Phytochemical Screening, *In vitro* Evaluation of Antioxidant and Anti-inflammatory Effects of Ethanol Extract from *Averrhoa carambola* L. (Star fruit) leaves

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ABSTRACT:
During ancient times, naturally occurring herbs were a remedy for various ailments in the human body. Parts of *Averrhoa carambola* trees are comprehensively used as medicinal properties. *A. carambola* has lavished phytochemical properties such as alkaloids, glycosides, tannins, flavonoids, saponins, Carbohydrates, steroids, proteins & amino acid, which give rise to countless possessions like anticancer, antibacterial, anti-inflammatory, and antioxidant activity. The antioxidative capacity of *A. carambola* leaves ethanol extracts have been assessed using the DPPH and ABTS assay. For DPPH and ABTS assay the concentrations were measured by using absorbance at wavelength 517 nm and 734 nm respectively and the ethanol extracts of *A. carambola* leaves were considered with a 20 to 80 µg/ml range (4 concentrations). For DPPH assay, using % inhibition, 27.43% at the lowest concentration (20 µg/ml) and 54.95% at high concentration (80 µg/ml) shows antioxidant activity. For ABTS assay using % inhibition, 26.85% at the lowest concentration (20 µg/ml) and 66.80% at high concentration (80 µg/ml) shows antioxidant activity. The active extracts of *A. carambola* leaves demonstrate the anti-inflammatory effects of using BSA as a reagent. This assay measured the activity using 4 concentrations (20-80 µg/ml) 28.87% at 20 µg/ml and 58.75% at 80 µg/ml concentration attained the anti-inflammatory activity. In the membrane stabilization assay the results showed that ethanol extract at concentration range of 100-500 µg/ml protects significantly the erythrocyte membrane against lysis induced by hypotonic solution. At the concentration of 500µg/ml, ethanol extract showed maximum of 76.34% protection, whereas Aspirin at 500 µg/ml showed 80.65% inhibition of RBC hemolysis when compared with control. The study exhibits that the higher the concentration, the higher the activity. The extraction of these active compounds has active principles and activities on anti-inflammatory and antioxidant properties.
KEYWORDS: DPPH, ABTS, BSA, Antioxidant, Anti-inflammatory

INTRODUCTION:

Herbal medicine is the earliest form of healthcare known to mankind. Plants have a long history as medicinal treatments for human and illness: many of the modern drugs commonly used today are developed from plant derivative[1]. The World Health organization (WHO) estimated that nowadays, the population in developing countries use traditional medicine to meet their healthcare needs at almost 80%. According to the World Health Organization (WHO), there are 21,000+ plant species that have been used on a global scale for medicinal purposes[2]. In India, more than 100 genera consisting of around 2,500 plant species are being used in traditional medicine systems[3]. India is the second highest country in the exportation of medicinal plants both in terms of volume and value[4]. Moreover, India is one of the 12 mega biodiversity centers in world and has 16 different agro climatic zones[5,6].

Averrhoa carambola, belonging to the family of Oxalidaceae was historically employed as an appetite enhancer, diuretic and for anti-inflammatory, antidiarrheic and febrifugal activities[7,8].

Antioxidant supplements with the normal cells as well, this stuff are important for our fitness[9]. Gallic acid, one of the most abundant phenolics in plant extracts, and ellagic acid are some phenolic compounds which have been shown to exert anticancer[10], antiviral[11], antimicrobial[12] and anti-inflammatory[13] abilities. Reactive oxygen species (ROS) release, reduced levels of intrinsic cardiac antioxidants and ultimately oxidative stress are all associated with myocardial infarction[11].

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of inflammatory conditions, but their use may be associated with significant side effects, including gastric irritation and ulcer formation[12]. Marker compounds in synthesis or semi-synthesis of natural organic

compounds are useful tools for biological studies aiming at the recognition of new drug leads with specific therapeutic target(s) anywhere in a living system. Thus, this study presents a comparative evaluation of the *in vitro* anti-inflammatory effect of ethanolic extracts through its ability to inhibit protein denaturation and hypotonic solution-induced hemolysis.

