

## Evaluation an Aqueous Extract of Irradiated and Non Irradiated of *Orthosiphon Aristatus* on Zebrafish Embryo (*Danio Rerio*) through Acute Toxicity Assay

Santhra Segaran Balan<sup>1,\*</sup>, Punithaamalar Manokaram<sup>1</sup>, Sitell Maya<sup>1</sup>,  
Hasnah Bahari<sup>2</sup>, Mahathir Mohd Uzi<sup>1</sup>, Muhammad Danial<sup>1</sup>,  
Azrina Zainal Abidin<sup>1</sup>, Arapoc Daryl Jesus<sup>3</sup> and Mahardian Rahmadi<sup>4,5</sup>

<sup>1</sup>Department of Diagnostic and Allied Health Sciences, Faculty of Health and Life Sciences, Management and Science University, Shah Alam, Malaysia

<sup>2</sup>Departments of Human Anatomy, Faculty Medicine and Health Sciences, University Putra Malaysia, Serdang, Malaysia

<sup>3</sup>Malaysian Nuclear Agency, Malaysia

<sup>4</sup>Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Malaysia

<sup>5</sup>Department of Pharmacy, Universitas Airlangga Hospital, Malaysia

(\*Corresponding author's e-mail: santhra@msu.edu.my)

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### Abstract

Background: *Orthosiphon aristatus* has been usually related to be powerful in curing many illnesses inclusive of post-partum remedy, anti-influence, rheumatism and stopping osteoporosis as a result of menopause. Objectives: Thus, this study was designed to differentiate the toxicity effect of non-irradiated and irradiated *Orthosiphon aristatus* at different dosage of 3, 6, 9 and 12 kilogray (kGy) on zebrafish embryo. Method: Survival rate, hatching rate, heart beat rate and scoliosis were observed and data were analysed using SPSS 25.00 windows. Result: The lethal dose (LC50) of non-irradiated *Orthosiphon aristatus* is 381.81 µg/mL compare to irradiated extract at 3 % is (371.27 µg/mL), 6 % (311.03 µg/mL), 9 % (160.72 µg/mL) and 12 % (140.18 µg/mL). Hatchability of zebrafish embryo for *Orthosiphon aristatus* extract decrease in the higher dosage of irradiated sample compared to non-irradiated sample. No presence of scoliosis was observed in all dosage of irradiated and non-irradiated of *Orthosiphon aristatus*. The heartbeat of zebrafish embryo treated with irradiated *Orthosiphon aristatus* specifically 12 % show decrease at 250 µg/mL concentration. Remaining dosage of irradiated and non-irradiated *Orthosiphon aristatus* show average heartbeat around 120 - 160 bpm. Conclusion: As conclusion, irradiated and non-irradiated of *Orthosiphon aristatus* is safe to be consumed due to its pharmaceutical effect but it still exhibited mild toxicity effect on zebrafish embryo.

**Keywords:** *Orthosiphon aristatus*, Irradiation, Zebrafish embryo, Heartbeat, Hatching

### Introduction

Traditional medicine or herbal medicine is an alternative, complementary medicine widely used in many countries. It became popular because people believe that conventional medicine has beneficial elements. These natural sources can treat various diseases, such as allopathic medicines based on their mechanism of action. Therefore, the main difference between traditional and herbal medicine is that the former is chemically treated and scientifically proven to be safe to consume. The diagnosis is based on conventional medicine symptoms, whereas folk medicine takes a preventive approach, as it is obtained from natural sources [1]. In traditional medicine, heavy metals can ultimately have toxic effects over high and sustained intake, despite minimal side effects from a small number of chemicals [2].

The poisonous material comes primarily from plant, animal and microorganism sources. As a toxic agent, the poisonous substance is transmitted mainly through direct contact through various transmission routes. A toxicology test is appropriate for both allopathic and complementary medicines. And complementary medicine for finding some unknown consequences before signs and symptoms occur when ingested strongly. Herbal medicine's common harmful impacts include nausea, vomiting, weakness,

dizziness, hypotension, etc. The evaluation of safety by toxicity tests will show the protection and use of conventional medicine [3].

