ANTIVIRAL ACTIVITY OF CINNAMON ESSENTIAL OIL AND CINNAMALDEHYDE-DERIVED BENZIMIDAZOLE AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

DANTE MENDILLO FABROS JR

MASTER OF SCIENCE IN BIOTECHNOLOGY

MAEJO UNIVERSITY

2018

ANTIVIRAL ACTIVITY OF CINNAMON ESSENTIAL OIL AND CINNAMALDEHYDE-DERIVED BENZIMIDAZOLE AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

DANTE MENDILLO FABROS JR

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE
IN BIOTECHNOLOGY
GRADUATE SCHOOL MAEJO UNIVERSITY
2018

Copyright of Maejo University

ANTIVIRAL ACTIVITY OF CINNAMON ESSENTIAL OIL AND CINNAMALDEHYDE-DERIVED BENZIMIDAZOLE AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

DANTE MENDILLO FABROS JR

THIS THESIS HAS BEEN APPROVED IN PARTIAL FULFLLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE
IN BIOTECHNOLOGY

APPROVED BY **Advisory Committee**

Chair ....

(Associate Professor Dr.Wasin Charerntantanakul)

................../................../ ....

Committee ....

(Assistant Professor Dr. Saengtong Pongjareankit)

................../................../ ....

Committee ....

(Dr. Somkit Deejing)

................../................../ ....

Progrem Chair, Master of Science ....

(Associate Professor Dr. Wasin Charerntantanakul)

................../................../ ....

CERTIFIED BY GRADUATE SCHOOL ....

(Associate Professor Dr. Kriangsak Mengamphan)

Dean of Graduate School

................../................../ ....

ชื่อเรื่อง ANTIVIRAL ACTIVITY OF CINNAMON ESSENTIAL OIL AND CINNAMALDEHYDE-DERIVED BENZIMIDAZOLE AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

ชื่อผู้เขียน Mr.Dante Mendillo Fabros JR

ชื่อปริญญา วิทยาศาสตรมหาบัณฑิต สาขาวิชาเทคโนโลยีชีวภาพ

อาจารย์ที่ปรึกษาหลัก วศิน เจริญตัณธนกุล

บทคัดย่อ

การระบาดของโรคพีอาร์อาร์เอสในสุกรทำให้เกิดการริเริ่มค้นคว้าวิธีการรักษาที่ช่วยยับยั้งไวรัสที่เป็นสาเหตุของโรค (PRRSV) อย่างไรก็ตามวิธีการควบคุม PRRSV ที่มีการศึกษา พบว่ายังมีไม่มากนัก การใช้น้ำมันหอมระเหยเป็นแหล่งสังเคราะห์สารประกอบอินทรีย์ชนิดใหม่ได้รับการยืนยันว่าเป็นหนึ่งในแนวทางที่มีประสิทธิภาพในการยับยั้งการจำลองตัวของ PRRSV ดังนั้นการศึกษานี้จึงนำน้ำมันหอมระเหยจากน้ำมัน *Cinnamomum* (*Cinnamomum iners* Reinw. ex Blume และ *Cinnamomum burmannii* Blume) มาใช้เป็นสารตั้งต้นในการสังเคราะห์ Benzimidazole และทดสอบฤทธิ์ยับยั้งเชื้อ PRRSV ในหลอดทดลองโดยการสังเกตการณ์เกิดพยาธิสภาพของเซลล์ และการตรวจวิเคราะห์หาไตเตอร์ไวรัสโดยวิธี plaque assay โครงสร้างทางเคมีของอนุพันธ์ benzimidazole และ cinnamaldehyde ในน้ำมันหอมระเหยได้ทำการตรวจสอบโดยวิธี thin layer chromatography และ fourier-transform infrared spectroscopy ผลการศึกษาพบว่า benzimidazole จาก cinnamaldehyde ไม่มีฤทธิ์ในการยับยั้งการแบ่งตัวของเชื้อไวรัส ในขณะที่น้ำมันหอมระเหยจากอบเชยไม่มีฤทธิ์ยับยั้งเชื้อไวรัส PRRSV หรือมีฤทธิ์ยับยั้งในระดับปานกลาง (0%-22%) เมื่อทำการทดสอบในช่วง pre-infection และสามารถแสดงฤทธิ์ในการการยับยั้งเชื้อไวรัสได้ถึง 51% ในช่วงทดสอบ post-infection ในส่วนของการทดสอบโดย plaque assay พบว่า น้ำมันหอมระเหยจากอบเชยส่งผลให้การสร้าง plaque ของไวรัสลดลงถึง 42% ในช่วง pre-infection และไม่พบการลดลงของ plaque เมื่อทดสอบโดย benzamidazole ที่ได้จาก cinnamaldehyde ผลการศึกษานี้จึงชี้ให้เห็นว่าอนุพันธ์ benzimidazole มีข้อจำกัดเมื่อนำมาใช้เป็นสารต้าน PRRSV.

Title	ANTIVIRAL ACTIVITY OF CINNAMON ESSENTIAL OIL AND CINNAMALDEHYDE-DERIVED BENZIMIDAZOLE AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS
Author	Mr. Dante Mendillo Fabros JR
Degree	Master of Science in Biotechnology
Advisory Committee Chairperson	Associate Professor Dr. Wasin Charerntantanakul

ABSTRACT

Porcine reproductive and respiratory syndrome outbreak has initiated the search for inhibitory treatments and remedies against this virus (PRRSV). However, existing control strategies against *PRRSV* are still insufficient. The use of essential oil as a source to synthesize new organic drug compounds was verified as being an effective approach to impede PRRSV replication. Therefore, the present study extracted cinnamon essential oil from two *Cinnamomum* species i.e. *Cinnamomum iners* Reinw. ex Blume and *Cinnamomum burmannii* Blume, used it as substrate in benzimidazole synthesis and tested its inhibitory effect on PRRSV replication *in vitro* by cytopathic effect and plaque assays. Chemical structures of benzimidazole derivatives and cinnamaldehyde in the essential oil were confirmed by thin layer chromatography and fourier-transform infrared spectroscopy. Results showed that cinnamaldehyde-derived benzimidazole had no antiviral activity against PRRSV replication, while cinnamon essential oil itself showed no to moderate anti-PRRSV properties with 0% to 22% virus inhibition in pre-infection and up to 51% virus inhibition in post-infection assays. Plaque formation was reduced by cinnamon essential oil up to 42% in pre-infection and no reduction was observed by cinnamaldehyde-derived benzimidazole. These results suggest that benzimidazole derivative has limited use for anti-PRRSV control.

Keyword : cinnamon oil, benzimidazole derivative, porcine reproductive and respiratory syndrome virus

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the heavenly Father, my God, my Savior Jesus Christ for giving me extravagant love, full of strength and wisdom to accomplish this wonderful opportunity of writing research paper. It is true that He will not abandon nor forsake us in times of challenges.

I would like to express my deepest and grandest gratitude to my very supportive adviser, Assoc. Prof. Dr. Wasin Charerntantanakul, Chairperson of my Advisory Committee, for his guidance, assistance, patience and understanding to complete this research. No words can explain how much I overwhelm and thankful for the extravagant ideas, wisdom, knowledge and expertise that he shared all throughout this research. I am deeply gladdened and touched from the beginning that he accepted me for being part of his research team as master's degree student. His generosity, compassion, benevolence of kindness, and big-heartedness were extremely appreciated. Because of him, I have learned much about becoming a problem solver, more responsible and a better person, in and outside the world of academic research. It is a great privilege to work with the most excellent and finest professor of Maejo University. To other members of thesis committee, Asst. Prof. Dr. Saengtong Pongjareankit, Dr. Somkid Deejing, Assoc. Prof. Dr. Thapanee Sarakonsri of Chiang Mai University who also served as the external committee chairperson for this thesis, and to Asst. Prof. Dr. Paweena Poomsuthapol, Program Chair for Master of Science in Biotechnology, I would like to thank them for their words of encouragement, expert suggestions and constructive feedbacks to make the thesis content stronger and reliable.

