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Original Article

ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF *ARECA CATECHU* SEED EXTRACT IN SWISS ALBINO MICE USING EAC CELL LINE IN DIFFERENT CULTURE MEDIUM

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ABSTRACT

Objective: Areca nut is the dried ripe seeds of *Areca catechu*, belonging to family Palmae. Areca nut contains a number of alkaloids, belonging to pyridine and piperidine groups, derived from amino acid lysine. Arecoline a nicotinic acid-based alkaloid present which exerts sialagogue property. But the habit of chewing marketed gutka may cause oral leukoplakia, sometimes lead to squamous cell carcinoma. However, Based on the phytochemical compounds present, it is predicted that it must show antioxidant and may show cytotoxic activities.

Methods: The antioxidant activity (nitrite scavenging and hydrogen peroxide scavenging) and cytotoxic activity in two different medium were checked in EAC cell line using the swiss albino mice model.

Results: The study was also postulated an idea about the qualitative and quantitive analysis of *Areca catechu.* The plant extract showed good Nitrite and Hydrogen peroxide scavenging activity. The cytotoxicity study conducted in swiss albino mice, cell viability and IC50 value was checked. The cytotoxic activity of different concentrations of different fractions of plant extract was checked in two different medium, i.e., PBS and RPMI 1640. IC50 values for following fractions which were studied in PBS; as, 91.73±73 (µg/ml), 183±36.24 (µg/ml), 53.74±1.562 (µg/ml) for crude ethanolic extract, alkaloid fraction and flavonoid fraction respectively. IC50 value in RPMI 1640 medium obtained as; 44.18±1.09µg/ml, 54.27±0.2279µg/ml and 51.24±2.461µg/ml for crude ethanolic extract, Alkaloid and Flavonoid fraction, respectively.

Conclusion: *Areca* nut extract showed good scavenging activity depending on concentration. Relatively RPMI 1640 medium showed better cytotoxic activity than other mediums.

Keywords: EAC cell line, RMPI medium, Areca, Catechu, Anti-oxidant, Anti-cancer.

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INTRODUCTION

Areca nuts or more preciously *Areca catechu* seeds are used for addiction by almost all age groups in South Asian countries. From child ages to old people keeps some misperception about this, some feels it freshens breath, few believes it improves the digestive system if consumed with betel leaves [1]. But the ultimate scenario is something different. Chewing of Areca nut in different forms, whether with betel leaf or flavoured materials develops different health complications, i.e., Diabetes, cancer of oral cavity, pharynx, neck, oesophagus [2]. Areca nuts contains arecoline as alkaloidial component (though other few alkaloids i.e., arecoline, arecadine, guacine and guacoline are present) which promotes in parasympathetic cholinergic stimulation, resulting in increased alertness, salivation, anti-migraine etc. However, dependency on this habit causes insomnia, mood swinging, behavioural changes and palpitation [3, 4]. Arecoline the specific alkaloid of Areca nuts on nitration forms nitrosoguvacoline and 3-methyl nitrosomino pripionitrile. Metabolite of nitrosoguvacoline causes formation of cyanoethyl, which ultimately results DNA damage of squamaus cell [5]. However, several studies were carried out to evaluate several activities such as; Anti-nematodal, Anti-venom, modulation of phagocytosis activity and effect on catecholamine release was also studied [6].

Squamous cell carcinoma is specifically malignant neoplasia, that is seen to be occurred in lips and oral cavity and it includes even neck region which is highly lymphatic region. This mutated cell enters through neck region in the body system and metastasis takes place [7, 8]. In this present study, we have performed phytochemical screening, quantitative analysis of crude extract done using, UV visible spectrophotometer [7]. *In vitro*, antioxidant activity (NO scavenging and H_2O_2 scavenging assay) was checked, the cytotoxicity of *Areca catechu* extract in RPMI 1640 MEDIUM was checked using Ehrlich ascites carcinoma (EAC) cell line and compared with conventional cytotoxicity of *Areca catechu* in Phosphate Buffer Saline. The reason of selecting EAC cell line is; this specific experimental mouse model provides rapid proliferation with a shorter life span, it is having the high transplantable capability and it is quite similar to human tumors [9].