Toxicity study is significant to check any plant or herbs, and this is carried out to ensure that the plant or herbs do not contain any life-threatening components. The toxicity test using zebra fish became the choice of researcher nowadays. Zebra fish and its transparent embryos are among the appropriate models used to study the impact of toxicity on embryonic development. The toxicity test is used to examine the bioactive compounds of the sample. Used mammalian models have certain drawbacks at higher costs and prolonged performance, which are also ethically controversial. Genetically humans have significant parallels with the *Danio rerio* of genomic sequences and brain patterning. This makes zebra fish embryos a helpful tool to investigate other toxicology analysis diversions that produce an immediate result. The frequent use of zebra fish embryo is usually due to its short life span-speedy and influential outcomes [4,5]. Zebra fish embryos have a rapid process of embryogenesis and contain several eggs. Besides, these eggs are extraordinarily permeable and highly sensitive for chemical components penetration. The embryo is clear enough to adequately track embryogenesis progress with a regular light dissection microscope. Two of the key parameters based on the toxicity evaluation are survival rate, LC<sub>50</sub> commitment, hatching rate, scoliosis rate and heart rate [5].

*Orthosiphon aristatus* is known as Misai kucing and Kumis kucing in Malaysia. *Orthosiphon aristatus* is one of South-East Asia's most widely used traditional medicinals [6]. It is often referred to as the mint family. *Orthosiphon* sp, according to floral and calyx colors. The variety is categorized as white flowers (white range) and light purple flowers (purple range). There are more bioactive compounds in the purple variety than the green. Some scientific work has, however, used the white variety [7]. *Orthosiphon aristatu* is commonly used for treatment such as rheumatoid diseases, diabetes, asthma, tonsillitis, epilepsy, menstrual disorders, gonorrhoea, syphilis, kidney stones, gallstones, burns, measles, hepatitis and jaundice. His leaves were used as wellness tea in Europe and Japan. *Orthosiphon aristatus* is famous for the diuretic effect, which is higher than most other known diuretics. The chemical tests have shown 3 kinds of flavonoids and phenylpropanoids in various *Orthosiphon aristatus* extracts [6,7].

Gamma rays are also used on plants in developing agricultural and economically valuable varieties with high productivity potential. Gamma rays are also essential to establish essential characteristics of plants and to improve genetic variability. Gamma irradiation may be useful in modifying one or more physiological characteristics. Gamma rays were a significant factor in developing new, improved mutants capable of producing more substantial quantities of commercially essential metabolites [8]. Gamma rays of varying irradiation rates can damage or alter important plant cell components and may affect the morphology, anatomy, biochemistry and physiological conditions of plants differently. Irradiation was also widely used for mutation breeding in various crops and ornamental plants and was shown to encourage recessive gene expression and create new genetic changes. A previous study on gamma-ray on *Orthosiphon stamineus* showed that the total soluble protein and entire chlorophyll content declined significantly with the rise in gamma dose. Specially irradiated plantlets at high gamma-ray dosages were shown to significantly increase peroxidase activity [1]. Gamma radiation is the physically-operated pressure used to improve the quality and quantity of plant attributes. It is often used in the plant science sector as an impact from low-scale incitement to high-scale impairment. Bioactive mixtures, for instance, maybe activated under sufficiently phenolic and flavonoid radiation. Induced oxidative stress in cells that increases phenolic through the introduction of phenolic mixtures gamma illumination applies its mechanisms [8].

## Materials and methods

### Plant identification

The fresh *Orthosiphon aristatus* (Blume) Miq was collect from a local place in Jelebu, Negeri Sembilan, Pahang. The plant were sent for confirmation and the plant ID is MFI 0140/19.

### Sample preparation

Gamma irradiation of *Orthosiphon aristatus* plan takes place in a cobalt 60 irradiator at the Malaysia Nuclear Agency (Science, 2008). The *Orthosiphon aristatus* powder content was irradiated at varying gamma-ray doses at 3, 6, 9 and 12 kGy. The non-irradiated plant was used as an experimental model for these studies. Irradiated and non-irradiated *Orthosiphon aristatus* extraction was prepared using a hot aqueous method for zebra fish study. Five hundred mL distilled water and 50 g *Orthosiphon aristatus* (irradiated and non-irradiated samples) well mixed in Scott bottle and placed in a water bath at 72 °C. After 24 h; the mixture was filtered using Whatman paper No 1. Filtration is done 3 times to get an

excellent solution. The filtered sample was transferred to the falcon tube and store in the freezer  $-40^{\circ}\text{C}$  before undergoing the freeze-drying process [9,10]

