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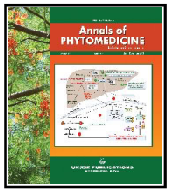

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Cytotoxic, apoptotic, anti-inflammatory and oxidative stress reduction potential of the defatted methanolic extract of the *Areca catechu* L. (Arecaceae) inflorescence in HepG2 cancer cell lines

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Abstract

The development of novel therapeutic agents with fewer adverse effects is crucial due to the persistent side effects of the current drugs, which are often required to treat both acute and chronic diseases characterised by inflammation and oxidative stress. This study investigated the anti-inflammatory and oxidative stress reduction potential of a defatted methanolic extract of *Areca catechu* L. inflorescence (ACME), rich in alkaloids and flavonoids, in lipopolysaccharide (LPS)-induced inflammatory and oxidative stress responses in HepG2 cancer cells. The MTT assay was used to evaluate the viability of HepG2 cells, which demonstrated a reduction in viability at various concentrations of ACME, with an IC_{50} value of 112.69 µg/ml. Further investigation on the apoptotic potential of ACME demonstrated that it triggered both early and late apoptosis, as well as necrosis in HepG2 cancer cells. *In vitro* anti-inflammatory studies showed that ACME at concentrations of 28, 56, and 112 µg/ml and equal concentrations of diclofenac significantly decreased the production of pro-inflammatory mediators (total COX, 5-LOX, MPO, iNOS, and NO) and cytokines (IL-1β, IL-6, and TNF-α) in LPS-stimulated HepG2 cells. ACME at a concentration of 112 µg/ml also improved cellular antioxidant status (SOD, CAT, GSH-Px, GSH, MDA, and total protein), reduced reactive oxygen species (ROS) levels, and maintained mitochondrial membrane potential (MMP). Moreover, ACME was found to significantly suppress LPS-induced activation of keap1, thereby preserving the expression of Nrf2, as confirmed by qRT-PCR. These findings suggest that ACME demonstrates significant anti-inflammatory and oxidative stress reduction potential, likely due to its effect on the Keap1-Nrf2 pathway.

1. Introduction

Inflammation and oxidative stress are two interconnected processes that play crucial roles in various diseases including cancer (Li *et al.,* 2016). These processes are essential for normal bodily functions in moderation, but can be detrimental when they persist or become uncontrolled. Inflammation is the body's natural response to harmful stimuli, such as pathogens, injuries, or irritants, and involves activation of the immune system, leading to increased blood flow and the production of immune cells and cytokines (Chen *et al.,* 2018; Pushpangadan *et al.,* 2015a; Ijinu *et al.,* 2022). Acute inflammation is a crucial defense mechanism that aids the removal of pathogens and facilitates tissue repair. However, chronic inflammation, in which the immune response persists over an extended period, can lead to tissue damage, and contribute to the development of various diseases including cancer (Furman *et al.,* 2019). Oxidative stress occurs when there is an imbalance between the production of free radicals (reactive oxygen species or ROS) and the ability of the body to neutralise them with cellular antioxidants (Pizzino *et al.,* 2017). This stress can damage cellular components, disrupt signaling pathways, and contribute to the initiation and progression of cancer. Additionally, the inflammatory microenvironment can stimulate ROS production and contribute to oxidative stress. These reactive molecules can damage DNA, leading to mutations that may initiate cancer growth (Kay *et al.,* 2019). Understanding the dynamic relationship between inflammation and cancer is of great significance in preventive medicine, diagnosis, and therapeutic interventions. It is not just about deciphering how chronic inflammation fuels carcinogenesis, but also explores promising avenues where interventions targeting inflammatory pathways could potentially disrupt or impede cancer progression. Targeting inflammation and oxidative stress pathways may offer potential therapeutic approaches.

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The relationship between medicinal plants and drug development is of paramount importance and has a rich historical context. Most modern medications are developed from plant-derived natural compounds (Pushpangadan *et al.,* 2015a, b; Mehrotra, 2021; Arif *et al.,* 2022). This study focused specifically on the inflorescence of *A. catechu*, and no prior research has reported this aspect of the plant. Most previous studies have concentrated on dried ripe nuts (areca nuts), locally known as *supari*. *A. catechu* is cultivated in the tropical regions of India and Southeast Asia and is considered a traditional herbal remedy. The Chinese Pharmacopoeia contains over 100 drug preparations that utilise areca nut as an ingredient to treat various conditions including parasitic diseases, dyspepsia, abdominal distension, pain, diarrhoea, oedema, and jaundice. The Chinese *Mingyi Bielu* and *Xinxiu Bencao* monographs mention the areca nut for its diuretic, digestion-promoting, and anti-parasitic properties, as well as for treating abdominal distension (Caius, 1934; Raghavan and Baruah, 1958; Amudhan *et al.,* 2012). In the Compendium of Materia Medica, areca nut is used as a treatment for dysentery, abdominal distension, and constipation. The Indian system of medicine also uses areca nuts as a popular digestive, astringent, and emmenagogue. In Cambodia, areca nuts are used to treat diarrhoea, dysentery, and liver disorders, whereas in Malaya they are used to treat lumbago and abdominal distension. Areca nut is also consumed as a daily food ingredient in Chinese Taipei, South, and Southeast Asia, and is considered the fourth most used addictive stimulant after caffeine, nicotine, and alcohol (Heatubun *et al.,* 2012). The immature fruit or pericarp of *A. catechu* is the primary constituent of betel quid, and chewing betel quid is strongly associated with ancient oriental culture (Lim and Kim, 2006; Benegal *et al.,* 2008; Ijinu *et al.,* 2023). Numerous studies have demonstrated that the various components of *A. catechu* possess a broad range of pharmacological properties, including gastroprotective, antioxidant, anti-inflammatory, anticancer, analgesic, anti-allergic, cardioprotective, antihyperlipidaemic, antidiabetic, neuroprotective, antimicrobial, and anti-parasitic effects (Peng *et al.,* 2015; Osborne *et al.,* 2017; Salehi *et al.,* 2020). Taking these findings and traditional usage into account, the present study aimed to investigate the cytotoxic, anti-inflammatory, and oxidative stress-reducing potential of a defatted methanolic extract of *A. catechu* inflorescence in HepG2 cancer cell lines induced by LPS.

2. Materials and Methods

2.1 Drugs and chemicals

Lipopolysaccharide (*Escherichia coli*, serotype 0111:B4), 3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT; M-5655), 2',7'-dichlorofluorescin diacetate (DCFDA), acridine orange, and ethidium bromide were purchased from Sigma-Aldrich, USA. Merck (India) supplied all biochemical reagents, while Nunc (Thermo Fisher Scientific, USA) provided tissue culture plates and flasks. Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM), foetal bovine serum (FBS), penicillin, and streptomycin were procured from Gibco (Thermo Fisher Scientific, USA). The ELISA kits were purchased from Cayman Chemical Co. (USA). The MitoPotential Assay Kit was obtained from EMD Millipore (USA). All remaining solvents, chemicals, and reagents were of analytical grade.

2.2 Inflorescence extract preparation

Fresh *A. catechu* inflorescences were obtained from the Southern Western Ghats region of Thiruvananthapuram, Kerala, India (8.5241°N, 76.9882°E). The plant material was authenticated by Dr. P. Pushpangadan of the Amity Institute for Herbal and Biotech Products Development, Thiruvananthapuram. The fresh inflorescence weighed 2200 g and was subsequently dried and powdered to yield 407 g. The powdered sample was defatted with petroleum ether (40-60) and further extracted with methanol using a Soxhlet extractor for 24 h. The resulting methanolic extract (ACME) was concentrated using a rotary evaporator and stored at 4°C until further use (Ijinu *et al.,* 2016).

2.3 Cell culture

HepG2 cells were obtained from the National Centre for Cell Sciences, India, and cultured in DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and an antibiotic solution containing penicillin (100 U/ml), streptomycin (100 µg/ml), and amphoteracin B (2.5 µg/ml). Cells were maintained at 37°C and 5% CO_2. After 24 h, the growth medium was removed and freshly prepared ACME in DMEM was serially diluted two-fold (100, 50, 25, 12.5, and 6.25 µg in 500 µl of DMEM), and 100 µl was added in triplicate to the respective wells and incubated at 37°C in a humidified 5% CO_2 incubator. The untreated control cells were maintained (Chithra *et al.,* 2020).

2.4 Cell viability assay by microscopy

The entire plate of cells was observed after 24 h of treatment using an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD Camera, Japan), and microscopic observations were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolisation in the cytoplasm of the cells, were considered as indicators of cytotoxicity/apoptosis (Chithra *et al.,* 2020).

2.5 MTT assay

After a 24 h incubation period, the contents of the wells were removed, and 30 µl of reconstituted MTT solution (0.5 mg/ml) was added to all test and cell control wells. The plates were shaken gently and incubated at 37°C in a humidified incubator with 5% CO_2 for 4 h. Following the incubation period, the supernatant was removed and 100 µl of MTT solubilisation solution (dimethyl sulfoxide) was added to the wells, which were then gently mixed by pipetting to solubilise the formazan crystals. The absorbance of the formazan solution was measured at 540 nm wavelength using an ELISA microplate reader (Erba LisaScan II, Germany). The optical density of the formazan produced in untreated control cells was designated as 100% viability (Talarico *et al.,* 2004).

2.6 Determination of apoptosis

After ensuring adequate confluence of HepG2 cells, ACME was added at an IC_{50} concentration of 112 µg/ml, and the cells were incubated for 24 h. Following incubation, cells were washed with cold PBS and stained with a mixture of acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) at room temperature for 10 min. The stained cells were washed twice with 1X PBS and observed under a fluorescence microscope using a blue filter on an Olympus CKX41 with an Optika Pro5 CCD Camera (Japan) (Zhang *et al.,* 1998). Control cells that were not treated were maintained.

