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Research Article

In-vitro and *In-silico* Studies on the Anti-inflammatory Properties of Arecoline from *Areca catechu* L. Nut

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Abstract

The development of anti-inflammatory medications that target NF-κB has received a lot of attention because NF-κB activation is also strongly linked to inflammatory illnesses. Therefore, the goal of the current study is to examine the anti-inflammatory properties of *Areca catechu* L. nut extract. The stability of the red blood cell membrane, *in silico* analysis, and *in vitro* denaturation of albumin were all investigated. An anti-inflammatory impact on albumin denaturation inhibition and HRBC membrane stability at 1000 g/mL, comparatively low IC_{50} value than normal, was seen in preliminary investigations utilising a single dose (10 mg/ml) of *Areca catechu* L. nut and its corresponding component arecoline. Arecoline's additional affinity for nuclear kappa factor B. These findings support the medical use of *Areca catechu* L. nut in inflammatory illnesses by demonstrating its anti-inflammatory.

Keywords: *In-vitro; In-silico*; Anti-inflammatory; Arecoline; NF-κB; Albumin Denaturation Inhibition; HRBC

Introduction

One of the Palmaceae family's well-known fruit plants is *Areca catechu* [1]. The pharmacological qualities of the *Areca catechu* L. nut, especially its anti-inflammatory benefits, have been widely researched. The primary components of *Areca catechu* L. nut include polyphenols, fat polysaccharides, fibre, and protein. In addition to these, nuts also contain arecoline (0.01-0.7%) and other alkaloids, such as arecadine, guvacoline, and guvacine, in trace levels. There are only trace levels of catechin, leucopelargonidin, and leucocyanidin in the mature *Areca catechu* L. nut polyphenols, which are primarily made up of polymers like leucocyanidins. Inflammation is a significant response to injury, illness, or destruction and is manifested by heat, redness, discomfort, swelling, and abnormal physiological processes [2]. Acute inflammation and chronic inflammation are two different types of inflammation [3]. The body's initial reaction to harmful stimuli is acute inflammation, which is brought on by an increase in the flow of plasma and leukocytes from the blood into the wounded tissues. Existing cells in the tissues start the process of acute inflammation. Induced by the effects of the numerous inflammatory mediators, this is characterised by substantial vascular alterations, including vasodilatation and enhanced capillary permeability [4]. Chronic inflammation is a protracted inflammatory response that causes a progressive change in the types of cells at the site of inflammation and is characterised by the simultaneous destruction and healing of the tissues affected by the inflammatory process [5].

When microorganism infections begin, the immune system is aroused and tries to stop inflammatory reactions brought on by disrupted cellular activities [6]. Living tissues respond to injury by inflaming themselves, and this process involves both systemic and local responses [7]. The complex process of inflammation is controlled by transcription factors, cytokines that

promote inflammation, adhesion enzymes, and other mediators [6,8]. A significant component of bacterial cell walls called lipopolysaccharide (LPS) stimulates immune cells and causes the release of inflammatory mediators like nitric oxide (NO) [9]. As a result, herbal remedies may reduce inflammation by inhibiting mediators including NF-B, iNOS, and COX-2. NF-κB is an inducible transcription factor. After its activation, it can activate transcription of various genes and thereby regulate inflammation. Antioxidants are likely to inhibit NF-κB by scavenging reactive oxygen intermediates involved in the NF-κB pathway. The nuclear factor-kappa B (NF-B) transcription factor, which is activated and translocates to the nucleus in response to inflammatory stimuli, controls the activity of these enzymes [10]. Both chronic and acute inflammation models using LPS-stimulated RAW 264.7 cells and carrageenan-induced inflammation are frequently used [11]. There is proof that the immunological response is what causes iNOS and COX-2 to be produced [12].

Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for the management of inflammatory conditions such as rheumatoid arthritis and other infectious diseases. The perceived efficacy, low incidence of serious side effects or relative safety, compared to the synthetic alternatives, as well as the affordability of plant-derived drugs make this search worthwhile. In addition, the ethnopharmacological uses of many medicinal plants extensively as crude extracts or as pure compounds, have generated considerable interest as it relates to the treatment of various medical conditions including chronic inflammatory diseases. It is widely acknowledged that medicinal plants are an important source of novel chemicals with potential therapeutic benefits. Due to their low cost and few side effects, the usage of natural goods and herbal medicines has increased recently [13]. Numerous organic plant components have been utilised for ages in Ayurvedic medicine to block inflammatory pathways with few negative effects [14]. With more than 80 % of the world's population currently relying on plant-derived medicines for their primary healthcare needs, screening of these plants for potential anti-inflammatory compounds could be a step toward the discovery of safer and more effective compounds [15-18].

The main action of anti-inflammatory agents is the inhibition of cyclooxegenase enzymes which are responsible for the conversion of arachidonic acid to prostaglandins. Due to the substrates' inability to connect to the active site, enzyme activity is lost [19]. The erythrocyte membrane is similar to the lysosomal membrane, and its stabilisation means that the extract may as well stabilise lysosomal membranes [20]. Since human red blood cell (HRBC) membranes are similar to these lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis was taken as a measure in estimating the anti-inflammatory property of extract of *Areca catechu* L. nut. Thus, Human red blood cell membrane stabilization (HRBC method) [21] has been used as a method in estimating the anti-inflammatory property.

Materials and Methods

Chemicals

Arecoline, dichlofenac, toluene, methanol, ethanol and alsevers solution are purchased from Eswarr Scientific and Co., Karumandapam, Trichy-620 001.

Collection and authentification of plant materials

Healthy un ripened *Areca catechu* L. nuts were collected from Kollam district of Kerala, India. It was dehusked and dried for three weeks. The dried seeds were powdered. The plant *Areca catechu* and *Areca catechu* L. nut were authenticated by JNTBGRI, Thiruvananthapuram, Pin 695 562, Kerala, India and voucher specimens (Specimen Numbers TBGT/95955 and TBGT/95956) are deposited at the herbaria of the same research institute.

Methodology

Areca catechu L. nut extraction

200 g of clean, fresh *Areca catechu* L. nut was pressed at 800 rpm and clarified juice was obtained. Edible ethanol was added to the clarified juice till the final concentration of the ethanol was 75%. After overnight the supernatant juice solution was collected and subjected to low pressure to remove ethanol. The concentrated juice solution is passed through a solid phase column. Stationary phase contacting *Areca catechu* L. nut residues were washed by 3 to 10 times of its weight with water and cellulose for biolysis until the final concentration of cellulose after mixing reach 0.01%-0.8% and centrifuged at 6000 rpm to obtain residual extract. The juice extract is combined with residue extract and dehydrated by low temperature decompression and concentrated to obtain a solid mater which is pulverized by 30 to 120 meshes.

Albumin denaturation inhibition

The *Areca catechu* L. nut extract (crude and arecoline) and positive standards (dichlofenac) were prepared at a concentration of 0.1% each (1.0 mg/ml). A reaction vessel for each mixture was prepared consisted of 200 µl of egg albumin, 1400 µl of phosphate buffered saline, and 1000 µl of the test extract at different concentration. Distilled water instead of extracts was used as a negative control. Afterward, the mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. After cooling, their absorbances were measured at 660 nm and the data were processed by Spectra Manager system. The inhibition percentage of protein denaturation was calculated using the formula:

% Denaturation inhibition = (1−D/C)×100%

Where D is the absorbance reading of the test sample, and C is the absorbance reading without test sample (negative control).

HRBC anti inflammatory

Blood was collected from healthy volunteers and was mixed with equal volume of sterilized alsevers solution. This blood solution was centrifuged at 3000 rpm and the packed cells were separated. The packed cells were washed with isosaline solution and a 10% (v/v) suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property. Different concentrations of extract, reference sample and control were separately mixed with 1mL of phosphate buffer, 2 mL of hyposaline and 0.5 mL of HRBC suspension. All the assay mixtures were incubated at 37° C for 30 minutes and centrifuged at 3000 rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a spectrophotometer at 560 nm. The percentage of hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

Percentage protection= 100- (OD sample/ OD control) × 100%

Molecular docking

The three dimensional structure of the nuclear factor kappa-b (NF-κB) p50 homodimer (PDB 1NKF) were obtained from the Research Collaborator for Structural Bioinformatics (RCSB) Protein data bank (www.rcsb.org) respectively. A chain of target was pre-processed separately by deleting other chains B. Using Pymol software, water molecules and ligands already present in the proteins were removed; hydrogen atoms were added and saved in PDB format.

