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Screening for anti-inflammatory activity of extract and fractions of *Morinda lucida* stem bark and detection of bioactive secondary metabolite

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Abstract

Morinda lucida Benth (Rubiaceae) is a versatile plant used in traditional medicine for the treatment of a variety of ailments and the claims of its efficacy are particularly remarkable in the treatment of infections and immunoinflammatory disorders. This study evaluates the anti-inflammatory properties of methanolic stem bark extract and fractions of *M. lucida* and also identifies the phytochemicals responsible for its anti-inflammatory activity. The crude extract was subjected to liquid-liquid partitioning successively with n-hexane, ethylacetate, butanol and water. High performance liquid chromatography (HPLC) of the four fractions and Vacuum Liquid Chromatography fraction (VLC) of the promising fraction was evaluated. The effect of the fractions on egg albumen induced rat paw oedema were also evaluated. Anti-inflammatory activity of the fractions was further screened using xylene induced ear oedema models and human red blood cell membrane stabilization test. Ulcerogenic test on the normal stomach mucosa was also evaluated. The result of the egg albumen induced rat paw oedema showed that the butanol fractions maximally inhibited egg albumen induced effect at 400 mg/kg (70%) and 200mg/kg (67.5%) after 180 minutes compared to the positive control, ibuprofen (20mg/kg) with 100% inhibition after 180 minutes. The result of the xylene induced ear oedema showed that the inhibition produced by 100 µg/ear of the Butanol fraction (BF) was 56.67 % and was greater than inhibition produced by 200 µg/ear of ibuprofen (38.89 %). HPLC analysis of the fractions revealed the following phytochemicals; Cytreo-a-pyrone, Cytosporin- J and Waol A. Ulcerogenic test was negative at the doses of 200 mg/kg and 400 mg/kg of the fractions when compared with the indomethacin (positive control) at dose of 50 mg/kg. Human red blood cell membrane stabilization assay showed that BF-VLC 2 (Dichloromethane: methanol (8:2) VLC of Butanol fraction) exhibited concentration dependent inhibition of heat-induced haemolysis while other extract showed a non-concentration dependent inhibition of haemolysis when compared to the standard, ibuprofen. These findings suggest that the stem bark of *M. lucida* possess promising anti-inflammatory phytochemicals which justify its use in ethnomedicine.

Keywords: Haemolysis; *Morinda lucida*; Paw oedema; Ear edema; Phytochemicals

1. Introduction

Inflammatory diseases, such as rheumatism and arthritis, have continued to be a significant cause of debilitation, morbidity and mortality globally. Inflammation leads to serious and chronic problem in the human body [1]. Most available therapeutic agents for the treatment of inflammatory diseases lack specificity and had posed various adverse effects ranging from gastrointestinal irritations, gastric ulcer, nephrotoxicity, hypertension among others [2]. Active compounds from medicinal plants are preferred as drug candidates because of their efficiency, cultural acceptability

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and little or no side effects [3]. The biological activities of medicinal plants are due to combination of their bioactive compounds which are secondary metabolites with wide range of chemical structures and biological activities [4].

Morinda lucida Benth (Rubiaceae) is a versatile plant used in traditional medicine for the treatment of a variety of ailments and the claims of its efficacy are particularly remarkable in the treatment of infections and immunoinflammatory disorders [5].

M. lucida is used for treating diabetes, hypertension, cerebral congestion, dysentery, stomach-ache, ulcers, leprosy and gonorrhoea [6]. Stem bark, roots and leaves infusion of *Morinda lucida* is used as an antimalarial, antidiabetic and in treatment of jaundice, dysentery and diarrhoea [7]. Previous studies show that it is an important plant in traditional medicine. In this study, we evaluated the anti-inflammatory properties of fractions of stem bark extract of *Morinda lucida*.

2. Material and methods

2.1. Reagents, Chemicals and instruments

10% of tween 80, egg albumen, Ibuprofen, Methanol (JHD China), Xylene, Normal saline, Test tubes, Centrifuge, Spectrophotometer, Human blood, Stop watch, Water bath, Rotary evaporator RE300, (Stuart, Barloworld Scientific Ltd, Stone, Staffordshire, St15 Osa, UK); Weighing balance (Ohaus, China); and Vacuum Liquid Chromatography column.

