EFFECT OF HEAT STRESS ON TRANSCRIPTOMIC PROFILE AND PROTECTIVE EFFICACY OF DIETARY ANTIOXIDANTS IN BREEDER HENS

PHOCHARAPON PASRI

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ผลของความเครียดจากความร้อนต่อทรานสคริปโตมิกส์และประสิทธิภาพการป้องกันของสารต้านอนุมูลอิสระในไก่แม่พันธุ์

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

(Prof. Dr. Chaiyapoom Bunchasak)
Chairperson

(Assoc. Prof. Dr. Sutisa Khempaka)
Member (Thesis Advisor)

(Dr. Pascal Mermillod)
Member

(Assoc. Prof. Dr. Worapon Aengwanich)
Member

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Member

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Vice Rector for Academic
Affaire and Quality Assurance

(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural Technology

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ความเครียดจากความร้อนส่งผลกระทบต่อสมรรถนะการสืบพันธุ์และการผลิตไข่ในไก่แม่พันธุ์ โดยเฉพาะอย่างยิ่งในไก่สายพันธุ์ที่ไวต่อความร้อน การศึกษานี้มีวัตถุประสงค์เพื่อค้นหาพืชที่มีคุณสมบัติต้านอนุมูลอิสระเพื่อใช้ลดผลกระทบความเครียดจากความร้อน การใช้เทคนิคทรานสคริปโตมิกส์เพื่อระบุยีนที่ทนต่อความร้อนระหว่างไก่แม่พันธุ์ที่ปรับตัวต่อความร้อนได้ดีและไก่แม่พันธุ์ที่ไวต่อความร้อน การศึกษาผลของการเสริมสารต้านอนุมูลอิสระสังเคราะห์และไฟโตเจนนิกในอาหารไก่แม่พันธุ์ที่ไวต่อความร้อน (ไก่แม่พันธุ์ มทส.) เมื่ออยู่ในสภาวะความเครียดจากความร้อน การศึกษาครั้งนี้ประกอบด้วย 4 การทดลอง ดังนี้

การทดลองที่ 1 ทำการคัดเลือกพืชจำนวน 17 ชนิด เพื่อทดสอบสำหรับใช้เป็นสารต้านอนุมูลอิสระ โดยพบว่ากานพลู กากชาเขียว และผักแพว มีปริมาณฟีนอลิกและฟลาโวนอยด์รวมทั้งหมดสูงกว่าบรรดาพืชทั้งหมด 17 ชนิด เมื่อนำสารสกัดหยาบของพืชทั้งสามชนิดนี้ผสมร่วมกันในอัตราส่วน 1:1:1 (ปริมาตร:ปริมาตร:ปริมาตร) พบว่ามีการออกฤทธิ์กำจัดอนุมูลอิสระและยับยั้งไฮโดรเจนเปอร์ออกไซด์ในเซลล์มะเร็งตับเฮปจี2 (HepG2) ได้อย่างมีประสิทธิภาพ นอกจากนี้การผสมร่วมกันของพืชทั้งสามชนิดนี้ได้รับการพิสูจน์ที่ความเข้มข้น 0.021 ถึง 0.346 มิลลิกรัมต่อมิลลิลิตร มีความปลอดภัยต่อเซลล์

การทดลองที่ 2 ศึกษาผลกระทบของความเครียดจากความร้อนต่อการแสดงออกของยีนระดับทรานสคริปชันในเนื้อเยื่อต่อมสร้างเปลือกไข่ที่มีท่อกักเก็บอสุจิระหว่างไก่แม่พันธุ์ที่ปรับตัวต่อความร้อนได้ดีและไก่แม่พันธุ์ที่ไวต่อความร้อนภายใต้สภาวะความเครียดจากความร้อนแบบเฉียบพลัน (3 ซ้ำต่อกลุ่มทดลอง) ผลการทดลองพบว่ามีการแสดงออกของยีนที่แตกต่างกันทั้งหมด 387 ยีน ประกอบด้วย 159 ยีนมีการแสดงออกเพิ่มขึ้นและ 228 ยีนมีการแสดงออกลดลง การวิเคราะห์ Gene Ontology (GO) ที่ทำงานแตกต่างกันอย่างชัดเจนใน 15 GO term ส่วนใหญ่เกี่ยวข้องกับการสร้างโปรตีนกลุ่มแชเพอโรนและโคแชเพอโรนของ heat shock proteins การแสดงออกของยีน HSP และ DNAJ ที่เพิ่มขึ้น และการแสดงออกของยีน IL18R1, CCL19, ADH1C, TAT, CA9 และ CA6 ที่ลดลงเกี่ยวข้องกับกระบวนการเมแทบอลิซึม 6 กลุ่ม ใน Kyoto Encyclopedia of Genes and Genomes

อีกทั้งการศึกษาครั้งนี้พบว่าว่า HSPB8, DNAJ4, HSP90AA1 และ TAT เป็นยีนเครื่องหมายในเนื้อเยื่อต่อมสร้างเปลือกไข่ที่ระบุการตอบสนองความเครียดจากความร้อน

การทดลองที่ 3 ศึกษาประสิทธิภาพของสารต้านอนุมูลอิสระสังเคราะห์และไฟโตเจนนิกในไก่แม่พันธุ์ที่ไวต่อความร้อน (ไก่แม่พันธุ์ มทส.) จำนวน 100 ตัว ซึ่งแบ่งออกเป็น 4 กลุ่ม: กลุ่ม 1) อาหารควบคุมเลี้ยงที่อุณหภูมิปกติ; กลุ่ม 2) อาหารควบคุมเลี้ยงภายใต้ความเครียดจากความร้อน กลุ่ม 3) อาหารควบคุมที่เสริมสารต้านอนุมูลอิสระสังเคราะห์ (วิตามินซีและอี ซีลีเนียม และแอลคาร์นิทีน) ภายใต้ความเครียดจากความร้อน และกลุ่ม 4) อาหารควบคุมที่มีสารต้านอนุมูลอิสระไฟโตเจนนิก (กานพลู กากชาเขียว และผักแพว) เลี้ยงภายใต้ความเครียดจากความร้อน สารต้านอนุมูลอิสระทั้งในรูปแบบสังเคราะห์หรือไฟโตเจนนิกสามารถเปลี่ยนแปลงการแสดงออกของยีนเครื่องหมาย HSP90AA1 และ TAT ในเนื้อเยื่อต่อมสร้างเปลือกไข่ สารต้านอนุมูลอิสระทั้ง 2 แหล่งสามารถเพิ่มผลผลิตไข่และการฟักออก และช่วยลดอัตราการตายของตัวอ่อนระยะสุดท้าย นอกจากนี้สารต้านอนุมูลอิสระเหล่านี้ยังช่วยเพิ่มคุณสมบัติต้านอนุมูลอิสระในไข่แดง ตับ และเนื้ออกเมื่อเทียบกับไก่แม่พันธุ์ภายใต้ความเครียดจากความร้อนที่ไม่มีการเสริม อีกทั้งยังพบว่าการแสดงออกของยีนในตับกลุ่ม SOD, GSH-Px และ CAT เพิ่มขึ้นและยีน NF-KB, HSP 70 และ 90 ลดลงในแม่ไก่พันธุ์ที่ได้รับสารต้านอนุมูลอิสระจากทั้ง 2 แหล่ง

การทดลองที่ 4 ศึกษาผลของสารต้านอนุมูลอิสระสังเคราะห์และไฟโตเจนิกต่อสมรรถนะการสืบพันธุ์ คุณภาพไข่ สมรรถนะการเจริญเติบโตและความสามารถในการต้านอนุมูลอิสระของลูกไก่ โดยมีการใช้สัตว์ทดลองและอาหารทดลองเช่นเดียวกันการทดลองที่ 3 ผลการทดลองพบว่าสารต้านอนุมูลอิสระทั้ง 2 แหล่งสามารถช่วยเพิ่มค่า Haugh unit และรักษาน้ำหนักรังไข่และจำนวนฟอลลิเคิล ขณะที่สารต้านอนุมูลอิสระไฟโตเจนนิกสามารถเพิ่มสีของไข่แดงด้วย นอกจากนี้สารต้านอนุมูลอิสระทั้ง 2 แหล่งยังมีศักยภาพในการกำจัดอนุมูลอิสระ ลดการเกิดออกซิเดชันของไขมัน เพิ่มระดับการแสดงออกของยีน SOD, CAT และ GSH-Px และยับยั้งการแสดงออกของยีน HSP90 ในตับของลูกไก่

โดยสรุป การศึกษานี้ให้ข้อมูลการแสดงออกของยีนทั้งหมดในระดับทรานสคริปชันธระหว่างไก่แม่พันธุ์ที่ปรับตัวต่อความร้อนได้ดีและไก่แม่พันธุ์ที่ไวต่อความร้อน สารต้านอนุมูลอิสระสังเคราะห์หรือไฟโตเจนนิกมีประสิทธิภาพในการลดผลกระทบความเครียดจากความร้อนในไก่แม่พันธุ์ที่ไวต่อความร้อน และถ่ายทอดความสามารถในการต้านอนุมูลอิสระไปสู่ลูกไก่ได้

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์
ปีการศึกษา 2566

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

PHOCHARAPON PASRI : EFFECT OF HEAT STRESS ON TRANSCRIPTOMIC PROFILE AND PROTECTIVE EFFICACY OF DIETARY ANTIOXIDANTS IN BREEDER HENS. THESIS ADVISOR : ASSOC. PROF. DR. SUTISA KHEMPAKA, Ph. D., 172 PP.

Keyword: TRANSCRIPTOME/BREEDER HEN/HEAT STRESS/DIETARY ANTIOXIDANT/ ANTIOXIDANT ACTIVITY

Heat stress (HS) adversely affects breeder hens in reproductive ability and egg production, especially in heat-sensitive breeds. This study aimed to explore plants with antioxidant properties for potential use in mitigating HS effects. Transcriptomic techniques were also conducted to identify heat tolerance genes by comparing heat-adapted and heat-sensitive breeds. Synthetic and phytogenic antioxidants were supplemented in heat-sensitive breed diets (SUT breed) to address the HS challenge. This study was comprised of four experiments as follows:

Experiment 1, a total of 17 plant materials were screened for potential use as antioxidant substances. Notably, clove, green tea pomace, and Vietnamese coriander exhibited significant levels of total phenolic and flavonoid contents among the 17 plant materials. The combined crude extract of these three plants (in a 1:1:1 ratio, v:v:v) displayed high strong radical scavenging and effectively inhibited H_2O_2 in HepG2 cells. In addition, this combination proved to be safe within the concentration ranges of 0.021 to 0.346 mg/mL for cellular application.

Experiment 2, conducted a study on the effects of HS on the transcriptomic profile analyzed in the uterovaginal junction (UVJ) tissue containing sperm storage tubules (SSTs), comparing heat-adapted and heat-sensitive breeds under acute HS (three replicates of each). A total of 387 differentially expressed genes, including 159 upregulated and 228 downregulated genes, were observed. Gene Ontology (GO) analysis identified the top significant 15 GO terms that mostly involved chaperone and co-chaperone of heat shock proteins (HSPs) transcripts. The upregulated HSP and DNAJ gene families, and downregulated IL18R1, CCL19, ADH1C, TAT, CA9, and CA6 genes were associated with six significant metabolism pathways according to Kyoto Encyclopedia of Genes and Genomes analysis. HSPB8, DNAJ4, HSP90AA1, and TAT genes were identified as candidate gene markers in UVJ for the HS response.

Experiment 3, the efficacy of synthetic and phytogenic sources were investigated using one hundred heat-sensitive breeds (SUT breeder hens), which were divided into four treatments: (T1) basal diets under thermoneutral zone (TN); (T2) basal diets under HS; (T3) basal diets with synthetic antioxidants (a combination of vitamin C and E, Se, and L-carnitine) under HS, and (T4) basal diets with phytogenic antioxidant (a combination of clove, green tea pomace, and Vietnamese coriander powders) under HS. Either synthetic or phytogenic antioxidants have the potential to modulate the expression of HSP90AA1 and TAT candidate gene markers in UVJ tissue. Either of the antioxidants showed the potential to improve egg production and hatchability while reducing late-stage embryo mortality. Furthermore, these antioxidants increased antioxidant properties in yolk, liver, and breast meat compared to HS breeder hens without supplements. Furthermore, the expression of SOD, GSH-Px, and CAT genes in the liver was upregulated, whereas the expression of NF-KB and heat shock proteins 70 and 90 genes were downregulated in breeder hens that received either of antioxidant sources.

Experiment 4, this study aimed to assess the effect of synthetic and phytogenic sources on reproductive performance, egg quality, offspring growth performance, and antioxidant capability. The experimental birds and diets were the same as those in Experiment 3. The results indicated that both of the antioxidant sources enhanced the Haugh unit and maintained ovary weight and number of follicles. Phytogenic antioxidants are particularly effective in improving yolk color. Additionally, these antioxidant sources exhibited the potential in enhancing free radical scavenging, mitigating lipid peroxidation, elevating mRNA expression levels of SOD, CAT, and GSH-Px, and suppressing HSP90 in the livers of offspring.

In conclusion, this study emphasized the distinct transcriptomic profiles observed between heat-adapted and heat-sensitive breeds. Both synthetic and phytogenic antioxidants demonstrated the ability to alleviate the adverse effects of HS in heat-sensitive breeder hens and confer antioxidant benefits to their offspring.

School of Animal Technology and Innovation Student's Signature Phacharapon Pasri
Academic Year 2023 Advisor's Signature Sutisa Khempaka
Co-advisor's Signature

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Phocharapon Pasri

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

M	=	Molar
mM	=	Milli Molar
mg	=	Milligram
µg	=	Microgram
µL	=	Microliter
g	=	Gram
min	=	Minute
s	=	Second
mL	=	Milliliter
℃	=	Degree Celsius
FC	=	Fold change
h	=	Hour
FI	=	Feed intake
WI	=	Water intake
ADG	=	Average daily gain
BW	=	Body weight
FBW	=	Final body weight
EW	=	Egg weight
FCR	=	Feed conversion ratio
ADFI	=	Average daily feed intake
SOD	=	Superoxide dismutase
CAT	=	Catalase
GSH-Px,	=	Glutathione peroxidase
HSP70	=	Heat shock protein 70
HSP90	=	Heat shock protein 90
NF-κB	=	Nuclear factor-κB
TN	=	Thermoneutral zone
HS	=	Heat stress

LIST OF ABBREVIATIONS (Continued)

LYF	=	Large yellow follicles
SYF	=	Small yellow follicles
LWF	=	Large white follicles
EP	=	Egg production
DEGs	=	Differentially expressed genes
KEGG	=	Kyoto Encyclopedia of Genes and Genomes
GO	=	Gene ontology
T	=	Treatment
UVJ	=	Uterovaginal junction
SSTs	=	Sperm storage tubes
N	=	Newton
g/cm^3	=	Gram per cubic centimeter

CHAPTER I
INTRODUCTION

1.1 Introduction

In the context of global climate change, animal production will face increasingly challenging conditions, particularly heat stress episodes, that are known to have a negative impact on production parameters, and especially on reproduction in both broiler and layer breeds. Farm animal species present a wide range of resilience and adaptability potential in front of extreme environmental conditions, including heat stress (HS) (Barrett et al., 2019). High environmental temperature is one of the most important factors causing economic loss for poultry breeder flocks in subtropical and tropical areas. In breeder hens, an environmental temperature of 32 to 38°C could induce large amounts of reactive oxygen species (ROS) and interfere with the integrity of sperm membrane and DNA in sperm storage tubules (SSTs) at the oviduct of hens including consequently negatively influencing fertility, hatchability, embryo development, and offspring quality (Fouad et al., 2016; Beckford et al., 2020). This can be improved by controlling the temperature of animal housing, but this is costly, energy-consuming, and increasing greenhouse gas production. Another way to face this problem is to select more resilient animal strains that can adapt to high temperatures. This selection requires understanding the mechanisms by which some locally adapted breeds can overcome the problems caused by heat stress. Another way to overcome heat stress damage would be fed poultry with specific bioactive nutrients having antioxidant and ROS scavenging properties such as vitamin C, vitamin E, selenium (Se), manganese, zinc, phytochemical or phytogenic feed additives, etc. have also been receiving increased attention in recent years (Shakeri et al., 2020; Hu et al., 2019).

Thailand is located in a subtropical climate area, in which in the summer season the temperature can reach up to 41°C with high humidity is considered as key stress. Heat stress can be classified into 2 main categories, acute heat and chronic heat stress depending on the changing of ambient temperature and period of time in

heat exposure (Akbarian et al., 2016). The resistance to heat stress differs between chickens of different genetic backgrounds, the strains selected for rapid growth or high production are significantly more sensitive to high environmental temperature than those with slow-growing rates (Duangjinda et al., 2017). Two breeder broiler strains, heat-adapted (Leung Hang Kaeo breeder line) and heat-sensitive (Suranaree University of Technology (SUT) breeder line) are preserved by the avian research center of SUT in Thailand, and used for the production of crossbred meat chicken (Korat chicken) for supplying to a niche local market. However, the productive performance including low egg production, fertility, and hatchability often occurs at high temperatures, especially in SUT strain, while little effects have been found in the local breed. The investigation of gene markers by using transcriptomic analysis that can be used in feed modulation using antioxidant bioactive substances for better adaptation under HS will contribute to regulating the production and increase the efficiency and genetic gain (Gvozdanovic et al., 2023).

In female breeder hens, the uniqueness of the reproductive system as sperm storage tubules (SSTs) can store and preserve spermatozoa for prolonged periods after a natural mating or a single artificial insemination, for the period 14–21 days. The ability of sperm survival is associated with the function of SSTs, which importantly affects the fertility rate of hens by providing a suitable environment for the hen oviduct (Yang et al., 2020). Bakst and Bauchan (2015) and Das et al. (2006) reported that breeder hens potentially possess the capacity to influence the metabolic activity and motility of resident sperm. Additionally, they play a role in safeguarding the sperm from immune challenges within the hen oviduct. There is a question if a nutrient with antioxidant activity can improve sperm survival and overcome the negative impact of HS. Dietary antioxidants are anticipated to function as defense networks against oxidative stress in three areas: organelles, subcellular compartments, and the extracellular space (Horváth and Babinszky, 2018). Dietary vitamin C, vitamin E, Se, L-carnitine, and phytogenic feed additives were widely used in the feed industry. The combined supplementation of vitamin C, vitamin E, and Se can work together to improve antioxidant mechanisms (Surai and Kochish, 2019; Surai et al., 2019), productive performance, and antioxidant activity (Sandhanu et al., 2017; Horváth and Babinszky, 2018). In addition, the use of L-carnitine has also been

reported to enhance hatchability and quality of offspring (Wang et al., 2013; Awad et al., 2017). There was intense interest in phytogenic substances from herbs, spices, and other plants and their extracts were used in poultry diets under HS conditions due to their strong antioxidant properties (Reis et al., 2019). Polyphenols have been reported to upregulate heat shock proteins and antioxidant enzymes, resulting in the suppression of suppress reactive oxygen species (ROS) and interference with various components of HS responses (Hu et al., 2019; Saracila et al., 2021). Therefore, the development of bioactive nutrients with exert antioxidant function in SST would be an alternative strategy to overcome heat stress in broiler breeder hens, unfortunately, there is still a lack of research.

The transcriptomic technique has a high potential to identify and quantify the changing expression levels of each transcript during development and under different conditions. The researcher can use the different gene expression data as candidate gene markers to describe molecular processes in response to different environmental or physiological factors (Reuter et al., 2015) because the identified candidate genes and mechanisms linked to the response to HS could form the basis for enhancing the adaptability of poultry to challenging HS conditions (Lim et al., 2022). In the field of animal science research, transcriptomic techniques have been used to investigate gene profiles and identify candidate genes in cells, tissues, physiological fluids, and animal products in relation to the responses to heat stress (Liu et al., 2022). Interestingly, transcriptome analysis in the uterovaginal junction (UVJ) tissue of hens can identify candidate gene markers of hens which is used to explain the capacity in sperm storage duration within the SST of hens (Yang et al., 2021). The differential gene expressions of HSP25, HSPA5, HSPA8, GKN2, IL4I1, PDK4, TAT, CA, LHCGR, GPX, and ISGs in the UVJ containing SSTs between breeder hens under thermoneutral and HS conditions could provide the understanding of molecular pathways and networks within UVJ containing SSTs tissues for preventing heat stress-induced fertility loss in breeder hens (Kubota et al., 2023). In addition, the study of differential gene expression by using the transcriptomic technique was also applied in nutrition research to study the efficiency of dietary treatments in metabolism, digestion, absorption, biomarkers and individualized requirements of nutrients and function of nutrients, dietary synthetic and phytogenic antioxidants on

growth, reproduction, health, and antioxidant activity (Li et al., 2020; Pascual et al., 2022; Gvozdanovic et al., 2023). Even though the previous study did not report the effects of bioactive nutrients on gene expression in UVJ-containing SSTs, information on gene markers may assist in directing the development of precision feeding for antioxidant substances. In turn, this could improve the reproduction and production of breeder hens under HS conditions.

Therefore, this study aimed to identify relevant gene markers through transcriptomic analysis in UVJ containing SSTs of breeder hens subjected to HS, comparing heat-adapted breeds and heat-sensitive breeds. The investigation focused on tracking the progressive changes in selected gene candidates among heat-sensitive breeder hens under HS. In addition, this study investigated the effects of dietary antioxidants used either synthetic (a combination of vitamin E, vitamin C, Se, and L-carnitine) or phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) supplements to overcome heat stress-induced damage in female breeder hens on production, reproduction, prolong sperm survival in the oviduct, blood chemistry, antioxidant properties, gene expression, and the quality of offspring.

1.2 Research objectives

The objectives of this study were:

1.2.1 To investigate the total phenolic (TPC) and flavonoid contents (TFC), antioxidant capacities, and cytotoxicity in 17 edible plant materials from herbs, fruits, vegetables, and plant by-products available in Southeast Asia for future use in the feed industry.

1.2.2 To identify general gene expression under heat stress between heat-adapted and heat-sensitive breeder hens.

1.2.3 To evaluate the effect of dietary supplementation, either with synthetic or phytogenic antioxidant mixtures, on alleviating the deleterious impact of heat stress in heat-sensitive breeder hens, various parameters were examined. These include productive performance, blood chemistry, fertile period length of sperm, antioxidant properties, and the gene expression of heat-sensitive breeder hens.

1.2.4 To investigate the effect of either synthetic or phytogenic antioxidant sources in breeder hen diets on the antioxidant status and growth performance of their offspring.

1.3 Research hypotheses

1.3.1 Plant materials from herbs, fruits, vegetables, and plant by-products available in Southeast Asia can be used as phytogenic antioxidant substances.

1.3.2 The gene expression in uterovaginal junction containing sperm storage tubes targeted to heat stress can be different between heat-adapted and heat-sensitive breeder hens.

1.3.3 Dietary antioxidant supplement in heat-sensitive breeder hens under heat stress can enhance production and reproduction, prolong sperm survival in the oviduct, improve blood chemistry and antioxidant properties, and alter gene expression target to heat stress in uterovaginal junction containing sperm storage tubes and enhance growth performance and antioxidant activity of offspring.

1.3.4 Either synthetic or phytogenic antioxidants can be used to prevent oxidative reactions caused by HS, both sources exhibit comparable high antioxidant activity.

1.4 Scope of the study

In the context of global climate change, animal production will face increasingly challenging conditions, particularly heat stress episodes, that are known to have a negative impact on production parameters, and especially on reproduction. In this study, plant materials were screened as antioxidant feed additives to reduce the risk of oxidative stress in animals. Two broiler breeder strains (heat-adapted and heat-sensitive breeders) were used to acquire a better understanding of heat stress responses by transcriptomic analysis and to use this knowledge to propose innovative strategies of heat stress management by evaluating the effects of dietary supplementation with antioxidant substances under heat stress in heat-sensitive breeder and by analyzing the productive performance, fertile period length of sperm, blood chemistry, antioxidant properties, protein expression, and quality of offspring.

1.5 Expected benefits

1.5.1 This approach enables us to identify novel potential HS biomarkers in breeder hens, which can be used for the manipulation of adding dietary antioxidants in poultry diets aimed at improving heat stress resilience.

1.5.2 Applying this knowledge to propose innovative strategies for heat stress management through dietary supplementation with antioxidant substances.

1.5.3 This knowledge can lead to the development of new alternative antioxidant substances specially designed to address HS in the poultry industry.

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CHAPTER II

LITERATURE REVIEW

2.1 The impact of heat stress on the reproductive performance of breeder hens

In poultry production, heat stress (HS) can result in high economic losses by reducing egg production, fertility, hatchability, and increasing mortality. High temperatures can induce heat stress (HS) and cause a cellular imbalance between the production of reactive oxygen species (ROS) and antioxidant protective systems, thus further stimulating ROS production, which is responsible for various types of oxidative damage, such as lipids and protein oxidation. In poultry, an environmental temperature of 32 to 38°C can induce the production of large amounts of ROS that can compromise the integrity of sperm DNA in uterovaginal sperm storage tubules (SSTs) and, consequently, negatively influence fertility and embryo development. Hu et al. (2019) reported that HS has a greater influence on laying hens compared to broilers, which is primarily attributable to metabolic distinctions and the heightened heat stress production exhibited by laying hens as opposed to broilers as shown in Figure 2.1.

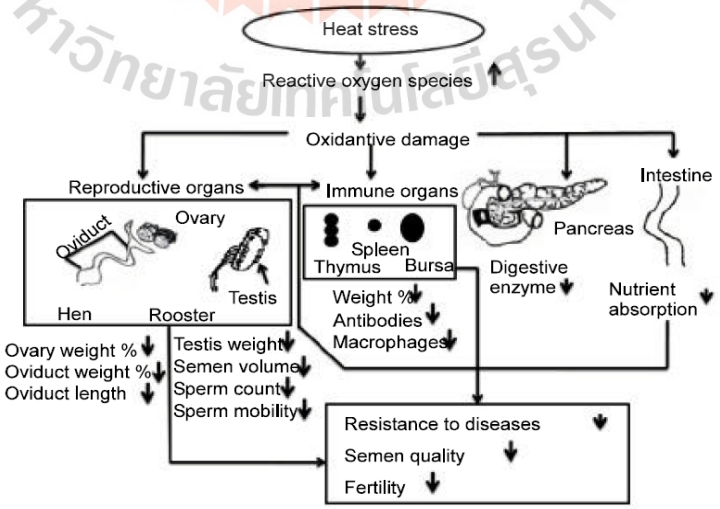

Figure 2.1 Effect of heat stress (HS) on immunity, semen quality, and fertility (Fouad et al. 2016).

2.2 Role of synthetic antioxidant substances on antioxidant capacity

Heat stress can be acute, brutal, and result in short exposure to high temperatures, or become chronic after long-term exposure to elevated temperatures. The mechanisms involved in the resistance against these two types of HS are different and involve the participation of different antioxidant enzymes. Under acute heat stress, the ROS levels in the body are rapidly increased and the antioxidant enzyme system also responds rapidly, by increasing the activity of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) to eliminate free radicals generated under HS. Besides the antioxidant enzyme system, there are non-enzymatic antioxidant defenses which include vitamin C, vitamin E, glutathione, carotenoid, and some microelements such as copper, zinc, selenium, and manganese (Mishra and Jha, 2019). Vitamin E serves as a crucial antioxidant within biological systems, which is capable of traversing lipid bilayer membranes to interact with vitamin C and other antioxidant mechanisms and effectively prevent lipid oxidation. Vitamin C has the ability to modulate the release of glucocorticoids and alleviate cellular damage induced by heat stress (Mishra and Jha, 2019). Zinc is a co-factor of the copper–zinc superoxide dismutase which plays a vital role in antioxidant defense systems (Singh et al., 2016). Glutathione peroxidase is a selenium-dependent enzyme via the metabolism of selenium which is required when poultry is subject to HS. Insufficient selenium levels lead to a decrease in the activity of GSH-Px (Mishra and Jha, 2019). Previous research reported the beneficial effects of coenzyme Q10 and vitamin C which induces a reduced heat shock protein expression and damage to primary myocardial cells during heat stress. In addition, indicators of oxidative stress, such as malondialdehyde (MDA), SOD, and lactate dehydrogenase (LDH), decreased after Q10 and vitamin C treatment (Xu et al., 2017). Our previous studies showed the potential effects of vitamin E, C, and selenium in broiler breeders and laying diets on the enhancement of antioxidant activity and productive performance (Samdangchai et al., 2015; Pasri et al., 2016). The antioxidant network is shown in Figure 2.2. In addition, L-carnitine is a water-soluble quaternary amine and has an antioxidant property. The important role of L-carnitine is to decrease the availability

of lipids for peroxidation by promoting the transport of long-chain fatty acids across the inner mitochondrial membrane for β-oxidation (Salmanzadeh, 2011).

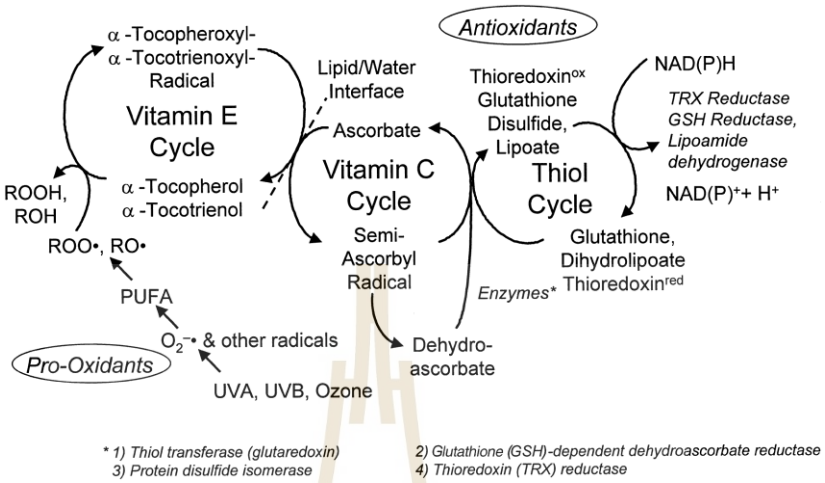

Figure 2.2 The antioxidant network showing the interaction between vitamin E, vitamin C, and thiol redox cycles (Pacer et al., 2001).

2.3 Effect of dietary vitamin C, vitamin E, selenium, and L-carnitine supplementation in poultry diets

A supplement of vitamin C at 200 to 1500 mg/kg in hen diets improved ($P<0.05$) egg weight, egg contents, and hatchability and also improved feed intake, feed conversion ratio, growth rate, and live weight of offspring at 7 weeks of age (Adesola et al., 2012). While hens were reared under heat stress conditions (38°C and 65% RH), feeding with 200 mg/kg of vitamin C, 150 mg/kg of vitamin E, and 1000 mg/kg of their combination can improve productive performances, fertility rate, and egg quality (Attia et al., 2011), including liver weights, spleen weights, thyroid gland weights, ovary weights, oviduct weights and oviduct lengths ($P<0.05$) (Attia et al., 2016). Jena et al. (2013) showed that lowered malondialdehyde (MDA) levels, higher activities of SOD and CAT enzymes, and higher ferric-reducing antioxidant power (FRAP) activities improve in broiler breeder hen diets supplemented with vitamin E (250 mg or 500 mg/kg) or vitamin C (200 mg or 400 mg/kg) alone or in combinations during the summer ($P<0.05$).

Vitamin E, selenium (Se), and their combination have effects on female breeder hen functions when it was determined that there were vitamin E and glutathione (GHS) accumulation in the vagina, uterovaginal junction, and uterus tissue of hen oviduct by dietary vitamin E supplementation (Breque et al., 2006). In another study, the addition of 150 mg/kg of DL-α-tocopherol acetate in Indian native Kadaknath hens resulted in higher sexual maturity, egg production, and fertility than 300 mg/kg of DL-α-tocopherol acetate (Biswas et al., 2010) and the study of Jiang et al. (2013) reported that hens fed dietary vitamin E at 200 mg/kg in diets can increase SOD, GSH-Px and decrease MDA in both the serum and the yolk. The bioavailability of organic Se sources in poultry diets was more efficient than inorganic sources, but the supplementation of DL-selenomethionine, sodium selenite, and seleno-yeast at 0.30 mg/kg in laying hen diets had positive effects on increasing antioxidant activity in plasma (Jing et al., 2015). Dietary Se-enriched yeast at 0.10 to 0.30 mg/kg in female broiler breeder diets often results in an enhanced percentage of fertility and hatchability and also transfers Se deposition to 1-day-old chicks (Osman et al., 2010; Yuan et al., 2011). In addition, the supplementation of vitamin E at 150 or 250 mg/kg combined with Se at 0.15 mg/kg in female turkey diets showed that the fertility and hatchability rate was higher including an increase in the survivability rate in offspring (Adebiyi et al., 2014; Sandhanu et al., 2017). Laying hens that were fed a combination of 125 mg/kg of vitamin E and 0.50 mg/kg of Se supplementation in diets protected cell membranes by increasing the activity of enzymatic antioxidants against free radicals (Celebi, 2019).

There are many previous studies on L-carnitine supplementation in breeder hen diets which found that L-carnitine at 400 to 500 mg/kg increased the weight of newly hatched chicks (Salmanzadeh, 2011). Awad et al. (2016) reported that the improvement of productive and reproductive traits of ducks in the supplemented 300 – 600 mg/kg L-carnitine in breeder hens under summer conditions was better than the control group. Furthermore, L-carnitine supplementation at levels of 100 mg/kg also reduces MDA and increases SOD and GSH-Px activity in the heart tissue of broilers reared in a low-temperature environment (Wang et al., 2013).

