Establishment of hydroponic root culture systems of *Rhinacanthus nasutus* and increased rhinacanthin production using elicitation technique

Thongtham Suksawat

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Pharmaceutical Sciences

Prince of Songkla University

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Candidate

ชื่อวิทยานิพนธ์ การสร้างระบบเพาะเลี้ยงรากทองพันชั่งแบบไฮโดรโปนิกและการเพิ่มการสร้างสารไรนาแคนตินโดยใช้เทคนิคอิลิศิเตชัน

ผู้เขียน นายธงธรรม สุขสวัสดิ์

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บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของส่วนต่างๆ ของพืชและระยะเวลาที่เก็บเกี่ยวต่อการสร้างสาร rhinacanthin-C, rhinacanthin-D และ rhinacanthin-N จากวัตถุดิบสมุนไพร รวมทั้งยังศึกษาคุณภาพของวัตถุดิบสมุนไพรที่มีจำหน่ายจากที่ต่างๆและผลิตภัณฑ์เพื่อสุขภาพจากชาชงทองพันชั่งในในแง่ของปริมาณสาร rhinacanthins จากนั้นยังมีการพัฒนาการสร้างระบบไฮโดรโปนิกส์ของต้นทองพันชั่งที่สามารถได้วัตถุดิบสมุนไพรที่มีปริมาณสูง ร่วมกับการศึกษาการใช้เทคนิคอิลิซิเตชั่นเพื่อกระตุ้นการสร้างสาร rhinacanthins ในระบบไฮโดรโปนิกส์ของทองพันชั่งดังกล่าว

ในบรรดาส่วนต่างๆ ของต้นทองพันชั่งตลอดฤดูกาลเก็บเกี่ยว พบว่าส่วนรากมีปริมาณสาร rhinacanthins มากที่สุด รองลงมาคือ ส่วนใบ โดยส่วนอื่นๆ มีปริมาณสาร rhinacanthins ค่อนข้างต่ำ โดยราก (4.91% w/w) และใบ (4.42% w/w) ทองพันชั่งมีสาร rhinacanthins สูงที่สุดในเดือนกันยายน และต่ำที่สุดในเดือนมีนาคม (3.73% และ 3.18% w/w ตามลำดับ) และพบว่าตัวอย่างวัตถุดิบทองพันชั่งในรูปแบบผงทั้งหมด 10 ตัวอย่าง และผลิตภัณฑ์ชาชงทองพันชั่ง 2 ตัวอย่าง มีปริมาณสาร rhinacanthins ค่อนข้างต่ำ ในช่วง 0.14 ถึง 0.55% w/w ซึ่งมีปริมาณใกล้เคียงกับวัตถุดิบทองพันชั่งจากส่วนเหนือดิน (0.27-0.53% w/w)

การศึกษาสภาวะที่เหมาะสมต่อระบบไฮโดรโปนิกส์ของทองพันชั่ง พบว่าการให้อากาศ (4.5 ลิตรต่อนาที) ในระบบเพาะเลี้ยงที่มีอาหารเพาะเลี้ยงชนิด Murishge and Skoog ที่ความเข้มข้น 10% ในภาชนะเพาะเลี้ยงชนิดทึบแสงสามารถเพิ่มปริมาณผลผลิตรากทองพันชั่งแห้ง (143.31 มก./ต้น) และปริมาณสาร rhinacanthins (2.21% w/w) ได้อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับระบบที่ไม่มีการให้อากาศและภาชนะเพาะเลี้ยงชนิดโปร่งแสง นอกจากนี้ความเข้มข้นของอาหารเพาะเลี้ยงที่ 20% สามารถเพิ่มปริมาณผลผลิตรากทองพันชั่ง (247.03 มก./ต้น) และปริมาณสาร rhinacanthins (2.65% w/w) ได้อย่างมีนัยสำคัญ การเก็บเกี่ยวที่ระยะเวลาสามเดือนจะให้ผลผลิตรากทองพันชั่งเท่ากับ 142.91 มก./ต้น และมีปริมาณสาร rhinacanthins เท่ากับ 2.70% w/w และหากเพิ่มระยะเวลาการเก็บเกี่ยวเป็น 5 เดือน จะทำให้ผลผลิตรากทองพันชั่งและปริมาณสาร rhinacanthins

เพิ่มขึ้นเป็น 232.07 มก./ต้น และ 3.04% w/w ตามลำดับ นอกจากนี้ แสงสีน้ำเงิน-แดงยังช่วยเพิ่มผลผลิตรากทองพันชั่งและปริมาณสาร rhinacanthins ได้ถึง 250.47 มก./ต้น และ 3.31% w/w ตามลำดับ

ในการศึกษาการใช้เทคนิคอิลิซิเตชั่นในระบบเพาะเลี้ยงไฮโดรโปนิกส์ของทองพันชั่ง พบว่าการใช้สาร chitosan ที่ความเข้มข้น 0.15 มก./มล. เป็น elicitor สามารถเพิ่มการสร้างสาร rhinacanthins ได้ถึง 6.10% w/w ซึ่งมากกว่ากลุ่มควบคุมถึง 2.24 เท่า นอกจากนี้ การใช้สารสกัดเชื้อราชนิด *Trichoderma harzianum* ที่ความเข้มข้น 1 มก./มล. ยังเพิ่มการสร้างสาร rhinacanthin ได้ถึง 2.22 เท่า (6.04% w/w DW) เมื่อเทียบกับกลุ่มควบคุม ในขณะที่การใช้สาร lawsone ที่ความเข้มข้น 6 μM สามารถเพิ่มการสร้างสาร rhinacanthins (4.19% w/w DW) ได้น้อยกว่าสาร chitosan และสารสกัด *T. harzianum* แต่ยังมากว่ากว่าในกลุ่มควบคุม จากการศึกษาปัจจัยของระยะเวลาในการกระตุ้นการสร้างสาร rhinacanthins ด้วย elicitor พบว่าสาร chitosan ที่ความเข้มข้น 0.15 มก./มล. ไม่ส่งผลต่อปริมาณผลผลิตรากทองพันชั่งแต่สามารถกระตุ้นการสร้างสาร rhinacanthins ในรากถึง 6.10% w/w (ใช้เวลาในการกระตุ้น 48 ชม.) และในใบถึง 5.44% w/w (ใช้เวลาในการกระตุ้น 24 ชม.) และสารสกัด *T. harzianum* ที่เวลา 24 ชม. สามารถกระตุ้นการสร้างสาร rhinacanthins ในรากถึง 2.32 เท่า (6.04% w/w) และในใบถึง 1.50 เท่า (6.66% w/w) ในขณะที่สาร lawsone สามารถกระตุ้นการสร้างสาร rhinacanthins ในรากได้เพียง 4.55% w/w และในใบได้เพียง 5.17% w/w โดยใช้เวลาในการกระตุ้น 72 ชม.

ในการศึกษาการใช้เทคนิคอิลิซิเตชั่นแบบ dual elicitation ในระบบเพาะเลี้ยงไฮโดรโปนิกส์ของทองพันชั่ง พบว่าการกระตุ้นด้วยสาร chitosan และสารสกัด *T. harzianum* พร้อมกันโดยใช้เวลาในการกระตุ้น 72 ชม. ทำให้มีการสร้างสาร rhinacanthins ในรากเท่ากับ 4.51% w/w และในใบเท่ากับ 5.76% แต่การใช้เทคนิคอิลิซิเตชั่นแบบ sequential dual elicitation โดยเริ่มจากการกระตุ้นด้วยสารสกัด *T. harzianum* เป็นเวลา 48 ชม. แล้วกระตุ้นต่อด้วยสาร chitosan เป็นเวลา 72 ชม. พบว่าสามารถกระตุ้นการสร้างสาร rhinacanthins ในรากได้ถึง 9.65% w/w และในใบได้ถึง 7.59% w/w โดยที่การกระตุ้นด้วยสาร chitosan เป็นเวลา 24 ชม. แล้วกระตุ้นต่อด้วยสารสกัด *T. harzianum* เป็นเวลา 96 ชม. ไม่ส่งผลต่อการเพิ่มการสร้างสาร rhinacanthins

การศึกษานี้ชี้ให้เห็นถึงแนวทางที่เหมาะสมต่อการผลิตวัตถุดิบสมุนไพรทองพันชั่งโดยเฉพาะในช่วงฤดูฝนโดยใช้รากหรือใบจะทำให้มีความเข้มข้นของสาร rhinacanthins สูงที่สุด นอกจากนี้ได้มีการพัฒนาระบบไฮโดรโปนิกส์จากทองพันชั่งร่วมกับการใช้เทคนิคอิลิซิเตชั่นโดยการใช้สารสกัด *T. harzianum* และสาร chitosan เพื่อเพิ่มปริมาณการสร้างสาร rhinacanthins ทั้งในรากและใบทองพันชั่ง เพื่อใช้ทดแทนวัตถุดิบทองพันชั่งที่ขาดแคลนและมีคุณภาพต่ำ

คำสำคัญ : ทองพันชั่ง, วัตถุดิบสมุนไพร, ไฮโดรโปนิกส์, อิลิซิเตชั่น, ไตรโคเดอร์มา, ไคโตซาน

Thesis Title Establishment of hydroponic root culture systems of *Rhinacanthus nasutus* and increased rhinacanthin production using elicitation technique

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Major Program Pharmaceutical Sciences
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ABSTRACT

The purpose of the study was to determine how *Rhinacanthus nasut*us natural growth parts and harvesting times affected the production of rhinacanthin-C, -D, and -N in plant raw materials. In addition, a study of the quality of commercially available plant raw materials and tea infusion health products from *R. nasutus* in terms of rhinacanthin content was carried out prior to establish hydroponic root culture systems of *R. nasutus* that produce high root biomass and a high content of rhinacanthin-C, -D, and -N. Elicitation techniques were used to improve rhinacanthin production in the hydroponic system of *R. nasutus*.

The rhinacanthin content in different parts of the plant varied significantly. Roots consistently had the highest total rhinacanthin levels, with September harvests yielding the highest concentrations (4.91% w/w). In contrast, leaves contained the second-highest levels of rhinacanthins (4.42% w/w) during the same season. In March, both roots and leaves exhibited lower rhinacanthin levels (3.73% and 3.18% w/w, respectively). On the contrary, *R. nasutus* powders sourced from ten distinct suppliers and two tea products demonstrated relatively low total rhinacanthin concentrations, spanning from 0.14% to 0.55% w/w. These concentrations were similar to those observed in powders derived from aerial components, falling within the range of 0.27% to 0.53% w/w.

According to the optimization of *R. nasutus* hydroponics, aeration (4.5 L/min) through 10% MS in light-protected growing containers significantly increased dried root biomass (143.31 mg/plant) and total rhinacanthin content (2.21% w/w) compared to those without aeration and in translucent growing containers. An appropriate MS

concentration of 20% was found to considerably enhance root biomass (247.03 mg/plant) and rhinacanthin content (2.65% w/w). Based on the harvesting time, the plant grown for three months produced sufficient root biomass (142.91 mg/plant) and rhinacanthin content (2.70% w/w). Notably, increasing the harvesting duration resulted in an increase in root biomass and rhinacanthin content, which reached 232.07 mg/plant and 3.04% w/w at five months, respectively. Additionally, blue-red lighting increased root biomass and rhinacanthin content up to 250.47 mg/plant and 3.31% w/w, respectively.

The addition of 0.15 mg/mL chitosan boosted rhinacanthin production to 6.10% dry weight (DW). The productivity obtained was 2.24 times more than that produced with untreated root (2.72% w/w DW). Furthermore, a 1 mg/mL *T. harzianum* treatment dosage increased rhinacanthin synthesis up to 2.22-fold (6.04% w/w DW) compared to untreated root. While lawsone (6 μM) treatment modestly increased rhinacanthin production to (4.19% w/w DW) compared to untreated root. Chitosan, *Trichoderma harzianum*, and lawsone were the most effective elicitors for increasing rhinacanthin production in a hydroponic system of *R. nasutus*.

In sequential dual elicitation, the root and leaf dry weights were unaffected by any of the treatments. Chitosan at 0.15 mg/mL for 48 h increased rhinacanthin production by 2.24-fold to 6.10% w/w dry weight (DW). The treated leaves generated a substantial quantity of rhinacanthin (5.44% w/w DW) after 24 h of chitosan treatment. Furthermore, a 24 h treatment with 1 mg/mL *T. harzianum* boosted rhinacanthin production up to 2.22-fold (6.04% w/w DW) in roots and 1.5-fold (6.66% w/w DW) in leaves. While 72 h of lawsone (6 M) treatment boosted rhinacanthin production in roots by (4.55% w/w DW) and leaves by 1.13-fold (5.17% w/w DW). During simultaneous dual elicitation, rhinacanthin production was maximum in roots at 72 h, reaching up to 4.51% w/w DW and 5.76% w/w DW in leaves. When chitosan was applied to *T. harzianum*-treated roots for 72 hours, the highest rhinacanthin production (9.65%w/w DW) was observed.

The current study indicated the best approach for obtaining *R. nasutus* herbal raw material during the rainy season by employing roots or leaves with the maximum rhinacanthin concentration. *R. nasutus* hydroponics were developed and improved to

overcome raw material scarcity and poor quality. Furthermore, the elicitation strategy utilizing *T. harzianum* and chitosan yielded a significant rhinacanthin concentration.

Keywords: *Rhinacanthus nasutus*, herbal raw materials, elicitation, *Trichoderma harzianum*, chitosan

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Thongtham Suksawat

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

B5	Gamborg's B5 medium
BA	6-Benzylaminopurine
BQL	Below quantification limit
DW	Dry weight
ECSAC	Environment Control System for Automatic Cultivation
EIMS	Electron ionization mass spectroscopy
HPLC	High performance liquid chromatography
IBA	Indole-3-butyric acid
IUPAC	International Union of Pure and Applied Chemistry
LAH	Lithium aluminium hydride
LED	Light-emitting diode
LOD	Limit of detection
LOQ	Limit of quantification
MeJA	Methyl jasmonate
MS	Murashige and Skoog
NaAlg	Sodium alginate
NAA	Naphthalene acetic acid
NFT	Nutrient film technique
NLEM	Thai National List of Essential Medicines
NMR	Nuclear magnetic resonance

ODS	Octadecyl-silica
OSB	O-succinylbenzoic acid
PAL	Phenylalanine ammonia lyase
PKC	Phospholipase C/protein kinase C
RC	Rhinacanthin-C
RD	Rhinacanthin-D
RN	Rhinacanthin-N
SA	Salicylic acid
SE	Standard error
TLC	Thin layer chromatography
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Rationale

The ethnomedicinal plant *Rhinacanthus nasutus* (L.) Kurz. (Acanthaceae family) has been utilized for the treatment of dermatological conditions such as dermatophyte infection and eczema (Brimson et al., 2020). Roots and leaves are the recommended parts of this plant due to a high content of the major active compounds, namely rhinacanthin-C (RC), -D (RD), and -N (RN) (Fig. 1). These rhinacanthins were reported to exhibit antifungal (Panichayupakaranant et al., 2009), anti-inflammatory (Tewtrakul et al., 2009b), antiallergic (Tewtrakul et al., 2009a), antibacterial (Puttarak et al., 2010), anti-Alzheimer (Chuang et al., 2017), anti-tumor (Boueroy et al., 2018), hypoglycemic and hypolipidemic (Shah et al., 2019), and anti-Parkinson effects (Saleem et al., 2021). Concerns regarding the inefficacy, adverse effects, and limited availability of numerous conventional pharmaceuticals, coupled with financial constraints, persist in driving the increasing attention toward medicinal plants and their extracts as potential alternative therapies in various global regions. In recent times, natural compounds have constituted a significant portion of contemporary therapeutic drugs. The secondary metabolism in plants serves as a prime example of how naturally occurring bioactive substances enrich biodiversity. Nevertheless, the phytochemical content is subject to variation due to numerous factors, including plant species, growth conditions, environmental influences, age or time of harvest, and the specific plant parts used (Fong, 2002; Pandey and Savita, 2017). In the creation of herbal products, it is crucial to utilize suitable plant raw materials to validate the effectiveness of medicinal products. The World Health Organization (WHO) has issued directives stipulating that particular components of medicinal plants should be collected during the correct seasons to guarantee the production of top-quality raw materials and commercial products (WHO, 2019). Ensuring the efficacy of herbal medicines requires a comprehensive understanding of optimal harvesting procedures. This includes recognizing which plant parts and seasons yield the highest concentrations of active

compounds. These variations in quality can significantly influence the effectiveness of healthcare products. In the case of *R. nasutus*, both its roots and leaves are employed for health benefits, with the roots containing a greater quantity of rhinacanthins compared to the leaves (Panichayupakaranant et al., 2006). However, due to the difficulty of harvesting the roots of this plant, they are not commercially accessible. Most commercially accessible *R. nasutus* materials are aerial components, which have extremely low rhinacantin concentrations. Essentially, the required quantity of active compounds in plant materials affects the efficacy of herbal medicines (Suksawat and Panichayupakaranant, 2023). As a result, the roots are an appropriate plant raw material for an *R. nasutus*-based treatment. *In vitro* root cultures of *R. nasutus* were recently established, although the root biomass and rhinacanthin concentration were relatively low (Cheruvathur et al., 2015; Cheruvathur and Thomas, 2014; Panichayupakaranant and Meerungrueang, 2010). Furthermore, herbal materials are commonly contaminated with heavy metals and other hazardous compounds, preventing them from obtaining quality clearance in accordance with World Health Organization (WHO) raw herb purity requirements (WHO, 2019). A hydroponics plant is currently being utilized to increase the quality of herbal material for industrial processing (Suryawanshi, 2021). It is a simple approach for producing herbal materials obtained from roots. There are various advantages of employing hydroponics, including a more organized and uniform production system under regulated circumstances. Furthermore, hydroponics can make it simpler to employ biotic and abiotic elicitors to increase the production of a certain metabolic pathway (Gontier et al., 2002). In this regard, a key benefit of hydroponics is ability to expose the plants to stressors that may produce an increase in secondary metabolite levels. To increase secondary metabolite production in the hydroponic environment, an elicitation technique might be applied.

Elicitation studies can be challenging since plants only respond to specific elicitors and hence promote secondary metabolite production. Additionally, plant defense responses to elicitors should be investigated in terms of elicitor types, elicitor concentrations, and exposure duration. Methyl jasmonate (MeJA) and salicylic acid (SA), natural plant stress mediators which could substantially accelerate plant synthesis of pharmaceutically active compounds, is one of the most renowned elicitors. MeJA

successfully boosted rhinacanthin production in *R. nasutus* cells cultured in Murashige and Skoog (MS) liquid media *in vitro*, although this approach had not previously been used in hydroponic cultivation (Cheruvathur and Thomas, 2014). Chitosan is a nontoxic, natural, and biodegradable polymer that has been demonstrated to stimulate plant growth as well as to elicit defensive reactions in plants, both of which necessitate changes in plant metabolite profiles (Sathiyabama et al., 2016). Studies have reported that the application of chitosan and lawsone can enhance the production of plumbagin, a naphthoquinone, from Plumbago indica root cultures by a factor of up to 6.6 times when compared to root cultures that were left untreated (Jaisi and Panichayupakaranant, 2017). Polymerization degrees of alginate oligomers ranging from tetramer to hexamer demonstrated a substantial growth-promoting and elicitor effect on plants (Golkar et al., 2019). *Trichoderma harzianum* provides a variety of advantages for hydroponic systems, including the ability to protect plants from pathogenic microorganisms. It has also been employed in several elicitation approaches for secondary metabolite accumulation (Manganiello et al., 2018). Furthermore, simultaneous and sequential dual elicitation may be more effective than employing only one elicitor to stimulate rhinacanthin production. Previous research on combination and sequential elicitation of aloe-Emodin production in *Cassia tora* root cultures has been reported (Teptat et al., 2020). However, using the elicitation strategy in hydroponically produced plants remains challenging.

The aim of this study is to investigate the impacts of plant parts and harvesting seasons on the levels of RC, RD, and RN in plant raw materials in order to identify an effective *R. nasutus* harvesting technique. Furthermore, an evaluation of the rhinacanthin content in commercially available plant raw materials and health products derived from R. nasutus was undertaken to determine their quality. In order to obtain high-quality herbal raw materials, *R. nasutus* hydroponics was established as an alternate source for its roots. Aeration, type of growing container, nutrient concentrations, harvesting periods, and light sources were also studied to improve root biomass and rhinacanthin content in hydroponics. Subsequently, an elicitation study was carried out utilizing MeJA, SA, chitosan, lawsone, and sodium alginate, and *T. harzianum* as elicitors to improve rhinacanthin production in an *R. nasutus* hydroponic

system in terms of elicitor concentrations and elicitor duration. Furthermore, simultaneous and sequential dual elicitation on an *R. nasutus* hydroponics was investigated.

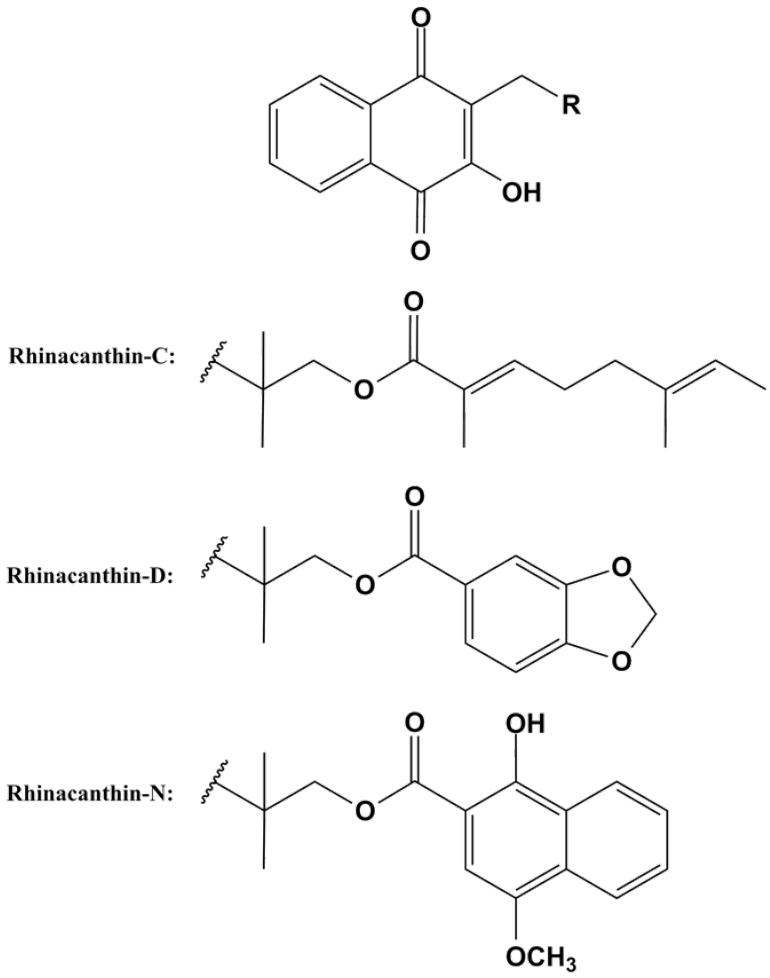

Figure 1 Chemical structures of rhinacanthin-C, rhinacanthin-D, and rhinacanthin-N

1.2 Objectives

The main objectives for this investigation are:

2.1. To study effect of natural growth *R. nasutus* parts and harvesting periods on production of RC, RD, and RN in plant raw materials.

2.2. To study quality of commercially available plant raw materials and tea infusion health products from *R. nasutus* in term of rhinacanthin content

2.3. To establish hydroponic root culture systems of *R. nasutus* that produce high root biomass and high content of RC, RD, and RN

2.4. To obtain the elicitation and substrate feeding conditions that increase production of rhinacanthin in the hydroponic culture systems of *R. nasutus*

2.5. To study effect of simultaneous and sequential dual elicitation of selected elicitors on rhinacanthin production of *R. nasutus*

CHAPTER 2
REVIEW OF LITERRATURES

The present study focused on an establishment of the hydroponic root culture systems of *R. nasutus*, which increased in rhinacanthin production using elicitation. In this section, the documents that are related to this research have been reviewed as follows.

