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A Study of Antioxidant Activity of *Averrhoea Carambola* L. (Starfruit) In Experimental Animal Receiving High Fat Diet.

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Abstract:
Introduction*: Increased production of reactive oxygen species (ROS) and down regulation of the endogenous antioxidant response can cause increased incidence of atherosclerosis. Formation of atherosclerosis is strongly associated with chronic oxidative stress. Antioxidants can stabilize free radicals before they attack cells. For maintenance of optimal cellular and systemic health and well-being antioxidants are absolutely necessary. Our study has been done to see the effect of ethanolic extract of leaves of Averrhoea carambola L. (EELAC) on serum antioxidant level in RABBITS which are receiving high fat diet*
Result: *In our study we have observed that after regular administration of high fat diet up to twelve weeks in rabbits of Group B, there was significant (p<0.0001) decrease in catalase activity (183.6 ± 2.19) compared to the Group A, receiving normal diet (249.0 ± 2.47). In the rabbits receiving EELAC,there was significant (p<0.0001) increase in catalase activity in comparison to the Group B(experimental control).*
There was also a significantly higher level of MDA (7.278±0.09002) in theGroup B than the Group A (5.076 ±0.1431). After administration of EELAC, we found that there was significant (p<0.0001) reduction in the levels of MDA in comparison to the Group B. MDA level was also significantly (p<0.0001) decreased in the Group D, receiving standard drug (4.690±0.1457 nmol/ml) in comparison to the Group B.
Discussion*: It has been seen that higher the doses of ethanolic extract, better the total antioxidant activity in the rabbits.*
Key-word: *antioxidant, Averrhoea Carambola L, ethanolic extract*

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I. Introduction

Atherosclerosis is the major cause of death and premature disability in developed societies. The cellular components in an atherosclerotic plaque are – foam cells (which are transformed macrophages) and smooth muscle cells filled with cholesteryl esters.[1]

There is increased production of reactive oxygen species (ROS) and down regulation of the endogenous antioxidant response under smoking, hypertension, hyperglycaemia, and hyperlipidemia states. The oxidation of LDL increases and there is impairment of endothelial functions during oxidative stress. Formation of atherosclerosis is strongly associated with chronic oxidative stress. [2]

The production of free ROS occurs from the endothelial cells, the smooth muscle cells and the adventitial cells. The production of free ROS also increases due to diabetes mellitus (DM), age, and nitrate intolerance.[3]

Antioxidants can stabilize free radicals before they attack cells. For maintenance of optimal cellular and systemic health and well-being antioxidants are absolutely necessary.[4]

Averrhoea carambola*L**.(commonly known as star fruit) belongs to theOxalidaceae*** family and bears a great significance in traditional medicine. Modern researches have revealed that star fruit has number of health benefits; anti-inflammatory activity, antimicrobial and antifungal activity,antitumor activity, antiulcer activity, negative inotropic and chronotropic effect, hypotensive activity, hypoglycaemic activity, hypocholesterolaemia, hypolipidemic activity, analgesic activity and antioxidant activity.[5,6,7]

There is a paucity of literature and studies regarding the anti- oxidant properties of ***Averrhoea carambola*** **Leaves**. Considering this the present study has been undertaken.

II. Aims and objectives

The aim of our study is to:
1. Determine the antioxidant activity of ethanolic extract of leaves of *Averrhoea carambola* L. in RABBITS receiving high fat diet.

To full fill the aim of our study we chose two objectives:
a) To estimate Catalase activity
b) To estimate the Malondialdehyde level in body

III. Material & methods:

The present study, "A STUDY OF ANTIOXIDATIVE ACTIVITY OF *AVERRHOEACARAMBOLA* L. (STAR FRUIT) IN EXPERIMENTAL ANIMAL RECEIVING HIGH FAT DIET." was conducted in the department of Pharmacology, Assam Medical College and Hospital, Dibrugarh

A) **DRUGS USED IN THE STUDY**:
1) Ethanolic extract of leaves of *Averrhoea carambola* L.
2) Atorvastatin was obtained from Lupin LTD, kartholi , Jammu.
3) Vehicle: Distilled water.