2.4 The use of phytogenic supplementation in poultry research and the role of phytogenic substances on antioxidant activity

Phytogenic substances are synthesized from secondary plant metabolites in plants that are used in natural substances or polyphenols. They have been called “Phytogenic feed additives” and are used in animal feed. Polyphenols contain several phenolic and flavonoid compounds, including catechins, flavanols, flavadiols, flavonoids, eugenol, carvacrol, thymol, alkaloids, tannins, cyanogenic and glycosides which are derived from herbs, spices, other plants, and their extracts, such as essential oils. The property of phytogenic feed additives has various functions that are associated with the mechanism of antioxidant, productive performance, stimulating animal digestive systems, antimicrobial, antifungal, antiparasitic, and anti-inflammatory properties depending on each type of natural phytogenic product and its objectives (Radwan et al., 2008; Al-Harthi, 2014; Hosseini-Vashan et al., 2015; Abou-Elkhair et al., 2018; Kamboh et al., 2018; Reis et al., 2019; Zdanowska-Sasiadek et al., 2019). Banning antibiotics and hot climate changes have a negative effect on the poultry industry, therefore the use of natural substances may help to reduce the amount of antibiotics and other deleterious chemicals as growth promoters and therapeutic agents, which all farmers should have access to. (Herve et al., 2019).

Phytogenic substances with antioxidant activity could also be a solution for overcoming heat stress in poultry. Polyphenols are commonly found in a variety of plants and have been used for various purposes because of their strong antioxidant ability (Hu et al., 2019). Most bioactive polyphenol compounds have been assessed partly through biological properties and bioavailability, especially strong radical scavenging activities (Lee et al., 2019) for their antioxidant ability which is dependent on the quantity and quality of polyphenol compounds in each plant species that act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin (Kratchanova et al., 2010). Polyphenols elevate the expression of stress response proteins such as heat shock proteins and antioxidant enzymes, which can suppress ROS and interfere with many players of HS responses (Figure 2.3) (Hu et al., 2019). In addition, the ability of phenolic substances on antioxidant activities occurs when there is a chemical structure of several hydroxyl

groups on one or more aromatic rings and which can stimulate Keap1-Nrf2 complexes by modifying cysteine residues in Kelch-like ECH-associated protein 1, leading to the translocation of Nrf2 into the nucleus after Nrf2 binds to an antioxidant electrophile/antioxidant response element (EpRE/ARE) sequence, resulting in the upregulation of cellular antioxidant enzymes such as SOD, CAT, GSH-Px, GR, and GST, etc. (Saracila et al., 2021) as shown in Figure 2.4.

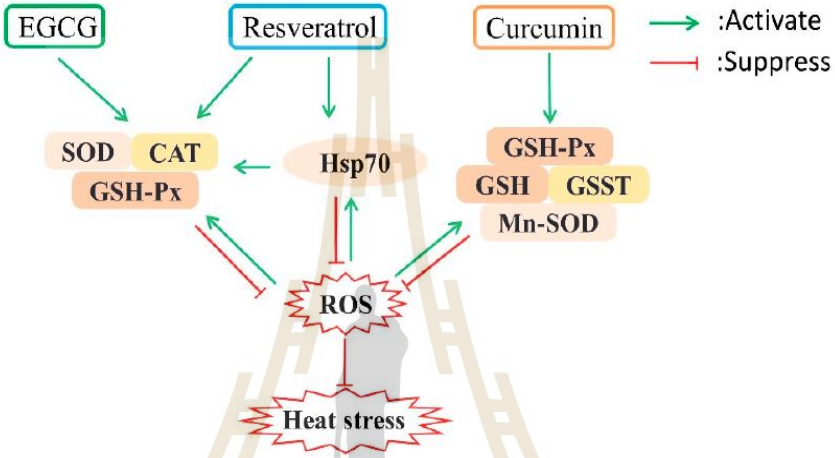

Figure 2.3 Potential mechanisms underlying the protective effect of polyphenols against heat stress (Hu et al., 2019).

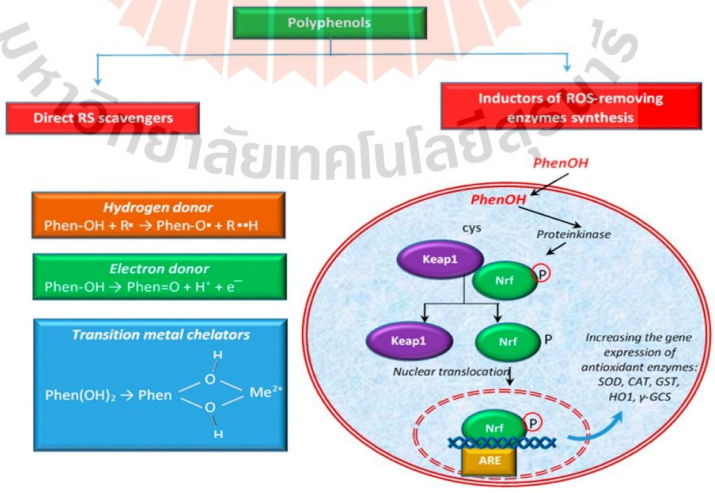

Figure 2.4 Role of polyphenols on scavenging reactive oxygen species and stimulating antioxidant enzyme (Saracila et al., 2021).

2.5 Effects of phytogenic substances from cloves, green tea, and Vietnamese coriander in diets on poultry performances

Cloves (*Syzygium aromaticum* L.) are one of the most efficient antioxidant medicinal herbs which in clove oil consists of various bioactive substances such as eugenol, isoeugenol, caryophyllene, α-humulene, and eugenyl acetate. The flowers, stems, and leaves of the clove tree as well as clove oil are widely used as alternative feed additives for humans and animals for antibacterial, digestion stimulation, antifungal, anti-inflammatory, anticarcinogenic, antiparasitic, and antioxidant properties (Alizadeh et al., 2015). The application of clove bud supplementation exhibited important values on growth performances, blood biochemistry, and remarkable antioxidant capacity of broiler chickens (Mahrous et al., 2017) as shown in Table 2.1. The supplementation of clove oil and clove leaf meal acts as an effective growth promoter in laying hens, especially in improving egg production and maintaining reproductive performance (Şehitoglu and Kaya, 2021; Olateju et al., 2022). The enhancing growth and productive performances that result from supplemented phytogenic feed additives in diets are due to some herbs that promote the development of follicles and immunity by inducing gastric secretion and digestive enzyme activities. The antimicrobial action of some phytogenic feed additives (ginger, mint, cloves, pepper, turmeric, tea) has an effect on decreasing pathogenic and increasing lactic acid bacteria by which hens can absorb nutrients via stimulating lactic acid bacteria (Zhao et al., 2011).

Green tea (*Camellia sinensis*) is well known for its excellent benefits and antioxidant polyphenols which mainly induce catechins, theaflavins, thearubigins, flavonols, caffeine, and other phenolic compounds. These green tea polyphenols can scavenge free radicals and prevent cells from damage (Luo et al., 2020). In addition, natural components of tea polyphenols also have other functions with anti-inflammatory, anti-carcinogenic, antiviral, and antibacterial properties (Lee et al., 2019). The effects of green tea powder or green tea polyphenols on poultry performances are presented in Table 2.2. Chen et al. (2021) suggest that 1% green tea powder in indigenous chicken breed diets enriches the PUFA content in egg yolks, improves the overall taste, and changes its processing characteristics. Green tea powder at 0.75% in laying hen diets improves average daily feed intake, feed conversion ratio, albumen

height, and Haugh units, while a high hen-day egg production rate was found in laying that for hens fed 0.5% green tea powder (Li et al., 2023). The addition of 300 mg tea polyphenols/kg in laying hen diets had a greater positive effect in decreasing the egg yolk cholesterol content and enhancing the antioxidant capacity, while there was no negative effect on production performance (Wang et al., 2021). In addition, bioactive compounds in tea polyphenols are transferred from feed into eggs leading to high epigallocatechin gallate and catechin deposition in yolk (Fan et al., 2021).

Table 2.1 Effects of clove powder supplementation in diets on poultry performances.

Treatment	Results	References
0, 0.5, 1, and 1.5 g clove bud/kg in broiler diets	Improved body weight gain, feed conversion ratio, and protein efficiency ratio Increased total serum protein, globulin, IgG, IgM, INF-γ, IL-10, muscle GSH levels, SOD, and GST activities by clove bud treatment	Mahrous et al. (2017)
0, 50, 100, and 150 mg clove oil/kg in laying hen diets	Improved feed conversion ratio and increased egg production by clove oil treatment Decreased lipid peroxidation by 150 mg clove oil/kg	Şehitoglu and Kaya (2021)
0%, 0.25%, and 0.50% clove leaf meal in laying hen diets	Enhanced shell thickness, yolk height, yolk index, and egg mass Increased weight of entire reproductive tract, ovary, length of infundibulum, magnum, isthmus, uterus, and vagina	Olateju et al. (2022)

Persicaria odorata (Lour.) or Vietnamese coriander is generally known as Pakpaw in Thailand. Vietnamese coriander has various bioactive compounds such as rutin, catechin, quercetin, kaempferol, isorhamnetin, and essential oil which show antimicrobial, anti-inflammatory, antitumor, and antioxidant activities (Rebickova et al., 2020). Their bioactive compounds are powerful antioxidants that can inhibit lipid peroxidation, prevent free radicals, chelate heavy metal agents, and stimulate antioxidant enzymes (Ahongshangbam et al., 2014). Ooi et al. (2018) found that 1% of Vietnamese coriander leaf powder in broiler diets could improve egg weight and egg production as well as the reduction of fecal pH and fecal *Entero bacteriaceae* (Table 2.3). The linear increase in the level of Vietnamese coriander leaf powder from 2–8 g in the diet has many positive effects, such as, increasing body weight gain, improving feed conversion ratio, and decreasing serum activity of aspartate aminotransaminase and alanine aminotransaminase, and the serum levels of glucose, cholesterol, triglycerides, urea, and creatinine (Basit et al., 2020a). Basit et al. (2020b) suggested that 8 g of Vietnamese coriander leaf powder/kg in broiler diets modulated the intestinal microarchitecture and enhanced nutrient digestibility, resulting in maximum body weight gain. Moreover, broilers that were fed Vietnamese coriander 600 mg/kg in diets showed a lower fat percentage and lipid peroxidation levels in breast meat and a better growth performance compared to the control group (Glinubon et al., 2022).

Table 2.2 Effects of green tea powder or green tea polyphenol supplementation in diets on poultry performances.

Treatment	Results	References
Control diets and 1% green tea powder in indigenous chicken breeds	Enriched the PUFA content in egg yolks, improved the overall taste, and changed the processing characteristics Increased radical scavenging activity	Chen et al. (2021)
0.02%, 0.05%, 0.09%, 0.14%, 0.19% and 0.24% tea polyphenol in laying hen diets	Increased epigallocatechin gallate and catechin accumulation in the egg yolk	Fan et al. (2021)
0, 150, 200, 250, 300, and 350 mg tea polyphenols/kg in laying hen diets	Decreased plasma triglyceride, total cholesterol, and low-density lipoprotein cholesterol level by tea polyphenol treatment Enhanced activity of serum glutathione peroxidase by 300 mg tea polyphenols/kg Decreased serum methane dicarboxylic aldehyde by 300 mg tea polyphenols/kg	Wang et al. (2021)
0, 0.5, 0.75, and 1.0% green tea powder in laying hen diets	Increased hen-day egg production rate in 0.5% green tea powder group Improved average daily feed intake, feed conversion ratio, albumen height, and Haugh units in 0.75% green tea powder group	Li et al. (2023)

Table 2.3 Effects of Vietnamese coriander powder supplementation in poultry diets.

Treatment	Results	References
1% of Vietnamese coriander leaf powder in broiler diets	Enhanced hen-day egg production and egg weight Reduced fecal pH and fecal *Entero bacteriaceae* counts	Ooi et al. (2018)
0, 2, 4, and 8 g *Persicaria odorata* leaf meal/kg in laying hen diets	Increased body weight gain and decreased feed conversion ratio by increasing the level of *Persicaria odorata* leaf meal Decreased serum activity of aspartate aminotransaminase and alanine aminotransaminase, and serum levels of glucose, cholesterol, triglycerides, urea, and creatinine	Basit et al. (2020a)
0, 2, 4, and 8 g *Persicaria odorata* leaf meal/kg in broiler	Increased body weight gain, modulated the gut architecture, enhanced nutrient digestibility by 8 g *Persicaria odorata* leaf meal/kg	Basit et al. (2020b)
0, 200, 400, and 600 mg Vietnamese coriander extract/kg in broiler diets	Showed the lowest fat percentage in 600 mg Vietnamese coriander extract/kg Decreased the TBARS value of breast meat and improved growth performance by 600 mg Vietnamese coriander extract/kg	Glinubon et al. (2022)

2.6 Effects of phytogenic supplementation on antioxidant activity of poultry under heat stress conditions

The negative impacts of the deficiency of the antioxidant mechanism in poultry because of heat stress are well known. The supplementation of phytogenic feed additives in diets has been used to alleviate the negative effects of heat stress (Habibian et al., 2014; Perai et al., 2015). Previous studies found that most phytogenic antioxidant substances are mostly supplemented in broiler and laying hen diets under heat stress and improve the total antioxidant capacity (T-AOC), glutathione (GSH), glutathione peroxidase (GSH-PX), superoxide dismutase (SOD) and catalase (CAT), while lipid peroxidation (MDA) and nitric oxide (NO) are reduced in blood and tissues (Table 2.4) (El-Maaty et al., 2014; Hosseini-Vashan et al., 2015; Ibtisham et al., 2019; Reis et al., 2019; Nawab et al., 2019). Herbs, spices, and other plants can be classified into stronger and weaker antioxidant groups which have potential in antioxidant mechanisms related to chemical properties, site of action, and supplementation levels in each phytogenic antioxidant substance. Phenolics and flavonoids are mainly contained in herbs, vegetables, medicinal plant spices, and fruits and contain efficient natural antioxidant substances (Zdanowska-Sasiadek et al., 2019).

Table 2.4 Effects of phytogenic supplementation on antioxidant activity of poultry under heat stress conditions.

Condition	Type of phytogenic substances	Results of antioxidant activities	Bioactive compound
TN (28°C, 50% RH) CHS (38°C, 90% RH, 4 h/day)	Basal diets (control) 0.050% Cinnamon powder 0.050% Turmeric powder 0.050% Ginger powder 200 mg of Ascorbic acid	Improved SOD and GSH and reduced MDA in plasma with cinnamon, turmeric, and ginger powder or ascorbic acid	Gingerol, gingerdione, and shogaols (El-Maaty et al., 2014)
TN (21°C, 55% RH) CHS (34±1°C, 55% RH, 5 h/day)	Basal diets (control) 0% Dried tomato pomace (DTP) 3% DTP 5% DTP	Enhanced GSP-PX and SOD, Reduced MDA in plasma by 5% DTP	3 and 5% of DTP contains 420 and 708 mg lycopene/kg diet, respectively (Hosseini-Vashan et al., 2015)
Temperature at 29 °C and 37°C during the experimental period	0% of grape pomace flour (GPF) 1% of GPF 2% of GPF 3% of GPF	Increased SOD, T-AOC, and GSH-PX in serum by 1-3% of GPF Increased T-AOC and decreased MDA of yolk in 1-3% of GPF	58.5 mg of resveratrol per g of GPF (Reis et al., 2019)

Table 2.4 Effects of phytogenic supplementation on antioxidant activity of poultry under heat stress conditions (continued).

Condition	Type of phytogenic	Results of antioxidant activities	Bioactive compound
Thermoneutral group (NC) at 22–28°C, heat-stressed control group (HC) at 32–38°C, 60-80% HR	NC HC HC + 1% Ginger powder HC + 0.332% Chinese herbal medicine HC + 0.1% Ginger powder + 0.332 g/kg Chinese herbal medicine	Improved SOD, NO, T-AOC, MDA, and GSH-PX in serum by supplemented groups Improved CAT in serum by HC + Chinese herbal medicine and ginger powder + Chinese herbal medicine	Ginger: gingerol, gingerdione, and shogaols; Chinese herbal medicine: vitamins, lipids, amino acids proteins, trace elements (Ibtisham et al., 2019)

2.7 The application of the transcriptomic technique in animal research

A few years ago, scientists developed special techniques to identify the expression levels of genes in mRNA transcripts of living organisms by using a transcriptomic technique with Northern blotting, real-time quantitative reverse transcription PCR (RT-PCR), microarray and RNA-sequencing (RNA-seq). Due to gene quantity changes rapidly their expression level through the regulation of different environmental changes (temperature, humidity, diseases, and nutritional status) and physiological factors (age, breed, growth rate, production, and egg quality) (Zampiga et al., 2018). The transcriptomic technique has a high potential in identifying gene markers in biological samples such as cells, tissue, and body fluids of animals including animal products. Researchers can use gene marker data to describe molecular processes in response to different environmental and physiological factors (Zdunczyk and Pareek, 2008). Nutrients are one of the environmental factors that influence the phenotypic responses of animals which are "nutrigenomics" (Kaput et al., 2005) (Figure 2.5). In animal nutrition research the transcriptomic technique has been applied to investigate the efficiency of dietary treatments. There are many objectives in the application of the transcriptomic technique in nutrition research as follows (Wang et al., 2006):

- Composition, characteristics, and efficiency of diet
- Metabolism and regulation of nutrients
- Functions of nutrients and phytochemicals in growth, reproduction, and health
- Digestion and absorption of nutrients
- Biomarkers and individualized requirements of nutrients
- Gene profiles and characteristics in cells, tissues, and physiological fluids
- molecular mechanisms of tissue damage or metabolic changes caused by environmental stress

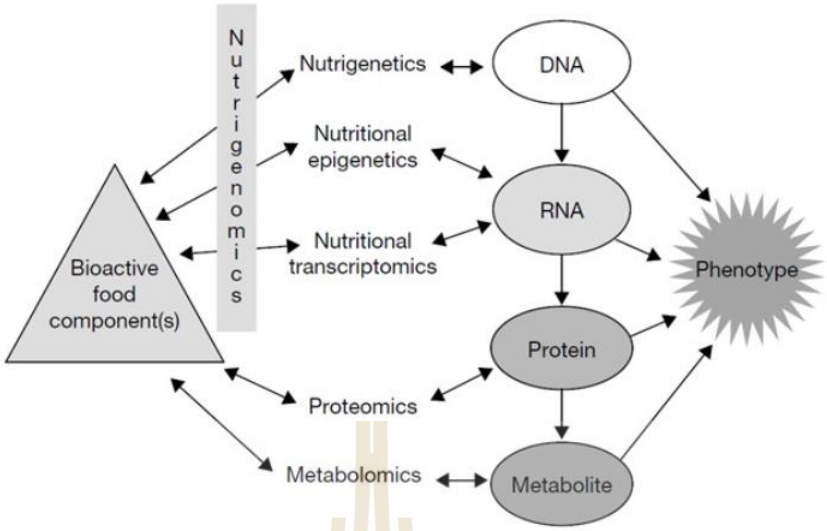

Figure 2.5 The influence of bioactive nutrients on phenotype responses (Tai and Gillies, 2007).

2.8 Transcriptome responses to heat stress for gene marker identification in poultry research

Heat stress can induce a change in the physiology and metabolism of poultry. The transcriptome technique has been used to study the function of molecule responses and chaperone changes to HS. Therefore, classifying the differentially expressed genes (**DEGs**) and gene marker identification under HS conditions may be beneficial to understanding the role of HS in poultry performances or production which leads to improving the ability of broilers to resist and alleviate the negative effect of HS (Kim et al., 2021; Lim et al., 2022). The transcriptome comparative analysis between native and commercial chicken breeds to HS response found that the expression of PT1A and ANGPTL4 genes in native chickens, and HSP90B1 and HSPA5 genes in commercial chickens could be potential candidate genes involved with HS. These genes exhibited enriched pathways related to metabolism activity and inflammatory reactions (Sánchez et al., 2022). Cheng et al. (2018) investigated global genes in the small yellow follicle tissues of chickens in response to acute HS and revealed that a total of 176 DEGs were identified between 25°C and 55% RH as a control group and 36°C and 55% RH for 4h as an HS group. The upregulated HSP25, HSP70, HSP90aa1, HSD17B1, and PrDX4 genes in the small yellow follicle tissues after

acute HS indicated the presence of mechanisms that are responsive and capable of safeguarding cells against apoptosis and oxidative damage. Furthermore, transcriptome analysis in the uterovaginal junction (UVJ) containing sperm storage tubules (SSTs) in breeder hens under thermoneutral (23°C) and heat stress (36°C for 6 h) indicated that the different gene expressions of HSP25, HSPA5, HSPA8, GKN2, IL4I1, PDK4, TAT, CA, LHCGR, GPX, and ISGs were associated with protein processing in endoplasmic reticulum, neuroactive ligand–receptor interaction, biosynthesis of amino acids, ferroptosis, and nitrogen metabolism pathways, which provide an understanding of the molecular pathways and networks within UVJ containing SST tissues for preventing heat stress-induced fertility loss in breeder hens. These candidate genes were suitable for further investigation in SSTs in the UVJ tissues of HS hens and provided an understanding of the molecular pathways and networks within UVJ-containing SST tissues for preventing heat stress-induced fertility loss in breeder hens (Kubota et al., 2023). Thus, transcriptome analysis could be useful to help understand the HS response in poultry.

2.9 The study of gene markers involved in reproductive performance traits of the female breeder hens

Transcriptomic analysis using RNA-seq technology has high accuracy, a short turnaround time, and the ability to process large sample volumes. RNA-seq technology is now the predominant method for identifying variations in gene expression among individuals in diverse developmental states. Its application has been widespread in the field of animal research (Zhang et al., 2014; Reuter et al., 2015). Zhang et al. (2021) studied the differentially expressed genes (**DEGs**) between high and low egg-laying rates in female breeder hens to identify essential candidate genes related to the egg-laying rate. The results showed that there were 235 DEGs in the ovarian tissues between high and low egg-laying rates. Gene Ontology (**GO**) analysis and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) analysis revealed that upregulated 209 DEGs and downregulated 26 DEGs were significantly enriched in the longevity regulating multiple species pathway, estrogen signaling pathway, and PPAR signaling pathway. The expression of FOXA2, MED37D, RXFP2, INSL3, and HSP70

genes were identified as essential candidate genes related to the egg-laying rate which can be used as base information for improving the egg production of laying hens. Transcriptome analysis of the uterus of laying hens demonstrated that out of a total of 12,253 genes, the expression of MEPE, BPIFB3, RARRES1, WAP, PER2, CRY2, CRY1, CLOCK, BMAL1, JUN, FOS, and genes for mitochondrial oxidative phosphorylation, active transport, and energy metabolism indicated that these genes changed their expression throughout eggshell formation supporting their importance (Pertiñez et al., 2020). The study by Yang et al. (2020) showed that transcriptome analysis in the uterovaginal junction (UVJ) tissue had a total of 574 DEGs, including 266 upregulated and 308 downregulated DEGs. These gene expressions of COMP, CTGF, IMPG2, PCOLCE, POSTN, RSPO3, AREG, RAMP3, SFRP1, and SSTR1 by GO analysis are mainly enriched in heparin binding, glycosaminoglycan binding, and response to estradiol and ion transport, whereas a KEGG analysis showed that CACNA1G, PDE1C, PDGFRB, SLC8A1 B3GNT7, CSGALNACT1, GLCE, and ST3GAL1 genes were associated with a calcium signaling pathway and glycosaminoglycan biosynthesis. This finding also indicated that the HIP1, PDE1C, and calcium-related genes in UVJ-containing sperm storage tubules (**SST**) were candidate genes to refer to capacity in sperm storage duration within the SST of hens. The outcome of the transcriptomic study in characteristic candidate genes and mechanisms associated with the reproductive performance of hens may be useful for a comprehensive understanding of the mechanism.

These gene markers, as previously mentioned, can provide the basic information to reflect the important mechanisms and contributions to the development of productive performance in female breeder flocks and they help to guide the development of individualized requirements of nutrients to optimize uterine fluid and egg composition, including the enhancement of heat stress tolerance of chickens (Wang et al., 2006). Daryabari et al. (2014) have studied the oviductal expression of avidin and avidin-related protein-2 in broiler breeder hens and found that they are increased by supplementary biotin. They concluded that sustaining the sperm in the SST may require avidin and perhaps avidin analogs (Foye-Jackson et al., 2011). Akazawa et al. (2019) and Da Silva et al. (2019) also indicated that after fertilization, chicken embryonic development utilizes the nutritional and

functional molecules from the egg white and yolk under the regulation of its genes. Thus, supplementation with bioactive substances such as bioactive amino acids, fatty acids, polyphenols, antioxidants, vitamins, minerals, and prebiotics, etc. via the maternal diet may enhance fertility and the hatchability rate.

All of the above-mentioned comparative studies are important in the suitable identification of gene profiles for use as a database in enhancing reproductive performance, fertility, and hatchability of female breeder hens. A deeper insight and understanding of gene functions that result in sperm survival in SST, embryo development, ovary composition, and the effect of heat stress on reproductive performances can be useful to predict the specific characteristics of female breeder hens in each breed or condition. As a result, the concept of modification of gene expression to change function has been developed. In this study, we would like to use bioactive nutrients in poultry diets to modulate gene targets in UVJ tissue containing SST, when female breeder hens were affected by heat stress conditions. Therefore, the feed formulation development for female breeder hens under heat stress should take into account the gene targets together with a knowledge of bioactive nutrients or dietary properties to combine them in a relationship that is appropriate to each objective.

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CHAPTER III

ANTIOXIDANT PROPERTIES AND CYTOTOXIC EFFECTS OF SELECTED EDIBLE PLANTS IN SOUTHEAST ASIA FOR FURTHER USE AS PHYTOGENIC ANTIOXIDANT ADDITIVES

3.1 Abstract

Excessive free radicals in human and animal bodies can cause oxidative stress (OS) which damages cells and tissues. Plant materials with high antioxidant potential would resolve the OS problem. Thus, this study proposed to investigate the total phenolic (TPC) and flavonoid contents (TFC), antioxidant capabilities, and cytotoxicity in 17 edible plant materials from herbs, fruits, vegetables, and plant by-products available in Southeast Asia for future use in the food or feed industry. Among 17 plant materials, *Syzygium aromaticum* (clove), *Camellia sinensi* (green tea pomace) from the beverage industry, and *Persicaria odorata* (Vietnamese coriander) showed a prominent amount of TPC and TFC. These three plants and their combination (1:1:1 ratio, v:v:v) also possessed a remarkable antioxidant function in terms of DPPH, ABTS, and FRAP, as well as showing a strong ROS inhibition through HepG2 cells. The cytotoxicity test of the crude extract of clove, green tea pomace, and Vietnamese coriander, or their combination can be used between 0.032 and 0.255, 0.011 to 0.088, 0.022 to 0.178 and 0.021 to 0.346 mg/mL, respectively, without impeding cell viability. A combined mixture of clove, green tea pomace, and Vietnamese coriander revealed the synergistic properties of antioxidants and cell safety. This indicates that there is a potential use of various antioxidant bioactive compounds in plant materials tested for use as phytogenic antioxidant additives

Keywords: Phytogenic substance; Natural antioxidant; Edible plant; Antioxidant capacity; Oxidative stress

3.2 Introduction

Free radicals in human and animal bodies are generally produced either from normal essential metabolic processes in the body or from external sources such as exposure to cigarette smoking, alcohol, radiation, and environmental toxins in humans (Xu et al., 2017), or intensive genetic selection towards rapid growth and high yield, high temperature coupled with humidity, improper diets and management factors in animals (Barrett et al., 2019). Excess free radicals can induce oxidative stress (OS) which leads to the damage of cells and tissues. This can negatively impact the development of various types of chronic and degenerative diseases of humans (Tan et al., 2020), as well as, productivity and reproduction, nutrient availability, and immunity in animals (Torki et al., 2021). Although synthetic antioxidants such as vitamin E, vitamin C, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), glutathione, coenzyme Q10, L-carnitine, selenium or zinc, etc. are widely used in food or feed in order to scavenge harmful OS (Herve et al., 2019; Liu et al., 2020), however, natural antioxidants from plant or phytogenic materials are also becoming of increasing interest nowadays. In particular, plant-based materials that contain phenolic compounds are exposed to high antioxidant activities with the potential to alleviate the adverse effects of OS induced diseases (Saracila et al., 2021). The antioxidant capacity depends upon the quantity and quality of phenolic compounds in each plant species which can perform as reducing agents, single oxygen quenchers, hydrogen donors, metal chelators, ferryl hemoglobin reducers and which can inhibit enzymes associated with the formation of free radicals (Altemimi et al., 2017). Therefore, an investigation of novel phytogenic antioxidants should provide some useful knowledge to solve OS problems.

Plants, fruits, vegetables, spices, and herbs are mainly a source of natural antioxidants (Xu et al., 2017; Alqethami and Aldhebiani, 2021) which are increasingly attractive due to safety concerns and which are also cheaper compared to synthetic antioxidants (Stankovic et al., 2016). Sufficient exogenous antioxidant intake from natural sources has been reported to relieve the negative effects of OS by preventing the propagation of an oxidative chain reaction, adsorbing and neutralizing free radicals, quenchers of singlet oxygen, and reducing agents in humans (Xu et al., 2017).

There is still little information on plant materials as well as their by-products which have a high antioxidant potential, especially for animals which could benefit from recycling by-products to reduce environmental pollution (Tsiplakou et al., 2021). In particular, in Thailand which is located in a subtropical area, the temperature reaches up to 40°C in the summer season, leading to an increase in OS and reduced animal production. Therefore, phytogenic antioxidant properties would resolve the OS problem. In our research, 17 edible plants including by-products from the beverage industry were selected according to several previously reported antioxidant properties. For example, *Camellia sinensis* (green tea), *Curcuma longa* L. (tumaric), *Syzygium aromaticum* (cloves), *Mentha piperita* L (peppermint*), Persicaria odorata* (Vietnamese coriander) or grape seed contain antioxidant phenolic compounds, which are most effective against the production of free radicals and the strongest radical scavenging activity (Basit et al., 2020; Liu et al., 2020).

Therefore, the purpose of this study was to investigate the phenolic and flavonoid contents, antioxidant capacities and cytotoxicity in herbs, fruits, vegetables and plant by-products available in Southeast Asia which could be suitable for future use.

3.3 Materials and methods

3.3.1 Plant material preparation and extraction

Plant extraction was slightly modified from a previous method (Ngo et al., 2017). A total of 17 plant materials, including local Thai vegetables, edible plants, herbs, spices, fruits, or plant by-products from the beverage industry (Table 3.1), were dried at 40–50°C until the moisture level was <10% and subsequently ground to approximately 1 mm. Two g of each plant material were weighed in a centrifugal tube then 40 mL of 50% ethanol (ethanol:DI water, 50:50, v:v) were added. The mixtures were put in an incubator shaker at room temperature, at 100 rpm for 1 h, sonicated for 30 min at 35°C, centrifuged at 4,500 rpm for 10 min, and filtered via Whatman filter paper No. 1. This extraction procedure was performed twice with a fresh solvent. Then each extraction solution was set to 100 mL with 50% ethanol and maintained in darkness at 4°C until it was used.

Table 3.1 Plant materials mostly containing natural antioxidant substances found in Southeast Asia.

Scientific/ Common name	Parts of plants
Plants	
Tiliacora triandra Colebr. Diels (Yanang)	Leaves
Persicaria odorata (Vietnamese coriander)	Leaves
Hibiscus sabdariffa L. (Jamaica Sorrel, Red sorrel, Roselle, Rozelle)	Flowers
Opuntia ficus indica L. (Indian fig opuntia, Barbary fig, Cactus pear, Prickly pear, Spineless cactus)	Leaves
Sida acuta Burman. f. (Sida, Stubborn weed)	Leaves
Herbs	
Centella asiatica (L.) Urban. (Gotu kola, Asiatic pennywort)	Leaves
Mentha piperita[1]	Leaves
Mentha piperita[2]	Leaves
Mentha piperita[3]	Leaves
Spices	
Syzygium aromaticum (L.) (Clove)	Flowers
Curcuma longa L. (Turmaric)	Roots
By-product from beverage industry	
Vitis vinifera L. (Grape pomace)	Skin and Seeds
Morus alba L. (Mulberry pomace)	Skin and Seeds
Camellia sinensis (Black tea pomace)	Stalk and Leaves
Camellia sinensis (Green tea pomace 1)[4]	Stalk and Leaves
Camellia sinensis (Green tea pomace 2)[5]	Stalk and Leaves
Coffea arabica L. (Coffee pomace)	Seeds

[1,2]*Mentha piperita* L. derived from companies 1 and 2 under hydroponic culture system, respectively. [3]*Mentha piperita* L. derived from general sale on the local market. [4,5]*Camellia sinensis* (green tea pomace) derived from beverage shop and beverage industry, respectively.

3.3.2 Determination of total phenol and flavonoid contents

The total phenol contents (TPC) in the extraction solution were analyzed using a modified method (Komes et al., 2011). The 20 µL extraction solution was placed into a 96-well microplate and blended with 100 µL of folin–ciocalteu reagent, which was diluted 10 fold with DI water. After 5 min of incubation, 75 µL of 75% sodium carbonate solution was added and incubated in the darkness for 2 h. The wavelength at 740 nm was used to detect the absorbance of the reaction using a microplate spectrophotometer (Thermo ScientificTM, MultidkanTM GO, Japan). Gallic acid was used as a concentration standard at 5–100 µg/mL for the calibration curve, in which 50% ethanol was used as a blank. The data were calculated as mg of dry weight sample/mg gallic acid equivalents (mg GAE/g DW). The measured values were carried out in triplicate. The total flavonoid content (TFC) was assessed as per previous procedure (Komes et al., 2011). The 50 µL of extraction solution was mixed well with 70 µL of DI water, and 15 µL of 5% sodium nitrite solution in a 96-well microplate, and the mixture was allowed to infuse for 5 min. The mixture was then added with 15 µL of 10% aluminum chloride solution. After 6 min of incubation, 100 µL of 1 M sodium hydroxide solution was supplemented and incubated for 10 min, then measured at a wavelength of 510 nm, and 50% ethanol was used as a blank. The quercetin was used as a concentration standard (5–100 µg/mL). The TFC content was calculated and reported as mg quercetin equivalents/g dry weight sample (mg QE/g DW).

