) increased the activity of SOD, CAT and GPx after ulcer induction. A significant (P <0.05) decrease was observed in MDA after treatment. The extracts were found to provide a dose-dependent protection against indomethacin-induced gastric ulcer by averting the deep necrotic lesions of the gastric epithelium, by preserving normal antioxidant enzymes activities and by inhibiting the lipid peroxidation in gastric mucosa. The anti-ulcerogenic activity of *H. Madagascaariensis* might be due to its antioxidant effects. The methanol extract exhibited better anti-ulcerogenic effect against indomethacin induced gastric ulcer than that obtained for the chloroform extract.

*Keywords*: *Harungana Madagascaariensis,* anti-ulcerogenic, indomethacin, antioxidant

**INTRODUCTION:**

“Currently, various steroidal and non-steroidal anti-inflammatory drugs (NSAID) are being used to treat inflammatory diseases. Most important adverse effect of NSAID is gastric ulceration” [1]. Even though gastric antisecretory drugs-H2 blockers, anticholinergic agents, proton pump inhibitors (such as Omeprazole, cemetidine, indomethacin) are effective in preventing NSAID associated peptic ulceration, they are not without side effects. [2]. “It is also claimed that free oxygen radicals plays an important role in the pathogenesis of gastric damage caused by NSAID” [3]. “For the prevention of such damage, gastric cells posses an enzymatic antioxidants defence system, which could neutralize the harmful effect of free radicals. But excessive generation of free radicals, resulted from long term use of NSAID, enhanced the lipid peroxidation process and attenuated the activities of the antioxidants defence system” [4]. “Although many available synthetic drugs are used to treat gastric ulcers, most of them produce several undesirable reactions when used for a long term” [5]. Due to the side effects of synthetic drugs, researchers are in desperate need to develop safer drugs as well as natural products possessing antioxidant and anti-ulcerogenic properties.

 “*Harungana Madagascariensis* (Hypericaceae), leaf is one such herbal drug currently undertaken in this study primarily to explore *in vivo* and *in vitro* antioxidant and the anti-ulcerogenic potentials in animal studies. *Harungana madagascariensis* (L.) is an indigenous medicinal plant in Nigeria. It is commonly found in savannah regions. The Plant has many common names such as; English (orange-milk tree, blood tree); Hausa (alillibar); Igbo (oturu) and Yoruba (elepo). It has been reported that H. madagascariensis has been used in the treatment of leprosy, jaundice, ulcers, asthma etc” [6]. “The bark and root decoctions are remedy for dysentery and piles” [7- 8]. “It relieves stomach-ache, painful menstrual problems, dysmenorrhea, miscarriage and sterility” [9- 10]. “The leaf and roots of *H. Madagascariensis* have been shown to have hypoglycemic effects, lowering blood glucose levels in diabetes mellitus” [11], and others like anti-inflammation[12], antihepatotoxicity [13] and antimicrobial activities [14] and anticonvulsant activity[15].

“Besides, it has been stated that the leaves of *H. Madagascariensis* contain profound amount of alkaloids, glycosides, flavonoids and polyphenolics” [16- 17] which possess significant activity against inflammation and ulcer [18].  The use of H. madagascariensis in the treatment of ulcer has not been validated scientically as of the of this research, therefore the aim for the study.

1. **Methodology**
	1. **Plant Material**

The fresh leaves of *Harungana madagascariensis* were collected from Adada river in Nsukka, Enugu State and identified by Mr. Alfred Ozioko of Bioresource Development and Conservation Programme (BDCP) Research Centre, Nsukka, Enugu State Nigeria.

**2.1. CHEMICALS AND REAGENTS**

All chemicals used in this study were of analytical grade and products of Sigma Aldrich, (USA), British Drug House (BDH) England, Burgoyne, (India), Harkin and Williams, (England), Qualikems (India), Fluka (Germany), May and Baker, (England). Reagents used for all the experiments were commercial kits and products of Randox, (USA) and Teco (TC), (USA).