2.7 Analysis of pro-inflammatory mediators

HepG2 cells were grown to 60% confluence and activated with 1 µl of 1 µg/ml LPS. Next, the cells were treated with different concentrations of ACME (28, 56, and 112 µg/ml), which were selected based on the IC_{50} value and the corresponding diclofenac sodium standard. Following a 24 h incubation period, the cells were harvested with ice-cold PBS and lysed using a buffer solution containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Lysis was performed on ice for 15 min. Subsequently, the insoluble fraction was separated by centrifugation at 12,000 g for 15 min at 4°C (Chithra *et al.,* 2020). The resulting supernatant comprising the whole-cell lysate was collected, and the cell lysate levels of total cyclooxygenase (Walker and Gierse, 2010), 5-lipoxygenase (Axelrod *et al.,* 1981; Yang *et al.,* 2017), myeloperoxidase (Bradley *et al.,* 1982; Pulli *et al.,*2013), inducible nitric oxide synthase (Salter *et al.,* 1991; Krasuska*et al.,* 2016), and nitrite (Bryan and Grisham, 2007) were determined.

2.8 Analysis of pro-inflammatory cytokines and PGE_2

The effect of equal concentrations of ACME (112 µg/ml) and diclofenac sodium on the production of pro-inflammatory cytokines (IL-1α, IL-6, and TNF-α) and PGE_2 in LPS-treated (1 µl of 1 µg/ml) HepG2 cells after 24 h was assessed by collecting the culture supernatants. Concentrations in the supernatants were measured using a commercial ELISA kit following the manufacturer's instructions.

2.9 Cellular antioxidant status

Following a 24 h activation period with LPS (1 µl of 1 µg/ml), HepG2 cells were treated with ACME (112 µg/ml). After treatment, the cells were trypsinised with trypsin-EDTA solution, collected, and transferred to microcentrifuge tubes. The tubes were then centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and the pellet was suspended in 200 µl of lysis buffer (0.1 M Tris, 0.2 M EDTA, 2 M NaCl, and 0.5% Triton). The samples were incubated at 40°C for 20 min. Cell lysates were used to study superoxide dismutase (SOD; Haida and Hakiman, 2019), catalase (CAT; Takahara *et al.,* 1960; Weydert and Cullen, 2010), glutathione peroxidase (GSH-Px; Agergaard and Jensen, 1982; Molina-Jijón *et al.,* 2012), glutathione (GSH; Moron *et al.,* 1979; Young *et al.,* 2010), malondialdehyde (MDA; Ohkawa *et al.,* 1979; González-Montelongo *et al.,* 2010), and total protein (TP; Lowry *et al.,* 1951; Hossen *et al.,* 2017) levels. All values were normalised to the protein content of each sample.

2.10 Intracellular ROS production

The ROS-scavenging activity of ACME was assessed using the oxidant-sensitive probe DCFDA, as described by Kang *et al.* (2012). First, HepG2 cells were seeded in a 96-well plate at a concentration of 5000-10000 cells per well and incubated for 24 h. Upon attaining a confluence 40-70%, the cells were exposed to LPS (1 µl of 1 µg/ml) for 1 h, followed by treatment with ACME (112 µg/ml) for 24 h in a CO_2 incubator. Subsequently, the cells were rinsed with PBS and incubated with 50 µl DCFDA (100 µM DCFDA in DMEM + 1% FBS) for 30 min in the dark at 37°C and 5% CO_2. Afterwards, the surplus dye was eliminated through PBS washing, and DCF fluorescence was quantified using a Qubit 3.0 instrument (Life Technologies, USA) with an excitation wavelength of 470 nm and an emission wavelength of 635 nm. LPS alone was used as a positive control, whereas untreated cells served as the control. The fluorescence was imaged using a fluorescence microscope (Olympus CKX41 microscope equipped with an Optika Pro5 CCD Camera, Japan), and the relative intensity was measured using ImageJ analysis software.

2.11 Mitochondrial membrane potential (Δψm)

ACME (112 µg/ml) was added to LPS-stimulated HepG2 cells (1 µl of 1 µg/ml) and incubated for 24 h at 37°C in a humidified incubator with 5% CO_2 after achieving sufficient confluence. Untreated control cells and LPS control wells were maintained. After incubation, the cells were trypsinised and subjected to flow cytometry according to the following procedure. The working solution was prepared by diluting Muse™ MitoPotential Dye 1:1000 in 1X assay buffer. After centrifugation, the cells were resuspended in 1X assay buffer and 95 µl of MitoPotential working solution was added. It was then mixed thoroughly by pipetting and incubating the cells for 20 min at 37°C in a CO_2 incubator. After incubation, 5 µl of Muse MitoPotential 7-AAD was added to each well and mixed thoroughly by pipetting up and down or vortexing for 3-5 s, followed by incubation for 5 min (Sini *et al.,* 2018). The samples were loaded onto a flow cytometer (Muse Flow cytometer, Millipore, USA) and events were acquired after gating and correlated with the controls.

2.12 Total RNA extraction, cDNA synthesis and qRT-PCR

HepG2 cells were stimulated with LPS and treated with ACME (112 µg/ml) for 24 h at 37°C in a humidified incubator with 5% CO_2. Subsequently, total RNA was isolated using TRIzol reagent, according to the manufacturer's instructions, and the purity and concentration of the isolated RNA were determined. A cDNA preparation kit was used to synthesise template cDNA. RT Easy mix (5 µl), oligo dT (0.5 µl), and RNA template (2 µl; 0.5 µg of total RNA) were added to an RNAse-free tube, and the total reaction volume was adjusted to 10 µl with the addition of sterile distilled water. The solution was mixed by gentle pipetting and a thermal cycler (Eppendorf Master Cycler) was used for cDNA synthesis.The following cycling conditions were used: 20 min at 42°C and 5 min at 85°C. Specific primers were used to measure gene expression at the mRNA level, including Nrf2 (F: 5'-CAC ATC CAG TCA GAA ACC AGT GG-3', R: 5'-GGA ATG TCT GCG CCA AAA GCT G-3'), Keap1 (F: 5'-GAT CGG CTG CAC TGA ACT G-3', R: 5'-GGA CTC GCA GCG TAC GTT-3'), and GAPDH (F: 5'-ACT CAG AAG ACT GTG GAT GG-3', R: 5'-GTC ATC ATA CTT GGC AGG TT-3'). qRT-PCR analysis was performed using SYBR Green Master Mix on a Light Cycler 96 (Roche) with the following program: 95°C (2 min), 95°C (10 s), 58°C (1 min), followed by extension at 72°C (1 min/kb) for 40 cycles. All reactions were performed in triplicate and the relative fold expression of each gene was calculated using the 2(-δδ (T)) method (Schmittgen and Livak, 2008) and corrected for amplification efficiency using GAPDH as the housekeeping gene.

2.12 Statistical analysis

Data were analysed using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). Two-way analysis of variance (ANOVA) with Tukey's multiple comparison test and one-way ANOVA with Tukey's multiple comparison test were performed. $p \leq 0.0001$, $p \leq 0.001$, $p \leq 0.01$, and $p \leq 0.05$ were considered statistically significant; ns was not significant ($p > 0.05$). Experimental data are presented as mean ± standard deviation (n=3).

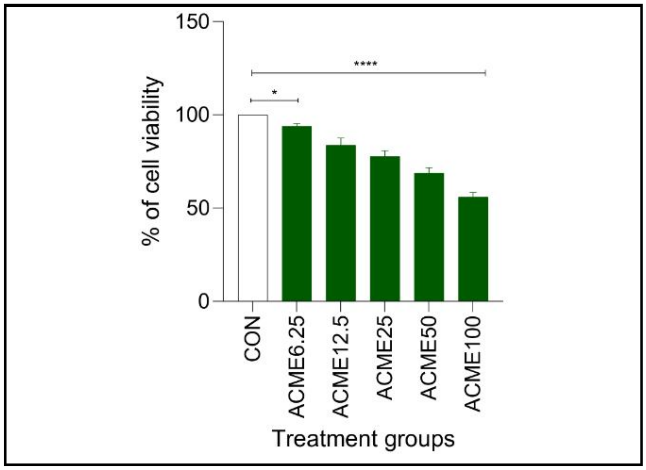
3. Results

3.1 Preliminary phytochemical analysis

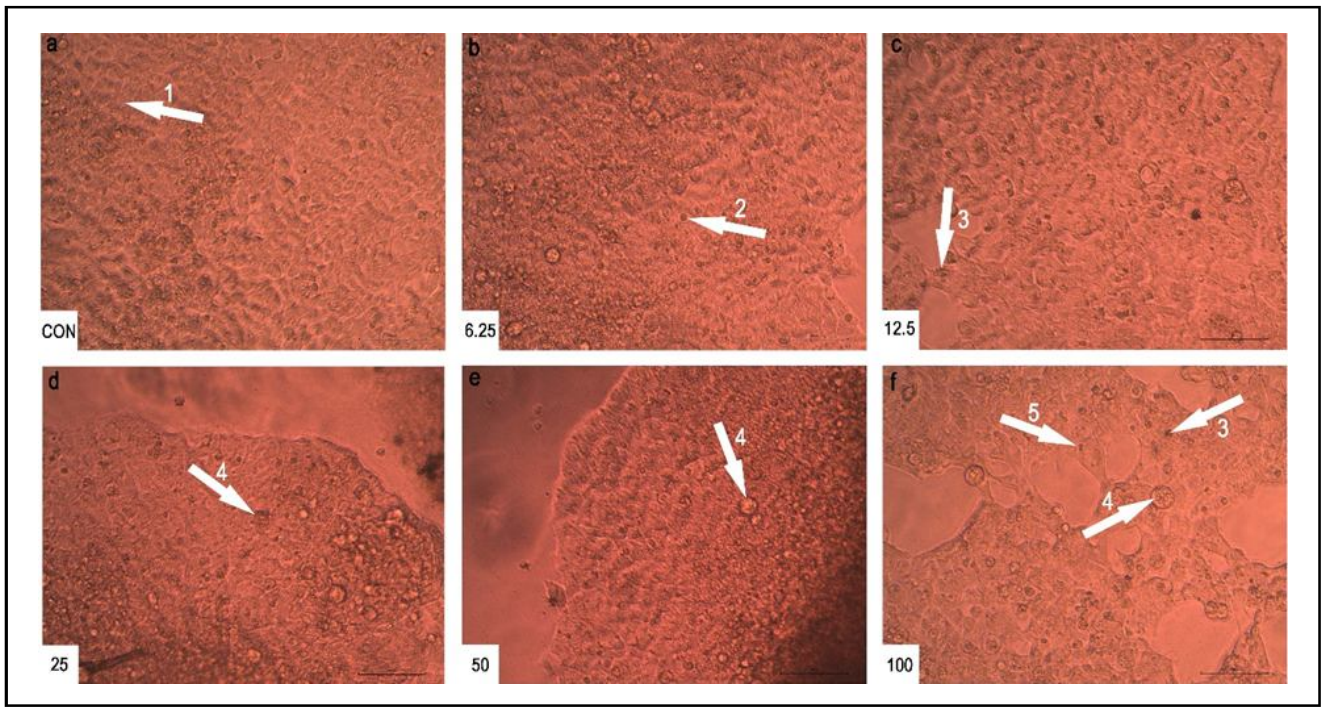
Preliminary phytochemical analysis of ACME indicated the presence of steroids, terpenoids, phenolics, flavonoids, alkaloids, and glycosides.