To Dr. Uthumporn Kankeaw and to the rest of her research team advisees, Miss Suwanna and Miss Benjamaporn from the Department of Chemistry thank you for teaching and assisting me in the chemistry experiment of my thesis. Thanks also to the staff of Department of Fisheries Technology and Aquatic Resources who let me used their machine for cytotoxicity testing.

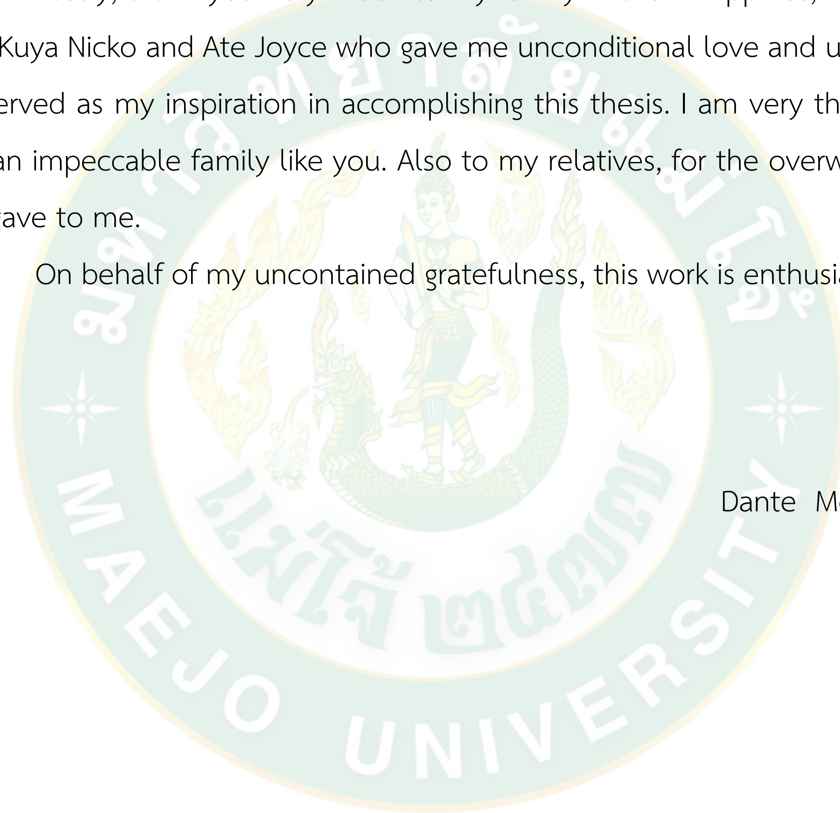
Special thanks to Miss Wilawun Ruansit and Miss Reunkaew Praphrute as they taught me styles and strategies on the technicalities of my experiment. I'm so blessed

to have them by my side and gave me much passionate help and encouragement in able to succeed. Thank you for the help of making every day process easier to complete my research.

Thank you to Ma'am Diana who helped me for my master's degree enrollment. This is not possible without your helping hands. To all Filipino graduate students (Ma'am Leah, Ma'am Ann, Sir Efren) and to internship students I have bonded, your backing and help in times of struggles and difficulties were also very appreciated.

Lastly, thank you very much to my family in the Philippines, Nanay Tess, Tatay Julio, Kuya Nicko and Ate Joyce who gave me unconditional love and unending support, you served as my inspiration in accomplishing this thesis. I am very thankful for having such an impeccable family like you. Also to my relatives, for the overwhelming support they gave to me.

On behalf of my uncontained gratefulness, this work is enthusiastically for all of you.



Dante Mendillo Fabros JR

TABLE OF CONTENTS

	Page
ABSTRACT (THAI)	C
ABSTRACT (ENGLISH)	D
ACKNOWLEDGEMENTS	F
TABLE OF CONTENTS	H
List of Tables	J
List of Figures	K
CHAPTER 1 INTRODUCTION	1
Objectives	3
CHAPTER 2 REVIEW OF RELATED LITERATURE	5
Porcine reproductive and respiratory syndrome virus	5
Anatomy and Morphology	5
Virus stability	7
PRRSV Replication	7
Pathogenesis	8
Clinical Signs and Epidemiology	10
Cinnamon essential oil	12
Bioactive properties of benzimidazole derivatives	14
Benzimidazole synthesis by *o*-phenylenediamine and aldehyde	15
CHAPTER 3 METHODOLOGY	17
Cinnamon oil extraction	17
Synthesis of benzimidazole	17

TLC and FT-IR 17

Cultivation of cells and virus 18

Cytotoxicity test by CV (crystal violet) staining assay and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 18

Anti-PRRSV screening by end-point dilution/cytopathic (CPE) assay 19

Pre-infection 19

Post-infection 19

Percentage virus inhibition analysis 20

Determination of viral plaque formation 20

CHAPTER 4 RESULTS AND DISCUSSION 21

Cinnamon oil yield 21

Yield of derived benzimidazole and composition 23

FT-IR spectrum analysis of synthesized benzimidazole derivative 27

Evaluation of least cytotoxic concentration of cinnamon essential oil and benzimidazole derivative 27

Antiviral assay 30

CHAPTER 5 SUMMARY, CONCLUSION AND RECOMMENDATION 36

REFERENCES 38

APPENDICES 60

Appendix A. Tables of data 60

Appendix B. Figures 69

Appendix C. Calculations 76

CURRICULUM VITAE 79

List of Tables

Page

Table 1 Essential oil extraction of cinnamon bark of *C. iners* 21

Table 2 Essential oil extraction of cinnamon bark of *C. burmannii* 21

Table 3 Yield of synthesized benzimidazole derivative from *C. iners*' cinnamon oil.. 23

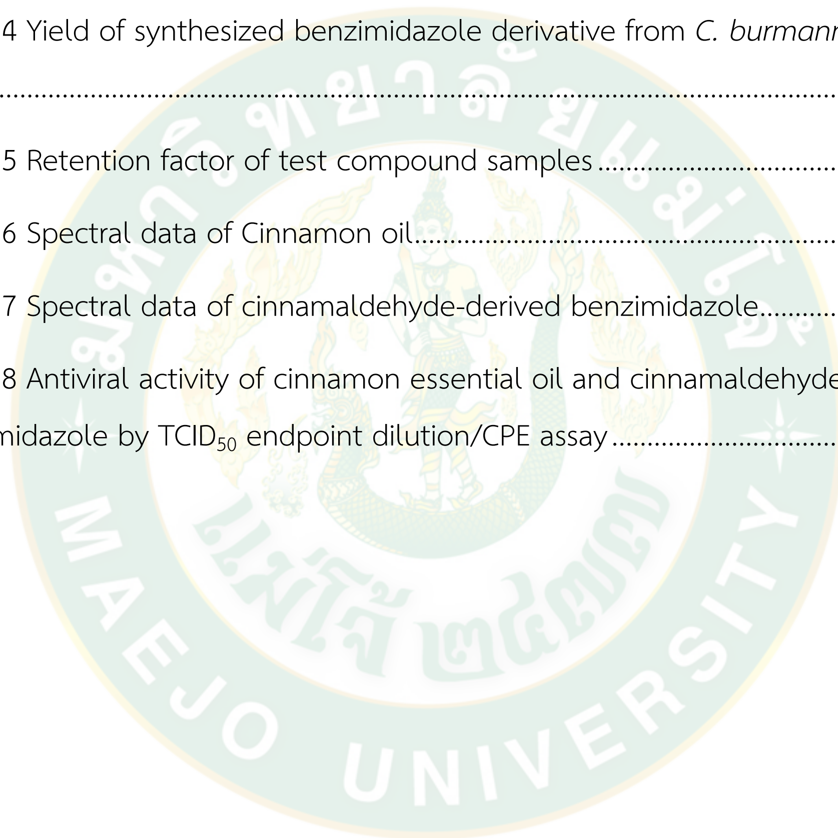
Table 4 Yield of synthesized benzimidazole derivative from *C. burmannii*'s cinnamon oil 23

