Cytotoxic, embryotoxic, insecticidal and anti-microbial activities of standardized *Areca catechu* nut

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**Abstract:** The study aimed at evaluating various biological actions of widely consumed *Areca catechu* nut. The nut’s ethanolic extract exhibited cytotoxicity (lung cancer cell line), embryotoxicity (chick embryo), phytotoxicity (*Lemna minor*), insecticidal (*Rhyzopertha dominica*), anti-bacterial (*Pseudomonas aeruginosa*), anti-fungal (*Microsporum canis*) and mitogenic (human blood lymphocytes) actions. The standardization results revealed presence of 1.7 µg arecoline per mg of extract. In conclusion, the *Areca* nut is endowed with both harmful and beneficial biological actions. Keeping in view its wide consumption and ease of availability, the aforesaid information should be channelized for health and agricultural benefits.

**Keywords:** Areca nut, cytotoxicity, embryotoxin, insecticidal, weedicide, anti-microbial.

**INTRODUCTION**

Medicinal plants have been used for treatment of various diseases for thousands of years such as use of licorice (*Glycyrrhiza glabra*) and poppy capsule latex (*Papaver somniferum*) were found on the clay tablets of Mesopotamia (Chin et al., 2006). They also provide potential lead compounds used in modern allopathic medicines such as morphine (Brownstein, 1993), acetylsalicylic acid (Mahdi et al., 2006) and taxol (*Taxus brevifolia*) (Wall and Wani, 1996). The use of herbs constitutes major part of Traditional Chinese Medicine (TCM), Indian medicine (Ayurveda) and Unani system (Eastern medicine). In addition to these well established systems, use of alternative medicine is expanding rapidly (Shankar and Liao, 2004, Singh, 2005) and WHO estimates that approximately 80% of global population rely on it (Cass, 2004).

The *Areca catechu* nut is a popular chewing nut used for mastication in various parts of the world, including Indo-Pak subcontinent. In traditional remedies, Areca nut is used for helminthes, visceral infections, diarrhea, dysentery, burns, ulcers, cardiac and nerve strength (Raghavan and Baruah, 1958). Additionally, the nut has been reported to possess various pharmacological actions such as anti-depressant (Abbas et al., 2013, Dar and Khatoon, 1997, Dar and Khatoon, 2000), learning and memory enhancer (Nieschulz, 1967), anti-inflammatory and analgesic (Khan et al., 2011), anti-oxidant (Kumar et al., 2012), anti-hyperlipidemic (Jeon et al., 2000), hypoglycemic (Huang et al., 2013), immunomodulator (Chang et al., 2006) as well as anthelmintic (Tye and Nelson, 1952). However, it has also been associated with various adverse effects such as genotoxicity (Lai and Lee, 2006), embryotoxicity (Sinha and Ramesha Rao, 1985) and carcinogenicity (Tseng et al., 2013). The phytochemical analysis of *Areca* nuts indicated the presence of alkaloids, polyphenols, fats, saponins, carbohydrates, amino acids and minerals. Among which, arecoline (alkaloid) in particular has been considered as an active constituent underlying most of the biological activities (Nelson and Heischober, 1999).

In current study, ethanolic extract of *A. catechu* nut was evaluated for cytotoxic, embryotoxic, insecticidal, phytotoxic and anti-microbial actions.

**MATERIAL AND METHODS**

**Chemicals**

The following chemicals were used: Fetal bovine serum, imipenum, parquat, penicillin, streptomycin, potassium chloride, RPMI-1640, sulforhodamine-B, tris-base and trichloroacetic acid (Sigma, USA); Colcemid and phytohemaglutin (Gibco, USA); Glacial acetic acid and methanol (Lab scan) and Geimsa stain (Invitrogen, USA). Ultra pure distilled water was used during the study.

**Extraction of *Areca catechu* nut**

*A. catechu* nuts (10kg) were purchased from the local market (Firdous market, Lalo-khait, Karachi, Pakistan). After grinding, powdered nuts (9.5kg) were soaked (6 days) in 17 liters of ethanol-water (7:3), shaken daily and observed for any fungal growth. After filtration (muslin cloth), rotary evaporation was performed to concentrate the filtrate which was freeze-dried yielding ethanolic extract (319g), as described earlier (Abbas et al., 2013).
The ethanolic extract was standardized against arecoline. Standardization of Areca catechu nut ethanolic extract. Cytotoxic, embryotoxic, insecticidal and anti-microbial activities of standardized Areca catechu nut extract and arecoline were prepared at the strengths of 180:20) was used at flow rate of 0.5 ml/min. Briefly, the extract and arecoline were prepared at the strengths of 1 mg/ml and 0.4mg/ml, respectively followed by injection (5 µl) into HPLC for analysis. The area under the curve (AUC) was used to quantify the amount of arecoline present in the extract.