### Serial dilution

The serial dilution method was used to obtain a series of *Orthosiphon aristatus* extract samples with different concentrations. The stock solution was prepared by diluting 0.1 g of *Orthosiphon aristatus* extract with 1000  $\mu\text{L}$  of distilled water. From the stock solution, seven (7) successive series of concentration were produced which were 500, 250, 125, 62.5, 31.25, 15.625 and 7.81  $\mu\text{g}/\text{mL}$ . On the other hand, as for the control treatment, the 500 mg dose of paracetamol was used as a positive control. In contrast, distilled water was used as a negative control [9,10].

### Zebra fish embryo assay

After 24 h post-fertilization (24 hpf), 1 healthy zebra fish embryo was transferred into 96 well plates using a sterile pasture pipette. After transfer each embryo in well, treatment was given by pipetting the irradiated sample into each 96 well with different concentrations. The observation was carried out during 24, 48 and 72 h after treatment. Inspection is done under the inverted or dissection microscope and focuses on the hatching rate, the heartbeat of zebra fish, survival rate and significant scoliosis [9,10].

### Data analysis

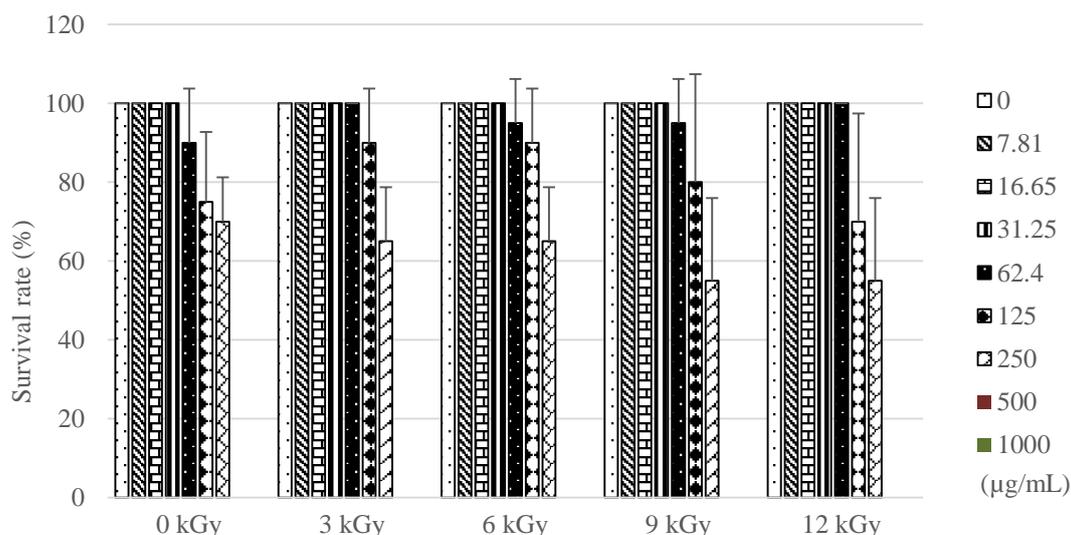
Data were analyzed using the software SPSS 25.0 version. All data were analyzed for normality test. The One-way Analysis of Variance (ANOVA) and linear regression was used to obtain data. All the data interpreted with  $p < 0.05$  as significant. The lethal concentration at 50 % ( $\text{LC}_{50}$ ) of each different dose of kGy extract of *Orthosiphon aristatus* was calculated through linear regression. The value was used to determine the mortality of zebra fish. All the data were interpreted as mean  $\pm$  SD.

