

## Comet Assay to Test Antioxidative Effects of Extracts from Different Parts of *Areca catechu* L.

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### ABSTRACT

**Objective:** Various parts of *Areca catechu* L. (family Arecaceae) have been used for chewing or as food in many communities throughout Thailand. Effects of aqueous and various organic extracts of different parts of this plant on oxidative DNA damage in human hepatocarcinoma HepG2 cells was compared with butylated hydroxyl toluene (BHT) using the comet assay.

**Method:** Hydrogen peroxide was used as DNA damage inducing agent. Comparison of a series of organic extracts from the areca seed and nut husk of this plant was performed.

**Results:** Incubation of 0.1% methanol and CH<sub>2</sub>Cl<sub>2</sub> extract of areca nut husk showed a significant inhibition of comet formation while other solvent extracts did not. All organic extracts of areca seed did not show an antioxidative effect. The untreated and boiled extracts of other parts of *Areca catechu*, i.e., flower bud, bloomed flower and leaf bud exhibited no antioxidative activity.

**Conclusion:** The methanolic and methylene chloride extracts of *Areca catechu* nut husk exerted antioxidant activity while extracts of other parts, e.g., seed, leaf bud, flower bud and bloomed flower, did not.

**Keywords:** Comet assay, *Areca catechu*, extract, antioxidative

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### Introduction

The single cell gel electrophoresis (SCGE) assay or comet assay is a new technique to detect DNA damage. This rapid, sensitive and relatively simple method could detect DNA damage at the level of individual cells. It combines the simplicity of biochemical techniques for detecting DNA single strand breaks (strand breaks and incomplete excision repair sites), alkali-labile sites, and cross-linking, with the single cell approach typical of cytogenetic assays. The comet assay could also determine the extent of oxidative DNA damage due to peroxide generator such as hydrogen peroxide.<sup>1</sup>

Comet assay has been found highly effective in bio-monitoring of natural compounds.<sup>2</sup> Natural compounds in herbal extracts possess varied antioxidative efficiency to reserve the DNA damage. When damaged, DNA strands were broken into fragments and then migrated towards the anode during electrophoresis. The extent of DNA damage was then quantified using normalized tail length. This normalized tail length of the comet indicated the amount of damage as longer tails indicated that the strand breaks were frequent and that the DNA was fragmented to several smaller molecules. One hundred comets on each slide were scored according to the length and relative intensity of fluorescence in the tail

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was compared to the head and expressed as tail to head ratio. Lower ratio suggested lower level of DNA damage or higher antioxidant activity.

*Areca catechu* L. (family Arecaceae) or betel nut tree is a species of palm which grows in much of the tropical Pacific, Asia and some part of east Africa. The betel nut, seed of *Areca catechu* contains alkaloids such as arecaine and arecoline, which when chewed is intoxicating and also slightly addictive. The concentration of the principal constituents of areca nut, polyphenols, flavonoids and alkaloids decrease with the maturity of the nut.<sup>2</sup> The alkaloid of areca nut, arecoline has a potent muscarinic action<sup>4</sup> and has a potential for an application in Alzheimer's disease.<sup>5</sup> An antioxidative effect of areca nut was also found.<sup>6</sup> Other four nitroso compounds namely N-nitrosoguvacoline, N-nitrosoguvacine, 3-(N-nitrosomethylalmino) propionaldehyde and 3-(N-nitroso-methylalmino) propionitrile, have been isolated from the aqueous extract of the areca nuts. They have been found to be cytotoxic and genotoxic to human buccal epithelial cells and could also produce pancreatic, lung, nasal and liver tumors in rats.<sup>7-9</sup> An oxidative stress or free radical might cause many diseases such as cardiovascular diseases, diabetes mellitus, cancer, etc. This preliminary study suggests that inhibition of the oxidative stress of areca extracts would lead to protection and/or treatment of diseases associated with aging.

The solvent extraction is a basic method to separate compounds from plants and other products. It is performed using water and organic solvents to extract a substance from herbal plant. This study aimed to determine antioxidative activity of extracts from various parts of *Areca catechu*. Antioxidative activity between untreated and boiled parts of *A. Catechu* was also compared.