2.2. Experimental animals

Albino Swiss mice (15 – 25 g) and Wistar rats (120-150 g) of both sexes were obtained from the colony breed of the animal house of the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu, Anambra State. Animals were handled in compliance with the national institute of health guidelines for the care and use of laboratory animals (Pub. No. 85-23, revised 1985) as approved by the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu, Anambra State.

2.3. Plant collection and authentication

Stem bark of *Morinda lucida* was collected from Abakiliki in Ebonyi state. The Plant material was authenticated by Mr. Alfred Ozioko, a plant taxonomist in Centre for Ethnomedicine and drug development, a subsidiary of Bio-resources Development and Conservation Program (BCDP), Nsukka, Enugu State.

2.4. Plant extraction, fractionation and isolation

About 1 kg of the pulverized stem bark was extracted in 7.5 L of methanol by cold macerated for 72 h with intermittent shaking. The filtrate recovered was concentrated to dryness using rotary evaporator at 50°C. The crude extracts were subjected to liquid-liquid fractionation to obtain n-hexane, ethyl acetate, butanol and water fractions. All of the fractions obtained were concentrated *in vacuo* using rotary evaporator set at a revolution of 70 RPM and a temperature of 50°C. Analytical HPLC was carried out on the four fractions with a Dionex P580 HPLC system coupled with a photodiode array detector (UVD340S) using methanol and nanopure water as the mobile phase. UV Detection of Compounds was done at 235, 254 and 340 nm and identified based on their peak similarity with data in the inbuilt library. From the bioassay conducted, the most active fraction (butanol) was subjected to vacuum liquid chromatography (VLC) using 500 mL of various ratios of dichloromethane and methanol 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10.

2.5. Acute toxicity studies

Acute toxicity studies were done to determine the median Lethal Dose (LD50) of the fractions. This assessment was carried out using the standard method (8) with little modifications. The mice were placed in four groups of three animals in each group and the fractions (HF, EF, BF and WF) at doses of 2000mg/kg, 3000mg/kg, 400mg/kg and 5000mg/kg were administered orally. The treated animals were observed for 24 h for any behavioral changes as well as mortality.

2.6. Anti-inflammatory assays

2.6.1. Egg albumin induced paw oedema test

This test was done using standard method [5]. Fifty albino rats of both sexes were randomly allocated into ten groups of five animal each as follows: group 1, control, group 2 and group 3 received 200 and 400 mg/kg of n-hexane fraction, group 4 and group 5 received 200 and 400 mg/kg of ethyl acetate fraction, group 6 and group 7 received 200 and 400

mg/kg of butanol fraction, group 8 and group 9 received 200 and 400 mg/kg of water fraction and group 10, positive control received 20mg/kg of ibuprofen. Thirty minutes after administration of different treatments, initial paw diameter (0 h) was measured with the aid of a cotton thread wrapped around the perimeter of the hind paw. Thereafter, 0.1 mL of fresh egg albumin was injected into the sub plantar region of the left hind paw of animal and subsequent paw diameter were measured and recorded at 60, 120 and 180 mins. Paw oedema was estimated as the difference between the paw diameter at the zero hour (V_0) and the paw diameter at other time intervals (V_t) after the administration of the fresh egg albumin. Percentage inhibition of the paw oedema in the test group was calculated relative to paw oedema in control group at various time points.

2.7. Effect of fractions on xylene induced topical oedema

This test was carried out using the standard method [5] with little modification. A total of fifty five albino mice of both sexes were randomized into eleven groups of five animals each as follows; Group 1 control (received xylene 50 μ L/ear), Group 2-3 (received Ibuprofen 100 μ g/ear and 200 μ g/ear), Group 4-5 (received 100 μ g/ear and 200 μ g/ear of butanol fraction), Group 6-7 (received 100 μ g/ear and 200 μ g/ear of BF- VLC 2, DCM: Methanol, 8:2), Group 8 -9 (received 100 μ g/ear and 200 μ g/ear of BF- VLC 3, DCM: Methanol, 4:6), Group 10- 11 (received 100 μ g/ear and 200 μ g/ear of BF- VLC 4, DCM: Methanol, 7:3). After grouping, the test samples were applied to the anterior (outer) surface of the mice right ear, while oedema was induced by the application of 50 μ L of xylene on the posterior surface of the same right ear. After two hours, the mice were sacrificed by cervical dislocation and both left and right ears were removed. Circular portions (4 mm) of both the right (treated) and the left (untreated) ear were punched out with the aid of a cork borer and weighed using analytical weighing balance. Oedema was quantified as the weight difference between right and left ear plug of the animals. Percentage Inhibition of oedema in the test groups were calculated relative to oedema of the control group.