## Results and discussion

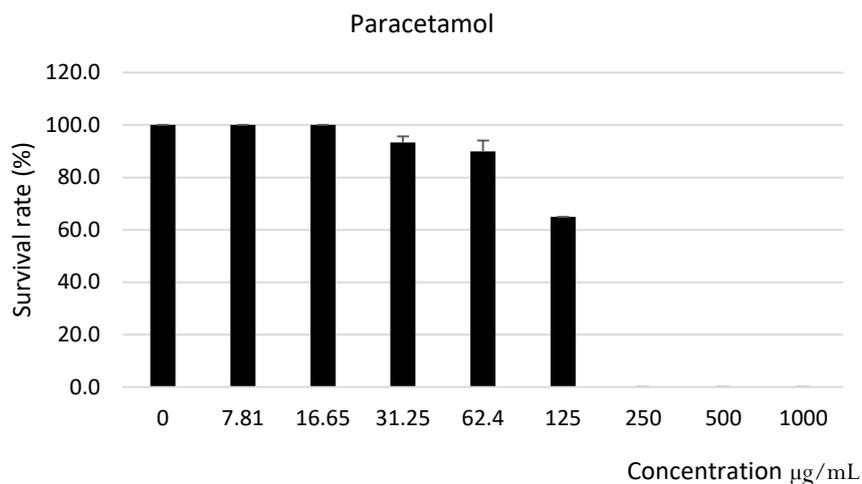
### Results

#### Effect of irradiated *Orthosiphon aristatus* extract on the survival rate

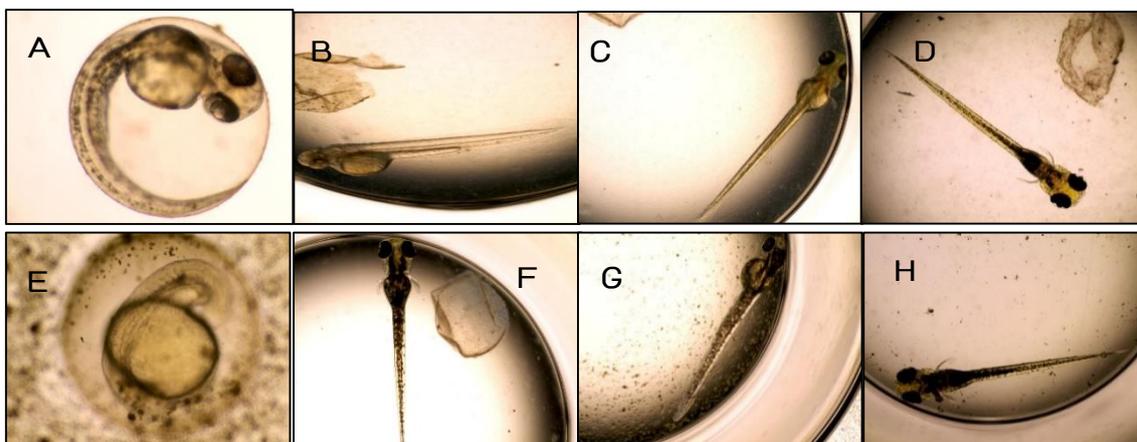
The survival rate of embryo tested on different gamma radiation show that increased in survival rate when the concentration of the extract decreased. No survival of embryo seen in 500 - 1000  $\mu\text{g}/\text{mL}$  for all irradiated and non-irradiated extract of *Orthosiphon aristatus*. Irradiated extract at 3, 6, 9 and 12 kGY, the higher survival rate has seen in 7.81  $\mu\text{g}/\text{mL}$  and gradually decreased in survival rate at 62.4 - 250  $\mu\text{g}/\text{mL}$ . For non-irradiated (0 kGY) extract of *Orthosiphon aristatus*, a similar pattern of survival has seen as an irradiated extract (**Figure 1**). As for the paracetamol, the survival rate decreased as the concentration increased (**Figure 2**). The growth of zebrafish embryo for irradiated and non-irradiated shown in **Figure 3**.



**Figure 1** Effect of irradiated and non-irradiated of *Orthosiphon aristatus* extract on survival rate of zebrafish embryo against different concentration. Data were expressed at mean  $\pm$  SD.



**Figure 2** Effect of paracetamol on survival rate of zebrafish embryo. Data were expressed at mean ± SD.



**Figure 3** Non-irradiated extract of *Orthosiphon aristatus* zebrafish embryo at 24 h post fertilization (A), 48 h (B), 72 h (C) and 98 h (D) observed under 10× magnification of inverted microscope. Irradiated extract of *Orthosiphon aristatus* zebrafish embryo at 24 h post fertilization (E), 48 h (F), 72 h (G) and 98 h (H) observed under 10× magnification of inverted microscope.

