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Identification of bioactive compounds with anti-inflammatory potential in the methanolic fruit extract of *Areca catechu* L. (Palmaceae)

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The present study was aimed to investigate the anti-inflammatory effect of methanolic fruit extract of *Areca catechu* L. (Arecanut) on lipopolysaccharide (LPS) stimulated RAW 264.7 cells and identification of bioactive components by liquid chromatography-mass spectrometry (LC-MS) analysis. The Arecanut extract showed maximum scavenging potential against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical with half maximal scavenging concentration value of 0.264 ± 0.073 µg/mL compared to BHA with 0.553 ± 0.082 µg/mL. The anti-inflammatory effect was investigated based on interleukin 6 (IL-6) production in LPS stimulated RAW 264.7 cells. The methanol extract of Arecanut showed a significantly higher inhibitory effect on IL-6 production, and it was further analyzed for the characterization of active compounds by LC-MS. LC-MS revealed the presence of 10 active phytoconstituents, including alkaloids, flavonoids, terpenoids, and glycoside. From the results, it is evident that methanol extract of Arecanut has an anti-inflammatory property and contains various phytochemicals and is recommended as a fruit of pharmaceutical importance.

Keywords: Antioxidant, Interleukin-6, Liquid chromatography-mass spectrometry (LC-MS)

Arecanut is the fruit of oriental palm *Areca catechu* L. (Palmaceae family) grown in India, Malaysia, Taiwan, and other Asian countries for their economic needs[1]. Traditionally, Arecanuts are mostly used for mastication and are reported to have lots of medicinal properties[2]. It has antioxidant[3], anti-bacterial[4], anti-fungal[5], anti-diabetic[6], antilipidemic[7], anti-aging[8], anti-migraine[9], anti-depressant[10] and hepatoprotective activities[11]. World Health Organization in 2009 has specified the medicinal properties of Arecanut[12]. It is also reported that the major six alkaloids present in Arecanut are responsible for its drug likeness properties[13]. The anti-inflammatory effect of Arecanut is well established in previous studies[14,15], however, there is no reported evidence is available for its attenuating effect on the production of proinflammatory cytokines by inflammatory cells.

Inflammation is an immune system response triggered by different stimuli, including chemical exposure, infection, tissue damage or other foreign substances[16]. However, excessive or inappropriate inflammatory responses are involved many diseases, for example, ulcerative colitis, Alzhemer's, dermatitis, cardiovascular diseases, cancer, inflammatory bowel disease, osteoarthritis and rheumatoid arthritis[17]. Macrophages are major inflammatory cells play vital roles in inflammation, and produce many kinds of inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, nitric oxide (NO) and prostaglandins[18]. Lipopolysaccharide (LPS), a component of gram negative bacteria cell wall, induces a number of cellular responses that act as strong stimulators of inflammatory responses in macrophages[19]. Thus, the regulation of macrophage activation might have therapeutic potential and is a powerful model for studying inflammation that is mediated by inflammatory cytokines from activated macrophages. The present study evaluates the effect of methanolic extract of Arecanut on the production of proinflammatory cytokine IL-6, an important target in the treatment of many inflammatory diseases, in LPS stimulated macrophages. Besides, the active compounds present in Arecanut was determined by LC-MS technique.

Materials and Methods

Chemicals

RAW 264.7 macrophage cell line was purchased from National center for cell science (NCCS), Pune, India. Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, Fetal Bovine Serum (FBS), Antibiotic solution (Penicillin and

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Streptomycin), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) are obtained from Himedia Ltd, India. Lipopolysaccharide (*Escherichia coli* O111: B4) was bought from Sigma Aldrich, St. Louis, USA. Mouse IL-6 ELISA kit was from Bio-Legend, USA. All the other chemicals were analytical grade and obtained from Merck Ltd, India.