Table 5 Retention factor of test compound samples 25

Table 6 Spectral data of Cinnamon oil 25

Table 7 Spectral data of cinnamaldehyde-derived benzimidazole 27

Table 8 Antiviral activity of cinnamon essential oil and cinnamaldehyde-derived benzimidazole by $TCID_{50}$ endpoint dilution/CPE assay 31



List of Figures

Page

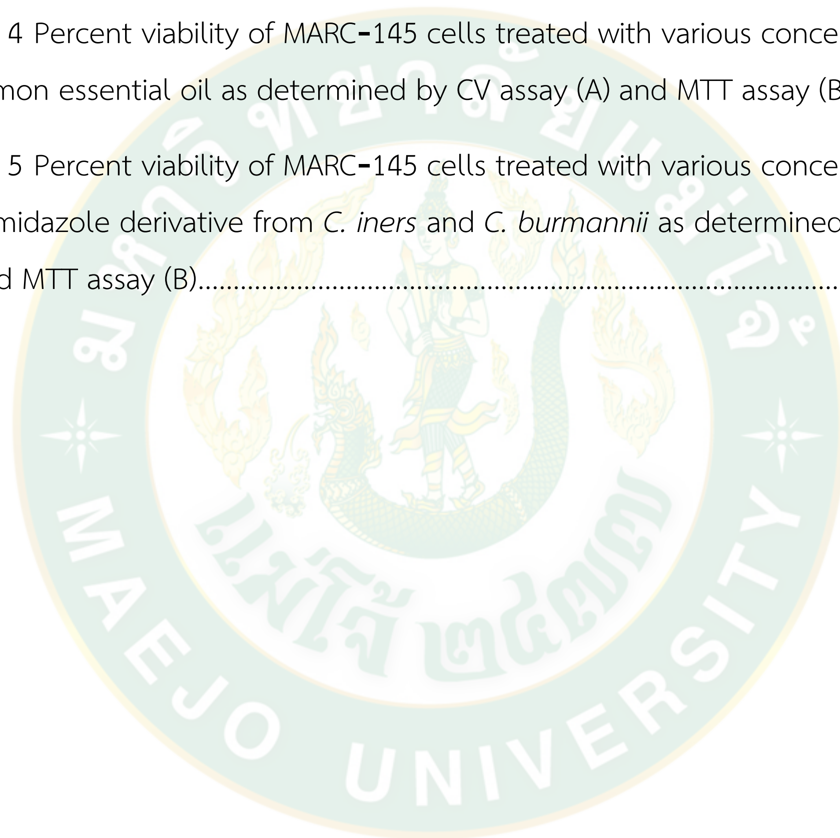
Figure 1 PRRSV structure and its genome 6

Figure 2 *o*-dinitrogen compound structure 15

Figure 3 Benzimidazole ring system.. 15

Figure 4 Percent viability of MARC-145 cells treated with various concentrations of cinnamon essential oil as determined by CV assay (A) and MTT assay (B).................... 29

Figure 5 Percent viability of MARC-145 cells treated with various concentrations of benzimidazole derivative from *C. iners* and *C. burmannii* as determined by CV assay (A) and MTT assay (B).. 30



CHAPTER 1

INTRODUCTION

The worldwide prevalence of porcine reproductive and respiratory syndrome (PRRS) is one of the major problems of swine industry resulting to global economic loss. The etiologic agent of this swine disease namely porcine reproductive and respiratory syndrome virus (PRRSV) is the cause of severe respiratory and reproductive failure (Stevenson and Torremorell, 2012). PRRSV is an enveloped, positive-sense RNA virus which belongs to the genus Arterivirus, order *Nidovirales*, family Arteriviridae (Faaberg *et al.,* 2012). The viral structure appears to be oval-shaped or roughly spherical particle of 50-60 nm in diameter and the genome comprises 11 known open reading frames (ORFs) (Lunney *et al.,* 2016). *In vivo* experiments demonstrated that the primary target of PRRSV infection is highly restricted to porcine alveolar macrophages (Duan *et al.,* 1997). After severe infection, the virus succeeds immunosuppression, which results in reproductive illness, respiratory ailment, abortion and death. The existing antiviral strategies against PRRSV are vaccination and biosecurity. Vaccination provides partial protection against clinical disease, primarily due to a high genetic variation among PRRSV isolates (Du *et al.,* 2017).

The emergence of drug-resistant viruses was led by the augmented availability and exploitation of antiviral drugs. Numerous source of medicinally valuable herbs and trees rest unexplored. However, essential oils and extracts from a wide-ranging variety of plants have long been used for medicinal purposes (Srisukh *et al.,* 2012). Usually, essential oil consists of bioactive compounds responsible for its organoleptic properties. One of the commercially available is the cinnamon essential oil which most contains cinnamaldehyde (Friedman *et al.,* 2000).

The bioactive compound composition of cinnamon essential oil varies depending on the part of the plant used in the extraction. Generally, cinnamon oil contains 80-90% cinnamaldehyde and with slight or no eugenol. Other compounds that can be found in cinnamon essential oil include condensed tannins, cinnamyl acetate, benzaldehyde, linalool, and limonene (Kim *et al.,* 2015; Shan *et al.,* 2007).

Cinnamaldehyde is an organic compound synthesized naturally in *Cinnamomum* trees including *Cinnamomum iners* Reinw. ex Blume and *Cinnamomum burmannii* Blume, belonging to the family *Laureaceae*. It is a viscous fluid, pale yellow in appearance that gives cinnamon its aroma and flavour. Chemically, cinnamaldehyde (C_9H_8O, 3-phenyl-2-propenal) is the major component of cinnamon essential oil revealed by high-performance liquid chromatography (HPLC) and Fourier-transform infrared (FT-IR) spectroscopy (Adinew, 2014; Li *et al.,* 2013; Wong *et al.,* 2014). The biological activities of this compound reportedly include antiviral (Fabra *et al.,* 2016), antimicrobial (Yossa *et al.,* 2014), anti-inflammatory (Muhammad *et al.,* 2015), antioxidant (Naveena *et al.,* 2014), antispasmodic (Jaafarpour *et al.,* 2015), anti-urease (Lee *et al.,* 2005), anti-cancer (Vangalapati and Prakash, 2013) and hyperglycemic (Camacho *et al.,* 2015). However, antimicrobial activity of cinnamaldehyde is extensively studied but there is only limited knowledge on its antiviral property (Liu *et al.,* 2015). In addition, the antipathogenic property of cinnamaldehyde extracted from *Cinnamomum verum* J .Presl and *Cinnamomum cassia* Blume has been proven by several reports conducted (Ooi *et al.,* 2006; Ouattara *et al.,* 1997; Wong *et al.,* 2008). Cinnamaldehyde from *Cinnamomum verum* J. Presl and *Cinnamomum osmophloeum* inhibits pro-inflammatory IL-1β (interleukin-1beta), IL-6 (interleukin-6) production, and suppresses iNOS (nitric oxide synthase) and COX-2 (cyclooxygenase-2). These findings conclude the anti-inflammatory effects of cinnamaldehyde (Koh *et al.,* 1998; Zhao *et al.,* 2011). Generally, the chemical property of cinnamaldehyde is unstable *in vivo* hence unstable in rat blood with a half-life of 4-minutes. (Li *et al.,* 2017; Yuan *et al.,* 1992). To overcome this problem, cinnamaldehyde derivatives were synthesized. Li *et al.* (2017) demonstrated the antiviral potential α-bromo-4-methyl-cinnmaldehyde and α-bromo-4-chloro-cinnamaldehyde effectively reduced the viral titer of coxsackievirus (CBV3) in Hela cells. Antiviral activities of cinnamaldehyde were significantly increased when cinnamaldehyde was brominated and chlorinated also resulting with low toxicity .

