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Phytochemical, antioxidant, antimicrobial and receptor binding activities of the methanolic extract from the testa of Artocarpus heterophyllus Lam.

Repon Kumer Saha[1*]; Maha Jamiruddin[1]; Srijan Acharya[1]; Priyanka Roy[2]
[1]*Department of Pharmacy, East West University, Dhaka, Bangladesh.*
[2]*Dhaka Medical College, Dhaka, Bangladesh.*
*reponsaha@yahoo.com - drks@ewubd.edu

Abstract

Artocarpus heterophyllus is the most widespread species of the genus. The objective of the present work is to determine the functional compounds and their properties present in the methanolic extract of testa of *Artocarpus heterophyllus* Lam.

Standard in vitro procedures were used to screen possible phytochemical, antimicrobial and biomedical activities. Thin layer chromatography, ultra-violet spectroscopy and HPLC were used to detect the presence of various types of compounds in testa. Antioxidant effects were measured by DPPH scavenging assay and total reducing assay.

Receptor binding activities was performed by hemagglutination inhibition assay. Anti-inflammatory assay was also investigated. Disc diffusion assay was performed to show the antibacterial effect using gram positive, gram negative strains of bacteria and fungi. The optical density of bacterial inhibition was also measured.

The testa contains higher amount of polyphenols and flavonoids. It showed stronger antioxidant, membrane stabilization activity, and antimicrobial activities. It also showed moderate hemagglutination inhibition activities. Therefore, testa of *Artocarpus heterophyllus* contains medicinal active components in different ratio.

KEY WORDS: *ARTOCARPUS HETEROPHYLLUS*, ANTIMICROBIAL ACTIVITY, ANTIOXIDANT ASSAY, ANTI-INFLAMMATORY ASSAY, HEMAGGLUTINATION

Introduction

Jackfruit or *Artocarpus heterophyllus* Lam.(syns. *A.philippinensis* Lam., *A. maxima* Blanco, *A. brasiliensis* Gomez), of the family Moraceae, is also called kanthal in Bengali, panasa in Sanskrit, palaa in Tamil. It is the most widespread species of the genus and found in the home gardens and evergreen forests of the western hills of India, Sri Lanka, and the Deccan plain of Bangladesh [2]. Each jackfruit can contain between 100 and 500 seeds [9], and each seed is enclosed in a yellowish, juicy sheath with a strong flavor. The seed is kidney-shaped and is 25 to 30 by 15 to 20 mm with unequal cotyledons. It has thin and leathery testa. The testa is rather thick, tough, parchment-like, and crinkly when dry. The inner seed coat or tegmen is a thin, brownish membrane [2]. In general, fresh seeds are considered high in starch, low in calcium and iron; good sources of vitamins B1 and B2. They (per 100g) contain 51.6-57.77g moisture, 6.6g protein, 0.4g fat, 38.4g carbohydrates, 1.5g fiber, 1.25-1.5g ash, 0.05-0.55mg calcium, 0.13-0.23mg phosphorus and 0.002-1.2mg iron. Whereas, dried seeds (per 100g) contain 2.96% ash, 0.13% calcium, 0.54% phosphorus and 0.005% iron [8]. Raw jackfruit seeds are indigestible due to the presence of a powerful trypsin inhibitor. This element is destroyed by boiling or baking. The seeds are sweet, diuretic, and constipating. The Chinese consider Jackfruit pulp and seeds tonic, cooling and nutritious, and to be useful in overcoming the influence of alcohol on the system. Starch extract from the seed is supposed to relieve biliousness, while the roasted seeds are considered to be aphrodisiac [8]. The decoction of seeds or bark is supposed to help in digestion while ripe fruits may be used as a natural laxative [3]. Fresh extract from seeds are also useful in the treatment of diarrhea and dysentery. Increased consumption of ripe jackfruit kernels alleviates vitamin A deficiency [2]. Seeds have antioxidant effects. The ethanolic extract of the defatted jackfruit seed and the pulp shown to be effective in ABTS and FRAP assays [15]. The crude extracts from the tegmen showed potential anti-tumor activity [11]. Different extracts of seeds showed effective antibacterial and antifungal activity [14]. A novel approach for the green synthesis of silver nanoparticles (AgNPs) from aqueous solution of silver nitrate ($AgNO_3$) by using *Artocarpus heterophyllus* Lam. seed powder extract (ASPE), as a reducing agent has been reported. Therefore, *A. heterophyllus* seed provides future opportunities in nanomedicine by tagging nanoparticles with jacalin [4].

In the present study, we are analyzing the phytochemicals present in testa of *Artocarpus heterophyllus*. We have shown the antioxidant, antibacterial, hemagglutination inhibition activity and anti-inflammatory activity of the methanolic extract of testa of *Artocarpus heterophyllus* collected from Bangladesh.

Materials and Methods

2.1. PLANT COLLECTION AND IDENTIFICATION

The seeds of the plant along with the testa were collected from the Reazuddin Bazar, Chittagong, Bangladesh during June, 2012 and identified by the taxonomist of the Bangladesh National Herbarium, Mirpur, Dhaka as *Artocarpus heterophyllus* Lam. A voucher specimen of the plant has been deposited (Accession No.: 38308) in the herbarium for further reference.