Collection of Plant material and preparation of extract

Mature *Areca catechu* L. fruits were collected from Kodungallor, Thrissur district, Kerala, India, and the botanical identity (Voucher number: 4754) was authenticated by Dr. Jomy Augustine, Head of the department, Postgraduate and research department of botany, St: Thomas College Pala, Kottayam, Kerala, India.

Only healthy fruits without infection or damage were chosen for experimental study. The husks were removed, nuts were sliced and shade dried for 30 days. The Arecanut was powdered and extracted with methanol in a Soxhlet apparatus at a temperature of below 45°C. The solvent from the extract was evaporated in a rotary evaporator at 45°C and lyophilized in a freeze drier at -85°C to dryness. The sample was stored at 4°C until experiments. The percentage yield of the extract was calculated as per the protocol described earlier[20].

$$\text{Yield of Extraction } (\%) = \frac{\text{DWe}}{\text{DWs}} \times 100$$

where DWe is the dry weight of sample extract after evaporation of solvent and DWs is the dry weight of the sample powder.

DPPH radical scavenging assay

The free radical scavenging potential of the methanol extract of Arecanut was carried out by DPPH assay as per the protocol described earlier with modifications[21]. Different concentrations of sample solutions ranging from 0.01-100 µg/mL were mixed 0.5 mM DPPH reagent and incubated in dark for 30 min. The colour change from dark purple to yellow was measured at 517 nm and the antioxidant activity was evaluated in terms of BHA equivalent. The percentage of free radical scavenging was measured by using the equation,

$$\%\text{scavengingactivity} = \frac{\text{The absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

From the percentage scavenging activity, effective concentration (EC_{50}) was calculated by using GraphPad Prism® version 5.03 software[22]. EC_{50} is the concentration of samples/BHA to scavenge 50% of DPPH radical present in the system.

Cell culture and treatments

RAW 264.7 cells were cultured in DMEM medium supplemented with 10% FBS and 0.1% Antibiotic solution and maintained at 37°C, 5% CO_2 in a CO_2 incubator. The dried methanol extract of Arecanut was dissolved in 100% DMSO and filtered through 0.22 µM syringe filter. The stock samples were serially diluted with cell culture media and added to cells at varying concentrations (0.01-100 µg/mL). The final concentration of DMSO in samples was less than 0.1%.

MTT cell viability assay

The effect of Arecanut extract on the viability of RAW 264.7 cells was determined by MTT assay as per the protocol described earlier with modifications[23]. RAW 264.7 cells were cultured in 96 well plate at a seeding density of 1×10^4 cells/well in DMEM medium containing 10% heat inactivated fetal bovine serum and incubated in a CO2 incubator (37°C, 5% CO_2) for 24 h. After incubation, the media was replaced with different concentrations (0.01, 0.1, 1, 10, and 100 µg/mL) of samples dissolved in culture media. Followed by overnight incubation, the cells were treated with MTT (0.5 mg/mL) in phosphate buffered saline for 4 h and formazan crystals formed were dissolved by 200 µL/well DMSO. Absorbance was measured at 570 nm with a reference wavelength at 650 nm using a multimode microplate reader (Thermo Fisher Scientific, USA). The percentage viability of cells was determined by using the equation, $A_T/A_C \times 100$, where A_T is the absorbance of test and A_C is the absorbance of control cells.

Quantification of proinflammatory cytokine

RAW 264.7 cells were seeded at a density of 1×10^6 cells/mL in a 48 well plate in DMEM medium containing 10% FBS and incubated in a CO_2 incubator. After 24 h, the cells were pretreated with 0.1, 1, 10 and 100 µg/mL of samples dissolved in culture media for 2 h. Lipopolysaccharide from *E. coli* (1 µg/mL) was added to stimulate the cells for 16 h and supernatants were collected[16]. The concentration of proinflammatory cytokine IL-6 was quantified in cell supernatant using ELISA kit, according to manufactures instructions. RAW 264.7 cells without drug treatment was used as control and fresh medium was taken as blank.