B) **DIET USED IN THE STUDY:**
1) Normal diet: Standard animal diet consisting of bengal gram,wheat, maize and carrot in sufficient quantity and water ad libitum.
2) High fat diet: Mixture of coconut oil (from Marico Industries Ltd, Mumbai) and vanaspati ghee (from Ruchi Industries, Mumbai) in a ratio of 2:3 (v/v) at a dose of 10 ml / kg body weight per day. (P.O.)[8] (Shyamala MP *et al*., 2003)

C) **COLLECTION OF MATERIAL**:
1. PLANT MATERIAL:
Leaves of *Averrhoea carambola* L.(starfruit) were collected from areas in and around Dibrugarh District.

2. EXPERIMENTAL ANIMALS:
Twenty-five number healthy New Zealand white rabbit (*Oryctolaguscuniculus*) of either sex weighing of 1.5–2.5 kg were taken from the CentralAnimal House, Assam Medical College (Reg.No. 634/02/a/CPCSEA, dated 19/05/2002).
The study was duly permitted by the Institutional animal ethics committee (IAEC), Assam Medical College, Dibrugarh vide approval no **IAEC/AMC/16 dated09/11/2017**. The study was conducted keeping in view with the CPCSEA(Committee for the Purpose of Control and Supervision on Experiments on Animals) guidelines.

D) METHOD OF PREPARATION OF ETHANOLIC EXTRACT OF LEAVES OF*AVERRHOEA CARAMBOLA* L. (EELAC):

The ethanolic extract of leaves of *Averrhoea carambola* L. was obtained by the method of percolation as described by SS Handa *et al*. (2008). The leaves were collected, washed and dried in shade on a drier table and grounded to fine powder in electric mixture and grinder. Sufficient amount of the powdered leaves was moistened with an appropriate amount of 90% ethanol and allowed to stand for approximately 4 h in a well closed container, after which the mass was packed and the top of the percolator was closed. Additional amount of 90% ethanol was added to form a shallow layer above the mass, and the mixture was allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then was opened and the liquid contained therein was allowed to drip slowly. Additional amount of 90% ethanol was added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc was then pressed and the expressed liquid was added to the percolate. Sufficient amount of 90% ethanol was added to produce the required volume, and the mixed liquid was clarified by standing followed by decanting.[9]

The extract was collected in glass petri dishes, further dried in a vacuum desiccator and finally stored in airtight glass containers in a refrigerator at 2-8 ºC for use in the experiments. A final yield was 150.6 grams of EELAC.

E) **METHOD OF PREPARATION OF HIGH FAT DIET**:

Edible coconut oil (from Marico Industries Ltd, Mumbai) and vanaspati ghee (from Ruchi Industries, Mumbai) were purchased from the market and a mixture of the two was prepared in a ratio of 2:3 v/v respectively as per method of Shyamala MP *et al.* (2003).[8]

Grouping and treatment schedule:

Group A: Normal control- Received normal diet.

Group B: Experimental control- Received high fat diet at a dose of 10mg/kg bodyweight per day mixed with normal diet.

Group C1: Test drug group- Received high fat diet mixed with normal diet plusethanolic extract of leaves of *Averrhoea carambola*L. (EELAC) at a dose of 200 mg/kg/day orally.

Group C2: Test drug group- Received high fat diet mixed with normal diet plusethanolic extract of leaves of *Averrhoea carambola*L. (EELAC) at a dose of 400 mg/kg/day orally.

Group D: Standard drug group- Received high fat diet mixed with normal dietplus Atorvastatin at a dose of 2.1mg/kg/day orally.[10]

All the animals used for the experiment were kept under observation for daily food intake. The drugs were administered to the animals in the doses given above orally, once daily, for 12 weeks by means of intragastric feeding tube in the volume of 5 ml/kg body weight.
At the end of 12 weeks, body weights of all animals were measured and they were kept fasting for 18 hours.

