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ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECT OF *ARECA CATECHU*

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ABSTRACT

Context: The present investigation provides proof for the effectiveness of Arecanut extract as an anti-inflammatory agent. Arecanut extract is a natural plant product mimic of peroxidase.

Objective: To explore the Anti-inflammatory activity of aqueous extract of *Areca catechu* L in carrageenan, dextran and formalin induced inflammation models in Swiss albino mice, by injection into the interdigital area, through the subplantar region of the paw. To explore the antioxidant effects of Arecanut extract on the *in-vitro* system.

Method: Treatment with aqueous extract at 250 mg/kg.bwt and 500 mg/kg.body weight and untreated group was started orally 1 hour prior to the subplantar injection of carrageenan. The paw volume was measured using vernier calipers, before and one hour after carrageenan injection. Similarly in the case of dextran, initial readings were taken on the first day, prior to Formalin administration. Day one readings were taken one hour post formalin administration. This was taken during seven consecutive days challenge period. The drug aqueous arecanut extract at 200mg/kg.bwt, 500 mg/kg.bwt produced reduction in inflammation of the paw produced due to carrageenan, formalin and dextran. *In-vitro* antioxidant studies showed that aqueous arecanut extract could inhibit superoxide radical production, could inhibit hydroxyl radicals, and could prevent lipid peroxidation. Arecanut extract could scavenge DPPH radicals and also ABTS. In FRAP assay, the reduction of ferric to ferrous is also seen in a concentration dependant manner.

Results: The present investigation provides proof for the effectiveness of treatment as an anti-inflammatory and antioxidant agent. Compared with the control group, the arecanut treated group showed free radical scavenging ability. Compared with the control group, the treatment of mice with Arecanut extract showed reduction in paw oedema in a dose dependent manner at 200 mg/kg.bwt and 500 mg/kg.bwt.

Discussion and Conclusion: Arecanut extract has some protective effects against peroxidative damage. It has anti inflammatory activity, thus it carries a great potential as a health product.

INTRODUCTION: Arecanut has main constituents like polyphenols, fat polysaccharides, fiber and protein. Besides these, nuts contain alkaloids, arecoline (0. 1-0. 7%) and others in trace amounts such as arecadine, guvacoline and guvacine. The polyphenols of ripe *Areca catechu* L., contain predominantly polymers like leucocyanidins as well as small amounts of catechin, leucopelargonidin and leucocyanidin. The polyphenolic substances of arecanut, termed tannins are responsible for the physiological action.

Free radicals and other reactive species generated by processes, such as ionizing radiation, toxic xenobiotics, inflammation and metabolites of membrane lipid transformation have been implicated in many human diseases [1]. They damage lipids, proteins and deoxyribonucleic acids with consequent effects ranging from cell death to neoplasia [2]. Reactive Oxygen Species (ROS) are biologically important, damaging molecules such as lipids, DNA or proteins and are involved in the pathogenesis of degenerative diseases [3].

Organism possess several antioxidant systems, enzymatic and non enzymatic, that are very important for the prevention of oxidative stress [4]. Given the multiplicity of antioxidant pathways, their centrality in the prevalence of oxidant stress and the influences of lifestyle and nutritional supplements on an individual's antioxidant capacity, it is important to be able to quantitatively measure the total antioxidant or antioxidant power within herbal or plant extracts [5]. Removal of the oxygen free radicals, enzymatically or non enzymatically has been shown to reduce the incidence of cancer in the animals as well as in clinical trials [6]. The antioxidant activity of arecanut is worth investigating, as there are reports that epicatechin protects human fibroblasts from oxidative stress induced cell death involving caspase-3 activation [7] and in this report an attempt has been made to study in detail, the *in-vitro* antioxidant activity of aqueous arecanut extract.

On the basis of this consideration, the current work is aimed at evaluating whether arecanut treatment has protective effect on chemically induced inflammation. Our hypothesis is that, the use of areca nut extract may make it possible to prevent and/or alleviate the oxidative stress and inflammation in patients, who have a potential towards oral cancer.