3.2 Cell viability assay

The MTT method was employed to assess cytotoxicity in HepG2 cells, revealing a notable dose-dependent reduction in cell viability, especially at higher concentrations of ACME (25, 50, and 100 µg/ml), compared to the control group ($p<0.0001$). At 100 µg/ml, ACME showed only 55.93% viability after 24 h of incubation at 37°C (Figure 1). Notably, lower doses of ACME (6.25 and 12.5 µg/ml) did not induce significant cytotoxic effects. The IC_{50} value was found to be 112.69 µg/ml. Thus, concentrations at or below the IC_{50} were considered safe and were selected for use in subsequent experiments.



Figures 1: Viability of HepG2 cells treated with different concentrations of ACME (6.25-100 µg/ml).



Figures 2a-f: The photomicrographs of the MTT assay of HepG2 cancer cells treated with various concentrations of ACME (6.25-100 µg/ml) examined using an inverted phase-contrast microscope (arrows: 1 normal cells, 2 apoptotic cells, 3 chromatin condensation, 4 membrane blebbing, and 5 apoptotic bodies).

3.3 Cell viability examination by microscopy

After a 24 h incubation with ACME, the morphology of HepG2 (Figures 2a-f) cells was observed under an inverted phase-contrast microscope, revealing significant changes in HepG2 cells treated with higher doses of ACME. These changes include membrane blebbing, chromatin condensation, and the formation of apoptotic bodies.

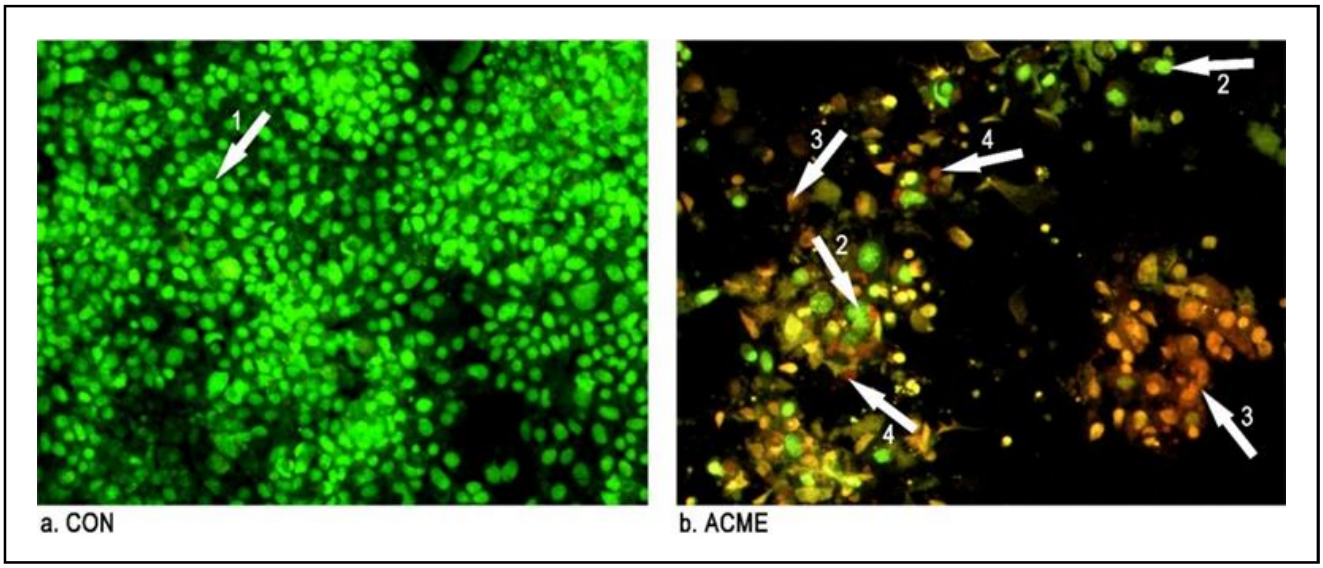
3.4 Apoptosis in HepG2 cells

Untreated HepG2 cells exhibited a uniformly circular nucleus positioned centrally within the cell (Figure 3a). Upon treatment with 112 µg/ml ACME, cells undergoing early apoptosis, late apoptotic processes, and necrosis were observed (Figure 3b). Early-apoptotic cells exhibited yellow-green fluorescence with condensed or fragmented chromatin when stained with acridine orange, while late-apoptotic cells displayed orange fluorescence with chromatin condensation or fragmentation when stained with ethidium bromide. Cells that have fully taken up ethidium bromide are considered necrotic and exhibit uniformly orange-stained nuclei.

3.5 Effect of ACME on pro-inflammatory mediators

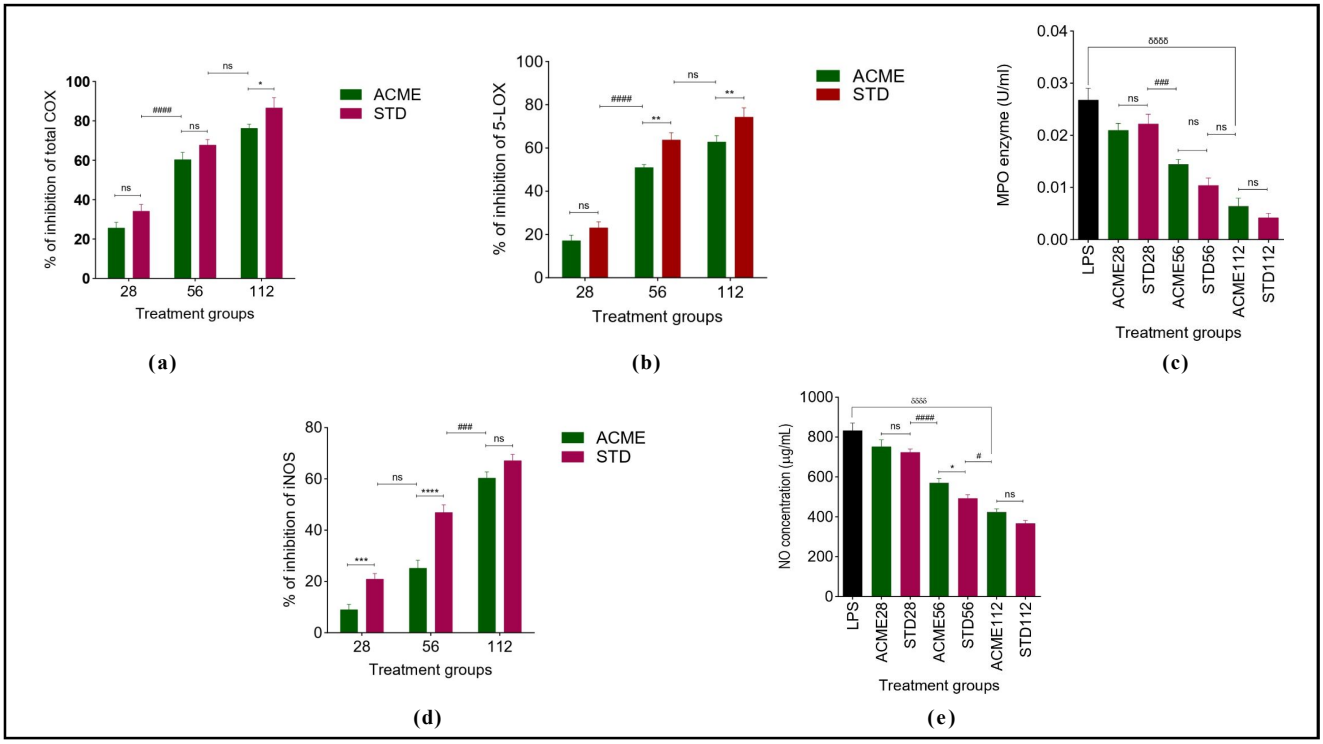
In HepG2 cells that were treated with LPS, it was observed that both the levels of total COX and 5-LOX activity were elevated. However, upon subsequent treatment with varying concentrations of ACME

(28, 56, and 112 µg/ml), a dose-dependent reduction in both total COX and 5-LOX activity was observed (Figures 4a, b).The high-concentration-treated group (112 µg/ml) showed significant inhibition, with a 76.26% reduction in total COX and 62.89% reduction in 5-LOX activities. Notably, diclofenac showed 86.66% and 74.38% inhibition of total COX and 5-LOX, respectively.



Figures 3a-b: Detection of apoptosis in HepG2 cells after treatment with ACME (IC_{50} = 112 µg/ml). Phase-contrast microscopy showed 1 normal cell, 2 early-apoptotic cell, 3 late-apoptotic cell and 4 necrosis.

The level of MPO in HepG2 cells increased considerably following treatment with LPS. However, when the cells were treated with ACME and diclofenac at the same concentrations (28, 56, and 112 µg/ml), a significant dose-dependent inhibition of MPO production was observed compared with the LPS control group ($p \leq 0001$) (Figure 4c). Notably, no discernible differences were observed between the ACME and diclofenac sodium groups, that is, both groups showed almost equal activity.