* + 1. **Preparation of Plant Material**

The fresh leaves of *Harungana madagascariensis* were air-dried, milled into coarse powder using a mechanical grinder. 1655g of the plant was macerated in a mixture of methanol and chloroform (2:1) for 48 hours in a maceration flask. The mixture was filtered with Whatman No.1 filter paper. The filtrate was partitioned using a 20% distilled water of the total volume of the filtrate, to obtain two layers that were separated using a separation funnel. The lower layer designated chloroform extract and the upper layer was designated methanol extract. They were concentrated using a rotary evaporator at an optimum temperature range of 45–50OC. The concentrated extracts were kept in refrigerator at a temperature of 2 to 40oC.

* 1. ***IN-VITRO* ANTIOXIDANTS ASSAYS**

 Determination of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the chloroform and methanol extracts of *H. madagascariensis* leaf.

DPPH radical scavenging activity of the methanol and chloroform extracts of *Harungana madagascariensis* leaves. 1, 1-diphenyl-2-picrylhydrazyl(DPPH) radical scavenging assay on both extracts were evaluated using the method of [19] with slight modifications.

**2.2.1 Determination of Total Antioxidant Capacity of the Chloroform and Methanol Extracts of *H. madagascariensis* leaf:** Total antioxidant capacity of the extracts was determined using the phosphomolybdate method as described by [20] using ascorbic acid as a standard drug.

**2.2.2 Determination of ferric reducing power activity of the chloroform and methanol extracts of *H. madagascariensis* leaf**

Ferric reducing power of the extracts was determined by the method of [21]

**2.3 *In vivo* antioxidants assays**

**2.3.1 Determination of catalase (CAT) concentration in Chloroform and Methanol extracts of *H. Madagascariensis* leaves**

This was done according to the method of This was determined according to the method of [22]. **Procedure**

Hydrogen peroxide (2 ml) and 2.5 ml of phosphate buffer were added in a beaker. Adequately, each of the sample (0.5 ml) was added and mixed. A portion of the reaction mixture (1 ml) was added to 2 ml of dichromate acetic acid reagent. The absorbance was read at 540nm.

**Calculation**

Catalase activity was calculated using the equation below:

$$Catalytic concentration (iU/L)=\frac{log⁡[abs 1/abs 2] X 0.23}{0.00693}$$

**2.3.2 Determination of superoxide dismutase (SOD) concentration in Chloroform and Methanol extracts of *H. Madagascariensis* leaves**

This was determined using the method of [23].

**Procedure**

A quantity of adrenaline (0.01) was dissolved in 17 ml of distilled water, 0.1 ml of serum and 0.9 ml of phosphate buffer (pH 7.8) was taken in triplicates in 2.5 ml buffer. A volume of adrenaline solution (0.3 ml) was added and mixed in the cuvette. The absorbance was taken at 480nm at 30 seconds intervals for five (5) times. The change in of absorbance was used to determine superoxide dismutase activity.

**2.3.3. Determination of Glutathione concentration concentration in Chloroform and Methanol extracts of *H. Madagascariensis* leaves**

This was determined according to the method of [24]. A volume of the sample (0.1 ml) was mixed with 0.9 ml of normal saline in a beaker. Sodium sulphate (0.02 ml) was added, shaken and allowed to stand for 2 minutes at room temperature. A volume of 20% lithium sulphate (0.02 ml), 0.2 ml of 20% Na2CO3 and 0.2 ml of phosphor-18-tungstic acid were also added to the beaker, shaken and allowed to stand for 4 minutes while observing it for maximum colour development. A volume (2.5 ml) of 2% sodium sulphate was also added and the absorbance was taken at 680nm within 10 minutes, a blank (0.1 m H2O) was also set up. Glutathione concentration was calculated from a standard cystein curve.

**2.3.4 Determination of Lipid Peroxidation (Malondialdehyde)**

Lipid peroxidation was determined by measuring spectrophotometrically the level of the lipid peroxidation product, malondialdehyde (MDA) as described by [25].