***Effect of Orthosiphon aristatus extract on LC<sub>50</sub> values***

The LC<sub>50</sub> value of the irradiated and non-irradiated samples of *Orthosiphon aristatus* plotted using mortality ratio versus the log concentration. Non-irradiated of *Orthosiphon aristatus* show 381.81 µg/mL, and the extract with the lower concentration than this value consider safer for consumed by zebrafish embryo. For sample irradiated 3 % kGy the LC<sub>50</sub> is 371.27 µg/mL, follow by 6 % 311.03 µg/mL, 9 % 160.72 µg/mL and finally 12 % 140.18 µg/mL. This show the irradiated extract of *Orthosiphon aristatus* were also safer to consume by zebrafish embryo. The paracetamol show LC<sub>50</sub> is 203.83 µg/mL.

**Table 1** The lethal concentration 50 (LC<sub>50</sub>) value of test extract and control solution examined on zebrafish embryo.

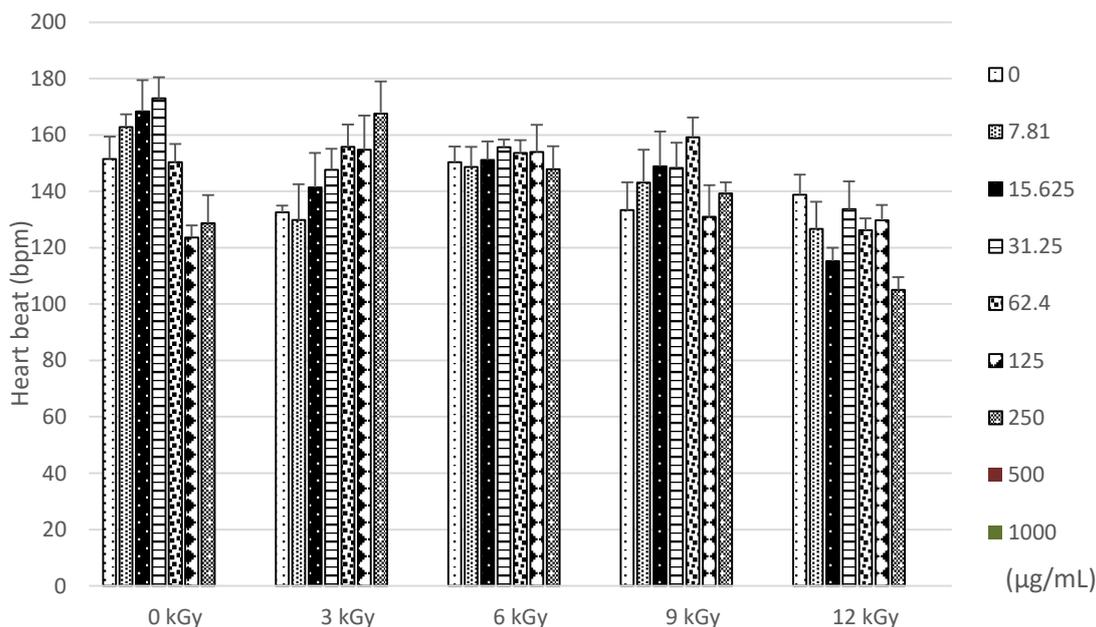
Dose of irradiated and non-irradiated samples	LC <sub>50</sub> (µg/mL)
0 kGy	381.81
3 kGy	371.27
6 kGy	311.03
9 kGy	160.72
12 kGy	140.18
Positive (Paracetamol)	203.83
Negative (Distilled Water)	-

**Effect of *Orthosiphon aristatus* extract on scoliosis**

Based on the toxicity test done using the irradiated and non-irradiated sample on zebrafish embryo, no scoliosis seen. High concentration and low concentration also not show any scoliosis in zebrafish embryo. No scoliosis not observed in positive control (paracetamol).

**Effect of *Orthosiphon aristatus* extract on heartbeat**

Toxic effect of irradiated and non-irradiated of *Orthosiphon aristatus* evaluated on observation of zebrafish embryo heartbeat. The heartbeat of zebrafish embryo for irradiated and non-irradiated not seen in 1000 and 500 µg/mL due to no zebrafish embryo survive in this concentration. Based on the result heartbeat of zebrafish embryo for non-irradiated *Orthosiphon aristatus* show same range in all concentration from 0 - 250 µg/mL. The higher heartbeat observed at 15.62 - 31.25 µg/mL. For irradiated *Orthosiphon aristatus* heartbeat of zebrafish embryo are similar for 3, 6, 9 and 12 kGy. Average heartbeat for irradiated *Orthosiphon aristatus* is around 120-1-070 bpm for all the concentration.