2.1 *R. nasutus* and its phytochemicals

2.2 Rhinacannthin-C

2.3 Pharmacological activities of rhinacanthin-C

- 2.3.1 Anti-bone resorption activity
- 2.3.2 Anticancer activity
- 2.3.3 Antidiabetic activity
- 2.3.4 Anti-inflammatory activity
- 2.3.5 Anti-Parkinson activity
- 2.3.6 Hepatoprotective activity
- 2.3.7 Immunomodulatory activity
- 2.3.8 Neuroprotective activity
- 2.3.9 Other potential use of rhinacanthin-C

2.4 Toxicity assessment of rhinacanthin-C

2.5 Metabolism and pharmacokinetic of rhinacanthin-C

2.6 Qualitative HPLC analysis of rhinacanthins

2.7 Extraction and standardization of rhinacanthin-C

2.8 Tissue cultures of *R. nasutus*

2.9 Hydroponics

2.10 Elicitations

2.1 *R. nasutus* and its phytochemicals

R. nasutus or *R. communis* Nees. (Thai name: Thong Phan Chang; English name: Snake jasmine) is a medicinal plant native to Thailand and Southeast Asia. The plant is well known in traditional medicines for the treatment of various diseases including dermatophytes and DM (Brimson and Tencomnao, 2014). In Thailand, *R. nasutus* has been traditionally used for treatment of dermatophytes and has been listed in Thai Primary Health Care guidelines as an essential herb. *R. nasutus* roots and leaves are topically applied to treat skin infections. However only a tincture made from *R. nasutus* leaf extract has been approved in Thai National List of Essential Medicines (NLEM, 2020).

Most of the traditional therapeutic uses of *R. nasutus* have been scientifically validated and it has been reported that the majority of the bioactivities are due to the naphthoquinone esters, namely rhinacanthins. Various naphthoquinone esters that have been found in *R. nasutus*, including rhinacanthin-A, -B, -D, -G, -H, -I, -J, -K, -L, -M, -N, O, -P, and -Q (Fig. 1) and their reported biological activities are summarized in Table 1. The most bioactive constituent is rhinacanthin-C, which may have potential as a drug development candidate. However, it is not yet available commercially. These compounds are mainly accumulated in the roots and leaves (Panichayupakaranant et al., 2006).

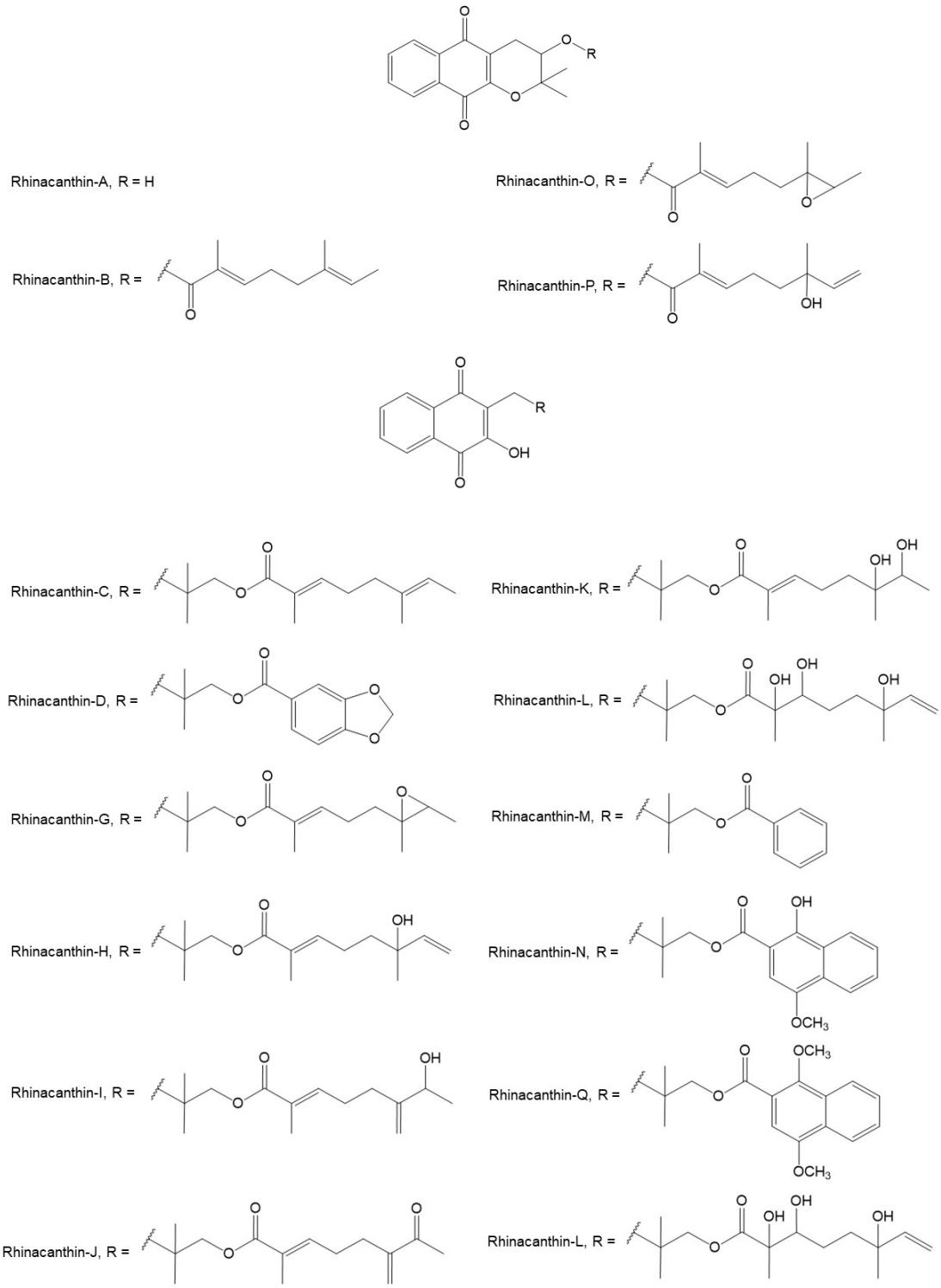

Figure 2 Chemical structures of naphthoquinone esters obtained from *R. nasutus*

Table 1 Biological activities of *R. nasutus* extracts and its naphthoquinones esters

Phytochemicals	Biological activities	References
Rhinacanthin-C, RRE	Analgesic	(Bhusal et al., 2014)
Rhinacanthin-C, -D, -N	Antiallergic	(Tewtrakul et al., 2009a)
Rhinacanthin-C, -N, -Q, RRE	Antibacterial	(Puttarak et al., 2010; Siripong et al., 2006)
Rhinacanthin-C, -D, -N, RRE	Antifungal	(Panichayupakaranant et al., 2009)
Rhinacanthin-C, -D, -N, RRE	Anti-inflammatory	(Bhusal et al., 2014; Tewtrakul et al., 2009a, b)
Rhinacanthin-C, RRE	Antioxidant	(Rao et al., 2012; Shah et al., 2017c)
Rhinacanthin-A, -B, -C, -G, -H, -I, -K, -M, -Q	Antiplatelet	(Wu et al., 1998)
Ethanol and aqueous extracts, rhinacanthin-C, -N, -Q, liposomal formulations of rhinacanthin-C, -N, -Q,	Antiproliferative	(Boueroy et al., 2018; Bukke et al., 2011; Gotoh et al., 2004; Siripong et al., 2007; Siriwatanametanon et al., 2010)
Rhinacanthin-M, -N, -Q	Antitumor	(Bukke et al., 2011)
Rhinacanthin-C, -D, -N, -Q	Antiviral	(Ngoc et al., 2019; Sendl et al., 1996)
Methanol extract, rhinacanthin-A, -B, -C, -D, -G, -H, -I, -K, -M, -N, -Q	Cytotoxic	(Wu et al., 1998)
Methanol extract, rhinacanthin-C	Anti-hyperlipidemic	(Rao et al., 2011; Shah et al., 2019)
Aqueous and 80% ethanol extracts	Immunomodulatory	(Punturee et al., 2005)
Rhinacanthin-C	Neuroprotective	(Chang et al., 2016; Chuang et al., 2017)
Rhinacanthin-C, -D, -N, RRE	Antidiabetic	(Shah et al., 2017b; Shah et al., 2019)
Rhinacanthin-C, RRE	Renoprotective	(Zhao et al., 2019)
Rhinacathin-C	Anti-Parkinson	(Saleem et al., 2021)

RRE: Rhinacanthins-rich *R. nasutus* leaf extract

Research into the phytochemical composition of *R. nasutus* has revealed that its major constituents include flavonoids, steroids, terpenoids, anthraquinones, lignans, and notably, naphthoquinone analogues. Specifically, various naphthoquinones, such as rhinacanthins A, B, C, D, G, H, I, J, K, L, M, N, O, P, and Q, have been isolated from both the leaves and roots of *R. nasutus* (Table 1) (Wu et al., 1998). In addition, rhinacanthone was isolated from leaves and stems. Dehydro alpha-lapachone was uniquely isolated from roots (Sendl et al., 1996). Rhinacanthin-E and rhinacanthin-F, two lignans, have been successfully extracted from the aerial parts of the plant. Additionally, various benzenoid compounds including p-hydroxy-benzaldehyde, vanillic acid, syringic acid, 2-methoxy-propionolphenol, methyl vanillate, and syringaldehyde have been isolated from the leaves, roots, and stems of *R. nasutus* (Jayapriya, 2015). Anthraquinone compound 1, 2-methyl from leaves and stem were additionally found. Triterpenoids, amyrin, glutinol and lupeol were obtained from roots of *R. nasutus* (Wu et al., 1995). Wogonin, oroxylin-A, rutin were specifically found in flower. Stigmasterol and β-sitosterol were accumulated in roots. Chlorophyll and methylphecophrorbide-A were identified in leaves and stems. Coumarins (+)-pracruptorin and umbelliferone, 2,6-dimethoxy benzoquinone were reported to be isolated from roots, leaves and stems (Subramanian et al., 1981). Additional compounds present in the plant material include carbohydrates, such as methyl-α-D-galactopyranoside, as well as quinol compounds like 4-acctony 1-3,5-dimethoxy-p-quinol. Furthermore, glycoside compounds like sitosterol-β-D-glucopyranoside, stigmasterol-β-D-glucopyranoside, 3,4-dimethylphenol-β-D-glucopyranoside, and 3,4,5-dimethylphenol-β-D-glucopyranoside have been detected in both the leaves and stems (Wu et al., 1998). Some TLC system have been described for detection of rhinacanthin-C in the extracts of root culture. The TLC (Thin-Layer Chromatography) setup employed a Silica gel $60F_{254}$ plate as the stationary phase, with various mobile phase mixtures including dichloromethane: hexane (4:1), ethyl acetate: hexane (3:7), and dichloromethane: petroleum ether (4:1). Standard rhinacanthin-C was used as the standard marker (Panichayupakaranant, 2017).

2.2 Rhinacanthin-C

Rhinacanthin-C, or its IUPAC designation as [3-(1-hydroxy-3,4-dioxonaphthalen-2-yl)-2,2-dimethylpropyl](2E,6E)-2,6-dimethylocta-2,6-dienoate (Fig. 2), is a member of the 1,4-naphthoquinone ester group with the chemical formula of $C_{25}H_{30}O_5$ and a molecular weight of 410.5. Rhinacanthin-C was first identified using the Electron Ionization Mass Spectroscopy (EIMS) and Nuclear Magnetic Resonance (NMR) techniques (Sendl et al., 1996). Rhinacanthin-C has a reddish-yellow semisolid oil appearance and can be dissolved in chloroform, ethanol, ethyl acetate, methanol, and hexane. It contains one hydrogen bond donor and five hydrogen bond acceptors (PubChem, 2023).

Rhinacanthin-C is naturally found in *R. nasutus*. It is markedly accumulated in the roots (3.4-4.1% w/w) and leaves (2.5-3.2% w/w) of this plant (Suksawat and Panichayupakaranant, 2023). Currently, the biosynthetic pathway of rhinacanthin-C is not yet fully described. Nonetheless, there was a review of the rhinacanthin biogenesis. Briefly, the chemical structure of rhinacanthin-C composes of two core skeletal, the naphthoquinone and isoprenyl units. The biogenetic perspective of its naphthoquinone unit appears to be implicated in the *o*-succinylbenzoate (OSB) pathway, beginning with the isomerization of chorismic acid to isochorismic acid. Following pyruvic acid removal and dehydration, the intermediate OSB is produced using OSB synthase enzyme. The formation of a coenzyme A Claisen-like condensation occurs when an ester reacts with OSB, allowing ring formation. Tautomerization, alkylation, decarboxylation, oxidation, and hydroxylation accelerate the transformation of previous product, 1,4-dihydroxynaphthoic acid into isoprenylated hydroxynaphthoquinone. An isoprenyl unit on the isoprenylated hydroxynaphthoquinone can be spontaneously reorganized into epoxide species and cyclized via an ether linkage, followed by hydrolysis and pinacol-pinacolone rearrangement, yielding an aldehyde hydroxynaphthoquinone. Aldehyde hydroxynaphthoquinone can be converted to the alcohol component of naphthoquinone ester by reduction. At this point, the naphthoquinone unit from rhinacanthin-C is more likely to be synthesized via the OSB pathway since the end result of the OSB pathway is isoprenylated hydroxynaphthoquinone, which is subsequently synthesized into the naphthoquinone unit that is a core structure of rhinacanthin-C.

The isoprenyl unit of rhinacanthin-C appears to be produced via the mevalonate route. The last metabolic process for generating rhinacanthin-C is prenylation of monoterpene to the naphthoquinone unit by esterification. As a result, rhinacanthin-C might be produced via the shikimate (OSB) route in conjunction with the mevalonate pathway. However, a thorough examination of the rhinacanthin-C production route is still required. Since, the OSB pathway is found in plastids and the mevalonate pathway is found in the cytoplasm, the biogenetic pathway of rhinacanthin-C appears to be found at chlorophylls in *R. nasutus* leaves. Upon the synthesis of rhinacanthin-C, it can be transported and stored within the roots. This accumulation of rhinacanthin-C is notably prominent in the roots of *R. nasutus* (Panichayupakaranant et al., 2021).

In addition to the naturally occurring biosynthesis, Awai et al. (1995) and Gotoh et al. (2004) have reported two methods for chemical synthesis of rhinacanthin-C. The first method utilized 2 precursors for constructing the naphthoquinone core structure and terpenoid side chain. Rhinacanthone was used to prepare the naphthoquinone core by undergoing ring opening to give 2-hydroxy-3-(3-hydroxy-2,2-dimethylpropyl)-1,4-naphthoquinone, while (*E*)-6,6-dimethoxy-3-methyl-2-hexene was reduced using lithium aluminium hydride (LAH) and subsequently subjected to the Wittig reaction to obtain (2*E*, 6*E*)-2,6-dimethyl-2,6-octadienoyl chloride as a terpenoid side chain. These two core structures were used for chemoselective esterification to from rhinacanthin-C (Awai et al., 1995). The second method utilized two precursors, 1,2,4-trimethoxynaphthalene for constructing the naphthoquinone core structure and monoterpene carboxylic acid for constructing the monoterpene sidechain. The metalation-substitution and LAH reduction reaction of 1,2,4-trimethoxynaphthalene produced 3-substituted-1,2,4-trimethoxynaphthalene derivative. Monoterpene side chain was generated using silyl ether exposed to tetrabutylammonium fluoride and subsequently subjected to the Swern oxidation of 4-methyl-trans-4-hexenol to produce monoterpene carboxylic acid. Then these two precursors were condensed with water-soluble carbodiimide and subsequently underwent demethylation to obtain rhinacanthin-C obtained (Gotoh et al., 2004). Despite the methods for the chemical synthesis of rhinacanthin-C have been established, the multiprocessing manufacturing is required. Therefore, its cost effectiveness remains a challenging issue.

2.3 Pharmacological activities of rhinacanthin-C

Based on variety of the medical uses of *R. nasutus* as well as identification of rhinacanthin-C as its major active ingredient, interventions have been studied on diverse pharmacological activities of rhinacanthin-C, *in vitro* and *in vivo*. The recently reported pharmacological activities of rhinacanthin-C on anti-bone resorption, anticancer, antidiabetic, anti-inflammatory, anti-Parkinson, hepatoprotective, immunomodulation, and neuroprotection are illustrated in and some of its mechanisms of action are described as follows.

2.3.1 Anti-bone resorption activity

Many bone-related diseases, including osteoporosis and rheumatoid arthritis, usually have abnormal bone lysis. Osteoclasts, or bone-breaking cells, serve as an important part in this process. Receptor activator of nuclear factor-B ligand (RANKL) is an important protein that promotes osteoclast development and activation. Rhinacanthin-C has been reported to possess anti-bone resorption properties by inhibiting the formation of osteoclasts, along with anti-inflammatory effects. This suggests its potential utility in addressing abnormal bone degradation caused by inflammatory bone resorption. The molecular mechanisms underlying its anti-osteoclastogenic effects involve the inhibition of the interaction between TRAF6 and TAK1, which is induced by RANKL. This inhibition subsequently leads to the activation of MAPKs/NF-B signaling pathways. In turn, this activation results in the suppression of c-Fos and NFATc1, both of which play crucial roles in regulating the transcription of genes associated with osteoclast differentiation (Tomomura et al., 2015). Rhinacanthin-C also inhibited RANKL-induced osteoclast development and bone resorption in rat calvaria. The results from this study indicate that rhinacanthin-C might be an alternative therapeutic agent for treatment of abnormal bone lysis caused by inflammatory bone resorption.

2.3.2 Anticancer activity

Chemotherapy is an important therapeutic option for various cancer diseases, but cancer cells can acquire drug resistance through a variety of cellular defense mechanisms. Chemotherapeutic failure is often due to transporter-mediated multidrug resistance. This can lead to higher doses of drugs being needed, which can result in increased toxicity and an unsuccessful outcome. Multidrug resistance (MDR) in cancer patients is primarily induced by the overexpression of drug efflux transporters, such as

P-glycoprotein (P-gp). These proteins aggressively release their drug substrates from cells, thus reducing the efficacy of treatment. Suppressing P-gp function in cancer cells could avoid drug tolerance and improve chemotherapy effectiveness. Downregulation of P-gp mRNA in cancer cells may be a strategy for reversing MDR to chemotherapy. In addition, targeting on the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase-protein kinase B/Akt (PI3K/Akt) signaling pathways can improve the efficacy of conventional chemotherapeutic treatments.

In a recent study, it was disclosed that rhinacanthin-C enhanced the cytotoxic effects of doxorubicin in human breast cancer cells (MCF-7) and doxorubicin-resistant breast cancer cells (MCF-7/DOX). This enhancement was achieved by inhibiting the functions of multidrug resistance-associated protein 2 (MRP2) and P-glycoprotein (P-gp) (Chaisit et al., 2017). This implies that rhinacanthin-C may have the capability to enhance the sensitivity of human breast cancer cells to doxorubicin treatment, signifying its potential utility as a chemosensitizer in cancer therapy. Furthermore, rhinacanthin-C exhibited the ability to reduce P-glycoprotein (P-gp) expression in MCF-7/DOX cells by inhibiting the Akt/NF-κB signaling pathway and downregulating Y-box binding protein-1 (YB-1) expression (Chaisit and Jianmongkol, 2022). In the context of human breast cancers, YB-1 is closely linked to rapidly proliferating tumors. Consequently, the inhibition of YB-1 expression leads to a decrease in the growth rate of breast cancer cells. Additionally, rhinacanthin-C demonstrated the ability to induce apoptosis in MCF-7/DOX cells through mechanisms involving heightened intracellular reactive oxygen species (ROS) levels and the suppression of cell survival pathways mediated by the MAPKs and Akt/GSK-3β/Nrf2 signaling pathways (Chaisit and Jianmongkol, 2021).

Cholangiocarcinoma is a life-threatening liver cancer that has metastasized throughout the human body. Cholangiocarcinoma cell invasion is typically promoted by the breakdown of extracellular matrix via proteolytic enzymes, such as MMP-2 and MMP-9. MMP-2 and MMP-9 are enzymes that exhibit high expression levels in various tumor types, and they serve as crucial facilitators of cancer invasion and metastasis. The suppression of tumor growth, angiogenesis, invasion, and the distant spread of cancer (metastasis) have been associated with the inhibition of MAPK signaling pathways. Notably, in the case of rhinacanthin-C, it has been reported to reduce MMP-

2 protein expression in human cholangiocarcinoma cells and to decrease the viability of these cells in a manner dependent on both the duration of exposure and the dosage administered (Boueroy et al., 2018). Thus, rhinacanthin-C may have therapeutic potential in the treatment of cholangiocarcinoma.

2.3.3 Antidiabetic activity

Type 2 Diabetes mellitus is a major worldwide health issue that is defined by a significant increase in blood sugar levels (hyperglycemia) as well as some severe complications, such as, hyperlipidemia, nephropathy, neuropathy, and cardiovascular disorders. Diabetes is characterized by hyperglycemia due to deficiency of insulin secretion, defective insulin action, or both (Suryasa et al., 2021). In diabetic rats, the administration of rhinacanthin-C has been shown to have significant effects. It resulted in a substantial decrease in fasting blood glucose, HbA1c, and lipid profile levels, while concurrently increasing serum insulin and the levels of pancreatic antioxidant enzymes. Furthermore, rhinacanthin-C mitigated pancreatic histopathological changes and lowered the levels of TNF-α, Ikkβ, and caspase-3 in diabetic rats (Adam et al., 2016).

Rhinacanthin-C coupled molecularly with superoxide dismutase and glutathione peroxidase, which leading to resolved blood lipid, liver and kidney profiles. Furthermore, rhinacanthin-C recovered diabetic pancreas intracellularly through islets of Langerhans, pancreatic cell which regulate blood sugar level (Adam et al., 2016; Shah et al., 2019). Therefore, rhinacanthin-C could be used potentially in pancreatic and other diabetic complications.

Another diabetic complication, diabetes nephropathy (DN), is a severe complication that affects more than 40% of people and is a leading cause of end-stage renal failure worldwide. Scarring of the glomerulus, albuminuria, tubulointerstitial renal fibrosis, and a decrease in kidney function represent all functional and structural abnormalities attributed with DN. The treatments which improve the antioxidant defense system and inhibit the release of pro-inflammatory mediators may be useful in managing or avoiding diabetic kidney failure. Diabetes-related kidney injury was alleviated by rhinacanthin-C by reducing oxidative stress and inflammation. It also boosted antioxidant enzyme amounts in the kidney (Zhao et al., 2019). Thus, rhinacanthin-C also might be used as a medicinal or dietary supplement to help avoid DN.

Oxidative stress and hyperglycemia are other factors, which intensify diabetic complications through accelerating the formation of advanced glycation end products (AGEPs). In which, the overproduction of free radicals can be reduced by antioxidants. Diabetic complications are mostly caused by nonenzymatic glycation reactions between proteins and reducing sugars in chronic hyperglycemic conditions. Plant extracts and isolated phytochemicals have emerged as promising alternatives to conventional drugs in inhibiting the formation of AGEPs (Advanced Glycation End Products). They have also demonstrated potential as antidiabetic agents by means of their antioxidant properties and their ability to combat AGEPs. Specifically, rhinacanthin-C has demonstrated substantial abilities in scavenging superoxide and impeding albumin glycation. These findings underscore its potential utility in addressing a range of chronic conditions, with a particular emphasis on diabetes. According to kinetic analyses, rhinacanthin-C displayed potent antioxidant activity *via* the ErCi mechanism, with an IC_{50} value of 8.0 μg/mL, an antioxidant capacity of 39439 M^{-1}, and a binding constant of 45709 M^{-1}. Rhincanthin-C exhibited glycation inhibitory activity, with IC_{50} values of 37.3 μg/mL, which could be explained that rhinacanthin-C tend to bind and mask various sites of albumin residue (Zhao et al., 2019). Rhinacanthin-C therefore, has demonstrated their therapeutic utility in different chronic diseases, particularly diabetes complications.