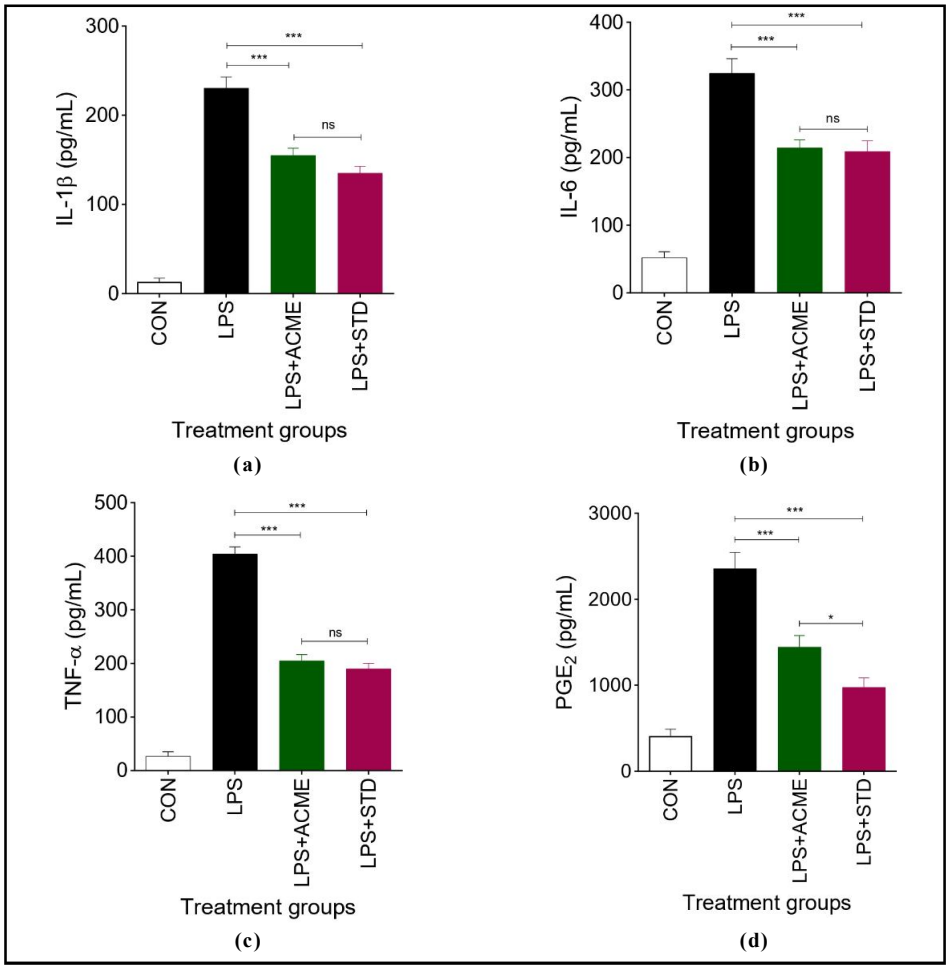
Figures 4a-e: ACME at different concentrations showed inhibitory effects on total COX, 5-LOX, MPO, iNOS, and NO activities in LPS-induced HepG2 cells. Values are presented as mean ± SD (n=3); ns = non-significant (p>0.05); **, ####, δδδδ $p \leq 0.0001$; ***, ### $p \leq 0.001$; ** $p \leq 0.01$ and *, # $p \leq 0.05$.**

The results demonstrated that both ACME and diclofenac effectively suppressed LPS-induced iNOS production in HepG2 cells in a dose-dependent manner (Figure 4d). The highest concentrations of ACME and diclofenac (112 µg/ml) displayed inhibition of 60.36% and 67.20%, respectively. Furthermore, the treatment of HepG2 cells with LPS resulted in a considerable increase in NO production, which was significantly reduced by ACME and diclofenac at their highest concentrations (112 µg/ml). The decrease in NO concentration was statistically significant ($p \leq 0001$) compared with that in the LPS control group (Figure 4e).

3.6 Effect of ACME on pro-inflammatory cytokines and PGE_2

Exposure of HepG2 cells to LPS resulted in a notable increase in the synthesis of pro-inflammatory cytokines, including IL-1β ($p \geq 0.001$), IL-6 ($p \geq 0.001$), and TNF-α ($p \geq 0.001$), relative to the control group. However, upon treatment with 112 µg/ml ACME, there was a significant decrease in the levels of these cytokines compared with the LPS control group ($p \geq 0.001$ for all cytokines, Figures 5a-c). Similarly, treatment with equal concentrations of diclofenac also significantly reduced the production of IL-1β, IL-6, and TNF-α ($p \geq 0.001$ for all cytokines).

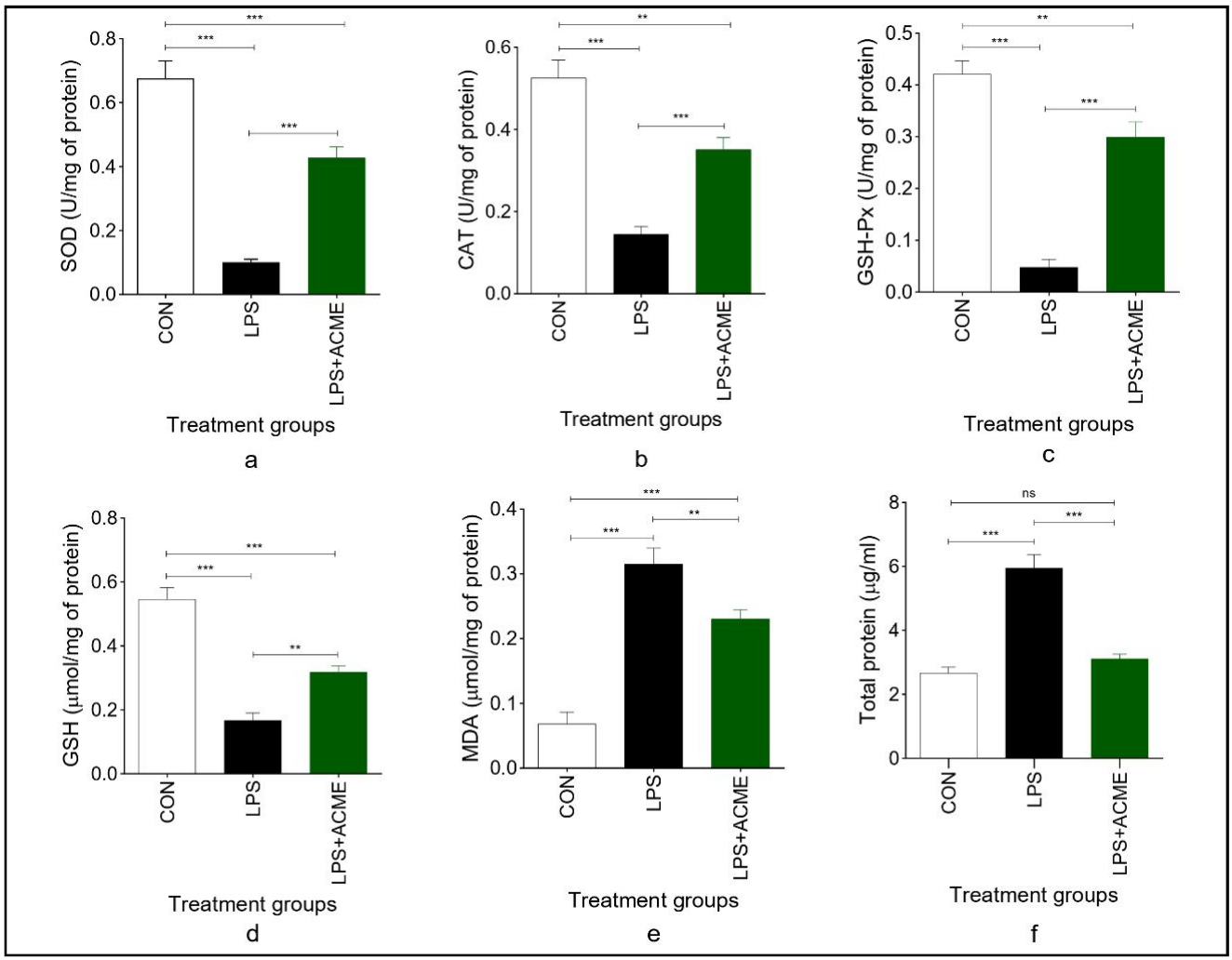
The PGE_2 levels in the culture medium of LPS-treated cells were notably higher than those in the normal control group ($p \geq 0.001$). Nonetheless, when HepG2 cells were treated with ACME and diclofenac at a concentration of 112 µg/ml, there was a significant reduction ($p \geq 0.001$) in PGE_2 levels compared with the LPS control group (Figure 5d).



Figures 5a-d: ACME regulates the production of IL-1β, IL-6, TNF-α, and PGE_2 in LPS-induced HepG2 cells, as determined by ELISA. Values are presented as mean ± SD (n=3). ns = non-significant ($p > 0.05$); *** $p \geq 0.001$ and * $p \geq 0.05$.

3.7 Effect of ACME on cellular antioxidant status

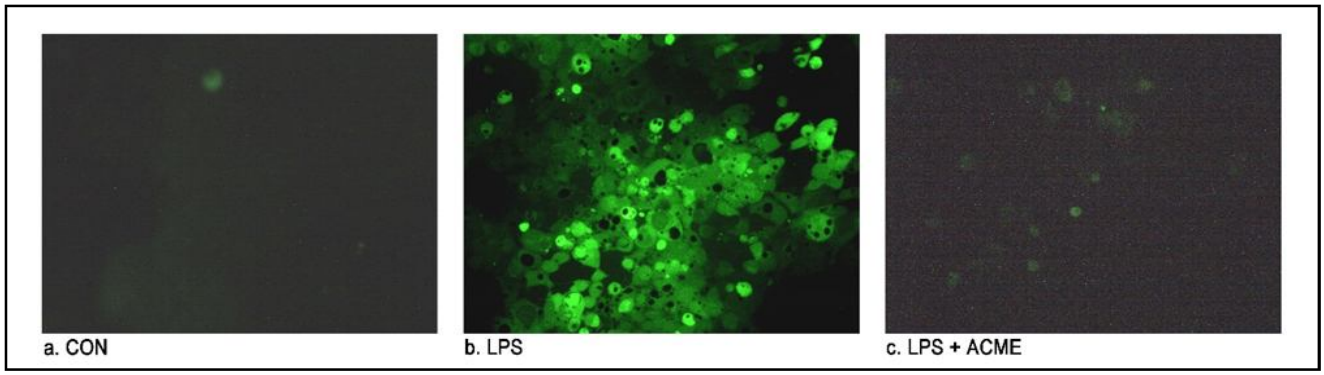
In HepG2 cells, compared to the normal control group, the activities of SOD, CAT, GSH-Px, and GSH in the cell lysate of the LPS control group were significantly decreased ($p \geq 0.001$ for all markers). Treatment with 112 µg/ml ACME resulted in significant improvements in SOD ($p \geq 0.001$), CAT ($p \geq 0.001$), GSH-Px ($p \geq 0.001$), and GSH ($p \geq 0.01$) levels compared to those in the LPS control group (Figures 6a-d). The results further indicated that the levels of MDA and total protein in the cell lysate of the LPS control group were significantly increased ($p \geq 0.001$) compared to the normal control group. However, upon treatment with ACME at a concentration of 112 µg/ml, there was a notable reduction in MDA ($p \geq 0.01$) and total protein levels ($p \geq 0.001$) (Figures 6e and f) compared with the LPS control group.