LC-MS analysis

The samples were dissolved in MS grade acetonitrile and filtered through 0.22 μM syringe filter. The analysis was done in a Waters Binary HPLC system connected to AQUITY QDa detector with an electrospray ionization (ESI) interface. The system was controlled with Empower 3 software. The separation was carried out in a reverse phase C_{18} column using water (Solvent A) and acetonitrile (Solvent B) containing 0.1% formic acid as mobile phase. The elution was carried out in an isocratic ratio of 60:40 (water: Acetonitrile). The source voltage, desolvation temperature, and capillary voltage were 0.8 kV, 600°C, and 15kV, respectively, and a scan range of m/z 100-600 was recorded in positive ionization mode. The flow rate was 0.5 mL/min and run time was set to be 10 min. For compound identification databases, ChemSpider (http://www.chemspider.com), PubChem (https://pubchem.ncbi.nlm.nih.gov), and NORMAN Mass bank (https://massbank.eu/MassBank) were used.

Statistical analysis

The data obtained were expressed as mean ± SD of three independent experiments. The significant difference among the control and treated groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test using graph pad prism software. Statistical significance was set at a level of $P< 0.05$ were,*$P< 0.05$, **$P< 0.01$ and ***$P< 0.001$.

Results

The free radical scavenging potential of Arecanut

The percentage yield obtained from soxhlet extraction of Arecanut was found to be 16.61%. DPPH assay was done for measuring the free radical scavenging potential of Arecanut. The methanol extract exhibited a dose dependent activity which indicates that DPPH radical scavenging activity was increased proportionately to the increase in the concentration of the extracts. Additionally, the EC_{50} value obtained for Arecanut was 0.264 ± 0.073 μg/mL and that of BHA standard was 0.553 ± 0.082 μg/mL, respectively, which indicates a strong radical scavenging potential of Arecanut compared to positive control BHA.

Effect of methanol extract of Arecanut on viability of RAW 264.7 cells

To ensure that the cells were healthy before performing the bioactivity assays and the tested concentrations were not toxic to the RAW 264.7 cells, cell viability after 24 h treatment with various concentrations of the Arecanut extract (0.01-100 μg/mL) was determined. The results presented in (Fig. 1) indicates no significant cytotoxicity at all tested concentrations (0.01-100 μg/mL). From the viability results, four sample concentrations 0.1, 1, 10, and 100 μg/mL were selected for further anti-inflammation experiment.

Effect of Arecanut on LPS induced IL-6 production by LPS stimulated RAW 264.7 cells

The RAW 264.7 cells were stimulated with LPS in the presence or absence of Arecanut extract, and the levels of IL-6 production was measured by ELISA. As shown in Figure 2, LPS treatment resulted in a

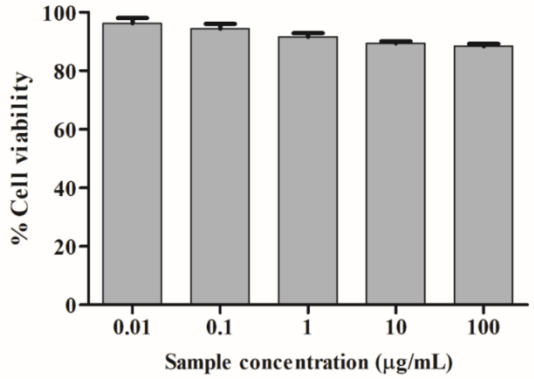


Fig. 1 — Effect of Arecanut extract on the viability of RAW 264.7 cells. Cells were exposed to different concentrations of extract (0.01-100 μg/mL) for 24 h and viability was determined by MTT assay. All the values were expressed as mean±SD of three independent experiments (n=3).

