IJPSR (2016), Vol. 7, Issue 4 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 12 October, 2015; received in revised form, 24 November, 2015; accepted, 16 January, 2016; published 01 April, 2016

EVALUATION OF THE ANTIMICROBIAL, ANALGESIC AND ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT OF THE LEAVES OF *AVERRHOA CARAMBOLA*

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Key words:

Evaluation, Antimicrobial, Paracetamol

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ABSTRACT: Aim: Evaluation of the antimicrobial, analgesic and antioxidant activity of ethanolic extract of the leaves of *Averrhoa Carambola*. **Materials and methods:** To test the antimicrobial activity the organisms used were *S. aureus, Klebsiella sp, E. coli* and *P. aeruginosa, C. albicans*. Zone of inhibitions produced by sensitive organisms were demarcated by a circular area of clearing around plant extract impregnated discs and were compared with zone of inhibitions of positive controls. Analgesic activity was tested by two methods: acetic acid induced writhing test and Eddy's hot plate mediated pain reaction. The animals were divided into 5 groups: Group I (Normal control), Group II (Standard drug), Group III, IV, V (Etahnolic extract of *A.carrambola* in the doses of 100, 200 and 400mg/kg respectively). To test the antioxidant activity, the mice were divided into 6 groups containing. Paracetamol in the dose of 250mg/kg p.o was administered to all the groups except the first group, which was taken as the normal control, for 10 days to induce oxidative stress. Silymarin in the dose of 25mg/kg p.o was given to the third group as a standard antioxidant. Group IV, V and VI received extract in the dose of 200mg/kg, 400mg/kg and 800mg/kg respectively. **Result:** Mean±SEM values were calculated for each group. The data were analyzed using ANOVA and post analysis was done by Dunnett's test. Results were found to be significant ($p<.05$). **Conclusion:** The results of the present study revealed the antimicrobial, analgesic and antioxidant activity of the leaf ethanolic extract of *A.carrambola*.

INTRODUCTION: Herbal medicine is the oldest form of healthcare known to mankind. Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them.[1] Herbal medicines have often retained popularity for historical and cultural ingredients and are used primarily for treating mild and chronic ailments. India has an ancient heritage of traditional medicines; Materia Medica of India provides lots of information on the folklore practices and traditional aspects of therapeutically important natural products.[2]

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(4).1716-23

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (4).1716-23

The species in the genus *Averrhoa* are the only woody, tree-like plants belonging to the Oxalis family Oxalidaceae. The Oxalis family has nearly 900 species; most are herbaceous perennials or annuals native from tropical and semitropical locations though a number also grow in other parts of the world. *Averrhoa* has sometimes been placed in the family Averrhoaceae.

The genus Averrhoa contains two species: Bilimbi (*Averrhoa bilimbi*. L) and Carambola (*Averrhoa carambola* L). *Averrhoa carambola* is considered most important between the two species. [3] The generic name is after Averroes, the widely known Arabian physician, astronomer and philosopher of 12th century. The specific name 'carambola', is said to have come from Malabar and was adopted early by the Portuguese. [4]

Scientific Name: *Averrhoa carambola*

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta -Vascular plants

Superdivision: Spermatophyta

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Rosidae

Order: Geraniales

Family: Oxalidaceae – Wood-Sorrel family

Genus: Averrhoa Adans. – averrhoa

Species: *Averrhoa carambola* L. – carambola [5]

This study was proposed to evaluate the antimicrobial, analgesic and antioxidant activity of *A.carrambola* leaves as many studies with the fruits of this plant has already been done.

MATERIALS AND METHODS:

Plant material:

The *A.Carrambola* leaves collected during December, 2014 in and around Guwahati, Assam were authenticated by Department of Botany, Gauhati University. The voucher specimens were kept in the Department of Botany, Gauhati University, Assam, India.