Controlling obesity involves two crucial strategies: inhibiting adipogenesis and stimulating glucose uptake in adipose and muscle tissues. Rhinacanthin-C has demonstrated a significant ability to stimulate glucose uptake, particularly in muscle cells, indicating its potential in obesity management. In adipocytes, it also had a strong antiadipogenic impact (Shah et al., 2017a). Rhinacanthin-C, thus could be applied as an efficient adjunctive treatment for obesity related T2DM.

Inhibiting α-glucosidase is an effective strategy for controlling postprandial glucose in type 2 diabetes, but commercially accessible inhibitors such as acarbose, voglibose, and miglitol are costly, possess gastrointestinal side effects, and are related to cardiac risks. Rhinacanthin-C inhibited α-glucosidase in a noncompetitive manner, while low-concentration combinations of both rhinacanthin-C and acarbose inhibited α-glucosidase in an additive synergistic manner (Shah et al., 2017b). Therefore rhinacanthin-C is recommended to be used as an α-glucosidase inhibitor in combination

with acarbose, implying that it could be used clinically to decrease the dose and side effects associated with acarbose treatment.

2.3.4 Anti-inflammatory activity

The anti-inflammatory and analgesic effects of rhinacanthin-C were examined using the Writhing test heated plate and the formalin test. An acetic acid-induced writing test revealed that the action of rhinacanthin-C is comparable to that of the normal medication Indomethacin, a nonsteroidal pain reliever. The formalin test showed that rhinacanthin-C can be an effective painkiller. To further validate the anti-inflammatory effects of rhinacanthin-C, additional methods involving carrageenan-induced edema and cotton pellet-induced granuloma in rats were employed. These methods help substantiate rhinacanthin-C's capacity to inhibit edema and granuloma formation. The results confirmed rhinacanthin-C's ability to treat acute inflammatory illnesses. Rhinacanthin-C, on the other hand, was ineffective against the hot plate test. It is possible that rhinacanthin-C has analgesic and anti-inflammatory properties in animal models. Depending on the dosage, rhinacanthin-C analgesic action may be carried by central or peripheral processes. Their anti-inflammatory activity can be attributed at least to the inhibition of prostaglandin synthesis (Bhusal et al., 2014).

2.3.5 Anti-Parkinson activity

Parkinson's disease is the second most common neurodegenerative ailment, characterized by signs of decreased motor and non-motor coordination. Parkinson patients' neurodegeneration is caused by dopaminergic loss and oxidative stress, which is mostly caused by mitochondrial malfunction. Although contemporary Parkinson's disease treatments include levodopa, a dopamine agonist for improving motor instability, amantadine, an anticholinergic, monoamine oxidase-B inhibitor, and β-blockers, they are associated with substantial side effects such as muscular dyskinesia. As a result, research on natural products for Parkinson treatment has been challenging for disease progression and symptom relief with limited side effects. Rhinacanthin-C improved Parkinson motor symptoms or cataleptic effects in a dose-dependent manner (5-20 mg/kg in mice). Furthermore, rhinacanthin-C improved brain morphology by restoring, dopamine, norepinephrine, and serotonin levels, neurotransmitter relating to Parkinson disorder. In Parkinson induced blood profile, rhinacanthi-C also recover and optimize levels of glutathione, catalase, and superoxide dismutase, while the pakinson

related mediators including malondialdehyde and nitrite, free radical indicators, were significantly decreased. (Saleem et al., 2021). The anti-Parkinson's potential of rhinacanthin-C is an anticipating approach to Parkinson's disease prevention and treatment.

2.3.6 Hepatoprotective activity

Nonalcoholic fatty liver disease (NAFLD) is a liver disorder characterized by fat buildup in hepatocytes that can progress to cirrhosis and hepatocellular carcinoma. NAFLD affects roughly 1.7 billion people globally and is on the rise as a result of the rise in overweight and obesity. The processes underlying NAFLD are unknown, but insulin resistance and high levels of fatty acids in the blood are critical to its development. Rhinacanthin-C shows promise in addressing Non-Alcoholic Fatty Liver Disease (NAFLD) by significantly reducing body weight and the liver-to-body ratio, inhibiting liver inflammation and fat buildup, and enhancing insulin sensitivity. Rhinacanthin-C also increased the production of adiponectin and adiponectin receptor in the liver while decreasing the expression of the NAFLD related factors including MCP-1, TNF-α, and IL-6. Rhinacanthin-C alleviated insulin resistance and lipid accumulation in NAFLD *via* activation of the AMPK/SIRT1 and SREBP-1c/Fas/ACC pathways (Gong et al., 2023). As a result, rhinacanthin-C could protect against NAFLD formation in a dose-dependent manner by lowering body weight and glucose levels, improving liver physiological changes, and restoring NAFLD metabolic markers.

2.3.7 Immunomodulatory activity

Monocytes, macrophages, and polymorphonuclear leucocytes (PMNs) are essential parts of the natural immune system which assist in the elimination of pathogens such as bacteria and fungi. The immune system maintains homeostasis and can be altered by pathogens, but innate immunity provides protection. Phagocytic cells produce reactive oxygen species in order to kill pathogens, but excessive production can cause inflammation and disease. Rhinacanthin-C has a significant inhibitory impact on phagocytic activities, indicating that it can impair the immune system's ability to engulf and eradicate invading pathogens. Rhinacanthin-C at 100.0 and 6.25 μg/mL substantially reduced phagocytosis by 26.40% and 30.59%, respectively. control. A dose-dependent reduction of myeloperoxidase (MPO) activity of rhinacanthin-C was also noticed (Chaisit and Jianmongkol, 2021). Therefore, rhinacanthin-C could be used

as an immunomodulatory agent to treat conditions where an overactive immune response is detrimental.

2.3.8 Neuroprotective activity

An increasing prevalence of neurodegenerative diseases such as Alzheimer's, Parkinson's, multiple sclerosis, neuroinflammation and amyotrophic lateral sclerosis, which have become significant health problems as the world's population has aged, with limited treatment options available. Neurodegenerative disorders are linked to oxidative stress and glutamate toxicity, involving various forms of cellular degeneration, such as apoptosis, autophagy, and necrosis. Rhinacanthin-C has shown neuroprotective properties in hippocampal neural cells by inhibiting these processes of apoptosis, necrosis, and autophagy. Furthermore, rhinacanthin-C enhances and restores cell survival by augmenting the activity of autophagy and elevating the expression of ERK, CHOP, and LC3B proteins (Rakkhittawattana et al., 2022). Rhinacanthin-C thus has potential as a neuroprotective agent for the treatment of neurodegenerative diseases.

Neuroinflammation refers to an inflammatory reaction that takes place within the central nervous system (CNS). It is marked by the infiltration of immune cells, activation of glial cells, and the release of inflammatory mediators. In this context of neuroinflammation, microglia, which are the resident phagocytes within the CNS, play a central and crucial role. Rhinacanthin-C has shown to alleviate inflammation events by inhibiting inflammatory activities such as amyloid-peptide (Aβ)-induced production, lipopolysaccharide (LPS)-activated nitric oxide generation, inducible nitric oxide synthase (iNOS) expression, and NF-κB signaling.

Rhinacanthin-C demonstrated the ability to suppress neuroinflammation in microglia cells by reducing the generation of nitric oxide (NO) and the mRNA levels of iNOS, IL-1β, CCL-2, and CCL-5 (Chuang et al., 2017). Rhinacanthin-C exhibits neuroprotective qualities, making it a potential therapeutic candidate for conditions associated with neuroinflammation.

Acute brain ischemia following subarachnoid hemorrhage (SAH) are crucial neuralgic disorders. Multiple systems and processes add to the pathogenesis of vasospasm but attempts to treat this disease have been unsuccessful. High-mobility group box-1 (HMGB-1) is implicated in cortical inflammation and may play a part in SAH-induced brain apoptosis. Rhinacanthin-C decreased the expression of pro-

inflammatory cytokines and HMGB-1 in an early brain damage model after subarachnoid bleeding (Chang et al., 2016). This indicates that rhinacanthin-C may have therapeutic benefits in decreasing neuro-inflammation and improving outcomes in SAH patients.

2.3.9 Other potential use of rhinacanthin-C

The continuous use of synthetic pesticides in mosquito control is thought to encourage resistance and impede disease management. Pyrethroid tolerance, for example, is common in Anopheles species. The activity of detoxification enzymes, including the heme-containing cytochrome P450 monooxygenase, is upregulated as a synthetic pesticide tolerance mechanism. The enzymes CYP6AA3 and CYP6P7 have been identified as being implicated in pyrethroid resistance in *Anopheles minimus* mosquitos. Rhinacanthin-C displayed reversible inhibition of both CYP6AA3 and CYP6P7 enzymes, and this inhibition exhibited specific characteristics related to time, concentration, and NADPH dependency. Furthermore, rhinacanthin-C made CYP6AA3 and CYP6P7-expressing cells more susceptible to cypermethrin neurotoxicity (Pethuan et al., 2014). As a result, it is possible that the combined inhibition effect of rhinacanthin-C compounds on mosquito cytochrome P450 enzyme and synergistic impact on cypermethrin cytotoxicity could be helpful for resistance approaches in mosquito vector control.

2.4 Toxicity assessment of rhinacanthin-C

Natural products are often less hazardous than synthetic products. Yet, a rising number of case reports reveal acute or chronic intoxications caused by natural product use. Hence, assessing the toxicity of natural materials facilitates in the identification of toxicants for which alternative treatments are available. Before clinical application, toxicity can be evaluated using both animal and non-animal models. Animal models are considered the gold standard in toxicological investigations because they frequently closely resemble human toxicity, and importantly, they encompass pharmacokinetic and ADME (Absorption, Distribution, Metabolism, and Excretion) profiles. Nonetheless, the use of *in silico* modeling in toxicity assessment is a potential endeavor in innovative drug discovery since it typically speeds up the process and reduces costs.

Although no conclusive evidence of rhinacanthin-C toxicity has been established, an *in vitro* toxicity investigation of a chloroform extract of *R. nasutus* root

in the Raji cell line revealed no significant harm (Murakami et al., 1993). In addition, rhinacanthin-C at 0.125 μM has shown rhinacanthin-C exhibited non-neurotoxic damage to rat hippocampus neurons (Chuang et al., 2017). Furthermore, *R. nasutus* extracts has been revealed that it shows there is an *in vivo* toxicity investigation of in mice. There was no significant toxicity reported after subcutaneous injection and feeding 10 μg/kg of *R. nasutus* ethanol and water extracts containing rhinacanthin-C through a gastric tube. In *in vivo* research of the chemoprotective impact of rhinacanthin-C found that rhinacanthin-C (25-50 mg/kg/day) had no effect on AST and ALT levels in mice. Extended and excessive usage of rhinacanthin-C should be avoided in patients with hepatic problems (Siripong et al., 2010). Additional toxicity information from *in silico* calculations of rhinacanthin-C has achieved a toxicity profile as non-toxic, non-lethal, and range in category III of acute oral toxicity (LD50 = 500-5000 mg/kg), which is more than 10 times greater than the therapeutic dose of rhinacanthin-C (Zhao et al., 2019). Although rhinacanthin-C has demonstrated diverse cytotoxicity activities, the majority of them were specific to tumor or parasite cells. Continuous administration of rhinacanthin-C, on the other hand, may overcome the unique bioactivity of rhinacanthin-C and be toxic to normal cells.

2.5 Metabolism and pharmacokinetic of rhinacanthin-C

In the context of administering natural products alongside therapeutic agents, the study of their metabolism and pharmacokinetics assumes a critical role in understanding herb-drug or herb-herb interactions. Notably, the inhibition and activation of drug-metabolizing enzymes and drug transporters are key mechanisms of interference that can give rise to concerns related to both the effectiveness and safety of such combinations.

The pharmacokinetics of Rhinacanthin-C are closely linked to drug transporter proteins and drug-metabolizing enzymes. It has been observed that rhinacanthin-C inhibits various transport proteins, including P-glycoprotein (P-gp) (with an IC_{50} of 5.20 M in Caco-2 and a 1.38-fold increase in MCF-7), multidrug resistance-associated protein 2 (MRP2) (resulting in a 1.18-1.65-fold effect in MCF-7), and breast cancer resistance protein (BCRP) (with an IC_{50} of 0.83 M). Moreover, inhibitory effect from rhinacanthin-C on those proteins were reversible. Rhinacanthin-C exhibits inhibitory characteristics towards drug-uptake proteins, specifically affecting organic anion-

transporting polypeptide B1 (OATP1B1) with an IC_{50} of 0.70 μM and OATP1B3 with an IC_{50} of 3.95 μM. Furthermore, pharmacokinetic studies have elucidated that rhinacanthin-C exerts an influence on Phase I drug-metabolizing enzymes, encompassing a range of human CYP isoforms. This includes CYP2C8 (with an IC_{50} of 4.56 M), 2C9 (with an IC_{50} of 1.52 M), 2C19 (with an IC_{50} of 28.40 M), 3A4 (with an IC_{50} of 53 M), and 3A5 (with an IC_{50} of 81.20 M). Rhinacanthin-C exhibited no impact on the CYP isoforms CYP1A2, 2B6, 2D6, and 2E1 (Dunkoksung et al., 2019; Pouyfung et al., 2014). Unfortunately, data on the influence of rhinacanthin-C on placental CYP1 activity and CYP1A1 and ABCG2 expression levels in placental are few. Rhinacanthin-C selectively and permanently inhibited CYP2A6 and 2A13 via coumarin 7-hydroxylation in concentration, time, and NADPH-dependent ways (Wang et al., 2021; Wongwanakul et al., 2013). Therefore, it is important to consider herb-drug interactions when rhinacanthin-C is used in conjunction with other conventional medications.

Rhinacanthin-C pharmacokinetic and toxicity profiles were also reported utilizing computational analyses. Rhinacanthin-C demonstrated drug candidate behavior according to Lipinski's rule. According to ADMET simulations, rhinacanthin-C easily crosses the blood-brain barrier. Rhinacanthin-C demonstrated oral bioavailability and intestinal absorption. P-gp transfers the medication from cells to the gut, causing drug bioavailability to be reduced. *In silico* studies revealed that rhinacanthin-C is a strong inhibitor of P-gp, resulting in high bioavailability. Recently, the *hERG* gene has been investigated as a potential target in preclinical drug development. Moreover, rhinacanthin-C has a low affinity for the inhibition of hERG, which increases its drugability. Furthermore, the ADMET results demonstrated rhinacanthin-C affinity as a CYP450 3A4 substrate, and its terminal methyl group and aromatic portion appear to be vulnerable to oxidation (Zhao et al., 2019). Although *in vitro*, *in vivo,* and in computational experiments may indicate that rhinacanthin-C is safe for various treatments, clinical research is required to confirm the safety.

2.6 Quantitative analysis of rhinacanthins

Determination of biomarker content in samples is critical step for analysis in phytochemical study. Rhinacanthins including RC, RD, and RN are biomarker in *R. nasutus*. One of the most technique used for phytochemical analysis is UV and HPLC

technique. A straightforward method for semi-quantitatively assessing the overall content of rhinacanthins in *R. nasutus* extracts, quantified as rhinacanthin-C, has been developed using a UV spectrophotometric technique (Panichayupakaranant et al., 2006). However, this method possessed low sensitivity and specificity. Hence, a validated quantitative HPLC method has been subsequently developed to determine the levels of rhinacanthin-C, -D, and -N in *R. nasutus* extracts (Panichayupakaranant et al., 2009). The HPLC method employed in this study utilized a TSK-gel ODS-80Ts column (5 μm, 4.6 × 150 mm i.d.) as the stationary phase, eluted with a mobile phase consisting of methanol and 5% aqueous acetic acid (80:20, v/v), at a flow rate of 1 mL/min, and chromatographic detection was conducted at a wavelength of 254 nm. The limit of detection and quantification for all rhinacanthins were determined to be 0.75 and 3.0 μg/mL, respectively. A high degree of accuracy (the recoveries of the method was 94.3-100.9%) as well as precision (R.S.D. values less than 5%) were also achieved with a good linearity (correlation coefficient ≥ 0.9999). Based on the HPLC analysis of *R. nasutus* leaf extract, rhinacanthin-C was the major naphthoquinone ester (1.9% w/w), while rhinacanthin-D (0.16% w/w) and rhinacanthin-N (0.07% w/w) were the minor ones (Panichayupakaranant et al., 2009).

2.7 Extraction and standardization

Medicinal plants are the most abundant resource of medications used in ancient systems of medicine, contemporary medicines, nutraceuticals, dietary supplements, and chemical pharmacophores for synthesized drugs. The World Health Organization (WHO) encourages, advises, and promotes traditional treatments in national health care systems because they are readily accessible, low-cost, and safe (Kumari and Kotecha, 2016). The extraction is the first process in obtaining a desired bio-active chemical from plant sources. Yet, there is a distinction between providing a pure phytochemical and the same chemical in the form of a plant extract. It applies in the context of chemical complexity if the pharmacological impact of a chemical combination is larger than the arithmetic sum of the effects of individual components. This is the foundation for making an active constituent-enriched herbal extract. Because bio-active chemicals are generated in tiny amounts in plants and embedded within the plant matrix, often complexed with other compounds in the plant, the extraction process is critical. Rhinacanthin-C, for example, is not commercially available. Moreover, the purification

of rhinacanthin-C is a multistage process that takes a substantial number of hazardous solvents as well as time and energy, raising the expense of manufacturing. Another strategy that may have a synergistic impact is to use rhinacanthins enriched extract (RRE), a semi-purified extract of *R. nasutus*.

Although ethyl acetate tends to be the optimum solvent for *R. nasutus* extraction, it is an organic solvent that may be detrimental to humans and the environment. As a result, a simple green extraction and fractionation technology that is environmentally friendly and suited for pharmaceutical applications in terms of safety and cheap cost is developed. The process solely utilized green solvents, such as ethanol, water, or natural polymers. A microwave-assisted extraction additionally was utilized, which consumed less energy and took less time, and a stage of solvent evaporation prior to the fractionation procedure was avoided (Panichayupakaranant et al., 2009).

Since rhinacanthins are anionic compounds, an anion exchange column chromatography was implemented to enrich the rhinacanthin content in RRE preparation. Also, undesirable compounds in the crude extract, such as pigments including chlorophylls and carotenoids, or sticky resin, might be washed away during the enrichment process. RRE and its standard markers, notably rhinacanthin-C, have been confirmed in a range of bioactivities to establish their applicability and standardization.

RRE was prepared and standardized to contain at least 70% w/w (73.7% w/w) total rhinacanthin. RRE demonstrated comparable α-glucosidase inhibitory action (IC_{50} of 25.0 μg/mL) to rhinacanthin-C (IC_{50} of 22.6 μg/mL). In an enzymatic kinetic analysis employing the Lineweaver-Burk plot, RRE was revealed to have noncompetitive α-glucosidase inhibitory activity. RRE was investigated for its glucose uptake stimulatory and antiadipogenic properties. RRE (20 μg/mL) stimulated glucose uptake in 3T3-L1 adipocytes in the same way that rhinacanthin-C (20 μg/mL) achieved (Shah et al., 2017b). Moreover, in L6 myotubes, only a small amount of RRE (2.5 μg/mL) stimulated glucose absorption by up to 80%. In terms of antiadipogenic action, RRE decreased adipogenesis in 3T3-L1 adipocytes in a dose-dependent manner, similar to rhinacanthin-C (Shah et al., 2017a). RRE additionally possessed anti-diabetic properties *via* superoxide scavenging and antiglycation properties. RRE was a potent

antioxidant with an IC_{50} of 8 μg/mL via the E_rC_i mechanism. RRE has potent anti-glycation properties equivalent to rhinacanthin-C, in antiglycation assays with IC_{50} values of 39.7 and 37.3 μg/mL, respectively (Shah et al., 2017c). In nicotinamide-streptozotocin-induced diabetic rats, the same batch of RRE was investigated for anti-hyperglycemic and anti-hyperlipidemic activities. RRE (15 mg/kg, comparable to rhinacanthin-C) dramatically reduced fasting blood glucose and HbA1c while increasing insulin levels in normal rats. Moreover, RRE has been shown to normalize lipid, liver, and renal blood profiles. Furthermore, after RRE treatment, diabetic rats' islets of Langerhans were recovered. RRE has anti-diabetic effects equivalent to rhinacanthin-C, the bioactive marker (Shah et al., 2019). In streptozotocin-nicotinamide-induced diabetic nephropathy rats, RRE also showed protective effect against diabetic nephropathy. RRE (24 mg/kg/day) and rhinacanthin-C (15 mg/kg/day) decreased renal index, oxidative stress biomarkers, and pro-inflammatory cytokines while enhancing renal glutathione, superoxide dismutase, and catalase. RRE has been proposed to treat diabetic nephropathy by inhibiting oxidative stress and inflammation. As a result, RRE also exhibits biological activity similar to rhinacanthin-C (Zhao et al., 2019). RRE is therefore an appropriate *R. nasutus* extract for the production of pharmaceutical preparations for anti-diabetic products. The extracts utilized in future research and applications should be standardized to a total rhinacanthin content of no less than 70% w/w or no less than 60% rhinacanthin-C w/w based on the adequate biological activity of RRE.

2.8 Tissue cultures of *R. nasutus*

Medicinal plant tissue culture is a technique using cells, tissues or organs from plants to grow in nutrient sterility and controlled at various conditions such as temperature, light sources and humidity (Barz et al., 2012). The main principle applied for medicinal plant tissue culture is totipotency (Zhou and Wu, 2006). Plant cells retain totipotency and developmental plasticity even after differentiation. They possess the capacity to undergo dedifferentiation, proliferation, and subsequent regeneration into mature plants when provided with suitable culture conditions (Sidhu, 2011). There are five types of plant cultures including seedlings, embryos, organ, cells and protoplasts. Plant tissue cultures can be propagated in a liquid medium or in a sold (agar) medium containing the growth nutrients (Sharma et al., 2010). Most of the studies on secondary

metabolite production in plant tissue cultures have been carried out in liquid of semisolid cultures, predominantly with cell suspension cultures, mainly because liquid cultures provide uniform culture conditions, support more rapid growth, and are more feasible for large-scale applications (Vanisree et al., 2004). The media for plant cell cultures are usually composed of several macro-inorganic elements (N,P,K,Mg^{2+} and Ca^{2+}) and microelements (Fe^{2+}, Mn^{2+}, Zn^{2+}, Mo^{2+}, Cu^{2+} and B^{3+}), few organic nutrients (vitamins and amino acids), plant growth regulators (phytohormones), and the carbon source (sucrose or some other carbohydrate) composition has unique effects on the cell growth and secondary metabolite accumulation, and needs to be optimized for specific cell lines and production steps (Gamborg et al., 1976). In addition, cell differentiation (primary metabolism) and secondary metabolite biosynthesis often require various nutrients, as many secondary metabolites are not associated with growth. A two-stage culture process is more suitable, with the first stage using a culture medium devised for maximum cell proliferation, and the second stage for maximum secondary metabolite accumulation (Singh and Dwivedi, 2014). Example of using plant tissue culture technique is callus induction and organogenesis of *Triticum aestivum* or wheat using different plant regulators (Ali et al., 2009). Another study using medicinal plant tissue culture technique to improve secondary metabolite production. *Hypericum hirsutum* was growth using plant tissue culture technique to enhance hypericins production (Yue et al., 2016).

Recently, research has been conducted on the establishment of tissue cultures of *R. nasutus* in order to use them as an alternative source of the natural plant. Cell suspension cultures of *R. nasutus* were initiated from the callus culture and cultivated in a liquid medium known as Gamborg's B5 (B5). This medium was supplemented with 2.0 mg/L of 6-benzylaminopurine (BA) and 0.5 mg/L of indole-3-butyric acid (IBA). Regrettably, these cell suspension cultures did not yield any rhinacanthin-C production. Various strategies, including medium manipulation and elicitation techniques, were explored to induce rhinacanthin production in the cell suspension cultures. Unfortunately, neither approach proved successful, indicating that the dedifferentiation of *R. nasutus* cell suspension culture led to a loss of rhinacanthin production potential.