2.2. EXTRACTION OF THE PLANT MATERIAL

The seeds along with testa were shade-dried and later dried testae were separated from the seeds. The testae were grinded (50g) and macerated in 200ml of methanol for 8 days. The extract was concentrated with a rotary evaporator (IKA, Germany) at low temperature (50-55 °C) and reduced pressure. The extract was stored at 4 °C until used.

2.3. THIN LAYER CHROMATOGRAPHY ANALYSIS

The extract was analyzed by performing TLC to determine the composition of the extract. TLC was done under polar basic solvent consisted of ethyl

acetate, ethanol, and water (8:1.2:0.8). After completion of TLC, the plate was exposed to UV light for compound detection and identification. For charring the plate was exposed to 10% sulphuric acid solution, dried and then heated to 80-90°C for charring purpose. This helped in the fixation of spot and allowed it to be prominently visible. For detection of flavanoids the plate was dipped into 0.04% DPPH solution and dried while keeping in a dark place. For detection of polyphenols the plate was washed with Folin-ciocalteu reagent and dried.

2.4. CHEMICAL ANALYSIS OF THE EXTRACT

Ultraviolet (UV) spectroscopy of the extract was performed within 200nm to 400nm wavelength using a Lambda UV spectrophotometer (Shimadzu, Japan) [13].

The HPLC analysis of methanolic extract was carried out with chromatographic system (Shimadzu Prominence high-performance liquid chromatograph, Japan) consist of autosampler (SIL-20A) and SPD-M20A photodiode array detector [8]. The mobile phase consists of acetonitrile to water (10:90 v/v) and the separation was performed by using isocratic mode at 30°C temperature, elution performed at a flow rate of 1 ml/min. The 40ml sample (1mg in 1ml acetonitrile) was run for 10min. and detection was done at 281 nm by UV detector. The chromatographic data was recorded and processed.

2.5. DETERMINATION OF TOTAL PHENOLIC CONTENT

The total phenolic content of the extract was determined using Folin-Ciocalteu method using Gallic acid as standard [1]. The extract (0.5 ml) was mixed with 5ml 10% Folin- Ciocalteu reagent (Merck, Germany) and was allowed to standing for 5 min in dark place. Aqueous Na_2CO_3 (4 ml, 1 M) were then added and the mixture was allowed to stand for 15 min. The absorbance of the resulting blue color was measured at 765 nm. The total phenolic contents were determined using a standard curve prepared with Tannic acid. The estimation of the phenolic compounds was carried out in triplicate. The results were mean ± standard deviations and expressed as milligram of Tannic acid equivalent/g of extract.

2.6. TOTAL FLAVONOID ASSAY

An aliquot (0.1 ml) of methanolic extract was added to 6ml of deionized water and then 0.45 ml 5% (w/v) $NaNO_2$ and incubated for 6 min [5]. 0.45 ml 10% (w/v) $AlCl_3$ and 6 ml 4 %(w/v) NaOH was added and the total volume was made up to 15 ml with distilled water. The absorbance was measured at 510 nm by using visible spectrophotometer. The results were expressed as mg quercetin equivalents/g. The experiments were triplicated.

2.7. DPPH RADICAL SCAVENGING ACTIVITY

The free-radical scavenging activity of the methanolic extract was measured by decrease in the absorbance of methanolic solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl) [12]. The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the extracts. Different concentrations of the extract (0.2, 0.4, 0.5 µg/ml, in methanol) were added at an equal volume (10ml) to methanol solution of DPPH (400 µg /ml). Different concentrations of L-Ascorbic acid (2-10 mg/ml) were used as the standard antioxidant. After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and converted into the percentage antioxidant activity using the following equation: DPPH antiradical scavenging capacity (%) = (Absorbance of sample – Absorbance of blank) × 100/Absorbance of blank. Methanol plus different concentration of plant extract solution was used as a blank, while DPPH solution plus methanol was used as a control. IC50values denote the concentration of the sample required to scavenge 50% of DPPH radicals.

2.8. TOTAL REDUCING ASSAY

Various amount of extracts (0-200 mg) and L-

ascorbic acid (0-1000 mg) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%); the mixture was incubated at 50°C for 20 min [17]. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. L-ascorbic acid was used as a standard.

2.9. ANTIMICROBIAL ASSAY

In order to screening the antimicrobial assay, four different bacterial strains of gram negative, four different strains of gram positive bacteria and three different strains of fungi were used to carry out this assay. The gram-negative bacterias are *P. aureus, V. mimicus, S. typhi, E. coli*; the gram-positive bacterias are *S. lutea, S. aureus, B. cereus, B. subtilis*; the fungi are *C. albicans, A. niger, S. cerevisiae*. Nutrient agar was used as the culture media. Stocks of these bacterial solutions were revived in nutrient agar by incubating at 37°C for 24 hrs. A single disk diffusion method was used to assess the presence of antimicrobial activities of the methanolic testa extract of jackfruit. Whatman's filter paper was punched, and 6 mm disks were collected in a beaker. The beaker was covered with foil paper and autoclaved. 20 µl of extract (40 mg/ml) were loaded per disk. The revived test organisms were plated onto nutrient agar plates. The disks were then placed equidistant on all plates for all extracts. Standard disc (Himedia, India) of Erythromycin (15 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. After incubation at 37°C for 24 hours, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm.