3.3.3 Determination of antioxidant activity

3.3.3.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH•) scavenging activity assay

The $DPPH^{\bullet}$ radical scavenging was detected in accordance with a previous protocol (Nuengchamnong et al., 2009). The reaction between 75 µL of each extraction solution and 150 µL of 0.6 mM DPPH in ethanol was incubated in darkness for 30 min and the absorbance was assessed at 517 nm.

3.3.3.2 Scavenging activity assay of 2, 2'-azinobis-(3- ethylbenzothiazoline -6-sulfonic acid) ($ABTS^{\bullet+}$) radical

The scavenging activity of the $ABTS^{\bullet+}$ radical was measured following Re et al. (1999) procedure. $ABTS^{\bullet+}$ stock solution was prepared from 7.4 mM

of ABTS and 2.6 mM of potassium persulfate in 10 mM phosphates buffer solution (pH 7.4) and stored in the dark at 4°C for 12 to 16 h. Prior to use, the $ABTS^{\bullet+}$ stock solution was diluted with a 10 mM cooled phosphate buffer to achieve an absorbance value of 0.70±0.02 at 734 nm. 20 µL of extracted solvents were put into 96-well microplates and reacted with 180 µL of $ABTS^{\bullet+}$ reagent and the wavelength of 734 nm was used to determine the reaction after 6 min inoculation. Trolox (25–1000 µM/mL) was used as the standard. The values for radical scavenging in both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ were represented as inhibition (%) = [(absorbance of control – absorbance of sample) / (absorbance of control)] × 100, then compared to the standard curve and reported in mM equivalent trolox/g dry weight sample (mM TE/g DW).

3.3.3.3 The ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out using the Benzie and Strain (1996) method. The working FRAP reagent was freshly assembled before use by adding 5 mL of 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine in 40 mM hydrochloric acid, 5 mL of 20 mM iron chloride, and 50 mL of 0.3 M acetate buffer (pH 3.6), respectively, then incubated at 37°C for 15 min. The 200 µL FRAP reagent was combined with 20 µL of plant extraction solvents in 96 well microplates and incubated for 30 min, the absorbance was then measured at 593 nm. A standard calibration curve was determined according to the concentration of 25–1000 µM Trolox/mL compared to the radical scavenging activity of the extracted solvents.

3.3.4 Assessments of the combination of the three selected plant materials with high antioxidant capacity

The three plant materials (clove, green tea pomace from the beverage industry, and Vietnamese coriander) with the highest antioxidant activity of DPPH, ABTS, and FRAP were selected. The extraction solution stocks of clove, green tea pomace, and Vietnamese coriander obtained from the part 2.1 which contained TPC 3.53, 2.31, and 1.26 mM GAE/mL, respectively, were split into 1, 2, and 3 proportions and mixed in a total of 25 combinations (v:v:v) and stored in darkness at 4°C until assessed. The combined ratios were calculated using the formula: n^A as n = number of herbal plants (three types) and A = number of proportions (3 levels), then calculated as 3^3 = 27

combinations of which the 2:2:2 and 3:3:3 merged parts were not selected in this analysis because they had the same ratio e.g. 1:1:1.

3.3.5 The assessments of selected plants on cell culture

3.3.5.1 Cell lines and culture medium

A human hepatoma cell line (HepG2) was cultured in a 96 well microplate at 7,000 cells/well with Dulbecco's modified eagle's medium (DMEM) added with 10 mg/mL of fetal bovine serum and 1 mg/mL of antibiotic. HepG2 cells were cultured in a CO_2 incubator with 5 mg/mL of CO_2 at 37°C for 24 h.

3.3.5.2 Plant extraction for cell culture

Three selected plant materials and their combination (clove, green tea pomace from the beverage industry, and Vietnamese coriander, 2 g of each) were extracted with 50% ethanol and evaporated at 80 rpm 40°C, until approximately 10 mL remained, then freeze-dried (Gamma 2–6 LSC, Christ, UK). The crude weight was recorded and then the mixture was dissolved with 10 mL of dimethyl sulphoxide (DMSO) and purified via a 0.2 µm syringe filter.

3.3.5.3 Cytotoxicity test

Cytotoxicity studies were conducted to assess the HepG2 cell viability in plant materials with strong antioxidant properties using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (Angius and Floris, 2015). HepG2 cells were treated with 100 µL of various concentrations of crude extract of clove at 0.032, 0.064, 0.128, 0.225, 0.510, and 1.020 mg/mL, or green tea pomace at 0.011, 0.022, 0.044, 0.088, 0.175, and 1.350 mg/mL, or Vietnamese coriander at 0.022, 0.044, 0.089, 0.178, 0.355, and 0.710 mg/mL, or their combination at 0.021, 0.043, 0.087, 0.173, 0.346, and 0.692 mg/mL for 24 h. Then 100 µL of 0.5 mg/mL MTT solution was mixed and incubated in darkness for 2 h, the supernatant was then detached from each well, and 100 µL of DMSO was suspended in formazan crystals prior to an absorbance measurement at 570 nm. The percentages of HepG2 cell viability were expressed as % cell viability = (absorbance of sample/absorbance of control) × 100.

3.3.6 Assessment of cellular radical scavenging activity

The HepG2 cells were treated with 100 µl per well of each of the two selected appropriate concentrations of clove (0.032 and 0.064 mg/mL), green tea pomace (0.001 and 0.022 mg/mL), Vietnamese coriander (0.022 and 0.044 mg/mL), and

their mixture (0.087 and 0.173 mg/mL), as these values produced the most beneficial effects on the percentage of cell viability based on the cytotoxicity test results and 10 mM N-acetylcysteine (NAC, positive control) in 100 mg/mL DMEM, and incubated for 24 h before a cellular ROS scavenging measurement was conducted using a 2'-7' di chlorofluorescin diacetate (DCFH-DA) fluorescence dye assay. Subsequently, 100 µL of 1 mM H_2O_2 which served as an intracellular radical was mixed with the treated cells in a 96-well microplate for 30 min while H_2O_2 was incubated with untreated cells as a negative control. The HepG2 cells were then stained with DCFH-DA at a final concentration of 2 µM for 30 min and finally examined by fluorescence spectrophotometry at a wavelength of 530 nm (Wang and Joseph, 1999).

3.3.7 Statistical analysis

The statistical data were analyzed by ANOVA in CRD of SPSS 16.0 software (SPSS Inc, 2007). Significant differences among treatments were assessed by Tukey. A significance level at $P < 0.05$ was used.

3.4 Results

3.4.1 Total phenolic and total flavonoid contents in plant materials

The TPC and TFC in 17 plant materials varied considerably (Table 3.2). The highest TPC and TFC were presented in Vietnamese coriander, followed by green tea pomace (beverage shop and beverage industry) and cloves, with a concentration ranging from 63.06 to 176.97 mg GAE/g DW and 135.07 to 360.50 mg QE/g DW ($P < 0.05$), respectively. Although *Curcuma longa* L. had a high TFC of 184.07 GAE/g DW compared to green tea pomace from beverage shop and Vietnamese coriander, these plants were lower in TPC ($P < 0.05$). Other plants in this study also contained median TPC values such as *Mentha piperita* L. from a local market, *Centella asiatica* (L.) Urban and *Camellia sinensis* (black tea pomace) at 38.63, 39.00, and 48.73 mg GAE/g DW, respectively. While the median TFC values were also found in *Mentha piperita* L. from company 2, *Camellia sinensis* (black tea pomace), *Mentha piperita* L. from company 1, *Centella asiatica* (L.), and Mentha piperita L. from a local market at 70.78, 70.78, 86.92, 134.78 and 146.50 mg QE/g DW, respectively ($P < 0.05$). The remaining plants which are not named here contain TPC and TFC from 4.58 to 30.48 mg GAE/g DW and 3.30 to 36.49 mg QE/g DW, respectively ($P < 0.05$).

3.4.2 Antioxidant properties

The antioxidant activity of the 17 plant materials is presented in Table 3.2. The results showed that cloves and green tea pomace of both sources showed a remarkably significant ability to function as an antioxidant, which exhibited the highest scavenging capacity of DPPH, ABTS, and FRAP compared to other plant materials ($P < 0.05$). All plant materials in this study possessed free radical scavenging properties, ranging from 4.48 to 94.95% or 12.85 to 293.42 mM TE/g DW DPPH, 46.38 to 279.50 mM TE/g DW ABTS, and 15.76 to 720.19 mM TE/g DW FRAP. Although the TFC values of *Curcuma longa* L. and *Mentha piperita* L. (from a local market) were greater than Vietnamese coriander, their DPPH, ABTS and FRAP scavenging capacity values were less potential (Table 3.2, $P < 0.05$). In addition, the scavenging capacity of DPPH and FRAP of *Camellia sinensis* (black tea pomace), *Centella asiatica* (L.) Urban. and *Morus alba* L. (Mulberry pomace) were lower than the cloves, green tea pomace, and Vietnamese coriander, but their potential was higher compared to other plants such as *Curcuma longa* L., *Sida acuta Burm*. f., *Hibiscus sabdariffa* L. or *Vitis vinifera* L. ($P < 0.05$).

Various combination ratios of cloves, green tea pomace, and Vietnamese coriander, and their effects on antioxidant capacity are presented in Table 3.3. The eight portions with the highest antioxidant ability to scavenge the free radicals were green tea pomace, cloves, and Vietnamese coriander in combinations of 1:1:1, 1:2:1, 1:2:2, 1:3:1, 2:2:1, 2:3:1, 3:3:1 and 3:3:2 (v:v:v), all of which showed high antioxidant properties through the DPPH, ABTS, and FRAP scavenging ($P < 0.05$). A high DPPH scavenging value was observed in the ratio of 1:3:1, 1:2:1, 2:3:1, 3:3:1, 3:3:2, 1:3:2 and 1:2:1, respectively, while a high FRAP scavenging value was also found in the ratio 1:1:1, followed by 2:3:1, 1:2:1, 3:3:1, 2:2:1 and 3:3:2, respectively ($P < 0.05$). However, these combination ratios reported for both DPPH and FRAP scavenging values showed no significant difference ($P > 0.05$), except the ratio of 2:21 and 3:3:1 in ABTS scavenging ($P < 0.05$). In this study, cloves, green tea pomace, and Vietnamese coriander in the ratio 1:1:1 (v:v:v) were selected for further assessments, as this proportion reduces the use of cloves and green tea pomace relative to other ratios.

Table 3.2 Total phenolic and flavonoid contents and antioxidant capacity of 17 extracted plant materials.

Items	TPC[1]	TFC[2]	DPPH[3]		ABTS[4]	FRAP[5]
	mg GAE/ g DW[6]	mg QE/ g DW	% Inhibition	mM TE/ g DW	mM TE/ g DW	mM TE/ g DW
Plants						
Sida acuta Burm. f.	14.30^{k}	27.46^{gh}	27.15^{j}	83.39^{l}	235.69^{bc}	96.04^{k}
Hibiscus sabdariffa L.	16.54^{jk}	17.62^{h}	44.62^{g}	137.51^{h}	208.51^{d}	124.85^{j}
Persicaria odorata Lour.	63.06^{d}	135.07^{d}	89.64^{b}	276.98^{b}	277.80^{a}	315.28^{d}
Tiliacora triandra Colebr. Diels	9.77^{l}	17.09^{h}	17.97^{k}	54.94^{k}	136.91^{ef}	55.57^{m}
Opuntia ficus indica L.	4.58^{m}	3.30^{i}	4.48^{l}	12.85^{l}	46.38^{h}	15.76^{n}
Herbs						
Centella asiatica (L.) Urban.	39.00^{f}	134.78^{d}	72.86^{d}	224.98^{d}	277.19^{a}	231.90^{f}
Mentha piperita L.[7]	30.48^{g}	86.92^{e}	46.81^{g}	144.30^{g}	223.19^{cd}	160.71^{h}
Mentha piperita L.[8]	25.34^{h}	70.78^{f}	46.17^{g}	142.31^{g}	216.03^{d}	138.00^{i}
Mentha piperita L.[9]	38.63^{f}	146.50^{cd}	61.46^{f}	189.68^{f}	266.76^{a}	246.33^{e}
Spices						
Syzygium aromaticum (L.)	176.97^{a}	360.50^{a}	94.23^{a}	291.19^{a}	275.37^{a}	720.19^{a}
Curcuma longa L.	17.41^{j}	184.07^{b}	40.73^{h}	125.45^{h}	243.94^{b}	93.76^{k}
Byproduct from beverage industry						
Camellia sinensis (Green tea pomace 1)[10]	71.91^{c}	149.07^{c}	94.95^{a}	293.42^{a}	278.86^{a}	451.00^{c}
Camellia sinensis (Green tea pomace 2)[11]	115.85^{b}	189.78^{b}	93.84^{a}	289.98^{a}	279.50^{a}	528.09^{b}
Camellia sinensis (Black tea pomace)	48.73^{e}	70.78^{f}	85.96^{c}	265.56^{c}	277.07^{a}	242.57^{e}
Morus alba L.	20.94^{i}	34.02^{g}	63.90^{e}	197.23^{e}	275.62^{a}	192.85^{g}
Coffea arabica L.	11.75^{l}	36.49^{g}	29.26^{j}	89.92^{j}	127.56^{f}	92.38^{k}
Vitis vinifera L.	10.92^{l}	28.80^{gh}	31.60^{i}	97.17^{i}	149.53^{e}	74.23^{l}
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Pooled SEM	4.7483	9.6904	3.4580	10.7120	8.2395	20.540

$^{a-n}$ Means within each column with different superscripts are significantly different ($P < 0.05$). [1]TPC: Total phenolic content. [2]TFC: Total flavonoid content. [3]DPPH: 2,2-Diphenyl-1-picrylhydrazyl scavenging activity assay. [4]ABTS; 2,20 -azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt scavenging activity assay. [5]FRAP: Ferric reducing antioxidant power. [6]DW: Dry weight of sample. [7,8]*Mentha piperita* L. derived from companies 1 and 2 under hydroponic culture system, respectively. [9]Mentha piperita L. derived from general sale on the local market. [10,11]*Camellia sinensis* (green tea pomace) derived from beverage shop and beverage industry, respectively.

Table 3.3 Various combination ratios of extracted *Camellia sinensis* (green tea pomace), *Syzygium aromaticum* (clove), and *Persicaria odorata* (Vietnamese coriander) on antioxidant capacity.

The combination ratios[1], v:v:v			DPPH[2]		ABTS[3]	FRAP[4]
Green tea pomace	Clove	Vietnamese coriander	% Inhibition	mM TE/ g DW[5]	mM TE/ g DW[5]	mM TE/ g DW[5]
1	1	1	34.44^{fgh}	353.16^{fgh}	552.64^{abcdef}	638.85^{a}
1	1	2	30.81^{ij}	315.66^{ij}	$485.09^{bcdefghi}$	583.04^{bc}
1	1	3	27.12^{lm}	277.62^{lm}	430.08^{fghi}	540.57^{de}
1	2	1	41.28^{b}	423.82^{b}	$522.71^{abcdefgh}$	596.80^{bc}
1	2	2	36.94^{de}	378.98^{de}	595.92^{abc}	521.86^{ef}
1	2	3	33.80^{gh}	346.58^{gh}	$528.37^{abcdefgh}$	503.43^{fg}
1	3	1	43.62^{a}	447.93^{a}	599.56^{abc}	576.38^{bc}
1	3	2	39.93^{b}	409.90^{b}	546.17^{abcdef}	441.05^{ij}
1	3	3	36.31^{def}	372.50^{def}	$528.38^{abcdefgh}$	417.52^{ijk}
2	1	1	32.60^{hi}	334.14^{hi}	$474.17^{cdefghi}$	377.61^{k}
2	1	2	28.13^{kl}	288.03^{kl}	461.22^{defghi}	414.14^{jk}
2	1	3	26.88^{lm}	275.07^{lm}	407.42^{ghi}	4811.10^{gh}
2	2	1	37.81^{cd}	388.02^{cd}	$489.51^{bcdefghi}$	585.45^{bc}
2	2	3	30.92^{ij}	316.82^{ij}	452.33^{efghi}	532.86^{def}
2	3	1	41.15^{b}	422.43^{b}	650.94^{a}	609.61^{ab}
2	3	2	36.08^{def}	370.16^{def}	616.96^{ab}	325.19^{lm}
2	3	3	32.07^{hj}	335.21^{hi}	5952.69^{abcd}	318.10^{m}
3	1	1	29.34^{jk}	300.57^{jk}	430.48^{fghi}	319.38^{m}
3	1	2	27.70^{klm}	283.57^{klm}	377.09^{i}	329.43^{lm}
3	1	3	25.77^{m}	263.70^{m}	394.89^{hi}	359.38^{l}
3	2	1	35.53^{efg}	364.42^{efg}	572.86^{abcde}	450.52^{hi}
3	2	2	32.99^{h}	338.18^{h}	430.08^{fghi}	426.00^{ijk}
3	2	3	30.40^{j}	311.51^{j}	410.26^{ghi}	419.48^{ijk}
3	3	1	39.66^{bc}	407.14^{bc}	$516.24^{bcdefgh}$	588.57^{bc}
3	3	2	37.85^{cd}	388.44^{cd}	$541.72^{abcdefg}$	562.09^{cd}
P-value			0.0001	0.0001	0.0001	0.0001
Pooled SEM			0.5019	5.1836	8.6956	9.0763

[a - m] Means within each column with different superscripts are significantly different ($P < 0.05$). [1]Green tea pomace from beverage industry in portions 1, 2, and 3 in the mixture; Clove in portions 1, 2, and 3 in the mixture; Vietnamese coriander in portions 1, 2, and 3 in the mixture, respectively. [2]DPPH: 2,2-Diphenyl-1-picrylhydrazyl scavenging activity assay. [3]ABTS: 2,2' - azino- bis (3- ethylbenzothiazoline- 6- sulphonic acid) diammonium salt scavenging activity assay. [4]FRAP: Ferric reducing antioxidant power. [5]DW: Dry weight of sample.

3.4.3 Cytotoxicity test and intracellular radical scavenging activity

The results of the cytotoxicity assessments on the percentage viability of HepG2 cells (Figure 3.1) showed that the crude extract content of cloves ranged from 0.032 to 0.255 mg/mL (Figure 3.1A), while green tea pomace ranged from 0.011 to 0.088 mg/mL (Figure 3.1B) and Vietnamese coriander from 0.022 to 0.178 mg/mL (Figure 3.1C) which showed a non-toxic dose in the HepG2 cells. The combined extract (1:1:1, v:v:v) was used at a concentration of 0.021 to 0.346 mg/mL ($P < 0.05$) (Figure 3.1D) which indicates that a higher dose of mixed plants will still be capable of encouraging the cell viability of HepG2 cells compared to individual plants.

Assessment of H_2O_2 scavenging activity using HepG2 cells (Figure 3.2) revealed a concentration of 0.032 to 0.064 mg/mL for the crude clove extract with significantly higher antioxidant capacity compared to the negative control composed of H_2O_2 ($P < 0.05$). Furthermore, a concentration at 0.032 to 0.064 mg/mL of crude clove extract revealed a significant effect on the scavenging of the H_2O_2 radical in the negative control, although it still had less potential than positive control composed of N-acetylcysteine (NAC) ($P < 0.05$) (Figure 3.2A). In the case of the crude green tea pomace extract, its concentration levels of 0.022 mg/mL resulted in a significant free radical scavenging in the negative control composed of H_2O_2 ($P < 0.05$). This was similar to cloves in which the antioxidant capacity was still lower compared to positive control ($P < 0.05$) (Figure 3.2B). Vietnamese coriander at 0.022 and 0.044 mg/mL showed no effect on ROS scavenging under either normal conditions or when induced with H_2O_2 (negative control) ($P > 0.05$) (Figure 3.2C). Interestingly, the combined mixture of plant extraction at 1:1:1 (v:v:v) revealed 0.087 to 0.173 mg/mL concentration with the strongest ROS inhibition with an efficiency equivalent to positive control (Figure 3.2D).

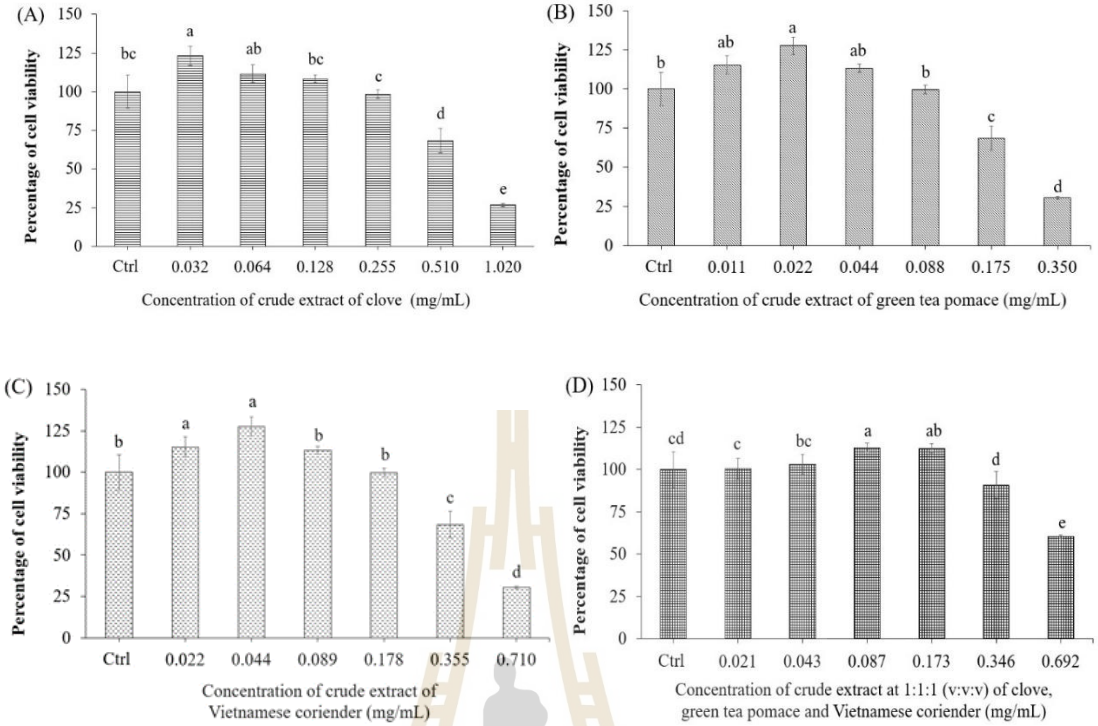

Figure 3.1 Cytotoxicity assessment of *Syzygium aromaticum* (clove) (A), *Camellia sinensis* (green tea pomace) (B), *Persicaria odorata* (Vietnamese coriander) (C), and their combination at 1:1:1 (v:v:v) (D) using MTT assay in HepG2 cells. The different letters are significantly different at $P < 0.05$.

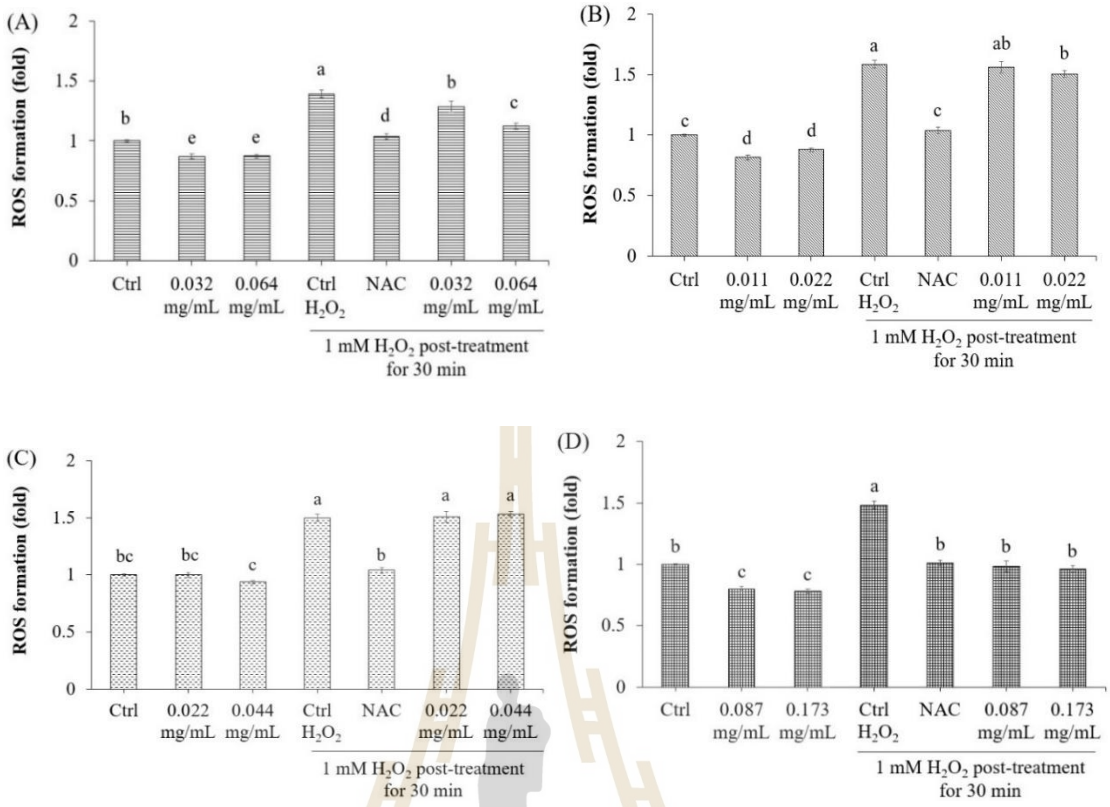

Figure 3.2 Reactive oxygen species (ROS) scavenging activity of *Syzygium aromaticum* (clove) (A), *Camellia sinensis* (green tea pomace) (B), *Persicaria odorata* (Vietnamese coriander) (C), and their combination at 1:1:1 (v:v:v) (D) using DCFH-DA assay in HepG2 cells. Control (Ctrl): HepG2 cells treated with plant extracts without H_2O_2 induction; Negative control (Ctrl H_2O_2): HepG2 cells induced with H_2O_2; Positive control: HepG2 cells treated with N-acetylcysteine (NAC). The different letters are significantly different at $P < 0.05$.

3.5 Discussion

Phenolic compounds are secondary metabolites of plants, which can be categorized into several characteristics (phenolic acids, flavonoids, stilbenes, coumarins, lignans, and tannins), while flavonoids are a large category of phenolic widely distributed in plants which have more than 8,000 metabolites. The quantity of TPC and TFC has been shown to be associated with potential multi-purpose functional uses, especially antioxidant activities (Aksoy et al., 2013; Valdés et al., 2015). In this study, Vietnamese coriander, green tea pomace from the beverage industry, and cloves

showed a prominent value of TPC (63.06, 115.85, and 176.97 mg GAE/g DW, respectively) and TFC (135.07, 189.78, and 360.50 mg QE/g DW, respectively). These plants can help to avoid the damage caused by free radicals in both humans and animals. Adaramola and Onigbinde (2016) also indicated that clove bud extracts with 80% methanol contain 170.90 mg GAE/g DW of TPC and 318.67 mg QE/g DW of TFC. While clove extraction with 50% ethanol contained less TPC content (167.22 mg GAE/g DW) and TFC (31.78 mg QE/g DW) (Muzolf-Panek and Stuper-Szablewska, 2021), these values are also lower than we reported (176.97 mg GAE/g DW in TPC and 360.50 mg QE/g DW in TFC). Green tea leaves contain TPC 148–243 mg GAE/g DW and TFC 358.9 mg QE/g DW (Yadav et al., 2020). These values are higher than those for green tea pomace with leaves and stalks, which is a by-product from the beverage industry used in this study. It is interesting to note that the by-product still remains abundant in phenolic and flavonoid compounds which demonstrate a potential use as a natural antioxidant in animal feed. According to Nugboon and Intarapichet (2015), the leaves of Vietnamese coriander extracted with 96% ethanol contained TPC and TFC approximately 389.00 ug GAE/g DW and 62.24 ug catechin/g DW, which had a higher TPC and a lower TFC than the present study. However, the difference in TPC and TFC yield in plant materials may depend on solvents and extraction methods (Sulaiman et al., 2015), including the planting area and conditions, harvest time, geography, environmental factors, and the species and parts of the plant (Kratchanova et al., 2010; Perez-Ochoa et al., 2022).

Of the 17 plants investigated, it was observed that cloves, green tea pomace, and Vietnamese coriander possessed a remarkable antioxidant function using the DPPH, ABTS, and FRAP assays. Cloves are recognized to have strong antioxidant activity as they contain numerous bioactive compounds in their oil such as eugenol, isoeugenol, caryophyllene, a-humulene, and eugenyl acetate (Sehitoglu and Kaya, 2021). Gulcin et al. (2012) indicated that clove oil (0.015, 0.030, and 0.045 mg/mL) can scavenge $DPPH^{\bullet}$ and ferric ions (Fe^{3+}) reduce power better than BHT, a-tocopherol, BHA and trolox, respectively. It also has a high potential for $ABTS^{\bullet+}$ scavenging which is better than a-tocopherol and trolox. In green tea, the major phenolic compounds are catechins which demonstrate the removal activity of free radicals of $DPPH^{\bullet}$ and $O^{\bullet}_2$ *in vitro*

method (Abbas and Wink, 2014). Vietnamese coriander contains various compounds in leaves such as rutin, catechin, quercetin, kaempferol, and isorhamnetin including terpenoids, sterols, steroids, phenols, and coumarins (Yanpirat and Vajrodaya, 2015). Its antioxidant activity is potent in terms of DPPH, FRAP, and TBARS (Nugboon and Intarapichet, 2015).

Intracellular radical scavenging activity of the combination of cloves, green tea pomace, and Vietnamese coriander in this study showed positive synergistic antioxidant effects which were better than individual plants. This is probably due to the combination of diverse functions of phenolic compounds. According to Hassimotto et al. (2005) and Ibtisham et al. (2019), using a combination of plants composed of various compounds can show synergistic and antagonistic effects on antioxidant capacities. A combination of medicinal plants (parsley, buckthorn, mint, caraway) in cookies demonstrated an association with high antioxidant activity and oxidative stability by improving $O_2^{-\bullet}$, $OH^{\bullet}$, $DPPH^{\bullet}$ scavenging, reducing activity and chelating activity on Fe^{2+} in comparison with individual plant (Misan et al., 2011).

Previous studies have indicated that the responses of bioactive compounds to antioxidant activity in green tea and cloves are composed primarily of catechin and eugenol, respectively (Alfikri et al., 2020; Chen et al., 2021). The prominent antioxidant activity of Vietnamese coriander may relate to gallic acid, quercetin, ferulic acid, and apigenin (Ahongshangbam et al., 2014). In this study, we also measured the chemical constituents of cloves, green tea pomace, Vietnamese coriander, and their combination, which indicated that the main bioactive compounds were eugenol and eugenyl acetate, as well as gallic acid, catechin, ellagic acid, quercetin, and kaempferol (data not shown). It is interesting to note that although green tea pomace is a by-product from a beverage, the quercetin, gallic acid, and catechin contents still remain in a high quantity (data not shown). According to the report of Bernatoniene and Kopustinskiene (2018), catechins can interact with membranes via adsorption or penetration into the lipid bilayers. The antioxidant activity of eugenol and one of its isomers, isoeugenol, are powerful enough to stop the initiating factor of lipid peroxidation by forming complexes with reduced metals and decreasing the formation of an iron-oxygen chelates complex. Eugenol also donates a hydrogen atom and stabilizes the phenoxyl radicals to inhibit oxidation (Nejad et al., 2017).

The individual crude extracts of cloves, green tea pomace from beverage industry, and Vietnamese coriander, or their combination, can be used within the range of 0.032 to 0.255, 0.011 to 0.088, 0.022 to 0.178, and 0.021 to 0.346 mg/mL, respectively, without adversely affecting the viability of the cells. According to Dwivedi et al. (2011), clove oil at 300 µl/mL showed 40–80% of cell deaths. In addition, it was found that the cell viability of human epidermoid cancer cells (Hep-2) decreased by 50% (IC50) after exposure to 500±10.2 µg/mL of clove oil (Kouidhi et al., 2010). The safe level of cloves presented in this study is likely to remain a significant gap to reach the viability of toxic cells compared to previous studies. The cytotoxicity of cloves is possibly due to the high concentration of eugenol (Yeddes et al., 2022). In addition, the World Health Organization has recommended safe doses of clove buds should be at 2.5 mg/kg daily of human body weight (Gulcin et al., 2012). This consumption level can decrease many health risks such as reducing lipid peroxidation and increasing endogenous redox enzymes (Batiha et al., 2020). In the case of green tea, excessive concentrations of 0.25 mg/mL of green tea extract were observed to affect the viability and apoptosis of HepG2 (Sun et al., 2020). Although this study revealed that a broader range of green tea pomace (0.0 11–0.088 mg/mL) is still safe for cell viability, it is probably a by-product and lesser bioactive contents remain. Our results showed no cytotoxicity of Vietnamese coriander between concentrations of 0.022–0.178 mg/mL. Somparn et al. (2014) reported that rats which received a crude extract of Vietnamese coriander at 400 and 800 mg/kg BW/day did not show any change in liver or kidney histology. In addition, Chansiw et al. (2019) reported that the leaf and stem extract of Vietnamese coriander at concentrations of 50, 100, and 200 lg/mL had no cytotoxicity and that cell viability remained greater than 70%. In this study, a higher dose of the plant mixture is likely to have been less toxic for cell viability compared to a single plant. This is probably due to some bioactive compounds in each plant having been diluted which would make the cells able to tolerate higher concentrations. In addition, all plant materials used in this study are edible plants, so the results obtained on the cytotoxicity test also confirm their potential for use as antioxidant additives for animals.