However, the medium manipulation technique did result in the establishment of *R. nasutus* root cultures. To stimulate root formation in *R. nasutus*, solid B5 medium

supplemented with 0.1 mg/L of IBA was initially used. Following subsequent modifications to the growth medium, it was established that employing Murashige and Skoog (MS) medium supplemented with 3.0 mg/L of indole-3-butyric acid (IBA) proved to be the most efficient method for promoting increased root formation. Nevertheless, the root cultures only yielded a minimal amount of rhinacanthin-C (0.003% w/w).

However, when these root cultures were transferred to a semisolid medium containing 4 g/L of agar and possessing the same MS composition, there was a notable increase in rhinacanthin production. This included the production of rhinacanthin-C, -D, and -N, with respective contents of 0.34%, 0.007%, and 0.007% w/w. (Panichayupakaranant and Meerungrueang, 2010).

Hairy root cultures of *R. nasutus* have been established from leaves and stems using *Agrobacterium rhizogenes* induction. The hairy roots were initiated from leaf explant, with more than 73% survival rate. Schenk, Hildebrandt and MS and woody plaint media were investigated for root biomass and rhinacanthin-C, -D and -N productions. MS found to be the most suitable nutrient for the root culture, which produced average root biomass of 0.89 g/flask and rhinacanthin-C, -D and -N of 3.8, 0.43 and 0.18 mg/g, respectively. Moreover, higher root biomass (1.41 g/flask) and rhinacanthin-C (4.4 mg/g), -D (0.69 mg/g) and -N (0.21 mg/g) productions were found in 4% sucrose-added MS (Cheruvathur et al., 2013).

A study on plant tissue culture of *R. nasutus* using different nutrients and plant growth regulators with different concentrations has exhibited that the most appropriate nutrient and plant growth regulators combinations were MS supplemented with indole-3-butyric acid (IBA) (3 mg/l) and 30 g/l sucrose added. This nutrient and plant growth regulator induced rhinacanthin-C, -D and -N productions of 3.45, 0.07 and 0.07 mg/DW, respectively. Moreover, there has been reported that the root induction and rhinacanthin productions were inhibited by light (Cheruvathur et al., 2015; Cheruvathur et al., 2013; Cheruvathur and Thomas, 2014; Johnson et al., 2005).

There was a study on establishment of callus mediated plant regeneration for *R. nasutus*. MS medium supplemented with 2,4-dichlorophenoxy acetic acid (6.78 µM) has found to exhibit maximum callus proliferation. Shoot regeneration was performed using friable and semi-friable callus (4-weel-old). Stems- and leaves-derived callus

provide maximum percentage of shoot regeneration for 74.9 ± 2.42% and 82.9 ± 1.39%, respectively. Excision and multiplication of regenerated shoots were supplemented with MS medium and benzyl amino purine (3.1 μM). Root initiation from differentiated shoots was performed with half concentration of MS medium supplemented with indole-3-butyric acid (9.84 μM) and sucrose (3%) for 15-day-old culture. Transferring regenerated plantlets to soil exhibited a survival rate of 68% (Johnson et al., 2005).

Micropropagation study of *R. nasutus* has been achieved by culturing nodal segments from natural occurring plants supplemented with MS medium. Nodal explants produced multiple shoots for 8 shootlets/explant supplemented with 2.22 μM of 6-benzyladenine. The shoots obtained at maximum length on the medium containing 0.44 μM of 6-benzyladenine. For rooting of the excised shoots, indolyl 3-butyric acid (4.92 μM) with half concentration of MS medium was optimally achieved. Rooted plants were achieved at 75% survival rate in pots after hardening (Johnson et al., 2002).

2.9 Hydroponics

Soilless cultivation, commonly referred to as hydroponics, has been a practiced method for several decades in the field of horticulture. Hydroponics involves the cultivation of plants using a mineral nutrient solution dissolved in a water-based solvent (Jensen, 1997). In a hydroponic system, the nutrients employed can consist of either artificial nutrient solutions or chemical fertilizers. A hydroponic nutrient solution is a liquid mixture primarily composed of inorganic ions originating from soluble salts containing essential elements crucial for the growth of higher plants. At times, organic compounds like iron chelates may also be incorporated into these solutions. Essential elements serve vital physiological functions, and the absence of any of these elements can impede the complete life cycle of a plant. Therefore, the essential elements generally deemed indispensable for most plants encompass carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, iron, copper, zinc, manganese, molybdenum, boron, chlorine, and nickel.

Furthermore, there are specific miscellaneous elements, such as sodium, silicon, vanadium, selenium, cobalt, aluminum, and iodine, which may be essential for certain plants that produce distinct secondary metabolites. However, the core nutrient composition is typically classified into macronutrients, including nitrogen, phosphorus,

potassium, calcium, magnesium, and sulfur, and micronutrients. The electrical conductivity and osmotic pressure should be concerned in hydroponic system of particular plant. Hydroponics provide several advantages, such as a decrease in water consumption (Douglas, 1985). Hydroponic system has been applied continuously in herbal industry scale to provide high quality of herbal raw material. Herbal hydroponic system provide herbal growing with no insecticide and re-harvestable. Moreover, leaves, root and rhizomes can be harvested in herbal hydroponic system (Jones Jr, 2016). For instance, roots and rhizomes of *Arctium lappa* (burdock) was growth using hydroponic systems, perlite-hydroponics, nutrient film technique (NFT), ebb and flow and aeroponics. Burdock root was harvested at highest yield when grow with aeroponics (227 g/unit) (Hayden, 2006).

2.10 Elicitations

Elicitation technique is the technique basically used to enhance or induce secondary metabolite production from plants. Different plants require different elicitation techniques to increase their secondary metabolite production (Narayani and Srivastava, 2017). Substances or factors used in elicitation is called elicitors. Elicitors can be classified accordingly to their origins which are abiotic and biotic elicitors (Eilert, 1987). Abiotic elicitors are the substances or factor which are not derived from biological origin such as inorganic salt (Cu, Cd or Ca ions) and physical factors (temperature, pH, osmotic stress, UV or wounding). On the other hand, biotic elicitors are obtained from biological origin such as yeast or bacteria extracts and polysaccharides extracted from microbial cell wall (chitosan, alginate, pectin, cellulose, chitin and glucan) (Wang and Wu, 2013). Mechanisms of elicitations from most elicitors can be basically explained that elicitors, substances or factors act as the stress which stimulate defensive mechanism which mostly produce secondary metabolite through intracellular signaling pathways (Hussain et al., 2012). For instance, high humidity in rain forest region stimulates some plant to produce secondary metabolite against microorganism. Moreover, it has been reported that *Trichoderma viride*, as a biotic elicitor stimulated *Catharanthus roeus* to produce ajmalicine, an antifungal biomarker (166 µg/g DW) more than control group (79 µg/g DW) for 2.1 times (Namdeo, 2007). Applying elicitation technique to different plants has to be concerned

of several factor such as plant age, elicitor concentration, elicitation period and harvesting period.

A study about plumbagin production enhancement from *Plumbago indica* was done. Different concentrations of chitosan, a biotic elicitor was used to treat *P. indica* with different elicitation period. The results were found that chitosan at 150 mg/l treated *P. indica* root (14 day-old) for 72 hours enhance plumbagin production up to 6.6 times (13.08 mg/g DW) compared to control group (1.97 mg/g DW) (Jaisi and Panichayupakaranant, 2017).

Twelve types of biotic and biotic elicitors were further used to enhance plumbagin production from *P. indica* for both intracellular and extracellular productions. Most of elicitors have no effect to *P. indica* root biomass. However, chitosan (250 mg/l) and L-alanine (150 mg/l) decrease root biomass of *P. indica* about 40% compared to control group. $AgNO_3$ (150 mg/l) stimulated plumbagin production 4 times (7.6 mg/g DW) higher than control group (1.9 mg/g DW). $AgNO_3$ stimulates plumbagin production through intracellular production. Unlikely, chitosan (150 mg/l), L-alanine (5 mM), and 1-naphthol (50 μM) increased plumbagin production in extracellular manner for 1.6, 6.9 and 5.7 mg/g DW, respectively. Moreover, they stimulated plumbagin production from *P. indica* in both intracellular and extracellular pattern as 12.5, 12.5 and 9.4 mg/g DW, respectively (Jaisi and Panichayupakaranant, 2016).

L-alanine was further examined for plumbagin production enhancement. The results were shown that after *P, indica* (14 day-old) was treated with L-alanine for 36 hours, plumbagin production was increased up to 9 times (14.4 mg/g DW) compared to control group (1.6 mg/g DW). According to the results, L-alanine enhanced plumbagin production from *P. indica* more than chitosan did, however, molecular mechanism of elicitation of both compounds are not clearly investigated. Co-feeding of chitosan and L-alanine has not done to confirm synergistic effect on elicitation (Jaisi and Panichayupakaranant, 2020).

For low-cost elicitation technique, heat shock and ultrasonic technique were used to enhance plumbagin production from *P. indica*. 10 minutes of heat shock stimulated plumbagin production up to 5 times (5.51 mg/g DW) compared to controlled group (1.14 mg/g DW). Ultrasonic and combined technique did not enhanced

plumbagin production from *P. indica* roots compared to control group (Jaisi and Panichayupakaranant, 2016).

It can be suggested that both biotic and abiotic elicitor can enhance secondary metabolite production in particular plant. Hence, treating both elicitor types in *R. nasutus* root for enhancing rhinacanthins production is still challenging. There was a study about *R. nasutus* root culture. Plant growth regulators and elicitors were used for *R. nasutus* root culture and rhinacanthin production. Auxins, plants growth regulators which consist of IBA and alpha-naphthalene acetic acid (NAA) and elicitors, methyl jasmonate (MeJA) and salicylic acid (SA) were used in the study. The results were shown that IBA (2.5 μM) and NAA (2.5 μM) produce highest root biomass of *R. nasutus* (4-week-old) compared to control group. However, rhinacanthin-C production was highest at 6-week harvesting period. Although, MeJA and SA tended to decrease *R. nasutus root biomass*, rhinacanthin-C (6.3 mg/g, 1.7-fold), rhinacanthin-D (1.1 mg/g, 2.5-fold) and rhinacanthin-N (0.61 mg/g, 3.5-fold) were produced higher in *R. nasutus* root culture treated with MeJA and SA for 7 days compared to control group (Cheruvathur and Thomas, 2014).

CHAPTER 3
MATERIALS AND METHODS

3.1. Chemicals

3.1.1. Chromatographic reagents

Methanol (HPLC grade), acetic acid (analytical grade), ethyl acetate (analytical grade), and ethanol (analytical grade) were obtained from Labscan Asia (Bangkok, Thailand). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

3.1.2. Standards

RC, RD, and RN were purified as previously described (Bhusal et al., 2014). RC (> 98% purity) has reddish yellow oily appearance. RD (> 98% purity) and RN (> 98% purity) have red and green crystalline form, respectively.

3.1.3. Hydroponics and elicitation

Murashige and Skoog basal salt mixture M524 was purchased from PhytoTech Labs (KS, USA). Chitosan (trc, Canada), MeJA (Sigma-Aldrich, USA), NaAlg (Sigma-Aldrich, USA), SA (Sigma-Aldrich, USA), lawsone (Sigma-Aldrich, USA) and *Trichoderma harzianum* (iLAB, Thailand) were analytical laboratory grade. Other chemicals used were analytical grade and purchased from Sigma (St. Louis, MO) or MERCK (Darmstadt, Germany).

3.1.4. Instruments

Herb grinder (SK300 Rostfrei, Retsch®, Germany), hot air oven (Venticell il easy, Venticell, Germany), laboratory ultrasonic cleansing bath (S100H Elmasonic, Elma, Germany), household microwave (EMS3085X, Electrolux, Thailand) were used in extraction process. HPLC was operated on Shimadzu® (Shimadzu® LC-20A series) with a quaternary pump, autosampler, and photodiode array detector (λ = 210 nm). Analytical reverse-phase column (Phenomenex ODS®, stainless steel column, phase C18 (octadecyl), 150 mm x 4.6 mm column, 5 μm, 100 °A, Phenomenex ®, USA) was

used in the analytical process of rhinacanthin determination. The rotary evaporator (Heidolph, Germany) was used for removing solvent. The 2 digits analytical balance (TE3102S, Sartorius, USA), and 4 digits analytical balance (Explorer (Eo2140), OHAUS, USA) were used to weigh the samples and materials. Environment Control System for Automatic Cultivation (ECSAC, Thailand), which is a system that controls the transpiration potential (vapor pressure deficit) of plants in the ranges of 0.5 – 1.5 KPa. The controlled system consisted of a temperature of 25 ± 2 ºC and relative humidity of 1.5 ± 0.5 KPa, with a sensor for measuring the environment inside the system as well as a misting pump and a heating system to control the internal environment, provided a light intensity of approximately 18,000-22,000 lumens were used to control hydroponic conditions. The stirrer and hotplate (SLR, SCHOTT-Gerate GmbH, Germany) and pH meter (Laqua 9615S, Horiba, Thailand) were used to prepare medium and elicitor solutions.

3.2. Determination of effects of natural growth *R. nasutus* parts and harvesting periods on production of RC, RD, and RN in plant raw materials

3.2.1. Plant materials

Six *R. nasutus* plant parts, including leaves, flowers, roots, green twigs, brown twigs, and aerial parts (leaves and stems) (Fig. 3), were used to determine the effect of plant parts, while six harvesting periods, including January, March, May, July, September, and November, were used to determine the effect of harvesting periods. The gathered plant parts were rinsed with tap water before being dried in a hot air oven at 60°C for 24 h. All samples were ground and sieved through a No. 20 mesh before being extracted and quantitatively HPLC analyzed for RC, RD, and RN.

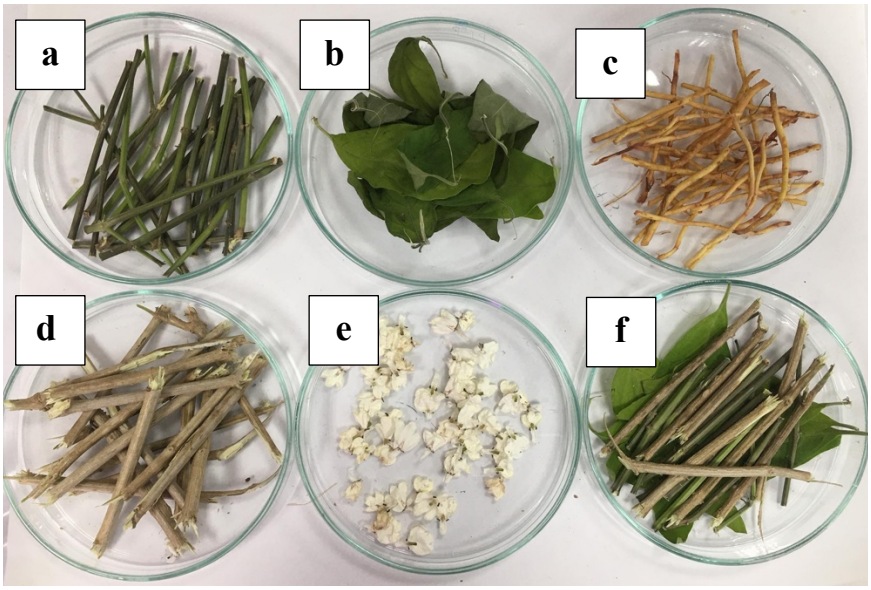

Figure 3 Different parts of R. nasutus; green twigs (a), leaves (b), roots (c), brown twigs (d), flowers (e), and aerial parts (f)

3.2.2 Extraction

The microwave extraction method previously described was applied (Shakya, 2015). In brief, the plant powders (100 mg) were microwave extracted using ethyl acetate (50 mL). The extraction conditions were as follows: a microwave frequency of 2,450 MHz and a power of 450 watts, respectively, at 72 2 ºC for three cycles. (1 cycle; power-on for 45 s, and then power-off for 30 s, with stirring) (Fig. 4). The extracts were then filtered and dried using a rotary evaporator set at 40°C. Table 2 shows the extraction efficiency. The extracts were then HPLC analyzed for rhinacanthin concentration. The experiment was carried out three times.

Table 2 Extraction efficiency (%yield) of sample extraction

Parts	Extraction efficiency (%, DW)
Roots	45.00 ± 1.20
Leaves	26.46 ± 1.71
Aerial parts	38.05 ± 2.10
Green twigs	18.81 ± 0.29
Brown twigs	25.47 ± 0.84
Flowers	21.88 ± 0.40
Raw materials	16.90 ± 1.31 – 40.09 ± 0.76

The extractions were performed in triplicate.

The extraction efficiency of 10 raw materials was provided in the range.

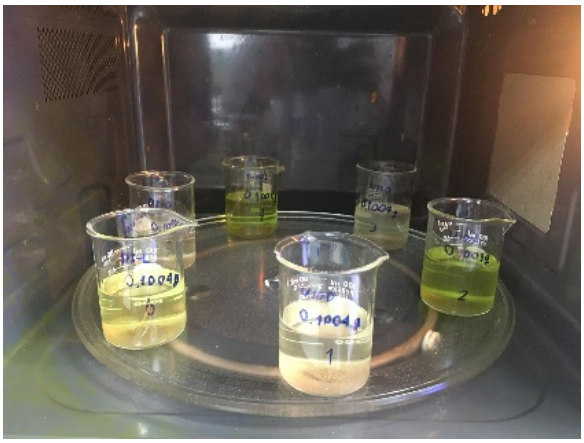

Figure 4 Extraction of *R. nasutus* using microwave extraction technique

3.3. Determination of quality of commercially available plant raw materials and tea infusion health products from *R. nasutus* in term of rhinacanthin content

3.3.1 Plant materials

Raw materials for *R. nasutus* powders were gathered from ten different provinces (Table 3), including I and III from Nakornpathom, II from Nakon Ratchasima, IV from Nonthaburi, V from Bangkok, VI from Pathum Thani, VII and X from Chon Buri, VIII from Chanthaburi, and IX from Buriram (Fig. 5). *R. nasutus*-

infused teas, RN-tea I and RN-tea II, were purchased from two suppliers in the provinces of Nakornpathom and Lampang, respectively (Fig. 6).

Table 3 Commercially available raw material and tea infusion health product information

Sample	Suppliers	Provinces	Coordinates
Commercially available raw materials*:			
I	PopayaHerb	Nakornpathom	14.01212, 100.02552
II	SEPT Chemicals and packaging	Nakon Ratchasima	14.92239, 102.06077
III	Creamryhomemade	Nakornpathom	14.01212, 100.02552
IV	Baanjaophaya	Nonthaburi	13.92663, 100.38741
V	A&A service	Bangkok	13.79678, 100.65989
VI	Kingkajorn	Pathum Thani	14.02954, 100.59170
VII	TPC HERB	Chon buri	13.28864, 101.24721
VIII	Chakkrawatherb	Chanthaburi	12.81841, 102.07689
IX	IIherb	Buriram	14.96048, 103.03944
X	Morchern	Chon buri	13.28864, 101.24721
Tea infusion health products**:			
RN-tea I	Thaiherbbiz	Nakornpathom	14.01212, 100.02552
RN tea II	Lampangherb	Lampang	18.24627, 99.49989

Time of harvesting and parts of raw materials were not informed by the suppliers.

*Commercially available raw materials were purchased as fine powders

**Tea in fusion health products were purchased as coarse powders in tea bags.

Figure 5 *R. nasutus* raw materials obtained from ten suppliers (I-X)

3.3.2. Extraction of rhinacanthins in raw material and tea products

Method 3.2.2 was applied to extract raw materials and tea powders. While tea infusions were prepared from *R. nasutus* teas, RN-tea I and RN-tea II (three batches of each product) as follows: infuse the tea bag (20 g/bag) in 250 mL of boiling water (100 °C) for 5 minutes. Both ethyl acetate extracts and tea infusions were then dried using a rotary evaporator at 50 °C before being submitted to quantitative HPLC analysis of RC, RD, and RN. The experiment was carried out three times.

Figure 6 Two commercially available teas of *R. nasutus* (brand a and b)

3.4. Establishment of hydroponic root culture systems of *R. nasutus* that produce high root biomass and rhinacanthin content

3.4.1. Plant materials

R. nasutus shoots were obtained from plants that had been cultivated for two years in the Botanical Garden of Prince of Songkla University's Faculty of Pharmaceutical Sciences (Fig. 7). Its voucher specimen (specimen number. 001 18 14) was identified by Pharkphoom Panichayupakaranant and arranged at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Figure 7 Shoots of *R. nasutus*

3.4.2 Establishment of hydroponics of *R. nasutus*

Static hydroponic system was used in all experiments). Briefly, *R. nasutus* shoots (12 - 15 cm tall) were rinsed with tap water, then 2% sodium hypochlorite (15 minutes), and deionized water (10 minutes), before being taken to a growth container (11,000 cm^3) for root initiation (20 plants/growing container). Roots of *R. nasutus* were started in deionized water (8 liters) and assigned to the Environment Control System for Automatic Cultivation (ECSAC, Thailand), which is a system that manages the transpiration potential (vapor pressure deficit) of plants in the 0.5 - 1.5 KPa range. The controlled system featured a temperature of 25 ± 2°C and a relative humidity of 1.5 ± 0.5 KPa, as well as a sensor for detecting the environment within the system, a misting pump, and a heating system to manage the interior environment. The system produces approximately 18,000-22,000 lumens of light. Roots started growing within 7 days in such conditions to develop into plantlets (Fig. 8).

Figure 8 Hydroponics system of *R.nasutus*

3.4.3 Effect of aeration on root biomass and rhinacanthin production

R. nasutus plantlets (7 days old) were placed into a translucent plastic growth container containing 8 L of 10% w/v Murashige and Skoog (MS) liquid medium (pH = 5.4) (without sucrose), with or without aeration, at a flow rate of 4.5 L/min (Fig. 9). There were 20 plantlets in each growth container. The hydroponics were run under ECSAC conditions. To keep the hydroponics system operating properly, 10% MS was replenished every 4 weeks to maintain nutrient level in the growing container. After three months, the roots were harvested and dried in a hot air oven at 60ºC for 24 h. The dried root biomass (mg/plant) was measured, and the contents of the dried roots' RC, RD, and RN were assessed using an HPLC technique. The experiment was repeated three times.

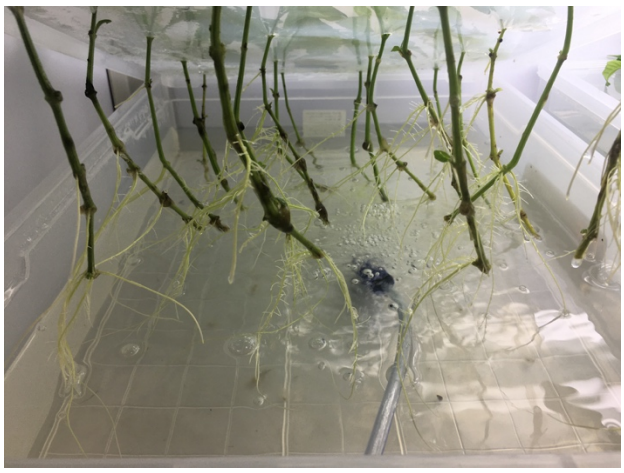

Figure 9 Hydroponics of *R. nasutus* with aeration

3.4.4 Effect of growing containers on root biomass and rhinacanthin production

Plantlets (7 days old) were grown in 10% MS (pH 5.4) in either light protected or translucent containers (20 plants/container) with aeration (4.5 L/min) (Fig. 10). ECSAC conditions were used to cultivate the plants. Every four weeks freshly made 10% w/v MS was replaced. After three months, the roots were harvested and dried at 60ºC. The dry root biomass was measured. A HPLC technique was used to determine the contents of RC, RD, and RN. The experiment was repeated three times.