2.8. Ulcerogenic test

The study was carried out using standard method [9] with little modification. Forty albino mice (20.00 \pm 3.00 g) of both sexes were fasted for 18 h before the experiment and randomized into eight groups of 5 animals each and were treated as follows: group 1, control (received distilled water 10ml/kg), group 2: Indomethacin (received 50mg/kg), group 3-4: Butanol fractions (received 200mg/kg and 400mg/kg), group 5-6: BF VLC -2, DCM: Methanol, 8:2 (received 200mg/kg and 400mg/kg), group 7-8: BF- VLC 4, DCM: Methanol, 7:3 (received 200mg/kg and 400mg/kg). Four hours after administration, the animals were sacrificed by cervical dislocation. The stomach was removed and cut along the larger curvature and the mucosal surface was exposed. The mucosa was washed with normal saline and observed for presence or absence of lesion (ulcers). The ulcer index was scored using the following arbitrary scale: No lesion (normal mucosal appearance) =0, Spot ulcer =1, Haemorrhagic streak =2, Deep ulcer =3 and Perforation =4

2.9. Human red blood cell stabilization test

2.9.1. Preparation of erythrocyte suspension

This assay was carried out using the standard method [10] with little modification. The stock solutions (50 μ g, 100 μ g and 200 μ g and 400 μ g) of the fractions were prepared according to the following groupings: group 1: control, group 2: indomethacin, group 3: butanol fraction, group 4: BF-VLC 1(DCM: Methanol, 3:7), group 5: BF-VLC 2 (DCM: Methanol, 8:2), group 6: BF-VLC 3 (DCM: Methanol, 4:6), group 7: BF-VLC 4 (DCM: Methanol, 7:3) and group 8: BF-VLC 5 (DCM: Methanol, 6:4)). Venous blood sample were collected from a volunteer into an EDTA tubes and centrifuged at 400 rpm for 5 min. The supernatant was removed and the residue (red cells) was washed with normal saline three times until the supernatant became clear. The red cells were reconstituted with isotonic buffer of pH 7.4.

2.9.2. Heat Induced Haemolysis

Two milliliters (2 mL) of various concentrations of the fractions were prepared in duplicate with isotonic buffer solution. Thereafter, 20 μ L of the erythrocyte suspension was added each tube and was mixed gently. The mixture was heated in the water bath at 54°C for 30 min. The reaction was allowed to cool and then centrifuged at 1500 rpm for 3 min. The absorbance of the supernatant was taken using the spectrophotometer at 540 nm. Percentage Inhibition of the test groups were calculated relative to that of the control group.

2.10. Statistical Analysis

All data obtained were expressed as Mean \pm SEM (standard error of mean). The results were analyzed using SPSS version 16 and presented as Mean \pm SEM. Significance between control and extract treated groups were determined

using student *t*-test and one-way Analysis of variance (ANOVA). Differences between means were considered statistically significant at $P < 0.05$.

3. Results

3.1. Acute toxicity

There were no sign of toxicity or death following treatment with various doses of the fraction (up to 5000mg/kg) 24 hours after treatment of mice. No toxicity or death were found after three days observation period.

3.2. Anti-inflammatory results

3.2.1. Effect on egg Albumin Induced paw oedema

Administration of 200 and 400 mg/kg doses of the fractions causes a reduction in paw oedema when compared to control group. Butanol fraction elicited a dose dependent inhibition against egg albumin induced paw oedema that was more marked than other groups. Each of the fractions showed better and more significant activity at the 180 min (Table 1).

3.3. Effect of butanol fraction (BF) and butanol VLC fractions on xylene-induced ear oedema

The fractions exhibited a strong inhibition of topical inflammation induced by xylene on the mice ears. These inhibitions were significant ($p < 0.05$) when compared to the control group. Among the fractions, butanol fraction (BF) exerted the highest activity while BF-VLC 4 fraction (DCM: Methanol, 7:3) produced the least activity (Figure 1).