Benzimidazole is a bicyclic isosteric compound with purine nuclei and indole. Physiochemical backbone structure of benzimidazole is the integral part of its

biological functionalities. However, numerous types of pharmacodynamics and pharmacokinetic properties have been linked with its derivatives (Amari *et al.,* 2002). As a multifunctional core nucleus of many compounds, benzimidazole plays an essential role in synthesis and development of therapeutic agents to elicit various biological functionalities. Benzimidazole drugs have been commercially available such as anthelmintic (albendazole), antiviral (anviradine), antitumor (bendamustine), anti-inflammatory (benoxaprofen analog) and antihistaminic (albenzole) (Bansal and Silakari, 2012). To improve the pharmacological activities, various derivatives of benzimidazole were synthesized although some of the readily synthesized compounds have found very robust application in medical treatments. For instance, Kankeaw and Rawanna (2015) reported the synthesis of derived-benzimidazole by the condensation between 1,2 phenylene diamine and aldehyde citronellal. This affords the product without Schiff base compound as by product in order to improve pathogenic activities.

Various benzimidazole derivatives have been evaluated for their antiviral activities .The antiviral properties of eighty-six benzimidazole derivatives against ten selected RNA and DNA viruses were screened (Tonelli *et al.,* 2010). Among those viruses, CVB-5 (coxsackie B-5), RSV (respiratory syncytial virus), BVDV (bovine viral diarrheal virus) and Sb-1 (poliovirus sabin strain 1) were significantly affected. Also derivatives of 2-phenylbendizimadole have been reported to have antiviral activities against CVB-2, BVDV, Sb-1, HSV-1 (herpes simplex virus) and YFV (yellow fever virus) while HIV-1 (human immunodeficiency virus) and VSV (vesicular stomatitis virus) were not affected (Tonelli *et al.,* 2010).

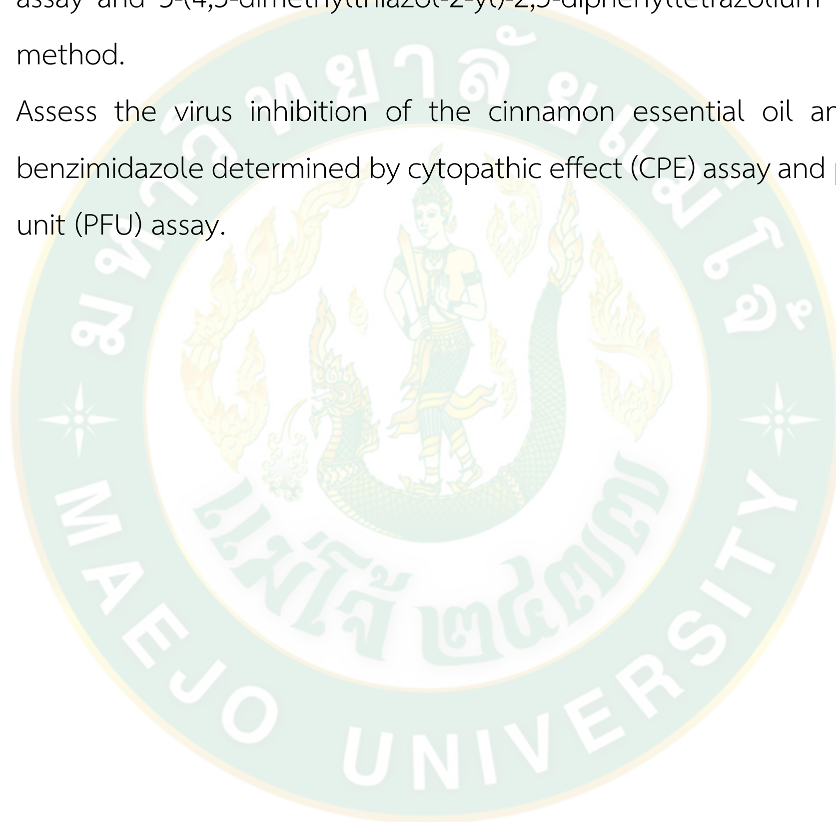
Despite several reports on the antiviral properties of cinnamon essential oil and benzimidazole against many viruses, study on their anti-PRRSV activity has not been elucidated. Therefore, the current study evaluates the antiviral activity of cinnamon essential oil and cinnamaldehyde-derived benzimidazole against PRRSV grown *in vitro* on MARC-145 cells.

Objectives

This study sought to investigate the antiviral activity of cinnamon essential oil and its derived-benzimidazole against PRRSV grown *in vitro* on MARC-145 cells.

Specifically, the objectives of this study will:

1. Extract and identify the cinnamon oil percentage yield from *C. iners* and *C. burmannii* tree bark by hydrodistillation
2. Synthesize derived-benzimidazole by mixing 1,2-phenylene diamine and cinnamaldehyde from cinnamon essential oil confirmed by thin layer chromatography (TLC) and fourier transform infrared spectroscopy (FT-IR).
3. Determine the percentage viability of MARC-145 cells treated with cinnamon essential oil and its derived-benzimidazole tested by crystal violet (CV) staining assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.
4. Assess the virus inhibition of the cinnamon essential oil and its derived-benzimidazole determined by cytopathic effect (CPE) assay and plaque forming unit (PFU) assay.