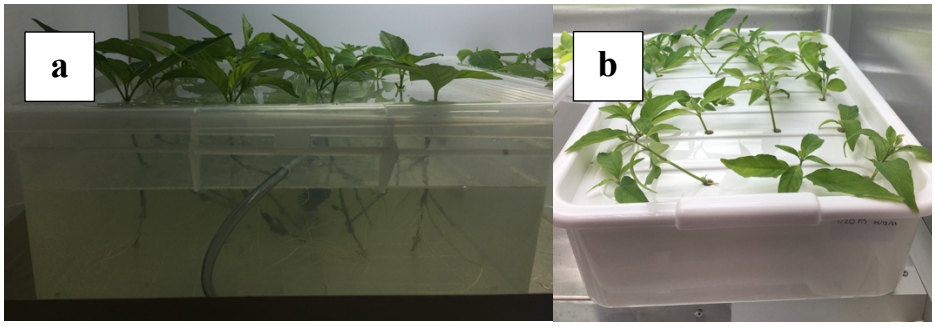

Figure 10 Translucent containers (a) and light protected growth container (b)

3.4.5. Effect of MS concentrations on root biomass and rhinacanthin production

The effect of MS concentrations on root biomass and rhinacanthin production has been examined using 5%, 10%, and 20% MS (pH 5.4) and purified water. Under ECSAC conditions, the plantlets (7 days old) were cultivated in light-protective containers (20 plants/container) with aeration (4.5 L/min). Every four weeks, freshly

produced MS medium and purified water were replenished. After three months, the roots were harvested and dried at 60ºC. The dried root biomass was measured, and the RC, RD, and RN contents were analyzed using an HPLC technique. The experiment was repeated three times.

3.4.6 Effect of harvesting periods on root biomass and rhinacanthin production

Under ECSAC conditions, the plantlets (7 days old) were cultivated in 20% MS (pH 5.4) in light protecting containers (20 plants/container) with aeration (4.5 L/min). Every four weeks freshly made 20% MS was replenished (Fig. 11). The roots were harvested at 2, 3, 4, 5, and 6-months of growth and dried at 60ºC. The dried root biomass was measured, and the RC, RD, and RN contents were analyzed using an HPLC technique. The experiment was repeated three times.

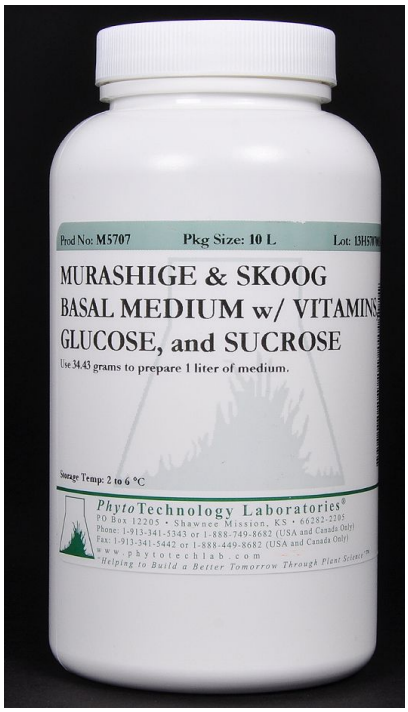

Figure 11 Murashige and Skoog nutrient

3.4.7. Effect of light sources on root biomass and rhinacanthin production

The effect of light sources on root biomass and rhinacanthin production was examined using different light-emitting diode (LED) lights, including white light and blue-red light (450-660 nm), with light intensities ranging from 18,000 to 22,000

lumens (Fig. 12). Under ECSAC conditions, the plantlets (7 days old) were cultivated in 20% MS (pH 5.4) in light protecting containers (20 plants/container) with aeration (4.5 L/min). Every four weeks freshly made 20% MS was changed. After three months, the roots were harvested and dried at 60ºC. The dried root biomass was measured, and the RC, RD, and RN contents were analyzed using an HPLC technique. The experiment was repeated three times.

Figure 12 ECSAC of *R. nasutus* with blue-red LED light

3.4.8. Extraction

The dried roots were pulverized and sieved with a No. 20 sieve (Fig. 13-15). Microwave-assisted extraction was used to extract the root powder (100 mg) with 50 mL of ethyl acetate. The extraction conditions were as follows: a microwave frequency and power of 2,450 MHz and 450 watts, respectively, at 72 ± 2ºC for three cycles (1 cycle = 45 s power-on, 30 s power-off). The extract was filtered and dried using a rotary evaporator set at 40ºC. In a volumetric flask, the dried extracts were reconstituted with methanol and the volume was adjusted to 10 mL before filtering through a 0.45 m membrane filter. The experiment was repeated three times.

Figure 13 Harvested roots of *R. nasutus*

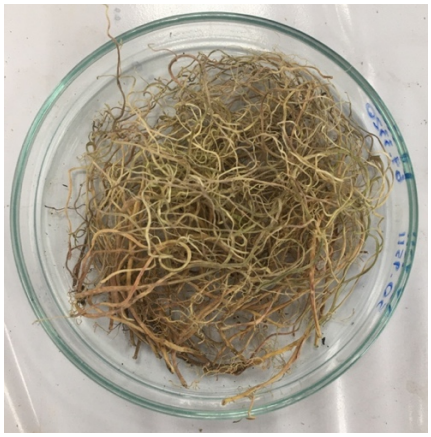

Figure 14 Dried roots of *R. nasutus*

Figure 15 Grinded powder of *R. nasutus* roots

3.5. Effect of elicitation and substrate feeding conditions on production of rhinacanthin in the hydroponic root culture systems

3.5.1. Establishment and maintenance of hydroponic system of *R. nasutus*

Previous on *R. nasutus* hydroponic system was followed from method 3.4.2. In brief, two-year-old *R. nasutus* shoots (3 nodes 12 - 15 cm tall) were harvested and rinsed with tap water (10 min), 2% NaClO (15 min), and deionized water (10 min) before being transferred to a growth container for root initiation (20 plants/growing chamber) in ECSAC. *R. nasutus* roots were planted in deionized water (8 liters) for 16 h under regulated conditions of 25 ± 2ºC and LED white light (230 mol/s/m^2). Roots were grown for 7 days under such conditions. The initiated roots were moved into a light-protective container. Each growth chamber received aerated air at a rate of 4.5 L/min/chamber. Each growth container has 8 liters of 20% MS medium in it. The pH of the nutrition solution was adjusted to 5.4. Every four weeks, the nutritional solution was replaced with the same nutrient concentration. The culture was carried on under the former conditions. Roots were grown in such conditions for 12 weeks.

3.5.2. Elicitor preparation

Elicitor stock solutions of chitosan, *Trichoderma harzianum* (1.9 x 10^8 cfu/g), sodium alginate, and lawsone were prepared in deionized water, while those of MeJA and SA were prepared in 50% (v/v) ethanol. The final concentrations of each elicitor were created in accordance with Table 12 The effect of elicitor types, concentrations, and elicitation times were determined.

3.5.3. Determination of the effect of elicitor types and concentrations

Twelve-week-old *R. nasutus* hydroponic cultivars, 20 plants per tray, were transplanted into an 8-liter growth tray containing 7 L deionized water. They were elicited using five distinct elicitor types at three different concentrations. In each experiment, the control cultures received an equal amount of water or ethanol. After 48 h of treatment, the roots were harvested, and the biomass dry weight measurement, extraction using method 3.4.8, and quantitative HPLC analysis of RC, RD, and RN were carried out.

3.5.4. Determination of the effect of contact time of elicitors on growth and rhinacanthin production from leaves and root of *R. nasutus*

Elicitor stock solutions of chitosan, lawsone, and *Trichoderma harzianum* (1.9 x 10^8 cfu/g) were generated in deionized water. According to earlier method, *R. nasutus* hydroponic cultivar (12-week-old), 20 plants per tray, were put into an 8-liter growth tray containing 7 L deionized water. They were elicited using three distinct elicitor kinds at three different concentrations. In each experiment, the control cultures received an equal amount of water or ethanol. After 24, 48, and 72 hours of treatment, the roots and leaves were harvested, and the biomass dry weight measurement, extraction using method 3.4.8, and quantitative HPLC analysis of RC, RD, and RN were done.

3.6. Effect of simultaneous and sequential dual elicitation of selected elicitors on rhinacanthin production

3.6.1. Establishment and maintenance of hydroponic system of *R. nasutus*

Method 5.1 was applied to establish and then operate the *R. nasutus* hydroponic system prior to conducting simultaneous and sequential dual elicitation.

3.6.2. Simultaneous dual elicitation treatment

Chitosan (0.15 mg/mL) and *T. harzianum* (1 mg/mL) were introduced to a 3-month-old *R. nasutus* hydroponic cultivar. The most optimal contact time was determined by collecting the leaves and roots after 24, 48, 72, 96, and 120 hours. The collected leaves and roots were extracted using method 3.4.8 and quantitative HPLC for rhinacanthin content (RC, RD, and RN) measurements, respectively.

3.6.3. Sequential dual elicitation treatment

For sequential elicitation, 0.15 mg/mL chitosan or 1 mg/mL *T. harzianum* were initially administered for 48 hours to a 3-month-old *R. nasutus* hydroponic cultivar. The chitosan-treated cultivar was then treated with 1 mg/mL *T. harzianum* during 24, 48, 72, and 96 h. For 24, 48, 72, and 96 h, and 0.15 mg/mL chitosan was administered to a *T. harzianum*-treated cultivar. The collected leaves and roots were extracted using

method 3.4.8 and quantitative HPLC for rhinacanthin content (RC, RD, and RN) measurements, respectively.

3.7. Quantitative HPLC method

HPLC analysis was performed using the previously described validated method (Panichayupakaranant et al., 2009) with minor changes. In summary, the procedure was carried out using a Shimadzu HPLC system (Model LC-20, Shimadzu, Tokyo, Japan) equipped with an LC-20AD pump, a SIL-20A autosampler, and an SPD-M20A photodiode-array detector (Fig. 16). The chromatographic system was run on a 150 mm x 4.6 mm Phenomenex ODS column at a flow rate of 1 mL/min with a solution of methanol and 5% aqueous acetic acid (80: 20, v/v). The quantitative UV detection wavelength was set at 254 nm.

The calibration curves were generated using the standard RC (12.5 - 200 µg/mL), RD (3.125 - 50 µg/mL), and RN (3.125 - 50 µg/mL). The calibration curves of RC, RD, and RN were established as $y = 31847x + 18991$ ($r^2 = 1$), $y = 50376x - 66641$ ($r^2 = 0.9999$), and $y = 51858x - 75959$ ($r^2 = 0.9997$), respectively.

Figure 16 High performance liquid chromatography instrument

3.8. Statistical analysis

The data was reported in the form of mean standard error (SE). The significance of the data was determined using one-way ANOVA, followed by Tukey's test. When $p < 0.05$, the results are considered statistically significant.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Effects of plant parts on rhinacanthin content

Table 4 shows data from HPLC analysis of rhinacanthin levels (Fig. 17 and 18), including RC, RD, and RN, of various plant parts collected in January. The total rhinacanthin content of the plant was highest in the roots (3.86%, w/w) and leaves (3.46%, w/w), while the aerial parts had the lowest total rhinacanthin content (0.39%, w/w). Furthermore, RC was shown to be the major naphthoquinone, with RD and RN representing the minor naphthoquinones found in *R. nasutus* roots and leaves. However, the concentrations of rhinacanthins in green and brown twigs, as well as flowers, were lower than the quantification limit.

The results showed that RC, RD, and RN were predominantly collected in the roots of *R. nasutus*, followed by the leaves (Table 3). This interpretation is consistent with the recommendation for the use of *R. nasutus* parts in Thai traditional medicine (Brimson et al., 2020), which suggests specific plant parts for illness therapy. Although it has been postulated that the biogenetic process of RC may include certain chlorophyll membrane associated enzymes in the O-succinylbenzoic acid (OSB) pathway, once RC is biosynthesized, it is translocated and accumulates in the roots (Panichayupakaranant et al., 2021). This might explain why the content of rhinacanthins found in the roots and leaves was higher than in the aerial parts of *R. nasutus*, where the amount found was remarkably low. Aerial parts may have contained low rhinacanthin levels because they contained not just leaves but also stems or twigs with low rhinacanthin levels. As a result, merging stems or twigs may reduce the concentration of rhinacanthin in aerial portions. Other substances identified in the stems or twigs besides rhinacanthins were methylphecophrorbide (sterols), pracruptorin and umbelliferone (coumarins), and 2,6-dimethoxy benzoquinone (benzoquinone), while wogonin, oroxylin-A, and rutin were detected in the flowers (Bukke et al., 2011).

Table 4 Rhinacanthin content of various parts of *R. nasutus* harvested in January

Compounds	Rhinacanthin content (%, w/w DW*)					
	Roots	Leaves	Aerial parts	Green twigs	Brown twigs	Flowers
RC	3.43 ± 0.19[a]	2.60 ± 0.19[b]	0.23 ± 0.01[c]	BQL	BQL	BQL
RD	0.15 ± 0.01[a]	0.71 ± 0.05[b]	0.13 ± 0.00[a]	BQL	BQL	BQL
RN	0.25 ± 0.01[a]	0.16 ± 0.01[b]	0.02 ± 0.00[c]	BQL	BQL	BQL
Total	3.86 ± 0.27[a]	3.46 ± 0.24[b]	0.39 ± 0.01[c]	BQL	BQL	BQL

* Dry weight of plant powders, RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N, BQL = below quantification limit. LOQ = 3.0 μg/mL.

Mean values within the same row that were labelled with different letters are significantly different ($p < 0.05$).

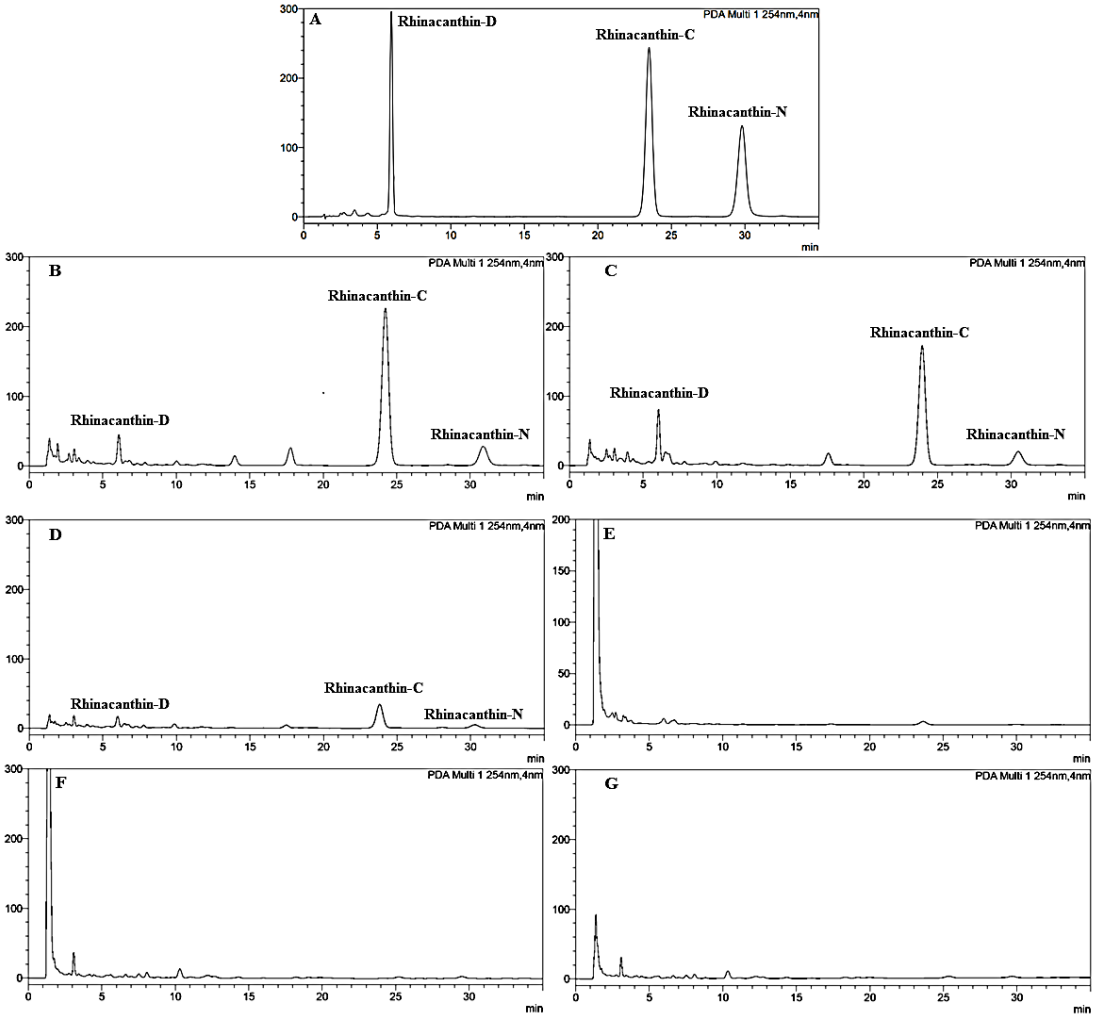

Figure 17 HPLC chromatograms of the authentic rhinacanthin-C, -D, and -N (A) and ethyl acetate extracts from roots (B), leaves (C), aerial parts (D), green twigs (E), brown twigs (F), and flowers (G) of *R. nasutus*

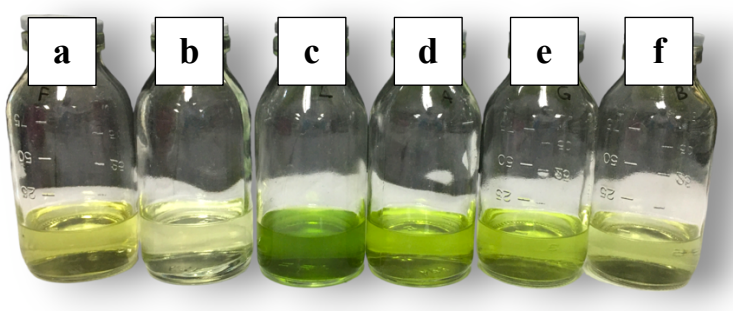

Figure 18 Ethyl acetate extracts of *R. nasutus* parts; roots (a), flowers (b), Leaves (c), aerial parts (d), green twigs (e), and brown twigs (f)

4.2. Effect of harvesting periods on rhinacanthin content

Table 5 shows the levels of RC, RD, and RN in *R. nasutus* roots, leaves, and aerial parts gathered across six harvesting periods. Across all harvesting seasons, the roots contained the highest total rhinacanthin content (3.73-4.91%, w/w), followed by the leaves (3.18-4.42%, w/w), and the aerial parts (0.27-0.53%, w/w). Rhinacanthin levels in roots and leaves produced from May to November, with the maximum amount reported in September. In contrast, the content of rhinacanthin in both parts was slightly lower from January to March. Notably, the ratios of RC, RD, and accumulated in each component were roughly comparable throughout the year.

Rhinacanthins were as well accumulated in the roots, followed by the leaves, during all harvesting seasons, whereas levels in the aerial portions remained low (Table 5). As a result, the roots and leaves are high-quality raw materials that should be exploited in the development of further *R. nasutus* health products. However, optimum harvesting seasons for *R. nasutus* root and leaves were shown to range from May to November, with September being the most ideal harvesting period due to the maximum concentration of rhinacanthins. These findings are congruent with a recent study, which found that rhinacanthins were substantially accumulated in *R. nasutus* roots and leaves, especially before the plant blooms (July to October) but were less accumulated from January to April (Panichayupakaranant et al., 2006). Rhinacanthin concentration was reduced from January to March, most likely owing to the seasons of winter and summer, which reduce natural water availability. *R. nasutus*, on the other hand, created and accumulated more rhinacanthins during the period of rainfall as a defensive mechanism. This idea has been supported by Harzra et al., who claim that biotic influences on host plants increase secondary metabolite synthesis during the rainy season (Hazra et al., 2021). Furthermore, some phytochemicals are generated in smaller quantities during the drought season; for example, Tibetan wild barley (*Hordeum vulgare* L. ssp. spontaneum) generates less flavonoid during the drought season (Ahmed et al., 2015).

Table 5 Rhinacanthin content of in the roots, leaves and aerial parts of *R. nasutus* harvested in different harvesting periods

Parts/Compounds	Rhinacanthin content (%, w/w DW*)					
	January	March	May	July	September	November
Roots:						
RC	3.43 ± 0.19^{a}	3.38 ± 0.15^{a}	3.79 ± 0.06^{b}	3.97 ± 0.09^{c}	4.11 ± 0.07^{c}	3.80 ± 0.07^{b}
RD	0.15 ± 0.01^{a}	0.14 ± 0.00^{a}	0.18 ± 0.00^{b}	0.25 ± 0.00^{c}	0.30 ± 0.01^{e}	0.22 ± 0.02^{f}
RN	0.25 ± 0.01^{a}	0.21 ± 0.01^{b}	0.31 ± 0.02^{c}	0.43 ± 0.03^{d}	0.51 ± 0.02^{e}	0.33 ± 0.01^{c}
Total	3.86 ± 0.27^{a}	3.73 ± 0.17^{a}	4.29 ± 0.09^{b}	4.64 ± 0.11^{c}	4.91 ± 0.09^{d}	4.34 ± 0.08^{b}
Leaves:						
RC	2.60 ± 0.19^{a}	2.52 ± 0.05^{a}	2.82 ± 0.07^{b}	2.96 ± 0.06^{b}	3.21 ± 0.10^{c}	2.84 ± 0.04^{b}
RD	0.71 ± 0.05^{a}	0.51 ± 0.01^{b}	0.89 ± 0.05^{c}	0.97 ± 0.06^{cd}	0.98 ± 0.03^{cd}	0.83 ± 0.01^{c}
RN	0.16 ± 0.01^{a}	0.15 ± 0.00^{ab}	0.16 ± 0.00^{abc}	0.22 ± 0.02^{d}	0.24 ± 0.00^{d}	0.17 ± 0.01^{ac}
Total	3.46 ± 0.24^{a}	3.18 ± 0.07^{b}	3.87 ± 0.08^{c}	4.15 ± 0.03^{d}	4.42 ± 0.09^{e}	3.84 ± 0.02^{cf}
Aerial parts:						
RC	0.23 ± 0.01^{a}	0.19 ± 0.03^{ab}	0.22 ± 0.01^{ab}	0.24 ± 0.01^{ac}	0.25 ± 0.01^{ac}	0.37 ± 0.00^{d}
RD	0.13 ± 0.00^{a}	0.06 ± 0.04^{b}	0.06 ± 0.00^{b}	0.09 ± 0.00^{c}	0.09 ± 0.01^{c}	0.12 ± 0.00^{a}
RN	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.05 ± 0.00^{b}	0.05 ± 0.00^{b}	0.05 ± 0.00^{b}
Total	0.39 ± 0.01^{a}	0.27 ± 0.01^{b}	0.30 ± 0.01^{b}	0.39 ± 0.01^{a}	0.39 ± 0.02^{a}	0.53 ± 0.00^{c}

* Dry weight of plant powders, RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N.

Mean values within the same row that were labelled with different letters are significantly different ($p < 0.05$).

4.3. Rhinacanthin content of *R. nasutus* raw materials obtained from ten suppliers

HPLC chromatograms of ethyl acetate extracts of 10 commercially available *R. nasutus* raw materials (I - X) are shown in Fig. 19 and 20. The similarity of HPLC profiles was close to 1.0 based on the HPLC chromatograms. The total amount of rhinacanthins in all samples was found to be in the range of 0.18% - 0.55%, w/w, according to the quality evaluation research (Table 6). Furthermore, the RC, RD, RN ratios in all samples were comparable. Among them, the raw material received from supplier I in Nakornpathom province had the highest level of rhinacanthins (0.55%, w/w), while that obtained from supplier VII in Chon Buri province had the lowest rhinacanthin content (0.18%, w/w).