MATERIALS AND METHODS:

Collection and authentication of the plant:

The leaves of *Averrhoa carambola*, belonging to the Oxalidaceae family, were collected from A Block in Baksa, Assam. Dr. Souravjyoti Borah, Assistant Professor in the Department of Botany at Gauhati University, Assam, identified and authenticated the plant.

Plant extraction:

The leaves of Averrhoa carambola were dried in the laboratory using air and ground to a coarse powder. The plant material was then extracted continuously by hot Soxhlet extraction method[13]. The first extraction employed a solvent of 500 ml petroleum ether, and the extraction lasted for 48 hours in Soxhlet extraction. The extract was obtained, and the solvent was evaporated in rotary vacuum evaporator at 40°C. After the petroleum ether extraction, the marc was extracted with 500 ml of 95% ethanol. The second extraction also took 48 hours, and the ethanol extract thus obtained was also collected and evaporated at 40°C utilizing the rotary vacuum evaporator.

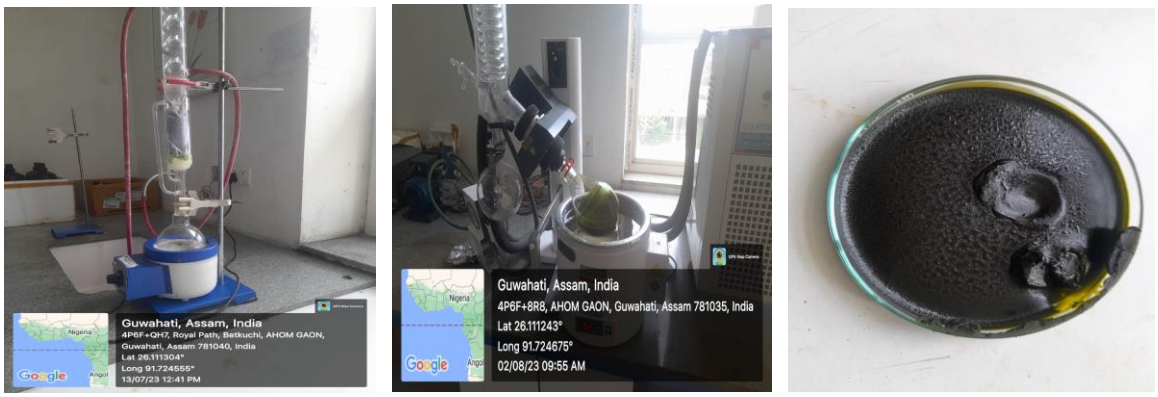

Figure 1: Soxhlet extraction of *Averrhoa carambola* powdered leaves

Preliminary Phytochemical Screening: The crude extracts of *Averrhoa carambola* were subjected to various qualitative phytochemical screenings to detect the presence of different secondary metabolites, including alkaloids, glycosides, tannins, phytosteroids, terpenoids, carbohydrates, proteins, amino acids, and others[14].

Total Phenol and Flavonoid Content Estimation[15]:

The total phenolic content will be determined using the method by Siddhuraju and Becker (2003). Triplicate 50 µL samples of extracts in their respective organic solvents at mg/mL concentrations will be added into test tubes, and volume will be made up to 1 mL with distilled water. And from tube 1, add one after the other 0.5 mL of Folin-Ciocalteu phenol reagent diluted 1:1 with water, and 2.5 mL 20% sodium carbonate solution. The test mixture will be vortexed and then incubated in the dark for 40 minutes. Then the test tubes will be passed through a spectrophotometer at 725 nm wavelength against the reagent as a blank. The results will be expressed in terms of gallic acid equivalents (GAE).