MATERIALS AND METHODS

Collection and preparation of extract

Areca catechu nuts were collected from the local market of Kolkata in the month of June. The plant products were identified by The Botanical Survey of India, Botanical Garden, and Howrah-711103. A voucher specimen No. CNH/(59g//Tech. II/46) has been preserved in the laboratory for future reference. After identification of the nuts, they were grinded to obtain the powdered form using the mechanical grinder available in the laboratory. The powdered product was extracted using pure ethanol as solvent, following two weeks of soxohelation process [10, 11]. The obtained extract contains excess solvent and the product was subjected to remove excess solvent under reduced pressure using rotary vacuum evaporator. Another technique of rapid evaporation was carried out using water bath heating; crude extract was obtained and calculated. The weighed amount of % yield was calculated as 19.23%.

Phytochemical screening

An urgent need of phytochemical screening was sensed to confirm the presence of different chemical compounds. The identification of following compounds was carried out by specific experimental models, i.e., steroids [12-14], flavonoids [15-17], saponins [18], alkaloids/Mayer's test [19] and Dragendorff's Test [20], tannins [21], glycosides-Cardiac Glycosides(Keller-Killani test) [22, 23]. The entire phytochemical analysis and screening were enlisted and summarized.

Quantitative analysis

After confirming the presence of different phytochemical groups, it was necessary to establish for quantification of the present compounds. Quantification was carried out using UV vis spectroscopy. The total phenolic content was measured by the Folin-Ciocalteu method, [25] total tannin was quantified by the Folin-Ciocalteu method [26] and total alkaloid and flavonoid estimated by standard procedure [27].

***In vitro* antioxidant assay**

Tissue or cellular oxygenation is a vital cause for different health-related disorders, which includes cancer too. A herbal therapeutic option includes an additional antioxidant activity which believed to have superior treatment regimen compared to others [28]. Reactive oxygen species (ROS) is formed in biological system because of metabolic reactions, which contributes different immunopathological disorders. Production of ROS may contribute in genetic mutation, which may interfere in impairment of immunity. It has been found that overproduction of different ROSs vis. hydroxyl radical (. OH), hydrogen peroxide (H_2O_2), superoxide anions (O2-) and nitric oxide (NO.) are generally considered for the pathophysiology of health-related disorders [29]. In this present study, Nitrite scavenging assay and Hydrogen peroxide scavenging assay techniques were performed to evaluate its antioxidant potential.

Nitrite scavenging assay

In vitro NO scavenging assay was performed in order to confirm the antioxidant activity of *Areca* nuts. Sodium Nitroprusside was used as a source of NO radical, 10 mmol of Sodium nitroprusside in PBS (0.02M) was mixed with different concentrations of plant extracts which is followed by incubation at 25 °C for 30 min. During incubation phase NO is generated in the mixture. Afterward, 1.5 ml of incubation solution was further mixed with 1.5 ml Gries solution. The chromophore formed there was measured using UV visible spectroscopy at wavelength 546 nm [30, 31].

Hydrogen peroxide scavenging assay

H_2O_2 scavenging activity was measured in terms of determination of antioxidant potential. The principle of the assay was to find out the amount of scavenging potential against a molecule based on intrinsic absorption of H_2O_2 and measured by using UV spectra. 1 ml of different concentrations (20-100µg/ml) of plant extract was mixed with 2 ml of hydrogen peroxide solution of 10 mm and maintaining the pH phosphate buffer (50 mmol) of pH 7.4. The solution was incubated for 30 min at 25˚C. Absorbance was recorded at wavelength of 230 nm using UV visible spectroscopy [32-34].