Figures 6a-f: ACME improved the production of SOD, CAT, GSH-Px, and GSH and decreased the levels of MDA and total protein in LPS-induced HepG2 cells.Values are presented as mean ± SD (n=3). ns = non-significant (p>0.05); *** $p \geq 0.001$ and ** $p \geq 0.01$.

3.8 Effect of ACME on ROS production

In the HepG2 cell line, ROS generation in the cell supernatant was visualised using a fluorescence microscope (Figures 7a-c) and quantified using a fluorimeter (Figure 8). LPS treatment led to an increase in ROS levels in HepG2 cells compared to those in the normal control group ($p \geq 0.0001$). However, a statistically significant ($p \geq 0.0001$) decrease in ROS levels was observed in the group treated with ACME at a concentration of 112 µg/ml compared to the LPS control.



Figures 7a-c: ACME inhibits ROS generation in LPS-induced HepG2 cells using the DCFDA method. a. Normal control group, b. LPS control, and c. LPS+ACME (112 µg/ml).

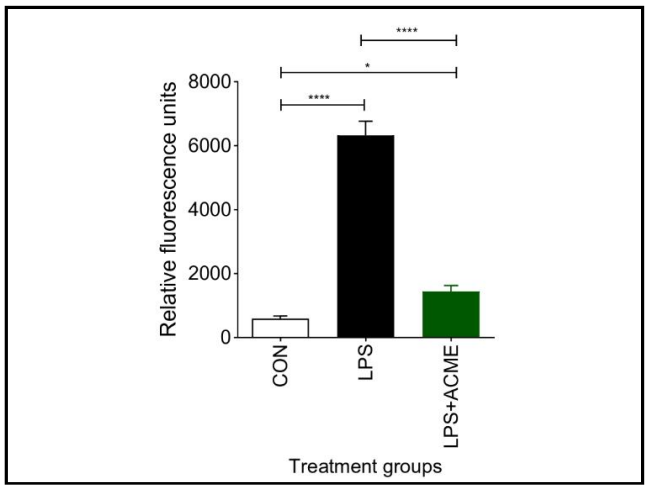


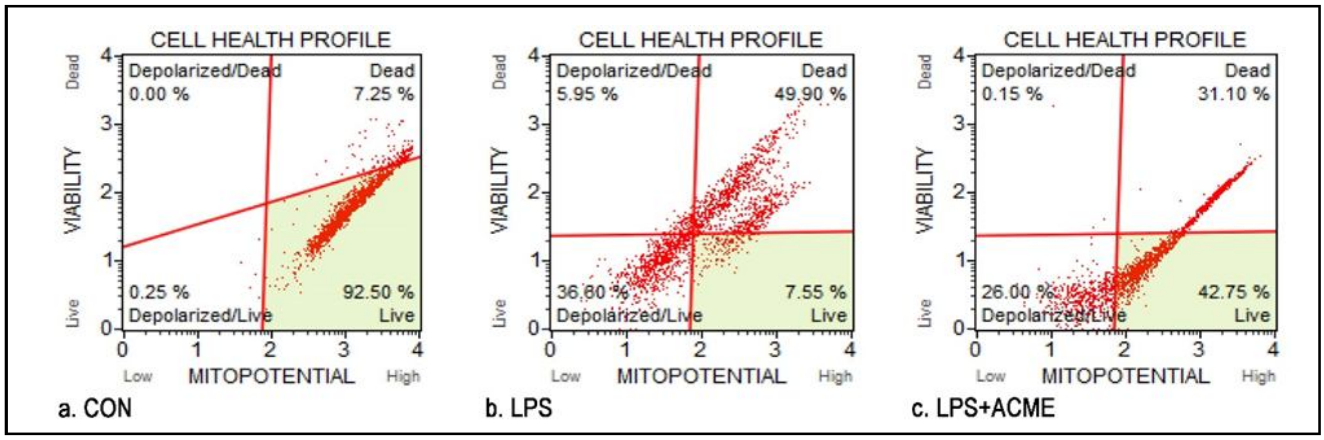
Figure 8: Determination of cellular ROS production in LPS-induced HepG2 cells using DCFDA and expressed in relative fluorescence units. Values are presented as mean ± SD (n=3). *** $p \geq 0.001$ and * $p \geq 0.05$.

3.9 Effect of ACME on mitochondrial membrane potential

The treatment of HepG2 cells with LPS resulted in a substantial proportion of cells undergoing depolarisation, accounting for 42.55% of the total cells. However, a marked decrease in the number of depolarised cells (26.15%) was observed in cells treated with 112 µg/ml ACME (Figures 9a-c and Figure 10).

3.10 Expression of Nrf2 and Keap1 genes

The expression levels of Keap1 and Nrf2, which are involved in oxidative stress response, were analysed relative to each other (Figures 11a-c). Upon LPS stimulation, Keap1 expression was upregulated (1.9908-fold increase), while Nrf2 expression was downregulated (0.1173-fold decrease) compared with the control. However, treatment with ACME resulted in downregulation (0.3883-fold decrease) of Keap1 expression and upregulation (1.8909-fold increase) of Nrf2 expression.



Figures 9a-c: ACME reduced mitochondrial depolarisation in LPS-induced HepG2 cells as analysed by flow cytometry. a. Normal control group b. LPS control and c. LPS+ACME (112 µg/ml).

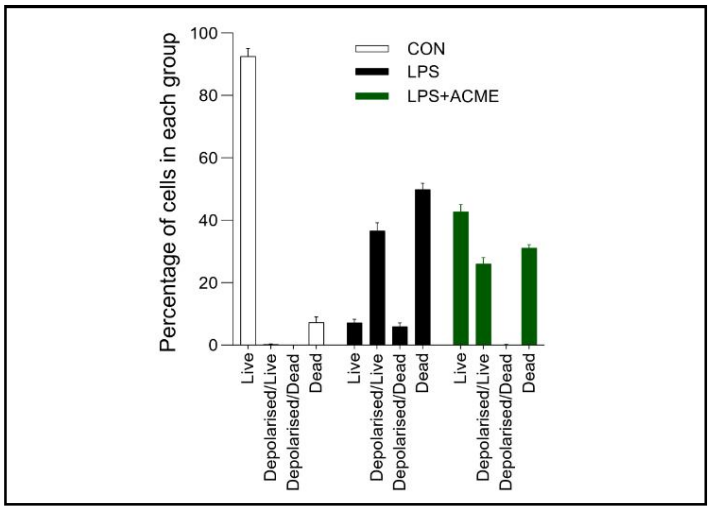
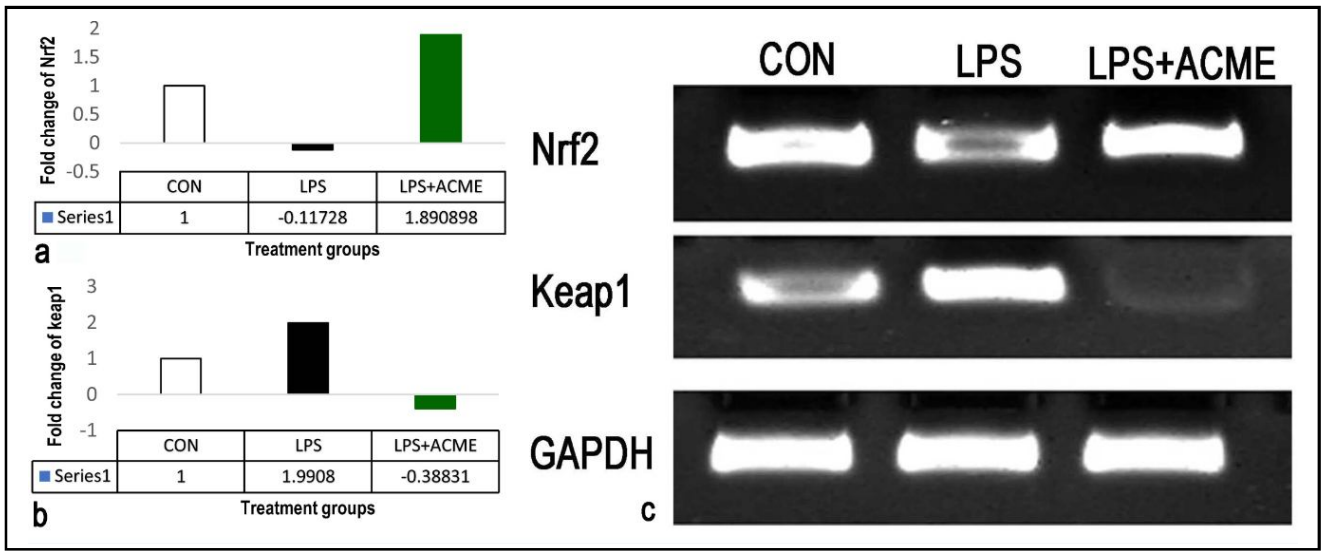


Figure 10: Quantitative results for live, dead, and depolarised HepG2 cells. Values are presented as mean ± SD (n=3).