The objective of this study is to evaluate the antioxidant and anti-inflammatory activity of arecanut. Lipid soluble (most importantly Vitamin E) and water soluble vitamins (vitamin C), Uric acid, bilirubin, thiols and glutathione antioxidants are involved in these processes.

On the contrary, there are reports that have not been scientifically tested, that oral cancer can occur in arecanut chewers, without a tobacco habit. There are studies [8] that illustrate the importance of arecanut habit with tobacco, in the development of oral cancer. Some of these showed signs of OSF- Oral Submucous Fibrosis.

Yet, detailed epidemiological and experimental findings have not backed these reports. The antioxidant activity of aqueous arecanut extract has no connection with the Oral Submucous Fibrosis that may lead to oral cancer.

Catechin, the major polyphenols present in arecanut has been shown to inhibit chemical carcinogenesis [9]. Catechin has been shown to inhibit mammalian collagense and thereby increase collagen accumulation [10, 11].

The present study was carried out to determine the effect of the aqueous extract of arecanut to scavenge free radicals generated or produced *in-vitro* and thereby evaluate the antioxidant potential of this extract. A close link exists between the occurrence of inflammation and free radicals, many antioxidants have been reported to exert anti-inflammatory effects. For example, a small molecule mimic of SOD, could inhibit carrageenan induced paw oedema and hyperalgesia [12].

MATERIALS AND METHODS:

- **Reagents:** Tri-pyridyl-S-triazine (TPTZ), Nitro-blue-tetrazolium (NBT), Riboflavin and deoxyribose were bought from HI-Media. DPPH-1, 1-Diphenyl-2-Picryl Hydrazyl, ABTS 2, 2-Azo bis -3 ethylbenzthiazoline-6-sulfonic acids and Carrageenan were bought from Sigma Aldrich (St. Louis, MO, USA). Carboxy Methyl Cellulose (CMC) and formalin was purchased from Merck. All other chemicals used were of analytical reagent grade.

- **Animals:** Female Swiss Albino mice (20-25 g) used in the study, were purchased from Small Animal Breeding Station, Mannuthy, Kerala. Female mice were readily available in multiple numbers and they were housed in well ventilated cages, under controlled conditions of light and humidity. They were provided with normal mouse chow and water *ad libitum.* All the animal studies were conducted with approval from the Institutional Animal Ethics Committee and as per the rules of CPSCEA, India.

- **Preparation of the Extract:** Tender arecanuts (*Areca catechu* L.) were collected from coastal areas in the monsoon preshower period. 100 g of tender arecanuts were cut into small pieces, and boiled in 500 ml distilled water for 1 hr. This was evaporated to dryness on steam water bath and was diluted with water to desired concentrations and used for the experiment. The yield was found to be 6.4%, which was the amount for every 100 g of tender arecanut kernels, boiled in 500 ml distilled water.

- ***In-vitro* Antioxidant Activity:**

  - **Determination of Superoxide Radical Scavenging Activity**: Superoxide radical Scavenging Activity was determined by the Nitro Blue Tetrazolium Reduction Method [13]. The reaction mixture contained Ethylene Diamine Tetraacetic Acid (6µm), Sodium Cyanide (3µg), Riboflavin (2µm), NBT (50µm), various concentrations of the extract (1 to 60µg/ml) and phosphate buffer (pH 7.8) in a final volume of 3ml. The tubes were uniformly illuminated with an incandescent lamp for 15 min and the Optical Density was measured at 560nm, before and after illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes.

