BIOACTIVITY AND QUALITATIVE ASSESSMENT OF ARECA NUT (*Areca catechu* L.)

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ABSTRACT: *Cancer is a global threat whose treatment is coupled with side effects ultimately affecting the patients' quality of life. One potential herbal remedy for cancer is Areca nut (Areca catechu L). Areca seed in the form of betel quid is a masticatory indulgence since the ancient period with ethnobotanical uses ranging from social to medicinal. This research evaluates the antioxidant and cytotoxic properties, of a local variety of Areca nut and to find out if the corresponding phytochemicals responsible for the abovementioned biological effects are present in Areca nut extract. Evaluation of the antioxidant activity was done using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) photometric assay. Results revealed that the half-maximal inhibitory concentration of the Areca nut extract was lower than 5 ppm indicating a scavenging activity equally strong as Vitamin C. Cytotoxicity test of the Areca nut extract against normal human blood lymphocytes showed no inhibition on cell proliferation and no significant effect on cellular metabolism, hence non-toxic. Moreover, when Gas Chromatography-Mass Spectrometry was employed, twenty-seven (27) possible bioactive compounds were identified, and eleven (11) of these compounds were known antioxidants. This study then corroborates the ethnomedicinal use of the seed of A. catechu as an antioxidant.*

Keywords: Betel nut, antioxidant, cytotoxic, GC-MS, masticatory

1. INTRODUCTION

One in every six death worldwide is due to cancer, thus in 2018, the World Health Organization identified cancer as the leading cause of mortality and morbidity worldwide, accounting for an estimated 9.6 million deaths [1], and projections resulted to a possible increase to 11.5 million deaths in 2030 [2]. The global cancer statistics revealed that lung, breast, colorectal, and prostate cancers were the most common cancers worldwide [3].

When one is diagnosed with cancer, various treatments are employed, which include surgery, radiation, chemotherapy, and other therapies. Cancer treatment usually depends on various factors including the type of cancer location, health status of the patient, and how advanced the cancer is. Some cancer patient is cured or the progression thereof is arrested with just one treatment but others need to be subjected to a combination of treatments, like surgery with chemotherapy and/or radiation therapy, immunotherapy, targeted therapy, or hormone therapy [4]. In most treatments, cancer cells are directly removed or they are deprived of the signals needed for survival. Other treatments work by stimulating the body's own defenses against the cancer cells [5].

Cancer treatments are said to be associated with a number of side effects most especially the damaging effect of the treatment to the healthy cells. Side effects can be different for each person, and for different medicines and kinds of treatment. In chemotherapy, for instance systemic toxicity might happen, especially if the patient is immunocompromised and worst, if the tumorous growth or the cancer cells are not completely wiped out [6].

Nowadays, people tend to utilize natural remedies or alternative medicines to treat different health conditions including cancer. One commonly used complementary and alternative therapy by people with cancer is herbal medicines. A couple of studies observed that as many as 6 out of every 10 people with cancer (60%) use herbal remedies or their derivative phytocompounds. Numerous studies have reported on the beneficial effects of herbal medicines especially on cell survival, immune modulation, and quality of life (QOL) of cancer patients [7].

The seeds of the oriental palm or Areca/Betel nut (*Areca catechu*) known for various utilization is the subject of this study. The Oriental palm is widely distributed in Asia, the Pacific, and East Africa, but the plant is believed to be a native of Sri Lanka, West Malaysia, and Melanesia [8]. The seed of the mentioned plant is a masticatory substance and is popularly consumed by people in numerous islands in the Pacific and Asia and ranked as the fourth most popular psychoactive substance in the world, after nicotine, alcohol, and caffeine [9].

The practice of betel chewing used to be prevalent throughout the Philippines from the mountains in the north to the Muslim communities in the south. The prevalence of betel nut chewing, especially by the tribal groups in the Philippines has both social and health dimensions. Betel nut in the form of betel quid (BQ) was an item of hospitality and friendship, a social lubricant preferred during social encounters, like tea, coffee or wine, or marijuana in more recent times. In central or southern Mindanao, betel chewing is even a ritualistic custom among the Meranao, Maguindanao, Bagobo, and Tausug groups [10].