## Materials and Methods

### Chemicals

Butylated hydroxytoluene (BHT) (CAS 128-37-0) was obtained from P.C. Drug Co., Thailand. Hydrogen

peroxide (H<sub>2</sub>O<sub>2</sub>) (CAS 7722-84-1, lot #AF404317) was purchased from Ajax Finechem, Australia, agarose (CAS 9012-36-6, lot no. 78K2170) from Gibco BRL, USA, and dimethyl sulfoxide (DMSO) (CAS 67-68-5, lot no. 33530) from Sigma-Aldrich Laborchemikalien GmbH, Germany. Tris (CAS 77-86-1, batch no. 57829) was purchased from Scharlau Chemie S.A., Spain. All tissue culture biologicals were purchased from GibThai (Bangkok, Thailand). All other chemicals were AR grade and purchased from Merck (Darmstadt, Germany).

### Plant material and extraction

Different parts of *Areca catechu* i.e. flower bud, bloomed flower, leaf bud, seed and nut husk were collected from Nakhon Pathom, Thailand, in October 2006. The voucher specimens were identified by Dr. Uthai Sotanaphun, Department of Pharmacognosy, Silpakorn University in Nakhon Pathom, Thailand and they were deposited in this department.

Different parts of *Areca catechu* were washed with distilled water before hot-air drying at 55 °C for 72 h. They were then passed through sieve no. 60 and kept at 4 °C before use. The 250 g dried seed and nut husk powder of areca nut tree were subsequently macerated in 1,500 mL with an order of various organic solvents. The sequential extraction for each solvent was 24 h, starting from petroleum ether, dichloromethane, ethyl acetate, methanol and water. The overnight percolate was filtered through no. 1 Whatman® filter paper until clear. The extracts were then subject to evaporation using a rotary evaporator (Buchi R205, Germany) until dry, and subsequently collected and weighed. Unboiled and boiled leaf bud, flower bud and bloomed flower were squeezed and their juices were individually filtered through no. 1 Whatman® filter paper until clear. Dried extract was dissolved in DMSO for further experiment.

### Cell culture

Human hepatocarcinoma cells (HepG2) were maintained in minimal essential medium (MEM) with 10% fetal bovine serum at 37 °C in 5% CO<sub>2</sub> humidified

incubator. Cells were sub-cultured every 5 - 7 days in the absence of antibiotics.

### Comet assay

In single cell gel electrophoresis (SCGE) experiments, after subculture of at least  $1 \times 10^5$  cells suspension with more than 99% pre-determined viability, cell culture was subject to oxidative DNA damage induced by incubation with 40  $\mu\text{M}$  of hydrogen peroxide for each reaction. Incubation was performed at 37 °C in a microcentrifuge tube in the absence or presence of various organic extracts of *Areca catechu* for 30 min. Comet slides for electrophoresis were made in-house by coating one side with 0.5% w/v normal agarose in water and left until gel setting.

Each experiment consisted of the following 4 reactions; 1) a control in which no  $\text{H}_2\text{O}_2$  was added, 2) a reaction with 40  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$ , 3) a positive control reaction by pre-incubation of the cells with 1  $\mu\text{M}$  of BHT in DMSO along with  $\text{H}_2\text{O}_2$ , and 4)  $\text{H}_2\text{O}_2$  reaction with 0.1% w/v of each extract (5-6 extracts with different solvents and different parts of *A. catechu*).

The 40  $\mu\text{M}$   $\text{H}_2\text{O}_2$  treatment could induce the apparent apoptotic cell death which was characterized by tailing of the nucleus in comet assay. DMSO concentration in final reaction volume was always kept below 2% v/v according to Uhl et al.<sup>1</sup> Cells were incubated with designated extracts (0.001, 0.005, 0.01 and 0.1% w/v) in 40  $\mu\text{L}$  final reaction volume containing 1% low melting agarose (LMA) and later applied over the slide to allow solidification at 4 °C, followed by another layer of 0.5% LMA. The slide was dipped into lysing solution at 4 °C for at least 1 h, then transferred into electrophoresis chamber containing electrophoresis buffer for at least 20 min before running. Electrophoresis was done at 4 °C under 300 mA, 25 V for 40 min. After 2 times of neutralization, the slide was then immersed into absolute ethanol for 5 min and dried at room temperature. DNA was stained with ethidium bromide (20  $\mu\text{g}/\text{mL}$ ) and observed under a fluorescence microscope (Nikon

Eclipse TE2000-S, excitation filter BP 543/10 nm, emission barrier filter 590 nm).