Brine shrimp (Artemia salina) lethality assay
Areca nut extracts (10, 100 and 1000µg/ml) were prepared in 5ml of brine containing 10 shrimps each. The vials were incubated (37°C) for 24h followed by counting of survivors. The anticancer drug etoposide (podophyllotoxin, 1-10 µg/ml) was used as positive control. All doses were tested in triplicate (Meyer et al., 1982).

Cytotoxicity assay
Human lung cancer cell line (NCI-H460) was used to assess cytotoxicity using sulforhodamine-B assay (Skehan et al., 1990, Monks et al., 1991). The cells (10000 cells/100µL) were plated in 96-well plate, incubated (37 °C in a humidified 5% CO₂) for 24 h to obtain monolayer. Different concentrations of extract (1, 10, 50, 100 and 250 µg/mL) were added in each well and incubated. After 48 h, cold TCA (50µL, 50%) was added gently and left at room temperature for 30 minutes. This was followed by washing with distilled water and air dried overnight. The SRB solution (0.4 % w/v in 1% acetic acid) was added to each well. After 10 min, the unbound stain was washed with acetic acid (1%) and left for drying at room temperature. The protein bound stain was solubilized with tris-base (100µL/well, pH 10.2), shaken (5 minutes) and absorbance was measured at 515 nm, using a micro plate reader.

Mitotic index assay using human lymphocytes
The assay was performed as described earlier (Eroglu et al., 2010, Moorhead et al., 1960). Human venous blood (5 ml) was collected from healthy individuals in the sodium heparin vacutainer. The blood (0.5 ml) was transferred to the tubes containing supplemented RPMI-1640 (87.5%), 1 % of L-glutamine (2mM), penicillin (100U/ml), streptomycin (100µg/ml), fetal bovine serum (10%) and phytohemaglutinin (1.5%). After incubation for 24h, different concentrations of Areca nut ethanolic extract (1, 10 and 25µg/ml) were added. After 46h of incubation, colcemid solution (10µg/ml, 100µl) was introduced in each tube, mixed well and incubated for further 2h. Centrifugation (1000 rpm for 8 minutes) was performed and pellet was re-suspended in pre-warmed (37°C) hypotonic solution (KCl solution 75mM, 5ml) with continuous shaking to avoid clumping. After incubation at 37°C for 15 minutes, it was centrifuged and pre-cold fixative solution (methanol: glacial acetic acid, 3:1) was added to pellet (5ml) with shaking followed by centrifugation (1000 rpm for 8 minutes). This step was repeated till the pellet was clear. Finally, it was re-suspended in fixative solution (0.5ml) and 2-3 drops were dropped from a distance of 1 feet, at an angle of 45° onto a pre-cleaned and chilled glass slides. After drying, slides were air dried and stained with Giemsa (2%) for 5 minutes. A proportion of metaphases were observed microscopically, counted and mitotic index was calculated as follows:

\[
\text{Mitotic index (MI)} = \frac{\text{Number of metaphase stage}}{\text{Total number of lymphocytes nuclei}} \times 100
\]

Embryotoxicity assay
Chick embryotoxicity was carried out according to (Gilani and Chatzinoff, 1981, Henshel et al., 2003). Briefly, the egg shell surface at the blunt end of fertilized chicken egg (Babcock B 300V) was cleaned with 70% ethanol prior to administration of Areca nut extract (0.3, 0.6 and 0.9mg/gm of egg) using airspace technique. After 48h of incubation, the embryos were mounted (0.9% saline) as per protocol outlined (Henshel et al., 2003) and observed for gross developmental abnormalities using microscope.

