博士論文要約（Summary）

入学年 Entrance Year：　令和 2 年
連合農学研究科
専攻 Course：　応用生命科学専攻
氏名 Name：　易　書瀚

タイトル **Title**	Study on the functionality and safety of areca nut（*Areca catechu* L.）, a chewing food　（咀嚼嗜好品である檳榔子の機能性と安全性に関する研究）

キーワード Key word（　Areca nut　）　（　Antioxidant　）　（　Anti-obesity　）

Chapter 1

1.1 Overview of areca nut

Areca nut, originating in Southeast Asia, has been used as plant medicine in China and India for thousands of years. Consumed in various regions, it has addictive properties and is considered a commercial crop. However, high consumption can lead to diseases.

1.2 Toxicological studies of areca nut

Areca nut consumption has been linked to oral submucous fibrosis (OSF), oxidative stress, and inflammation, which contribute to the development of various diseases. Areca nut extract has been found to increase the expression of inflammatory factors, such as COX-2 and VEGF, and induce ROS in cells. Areca nut extract can also induce DNA damage by inhibiting p27, a kinase inhibitor that regulates cell cycle responses. Areca nut extract has also been found to negatively affect sperm motility in humans, leading to decreased sperm count and activity. Additionally, areca nut is the fourth most widely consumed addictive substance globally, with arecoline activating muscarinic acetylcholine receptors, similar to nicotine addiction.

1.3 Biological activity of areca nut

Areca nut extracts have been found to have antibacterial, antiviral, anti-aging, antioxidant, antidepressant, and cholesterol-lowering effects. They have been found to inhibit Staphylococcus aureus, Escherichia coli, Salmonella enterica, and Enterobacter aerogenes. They also have antioxidant properties, activating the Nrf2/HO-1 pathway and inhibiting LPS-induced inflammation. Areca nut's polyphenols have ROS scavenging and antioxidant properties. Additionally, areca nut extract has been found to reduce triglyceride uptake and plasma lipid concentrations in rats.

1.4 Resarch contens in this study.

This study investigates the bioactivities of areca nut, including polyphenols and alkaloids. Whole areca nut extract (ANE), areca nut polyphenol (ANP), and arecoline (ARE) were prepared and tested for their antioxidant and anti-inflammatory properties. ANP reduced ROS and inflammation in lipopolysaccharides-stimulated mouse RAW264.7 cells. ANE also reduced WD-induced dyslipidemia in mice. The study provides scientific evidence for the safety and functionality of areca nut.

Chapter 2

2.1 Introduction and Purpose

Areca catechu L., a tropical fruit, has been used in traditional Chinese and Ayurvedic medicine for its addictive properties. However, it has been classified as a class I carcinogen due to its high alkaloids. This study focuses on the antioxidant effects of areca nut polyphenols, which have been shown to remove free radical reactive oxygen species (ROS) from the body. The study uses RAW264.7 cells as a model to investigate the role of areca nut polyphenols in cellular antioxidant and anti-inflammatory activities.

2.2 Materials and method

The study used areca nut polyphenols (ANP) to prepare antioxidant activity assays in LPS-stimulated RAW264.7 macrophages. The areca nut was obtained from Wanning city, Hainan, China and the crude polyphenols were extracted. The total polyphenol contents were estimated at 80% by the Folin-Ciocalteu method. The cells were cultured in DMEM containing 10-20% FBS and 1% antibiotic at 37°C in 5% CO2 humidified air. Cell viability was assessed using MTT cell proliferation and cytotoxicity assay kits. Cellular ROS were detected using the fluorescent probe DCFH-DA and fluorescence inverted microscope observation. Western blotting was performed on the lysate, and protein content was determined using a protein assay kit. Transcriptome analysis was performed on the lysate, and differential gene analysis was performed using DESeq2. Data were analyzed using one-way ANOVA and differences among treatment groups were evaluated using least significant difference (LSD) and Duncan's multiple range tests.