  - **Determination of Hydroxyl Radical Scavenging Activity:** Hydroxyl radical scavenging Activity was measured by studying the competition between deoxyribose and test compound for hydroxyl radicals generated by Fenton Reaction. The reaction mixture contained deoxyribose (2.8mM), Ferric Chloride (0.1mM), Ethylene Di Amine Tetraacetic Acid (0.1m M), Hydrogen Per Oxide (1mM), Ascorbic Acid (0.1 mM), Phosphate Buffer (KH_2PO_4-KOH) buffer (20 mM, pH 7.4) [14] and various concentrations of the extract (5 to 200 µg/ml) in a final volume of 1 ml. The hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the compounds for hydroxyl radicals generated from Fe^{3+}/ascorbate EDTA/ H_2O_2 system. The reaction mixture was incubated for 1h at 37°C. Deoxyribose degradation was measured as ThioBarbituric Acid Reacting Substance (TBARS) and percentage inhibition was calculated [15].

  - **Determination of Inhibition of Lipid Per oxidation**: Reaction mixture (0.5 ml) containing rat liver homogenate (0.1 ml, 25%w/v), ferrous (0.16mM) and Ascorbic Acid (0.06 mM) were incubated for 1 hr at 37°C in the presence and absence of the extracts (30 to 200µg/ml). The lipid peroxide formed was measured by TBARS formation and percent inhibition was calculated [16].

  - **Determination of Diphenyl -2-Picryl Hydrazyl (DPPH) Radical Scavenging activity:** DPPH in its radical form has an absorption peak at 515 nm, which disappeared upon reduction by an antioxidant compound 250µg/L [16] (Aquino R *et al*, 2001). Different concentrations of the extract (0.2 to 10µg/ml) were added to 1.5 ml of freshly prepared DPPH solution (0.25g/L in methanol) After 20 minutes the absorbance was measured at 515 nm, the percentage inhibition was calculated by comparing with the control.

  - **Determination of Ferric Reducing Antioxidant Power (FRAP) assay:** The freshly prepared FRAP reagent containing 2.5 ml 10 mM TPTZ (Tri-pyridyl-S-Triazine) solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. 900 µl FRAP reagent was mixed with 90µl distilled water and 30µl different concentrations of arecanut extract (1- 20µg/ml), were added and the reaction mixture was then incubated at 37°C for 30 min and absorbance change was recorded at 595 nm.

  - **Determination of ABTS Radical Scavenging Activity:** ABTS (Di ammonium salt) Radical Scavenging Activity of the extract was

determined using ferryl myoglobin/ABTS protocol [17]. Metmyoglobin was prepared by mixing equal amounts of myoglobin (Mb III) and potassium ferricyanide solutions (740µM).The reaction mixture (2 ml) contained ABTS (150µM), MbIII (2.25µM), varying concentrations of Arecanut extract (5-25µg/ml) and Phosphate Buffered Saline (PBS). The reaction was initiated by adding 75mM H_2O_2 and lag time in seconds was recorded before absorbance of ABTS at 734 nm began to increase.

- **Determination of Anti-Inflammatory Activity**: Carrageenan induced paw oedema in mice, Dextran induced paw oedema in mice and Formalin induced paw oedema in mice.

- **Carrageenan induced Paw Oedema in mice**: Animals were divided into three groups of six animals in each group. In all groups, the inflammation was induced by subplantar injection of 0.02 ml of freshly prepared 1.5 mg/ml carrageenan (1.5%) in normal saline, in right hind paw of mice. One group was kept as the control, the second group received 250 mg/kg bodyweight and the third group received 500 mg/kg b.wt of the aqueous extract orally 1 hour prior to the subplantar injection of carrageenan. The paw thickness was measured using vernier calipers before and 1 hour after carrageenan injection. Readings were taken every half hour for eight hours.

- **Dextran induced Paw Oedema in mice**: Experiment was same as described above, except that single dose of 0.02 ml of 1% Dextran in 0.1% Carboxy Methyl Cellulose (CMC). Carboxy Methyl Cellulose was used as the inducer of inflammation. The paw thickness was measured using vernier calipers 1 hour before and after carrageenan injection. Readings were taken every half hour, for eight hours.