3.4. Effects of fractions of *Morinda lucida* on heat induced haemolysis

Table 1 The Effect of fractions on Egg Albumin Induced Paw Oedema

Group	Dose (mg/kg)	Increase in paw oedema (cm) at time T(min)			
		T30	T60	T120	T180
Gp1 (Control)	-	0.78 ± 0.02 (-)	0.68 ± 0.07 (-)	0.46 ± 0.06 (-)	0.40 ± .06 (-)
Gp2 (N-hexane fraction)	400	0.50 ± 0.05 (35.90)	0.68 ± 0.04 (0.00)	0.33 ± 0.05 (28.26)	0.27 ± 0.10 (32.50)
Gp3 (N-hexane fraction)	200	0.27 ± 0.06 (65.38)	0.50 ± 0.07 (26.47)	0.27 ± 0.10 (41.30)	0.17 ± 0.10 (57.50)
Gp4 (Ethylacetate Fraction)	400	0.37 ± 0.15 (52.56)	0.33 ± 0.15 (51.47)	0.33 ± 0.10 (28.26)	0.20 ± 0.03 (50.00)
Gp5 (Ethylacetate Fraction)	200	0.40 ± 0.12 (48.71)	0.43 ± 0.06 (36.76)	0.17 ± 0.03 (63.04)	0.20 ± 0.09 (50.00)
Gp6 (Butanol Fraction)	400	0.42 ± 0.05 (46.15)	0.40 ± 0.08 (41.18)	0.26 ± 0.05 (43.48)	0.12 ± .04 (70.00)
Gp7 (Butanol Fraction)	200	0.44 ± 0.07 (43.60)	0.43 ± 0.08 (36.76)	0.40 ± 0.07 (13.04)	0.13 ± 0.06 (67.50)
Gp8 (water Fraction)	400	0.45 ± 0.07 (42.31)	0.40 ± 0.03 (41.18)	0.20 ± 0.05 (56.52)	0.17 ± 0.00 (57.50)
Gp9 (Water Fraction)	200	0.48 ± 0.03 (38.46)	0.50 ± 0.06 (26.47)	0.33 ± 0.06 (28.26)	0.23 ± 0.09 (42.50)
Gp10 (Ibuprofen)	20	0.34 ± 0.15 (56.41)	0.25 ± 0.05 (63.24)	0.20 ± 0.10 (56.52)	0.00 ± 0.00 (100)

Values in parenthesis represent the inhibition (%) of oedema
Values are presented as mean ± Standard error of mean (SEM), student's *t*-test (N=5)

The fractions exhibited a strong inhibition of heat-induced haemolysis of human erythrocyte. These inhibitions were significant ($p < 0.05$) when compared to that of the control group. Among the fractions, BF-VLC 4 fraction exerted the highest concentration dependent inhibition of haemolysis while BF-VLC 3 (DCM: Methanol, 4:6) produced the least activity when compared to the standard, ibuprofen (Figure 2).