**2.4 Anti-ulcerogenic Test**

**2.4.1 Indomethacin- induced ulcer**

This determination was carried out using the method of [26]. Twenty adult rats randomly divided into 5 groups of 4 rats each were deprived of food for 18 hours and administered orally with normal saline and varying doses of the (Chloroform and Methanol). The extracts and drug used were freshly prepared as a suspension in 3% tween 80 and administered orally to the animals in 5 ml/kg doses. Group 1(normal control) was administered 3 % tween 80 (2 ml/kg). Groups II, III and IV were treated with 100, 200, and 400 mg/kg of the respectively. Group V (reference group) was administered 100 mg/kg cimetidine, a standard anti-ulcer drug. Thirty minutes later, 50 mg/kg of indomethacin was administered (p.o) to the rats. After 8 hours, each animal in the groups was sacrificed by chloroform anesthesia and the stomach removed and opened along the greater curvature, rinsed with water and pinned flat on a board. Erosions formed on the glandular portions of the stomach were counted and the ulcer index calculated as described by Main and Whittle (1993). The ulcer was usually counted and scored 0= no ulcer; 1= superficial ulcer; 2 = deep ulcer; 3= perforations. The sum of all the lesions/ulcers in all the animals for each group (total ulcer score) was used to calculate the ulcer index. The percent ulcer inhibition was calculated relative to control as follows:

$$\% ulcer inhibition \left(\% U.I\right)=1-\frac{Ut}{Uc} X 100$$

Where Ut and Uc represents the ulcer index of the treated and that of the control group respectively.

NB: The serum of the ulcerated rats was used for *in vivo* antioxidant studies

**2.5 Statistical Analysis**

The results obtained were statistically analysed using the Statistical product and Service Solutions 20.0 and the results expresses as mean ± standard deviation. Significant differences in the results were established by one-way analysis of variance (ANOVA), and the acceptable level of significance was P< 0.05 for all the results.

**RESULTS**

**3.1:** Effect ofmethanol and chloroform extracts of *H.madagascariensis* leaves on DPPH radical scavenging activities

As shown in Table 1, the methanol and chloroform extracts of *H.madagascariensis* leaves at different concentrations (15.7-1000 µg/ml) as well as the standard antioxidant agent, ascorbic acid, showed high DPPH radical scavenging activities. The result indicates that as the concentrations increase the percentage inhibition of the extracts increase.

**Table 1: Percentage inhibition of DPPH radical by methanol and chloroform extracts of *H. madagascariensis* leaves**

|  |  |  |  |
| --- | --- | --- | --- |
| Concentration (µg/ml) | % inhibitionMethanol | % inhibitionChloroform | % inhibitionAscorbic acid |
| 15.7 | 61.6 |  47.5 | 74 |
| 31.3 | 70.1 |  50.1 | 83.3 |
| 62.5 | 73.3 |  54.5 |  87.2 |
| 125 | 82.6 |  56.7 | 88.8 |
| 250 | 88.2 |  61.9 |  93 |
| 500 | 91.2 |  66.8 |  94 |
| 1000  | 93 |  72.5 | 95.7 |
| EC50  | 1.171  |  1.780 | 0.925 |

**3.2: Percentage inhibition of TAC of the methanol and chloroform extracts of *H. madagascariensis* leaves** .In the TAC assay, both extracts scavenged free radical, as indicated by their % inhibitions which peaked at 1000µg/ml. Total antioxidant inhibitory activities of methanol and chloroform extracts increased, though the increase were not concentration dependent. Methanol extract had 41.9, 53.9, 68.8, 63.8, 70.5 and 72%. Chloroform had 37, 50, 68.4, 44.7, 63.3, 63.2 and 97.9% while Ascorbic acid had 56, 78.3, 84.5, 85.4, 86.4, 82.9, and 92.6% inhibitions as shown in Table 2.

**Table 2: Total antioxidant capacity of the methanol and chloroform extracts of *H.***

***madagascariensis* leaves**

|  |  |  |  |
| --- | --- | --- | --- |
| Concentration (µg/ml) | % inhibitionMethanol | % inhibition Chloroform | % inhibitionAscorbic acid |
| 15.7 | 41.9 | 37 | 56 |
| 31.25 | 53.9 | 50 | 78.3 |
| 62.5 | 68.8 | 68.4 | 84.5 |
| 125 | 63.8 | 44.7 | 85.4 |
| 250 | 70.5 | 63.3 | 86.4 |
| 500 | 72 | 63.2 | 82.9 |
| 1000 | 74.2 | 79.9 | 92.6 |

As shown in table 3, the methanol extract of *H. madagascariensis* leaves showed higher reducing capability compared to the chloroform extract in all the concentrations used for FRAP investigation. This is shown in the number of Fe3+ ions reduced to Fe2+ and the quantity reduced in µMFe2+/g, which was concentration dependent. The reducing ability of the extracts was found to be significantly (p < 0.05) lower than that of the standard drug, ascorbic acid in most of the concentrations used.