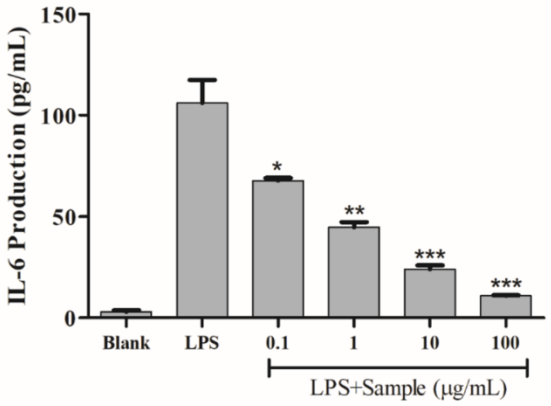


Fig. 2 — Effect of Arecanut extract on the product on the production of IL-6 production. The RAW 264.7 cells were pretreated with different concentrations of Arecanut extracts for 2 h and then exposed to 1 μg/mL of LPS for 16 h. the levels of IL-6 released into the cell supernatant was determined by ELISA. Data shows mean±SD of three independent experiments (n=3)., *$P< 0.05$, **$P< 0.01$ and ***$P< 0.001$ indicate significant differences from LPS stimulation value

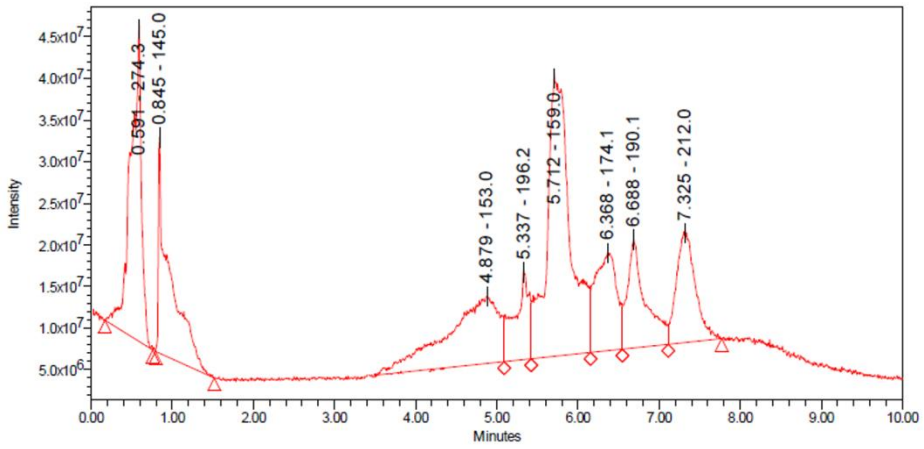


Fig. 3 — Total ion chromatogram in positive ionization mode

significant increase in IL-6 production as compared to the cells without LPS stimulation (Blank). However, pre-treatment of cells with Arecanut extract showed a dose dependent inhibition on IL-6 production. Arecanut extract at tested concentrations 0.1, 1, 10 and 100 µg/mL inhibited IL-6 production by 35.97±1.59, 57.50±3.68, 77.34±2.54, 89.64±0.24%, respectively.