Insecticidal assay
The contact toxicity method (impregnated filter paper test) was used as described earlier (Sighamony et al., 1986). Briefly, the Areca nut extract (1000µg/cm²) and permethrin (239.5µg/cm²) were loaded on the filter paper, placed in Petri plates and the solvent (ethanol) was allowed to evaporate. After 24h, the insects (Callosbruschus analis, Rhizopertha dominica, Sitophilus oryzae and Tribolium castanum insects) of uniform age and size were placed on filter paper. After 24 h of incubation growth chamber (27°C and 50% humidity), mortality was noted.

Phytoxic assay
The Lemma minor (Duckweed) bioassay was used for phytoxic evaluation (Mclaughlin et al., 1998) of Areca nut ethanolic extract. Briefly, 10 plants (containing 2-3 rosettes) were subjected to nutrient medium containing nut extract (10, 100 or 1000µg/ml) and placed in the growth chamber (27±1°C). On 7th day, the numbers of fronds were counted.

Antibacterial and antifungal assays
The agar well diffusion assay (Holder and Boyce, 1994) was performed to evaluate the effect on Areca nut on Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Shigella flexenari and
Salmonella typhi. The nutrient broth was inoculated with respective bacterial culture. After 24h of incubation, broth (0.6ml) was mixed with molten agar (60ml) and poured in Petri plates. After solidification, the wells were made with the help of sterile cork borer. The extract (300 µg/100 µl) was poured into these wells and incubated. After 24h, the zone of inhibition was measured. A broad spectrum β-lactam antibiotic, imipenem (10 µg/disc) was used as standard drug.

STATISTICAL ANALYSIS

The data is represented as mean ± SEM. Differences between various means were computed by one-way ANOVA using SPSS 10. Asterisk(s) indicate levels of significance i.e. *p<0.05, **p<0.01 and ***p<0.005 as compared to respective control.

RESULTS

Standardization of Areca catechu nut ethanolic extract

The retention time of arecoline was found to be 1.15 minutes (fig. 1). The extract contained 1.7 µg of arecoline per mg of extract.

Table 1: Growth inhibitory and cytotoxic effects of A. catechu nut extract against human lung cancer cell line (NCI-H460).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Growth inhibition (+)/Cytotoxic (-) (%)</th>
<th>GI50 (µg/ml)</th>
<th>TGI (µg/ml)</th>
<th>LC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areca nut extract (µg/ml)</td>
<td>1.0</td>
<td>+1.0 ± 0.1</td>
<td>60 ± 2.0</td>
<td>90 ± 3.0</td>
<td>250 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+2.0 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>+33 ± 2.0***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-16 ± 2.5***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-50 ± 3.0***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin (µM)</td>
<td>0.01</td>
<td>+1.0 ± 1.0</td>
<td>0.3 ± 0.08</td>
<td>3.8 ± 0.9</td>
<td>9.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>+37 ± 9.0**</td>
<td>(0.17 ± 0.05)</td>
<td>(2.2 ± 0.7)</td>
<td>(5.4 ± 0.7)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>+81 ± 8.0***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-27±3.0***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-54 ± 2.0***</td>
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</tr>
</tbody>
</table>

GI50 = 50 % growth inhibition of the cells, TGI = Total growth inhibition, LC50 = 50 % killing of the cells, Asterisk indicates level of significance (p< 0.01** and p< 0.001***). For comparison with the Areca extract, values within parenthesis have been expressed in µg/ml.

Table 2: Effect of A. catechu nut ethanolic extract on the mitotic index of human lymphocytes

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Number of metaphases</th>
<th>Mitotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>220</td>
<td>3.6 ± 0.12**</td>
</tr>
<tr>
<td>10</td>
<td>250</td>
<td>4.1 ± 0.21***</td>
</tr>
<tr>
<td>25</td>
<td>253</td>
<td>4.2 ± 0.06***</td>
</tr>
</tbody>
</table>

Total cells observed = 6000, Asterisk indicates level of significance (p<0.01** and p<0.001*** as compared to mitotic index of control (2.6 ± 0.09%)

Table 3: Effect of A. catechu nut ethanolic extract on chick embryo toxicity

<table>
<thead>
<tr>
<th>Growth indicators</th>
<th>A. catechu nut ethanolic extract (mg) / gm of egg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Somites number (pairs)</td>
<td>19</td>
</tr>
<tr>
<td>Heart</td>
<td>+</td>
</tr>
<tr>
<td>Curvature</td>
<td>+</td>
</tr>
<tr>
<td>Optic cup</td>
<td>+</td>
</tr>
</tbody>
</table>

Present (+) or absent (-)
Cytotoxic, embryotoxic, insecticidal and anti-microbial activities of standardized Areca catechu nut

Fig. 1: Standardization of Areca catechu nut ethanolic extract against arecoline. The Areca nut ethanolic extract was standardized against arecoline using HPLC-DAD. Chromatograms of: (a) Standard arecoline (1 mg/2.5 ml, retention time = 1.15 min) and (b) Areca nut extract (1 mg/1 ml) showing an arecoline peak at 1.15 min, which is equivalent to 1.7 µg per mg of extract.