Extraction procedure:

Shade dried leaves (200 g) were coarsely powdered and subjected to continuous Soxhlet extraction. The extraction was done using ethanol. The jelly like extract of the leaves was collected in a petridish. A final yield of 40.5 gm was obtained. The percentage yield of *A.carrambola* was 20.25% (w/w) with respect to the original dried powder. The extract was stored in a refrigerator at 4°C in labeled air-tight containers for further use. The dried extracts were dissolved in dimethyl sulphoxide (DMSO) and subjected to antibacterial activity.

Drugs and chemicals used in the study:

Antibiotic discs from, Pentazocine Ampoule (30mg/ml. obtained from Ranbaxy Pharmaceuticals), Aspirin obtained from USV Ltd, Glacial acetic acid obtained from Fisher Scientific Ltd., Silymarin from Admac Formulations, Paracetamol from Dr. Reddy's laboratories Ltd.

Animals and acute toxicity study:

The animals were taken from the Department of Pharmacology, Gauhati Medical College & Hospital. This test was done using 20-25gm of healthy albino mice of either sex in the Department of Pharmacology of Gauhati medical college. They will be housed in standard laboratory condition at 25ºC and fed on standard diet and water *ad libitum*

The present study was conducted in the department of Pharmacology, Gauhati Medical College, Guwahati after getting approval from institutional Animal Ethics Committee.

The acute toxicity study was carried out as per OECD guidelines 425. The mice were randomly selected, marked to permit individual identification, and kept in their cages for seven days prior to dosing to allow for acclimatization to the laboratory conditions. Animals were fasted prior to dosing (food but not water was withheld for 3-4 hours). The fasted body weight of each animal was determined and the dose was calculated according to the body weight. Following the period of fasting, the first animal was dosed at 175 mg/kg body weight with EEAC by gavage. Food was withheld for a further 1-2 hours. The animal was observed for mortality for 48 hours and then a second animal was dosed at 550 mg/kg body weight by gavage. Again after 48 hours of the second dosing a third animal was dosed at 2000 mg/kg and observed for the next 48 hours for mortality. All the above animals were observed for a full observation period of 14 days. The animals were found to be alive at 2000 mg/kg.[6]

Ethical Approval:

The study protocol was approved by the Institutional Ethics Committee of Gauhati Medical College & Hospital (IEC No.MC/05/2015/12).

Evaluation of Antimicrobial Activity:

Test organisms:

The organisms used for the test were *Staphylococcus aureus* (*S. Aureus), Klebsiella sp, Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), *Candida albicans* (*C.albicans*). All the stock cultures were obtained from Department of Microbiology, Gauhati Medical College, Guwahati, Assam, India.

Preparation of plant extract impregnated discs:
Whatman filter paper no.1 was used to prepare discs of 5mm. They were sterilized by autoclaving and subsequently dried at 80ºC for an hour in hot air oven. The discs were impregnated with ethanolic extracts of *A.carrambola*. The produced discs (each one) have the ability to absorb about 0.01ml.

A**ntimicrobial testing:**
Antimicrobial testing was done by the modified agar disc diffusion method originally described in 1966 by Kirby-Bauer. Bacterial strains were cultured overnight in Nutrient agar (HiMedia, Mumbai) at 37±2°C. Overnight grown culture of microorganisms was used for inoculums preparation. A loopful of isolated colony was inoculated in 4ml of Peptone water (HiMedia, Mumbai) at 37°C for 2 h. The turbidity of resulting suspension was compared to 0.5 McFarland turbidity standards. The level of turbidity was equivalent to approximately 3.0×10^5 cfu/ml. The Mueller Hinton Agar media (HiMedia, Mumbai) was prepared and poured into Petri dishes. Once the media solidifies it was then inoculated with microorganism suspended in peptone water.

