**Studies on the anti-inflammatory properties of arecoline from**

**areca catechu L. nuts in vitro and in silico**

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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 IC50 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 [8,9]. A significant component of bacterial cell walls called lipopolysaccharide (LPS) stimulates immune cells and causes the release of inflammatory mediators like nitric oxide (NO) [10]. 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 [11]. Both chronic and acute inflammation models using LPS-stimulated RAW 264.7 cells and carrageenan-induced inflammation are frequently used [12]. There is proof that the immunological response is what causes iNOS and COX-2 to be produced [13].

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 [14]. Numerous organic plant components have been utilised for ages in Ayurvedic medicine to block inflammatory pathways with few negative effects [15]. 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 [16-19].

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 [20]. The erythrocyte membrane is similar to the lysosomal membrane, and its stabilisation means that the extract may as well stabilise lysosomal membranes [21]. 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) [[22]](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3609167/#b2) 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 & 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.

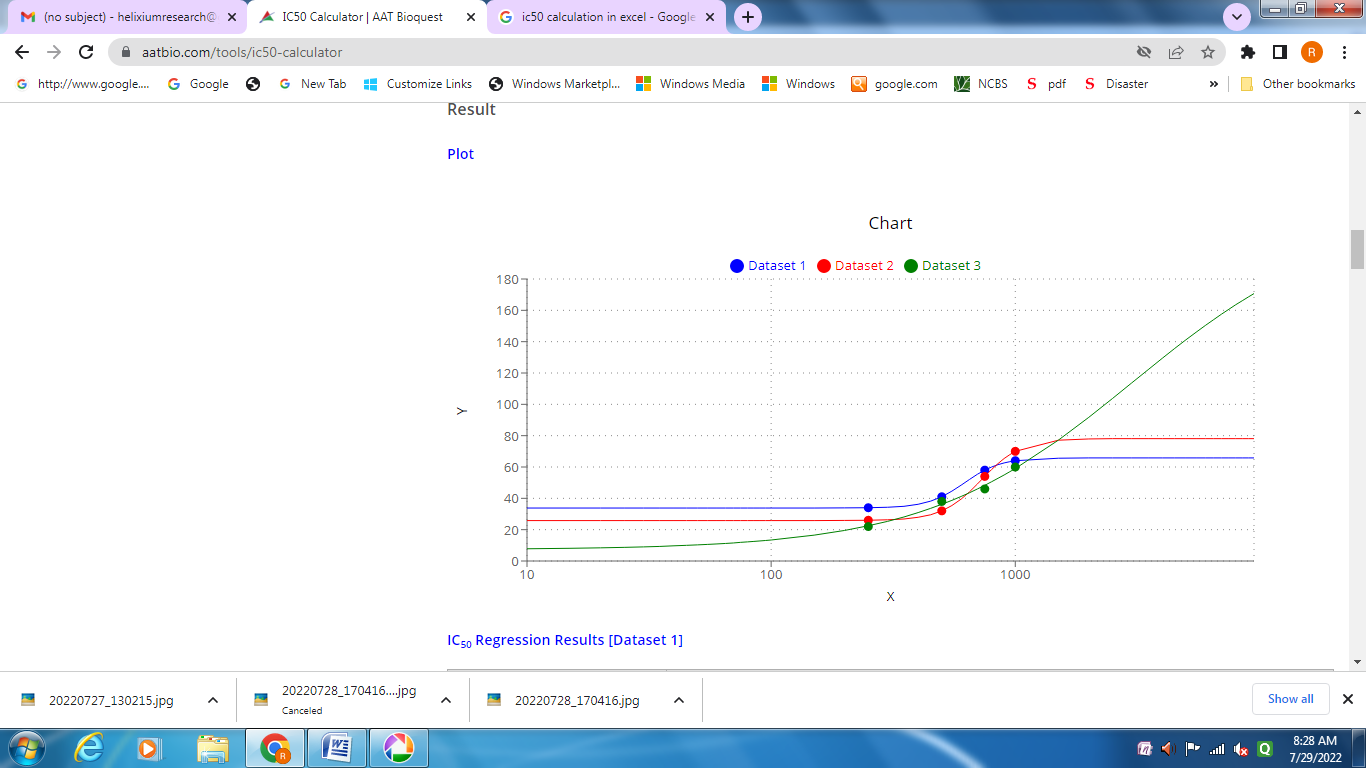
**RESULTS AND DISCUSSION**

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 [23]. 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 [24] .The value of IC50 of extract is 618 µg, arecoline was 728 µgand 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 favorable protein-ligand interactions with 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 [25].