Catalase:

Catalase is a common antioxidant enzyme that is present in all living tissues that utilises oxygen. This enzyme uses manganese, or iron as a co factor**.** Catalase (CAT) is a tetrameric protein of 240 kilodalton (kDa), with four similar subunits and is encoded by ctt1 gene mapping to chromosome 11.The weight of each polypeptide subunit is 60 kDa and contains a single ferri protoporphyrin. This enzyme is primarily present in the peroxisomes but absent in mitochondria of mammalian cells. The only exception is the heart of rat where it is present in the mitochondria. [10]

Each subunit of catalase contains an active site heam group buried deep within the structure, but which is accessible from the surface through hydrophobic channels. The very rigid,stable structure of catalases is resistant to unfolding, whichmakes them uniquely stable enzymes that are more resistant to $p^{H,}$ thermal denaturation and proteolysis than most other enzymes. Their stability and resistance to proteolysis is an evolutionary advantage, especially since they are produced during the stationary phase of cell growth when levels of proteases are high and there is a rapid rate of protein turnover.[11] Catalases facilitates the degradation of hydrogen peroxide into water and oxygen. The activity of catalase itself can be drastically reduced in case of pathological conditions,where the generation of superoxide radicals can reach up to 3 nmol/min. [12]

ENZYMATIC ASSAY OF CATALASE USING SPECTROPHOTOMETER:

PRINCIPLE:

Catalase

$2\ H_2\ O_2 \rightarrow\ 2H_2\ O + O_2$

CONDITIONS:

Temperature = 25 ºC

$P^{H\,=}$ 7.0

A240nm,Light path = 1cm

METHOD: Continuous Spectrophotometric rate determination

REAGENTS:

A) 50mM Potassium phosphate buffer P^H 7.0 at 25 ºC (Prepare 200 ml in deionized water using Potassium phosphate,Monobasic,Anhydrous, Adjust to P^H 7.0 at 25 ºC using 1M KOH).

B) 0.036% (W/W) Hydrogen peroxide solution ($H_2\ O_2$) [substrate solution] (prepare in reagent A using

hydrogen peroxide,30% (w/w), determine the A240nm of this solution using reagent A as a blank. The A240nm should be between 0.550 and 0.520 absorbance unit. Add hydrogen peroxide to increase the absorbance and reagent A to decrease the absorbance).

C) Catalase solution
(Immediately before use prepare a solution containing 50- 100 units per ml in cold Reagent A.)

Malondialdehyde (MDA):

MDA is usually associated with the pathogenesis of various diseases such as atherosclerosis, stroke and Graves' disease.[13]According to the study of Bhale D. V. et al, Obesity is associated with increased level of Malondialdehyde (MDA)[14].

Analysis of MDA:

One of the useful methods to predict oxidative stress level is the determination of MDA in blood or tissue homogenetes. MDA is included in Thiobarbituric Acid Reactive Substances (TBARS). Out of different techniques to measure MDA levels in serum, plasma or tissues Thiobarbituric acid assay (TBA) is the commonly used technique.[15]

One of the widely used biomarker for oxidative stress is MDA. Themolecular formula ofMDA is C3H4O2, molar mass is 72.02g $mol^{-1.}$ MDA is appeared to be solid and needle like. Unstable lipid peroxidase which is derived from poly unsaturated fatty acid readily decomposes to form a compound series that include MDA. MDA is mutagenic, tumourogenic and highly reactive three-carbondialdehyde produced during peroxidation of polyunsaturated fatty acid and arachidonic acid metabolism.Also, during the breakdown of prostaglandin endoperoxide (PGH2) into12-hydroxyheptadecatrienoate (HHT) MDA is generated. MDA takes part in different biological reactions like covalent binding to proteins, RNA and DNA. [16]

According to various studies it is reported that, MDA acts as a signalling messenger and it regulates the islet glucose-stimulated insulin secretion (GSIS) through Wnt pathway.It is also reported that by upregulating specificity protein-1 (*Sp1*) gene expression andSp1 and Sp3protein levels MDA induced collagen-gene expression in hepatic stellate cells.As an end product of arachidonic acid and largepoly unsaturated fatty acid (PUFA) decomposition, MDA is generated through enzymatic and nonenzymatic process. MDA production through enzymatic process is well known. MDA is more chemically stable, membrane permeable than ROS. MDA is less toxic than 4-hydroxynonenal (HNE). [15]