The flavonoid content of the extracts will be determined using the method proposed by Zhishen et al. (1999). For every extract, 500 µL (1 mg/mL in their corresponding organic solvent) will be added to different test tubes, along with 2 mL of distilled water. A blank will be prepared using 2.5 mL of distilled water. Then, 150 µL of 5% $NaNO_2$ will be added to each of the two test tubes, which will then be incubated at room temperature for 6 minutes. After the incubation, 150 µL of 10% $AlCl_3$ will be added in the same two test tubes and the blank, which would again be incubated for 6 minutes. Then, all the test tubes would have 2 mL of 4% NaOH added to them and brought up to a final volume of 5 mL with distilled water. The extracts will be vortexed vigorously and allowed to stand at room temperature for 15 minutes. The intensity of pink colour coming as an indication of flavonoids shall be determined in a UV spectrophotometer at 510 nm. Rutin will be used as the standard for the quantification of flavonoids. The experiments shall be carried out in triplicate, and the results shall be expressed in terms of Rutin equivalents in RE.

***In-vitro* antioxidant assay:**

In accordance with established protocols, two assay methods were employed to evaluate the antioxidant activity of the extracts *in-vitro*.

DPPH radical scavenging activity[16]:

In order to evaluate the free radical scavenging activity, DPPH was used at a concentration of 0.1 mM in 1 mL of the ethanol solution. The solutions were mixed with 3 mL of the tested extract at four different concentrations (10, 20, 40, and 80 µg/mL) followed by keeping those mixtures in the dark at room temperature for 30 minutes. Then, absorbance at 517 nm was measured using a UV-Visible spectrophotometer. An appropriate blank was added to each experiment, and positive

control without the extract was performed in parallel. Ascorbic acid at different concentrations served as standard. The activity of scavenging of the extract was calculated as percentage DPPH radicals scavenged (I%) by the following formula:

% Inhibition = [(A control-A sample) /A control] x 100

Where 'A sample' represents the absorbance of the sample solution and 'A control' represents the absorbance of the control solution.

ABTS radical scavenging activity[17]:

The scavenging activity of the ABTS radical was carried out by using the method described by Re et al. (1999). This entails the mixing of 5 mL of ABTS stock solution with 5 mL of 2.45 mM potassium persulfate, followed by incubation in the dark at room temperature for 16 hours. The solution obtained was diluted to the point that it had an absorbance of 0.700 ± 0.020 at 734 nm and was equilibrated at 30°C before use. Plant extracts, prepared at various concentrations and diluted by adding DMSO, were utilized as sample solutions. For each assay, 5 µL of sample solution was mixed with 195 µL of ABTS+ solution, incubated at room temperature for 6 minutes and read its absorbance at 734 nm. Ascorbic acid at variable concentrations was used to obtain standard curves, while blanks were run for each assay. The ability to scavenge ABTS was expressed as IC_{50} (µg/mL). Calculations were done using the following percent inhibition formula:

% Inhibition = [(A control-A sample) /A control] x 100

Where 'A sample' is the absorbance of a sample solution, and 'A control' is the absorbance of the control solution[24].

***In-vitro* anti-inflammatory assay:**

In accordance with established protocols, two assay methods were utilized to evaluate the anti-inflammatory activity of the extracts *in-vitro*.

Inhibition of protein denaturation assay[18]:

Denaturation of proteins will lead to loss of biological function in protein molecules and has been related to the development of inflammatory conditions such as rheumatoid arthritis, diabetes, and cancer. Therefore, substances that could prevent protein denaturation may prevent inflammatory disorders. In this assay, either egg albumin or BSA is used as the protein. To create denaturation, the reaction mixture is kept in a water bath at 70°C for 10 minutes. The concentrations used in the reaction mixture include 100 to 500 µg/mL plant extract to be combined with 1000 µL, 200 µL of egg albumin or 450 µL of BSA in 5% w/v aqueous solution, and 1400 µL of phosphate-buffered saline. Negative control is also made by replacing the extract with distilled water. It is then incubated at 37°C for 15 minutes and then heated at 70°C for 5 minutes. After this, it is cooled under running tap water and measured for absorbance at 660 nm. A positive control is comprised of acetyl salicylic acid, diclofenac sodium, ibuprofen, or indomethacin. The experiment will be considered repeatedly thrice and the percentage inhibition of protein denaturation will be calculated by using following equations:

% Inhibition of denaturation = [(A control-A sample) /A control] x 100

Where 'A sample' is the absorbance of a sample solution, and 'A control' is the absorbance of the control solution.