- **Formalin Induced Paw Oedema in mice**: Experiment was conducted as same as described above, except that a single dose of 0.02 ml of Formalin (2%) was used as the inducer of inflammation. The extract was administered orally, once daily for six consecutive days. Initial readings were taken on the first day prior to the Formalin administration. Day 1 readings were taken 1 hour post formalin administration. Readings were taken for seven consecutive days.

- **Statistical Analysis:** Values were presented as mean and standard deviation. Repeated measures for ANOVA were done. Analysis was done separately for each group (control, 250mg, and 500mg). The software package used is SPSS (Statistical package for social sciences, Version 13). Level of significance - <0.05.

RESULTS:

- **Effect of arecanut extract on the inhibition of superoxide radical production**: Aqueous extract of arecanut was found to scavenge the super oxides, generated by riboflavin photo-reduction method in a concentration dependant manner. The concentration needed for 50 % scavenging of superoxide was 19.5µg/ml (**Table 1**).

- **Effect of arecanut extract on the inhibition of Hydroxyl Radicals**: Degradation of deoxyribose by hydroxyl radicals generated by Fe^{3+}/ascorbate/EDTA/H2O2 system was found to be inhibited by arecanut extract and the concentration of the extract needed for 50 % inhibition was found to be 161µg/ml (Table 1).

- **Effect of arecanut extract inhibition of Lipid Peroxidation:** Lipid peroxide induced by Fe^{3+} /ascorbate in rat liver homogenate was found to be inhibited and the concentration needed for 50% inhibition was 28µg/ml (Table 1). Graph is depicted on **Fig. 1D**.

- **Effect of arecanut extract on DPPH Radical Scavenging Activity:** The aqueous extract of arecanut was found to scavenge the DPPH radicals generated. The concentration needed for 50% scavenging of the radicals was 95µg/ml (Table 1). Graph is depicted in **Fig. 1A**.

- **Effect of arecanut extract on ABTS Radical Scavenging Activity**: Arecanut extract showed a concentration dependant increase in ABTS radical scavenging Activity. The concentration

needed for 50 % inhibition of ABTS radicals was 9.5µg/ml. These *in-vitro* studies indicate that arecanut extract could scavenge a variety of oxygen radical's in vitro concentrations comparable to that produced by natural antioxidants. Graph is depicted in **Fig. 1C**.

- Effect of arecanut extract on Ferric Reducing Antioxidant Power: Arecanut extract showed a concentration dependant increase in reducing ferric to ferrous. The concentration is shown to be 0.247µg/ml. Graph is depicted in Fig. 1B.

- **Effect of arecanut extract on inflammation of paw oedema:** The anti-inflammatory studies showed dose dependant reduction in paw oedema, depicted in **Fig. 1A, 1B, 1C**.

TABLE 1: FREE RADICAL SCAVENGING ACTIVITY OF *ARECA CATECHU* EXTRACT AND COMPARISON WITH GINGER EXTRACT

Concentration Needed For 50% Inhibition		
	Areca catechu (µg/ml)	Ginger extract (µg/ml)
Superoxide Radical Scavenging	19	22
Hydroxyl Radical	55	150
Inhibition of Lipid Peroxidation	71.875	30
DPPH Radical Scavenging	0.4	7
FRAP Assay	0.247	-
ABTS radical	9.5	2.75