2.10. OPTICAL DENSITY MEASUREMENT OF BACTERIAL INHIBITION

Minimum inhibition concentration of the extract against *Vibrio mimicus* was determined according to [16] with slight modifications. 25ml of bacterial broth from stock were added into 15ml of nutrient broth. 2ml of this broth was taken as positive control. Various amounts of extracts (60-500 mg) in 1 mL of 0.9% saline water were mixed with 2 ml bacterial broth and were left in the incubator at 37°C. For negative control, 2ml broth was taken. Using ELISA plate reader set at 340nm, readings of 100ml of each sample were taken after 16hrs and 34hrs. The results were triplicated.

2.11. HEMAGGLUTINATION INHIBITION ASSAY

The hemagglutination assay was performed in 96-well microtiter plates [6] in a final volume of 100 µL containing 50 µL of 1% suspension of human erythrocytes (blood groups A, B, AB) previously washed with 0.15 M NaCl and 50 µL of two-fold serially diluted methanolic testa extract in 0.9% NaCl. After gentle shaking, the plate was kept at room temperature for 30 min. The reaction was compared with negative control (50 ml of 0.9% NaCl and 50 ml of blood cell). Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity. The intensity of hemagglutination was determined from the extent of deposition.

2.12. IN-VITRO ANTI-INFLAMMATORY TEST (ERYTHROCYTES MEMBRANE STABILIZATION METHOD)

Blood was collected from the healthy volunteers and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride in water) [7]. The blood was centrifuged at 3000 rpm and packed cells were washed with isosaline (0.85%, pH 7.2) and a suspension was made with isosaline (10% v/v). The assay mixture contained 1ml of phosphate buffer

(0.15M, pH 7.4), 2ml of hyposaline (0.36%), 0.5ml of HRBC suspension and 1ml of various concentrations of the extract. Diclofenac sodium was used as reference drug. In the control solution instead of hyposaline, 2ml of distilled water was added. The mixtures were incubated at 37°C for 30min and centrifuged at 3000rpm for 5 min. The absorbance of the supernatant solution was read at 560nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100%. The percentage of HRBC membrane stabilization was calculated using the formula: Percentage membrane stabilization = 100 – [(O.D of drug treated sample/O.D of control) *100].

Results and Discussions

3.1. PHYTOCHEMICAL SCREENING

Polar basic solvent consisted of ethyl acetate, ethanol, and water (8:1.2:0.8). The separation performed by the polar basic solvent is shown here in Figure 1. TLC plates were seen under UV light and found some compounds separated at the bottom of the plates. Charring with H_2SO_4 in high temperature the separated compounds transformed into black colour indicates the presence of organic compounds in the sample. Staining the plate with FC-reagent the colour of the separated compounds changed into bluish colour indicates the presence of polyphenols in the samples. Staining the plate with DPPH solution the colour of the separated compounds changed into yellow colour indicates the presence of flavonoids in the sample. The separation in polar solvent system has been shifted from the bottom to the top of the stationary TLC plates.

UV spectroscopic analysis of the methanolic extract of testa of *Artocarpus heterophyllus* is shown in Figure 2. The maximum absorbance wavelength for the extract was at 281nm.

The methanolic extract of *Artocarpus heterophyllus* testa extract chromatogram (Figure 3) shows different constituents at various retention time (2.139 and 3.102 min.). These two peaks represent the main constituents present in the testa.

Thin layer chromatography (TLC) is used to separate mixtures of compounds. TLC provided a qualitative idea about the components that are present in the crude mixture of the methanolic extract of testa. The developed plates were tested with DPPH and turned in yellow colour ensures that the extracts possess any antioxidant activities. Folin-Ciocalteu Reagent was used to test the developed TLC plates and found bluish colour indicates the presence of phenolic and polyphenolic antioxidants present in the extract (Figure 1). HPLC is a sensitive and accurate tool that widely used for the quality assessment of plant extract and its derived product. This method can be coupled to NMR analyses in order to completely identify the compounds detected (Figure 3) and get some insight into their structure.

3.2. TOTAL PHENOLIC AND FLAVONOID CONTENT ASSAY

For quantitative analysis of polyphenols a standard curve was established by using different concentrations of Tannic acid and using Beer-Lambert`s law in a concentration dependent manner. The equation found was: Y= 19.319X; where, R^2 = 0.9689. From the standard curve, the total phenolic compounds as tannic acid equivalent (TAE) present in extract was 280 mg/g.

In case of flavonoid quantification a standard curve [1] was used, where the equation is y = 0.0067x + 0.0132, R^2= 0.999. From the standard curve, the amount of quercetin present in the extract was 41.8 mg/g of quercetin equivalent per gram of sample.

The Folin–Ciocalteu reagent is used for the colorimetric in vitro assay of phenolic and polyphenolic antioxidants. Flavonoids are all ketone-containing compounds and are secondary plant metabolites. They not only have various physiological functions in plants but they are also potent antioxidants. Measuring the content of flavonoids and phenols in plant samples can give us a measure of their antioxidant potential. In order to investi-

gate the total potent antioxidants in the extract we performed total phenolic and flavonoid content. We found that the testa contained a significant amount of phenols and flavonoids. Therefore, testa of *Artocarpus heterophyllus* has a potential to show antioxidant activities.

3.3. DPPH RADICAL SCAVENGING ACTIVITY AND TOTAL REDUCING ASSAY

From the analyses of Figure 4, we found the scavenging effect of the extract in a concentration dependent manner. The 50% inhibitory concentration (IC50) value of the extract and ascorbic acid was 0.000431 mg/ml and 1.295 mg/ml respectively. Therefore, the testa of *Artocarpus heterophyllus* showed stronger radical scavenging activities than that of ascorbic acid.