2.3 Results

The study investigates the antioxidant activity of ANP in LPS-stimulated RAW264.7 macrophages. Cell viability was increased in a time- and dose-dependent manner, but cell toxicity was observed when the time and concentration reached 15 hours and 640 μg/mL. ANP was found to eliminate intracellular ROS in RAW264.7 cells, with LPS significantly increasing ROS levels compared to the Con group. The addition of ANP dose-dependently reduced ROS levels induced by LPS.ANP significantly enhanced Nrf2 and HO-1 levels from 40 μg/ml to 320 μg/ml of ANP for 12 hours. The molecular mechanisms of antioxidant activity were evaluated using high-throughput RNA-sequencing technology. Differentially expressed genes (DEGs) were compared between the 160 / LPS group and the 320 / LPS group, with treatment targeting mitochondrial organization, kinase activity, and transferase activity. Further, pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed that the pathways of 160 / LPS had 9 pathways related to the MAPK pathway, while the 320 / LPS group had 12 pathways associated with the MAPK pathway. The protein-protein interaction network (PPI) results were based on DEGs, and the inhibitory effect of ANP on the MAPK pathway was demonstrated. ANP showed significant inhibition of the phosphorylation of MAPKs induced by LPS in a dose-dependent manner, with significant inhibition at 320 μg/ml of ANP.

2.4 Discussion

Areca nuts, a popular tropical fruit in Asian areas, are rich in polyphenols and have been found to have antioxidant and antibacterial properties. A study using a macrophage-like cell line, RAW264.7,

investigated the antioxidant activity of ANP and its anti-inflammatory activities. ANP showed significant inhibitory effects on LPS-stimulated ROS and increased Nrf2 and HO-1 levels. It also downregulated the activation of the MAPK signaling pathway, inhibiting oxidative stress and inflammation. ANP also influenced mitochondria, kinase activity, and transferase activity, suggesting it may alleviate oxidative stress by regulating mitochondrial activity and protease activity. ANP also affected pathways related to diseases, NAFLD, virus infections, and bacterial infections, most of which are related to the MAPK pathway. The cellular senescence pathway and the MAPK pathway interacted, with Mapk3 playing a key role in cellular senescence and Erk1/2 playing a key role in cellular senescence due to smoking.

2.5 Conclusions

In conclusion, our work demonstrated that ANP reduced LPS-stimulated oxidative stress through MAPK-mediated Nrf2/HO-1 signaling pathway. These data provide the molecular bases for understanding the antioxidant activity of ANP.

Chapter 3

3.1 Introduction and Purpose

Areca nut, a chewing food in Asian areas, is classified as a class I carcinogen by the IARC due to its association with oral diseases. Areca nut alkaloids, such as arecoline, have been found to inhibit adipogenic differentiation and induce lipolysis. Polyphenol, a major component of areca nut, has antioxidant, anti-inflammatory, and anti-atherosclerotic effects. Obesity is a global health problem, with unhealthy diets leading to chronic diseases like NAFLD. The areca nut and its polyphenols may prevent unhealthy diet-induced disorders, which may be regulated by gut microbiota. To determine the components contributing to areca nut biofunctions, the study created whole ANE, ANP, and ARE as experiment materials.

3.2 Materials and method

The study focuses on the preparation of whole areca nut powder and its polyphenols, as well as ARE, and their effects on mice. Raw areca nut was obtained in Wanning City, Hainan Province, China, and areca nut powder and ANP were prepared using a method that involved freezing and pulverizing the nuts. The major polyphenols in ANE were identified as catechins and proanthocyanidin B1. The study used male C57BL/6N mice, which were raised individually in cages with controlled light and temperature. The mice were fed different diets, with varying levels of polyphenols and alkaloids. Serum biochemical indicators were measured, and liver lipid rate and histomorphology were analyzed. Western blotting analysis was performed on liver tissue, and gut microbiota analysis was performed using fetal DNA extract and 16S rRNA gene sequencing. The data were analyzed using one-way ANOVA, and differences among treatment groups were evaluated using least significant difference and Duncan's multiple range tests.