The filter paper discs were impregnated with the extracts and placed individually on the Mueller-Hinton agar with flamed forceps and gently pressed down to ensure contact with the agar surface. The discs were placed far enough from each other to avoid both reflection waves and overlapping rings of inhibition. DMSO was used as negative control. The experiment was performed at four different concentrations (50, 100, 200 and 400mg/ml). The test was carried out five times to eliminate any error. The Petri dishes were incubated for 24 h at 37±2°C for the bacteria. Sabouraud agar was used for *C.albicans* and tested by the same method as described above.

Zone of inhibitions produced by sensitive organisms were demarcated by a circular area of clearing around plant extract impregnated discs and were compared with zone of inhibitions of positive controls (Gentamicin 10µg, Ceftazidime 30µg and Fluconazole 25µg HiMedia, Mumbai) and negative controls (DMSO). The tests were repeated five times to ensure reliability.

Evaluation of Analgesic Activity:
Acetic acid induced writhing test:
Male swiss albino mice were divided into five groups containing six animals each.

Group I: Control, received normal saline at a dose of 10ml/kg + Acetic acid 0.6% v/v 10ml/kg i.p.

Group II: Aspirin 100mg /kg p.o + Acetic acid 0.6% v/v 10ml/kg i.p.

Group III: *A.carambola* extract 100mg/kg p.o + Acetic acid 0.6% v/v 10ml/kg i.p

Group IV: *A.carambola* extract 200mg/kg p.o + Acetic acid 0.6% v/v 10ml/kg i.p.

Group V: *A.carambola* extract 400mg/kg p.o + Acetic acid 0.6% v/v 10ml/kg i.p.

After administration of acetic acid the number of writhes (abdominal muscle contractions) were counted over a period of 15 mins.

Eddy's hot plate mediated pain reaction:
Male swiss albino mice were divided into five groups containing six animals each.

Group I: Normal control, received normal saline at a dose of 10ml/kg.

Group II: Pentazocine 10mg /kg p.o.

Group III: *A.carambola* extract 100mg/kg p.o.

Group IV: *A.carambola* extract 200mg/kg p.o.

Group V: *A.carambola* extract 400mg/kg p.o.

Then the animals were placed on the hot plate maintained at 55±1̊c.The pain threshold was considered to be reached when the animals lift and lick their paws or attempt to jump out of the hot plate. The time taken for the mice to react in this fashion was obtained using a stop watch and noted as the basal reaction time. The latency is recorded before and after 30, 60 and 120 min following oral administration of the standard or the test compound. [7]

Evaluation of Antioxidant Activity:

The mice were divided into six groups containing six animals each. Paracetamol in the dose of 250mg/kg was administered orally per day to all the groups except the first group, which was taken as the normal control, for 10 days to induce oxidative stress. Silymarin in the dose of 25mg/kg p.o [8] was given to the third group along with paracetamol as a standard antioxidant. Group IV, V and VI were given ethanolic extract of *A.carrambola* in the dose of 200mg/kg, 400mg/kg and 800mg/kg respectively along with paracetamol for 10days. The second group was taken as the disease control and received only paracetamol.

Group I: Normal control, received normal saline at a dose of 10ml/kg.

Group II: Recieved Paracetamol 250mg/kg p.o per day for 10days.

Group III: Silymarin 25mg/kg,p.o + Paracetamol 250mg/kg p.o per day for 10days.

Group IV: Ethanolic extract of *A.carrambola* 200mg/kg p.o + Paracetamol 250mg/kg p.o per day for 10days.

Group V: Ethanolic extract of *A.carrambola* 400mg/kg p.o + Paracetamol 250mg/kg p.o per day for 10days.

Group VI: Ethanolic extract of *A.carrambola* 800mg/kg p.o + Paracetamol 250mg/kg p.o per day for 10days.

After 10 days, the animals were sacrificed under ether anaesthesia 24 hrs after the last administration. Liver samples were immediately removed and stored at -80 °C until analysis.