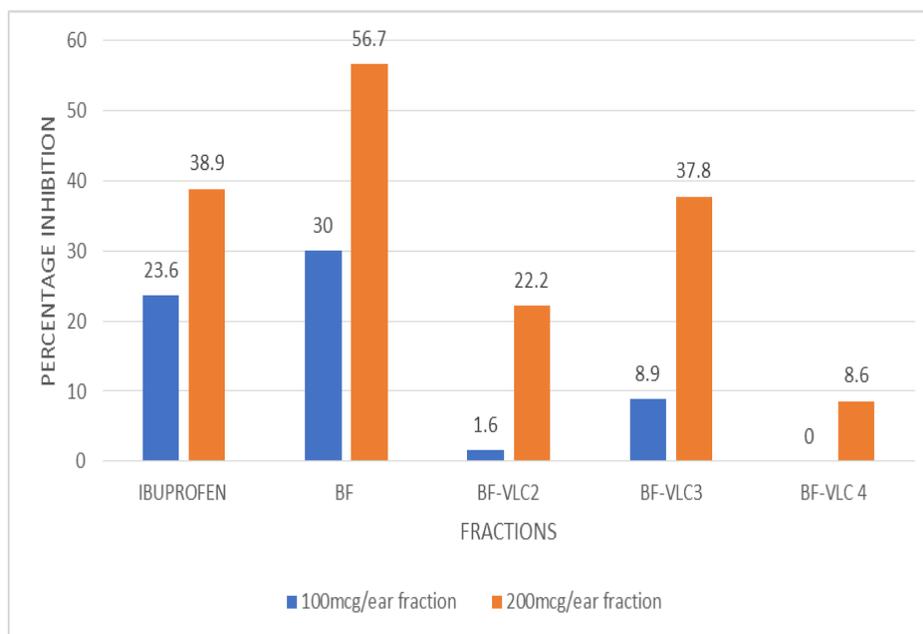


Figure 1 Percentage inhibition of n-hexane, ethyl acetate, n-butanol and aqueous stem bark fractions of *M. lucida* on xylene induced ear oedema. $P < 0.05$ compared with negative control

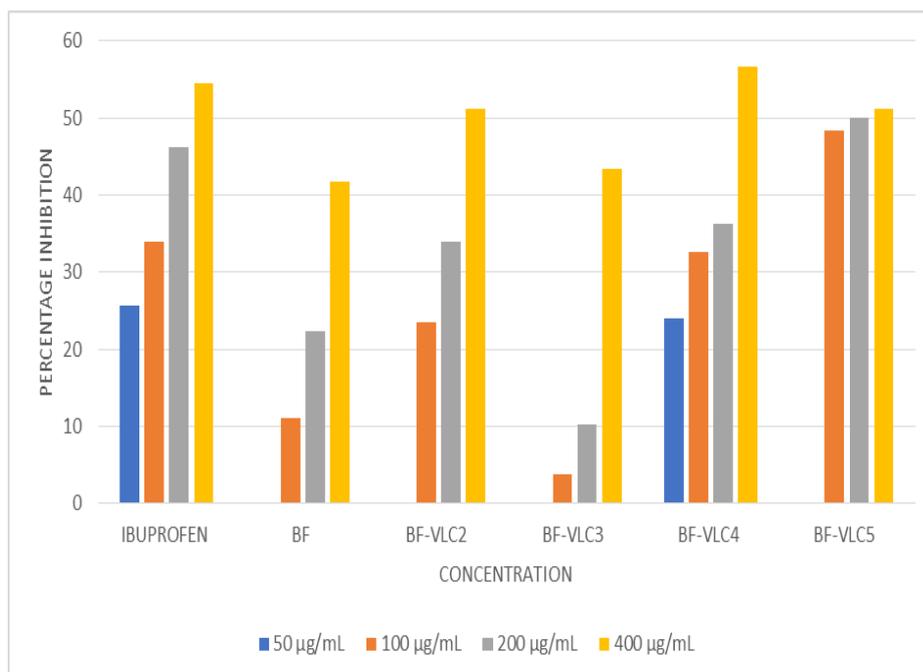


Figure 2 Effects of fractions of *Morinda lucida* on heat induced haemolysis. $P < 0.05$ compared with negative control

3.5. Effects on gastric mucosa integrity

There were spot ulcers in indomethacin-treated group. However, administration of the butanol fraction (BF), BF-VLC 2 (DCM: Methanol, 8:2) and BF-VLC4 (DCM: Methanol, 7:3) did not produce gastric lesions (Table 2).

Table 2 Effects on gastric mucosa integrity.

Treatment groups	Dose (mg/kg)	Ulcer score
Group 1: (Control)	Distilled water (10ml/kg)	0.00 ± 0.00
Group 2: (Indomethacin)	50	1.00 ± 0.00
Group 3- 4: (BF)	200	0.00 ± 0.00
	400	0.00 ± 0.00
Group 5-6: (BF-VLC2)	200	0.00 ± 0.00
	400	0.00 ± 0.00
Group 7-8: (BF-VLC4)	200	0.00 ± 0.00
	400	0.00 ± 0.00

Values are expressed as Mean ±SEM of sample replicates (n=5)

Scoring of ulcers: Normal colored stomach (0); Red coloration (1); Spot ulcer (2); Hemorrhagic speak (3); Deep ulcers (4); Perforation (5). HPLC chromatogram and UV spectrals of major compounds detected in the fractions Figure 3 - 5 Show Cytreo-a-pyrone, Cytosporin- J and Waol A as the major compound in the fractions.