Cytotoxicity study of *Areca* nuts fractions in PBS

Cytotoxicity of *Areca* nuts of different fractions were observed in EAC (*Ehrlichs Ascites Carcinoma*) cell line, which was collected from intraperitoneal cavity of Swiss albino mouse. However, the EAC cells were injected in healthy mice in animal house (IAEC Reg. No. 1439/PO/a/11) before 9-10 d from collection until solid tumour is formed and which is seen around peritoneal cavity. The cells were taken out and kept in eppendorf tube, stored in ice bath maintained at-4±1 °C, to slow down protease activity and to control apoptosis [34]. A stock sample of 1µg/µl (in Phosphate buffer solution) with crude extract of *Areca catechu* was prepared, and with the prepared stock five different samples were prepared in a differently marked glass tube at concentrations of 0 (control), 20, 50, 100, 200 µg/ml. EAC was added (50 µl) in each experimental groups, volume was made up to a definite ratio with PBS and was kept in CO_2 incubator for 2 h at 37±1 °C. *In vitro* cell exclusion assay was performed with the addition of PBS along with the incubated solution with 10 µl of trypan blue (an azo dye for staining dead cells) in an eppendorf tube and was mixed well and cells were counted accordingly under the microscope [35, 36]. IC50 values were calculated using graph pad prism software by extrapolating from the dose-response graph. Cytotoxic responses at different concentrations for different fractions were represented statistically.

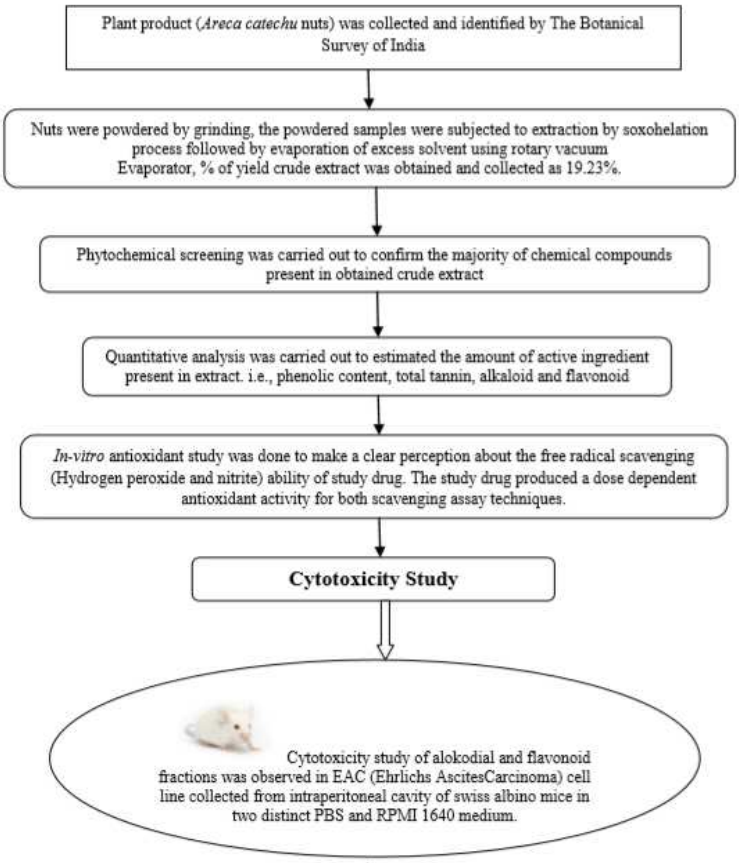

Fig. 1: Schematic representation of the process flow

Cytotoxicity study of *Areca* nuts fractions in RPMI 1640 medium

Culture and collection of Ehrlichs Ascites carcinoma cell lines (EAC) were the same as described in earlier section. Furthermore, five different concentrations of different treatments i.e., 0 (control), 20, 50, 100, 200 µg/ml were prepared in pre-labeled glass tubes from the stock sample of 1µg/µl. 50 µl of EAC was added in each experimental groups [34]. Further, the volume of each glass tube was adjusted with PBS, and was incubated in CO_2 incubator for 2 h at 37±1 °C. *In vitro* cell exclusion assay was performed upon addition of 1000 µl of RPMI 1640 medium with Foetal Bovine Serum and 150 µl of EAC cell lines and then observation was recorded after incubation as discussed earlier [37, 38]. IC50 values of different fractions (alkaloidal and flavonoid) were calculated using graph pad prism software and graphical and statistical presentation carried out for different concentrations.