Liver was excised and homogenized in phosphate buffer (1:10 ratio). Homogenate was centrifuged at 10,000 rpm at 4°C for 30 min using DuPont Sorvall ultracentrifuge (USA). The resulting supernatant was used for the estimation of lipid peroxidation product Malonyldialdehyde (MDA), antioxidant parameters Reduced glutathione (GSH), Catalase and Superoxide dismutase (SOD).

Homogenate (60µl) and 1% trichloroacetic acid (60µl) mixed and kept at 4^oC for 30 min. The homogenate is centrifuged at 1000 rpm. For 5 min. at 4^oC. Supernatant collected and used for GSH assay.

Superoxide dismutase (SOD) Assay kit (Cat. No. CS 19160) manufactured by Sigma-Aldrich, was used for the quantitative in vitro determination of Superoxide dismutase (SOD) in sciatic nerve. SOD assaying by utilizing highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the IC50 (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. The absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 440 nm.

Catalase assay kit (Cat. No. CAT100) manufactured by Sigma-Aldrich, was used for quantitative determination of Catalase activity in sciatic nerve of rats. The assay method for catalase is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase. First, the catalase converts hydrogen peroxide to water and oxygen (catalatic pathway) and then this enzymatic reaction is stopped with sodium azide. An aliquot of the reaction mix is then assayed for the amount of hydrogen peroxide remaining by a colorimetric method. The colorimetric method uses a substituted phenol (3,5-dichloro-2-hydroxybenzenesulfonic acid), which couples oxidatively to 4-aminoantipyrine in the presence of hydrogen peroxide and horseradish peroxidase (HRP) to give a red quinoneimine dye (N-(4-antipyryl) - 3 - chloro - 5 - sulfonatep-benzoquinone-monoimine) that absorbs at 520 nm. MDA is a product of lipid peroxidation that appears to be produced in relatively constant proportion from the breakdown of polyunsaturated fatty acids. Quantification of MDA is done by reaction with TBA (Thiobarbituric Acid) and measurement of the pink chromophore produced. It was estimated by

measuring the formed malondialdehyde (MDA) colorimetrically using method described by Zhang *et al*. (2004). [9]

Statistical Analysis: One-way ANOVA with Dunnett's posthoc test was used for the determination of statistical significance as appropriate. Differences of a *p* value of less than 0.05 was considered statistically significant. Graph pad prism Version 5 was used in the statistical analysis.

RESULTS AND OBSERVATIONS:

TABLE 1: ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACT *A.CARRAMBOLA* AGAINST DIFFERENT MICROORGANISMS

	Staphylococcus Aureus	***Escherichia Coli***	***Klebsiella Spp***	***Pseudomonas Spp***	***C.albicans***
EEAC-400 mg/ml	16.04±0.2856	15.4±0.2645	17.9±0.158	18±0.2549	17.9±0.1516
EEAC-200 mg/ml	13.12 ± 0.2266	13.3 ± 0.187	14.14±0.2378	14.28± 0.2835	14.26±0.2204
EEAC-100 mg/ml	10.12 ± 0.1984	10.42 ± 0.22	10.16±0.1938	11.24± 0.2063	10.66 ± 0.186
EEAC-50 mg/ml	6.6 ± 0.1788	7.94±0.1536	6.92 ± 0.1743	7.18 ± 0.2477	7.18 ± 0.2781
GENTA-10 µg	25.06 ± 0.2501	---	---	---	---
CEFTA-30µg	---	25.94±0.2314	28.74±0.2226	30.36± 0.1503	---
FLUCONAZOLE-25µg	---	---	---	---	27.26±0.2014

Values are expressed as Mean ± SEM (n=5);
One Way ANOVA followed by Dunnett's multiple comparison tests is done.
p<0.05 when compared to standard positive control.

TABLE 2: ANALGESIC ACTIVITY OF ETHANOLIC EXTRACT *A.CARRAMBOLA* BY ACETIC ACID INDUCED WRITHING TEST.