Identification of bioactive compounds by Liquid chromatography-Mass spectrometry

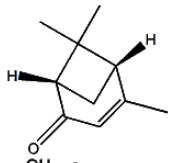
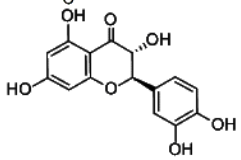
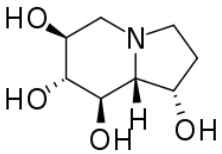
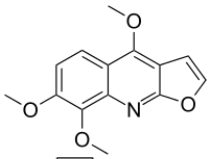
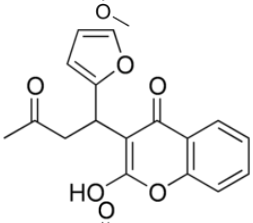
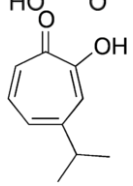
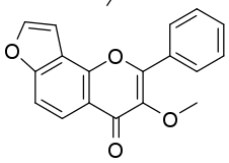
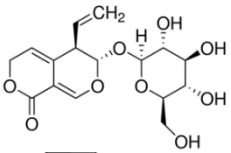
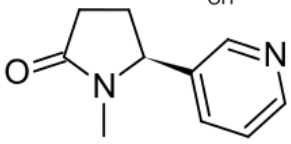
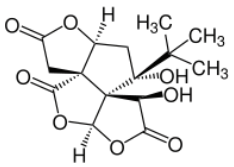
The chemical composition of methanol extract of Arecanut was determined by LC-MS method. The obtained total ion chromatograms in positive ionization mode are shown in (Fig. 3). The compound identification was done by comparing the retention time and molecular mass of compounds with available pieces of literature and databases (ChemSpider, NORMAN Mass Bank, and Pubchem). The LC-MS analysis determined 10 compounds in methanol extract of Arecanut (Table 1), including 3 alkaloids: Castanospermine, Skimmianine, and Cotinine, 2 flavonoids: Dihydroquercetin, and Karanjin, 2 terpenoids: Hinokitol and Bilobalide, 1 glycoside: Gentiopicroside, 1 terpene: Verbenone and 1 coumarin: Fumarin.

Discussion

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO) are highly reactive chemical species generated as an imbalance between formation and neutralization of prooxidants. Excess production of ROS cause oxidative damage to lipids, proteins, and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans[24]. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available synthetic antioxidants reported to be toxic to human beings[25]. Therefore, the natural antioxidants available from plant extracts or isolated products of plant origin are in high demand for application as nutraceuticals, bio-pharmaceuticals, as well as food additive because of consumer preference. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a reliable method to determine the antioxidant activity of plant extracts. DPPH is a stable free radical which dissolves in methanol and shows characteristic absorption between 515-520 nm. When a compound with antioxidant activity scavenges free radicals by hydrogen donation, the DPPH solution becomes lighter in colour[26]. Generally, the DPPH scavenging activity is quantified in terms of percentage scavenging of the pre-formed free radical by antioxidants, and the effective concentration (EC_{50}) is a widely used parameter to express the antioxidant potential and to compare the activity of different compounds[22]. In the present study methanol extract of Arecanut showed a significant DPPH radical scavenging activity (EC_{50} = 0.264 ± 0.073 µg/ mL) compared to positive control BHA (EC_{50} = 0.553 ± 0.082 µg/mL). The result indicated the potential antioxidant activity of Arecanut extract, hence it could be useful for treating radical-related pathological damage.

Macrophage cells play a significant role in the development of inflammatory diseases that are associated with excessive production of proinflammatory cytokines. They were important targets for studying cellular immunity, phagocytosis, and inflammation[27]. The levels of proinflammatory mediators and cytokines produced by macrophages are reflective

Table 1 — Phytochemical compounds identified in the methanol extract of Arecanut

Retention time	Molecular formula	Calculated mass (*m/z*)	[M+H] (*m/z*)	Predicted compound	Structure	Class of compound
0.515	$C_{10}H_{14}O$	150.104	151.112	Verbenone		Terpene
0.952	$C_{15}H_{12}O_7$	304.058	305.066	Dihydroquercetin		Flavonoid
1.974	$C_8H_{15}NO_4$	189.100	190.108	Castanospermine		Alkaloid
2.223	$C_{14}H_{13}NO_4$	259.084	260.092	Skimmianine		Alkaloid
3.548	$C_{17}H_{14}O_5$	298.084	299.092	Fumarin		Coumarin
5.630	$C_{10}H_{12}O_2$	164.083	165.093	Hinokitol		Terpenoid
6.126	$C_{18}H_{12}O_4$	292.073	293.082	Karanjin		Flavonoid
6.871	$C_{16}H_{20}O_9$	356.110	357.119	Gentiopicroside		Glycoside
7.158	$C_{10}H_{12}N_2O$	176.094	177.103	Cotinine		Alkaloid
8.585	$C_{15}H_{18}O_8$	326.100	327.108	Bilobalide		Terpenoid