But while betel nut is an important cultural and social component of the tradition in many countries, growing evidence points to serious health effects of regular use. Several diseases ranging from simple to serious ones is associated with the habitual mastication of Areca nut. Some of these are cancers of the mouth and esophagus, systemic effects on the brain, heart, lungs, gastrointestinal tract, and reproductive organs. It was also noted that the seed can aggravate pre-existing conditions such as neuronal injury, myocardial infarction, cardiac arrhythmias, hepatotoxicity, asthma, central obesity, type II diabetes, hyperlipidemia, metabolic syndrome, etc. Areca nut also interferes with endocrine function resulting to endocrine disorders such as hypothyroidism, prostate hyperplasia, and infertility. Studies further documented the effect on the immune system leading

to suppression of T-cell activity and decreased release of cytokines and the harmful effects on the fetus when used during pregnancy [11].

Ironically, the World Health Organization in 2009 has listed around 25 beneficial properties of Areca nut but at the same time classifies Areca nut as a carcinogen [12, 13]. The ill-effects reported in association with Areca nut chewing can be attributed to other factors like the role of other ingredients added when preparing betel quid, the quality of Areca nuts (including contaminations and adulterations) used for making different preparations of the chewing product, and the high doses or the manner of administration Ironically, these factors were not taken into consideration or discussed at all in most of the publications that showed that Areca nut chewing is dangerous. Other contradictory reports even claim that Areca nut is not cancerous but can cure cancer [14]. In the Philippines, Meranaos claimed that betel nut has anticancer activity. Amidst these conflicting claims, hence this research was conceived. The main aim of this study is to provide scientific backing to Areca nut folkloric medicinal claim by assessing antioxidant and cytotoxicity effects and to provide a biochemical basis for the cellular responses.

2. EXPERIMENTAL DETAILS

Collection of Plant material

Around 300 seeds of Areca nut purchased from the street market in Marawi City, were washed with water and then cut into four exposing the seed. The seeds were then air-dried for three weeks. After drying, the seeds were diced into small pieces (about 1cm) and were pulverized by a mechanical blender. The crushed seeds were then stored in an airtight container and kept at room temperature.

Preparation of Crude Ethanolic Extract

Approximately 200 grams of Areca nuts were blended using a food processor. Powdered Areca nut seeds were added to 2 liters of 98% ethanol and were kept for 48 hours in a temperature below 25 oC. Ethanol was used as a solvent in the extraction since most of the polar compounds are easily eluted by this kind of solvent. The extract was filtered first using cheesecloth, followed by a Whatman filter paper No.1. The filtration lasted for three days using three funnels simultaneously. The filtrate was made ethanol-free by evaporating it using a rotary-evaporator at 45 ^{o}C. The obtained crude extracts were stored in vials for antioxidant (DPPH/free radical scavenging activities), cytotoxicity test, and GCMS analysis.

Evaluation of Antioxidant activity by DPPH radical scavenging method

Evaluation of free radical scavenging activity of ethanolic seed extract of the Areca nut was done by quantifying the 1,1-diphenyl-2-picryl hydroxyl (DDPH). About 0.1 ml solution of DPPH in ethanol was prepared and was added to 3 ml of extract in ethanol at different concentrations (0 (control), 5,10, 20, 30, 50, 100, 200 and 300 ppm). Vigorous shaking of the mixture followed this was allowed to stand at room temperature for 30 minutes., Measurement of the absorbance was done with the use of a spectrophotometer (UV-VIS Shizmadzu) at 517 um and the reference standard compound used was ascorbic acid. The set-up was replicated thrice. The IC_{50} value of the sample (s the concentration of the sample required to inhibit 50% of the DPPH free radical) was calculated using the log dose inhibition curve. Lower absorbance reading indicated higher free radical activity. The percent DPPH scavenging effect was calculated using the following:

$$\%\, inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100\%$$

In vitro MTT Cytotoxicity Assay for Lymphocyte Proliferation

MTT (3-(4,5-dimethyl thiazoyl)-2,5diphenyltetrazolium bromide) assay was used for measuring cell survival [15]. The crude extract was filtered through a 0.1 µm PES syringe filter disc in a centrifuge tube. Thirty (30) µm of the treatment solutions were dispensed to three microcentrifuge tubes. Two hundred seventy (270) µm of the cell-cultured suspension was added to the microcentrifuge tubes containing the treatments and incubated at 37 ºC with 5% CO2 for 24±3 hours. After the 24 hours incubation period, the treated cultures were separated for cell counting. Seven (7) microliters of incubated culture were added to 7 µl of trypan blue, and 7 µl of the mixed solution was placed in a hemacytometer. The number of dead and live lymphocytes were counted in all 25 squares within the 1 mm center grid. Cell density (number of cells per ml) is computed using the following formula:

$$\#\text{cells/ml} = \frac{\#\text{ of cells total}}{\#\text{ of 1 mm squares}} \times 10^4 \times \text{original dilution}$$

Where dilution factor is (§dilution factor = 2)

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Determination of the active compounds present in the ethanolic extract of *Arecha catechu* seeds was done using the protocol of Chipiti *et al. (2015)* for GC-MS analysis with modifications [16]. The extract was diluted with chloroform and subjected to Agilent Technologies 7890AGC system coupled with (an Agilent) 5975C Mass Selective Detector. An HP-5MS capillary column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness) was applied. The carrier gas was helium with the injector temperature set at 320 °C. The initial oven temperature was at 70 °C which was programmed to increase to 280 °C at the rate of 10 °C/min with a hold time of 4 min at each increment. Injections of 1 µL were made in split mode with a split ratio of 100:1.

The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 °C, quadrupole temperature 150 °C, solvent delay 3 min, and scan range 33 - 550 amu. The compounds were identified by direct comparison of the mass spectrum of the analyzer at a particular retention time to that of a reference standard provided by the National Institute of Standards and Technology (NIST) library. The total GC-MS running time was 45 minutes. An 80% similarity index was considered significant.

3. RESULTS AND DISCUSSION

Areca nut extract ((IC_{50}=<5ppm) is a potential source of antioxidants (Table 1), with a similar effect to Vitamin C (IC50=1.74ppm). The range of the scavenging activity of Areca nut is similar - much stronger compared to Vitamin C. This indicates that the extract has a strong antioxidant activity evidenced by the ability to inhibit free radicals even at lesser concentrations. It must be recalled that DPPH assay is a measure of the ability of the extract to donate hydrogen to the DPPH radical resulting in bleaching of the DPPH solution [17].

Table 1. Antioxidant properties of the extract of A. catechu L.

Extract Concentration (ppm)	**Percent Inhibition (%)**	**Vitamin C Concentration** (ppm)	**Percent Inhibition (%)**
Control	0	Control	0
5	94.47	2	60.07
10	94.21	3	83.91
20	93.18	4	87.73
30	93.95	5	91.55
50	94.98	10	94.56
100	94.85	20	**94.44**
200	94.85		
300	**95.11**		
IC_{50}=**5 ppm**		IC_{50}=**1.74 ppm**	

Other plant parts of Areca nut also exhibited good antioxidant activities [18]. The seed and husk are known to be a good source of antioxidants which are rich in Vitamin C and other phenolic compounds known to help prevent or delay products from oxidization through scavenging free radicals and lowering oxidative stress, thus slowing down the aging process and the severity of various diseases such as cardiovascular diseases, cancers, neurodegenerative disorders inflammatory diseases, arthritis, eczema, diabetes, and gastrointestinal inflammatory diseases [19].

Among the free radicals, DPPH is said to be the most stable compared to in vitro generated free radicals like hydroxyl radical and superoxide anion. This advantage of DPPH is due to its being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colorless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm band [19].

The antioxidant activity of extracts from *A. catechu* is expressed in terms of percentage of inhibition (%) and IC_{50} values (µg/ml) (Fig. 1). One important finding is the fact that plants natural antioxidants were found out to not induce side effects, while synthetic antioxidants seem to pose some genotoxic effect [20]

The cytotoxic potential of *A. catechu* ethanolic extract on normal human lymphocytes also showed that the administered concentration of 1mg/ml of the extract resulted to an average of 85.5% live cells. Out of 59.67×10^4 cells/mL there were only 51.00×10^4 cells/ml. The result suggests that Areca just like *Andrographis paniculata* is not toxic if used singly, hence generally perceived as safe for therapy [21].