MAEJO UNIVERSITY

CHAPTER 2

REVIEW OF RELATED LITERATURE

Porcine reproductive and respiratory syndrome virus

Anatomy and Morphology

PRRSV is a positive sensed and enveloped RNA virus, a member of family Arteriviridae along with simian hemorrhagic fever virus (SHFV), equine arteritis virus (EAV) and mouse lactate dehydrogenase-elevating virus (LDV) (Snijder *et al.,* 2013). Arteriviridae family alongside with Coronaviridae, Roniviridae, Mesoniviridae with the same gene orientation and replication process, were placed under order Nidovirales (Cavanagh, 1997). The whole PRRS virion structure sized approximately 50-60 nm in diameter that appears to be oval-shaped or roughly spherical (Spilman *et al.,* 2009). The formed virion particle consists of genome RNA packed by homodimer double layered protein N (nucleocapsid) surrounded by glycoproteins and membrane proteins implanted onto a lipid bilayer envelop (Fang and Snijder, 2010). PRRSV genome RNA have approximately 15 kb in size situated between untranslated regions; 5’-UTR and 3’UTR with methylated cap and polyadenylated tail respectively, which contains 11 ORFs. The known ORFs are the following; ORF1a, ORF1a’-TF, ORF1b, ORF2a, ORF2b, ORF3-5, ORF5a, ORF6 and ORF7. ORF1a and ORF1b constitute the third quarter of the genome that encodes long non-structural polyproteins pp1a and pp1b whereas ORF1B is expressed by a -1 ribosomal frameshift. These large polyproteins then cleaved into 16 known nsps (nsp1α and β, nsp2-6, nsp2TF, nsp2n, nsp7α and β, nsp8-12). ORF2-4 code for minor structural components i.e. GP2, E, GP3, and GP4. ORF5, ORF5a, ORF6 and ORF-7 expressed 3 major structural proteins i.e. GP5, M, N, and a minor protein ORF5a. However, ORF5a interaction and orientation in the virion structure still needs to be elucidated (Firth *et al.,* 2011; Johnson *et al.,* 2011). Overall, membrane associated glycoproteins (GP2a, GP3-5), unglycosylated membrane proteins (E, ORF5a, M) and nucleocapsid complete the virion structure (Fang and Snijder, 2010; Lunney *et al.,* 2016; Snijder *et al.,* 2013). The recent discovered ORF (TF) in the central region of ORF1a encodes nsp2F and nsp2N (Fang *et al.,* 2012; Li *et al.,* 2014). The lipid bilayer

envelop embedded with major proteins M and GP5 comprises at least half the amount of viral protein. Heterodimer M with GP5 form a disulphide bond linked cysteine residues (Figure 1) (Ko *et al.,* 2015; Verheije *et al.,* 2002). On the other hand, minor glycoproteins (GP2-3, E) form a multimeric complex incorporated on the lipid envelop (Das *et al.,* 2010; Wissink *et al.,* 2005).

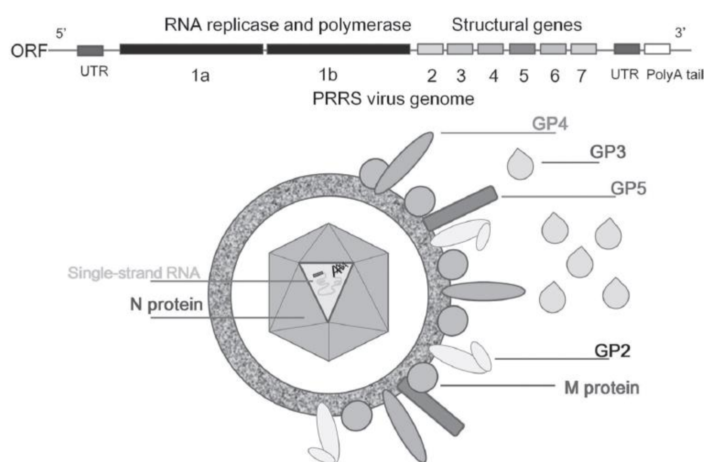


Figure 1 PRRSV structure and its genome

There are two PRRSV genotypes such as type 1 isolated from Europe with prototype Lelystad and type 2 (prototype VR 2332) originally isolated in North America. (Cha *et al.,* 2006; Stadejek *et al.,* 2013). It was reported that these two PRRSV genotypes with antigenic and serological differences were 60 % nucleotide identical as revealed by genomic sequence analysis (Allende *et al.,* 1999; Wensvoort *et al.,* 1992). Moreover, the non-glycosylated protein E of type 2 PRRSV was not incorporated with minor glycosylated multimeric protein complex in the lipid envelop (Das *et al.,* 2010; Wissink *et al.,* 2005). All eight relatively small genes (ORF2a-ORF7) have both 5' and 3' terminal sequences overlapping with neighbouring genes except for type 2 PRRSV where its ORF4 and ORF5 were not overlapping (Lunney *et al.,* 2016). It was also reported that PRRSV isolated in China with genomic analysis belongs to genotype 2 compatible to highly pathogenic strain (Zhou *et al.,* 2012). PRRSV is considered highly mutable RNA virus with significant genetic variability within its two types based on ORF5 phylogenetic analysis (Brar *et al.,* 2015; Murtaugh *et al.,* 2010).

Virus stability

PRRSV is prone to heat but stable at temperatures 4°C and -70°C (Benfield *et al.,* 1992). With pH 6.5-7.5, the virus is also stable but its infectivity will be reduced outside this pH range (Bloemraad *et al.,* 1994; Van Alstine *et al.,* 1993).

Solvents such as chloroform and ether disrupts the PRRSV lipid bilayer envelop. Also 0.0075% iodine and 0.0063% quaternary ammonium compounds can inactivate the virus (Shirai *et al.,* 2000). Complete inactivation of PRRSV can be accomplished with 0.03% chlorine or using ultraviolet upon 10 minutes of exposure (Dee *et al.,* 2011; Shirai *et al.,* 2000).

PRRSV Replication

Macrophage or monocytic lineage is the restriction of PRRSV cell tropism. The primary target cell is the fully differentiated porcine alveolar macrophages (PAMs) for PRRSV infection (Lawson *et al.,* 1997; Park *et al.,* 2008). *In vitro* propagation can be done using non-porcine cell lines aside from blood monocytes and PAMs. MA104 cell line derived from African green monkey was routinely used for viral *in vitro* propagation as well as its subclones MARC-145 and CL2621. On the other hand, expression of Sn (sialoadhesin) receptor is absent on MA104 cell line. Therefore, Van Breedam *et al.* (2010a) and Nauwynck *et al.* (2012) conclude that virus propagation on MA104 cell line can lead to structural protein mutation resulting to virus infectivity increase.

The entry of PRRSV was achieved by receptor-mediated endocytosis. Initially, the virus binds to the host cell via heparin-sulphate GAGs (glycosaminoglycans) (Delputte *et al.,* 2005) after interaction of the virion to the cell surface, internalization may occur by the involvement of sialoadhesin (Sn or CD169) receptor binding to sialic acid that is present on glycosylated membrane protein M/GP5 heterodimer complex (Van Breedam *et al.,* 2010b; Vanderheijden *et al.,* 2003). However, recent study using CD169 gene knockout pigs demonstrated that the intact Sn is not required for the attachment and/or internalization of PRRSV (Prather *et al.,* 2013). Hence, PRRSV enters the cell through clathrin-mediated endocytosis (Nauwynck *et al.,* 1999). The endosome internalized with the virus then co-localized with CD163, a member of scavenger receptor cysteine-rich family (Van Gorp *et al.,* 2009). Recent study determines CD163 as the major receptor to mediate viral internalization and disassembly (Van Breedam

et al., 2010a). Drop in pH inside the endosome together with CD163 receptor interaction uncoats the virus, thus, releasing its genome to the cytosol (Van Gorp *et al.,* 2009; Van Gorp *et al.,* 2008). Das *et al.* (2010) demonstrated the interaction of GP2 and GP4 glycosylated membrane proteins to CD163 but the exact mechanism and CD163 role in the uncoating process is not yet clear. Moreover, it was reported that CD163 was identified as initiation key factor of PRRSV infection (Calvert *et al.,* 2007).