ESTIMATION OF SERUM MALONDIALDEHYDE (MDA)USINGCOLORIMETER:

PRINCIPLE:

Auto-oxidation of unsaturated fatty acids (FA), involves the formation of semi-stable peroxidase, which then undergo a series of reaction to form short chain aldehyde like MDA. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) with the elimination of two molecules of water to yield pink crystalline pigment with absorption maximum at 535nm.

REAGENTS:

75mg of TBA was dissolved in 15% Trichloro acetic acid (TCA), to which 2.08ml of 0.2N Hydrochloric acid (HCL) was added, the volume was made up to 100ml using 15% TCA.

IV. Results

1) EFFECT OF EELAC ON CATALASE ACTIVITY IN RABBITS FED WITHHIGH FAT DIET

The catalase activity of rabbits belonging to the Group A (Normal control), Group B (Experimental control), Group C1 (Test drug 200 mg/kg), Group C2 (Test drug 400 mg/kg) and Group D (Standard drug) were 249.0 ± 2.47, 183.6 ± 2.19, 276.8 ±4.42, 296.7 ± 2.27, 249.2 ± 5.05 µmol/min/ml respectively. The percentageof increase in catalase activity in Group C1, Group C2 and Group D were 50.76%,61.6% and 35% respectively as compared to Group B.

Table1: EFFECT OF EELAC ON CATALASE AT THE END OF 12TH WEEK OFEXPERIMENT

GROUPS	CATALASE(µmole/min/ml)
Group A (Normal control)	249.0 ± 2.47

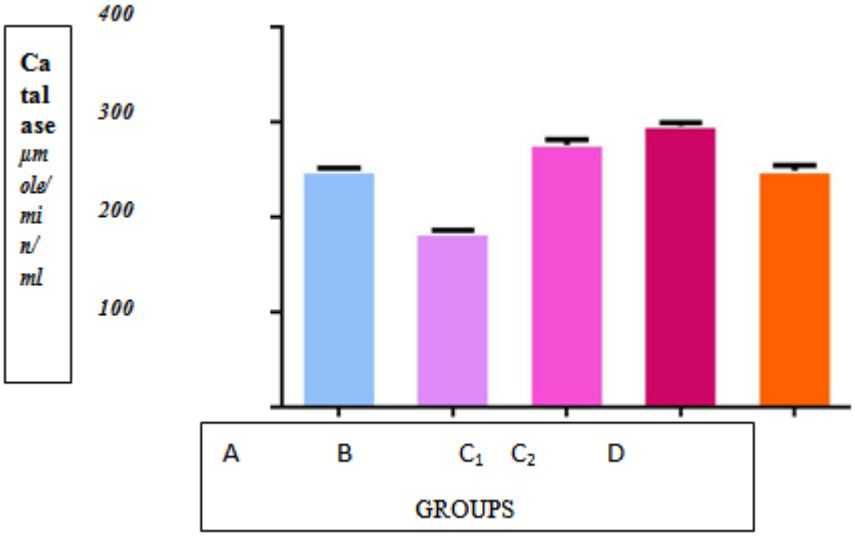
Group B (Experimental control)	183.6 ± 2.19 [a]
Group C_1 (Test drug 200 mg/kg)	276.8 ± 4.42[a b]
Group C_2 (Test drug 400 mg/kg)	296.7 ± 2.27[a b c]
Group D (Standard drug)	249.2 ± 5.05[b c d]
F ANOVA df *p*	149.4 4, 20 <0.0001

Values are expressed as MEAN SEM (n=5)

One Way ANOVA followed by Bonferroni's Multiple Comparison test is done. [a]p<0.05, when compared to the Group A (Normal control).