Membrane stabilization method:

The membrane stabilizing activity of the extracts can be assessed using it for evaluating the effects in the context of heat-induced hemolysis and hypotonic solution-induced hemolysis on erythrocytes taken from humans, rats, or mice. Since the erythrocyte membrane is equivalent to the lysosomal membrane, a stabilization effect on erythrocyte membrane by the extracts may be conceived to affect the lysosomal membrane stabilization in turn.

Hypotonic solution induced hemolysis[18]:

This experiment is carried out with a hypotonic solution which may be hypo saline (50 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4) or distilled water. The reaction mixture consists of an erythrocyte suspension, plant extract, and hypotonic solutions. A control is prepared by excluding the plant extract. Acetyl salicylic acid, indomethacin, or diclofenac can be referred as standard drugs. The mixture is incubated at 37°C for 30 minutes and then centrifuged at 3000 rpm for 20 minutes. The hemoglobin content in the supernatant is then measured spectrophotometrically at 560 nm. The percentage of red blood cell membrane stabilization or protection is calculated using equations below:

% inhibition of hemolysis = [(OD1- OD2)/OD1] × 100

Where, OD1 represents the optical density of the control containing only the hypotonic-buffered saline solution, and OD2 represents the optical density of the test sample in the hypotonic solution.

RESULTS AND DISCUSSION:

Preliminary phytochemical screening:

Phytochemical screening indicated the presence of alkaloids, glycosides, tannins, flavonoids, saponins, carbohydrates, steroids, proteins, and amino acids in the leaves of Averrhoa carambola; results summarized in Table 1.

Table 1. Preliminary phytochemical analysis of petroleum ether and ethanolic extract

Sl No	Test	Petroleum ether	Ethanol
1	Test for alkaloids		
	1. Dragendorff's test	- ve	+ve
	2. Hager's test	- ve	+ve
	3. Wagner's test	- ve	+ve
	4. Mayer's test	- ve	+ve
	5. Tannic acid test	- ve	+ve
2	Test for glycosides		
	1. Borntrager trest	- ve	+ve
	2. Legal's test	- ve	+ve
	3. Keller-Killani test	- ve	+ve
3	Test for steroid		
	1. Salkowski test	- ve	+ve
4	Test for tannins		
	1. Ferric chloride test	- ve	+ve
	2. Lead acetate test	- ve	+ve

	3. Potassium permanganate test	- ve	+ve
5	Test for flavonoids 1. Shinoda test 2. Lead acetate test & NaOH test	 - ve - ve	 +ve +ve
6	Test for Fats & Oils 1. Saponification test	 +ve	 -ve
7	Test for proteins & amino acid 1. Nitric acid test 2. Ninhydrin test	 - ve - ve	 +ve +ve
8	Test for carbohydrates 1. Molisch's test 2. Fehling's test 3. Benedict's test	 - ve - ve -ve	 +ve -ve +ve

Results of total phenolic content:

Various studies have shown that phenolic compounds are essential for human health because of their antioxidant activity. The total phenolic content in the A. carambola ethanolic extract was established to be 8.323 mg of gallic acid equivalents per gram of extract using the following regression equation ($y = 0.0084x + 0.0856$; $R^2 = 0.97$). A standard calibration curve was prepared at 765 nm, as shown in Fig. 1, by taking known concentrations of gallic acid.