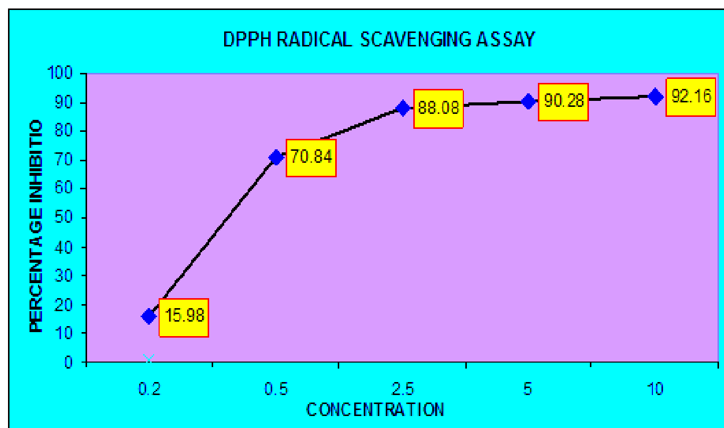

FIGURE 1(A): DPPH RADICAL SCAVENGING ASSAY

CONCENTRATION (µg/ml)	% INHIBITION
0.2	15.98
0.5	70.84
2.5	88.08
5	90.28
10	92.16

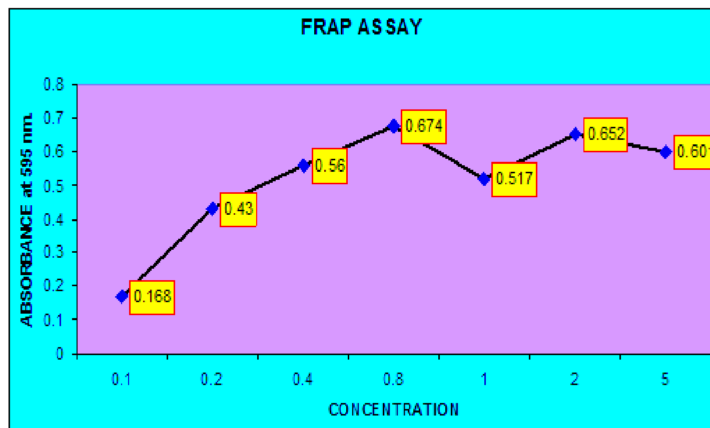

FIGURE 1(B): FRAP ASSAY

CONCENTRATION (µg/ml)	ABS at 595 nm
0.1	0.168
0.2	0.43
0.4	0.56
0.8	0.674
1	0.517
2	0.652
5	0.601