3.6 Conclusions

This study demonstrated that out of a total of 17 plants, *Syzygium aromaticum* (clove), *Camellia sinensis* (green tea pomace) from the beverage industry, and *Persicaria odorata* (Vietnamese coriander) contained high levels of TPC and TFC. These 3 plants and their combination in a ratio of 1:1:1 (v:v:v) revealed powerful antioxidants in terms of DPPH, ABTS, and FRAP. In addition, the combination of cloves, green tea pomace, and Vietnamese coriander also possesses the synergistic ability to scavenge free radicals and non-cytotoxicity to cells relative to individual plants. This suggests the possibility of using plant materials as antioxidant feed additives to reduce the risk of OS in animals.

3.7 References

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CHAPTER IV
EFFECT OF HEAT STRESS ON TRANSCRIPTOMIC PROFILE AND PROTECTIVE EFFICACY OF DIETARY ANTIOXIDANTS IN BREEDER HENS

4.1 Abstract

Heat stress (HS) is a cause of occurring oxidant stress that negatively affects the reproductive performance of breeder hens, especially damaging sperm integrity in sperm storage tubules (SSTs) at the uterovaginal junction (UVJ) of the oviduct after artificial insemination or mating. However, the gene regulation and understanding of the molecular mechanisms in UVJ to HS response remain unelucidated. Thus, this study aimed to investigate the differentially expressed genes (DEGs) and molecular mechanisms in the UVJ of breeder hens under HS conditions (36°C for 6 h) between heat-adapted and heat-sensitive breeds by using transcriptomic analysis. The result of the transcriptomic analysis revealed a total of 387 DEGs, including 159 upregulated and 228 downregulated genes in heat-sensitive and heat-adapted breeder hens under HS. Gene Ontology (GO) analysis showed that the top 15 significant GO terms were highly enriched in biological processes, cellular components, and molecular function (adjusted $P < 0.05$) involving heat shock protein (HSP) transcripts. Kyoto Encyclopedia of Genes and Genomes analysis showed 6 significant pathways associated with the upregulated HSP and DNAJ gene families and downregulated IL18R1, CCL19, ADH1C, TAT, CA9, and CA6 genes ($P < 0.05$). HSPB8, DNAJ4, HSPH1, HSP90AA1, and TAT genes were confirmed to be the candidate genes in UVJ to HS response. Moreover, the effect of either synthetic (a combination of vitamin E, vitamin C, Se, and L-carnitine) or phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) antioxidants in heat-sensitive breeder hen diets on the change of candidate gene expression in the UVJ were examined. The result showed that either synthetic or phytogenic antioxidants altered the expression of HSP90AA1 and TAT genes in UVJ tissue of heat-sensitive breeder hens subjected to HS. In conclusion, this study could

provide insights into UVJ transcriptome changes between heat-adapted and heat-sensitive breeds under HS conditions and identify candidate genes that could be modulated by synthetic or phytogenic antioxidants in UVJ of HS in breeder hens.

Keywords: Heat stress; Transcriptomic analysis, Breeder hen, Uterovaginal junction, Sperm storage tubes; Dietary antioxidant

4.2 Introduction

High environmental temperature combined with high relative humidity is an impact cause of heat stress (**HS**) in the poultry industry, particularly in subtropical and tropical areas such as Thailand. Breeding selection for chicken economic traits with higher performance has resulted in chickens having difficulty in acclimatizing to stressful environmental conditions. The fast-growing commercial broilers or layers are more sensitive to HS than native chickens (Liu et al., 2022). In breeder hens, an environmental temperature causes reactive oxygen species (**ROS**) generation and develops into oxidative stress (**OS**). When sperms are stored in sperm storage tubules (**SSTs**) at the uterovaginal junction (**UVJ**) of the hen oviduct, OS can interfere with the integrity of sperm membrane and DNA, consequently impairing reproduction, increasing sperm mortality, and reducing fertility and hatchability (Breque et al., 2006; El-Deep et al., 2017). A comprehensive understanding of the molecular mechanisms by gene study with transcriptome analysis is essential to drive acclimation to HS in breeder hens in order to overcome these challenges of HS effectively (Lim et al., 2022).

HS affects the various cell cycles of chicken at cellular levels such as DNA repair mechanisms, transcription, translation, post-translational modifications, oxidative metabolism, membrane structure and function, and the unfolding or improper folding of proteins (Pritchett et al., 2023). The differential genetic backgrounds in chickens affected different heat responses (Soleimani et al., 2011). Perini et al. (2020) and Sadr et al. (2023) suggested that gene identification with a breed-specific expression between commercial and indigenous chickens can be used to identify candidate genes and molecular pathways associated with metabolism, immune system, and heat stress responses for further breeding fields and nutrigenomic study. The previous studies found that either commercial or indigenous chickens showed different upregulation of

heat shock proteins (HSPs) in various organs after HS exposure (Rimoldi et al., 2015). Broiler breeders under HS conditions were induced to increase levels of ROS, which resulted in the decreasing antioxidant and releasing of HSPs (Sánchez et al., 2022), whereas liver transcriptome analysis of Sánchez et al. (2022) revealed that HSP90B1 and HSPA5 were more highly expressed in commercial chicken breeds than native chicken breeds. HSPs are stress-modulated proteins that are upregulated or downregulated in response to high temperatures. The functions of HSP100, HSP90, HSP70, HSP60, HSP40, and small heat shock proteins (sHSP) are to protect organisms from HS damage and as intracellular chaperones for other proteins (De Maio and Vazquez, 2015). In addition, HSP70 and HSP90 in chicken have been extensively focused on due to their strong association with heat tolerance (Perini et al., 2020). Furthermore, the pro-inflammatory cytokines (e.g., IL6, IL1β, and TNF-α) expression could trigger a protective response to counter damage caused by heat stress in ovarian follicle tissues involved with the biological processes of reproduction, response to stress, and regulation of these responses (Cheng et al., 2018). However, there is still a lack of knowledge of HS impacts on changes in molecular mechanisms in UVJ tissues containing SSTs of HS-sensitive breeder hen bred by comparing RNA-Seq transcriptional profiling to excellent indigenous breeder hen models, that have a greater heat resistance capacity in being gene marker for feed management.

Certainly, the homeostasis mechanisms within the body play a critical role as endogenous cellular defense. These mechanisms may aid cells in managing stressful conditions by modulating pro-inflammatory cytokines, HSPs, and the antioxidant system (Jang et al., 2014). Vitamins C and E, selenium, L-carnitine, and phytogenic play an important role in alleviating cellular damage and preventing HS (Surai, 2014; Surai et al. 2019). Vitamin E, carotenoids, and Se in the maternal hen diets scavenged initial radicals and various transcription factors (NF-E2-related factor 2 and nuclear factor-kB), including synthesis and activated expression of protective molecules (thioredoxins, superoxide dismutase, and HSPs) (Surai et al., 2016). Combined feeding of Se and vitamin E in broilers exposed to high temperature increased tissue Se and vitamin E accumulation can decrease expression of HSP90, HSP70, and HSP60 mRNA levels (Kumbhar et al., 2018). The expression of HSP70 in the ovary and brain and interleukin (IL)-1β, IL-6, interferon (IFN)-γ, Toll-like receptor (TLR)-4, and HSP70 in the liver of heat-

stressed chickens were downregulated by dietary vitamin C or E supplementation (Jang et al., 2014; Balakrishnan et al., 2023), while dietary L-carnitine also demonstrated antioxidant and anti-inflammatory effects (Agarwal et al., 2018). Our previous study revealed the combined mixture of *Syzygium aromaticum* (clove), *Camellia sinensi* (green tea pomace) from the beverage industry and *Persicaria odorata* (Vietnamese coriander) consisted of various polyphenols such as eugenol, gallic acid, catechin, ellagic acid, quercetin, and kaempferol, which there are many properties like anti-inflammatory, antioxidant, antimicrobial, or antiviral etc. (Pasri et al., 2023). Quercetin and catechin in broiler diets could modulate heat shock transcription factor activity, thus inhibiting HSP70 expression (Sugito et al., 2020). In addition, tea polyphenols or common other polyphenol properties blocked nuclear factor-kB activation in response to diverse various inflammatory stimuli, which suppressed various pro-inflammatory cytokines expressions, such as IL-1β, IL-4, IL-6, IL-10, TNF-α, and IFN-γ (Liu et al., 2020; Pascual et al., 2022). However, limited information is available for the study of the combined effect of vitamins E and C, Se, L-carnitine, and phytogenic to alter target genes in HS response that are located in the UVJ containing SSTs of breeder hens.

Therefore, the aim of this study was to identify the relevant gene markers by transcriptomic analysis in UVJ containing SSTs of breeder hens subjected to heat stress between HS-adapted breed and HS-sensitive breed and study the progressive changes of selected gene candidate markers of HS-sensitive breeder hens under HS and dietary supplementation with antioxidant substances from synthetics (combination of vitamin E, vitamin C, Se, and L-carnitine) and phytogenic (combination of clove, green tea pomace, and Vietnamese coriander).

4.3 Materials and methods

4.3.1 Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of the Suranaree University of Technology (Nakhon Ratchasima, Thailand) (SUT-IACUC-012/2020).

4.3.2 Bird and sample collection

This study was divided into 2 trials, transcriptomic and antioxidant studies. In the transcriptomic study, a total of 50 heat-sensitive breeds (SUT breeder hens) and heat-adapted breeds (Leuang Hang Kao breeder hens) at 22 week of age, 25 hens per strain, were raised in individual cages with a size of 40 × 45 × 40 cm^3 (width × depth × height) and acclimated for 6 weeks in thermoneutral (TN, 23±1°C) room by using air conditioner. The breeder hens were fed 140 g/day of corn-soy basal diets following the NRC (1994) and Aviagen (2021) recommendations (15% crude protein, 2,800 kcal metabolizable energy/kg) as shown in Table 4.1 and free access to drinking water, under 16 hours of light per day at Suranaree University farm. At 28 wk of age, all breeder hens were moved to heat stress (HS) room with a controlled temperature at 36°C with a humidity of 40-70% for 6 hours using gas heater with thermostat-controlled equipment according to the modified method (Xie et al., 2014; Duangjinda et al., 2017). After 6 h of heat exposure, 12 breeder hens from each strain were randomly selected and killed by cutting the vein of the neck. The UVJ tissues containing SSTs were collected and frozen immediately in liquid nitrogen, then preserved at −80°C until further transcriptome analysis and gene validation analysis.

In the antioxidant study on altering gene markers, 100 SUT female breeder hens (33 weeks of age) were housed in individual cages and allotted into four treatment groups, each consisting of 25 females using Completely Randomized Design. Group 1 was raised in a TN room while groups 2, 3, and 4 were subjected to HS room for 4 h daily. There were four experimental diets as follows: T1) basal diets under TN, T2) basal diets under HS, T3) basal diets with combined synthetic antioxidants (200 mg of vitamin C/kg, 150 mg of vitamin E/kg, 0.30 mg of Se yeast/kg, and 150 mg of carnitine/kg) under HS, and T4) basal diets with 1% phytochemical antioxidants (a mixture of clove, green tea pomace, and Vietnamese coriander powder) under HS. All breeder hens were fed 140 g of feed (15% CP, 2800 kcal ME/kg) as shown in Table 4.1 and provided 16 h of light per day and access to water throughout the experimental period (33–52 weeks of age). At the end of the experiments, all breeder hens were slaughtered after heating at 36°C for 4 h, collected UVJ tissues containing SSTs in liquid nitrogen immediately, and stored at −80°C for further marker gene expression analysis.

Table 4.1 Ingredients and chemical composition of the basal diet.

	Female breeder hen diets	
	25-50 weeks of age	**After 50 weeks of age**
Ingredients (%)		
Corn	64.60	63.50
Soybean meal, 44 %CP	18.20	16.52
Full fat soybean meal	6.70	9.00
Calcium carbonate	8.50	8.90
Monocalcium phosphate	0.94	1.00
Salt	0.41	0.44
DL-Methionine	0.135	0.134
L-Lysine	-	-
L-Threonine	-	-
Premix	0.52[1]	0.52[1]
Analyzed compositions (%)		
Dry matter	93.06	93.10
Crude protein	16.02	16.20
Crude fiber	3.06	3.04
Ash	11.08	11.66
Ether extract	3.35	4.49
Calculated compositions (%)		
Metabolizable energy (kcal/kg)	2,800	2,800
Calcium	3.51	3.71
Total Phosphorus	0.53	0.54
Available phosphorus	0.31	0.32
Digestible Lysine	0.70	0.70
Digestible Methionine	0.35	0.35
Digestible Methionine + Cystine	0.57	0.57
Digestible Threonine	0.50	0.50

[1]Premix for breeder hens (0.52%) provided the following (per kg of diet) by withdrawing vitamin E and Se; vitamin A, 15,000 IU; vitamin D3, 3,750 IU; vitamin K3, 5 mg; vitamin B1, 2 mg; vitamin B2, 9.8 mg; vitamin B6, 4 mg; vitamin B12, 25 mg; pantothenic acid,

11.04 mg; nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 15.5 µg; choline chloride, 250 mg; Cu, 2.1 mg; Mn, 84 mg; Zn, 66.5 mg; Fe, 80 mg; I, 1.2 mg.

4.3.3 RNA extraction

Total RNAs of 12 UVJ tissue samples in each strain (heat-adapted and heat-sensitive breeds) were isolated individually by using RNeasy Mini Kit (Qiagen, Hilden, Germany). The quantity and quality of the extracted RNA were detected by using Nano Drop Spectrophotometer (NanoDrop 2000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA) and 1% agarose (w/v) gel electrophoresis. The 3 pooled RNA (one pool from 4 individual UVJ samples) per each strain was proceeded to construct an RNA-seq library. RNA integrity number (RIN) was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and RNA samples with RIN ≥8 were used for cDNA library preparation.

Total RNA was extracted from 8 UVJ tissue samples of breeder hens from each T1, T2, T3, and T4 by using NucleoSpin® RNA Midi kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and purified using a QIAamp spin column (Qiagen, Hilden, Germany). The extracted RNA from 2 individual UVJ samples were pooled and 4 replications were generated in each treatment, and the purity and quantification of RNA were measured, as previously described.

4.3.4 Transcriptome sequencing, data analysis, gene ontology and Kyoto encyclopedia of genes, and genomes pathway enrichment

Library preparation and subsequent RNA-seq were performed by Novogene Biotechnology Company (Novogene, Beijing, China). Libraries were sequenced on an Illumina Novaseq 6000 instrument (Illumina, San Diego, CA), and a 2 × 150 bp paired-end configuration was applied. The quality of the raw data was assessed and mapped to the chicken reference genome GRCg6a (GenBank Assembly ID: GCA_000002315.5) using Hisat2 version 2.0.5 (Kim et al., 2019). Reads mapped to each gene were counted using featureCounts version 1.5.0-p3 (Liao et al., 2014). DESeq2 R package version 1.20.0 (Love et al., 2014) was used to identify differential expression genes between heat-adapted and heat-sensitive breeder hens. False discovery rate was controlled using the Benjamini–Hochberg method. Transcripts with an absolute fold-change (FC) of ≥2 and adjusted values of $P < 0.05$ were considered as DEGs.

Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEGs were conducted using R package ClusterProfiler version 3.8.1 (Yu et al., 2012).

4.3.5 Validation of DEGs and marker genes via quantitative polymerase chain reaction

The primer sequences for heat shock protein family B (small) member 8 (HSPB8), DnaJ heat shock protein family (Hsp40) member A4 (DNAJA4), heat shock protein family H (Hsp110) member 1 (HSPH1), heat shock protein 90 alpha family class A member 1(HSP90AA1), tyrosine aminotransferase (TAT) and β-actin are presented in Table 4.2. National Center for Biotechnology Information Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the primers. A high-quality RNA sample was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and kept at −20°C for qPCR analysis. Then, the cDNA samples from heat-adapted and heat-sensitive breeds, T1, T2, T3, and T4 were used to analyze the DEGs validation and marker gene confirmation. For preparing the reaction's master mix, a total of 8 µL reaction mixture was made from 5 µL of SYBR Green, 0.4 µL of forward primer, 0.4 µL reverse primer, and 2.2 µL of H_2O_2 using the QuantiNova™ SYBR Green PCR kit (Qiagen, Hilden, Germany), then mixed with 2 µL of cDNA samples in a 96-well microplate. The quantification of the target and reference genes (β-actin) was analyzed in triplicate for each sample by the CFX96 real-time PCR system (BioRad, Hercules, California, USA). Quantitative PCR program contained a step of 40 cycles of denaturation at 95°C for 10 s, followed by annealing for 30 s and final extension at 60°C for 30 s. The quantitative PCR data was normalized using β-actin as the reference gene and calculated relative changes in gene expression using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The mean $2^{-\Delta\Delta_{CT}}$ values were converted to FC values, and qPCR results were compared to the RNA-seq results (Kubota et al., 2023).

Table 4.2 Primer sequences used in real-time PCR.

Gene	Primer sequences	Gene accession number
HSPB8	F-5'-TTCAAGCCTGAGGAGCTGACG-3'	NM_ 040685085.2
	R-5'-AAGGAGGCGAAGACAGTGATGG-3'	
DNAJA4	F-5'-AGTTGCTGCGCTGTCAGTAT-3'	NM_ 040680548.2
	R-5'-AGTTGGTTCTCAGCTGTGTGA-3'	
HSPH1	F-5'-CCCAGATGTCAAGAAAACAAGTGA-3'	NM_ 001159698.2
	R-5'-AGCTTCAATAGGCAGTTCCACA-3'	
HSP90AA1	F-5'-GCAGCAGCTGAAGGAATTTGA -3'	NM_ 001109785.2
	R-5'-GGAAGCTCTAAGCCCTCTTTTGT-3'	
TAT	F-5'-CCACAAATGATGAGGTCACG-3'	NM_ 025154347.3
	R-5'-TCTCGACAGGACTGGTAGCC-3'	
β-actin	F-5'- TTGGTTTGTCAAGCAAGCGG-3'	NM_205518.1
	R-5'- CCCCCACATACTGGCACTTT-3'	

Abbreviations: HSPB8, heat shock protein family B (small) member 8; DNAJA4, DnaJ heat shock protein family (Hsp40) member A4; HSPH1, heat shock protein family H (Hsp110) member 1; HSP90AA1, heat shock protein 90 alpha family class A member 1; TAT, tyrosine aminotransferase

4.3.6 Statistical analysis

The gene expression data from T1, T2, T3, and T4 was analyzed using ANOVA in a Completely Randomized Design (CRD) with SPSS 16.0 software. Tukey's test was applied to assess significant differences among treatments. Additionally, orthogonal contrasts were utilized to compare the following conditions: 1, thermoneutral (T1) vs. heat stress (T2, T3, T4); 2, non-supplementation (T2) vs. supplementation (T3, T4); and 3, synthetic (T3) vs. phytogenic (T4) antioxidants. A significance level at $P < 0.05$ was used (SPSS Inc. 2007).

4.4 Results

4.4.1 Quality of RNA-seq reads

Six RNA-sequencing libraries from heat-adapted breeder hen (HA 1, 2, 3) and heat-sensitive breeder hen (HS 1, 2, 3) groups were constructed with an average of 42,552,281 raw sequencing reads (Table 4.3). A sequence quality score greater than Q20 (percentage of bases with a Phred value ≥20) was obtained with an average clean reads rate of 97.83%. An average of 91.46% of high-quality reads were mapped to the reference genome for further gene expression analysis.

Table 4.3 RNA-sequencing reads and mapping rates in the uterovaginal junction tissues containing sperm storage tubules in breeder hens.

Sample[1]	Raw reads	Clean reads	Q20 (%)[2]	GC content (%)	Mapping rate (%)
HA1	35749329	34870468	97.74	51.54	91.02
HA2	53028745	51649094	97.89	51.30	91.27
HA3	44074508	42951594	97.81	51.11	91.68
HS1	39620749	38532168	97.80	50.92	91.35
HS2	45673797	44532395	97.85	51.27	91.72
HS3	37166560	36246626	97.87	51.14	91.70
Average	42552281	41463724	97.83	51.21	91.46

[1]Sample name represents the UVJ tissues containing SSTs from heat-adapted breeder hens (HA) and heat-sensitive breeder hens (HS) under heat stress.

[2]Q20 indicates the percentage of bases with a Phred value ≥20.

4.4.2 Significant DEGs

RNA-seq reads of UVJ tissues containing SST samples were compared between heat-adapted breeder hens and heat-sensitive breeder hens under HS. Volcano plots showed a total of 387 DEGs, including 159 upregulated and 228 downregulated genes, in heat-sensitive breeder hens under HS (Figure 4.1). All information on identified DEGs is presented in the supplementary Table 4.7. The eleven chaperone and co-chaperone of heat shock proteins (HSPs) such as HSPH1, HSP90AA1,

HSPA4L, HSPA4, HSPA8, HSPD1, DNAJA4, DNAJB4, DNAJA1, DNAJB1, and HSPB8 were remarkably upregulated in heat-sensitive breeder hens under HS (adjusted $P < 0.05$). The top 20 upregulated and downregulated genes were listed in Table 4.4.

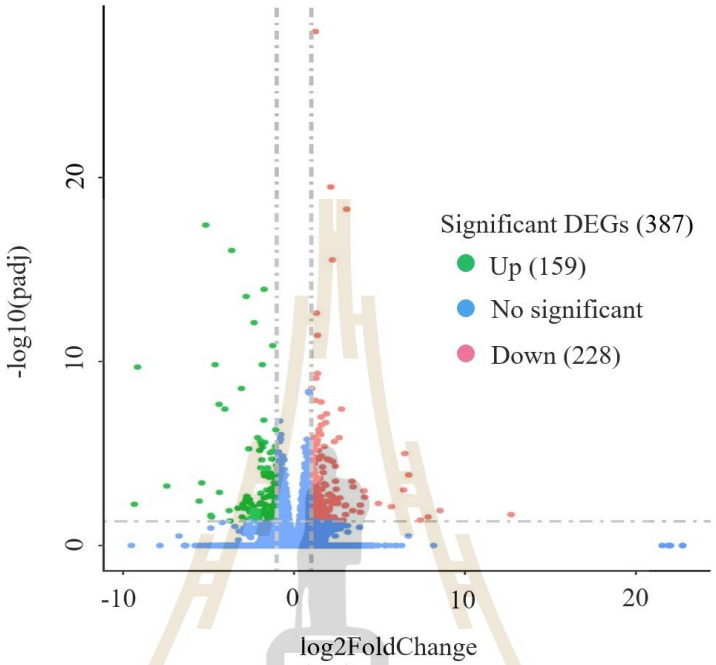

Figure 4.1 Volcano plot of differentially expressed genes (DEGs) in the uterovaginal junction tissues containing sperm storage tubules in heat-sensitive breeder hens compared to heat-adapted breeder hens under heat stress.

Table 4.4 Top 20 upregulated and downregulated differentially expressed genes (DEGs) in the uterovaginal junction tissues containing sperm storage tubules in heat-sensitive breeder hens compared to heat-adapted breeder hens under heat stress.

Ensemble Gene ID	Gene Name	log2 fold change	P values[1]	Regulated
ENSGALG00000035230	CSMD1	7.4505	0.000576733	up
ENSGALG00000007419	MASP1	4.8346	0.027482685	up
ENSGALG00000020836	FAM237A	4.0525	3.94E-08	up
ENSGALG00000026598	NXNL2	3.0012	0.005692156	up
ENSGALG00000010853	C8B	2.9594	0.00927185	up
ENSGALG00000039869	PYURF	2.8130	2.96E-14	up
ENSGALG00000007383	HSPB8	2.7986	0.003272468	up
ENSGALG00000036850	DNAJA4	2.7714	0.002262966	up
ENSGALG00000010936	MYF6	2.7109	0.041658513	up
ENSGALG00000017077	HSPH1	2.6227	0.003170131	up
ENSGALG00000009433	BAG3	2.5785	0.016161149	up
ENSGALG00000003369	KAT2A	2.3607	0.00830988	up
ENSGALG00000014976	GATA6	2.3428	7.54E-13	up
ENSGALG00000001141	HES5	2.2411	0.012864101	up
ENSGALG00000051280	DNAJB1	2.1370	0.01462694	up
ENSGALG00000010208	CAPS2	2.1237	1.44E-06	up
ENSGALG00000000184	SLC27A6	1.9708	2.20E-06	up
ENSGALG00000037082	ASCL1	1.9382	0.001492496	up
ENSGALG00000011341	UNC93A	1.9375	0.004926626	up
ENSGALG00000008916	DNAJB4	1.8844	2.35E-05	up
ENSGALG00000026392	PIPOX	−1.6424	4.63E-02	down
ENSGALG00000031776	NFASC	−1.6663	0.0000567	down
ENSGALG00000001214	PARD6A	−1.6716	0.007414563	down
ENSGALG00000013293	GLDN	−1.7116	3.67E-02	down
ENSGALG00000046192	CCL19	−1.8487	2.12E-07	down

[1]P value was obtained via the Benjamini–Hochberg method.

Table 4.4 Top 20 upregulated and downregulated differentially expressed genes (DEGs) in the uterovaginal junction tissues containing sperm storage tubules in heat-sensitive breeder hens compared to heat-adapted breeder hens under heat stress (Continued).

Ensemble Gene ID	Gene Name	log2 fold change	P values[1]	Regulated
ENSGALG00000034975	FUT7	−1.8741	0.039613539	down
ENSGALG00000038225	SEMA3E	−2.0196	0.001835848	down
ENSGALG00000025991	GLOD5	−2.0372	3.25E-02	down
ENSGALG00000002125	TMOD1	−2.0908	0.001565477	down
ENSGALG00000016620	PNOC	−2.1807	0.049223783	down
ENSGALG00000035725	SP8	−2.1929	0.048741033	down
ENSGALG00000006273	MYLK2	−2.2562	0.004494361	down
ENSGALG00000037148	CRH	−2.3764	0.005477453	down
ENSGALG00000030614	ADH1C	−2.4102	0.0000516	down
ENSGALG00000025821	CLDN16	−2.5090	4.46E-02	down
ENSGALG00000012869	OVA	−3.0116	0.006446308	down
ENSGALG00000050250	HIST1H46L2	−3.3922	0.00031843	down
ENSGALG00000012483	TMPRSS6	−4.9285	5.60E-03	down
ENSGALG00000015599	SOHO-1	−5.6889	0.007569389	down
ENSGALG00000051839	RF00012	−6.4645	0.0000101	down

[1]P value was obtained via the Benjamini–Hochberg method.

4.4.3 GO and KEGG pathway enrichment of DEGs

GO functional analysis revealed a total of 253 significantly enriched GO terms with three categories, including biological process, cellular component, and molecular function ($P < 0.05$; the supplementary Table 2.8). The top 30 most significantly enriched GO terms are shown in Figure 4.2. Of these, 15 GO terms were the most prominent enrichment (adjusted $P < 0.05$) that are associated with protein folding, complement activation, response to temperature stimulus, chaperone-mediated protein folding, de novo' protein folding, response to heat, complement activation, classical pathway, protein activation cascade, activation of immune response, and humoral immune response mediated by circulating immunoglobulin in

terms of biological process, chaperone complex in terms of cellular component, and heat shock protein binding, chaperone binding, unfolded protein binding, and ATPase regulator activity in terms of molecular function.

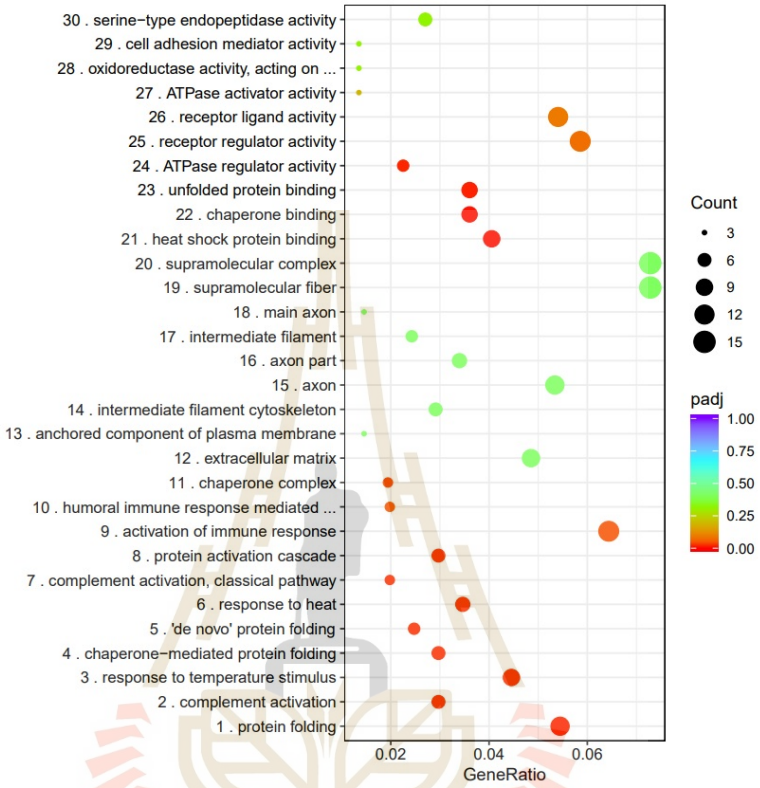

Figure 4.2 Top 30 enriched Gene Ontology (GO) terms of differentially expressed genes (DEGs) in the uterovaginal junction tissues containing sperm storage tubules in heat-sensitive breeder hens compared to heat-adapted breeder hens under heat stress. The size of the dots is positively correlated to the number of DEGs in the pathway.

Moreover, KEGG pathway analysis was performed to classify the biological pathways of the 387 DEGs. The result of the KEGG pathway exhibited 6 significant pathways ($P < 0.05$) (Table 4.5). Protein processing in endoplasmic reticulum pathway was associated with 5 DEGs having a higher expression of HSP90AA1, DNAJA1, HSPA4L, and HSPA8/HSPH1 genes. Cytokine-cytokine receptor interaction pathway was mainly related to the downregulation of ACVRL1, CCL19 (ENSGALG00000028256), CCL19 (ENSGALG00000046192), CNTF, and IL18R1, upregulation of GDF8, and AMH, especially

CCL19 (ENSGALG00000046192) had low fold change (FC) values at −1.84 (Table 4.4). Furthermore, the downregulated ADH1C, TAT, CA9, and CA6 genes are involved in tyrosine, phenylalanine, and nitrogen metabolism pathways, while NFASC and CLDN16 genes were enriched in cell adhesion molecules pathway.

Table 4.5 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways possibly affected by heat stress in the uterovaginal junction (UVJ) tissues containing sperm storage tubules (SSTs) in heat-sensitive breeder hens compared to heat-adapted breeder hens under heat stress.

Term	Count	P value	Gene name[1]
gga04060: Cytokine-cytokine receptor interaction	9	0.003788	**ACVRL1**, **CCL19,** **CCL19**, **CNTF**, **novel.112**, ENSGALG00000052151, **IL18R1**, GDF8, AMH
gga04141: Protein processing in endoplasmic reticulum	8	0.00491	novel.1269, HSP90AA1, ENSGALG00000031518, DNAJA1, HSPA4L, HSPA8/HSPH1, ENSGALG00000011715
gga00350: Tyrosine metabolism	3	0.011748	**ENSGALG00000036044**, **ADH1C**, **TAT**
gga00360: Phenylalanine metabolism	2	0.021575	**ENSGALG00000036044**, **TAT**
gga00910: Nitrogen metabolism	2	0.032069	**CA9**, **CA6**
gga04514: Cell adhesion molecules (CAMs)	5	0.038481	**ENSGALG00000028341**, **NFASC**, **CLDN16**, **ENSGALG00000031430**, **ENSGALG00000009355**

[1]The bolded font in the table represents the downregulated express genes in UVJ tissues containing SSTs in heat-adapted breeder hens compared to heat-sensitive breeder hens under heat stress.

4.4.4 qPCR validation

To confirm the accuracy of the identified DEGs results, qPCR analysis was used for gene validation. We selected 4 upregulated genes (HSPB8, DNAJA4, HSPH1, and HSP90AA1) and 1 downregulated gene (TAT). qPCR and RNA-seq results revealed the same DEG expression trends (Figure 4.3).

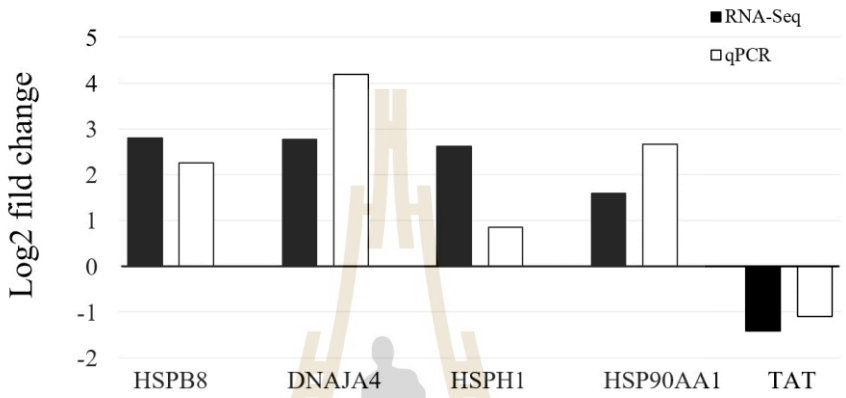

Figure 4.3 Quantitative polymerase chain reaction (qPCR) validation of 5 differentially expressed genes (DEGs) identified using RNA-sequencing (RNA-seq). The x-axis represents the genes, and the y-axis represents their mRNA expression levels expressed in fold-change (FC) values. Expression levels determined via RNA-seq and qPCR are represented by black and white fill columns, respectively. HSPB8, heat shock protein family B (small) member 8; DNAJA4, DnaJ heat shock protein family (Hsp40) member A4; HSPH1, heat shock protein family H (Hsp110) member 1; HSP90AA1, heat shock protein 90 alpha family class A member 1; TAT, tyrosine aminotransferase.