Figures 11a-c: The relative fold change in gene expression of Keap1 and Nrf2 in HepG2 cells exposed to LPS and treated with ACME, as determined through qRT-PCR analysis.

4. Discussion

The growing interest in medicinal plants for the prevention and treatment of diseases is attributed to their rich phytochemical contents, which possess various biological properties. The aim of this study was to evaluate the cellular toxicity and anti-inflammatory and oxidative stress-reducing properties of the methanol extract of *A. catechu* inflorescence (ACME). Preliminary analysis revealed that ACME contains significant amounts of alkaloids, tannins, and flavonoids. Conversely, earlier studies have reported that different parts of *A. catechu* contain pyridine-type alkaloids and tannin derivatives as unique secondary metabolites (Peng *et al.,* 2015). Arecoline, identified as the major alkaloid of *A. catechu*, and other alkaloids include arecaidine, guavacoline, guavacine, arecolidine, methyl nicotinate, ethyl nicotinate, and nicotine (Holdsworth *et al.,* 1998, Ijinu *et al.,* 2023). Flavonoids in *A. catechu* include isorhamnetin, luteolin, quercetin, chrysoeriol, liquiritigenin, and jacareubin (Zhang *et al.,* 2009). To evaluate the bioactivity, initial cytotoxicity assessments were conducted on HepG2 cancer cell lines, which revealed a dose-dependent increase in toxicity. In contrast, Abbas *et al.* (2018) found a differential effect of areca nuts against normal and cancerous cells. Another study highlighted that arecoline hydrobromide hinders the activity of the enzyme acetyl-CoA acetyltransferase, resulting in reduced cancer cell proliferation and suppressed tumour growth in mice (Fan *et al.,* 2016). The present study further explored the apoptotic potential of ACME in HepG2 cells. The findings showed that at a concentration of 112 µg/ml, ACME triggered early and late apoptosis, as well as necrosis, as observed using acridine orange and ethidium bromide staining. In an earlier investigation, Li *et al.* (2011) observed that arecoline at doses of 100 and 125 µg/ml triggered apoptosis in HaCaT cells. According to Wang *et al.* (2009), oligomeric procyanidin-rich extract from areca nut significantly induced apoptosis in splenic lymphocytes when administered at concentrations of 20-1000 µg/ml. Furthermore, an *in vivo* study conducted on albino mice revealed that arecoline administered at 5 mg/kg intraperitoneally for 14 days resulted in cell cycle arrest in splenic lymphocytes, whereas higher doses (10-20 mg/kg intraperitoneally for 14 days) induced apoptosis (Dasgupta *et al.,* 2006).

Chronic inflammation has been implicated in the development of cancer because of its potential to cause DNA damage and lead to mutations that can initiate or promote tumour growth (Kay *et al.,* 2019). Inflammatory cells also secrete pro-inflammatory mediators such as cytokines and chemokines, which can stimulate angiogenesis, support the growth and survival of cancer cells, and suppress the immune system's ability to recognise and destroy cancer cells (Aguilar-Cazares *et al.,* 2019; Greten and Grivennikov, 2019). Targeting pro-inflammatory mediators, such as COX, LOX, MPO, iNOS, and NO, and cytokines, such as IL-1β, IL-6, and TNF-α, is a promising strategy for the development of novel anti-inflammatory drugs. Furthermore, prolonged use of anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs), can have adverse effects. Therefore, there is an ongoing need for new drug candidates with minimal side effects. The results showed that ACME effectively inhibited the activities of total COX and 5-LOX in a dose-dependent manner, with comparable effects to diclofenac. ACME also effectively inhibited MPO production in a dose-dependent manner, with maximum inhibition observed at the highest concentration of ACME. In addition, ACME suppressed iNOS production, leading to reduced levels of nitric oxide. These results suggest that ACME has therapeutic potential for modulating inflammation by targeting key enzymes in the arachidonic acid metabolism pathway. These results also indicate that ACME may be a promising candidate for the development of novel anti-inflammatory drugs with minimal side effects.

Early reports showed that procyanidin-rich acetone extract of areca nut (0.1-1µg/ml) significantly downregulated the expression of 12-*O*-tetradecanoylphorbol-13-acetate induced COX-2 by inhibiting extracellular-signal-regulated kinase (ERK) phosphorylation in oral cancer cells. Furthermore, five days of treatment with acetone extract (1 and 10 mg/kg/day) suppressed carrageenan-induced inflammatory oedema and PGE_2 levels in rats (Huang *et al.,* 2010). Khan *et al.* (2011) reported that the methanol extract of areca nut and its water fraction (100 mg/kg) showed significant anti-inflammatory and analgesic effects in mouse and rat models *via* the suppression of PGE_2 and arachidonic acid expression. In addition, the hydroalcoholic

extract of areca nut at doses of 250 and 500 mg/kg suppressed plasma protein extravasation in models of both intravenous and topical nitroglycerin application *via* iNOS inhibition (Bhandare *et al.,* 2011). The action of pro-inflammatory cytokines results in the upregulation of pro-inflammatory genes, such as COX-2 and iNOS, consequently leading to augmented production of the pro-inflammatory mediators, PGE_2 and NO (Ahmad *et al.,* 2015). In the present study, LPS exposure significantly increased the production of IL-1β, IL-6, and TNF-α, intensifying inflammatory responses in HepG2 cells. However, the presence of ACME reduced this LPS-induced effect, suggesting a protective effect of ACME against inflammation in the HepG2 cells.

The effect of ACME on intracellular ROS formation in LPS-stimulated HepG2 cells was investigated using the DCFDA fluorescence dye. The results showed a significant increase in fluorescence intensity following LPS treatment, which was attenuated in the ACME-treated HepG2 cells. To further investigate mitochondrial alterations, changes in mitochondrial membrane potential ($\Delta\psi m$) were examined using Muse™ MitoPotential dye and flow cytometry. Mitochondrial membrane potential (MMP) is a crucial factor in both mitochondrial function and dysfunction, and even subtle changes have a significant impact on cell fate and overall cellular health (Logan *et al.,* 2016). LPS treatment has been shown to decrease MMP in various studies in various cellular systems (Zhang *et al.,* 2017). Therefore, restoration of MMP by ACME treatment may be an important regulatory factor for proper mitochondrial functioning and cell survival. Elevated levels of ROS and reduced glutathione content have been shown to have profound effects on mitochondrial function and various metabolic processes (Calabrese *et al.,* 2017). Glutathione, a natural antioxidant present in its reduced form, collaborates with GSH-Px and glutathione reductase (GR) to form a critical antioxidant defense system. Moreover, SOD and CAT counteract LPS-induced increases in ROS production in HepG2 cells (Chou *et al.,* 2018). MDA is a biomarker of oxidative stress and serves as a reliable indicator of lipid peroxidation (Hsouna *et al.,* 2019). The thiobarbituric acid-reactive substance method was used to assess the cellular MDA levels. When HepG2 cells were treated with LPS, a substantial increase in MDA and a reduction in the activity of antioxidant enzymes such as SOD, CAT, and GSH-Px, along with a significant decrease in GSH, were observed compared to the normal control group. ACME significantly reduced MDA levels, indicating an improvement in overall antioxidant status and a decrease in lipid peroxidation. Moreover, treatment with ACME improved SOD, CAT, GSH-Px, and GSH levels, suggesting that ACME can enhance both non-enzymatic and enzymatic reactions within the antioxidant defense system.The Keap1-Nrf2 pathway serves as a critical defence mechanism against oxidative and electrophilic stress. Keap1, a component of an E3 ubiquitin ligase, typically regulates Nrf2 activity by targeting it for degradation through ubiquitination. However, under stressful conditions, the sensor cysteines in Keap1 enable Nrf2 to evade ubiquitination, accumulate within the cell, and translocate to the nucleus where it activates antioxidant genes (Baird and Yamamoto, 2020). In this study, LPS-induced upregulation of Keap1 and downregulation of Nrf2 were significantly attenuated by ACME treatment.

The bioactivity of areca nuts has been the subject of extensive research, although the results of these studies have often been conflicting, leading to divergent opinions regarding its effects. While some investigations align with the findings of this study, others have proposed a potential association between betel nut consumption and an elevated risk of specific cancers, particularly oral cancer. Consistent comparisons between betel quid consumers and non-users have consistently revealed heightened risks, resulting in its classification as a group 1 carcinogen by the International Agency for Research on Cancer (Warnakulasuriya and Chen, 2022). The risk of oral cancer increases in tandem with the frequency and duration of betel quid consumption. This practice, which typically involves betel leaves, areca nuts, and slaked lime, has been linked to a higher incidence of oral cancers. In regions such as the Indian subcontinent and Taiwan, approximately half of the reported oral cancer cases are attributable to betel quid chewing. However, there are contrasting reports suggesting that specific compounds found in betel nuts, such as polyphenols, tannins, and flavonoids, may possess potential anticancer properties. As evidenced by numerous investigations, these compounds display antioxidant and anti-inflammatory characteristics, suggesting that they may play protective roles against cancer under certain circumstances, which is consistent with the findings of the present study.

5. Conclusion

The defatted methanolic extract of *A. catechu* inflorescence displayed multifaceted action in LPS-induced HepG2 cancer cell lines, showing cytotoxic, apoptotic, anti-inflammatory, and oxidative stress-reducing potentials. This underscores the importance of exploring botanical sources for novel treatments and offers hope for the relentless pursuit of effective cancer therapies.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

References

Abbas, G.; Kashif, M.; Mudassar, T.A.K.; Bhatti, H.A.; Haque, S.; Naqvi, S. and Farooq, A.D. (2018). Pak. J. Pharm. Sci., **31**(2):385-392.