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

|  |  |  |  |
| --- | --- | --- | --- |
| **Concentration (µg/mL)** | **Crude Extract** | **Arecoline** | **Dichlofenac** |
| 250 | 34 | 26 | 22 |
| 500 | 41 | 32 | 38 |
| 750 | 58 | 54 | 46 |
| 1000 | 64 | 70 | 60 |
| IC50 (µg) | 618 | 728 | 3021 |



%

Concentration µg/mL

**Figure 1. IC50 value of Areca catechu L. nut extract and standard arecoline**

**Table 2- Percentage of HRBC membrane stabilization**

|  |  |  |
| --- | --- | --- |
| **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 3- Binding affinity of Arecoline with nuclear Kappa factor B**

|  |  |  |
| --- | --- | --- |
| **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 4-Binding affinity of dichlofenac with nuclear Kappa factor B**

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

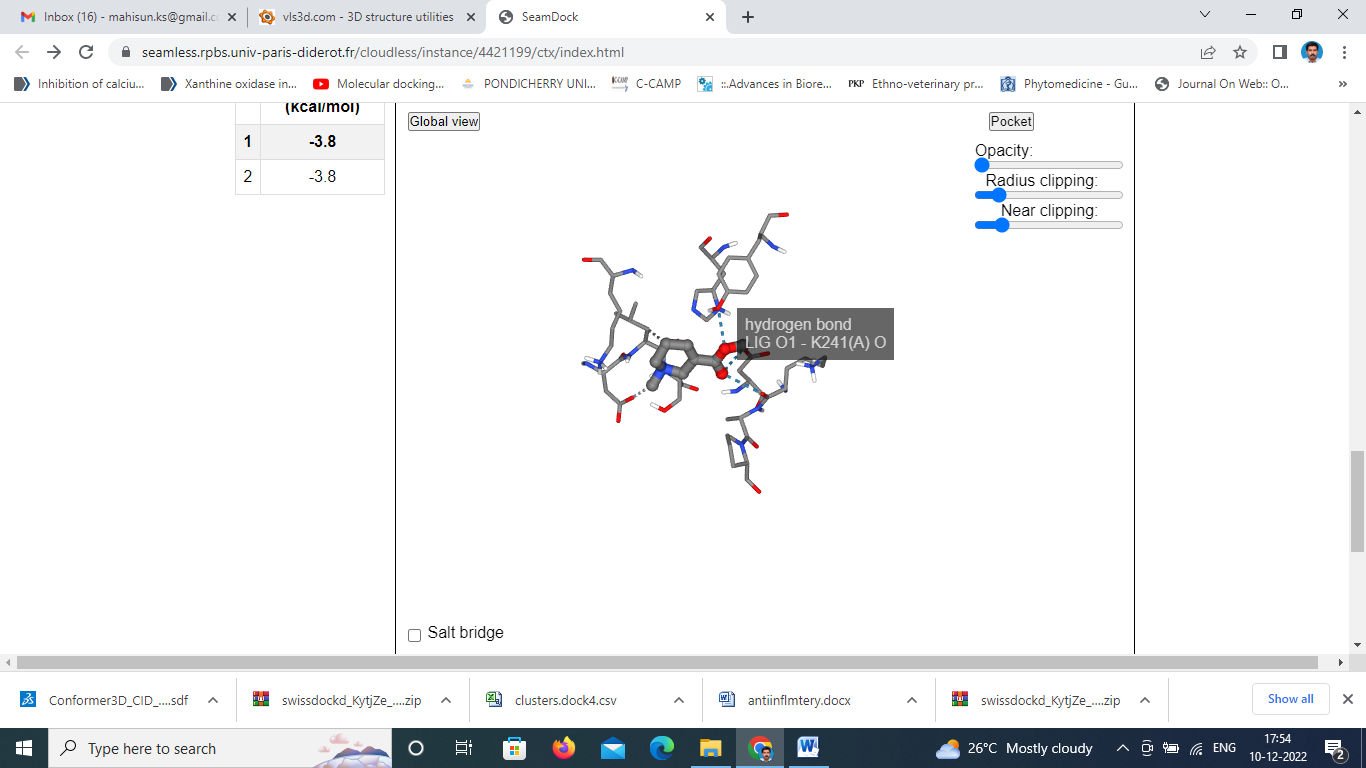
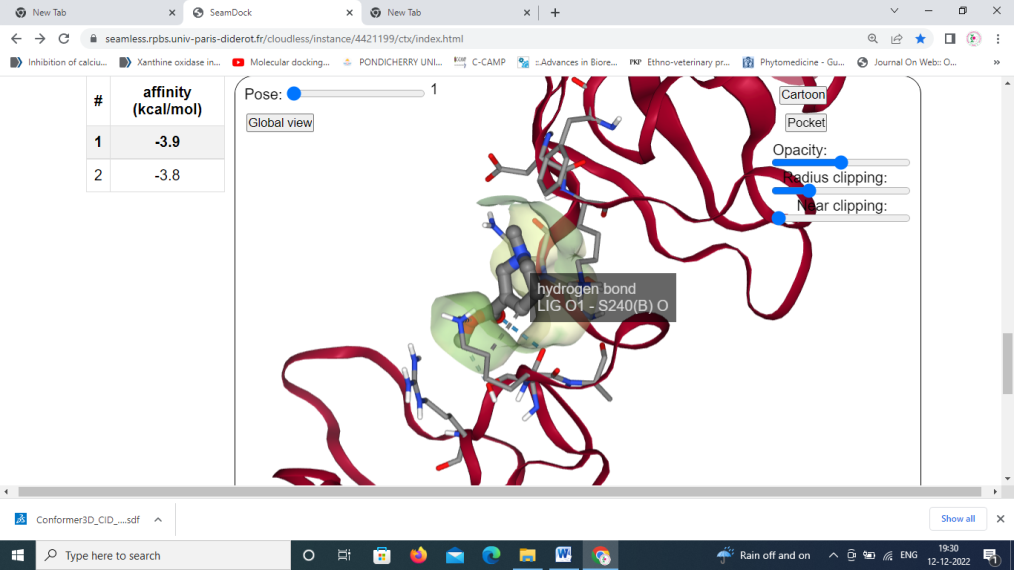
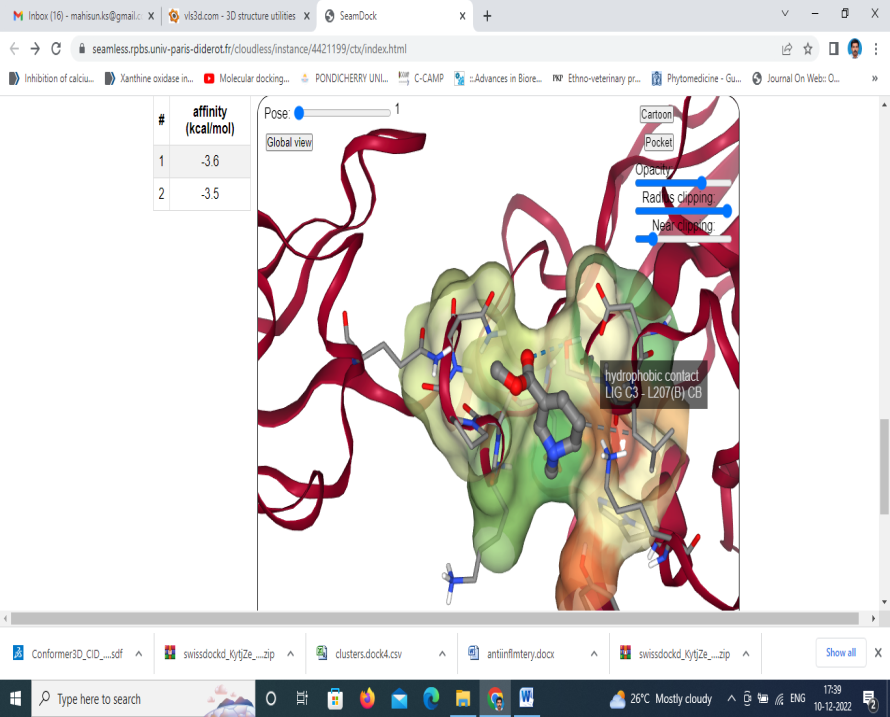


Figure 2.Formation of Hydrogen and hydrophobic bond between Arecolin with kappa factor B