In the current investigation, we additionally assessed the quality of 10 commercially available *R. nasutus* raw materials (I - X) in terms of rhinacanthin content. Based on HPLC chromatograms, the similarity of their HPLC profiles was close to 1.0, indicating that they were of similar quality. Furthermore, their HPLC chromatograms were identical to those of *R. nasutus* aerial parts, implying that they were produced from the aerial parts. Furthermore, a precise quality assessment research based on total rhinacanthin content found that their total rhinacanthin content was very low (0.18% - 0.55%, w/w) (Table 5) and similar to the rhinacanthin content of the aerial sections, indicating low raw material quality The quality of rhinacanthins varied according to cultivation and harvesting locations, as well as post-harvest processing and storage circumstances (Bansal et al., 2014). These findings also revealed that the majority of commercially accessible *R. nasutus* raw materials in Thailand are acquired from aerial portions, which meet the standard criteria of 1% (w/w) total rhinacanthins (Panichayupakaranant et al., 2006). As a result, the quality of *R. nasutus* raw material should be increased by collecting just the plant's roots and leaves.

Table 6 Rhinacanthin content of *R. nasutus* raw materials obtained from different suppliers

Suppliers	Rhinacanthin content (%, w/w DW*)			
	RC	RD	RN	Total
I	0.35 ± 0.00^{a}	0.14 ± 0.01^{a}	0.06 ± 0.00^{a}	0.55 ± 0.01^{a}
II	0.19 ± 0.00^{bc}	0.08 ± 0.01^{b}	0.05 ± 0.01^{bcf}	0.32 ± 0.01^{bc}
III	0.18 ± 0.00^{bc}	0.09 ± 0.01^{c}	0.04 ± 0.01^{bcd}	0.31 ± 0.01^{bc}
IV	0.18 ± 0.01^{bc}	0.07 ± 0.00^{b}	0.05 ± 0.00^{bcf}	0.30 ± 0.01^{c}
V	0.18 ± 0.01^{bc}	0.09 ± 0.01^{c}	0.05 ± 0.00^{bcf}	0.31 ± 0.01^{bc}
VI	0.20 ± 0.00^{b}	0.09 ± 0.01^{c}	0.04 ± 0.00^{cd}	0.33 ± 0.01^{b}
VII	0.10 ± 0.01^{d}	0.05 ± 0.00^{d}	0.03 ± 0.01^{e}	0.18 ± 0.01^{d}
VIII	0.12 ± 0.00^{e}	0.06 ± 0.01^{e}	0.04 ± 0.01^{d}	0.21 ± 0.01^{e}
IX	0.23 ± 0.00^{f}	0.07 ± 0.00^{b}	0.05 ± 0.01^{bc}	0.23 ± 0.01^{f}
X	0.30 ± 0.01^{g}	0.11 ± 0.00^{f}	0.06 ± 0.00^{af}	0.47 ± 0.01^{g}

* Dry weight of plant powders, RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N.

Mean values within the same column that were labelled with different letters are significantly different ($p < 0.05$).

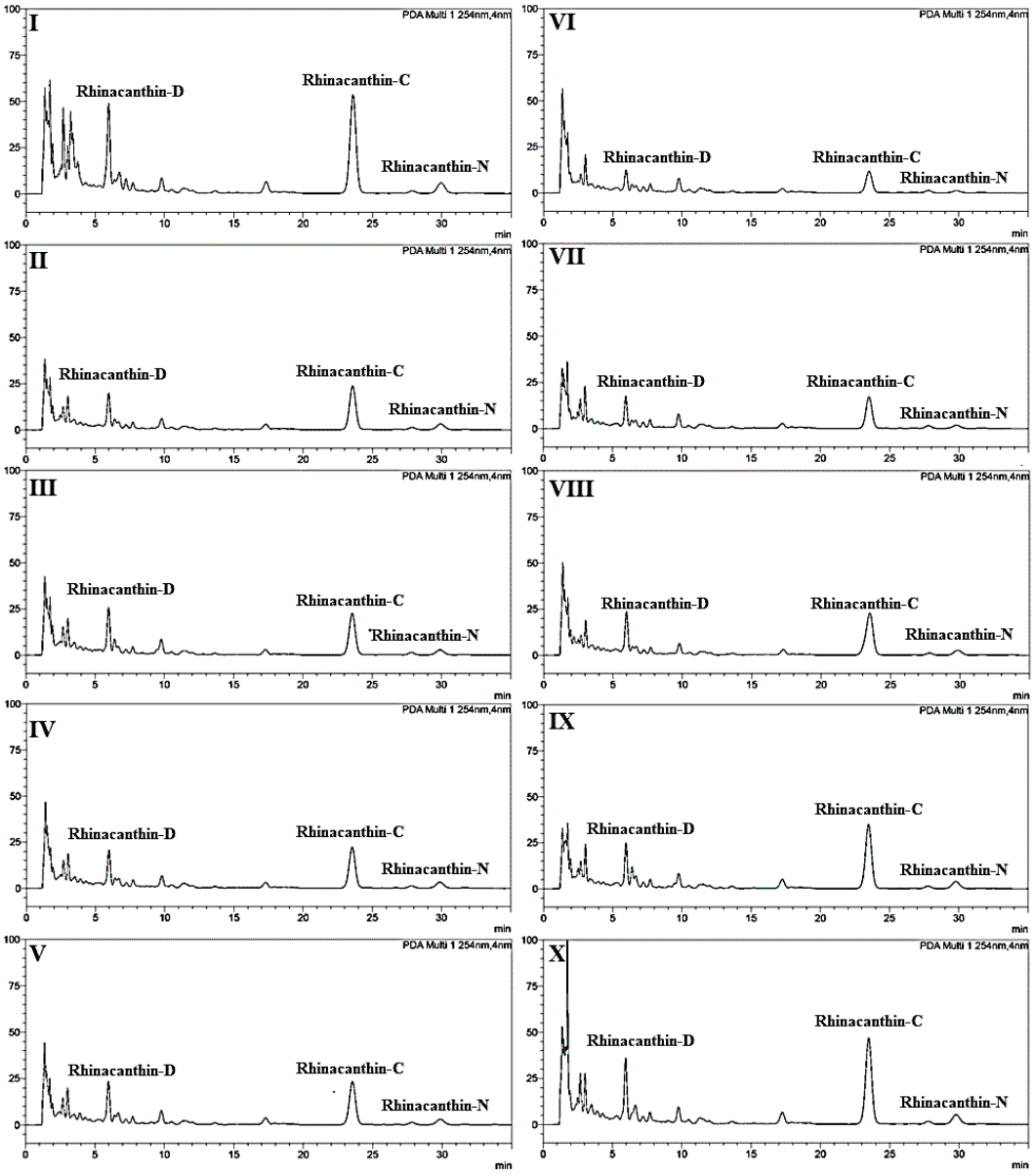

Figure 19 HPLC chromatograms of *R. nasutus* raw materials obtained from ten suppliers (I-X)

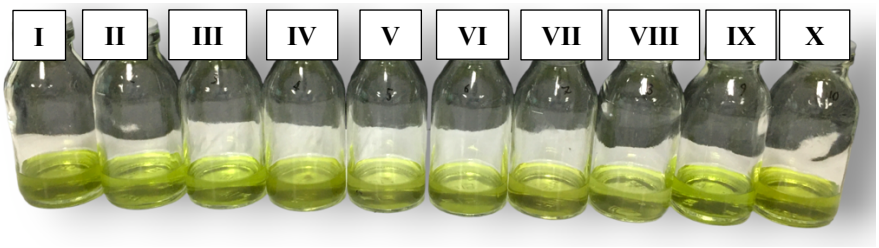

Figure 20 Extracts of *R. nasutus* raw materials obtained from ten suppliers (I-X)

4.4. Rhinacanthin content of two commercially available teas

Table 7 shows the rhinacanthin concentration of two commercially available teas (RN-tea I and RN-tea II). The total rhinacanthin concentration of both teas was relatively low (Fig. 21). Furthermore, the total rhinacanthin concentration of RN-tea I (0.30% - 0.38%, w/w) was more than twice that of RN-tea II (0.14% - 0.18%, w/w) and remained stable throughout three batches of each product. However, based on the HPLC method's detection and quantification limitations (LOD = 0.8 μg/mL and LOQ = 3.0 μg/mL) neither rhinacanthin was detected in either tea infusion.

The active compound content of herbal preparations is critical to their medicinal effectiveness. The amount of rhinacanthins in tea infusions derived from two commercially available *R. nasutus* health products was therefore measured in this investigation. Tea powders, including RN-tea I and RN-tea II, contained rhinacanthins in amounts ranging from 0.30% to 0.38% (w/w), indicating that they were derived from the aerial parts of *R. nasutus*. The results also revealed a variety in product quality due to varying raw material and/or manufacturing process quality. The rhinacanthin concentration of RN-tea I was two times the amount of RN-tea II. Furthermore, the content of rhinacanthins was constant throughout three batches of each product, indicating that their manufacturing techniques were consistent. Unfortunately, no rhinacanthins were discovered in the tea infusions made from either product. This finding might be explained in part by the fact that rhinacanthins are hydrophobic molecules that are insoluble in hot water. This data implies that if the target active components are rhinacanthins, tea infusion is not an appropriate *R. nasutus*-based herbal treatment. Because of their comparable non-polar characteristics, ethyl acetate has been identified as the best solvent for rhinacanthin extraction (Panichayupakaranant

et al., 2009). Ethyl acetate, on the other hand, is a hazardous chemical solvent that cannot be used in herbal medicines. As a result, ethanol, or other alternative green solvents such as glycerol, propylene glycol, and polyethylene glycol may be preferred solvents for future *R. nasutus* product development, including oral and topical treatments.

Table 7 Rhinacanthin content of ethyl acetate extracts from *R. nasutus* teas

Product/Batch no.	Rhinacanthin content (%, w/w DW*)			
	RC	RD	RN	Total
RN-tea I				
No. 04156104	0.28 ± 0.02^{a}	0.09 ± 0.00^{a}	0.01 ± 0.00^{a}	0.38 ± 0.02^{a}
No. 04156202	0.25 ± 0.00^{ab}	0.09 ± 0.00^{a}	0.01 ± 0.00^{a}	0.35 ± 0.01^{b}
No. 04156302	0.24 ± 0.01^{bc}	0.06 ± 0.00^{b}	0.01 ± 0.00^{a}	0.30 ± 0.01^{c}
RN-tea II				
060257DPD20902	0.10 ± 0.00^{d}	0.03 ± 0.00^{c}	0.01 ± 0.00^{a}	0.14 ± 0.00^{d}
061061DPD20902	0.12 ± 0.01^{e}	0.04 ± 0.00^{c}	0.02 ± 0.00^{b}	0.18 ± 0.01^{e}
062257DPD20902	0.11 ± 0.01^{de}	0.04 ± 0.01^{c}	0.01 ± 0.00^{a}	0.15 ± 0.01^{f}

* Dry weight of plant powders, RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N.

Mean values within the same column that were labelled with different letters are significantly different ($p < 0.05$).

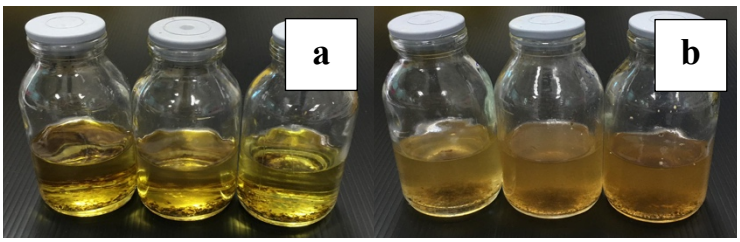

Figure 21 Extracts of two commercially available teas of *R. nasutus* (brand a and b)

4.5. Effect of aeration on root biomass and rhinacanthin production

An investigation of the influence of aeration (4.5 L/min) on root biomass and rhinacanthin production in the *R. nasutus* hydroponics system indicated that the aerated hydroponics system produced considerably more dry root biomass than the non-aerated version (Table 8) (Fig. 22). The findings are explained by the fact that the air supply in hydroponics provides dissolved oxygen, which promotes root activity and function as well as the rate of water and nutrient absorption. Furthermore, dissolved oxygen affects the development of root hairs (Schröder and Lieth, 2002). Furthermore, aerobic conditions encourage root growth and development due to the dispersion of water and air-occupied pore spaces (Jensen, 1997). The predominant rhinacanthin generated in the aerated (1.24% w/w) and non-aerated (0.97% w/w) hydroponics systems of *R. nasutus* was RC, whereas the minor compounds were RN (0.15 and 0.12% w/w, respectively) and RD (0.14 and 0.11% w/w, respectively) (Table 8).

Similarly, the roots in the aerated hydroponics system collected significantly higher rhinacanthins than the roots in the non-aerated system (Table 8). The findings suggest that dissolved oxygen influences not only root growth but also rhinacanthin production. Dissolved oxygen may influence gene and enzyme expression in secondary metabolite biosynthesis, thereby increasing secondary metabolite production (Abu-Shahba et al., 2021). For example, it influenced the allosteric regulation of tryptophan decarboxylase activity in *Catharanthus roseus* ajmalicine biosynthesis (Schlatmann et al., 1994), and it boosted *Panax ginseng* biomass and saponin production (Jeong et al., 2006). Our findings are consistent with earlier publications. However, it has been discovered that high dissolved oxygen increased *Taxus chinensis* biomass production while decreasing Taxol synthesis (Luo et al., 2001; Zhao et al., 2016). Further modification of the dissolved oxygen rate may be necessary to increase biomass and rhinacanthin production in the *R. nasutus* hydroponics system.

Table 8 Effect of aeration on dried root biomass and rhinacanthin content

	Root biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Aeration	66.61 ± 0.45[b]	1.24 ± 0.03[b]	0.14 ± 0.01[b]	0.15 ± 0.01[b]	1.52 ± 0.02[b]
Without aeration	47.75 ± 0.43[a]	0.97 ± 0.03[a]	0.11 ± 0.00[a]	0.12 ± 0.00[a]	1.20 ± 0.03[a]

*Calculated based on dried weight of root powders. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N, Total = Total rhinacanthins.

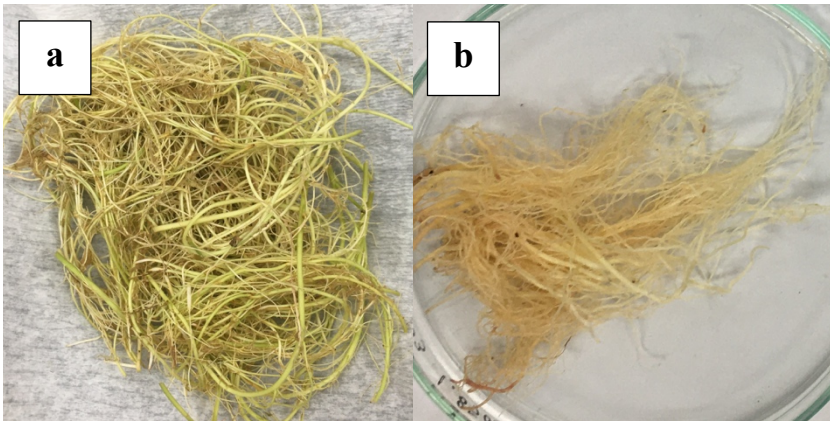

Figure 22 *R. nasutus* roots with (a) and without aeration (b), respectively

4.6. Effect of growing container on root biomass and rhinacanthin production

Natural plant root systems commonly develop in the dark, downward into the earth, and in the absence of light. Furthermore, positive gravitropism promotes root development in the dark along the gravity vector. As a result, light exposure to the roots has a direct impact on root organogenesis, orientation, and coloring (Su et al., 2017). The present research examined at how growing containers affected root biomass and rhinacanthin production in an *R. naustus* hydroponics system. When compared to transparent containers, the hydroponics system utilizing light-protected containers improved root development and dramatically increased all rhinacantin accumulation in

the roots (Table 9) (Fig. 23). According to light response in plants, or phototropism, roots often exhibit negative phototropism, which causes them to grow away from light (Fankhauser and Christie, 2015). As a result, light-protected containers were preferable for promoting the development of *R. nasutus* roots. Furthermore, when *R. nasutus* roots were grown in translucent containers and exposed to light, they became brown and developed slowly due to the toxicity of the oxidative products of phenolic chemicals formed in the roots. The specific rationale for how gravitropism influences metabolite levels has not yet been elucidated based on secondary metabolite synthesis. Constantly exposing the roots to light, on the other hand, may disturb the circadian cycle of root activity, leading to variations in secondary metabolite production (Carvalho and Castillo, 2018). Furthermore, light often reduces the stability of phenolic compounds, particularly naphthoquinones and anthraquinones (Alexandra et al., 2017). Due to light-accelerated oxidation and the decomposition of these chemicals, light allegedly reduced root development and naphthoquinone or anthraquinone concentration in several root cultures (Hu et al., 2019; Panichayupakaranant and Meerungrueang, 2010). As a result, in the *R. nasutus* hydroponics system, light exposure to the roots reduced both root growth and rhinacanthin accumulation.

Table 9 Effect of types of growing container on dried root biomass and rhinacanthin content

	Root biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Translucent container	66.28 ± 0.16[a]	1.41 ± 0.02[a]	0.16 ± 0.00[a]	0.17 ± 0.00[a]	1.74 ± 0.02[a]
Light-protected container	143.31 ± 1.20[b]	1.86 ± 0.03[b]	0.16 ± 0.00[a]	0.18 ± 0.00[a]	2.21 ± 0.04[b]

*Calculated based on dried weight of root powders. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N, Total = Total rhinacanthins.

Figure 23 *R. nasutus* roots growth in light-protective and translucent growing container

4.7. Effect of MS concentrations on root biomass and rhinacanthin production

The modification of nutrient types and concentrations is an approach for increasing plant growth and secondary metabolite synthesis. MS medium has been shown to be the best for establishing rhinacanthin-producing *R. nasutus* root cultures (Cheruvathur et al., 2015; Panichayupakaranant and Meerungrueang, 2010). This might be attributed to a high concentration of macronutrients and a sufficient nitrogen source ratio (NO_3/NH_4) of 3/1. In the present investigation, three concentrations of MS liquid medium (5%, 10%, and 20% v/v) and purified water were tested for their effects on the

growth and rhinacanthin content of *R. nasutus* hydroponics system roots. The outcomes are shown in Table 10 (Fig. 24). An increase in MS concentration increased root biomass and rhincanthin production significantly. Microbial contamination of the liquid medium was found during culture when the MS concentration was raised to 25%. As a result, 20% MS is appropriate for the *R. nasutus* hydroponics system, which needed a lower concentration of MS than the *in vitro* root cultures.

Table 10 Effect of nutrient concentration on dried root biomass and rhinacanthin content

Medium	Root biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Water	108.59 ± 2.01[a]	0.58 ± 0.04[a]	0.05 ± 0.00[a]	0.06 ± 0.00[a]	0.69 ± 0.04[a]
5% MS	126.58 ± 2.15[b]	1.02 ± 0.03[b]	0.08 ± 0.00[b]	0.11 ± 0.00[b]	1.21 ± 0.03[b]
10% MS	165.88 ± 3.01[c]	1.78 ± 0.06[c]	0.16 ± 0.01[c]	0.20 ± 0.01[c]	2.13 ± 0.08[c]
20% MS	194.44 ± 2.14[d]	2.20 ± 0.10[d]	0.20 ± 0.01[d]	0.24 ± 0.01[d]	2.65 ± 0.08[d]

*Calculated based on dried weight of root powders. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N, Total = Total rhinacanthins.

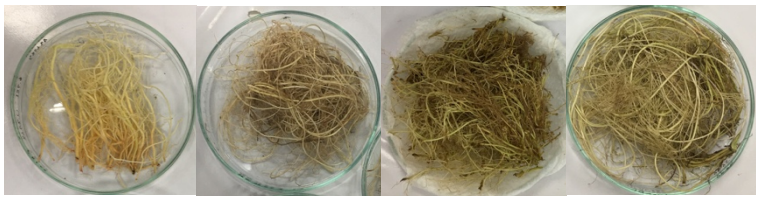

Figure 24 *R. nasutus* roots growth in water, 5%, 10%, and 20% MS nutrient, respectively

4.8. Effect of harvesting periods on root biomass and rhinacanthin production

In order to have an adequate yield, harvesting should be done after the plants are fully grown. Furthermore, plant age influences the quantity of secondary metabolites accumulated in plants (Isah, 2019). The influence of harvesting times on root biomass and rhinacanthin production of *R. nasutus* hydroponics system was investigated in the present research. The harvesting intervals were varied from two to six months, and an increase in harvesting period resulted in a substantial increase in dry root biomass and rhinacanthin concentration (Table 11) (Fig. 25). Rhinacantin

production was not impacted by seasonal variables, but by plant age, since the cultured parameters of *R. nasutus* hydroponics system, including liquid medium, temperature, humidity, oxygen supplement, and light, were regulated. At 5- and 6-months-old, the hydroponics roots reached the stagnant and decrease growth stages, respectively. The roots taken over a five-month period yielded the greatest total rhinacanthin content (3.04% w/w) and dry root biomass (232.07 mg/plant). However, the total rhinacanthin content of roots collected from 3- to 6-month-old hydroponics plants does not appear to differ considerably. A rhinacanthin production profile of hydroponic plants revealed that after 3 months, rhinacanthin accumulation in the roots reached a stable state and began to fall after 6 months. An acceptable harvesting period for the roots may be regarded between 3 and 5 months based on the overall rhinacanthin content.

Furthermore, a 3-month re-harvesting experiment was carried out using the same hydroponics plant to verify the repeatability of the hydroponics system. The results showed that the roots produced at 3 months had a dried root biomass of 142.53 ± 5.44 mg/plant and a total rhinacanthin content of 2.77 ± 0.03 %w/w, which were comparable to the first harvesting roots. The finding showed that the *R. nasutus* hydroponics system might be utilized for re-harvesting every three months of culture. It was also discovered that two cycles of three-month-period culture resulted in a total dried root biomass of 285.44 mg/plant, which was greater than that of six-month-period cultivation (219.68 mg/plant). To reduce the culture period to three months, a technique was implemented to increase the rhinacanthin product in three-month-old hydroponics plants utilizing blue-red light.

Table 11 Effect of harvesting periods on dried root biomass and rhinacanthin content

Period (months)	Root biomass (mg/plant)	Rhinacanthin content (% w/w)			
		RC	RD	RN	Total
2	121.63 ± 2.10[a]	1.81 ± 0.07[a]	0.16 ± 0.00[a]	0.20 ± 0.00[a]	2.17 ± 0.06[a]
3	142.91 ± 2.07[b]	2.24 ± 0.13[b]	0.21 ± 0.01[b]	0.25 ± 0.01[b]	2.70 ± 0.011[b]
4	164.49 ± 3.18[c]	2.41 ± 0.08[bc]	0.23 ± 0.01[c]	0.26 ± 0.01[bc]	2.90 ± 0.06[bc]
5	232.07 ± 5.28[d]	2.51 ± 0.21[c]	0.21 ± 0.01[b]	0.32 ± 0.01[c]	3.04 ± 0.17[c]
6	219.68 ± 2.37[e]	2.42 ± 0.02[bc]	0.23 ± 0.00[c]	0.26 ± 0.00[bc]	2.91 ± 0.02[bc]

*Calculated based on dried weight of root powders. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N, Total = Total rhinacanthins.