### Comet slide analysis

Antioxidant efficiency of extract was determined by measuring the shortening of comet tail that migrated forward to the anode during electrophoresis. Normalized tail length which represented frequency of DNA breakage and number of fragments was determined. One hundred comets on each slide were scored according to the length and relative intensity of fluorescence in the tail when compared to the head.<sup>9</sup> Comets were selected at random from each slide. On screen measurement of head and tail length was consistently performed. Tail to head ratios for each reaction was calculated.

### Statistical analyses

The normalized tail length to head ratios among different reactions were statistically tested using one-way ANOVA. Independent samples *t* test with adjustment for multiple comparisons were used for between-group comparisons.

## Results

### Anti-oxidative effects of *Areca catechu* L. nut husk extracts

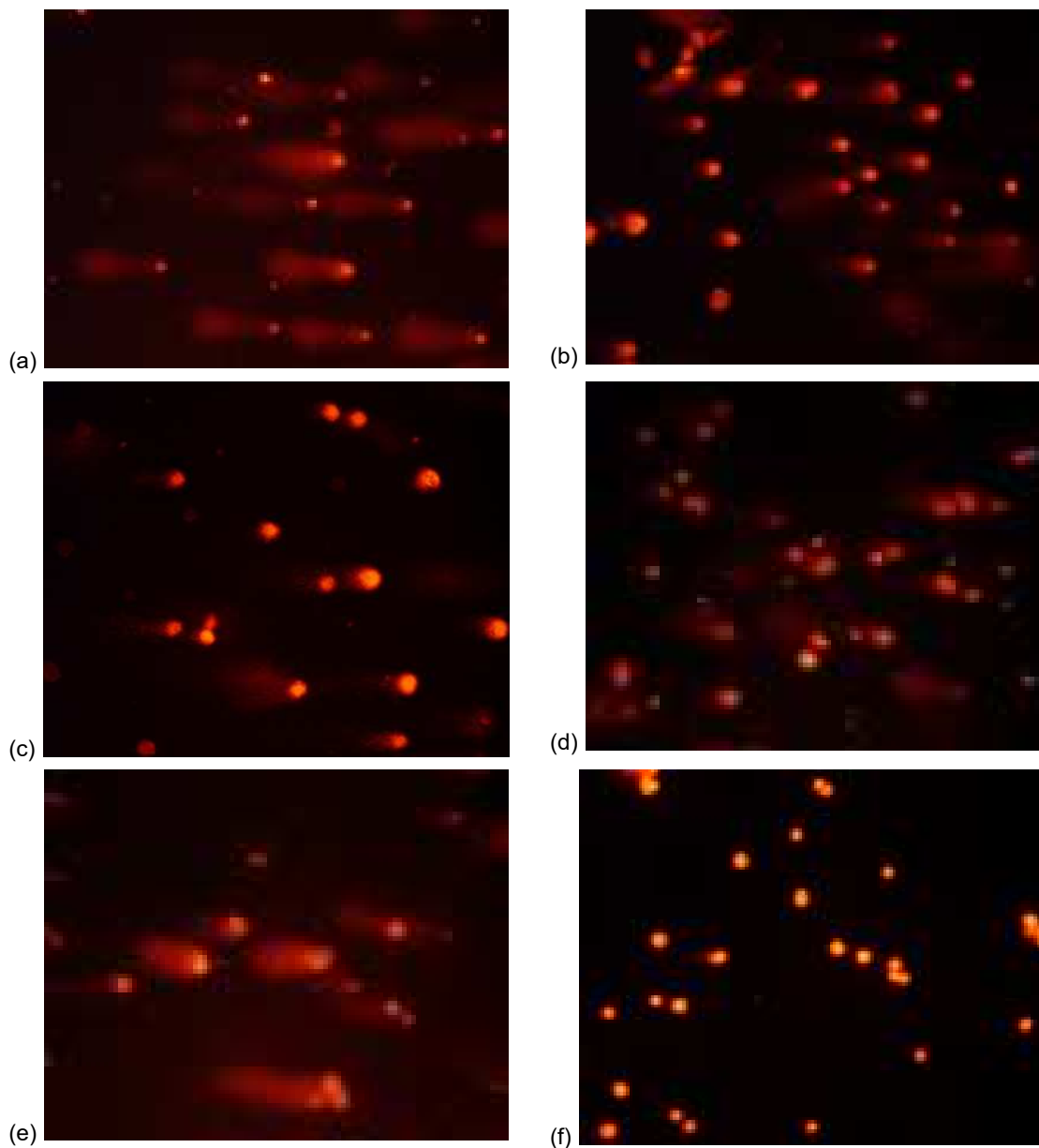
A comet assay was carried out to assess oxidative DNA damage induced with a free radical producer,  $\text{H}_2\text{O}_2$ , in order to screen for potential antioxidative compounds from areca nut tree extracts. Typical comet cells and results of inhibition associated with antioxidative effects of *Areca catechu* L. nut husk extracts were shown in Fig. 1.