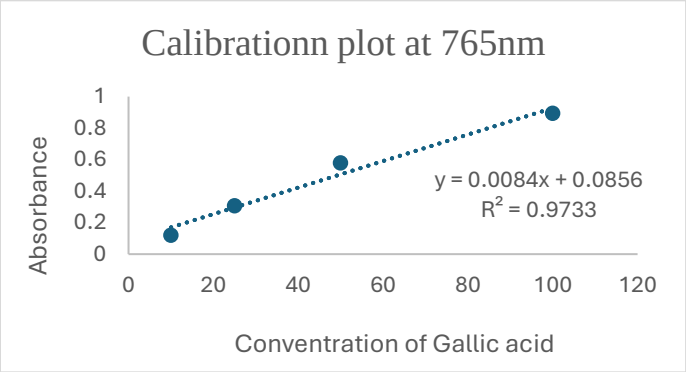

Figure 1: Calibration plot for phenolic determination

Results of total flavonoid content:

Flavonoids are probably the most important of all the natural phenols but certainly the most diverse and profuse of the groups of natural compounds. After constructing the calibration curve [y= 0.0102x - 0.0637 (R2=0.9693)], the results showed considerable flavonoid content in the ethanolic extract with 1.52 mg of quercetin equivalents per gram of extract.

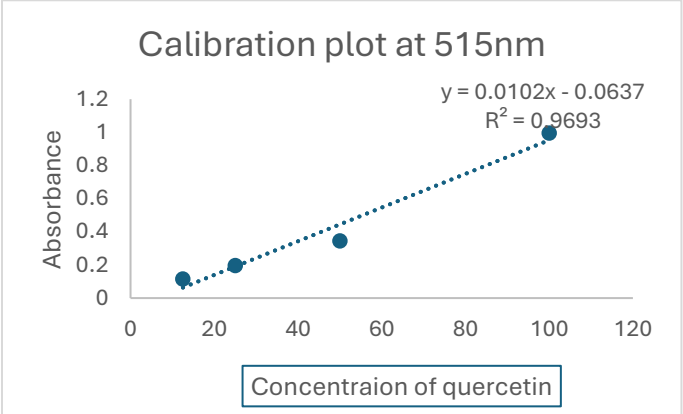

Figure 2: Calibration plot for flavonoid determination.

Results of DPPH radical scavenging activity:

Figure 3 Curves showing the dose-dependent DPPH radical scavenging activity of the ethanolic plant extract and the standard ascorbic acid. Percentage inhibition and IC_{50} values for plant sample and standard are provided in Table 1. For the plant extract, an inhibition level of 54.95% was observed at 80 µg/mL and its IC_{50} value was found to be 70.71 µg/mL. The inhibition level for the ascorbic acid standard at 80 µg/mL was at 85.59% inhibition value with an IC_{50} of 20.71 µg/mL.

Table 1: IC_{50} and % inhibition of Plant extract and Ascorbic acid for DPPH assay

Sample	**Concentration (µg/ml)**	**% Inhibition**	**IC_{50} (µg/ml)**
Averrhoa carambola	20	27.43	70.71
	40	34.31	
	60	45.65	
	80	54.95	
Ascorbic acid	20	48.88	20.71
	40	61.96	
	60	76.66	
	80	85.59	
Absorbance of the control: 0.78. Each test was carried out triplicate (n=3)			

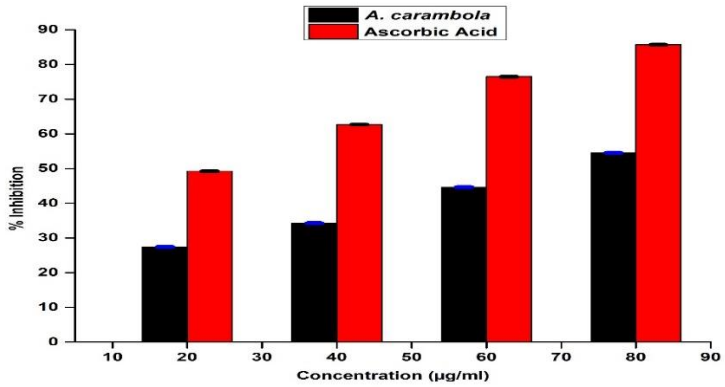

Figure 3: DPPH radical scavenging activity of Ascorbic acid and ethanol plant extract of *A. carambola*