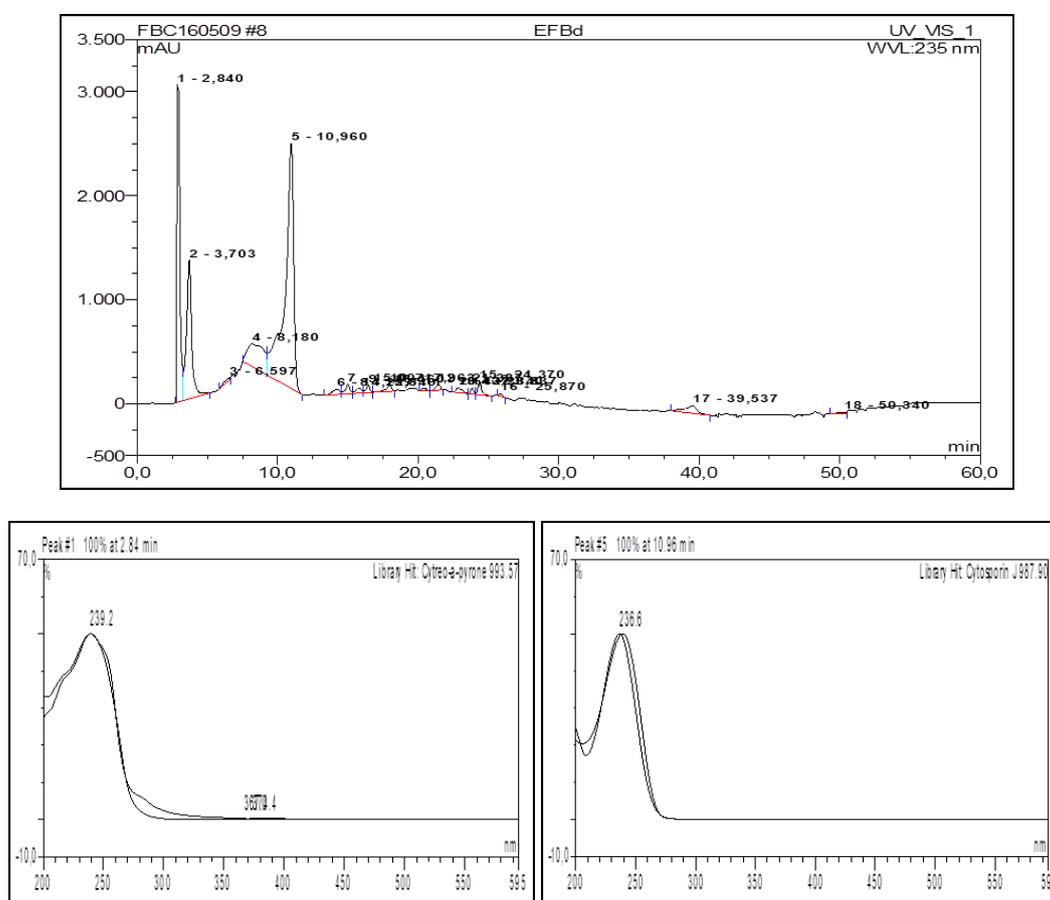


Figure 3 HPLC Chromatogram and UV- Spectra of major compounds detected in the butanol fraction (BF) of the methanolic stem bark extract of *Morinda lucida*. A= Cytreo-a-pyrone (Rt =2.84 min, 993.57), B= Cytosporin- J (Rt =10.96 min, 987.90).