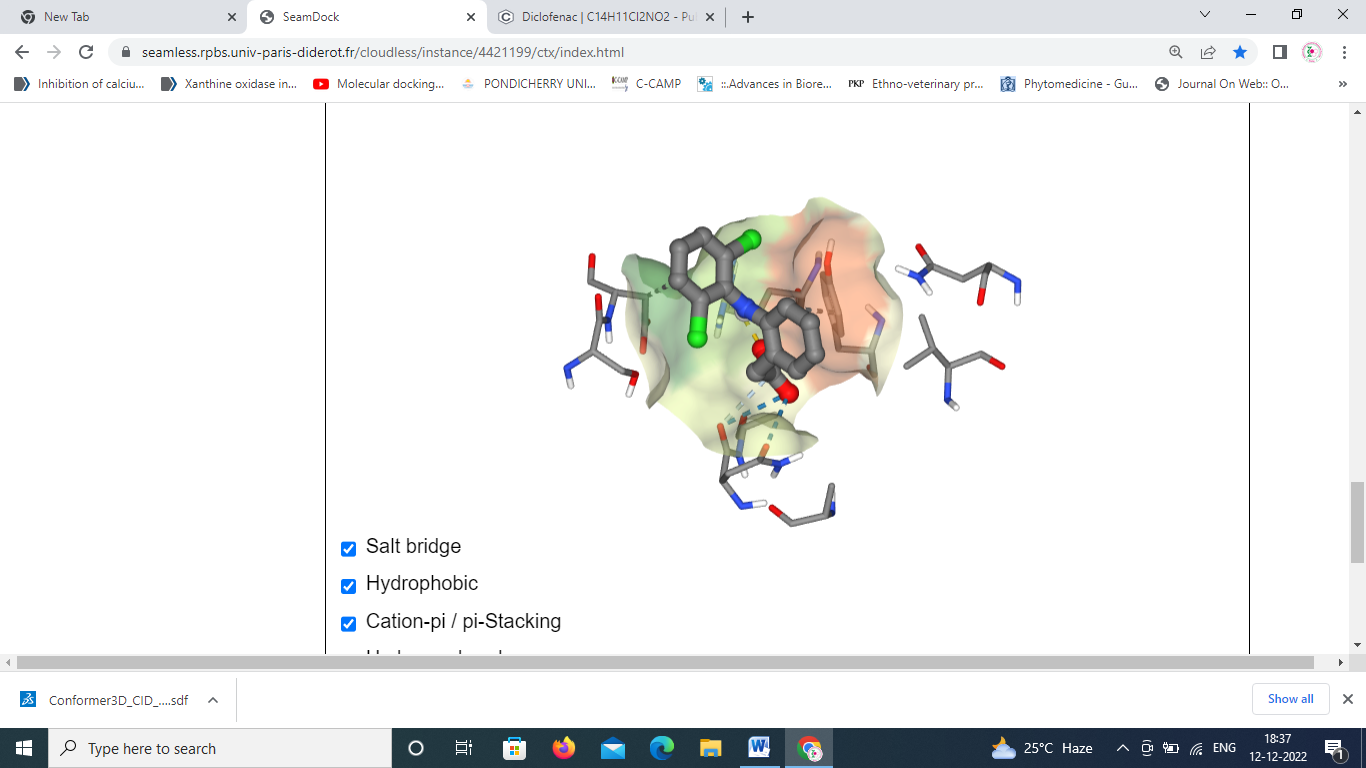
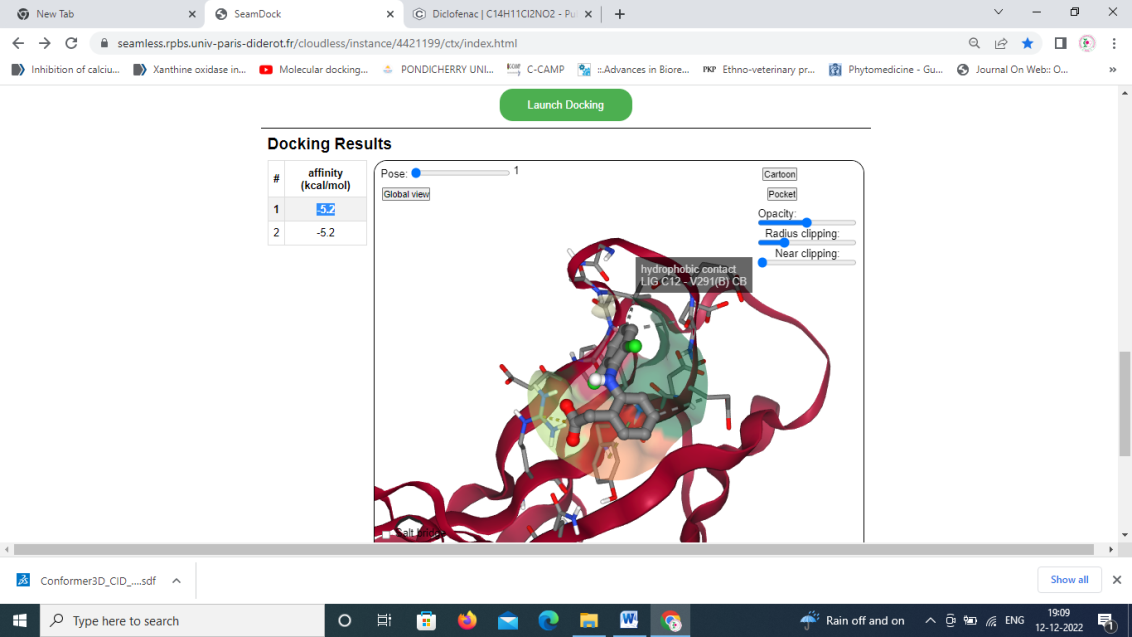