Table 2. Cytotoxic activity of A. catechu ethanolic extract to normal human lymphocytes

Treatment	Average # Live Cells	Average # Dead Cells	Average # Total Cells	Average # Live Cells
Supplemented **RPMI**[a]	61.56	4.00	65.56	93.90
Triton X – 100[b] 0.1%	0.00	56.56	56.56	0.00
DMSO[c] 2%	49.67	6.67	56.33	88.20
Areca nut 1 mg/ml	51.00	8.67	59.67	85.50

[a] Negative control. [b] Positive control. [c] Vehicle control

Meanwhile, qualitative assessment of the compounds detected by GCMS revealed several compounds with antioxidant activities and compounds with potential cytotoxic properties. A total of 11 compounds, some of which belonging to the polyphenols groups can act as strong antioxidants as they can prevent oxidative damage and reduce inflammation [22]. These compounds include 2-Phenylethanol *(Benzene ethanol)*, Neophytadiene, n-Tridecane, Nonadecane, Hexadecane , n- Hexadecanoic acid, ethyl ester, 2,4,4,6,6,8,8-Heptamethyl-1-nonene, Heneicosane, Hexadecanoic aid, butyl ester, Heptadecanoic acid, ethyl ester, and Disulfide, di-tert-dodecyl.

Polyphenolic compounds slow down auto-oxidation by inhibiting the formation of free radicals and other reactive oxygen species (ROS) or through interrupting the proliferation of the free radical by scavenging species that initiates peroxidation, breaking the autoxidative chain reaction, and reducing localized O_2 Concentrations [23]. They work as they affect enzyme activities, plasma, membranes, transcription factors *in vivo* [24], enhancing the total oxidant-scavenging capacities of human blood by binding to red blood cells [25].

Table 3. Bioactive compounds qualitatively identified from the ethanolic extracts of A. catechu L. using GC-MS analysis

	Name of Compound	**Formula**	**SI**	**Mol. Wt.**	**Reported Biological Properties**
1	Benzene ethanol (2-Phenylethanol)	$C_8H_{10}O$	98	122	Antimicrobial, Antiseptic, Disinfectant, Aromatic essence, Preservative in pharmaceutics and Perfumery (MeSH and DrugBank, 1976); *Saccharomyces cerevisiae* metabolite, plant metabolite, *Aspergillus*