After genome release, gene replication occurs in the host cell's cytoplasm. Proteins encoded from ORF1a and ORF1b will be synthesized before viral genome replication takes place. ORF1a translated directly from genomic RNA following the ORF1b translation through -1 programmed ribosomal frameshift upstream of ORF1a termination codon resulting to the extension of pp1a to pp1ab. The synthesized polyproteins (i.e. pp1a, pp1b) well then cleaved by internal proteinases to generate at least 14 nsps, which were assembled into replication and transcription factor complex (RTC) (Snijder and Meulenberg, 1998). RNA polymerase (RdRp) and RNA helicase were the major enzymes of RNA replication both encoded in ORF1b (nsp9 and nsp10) (Van Dinten *et al.,* 1996). With RTC, minus-strand RNA synthesis occurs to produce subgenomic (sg) negative-sense RNAs. These sg RNAs serve as the template in discontinuous positive-strand mRNA synthesis for the expression of structural protein genes located at the 3' proximal quarter of the genome (Conzelmann *et al.,* 1993; Pasternak *et al.,* 2006; Snijder and Meulenberg, 1998). Then, the generated viral RNAs were packed into nucleocapsid. Envelop was formed by budding from smooth intracellular membrane. The release of new virion from the cell was accomplished by exocytosis (Lunney *et al.,* 2016).

Pathogenesis

PRRSV infection is not persistent. But in swine industry production system, the average lifetime period of pigs is 180 days so it was considered as life-long lasting to the majority of the pigs. The infection principally occurs as subclinical infection associated as co-factor in different diseases like porcine respiratory disease complex (PRDC) and porcine circovirus associated disease (PCVAD) (Chand *et al.,* 2012). The usual host response to the single pathogen infection was altered when PRRSV interacts with other swine pathogens (e.g. porcine respiratory coronavirus, swine influenza virus,

Haemophilus parasuis) (Solano *et al.,* 1997; Van Reeth *et al.,* 1996). PRRSV suppresses the host immune defence that causes other pathogens to establish secondary and opportunistic infection leading to more severe and chronic diseases. Brockmeier *et al.* (2002) demonstrated that PRRSV was the most common virus isolated on PRDC infected pigs. Co-infection of PRRSV and *Bordettella bronchiseptica* worsens the clinical disease (Brockmeier *et al.,* 2000). Also, there were longer period and more severe lung pneumonia observed on *Mycoplasma hyopneumoniae* and PRRSV co-infected pigs (Thacker *et al.,* 1999). Similarly, lung lesions and more severe clinical symptoms were observed in PCVAD infected pigs compared to the single infection of PRRSV or porcine circovirus-2 (PCV-2) (Allan *et al.,* 2000).

Dissemination of susceptible macrophages throughout the body system mainly hints the PRRSV shedding by multiple routes (Pol *et al.,* 1991). PRRSV was found in blood, nasal secretions, semen, mammary gland secretion, urine, and feces (Rossow *et al.,* 1994; Swenson *et al.,* 1994; Wagstrom *et al.,* 2001; Wills *et al.,* 1997b). It has been reported that PRRSV nasal shedding was strain dependent, at least for type 1, and it may have the effect on the formation of aerosols infected with PRRSV (Frydas *et al.,* 2015; Frydas *et al.,* 2013). Transmission of virus by aerosol was strain dependent as studies showed that efficient aerosol transmission can be achieved depending on pathogenicity of the virus (Cho *et al.,* 2006; Cho *et al.,* 2007). On the other hand, contradicting reports on the presence of PRRSV in feces were recorded. Fecal swabs were observed positively with PRRSV (Christianson *et al.,* 1993) but some studies showed no detection of virus in feces (Rossow *et al.,* 1995). PRRSV can be also present in oral fluids (Kittawornrat *et al.,* 2010; Wills *et al.,* 1997a) with matching viral loads found in serum (Prickett *et al.,* 2008). Similarly, viral shedding in semen of the boar was extensively studied. This implies the high risk of transmission between the infected boars to the sows and gilts (Christopher-Hennings *et al.,* 1995a; Christopher-Hennings *et al.,* 1995b; Nielsen *et al.,* 1997; Yaeger *et al.,* 1993). In line with this, PRRSV was isolated on bulbourethral gland. Consequently, it can be a long term source of virus and viremia is not a sufficient indication of the possible contagiousness (Christopher-Hennings *et al.,* 1995a). Colostrum and milk of sows can be a PRRSV source to the litters but their connection to PRRSV transmission was only secondary infection

(Wagstrom *et al.,* 2001). These studies implicate the methods and routes of PRRSV transmission by either direct or indirect contact e.g. insemination, contaminated needles, vertical transmission, ingestion, and aerial.

The acute post-infection phase was described when there's high viral load in tissue and serum viremia was observed by 6-12 hours post infection that may last up to 28 days. Lung is the superior site of infection where viral replication in macrophages and dendritic cells in upper respiratory tract occurs (Duan *et al.,* 1997; Halbur *et al.,* 1995a; Halbur *et al.,* 1995b). Characterization of viral persistence was demonstrated when the virus was not detected already in lungs and blood because the virus replication site localized in lymphoid tissue. The virus can be isolated in lymph nodes for longer than 100 days post infection even though the pig has no longer displays clinical signs. This stage was when the virus can transmit easily to naïve pigs because continuous replication happens in regional lymph nodes triggering the efficient viral transmission through oral-nasal secretions and semen (Christopher-Hennings *et al.,* 2008; Rowland *et al.,* 2003)

Clinical Signs and Epidemiology

The virus occurred in Europe and North America at the same time during late 1980's (Stevenson and Torremorell, 2012), but study by bioinformatics analysis suggests that PRRSV existed a century ago (Forsberg, 2005). At present, PRRSV emerged from Europe with prototype Lelystad virus was designated as PRRSV type 1 and PRRSV type 2 for the virus VR-2332 from North America. Genomic sequence analysis revealed that these two genotypes of PRRSV were distantly related (Faaberg *et al.,* 2012). After the discovery of PRRSV, the virus was distributed globally from Canada, Germany, United Kingdom, Japan and throughout Asia (Cha *et al.,* 2006; Gilbert *et al.,* 1997; Larochelle and Magar, 1997). Recently, nucleotide analysis revealed that PRRSV isolated in south western China belongs to PRRSV genotype 2, the same as highly pathogenic strain (Zhou *et al.,* 2012). Type 1 PRRSV was more diverse than type 2 PRRSV as the result of studies conducted from different countries such as Latvia, Italy, Lithuania, Belarus and Russia (Forsberg *et al.,* 2002; Le Gall *et al.,* 1998; Stadejek *et al.,* 2006; Stadejek *et al.,* 2008; Stadejek *et al.,* 2013; Suárez *et al.,* 1996). PRRSV 1 subtypes 1-4 has been discovered because the differences in their ORF5 and ORF7 were

defensible and evident (Stadejek *et al.,* 2006; Stadejek *et al.,* 2008; Stadejek *et al.,* 2013). Since the discovery of PRRSV, Kuhn *et al.* (2016) stated that the broad developing apprehension on the evolution and discovery of Arteriviridae virus warranted the proposal in separating type 1 and 2 PRRSV into two different species.