**3.3: Ferric reducing power capacity of the methanol and chloroform extracts of *H.***

***madagascariensis* leaves**

**Table 3:** **Ferric reducing power capacity of the methanol and chloroform extracts of *H. madagascariensis* leaves**

|  |  |  |  |
| --- | --- | --- | --- |
| Concentration (µg/ml) | Methanol extract µMFe2+/g | Chloroform extract µMFe2+/g | Ascorbic acid µMFe2+/g |
| 31.5 | 16.5±1.5 | 3.44±0.83 | 19.71±0.1 |
| 62.5 | 34.7±1.44 | 6.81±0.56 | 39.2±0.83 |
| 125 | 78.8±2.25 | 17±3.38 | 84.87±3.9 |
| 250 | 183±17. | 30.75±1.15 | 107.2±12.4 |
| 500 | 198.1±1.07 | 64±8.53 | 243.3±41.2 |
| 1000 | 21.23±2.5 | 66.33±8.33 | 509±78.1 |

3.4: **Effect of methanol and chloroform extracts on indomethacin induced gastric ulcer in rats**

Data from Table 4 shows that indomethacin induced gastric ulcer in all the groups. Groups treated with chloroform extract had significant reductions (p <0.05) in the gastric ulcer formed as is shown from the significantly reduced ulcer lesion indices (ULI) of 2.0 ± 1.1, 1.55 ± 0.4, and 1.5± 0.4 obtained for 100, 200 and 400 mg/kg b.w of the extract respectively, when compared with that of the control (3.03 ± 0.4). The percentage inhibitions of ulcer, 33.9, 48.8 and 50.4% obtained for the various doses of the chloroform extract; 100, 200 and 400 mg/kg b.w respectively, were lower than the 71.1% inhibition obtained for the standard drug cimetidine.

The methanol extract treated rats showed significantly reduced ulcer lesion indices (ULI) of 1.4 ± 0.4, 1.38 ± 0.7 and 1.1± 0.9 obtained for the 100, 200 and 400 mg/kg b.w respectively, as compared to the 3.03 ± 0.5 ulcer index obtained for the control. The percentage ulcer inhibitions of 53.7, 57.9, and 63.6 % obtained for the methanol extract at 100, 200 and 400 mg/kg b.w respectively, were comparable with the 71.1 % inhibition obtained for the reference drug, cimetidine. Comparatively, the ulcer lesion indices of groups treated with 100, 200, and 400 mg/kg b.w of the methanol extract were significantly (p < 0.05) lower than those of the chloroform extract at equivalent doses. Thus, the percentage inhibitions produced by methanol extract were higher than those obtained for chloroform extract.

**Table 4: Effect of methanol and chloroform extracts of *H. madagascariensis* leaves on indomethacin induced gastric ulcer in rats**

|  |  |  |  |
| --- | --- | --- | --- |
| **Treatment** | **Dose (mg/kg)** | **Ulcer index** | **Percentage (%) ulcer inhibition** |
|  |  | Chloroform | Methanol | Chloroform | Methanol |
| Control (3% tween 80) | 2 ml |  | 3.03c | - | - |
| Extract |  100 | 2.00 ± 0.4b | 1.4 ± 0.4b | 33.9 | 53.7 |
| Extract | 200 | 1.55 ± 1.1ab | 1.38 ± 0.7a | 48.8 | 57.9 |
| Extract | 400 | 1.50 ± 0.4ab | 1.1 ± 0.9a | 50.4 | 63.6 |
| Cimetidine | 100 | 0.88 ± 0.5a | 0.88 ± 0.5a | 71.1 | 71.1 |

**3.5: Effect of methanol and chloroform extracts of *H. madagascariensis* leaves on antioxidant enzymes activities and lipid peroxidation product (MDA) of indomethacin induced ulcer rats**

Table 5 revealed that the extracts significantly (p < 0.05) increased the antioxidant enzymes activities of indomethacin induced ulcer in rats compared to the control group. This showed that the extracts have free radical scavenging capability. A significant decrease (p < 0.05) was observed in the malondialdehyde (MDA) concentrations of rats in the test groups treated with the extracts when compared with the untreated rats. However, no significant difference (p > 0.05) was observed in the MDA concentration of rats that received 400 mg/kg of both extract when compared to the standard drug.

