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Phytochemical and Pharmacological Evaluation of Extract of Morinda Citrifolia as Anti-Inflammatory Agent

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ABSTRACT:
Development of effective and economical nonsteroidal anti-inflammatory drugs (NSAIDs) with minimal or no gastrointestinal (GI) side effects is an area of importance in drug discovery pharmaceutical industry. Due to these side effects and health problems of existing anti-inflammatory therapies, natural anti-inflammatory supplements are becoming more popular and many scientific investigations have been concentrated on this area. In recent years, enormous deal of effort has focused on using available experimental techniques to verify natural antioxidant and anti-inflammatory drugs from natural product resources. Morinda citrifolia (Family: Rubiaceae) have been used since ancient times in the traditional medicinal systems like, Ayurveda, Siddha, Chinese and many other system of medicines in the treatment of various ailments. The fruits have been used topically in various conditions like, sprains, swellings, wounds and bruises. In present work Phytochemical and Pharmacological Evaluation of various Extract of Morinda Citrifolia fruit and stem were performed t proves as Anti-Inflammatory Agent. The In vitro bovine serum albumin denaturation inhibition assay of petroleum ether, chloroform, ethyl acetate, methanol and water extract of M citrifolia fruit, stem were performed. The result of M. citrifolia fruit extract (400 µg/ml) has more effect than M. citrifolia stem extract. The In vitro inhibitory effects of petroleum ether, chloroform, ethyl acetate, and methanol and water extract of both plant parts at different concentrations were measured on in-vitro enzymatic activities against COX-1 and COX-2 receptor. Methanol extract of M. citrifolia fruit extract was found more effective in all in vitro anti-inflammatory activity than other extract.

Introduction:
Inflammation is generally described as consisting of separate acute and chronic phases, though there is overlap between these processes. In the acute phase, leukocytes, primarily granulocytes, migrate along a chemotactic gradient to the site of injury in a carefully orchestrated effort that is mediated by cytokines and acute-phase proteins, with the objective of removing the inflammatory stimulus (e.g., infectious agent, foreign material) or cells damaged by injury and initiate healing **[1]**. Depending on the degree of injury, this acute cellular phase may be sufficient to resolve any damage. Chronic inflammation is reported to contribute to numerous diseases, including arthritis, asthma, atherosclerosis, autoimmune diseases, diabetes, and cancer, and to conditions of aging **[2]**. Acute inflammation is a short procedure, lasting from minutes to a few days, and its major features are leakage of plasma proteins or fluid and movement of leukocytes into an extravascular area. These cellular and vascular reactions are intermediated by chemical factors produced from cells or plasma and are responsible for the classic clinical symptoms of inflammation such as swelling, redness, pain, warmth, and loss of function **[3]**. Chronic inflammation can have several secondary consequences in biological response associated with enhanced risk of chronic diseases and disorders. Chronic inflammation in tissue usually happens when inflammatory responses are in the absence of an actual stimulus. It usually occurs through infections that are not resolved either within endogenous protection mechanisms or via some other resistance mechanism from host defences **[4]**. They can also happen from physical or chemical agents, which cannot be broken down, as well as from some kind of genetic susceptibility. Persistence of foreign bodies, continuous chemical exposures, recurrent acute inflammation, or specific pathogens are all crucial reasons for chronic inflammation **[5]**. The inflammation can be cure by addition of free radical as a molecule or atom that carries one or more unpaired electrons and is able to exist independently. Meanwhile, free radicals have an odd number of electrons; this makes them short lived, highly reactive, and unstable. **[6]**. Anti-inflammatory drugs include "biologicals" like anticytokine therapies which block the activity of various kinases and show a significant decrease in host defence toward infections. Due to these side effects and health problems of existing anti-inflammatory therapies, natural anti-inflammatory supplements are becoming more popular and many scientific investigations have been concentrated on this area. In recent years, enormous deal of effort has focused on using available experimental techniques to verify natural antioxidant and anti-inflammatory drugs from natural product resources. The proposed work has herbal drugs and their constituent derivatives are the prerequisite demand in every developing and developed country due to their medicinal properties and economic procurement. Morinda citrifolia (Family: Rubiaceae) have been used since ancient times in the traditional medicinal systems for various treatment. The successive solvent extraction of Morinda citrifolia fruits are investigated for phytochemical and pharmacological investigation.

