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

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Abstract: The study was 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 (Ayuveda) 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 nervine 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, paraquat, permethrin, penicillin/streptomycin, potassium chloride, RPMI-1640, sulforhodamine-B, tris-base and trichloroacetic acid (Sigma, USA); Colcemid and phytohemoglutinnin (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 aareca 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).

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Standardization of Areca catechu nut ethanolic extract

The ethanolic extract was standardized against arecoline using HPLC-DAD (Agilent technologies 1290 infinity, USA) containing rapid resolution HT-XBD C-18 column (3.0x50 mm, 1.8µ). The buffer (water and acetonitrile, 80:20) was used at flow rate of 0.5 ml/min. Briefly, the extract and arec oline 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, 1-10µg/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% CO_2) 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 phytohemoglutinnin (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 (37ºC) hypotonic solution (KCl solution 75mM, 5ml) with continuous shaking to avoid clumping. After incubation at 37ºC 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/cm^2) and permethrin (239.5µg/cm^2) were loaded on the filter paper, placed in Petri plates and the solvent (ethanol) was allowed to evaporate. After 24h, the insects (*Callosbruchus analis, Rhyzopertha dominica, Sitophilus oryzae* and *Tribolium castaneum* 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.

Phytotoxic assay

The *Lemna minor* (Duckweed) bioassay was used for phytotoxic 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, Escheriehia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Shigellaflexenari and*

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

Treatment	Concentration	Growth inhibition (+)/Cytotoxic (-) (%)	GI_{50} (µg/ml)	TGI (µg/ml)	LC_{50} (µg/ml)
Areca nut extract (µg/ml)	1.0 10 50 100 250	+1.0 ± 0.1 +2.0 ± 0.5 +33 ± 2.0*** -16 ± 2.5*** -50 ± 3.0***	60 ± 2.0	90 ± 3.0	250 ± 2.0
Doxorubicin (µM)	0.01 0.1 0.5 5.0 10	+1.0 ±1.0 +37± 9.0** +81± 8.0*** -27±3.0*** -54 ± 2.0***	0.3 ± 0.08 (0.17 ± 0.05)	3.8 ± 0.9 (2.2 ± 0.7)	9.3 ± 1.2 (5.4 ± 0.7)

GI_{50} = 50 % growth inhibition of the cells, TGI = Total growth inhibition, LC_{50} = 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

Concentration (µg/ml)	Number of metaphases	Mitotic index (%)
1	220	3.6 ± 0.12**
10	250	4.1 ± 0.21***
25	253	4.2 ± 0.06***

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

Growth indicators	*A. catechu* nut ethanolic extract (mg) / gm of egg			
	Control	0.3	0.6	0.9
Somites number (pairs)	19	19	14	9
Heart	+	+	-	-
Curvature	+	+	-	-
Optic cup	+	+	-	-

Present (+) or absent (-)

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 Pertri plates. After solidification, the wells were made with the help of sterile cork borer. The extract (300 µg/100µl) was poured into these the wells and incubated. After 24h, the zone of inhibition was measured. A broad spectrum β-lactam antibiotic, imipenem (10µg/disc) was used as standard drug.

The agar tube dilution protocol was performed (Cole, 1994) to observe the antifungal effect of Areca nut ethanolic extract on *Aspergillu sflavus*, *Candida albicans*, *C. glabrata*, *Fusarium solani* and *Microsporum canis*. Briefly, the Sabouraud dextrose agar (4%, 4ml) in screw caped tubes was autoclaved (121°C for 15 minutes), allowed to cool till 50°C and mixed with extract (400 µg/ml). After solidification, the tubes are inoculated with respective inoculums (4 mm diameter) from 7 days old culture. After incubation for 7 days (28-30°C and humidity 40% to 50%), the linear growth (mm) was noted.

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 1.7µg of arecoline per mg of extract.