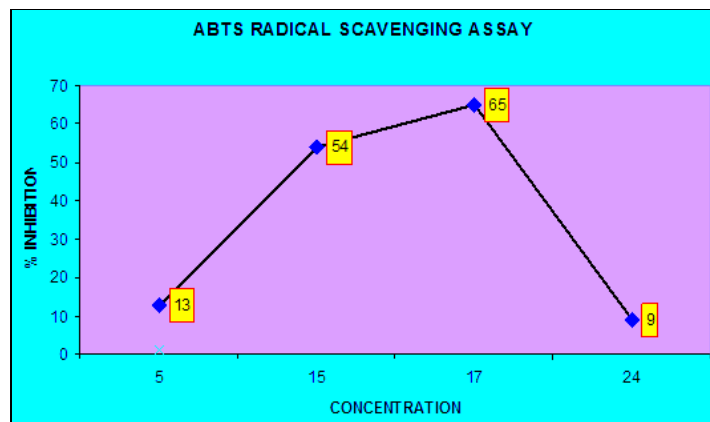

FIGURE 1(C): ABTS RADICAL SCAVENGING ASSAY

CONCENTRATION (µg/ml)	% INHIBITION
5	13
15	54
17	65
24	9

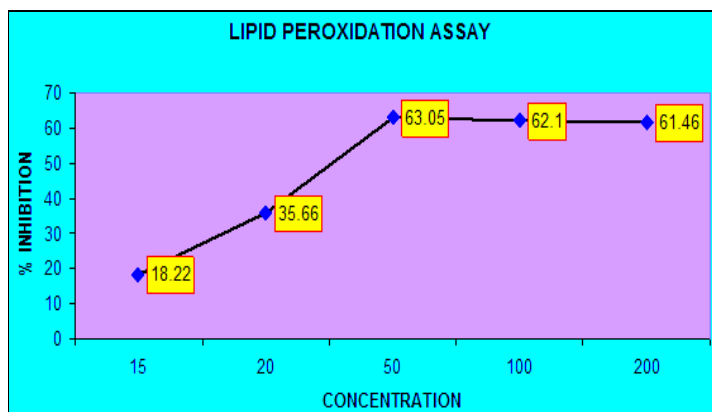

FIGURE 1(D): LIPID PEROXIDATION ASSAY

CONCENTRATION (µg/ml)	% INHIBITION
15	18.22
20	35.66
50	63.05
100	62.1
200	61.46

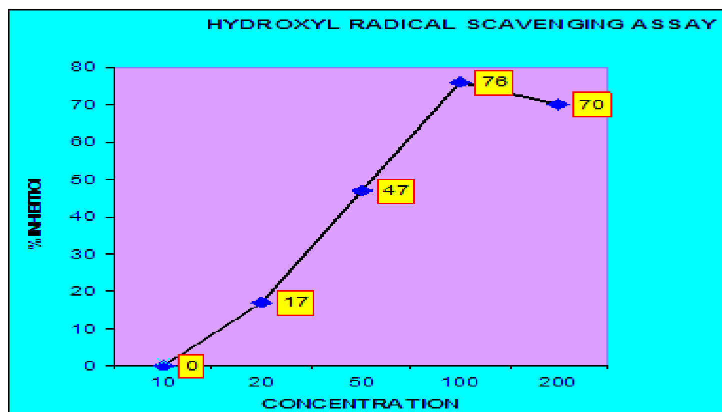

FIGURE 1(E): HYDROXYL RADICAL SCAVENGING ASSAY

CONCENTRATION (µg/ml)	% INHIBITION
5	12.65
20	26.2
50	44.84
100	77.14
200	95.08

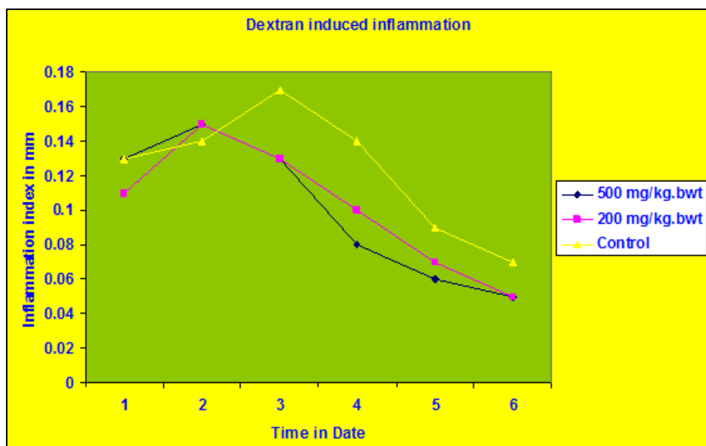

FIG. 2A

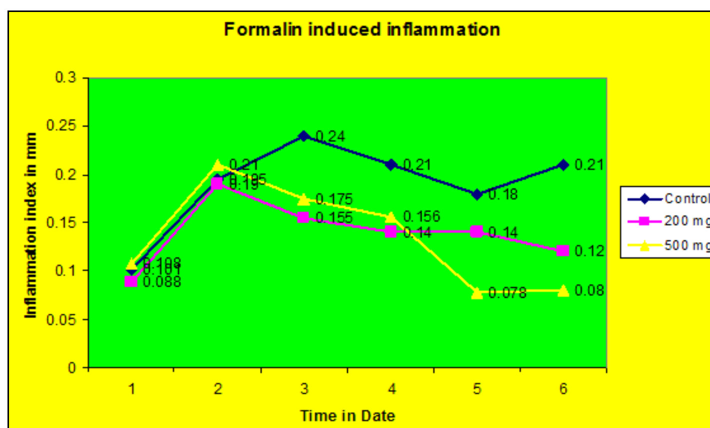

FIG. 2B

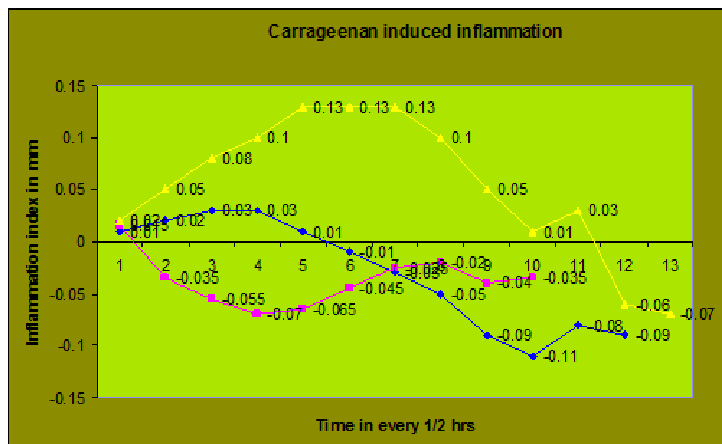

FIG. 2C

DISCUSSION: The antioxidant studies, in vitro showed that arecanut extract does not produce any oxidative stress and has significant antioxidant potential and could scavenge the free radicals effectively *in vitro*. These results are sufficient to validate the use of boiled dried aqueous arecanut extract, to scavenge free radicals, thus promoting and supplementing anti cancer therapy.

In-vitro studies, that were done to scavenge superoxide radicals, hydroxyl radicals, stable free radical, DPPH and ABTS showed 50% inhibition at 19.5µg/ml, 161µg/ml, 95µg/ml and 9.5µg/ml. Lipid peroxidation, induced in the in vitro system by Fe^{2+} ascorbate system in the rat liver homogenate in the presence and absence of extracts, showed the ability of arecanut extract to scavenge 50% free radicals at a concentration of 28µg/ml.

The ferric ion , reducing ability of the extract was seen and concentration for 50 % inhibition is 0.247µg/ml, as seen in FRAP assay. There are earlier reports that Arecanut extract and arecoline, reduced the extracellular release of superoxide anion in PMN (Poly morphonuclear leukocytes), the effects of arecanut extract and arecoline on intracellular levels of PMN showed, couple of interesting yet discrepant results.