4.4.5 Effect of dietary antioxidants on altering gene markers in UVJ tissue

The effect of antioxidant supplementation in the form of synthetic (vitamin C, vitamin E, Se, and L-carnitine) and phytogenic substances (clove, green tea pomace, and Vietnamese coriander) in breeder hen diets on altering gene markers in UVJ tissue is shown in Table 4.6. The expression of DNAJA4 and HSP90AA1 were upregulated in heat-sensitive breeder hens under heat stress without antioxidant supplementation, and that of TAT was downregulated ($P < 0.05$). In contrast, synthetic

and phytogenic antioxidant supplementations showed individual effects on HSPB8 and DNAJA4 gene expression, respectively ($P < 0.05$). The expression level of HSPB8 was downregulated under heat stress with synthetic antioxidant supplementation, while the expression level of DNAJA4 was downregulated under heat stress with phytogenic antioxidant supplementation. Although some genes showed expression levels under heat stress with antioxidant supplementation similar to those in thermoneutral condition (e.g., HSPB8, HSP90AA1, and TAT in T3 and DNAJA4 in T4), no significant differences were observed in all gene markers between antioxidant supplementation and non-supplementation under heat stress ($P > 0.05$).

Table 4.6 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on relative gene markers in the uterovaginal junction tissues containing sperm storage tubules.

Items	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
HSPB8	1.00	2.50	0.41	2.40	0.4524	0.171	0.104	0.020
DNAJA4	1.00^{b}	4.74^{a}	4.79^{a}	2.02^{ab}	0.7957	0.006	0.190	0.025
HSPH1	1.00	1.44	1.38	1.36	0.1607	0.282	0.859	0.962
HSP90AA1	1.00^{b}	2.17^{a}	1.12^{ab}	1.87^{ab}	0.2183	0.033	0.073	0.590
TAT	1.00^{a}	0.36^{b}	0.98^{ab}	0.46^{ab}	0.1564	0.250	0.138	0.700

$^{a-b}$Means within each row with different superscripts are significantly different ($P < 0.05$).

[1]T1, thermoneutral zone (23±1℃) + basal diet without supplementation; T2, heat stress condition (36±1℃, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1℃, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1℃, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1= thermoneutral (T1) vs. heat stress condition (T2, T3, T4); 2, non-supplement (T2) vs. supplement (T3, T4); 3, synthetic antioxidants (T3) vs. phytogenic antioxidants (T4).

4.5 Discussion

Breeder hens store sperm in SSTs in the UVJ and have the potential to modulate the motility and metabolic activity of the residing sperm (Yang et al., 2021a). HS in breeder hens could trigger detrimental impacts on cellular structure and function, leading to disruptions in transcription, RNA processing, oxidative metabolism, the integrity of various membranes, and reduction of productive and reproductive performances (Roushdy et al., 2018; Yang et al., 2021b). Transcriptome analysis can help to understand both gene candidate and regulatory mechanisms associated with HS response in UVJ tissues containing SSTs in different breeder hen breeds (Kubota et al., 2023), which can be used in the development of dietary antioxidant strategies to alleviate the negative effect of HS on the reproductive performance of breeder hens (Gvozdanovic et al., 2023). Our transcriptomic analysis identified 387 DEGs in UVJ tissues containing SSTs by comparison between heat-adapted and heat-sensitive breeder hens. Of these, 159 and 228 DEGs were upregulated and downregulated in heat-sensitive breeder hens, respectively. The upregulated chaperones and co-chaperones HSP and DNAJ gene families and the downregulated TAT gene were used as gene markers in heat-stressed hens supplemented with antioxidants under HS. Both synthetic and phytogenic antioxidants supplementations affect the expression levels of gene markers in UVJ tissues containing SSTs in heat-stressed breeder hens.

In this study, the differential transcriptome responses to heat stress in UVJ of breeder hens between heat-adapted and heat-sensitive breeder hens were the expression of 1 HSP110 (HSPH1), 1 HSP90 (HSP90AA1), 3 HSP70 (HSPA4L, HSPA4, and HSPA8), 1 HSP60 (HSPD1), 4 HSP40 (DNAJA4, DNAJB4, DNAJA1, and DNAJB1), and 1small HSP (HSPB8) genes ($P < 0.05$; Table 4.4 and the supplementary Table 4.7). Srikanth et al. (2019) explained that HSP family HSP70 and HSP90 were significantly upregulated in chickens at the colder (8–26 °C) than in hot and humid regions that were challenged acute (35°C for 5h) and chronic (3 days of 35°C for 8h/day). Whereas UVJ tissue of HS breeder hens upregulated HSPD1, HSPA2, HSPA4, HSPA4L, HSPA5, HSPA8, HSP90AA1, HSP90B1, HSPH, HSP25, and HSPB8 transcripts compared to breeder hen under thermoneutral zone (Kubota et al., 2023). Heat Shock Proteins (HSPs) are synthesized in cells in response to heat stress and several extrinsic environmental stressors. HSPs act as molecular chaperones to play vital roles in cell tolerance under HS conditions

through protein secretion, assembly, maintaining the integrity of structural proteins, folding, trafficking, protein degradation, and regulating transcription factors (Shehata et al., 2020). DNAJA4 is an HSP coding gene to involves facilitating ATP hydrolysis and facilitating the binding of Hsc70 (the constitutive form) to the aggregated protein (Slawinska et al., 2016). HSPH1 plays a crucial role in preventing the aggregation of denatured proteins within cells experiencing intense stress and there is a significant reduction in ATP levels. It also functions to hinder the ATPase and chaperone actions of HSPA8/HSC70, as indicated by similarity in function. HSP90AA1 serves as a molecular chaperone, facilitating the proper folding and ensuring the quality of a large number of client proteins (Balakrishnan et al., 2023). Hepatic transcriptome, Lim et al. (2020) found DNAJA4, DNAJB1, HSPB9, and HSPH1 in broiler chickens subjected to cyclic HS were increased in the higher growth and weight broiler at 32°C and 70% RH than thermoneutral (TN) broiler at 22°C and 60% RH, which could indicate the adaptive responses related to the acclimation mechanism via HSPs and antioxidant enzymes. Therefore, these results may indicate that the UVJ of heat-sensitive breeder hens responds to heat stress by inducing high HSP gene expression.

GO analysis identified that 387 DEGs were enriched for 253 functions like biological process, cellular component, and molecular function ($P < 0.05$; the supplementary Table 2.8). The liver transcriptome response to HS exhibited that commercial chicken breeds presented higher 38 GO terms of response to biotic stimulus, response to external stimulus, cell chemotaxis, defense response, immune system process, response to chemical, response to stress, humoral immune response, extracellular region, integral component of plasma membrane, intrinsic component of plasma membrane, extracellular space, cytokine receptor binding, organic acid binding, and secondary active transmembrane transporter activity than 17 GO terms of native chicken breeds, which indicated to less metabolism activity and inflammatory reactions in native chicken breeds (Sánchez et al., 2022). The previous study of Kubota et al. (2023) reported that Go-enrichment classes for the top 15 terms from transcriptome analysis between TN and HS breeder hens were mainly enriched protein folding, chaperone-mediated protein folding, “de novo” protein folding, inclusion body assembly, protein refolding, chaperone cofactor-dependent protein refolding, positive regulation of ATPase activity, “de novo” post-translational protein folding, chaperone

complex, heat shock protein binding, unfolded protein binding, chaperone binding, ATPase regulator activity, ATPase activator activity, and Hsp90 protein binding. In addition, Go annotation showed that differentially expressed genes in the small yellow follicles of hens after acute HS (36°C for 4h) were mainly associated with the molecular functions of catalytic activity and binding (Cheng et al., 2018). While the DEGs of UVJ containing SSTs involved in the term were different between heat-adapted and heat-sensitive breeder hens in this study. Of the 253 GO terms, 15 GO terms were remarkably enriched (adjusted $P < 0.05$, the supplementary Table 4.8). Of these, transcripts of HSP110 (HSPH1), HSP90 (HSP90AA1), HSP70 (HSPA8), HSP60 (HSPD1), and HSP40 (DNAJB4, DNAJB1, DNAJA4, and DNAJA1) were identified in 4, 3, 7, 6, and 10 in GO terms, respectively. These phenomena demonstrated higher expression of HSP110, HSP90, HSP70, and HSP60 in UVJ tissue containing SSTs of HS breeder hens that were involved in protecting cells against both heat and oxidative stress through various functions.

KEGG pathway analysis identified 6 pathways (*P < 0.05*) and the downregulated interleukin 18 receptor 1 (IL18R1) and C-C motif chemokine ligand 19 (CCL19) under HS were related to cytokine-cytokine receptor interaction (Table 4.5). HS could negatively effect on the immune function of chickens in a variety of pro-inflammatory cytokines (Zhang et al., 2018) and upregulation of HSPs also has prohibited the production of inflammatory cytokines during cellular stress (Ferat-Osorio et al., 2014). Cytokines play a critical role as mediators that facilitate communication between the neuroendocrine system and the immune system. HS or stress can activate the secretion of inflammatory molecules, leading to an inflammatory response. Additionally, stress may reduce T cell counts and impair cellular immunity function (Xie et al., 2013). CCL19 is a small cytokine in the CC chemokine family and stimulates the chemokine receptor CCR7 to bind with their cell targets. When CCL19 is expressed, it can present as one marker for the assessment of immune responses to infections (Wang et al., 2019). Forster et al. (2008) and Noor and Wilson (2012) have revealed that the chemokines CCL19 and CCL21 significantly contributed to the trafficking of T cells and dendritic cells into lymphoid tissue which was indicated as homeostatic chemokines. In chicken stress model, transcriptome profile in bursa of Fabricius revealed that the radical S-adenosyl methionine domain-containing 2 (RSAD2), chemokine (C-C motif) ligand 19 (CCL19), chemokine-like ligand 1 precursor (CCL4), and immune responsive 1 homolog (IRG1),

IL4I, and CCLI10 was downregulated by the increasing glucocorticoid (Zhang et al., 2018). The elevated glucocorticoid is a stress response in poultry to induces the breakdown of muscle protein and adipose tissue, promoting gluconeogenesis and improving stress resistance (Beckford et al., 2020). Ray et al. (1990) and Barnes (1998) reported that when glucocorticoid had been expressed, a variety of pro-inflammatory cytokines, including IL-1 β, IL-2, IL-4, IL-6, IL-8, IL-11, IL-12, GM-CSF, and TNF-α were downregulated. The downregulation of IL18R1 (FC = −1.10) and CCL19 (FC = −1.84) in our findings may indicate that they were severely influenced by HS to alter the immune function of heat-sensitive breeder hens via the cytokine-cytokine receptor interaction pathway.

In this study, KEGG pathway analysis also identified HSP90AA1, DNAJA1, HSPA4L, HSPA8, and HSPH1 genes belonging to the protein processing in endoplasmic reticulum which was upregulated by heat stress in chicken UVJ tissues containing SSTs ($P < 0.05$) (Table 4.5). HS response in chickens reduces the capacity of the protein folding and aggregation of unfolded and misfolded proteins within ER, particularly fast-growing chickens exhibit high sensitivity to heat stress and may not have the genetic potential to acquire thermal resilience. This ultimately disrupts cellular homeostasis and functionality (Fleming et al., 2017; Miao et al., 2022). Therefore, the upregulation of HSP genes in UVJ tissues of heat-sensitive breeder hens is more required for the regulation of misfolded and denatured proteins than in heat-adapted breeder hens.

According to the results of KEGG pathway, ADH1C via the tyrosine metabolism, TAT via the phenylalanine metabolism, and CA9 and CA6 via the nitrogen metabolism were changed by HS in UVJ tissues containing SSTs (Table 4.5). Under HS condition, hepatic transcriptomic profiles of broiler demonstrated to alter protein metabolisms, this functional change may result in reduced growth performance and protein retention (Kim et al., 2022). Tyrosine amino transferase (TAT) gene is usually expressed in the liver, poultry reproductive oranges, including testes, oviducts, and ovaries which acts as an invaluable enzyme to convert tyrosine to p-hydroxyphenylpyruvate in a transamination reaction in response to a pyridoxal phosphate (Rohr et al., 2000; Lim and Song, 2016). Liu et al. (2020) have hypothesized that the TAT gene showed notable enrichment in pathways associated with the synthesis of steroid hormones and these pathways are intricately linked to egg production in Muscovy ducks while other

research has indicated that TAT gene was induced by estrogen during the differentiation and development of the oviduct in chicks (Zhou et al., 2011). In the previous study, the highest productive duck also showed higher TAT gene expression than the lowest productive duck and it was possible that the TAT gene might play a regulatory role in the egg production traits of Muscovy ducks (Ju et al., 2023). Moreover, increased expression of the TAT gene was strongly linked to ovarian carcinogenesis of laying hens (Lim and Song, 2016) and the low level of TAT gene expression in UVJ tissues of breeder hens was associated with the HS effect (Kubota et al., 2023). In this study, we observed a downregulation of the TAT gene (FC = −1.41) in the UVJ tissues of HS breeder hens. The results imply that TAT had lower expression HS in heat-sensitive breeder hens, which might indicate to a negative effect of HS.

In addition, we observed that HS is associated with nitrogen metabolism in downregulating carbonic anhydrases-encoding genes (CA9 and CA6) in HS breeder hens (Table 4.5). Acute and chronic heat exposures induced low calcium, CO_2, HCO_3^-, and CO_3^- consequently respiratory alkalosis in the blood of laying hens resulting in weak eggshells (Barrett et al., 2019). CAs catalyze the reaction $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$, which these CA isozymes in both membrane-bound and cytoplasmic isozymes have an important function in facilitating HCO_3^- and H^+ transportation mechanisms, thus contributing to the regulation of acid-base balance (Halgrain et al., 2022). The upregulation of CAs gene in turkey SST can increase pH and bicarbonate stimulating sperm motility in oviducal lumen and may play a role in the duration of sperm storage (Holm and Ridderstråle 1998). Furthermore, Ma et al. (2014) found a reduction of CA and calcium-binding protein gene expression in shell glands of HS ducks, caused negatively on reproductive development, especially in reducing oviduct weight and length, and few large follicles. Kubota et al. (2023) reported that HS breeder hens showed a reduction of CA12 and CA6 genes in SST compared to breeder hens under thermoneutral conditions. The lower expression of CA9 and CA6 in UVJ of heat-sensitive breeder hens might affect the changing of biochemical properties in SST region and fertile period length of sperms.

Gene validation of this study, HSPB8, DNAJA4, HSPH1, HSP90AA1, and TAT genes were confirmed following the transcriptome analysis as gene markers relative to HS effects in UVJ tissue containing SSTs of heat-sensitive breeder hens (Figure 4.3). These

gene markers were used to further investigate the effect of synthetic and phytogenic antioxidants in heat-sensitive breeder hens under HS to alter those gene markers in UVJ tissue. Vitamin C, E, Se, L-carnitine, and phytogenic feed additives are well known nutritional strategies to ameliorate the adverse effects of HS (Balakrishnan et al., 2023). The results of this study HSP90AA1 in either synthetic or phytogenic antioxidants, HSPB8 in a synthetic antioxidant, and DNAJA4 in phytogenic antioxidants showed expression levels similar to breeder hens under TN conditions ($P > 0.05$; Table 4.6) and seem to be slightly downregulated than HS breeder hens without antioxidant supplementation ($P > 0.05$). HSP70 and HSP90 were downregulated in heat-stressed broilers fed with vitamin E/Se-supplemented diets (Calik et at 2022), vitamin C, and L-carnitine (Surai et al., 2019; Goel et al., 2022). While, phytogenic feed additives consisting of encapsulated essential oils, dried herbs and spices, and saponins had an effective downregulation of HSP70 (Hosseini et al., 2018; Greene et al., 2021) and expression levels of HSP70 and HSP90 were dramatically reduced during resveratrol feeding after challenged HS conditions in the chickens (Sahin et al., 2012). Vitamin C, E, Se, and L-carnitine play crucial roles within the antioxidative defense network, and their effectiveness is optimized when they work together synergistically (Leskovec et al., 2018; Surai et al., 2019). In addition, according to our previous studies demonstrated that the combination of clove, green tea pomace, and Vietnamese coriander at a 1:1:1 (v:v:v) ratio, containing eugenol, gallic acid, catechin, ellagic acid, quercetin, and kaempferol showed high efficacy in radical scavenging in terms of DPPH, ABTS and FRAP and cellular H_2O_2 inhibition (Pasri et al., 2023). The high expression of HSP genes may play a role in mitigating OS induced by HS which safeguards cells from both HS and OS by performing diverse functions, including the development of chicken thermal endurance, the modulation of apoptotic signaling pathways, and the regulation of cellular oxidative states (Shehata et al., 2020). However, the properties of synthetic and phytogenic antioxidants can eliminate the presence of ROS by enzymatic and nonenzymatic antioxidant defense mechanisms and free radical scavenging antioxidants, consequently downregulation of HSPs expression (Calik et al., 2022). Therefore, the supplementation of either synthetic or phytogenic in breeder hens under HS are trend to downregulate HSPs expression in UVJ tissue containing SSTs,

which is associated with the antioxidant properties of their bioactive compounds thereby reducing the demand for the HSPs response.

TAT expression in UVJ tissues of breeder hens can serve as a biomarker for tracking oviduct damage resulting from heat-induced stress (Kubota et al., 2023). In our results, TN breeder hens had higher TAT gene expression than HS breeder hens without supplementation and neither synthetic nor phytogenic antioxidants did not influence TAT gene expression (Table 4.6). However, Ju et al. (2023) have reported that the increased TAT gene expression was correlated with the highest egg production of Muscovy ducks. The TAT gene plays a crucial role in the regulation of estrogen-induced genes and is associated with reproductive organ developments of chickens, underscoring its significance in reproductive health and function (Rohr et al., 2000). In the nutritional study field, feeding high-zinc diets decreased concentrations of estrogen resulting in dramatic changes in size and formation of tubular glands in the chicken oviduct but chickens fed normal diets increased the concentration of estrogen in serum and expression of the TAT gene (Jeong et al., 2013). Some vitamins, minerals, or phytogenic plants as antioxidant feed additives have several biological properties in inducing estrogen hormones and reproductive encouragement (Attia et al., 2016; Amevor et al., 2021). Vitamin C, E, Se, and L-carnitine can improve reproductive performance through synergistic ability in the antioxidant system in subcellular compartments or the extracellular space. They protect proteins and lipids from oxidative damage and prevent cell membrane damage in breeder hens challenged with HS (Shakeri et al., 2020; Surai et al., 2019). For phytogenic, catechin, quercetin, or other polyphenols from plants are phytoestrogens and plays a key mechanism of action through estrogen receptor (ER) binding in mediating oestrogen action in the uterus (Moon et al., 2021; Amevor et al., 2021). Phytoestrogens can provide an essential phenolic ring to bind with estrogen receptors exhibiting a molecular weight the same as estradiol (E2), these compounds can serve as both agonists and antagonists of estrogen receptors in the oviduct to modulate reproductive function (Yuan et al., 2016). However, either synthetic or phytogenic antioxidants supplementations did not change TAT gene expression in this study although heat-stressed breeder hens supplemented with synthetic antioxidants showed the almost same TAT gene expression levels as breeder hens under thermoneutral conditions. It was thus interesting to further

investigate the effect of dietary antioxidants on TAT gene expression in breeder hens under HS.

4.6 Conclusions

To the extending of our understanding, this study is the first to provide information on the differences in the expression profile of genes in the UVJ tissue containing SSTs between heat-adapted and heat-sensitive breeder hens under HS. There was a total of 387 DEGs in the UVJ tissue that compared the HS responses of heat-adapted and heat-sensitive breeder hens. Significantly, GO functional analysis using 387 DEGs exhibited that the top 15 GO terms were notably enriched in the chaperones and co-chaperones of HSP and DNAJ gene families in heat-sensitive breeder hens. KEGG pathway analysis identified upregulated HSP and DNAJ gene families relative to protein processing in endoplasmic reticulum, downregulated IL18R1 and CCL19 genes relative to cytokine-cytokine receptor interaction, ADH1C, TAT, CA9, and CA6 relative to tyrosine, phenylalanine, and nitrogen metabolism which these changes might indicate that UVJ tissue was damaged by HS in heat-sensitive breeder hens than heat-adapted breeder hens. The different expressions of HSPB8, DNAJA4, HSP90AA1, and TAT genes can be used as potential gene markers relative to HS effects in UVJ tissue containing SSTs of heat-sensitive breeder hens. In addition, the supplementation of synthetic and phytogenic antioxidants has the potential to modulate the HSP90AA1 and TAT gene expression in UVJ tissue of heat-sensitive breeder hens subjected to HS which can indicate the ability of breeder hens to alleviate HS effects. Therefore, our results provided a valuable resource of transcriptomic data to explain the global repertoire of functional genes involved in HS effect on UVJ tissue containing SSTs of breeder hens. This gene marker identification can contribute to guidelines in applying dietary antioxidants for overcoming the HS effect, maintaining reproductive performance, and preventing sperms in the SST of breeder hens during HS exposure.

4.7 References

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CHAPTER V
SYNTHETIC AND PHYTOGENIC ANTIOXIDANTS IMPROVE PRODUCTIVE PERFORMANCE, ANTIOXIDANT ACTIVITY, GENE EXPRESSION, AND OFFSPRING QUALITY IN BREEDER HENS SUBJECTED TO HEAT STRESS

5.1 Abstract

This study aimed to investigate the efficacy of a synthetic source (a combination of vitamin E, vitamin C, selenium, and L-carnitine) and phytogenic sources (a combination of clove, green tea pomace, and Vietnamese coriander) in overcoming heat stress (HS) damage in female breeder hens on production, blood chemistry, sperm survival in the oviduct, antioxidant properties, gene expression, and quality of offspring. One hundred SUT female breeder hens were housed in individual cages and divided into four treatment groups: T1) basal diets in the thermoneutral (TN) zone; T2) basal diets under HS; 3) basal diets with synthetic antioxidants under HS; and T4) basal diets with phytochemical antioxidants under HS. The result revealed that HS condition had a negative effect on reducing final body weight, egg weight, and 1-day-old chick weight while increasing water intake and FCR and altered blood chemicals in breeder hens compared to TN breeder hens ($P < 0.05$). However, either synthetic or phytogenic antioxidants resulted in increased egg production and hatchability, while decreasing the number of late stages of embryo death during the incubation ($P < 0.05$). Furthermore, the synthetic antioxidants also improved the uniformity of chicks and reduced late-stage embryo death compared with phytogenic antioxidants ($P < 0.05$). HS breeder hens fed with either of the antioxidant sources exhibited higher antioxidant capacity in terms of DPPH and ABTS radical scavenging (in yolk, liver, and breast meat) and FRAP radical scavenging (in yolk and liver) and lower liver malondialdehyde than HS breeder hens fed with the control diet ($P < 0.05$). Additionally, the gene expression of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) in the liver was upregulated, whereas the expression of pro-inflammatory cytokines

(nuclear factor-KB) and heat shock proteins (HSP70 and HSP90) was downregulated in breeder hens that received both antioxidant sources ($P < 0.05$). Future investigations should focus on the potential for combinations of synthetic and phytogenic antioxidants in diets for HS breeder hens.

Keywords: Dietary antioxidant; Breeder hen; Heat stress; Antioxidant activity; Hatchability

5.2 Introduction

In the context of global climate change, poultry production faces increasingly challenging conditions, particularly during heat stress (HS) episodes. High environmental temperature of 32–38°C, coupled with high humidity, could induce large amounts of reactive oxygen species (ROS) that lead to oxidative stress (OS) in female breeder hens, which negatively affects the integrity of the sperm membrane and DNA in sperm storage tubules, egg production, egg quality, fertility, hatchability, and embryo development, causing economic losses (Ajakaiye et al., 2011; Fouad et al., 2016). Methods to overcome heat stress damage include supplementation with dietary antioxidants, such as vitamin C, vitamin E, selenium (Se), manganese (Mn), zinc (Zn), or phytogenic (Hu et al., 2019). In addition, the resolution of HS using a mixture of antioxidants from synthetic or natural sources that function at all levels of the antioxidant defense network can potentially alleviate the negative impacts of HS. Unfortunately, little information is available regarding the use of mixtures from synthetic or phytogenic sources in breeder hens.

Dietary antioxidants are expected to serve as antioxidant defense networks in three areas: organelles, subcellular compartments, and the extracellular space (Horváth and Babinszky, 2018). Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), serve as the first level of defense against ROH chain initiation. Optimum dietary levels of Mn, Cu, Zn, and Se act as redox catalysts, form integral components of active sites necessary for antioxidant function or serve as co-factors in the regulation (Leung, 1998). However, some ROS, particularly transition metal ions, may remain active, causing lipid peroxidation and

damage to DNA and proteins. To address this issue, second-level antioxidants such as vitamins A, E, and C, carotenoids, coenzyme Q10, and L-carnitine are required to break the chain and scavenge peroxyl radicals, preventing the propagation of lipid peroxidation. The third level of the antioxidant defense network works continuously to repair and remove ROS-damaged molecules (lipids, proteins, and DNA) through the actions of heat shock proteins (HSPs), methionine sulfoxide reductases, DNA repair enzymes, and phospholipases (Surai and Kochish, 2019; Surai et al., 2019). This necessitates nutritionists and feed formulators to find suitable antioxidants that can effectively support hens in coping with HS conditions (Surai et al., 2016).

Previous studies have reported a synergistic effect of dietary vitamins E and C in poultry during HS on antioxidant status (Jena et al., 2013) and productive performance (Ipek and Dikmen, 2014). The combination of vitamin E and Se in diets provides highly effective protection against OS and improves the production and reproduction of poultry in comparison to the use of individual antioxidants (Harsini et al., 2012; Horváth and Babinszky, 2018). The combination of vitamins E and C and Se in the diet can act synergistically as antioxidants to reduce HS and lipid peroxidation in poultry meat (Leskovec et al., 2019). Furthermore, the use of L-carnitine is gaining attention for its crucial role as a novel antioxidant via preventing DNA damage induced by ROS, stimulating antioxidant enzyme activities, and supporting energy production in laying hens (Surai, 2015; Agarwal et al., 2018). Dietary L-carnitine supplementation in poultry decreases malondialdehyde (MDA) levels and increases SOD, CAT, and GSH-Px activities under high stocking density stress (Çetin and Güçlü, 2019). In addition, some studies have reported improved egg production, hatchability, antioxidant activity, and offspring quality in laying hens and duck breeders fed L-carnitine (Salmanzadeh, 2011; Wang et al., 2013; Awad et al., 2017).

Phytogenic compounds consist of a variety of polyphenols derived from plant materials that can activate the expression of stress response proteins, such as HSPs and antioxidant enzymes, which then repress ROS and interfere with negative inducers in the HS response (Hu et al., 2019; Saracila et al., 2021). The most bioactive compound in cloves (*Syzygium aromaticum*) is eugenol, which is a potent natural antioxidant (Hemalatha et al., 2016). Supplementation of poultry diets with clove oil or powder can improve feed efficiency, egg production, immunity, and antioxidant activity

(Mahrous et al., 2017; Sehitoglu and Kaya, 2021). Green tea (*Camellia sinensis*), rich in catechins, exhibits antioxidant, antimicrobial, antifungal, and anticarcinogenic properties (Pinto et al., 2020). The supplementation of green tea powder or extract to breeder poultry diets has been shown to reduce egg yolk concentrations of MDA and improve fertility, hatchability, and sperm quality (Kara et al., 2016; Chen et al., 2021; Wang et al., 2021). Vietnamese coriander (*Persicaria odorata*) has a high phytochemical composition, especially of gallic acid, quercetin, ferulic acid, apigenin, and essential oils, which contribute to antioxidant and biological activities (Pawłowska et al., 2020). Supplementing broiler or laying hen diets with Vietnamese coriander leaf meal has shown beneficial effects on growth performance, digestibility, egg production, egg weight, and lipid peroxidation in meat (Ooi et al., 2018; Basit et al., 2020; Glinubon et al., 2022). In our previous *in vitro* study, we examined 17 edible plant materials; clove, green tea pomace, and Vietnamese coriander showed notably high levels of phenolic and total flavonoid content, as well as strong antioxidant activity in terms of DPPH, ABTS, and FRAP radical scavenging. Their equal-part combination (1:1:1 ratio, v:v:v) produced synergistic antioxidant properties and improved cell safety, making them suitable candidates as phytogenic antioxidant feed additives (Pasri et al., 2023).

Unfortunately, no information is available on the combined effects of vitamins E, C, Se, L-carnitine, and phytogenic antioxidants in breeder hens under HS that would be applicable to the development of a potential group of feed additives to alleviate the adverse effects of HS. Therefore, this study aimed to investigate the efficacy of two sources of antioxidants, synthetic (a combination of vitamin E, vitamin C, Se, and L-carnitine) and phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) in mitigating HS damage in breeder hens in terms of productive performance, antioxidant properties, gene expression, and quality of offspring.

5.3 Materials and methods

5.3.1 Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of Suranaree University of Technology (SUT-IACUC-012/2020).

5.3.2 Housing, birds, and experimental diets

This study maintained controlled temperature (°C) and humidity (%) levels according to the temperature and humidity stress indices for laying hens (Hy-line, 2016). A thermoneutral (TN) zone and chronic HS were implemented according to the methodology described by Duangjinda et al. (2017). The TN was set up at 23±1°C with a humidity of 40–70% by using an air conditioner. The HS room was kept at a temperature of 36±1°C for 4 h daily, throughout the experimental period from 38–46 weeks of age (from 1 pm to 5 pm) using a gas heater with thermostatically-controlled equipment, whereas during the remainder of the day, the temperature was maintained consistently under the conditions within the TN zone. The experimental periods were divided into an adaptation period from 33–38 weeks of age and an assessment of productive performance from 38–46 weeks of age.

A total of 100 female Suranaree University of Technology (SUT) breeder hens (33-week-old) were housed in individual cages with dimensions of 40 × 45 × 40 cm^3 (length × width × height) and divided into four treatment groups, each consisting of 25 females, using a completely randomized design. Group 1 was raised in a TN room, whereas groups 2, 3, and 4 were subjected to HS for 4 h daily. In this study, two sources of antioxidants (synthetic and phytogenic) were evaluated in female SUT breeder hens under HS conditions. The experimental diets consisted of four treatments: T1) basal diets under conditions of thermoneutrality, T2) basal diets under HS, T3) basal diets with combined synthetic antioxidant (200 mg of vitamin C/kg, 150 mg of vitamin E/kg, 0.30 mg of Se yeast/kg, and 150 mg of carnitine/kg) under HS as recommended by Ross 308 parent stock standard and previous studies (Çetin and Güçlü, 2019; Shakeri et al., 2020; Aviagen, 2021), and T4) basal diets with 1% phytogenic antioxidant (a mixture of clove, green tea pomace, and Vietnamese coriander powders, 1:1:1 ratio/w:w:w) under HS; this particular combination was previously evaluated in our laboratory (Pasri et al., 2023). Diets were formulated to meet the nutrient requirements according to the National Research Council 1994 (NRC, 1994) and Ross 308 parent stock standard recommendations (Aviagen, 2021) (15% CP, 2800 kcal ME/kg) (Table 5.1). All breeder hens were provided 16 h of light per day, received 140 g of feed daily, and had unrestricted access to water throughout the experimental period. Each experimental

diet was provided to breeder hens for approximately 5 weeks (33–38 weeks of age) prior to starting the trial.

Table 5.1 Ingredients and chemical composition of the basal diet.

	Female breeder hen diets	
	25-50 weeks of age	**After 50 weeks of age**
Ingredients (%)		
Corn	64.60	63.50
Soybean meal, 44 %CP	18.20	16.52
Full fat soybean meal	6.70	9.00
Calcium carbonate	8.50	8.90
Monocalcium phosphate	0.94	1.00
Salt	0.41	0.44
DL-Methionine	0.135	0.134
L-Lysine	-	-
L-Threonine	-	-
Premix	0.52[1]	0.52[1]
Analyzed compositions (%)		
Dry matter	93.06	93.10
Crude protein	16.02	16.20
Crude fiber	3.06	3.04
Ash	11.08	11.66
Ether extract	3.35	4.49
Calculated compositions (%)		
Metabolizable energy (kcal/kg)	2,800	2,800
Calcium	3.51	3.71
Total Phosphorus	0.53	0.54
Available phosphorus	0.31	0.32
Digestible Lysine	0.70	0.70
Digestible Methionine	0.35	0.35
Digestible Methionine + Cystine	0.57	0.57
Digestible Threonine	0.50	0.50

[1]Premix for breeder hens (0.52%) provided the following (per kg of diet) by withdrawing vitamin E and Se; vitamin A, 15,000 IU; vitamin D3, 3,750 IU; vitamin K3, 5 mg; vitamin B1, 2 mg; vitamin B2, 9.8 mg; vitamin B6, 4 mg; vitamin B12, 25 mg; pantothenic acid, 11.04 mg; nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 15.5 µg; choline chloride, 250 mg; Cu, 2.1 mg; Mn, 84 mg; Zn, 66.5 mg; Fe, 80 mg; I, 1.2 mg.

5.3.3 Blood chemical analysis

After the hens were subjected to HS for 4 h (38 weeks of age), 12 breeder hens from each treatment group were randomly selected for blood chemical analysis using an Abbott i-STAT 1 handheld blood gas analyzer (Abbott Point of Care Inc. IL, USA) equipped with a CG8+ cartridge (Abaxis item number 600–9001). This CG8+ cartridge performed various parameters such as partial pressure of carbon dioxide (PCO_2) and oxygen (PO_2), pH, saturation of oxygen (sO_2), concentration of bicarbonate ions (HCO_3^-), total concentration carbon dioxide (TCO_2), concentration ionized calcium (iCa), sodium (Na), potassium (K), glucose (Glu), hematocrit (Hct), hemoglobin (Hgb), and base excess (BE). Blood from each breeder hen was collected and quickly placed in a lithium heparin tube that should be used for analysis within 3 min. One hundred µL of blood was dropped into the CG8+ cartridge, which was subsequently inserted into the Abbott i-STAT 1 handheld blood gas analyzer (Barrett et al., 2019).