The methanol extracts of 8-month areca nut husk was found to have a dose-dependent inhibition of comet formation where significant protection of  $\text{H}_2\text{O}_2$ -induced DNA damage was observed at 0.1% w/v concentration ( $P < 0.05$ ).<sup>11</sup>

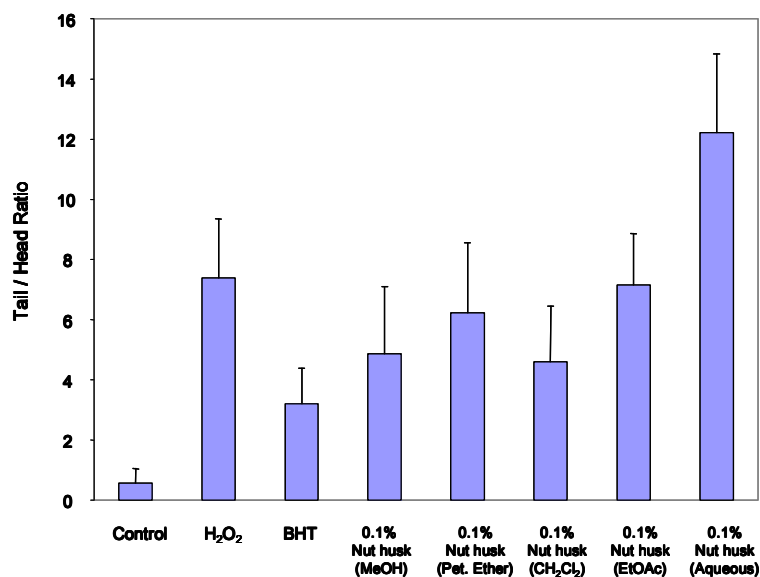
Other organic extracts, i.e., petroleum ether, ethyl acetate and aqueous extracts of areca nut husk, did not

show significant reduction of tail DNA of the cells treated with 0.1% w/v concentrations used in the assay (Fig. 2). However, one experiment with 0.1% w/v of the methylene chloride extract was able to reduce DNA

damage compared with 0.1% w/v of the methanol extract ( $P < 0.05$ ). Aqueous extract, on the contrary, significantly enhanced DNA damage ( $P < 0.05$ ).



**Figure 1** Single cell gel electrophoresis of HepG2 cells showing DNA damage (comet) (400x) after treatment with 40  $\mu\text{M}$   $\text{H}_2\text{O}_2$  alone (a), with 1  $\mu\text{M}$  BHT (b), with 0.1% w/v nut husk MeOH extract (c), with 0.1% w/v areca seed MeOH extract (d), with 0.1% w/v bloomed flower MeOH extract (e), and control (f).

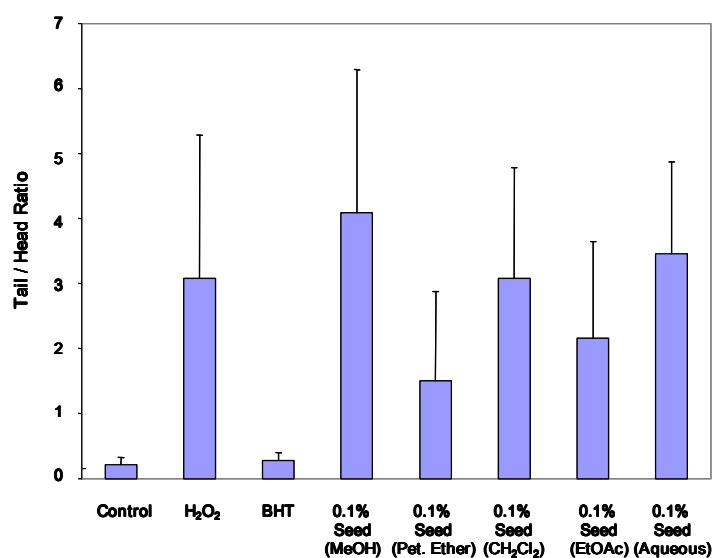


**Figure 2** Tail to head ratios corresponding to the anti-oxidative effects of various organic solvent extracts of areca nut husk on inhibition of comet cell formation.