Material and Methods: The pharmacognostic study of plant material was support for confirmation and determination of identity, purity and quality of a crude drug. The Morinda citrifolia fully grown plants stem and fruits for study were collected and dried under shade. Stem and fruits of Morinda citrifolia were powdered to 60# separately and stored in airtight containers and used for phytochemical and pharmacological studies.

Pharmacognostical studies

Macroscopical study: The macroscopical description of different parts of Morinda citrifolia plant include size, shape, nature of outer and inner surfaces, types of fracture, and organoleptic characters like color, odour, taste etc. were studied **[7]**.

Microscopical study (Transverse section of crude drug (fruit and stem): The Transverse sections were taken by placing the Morinda citrifolia between the thumb and four finger of the left hand. Using sharp razor held in the right hand, thin section was made the razor across the object in quick successions. The sections were transferred in to watch glass containing water, added chloral hydrate to these sections, boiled, filtered and the sections were stained with phloroglucinol and hydrochloric acid (1:1) and the same was mounted in glycerin and observed under low power. In order to supplement the descriptive part, photomicrographs in different magnification of all necessary cells and tissues were taken. For the purpose of studying crystals and starch grain photographs of unstained slide were taken. For normal histological purpose, sections were photographed under the bright field light.

Powder microscopy: The shade dried fruit of Morinda citrifolia fruit/stem were powdered and the powders passed through sieve no. 60# separately and individually then subjected to powder analysis. Each one of the three powders was taken to which few drops of chloral hydrate was added and heated for one to two minutes. The chloral hydrate was used to clear the tissues and for clarification. To the cleared powder, a few drops of 1:1 mixture of phloroglucinol and HCl was added and then it was finally mounted with glycerin. Lignified tissue acquired pink colouration. In order to study starch grains, powder were mounted with water and one to two drops of dilute iodine, while to observe the calcium oxalate crystals unstained sections were mounted only with water.

Physicochemical Evaluation: Physicochemical (Proximate) analysis helps to set up certain standard for dried crude drugs in order to avoid batch-to-batch variation and also to judge their quality. Their studies also give an idea regarding the nature of phytoconstituents present. Proximate analysis of Morinda citrifolia fruit/stem powders were carried out using methods prescribed in the Ayurvedic pharmacopoeia of India by subjecting them to various determinations like total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive value, water soluble extractive value, loss of moisture content and swelling index **[8]**

In vitro Anti-inflammatory Activity:

Human Red Blood Cell Membrane Stabilizing Activity: Inflammation is caused by inflammatory mediators released due to rupture of lysosomes which have membrane structurally similar to Human Red Blood Cell membrane. Protective effect of any extract/drug on heat or hypotonic saline induced erythrocyte lysis is a very good index of antiinflammatory activity any extract/drug. Erythrocyte lysis is determined by measuring the red colour intensity (hemoglobin released due to rapture) at 560 nm **[9]**. This method assesses the membrane stabilizing activity of various plants extract on erythrocyte against the osmotic pressure created using Alsever solution. Blood was collected from Peoples Medical College Blood Bank, Bhopal. The collected blood was mixed with equal volumes of Alsevers solution, centrifuged (3000 rpm), then the packed cells were washed with isosaline and suspension (10% v/v) was made. The drug and extracts samples were prepared by suspending the residue in hot water (concentration 50µg/ml to 250µg/ml). The assay mixture contained the drug/ extracts, phosphate buffer (1ml), 0.25% w/v hyposaline (2ml), Human Red Blood Cell membrane (0.5ml) suspension. Indomethacin was used as the reference drug and 2ml of distilled water as control. All the assay mixtures were incubated at 37°C for 30 minutes and centrifuged. The hemoglobin content in the supernatant solution of all assay mixtures was estimated using a UV spectrophotometer at 560 nm. The percentage haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100%. Percentage of protection was calculated using the following equation.

% of membrane stabilization = 100 - Atest / Acontrol x100

Where, Atest is the absorbance of the test solution and Acontrol is the absorbance of the control solution.