Total reducing assay of the extract in a concentration dependent manner was investigated in compared with ascorbic acid as shown in Figure 5. The testa of *Artocarpus heterophyllus* showed stronger reducing activities than that of ascorbic acid.

DPPH is a very good free radical scavenger for other radicals; therefore, reduction of DPPH upon addition gives a very good measure of antioxidant activity. On the other hand, total reducing power activity test is a good test to estimate the potential antioxidant activity of a sample. Substances that have reducing potential such as antioxidants react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferric ferrous complex that is prussian blue in color and that has absorption maximum at 700nm. We found that methanolic extract of the testa showed DPPH scavenging and total reducing activities.

3.4. ANTIMICROBIAL ASSAY

Different types of Gram-positive, Gram-negative bacteria and fungi were subjected in this test. The positive control used was erythromycin (15µg/disc) and the negative control used was methanol. The zones of inhibition for the microbes were measured in millimeters using a transparent ruler after 24hrs of incubation. The results are shown in Table 1.

In order to investigate the minimum inhibitory concentration (MIC) of the methanolic testa extract of *Artocarpus heterophyllus* required on *Candida albicans* infection a dose dependent disc diffusion assay was performed as shown in Figure 6. MIC was calculated at that concentration where minimum inhibition was seen. MIC of the extract for *Candida albicans* is 800mg/disk.

The disc-diffusion method is a means of measuring the effect of an antimicrobial agent against bacteria grown in culture. Crude methanolic testa extract of *Artocarpus heterophyllus* showed broad-spectrum antibacterial and antifungal activities. The extracts also showed the dose-response relationship of antifungal activities on *Candida albicans* strain. Therefore, testa of *Artocarpus heterophyllus* may be considered as a useful source for discovering a safe and novel antimicrobial compound.

3.5. OPTICAL DENSITY MEASUREMENT OF BACTERIAL INHIBTION

After plotting a log graph of concentration against the O.D. of bacterial growth after 36hrs (figure 7), R^2 value of 0.5573 was obtained.

The ELISA is a rapid test used for detecting and quantifying antibodies or antigens against viruses, bacteria and other agents. The results show dose dependent response.

3.6. HEMAGGLUTINATION INHIBITION ASSAY

Various concentrations of the extract (0-100mg/ml) were taken to investigate hemagglutination inhibition activity on different types of human blood groups. (Figure 8). The hemagglutination inhibitory concentration is listed in Table 2.

Many viruses attach to molecules present on the surface of RBCs. Hemagglutination inhibition assay was performed to investigate the receptor binding

affinity of the compounds present in the crude methanolic testa extract on human erythrocytes. It was observed that the extract has different binding affinity to the different receptors of erythrocytes and prevent agglutination. Hence, the results showed a possible benefits of *Artocarpus heterophyllus* testa extract as an antiviral therapeutics.

3.7. IN-VITRO ANTI-INFLAMMATORY TEST (ERYTHROCYTES MEMBRANE STABILIZATION METHOD)

The extract was subjected to the in-vitro anti-inflammatory test using diclofenac as a standard as shown in Figure 9. As the concentration of the extracts goes up, their membrane stabilizing capacity also goes up. This can be implied that the extracts have anti-inflammatory properties because they have antioxidant molecules such as flavonoids and polyphenols that prevent oxidative stress.

The percentage membrane stabilization of RBCs is considered to correlate to the anti-inflammatory activity. The RBC membrane is analogus to lysosomal membrane components. Hypotonicity brings about oxidative stress in the cell and promotes cell lysis. Therefore, stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents which can cause further tissue inflammation and damage, upon extra cellular release. In comparison with diclofenac sodium, the testa extract of *Artocarpus heterophyllus* showed a considerable effect on stabilizing the lysosomal membrane. (Figure 9).

Conclusion

Therefore, the present study on the methanolic testa extract of *Artocarpus heterophyllus* showed the potentiality of it as an antioxidant, receptor binding activity with RBCs, in vitro anti-inflammatory activities, antifungal activities and antibacterial activity. *Artocarpus heterophyllus* is the most widespread species of the genus in the tropical regions. We suggest that the antimicrobial activities of the testa is may be due to the presence of high amount of polyphenols and flavonoids present in the testa. Besides, the testa showed anti-inflammatory activity which may be induced due to its antioxidant property. Further research is required to isolate functional molecules present in the testa of *Artocarpus heterophyllus*.

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Microorganisms	Negative Control	Testa Methanolic	Erythromycin
		Zone of inhibition (in mm)	
Fungi			
Candida albicans	-	8	22
Aspergillus niger	-	10	26
Saccharomyces cerevisiae	-	9	28
Gram positive bacteria			
Sarcina lutea	-	-	25
Staphylococcus aureus	-	10	32
Bacillus cereus	-	9	21
Bacillus subtilis	-	10	20
Gram negative bacteria			
Pseudomonas aureus	-	8	29
Vibrio mimicus	-	10	26
Salmonella typhi	-	7	36
Escherichia coli	-	8	34

Table 1: Antimicrobial sensitivities of methanolic testa extract in disc diffusion assay