The schematic representation of the process flow is shown in fig. 1.

RESULTS AND DISCUSSION

The phytochemical screening was carried out in accordance with the approved methods described; the purpose of the screening was to ensure a basic knowledge on which the chemical groups are present in the extract. The obtained results summarized in table 1.

Table 1: Summarization of qualitative tests performed for *Areca catechu*

S. No.	Test performed	For chemical compound	Theoritical result	Practical result	Observation
01.	Shinoda Test	Flavonoid	Magenta red/crimson red	Magenta red/crimson red	Positive
02.	Alkaline test	Flavonoid	Yellow precipitate	Yellow precipitate	Positive
03.	Tannin test1	Tannin	Greenish black precipitate	Greenish black precipitate	Positive
04.	Tannin test2	Tannin	Yellow precipitate	Yellow precipitate	Positive
05.	Mayer's test	Alkaloids	Yellow precipitate	Yellow precipitate	Positive
06.	Dragendorff's test	Alkaloids	Orange-brown precipitate	Orange-brown precipitate	Positive
07.	Foam test	Saponin glycosides	Permanent foam seen	No permanent foam seen	Negative
08.	Lead acetate test	Saponin glycosides	White precipitate	White precipitate	Positive
09.	Borntrager's test	Anthraquinone glycosides	Rose pink colour	White colour	Negative
10.	Keller-killani test	Cardiac glycosides	Blue colour	Red colour	Negative
11.	Hydroxy-anthraquinone test	Hydroxy-anthraquinone glycosides	Red coloured solution	Red coloured solution	Positive
12.	Salwoski test	Steroids	Red precipitate	Red precipitate	Positive
13.	Salwoski test	Triterpenoids	Yellow precipitate	Red precipitate	Negative
14.	General Glycoside test	Glycosides	Type A>Type B	Type A>Type B	Positive

Quantitative analysis

Quantitative analysis was carried out to make a clear view regarding the amount of phytochemicals present in the plant extract. Moreover, quantification was needed to predict the therapeutic outcome. It was observed that pyrocatechol a phenolic compound is present at a maximum concentration, though other significant components like gallic acid, alkaloids, tannins are also present. Some quantifiable amount of flavonoid was also observed. Quantification of each group was repeated for at least three times. The comparative amounts of phytoconstituents present tabulated in table 2 and statistically presented in fig. 2 using Graph pad prism software.

Table 2: Mean, SD and SEM value of essential phytoconstituents present in *Areca catechu* extract

Statistical interpretation	Pyrocatecol	Galic Acid	Alkaloid	Tanins	Flavonoids
Mean	734.7	138.3	461.8	296.9	7.424
SD (Std Div)	4.509	0.4871	6.380	0.7284	0.8510
SEM(Std Error Mean)	2.603	0.2812	3.683	0.4205	0.4913

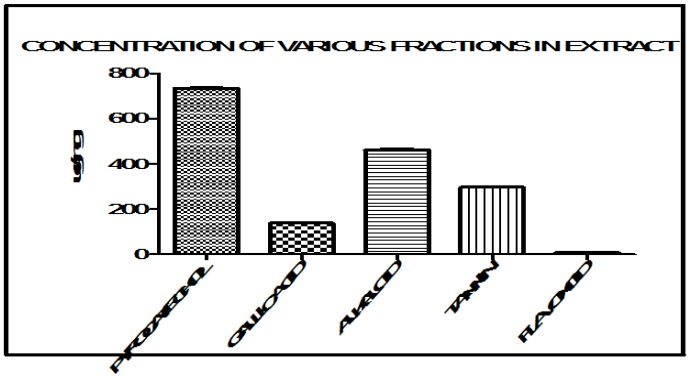

Fig. 2: Quantitative analysis of different phytoconstituents present in the extract

***In vitro* antioxidant assay**

Nitrite scavenging assay

NO is produced as metabolic by-product within the cell, which alter the cellular structure and finally leads to alteration function of the cell. NO within the cell may react with O^{2-} to produce peroxinitrate (ONO^{2-}). The assay principle was designed for inhibition of NO radical generated from sodium nitroprusside is measured by Griess reagent. The *in vitro* procedure resembles for determination of NO production by whole cells or enzymes within the body.