Figure 25 *R. nasutus* roots harvested at different periods (2, 4, 6 months)

4.9. Effect of light source on root biomass and rhinacanthin production

Light has a significant impact on plant development and secondary metabolite production. LEDs are a typical light source used to study how light affects plant growth and secondary metabolism. LEDs are appropriate for application because of their several benefits, including compact size, low cost, low heat, high intensity, great energy efficiency, and a specified target wavelength (Thoma et al., 2020). Blue and red lights are essential for plant growth and development, as well as secondary metabolism. Furthermore, as compared to monochromatic lighting, the mix of blue and red lights improved plant growth and secondary metabolite synthesis (Sng et al., 2021). As a result, the current study investigated the influence of blue-red light on root development and rhinacanthin production in a hydroponics system of *R. nasutus*. The results (Table 12) (Fig. 26) showed that hydroponics plants treated with blue-red light produced considerably moredry root biomass (250.47 mg/plant) and total rhinacanthin accumulation (3.31% w/w) than the white light treatment (194.44 mg/plant and 2.65% w/w, respectively).

It has been reported that blue light (340–520 nm) affected circadian rhythms, phototropism, stomata opening, compact growth, and intracellular positioning of chloroplasts to increase light absorption and result in growth promotion (Yu et al., 2010), whereas red light (620–750 nm) promoted cell elongation and the development of a larger leaf surface area (Kong et al., 2018).

In terms of secondary metabolism, blue light has been proposed to activate photoreceptor proteins such as cryptochrome and phototropin, resulting in additional signal transduction and the creation of secondary metabolites (Thoma et al., 2020). Furthermore, blue light has been shown to activate gene expression of chalcone synthase, which is implicated in the polyphenolic metabolic pathway in *Cyclocarya paliurus via* phenylalanine ammonia lyase (PAL) (Liu et al., 2018). Red light, on the other hand, modifies plant morphology and physiology while having little influence on secondary metabolism (Thoma et al., 2020).

Blue-red light also increased root development and rhinacanthin production, according to our findings. The blue-red light treatment of *R. nasutus* hydroponics system also resulted in increased root biomass and total rhinacanthin content than the white light treatment of 5-month-old plants.

Table 12 Effect of light sources on dried root biomass and rhinacanthin content

Medium	Root biomass (mg/plant)	Rhinacanthin content (% w/w)			
		RC	RD	RN	Total
White light	194.44 ± 2.14[a]	2.20 ± 0.10[a]	0.20 ± 0.01[a]	0.24 ± 0.01[a]	2.65 ± 0.08[a]
Blue-red light	250.47 ± 14.02[b]	2.75 ± 0.08[b]	0.25 ± 0.01[b]	0.31 ± 0.01[b]	3.31 ± 0.10[b]

*Calculated based on dried weight of root powders. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N, Total = Total rhinacanthins.

Figure 27 *R. nasutus* roots growth in white (a) and blue-red LED light (b), respectively

4.10. Determination of the effect of elicitor types and concentrations

The effects of elicitor types, concentrations, and elicitation periods on root biomass and rhinacanthin production (RC, RD, and RN) was examined. On week 12, which corresponded to the *R. nasutus* linear growth phase, all elicitors were employed to treat the *R. nasutus* hydroponic system. None of the elicitors had a detrimental influence on the hydroponic system's growth, viability, or biomass output (121.12-137.43 mg/plant) (Table 13) (Fig. 27 and 28). Several elicitors with sufficient concentrations used in the experiment promoted rhinacanthin production as compared to the non-treated cultivar.

Treatment with chitosan at varied concentrations (0.05, 0.15, and 0.25 mg/mL) increased all rhinacanthin production, including RC, RD, and RN (Table 12). Total rhinacanthin levels were considerably higher in 0.15 mg/mL chitosan-treated root cultures (6.10% w/w DW) than in untreated root cultures (2.72% w/w DW). Among the various chitosan concentrations applied, 0.15 mg/mL results in the largest accumulation of RC (4.96% w/w DW), RD (0.48% w/w), and RN (0.67% w/w). Chitosan concentrations of 0.05 and 0.15 mg/mL elevated rhinacanthin accumulation, but afterwards declined. Chitosan has previously been demonstrated to be a well-known exogenous promoter of secondary metabolite productions. It is a nontoxic, natural, and biodegradable polymer that has been shown to stimulate plant growth and provoke defensive responses in plants, both of which require changes in plant metabolite profiles (Sathiyabama et al., 2016). Chitosan has been reported to activate jasmonic acid, a signal molecule involved in defensive gene regulation and phospholipase C/protein kinase C (PKC) cascades (Rajestary et al., 2021). Furthermore, it has been reported to stimulate naphthoquinone productions in some plant tissue cultures, such as an increased plumbagin production in *Plumbago indica* root cultures (Jaisi and Panichayupakaranant, 2017) and an enhanced methylene-3,3′-bilawsone biosynthesis in *Impatiens balsamina* root cultures (Sakunphueak and Panichayupakaranant, 2010). Although the mechanism for this effect has not been determined, it may involve induced stimulation of the antioxidant defense machinery, stimulation of nitrogen metabolism, increased water uptake, reduction of transpiration, and essential nutrients *via* cell osmotic pressure adjustment (Sathiyabama et al., 2016). The ability of chitosan to specifically increase naphthoquinone production might be due to mimicking a natural defense response or stimulating the enzymes involved in the biosynthesis of naphthoquinones. Although the mechanisms of chitosan for eliciting naphthoquinone biosynthesis have not yet clarified, various mechanisms have been proposed, including stimulation of the antioxidant defense machinery, stimulation of nitrogen metabolism, increased uptake of water, and reduction of transpiration and essential nutrients through adjusting cell osmotic pressure

Table 12 shows how different *T. harzianum* concentrations influenced rhinacanthin production. Treatment with *T. harzianum* (1, 2 and 3 mg/mL) enhanced RC (4.10-5.53% w/w DW), RD (0.18-0.29% w/w DW), and RN (0.17-0.22% w/w

DW). The addition of *T. harzianum* at 1 mg/mL produced the highest rhinacanthin level and considerably increased total rhinacanthin (6.04% w/w DW) up to 2.22-fold higher than the control group. Natural soil bacteria called *Trichoderma* spp. have been shown to increase the manufacture of a number of bioactive phytochemicals. Higher nutrient absorption, stronger root growth and development, enhanced production, and improved tolerance to various stresses, including disease, are all typically caused by *Trichoderma* spp. These effects might be a result of *Trichoderma* spp. effector chemicals being delivered to plants during the contact. *Trichoderma* extracts may also produce mitogen-activated proteins, auxin, phenylpropanoids, and phytoalexins as well as release volatile and non-volatile chemicals that improve plant life and nutrient uptake as part of their biostimulant mechanisms (Iula et al., 2021; Siddaiah et al., 2017). However, the current research first identified *T. harzianum* elicitation of rhinacanthin production.

Rhinacanthin levels were affected by the lawsone treatment (Table 12). Only 3 and 6 M lawsone modestly increased rhinacanthin (4.08 and 4.19% w/w DW) production up to and 1.54-fold, respectively, compared to the control group. RC (3.10-3.56% w/w DW), RD (0.19-0.22% w/w DW) and RN (0.40-0.46% w/w DW) have increased in the same manner with different lawsone treatment compared to control. Lawsone, a naphthoquinone that occurs naturally, is only present in the leaves of *Lawsonia inermis* and *Impatiens balsamina*. The current work is the first to show that lawsone can increase secondary metabolite production in plants, particularly naphthoquinone. The particular method through which lawsone is elicited has not yet been discovered. However, lawsone's ability to induce oxidative stress by raising H_2O_2 production and antioxidative enzyme activity in plant cells may be related to its evoking potential for rhinacanthin formation (Kurtyka et al., 2016). Plants' redox homeostasis is widely recognized as a crucial component of auxin-mediated growth regulation. On the other hand, it is a subunit of the rhinacanthin core structure based on its 1,4-naphthoquinone core structure with an ortho-hydroxyl group substitution. Consequently, it could be a step in the biosynthesis of rhinacanthins. Additionally, it has been suggested that the shikimate pathways are used for the biosynthesis of lawsone and rhinacanthins (Panichayupakaranant et al., 2021). In this way, precursor feeding may be the method by which lawsone increases rhinacanthin production. Furthermore,

lawsone has not yet been reported to be an elicitor or precursor feeding to enhance secondary metabolite production.

Unfortunately, MeJa, SA, and sodium alginate had little effect on rhinacanthin production at all concentrations. Differential elicitor-contact periods, on the other hand, may have a significant influence on rhinacanthin production. According to the the MeJA and SA elicitation, results contradicted previous report that MeJA and SA were effective elicitors for increasing rhinacanthin production in *R. nasutus* root culture (Cheruvathur and Thomas, 2014). This might be because the elicitation duration for MeJA and SA has not yet been optimized. Further contact time optimization should be conducted.

Chitosan, *T. harzianum*, and lawsone might be exploited to perform additional research on contact time during elicitation in order to optimize for optimal rhinacanthin production circumstances. Furthermore, combining or sequentially applying these elicitors may increase rhinacanthin levels in *R. nasutus* hydroponic roots. In addition, since the elicitation strategy is successful in the *R. nasutus* hydroponic culture, it could be a good model-system for researching and identifying the genes involved in rhinacanthin biosynthesis, as well as the underlying mechanism for improving production through elicitation.

Table 13 Effect of elicitors and their concentrations on growth and rhinacanthin (RC, RD, and RN) production of *R. nasutus* hydroponic root system

Conc. & periods	Root biomass (g/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Control	125.26 ± 4.04[a]	2.18 ± 0.16[a]	0.24 ± 0.02[a]	0.30 ± 0.01[a]	2.72 ± 0.20[a]
Chitosan					
0.05 mg/mL	126.34 ± 6.10[a]	3.96 ± 0.18[b]	0.39 ± 0.01[b]	0.53 ± 0.02[b]	4.89 ± 0.20[b]
0.15 mg/mL	123.31 ± 8.26[a]	4.96 ± 0.27[c]	0.48 ± 0.01[c]	0.67 ± 0.02[c]	6.10 ± 0.09[c]
0.25 mg/mL	116.64 ± 3.47[a]	2.78 ± 0.03[d]	0.28 ± 0.01[ad]	0.38 ± 0.01[a]	3.44 ± 0.03[d]
Trichoderma harzianum					
1 mg/mL	134.45 ± 6.78[a]	5.53 ± 0.10[e]	0.29 ± 0.03[d]	0.22 ± 0.01[d]	6.04 ± 0.12[c]
2 mg/mL	123.39 ± 3.28[a]	4.10 ± 0.11[b]	0.20 ± 0.01[e]	0.18 ± 0.01[d]	4.48 ± 0.12[b]
3 mg/mL	124.32 ± 4.65[a]	4.16 ± 0.63[b]	0.18 ± 0.03[ef]	0.17 ± 0.03[d]	4.50 ± 0.69[b]
Methyl jasmonate					
100 µM	124.55 ± 2.32[a]	2.14 ± 0.06[a]	0.25 ± 0.02[ad]	0.21 ± 0.01[d]	2.60 ± 0.08[a]
200 µM	135.63 ± 3.05[a]	2.13 ± 0.15[a]	0.26 ± 0.02[ad]	0.21 ± 0.01[d]	2.60 ± 0.16[a]
400 µM	136.44 ± 7.26[a]	1.58 ± 0.06[f]	0.21 ± 0.01[ae]	0.17 ± 0.01[e]	1.96 ± 0.06[e]
Salicylic acid					
50 µM	133.01 ± 8.91[a]	2.82 ± 0.03[d]	0.11 ± 0.01[f]	0.30 ± 0.01[a]	3.24 ± 0.04[d]

Conc. & periods	Root biomass (g/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
100 µM	132.70 ± 9.39[a]	3.31 ± 0.02[g]	0.06 ± 0.01[g]	0.08 ± 0.01[f]	3.45 ± 0.02[d]
150 µM	121.12 ± 1.54[a]	3.19 ± 0.04[g]	0.05 ± 0.01[g]	0.06 ± 0.01[f]	3.31 ± 0.04[d]
Sodium alginate					
0.8 mg/mL	132.04 ± 7.41[a]	2.33 ± 0.16[a]	0.15 ± 0.02[f]	0.12 ± 0.01[f]	2.61 ± 0.01[a]
1.5 mg/mL	136.58 ± 8.11[a]	2.34 ± 0.27[a]	0.09 ± 0.02[fg]	0.12 ± 0.03[f]	2.54 ± 0.01[a]
3.0 mg/mL	136.11 ± 10.34[a]	2.49 ± 0.06[a]	0.13 ± 0.01[f]	0.05 ± 0.00[f]	2.67 ± 0.01[a]
Lawsone					
1.5 µM	137.43 ± 4.57[a]	3.10 ± 0.02[g]	0.19 ± 0.01[e]	0.40 ± 0.01[bc]	3.69 ± 0.02[d]
3.0 µM	133.79 ± 13.51[a]	3.96 ± 0.18[b]	0.21 ± 0.00[ae]	0.46 ± 0.02[bc]	4.08 ± 0.03[d]
6.0 µM	126.14 ± 7.32[a]	3.82 ± 0.25[b]	0.22 ± 0.03[ae]	0.41 ± 0.03[c]	4.19 ± 0.03[d]

*Calculated based on dried weight of root powders.

Note: RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N

Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$)

Figure 27 Non-treated roots of *R. nasutus*

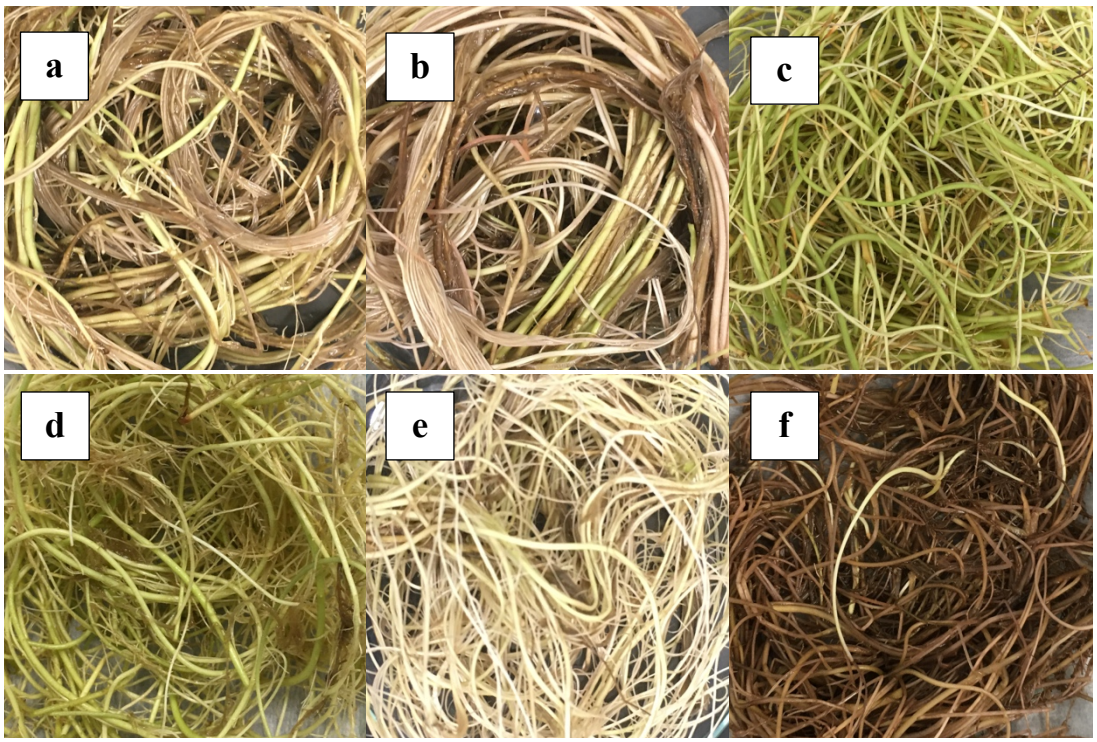

Figure 28 Roots of *R.nasutus* treated with control, chitosan (0.15 mg/mL) (a), *T. harzianum* (1 mg/mL) (b), MeJA (100 µM) (c), SA (100 µM) (d), sodium alginate (3.0 mg/mL) (d), and lawsone (6.0 µM) (f)

4.11. Effect of contact time of elicitors on growth and rhinacanthin production from leaves and root of *R. nasutus* hydroponics

The effects of elicitor contact time on root and leaf biomass and rhinacanthin production (RC, RD, and RN) was examined. On week 12, which corresponded to the

R. nasutus linear growth phase, all elicitors were employed to treat the *R. nasutus* hydroponic system. None of the elicitors had a detrimental influence on the hydroponic system's growth, viability, and biomass output (116.64 - 131.60 mg/plant for roots and 158.83-173.86 mg/plant for leaves) (Table 14 and 15). Different elicitors with suitable contact duration performed in the experiment improved rhinacanthin production as compared to the non-treated cultivar.

Elicitor contact time is a significant aspect of secondary metabolite synthesis. Extending the contact time might result in metabolic or physiological damage. As a result, determining the optimal contact time of chitosan, *T. harzianum*, and lawsone for rhinacanthin production in *R. nasutus* hydroponics was efficient. Different contact times for chitosan (0.15 mg/mL) impacted rhinacanthin production in roots (Table 14) (Fig. 29) and leaves (Table 15) (Fig. 30 and 31). The ratios of RC, RD, and RN in both roots and leaves were unaffected by chitosan contact time. An increase in contact time resulted in a large improvement in rhinacanthin production in roots. However, the most acceptable contact period was taken to be 48 h, as this increased the formation of total rhinacanthin up to 6.18% w/w DW that was 2.27-fold more than the control group (2.72% w/w DW). Contact times longer than 48 h, 72 h, resulted in a decrease in rhinacanthin production. As a result, 72 h of treatment was chosen as a suitable contact time for the elicitation. Interestingly, treated roots exhibited the highest rhinacanthin content at 24 h (5.44% w/w DW) compared to the control group (4.58% w/w DW). Decreasing of rhinacanthin production in treated leaves were detected after 24 h of treatment (5.44% w/w DW). Chitosan has been demonstrated to activate jasmonic acid, a signal molecule involved in defensive gene regulation and phospholipase C/protein kinase C (PKC) cascades (Rajestary et al., 2021). Increasing the chitosan contact duration resulted in tremendous progressive increase in rhinacanthin production in roots, culminating at 48 h, according to the data. Contact periods of more than 48 hours, on the other hand, result in a decline in rhinacanthin production after 72 h. This might be because functioning cells are toxic (Stasińska-Jakubas and Hawrylak-Nowak, 2022). The maximal level of rhinacanthin concentration in leaves, on the other hand, was seen at 24 h. The finding that chitosan showed less impact on rhinacanthin synthesis in treated leaves than in treated roots might be due to the leaves' lack of direct contact with the elicitor. Chitosan's ability to specifically increase rhinacanthin production by such

a large amount could be due to mimicking a natural defense response, or it could have enhanced the enzymes involved in rhinacanthin biosynthesis, similar to the enzymes involved in the phenylpropanoid pathway of other secondary metabolite biosynthetic systems.

An increase in *T. harzianum* contact time at 1 mg/mL in root resulted in an elevation in total rhinacanthin level. Rhinacanthin levels were considerably greater at 24, 48, and 72 h compared to the control group. Rhinacanthin content did not vary significantly between the 24 (6.11% w/w DW) and 48 h (6.04% w/w DW) elicitation periods. However, after 48 hours of treatment, 1 mg/mL *T. harzianum* seems to reduce rhinacanthin levels (4.25% w/w DW). Similarly, rhinacanthin content in leaves followed a similar trend to that of roots. When compared to the untreated group (4.58% w/w DW), rhinacanthin production increased up to 1.5-fold (6.66% w/w DW) after 24 h. Total rhinacanthin levels decreased after 48 h of contact time with *T. harzianum* (6.10% w/w DW). Rhinacanthin levels in *T. harzianum* leaves and roots reached a peak within 24 h of elicitation. Since then, *Trichoderma* treatment has been widely employed as an anti-pathogenic agent that may be involved in plant response (Rajani et al., 2021). *Trichoderma* spp., according to earlier study, promotes the production of glucosinolates, which have an anti-pathogenic impact in plants (Iula et al., 2021). *R. nasutus* may respond similarly to how *T. harzianum* enhances rhinacanthin synthesis in *R. nasutus* through pathogen imitation.

Rhinacanthin concentrations in roots increased with increased contact duration between 24, 48, and 72 h in lawsone (6 μM) treatment. The maximum rhinacanthin production was observed after 72 h of lawsone-contact time, which was up to 1.7-fold (4.55% w/w DW) higher than the control group (2.72% w/w DW). Similarly, lawsone-treated leaves showed a rising tendency from 24 to 72 h. Rhinacanthin levels were 1.13-fold (5.17% w/w DW) higher after 72 h of treatment compared to the control. In the experiment, Lawsone was used as a hypothesized precursor in the rhinacanthin biogenetic pathway or as an elicitor in the protective mechanism. The study was discovered that lawsone improved rhinacanthin production at all contact times ranging from 24 to 72 h. To enhance rhinacanthin production by lawsone, precursor feeding, or elicitation may be utilized. More study is necessary to corroborate the theory. However, it has a lower elicitor potential than chitosan and *T. harzianum*.

These findings suggest that chitosan and *T. harzianum* are novel elicitors of rhinacanthin synthesis in *R. nasutus* hydroponics. Although the cost of chitosan and *T. harzianum* are comparable, the *T. harzianum* elicitation technique was found to be shorter than that of chitosan. As a result, it is presumed that *T. harzianum* is the most effective elicitor of rhinacanthin production in *R. nasutus* hydroponics.