Blood groups	Testa methanolic Conc. (mg/ml)
A +ve	12.5
B +ve	100
AB +ve	100

Table 2: Hemagglutination inhibitory concentration of methanolic testa extract

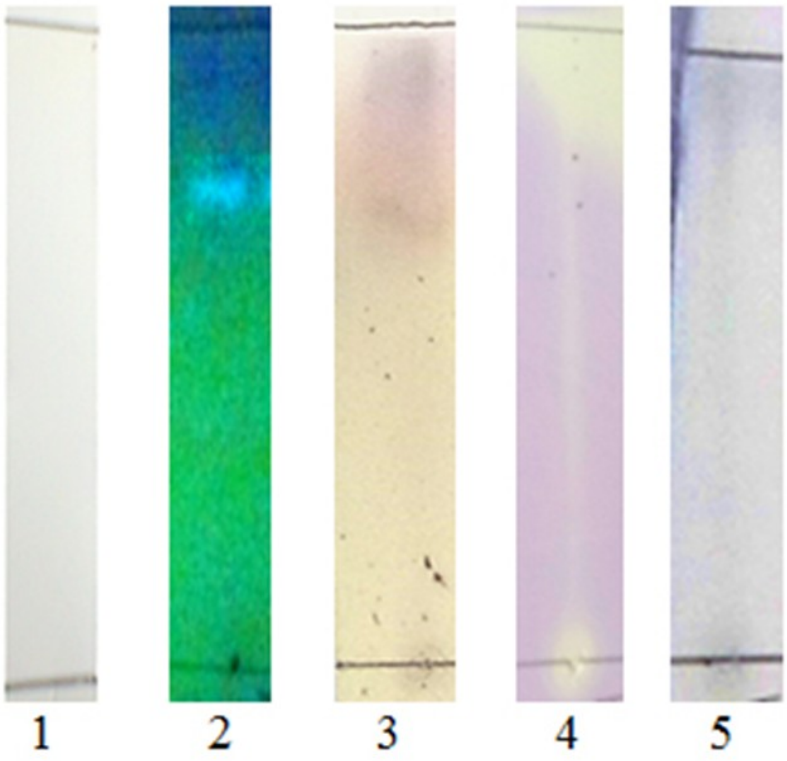

Figure 1: Separation of methanolic testa extract of *Artocarpus heterophyllus* in TLC system using ethyl acetate, ethanol, and water (8:1.2:0.8) solvent system. Key: 1= Normal view; 2= Ultra-Violet view; 3= Sulphuric acid charring view; 4= DPPH staining view; 5= FC reagent staining view