Before the discovery of the etiological agent of PRRS, the disease was formerly named as blue ear disease, porcine epidemic abortion and respiratory syndrome, swine infertility and respiratory syndrome, swine reproductive and respiratory syndrome, pig plague 89 and disease 89 (Goyal, 1993; Keffaber, 1989; Wensvoort *et al.,* 1991). In general, the clinical symptoms appear mild to acute respiratory disease in pigs and reproductive disorders in sows. The clinical representation of PRRSV infection depends on various factors such as age, pregnancy status, gestation trimester of sows and gilts, immune status, environmental factors, virus strain, genetic susceptibility and co-infection with other swine pathogens (Rossow, 1998; Stevenson and Torremorell, 2012). PRRSV infected sows exhibited lack of appetite, anorexia, abortion, birth of dead or weak piglets, mummified fetus, and temporary blue discoloration of ears vulva or abdomen (Terpstra *et al.,* 1991). Weaned pigs show signs like pneumonia, fever, lethargy and insufficient weight gain or inappropriate weight loss. Gross lesions depend on virus isolate, herd status and genetic representation while lung lesions occur by pulmonary consolidation merged with another swine pathogen infection present as subclinical symptom (Rossow, 1998). Different isolates of North American PRRSV genotype 2 vary on their clinical infection i.e. gross lesions, rectal temperature, lung lesions. Type 1 PRRSV infection represented by prototype Lelystad virus results to temporary pyrexia, tachypnea and dyspnea while its highly pathogenic strain outcomes anorexia, pyrexia, lethargy and labored breathing (Halbur *et al.,* 1995a; Halbur *et al.,* 1995b; Halbur *et al.,* 1996; Mengeling *et al.,* 1996).

It has been over 25 years since the discovery of PRRSV. Highly virulent strains have constantly evolved through time to cause various acute diseases that has quickly spread globally. In late 1990's, atypical PRRSV caused high percentage of swine abortion and death reported in USA (Mengeling *et al.,* 1998). Highly virulent strain MN1-8-4 similar to PRRSV genotype 2 was also reported in Canada and North Central USA (Han *et al.,* 2006). Simultaneously in China and Southeast Asia, highly pathogenic

variants of PRRSV were reported as subclinical infection with porcine high fever disease in all ages of pigs with severe respiratory disorder producing high percentage of mortality (Tian *et al.,* 2007). On the other hand, a highly pathogenic PRRSV 1 subtype 3 (Lena strain) was isolated in Belarus, Eastern Europe (Karniychuk *et al.,* 2010). Highly pathogenic strain infection is accompanied by abnormal host response, severe lung lesions and clinical symptoms (Han and Yoo, 2014; Karniychuk *et al.,* 2010). Stadejek *et al.* (2017) demonstrated the pathogenicity of various PRRSV 1 strains comparing subtype 1 Danish strain, subtype 2 Russian strain and subtype 2 Belarusian strain. Subtype 2 BOR59 Belarusian strain was highly pathogenic comparable to subtype 3 Lena strain and SU1-bel (Karniychuk *et al.,* 2010; Morgan *et al.,* 2013; Weesendorp *et al.,* 2013; Weesendorp *et al.,* 2014).

Cinnamon essential oil

Plant essential oil have wide variety of applications such as flavouring, cosmetics, industrial applications, pesticides, fragrance and aromatherapy. Essential oil has pharmacological and medicinal properties based on their chemical components (Bousbia *et al.,* 2009). Currently used essential oil that are available commercially worldwide is the cinnamon essential oil. *Cinnamomum* species tree bark is the main source of commercial cinnamon material based on its high essential oil and cinnamaldehyde content (Geng *et al.,* 2011; Li *et al.,* 2013).

Cinnamon was proven as non-toxic organic product and considered as convenient to manufacture at cheap price (Frydman-Marom *et al.,* 2011). It was recognized Generally Recognized as Safe (GRAS) by FDA (FDA, 2015) that makes it suitable as natural food additive and potential medicine. Essential oil contains concentrated volatile oils that are hydrophobic, lipophilic and carry distinct scent through various parts of plants and herbs (Bousbia *et al.,* 2009).

The source of cinnamon is from the bark of trees belonging to *Cinnamomum* sp under family Laureaceae is a genus of evergreen shrubs and trees. It comprises about 250-350 species distributed globally, dispersed in tropical and subtropical areas of Southeast Asia, Australia, North America, Central America, South America (Rana *et al.,* 2009; Wang *et al.,* 2009). *C. iners* Reinw. ex Blume and *C. burmannii* Blume are two

species widely used as raw material to produce cinnamon. The brief taxonomical features of *C. iners* were, the tree or small tree measured 4-12 m tall, stem circumference up to 14 cm in diameter, yellowish inner bark is smooth, twigs stout or slender, terete, 2-3 mm in diameter, apically terete to subangular, drying dark brown to black. Known as Indonesian cassia, *C. burmannii* tree can grow up to 20 m tall with stem sized 12-40 cm in diameter. Its bark is smooth, greyish brown with fragrant inner bark and yellowish sapwood. Twigs are slender, terete, 2-3 mm in diameter, apically subangular, glabrous, and dark brown to blackish. These taxonomical descriptions of various *Cinnamomum* sp. were reported by Wuu-Kuang (2011).

Several studies on cinnamon essential components have been reported. Zhang *et al.* (2016) found 92.40% cinnamaldehyde in cinnamon oil with small amount of benzaldehyde, styrene, di-acetone alcohol, benzylcarboxaldheyde, phenol, trans-cinnamic acid and octadecadienoic acid *C. zeylanicum* essential oil has 68.95% cinnamaldehyde, 9.94% benzaldehyde, 7.44% cinnamyl acetate, limonene 4.42% and eugenol 2.77% (Unlu *et al.,* 2010). In addition, Li *et al.* (2013) identified 81.97% trans-cinnamaldehyde in *C. loureirii*, *C. verum* was 74.49% and 74.49% on *C. cassia* essential oil. Thus, cinnamaldehyde is the main compound of cinnamon essential oil (Shareef, 2011).

Pharmacological property of essential oil was attributed to their main component (Burt, 2004; Ojeda-Sana *et al.,* 2013), however, the bioactivity of essential oil resulted from the involvement of different compounds (Hussain *et al.,* 2010; Jantan *et al.,* 2008; Ojeda-Sana *et al.,* 2013). The bioactive properties of cinnamon essential oil and its principal component cinnamaldehyde have also been recorded. Liu *et al.* (2015) demonstrated that both cinnamaldehyde and volatile oil of cinnamon from *C. cassia* Presl. showed significant inhibitory effect on H1N1 influenza virus proliferation. It concluded that these compounds induce expression of IFN-B in MDCK cells by stimulating the TLR-7 and IRAK-4 pathway (Liu *et al.,* 2015). Cinnamon essential oil has antibacterial against representative gram positive and gram-negative bacteria, i.e. *S. aureus* and *E. coli* (Zhang *et al.,* 2016). Cinnamaldehyde is reported to be responsible for the antimicrobial activity of cinnamon oil (Kaskatepe *et al.,* 2016). Other pharmacological activities of cinnamon essential oil were anticarcinogenic (Unlu *et al.,*

2010) antifungal (Xing *et al.,* 2014), insecticidal (Jumbo *et al.,* 2014), anti-inflammatory (Tung *et al.,* 2008), nematicidal (Kong *et al.,* 2007), acaricidal and repellent effect (Oh, 2011).

Bioactive properties of benzimidazole derivatives

Benzimidazole ring system (Figure 2) is one of the most common heterocyclic pharmacophores. This chemical substructure is called "privileged" because of their broad prevalence in many integral cellular constituents and bioactive compounds. Benzimidazole structure and its ligands have been widely studied but the major concern is their pharmacological and pharmaceutical properties (Alaqeel, 2017). Typically, the benzimidazole nucleus is the major structural component of Vitamin B12 (O'Neill *et al.,* 2001). Substituted benzimidazole derivatives have established uses and applications in variety of therapeutics. Several bioactive functionalities of derived benzimidazole were antihypertensive (Naik *et al.,* 2010), anti-inflammatory (Grivennikov *et al.,* 2010), antimicrobial (Ansari and Lal, 2009), antiviral (Starčević *et al.,* 2007), anticancer (Sondhi *et al.,* 2010), antiprotozoal (Valdez-Padilla *et al.,* 2009), anthelmintic (Köhler, 2001), analgesic and gastric ulcerogenic effects (Gaba *et al.,* 2010).