[b]p<0.05, when compared to the Group B (Experimental control). [c]p<0.05, when compared to theGroup C1 (Test drug200 mg/kg). [d]p<0.05, when compared to theGroup C2 (Test drug 400 mg/kg).

Fig: 1



2) EFFECT OF EELAC ON MALONDIALDEHYDE (MDA) IN RABBITS FEDWITH HIGH FAT DIET

At the end 12 week of drug administration, the serum malondialdehyde (MDA) levels in rabbits belonging to the Group A (Normal control), Group B (Experimental control), Group C1 (Test drug 200 mg/kg), Group C2 (Test drug 400 mg/kg) and Group D (Standard drug) were-5.076 ±0.1431, 7.278±0.09002, 5.736±0.2423, 5.010±0.1207, 4.690±0.1457 nmol/ml respectively. The percentage of reduction of malondialdehyde (MDA) in Group C1, Group C2 and Group D were 21.87%, 31.62% and 35.55% respectively as compared to Group B.

TABLE 2: EFFECT OF EELAC ON MALONDIALDEHYDE AT THE ENDOF 12TH WEEK OF EXPERIMENT

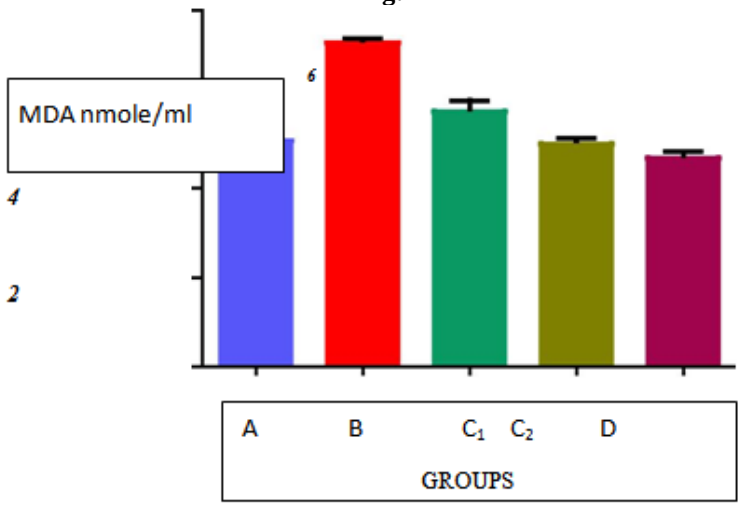
GROUPS	MDA (n mol/ml)
Group A (Normal control)	5.076 ± 0.1431
Group B (Experimental control)	7.278±0.09002[a]
Group C_1 (Test drug 200 mg/kg)	5.736±0.2423[b]
Group C_2 (Test drug 400 mg/kg)	5.010±0.1207[bc]
Group D (Standard drug)	4.690±0.1457[bc]
F ANOVA df *P*	43.42 4, 20 <0.0001

Values are expressed as MEAN SEM (n=5)

One Way ANOVA followed by Bonferroni's Multiple Comparison test is done. [a]p<0.05, when compared to the Group A (Normal control).

[b]p<0.05, when compared to the Group B (Experimental control). [c]p<0.05, when compared to theGroup C1 (Test drug 200 mg/kg).

Fig:2



V. Discussion:

Oxidative stress plays an important role in the atherogenic process. The aim of the present study was to evaluate antioxidative activity of ethanolic extract of leaves of ***Averrhoea carambola*L. *(STARFRUIT)*(EELAC)** in rabbits, receiving high fat diet in comparison to a standard hypolipidemic agent Atorvastatin and normal rabbits that received normal diet alone. The results of above-mentioned groups were compared with Group B (experimental control) that received only high fat diet. The purpose of the present study was to evaluate the action of EELAC on the catalase activity and malondialdehyde (MDA) levels which are markers of oxidative stress.