5.3.4 Productive performance measurements

Productive performance parameters were measured over 8 weeks from 38–46 weeks of age. Body weight was recorded at the beginning and end of the experiment. Daily records were made for the number of eggs, egg weight, feed intake, and water intake in each treatment throughout the experimental period and were used to calculate egg production, feed conversion ratio, average egg weight, average daily feed intake, and water intake.

The length of the fertile period of sperm was determined from eggs collected over 21 days (38–41 weeks of age). Semen samples were collected from 60 Lueng Hang Khao breeder males by pooling and diluting with Beltsville poultry semen extender II (1:1/v:v) prior to artificial insemination. The breeder hens were artificially inseminated for two days continually (0.1 mL of pooled semen/time) and then the hens in groups 2, 3, and 4 were exposed to HS at 36°C for 4 h/day. From day 3, eggs

from all treatments were collected over 21 days and stored in a cool room at 15°C. Every 7 days, these eggs were then placed in an automatic incubator (Model 192, Petersime Incubation Equipment Co., Ltd., Zulte, Belgium) with optimal conditions at 37.67±0.20°C and 62–65% relative humidity at the hatchery of the University farm. The fertile period of the sperm was determined by candling on day 7 of incubation. When the infertile eggs were broken, the germinal disc region was monitored for embryonic development. The number of days for the fertile period of sperm was counted from the last day of the fertile egg prior to a sequence of three consecutive days with detected infertile eggs (Biswas et al., 2010; Ahammad et al., 2013).

Fertility, hatchability, and embryonic mortality rate were assessed from 41–46 weeks of age. Each breeder hen was artificially inseminated twice per week with pooled semen in the afternoon and induced daily with heat stress at 36°C for 4 h. Eggs in each treatment were then collected daily and stored in a cool room at 15°C. Each week, the eggs were incubated in an automatic incubator for 21 days. Fertility, early, and late embryonic mortality rates were detected by candling on days 10 and 18, whereas hatchability was measured on day 21 of incubation by counting the number of 1-day-old chicks. Productive performances were calculated using formulae (Salmanzadeh, 2011; Urso et al., 2015): Fertility (%) = [(number of fertilized eggs/total eggs set) × 100]; Early embryonic mortality rate (%) = [(number of fertilized eggs before day 10/total eggs set) × 100]; Late embryonic mortality rate (%) = [(number of fertilized eggs after day 10/total eggs set) × 100]; Hatchability (%) = [(number of day-old chicks/fertilized eggs) × 100], and Total hatchability (%) = [(number of 1-day old chicks/total eggs set) × 100].

5.3.5 Sample collection and sample extraction for antioxidant activity

At the end of the experiments, 25 breeder hens were randomly selected and slaughtered after the birds were heated in their cages at 36°C for 4 h. Liver and breast tissues were collected, immediately frozen in liquid nitrogen, and stored at −80°C until further gene expression and antioxidant activity analyses. Two grams each of egg yolk, liver, and breast tissue were extracted with 2 mL of 99% ethanol in a centrifugal tube. The samples were ground for 20 s using an ultra-homogenizer and then centrifuged at 12,000 × *g* at 4°C for 10 min. The supernatants were used to estimate the antioxidant activity.

5.3.5.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH•) scavenging activity assay

DPPH• scavenging activity was determined according to the method described by Nuengchamnong et al. (2009). For each sample extraction (100 µL), 100 µL of 0.6 mM DPPH in ethanol was added to a 96-well microplate. The mixture was gently shaken and incubated in the dark for 30 min. The absorbance of the reaction mixture was measured at 517 nm using a microplate spectrophotometer (Thermo ScientificTM, MultidkanTM GO, Japan). Ethanol was used as a reagent blank instead of the sample. The DPPH• scavenging activity was calculated as: Inhibition (%) = [(absorbance of blank – absorbance of sample)/(absorbance of blank)] × 100. All measurements were performed in triplicate.

5.3.5.2 Scavenging activity assay of 2, 2'-azinobis-(3-ethylbenzothiazoline -6-sulfonic acid) (ABTS•+) radical

The $ABTS^{\bullet+}$ cation radical assay was conducted as described by Re et al. (1999). The $ABTS^{\bullet+}$ stock solution was prepared by mixing 7.4 mM ABTS with 2.6 mM of potassium persulfate in a 10 mM phosphate buffer solution at pH 7.4. The mixture was then left to react overnight (12–16 h) in the dark at 4°C. Before starting the reaction, the freshly prepared $ABTS^{\bullet+}$ stock solution was adjusted to an absorbance value of 0.70±0.02 at 734 nm by dilution with a 10 mM cooled phosphate buffer. For the assay, 180 µL of $ABTS^{\bullet+}$ working solution was added to 20 µL of the extracted sample solvents in a 96-well microplate.

After 6 min of inoculation at room temperature, absorbance at 734 nm was measured using a microplate spectrophotometer. The absorbance of the blank was measured using ethanol used in the reaction. The $ABTS^{\bullet+}$ cation radical was calculated using the following equation: Inhibition (%) = [(absorbance of blank – absorbance of sample)/(absorbance of blank)] × 100. The results were compared to the standard curve and reported in mM equivalent trolox/g sample weight (mM TE/g sample).

5.3.5.3 The ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted according to the method described by Benzie and Strain (1996). The working FRAP reagent required fresh preparation before use, consisting of 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-Tris (2-

pyridyl)-s-triazine in 40 mM hydrochloric acid, and 20 mM iron chloride, mixed at a ratio of 10:1:1/v:v:v. The reagent was incubated at 37°C for 15 min. The 200 µL of the FRAP working reagent was added to 20 µL of sample extraction solvents in a 96-well microplate and incubated for 30 min, and the absorbance was then measured using a microplate spectrophotometer at 593 nm. The FRAP value was calculated using a calibration curve of Trolox (25–100 mM/mL) and the results were reported as mM Trolox equivalents per gram of sample weight (mM TE/g sample).

5.3.6 Thiobarbituric acid reactive substances (TBARs)

Egg yolk, liver, and breast tissue (2 g) were homogenized with 6 mL of deionized water and 34 µL of 7.2% butylated hydroxytoluene (BHT) in ethanol using ultra-homogenizer for 40 s. Subsequently, 2 mL of the homogenized sample was mixed with 4 mL of TBA-TCA solution (20 mM TBA in 15% TCA) in a 15 mL tube and boiled at 95°C for 20 min in an ultrasonic bath (Ultrasonic Cleaner 3200 EP S3, Soltec, Italy, 40 KHz and 180 W). After cooling, the mixture was centrifuged at 5,000 × g for 10 min at room temperature. The supernatant (200 µL) was then transferred to a 96-well microplate, and absorbance readings were taken at 532 nm. To quantify lipid peroxidation, MDA was used as a standard at concentrations from 5–40 µM. The TBARs value was expressed as MDA equivalents per gram of sample weight (µM MDA/g sample) and determined based on the calibration curve of MDA (Grotto et al., 2009).

5.3.7 Hepatic gene expression

Total RNA was extracted from liver tissue using QIAamp® DNA Stool Mini kits (Qiagen, Hilden, Germany) and purified using a QIAamp spin column (Qiagen, Hilden, Germany). RNA purity and quantification were assessed using a Nanodrop spectrophotometer at 260 nm/280 nm. Subsequently, 1 µg of high-quality RNA sample was applied for complementary DNA (cDNA) synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). For real-time polymerase chain reaction (PCR), each reaction's master mix (8 µL) contained 5 µL of SYBR Green, 0.4 µL of forward primer, 0.4 µL reverse primer, and 2.2 µL of H_2O_2 and then added 2 µL of cDNA samples in a 96-well microplate. The real-time PCR was performed using the QuantiNova™ SYBR Green PCR kit (Qiagen, Hilden, Germany) and analyzed in triplicate as described by Humam et al., (2019). The primer sequences for SOD, CAT, GSH-Px, nuclear factor-κB (NF-κB), heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), and β-actin are

presented in Table 5.2. Reverse transcription-quantitative real-time PCR (RT-qPCR) was accomplished using the CFX96 real-time PCR system (BioRad, Hercules, California, USA). The RT-qPCR reactions were conducted as follows: initial heat activation at 94°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and final extension at 72°C for 30 s. Relative quantification of the target gene expressions was normalized using β-actin as the reference gene and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 5.2 Primer sequences used in real-time PCR.

Gene	Primer sequences[1]	Accession No.
SOD	F-5'-CACTGCATCATTGGCCGTACCA-3'	NM_001031215.1
	R-5'-GCTTGCACACGGAAGAGCAAGT-3'	
CAT	F-5'-TGGCGGTAGGAGTCTGGTCT-3'	NM_205064.1
	R-5'-GTCCCGTCCGTCAGCCATTT-3'	
GSH-Px	F-5'-GCTGTTGCCTTCCTGAGAG-3'	NM_001277853.1
	R-5'-GTTCCAGGAGACGTCGTTGC-3'	
HSP70	F-5'-GATCTGGGCACCACGTATTCT-3'	FJ217667.1
	R-5'-GGTTCATTGCCACTTGGTTCTT-3'	
HSP90	F-5'-ACACATGCCAACCGCATTTA-3'	NM_001109785.1
	R-5'-CCTCCTCAGCAGCAGTATCA-3'	
NF-κB	F-5'-GAAGGAATCGTACCGGGAACA-3'	NM_205134
	R-5'-CTCAGAGGGCCTTGTGACAGTAA-3'	
β-actin	F-5'-TTGGTTTGTCAAGCAAGCGG-3'	NM 205518.1
	R-5'-CCCCCACATACTGGCACTTT-3'	

[1]Data form reference Chiang et al. (2009), Ahmadipour and Khajali, (2019), and Madkour et al. (2021)

5.3.8 Statistical analysis

Statistical analysis of the data was performed using analysis of variance in a completely randomized design (CRD) with SPSS software (version 16.0). Tukey's test was used to assess significant differences among treatments. Additionally, orthogonal

contrasts were used to compare the following conditions: 1, thermoneutral vs. heat stress conditions; 2, non-supplement vs. supplement; and 3, synthetic vs. phytogenic antioxidants. A significance level of $P < 0.05$ was used (SPSS Inc, 2007).

5.4 Results

5.4.1 Productive performances

According to the orthogonal contrast test, HS breeder hens had lower final body weight (FBW), egg weight (EW), and 1-day-old chick weight, but higher hatchability and water intake (WI) than TN hens ($P < 0.05$) (Table 5.3). Either synthetic or phytogenic antioxidant supplementation groups showed increased egg production (EP) and hatchability as well as reduced feed intake (FI), WI, EW, early-dead and late-dead embryos, and 1-day-old chick weight compared to the non-supplemented group ($P < 0.05$). However, no differences were observed in any of the measured parameters between synthetic and phytogenic antioxidants ($P > 0.05$), except for chick uniformity, which was higher in the synthetic antioxidant group than in the phytogenic antioxidant group ($P < 0.05$). Interestingly, based on the Tukey analysis, it was found that the supplementation of antioxidants in the HS group could increase EP and reduce feed conversion ratio (FCR), similar to that in the TN group ($P > 0.05$). In addition, supplementation with either antioxidant resulted in higher hatchability compared to the TN group ($P < 0.05$). However, a reduction in FBW, FI, EW, and 1-day-old chick weight was observed in HS breeder hens that received phytogenic antioxidants compared to TN hens ($P < 0.05$), whereas no such differences, except EW, were observed for synthetic antioxidants ($P > 0.05$).

5.4.2 Blood chemistry parameters

Based on orthogonal contrasts, breeder hens subjected to HS had lower blood values of PCO_2, PO_2, BE, HCO_3^-, TCO_2, and iCa, whereas pH and sO_2 were higher than the TN breeder hens ($P < 0.05$) (Table 5.4). In addition, HS led to decreased blood Na, K, Hct, and Hb and increased blood Glu compared to TN ($P < 0.05$). However, under HS conditions, supplementation with either synthetic or phytogenic antioxidant sources did not alter any of the blood parameters compared to the non-supplementation group ($P > 0.05$), except for K, which was lower in the phytogenic antioxidant group than in the synthetic antioxidant group ($P < 0.05$). In addition, the Tukey tests revealed

that HS breeder hens supplemented with phytogenic antioxidants had lower blood Na and K levels than the TN group ($P < 0.05$), whereas no statistically significant differences were observed for synthetic antioxidants ($P > 0.05$).

Table 5.3 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on productive performances.

Items	Treatments[1]				Pooled SEM	Contrasts[2]		
	T1	T2	T3	T4		1	2	3
IBW (g)	2795.21	2891.37	2747.18	2785.16	26.644	0.836	0.057	0.614
FBW (g)	3314.48^{a}	3176.57^{ab}	3102.67^{ab}	3048.19^{b}	34.971	0.013	0.215	0.571
FI (g/day/hen)	137.20^{a}	137.22^{a}	135.92^{ab}	135.56^{b}	0.2673	0.103	0.025	0.634
FCR	2.36^{b}	2.51^{a}	2.45^{ab}	2.44^{ab}	0.0164	0.006	0.097	0.927
WI (mL/day/hen)	290.60^{b}	406.57^{a}	370.27^{a}	372.05^{a}	7.7241	0.001	0.015	0.913
EP (%)	88.64^{a}	83.50^{b}	88.62^{a}	89.78^{a}	0.6707	0.372	<0.001	0.524
EW (g)	64.61^{a}	63.83^{b}	62.94^{c}	62.92^{c}	0.0943	<0.001	<0.001	0.925
Fertility (%)	98.86	98.43	99.34	99.14	0.1925	0.798	0.086	0.709
Hatchability (%)	87.63^{b}	87.79^{b}	91.81^{a}	91.71^{a}	0.6394	0.046	0.009	0.957
Fertile period length of sperm (day)	15.04	14.64	14.5	14.33	0.157	0.141	0.556	0.707
Day old chick weight (g)	46.30^{a}	45.55^{a}	44.89^{ab}	43.66^{b}	0.3195	0.009	0.037	0.074
Early dead (%)	2.06^{b}	4.70^{a}	2.76^{ab}	2.67^{ab}	0.3512	0.096	0.022	0.267
Late dead (%)	5.85^{ab}	6.16^{a}	2.83^{c}	3.27^{b}	0.5029	0.123	0.011	0.744
Abnormal chicks (%)	2.55	1.38	1.02	2.21	0.3215	0.172	0.769	0.192
Chick uniformity (%)	75.85^{ab}	76.39^{ab}	82.23^{a}	70.94^{b}	1.6265	0.833	0.953	0.007

$^{a-c}$Means within each row with different superscripts are significantly different ($P < 0.05$).
[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic.

[2]Orthogonal contrasts: 1, thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2, non-supplement (T2) vs. supplement (T3, T4); 3, synthetic (T3) vs. phytogenic antioxidants (T4).

Table 5.4 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on blood chemistry.

Items	Treatments[1]				Pooled SEM	Contrasts[2]		
	T1	T2	T3	T4		1	2	3
pH	7.37[b]	7.47[a]	7.46[a]	7.46[a]	0.007	<0.001	0.458	0.859
PCO_2 (mmHg)	50.73[a]	36.90[b]	36.51[b]	36.82[b]	0.9762	<0.001	0.886	0.867
PO_2 (mmHg)	40.09[a]	37.36[b]	36.72[b]	37.18[b]	0.3859	<0.001	0.627	0.641
BE (mmol/L)	4.72[a]	3.17[ab]	2.55[b]	2.41[b]	0.2842	0.002	0.299	0.85
HCO_3^- (mmol/L)	29.94[a]	26.84[b]	26.35[b]	26.20[b]	0.3098	<0.001	0.368	0.842
TCO_2 (mmol/L)	31.33[a]	27.94[b]	27.44[b]	27.35[b]	0.3098	<0.001	0.406	0.902
sO_2 (%)	70.17[b]	77.29[a]	76.11[a]	77.05[a]	0.7313	<0.001	0.662	0.609
iCa (mmol/L)	1.78[a]	1.53[b]	1.51[b]	1.58[b]	0.0263	<0.001	0.860	0.225
Na (mmol/L)	148.35[a]	146.43[ab]	146.37[ab]	146.00[b]	0.3218	0.004	0.744	0.687
K (mmol/L)	5.13[a]	4.92[ab]	5.10[ab]	4.86[b]	0.0348	0.023	0.448	0.011
Glu (mg/dL)	229.77[b]	241.17[a]	246.16[a]	246.47[a]	1.6465	<0.001	0.159	0.947
Hct (%PCV)	28.27[a]	25.64[b]	25.72[b]	25.47[b]	0.3174	<0.001	0.942	0.781
Hgb (g/dL)	9.62[a]	8.71[b]	8.75[b]	8.66[b]	0.1076	<0.001	0.966	0.760

[a-b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1, thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2, non- supplement (T2) vs. supplement (T3, T4); 3, synthetic (T3) vs. phytogenic antioxidants (T4).

Abbreviations: PCO^2, Partial pressure of carbon dioxide; PO_2, Partial pressure of oxygen; BE, Base excess; HCO_3^-, concentration of bicarbonate ions; TCO_2, total concentration carbon dioxide; sO_2, saturation of oxygen; iCa, concentration ionized calcium. Na, sodium; K, potassium; Glu, glucose; Hct, hematocrit; Hgb, hemoglobin.

5.4.3 Antioxidant activities in liver, breast, and yolk

Results from the orthogonal contrast tests revealed that the HS breeder hen groups that received either of the antioxidant sources had higher antioxidant activities in terms of DPPH, FRAP, and ABTS radical scavenging in all samples (except for FRAP in breast tissue), as well as a decrease in egg yolk MDA compared to the non-supplementation group during HS ($P < 0.05$; Table 5.5). In this context, synthetic antioxidants showed higher DPPH and FRAP radical scavenging activity in the egg yolk and lower MDA levels in all samples compared to those fed with phytogenic antioxidants ($P < 0.05$). The Tukey test indicated that the supplementation of synthetic antioxidants in HS breeder hen diets enhanced the values of DPPH and FRAP, while reducing MDA in the egg yolk and liver compared to the TN group ($P < 0.05$).

Table 5.5 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on antioxidant activity in egg yolk, liver, and breast.

Items	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
DPPH (%)								
Egg yolk	17.23^{c}	18.20^{c}	47.03^{a}	22.18^{b}	2.2021	<0.001	<0.001	<0.001
Liver	81.82^{b}	86.24^{ab}	87.48^{a}	86.43^{ab}	0.7426	0.003	<0.001	0.567
Breast	19.49^{ab}	18.60^{b}	21.05^{a}	21.35^{a}	0.3478	0.212	0.004	0.733
FRAP (mM TE/g sample)								
Egg yolk	0.42^{b}	0.36^{b}	0.71^{a}	0.40^{b}	0.0299	0.072	<0.001	<0.001
Liver	1.87^{b}	2.14^{b}	2.49^{a}	2.53^{a}	0.0628	<0.001	0.001	0.676
Breast	0.23	0.29	0.29	0.29	0.0111	0.023	0.962	0.934
ABTS (mM TE/g sample)								
Egg yolk	5.46^{a}	5.18^{b}	5.43^{a}	5.42^{a}	0.0336	0.052	0.001	0.921
Liver	35.56^{ab}	34.18^{b}	35.23^{ab}	37.28^{a}	0.4011	1.000	0.027	0.051
Breast	40.28^{ab}	38.29^{b}	45.37^{a}	45.02^{a}	0.8393	0.103	<0.001	0.855
MDA (uM/g sample)								
Egg yolk	28.14^{ab}	32.74^{a}	10.76^{c}	23.94^{b}	1.6502	0.001	<0.001	<0.001
Liver	10.92^{a}	10.90^{a}	8.65^{b}	11.53^{a}	0.3397	0.398	0.274	0.002
Breast	8.26	8.71	7.74	8.68	0.1433	0.716	0.134	0.015

$^{a-c}$Means within each row with different superscripts are significantly different ($P < 0.05$).
[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1, thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2, non- supplement (T2) vs. supplement (T3, T4); 3, synthetic(T3) vs. phytogenic antioxidants (T4).

5.4.4 Gene expression in the liver

The orthogonal contrasts revealed the upregulation of SOD and GHS-Px genes, along with the downregulation of HSP70 and HSP90 genes in HS breeder hens compared to those in TN breeder hens ($P < 0.05$; Table 5.6). However, no differences in the expression of the CAT and NF-KB genes were found between the HS and TN groups ($P > 0.05$). During HS, it was found that either synthetic or phytogenic antioxidants could alter gene expression in the liver by upregulating SOD, CAT, and GSH-Px and downregulating NF-KB, HSP70, and HSP90 compared to non-supplementation ($P < 0.05$). It is interesting to note that, based on the Tukey test, that phytogenic antioxidants were found to induce a higher degree of expression in the GSH-Px gene compared to synthetic antioxidants ($P < 0.05$).

Table 5.6 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on gene related to antioxidant enzyme, pro-inflammatory cytokines, and heat shock proteins expressions.

Items	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
SOD	1.00^{b}	0.71^{c}	1.56^{a}	1.45^{a}	0.0001	<0.001	<0.001	0.121
CAT	1.00^{ab}	0.35^{b}	1.49^{a}	1.54^{a}	0.0240	0.672	0.003	0.907
GHS-Px	1.00^{c}	0.76^{c}	2.40^{b}	5.42^{a}	0.0001	<0.001	<0.001	<0.001
NF-KB	1.00^{ab}	1.25^{b}	0.64^{ab}	0.42^{a}	0.5257	0.285	0.009	0.381
HSP70	1.00^{b}	1.11^{b}	0.16^{a}	0.15^{a}	0.2252	<0.001	<0.001	0.799
HSP90	1.00^{b}	1.01^{b}	0.20^{a}	0.17^{a}	0.0058	0.011	0.001	0.872

[a-c]Means within each row with different superscripts are significantly different ($P < 0.05$).
[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1 C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1, thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2, non- supplement (T2) vs. supplement (T3, T4); 3, synthetic (T3) vs. phytogenic antioxidants (T4).

5.5 Discussion

Exogenous dietary antioxidants are widely accepted as effective substances for mitigating the adverse effects of HS on breeder hens in terms of favorable productive and reproductive performance, immunity, embryonic development, and antioxidant activity (Ibtisham et al., 2019; Amevor et al., 2021; Darmawan et al., 2022). Our results revealed the beneficial effects of synthetic (vitamins C, E, Se, and L-carnitine) and phytogenic (clove, green tea pomace, and Vietnamese coriander) antioxidants on egg production, hatchability, embryo development, and antioxidant activity of breeder hens under HS.

In this study, either synthetic or phytogenic antioxidant sources were found to improve egg production and hatchability in breeder hens subjected to HS. However, in the orthogonal contrast test, the FI in the HS hen groups supplemented with antioxidants was lower than that in the HS hen group without supplementation or in the TN hen group. This resulted in insufficient nutrient uptake, but these groups of hens were still able to maintain egg production. This is probably because birds anabolize fat stored in the body to conserve the nutrients needed for maintenance and production, which can be confirmed by the reduced BW of the HS hens. In general, HS poultry have a higher maintenance energy requirement for thermoregulation. However, the decrease in FI during HS results in sufficient nutrient intake, as chickens compensate by breaking down glycogen or fat stored through gluconeogenesis (Nawaz et al., 2021; Jastrebski et al., 2017). In addition, Xie et al. (2015) reported that elevated plasma glucose levels in HS broiler breeders indicate changes in carbohydrate and lipid metabolism for maintaining the metabolic rate, which is consistent with our results, which revealed increased blood glucose in HS breeder hens. In addition, this study found reductions in PCO_2, PO_2, BE, HCO_3^-, TCO_2, and iCa, together with an increase in pH and sO_2 in the blood of HS breeder hens.

The current study indicates that either of the antioxidant sources plays interconnected roles in metabolic pathways, which effectively assists breeder hens in

combatting the negative effects of HS. During HS, vitamin C serves as a co-factor for dopamine beta-hydroxylase, converting dopamine into norepinephrine in neural tissues during the HS, promoting gluconeogenesis, which indirectly causes an increase in heart rate, blood pressure, blood glucose, and skeletal muscle blood flow (Shakeri et al., 2020). Vitamin E prevents liver damage and maintains vitellogenin synthesis, which is important for yolk formation and the consequences of egg production (Khan et al., 2011). L-carnitine is crucial for transferring long-chain fatty acids from the cytoplasm to the mitochondrial matrix for β-oxidation and energy production, facilitating the anabolism of stored fat in the body of the bird, which ultimately provides energy for follicular development and egg production (Zhai et al., 2008; Awad et al., 2017). Selenium is essential for optimal poultry performance during HS, indirectly regulating triiodothyronine (T3) and thyroxine (T4) hormones, which affect the metabolic rate, protein synthesis, and nutrient metabolism (Shakeri et al., 2020).

The improved hatchability and antioxidant capacity observed in breeder hens that received dietary phytogenic antioxidants in the current study can be attributed to the deposition of bioactive antioxidant compounds. In phytogenic sources (clove, green tea pomace, and Vietnamese coriander), several bioactive compounds are associated with antioxidant properties, such as eugenol (from clove), catechin (from green tea pomace), and catechin, quercetin, kaempferol, and ellagic acid (from Vietnamese coriander). These polyphenols are potent antioxidants because their chemical structure contains more than two hydroxyl groups (PhenOH), which allow them to break bonds and release hydrogen and electrons. This effectively eliminates excess ROS (such as O_2, H_2O_2, $OH^{\bullet}$, $RO^{\bullet}$, and $RO_2^{\bullet}$) and transforms them into a phenoxyl radical (PhenO), which is more stable and less likely to initiate chain reactions than the initial radicals. Polyphenols can also transfer electrons to bind to metal-ion free radicals (such as Fe^{2+}, Cu^{2+}, or Cu^{+}) (Saracila et al., 2021). Dietary catechins or tea polyphenols are transferred from the blood to the ovaries, magnum, and other organs, providing antioxidant capacity and stability for polyunsaturated fatty acids (PUFA) in eggs (Ariana et al., 2011). Chen et al. (2021) observed catechin deposition in chickens fed with green tea, which led to increased DPPH, ABST, and OH radical scavenging activities. Additionally, clove oil or buds with high eugenol levels showed the potential to decrease MDA in eggs and increase the enzyme activity of reduced glutathione (GSH),

SOD, and glutathione S-transferase (GST) in the breast muscle (Mahrous et al., 2017; Sehitoglu and Kaya, 2021). Feeding broilers with a mixture of herbal extracts (mulberry leaf, Japanese honeysuckle, and goldthread) resulted in the accumulation of phenolic compounds in breast tissue and demonstrated the potential to increase DPPH and ABST and reduce TBARs (Jang et al., 2008). In the current study, various bioactive polyphenol compounds, such as eugenol, catechin, quercetin, kaempferol, and ellagic acid, were detected, all of which could complement the mechanisms of action to eliminate ROS. However, there are limitations to the metabolism and bioavailability of polyphenols, as some are poorly absorbed in the small intestine and require enzymatic hydrolysis by gut microbes (Abd El-Hack et al., 2022). The rapid absorption and elimination of polyphenols is a major factor leading to their low accumulation in tissues in comparison to synthetic antioxidants. Although the current study did not measure the deposition of phytogenic bioactive compounds in tissues, the reduced antioxidant properties, reflected by lower DPPH and FRAP in egg yolk, coupled with elevated MDA levels in egg yolk, liver, breast meat, may indicate inferior deposition of phytogenic compared to synthetic antioxidants. Thus, frequent supplementation could potentially improve their biological activity (Hidalgo et al., 2012).

Although egg production was maintained by either of the antioxidant sources, there was an observed reduction in egg weight. HS is known to reduce FI in poultry, resulting in insufficient nutrient intake, particularly protein, which leads to a decrease in egg weight. Khatibi et al. (2021) revealed that diets with crude protein levels of 15.0–15.5% for laying hens in a subtropical climate can improve production, egg weight, and egg mass compared to diets with 14.2–14.7% CP. Although both antioxidants in this study reduced egg weight compared to the control and HS without supplementation, the weight was still 50–70 g, which is suitable for hatching, as per the Cobb 500 or Ross 308 breeder guidelines. In general, egg weight is associated with chick weight, indicating that either of the antioxidant sources can help maintain egg weight, resulting in a normal chick weight, which benefits the poultry industry.

Neither the HS conditions nor antioxidant supplementation affected the fertility rate or fertile period length of the sperm. However, this phenomenon remains unclear, and related research on this subject is limited. HS can adversely affect the reproductive performance of both male and female poultry; however, its effects on male fertility,

specifically spermatogenesis, have been extensively studied (Fouad et al., 2016). In the current study, we attempted to minimize the sperm factor error by using artificial insemination techniques and pooling sperm. Based on our results, the fertility and fertile period length of sperm in breeder hens aged 47–50 weeks were 98–99% and 15 days, respectively. Notably, this fertility rate was higher than that of the Cobb 500 breeder hens (95%) (Cobb-Vantress Inc., 2018). This is consistent with previous reports in which the combination of vitamins C, E, and Se in ISA brown laying hen diets did not affect the fertility rate (77–80%) or fertile period of sperm (17 days), possibly because the hens reached their maximum productivity aligned with their genetic potential (Pasri et al., 2018).

Notably, the hatchability rate in HS hens supplemented with either antioxidant was higher than that in TN and HS hens without supplementation. This is consistent with the results of increased antioxidant activities (DPPH, ABTS, and FRAP radical scavenging) and decreased lipid peroxidation (MDA) in the egg yolk, liver, and breast tissues of breeder hens. Both sources of dietary antioxidants were observed to enhance hatchability, probably because the function of antioxidants that were deposited in the egg yolk was to eliminate ROS that occurred during embryonic metabolism. Embryonic tissues typically contain a high proportion of PUFA, rendering them vulnerable to lipid peroxidation (Zhai et al., 2008). ROS can also destroy other biological molecules, such as DNA, proteins, and carbohydrates, which are the leading causes of infertility and embryonic mortality (Surai et al., 2016). Various types of antioxidants have been observed in the egg yolk and tissues of chick embryos and contribute to the achievement of high-quality and viable chicks, including antioxidant enzymes, such as SOD, GSH-Px, and CAT; water-soluble antioxidants, such as vitamin C, taurine, L-carnitine, and glutathione; and fat-soluble antioxidants such as vitamin E, carotenoids, coenzyme Q, and Se (Urso et al., 2015; Surai et al., 2019).

The synergistic effects of a combination of various antioxidant sources, especially vitamin C, vitamin E, Se, and L-carnitine, have been reported (Abdel-Azeem et al., 2016; Leskovec et al., 2019; Shakeri et al., 2020). This indicates that a combination of these substances can function more effectively in the antioxidant defense network. Vitamin E plays an antioxidant role in cells and prevents the oxidation of low-density lipoproteins in cell membranes by donating electrons to lipid peroxyl radicals to stop

chain-breaking antioxidant reactions before interacting with other lipids (Ebeid, 2012). Vitamin C acts as a potent reducing agent in ROS scavenging by interacting with the tocopheroxyl radical and regenerating reduced tocopherol (Akbari et al., 2016). Selenium plays a crucial role in antioxidant activity as an essential coenzyme of GSH-Px, which facilitates the disposal of hydrogen peroxide generated after the superoxide is catalyzed by SOD in the cellular antioxidant defense network, contributing to the detoxification of lipid peroxides (Lykkesfeldt and Svendsen, 2007; Mishra and Jha, 2019).

Vitamin C and E supplementation in broiler breeder hen diets during the summer has been shown to improve FRAP activity and reduce MDA levels in erythrocytes compared to a control (Jena et al., 2013). Generally, MDA serves as a marker of oxidative stress and represents cell/tissue damage caused by lipid peroxidation, and its reduction is linked to the antioxidant defense system through antioxidant supplementation (Shakeri et al., 2020; Tang et al., 2022). It has also been reported that L-carnitine can prevent the formation of ROS in the mitochondria, such as xanthine oxidase, cytochrome p450, cyclooxygenase, lipoxygenase, nitric oxide synthase (NOS), and NADPH oxidases (NOXs), to maintain a normal electron transport chain during the elevated metabolic rates of rapid embryo development or HS exposure (Surai et al., 2016). The use of L-carnitine in female breeder hen diets has been shown to increase carnitine deposition in egg yolks and promote the utilization of yolk lipids for energy production during embryogenesis (Zhai et al., 2008; Awad et al., 2017).

Synthetic and phytogenic sources were found to upregulate the expression of gene-related antioxidant enzymes, such as SOD, CAT, and GSH-Px, in the liver. This result is consistent with the finding that vitamin E, vitamin C, and L-carnitine can activate the transcriptional factor activity of activator protein-1 (AP-1), nuclear factor erythroid 2 related factor 2 (Nrf2), and NF-κB DNA binding site; this activation helps in regulating the expression of adhesive molecules, cytokines, and antioxidant enzyme genes, ultimately providing additional protection in HS conditions (Surai, 2015; Min et al., 2018).

Selenium mainly regulates GSH-Px mRNA through the biological processes of selenoproteins, requiring a selenocysteine insertion sequence that incorporates a Se-specific elongation factor, selenocysteinyl-tRNA, and a selenocysteine insertion

sequence mRNA stem-loop structure into the Se-insertion complex during translation. This complex subsequently modulates a unique endonucleolytic cleavage site, resulting in increased GSH-Px mRNA expression (Weiss and Sunde, 1997; Puangmalee et al., 2020). Studies have reported that feeding chickens vitamin C, vitamin E, Se, and L-carnitine leads to increased expression of SOD, CAT, and GSH-Px mRNA in the liver, which is considered the initial step in the antioxidant defense against free radicals and superoxide (Elgendey et al., 2022).