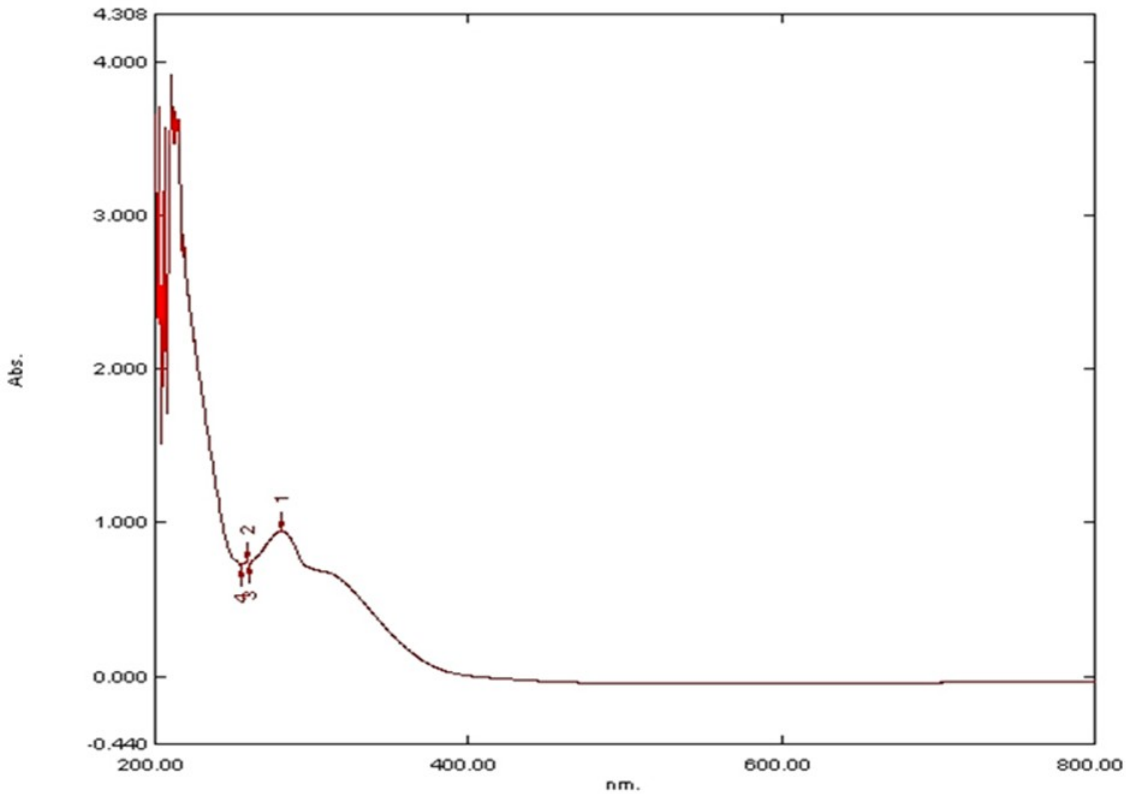

Figure 2: UV-scanning of methanolic testa extract

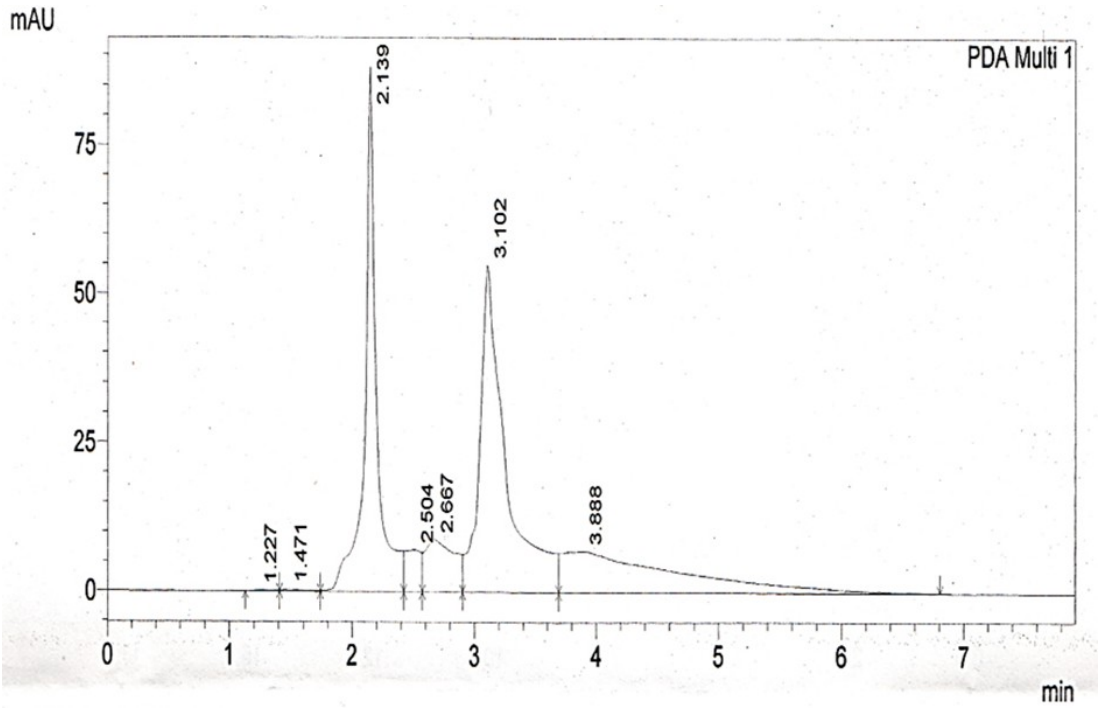

Figure 3: HPLC chromatogram of methanolic extract of testa of *Artocarpus heterophyllus* at 281 nm

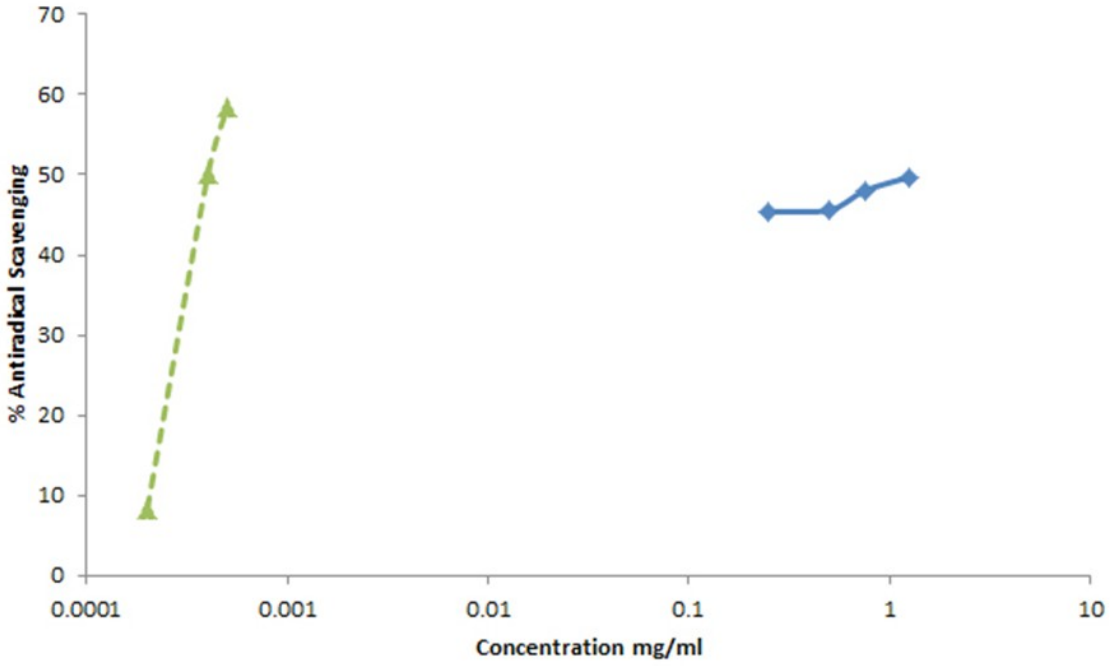

Figure 4: DPPH scavenging activity of the methanolic testa extracts of *Artocarpus heterophyllus*. Key: Solid rhombus line: Ascorbic acid; Dotted triangular line: Methanolic testa extract