The present antioxidant activity revealed that *Areca catechu* nut extract exhibited maximum NO scavenging potential. The dose-dependent radical scavenging activity was described using the standard curve in fig. 3.

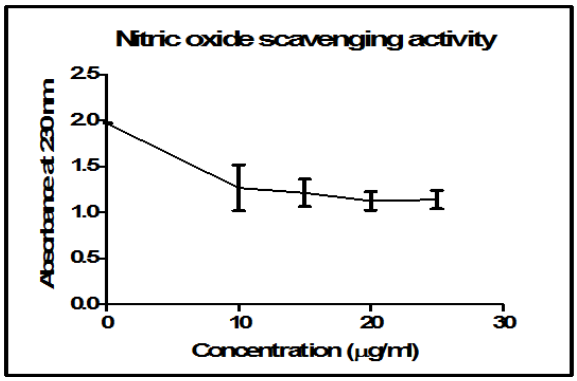

Fig. 3: Dose-dependent NO scavenging activity of ethanolic extract of *Areca catechu*

Hydrogen peroxide scavenging assay

H_2O_2 and OH^- are the important and vital free radicals which usually formed by auto-oxidation or partly by cellular metabolism. Though H_2O_2 itself a vital free radical responsible for the pathogenicity of several diseases, it also contributes in the formation of other few free radicals, such as OH, O^{2-} by different mechanism i.e., Fenton reaction, catalysed by enzyme myeloperoxidase. The purpose of this H_2O_2 scavenging assay was to confirm the ability of plant extract to scavenge the free radical [39]. However, it was observed that the ethanolic extract of *Areca* nuts had shown dose-dependent scavenging activity. Earlier in the chemical evaluation study, it was confirmed that the extract consists of flavonoids and phenolic compounds, which are known as good radical scavenger [39]. A dose-dependent scavenging activity was described in fig. 4.

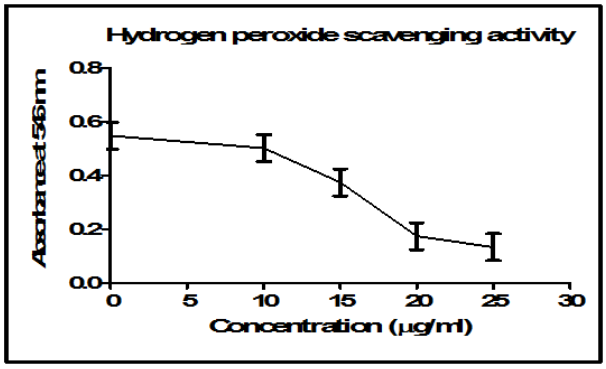

Fig. 4: Dose-dependent H_2O_2 scavenging activity of ethanolic extract of *Areca catechu*

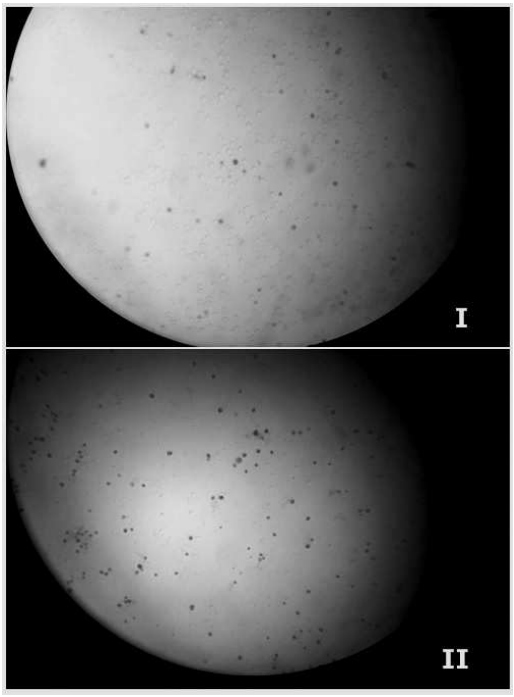

Fig. 5: Sample EAC cell line staining images demonstrating cytotoxicity of *Areca catechu* (Blue coloured= Dead cells) I:-Cytotoxicity of *Areca catechu* flavoinoidial fraction in PBS, II:-Cytotoxicity of *Areca catechu* flavoinoidal fraction in RPMI 1640 medium