Prediction of active site

Prediction of the active site is important in structure-based drug design. Co-ordinates of binding sites of the proteins were identified using the software UCSF chimera Docking. Molecular docking calculations were carried out with the aid of the software AutoDock 4.2 and binding energy of the protein—Schiff base adducts were obtained.

Results and Discussion

Anti-inflammatory activity of the *Areca catechu* L. nut extract was evaluated against denaturation of egg albumin method. Table 1 represents the percentage of albumin Denaturation of crude extract, arecoline and dichlofenac. The arecoline had showed the greatest inhibition capacity with 80.58±00.004%, followed by crude extract 66.52±0.02%. Meanwhile the anti-inflammatory activity of standard dichlofenac reference drugs showed higher inhibition capacity of 91.58±0.004%. NSAIDs prevent inflammation by blocking the cyclooxygenase, protease, enzyme activity. However, these drugs cause side effects of ulceration, hemorrhage, perforation and obstruction [22]. Denaturation of protein causes the production of autoantigens in conditions such as rheumatic arthritis, cancer and diabetes which are conditions of inflammation. Hence, by inhibition of protein denaturation, inflammatory activity can be inhibited [23]. The value of IC_{50} of extract is 618 µg, arecoline was 728 µg and standard was 3021 µg (Figure 1). The HRBC (human red blood cell) membrane is similar to lysosomal membrane, the study was undertaken to check the stability of HRBC membrane by the extracts to predict the anti-inflammatory activity *in vitro*. Aqueous extract of *Areca catechu* L. nut significantly and dose dependently inhibit HRBC haemolysis. The percentage of 94≥90≥89≥87≥87% respectively among 5, 25, 50, 100 and 200 µg/mL. Aspirin was taken as standard drug for the comparison and found 98% membrane stabilization under hypotonic condition (Table 2).

In silico docking simulation revealed that arecoline possessed good binding poses and favourable protein-ligand interactions with

Concentration (µg/mL)	Crude Extract	Arecoline	Dichlofenac
250	34	26	22
500	41	32	38
750	58	54	46
1000	64	70	60
IC_{50} (µg)	618	728	3021

Table 1: Percentage of anti-inflammatory among *Areca catechu* L. nut extract and standard arecoline.

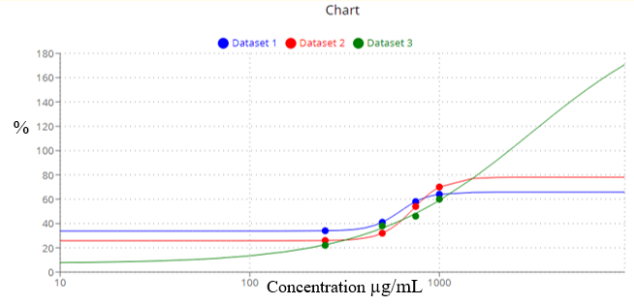

Figure 1: IC_{50} value of Areca catechu L. nut extract and standard arecoline.

Concentration (µg/mL)	% of HRBC lysis	Stabilization
5	6	94
25	10	90
50	11	89
100	13	87
200	13	87
Aspirin 200	2	98

Table 2: Percentage of HRBC membrane stabilization.

nuclear kappa factor and the data is given in Table 3. The O1, O2, C1 and C8 atoms of ligand interacted and produced hydrogen bond in both A and B chain residues and TYR 207 shows Hydrophobic interaction with Ligand. LEU, GLU, SER, ARG, GLY were found take part in the interaction with ligand (Figure 2). From the docking simulations, the promising pose with higher binding energy, ligand efficiency and intermolecular H-bonds was retained for detailed intermolecular interaction analysis. The predicted binding free energy of NF-κB1-genistein complex was found to be -6.8 kcal/mol (Table 3). Standard dichlofenac shows Hydrogen bond with ILE and the affinity was -5.4 (Table 4) along with some Hydrophobic and Ionic interaction (Figure 3). The nuclear factor NF-κB pathway has long been considered a prototypical proinflammatory signaling pathway, largely based on the role of NF-κB in the expression of proinflammatory genes. It was previously reported that arecoline have act as Inhibitors for Lung A549 and Leukemia K562 Cell Lines receptor [24].