**Figure 4** Effect of irradiated and non-irradiated of *Orthosiphon aristatus* extract on heartbeat of zebrafish embryo against different concentration. Data were expressed at mean ± SD.

## Discussion

### *Effect of irradiated Orthosiphon aristatus extract on the survival rate*

Result of the study indicate that the non-irradiated *Orthosiphon aristatus* extract has a higher survival rate compare to the different dose of irradiated *Orthosiphon aristatus*. This is because, although both extracts have the same compound or component, they [who?] believe that gamma radiation has been slightly changing an irradiated extract. Previous research revealed that, *Orthosiphon aristatus* rich with many bioactive properties, such as antioxidant, antimicrobial and anti-inflammatory, which each component play a vital role. It could be due to this gamma radiation slight change has happened in this bioactive compound. Due to that, the non-irradiated expose zebrafish embryo show higher survival rate compared to the irradiated sample. The low hatchability rate and delayed hatching process indicated the retardation of growth in zebrafish embryo. However, in this study, no delayed hatching was reported at lower concentration compares to a higher concentration. This due that higher concentration of extract has affected the chorionic membrane of zebrafish embryos do to chorionase enzyme activity [11].

### *Effect of Orthosiphon aristatus extract on LC<sub>50</sub> values*

The LC<sub>50</sub> value of *Orthosiphon aristatus* determined by the figure out the amount of toxicant that required to induce death of 50 % of the population of the zebrafish embryo test in it calculated using probit analysis. The LC<sub>50</sub> value of non-irradiated and irradiated of *Orthosiphon aristatus* extract to the zebrafish embryo reveal that it is less harmful to zebra fish embryos. In this study, the embryos immersed in different concentration of *Orthosiphon aristatus* and result revealed no morphological changes or defect in embryo or zebrafish larvae [12].

### *Effect of Orthosiphon aristatus extract on scoliosis*

Results of the study show no deformity observed for a non-irradiated or irradiated extract of *Orthosiphon aristatus*. Deformity present can observe in spinal curvature, and they are kyphosis, lordosis and scoliosis. All 3 deformity measured based on the spinal bending degree depend on toxicity level in the plant. If the plant has toxicity level higher, will course the AChE enzyme inhibit and will lead the neuromuscular coordination in zebrafish. The previous study shows that spinal deformities occur due to the reduction of collagen level in the spinal column, reduction of specific gene regulator, inhibition of protein tyrosine kinase 7 (ptk7) and also an alteration of amino acid composition [11,12].

### *Effect of Orthosiphon aristatus extract on heartbeat*

Average heart rate is essential and essential for growth and development of zebrafish embryo in later stages of life, but abnormality on heart rate will affect the heart function and also can cause severe development effect in zebrafish embryo. In this study, heart rate of zebrafish embryo exposed with irradiated and non-irradiated *Orthosiphon aristatus* is standard in all concentration expect in higher concentration. It shows that irradiated and non-irradiated *Orthosiphon aristatus* do not have cardiotoxicity effect on the heart rate of zebrafish embryo. The heart is the 1<sup>st</sup> organ develops in the zebrafish embryo and is vital for the development of zebrafish to be adult health fish. If the cardiac system develops very poorly, it may lead to abnormality or malformation of other organs in zebrafish. In overall even though *Orthosiphon* embryo *aristatus* rich with phenolic and flavonoids but it does not cause cardiotoxicity in zebrafish [11,13].

## Conclusions

This study shows that irradiated and non-irradiated *Orthosiphon aristatus* does not cause significant toxicity effect on the development of zebrafish embryo, and it depends to the time of expose and also the concentration of the extract. The median lethal concentration (LC<sub>50</sub>) of irradiated and non-irradiated *Orthosiphon aristatus* is varying with no abnormality in zebrafish embryo. Study indicates the irradiated and non-irradiated *Orthosiphon aristatus* is not harmful, but further research should be conducted in order to understand on specific reaction of non-irradiated *Orthosiphon aristatus*.

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