Cytotoxicity or cell viability

The cytotoxicity of a particular substance or molecule is determined to establish the anti-cancer activity. In oncology study, the aim is focused to emphasize "tumour chemosensitivity assays", "drug response assays", or "drug sensitivity assays" of a product [40, 41]; the present study on the anti-cancer activity of different fractions of *Areca* nuts lies on "drug response assay". Different concentrations of different fractions had shown different responses and represented as dose-%inhibition graphs. From the given graph, the IC50 value is calculated by extrapolating the graph at which concentration the viability of the cell was reduced by 50%.

Two different cytotoxicity study was designed depending upon different culture medias, i) EAC cells which were cultured in PBS only, ii) EAC cells which were cultured in additional RPMI 1640 medium. In both cases, the dead cells were identified by blue colour, as Trypan Blue was used as an indicator under an electronic microscope fig. 5.

The dose-response graph was plotted against the different concentrations of treatment groups with different fractions (shown in **fig. 6, 7 a**nd **8** respectively for crude ethanolic extract, alkaloidal fraction and flavonoid fraction). It was found that the IC50 values for different fractions studied in PBS were 91.73±73 (µg/ml), 183±36.24 (µg/ml), 53.74±1.562 (µg/ml) for crude ethanolic extract, alkaloid fraction and flavonoid fraction respectively. From the obtained results it is predicted that, as the IC50 value is lowest for flavonoid fraction, it is implied that the cytotoxicity or cell viability capability is more potent for flavonoid fraction as it has the potential for reducing the cell count to 50% at lowest concentration.

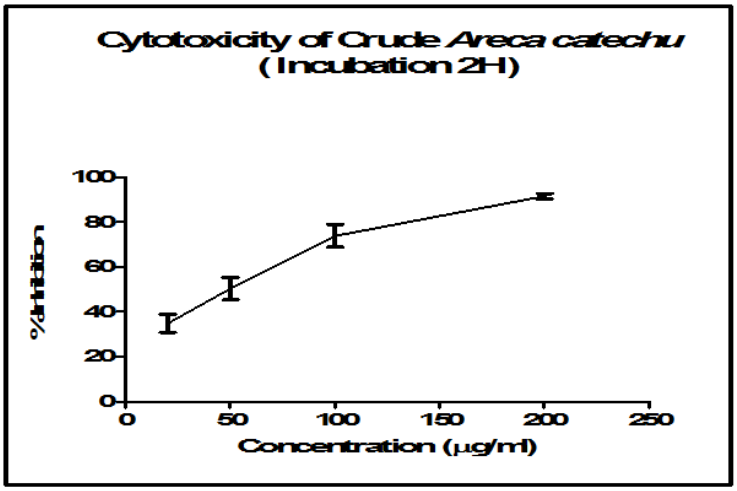

Fig. 6: Cytotoxicity of crude ethanolic extract of *Areca catechu* [IC50 value: 91.73±73 (µg/ml)]

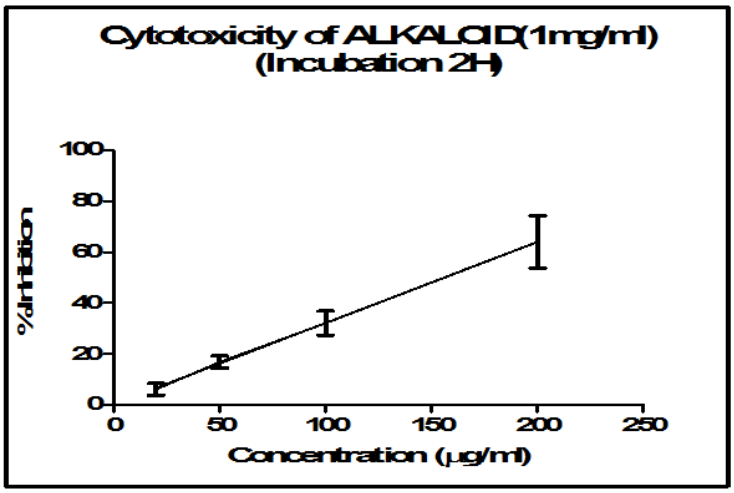

Fig. 7: Cytotoxicity of an alkaloidal fraction of *Areca catechu* [IC50 value: 183±36.24 (µg/ml)]