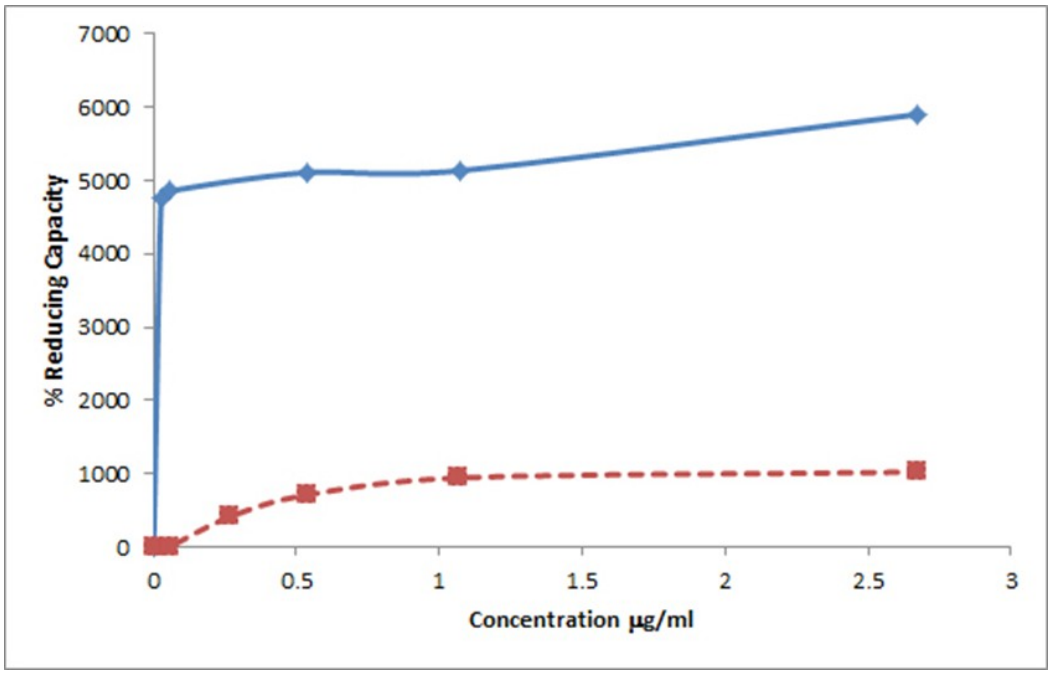

Figure 5: Total reducing activity of the methanolic testa extracts of *Artocarpus heterophyllus*. Key: Dotted rectangular line: Ascorbic acid; Solid rhombus line: Methanolic testa extract

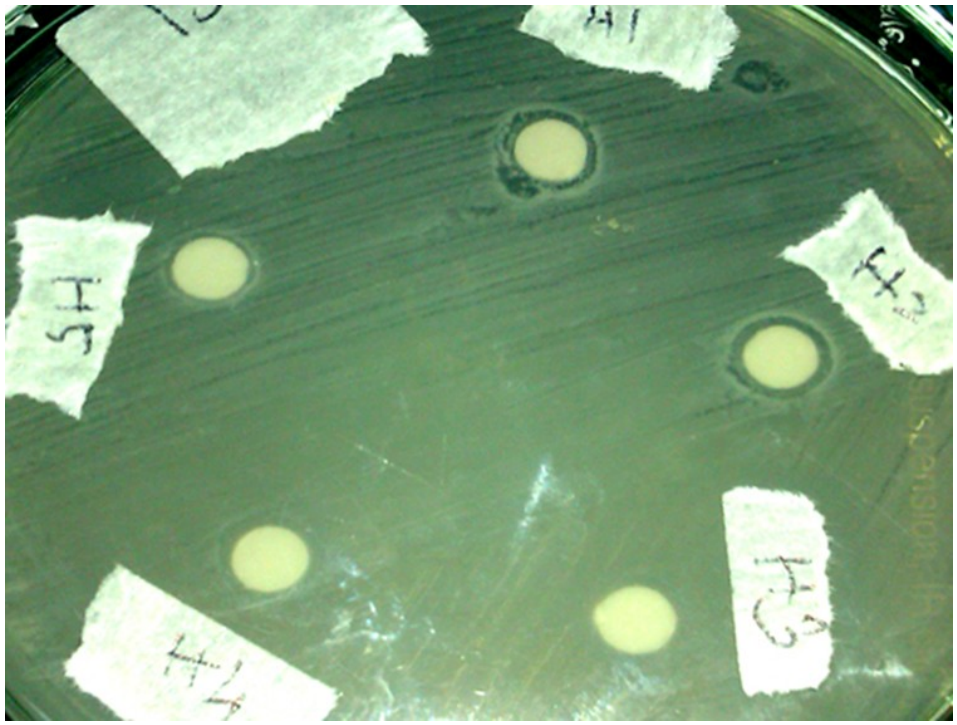

Figure 6: Minimum inhibitory concentration determination of methanolic testa extract on *Candida albicans* infection.