Table 14 Effect of elicitor contact times on growth and rhinacanthin (RC, RD, and RN) production in roots of *R. nasutus* hydroponics

Elicitors & Contact periods	Dried root biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Chitosan (0.15 mg/mL)					
24 h	125.88 ± 3.01[a]	3.95 ± 0.03[a]	0.41 ± 0.01[a]	0.52 ± 0.01[a]	4.89 ± 0.03[a]
48 h	123.31 ± 8.26[a]	4.96 ± 0.07[b]	0.48 ± 0.01[b]	0.67 ± 0.02[b]	6.12 ± 0.09[b]
72 h	116.64 ± 7.43[a]	4.58 ± 0.29[b]	0.43 ± 0.02[a]	0.59 ± 0.04[a]	5.61 ± 0.35[b]
T. harzianum extract (1.0 mg/mL)					
24 h	121.70 ± 4.99[a]	5.63 ± 0.08[c]	0.22 ± 0.02[c]	0.22 ± 0.01[c]	6.07 ± 0.08[b]
48 h	119.28 ± 3.14[a]	5.53 ± 0.10[c]	0.29 ± 0.03[c]	0.22 ± 0.01[c]	6.04 ± 0.12[b]
72 h	117.03 ± 6.46[a]	3.92 ± 0.08[a]	0.17 ± 0.01[d]	0.16 ± 0.01[d]	4.25 ± 0.08[c]
Lawsone (3.0 μM)					
24 h	131.60 ± 3.35[a]	3.56 ± 0.03[d]	0.13 ± 0.01[d]	0.36 ± 0.01[e]	4.15 ± 0.03[c]
48 h	121.01 ± 14.35[a]	3.59 ± 0.05[d]	0.22 ± 0.01[c]	0.41 ± 0.01[f]	4.22 ± 0.05[c]
72 h	128.82 ± 12.92[a]	3.86 ± 0.02[a]	0.25 ± 0.01[c]	0.46 ± 0.01[f]	4.57 ± 0.02[d]

Note: *Calculated based on dry weight of the roots, RC = rhinacanthin-C, RD = rhinacanthin-D, and RN = rhinacanthin-N. Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

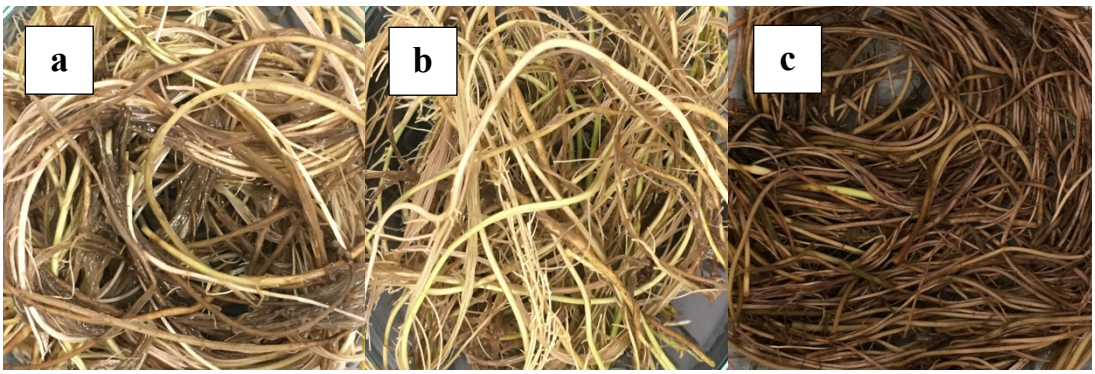

Figure 29 Roots of *R. nasutus* treated with chitosan (48 h) (a), *T. harzianum* (24 h) (b), and lawsone (48 h) (c)

Table 15 Effect of elicitor contact times on growth and rhinacanthin (RC, RD, and RN) production in leaves of *R. nasutus* hydroponics

Conc. & periods	Leaf biomass (g/plant)	Rhinacanthin content (% w/w)			
		RC	RD	RN	Total
Control	158.83 ± 8.76[a]	3.94 ± 0.03[a]	0.22 ± 0.01[a]	0.42 ± 0.01[a]	4.58 ± 0.03[a]
Chitosan (0.15 mg/mL)					
24 h	168.90 ± 11.41[a]	4.77 ± 0.11[b]	0.24 ± 0.01[a]	0.42 ± 0.01[a]	5.44 ± 0.11[b]
48 h	165.00 ± 13.04[a]	4.15 ± 0.03[a]	0.22 ± 0.03[a]	0.47 ± 0.01[b]	4.84 ± 0.03[c]
72 h	173.86 ± 15.62[a]	4.11 ± 0.39[a]	0.20 ± 0.02[a]	0.52 ± 0.03[c]	4.82 ± 0.42[c]
T. harzianum (1 mg/mL)					
24 h	168.90 ± 2.47[a]	5.72 ± 0.19[c]	0.30 ± 0.02[b]	0.64 ± 0.01[d]	6.66 ± 0.22[d]
48 h	165.00 ± 16.27[a]	5.84 ± 0.22[c]	0.23 ± 0.01[a]	0.51 ± 0.02[c]	6.58 ± 0.26[d]
72 h	173.86 ± 13.88[a]	4.96 ± 0.02[a]	0.48 ± 0.01[c]	0.62 ± 0.03[d]	6.10 ± 0.04[d]
Lawsone (6 μM)					
24 h	162.56 ± 14.25[a]	3.88 ± 0.01[a]	0.23 ± 0.01[a]	0.50 ± 0.01[c]	4.60 ± 0.02[c]
48 h	168.99 ± 13.47[a]	3.96 ± 0.11[a]	0.25 ± 0.01[a]	0.45 ± 0.01[b]	4.66 ± 0.11[c]
72 h	172.85 ± 11.89[a]	4.38 ± 0.07[d]	0.29 ± 0.01[b]	0.46 ± 0.01[b]	5.17 ± 0.07[bc]

Note: RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N

Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Figure 30 Non-treated *R. nasutus* leaves

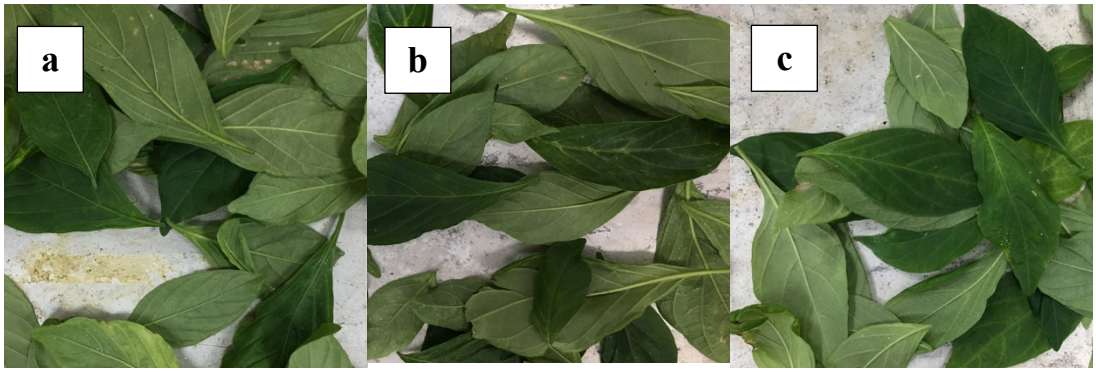

Figure 31 Leaves of *R. nasutus* treated with chitosan (48 h) (a), *T. harzianum* (24 h) (b), and lawsone (48 h) (c)

4.12. Simultaneous dual elicitation treatment

The chitosan and *T. harzianum* extract quantities in this investigation, as well as the elicitor contact times employed for the typical single elicitations, have all been previously optimized. Typically, elicitor contact times are crucial for phytoalexin synthesis and plant growth. So, for the dual elicitations, the elicitor contact times (24–96 h) for the second elicitors were likewise established. According to the dual elicitation techniques utilized in this work, none of the treatments had any impact on the biomass output of the plant's roots or leaves when compared to the control groups (Tables 15–20).

An increase in contact time between 24 and 96 h for chitosan-*H. harzianum* combination elicitation resulted in an increasing trend of total rhinacanthin levels (Table 17 and 18) (Fig. 32 and 33). Rhinacanthin production was observed to be maximum at 72 h in roots, up to 4.51% w/w DW compared to the control group (3.43%

w/w DW). Interestingly, rhinacanthin levels decreased after 96 hours of elicitation (3.88% w/w DW, 120 h). In treated leaves, a similar trend in rhinacanthin production was observed. Between 24 and 72 h, there was an increasing trend in rhinacanthin level (5.61-5.76% w/w DW) compared to the control group (5.35% w/w DW). When the elicitation duration exceeds 72 h, rhinacanthin production appears to diminish with time (5.00% w/w DW, 96 h and 4.71% w/w DW, 120 h). The enhanced production of naphthoquinone and anthraquinone by simultaneous dual elicitation using chitosan and another elicitor in the root cultures of *P. indica* and *C. tora* (Jaisi and Panichayupakaranant, 2020) and *C. tora* (Teptat et al., 2020), respectively, is contrary to the simultaneous dual elicitation effect in this study. It's still unclear why this phenomenon exists. Due to their toxicity, compatibility, and specificity, two or more elicitors may have different effects (Halder et al., 2019) Chitosan and *T. harzianum* extract may interact chemically, or they may be incompatible with one another or unstable, resulting in a reduction in the elicitor stimulating effect when they are used in tandem. Therefore, additional research is required to identify the composition of *T. harzianum* extract as well as the mechanisms of elicitation and chemical interaction between chitosan and *T. harzianum* extract.

Table 16 Effect of combined elicitation on growth and rhinacanthin (RC, RD, and RN) production in roots of *R. nasutus* hydroponics

Treatments	Root biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Untreated group	254.42 ± 2.76[a]	3.23 ± 0.01[a]	0.10 ± 0.01[a]	0.10 ± 0.01[a]	3.43 ± 0.01[a]
Chitosan (48 h)	244.59 ± 13.80[a]	6.29 ± 0.05[b]	0.23 ± 0.01[b]	0.22 ± 0.01[b]	6.75 ± 0.06[b]
T. harzianum extract (24 h)	250.81 ± 15.17[a]	6.37 ± 0.07[b]	0.24 ± 0.01[b]	0.23 ± 0.01[b]	6.84 ± 0.07[b]
Chitosan + *T. harzianum* extract					
24 h	244.85 ± 13.73[a]	3.90 ± 0.02[c]	0.14 ± 0.01[c]	0.12 ± 0.01[ac]	4.16 ± 0.02[c]
48 h	240.40 ± 24.46[a]	3.94 ± 0.09[c]	0.14 ± 0.01[c]	0.13 ± 0.01[c]	4.20 ± 0.09[c]
72 h	226.34 ± 1.59[a]	4.12 ± 0.17[cd]	0.14 ± 0.01[c]	0.13 ± 0.01[c]	4.39 ± 0.17[c]
96 h	228.89 ± 8.64[a]	4.45 ± 0.07[d]	0.14 ± 0.01[c]	0.13 ± 0.01[c]	4.71 ± 0.08[d]

Note: RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N

Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Figure 32 Roots of *R. nasutus* treated with chitosan and *T. harzianum* for 96 h

Table 17 Effect of combined elicitation on growth and rhinacanthin (RC, RD, and RN) production in leaves of *R. nasutus* hydroponics

Treatments	Leaf biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Untreated group	299.94 ± 11.54[a]	4.48 ± 0.05[a]	0.23 ± 0.01[a]	0.34 ± 0.01[a]	5.05 ± 0.05[a]
Chitosan (48 h)	301.34 ± 15.10[a]	5.95 ± 0.07[b]	0.16 ± 0.01[b]	0.42 ± 0.01[b]	6.53 ± 0.07[b]
T. harzianum extract (24 h)	305.50 ± 14.87[a]	6.18 ± 0.05[c]	0.36 ± 0.01[c]	0.47 ± 0.01[b]	7.06 ± 0.05[c]
Chitosan + *T. harzianum* extract					
24 h	303.32 ± 16.63[a]	5.14 ± 0.07[d]	0.26 ± 0.01[d]	0.36 ± 0.01[a]	5.76 ±0.08[d]
48 h	281.17 ± 6.50[a]	5.03 ± 0.14[d]	0.25 ± 0.01[d]	0.34 ± 0.01[a]	5.61 ± 0.14[d]
72 h	301.18 ± 13.72[a]	5.04 ± 0.24[d]	0.25 ± 0.01[d]	0.34 ± 0.01[a]	5.63 ± 0.24[d]
96 h	300.31 ± 15.07[a]	4.46 ± 0.04[a]	0.23 ± 0.01[a]	0.31 ± 0.01[c]	5.00 ± 0.04[a]

Note: RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N

Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Figure 33 Leaves of *R. nasutus* treated with chitosan and *T. harzianum* for 96 h

4.13. Sequential dual elicitation treatment

The highest total rhinacanthin concentration in the roots, up to 9.65% w/w, was obtained by sequential dual elicitation utilizing chitosan and *T. harzianum* extract, which was much greater than its typical single elicitations (Table 19). However, a prolonged 72-hour contact time with T. harzianum extract was necessary for this stimulating action. Rhinacanthin production in the roots appeared to decline as the elicitor contact time increased over 96 hours. Controversially, employing a contact period of 24 hours for the second elicitor, this dual stimulation resulted in the highest total rhinacanthin synthesis in the leaves, up to 7.05% w/w (Table 19). However, it did not differ substantially from the single elicitation with *T. harzianum* extract.

According to Hosseini et al. (2018), one way to hasten the intracellular integration of *T. harzianum* extract and increase rhinacanthin production in plant cells is through the permeability that chitosan increases (Hosseini et al., 2018). However, because chitosan did not directly contact the leaves and did not increase the permeability of plant cells to *T. harzianum* extract, it had less of an impact on the elevated rhinacanthin synthesis in the leaves. Trichokonins, a peptide produced by *Trichoderma* that has been proposed to function as a permeabilizer and plant immunological inducer (Kappel et al., 2020; Keswani et al., 2014), may be involved in the elicitation mechanisms of *T. harzianum.*

The innovative hydroponics of *R. nasutus* elicited with selected elicitors that produce high and consistent yield and rhinacanthin production (9.65% w/w, total

rhinacanthin level) may overcome in-field growing rhinacanthin farms (4.91% w/w, total rhinacanthin level), which commonly lack consistent production. Although the cost of setting up and maintaining *R. nasutus* hydroponics (28,000 baht/Rai/year) is comparable to that of soil-grown *R. nasutus* (26,000 baht/Rai/year), the production and quality of the product is significantly higher (1 Rai = 1,600 m^2). As a result, implementing hydroponics for high quality *R. nasutus* raw material is an achievable approach to overcoming raw material scarcity and low quality in the herbal market.

Table 18 Effect of sequential elicitation on growth and rhinacanthin (RC, RD, and RN) production in leaves of *R. nasutus* hydroponics

Treatments	Root biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Untreated group	254.42 ± 2.76[a]	3.23 ± 0.01[a]	0.10 ± 0.01[a]	0.10 ± 0.01[a]	3.43 ± 0.01[a]
Chitosan (48 h)	244.59 ± 13.80[a]	6.29 ± 0.05[b]	0.23 ± 0.01[b]	0.22 ± 0.01[b]	6.75 ± 0.06[b]
T. harzianum extract (24 h)	250.81 ± 15.17[a]	6.37 ± 0.07[b]	0.24 ± 0.01[b]	0.23 ± 0.01[b]	6.84 ± 0.07[b]
Pretreatment with chitosan (48 h) followed by *T. harzianum* extract					
24 h	248.78 ± 14.69[a]	6.39 ± 0.12[b]	0.21 ± 0.01[b]	0.20 ± 0.01[b]	6.81 ± 0.12[b]
48 h	242.24 ± 10.34[a]	7.05 ± 0.22[c]	0.23 ± 0.01[b]	0.21 ± 0.01[b]	7.49 ± 0.22[c]
72 h	236.32 ± 13.10[a]	9.04 ± 0.22[d]	0.33 ± 0.01[c]	0.28 ± 0.01[c]	9.65 ± 0.22[d]
96 h	246.79 ± 6.40[a]	8.89 ± 0.15[d]	0.32 ± 0.01[c]	0.27 ± 0.01[c]	9.48 ± 0.15[d]

Note: RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N

Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

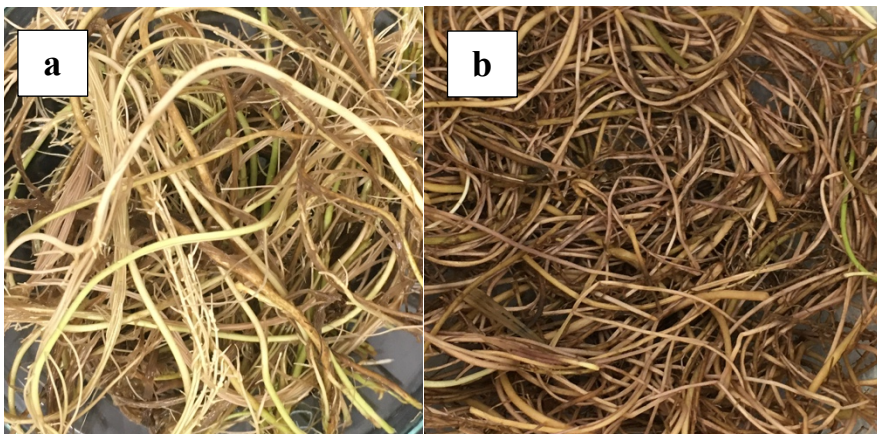

Figure 34 Roots of *R. nasutus* treated with chitosan (a) and chitosan-treated roots of *R. nasutus* treated with *T. harzianum* (48 h) (b)

Table 19 Effect of sequential elicitation on growth and rhinacanthin (RC, RD, and RN) production in leaves of *R. nasutus* hydroponics

Treatments	Leaf biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Untreated group	299.94 ± 11.54[a]	4.48 ± 0.05[a]	0.23 ± 0.01[a]	0.34 ± 0.01[a]	5.05 ± 0.01[a]
Chitosan (48 h)	301.34 ± 15.10[a]	5.95 ± 0.07[b]	0.16 ± 0.01[b]	0.42 ± 0.01[b]	6.53 ± 0.07[b]
T. harzianum extract (24 h)	305.50 ± 14.87[a]	6.18 ± 0.05[c]	0.36 ± 0.01[c]	0.47 ± 0.01[c]	7.06 ± 0.05[c]
Pretreatment with chitosan (48 h) followed by *T. harzianum* extract					
24 h	295.49 ± 9.00[a]	6.30 ± 0.05[c]	0.37 ± 0.01[c]	0.37 ± 0.01[c]	7.05 ± 0.06[c]
48 h	300.27 ± 16.43[a]	6.27 ± 0.10[c]	0.31 ± 0.01[d]	0.44 ± 0.01[b]	7.03 ± 0.10[c]
72 h	299.96 ± 15.62[a]	5.93 ± 0.07[b]	0.31 ± 0.01[d]	0.38 ± 0.02[c]	6.63 ± 0.11[b]
96 h	297.91 ± 13.69[a]	5.07 ± 0.11[d]	0.26 ± 0.01[a]	0.35 ± 0.01[c]	5.69 ± 0.11[d]

Note: RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N

Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

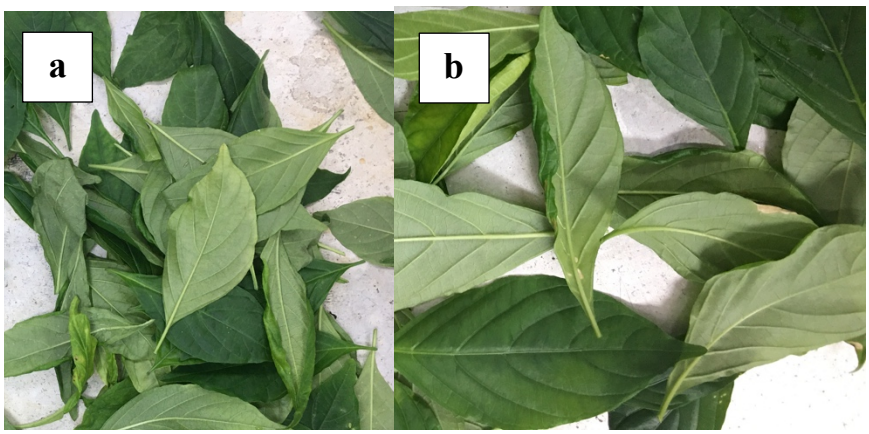

Figure 35 Leaves of *R. nasutus* treated with chitosan (a) and chitosan-treated leaves of *R. nasutus* treated with *T. harzianum* (24 h) (b)

Table 20 Effect of sequential elicitation on growth and rhinacanthin (RC, RD, and RN) production in roots of *R. nasutus* hydroponics

Treatments	Root biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Untreated group	254.42 ± 2.76[a]	3.23 ± 0.01[a]	0.10 ± 0.01[a]	0.10 ± 0.01[a]	3.43 ± 0.01[a]
Chitosan (48 h)	244.59 ± 13.80[a]	6.29 ± 0.05[b]	0.23 ± 0.01[b]	0.22 ± 0.01[b]	6.75 ± 0.06[b]
T. harzianum extract (24 h)	250.81 ± 15.17[a]	6.37 ± 0.07[b]	0.24 ± 0.01[b]	0.23 ± 0.01[b]	6.84 ± 0.07[b]
Pretreatment with *T. harzianum* extract (24 h) followed by chitosan					
24 h	247.43 ± 13.80[a]	6.47 ± 0.10[b]	0.28 ± 0.01[c]	0.23 ± 0.01[b]	7.03 ± 0.10[b]
48 h	236.16 ± 13.32[a]	6.86 ± 0.05[c]	0.24 ± 0.01[b]	0.22 ± 0.01[b]	7.32 ± 0.05[c]
72 h	247.58 ± 22.30[a]	5.87 ± 0.07[cd]	0.20 ± 0.01[d]	0.20 ± 0.01[b]	6.26 ± 0.07[d]
96 h	239.08 ± 20.63[a]	5.73 ± 0.09[d]	0.20 ± 0.01[d]	0.19 ± 0.01[b]	6.12 ± 0.09[d]

Note: RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N

Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Figure 36 Roots of *R. nasutus* treated with *T. harzianum* (a) and *T. harzianum* -treated roots of *R. nasutus* treated with chitosan (96 h) (b)

Table 21 Effect of sequential elicitation on growth and rhinacanthin (RC, RD, and RN) production in leaves of *R. nasutus* hydroponics

Treatments	Leaf biomass (mg/plant)	Rhinacanthin content (% w/w*)			
		RC	RD	RN	Total
Untreated group	305.17 ± 7.65[a]	4.48 ± 0.05[a]	0.23 ± 0.01[a]	0.34 ± 0.01[a]	5.05 ± 0.05[a]
Chitosan (48 h)	301.34 ± 15.10[a]	5.95 ± 0.07[b]	0.16 ± 0.01[b]	0.42 ± 0.01[b]	6.53 ± 0.07[b]
T. harzianum extract (24 h)	305.50 ± 14.87[a]	6.18 ± 0.05[c]	0.36 ± 0.01[c]	0.47 ± 0.01[b]	7.06 ± 0.05[c]
Pretreatment with *T. harzianum* extract (24 h) followed by chitosan					
24 h	289.57 ± 13.60[a]	6.34 ± 0.10[c]	0.31 ± 0.02[c]	0.45 ± 0.01[bc]	7.10 ± 0.11[c]
48 h	307.08 ± 16.20[a]	6.40 ± 0.13[c]	0.33 ± 0.01[c]	0.43 ± 0.03[bc]	7.16 ± 0.14[cd]
72 h	301.04 ± 11.56[a]	6.57 ± 0.08[de]	0.32 ± 0.01[c]	0.45 ± 0.01[bc]	7.34 ± 0.08[de]
96 h	298.46 ± 15.38[a]	6.79 ± 0.13[e]	0.34 ± 0.01[c]	0.47 ± 0.02[c]	7.59 ± 0.15[e]

Note: RC = rhinacanthin-C, RD = rhinacanthin-D, RN = rhinacanthin-N

Mean values within the same column that labelled with different letters are significantly different ($p < 0.05$).

Figure 37 Leaves of *R. nasutus* treated with *T. harzianum* and *T. harzianum*-treated roots of *R. nasutus* treated with chitosan (96)

CHAPTER 5
CONCLUSION

The present study revealed that RC was the most abundant active chemical in *R. nasutus* roots and leaves, whereas RD and RN were insignificant. These chemicals might be employed as bioactive indicators for *R. nasutus* raw material quality management. Because of the high amount of rhinacanthins in all harvesting times, good quality raw materials for further development of health products from *R. nasutus* should be collected from roots and leaves. Currently, the majority of commercially accessible raw materials and tea products from *R. nasutus* in Thailand are derived from aerial parts, which are of poor quality due to a lack of rhinacanthins. Furthermore, because rhinacanthins do not dissolve in hot water, tea infusion is not an achievable herbal therapy for *R. nasutus*. The current study contributed to an efficient *R. nasutus* hydroponic system that might be employed as an alternate supply of roots. Through adjustments of aeration, growing container types, nutrient concentrations, harvesting periods, and light sources, the hydroponic system generated high and consistent quality root material. Furthermore, the cultivation period might be decreased with the roots taken every three months.

In the *R. nasutus* hydroponic system, the elicitation conditions for rhinacanthin production were optimized. The current study confirmed that chitosan, *T. harzianum*, and lawsone increased rhinacanthin synthesis. They were recently discovered to boost rhinacanthin production. Chitosan and *T. harzianum* have been further optimized for rhinacanthin synthesis in *R. nasutus* hydroponics by time of elicitation. Although the costs of chitosan and *T. harzianum* are comparable, the *T. harzianum* elicitation approach was discovered to be shorter. As a result, *T. harzianum* is thought to be the most effective elicitor of rhinacanthin production in *R. nasutus* hydroponics. Moreover, the elicitor combination and sequential elicitation were optimized for *R. nasutus* hydroponic system. The current investigation demonstrated the augmentation of rhinacanthin production by chitosan treatment prior to *T. harzianum* elicitation at optimal concentrations and contact periods, indicating the most effective approach for inducing rhinacanthin synthesis without a detrimental effect on root biomass. They were recently reported to boost rhinacanthin production. Furthermore, it may be

beneficial in increasing rhinacanthin production in *R. nasutus*, which may be used to produce herbal raw materials for health supplements or herbal goods. In conclusion, *R. nasutus* hydroponics with solitary elicitation or sequential elicitation might be employed as a unique alternative in the industry for herbal raw materials once it has been researched on an industrial scale.

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List of Publication and Proceeding

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Patent

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