For crude extract and different fractions in RPMI 1640 medium

In vitro cytotoxicity studies in RPMI 1640 medium had shown IC50 value of Areca catechu crude extract of 44.18±1.09µg/ml, Alkaloid fraction of *Areca catechu* of 54.27±0.2279µg/ml. Flavonoid fraction of *Areca catechu* of 51.24±2.461µg/ml. However, in RPMI 1640 medium the extract and its different fraction had shown better cytotoxic outcome compared to that of PBS or normal saline. Though, it was an early prediction that, as RPMI 1640 supplemented with Bovine serum contains glutathione (a reducing agent), biotin, vitamin $B_{12,}$ etc and it is ideal for various ranges of cells lines. Additionally, the study objective in this study is justified, as it has shown cytotoxicity of study component; *Areca catechu* crude extract and its different fractions. It was also found that the cytotoxicity was greater when cultured in RPMI 1640 medium. The cytotoxicity of *Areca catechu* nut extract and alkaloidal and flavonoid fractions exhibited dose-dependent cell viability in EAC cells. Cell viability was summarized by standard curves (fig. 9, 10 and 11) made for each treatment group using Graph pad prism software.

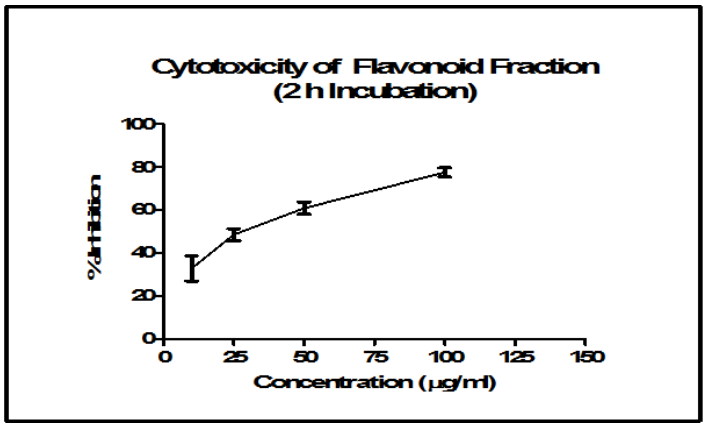

Fig. 8: Cytotoxicity of flavonoid fraction of *Areca catechu* [IC50 value: 53.74±1.562 (µg/ml)]

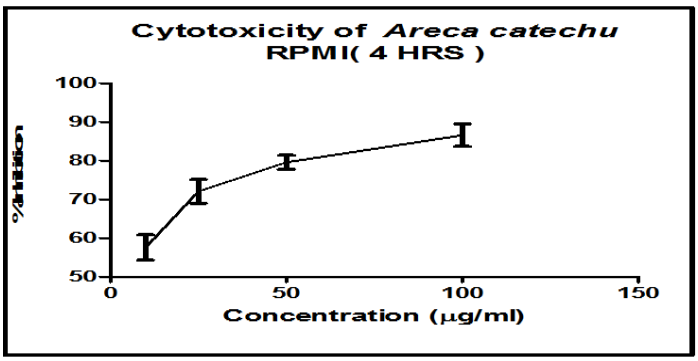

Fig. 9: Cytotoxicity of crude extract of *Areca catechu* in RPMI 1640 medium [IC50 value: 44.18±2.669 (µg/ml)]