Results of ABTS radical scavenging activity:

From the results of ABTS radical scavenging activity, it can be concluded that the ethanolic extract had good inhibition, but the highest inhibition was with the standard drug ascorbic acid. As mentioned in Table 2, it can be clearly observed that at various concentrations, the percentage of ABTS radical scavenging activity of the ethanolic plant extract is concentration dependent. At 80 µg/mL, the percentage inhibition of the leaf extract was 66.80%, while that of ascorbic acid at the same concentration stood at 83.85%. IC_{50} value for ascorbic acid was 24.75 µg/mL while the IC_{50} for the leaf extract was 57.22 µg/mL.

Sample	**Concentration (µg/ml)**	**% Inhibition**	**IC_{50} (µg/ml)**
Averrhoa carambola	20	26.85	
	40	38.33	

Table 2: IC_{50} and % inhibition of Plant extract and ascorbic acid for ABTS radical scavenging activity

	60	49.33	57.22
	80	66.80	
Ascorbic acid	20	47.95	24.75
	40	57.57	
	60	74.42	
	80	83.85	
Absorbance of the control: 0.7. Each test was carried out triplicate (n=3)			

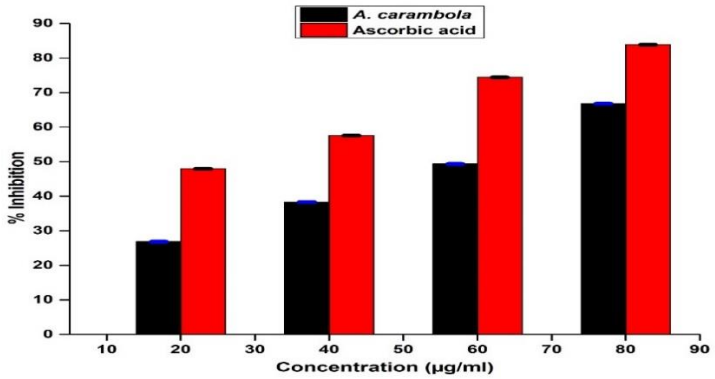

Fig 4: ABTS radical scavenging activity of Ascorbic acid and ethanol plant extract of *A. carambola*

Results of Inhibition of protein denaturation assay:

The results from the albumin denaturation inhibition assay indicated that the extracts possess anti-inflammatory potential, as they prevented albumin from being denatured by hypotonic solutions and heat. There was greater inhibition of ethanolic extract and highest inhibition by the standard drug aspirin. For the plant extract and aspirin, the percentage inhibition of albumin denaturation at concentrations of 20, 40, 60, and 80 µg/mL was found to be, respectively, 28.87, 39.57, 49.96%, and 58.75% for the extract, and 48.29%, 54.80%, 63.83%, and 75.93% for aspirin, as presented in Table 3. The IC_{50} value indicated to be 61.60 µg/mL for the plant extract and 26.85 µg/mL for aspirin. The inhibition response was dose-dependent for aspirin and dose-independent for the extracts. They existed linear relations of the concentrations of the plant extract with aspirin and percentage albumin denaturation inhibition, respectively, since it was shown by the regression equation, y=0.5021x+19.07, y=0.4576x+37.713, respectively, and correlation coefficient, R2=0.9985, 0.9805.

Table 3: IC_{50} and % inhibition of Plant extract and Aspirin for protein denaturation assay

Sample	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)
Averrhoa carambola	20	28.87	61.60
	40	39.57	
	60	49.96	
	80	58.75	
Aspirin	20	48.29	26.85
	40	54.80	
	60	63.83	
	80	75.93	
Absorbance of the control: 0.86. Each test was carried out triplicate (n=3)			