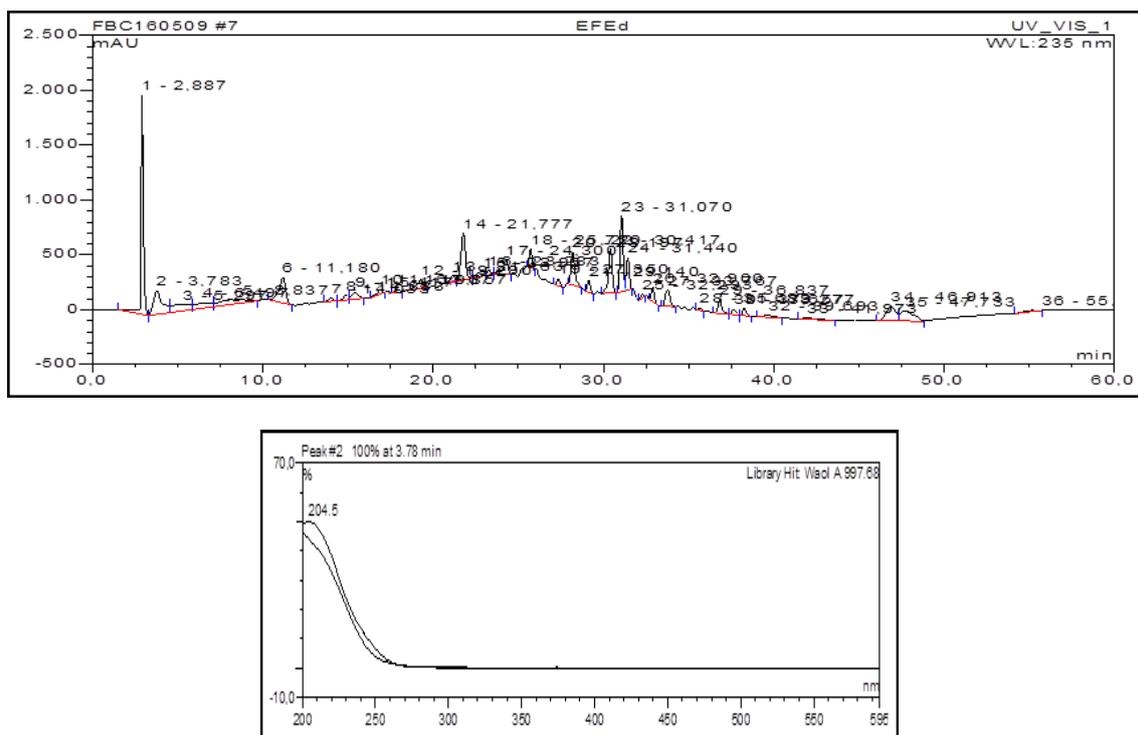


Figure 4 HPLC Chromatogram and UV- Spectra of major compounds detected in the ethyl acetate fraction (EF) of the methanolic stem bark extract of *Morinda lucida*. C= Waol A (Rt =3.78 min, 997. 68)