In the presence of CB/ FMLP (Cytochalasin B and f Met-Leu-Phe), treatment of Human polymorphonuclear leukocytes, the intracellular levels of ROS in arecanut extract treated PMN decreased, when compared with HBSS treated PMN (Hank's Balanced Salt Solution) [18]. Ginger is used as a control, as it effectively boost the effectiveness of a network of antioxidants vitamin C, vitamin E, Glutathione, lipoic acid and coenzyme Q-10.

Arecanut extract was effective in reducing the inflammatory oedema, induced by all the three oedematogenic agents.

Anti-inflammatory activity of the extract was in a dose dependant manner. The extract at a concentration of 500 mg/kg b.wt showed significant inhibition of oedema, induced by the chronic inflammatory agent (formalin). This review will add on to the existing research generated, into the path physiology of inflammation, enabling various aspects of the inflammation response to be targeted. Further investigations using various other animal models should be pursued, followed by clinical trials and thereby present recommendations for management of inflammation.

CONCLUSIONS: The antioxidant studies, *in-vitro* showed positive results. These results, are sufficient by itself, to validate the use of boiled dried aqueous Arecanut extract, to scavenge free radicals, thus promoting and supplementing anticancer therapy. Invitro studies, that were done to scavenge superoxide radicals , hydroxyl radicals, stable free radical DPPH and ABTS showed 50 % inhibition at 19.5µg/ml, 161µg/ml, 95 µg/ml and 9.5µg/ml. Lipid peroxidation, induced in the *in-vitro* system by Fe^{3+} ascorbate system in the rat liver homogenate in the presence and absence of extracts, showed the ability of Arecanut extract to scavenge 50% free radicals at a concentration of 28µg/ml.

The ferric ion reducing ability of the extract was seen and the concentration for 50% inhibition is 0.247µg/ml, seen in FRAP assay. The anti inflammatory studies show dose dependent reduction in paw oedema, at 200 mg and 500 mg per kg body weight.

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