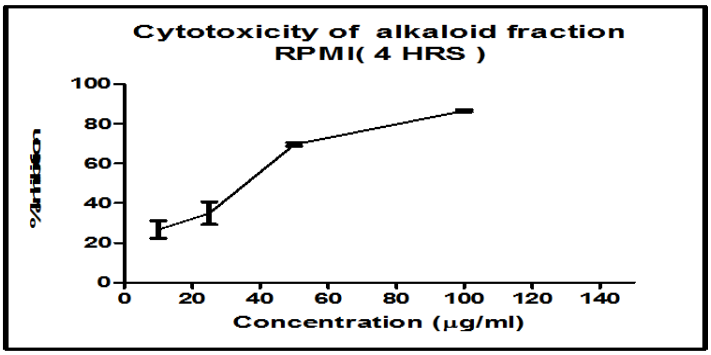

Fig. 10: Cytotoxicity of alkaloidal fraction of *Areca catechu* in RPMI 1640 medium [IC50 value: 54.27±0.3224 (µg/ml)]

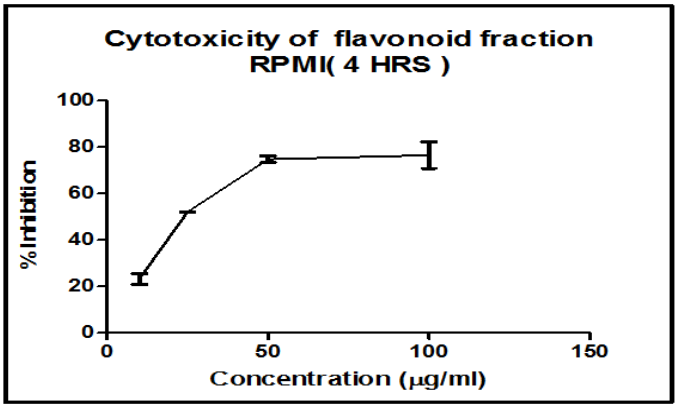

Fig. 11: Cytotoxicity of flavonoid fraction of *Areca catechu* in RPMI 1640 [IC50 value: 51.24±3.481 (µg/ml)]

The prediction and establishment of antioxidant activity depends on various parameters. It seems very difficult to illustrate the anti-oxidant potential by only few assay methods. Although, *Areca catechu* nut ethanolic extract had shown anti-oxidant activity but the potency may be confirmed after implementing more organic radical procedures. Generally, antioxidants may be of two types, such as; polar and non-polar; whether the molecule will act as electron-donating or hydrogen donating mechanism would be confirmed after evolution only.

Oral cancer is nowadays has become a threat towards society [42]. Oral cancer involved the genetic mutation including of tongue, gum, oral cavity etc. The reason behind it has been a topic of worry because people around the world are not aware of the severity of this disease. If it remains untreated for a while it propagated towards metastatic stage through lymph nodes. The stage is also somewhat known as tumor lymph metastasis of oral carcinoma [43, 44]. An early detection may be carried out with a biomarker. The common biomarkers for detection conventionally are; Ki-67 and P-53 [45,46]. The oral cavity including gum and tongue is composed of squamous tissue, which has decent permeability and having a characteristic to pass small molecules through membranes via filtration or diffusion. The objective of the study was to evaluate the cytotoxicity involving EAC cell line, which is a commonly used reference cell line for solid tumor carcinomas and sarcomas. Two different mediums were used; PBS and RPMI 1640. It was further observed that EAC cultured in RPMI 1640 medium had shown a better cytotoxic activity. However, before concluding it's important to undergo a safety evaluation of crude extract and fractions. In the future, other organotoxicity studies with histopathological findings may be employed for briefing the rationale of the treatment.

CONCLUSION

From this study, it was concluded that *Areca catechu* nut extract shows maximum nitric oxide scavenging activity and this scavenging activity depends on the dose of the nut extract. It was also concluded that extract contains flavonoid and the phenolic compound which showed dose-dependent good radical scavenger activity and this extract and its composition showed better cytotoxic activity in RPMI 1640 medium than PBS and normal saline medium. This cytotoxic and antioxidant scavenging activity of *Areca catechu* nut extract inhibits the squamous cell carcinoma.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors hereby declare that they have no conflicts of interest either to disclose.

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