Moresco HH *et al.* (2012), has reported that ***Averrhoea carambola*L.** leaves contain high amount of phenolic compounds, mainly flavonoids. Results of a study showed that leaves of *Averrhoea carambola*L. contains; β-sitosterol and apigenin-6-*C*-β-L-fucopyranoside , two *C*- glycosyl flavones, and apigenin-6-*C*-(2”-*O*-α-Lrhamnopyranosyl)-β-L-fucopyranoside. [17]

In our study we have observed that after regular administration of high fat diet up to twelve weeks in rabbits of Group B, there was significant (p<0.0001) decrease in catalase activity (183.6 ± 2.19) compared to the Group A (249.0 ± 2.47). In the rabbits of Group **C1**receiving 200 mg/kg body weight/day p.o EELAC,there was significant (p<0.0001) increase in catalase activity in comparison to the Group B (experimental control). The percentage of increase in catalase activity in Group **C1** was 50.76%, as compared to Group B (Experimental control). In case of Group **C2**, which received 400 mg/kg body weight/day EELAC also showed significant increase in catalase activity in comparison to the Group B (experimental control). In thisgroup the percentage of increase of catalase activity was found to be 61.6% in comparison to the Group B (experimental control). We also found increased catalase activity in the Group D (35%) in comparison to the Group B (experimental control). It was noticed that the percentage of increase in catalase activity in test drug groups receiving EELAC 200 and 400mg/kg body weight/day (Group **C1** and Group **C2)** were higher than the Group D (standard drug). Enhanced catalase activity showed higher antioxidant activity of the *Averrhoea carambola* L. leaf extract than atorvastatin.

There was a significantly higher level of MDA (7.278±0.09002) in theGroup B than the Group A (5.076 ±0.1431). After administration of 200 and 400 mg/kg/day of EELAC, we found that there was significant (p<0.0001) reduction in the levels of MDA in the Group **C1**and Group **C2** (5.736±0.2423 nmol/ml and 5.010±0.1207 nmol/ml respectively) in comparison to the Group B. MDA level was also significantly (p<0.0001) decreased in the Group D (4.690±0.1457 nmol/ml) in comparison to the Group B. The percentage of reduction of malondialdehyde (MDA) in test drug groups receiving EELAC 200mg/kg body wt/day and 400 mg /kg body weight/day (Group **C1**and Group **C2)** were 21.87% and 31.62% respectively as compared to Group B(experimental control).In the Group D the percentage of reduction of MDA level was 35.55 % as compared to the Group B. The percentage of reduction of MDA level in the Group D was more than the two test drug groups.

Oxidants are products of the inflammatory response and also derived from normal aerobic metabolism. They are mostly of the nature of "free radicals" whichare highly reactive molecules and can cause dyslipidaemia, coronary artery disease, atherosclerosis and many other diseases. In human, catalase and superoxide dismutase are enzymes and act as antioxidants.[18]

Various experimental studies on animals and human cells demonstrated the effectiveness of antioxidants against oxidative stress. Increased production of catalase (CAT) gives protection of endothelium of human aorta against apoptosis, which is caused by the oxidized forms of low-density lipoproteins. Maksimenko A V *et al.* (2012) have demonstrated that the atherosclerotic process is inhibited byincreased expression of catalase (CAT) or CAT together with SOD-1 in mice lacking apolipoprotein E (ApoE/).[19]

From the results of the present study it was observed that thetwo test drug groups (Group **C1**andGroup **C2)**and Group Dshowed significant rise in catalase activity which indicates protection against lipid peroxidation.

MDA is produced during the peroxidation of polyunsaturated fatty acids and it is an important indicator of lipid peroxidation.[103]In a study conducted by Dhingra D *et al.* (2014) found that high fat diet or fructose significantly increases the levels of liver MDA and decreases the GSH levels. [20]

Rabbits treated with ***Averrhoea carambola*L.** leaf extract and atorvastatin showed significant protection against lipid peroxidation which was indicated by decreased level of MDA.

VI. Conclusion:

The antioxidative and antiatherosclerotic action of*AVERRHOEA CARAMBOLA* L. may be due to the bioactive phytochemical presentin it, which exert a positive effect on serum lipids by controlling different steps of lipid absorption, its transport, metabolism and free radical scavenging effect.

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