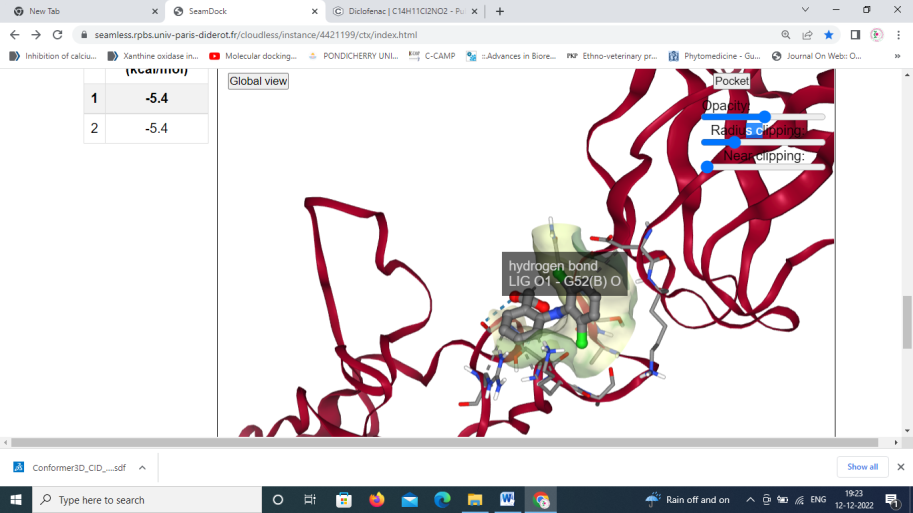