Bovine Serum Albumin Denaturation Inhibition Assay: The compounds that inhibit the denaturation of proteins in vitro may be used as anti-inflammatory agents (inflammation cause denaturation of proteins). Bovine Serum Albumin (BSA) may be used for denaturation inhibition study. When BSA is heated, it undergoes denaturation and expresses antigens associated with type III hypersensitive reaction associated with chronic inflammatory diseases. Thus drug/compounds that stabilize proteins from denaturation may possess anti-inflammatory activity **[10]**. The reaction mixture (3ml) contained, test solution (50 µl) of various concentarion prepared in methanol (100, 200, 400µg/ml), 5% w/v BSA (450µl) was added to all the above test tubes. For control tests,50 µl of distilled water instead of test solution. The test tubes were incubated at 37ºC for 20 minute and then heated at

57ºC for 3 minutes. After cooling the test tubes, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. The absorbance of these solutions was determined by using spectrophotometer at a wavelength of 660nm.

Extracts were dried in vacuum oven and re-dissolved in iso saline seperately. Different concentrations (100, 200, 400µg/ml) of extracts were prepared and added to 1 % BSA solution (1.8 ml). The pH of mixture was adjusted to 6.5 using 1N Hydrochloric acid and the mixture was incubated at 37 °C for 30 min and then heated upto 50°C for 10 minute, then cooled, following the absorbance was measured at 660 nm. Diclofenac sodium was used as the standard and a solution without extract or drug was considered as the control. The experiment was performed in triplicates. The relative percentage inhibition of protein denaturation was calculated as.

% Protein denaturation inhibition = [(Absorbance of control – Absorbance of test)/Absorbance of control] × 100

Result and Discussion:
The pharmacognostic study of plant material was support for confirmation and determination of identity, purity and quality of a crude drug. Morinda citrifolia is also an evergreen shrub or small crooked tree with a conical crown, 3-8(-10) m tall, with a deep taproot; bark greyish or yellowish-brown, shallowly fissured, glabrous; branchlets quandrangular. Leaves were opposite and simple, elliptic-lanceolate, (10-)15-50 cm x 5-17 cm, entire, acute to shortly acuminate at apex, cuneate at base, pinnately nerved, glabrous; petioles 0.5-2.5 cm long; stipules variable in size and shape, broadly triangular. Fruit an ovoid syncarp of yellow-white., pyramidal, 2-seeded drupes, 4-11 cm x 2-3 cm, yellow-white **(Table 1 and Figure 1)**. Microscopic evaluation of the powder showed the presence of single acicular calcium oxalate crystals, lignified cells, starch cells and oil globules. The transverse section of fresh Morinda citrifolia fruits showed a single layered epidermis and mucilaginous hypodermis region containing oil glands. The mesocarp was identified by the presence of vascular bundles **(Figure 3)**. Transverse section of Morinda citrifolia stem in outer most region was found to contain 2-4 layers of closely packed cells having greenish colour in cork. Cortex contains 2-5 layers of collenchyamatous cells and 3-8 layers of loosely arranged parenchymatous. Microscopic evaluation of the powder showed the presence of single acicular calcium oxalate crystals, lignified cells, starch cells and oil globules **(Figure 4)**. M citrifolia fruit and stem: Total ash of M citrifolia fruit and stem were found 7.4% and 8.7% respectively. Water soluble ash was found 3.91 % and 4.23 % whereas 0.89% and 0.97 % respectively for M citrifolia fruit and stem. The ethanol soluble extractive values were found to be 10.4 % and 7.2 % w/w respectively and The ethanol soluble and water-soluble extractive values were found to be 14.2 % and 9.8 % w/w respectively for M citrifolia fruit and stem. The moisture content of the powder estimated as percentage loss on drying (LOD) was found to be 6.2 % w/w for fruit and 5.6 % w/w for stem of M citrifolia **(Table 2)**.