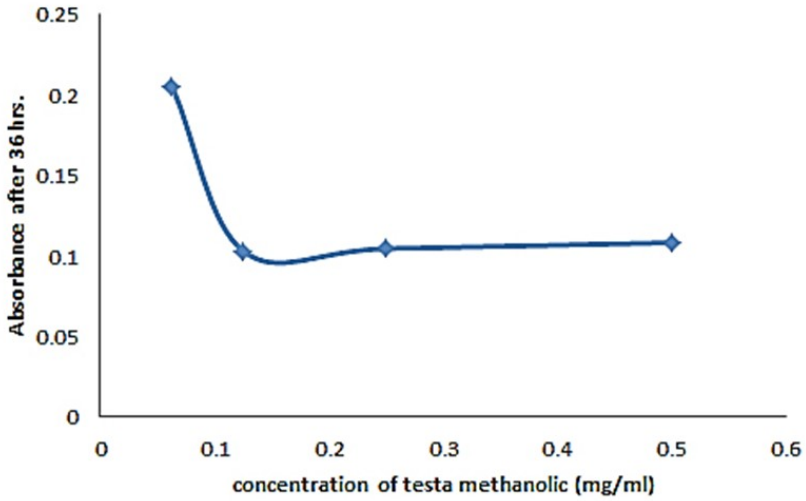

Figure 7: Optical density measurement of bacterial inhibition induced by methanolic testa extract.

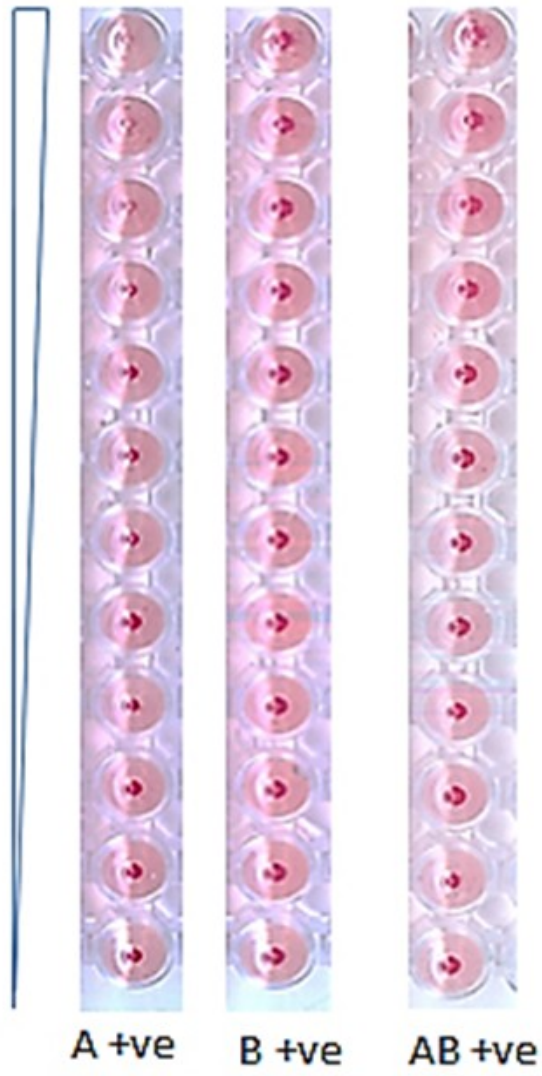

Figure 8: Hemagglutination inhibitory concentration determination of methanolic testa extract

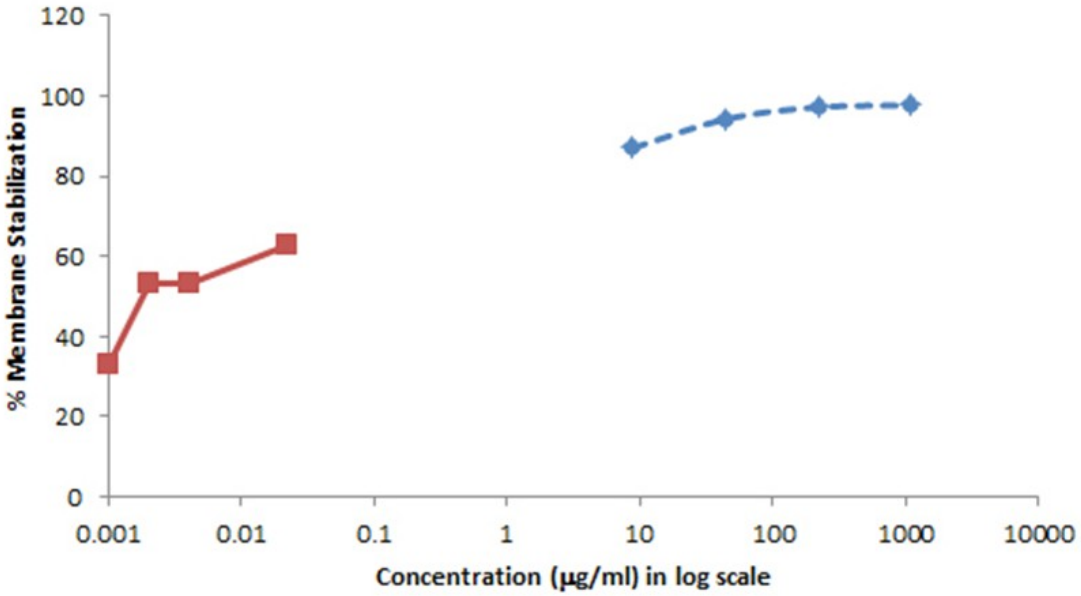

Figure 9: In-vitro anti-inflammatory activity of the methanolic testa extracts of *Artocarpus heterophyllus*. Key: Dotted rhombus line: Diclofenac sodium; Solid rectangular line: Methanolic testa extract