3.3 Results

The study investigates the effects of areca nuts and their ingredients on preventing weight gain due to dietary changes. Mice were fed three different samples of ANE, ANP, and ARE for 12 weeks. Results showed that the final body weights of all areca nut groups were significantly decreased compared to the WD group. ANP most significantly decreased body weight in the three sample groups, while WD-induced liver weight and epididymal fat were significantly decreased by ANP. The areca nut was found to ameliorate WD-induced overweight, with ANP acting as a master ingredient for this effect. The liver's total lipid rate was increased by WD, and significantly reduced by ANE and ANP, but not ARE. Hepatic H&E staining results indicated that lipid droplets were markedly increased in WD and markedly reduced in ANP, although ANE and ARE showed a reduced trend but not as clear as ANP. The areca nut and its ingredients also improved WD-induced dyslipidemia by reducing the accumulation of lipid droplets in the liver and improving liver injury. The areca nut and its ingredients also reduced WD-induced T-Cho levels, which were found to be inhibited by ANE, ANP, and ARE. Fecal samples were collected at the end of the experiment, and the areca nut and its ingredients showed different effects on mice gut microbiota. *Akkermansia* was significantly increased by ANP and decreased by ARE, compared to ND. ANE and ANP decreased the abundance of *Ruminococcus*, but not in ARE compared with WD. Pearson correlation analysis revealed significant negative correlations between the gut microbiome community and serum biochemical profiles. ANP had a promotive effect on probiotics such as *Akkermansia* and *Ruminococcus*, and an inhibitory effect on pathogenic bacteria such as g_*Sutterella* and f_*Desulfovibrionaceae*;g_.

3.4 Discussion

Areca nut, a tropical fruit, contains bioactive polyphenols and ARE, which can cause ROS accumulation, leading to cholesterol accumulation in the liver, leading to NAFLD. A study assessed the effects of whole areca nut extract, polyphenol extract, and ARE on a WD-induced NAFLD mouse model. Results showed that ANP ameliorated morphological traits, liver lipid rate, and accumulation of lipid droplets in the liver, which are related to being overweight and NAFLD. ANP also improved WD-induced dyslipidemia by inhibiting WD-induced cholesterol synthesis by increasing p-AMPKα expression. The areca nut and its ingredients also showed different effects on mouse gut microbiota, with ANP increasing the abundance of *Akkermansia* and decreasing ARE. ARE, the primary active ingredient responsible for the central nervous system effects of the areca nut, was harmful for gut microbiota. The study concluded that areca nut crude containing both polyphenol and ARE had a potential preventive effect on obesity, liver injury, and gut microbiota.

3.5 Conclusions

In conclusion, our works show that ANE and ANP ameliorated WD-induced obesity via activating p-AMPKα to inhibit the SREBP2 and HMGCR expressions and by improving the gut microbiota. On the other hand, ARE had an adverse effect on the gut microbiota. These data provide evidence for understanding the polyphenol benefit function and ARE side effect in the areca nut.

Chapter 4

4.1 General discussion

The study explores the antioxidant capacity and preventive effects of areca nut polyphenols on metabolic syndrome, focusing on lipid and cholesterol metabolism and changes in the gut microbiota. The research, which is widely consumed as a chewing habit and phytomedicine, aims to understand the safety and functionality of areca nut. Areca nut, a carcinogen, has various pharmacological activities, including antibacterial, antiviral, antioxidant, antidepressant, anti-Alzheimer's, and cholesterol-lowering effects. A preliminary experiment was conducted using the RAW264.7 cell model, where LPS stimulates ROS production, leading to oxidative stress and inflammation. The results showed that areca nut polyphenols can effectively scavenge excessive ROS, exert antioxidant properties, and reduce LPS-induced inflammatory response. However, the study has limitations, including the availability of mixtures of areca nut polyphenols, the comparison between natural plant extracts and synthetic compounds, and the lack of investigation into the effect of areca nut's coarse fiber structure on human oral consumption.

4.2 General conclusion

In summary, this study revealed that areca nut polyphenols can attenuate LPS-induced oxidative stress and inflammation. Areca nut polyphenols can also inhibit diet-induced metabolic syndrome and gut microbiota dysregulation. Furthermore, they can enhance the gut microbiota and stimulate the growth of beneficial bacteria. Although arecolin can lower body weight, it does not ameliorate the metabolic disorders induced by WD and has an adverse effect on gut microbiota. This implies that the areca nut polyphenols account for the biological effects of areca nut. These findings provide new insights into the safety and functionality of areca nut.

4.3 Prospects

The areca nut is a widely chewed food that has been controversial due to the hazards of its biological components. After determining that the main harmful substance in areca nut is alkaloids. It is possible to develop alkaloid-free areca nut, imitating decaffeinated coffee. In this way, we can provide a direction for the development and exploitation of areca nut.