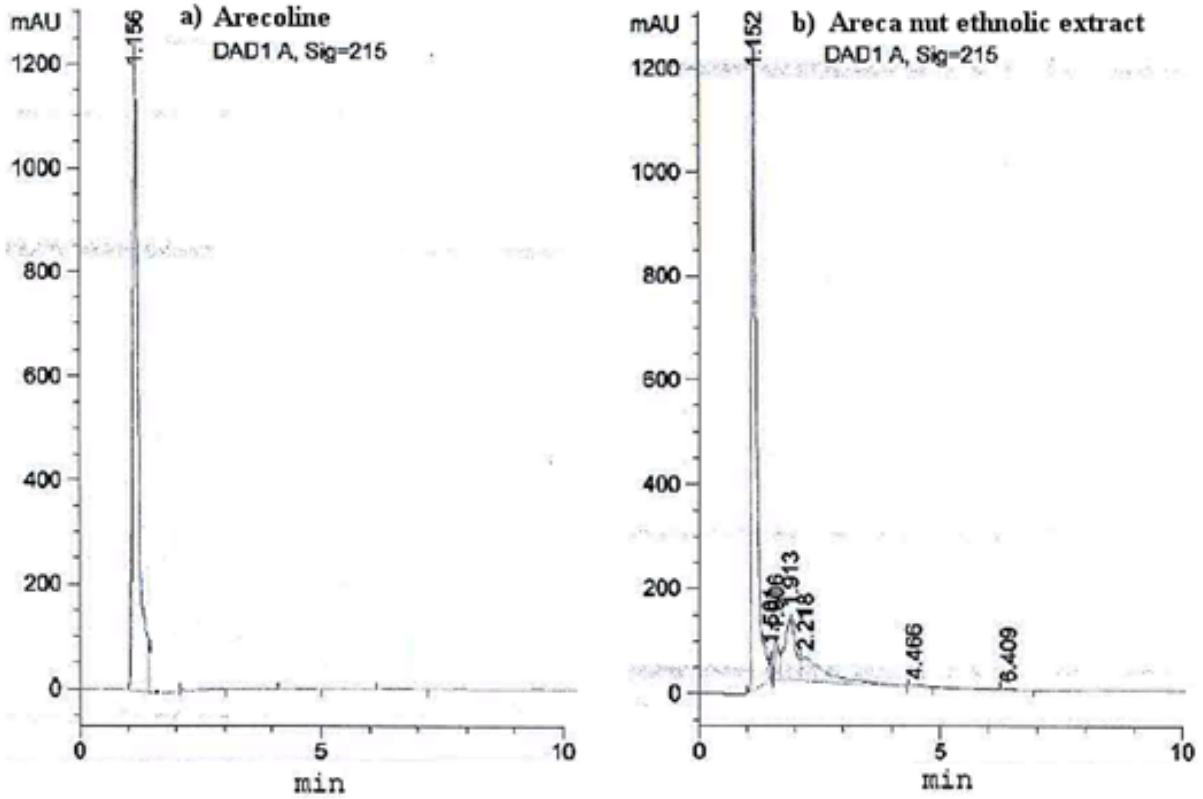

Fig. 1: Standardization of *Areca catechu* nut ethanolic extract against arecoline. The Areca nut ethanolic extract was standardized against arecoline using HPLC-DAD. Chromatograms of: (a) Standard arecoline (1 mg/2.5 ml, retention time = 1.15 min) and (b) Areca nut extract (1 mg/1 ml) showing an arecoline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

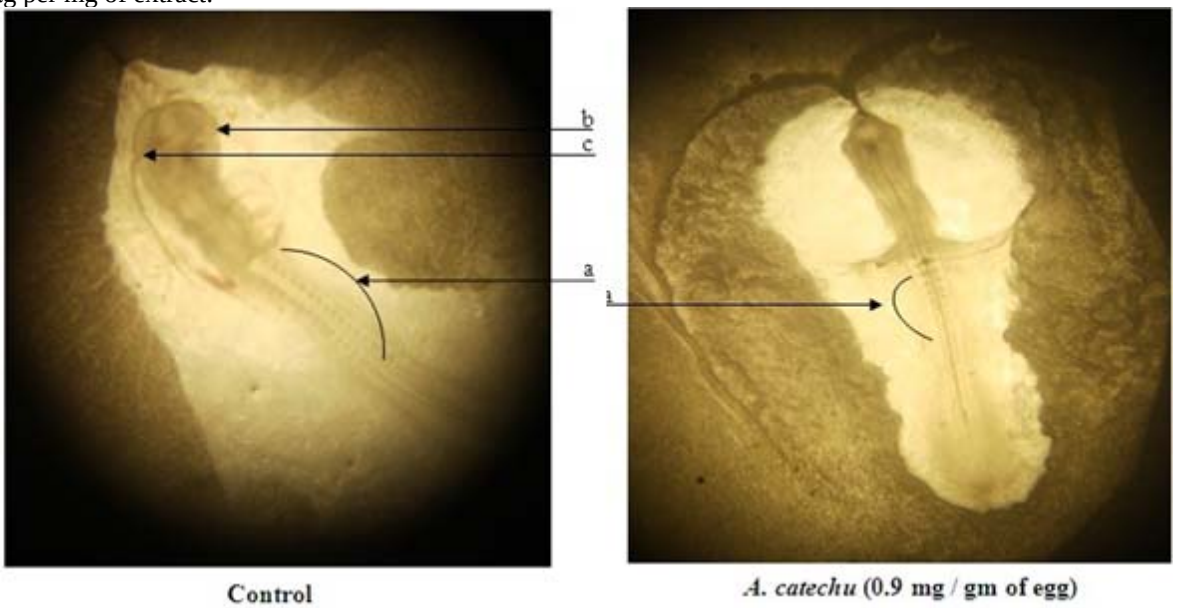

Fig. 2: The effect of *Areca catechu* nut on chicken embryos. The figure depicted the effect of *A. catechu* on chicken embryo development at 48 hrs / chicken embryo observations at 48 showing: a) Somites number (19 pairs in control and 9 pairs in treated), b) optic bud and c) curvature. The Areca nut 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 LD_{50} is greater than 1000 µg/ml. The standard drug etoposide exhibited an LD_{50} 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 (GI_{50} =60µg/ml; table 1). The total growth inhibition (TGI) was observed at 90µg/ml, whereas LC_{50} 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/cm^2) 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 lemna 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. subtillus* 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 LC_{50}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 lympohocyte (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 LD_{50} (>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 *Rhyzopertha 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 *Lemna 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 imipenum) 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.

Areca nut ethanolic extract demonstrated cytotoxic (lung cancer cell line), embryotoxic, weedicidal, insecticidal, antibacterial and antifungal activities. Hence, precautionary measures should be taken by the consumers to minimize associated adverse effects and avoid its use during pregnancy. In parallel, the positive effects should be channelized for health and agriculture benefits. Hence, widely consumed and easily available nut is biologically active and merits thorough investigations for isolation and identification of underlying active principles.

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