Fig. 2: The effect of Areca catechu nut on chicken embryos. The figure depicted the effect of A. catechu on chicken embryo development at 48 hrs / chicken embryo observations at 48 showing: a) Somites number (19 pairs in control and 9 pairs in treated), b) optic bud and c) curvature. The Areca nut treatment causes significant reduction in somites number, while the curvature and optic nerve failed to develop.
Brine shrimp lethality
The highest tested dose of Areca nut extract i.e. 1000 µg/ml caused 40% lethality. Hence, the LD₅₀ is greater than 1000 µg/ml. The standard drug etoposide exhibited an LD₅₀ value of 7.5µg/ml.

Cytotoxicity of NCI-H460 cell line
Areca nut extract did not cause toxicity at low concentrations (1 and 10µg/ml), however, at 50 and 100 µg/ml, it inhibited the growth of cells (GI₅₀ =60µg/ml; table 1). The total growth inhibition (TGI) was observed at 90µg/ml, whereas LC₅₀ was found to be 250µg/ml. The anticancer drug doxorubicin appeared to be 27 times more cytotoxic than Areca nut extract.

Mitotic index of human lymphocytes
The Areca nut ethanolic extract (1, 10 and 25µg/ml) caused dose dependent increase (1.38x, 1.57x and 1.6x) in the mitotic index of human whole blood lymphocytes as compared to control (2.6±0.09 %, table 2).

Chick embryotoxicity
At the given dose of 0.3mg/gm of egg, no toxic effect was seen in chick embryo. The appearance of somite (bilaterally paired blocks of mesoderm along the neural tube which gives rise to vertebral column, skeletal muscle, cartilage, tendons and skin) number, heart, curvature and optic nerve were similar to that of control. However, morphological abnormalities were evident at 0.6 and 0.9 mg after 48 hrs of treatment. Somite number was found to be reduced by 26% and 52%, respectively. At both doses, the heart, curvature and optic nerve failed to develop (table 3; fig. 2).

Insecticidal activity
The Areca nut extract (1000µg/cm²) caused 25 and 50% mortality of T. castaneum and R. dominica insects, respectively. No mortality was observed in C. analis and S. oryzae.

Phytotoxicity of lemma minor
At given dose of 10µg/ml, the extract did not affect the number of fronds. However, respective inhibition of 35 and 50% was observed at 100 and 1000µg/ml.

Antibacterial and antifungal activities
The Areca nut extract had no effect on S. typhi and S. aureus. However, zone inhibition of 9mm against B. subtiliss and approximately 15mm against E. coli, P. aeruginosa, and S. flexenari was observed.

In case of fungi, Areca nut extract (400µg/ml) caused percent inhibition of ~25% against A. flavus and F. solani; while 35% for M. canis. It had no effect on the growth of C. albicans and C. glaberata.

DISCUSSION
A. catechu nut is the 4th most commonly used psychoactive substance in the world (Gupta and Ray, 2004). An approximately 600 million people use it in different parts of the world, especially Indo-Pakistan subcontinent (Nelson and Heischober, 1999). Keeping in view its wide consumption and availability, the present study was aimed at evaluating its potential interaction with biological systems.