					metabolite, and plant growth retardant (ChEBI, 2014)
2	Neophytadiene	$C_{20}H_{38}$	83	278	Antimicrobial (Yi *et al.*, 2001)
3	Arecaidine (3-Pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-methyl-,methyl ester)	$C_8H_{13}NO_2$	96	155	Carcinogenic (Duke, 2016), Antihelmintic (Yusuf and Yong, 2002); Ganglionic stimulant, Parasympathomimetic, Vermifuge, Euphoriant (MeSH and DrugBank, 2018); Muscarinic agonist (ChEBI, 2016); treatment for Alzheimer's (Christie *et al.*, 1981)
4	n-Tridecane	$C_{13}H_{28}$	96	184	Antimicrobial (Khan *et al.*, 2016); Insect repellent, Predator deterrent (Zarbin *et al.*, 2000)
5	2,4-Hexadienedioic acid, 3-methyl-4-propyl-, dimethyl ester, (Z,E)-(CAS)	$C_{12}H_{18}O_4$	74	226	No reported functional property
6	Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)2-methyl-1,3-propanediyl ester	$C_{16}H_{30}O_4$	92	286	Volatile biomarker of lung cancer (Jia *et al.*, 2019); Food Packaging (Alam *et al.*, 2013)
7	Nonadecane	$C_{19}H_{40}$	94	268	Antioxidant (Tualeka *et al.*, 2016)
8	Sulfurous acid, cyclohexylmethyl heptyl ester	$C_{14}H_{28}O_3S$	78	276	No reported functional property
9	4-Octen-3-ol, 2,2-dimethyl	$C_{10}H_{20}O$	86	156	Prominent odor volatile produced by fungi (Kaminski *et al.*, 1974); Potent mosquito attractant (Takken and Kline, 1989)
10	4-Octen-3-ol, 2,2-dimethyl	$C_{10}H_{20}O$	81	156	Prominent odor volatile produced by fungi (Kaminski *et al.*, 1974); Potent mosquito attractant (Takken and Kline, 1989)
11	n-Hexadecane	$C_{16}H_{34}$	95	226	Antibacterial (Gerge *et al.*, 2013; Rahimian*et al.*, 2014); Antifungal (Duraipandiyan *et al.*, 2014; Abdul Kaffoor *et al.*, 2017); Antioxidant (Gergel *et al.*, 2013; Abdul Kaffoor *et al.*, 2017)
12	2,6,10,14-Tetramethylhexadecane	$C_{20}H_{42}$	94	282	No reported functional property
13	Benzeneacetic acid, 2-phenylethyl ester	$C_{16}H_{16}O_2$	96	240	Perfumery flavoring agent and Food Additives (PubChem, 2019)
14	Sulfurous acid, cyclohexylmethyl pentadecyl ester	$C_{22}H_{44}O_3S$	86	388	Pesticide activity (Gore *et al.*, 2000)
15	Sulfurous acid, cyclohexylmethyl hepthyl ester	$C_{14}H_{28}O_3S$	79	276	Disinfectants (Duke, 2016)
16	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	96	284	Antioxidant (Brindha and Sivasubramanian, 2013; Abdennebi *et al.*, 2015); Antibacterial (Rahimian *et al.*, 2014); Antifungal and Antitumor (Agarwal and Tyagi, 2016); Anticancer (Brindha and Sivasubramanian, 2013); Hypocholesterolemic (Abdennebi *et al.*, 2015) Nematicide, Pesticide, Antiandrogenic flavor, Hemolytic, Alphareductase inhibitor (Sudha *et al.*, 2013)
17	2,4,4,6,6,8,8-Heptamethyl-1-nonene	$C_{16}H_{32}$	83	224	Antioxidant activity (Khan *et al.*, 2016)
18	Heneicosane	$C_{21}H_{44}$	96	296	Antibacterial, Oviposition attractant pheromone (Arora and Meena, 2018)
19	1-Ethylsulfanylmethyl-2,8,9-trioxa-5-aza-1-sila-bicyclo[3,3,3]undecane	$C_9H_{19}NO_3SSi$	85	249	No reported functional property
20	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	93	308	Hypocholesterolemic, Nematicide, Antiarthritic, Hepatoprotective Antiandrogenic, Hypocholesterolemic, 5-Alpha reductase inhibitor, Antihistaminic, Anticoronary, Insectifuge, Antieczemic, Antiacne (Sudha *et al.*, 2013)
21	Ethyl Oleate	$C_{20}H_{38}O_2$	87	310	Pharmaceutical drug preparations involving lipophilic substances such as steroids, lubricant, and a plasticizer and food additive (Sulochana *et al.*, 2016)
22	Hexadecanoic aid, butyl ester	$C_{20}H_{40}O_2$	94	312	Antioxidant, hypocholesterolemic, antiandrogenic, flavor, nematicide, hemolytic5 alpha-reductase inhibitor (Arora and Meena, 2018)
23	Heptadecanoic acid, ethyl ester (CAS) Ethyl n-heptadecanoate	$C_{19}H_{38}O_2$	92	298	Antimicrobial (Shobier *et al.*, 2005)
24	Tetratriacontane	$C_{34}H_7O$	92	478	No reported functional property
25	1-Docosanol, acetate	$C_{24}H_{48}O_2$	96	368	No reported functional property
26	Disulfide, di-tert-dodecyl	$C_{24}H_{50}S_2$	84	402	Antiviral (H Katz *et al.*, 1992)
27	Sulfurous acid, cyclohexylmethyl octadecyl ester	$C_{25}H_{50}O_3S$	84	430	No reported functional property

4. CONCLUSIONS

Results of this study affirm that the folkloric medicinal utilization of Areca nut has a biological and chemical basis. Results further revealed that the Areca nut ethanolic extract possesses compounds that can be used for the development of antioxidant and anti-cancer drugs. This study further proves that consumption of Areca nut seeds is safe.

4. REFERENCES

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