The extraction of the coarse powder of the fruit and stem of Morinda citrifolia were subjected to successive solvent extraction using solvents of ascending polarity. After extraction the percentage yield of each extract was calculated with reference to the air dried drug used for the study. The percentage yield and other characteristic features of the extracts are tabulated in **Table 3**. The qualitative phytochemical screening of the petroleum ether, chloroform, ethyl acetate, methanol and water extracts of the fruit and stem of Morinda citrifolia were carried out. The presence of alkaloids, carbohydrate, reducing sugars, glycosides like anthraquinones, flavanoids, saponins, tannins, phenolic compounds, fixed oils, fats, proteins, amino acids and sterols was investigated for qualitative phytochemical screening. Preliminary phytochemical analysis of M. citrifolia fruits / stem was investigated for the presence of various secondary metabolites using different solvents. Aqueous extract of M. citrifolia fruits showed the presence of carbohydrate, protein, alkaloids, saponin, glycosides, tannins, flavonoids and steroids. Methanol extract showed the presence of all the tested metabolites except saponins. Proteins were not identified in the methanol extract. In ethyl acetate extract, alkaloids were not detected; Carbohydrates, proteins, tannins and steroids were present in chloroform extract, while flavonoids, saponins, glycosides and alkaloids were absent. The in-vitro Anti-inflammatory Activity by HRBC Membrane Stabilization Method of M. citrifolia fruit was estimated. The petroleum ether, chloroform, ethyl acetate, methanol and water extract of M. citrifolia fruit at different concentrations (50, 100, 150, 200, 250 µg/ml) showed significant ($p<0.0001$) stabilization towards HRBC membrane. In this study the methanol extract of M. citrifolia fruit extract (250 µg/ml) displayed potent action (55.65 % of membrane stabilization) compared to other extracts of M. citrifolia fruit **(Table 5)**. M. citrifolia stem containing petroleum ether, chloroform, ethyl acetate, methanol and water extract of M. citrifolia stem at different concentrations (50, 100, 150, 200, 250 µg/ml) showed significant ($p<0.0001$) stabilization towards HRBC membrane. In this study the methanol extract of M. citrifolia stem extract (250 µg/ml) showed potent action (54.98 % of membrane stabilization) compared to other extracts of M. citrifolia stem **(Table 6)**.

Summary and conclusion:
The present study was undertaken to investigate the anti-inflammatory activity of Morinda citrifolia an Indian medicinal plants. The discovery of inflammatory inhibitors from natural origin will present an opportunity for a medicinal chemist to design novel, structurally diverse selective inflammatory inhibitors. The pharmacognostic study of plant material was support for confirmation and determination of identity, purity and quality of a crude drug and both plants Morinda citrifolia was showed valuable herb for the

study. The coarse powder of the fruit and stem of Morinda citrifolia was subjected to successive solvent extraction using solvents of ascending polarity. The In vitro bovine serum albumin denaturation inhibition assay of petroleum ether, chloroform, ethyl acetate, methanol and water extract of M citrifolia fruit, stem was performed. The result of M. citrifolia fruit extract (400 µg/ml) has more effect than M. citrifolia stem extract. The In vitro inhibitory effects of petroleum ether, chloroform, ethyl acetate, and methanol and water extract of both plant parts at different concentrations were measured on in-vitro enzymatic activities against COX-1 and COX-2 receptor.

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Table 1: Organoleptic identification of M . citrifolia fruit and stem

S. No.	Parameters	M . citrifolia	
		Fruit	Stem
1	Color	Yellow-white.	Centre part white in colour
2	Odor	Acrid and pungent	Pungent
3	Shape	Ovoid elongated in shape	Cylindrical
4	Taste	Sweet	Characteristic
5	Size	4-11 cm	1 cm thick
6	Foreign Organic matter	NO	NO

Table 2: Physicochemical parameters of Morinda citrifolia fruit and stem

S. No.	Physicochemical parameter values (% w/w)	Fruit (% w/w)	Stem (% w/w)
1	Total ash	7.4	8.7
2	Water soluble ash	3.91	4.23
3	Acid insoluble ash	0.89	0.97
4	Foreign organic matter determination	1.12	1.5
5	Ethanol soluble extractive	10.4	7.2
6	Water-soluble extractive	14.2	9.8
7	Loss on drying (%)	6.2	5.6