Interaction	Amino acid	Affinity (kcal/mol)
Hydrogen bond	GLU	-6.8
	ARG	-5.2
	GLY	-5.1
	Ser	-5.8
Hydrophobic	TYR	-5.2

Table 3: Binding affinity of Arecoline with nuclear Kappa factor B.

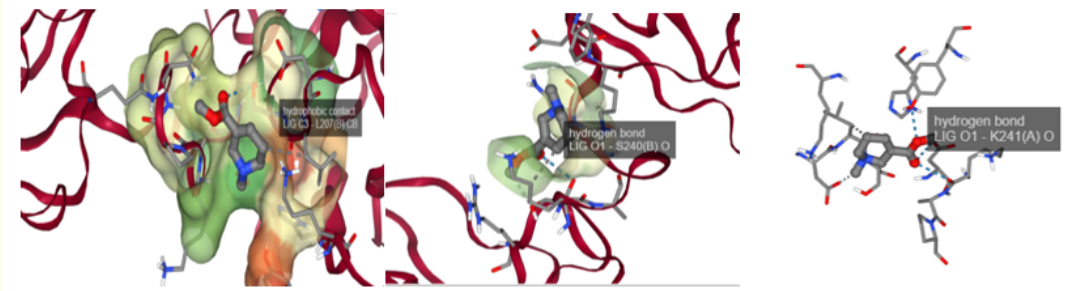

Figure 2: Formation of Hydrogen and hydrophobic bond between Arecolin with kappa factor B.

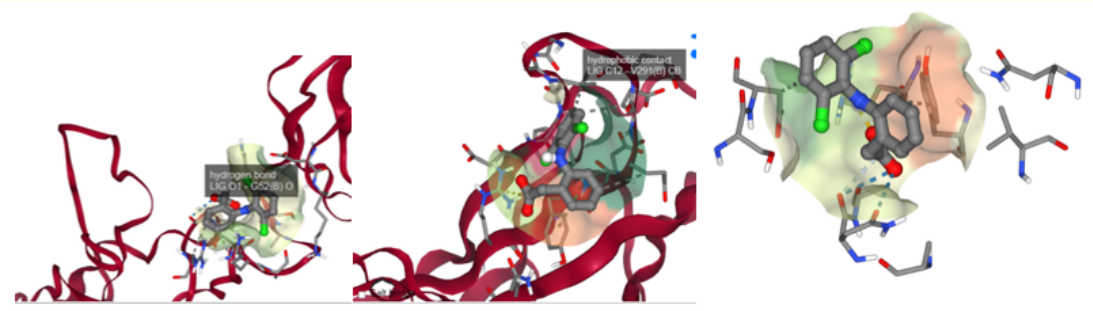

Figure 3: Formation of Hydrogen and hydrophobic bond between dichlofenac with kappa factor.

Interaction	Amino acid	Affinity (kcal/mol)
Hydrophobic	GLY	-5.2
	LEU	-5.2
Ionic	LEU	-4.7
Hydrogen	ILE	-5.4

Table 4: Binding affinity of dichlofenac with nuclear Kappa factor B.

Conclusion

In preliminary studies using a single dose (10 mg/ml) of *Areca catechu* L. nut and its related component arecoline, an anti-inflammatory effect on albumin denaturation inhibition and HRBC membrane stability at 1000 g/mL, comparatively low IC_{50} value than usual, was observed increased affinity of arecoline for nuclear kappa factor B. From this we can conclude that the anti-inflammatory characteristics of *Areca catechu* L. nut, which is a seed used medically to treat inflammatory diseases. Since NF-κB activation is closely related to inflammatory diseases, there has been a lot of interest in the development of anti-inflammatory drugs that target NF-κB. This leads to a wide area of research in the field of different types of inflammatory illness by *in-vivo* studies.

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