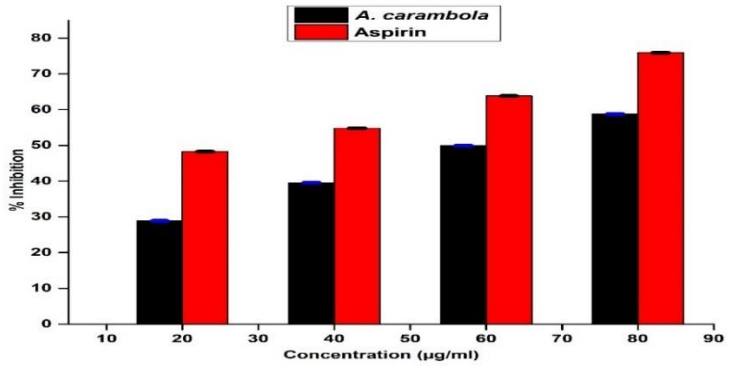

Figure 5: Inhibition of albumin denaturation percentage of ethanol plant extract of A. carambola and aspirin

Results of hypotonic solution induced haemolysis:

Results showed that the ethanol extract at concentrations between 100-500 µg/mL prominently protected the erythrocyte membrane from hypotonic solution-induced lysis (Table 4). Aspirin, similarly, at concentrations of 100-500 µg/mL afforded considerable protection against hypotonic solution-induced damage. Maximum protection was shown by ethanol extract at a concentration of 500 µg/mL, whereas aspirin at the same concentration showed 80.65% inhibition of hemolysis of RBC compared with the control.

Table 4: Effect of ethanol plant extract on hypotonicity induced haemolysis of erythrocyte

Sample	**Concentration (µg/ml)**	**% Inhibition**
Averrhoa carambola	100	32.26
	200	50.54
	300	55.91
	400	63.44
	500	76.34
Aspirin	100	48.39
	200	58.06
	300	66.67
	400	73.12
	500	80.65
Absorbance of the control: 0.31. Each test was carried out triplicates (n=3)		

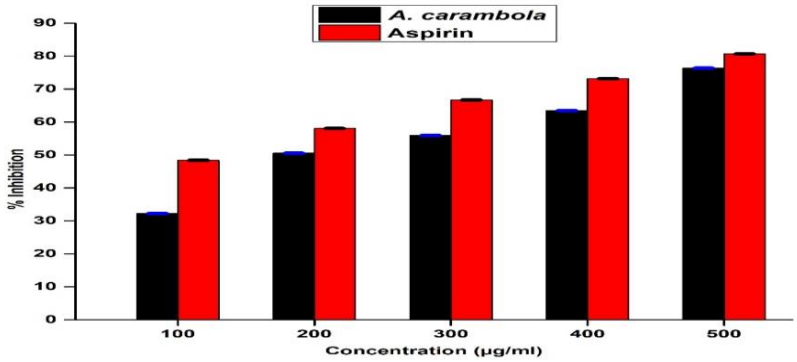

Fig 6: Effect of ethanol plant extract of *A. carambola* and aspirin on hypotonicity induced haemolysis of erythrocyte

CONCLUSIONS:

The percentage yields for the petroleum ether and ethanolic extracts of Averrhoa carambola leaves were at 12% and 20%, respectively. Phytochemical screening of the ethanolic extract revealed bioactive components such as alkaloids, glycosides, tannins, phyto steroids, terpenoids, carbohydrates, proteins, amino acids, and flavonoids. Such a spectrophotometric method used in this experiment is sensitive and reliable in quantifying DPPH and ABTS scavenging activities, hence showing potential antioxidant activity of the phytoextract. Similar results were also observed in the inhibition assay involving albumin denaturation where extracts demonstrated anti-inflammatory activity since they acted to protect albumin from denaturation when exposed to hypotonic solutions and heat. A high percentage inhibition was shown by the ethanolic extract, and the highest percentage inhibition was given by aspirin, the standard drug used. Inhibition caused by aspirin was dose-dependent, whereas that by extracts was dose-independent. The results of the study indicate ethanol extracts of A. carambola possess in vitro anti-inflammatory properties that could result from widespread presence of polyphenols, steroids, and terpenoids. The extract

fractions have an inhibitory activity like free radical scavengers or inhibitors and possibly could serve as primary oxidants, which inhibit heat-induced denaturation of albumin and stabilize red blood cell membranes. This series of results could lead to promising compound *A. carambola* toward a drug development for a strong active substance against many diseases and conditions, such as cancer, neurological disorders, aging, and inflammation.