Table 3: Physicochemical parameters of Morinda citrifolia Fruit and stem

S. No.	Extract	Fruit Yield (%W/W)	Stem Yield (%W/W)
1	Petroleum ether	6.5	4.9
2	Chloroform	12.2	8.9
3	Ethyl acetate	9.7	7.6
4	Methanol	18.3	12.3
5	Water	17.5	11.7

Table 4: Phytochemical analysis of Morinda citrifolia fruit extracts

Tests of Phytoconstituents	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Water extract
1. Alkaloids					
a) Mayer's reagent	-	-	-	+	-
b) Dragendorff's reagent	-	-	-	+	-
2. Flavonoids					
a) Shinoda test	-	-	+	+	-
3. Saponins					
a) Froth test	-	-	-	+	+
4. Carbohydrate					
a) Molisch's test	-	-	+	+	+
c) Test for gums	-	-	+	+	+
d) Test for mucilage	-	-	+	+	+
5. Phytosterols					
a) Libermann-Burchard test	+	+	-	-	-
c) Salkowski reaction:	+	+	-	-	-
6. Tannins and Phenolic					
a) With Lead acetate	-	-	+	+	+
7. Cardiac glycoside					
a) (a) Borntrager's test	-	-	+	+	+
b) Legal's test	-	-	+	+	+
8. Coumarins					
a) With ammonia	-	-		-	-
b) Hydroxylamine HCl	-	-		-	-
9. **Proteins**					
a) Biuret test	-	+	+	+	-
10. Triterpens					
a) Vanillin sulphuric acid	+	+		-	-
	Where +: Present, - : Absent				

Table 4: Phytochemical analysis of Morinda citrifolia stem extracts

Tests of Phytoconstituents	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Water extract
1. Alkaloids					
a) Mayer's reagent	-	-	-	+	-
b) Dragendorff's reagent	-	-	-	+	-
2. Flavonoids					
a) Shinoda test	-	-	+	+	-
3. Saponins					
a) Froth test	-	-	-	+	+
4. Carbohydrate					
a) Molisch's test	-	-	+	+	+

c) Test for gums	-	-	+	+	+
d) Test for mucilage	-	-	+	+	+
5. Phytosterols					
a) Libermann-Burchard test	+	+	-	-	-
c) Salkowski reaction:	+	+	-	-	-
6. Tannins and Phenolic					
a) With Lead acetate	-	-	+	+	+
7. Cardiac glycoside					
a) (a) Borntrager's test	-	-	+	+	+
b) Legal's test	-	-	+	+	+
8. Coumarins					
a) With ammonia	-	-		-	-
b) Hydroxylamine HCl	-	-		-	-
9. **Proteins**					
a) Biuret test	-	+	+	-	-
10. Triterpens					
a) Vanillin sulphuric acid	+	+		-	-
	Where +: Present, - : Absent				

Table 5: In vitro anti-inflammatory activity of various extracts of Morinda Citrifolia fruit extracts by HRBC method

Sample	Concentration (µg/ml)	% inhibition	EC_{50} (µg/ml)
Indomethacin	50	34.79	120.58
	100	45.17	
	150	55.82	
	200	65.81	
	250	78.89	
Petroleum ether extract	50	16.59	378.5
	100	22.54	
	150	25.14	
	200	32.82	
	250	36.55	
Chloroform extract	50	14.59	362.25
	100	21.74	
	150	26.89	
	200	32.35	
	250	35.78	
Ethyl acetate extract	50	14.36	343.26
	100	21.44	
	150	25.98	
	200	32.24	
	250	36.87	

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Methanol extract	50	25.89	220.73
	100	33.23	
	150	41.78	
	200	49.8	
	250	55.65	
Water extract	50	21.19	256.43
	100	27.11	
	150	33.25	
	200	40.27	
	250	42.25	

Values are Mean ±SEM ,n=3,(***p value <0.0001) - highly significant compared to control.

Table 6: In vitro Anti-inflammatory Activity of Various Extracts of Morinda citrifolia stem extracts by HRBC Method.