of the degree of inflammation, and are used to evaluate the anti-inflammatory effects of pharmacological agents[28]. In the present study, LPS was used to induce a proinflammatory reaction in RAW 264.7 macrophages for determining the anti-inflammatory activity of Arecanut. The results showed that methanol extract of Arecanut significantly inhibited the production of inflammatory mediator IL-6, at all concentrations studied, in LPS induced RAW 264.7 macrophage cell lines. IL-6 is the primary mediator of local inflammation and sepsis that promotes the differentiation of T lymphocytes and B lymphocytes and release other proinflammatory cytokines because of its pleiotropic effects[29]. As Arecanut extract showed the potent anti-inflammatory activity by inhibiting the production of IL-6 cytokine, LCMS analysis was carried to identify the bioactive compounds present in the extract.

10 compounds such as Verbenone, Dihydroquercetin, Castanospermine, Skimmianine, Fumarin, Hinokitol, Karanjin, Gentiopicroside, Cotinine and Bilobalide were identified by LC-MS analysis, of which dihydroquercetin[30], castanospermine[31], Skimmianine[32], bilobalide[33], karanjin[34], Hinokitol[35], Gentiopicroside[36] and Cotinine[37] are previously reported to have anti-inflammatory effects. Dihydroquercetin is a potent flavonoid possess significant anti-inflammatory activity in RAW 264.7 cells *via* activation of AMPK/Nrf2/HO-1 signal axis. In addition dihydroquercetin pretreatment could protect mice against the inflammation and mortality associated with endotoxemia[38]. Castanospermin, an indolizidine alkaloid have been shown to `inhibit brain inflammation with the resulting appearance of delayed neuronal disorders, including seizures and paralysis in an Ifnar1$^{-/-}$ mouse[31].

The anti-inflammatory compound Skimmianine present in *Zanthoxylum zanthoxyloides* inhibit neuro-inflammation in LPS-and hemozoin-activated BV-2 microglia by targeting the activation of both NF-κB and NLRP3 inflammasome[32]. Hinokitol, a monoterpenoid exerts a spectrum of biological activities including anti-inflammatory[35], antibacterial, antifungal, antioxidant and anticancer activity[39]. Karanjin is a furano flavonol possess anti-inflammatory effect in rat model by the inhibition of lipoxygenase-1 and 5-LOX. In addition, anti-ulcer property of karanjin by the inhibition of H+, K+-ATPase by *in vitro* studies using sheep parietal cells and *in vivo* using ulcer induced adult rat models has been reported[34]. Gentiopicroside belongs to glycosides shows anti proliferative activity, wound healing activity, anti-hepatotoxic, and analgesic effects. The anti-inflammatory activity of gentiopicroside was associated with the down-regulation of inflammatory cytokines, such as NO, PGE_2, and IL-6, and the suppression of iNOS and COX-2[36]. Cotinine is an alkaloid that inhibits the pro-inflammatory response initiated by multiple cell surface Toll-like receptors in monocytic THP cells[37]. Bilobalide, a terpenic trilactone has been shown to inhibit M1 macrophage polarization through the NF-κB signaling pathway[33].Overall, the methanol extract of Arecanut possess antioxidant and anti-inflammatory activities and indicated the presence of a large variety of phytoconstituents.

Conclusion

The fruits of *Areca catechu* L. (Palmaceae family) possess high level of antioxidant activity and thus can be considered as new source of natural antioxidant. It has a strong anti-inflammatory effect in a dosage-based mode, which is non-toxic to the cells. As inflammation is a double sworded mechanism, the Arecanut extract can be effectively used to treat the inflammation due to the overproduction of IL-6. The compounds identified from methanol extract of Arecanut can be further isolated and can extend the studies for the drug development in the future against inflammation and associated diseases.

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Conflict of interest

All authors declare no conflict of interest.

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