Agergaard, N. and Jensen, P.T. (1982). Procedure for blood glutathione peroxidase determination in cattle and swine. Acta Vet. Scand., **23**(4):515-527. https://doi.org/10.1186/bf03546770

Aguilar-Cazares, D.; Chavez-Dominguez, R.; Carlos-Reyes, A.; Lopez-Camarillo, C.; Hernadez de la Cruz, O.N. and Lopez-Gonzalez, J.S. (2019). Contribution of angiogenesis to inflammation and cancer. Front. Oncol., **9**:1399. https://doi.org/10.3389/fonc.2019.01399

Ahmad, S.F.; Attia, S.M.; Bakheet, S.A.; Zoheir, K.M.A.; Ansari, M.A.; Korashy, H.M.; Abdel-Hamied, H.E.; Ashour, A.E. and Abd-Allah, A. R. A. (2015). Naringin attenuates the development of carrageenan-induced acute lung inflammation through inhibition of NF-κB, STAT3 and pro-inflammatory mediators and enhancement of IλBα and anti-inflammatory cytokines. Inflammation, **38**(2):846-857. https://doi.org/10.1007/s10753-014-9994-y

Amudhan, M.S.; Begum, V.H. and Hebbar, K.B. (2012). A review on phytochemical and pharmacological potential of *Areca catechu* L. seed. Int. J. Pharm. Sci. Res., **3**:4151-4157. http://dx.doi.org/10.13040/IJPSR.0975-8232.3(11).4151-57

Arif, S.; Sharma, A. and Islam, M.H. (2022). Plant derived secondary metabolites as multiple signalling pathways inhibitors against cancer. Ann. Phytomed., **11**(1):189-200. : http://dx.doi.org/10.54085/ap.2022.11.1.18

Axelrod, B.; Cheesbrough, T.M. and Laakso, S. (1981). Lipoxygenase from soybeans. In: Methods in enzymology (ed. Hash, J.H.), Vol. **71**, Elsevier, pp:441-451. https://doi.org/10.1016/0076-6879(81)71055-3

Baird, L. and Yamamoto, M. (2020). The molecular mechanisms regulating the Keap1-Nrf2 pathway. Mol. Cell Biol., **40**(13):e00099-20. https://doi.org/10.1128/MCB.00099-20

Benegal, V.; Rajkumar, R.P. and Muralidharan, K. (2008). Does areca nut use lead to dependence? Drug Alcohol Depend., **97**(1-2):114-121. https://doi.org/10.1016/j.drugalcdep.2008.03.016

Bhandare, A.; Kshirsagar, A.; Vyawahare, N.; Sharma, P. and Mohite, R. (2011). Evaluation of anti-migraine potential of *Areca catechu* to prevent nitroglycerin-induced delayed inflammation in rat meninges: Possible involvement of NOS inhibition. J. Ethnopharmacol., **136**(1):267-270. https://doi.org/10.1016/j.jep.2011.04.039

Bradley, P.P.; Priebat, D.A.; Christensen, R.D. and Rothstein, G. (1982). Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker. J. Invest. Dermatol., **78**(3):206-209. https://doi.org/10.1111/1523-1747.ep12506462

Bryan, N.S. and Grisham, M.B. (2007). Methods to detect nitric oxide and its metabolites in biological samples. Free Radic. Biol. Med., **43**(5):645-657. https://doi.org/10.1016/j.freeradbiomed.2007.04.026

Caius, J.F. (1934). The medicinal and poisonous palms of India. J. Bombay Nat. Hist. Soc., **37**:917-941.

Calabrese, G.; Morgan, B. and Riemer, J. (2017). Mitochondrial glutathione: Regulation and functions. Antioxid Redox Signal., **27**(15):1162-1177. https://doi.org/10.1089/ars.2017.7121

Chen, L.; Deng, H.; Cui, H.; Fang, J.; Zuo, Z.; Deng, J.; Li, Y.; Wang, X. and Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. Oncotarget, **9**(6):7204-7218. https://doi.org/10.18632/oncotarget.23208

Chithra, M.A.; Ijinu, T.P.; Kharkwal, H.; Sharma, R.K.; Pushpangadan, P. and George, V. (2020). Phenolic rich *Cocos nucifera* inflorescence extract ameliorates inflammatory responses in LPS-stimulated RAW264.7 macrophages and toxin-induced murine models. Inflammopharmacology, **28**(4):1073-1089. https://doi.org/10.1007/s10787-019-00620-6

Chou, S. T.; Lai, C. C.; Lai, C. P. and Chao, W. W. (2018). Chemical composition, antioxidant, anti-melanogenic and anti-inflammatory activities of *Glechoma hederacea* (Lamiaceae) essential oil. Ind. Crops Prod., **122**:675-685. https://doi.org/10.1016/j.indcrop.2018.06.032

Dasgupta, R.; Saha, I.; Pal, S.; Bhattacharyya, A.; Sa, G.; Nag, T.C.; Das, T. and Maiti, B.R. (2006). Immunosuppression, hepatotoxicity and depression of antioxidant status by arecoline in albino mice. Toxicology, **227**(1-2):94-104. https://doi.org/10.1016/j.tox.2006.07.016

Fan, J.; Lin, R.; Xia, S.; Chen, D.; Elf, S. E.; Liu, S.; Pan, Y.; Xu, H.; Qian, Z.; Wang, M.; Shan, C.; Zhou, L.; Lei, Q.-Y.; Li, Y.; Mao, H.; Lee, B.H.; Sudderth, J.; DeBerardinis, R.J.; Zhang, G.; Owonikoko, T.; Gaddh, M.; Arellano, M.L.; Khoury, H.J.; Khuri, F.R.; Kang, S.; Doetsch, P.W.; Lonial, S.; Boggon, T.J.; Curran, W.J. and Chen J. (2016). Tetrameric acetyl-CoA acetyltransferase 1 is important for tumor growth. Mol. Cell, **64**(5):859-874. https://doi.org/10.1016/j.molcel.2016.10.014

Furman, D.; Campisi, J.; Verdin, E.; Carrera-Bastos, P.; Targ, S.; Franceschi, C.; Ferrucci, L.; Gilroy, D.W.; Fasano, A.; Miller, G.W.; Miller, A.H.; Mantovani, A.; Weyand, C.M.; Barzilai, N.; Goronzy, J. J.; Rando, T. A.; Effros, R.B.; Lucia, A.; Kleinstreuer, N. and Slavich, G.M. (2019). Chronic inflammation in the etiology of disease across the life span. Nat. Med., **25**(12):1822-1832. https://doi.org/10.1038/s41591-019-0675-0

González-Montelongo, R.; Gloria Lobo, M. and González, M. (2010). Antioxidant activity in banana peel extracts: Testing extraction conditions and related bioactive compounds. Food Chem., **119**(3):1030-1039. https://doi.org/10.1016/j.foodchem.2009.08.012

Greten, F.R. and Grivennikov, S.I. (2019). Inflammation and cancer: Triggers, mechanisms, and consequences. Immunity, **51**(1):27-41. https://doi.org/10.1016/j.immuni.2019.06.025

Haida, Z. and Hakiman, M. (2019). A comprehensive review on the determination of enzymatic assay and nonenzymatic antioxidant activities. Food Sci. Nutr., **7**(5):1555-1563. https://doi.org/10.1002/fsn3.1012

Heatubun, C. D.; Dransfield, J.; Flynn, T.; Tjitrosoedirdjo, S.S.; Mogea, J. P. and Baker, W. J. (2012). A monograph of the betel nut palms (*Areca*: Arecaceae) of East Malesia: Monograph of East Malesian*Areca*. Bot. J. Linn. Soc., **168**(2):147-173. https://doi.org/10.1111/j.1095-8339.2011.01199.x

Holdsworth, D.K.; Jones, R.A. and Self, R. (1998). Volatile alkaloids from *Areca catechu*. Phytochemistry, **48**(3):581-582. https://doi.org/10.1016/s0031-9422(98)00016-8

Hossen, M.S.; Tanvir, E.M.; Prince, M.B.; Paul, S.; Saha, M.; Ali, M.Y.; Gan, S.H.; Khalil, M.I. and Karim, N. (2017). Protective mechanism of turmeric (*Curcuma longa*) on carbofuran-induced hematological and hepatic toxicities in a rat model. Pharm. Biol., **55**(1):1937-1945. https://doi.org/10.1080/13880209.2017.1345951

Hsouna, A.B.; Gargouri, M.; Dhifi, W.; Ben Saad, R.; Sayahi, N.; Mnif, W. and Saibi, W. (2019). Potential anti inflammatory and antioxidant effects of *Citrus aurantium* essential oil against carbon tetrachloride mediated hepatotoxicity: A biochemical, molecular and histopathological changes in adult rats. Environ. Toxicol., **34**(4):388-400. https://doi.org/10.1002/tox.22693

Huang, P.L.; Chi, C.W. and Liu, T.Y. (2010). Effects of *Areca catechu* L. containing procyanidins on cyclooxygenase-2 expression *in vitro* and *in vivo*. Food Chem. Toxicol., **48**(1):306-313. https://doi.org/10.1016/j.fct.2009.10.014

Ijinu, T. P.; Latha, P. G.; George, V. and Pushpangadan, P. (2016). Total phenolic and flavonoid contents and *in vitro* antioxidant potential of methanolic extract of *Blepharismaderaspatensis* (L.) B. Heyne ex Roth whole plant. Ann. Phytomed., **5**(1):85-90.

Ijinu, T.P.; Manikantan, K.; Aneeshkumar, A.L.; Soorya, V.; Latha, P.G.; George, V. and Pushpangadan, P. (2022). Ameliorative effect of the ethnomedicinal plant *Blepharismaderaspatensis* (L.) B.Heyne ex Roth on lipopolysaccharide-induced acute liver inflammation and oxidative stress in Wistar rat model. Phytomed. Plus, **2**(2), 100247. https://doi.org/10.1016/j.phyplu.2022.100247

Ijinu, T.P.; Sasikumar, P.; Aswany, T.; Jijymol, K.K.; Mustak, M.S. and Pushpangadan, P. (2023). Phytochemical constituents and pharmacology of *Areca catechu* L., In: Phytochemical composition and pharmacy of medicinal plants (ed. Pullaiah, T.), vol. 2, Apple Academic Press, USA, pp:357-371.