Treatment groups	**Number of writhes (per 15 minutes)**	**Percentage inhibition (no.of wriths in control - no. of wriths in test group/no. of wriths in control)×100**
GROUP I	66.5±1.3844	
GROUP II	18.1±0.8724	72%
GROUP III	45±1.1255	32%
GROUP IV	35±1.1547	47%
GROUP V	28.3±0.7149	57%

Values are expressed as Mean ± SEM (n=6);
One Way ANOVA followed by Dunnett's multiple comparison tests is done.
p<0.0001 when compared to the control
p<0.0001 when compared to the standard

TABLE 3: ANALGESIC ACTIVITY OF ETHANOLIC EXTRACT *A.CARRAMBOLA* BY EDDY'S HOT PLATE MEDIATED PAIN REACTION.

Groups	**Reaction time in seconds(mean±sem)**		
	30 min	**60 min**	**120 min**
GROUP I	3.7±O.1807	3.85±0.1335	4.13±0.2361
GROUP II	11.8±0.1861	12.01±0.1922	13.21±0.174
GROUP III	7.56±0.1605	7.21±0.1701	8.05±0.1927
GROUP IV	8.8 ± 0.1922	8.71±0.1814	9.28±0.2039
GROUP V	10.2 ± 0.1633	10.06 ± 0.1706	10.35 ± 0.1477

Values are expressed as Mean ± SEM (n=6);
One Way ANOVA followed by Dunnett's multiple comparison tests is done.
p<0.0001 when compared to the Normal control group.
p<0.0001 when compared to the standard group.

TABLE 4: ANTIOXIDANT ACTIVITY OF ETHANOLIC EXTRACT *A.CARRAMBOLA*

	SOD U/mg protein	CATALASE µM/min/ml	GSH µM/mg protein	MDA µM/mg protein
GROUP I	4.83±0.0714	29.01±0.1956	50.08±0.2773	7.45±0.1175
GROUP II	2.41±0.1077	10.93±0.1686	22.7±0.3018	21.16±0.2076
GROUP III	4.36±0.1021	27.33±0.1282	46.5±0.1706	9.45±0.1408
GROUP IV	3.8±.1633	16.3±0.2205	27.3±0.1833	16.4±0.129
GROUP V	4.08±0.0792	21.05±0.1765	36.2±0.2056	14.3±0.1688
GROUP VI	4.46±0.1904	26.41±0.1869	43.3±0.1183	11.58±0.1351

Values are expressed as Mean ± SEM (n=6);
One Way ANOVA followed by Dunnett's multiple comparison tests is done.
$p<0.0001$ when compared to the disease control group.