**Table 5: Effect of methanol and chloroform extracts on antioxidant enzymes activities and lipid peroxidation product (MDA).**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Treatment (mg/kg)** | **SOD (IU/L)** | **CAT (U/ML)** | **GPX (IU/L)** | **MDA (mg/dl)** |
| Control | 11.86±0.09a | 0.88±0.02a | 8.03±0.02a | 4.44±0.06a |
| Methanol 100 | 12.42±0.01b | 1.12±0.02b | 8.28±0.05b | 2.50±0.01c |
| 200 | 12.85±0.03c | 1.18±0.05b | 8.38±0.02c | 2.13±0.10b |
| 400 | 13.30±0.20d | 1.19±0.08b | 8.69±0.10d | 2.03±0.05b |
| Chloroform100 | 12.04±0.04a | 0.93±0.03b | 8.08±0.02b | 2.57±0.02b |
| 200 | 12.48±0.10b | 1.04±0.01c | 8.11±0.01b | 2.47±0.03b |
| 400 | 12.80±0.21c | 1.09±0.01d | 8.17±0.01c | 2.38±0.01b |
| Cimetidine100 | 13.40±0.27d | 1.30±0.04e | 9.12±0.06d | 1.33±0.38b |

Means with different superscript are significantly different at (P < 0.05), while mean values with the same superscript are not significantly different (P > 0.05).

**4.0: DISCUSSION**

The study investigated the in vitro antioxidant potential of the leaf extracts (chloroform and methanol) of *H. madagascariensis*. Antioxidants protect cells from damage caused by free radicals, which can lead to lipid peroxidation and cell injury. The antioxidant activity was measured using the DPPH radical scavenging assay, which detects the ability of a substance to neutralize free radicals. Both extracts showed dose-dependent scavenging activity. The chloroform extract inhibited 47.5% to 72.5%, while the methanol extract inhibited 61.6% to 93%. The potency of the extracts was determined by their EC50 values, which represent the concentration needed to inhibit 50% of the free radicals. The EC50 for the chloroform extract was 1.780 µg/ml, and for the methanol extract, it was 1.171 µg/ml, compared to ascorbic acid, which had an EC50 of 0.925 µg/ml. This results are in line with [28]. The study also evaluated the total antioxidant capacity (TAC) and reducing power of *H. madagascariensis* extracts. The methanol extract showed higher TAC (41.9% to 74.2%) than the chloroform extract (37% to 79.9%), indicating that polar solvents are more effective in extracting antioxidant compounds like phenolics and flavonoids. This results are in accordance with [29]The FRAP assay, which measures reducing power, also showed stronger activity in the methanol extract (16.5 to 198 µM Fe2+/g) compared to the chloroform extract (3.44 to 66.3 µM Fe2+/g). This suggests the methanol extract has higher antioxidant potency, likely due to its higher phenolic content and reducing properties. Additionally, a significant decrease in enzymatic antioxidants was observed after ulcer induction, possibly due to excessive reactive oxygen species (ROS) consumption, which impaired antioxidant defense systems like glutathione (GSH), catalase, and superoxide dismutase. This reduction in antioxidant enzymes may increase sensitivity to free radical-induced damage. The study found that a reduction in antioxidant enzyme activities (SOD, CAT, GPx) could lead to harmful effects due to increased superoxide and hydrogen peroxide levels. However, pre-treatment with *H. madagascariensis* extracts boosted the activity of these enzymes, suggesting that the extracts might help neutralize free radicals or enhance antioxidant enzyme production, thereby protecting cells from oxidative stress. These results agreed with [30]. The study also highlighted that long-term use of NSAIDs can cause gastrointestinal ulcers by either damaging the mucosal barrier or decreasing protective prostaglandin production. In this context, *H. madagascariensis* extracts helped to reduce ulcer formation, with the methanol extract showing the most significant effect at 400 mg/kg, likely due to its high flavonoid content.

**Conclusion:** The methanol and chloroform fraction of *H. madagascariensis* shows promising antioxidant and anti-ulcerative properties when given orally in moderate doses to experimental rats. Thus, this plant could be considered a therapeutic option for individuals experiencing ulcer, in addition to its other values.

**Ethical Approval**

Animal Ethic committee approval has been collected and preserved by the author(s)

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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