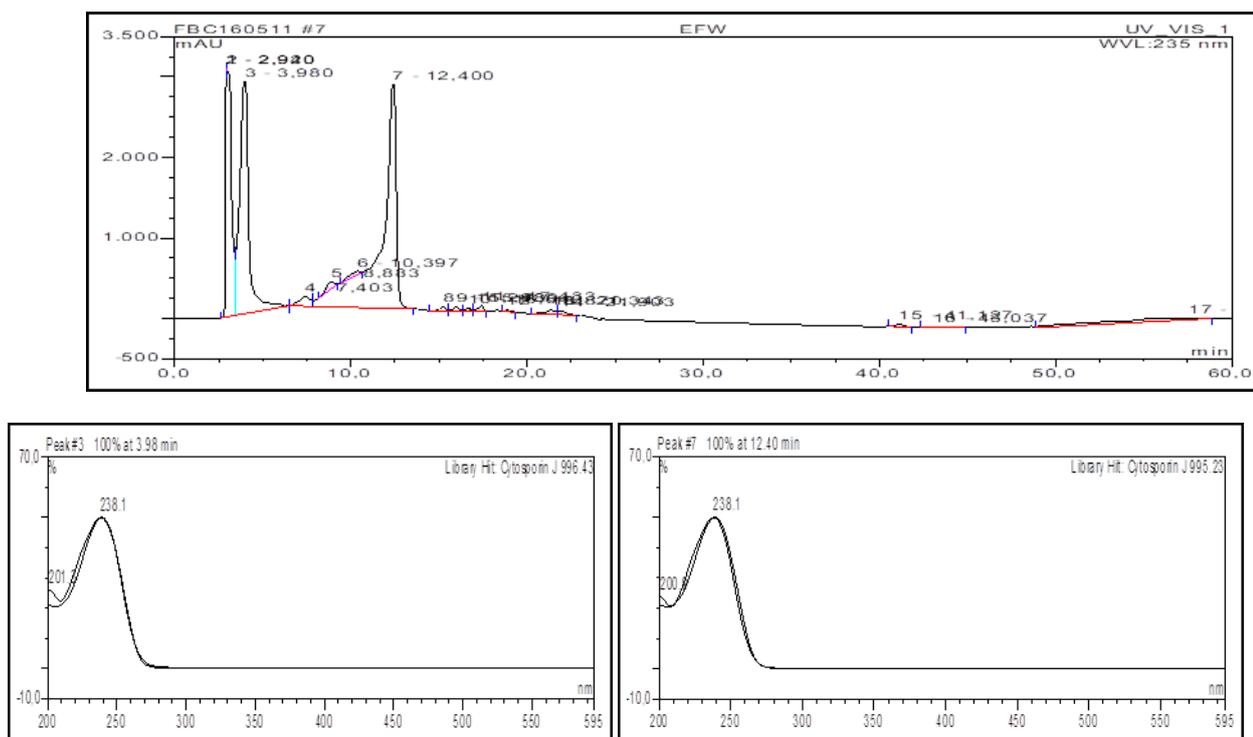


Figure 5 HPLC Chromatogram and UV- Spectra of major compounds detected in the water fraction (WF) of the methanolic stem bark extract of *Morinda lucida*. D =E= *Cytosporin J* (3.98 mins, 996.43; 12.40mins, 995.23)

4. Discussions

Inflammatory disorders constitute a major global health challenge. Although synthetic drugs for the alleviation of these disorders are dominating the market, their adverse effects raise a lot of concerns [11]. Safety and efficacy govern scientific exploration of natural product as alternative anti-inflammatory agents. The present study therefore evaluated the anti-inflammatory properties of methanolic stem bark extract and fractions of *M. lucida*. In the current study, acute toxicity test revealed the absence of toxic symptoms or mortality at 5000 mg/kg dose, suggesting that *M. lucida* stem bark fraction is safe in the treatment of acute inflammatory conditions. From the anti-inflammatory results, significant reductions in paw oedema by the fractions especially at 180 min, suggest their ability to inhibit acute phase of inflammatory responses which is characterized by the release of inflammatory mediators associated with the first phase (histamine and serotonin) and the mediators associated with the second phase (prostaglandin and bradykinins). Secondary metabolites of this plant have been reported as an enhancer of immune-restoration and upregulates the expression of cytokines and immune-stimulatory markers [5]. In the present investigation, fractions significantly inhibited the xylene -induced increases in ear weight. This inhibition capacity of the fractions can be regarded as the evidence of anti-inflammatory efficacy through reducing vasodilation and improving oedematous condition which also suggest the inhibition of phospholipase involved in the pathophysiology of inflammation due to xylene [12]. Application of xylene is known to cause irritation of living tissues, increase in prostaglandin E₂ production and oedema production at the site of inflammation. From the result of this study, inhibition of xylene induced ear oedema by the fraction suggests that they possess activity against acute inflammation and could be useful in management of skin- related inflammatory disorder. The human red blood cell membrane stabilization results show that butanol fraction (BF) and butanol sub-fraction at concentrations of 50, 100, 200 and 400 µg/ml protected significantly ($p < 0.01$) the erythrocyte membrane against lysis induced by heat. Prolonged administration of NSAIDs is commonly associated with gastrointestinal bleeding and peptic ulcer [13]. Based on our findings, there were no gastric lesions in the group treated with the fractions compared with indomethacin, a reference NSAID. This suggest that the fractions are not associated with gastrointestinal irritation and could be a better choice in the management of inflammatory disorder than conventional NSAIDs. A study conducted by group of scientists [14] revealed that damage to the gastric mucosa of a normal stomach can only occur when expression of both COX - 1 and COX - 2 are inhibited. This also suggest that the possible mechanism of anti-inflammatory activity is not directly associated with COX-1 inhibition but COX- 2. Some of the compounds revealed by the HPLC results of the butanol fractions (Cyteo- a- pyrone and Cytosporin J) may be responsible for the observed anti-inflammatory properties.

5. Conclusion

From this study, we discovered that the fraction of *M. lucida* elicited anti-inflammatory activities due to their bioactive secondary metabolites. This study revealed that the isolated compounds in the butanol fraction could be contributing to the anti-inflammatory effects of *butanol fractions of the stem bark of Morinda lucida* which justifies its importance in traditional medicines in the management of inflammatory conditions. This may also justify its use in enhancement of immune-restoration and upregulates the expression of cytokines and immune-stimulatory markers.

Compliance with ethical standards

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Disclosure of conflict of interest

The author declares no conflict of interest.

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