Lung cancer is a leading cause of death worldwide with an estimate of one million deaths per year (Sun et al., 2007). Therefore, the Areca nut extract was also screened for its cytotoxic activity against human lung cancer line (NCI-H460). The extract appeared to be active against lung cancer with an LC₅₀ value of 250 µg/ml (table 1) and merits further investigation for identification of lead molecules and its mode of action. Notably, plants are an important source of several clinically used anticancer drugs such as vinblastine and vincristine (Catharanthus roseus L.), topotecan and irinotecan (Typhonium divaricatum) etoposide (Podophyllum) and paclitaxel (Taxus brevifolia Nutt) (Cragg and Newman, 2005). Literature revealed that Areca nut is a carcinogen; the action primarily attributed to nitrosamines (Prokopczyk et al., 1988), copper (Raja et al., 2007) and aflatoxins (Chaturvedi and Chaturvedi, 1995). This is in contradiction with our findings against NCI-H460 cell line. One possibility for this outcome is that the nuts used in our work were free from fungal contamination and copper contents. Furthermore, it did not underwent the chewing process, which is required for formation of nitrosamines (Prokopczyk et al., 1988). Moreover, the presence of both pro-cancer and anti-cancer constituents cannot be ignored and merits further investigation for isolation and identification of anticancer agents.

Mitotic index is commonly used to measure cellular proliferation. Any change in this index is suggestive of cellular toxicity. In present study conducted on human blood lymphocyte (Kulling et al., 1999), the enhanced mitotic index, as evident by concentration dependent increase in metaphase, was observed (table 2). Literature revealed the aneugenic class of anticancer drugs (e.g. melphalan) has the ability to increase mitotic index (Efthimiou et al., 2013). Hence, it can be presumed that Areca nut possesses aneugenic contents and merits further investigation for their identification.

Brine shrimp lethality assay, is a widely used test to evaluate the cytotoxic potential of substances (Meyer et al., 1982). In this test, the Areca nut ethanolic extract was found to be non-toxic as suggested by high LD₅₀ (>1000 µg/ml). Keeping in view aforementioned effect against cancer cell line, this outcome is suggestive of differential effect of Areca nut against normal and cancerous cell.
Areca nut is reported to be an embryotoxin via delaying skeletal maturity (Sinha and Ramesha Rao, 1985). More recently, the Areca nut alkaloid i.e. arecoline was reported to inhibit the myogenic differentiation thereby leading to births with lower weights (Chang et al., 2012). In similar lines, our data also showed growth retardation of chick embryos (table 3, fig. 2). Literature revealed that the essential oils can be attribute to the embryotoxic effect of plants (Domaracky et al., 2007), thereby emphasizing detailed studies on Areca nut essential oils and their effect on chick and rodent development. Importantly, several plants such as Ginkgo biloba (Baron-Ruppert and Luepke, 2001), Indigofera suffruticosa (Leite et al., 2004) and Lantana camara (Mello et al., 2005) exhibited toxic effect on the growth and development of embryo and hence their consumption should be either minimized or avoided during pregnancy.

Pests are one of the important threats to the stored dietary substances such as Rhizopertha dominica (grain pest) (Oppert and Morgan, 2013). Our data revealed that it is sensitive to the Areca nut extract. Hence, the nut offers economical solution towards pest control in warehouse. Literature revealed that the essential oils (Kim et al., 2003) and toxic proteins i.e. lectins (Carlini and Grossi-De-Sá, 2002) possesses insecticidal action. Furthermore, poor agricultural productivity / yield are also attributed to weeds. Areca nut extract inhibited growth of Lemma minor weed with an IC$_{50}$ value of 1000 µg/ml (Mclaughlin et al., 1998), thereby providing a useful means of natural nut extract weedicide.

Microbes (bacteria and fungi) are a cause for large number of diseases in humans, animals and plants. With the advent of penicillin (1928) from mold Penicillium, natural products have led to discovery of many antimicrobial agents (Shahid et al., 2009). Among 109 new antibacterial drugs, approved in the period 1981-2006, ~70% originated from natural products (Newman, 2008). Keeping in view the emergence of new pathogens and their ability to develop drug resistance, antimicrobial drug development is the continuous process. In the present study, nut extract was found to be most effective (50 % of standard imipenem) against P. aeruginosa thereby demonstrating its potential against pneumonia and nosocomial infections (Suzuki et al., 2013). In similar lines, Areca nut was reported to be active against Streptococcus mutans via anti-glucosyltransferase activity and mediated by fatty acids and procyanidins contents (Hada et al., 1989). In case of antifungal activity, extract (400 µg/ml) was most active against M. canis thereby offering a potential treatment option for tinea capitis (fungal infection of scalp leading to bald patches) (Ginter-Hanselmayer et al., 2004) and may serve as an alternate for healthy hair growth.

REFERENCES


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