Polyphenols exert their influence on gene expression in the liver of breeder hens through an indirect mechanism involving the synthesis of ROS-removing enzymes, which can stimulate the Keap1–Nrf2 complex by modifying cysteine residues in Kelch-like ECH-associated protein 1, leading to the translocation of Nrf2 into the nucleus. After that, Nrf2 binds to the antioxidant electrophile/antioxidant response element (EpRE/ ARE) sequence (Lee et al., 2017; Saracila et al., 2021), resulting in the upregulation of cellular antioxidant enzymes, such as SOD, CAT, GSH-Px, and GST (Hosseini-Vashan et al., 2016; Bernatoniene and Kopustinskiene, 2018). Interestingly, our study revealed that phytogenic antioxidants have a greater capacity to upregulate GSH-Px gene expression than synthetic antioxidants do. This difference is likely attributable to the fact that synthetic antioxidants contain only one type of Se, which is the main precursor for GSH-Px synthesis, whereas the phytogenic antioxidants found in cloves, green tea pomace, and Vietnamese coriander consist of a variety of polyphenols with antioxidant properties, including eugenol, gallic acid, catechin, ellagic acid, quercetin, and kaempferol.

The characteristic chemical structures of certain polyphenols can also activate antioxidant enzymes via modification of the transcription pathway (Saracila et al., 2021). Epigallocatechin gallate from green tea exhibits outstanding antioxidant activity in poultry, surpassing that of vitamin E by 25 times and vitamin C by 100 times (Abd El-Hack et al., 2020). In vitro tests have demonstrated that clove oil exhibits pronounced positive effects on antioxidant properties, including radical scavenging of DPPH, ABTS, H_2O_2, superoxide anion radicals, and chelating activities, compared to butylated hydroxyanisole (BHA), BHT, tocopherol, and trolox (Gulcin et al., 2012). This can be attributed to the presence of natural antioxidants, essential fatty acids, and lipid-soluble bioactive molecules typically found in clove oil (Sehitoglu and Kaya, 2021).

The bioactive compounds present in *Polygonum odoratum* L., such as flavonoids, alkaloids, phenolic compounds, and tannin, showed moderately potent antioxidant activity (50.25±0.61 mg/mL) compared to vitamin E (14.79±0.78) and BHT (19.71±0.79 mg/mL) (Somparn et al., 20.14). Ibtisham et al. (2019) reported that incorporating a mixture of Chinese herbal medicine and ginger powder in layer hen diets resulted in improved antioxidant capacity compared to a single form. Although the combination of clove, green tea pomace, and Vietnamese coriander has not been studied in vivo before, our previous in vitro testing indicated synergistic antioxidant properties and improved cell safety (Pasri et al., 2023), aligning with the current results. Therefore, our finding highlights the synergistic effects of various bioactive compounds in phytogenic sources, suggesting their potential contribution to serving as an antioxidant defense in all three areas (organelles, subcellular compartments, and the extracellular space).

NF-ΚB, HSP70, and HSP90 mRNA expression were found to be down-regulated in HS hens receiving synthetic and phytogenic antioxidants compared to HS hens without supplementation. In general, when animals are exposed to HS, two possible mechanisms are involved in homeostasis that make the body tolerant to HS. First, in response to HSPs, cells promote the expression of HSP genes to protect against cell damage, particularly HSP70, which plays an important role in the HS response in chickens (Soleimani et al., 2011). HSP70 in cells also helps maintain protein refolding, promotes the degradation of misfolded proteins, and reduces cell inflammation (Varasteh et al., 2015; Xu et al., 2018). Second, the oxidative stress response is a defense system against HS, and the gene playing a key role in this regulation is Nrf2 (Lian et al., 2020; Surai et al., 2021). Both response systems have been reported to improve antioxidant capacity, reduce lipid oxidation, and increase digestive enzyme activity in the gastrointestinal tract (Shehata et al., 2020). Hence, dietary antioxidants (e.g., vitamins C and E, Se, catechin from green tea, quercetin from Vietnamese coriander, and other phytochemicals) may inhibit NF-ΚB binding to inflammation-related genes. This in turn, can lower the expression of pro-inflammatory cytokines and decrease the levels of HSPs (HSP60, HSP70, and HSP90), which serve as mediators for inducing NF-ΚB expression (Jang et al., 2014; Akbarian et al., 2016; Bernatoniene and Kopustinskiene, 2018; Kumbhar et al., 2018; Chansiw et al., 2019; Manaig et al., 2022).

5.6 Conclusions

This study revealed the benefits of a combination of synthetic antioxidants (vitamins C and E, Se, and L-carnitine) and phytogenic antioxidants (clove, green tea pomace, and Vietnamese coriander) in breeder hens exposed to HS. Both sources of antioxidants individually demonstrated significant improvements in egg production and hatchability and reduced embryo mortality. In addition, either of the antioxidant sources alleviated the adverse effects of HS by increasing antioxidant defenses, as evidenced by elevated DPPH, ABTS, and FRAP radical scavenging, along with reduced lipid peroxidation in yolk and tissues. Furthermore, there is an up-regulation in the relative expression of SOD, CAT, and GSH-Px mRNA and a down-regulation of NF-KB, HSP70, and HSP90 mRNA expressions in the liver. These findings suggest there may be benefits to be observed if synthetic and phytogenic antioxidants are combined in HS breeder hens. This knowledge may lead to the development of innovative strategies for HS management by integrating dietary supplements with antioxidants.

5.7 References

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CHAPTER VI

ALLEVIATING HEAT STRESS ON BREEDER HENS: EFFECT Of DIETARY ANTIOXIDANT SUPPLEMENTATION ON REPRODUCTIVE PERFORMANCE, EGG QUALITY, OFFSPRING GROWTH, AND ANTIOXIDANT CAPACITY

6.1 Abstract

The purpose of this study was to investigate the effect of antioxidant compound substances derived from either synthetic (a combination of vitamin C and E, Se, and L-carnitine) or phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) sources in breeder hen diets under heat stress (HS) on reproductive performance, egg quality, and offspring growth performance and their antioxidant capacity. One hundred SUT female breeder hens were randomly divided into four treatments, each comprising 25 females. The hens were provided with either a basal diet or a basal diet supplemented with synthetic and phytogenic antioxidants. The experiment was conducted in two environments: the thermoneutral (TN) condition at 23±1°C and the HS condition at 36±1°C for 4 h. Reproductive performance and gg quality were conducted during 46–52 weeks of breeder hen ages. Offspring liver samples were collected from 1-d-old chicks, and a total of 132 chicks/ treatment (6 replications of 22 chicks each) were assessed for growth performance over a 21-d period. The results showed that either synthetic or phytogenic antioxidants can improve the Haugh unit and maintain the ovary weight, and large and small yellow follicles ($P < 0.05$). Although the body weight (BW) of offspring from either antioxidant source was initially low on day 1, it subsequently increased until reaching levels comparable to those in the TN and HS without supplementation by 3 weeks of age ($P > 0.05$). Either antioxidant source can improve free radical scavenging, reduce lipid peroxidation, upregulate the relative expression of SOD, CAT, and GSH-Px mRNA, and downregulate HSP90 in offspring liver ($P < 0.05$). In conclusion, this study highlights the beneficial effect of a combination of either synthetic or phytogenic antioxidants against

the negative effect of HS in female breeder hens and their transgenerational antioxidant properties passed on to offspring.

Keywords: Dietary antioxidant; Phytogenic substance; Breeder hen; Heat stress; Chick offspring

6.2 Introduction

Female breeder hens are vital in breeding programs for producing healthy broiler chicks with high growth potential (Surai et al., 2016). Nowadays, breeder hens face the challenge of high environmental temperatures, particularly in tropical or subtropical countries, leading to heat stress (HS) which adversely affects reproduction, production, hatchability, embryonic development, and offspring growth performance (Zhu et al., 2017). As the chicken embryo develops externally, the nutrient composition of the eggs plays a crucial role in fulfilling the requirements of the embryo. This composition directly affects the structure, physiology, metabolism, and overall health of the developing embryo. Therefore, effective management of breeder hen diets is necessary to maximize high-quality egg production, improve hatchability, and ensure that their offspring possess the potential to withstand initial field challenges and achieve performance goals (Chang et al., 2016).

Dietary antioxidants are among the nutrients of interest to mitigate the HS in poultry. It has been proven that combining various antioxidants can yield synergistically beneficial effects on the antioxidant system in both breeder hens and their offspring, contributing to the protection of overall cellular health (Surai et al., 2016). Dietary vitamins, minerals, and phytogenic antioxidants play a crucial role in serving as antioxidant deposits in egg yolk, thereby promoting embryo development, health status, and offspring growth. This is particularly important for enhancing antioxidant capacity in breeder hens facing HS (Shakeri et al., 2020; Amevor et al., 2021). The supplementation of vitamin C, vitamin E, selenium (Se), and L-carnitine in poultry diets, either individually or in combination, showed synergetic effects on the antioxidant defense system within organelles, subcellular compartments, and extracellular space (Horváth and Babinszky, 2018; Leskovec et al., 2019). Supplementing layer hen diets with a combination of vitamin C and E under HS has been shown to enhance

reproductive organs, particularly by increasing the size and weight of follicles and oviducts (Attia et al., 2016). Maternal diets enriched with vitamin E and Se have demonstrated effective deposition in egg yolk, thereby enhancing the antioxidant defense system within the developing tissues of offspring chicks, which contributes to improved growth performances observed in the offspring (Yang et al., 2019; Xia et al., 2022). The supplementation of maternal diet with Se resulted in an increased Se content in egg yolk, leading to the upregulation of glutathione peroxidase gene expression, enhanced total antioxidant capacity, and improved abilities to inhibit hydroxyl radicals and reduce malondialdehyde (MDA) in the liver of 1 d old chicks (Wang et al., 2021). Additionally, the use of L-carnitine, ascorbic acid, or their combinations have been shown to improve egg quality, relative ovary weights, and oviduct length in hens (Hassan et al., 2011).

Phytogenic is derived from edible plants, herbs, fruit, or plant by-products with antioxidant capacity and has gained significant attention as an additive in poultry diets to counteract oxidative stress-induced damage, particularly in the HS condition (Reis et al., 2019). The phytogenic sources are rich in polyphenols, including phenolic acids, flavonoids, stilbenes, coumarins, lignans, and tannins, while their bioactive compounds play an important role in functions such as anti-inflammatory, antimicrobial, antioxidant, stimulation of animal digestive system, and immunomodulation (Herve et al., 2019). The supplementation of phytogenic in laying or breeder hen diets has been shown to improve egg quality, productivity and reproductive performance, and enhance viability physical condition, antioxidant status, and growth of offspring during the post-hatching period (Barbe et al., 2020; Pliego et al., 2022). In our previous in vitro study, we found that the combination of *Syzygium aromaticum* (clove), *Camellia sinensi* (green tea pomace) derived from the beverage industry, and *Persicaria odorata* (Vietnamese coriander) exhibited strong free radical scavenging activity, synergistic antioxidant properties and enhanced cellular safety (Pasri et al., 2023). The supplementation of clove leaf meal to laying hen diets has shown the potential to promote the development of the entire reproductive tract and ovaries (Olateju et al., 2022). Sehitoglu and Kaya (2021) reported that feeding laying hens with clove oils containing eugenol resulted in a reduction of lipid peroxidation in egg yolk. Furthermore, it has been reported that catechin flavonoids present in green tea have

the potential to improve reproductive performance, egg quality, yolk oxidative stability, and reproductive performance in breeder quails (Kara et al., 2016). Vietnamese coriander consists of flavonoids and a high concentration of essential oils that exhibit antiviral, antifungal, antimicrobial, and antioxidant effects (Christapher et al., 2015). The supplementation of Vietnamese coriander leaf meal in broilers or laying hens has been observed to primarily modulate intestinal microarchitecture and enhance egg production and egg weight (Ooi et al., 2018; Basit et al., 2020). However, there has been limited research on the effects of various antioxidant sources, including both synthetic and phytogenic sources, in breeder hen diets under HS, especially with regard to their potential impact on reproductive performance, offspring quality, and antioxidant status.

Therefore, this study aimed to investigate the effect of antioxidant sources derived from either synthetic (a combination of vitamin E, vitamin C, Se, and L-carnitine) or phytogenic sources (a combination of clove, green tea pomace, and Vietnamese coriander) when used as a supplementation in breeder hen diets under HS on reproductive performance, egg quality, offspring growth performance, and their antioxidant capacity.

6.3 Materials and methods

6.3.1 Ethics statement

The animal experiments were approved by the Animal Care and Use Committee of Suranaree University of Technology (SUT-IACUC-012/2020).

6.3.2 Birds, experimental design and diets

A total of 100 SUT female breeder hens at 46 weeks of age were individually weighed and randomly divided into 4 treatments of 25 females each, housed in individual cages (40 cm length × 45 cm width × 40 cm height). The breeder hens in treatment 1 were housed in the thermoneutral room (TN) at 23 ± 1 °C with a humidity of 40 – 70 %, maintained by using an air conditioner. While breeder hens in treatment groups 2, 3, and 4 experienced daily heat stress (HS) from 46–52 weeks of age. This involved maintaining a controlled room temperature of 36±1°C for 4 h (from 1 pm to 5 pm), using a gas heater equipped with thermostat-controlled equipment. Subsequently, during rest periods, the hens were returned to conditions which were maintained for the same period as for TN (Duangjinda et al., 2017).

Experimental diets, synthetic antioxidants (a combination of 200 mg of vitamin C/kg, 150 mg of vitamin E/kg, 0.30 mg of Se yeast/kg, and 150 mg of carnitine/kg) and a 1% mixture of phytogenic antioxidants (a combination of clove, green tea pomace, and Vietnamese coriander powder in a 1:1:1 ratio/w:w:w) were used to evaluate breeder hens under HS. The efficacy of this phytogenic combination had been previously assessed in our laboratory (Pasri et al., 2023). The four experimental diets consisted of: T1) basal diets under TN; T2) basal diets under HS; T3) basal diets with synthetic antioxidants under HS, as recommended by Ross 308 parent stock standard and previous studies (Çetin and Güçlü, 2019; Shakeri et al., 2020; Aviagen, 2021); and T4) basal diets with 1% phytogenic antioxidants under HS. The basal diet for breeder hens was formulated to meet nutrient requirements according to NRC and Ross 308 parent stock standard recommendations (15% CP, 2800 kcal ME/kg) (NRC, 1994; Aviagen, 2021). The ingredient composition and calculations of the experimental diets are shown in Table 6.1. All breeder hens were fed 140 g with each experimental diet and provided with 16 h of light daily, including free access to water for four weeks before starting the trial. The experimental periods were divided into three phases, an assessment of physiological response and egg quality from 46–48 weeks of age, hatching egg collection for 1-d-old chick production from 48–51 weeks of age, and measurements of reproductive and internal organs at the end of the experiment.

6.3.3 Physiological measurements of breeder hens

The rectal temperature of each bird was measured with a digital thermometer approximately 4 h after heat exposure. The respiratory rate of the birds was recorded with the number of breaths per minute. The heart rate was also evaluated using a fetal heart detector, with the number of beats per minute recorded.

6.3.4 Egg quality measurements of breeder hens

Weekly egg quality assessments were conducted by collecting three eggs from each treatment group during the 46–48 weeks of age period. Egg-specific gravities were evaluated using sodium chloride water flotation method with gradations, followed by measuring the values of shell-breaking strength using digital force gauges (CHATILLON® DFGS50, Singapore). Subsequently, eggs were determined for weight, albumen height, yolk color, and Haugh unit continuously for three days using an Egg Multitester (EMT7300, Japan). The albumen, yolk, and shell components were

separated and weighed. Eggshell thickness was measured at three locations on the eggs using a micrometer caliper (Saleh et al., 2019; Mutlu and Yildirim, 2020).

Table 6.1 Ingredients and chemical composition of the basal diet.

	Female breeder hen diets		Chick diets
	25-50 weeks of age	After 50 weeks of age	0–3 weeks of age
Ingredients (%)			
Corn	64.60	63.50	55.42
Soybean meal, 44 %CP	18.20	16.52	25.00
Full fat soybean meal	6.70	9.00	15.00
Calcium carbonate	8.50	8.90	1.54
Monocalcium phosphate	0.94	1.00	1.60
Salt	0.41	0.44	0.50
DL-Methionine	0.135	0.134	0.29
L-Lysine	-	-	0.07
L-Threonine	-	-	0.08
Premix	0.52[1]	0.52[1]	0.50[2]
Analyzed compositions (%)			
Dry matter	93.06	93.10	93.01
Crude protein	16.02	16.20	21.55
Crude fiber	3.06	3.04	3.48
Ash	11.08	11.66	5.93
Ether extract	3.35	4.49	5.56
Calculated compositions (%)			
Metabolizable energy (kcal/kg)	2,800	2,800	2,934
Calcium	3.51	3.71	1.03
Total Phosphorus	0.53	0.54	0.74
Available phosphorus	0.31	0.32	0.48
Digestible Lysine	0.70	0.70	1.07
Digestible Methionine	0.35	0.35	0.57
Digestible Methionine + Cystine	0.57	0.57	0.84
Digestible Threonine	0.50	0.50	0.74

[1]Premix for breeder hens (0.52%) provided the following (per kg of diet) by withdrawing vitamin E and Se; vitamin A, 15,000 IU; vitamin D3, 3,750 IU; vitamin K3, 5 mg; vitamin B1, 2 mg; vitamin B2, 9.8 mg; vitamin B6, 4 mg; vitamin B12, 25 mg; pantothenic acid, 11.04 mg; nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 15.5 µg; choline chloride, 250 mg; Cu, 2.1 mg; Mn, 84 mg; Zn, 66.5 mg; Fe, 80 mg; I, 1.2 mg.

[2]Premix for chickens (0.5%) provided the following (per kg of diet); vitamin A, 15,000 IU; vitamin D3, 3,000 IU; vitamin E, 25 IU; vitamin K3, 5 mg; vitamin B1, 2 mg; vitamin B2, 7 mg; vitamin B6, 4 mg; vitamin B12, 25 mg; pantothenic acid, 11.04 mg; nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 0.155 mg; choline chloride, 250 mg; Cu, 1.6 mg; Mn, 60 mg; Zn, 45 mg; Fe, 80 mg; I, 0.4 mg; Se, 0.15 mg.

6.3.5 Reproductive and internal organ measurements of breeder hens

At the end of the experiments, 25 breeder hens were weighed and slaughtered. The ovary, oviduct, liver, and abdominal fat were separated and weighed, and the length of the oviduct was measured. The numbers of large yellow follicles (up to 10 mm diameter), small yellow follicles (5–10 mm diameter), and large white follicles (2–4 mm diameter) were measured using a micrometer caliper. The weight of the reproductive and internal organs was calculated and expressed as a percentage of the body weight (BW) of hens in each treatment, following the methods of Oke et al. (2016) and Saleh et al. (2019).

6.3.6 Growth performances and sample collection of offspring

At 50–52 weeks of age, breeder hens underwent artificial insemination twice a week in the afternoon, using 100 µL of freshly pooled semen sourced from 60 Lueng Hang Khao native breeder males (diluted with Beltsville poultry semen extender II at a 1:1, v/v). Eggs were collected daily and stored in a cool room at 15°C until the total count reached 250 hatching eggs per treatment. All collected eggs were incubated in an automatic incubator and hatcher (Model 192, Petersime Incubation Equipment Co., Ltd., Zulte, Belgium) at a temperature of 37.67±0.20°C and a relative humidity range of 62–65% for a duration of 21 days. The hatched chicks (Korat chicks) resulting from this incubation process were used for assessing the study on antioxidant activity and growth performance.

A total of 528 one-day-old Korat chicks, hatched from the eggs of SUT breeder females and Leung Hang Khao native breeder males (132 chicks per treatment) as previously mentioned, were weighed and allocated into 6 replications, with 22 chicks per replication. All chicks were reared in a litter pen (1.0 × 2.0 m) in an open housing under 16 h of light per day, with free access to the same feed and water at Suranaree University of Technology farm. The basal diets for the first 0–3 weeks of age were formulated according to the study of Korat chicken requirements containing 21% CP and 2900 kcal ME/kg diet, as outlined in the studies by NRC (1994), Maliwan et al. (2018), Tran et al. (2021), Maliwan et al. (2019) and Maliwan et al. (2022) as shown in Table 6.1. Growth performances, including average BW gain (g/bird), daily body weight gain (ADG) (g/bird/d), average daily feed intake (ADFI) (g/bird/day), and feed conversion ratio (FCR) were measured per cage weekly (Marcu et al., 2013).

6.3.7 Antioxidant capacity in offspring liver

6.3.7.1 Sample extraction for antioxidant activity

Liver tissue of eight 1-d-old chicks from each treatment were randomly collected and stored at −80°C for assessment of radical scavenging activity and lipid peroxidation. Two livers of 1-d-old chicks were pooled, resulting in four replications per treatment, and a 50 mg sample was homogenized in 1 mL of 99% ethanol. The homogenate was centrifuged for 10 min at 12,000 × g at 4°C. Finally, the supernatant was collected for the estimation of antioxidant activity.

6.3.7.2 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity assay

The DPPH•scavenging activity was determined using a modified method by Nuengchamnong et al. (2009). A 100 µL aliquot of the extracted sample was added to 100 µL of 0.6 mM DPPH in ethanol in a 96-well microplate. The mixture was gently stirred and set aside in the dark for 30 min to achieve color stabilization. The absorbance of the reaction mixture was measured at 517 nm using a microplate spectrophotometer (Thermo ScientificTM, MultidkanTM GO, Japan). Ethanol was used instead of the sample as the reagent blank. The percentage inhibition of the DPPH radical by the test samples was calculated as follows: inhibition (%) = [(absorbance of blank – absorbance of sample)/(absorbance of blank)] × 100. All determinations were carried out in triplicate.

6.3.7.3 The ferric reducing antioxidant power (FRAP) assay

The FRAP assay was measured spectrophotometrically according to the procedure developed by Benzie and Strain (1996). The fresh working FRAP reagent was prepared using 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine in 40 mM hydrochloric acid, and 20 mM iron chloride in the proportion of 10:1:1, v/v/v at 37 °C for 15 min. In the FRAP assay, 200 µL of the FRAP working reagent was pipetted and mixed with 20 µL of sample extract in 96 well microplates and kept at room temperature for 30 min. The absorbance of the resulting reaction was then immediately measured with a microplate spectrophotometer at 593 nm. The FRAP value of the sample extract was calculated using a calibration curve of Trolox (25-100 mM/mL), and the results are expressed as mM Trolox equivalents per gram of sample weight (mM TE/g sample).

6.3.8 Thiobarbituric acid reactive substances (TBARs)

The pooled liver tissue (80 mg) from 1-d-old chicks was extracted with 400 µL of DI water and 1 µL of 7.2% butylated hydroxytoluene (BHT) in ethanol for 40s using an ultra-homogenizer. The entire volume of the homogenized sample and 800 µL of TBA-TCA solution (20 mM TBA in 15% TCA) were mixed. The reaction mixture was heated at 95°C for 20 min in an ultrasonic bath (Ultrasonic cleaner 3200 EP S3, Soltec, Italy, 40 KHz and 180 W), followed by centrifugation at 5,000 × g at room temperature for 10 min. Finally, the supernatant (200 µL) was pipetted into a 96-well microplate and the absorbance was read at 532 nm. Malondialdehyde (MDA) solution with a final concentration ranging from 0–40 µM was used as a lipid peroxidation standard, and a standard curve was plotted. The TBARs of the sample was expressed as MDA equivalents per gram of sample weight (µM MDA/g sample) and derived from the basis of the calibration curve of MDA (Grotto et al., 2009).

6.3.9 Gene expression of offspring liver

In addition to collecting the livers of one-d-old chicks for measuring antioxidant activity, the livers from another eight chicks in each treatment were also collected and pooled, with two chicks per tube (replicate), before being rapidly frozen in liquid nitrogen and stored at −80°C, respectively. The mashed liver tissue was used to extract total RNA using NucleoSpin® RNA Midi kit (MACHEREY-NAGEL GmbH & Co.

KG, Düren, Germany) and subsequently purified with a QIAamp spin column (Qiagen, Hilden, Germany). Then, the RNA purity and quantification were checked using a Nanodrop spectrophotometer at 260 nm/280 nm. A high-quality RNA sample (1 µg) was then reverse-transcribed into complementary DNA (cDNA) using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). For the reverse transcription-quantitative real-time PCR (RT-qPCR) analysis, the reaction was performed in a final volume of 8 µL, consisting of 5 µL of SYBR Green, 0.4 µL of forward primer, 0.4 µL of reverse primer, and 2.2 of Nuclease-free water. This mixture was then mixed with 2 µL of cDNA samples in a 96-well microplate, using the QuantiNova™ SYBR Green PCR kit (Qiagen, Hilden, Germany) (Humam et al., 2019). The primer sequences for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), nuclear factor-**K**B (NF-**K**B), heat shock protein (HSP90), and β-actin are presented in Table 6.2. RT-qPCR was conducted using the CFX96 real-time PCR system (BioRad, Hercules, California, USA). The RT-qPCR program included an initial heat activation at 94°C for 10 min, followed by 40 cycles of denaturation for 10s at 95°C, annealing at 60–65°C for 30s, and a final extension at 72°C for 30 s. The relative concentration of mRNA for the target gene expressions was normalized with β-actin as the reference gene. The relative mRNA expression levels of SOD, CAT, GSH-Px, NF-**K**B, and HSP90 in liver were calculated using the 2-ΔΔCT method (Livak and Schmittgen, 2001). All reactions were measured in triplicate.

6.3.10 Statistical analysis

The statistical analysis of data was analyzed using ANOVA in CRD of SPSS 16.0 software (SPSS Inc, 2007). Significant differences among treatments were assessed by Tukey. A significance level at $P < 0.05$ was used. Orthogonal contrasts were also used in the comparison between 1, thermoneutral vs. heat stress conditions; 2, non-supplement vs. supplement; and 3, synthetic vs. phytogenic antioxidants.

Table 6.2 Primer sequences used in real-time PCR.

Gene	Primer sequences[1]	Gene accession number
SOD	F: CACTGCATCATTGGCCGTACCA	NM_205064.1
	R: GCTTGCACACGGAAGAGCAAGT	
CAT	F: TGGCGGTAGGAGTCTGGTCT	NM_001031215.1
	R: GTCCCGTCCGTCAGCCATTT	
GSH-Px	F: GCTGTTGCCTTCCTGAGAG	NM_001277853.1
	R: GTTCCAGGAGACGTCGTTGC	
HSP90	F-5'-ACACATGCCAACCGCATTTA-3'	NM_001109785.1
	R-5'-CCTCCTCAGCAGCAGTATCA-3'	
NF-κB	F-5'-GAAGGAATCGTACCGGGAACA-3'	NM_205134
	R-5'-CTCAGAGGGCCTTGTGACAGTAA-3'	
β-actin	F: TTGGTTTGTCAAGCAAGCGG	NM 205518.1
	R: CCCCCACATACTGGCACTTT	

[1]From: Ahmadipour and Khajali (2019).

6.4 Results

6.4.1 Physiological responses

The analysis of the orthogonal contrasts based on physiological responses indicated that breeder hens in the HS groups exhibited significantly higher rectal temperature, respiratory rate, and heart rate compared to the thermoneutral (TN) temperature group (Table 6.3, $P < 0.05$). The supplementation of either antioxidant source did not alter any of the physiological response measurements ($P < 0.05$).

Table 6.3 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress on physiological responses.

Parameters	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
Rectal temperature (˚C)	40.56[b]	42.10[a]	42.16[a]	42.27[a]	0.1015	0.001	0.479	0.573
Respiratory rate (times/minute)	41.10[b]	181.70[a]	186.00[a]	171.55[a]	7.9805	0.0001	0.767	0.215
Heart rate (times/minute)	157.88[b]	174.77[a]	174.33[a]	172.23[a]	2.2631	0.020	0.777	0.733

[a - b] Means within each row with different superscripts are significantly different (P<0.05).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1) thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2) non-supplement (T2) vs. supplement (T3, T4); 3) synthetic antioxidants (T3) vs. phytogenic antioxidants (T4)

6.4.2 Reproductive organ characteristics

In the orthogonal contrast comparison, breeder hen groups exposed to HS exhibited significantly reduced BW, numbers of large and small yellow follicles, liver weight, and abdominal fat compared to the TN breeder hen group ($P < 0.05$; Table 6.4). Although changes in oviduct weight and length, as well as large white follicles, were not influenced by either temperature conditions or dietary antioxidants, both synthetic and phytogenic sources were found to enhance ovary weight and the number of large and small yellow follicles in HS-breeder hens compared to non-supplementation ($P < 0.05$) and similar to TN breeder hens ($P > 0.05$). According to the Tukey test, breeder hen groups that received either antioxidant showed significantly lower abdominal fat than the TN group ($P < 0.05$). Interestingly, breeder hens that received phytogenic supplementation exhibited higher ovary weight than those without supplementation ($P < 0.05$).

Table 6.4 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on reproductive and internal organ characteristics.

Items	Treatments[1]				Pooled SEM	Contrasts[2]		
	T1	T2	T3	T4		1	2	3
Body weight (g)	3314.48[a]	3176.57[ab]	3102.67[ab]	3048.19[b]	34.971	0.013	0.215	0.571
Ovary weight (%BW)	1.78[ab]	1.64[b]	1.83[ab]	1.89[a]	0.0312	0.997	0.005	0.481
Oviduct weight (%BW)	2.29	2.15	2.25	2.29	0.0378	0.400	0.289	0.878
Oviduct length (cm)	69.15	69.8	70.1	70.2	0.6121	0.541	0.819	0.955
No. of LYF (>10 mm)	6.00[a]	5.28[b]	5.60[ab]	5.89[ab]	0.091	0.050	0.031	0.238
No. of SYF (5-10 mm)	12.31[a]	9.10[b]	10.25[ab]	11.38[ab]	0.3813	0.018	0.050	0.271
No. of LWF (2-4 mm)	28.76	28.66	30.23	30.3	0.9001	0.659	0.462	0.982
Liver weight (%BW)	1.88[a]	1.64[b]	1.65[b]	1.66[ab]	0.0301	0.001	0.828	0.930
Abdominal fat (%BW)	6.15[a]	5.77[ab]	4.93[b]	4.92[b]	0.1663	0.017	0.270	0.988

[a - b] Means within each row with different superscripts are significantly different ($P<0.05$).

Abbreviations: LYF, Large yellow follicles (up to 10 mm diameter); SYF, Small yellow follicles (5–10 mm diameter); LWF, Large white follicles (2–4 mm diameter).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1) thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2) non-supplement (T2) vs. supplement (T3, T4); 3) synthetic antioxidants (T3) vs. phytogenic antioxidants (T4).

6.4.3 Egg qualities

Egg weight (EW), Haugh unit, eggshell weight and thickness, and egg-specific gravity were significantly affected by the HS condition compared to the TN condition, as determined by an orthogonal contrast test ($P < 0.05$, Table 6.5). While albumen high and weight, yolk weight, and eggshell breaking strength in all treatments showed no significant differences influenced by either temperature conditions or dietary antioxidants ($P > 0.05$). However, a comparison of the orthogonal contrasts showed that both synthetic and phytogenic antioxidants could enhance yolk color and Haugh unit more effectively than the HS without supplementation for the breeder hen groups ($P < 0.05$). In addition, the Tukey test indicated that the breeder hens receiving phytogenic supplementation exhibited the most significant increase in yolk color ($P < 0.05$).

6.4.4 Growth performance of offspring

The effects of antioxidant supplementation in female breeder hen diets on the growth performance of offspring are shown in Table 6.6. It was observed that the initial BW of 1-d-old chicks in the TN and HS breeder hens without supplementation was higher than that of both antioxidant groups ($P < 0.05$). The decrease in BW was observed until 1 week of age, after that, there were no significant differences among the groups. ADG, ADFI, and FCR also showed no significant differences among the treatment groups at any period of age ($P > 0.05$).

Table 6.5 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on egg quality.

Parameters	Treatments[1]				Pooled	Contrasts		
	T1	T2	T3	T4	SEM	1	2	3
Egg weight (g)	65.15^{a}	64.51^{ab}	62.57^{b}	62.72^{b}	0.3990	0.035	0.054	0.897
Albumen high (mm)	8.30	8.43	8.39	8.72	0.0826	0.054	0.851	0.184
Yolk color	7.31^{b}	7.17^{b}	7.19^{b}	7.62^{a}	0.0435	0.847	0.017	0.0001
Haugh unit	90.87^{b}	91.95^{b}	94.87^{a}	96.59^{a}	0.3449	0.0001	0.0001	0.342
Albumen weight (g)	34.59	34.12	33.17	33.67	0.3507	0.251	0.412	0.620
Yolk weight (g)	18.17	19.00	18.01	17.90	0.1692	0.734	0.011	0.810
Eggshell weight (g)	6.74^{a}	6.53^{ab}	6.36^{ab}	6.32^{b}	0.0551	0.007	0.159	0.799
Eggshell thickness (mm)	0.36^{a}	0.34^{b}	0.33^{b}	0.33^{b}	0.0025	0.0001	0.354	0.776
Egg specific gravity (g/cm^{3})	1.1027^{a}	1.0994^{ab}	1.0991^{ab}	1.0990^{b}	0.0005	0.003	0.804	0.924
Eggshell breaking strength (N)	39.28	38.29	39.58	40.00	0.5724	0.993	0.290	0.810

$^{a - b}$ Means within each row with different superscripts are significantly different($P<0.05$).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1) thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2) non-supplement (T2) vs. supplement (T3, T4); 3) synthetic antioxidants (T3) vs. phytogenic antioxidants (T4).

Table 6.6 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on growth performance of offspring.