Figure 3.Formation of Hydrogen and hydrophobic bond between dichlofenac with kappa factor

**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 IC50 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.

**REFERENCE**

1. Khan S, Mehmood MH, Ali AN, Ahmed FS, Dar A, Gilani AH. Studies on anti-inflammatory and analgesic activities of betel nut in rodents.J Ethnopharmacol. 2011;135:654–661.
2. Dharmadeva S, Galgamuwa L S, Prasadinie C, Kumarasinghe N. *In vitro* anti-inflammatory activity of *Ficus racemosa* L. bark using albumin denaturation method. J Ayu 2018; 39(4), 239-242.
3. Ferrero-Miliani L, Nielson OH, Andersen PS, Girardin SE. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. *Clin Exp Immunol.*2007;147(2):227–235.
4. Okoli CO, Akah PA, Nwafor SV, Anisiobi AI, Ibegbunam IN, Erojikwe O. Anti-inflammatory activity of hexane leaf extract of Aspilia africana C.D. Adams. *J Ethnopharmacol.*2007;109(2):219–22.
5. Eming SA, Krieg J, Davidson JM. Inflammation in wound repair. Molecular and cellular mechanisms. *J Invest Dermatol.*2007;127(3):514–525.
6. Broz P, Monack DM. Molecular mechanisms of inflammasome activation during microbial infections. *Immunol Rev.*2011;243:174–190.
7. Hardmann Joel A, Limbard Lee E, Goodmann Alfred. *Pharmacological basis of therapeutics.*1998:1465
8. Broz P, Monack DM. Molecular mechanisms of inflammasome activation during microbial infections. Immunol Rev. 2011;243:174–190.
9. Walsh LJ. Mast cells and oral inflammation.Crit Rev Oral Biol Med. 2003;14:188–198.
10. Clària J, González-Périz A, López-Vicario C, Rius B, Titos E. New insights into the role of macrophages in adipose tissue inflammation and Fatty liver disease: modulation by endogenous omega-3 Fatty Acid-derived lipid mediators. Front Immunol. 2011;2:49.
11. Park CM, Song YS. Luteolin and luteolin-7-O-glucoside inhibit lipopolysaccharide-induced inflammatory responses through modulation of NF-κB/AP-1/PI3K-Akt signaling cascades in RAW 264.7 cells. *Nutr Res Pract.*2013;7:423–429.
12. Halici Z, Dengiz GO, Odabasoglu F, Suleyman H, Cadirci E, Halici M. Amiodarone has anti-inflammatory and anti-oxidative properties: an experimental study in rats with carrageenan-induced paw edema. *Eur J Pharmacol.*2007;566:215–221.
13. Zamora R, Vodovotz Y, Billiar TR. Inducible nitric oxide synthase and inflammatory diseases. *Mol Med.*2000;6:347–373.
14. Nostro A, Germanò MP, D'angelo V, Marino A, Cannatelli MA. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Lett Appl Microbiol.*2000;30:379–84
15. Maroon JC, Bost JW, Maroon A. Natural anti-inflammatory agents for pain relief. *Surg Neurol Int.*2010;1:80
16. Chatterjee, P, Chandra, S, Dev, P, Bhattacharya, S. Evaluation of anti-inflammatory effects of green tea and black tea: A comparative in vitro study. J. Adv. Pharm. Technol. Res., 3(2), 2012, 136-138.
17. Tatti, PN, Anitha, S, Shashidhara, S, Deepak, M, Bidari, S. Evaluation of in-vitro anti-denaturation activity of isolated compound of Buteamonosperma Bark. Pharma Sci. Monitor, 3(4), 2012, 2314-2320.
18. Dar, SA, Yousuf, AR, Ganai, FA, Sharma, P, Kumar, N, Singh, R. Bioassay guided isolation and identification of antiinflammatory and anti-microbial compounds from Urtadioica L. (Urticaceae) leaves. Afr. J. Biotechnol., 11(65), 2012, 12910-12920.
19. Khuda, F, Iqbal, Z, Khan, A, Zakiullah, Shah, Y, Ahmad, L, Nasir, F, Hassan, M, Ismail, Shah, WA. Evaluation of antiinflammatory activity of selected medicinal plants of Khyber Pakhtunkhwa, Pakistan. Pak. J. Pharm. Sci., 27(2), 2014, 365-368.
20. Handa SS, Khanuja SP, Longo G, Rakesh DD. *Technologies for Medicinal and Aromatic Plants. No. 66.* 1st ed. Italy: United Nations Industrial Development Organization and the International Centre for Science and High Technology; 2008.
21. Shenoy S, Shwetha K, Prabhu K, Maradi R, Bairy KL, Shanbhag T. Evaluation of antiinflammatory activity of Tephrosia purpurea in rats. *Asian Pac J Trop Med.*2010;3(3):193–195
22. Ejebe DE, Siminialayi IM, Emudainowho JOT, Ofesi U, Morka L. Analgesic and anti-inflammatory activities of the ethanol extract of the leaves of Helianthus Annus in Wistar rats. Asian Pac J Trop Med. 2010;3(5):341–347.
23. Sostres C, Gargallo CJ, Arroyo MT, Lanas A. Adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and coxibs) on upper gastrointestinal tract. Best Pract Res ClinGastroenterol. 2010;24:121–32.
24. Tak PP, Firestein GS 2001. NF-κB: A key role in inflammatory diseases. J Clin Invest 107:7–11
25. Ragab A.E, Badawy E.T, Aboukhatwa S.M, Kabbash A, Abo El-Seoud K.A. In Vitro Characterization of Inhibitors for Lung A549 and Leukemia K562 Cell Lines from Fungal Transformation of Arecoline Supported by In Silico Docking to M3-mAChR and ADME Prediction. Pharmaceuticals 2022, 15, 1171.