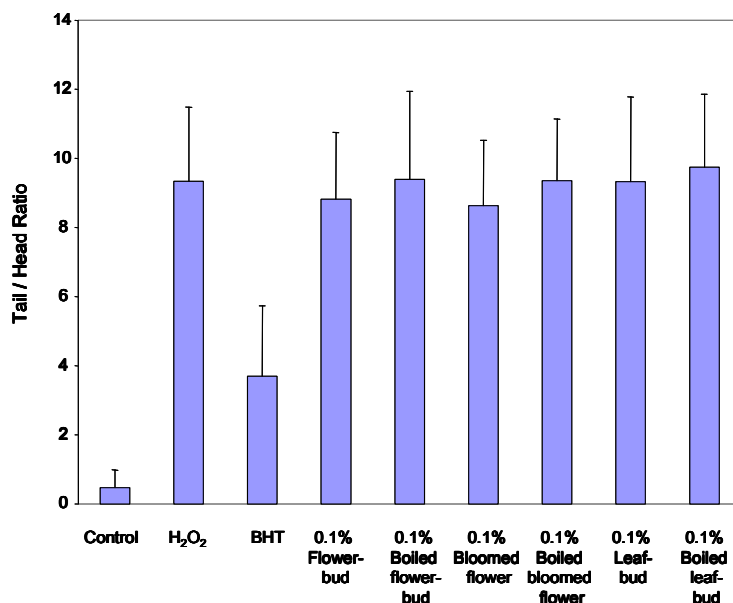
### Anti-oxidative effects of areca seed extracts, and boiled and unboiled flower bud, bloomed flower and leaf bud extracts

To determine effect of areca seed on antioxidation, various organic extracts of areca nut were tested. No extracts inhibited H<sub>2</sub>O<sub>2</sub>-induced DNA damage compared with BHT (Fig. 3). However, methanol and aqueous extracts showed an enhanced DNA damage. As shown

in Fig. 4, unboiled and boiled flower bud, bloomed flower and leaf bud aqueous extracts did not inhibit a DNA fragmentation. The similar average tail length of cell-treated with boiled or unboiled extracts and H<sub>2</sub>O<sub>2</sub> was evident.



**Figure 3** Tail to head ratios corresponding to the anti-oxidative effects of various organic solvent extracts of areca seed on inhibition of comet cell formation.



**Figure 4** Tail to head ratios corresponding to the anti-oxidative effects of aqueous extracts of flower bud, bloomed flower and leaf bud of areca with and without boiling on inhibition of comet cell formation.

## Discussion

Comet assay for DNA damage has been widely used for assessing antioxidant activity and DNA effects. The extracts that exhibited antioxidant activity might reduce hydrogen peroxide-induced nuclear fragmentation. Among various extracts, methanol and methylene chloride extracts of *Areca catechu* nut husk could result in inhibition of comet observed with HepG2 cells. These results may suggest that there may be more than one compound that exhibited antioxidation activity and these compounds have different polarity. Therefore, the antioxidative constituents in methanol and methylene chloride fractions from areca nut husk should be further characterized. All solvent extracts of areca nut or areca seed did not show antioxidative activity. On the contrary, aqueous and methanol extracts tended to enhance DNA damage. Although areca seed extract exhibited a strong scavenging effect against free radical<sup>10</sup>, its high level of polyphenolic compounds could potentially promote the HepG2 DNA damage. This has been related to many health hazard reports which examined the oxidative and

genotoxic effects of areca nut extract or areca quid in several assay systems.<sup>7-9</sup>

The aqueous extract of areca leaf bud, flower bud and bloomed flower showed similar results of no inhibition of comet formation. Boiling process which is the usual way of consuming these parts among Thai people might reduce this hazardous effect. Similarly, boiled parts of *Areca catechu* exhibited no antioxidative activity.

## Conclusion

In this study, the comet assay was used to evaluate antioxidant effect of *Areca catechu* extracts. The methanolic and methylene chloride extracts of *Areca catechu* nut husk exhibited an antioxidant activity. On the contrary, extracts of other parts of areca, e.g., seed, leaf bud, flower bud and bloomed flower did not show any antioxidative effect.

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