Items	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
Initial body weight (g/bird)								
	46.08[a]	46.08[a]	44.49[b]	44.49[b]	0.179	0.0001	0.0001	1.000
Body weight (g/bird)								
Week 0-1	93.08[ab]	95.05[a]	92.09[b]	91.71[b]	0.4537	0.402	0.003	0.731
Week 1-2	178.2	179.73	176.21	174.49	1.2125	0.628	0.155	0.625
Week 1-3	289.01	292.81	287.07	291.7	2.1348	0.772	0.736	0.472
Daily body weight gain (g/day/bird)								
Week 0-1	6.8	7.01	6.79	6.82	0.057	0.588	0.389	0.88
Week 1-2	9.43	9.55	9.42	9.32	0.0812	0.996	0.394	0.682
Week 1-3	11.6	11.87	11.57	11.79	0.1023	0.572	0.481	0.465
Daily feed intake (g/birds/day)								
Week 0-1	9.08	9.22	9.13	9.66	0.0867	0.17	0.211	0.685
Week 1-2	13.63	14.08	13.65	13.96	0.1001	0.242	0.271	0.285
Week 1-3	18.34	19.06	18.56	19.25	0.1676	0.107	0.698	0.145
Feed conversion ratio								
Week 0-1	1.33	1.37	1.34	1.35	0.0076	0.227	0.623	0.699
Week 1-2	1.44	1.47	1.45	1.5	0.0085	0.127	0.966	0.042
Week 1-3	1.58	1.6	1.6	1.63	0.0106	0.153	0.589	0.353

[a - b] Means within each row with different superscripts are significantly different ($P<0.05$).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1) thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2) non-supplement (T2) vs. supplement (T3, T4); 3) synthetic antioxidants (T3) vs. phytogenic antioxidants (T4).

6.4.5 Liver antioxidant capacity of offspring

The effects of antioxidant supplementation in breeder hen diets under HS on offspring liver antioxidant capacities are shown in Figure 6.1. Based on the orthogonal contrasts, the findings revealed that the supplementation of either antioxidant source in breeder hen diets under HS resulted in an enhancement of DPPH and FRAP scavenging capacity in the livers of 1-d-old chicks compared to both the TN and HS breeder hens without supplementation ($P < 0.05$). However, incorporating synthetic antioxidants into maternal diets in HS breeder hen groups resulted in greater DPPH and FRAP radical scavenging activity in the liver tissue of 1-d-old chicks compared to those fed with phytogenic antioxidants ($P < 0.05$). Orthogonal contrast tests also demonstrated a significant difference in the MDA level of 1-d-old chick liver, between the maternal synthetic and phytogenic antioxidant groups ($P < 0.05$). In addition, the Tukey test also revealed that maternal synthetic antioxidants were highly efficacious in increasing radical scavenging capacity compared to other treatment groups ($P < 0.05$) and reduced MDA levels compared to maternal HS without non-supplementation.

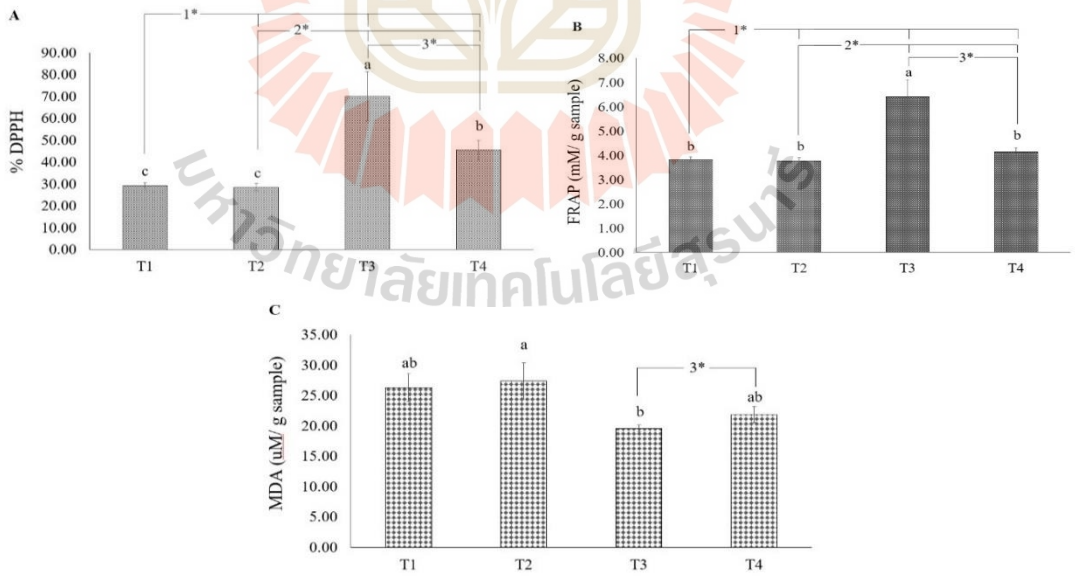

Figure 6.1 Effect of dietary antioxidant supplementation in breeder hens under heat stress on offspring liver antioxidant capacities of DPPH (A) and FRAP (B) radical scavenging, and malondialdehyde (MDA) (C).

The values with different superscript letters (a, b, c) in the figure indicate significant differences at $P < 0.05$.

Abbreviations: T1, thermoneutral zone (23±1 ℃) + basal diet without supplementation; T2, heat stress condition (36±2 ℃, 4 h/day) + basal diet without supplementation; T3, heat stress condition + basal diet with synthetic antioxidants; T4, heat stress condition + basal diets with phytogenic; DPPH, 2,2-Diphenyl-1-picrylhydrazyl scavenging activity assay; FRAP, The ferric reducing antioxidant power assay; MDA, malondialdehyde.

The superscript (*) in the figure represents different levels of significance at $P < 0.05$ based on orthogonal contrasts of 1, thermoneutral (T1) vs. heat stress condition (T2, T3, T4); 2, non-supplement (T2) vs. supplement (T3, T4); 3, synthetic antioxidants (T3) vs. phytogenic antioxidants (T4).

6.4.6 Gene expression in liver of offspring

The orthogonal contrast test revealed that under HS conditions, either of the dietary synthetic or phytogenic antioxidants upregulated the gene expression related to antioxidant enzymes (SOD, CAT, and GSH-Px) and HSP90 in the livers of offspring when compared to non-supplementation ($P < 0.05$), as shown in Table 6.7. Notably, the Tukey test revealed that maternal dietary synthetic antioxidants under HS significantly activated the gene expression of SOD and GSH-Px in the liver of 1-d-old chicks compared to the TN and HS conditions without supplementation ($P < 0.05$). In addition, each antioxidant source was capable of downregulating the expression of heat shock protein (HSP90), similar to the TN condition ($P > 0.05$). However, the expression of pro-inflammatory cytokines (NF-KB) was not influenced by either synthetic or phytogenic antioxidants ($P < 0.05$).

Table 6.7 Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on the relative mRNA levels of liver SOD, CAT, GSH-Px, NF-KB, and HSP90 in offspring.

Items	Treatments[1]				Pooled	Contrasts[2]		
	T1	T2	T3	T4	SEM	1	2	3
SOD	1.00^{b}	0.99^{b}	1.86^{a}	1.38^{ab}	0.1092	0.100	0.026	0.086
CAT	1.00^{ab}	0.64^{b}	1.66^{a}	1.22^{ab}	0.1487	0.452	0.007	0.142
GSH-Px	1.00^{bc}	0.74^{c}	2.05^{a}	2.01^{ab}	0.1905	0.060	0.001	0.902
NF-KB	1.00	0.82	1.30	1.09	0.1715	0.883	0.424	0.685
HSP90	1.00^{a}	3.98^{b}	1.34^{a}	1.59^{a}	0.3112	0.054	0.002	0.750

$^{a-b}$ Means within each row with different superscripts are significantly different ($P<0.05$).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic antioxidants.

[2]Orthogonal contrasts: 1) thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2) non-supplement (T2) vs. supplement (T3, T4); 3) synthetic antioxidants (T3) vs. phytogenic antioxidants (T4).

6.5 Discussion

It has been suggested that dietary synthetic and phytogenic antioxidants play an important role in reducing oxidative stress, including HS, which can alleviate the adverse effects on the reproduction and production aspects of breeder hens (Surai et al., 2016). Our results found that supplementing breeder hens under HS with either synthetic (a combination of vitamin C, E, Se, and L-carnitine) or phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) antioxidant sources can help maintain reproductive performances and improve the Haugh unit. In addition, phytogenic antioxidants provide additional benefits by enhancing yolk color. Interestingly, either synthetic or phytogenic antioxidants showed efficacy in elevating antioxidant capacities, upregulating genes related to antioxidant enzymes, and downregulating heat shock protein expressions in the liver of offspring.

In general, HS has been observed to exert a negative effect on the reproductive organs and follicle development of laying hens, leading to a decrease in egg production (Fouad et al., 2016; Liu et al., 2020). However, in this study, synthetic or phytogenic antioxidants were effective in maintaining the ovary weight and the number of large and small yellow follicles in breeder hens exposed to HS. This observation is in line with our previous findings, which indicated that either synthetic or phytogenic antioxidants improved egg production in HS breeder hens (Pasri et al., 2024). This is likely attributed to the effective activation of the mechanism utilizing body energy reserves (body fat) through gluconeogenesis for maintenance and production by either of the antioxidant sources. This phenomenon is correlated with lower liver and abdominal fat weights observed in HS groups supplemented with antioxidants compared to the TN and HS with non-supplementation groups. This effect may be attributed to the synergistic action of vitamin E, vitamin C, Se, and L-carnitine. Although there are limited previous reports on the combination of these compounds, individual functions of each have been reported. Dietary vitamin E has been reported to promote the release of egg yolk precursor proteins, vitellogenin, and triglyceride (very low-density lipoprotein) from the liver into the bloodstream by preventing damage to the cell membranes of hepatocytes caused by OS (Puthpongsiriporn et al., 2001; Ciftci et al., 2005). Additionally, the supplementation of vitamin E at 200 IU/kg in geese diets was found to enhance the secretion of follicle-stimulating hormones, estradiol, and progesterone, contributing to the stimulation of follicle development (Fu et al., 2022). The combination of Vitamin E and C showed the potential to increase estrogen and progesterone hormone levels, as well as to enhance the ovary and oviduct weight, including ovary length in HS laying hens (Attia et al., 2016). L-carnitine influences female reproduction by directly affecting oocyte quality and promoting energy production via β-oxidation. In addition, it indirectly regulates reproductive hormone levels through the hypothalamic–pituitary–gonadal axis, resulting in an increase in follicle-stimulating hormone and luteinizing hormone, while concurrently reducing prolactin levels (Agarwa et al., 2018). L-carnitine has also been shown to increase both egg weight and production in laying hens (Kazemi-Fard et al., 2015). Dietary Se was found to increase oviduct length, as well as oviduct and ovary weight in turkey hens aged 32–44 weeks of age (Ismail et al., 2016). Brennan et al. (2011) suggested that Se yeast has the

potential to accumulate in reproductive tissue, thereby influencing the transcripts of genes associated with respiratory complexes, ATP synthesis, protein translation, and metabolism in the oviduct of hens.

In this study, we also observed the beneficial effect of phytogenic antioxidants on both enhancing ovary weight and the number of large and small yellow follicles. The phytogenic antioxidants used in this study included clove, green tea pomace, and Vietnamese coriander powder. These sources are rich in phenolic and flavanol contents, along with various bioactive compounds such as eugenol, gallic acid, catechin, ellagic acid, quercetin, and kaempferol (Pasri et al., 2023). The supplementation of the phenolic compounds from grape seed (Liu et al., 2018), clove leaf meal containing saponin, flavonoid, phenol, and terpenoid (Olateju et al., 2022), and quercetin, either alone or in combination with vitamin E (Amevor et al., 2021), in hen diets showed significant effects on ovary weight, follicle F1–3 index, follicle diameter, oviduct, and follicle index as well as the prevention of follicle growth by inhibiting OS. The chemical structure of polyphenols is similar to that of estradiol, enabling them to function as both estrogen agonists and antagonists (Abid et al., 2020; Bhagwat et al., 2021). The function of these bioactive compounds may encompass decreasing ROS in reproductive organs, stimulating sex hormones, and promoting the production of egg yolk precursors by stimulating triglycerides, very-low-density lipoprotein, and vitellogenin in the bloodstream (Liu et al., 2020). In addition, various polyphenol compounds found in fruits, vegetables, herbs, seeds, and tea have the ability to activate estrogen, follicle-stimulating hormones, luteinizing hormones, and their respective receptor (estrogen receptor α and β, follicle-stimulating hormone receptor, and luteinizing hormone receptor) (Amevor et al., 2021).

The Haugh unit, a measure used to evaluate the internal quality of eggs, establishes a relationship between albumen height and egg weight. It provides a quantifiable mathematical assessment of egg albumen quality, which helps to estimate the extent of albumen degradation that can lead to early embryonic death (Hegab and Hanafy, 2019). In general, HS has been identified as a factor inducing elevated oxidative products in eggs, leading to lipid peroxidation and albumen degradation in laying hens (Gharaghani et al., 2015). The current study found that either of the antioxidant sources could enhance the Haugh unit in HS breeder hens to a greater extent compared to

those in TN and under HS without supplementation. This effect may be attributed to the specific functions or bioactive compounds present in both dietary antioxidant sources. Although there is limited literature on the combined effects of vitamin C, vitamin E, Se, and L-carnitine on the Haugh unit, there are reports indicating decreased egg yolk lipid peroxidation in laying hens when fed dietary antioxidants (such as vitamin E, C, and A, Se, and essential oil), leading to an improvement in the Haugh unit (Asadi et al., 2017; Beyzi et al., 2020). Se in laying hen diets can accumulate in both egg albumen and yolk, enhance antioxidant enzymes, and reduce lipid peroxidation and carbonyl group in eggs. This may provide protection against protein oxidation resulting from oxidative stress, preventing the conversion of amino acid residues in egg albumen, such as Lys, Met, and Cys into carbonyl derivatives, and these derivatives can affect the physicochemical and functional properties of egg albumen (Wang et al., 2010; Jing et al., 2015). It has been reported that supplementing hen diets with L-carnitine under HS can lead to an increase in both albumen weight and height (Celik et al., 2004; Kazemi-Fard et al., 2015). This observed increase is likely attributable to L-carnitine, which not only provides metabolizable energy but also facilitates the formation of β-ovomucin in the thick albumen gel, thereby contributing to the improvement of the Haugh unit. Green tea polyphenols (Xia et al., 2018; Chen et al., 2021) and quercetin (Liu et al., 2013) have been observed to increase albumin height and the Haugh unit. Laying hens fed green tea in diets showed elevated β -ovomucin, including β, α1, and α2 subunits of ovomucin proteins, resulting in increased albumin height and Haugh unit compared to the control group (Wang et al., 2018). In addition, catechin and epigallocatechin gallate in tea polyphenols increased albumen height and the Haugh unit by protecting the structure and function of the magnum from OS (Yuan et al., 2016).

The egg yolk color score increased only in the HS breeder hens fed phytogenic antioxidants, but not in the synthetic antioxidant group. This is likely associated with pigment compounds such as chlorophylls a, chlorophylls b, pheophytins a, pheophytins b, zeaxanthin, α and β-carotene, and lutein, along with the yellowish pigment quercetin found in clove, green tea pomace, and Vietnamese coriander (Abou-Elkhair et al., 2018; Chen et al., 2021; El-Saadany et al. 2022). Generally, yolk coloration is associated with the quantity and types of carotenoids consumed, which can be

transferred and deposited in the yolk. Hammershøj et al. (2010) noted that the enhancement of egg yolk color is influenced by a variety of pigment compounds present in dietary plant materials. Dietary green tea powder has been reported to influence the increased yolk color in laying hens (Chen et al., 2021). In addition, the yellowish pigment of quercetin was also noted to be responsible for increased yolk color (El-Saadany et al., 2022). Based on these findings, it can be assumed that bioactive compounds from phytogenic antioxidants are deposited and distributed in the ovary, egg yolk, and embryos.

In this study, we observed that BW of offspring from breeder hens under HS and supplemented with antioxidants was lower at both day 1 and day 7 of age compared to chickens from the TN and HS without antioxidant supplementation. This phenomenon is likely attributable to the fact that the EW of breeder hens in the HS groups supplemented with antioxidants was the lowest among the groups. It has been reported that EW is closely correlated with the BW of hatched chicks (Gunawardana et al., 2008). Our previous study showed that HS hens supplemented with either synthetic or phytogenic antioxidants showed a decrease in FI, resulting in a subsequent reduction in EW. Despite this, these groups of hens were still able to produce more eggs when compared to both the TN and HS groups (Pasri et al., 2024). Although the BW of offspring in either of the antioxidant groups was lower at the beginning and at 1 week of age, their BW showed a subsequent increase, reaching levels comparable to those in the TN and HS groups without supplementation by 3 weeks of age. This indicates that either of the antioxidant sources can contribute to the improvement of the BW of chicks after hatching. Although there is limited research on the use of this type of synthetic antioxidant on offspring performance, some studies have reported on their function through antioxidant capacity. Xia et al. (2022) observed that while maternal Se supplementation did not influence the BW of ducklings from day 1 to 2 weeks, it can improve health status via the antioxidant properties of Se. The addition of antioxidants such as carotenoids, vitamins E and C, L-carnitine, and Se in breeder hen diets has demonstrated beneficial effects on the survival rate and offspring health, and this improvement is attributable to the enhancement of the antioxidant system during early postnatal development (Surai and Fisinin, 2014; Abdel-Azeem et al., 2016; Oso et al., 2020). In addition, Ren et al. (2016) and Wang et al. (2020) have suggested that

supplementing maternal diets with elevated levels of vitamins or minerals beyond the standard requirements for egg production could improve the growth performance of offspring. Regarding phytogenic antioxidants, although numerous studies have extensively investigated their effect on laying or breeder hen diets, including performance, egg quality, and antioxidant capacity in 1-d old chicks (Kara et al., 2016; Saleh et al., 2019), unfortunately, the effects of maternal phytogenic antioxidants on post-hatch growth performance have been rarely reported. Barbe et al. (2020) observed an increase in chick BW at day 1 and beyond day 10 of age, correlating with an extended feeding duration and high levels of grape seed extract polyphenols in the diets provided to the breeder hens.

The high efficacy of dietary antioxidants is expected to contribute to antioxidant defense networks, covering three crucial levels: organelles, subcellular compartments, and extracellular space. This function encompasses inhibiting ROS formation, activating antioxidant enzymes, and repairing or removing damaged molecules (Horváth and Babinszky, 2018). The present study observed an increasing antioxidant capacity (DPPH and FRAP) and mRNA expression of SOD, CAT, and GSH-Px, along with a reduction in lipid peroxidation (MDA) in the liver of 1-d-old chicks. This suggests that bioactive compounds from both antioxidant sources accumulate in the egg yolk, serving as a protective barrier against cellular damage in all three areas throughout embryo development until hatching. Surai et al. (2016) reported the effective transfer of vitamin E and Se from feed to yolk and subsequently to embryonic tissues. A chicken embryo can retain a-tocopherol from egg yolk within the liver, where vitamin E serves as a major lipid-soluble antioxidant, scavenging free radicals and breaking the chain reaction of lipid peroxidation. The accumulation of Se in the egg yolk could increase Se concentration in the embryonic tissues, imparting enduring effects on maintaining antioxidant protection in the developing chick embryo and newly hatched chicks. Supplementing the diet of duck breeders with Se has been shown to enhance GSH-Px and decrease MDA in the livers of ducklings as GSH-Px plays a crucial role in cellular antioxidant defense by eliminating hydrogen peroxide and preventing the formation of lipid hydroperoxides (Xia et al., 2022). Even though numerous previous studies have indicated a limited ability of dietary L-carnitine and vitamin C to be transported from maternal diets into egg yolk, these compounds can still exhibit antioxidant properties

(Zhu et al., 2021; Rouhanipour et al., 2022). Adabi et al. (2011) revealed that supplementing laying hen diets with L-carnitine resulted in an elevated accumulation of carnitine accumulation in the egg yolk, contributing to the improvement of lipid peroxidation in the livers of chicks by reducing the availability of lipids for peroxidation, consequently leading to a decrease in MDA. Vitamin C has also been reported to eliminate peroxyl radicals in the aqueous phase before peroxidation occurs, thereby preventing cell lipid peroxidation of cell membranes (Cinar et al., 2014).

Interestingly, the maternal phytogenic antioxidants in this study increased the radical (DPPH and FRAP) scavenging ability and inhibited lipid peroxidation in the livers of 1-d-old chicks. This is in line with our reported observations in a previous study, where egg yolk from breeder hens fed with dietary phytogenic exhibited high antioxidant activities in terms of scavenging radicals such as DPPH, ABTS, and FRAP, and showed low MDA levels (Pasri et al., 2024). In general, the beneficial effect of phytogenic substances on increased free radical scavenging or reduced MDA level depends on the quantity of bioactive compounds that can be transferred to the yolk and subsequently to embryonic tissues (Abou-Elkhair et al., 2020). Our previous in vitro study found that the clove, green tea pomace, and Vietnamese coriander and their combination exhibited potent radical scavenging and hydrogen peroxide inhibition in HepG2 cells, which was linked to their phenolic and flavanol contents, along with key antioxidant bioactive compounds like eugenol catechin, quercetin, kaempferol, and ellagic acid in these three plants (Pasri et al., 2023). These findings suggest that certain bioactive compounds from phytogenic sources accumulate in the egg yolk and are subsequently distributed and deposited in chick tissues. Most of the bioactive compounds present in phytogenic substances are polyphenols, which are considered powerful antioxidants due to their chemical structure with two hydroxyl groups (PhenOH). This structural feature allows them to donate hydrogens or electrons, effectively eliminating excess ROS (such as O^2, H^2O^2, $OH^{\bullet}$, $RO^{\bullet}$, $RO_2^{\bullet)}$, and transfer electrons by binding to metal ion free radicals (such as Fe^{2+}, Cu^{2+} or Cu^{+}) (Saracila et al., 2021). Limited reports are available on the accumulation of polyphenols in the yolk and their subsequent transfer for utilization in embryo tissue. However, Kara et al. (2016) revealed that supplementing breeder quail diets with green tea could be transferred catechins, major bioactive compounds, into the egg yolk, contributing to

ROS scavenging activity and reducing lipid peroxidation. In addition, dietary green tea powder contains chlorophylls a and b, pheophytins a and b, and carotenoids, all of which exhibit high antioxidant activity and can be stored in egg yolk (Chen et al., 2021). Similarly, the reduction in yolk lipid peroxidation observed in laying hens fed diets containing clove oil may be attributed to the accumulation of clove oil, which contains eugenol compounds that possess antioxidant properties (Sehitoglu and Kaya, 2021). Although Vietnamese coriander has not been investigated for its potential to improve antioxidant activity in eggs and 1-d-old chicks in hen diets, considerable attention has been directed toward quercetin as a major bioactive compound in poultry feed additives (Basit et al., 2020).

In addition to the heightened activities of antioxidant enzymes in the liver of 1-d-old chicks, we observed that either of the antioxidant sources can provide unregulated relative expression of antioxidant enzyme genes such as SOD, CAT, and GSH-Px. Numerous studies have indicated that the increased expression of SOD, CAT, and GSH-Px enzymes or genes in the liver of 1-d old chicks is associated with a high level of antioxidant concentration in egg yolk (Ren et al., 2016; Barbe et al., 2020; Xia et al., 2022). Vitamin E and Se were reported to be deposited on the egg yolk to a greater extent than vitamin C and L-carnitine (Surai and Fisinin, 2019). In addition, Se has been reported to be deposited in both yolk and 1-d-old chick tissue, playing a crucial role in antioxidants by inducing the active site of the enzyme GSH-Px and resulting in the upregulating of GSH-Px in the livers of 1-d-old chicks (Surai et al., 2016; Oso et al., 2020). Vitamin E, vitamin C, and L-carnitine can activate transcriptional factor activity of activator protein-1 (AP-1), nuclear factor erythroid 2 related factor 2 (Nrf2), and nuclear factor-**K**B (NF-**K**B) DNA binding site. These influence the regions of adhesive molecules, cytokines, and antioxidant enzyme genes, which provides additional protection (Surai, 2015b; Min et al., 2018). Furthermore, the properties of polyphenols can indirectly stimulate the Keap1-Nrf2 complex by modifying cysteine residues in Kelch-like ECH-associated protein 1, leading to the translocation of Nrf2 into the nucleus, following which Nrf2binds to the antioxidant electrophile/antioxidant response element (EpRE/ARE) sequence (Lee et al., 2017; Saracila et al., 2021), resulting in the expression of cellular antioxidant enzymes such as SOD, CAT, GPx, GR, and GST etc. (Surai et al., 2016; Hosseini-Vashan et al., 2016; Bernetoniene and Kopustinskiene, 2018).

In this study, a reduction in the relative mRNA levels of HSP90 was observed in the livers of 1-d-old chicks whose maternal diets were supplemented with either synthetic or phytogenic antioxidants under HS. This reduction showed no significant difference compared to the TN condition. Heat shock proteins (HSPs) can serve as biomarker genes for oxidative stress (OS) damage, as they respond to increased generation of reactive oxygen species (ROS). Zhu et al. (2017) reported that maternal heat stress can induce OS in embryos, leading to elevated mRNA expression levels of HSP90 and HSP70 mRNA in the embryonic liver. When the cells were exposed to HS, accompanied by increased lipid peroxidase, HSPs might upregulate in response to cellular damage (Tedeschi et al., 2015). Thus, the increased HSPs expression levels during OS play an important role in maintaining the integrity of structural proteins, regulating transcription factors, preventing cell apoptosis, and promoting cell survival (Balakrishnan et al., 2023). In addition, the increased antioxidant activities of SOD, CAT, GSH-Px, DPPH, and FRAP in the liver of 1-d old chick, may contribute to scavenging a significant portion of the ROS, which could potentially inhibit the expression of HSP proteins, thereby enhancing cell survival. This result aligns with the findings of Xiao et al. (2016), who reported that Se supplementation in breeder hen diets increased GSH-Px mRNA expression and antioxidant enzyme activity in the livers of HS broiler embryos, as well as effectively decreasing the expression of HSP70. However, there is a scarcity of available information on the research investigating the effect of either a combination of vitamins E and C, Se, and L-carnitine or cloves, green tea pomace, and Vietnamese coriander in maternal breeder hen diets on altering the expression of HSP in offspring. Therefore, this study represents the first attempt to report the possible effects of either synthetic or phytogenic antioxidants which influence the antioxidant defense network of offspring in maternal breeder hens.

6.6 Conclusions

This study indicated the positive effects of either synthetic (a combination of vitamin C and E, Se, and L-carnitine) or phytogenic (a combination of cloves, green tea pomace, and Vietnamese coriander) antioxidants in breeder hens under HS. Both of the antioxidant sources were effective in improving the Haugh unit and maintaining reproductive performance in terms of ovary weight, and large and small yellow

follicles. In addition, the inclusion of phytogenic antioxidants was also found to enhance yolk color. The antioxidant capacity of offspring was positively influenced by either synthetic or phytogenic antioxidants in maternal diets by increasing free radical scavenging in terms of DPPH and FRAP radical scavenging, reducing lipid peroxidation (MDA), and upregulating the relative expression of SOD, CAT, and GSH-Px mRNA, as well as downregulating HSP90 expression in the livers of offspring. This indicates a link between synthetic or phytogenic antioxidants in breeder hen diets, which influences reproductive performances, accumulation in tissues or eggs, and the antioxidant defense system of offspring.

6.7 References

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CHAPTER VII

OVERALL CONCLUSION AND IMPLICATION

7.1 Overall conclusion

Heat stress (HS), characterized by high environmental temperatures ranging from 32–38°C and coupled with high humidity, has the potential to induce large amounts of reactive oxygen species that lead to oxidative stress in female breeder hens. This oxidative stress (OS) adversely affects the integrity of the sperm membrane and DNA in sperm storage tubules (SSTs), impacting various aspects of reproduction such as egg production, egg quality, fertility, hatchability, and embryo development, causing economic losses. Therefore, a comprehensive understanding of the mechanism of HS response associated with reproductive performance and sperm prevention in sperm storage tubes of breeder hens is crucial. In this study, various herbs, fruits, vegetables, and vegetable by-products were screened and evaluated for their ability as phytogenic antioxidant substances. Transcriptomic techniques were used to identify the mechanism and relevant gene markers in the uterovaginal junction (UVJ) containing SSTs of breeder hens subjected to HS between the heat-adapted breed and the heat-sensitive breed. In addition, the study investigated the effects of either synthetic (a combination of vitamin E, vitamin C, Se, and L-carnitine) or phytogenic (a combination of clove, green tea pomace, and Vietnamese coriander) antioxidants on heat-sensitive breeder hens to assess their potential in mitigating HS. The main results are summarized as follows:

7.1.1 Among a total of 17 screened plant materials, high levels of total phenolic and total flavonoid contents were found in *Syzygium aromaticum* (clove), *Camellia sinensis* (green tea pomace) sourced from the beverage industry, and *Persicaria odorata* (Vietnamese coriander)**.** These three plants, when combined in a ratio of 1:1:1, demonstrated potent antioxidant activity in terms of DPPH, ABTS, and FRAP radical scavenging. Furthermore, the combination of clove, green tea pomace,

and Vietnamese coriander demonstrated synergistic capabilities in scavenging free radicals and exhibited lower cytotoxicity to cells compared to individual plants.

7.1.2 This study is the first to provide insights into the different expression genes (DEGs) in the UVJ tissue containing SSTs between heat-adapted and heat-sensitive breeder hens under HS. A total of 387 DEGs indicated that the top 15 GO terms were notably enriched in the chaperones and co-chaperones of HSP and DNAJ gene families in heat-sensitive breeder hens associating with biological processes, cellular components, and molecular function. KEGG pathway analysis identified changes in protein processing in the endoplasmic reticulum, cytokine-cytokine receptor interaction, and tyrosine, phenylalanine, and nitrogen metabolism. These changes might indicate that UVJ tissue was more damaged by HS in heat-sensitive breeder hens than in heat-adapted breeder hens. The different expressions of HSPB8, DNAJA4, HSP90AA1, and TAT genes have the potential to serve as gene markers indicative of HS effects in the UVJ tissue containing SSTs of heat-sensitive breeder hens. Furthermore, the supplementation of either synthetic or phytogenic antioxidants appears to have the capacity to modify the expression of HSPB8, DNAJ4, HSP90AA1, and TAT genes in the UVJ tissue of heat-sensitive breeder hens exposed to HS, thereby mitigating the negative effects of HS.

7.1.3 The supplementation of either synthetic antioxidant (a combination of vitamins C and E, Se, and L-carnitine) and phytogenic antioxidants (a combination of clove, green tea pomace, and Vietnamese coriander) in the diets of heat-sensitive breeder hen exposed to HS could improve egg production and hatchability while reducing embryo mortality. Either of the antioxidant sources elevated DPPH, ABTS, and FRAP radical scavenging, along with reducing lipid peroxidation in yolk and tissues. In addition, either synthetic or phytogenic antioxidants demonstrated an up-regulation in the relative expression of SOD, CAT, and GSH-Px mRNA and a down-regulation of NF-kB, HSP70, and HSP90 mRNA expressions in the liver of HS breeder hens.

7.1.4 Either of the antioxidant sources in breeder hen diets indicated a positive effect in improving the Haugh unit and maintaining reproductive performance in terms of ovary weight, large yellow follicles, and small yellow follicles. Phytogenic antioxidants were found to increase yolk color. In addition, either synthetic or phytogenic antioxidants in maternal diets influenced an increase in free radical

scavenging, as indicated by DPPH and FRAP radical scavenging, a decrease in lipid peroxidation (MDA), and upregulation in the relative expression of SOD, CAT, and GSH-Px mRNA, as well as downregulation of HSP90 expression in the liver of offspring.

7.2 Implication

This present study demonstrated the possibility of using plant materials as antioxidant feed additives to reduce the risk of OS in animals, especially the combination of clove, green tea pomace, and Vietnamese coriander powder with high antioxidant capabilities and various polyphenol bioactive compounds. In general, the bioactive compounds in plant materials can vary based on genetic factors, environmental conditions, cultivation practices, harvesting, processing, etc. The utilization of extracts or purified forms would maintain consistent outcomes for animals; however, it may lead to increased costs compared to the use of the raw form. While either synthetic or phytogenic antioxidants can offer various benefits to both breeder hens and their offspring, the decreased feed intake during heat stress can result in reduced egg weight and, consequently, a decrease in the weight of 1-day-old chicks. To enhance this situation, adjusting the nutrient density in diets should be synchronized with the level of feed intake to guarantee an adequate nutrient supply during heat stress.

APPENDIX

Supplementary Table 4.7 Differentially expressed genes (DEGs) in the uterovaginal junction tissues containing sperm storage tubules between heat-adapted breeder hens and heat-sensitive breeder hens under heat stress.

Supplementary Table 4.8 Gene Ontology (GO) terms of differentially expressed genes (DEGs) in the uterovaginal junction tissues containing sperm storage tubules between heat-adapted breeder hens and heat-sensitive breeder hens under heat stress.

QR code for Supplementary files

BIOGRAPHY

Phocharapon Pasri was born on November 27th, 1989, in Lopburi, Thailand. In 2012, he obtained her Bachelor of Science in Animal Science and Agricultural Technology, Faculty of Animal Science and Agricultural Technology, Silpakorn University. In 2017, he received his Master of Science in Animal Production Technology, Institute of Agricultural Technology, Suranaree University of Technology. In 2017, he was awarded a scholarship by the Royal Golden Jubilee Ph.D. (RGJ-PHD) Programme (Grant number PHD/0165/2560) for his Doctor of Philosophy (Ph.D. degree) study in Animal Production Technology at the School of Animal Technology and Innovation, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima. During his doctoral study, he had an opportunity to go abroad for training in Cell Interactions and Fertility Unit at UMR de Physiologie de la Reproduction et des Comportements, National Research Institute for Agronomy and Environment (INRAe), INRA - CNRS - Tours University, 37380 Nouzilly France for 14 months (from 1st Jan 2022 to 28th Feb 2023).

During his Ph.D. study, he has published two articles "**Pasri, P.,** Mermillod, P., and Khempaka, S. (2023). Antioxidant properties and cytotoxic effects of selected edible plants in Southeast Asia for further use as phytogenic antioxidant additives. **Saudi J. Biol. Sci.** 30(5): 103631." and "**Pasri, P.,** Rakngam, S., Gérard, N., Mermillod, P., and Khempaka, S. (2024). Synthetic and phytogenic antioxidants improve productive performance, antioxidant activity, gene expression, and offspring quality in breeder hens subjected to heat stress. **Poult. Sci. 103(3)**: 103390."