Sample	Concentration (μg/ml)	% inhibition	EC_{50} (μg/ml)
Indomethacin	50	34.79	120.58
	100	45.17	
	150	55.82	
	200	65.81	
	250	78.89	
Petroleum ether extract	50	15.59	380.45
	100	21.54	
	150	24.14	
	200	31.82	
	250	35.55	
Chloroform extract	50	14.19	371.36
	100	21.24	
	150	25.99	

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	200	31.89	
	250	34.68	
Ethyl acetate extract	50	14.89	280.28
	100	21.78	
	150	26.08	
	200	32.98	
	250	37.07	
Methanol extract	50	25.56	231.75
	100	32.98	
	150	40.98	
	200	48.89	
	250	54.98	
Water extract	50	21.87	285.67
	100	27.98	
	150	33.96	
	200	40.86	
	250	42.99	

Values are Mean ±SEM ,n=3,(***p value <0.0001) - highly significant compared to control.

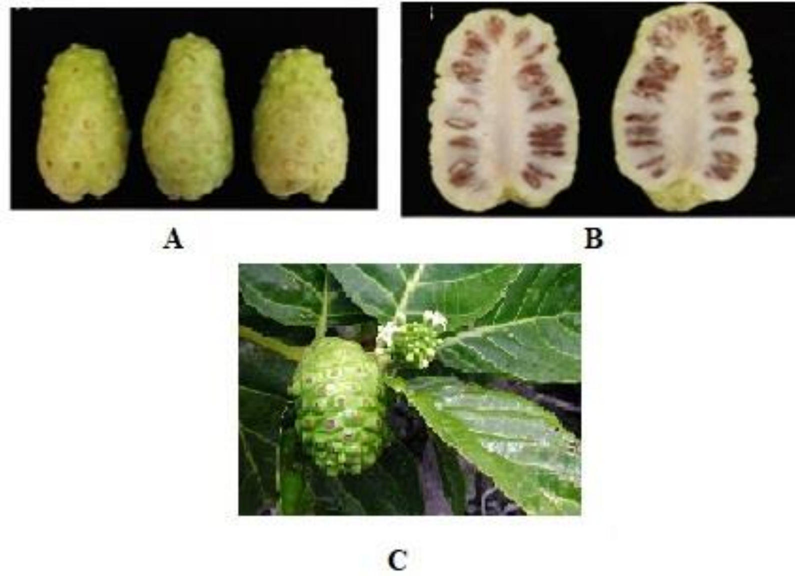

Fig. 1: Morinda citrifolia fruits

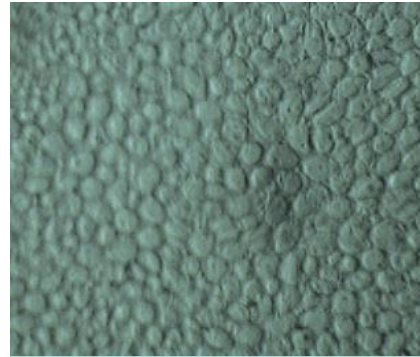

Fig. 2: Transverse section of fruit of Morinda citrifolia

Fig. 3: Transverse section of stem of Morinda citrifolia

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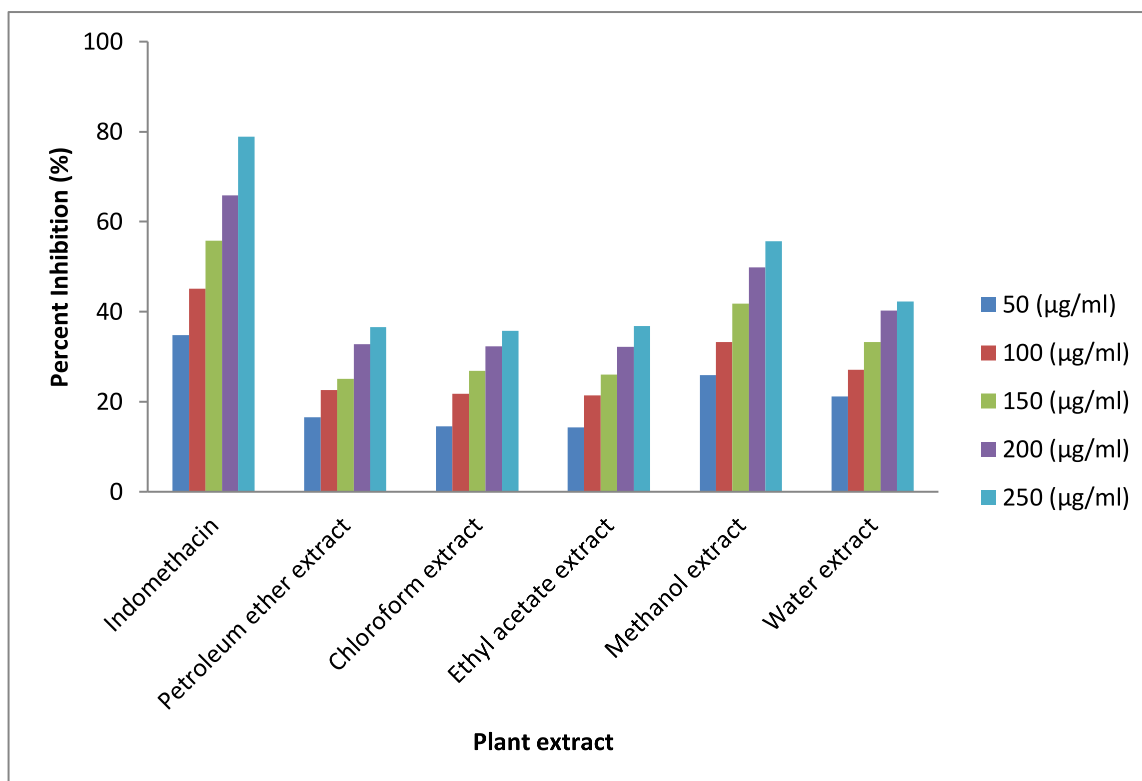

Fig. 4: In vitro anti-inflammatory activity of various extracts of Morinda citrifolia fruit by HRBC method

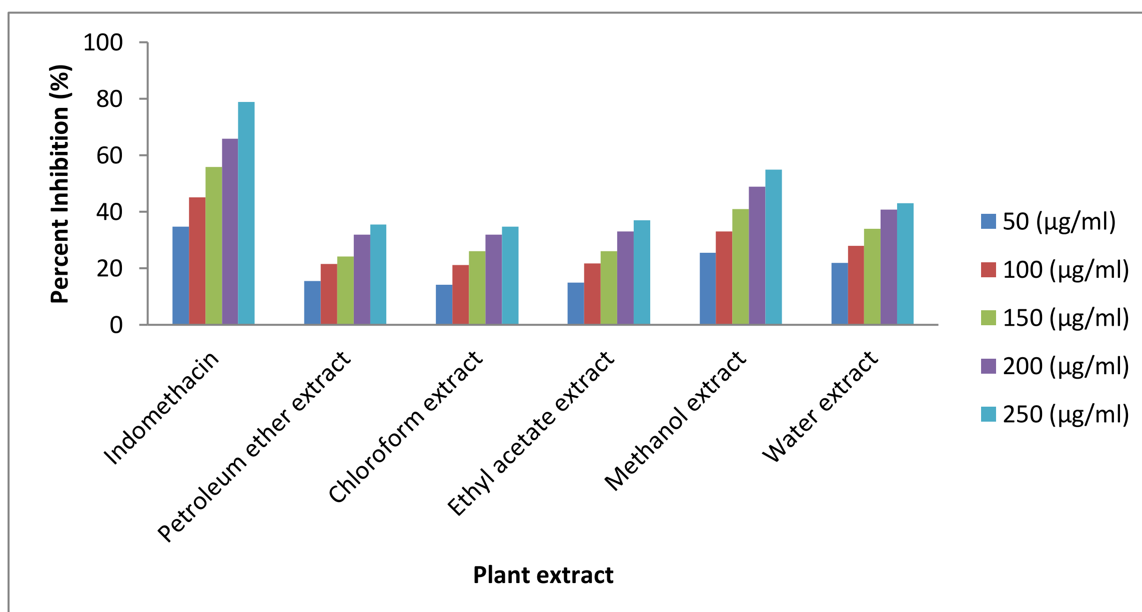

Fig. 5: In vitro anti-inflammatory activity of various extracts of Morinda citrifolia stem by HRBC method