REFERENCERS:

1. Kamboj, V. P. (2000). Herbal medicine. *Current Science, 78*(1), 35–39.

2. Chopra, R. N., Nayar, S. L., & Chopra, I. C. (1956). *Glossary of Indian medicinal plants.* Council of Scientific and Industrial Research.

3. Yadav, J. P., Kumar, S., & Siwach, P. (2006). Folk medicines used in gynecological and other related problems by rural population of Haryana. *Indian Journal of Traditional Knowledge, 5*(3), 323–326.

4. Gupta, M., Biswas, T. K., Saha, S., & Debnath, P. K. (2006). Therapeutic utilization of secretory products of some Indian medicinal plants: A review. *Indian Journal of Traditional Knowledge, 5*(4), 569–575.

5. Setia, G., Luthra, P., & Sharma, P. C. (2005). Siddha system: An ancient heritage of India. In *Proceedings of the National Seminar on Role of Medicinal and Aromatic Plants in Ayurvedic, Unani and Siddha Systems of Medicine* (pp. 11–14). Hissar.

6. Jain, J. B., Kumane, S. C., & Bhattacharya, S. (2006). Medicinal flora of Madhya Pradesh and Chhattisgarh – A review. *Indian Journal of Traditional Knowledge, 5*(2), 237–242.

7. National Tropical Botanical Garden. (2012, May 26). *Averrhoa carambola - Meet the Plants*. Retrieved from https://ntbg.org.

8. Warrier, P. K. (1993). *Indian medicinal plants: A compendium of 500 species (Vol. 5).* Orient Blackswan.

9. Yanai, N., Shiotani, S., Hagiwara, S., Nabetani, H., & Nakajima, M. (2008). Antioxidant combination inhibits reactive oxygen species mediated damage. *Bioscience, Biotechnology, and Biochemistry, 72*(12), 3100–3106.

10. Rangsriwong, P., Rangkadilok, N., Satayavivad, J., Goto, M., & Shotipruk, A. (2009). Subcritical water extraction of polyphenolic compounds from *Terminalia chebula* Retz. fruits. *Separation and Purification Technology, 66*(1), 51–56.

11. Rathore, N., Kale, M., John, S., & Bhatnagar, D. (2000). Lipid peroxidation and antioxidant enzymes in isoproterenol-induced oxidative stress in rat erythrocytes. *Indian Journal of Physiology and Pharmacology, 44*(2), 161–166.

12. Tripathi, K. D. (2008). *Essentials of medical pharmacology* (6th ed.). Jaypee Brothers Medical Publishers.

13. Murugan, R., & Parimelazhagan, T. (2013). Study of anti-nociceptive, anti-inflammatory properties and phytochemical profiles of *Osbeckia parvifolia* Arn. (Melastomataceae). *Industrial Crops and Products, 51*, 360–369.

14. Harborne, A. J. (1998). *Phytochemical methods: A guide to modern techniques of plant analysis.* Springer Science & Business Media.

15. Murugan, R., & Parimelazhagan, T. (2014). Comparative evaluation of different extraction methods for antioxidant and anti-inflammatory properties.

16. Dahake, A. P., Gagare, M., Jadhav, V. M., & Kadam, V. J. (2010). Antioxidant activity of methanolic extract of *Madhuca longifolia* bark. *Journal of Pharmacy Research, 3*(8), 1709–1711.

17. Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal, 21*(2), 143–152.

18. Sarveswaran, R., Jayasuriya, W. J. A. B., & Suresh, T. S. (2017). *In vitro* assays to investigate the anti-inflammatory activity of herbal extracts: A review.