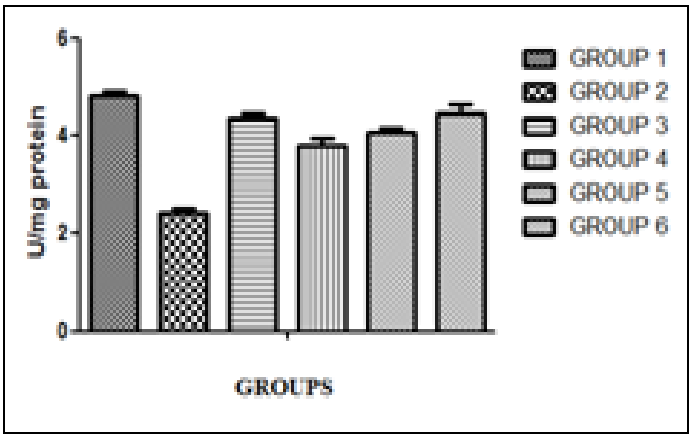

FIG. 1: LEVELS OF SOD

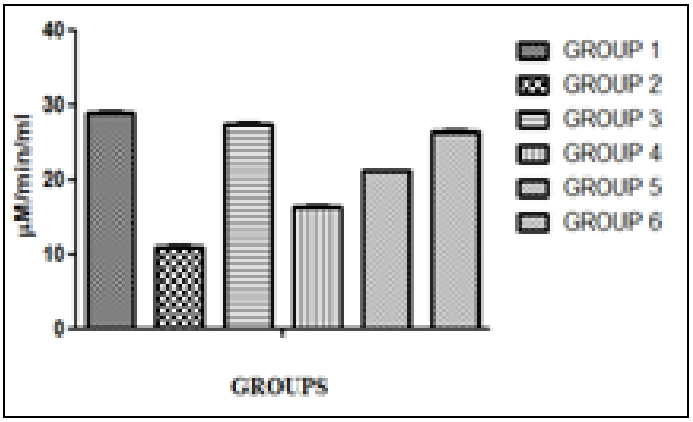

FIG. 2: LEVELS OF CATALASE

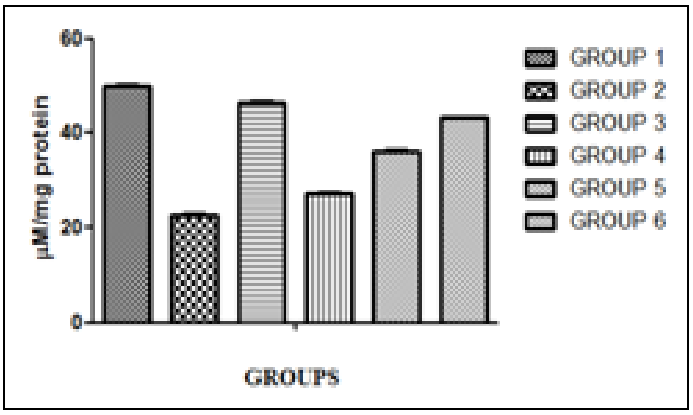

FIG. 3: LEVELS OF GSH

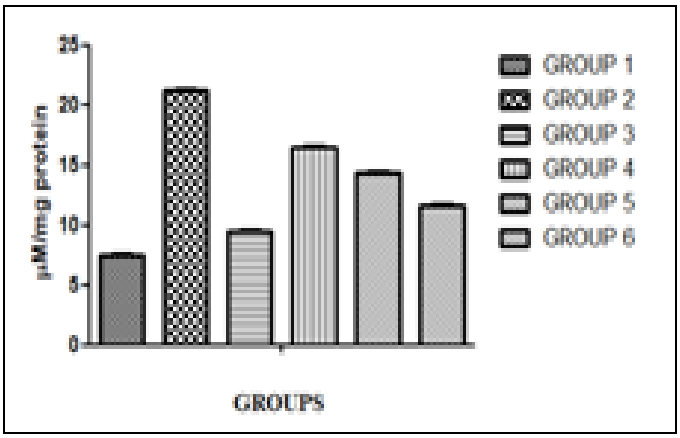

FIG 4: LEVELS OF MDA

DISCUSSION: The search for antimicrobials from natural sources has received much attention and efforts have been put in to identify compounds that can act as suitable antimicrobials agent to replace synthetic ones. Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganism. In the present investigation, ethanolic extract of *A. carrambola* was evaluated for exploration of their antimicrobial activity against certain Gram negative and Gram positive bacteria, fungus which were regarded as human pathogenic microorganism. Susceptibility of each plant extract was tested by serial microdilution method and agar well diffusion method was determined. Our preliminary investigation **(Table 1)** showed that the Ethanolic extract of *A. carrambola* was active against the locally isolated human pathogens like *Escherichia coli, Staphylococcus aureus, klebsiella, Pseudomonas aeruginosa* and *C.albicans* although the exact mechanism of action is not known.