Kang, M.A.; So, E.-Y.; Simons, A.L.; Spitz, D.R. and Ouchi, T. (2012). DNA damage induces reactive oxygen species generation through the H2AX-Nox1/Rac1 pathway. Cell Death Dis., **3**(1):e249-e249. https://doi.org/10.1038/cddis.2011.134

Kay, J.; Thadhani, E.; Samson, L. and Engelward, B. (2019). Inflammation-induced DNA damage, mutations and cancer. DNA Repair, **83**:102673. https://doi.org/10.1016/j.dnarep.2019.102673

Khan, S.; Mehmood, M.H.; Ali, A.N.A.; Ahmed, F.S.; Dar, A. and Gilani, A.-H. (2011). Studies on anti-inflammatory and analgesic activities of betel nut in rodents. J. Ethnopharmacol., **135**(3):654-661. https://doi.org/10.1016/j.jep.2011.03.064

Krasuska, U.; Ciacka, K.; Orzechowski, S.; Fettke, J.; Bogatek, R. and Gniazdowska, A. (2016). Modification of the endogenous NO level influences apple embryos dormancy by alterations of nitrated and biotinylated protein patterns. Planta, **244**(4):877-891. https://doi.org/10.1007/s00425-016-2553-z

Li, M.; Peng, J.Y.; Wu, Y.F.; Li, J.J. and Cao, Q. (2011). Apoptosis of HaCaT cells induced by arecoline. Int. J. Clin. Exp. Pathol., **31**:282-285.

Li, S.; Hong, M.; Tan, H.-Y.; Wang, N. and Feng, Y. (2016). Insights into the role and interdependence of oxidative stress and inflammation in liver diseases. Oxid. Med. Cell. Longev., **4234061**:1-21. https://doi.org/10.1155/2016/4234061

Lim, D.Y. and Kim, I.S. (2006). Arecoline inhibits catechuolamine release from perfused rat adrenal gland. Acta Pharmacol. Sin., **27**:71-79. 10.1111/j.1745-7254.2006.00233.x

Logan, A.; Pell, V.R.; Shaffer, K.J.; Evans, C.; Stanley, N.J.; Robb, E.L.; Prime, T.A.; Chouchani, E.T.; Cochemé, H.M.; Fearnley, I.M.; Vidoni, S.; James, A.M.; Porteous, C.M.; Partridge, L.; Krieg, T.; Smith, R.A.J. and Murphy, M. P. (2016). Assessing the mitochondrial membrane potential in cells and *in vivo* using targeted click chemistry and mass spectrometry. Cell Metab., **23**(2):379-385. https://doi.org/10.1016/j.cmet.2015.11.014.

Lowry, O.; Rosebrough, N.; Farr, A.L. and Randall, R. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**(1):265-275. https://doi.org/10.1016/s0021-9258(19)52451-6

Mehrotra, N. (2021). Herbs that heal: Nature's pharmacy endowed remedies for better health. Ann. Phytomed., **10**(1):6-22. http://dx.doi.org/10.21276/ap.2021.10.1.2

Molina-Jijón, E.; Zarco-Márquez, G.; Medina-Campos, O.N.; Zataraín-Barrón, Z.L.; Hernández-Pando, R.; Pinzón, E.; Zavaleta, R.M.; Tapia, E. and Pedraza-Chaverri, J. (2012). Deferoxamine pretreatment prevents Cr(VI)-induced nephrotoxicity and oxidant stress: Role of Cr(VI) chelation. Toxicology, **291**(1-3):93-101. https://doi.org/10.1016/j.tox.2011.11.003

Moron, M.; Depierre, J. and Mannervik, B. (1979). Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim. Biophys. Acta - Gen. Subj., **582**(1):67-78. https://doi.org/10.1016/0304-4165(79)90289-7

Ohkawa, H.; Ohishi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., **95**(2):351-358. https://doi.org/10.1016/0003-2697(79)90738-3

Osborne, P.G.; Ko, Y. C.; Wu, M.T. And Lee, C.H. (2017). Intoxication and substance use disorder to *Areca catechu* nut containing betel quid: A review of epidemiological evidence, pharmacological basis and social factors influencing quitting strategies. Drug Alcohol Depend., **179**:187-197. https://doi.org/10.1016/j.drugalcdep.2017.06.039

Peng, W.; Liu, Y.J.; Wu, N., Sun, T., He, X.Y., Gao, Y.X. and Wu, C.J. (2015). *Areca catechu* L. (Arecaceae): A review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. J. Ethnopharmacol., 164:340-356. https://doi.org/10.1016/j.jep.2015.02.010

Pizzino, G.; Irrera, N.; Cucinotta, M.; Pallio, G.; Mannino, F.; Arcoraci, V.; Squadrito, F.; Altavilla, D. and Bitto, A. (2017). Oxidative stress: Harms and benefits for human health. Oxid. Med. Cell. Longev., **8416763**:1-13. https://doi.org/10.1155/2017/8416763

Pulli, B.; Ali, M.; Forghani, R.; Schob, S.; Hsieh, K.L.C.; Wojtkiewicz, G.; Linnoila, J.J. and Chen, J.W. (2013). Measuring myeloperoxidase activity in biological samples. PloS One, **8**(7):e67976. https://doi.org/10.1371/journal.pone.0067976

Pushpangadan, P.; Ijinu, T.P. and George, V. (2015a). Plant based anti-inflammatory secondary metabolites. Ann. Phytomed., **4**(1):17-36.

Pushpangadan, P.; Ijinu, T.P.; Dan, V.M. and George, V. (2015b). Hepatoprotective leads from plants. Ann. Phytomed., **4**(2):4-17.

Raghavan, V. and Baruah, H.K. (1958). Areca nut: India's popular masticatory-history, chemistry and utilization. Econ. Bot., **12**(4):315-345. https://doi.org/10.1007/bf02860022

Salehi, B.; Konovalov, D.A.; Fru, P.; Kapewangolo, P.; Peron, G.; Ksenija, M.S.; Cardoso, S.M.; Pereira, O.R.; Nigam, M.; Nicola, S.; Pignata, G.; Rapposelli, S.; Sestito, S.; Anil Kumar, N.V.; de la Luz Cádiz-Gurrea, M.; Segura-Carretero, A.; Mishra, A.P.; Sharifi-Rad. M.; Cho, W.C.; Taheri, Y.; Setzer, W.N. and Sharifi-Rad, J. (2020). *Areca catechu*-From farm to food and biomedical applications. Phytother. Res., **34**(9):2140-2158. https://doi.org/10.1002/ptr.6665

Salter, M.; Knowles, R.G. and Moncada, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+} dependent and Ca^{2+} independent nitric oxide synthases. FEBS Lett., **291**(1):145-149. https://doi.org/10.1016/0014-5793(91)81123-p

Schmittgen, T.D. and Livak, K.J. (2008). Analysing real-time PCR data by the comparative C(T) method. Nat Protoc., **3**:1101-1108. https://doi.org/10.1038/nprot.2008.73

Sini, S.; Deepa, D.; Harikrishnan, S. and Jayakumari, N. (2018). Adverse effects on macrophage lipid transport and survival by high density lipoprotein from patients with coronary heart disease. J. Biochem. Mol. Toxicol., **32**(9): e22192. https://doi.org/10.1002/jbt.22192

Takahara, S.; Hamilton, H.B.; Neel, J.V.; Kobara, T.Y.; Ogura, Y. and Nishimura, E.T. (1960). Hypocatalasemia: A new genetic carrier state. J. Clin. Investig., **39**(4):610-619. https://doi.org/10.1172/jci104075

Talarico, L.B.; Zibetti, R.G.M.; Faria, P.C.S.; Scolaro, L.A.; Duarte, M.E.R.; Noseda, M.D.; Pujol, C.A. and Damonte, E.B. (2004). Anti-herpes simplex virus activity of sulfatedgalactans from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemiacrenulata*. Int. J. Biol. Macromol., **34**(1-2):63-71. https://doi.org/10.1016/j.ijbiomac.2004. 03.002

Walker, M.C. and Gierse, J.K. (2010). *In vitro* assays for cyclooxygenase activity and inhibitor characterization. In: Cyclooxygenases. Methods in molecular biology (ed. Ayoub, S.; Flower, R. and Seed, M.), vol. 644, Humana Press, pp:131-44. https://doi.org/10.1007/978-1-59745-364-6_11

Wang, C.C.; Huang, P. L.; Liu, T.Y. and Jan, T. R. (2009). Highly oligomeric procyanidins from areca nut induce lymphocyte apoptosis *via* the depletion of intracellular thiols. Toxicol. *In vitro*, **23**:1234-1241. https://doi.org/10.1016/j.tiv.2009.07.033

Warnakulasuriya, S. and Chen, T.H.H. (2022). Areca nut and oral cancer: Evidence from studies conducted in humans. J. Dent. Res., **101**(10):1139-1146. https://doi.org/10.1177/00220345221092751

Weydert, C.J. and Cullen, J.J. (2010). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat. Protoc., **5**(1):51-66. https://doi.org/10.1038/nprot.2009.197

Yang, L.; Wu, L.; Wu, D.; Shi, D.; Wang, T. and Zhu, X. (2017). Mechanism of transdermal permeation promotion of lipophilic drugs by ethosomes. Int. J. Nanomed., **12**:3357-3364. https://doi.org/10.2147/ijn.s134708

Young, D.; Fan, M. Z. and Mine, Y. (2010). Egg yolk peptides up-regulate glutathione synthesis and antioxidant enzyme activities in a porcine model of intestinal oxidative stress. J. Agric. Food Chem., **58**(13):7624-7633. https://doi.org/10.1021/jf1011598

Zhang, J.H.; Yu, J.; Li, W.X. and Cheng, C.P. (1998). Evaluation of Mn^{2+} stimulated and Zn^{2+} inhibited apoptosis in rat corpus luteal cells by flow cytometry and fluorochromes staining. Chin. J. Physiol., **41**(2):121-126.

Zhang, M.; Pan, H.; Xu, Y.; Wang, X.; Qiu, Z. and Jiang, L. (2017). Allicin decreases lipopolysaccharide-induced oxidative stress and inflammation in human umbilical vein endothelial cells through suppression of mitochondrial dysfunction and activation of Nrf2. Cell. Physiol. Biochem., **41**(6):2255-2267. https://doi.org/10.1159/000475640

Zhang, X.; Mei, W.L.; Zeng, Y.B.; Liu, J.; Dai, W.J. and Dai, H.F. (2009). Phenolic constituents from the fruits of *Areca catechu* and their anti-bacterial activities. J. Trop. Subtrop. Bot., **17**:74-76.

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