Acetic acid test is a visceral pain model produces a painful reaction and acute inflammation in the

peritoneal area. Release of arachidonic acid and biosynthesis of prostaglandin via cyclooxygenase pathway plays a role in the nociceptive mechanism of this test.[10] The analgesic effect of the tested compounds may be mediated through inhibition of cyclooxygenase and/or lipooxygenase (and other inflammatory mediators).[7] Aspirin offers relief from inflammatory pain by suppressing the formation of pain mediators in the peripheral tissues, where prostaglandins and bradykinins were suggested to play an important role in the pain process. Prostaglandins elicit pain by the direct stimulation of sensory nerve endings. [11] It is evident from the study that *A.carrambola* exhibits significant peripheral analgesic effect in mice comparable with standard **(Table 2)**.

The classic hot plate model was followed to evaluate the analgesic activity of *A.carrambola* leaf ethanolic extract. The hot plate model has been found suitable to investigate central antinociceptive activity because of several advantages, particularly the sensitivity to antinociceptives and limited tissue damage. [12] Proinflammatory mediators like prostaglandins and bradykinins were suggested to play an important role in analgesia.[13] The obtained results **(Table 3)** confirmed that leaf ethanolic extract at the dose 100, 200 and 400 mg/kg has a central analgesic effect, which was compared with reference drug (Pentazocine 10mg/kg).

The analgesic effect of *A.carrambola* might be attributed to the inhibition of the synthesis of some pro-inflammatory mediators, such as prostaglandins and cytokines. The anti-inflammatory effect may also be due to inhibition of either vascular event or cellular events or due to both in experimental rats.

Averrhoa carambola is a small, attractive, multistemmed, slow growing evergreen tree with a short trunk or a shrub, 5-7m of height or rarely, 10m high, spreading 20-25 ft in diameter. It has a bushy shape with many branches producing a broad, rounded crown. At the base, the trunk reaches a diameter of 15cm.[14, 15] Leaves are 15-25cm long, alternate, spirally arranged, ovate to ovate-oblong in shape, imparipinnate, shortly petiolate with 5-11 green pedant leaflets of 2-9cm long and 1- 4.5cm wide. The compound leaves are soft, pubescent, medium-green, smooth on the upper surface and whitish on the underside. The leaflets are reactive to light and tend to fold together at night; they are also sensitive to abrupt shock. They have purple to bright purple coloured flowers are produced in the axils of the leaves. The flowers are arranged in small clusters and each cluster is attached to the tree with red stalks. The flowers are small, about 6mm wide, pedicellate with 5 petals (having curve ends) and sepals.

The result of the treatment shows increase in enzymatic activity. Ethanolic extract of *A.carrambola* exhibit antioxidant activity **(Table 4)** and they are able to diminish the oxidative hepatic damage produced by paracetamol. Nowadays, changes in dietary habits by increasing the consumption of antioxidants in the diet are recommended. Therefore, when an organism is subjected to greater oxidative stress, it would have better antioxidant defense characteristics, thus counteracting the effects of any pro-oxidant.

CONCLUSION: In conclusion, the results of the present study revealed the antimicrobial, central and peripheral analgesic and antioxidant activity of the leaf ethanolic extract of *A.carrambola*. The data reported in this study confirms the traditional use of *A.carrambola* to treat various disorders. However, less information is available regarding the clinical study, toxicity study, phyto-analytical studies of this plant. With the availability of primary information, further studies can be carried out such as phyto-analytical studies, clinical trials, toxicity evaluation and safety assessments. The plant is pre-clinically evaluated to some extent; if these claims are scientifically and clinically evaluated then it can provide good remedies and help mankind in various ailments.

ACKNOWLEDGEMENT: The authors would like to thank Department of Microbiology, Gauhati Medical College and Hospital, Guwahati, Assam for their co-operation while testing the antimicrobial property of *A. Carrambola* extract.

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How to cite this article:
Phukan S and Ahmed A: Evaluation of the Antimicrobial, Analgesic and antioxidant activity of Ethanolic Extract of The Leaves of *Averrhoa Carambola*. Int J Pharm Sci Res 2016; 7(4): 1716-23.doi: 10.13040/IJPSR.0975-8232.7(4).1716-23.