Tonelli *et al.* (2014) stated that the various interacting structure of several benzimidazole derivatives consequents to their functionalities and modulates potency that may imply to antiviral specificity. Benzimidazole derivatives with antivirals depend on their chemical group position acting against RSV and HCV (Boido *et al.,* 2009). Series of non-halogenated, brominated, chlorinated benzimidazole derivatives have no significant antiviral activity against representative positive-sensed RNA viruses i.e. HIV-1, BVDV, YFV, and WNV (Budow *et al.,* 2009). Recently, non-nucleoside derivatives of benzimidazole have gained attention to fight viral proliferation because the past researches have been focused on the synthesis of nucleoside analogues to hinder virus replication (Beaulieu *et al.,* 2004; Budow *et al.,* 2009; Moussa *et al.,* 2016). Structure activity relationship study between the synthesized compound and its therapeutic activity is very significant. As such, Pan *et al* (2015) found that 2-substituted benzimidazole with N,N-dialkyl amine, m-methoxy and p-hydroxyl were the most promising anti-HIV-1 whereas derivative N,N-dialkyl amine is required for anti-HIV-1

replication. Additionally, a set of derived benzimidazole compounds were tested against HCV and inclusion of methyl group in the ring system showed significant anti-HCV property (Tsay *et al.,* 2013). It was also reported that pyridine at the C2 of benzimidazole ring have the most observed antiviral activity against coxsackie virus and echo virus (Starčević *et al.,* 2007).

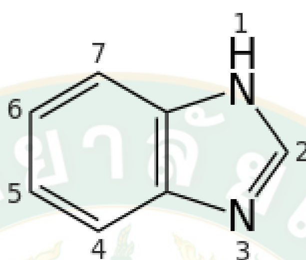


Figure 3 Benzimidazole ring system

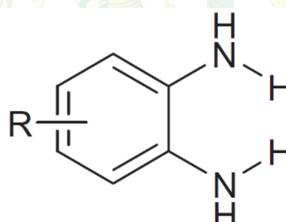


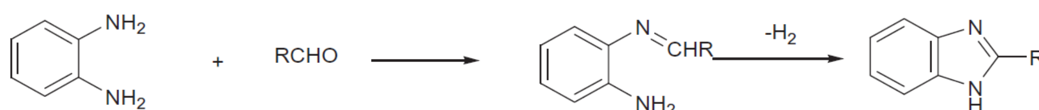
Figure 2 *o*-dinitrogen compound structure

Benzimidazole synthesis by *o*-phenylenediamine and aldehyde

Though all seven positions in the benzimidazole nucleus can be substituted with a variety of chemical entities, most of the biologically active compounds bear functional group at 1,2 and/or 5(or 6) positions (Bansal and Silakari, 2012).

Most reactions of benzimidazole synthesis involve starting material benzene ring derivative with *o*-dinitrogen compound (Figure 3). Generally, there are two major typical methods of 2-substituted benzimidazole synthesis. One is the condensation of *o*-phenylenediamine with carboxylic acids and their derivatives, under strong acid condition, microwave irradiation or high temperature (Beaulieu *et al.,* 2004; Saberi, 2015). The other one is also the condensation of o-phenylenediamine with an aldehyde compound in combination of oxidative cyclocondensation of Schiff bases (Asadipour *et al.,* 2013; Chakrabarty *et al.,* 2006; Gogoi and Konwar, 2006). The

condensation of *o*-phenylenediamine and aldehydyde will yield 2-substituted benzimidazoles under correct reaction conditions. However, the procedure modified by Kankeaw and Rawanna 2015 was adapted in this study where simple condensation of o-phenylenediamine and aldehyde (Scheme 1) is achieved without Schiff base in order to increase the bioactivity of the synthesized compound. Other compounds may also react with *o*-phenylenediamine to yield benzimidazole depending on the chemical reaction conditions employed. These compounds are acid anhydride, esters, amides, urea, acid chlorides, nitriles, ketones, potassium hydroxide and chloroform (Alaqeel, 2017; Rathod *et al.,* 2013).



Scheme 1 Condensation reaction of *o*-phenylenediamine and aldehyde

CHAPTER 3
METHODOLOGY

Cinnamon oil extraction

Hydrodistillation was conducted to extract the essential oil of *C. iners* and *C. burmannii* (Singh *et al.,* 2007). Dried cinnamon barks obtained from *C. iners* and *C. burmannii* were purchase from Warorot market, Chiang Mai, Thailand. One kg of cinnamon was mixed with 3 L of distilled water, and heated at 100°C for 8 h (Wong *et al.,* 2014). Using methylene dichloride, the volatile products were extracted thrice from the water phase (Singh *et al.,* 2007). Esssential oil structure was characterized by FT-IR spectroscopy.

Synthesis of benzimidazole

The benzimidazole derivative was synthesized by mixing 0.70 g of 1,2-phenylenediamine and 1.7 ml of cinnamon essential oil in 30 mL ethanol and refluxed for 2-6 h. The mixture was cooled for one day and filtered. The product was recrystallized with absolute ethanol. The melting point of benzimidazole derivative was measured using melting temperature apparatus. Temperature was recorded when the sample started to melt and when it was molten completely.

TLC and FT-IR

Infrared (IR) spectra of cinnamon oil and benzimidazole derivative were recorded on Perkin Elmer FT-IR spectrometer with salt plate and KBr pellets respectively. IR spectrum was reported in % transmittance. Cinnamon oil spectra were compared with cinnamaldehyde IR spectrum standard. The wave number region for the analysis was 4000-400 cm^{-1}. The purity of the compound was checked by TLC on precoated SiO_2 gel (HF254 200 mesh) aluminum plates (Merck) (Kankeaw and Masong, 2015).

Cultivation of cells and virus

Virus culture and cell line were obtained from Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University. It was maintained in MEM++ comprising minimum essential medium (Caisson Laboratories, Utah, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Capricorn Scientific GmbH, Germany), antibiotics/antimycotic penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml) (all from Gibco, New York, USA) to avoid microbial contamination in the culture. PRRSV was propagated in sub cultured MARC-145 cells grown in MEM++ at 37°C in a 5% CO_2 incubator. After four days of incubation, the frozen overnight virus cultures were thawed twice, centrifuged, and harvested. Supernatant was filtered through 0.22 µM filter (Minisart®, Sartorius, France) to remove unnecessary particles, cells and microbes. Harvested viruses were stored at -80°C. Viral titers were determined by 50% cell culture infectious dose ($TCID_{50}$) endpoint dilution assay after 96 h inoculation and adjusted to 10^6 $TCID_{50}$/ml prior to anti-PRRSV culture assay.

Cytotoxicity test by CV (crystal violet) staining assay and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

Cytotoxicity of cinnamon essential oil was tested by CV staining assay and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as previously described (Sun *et al.,* 2012). Approximately $1x10^5$ cells/ml MARC-145 cells were seeded in a flat-bottom 96-well plate (Nunc, Denmark). The essential oil was serially 2-fold diluted, added onto the wells, and cultivated at 37°C in humidified 5% CO_2 atmosphere for 96 h. After completion of incubation, the media were removed and the cells were washed with PBS thrice. Cells were fixed with acetone:methanol (60:40) solution at 4°C for 30 min. Then, 0.5% CV solution was added into each well and incubated at room temperature for 5 min, followed by rinsing with water. The wells then received Sorenson's buffer and were incubated at room temperature for 15 min. The optical density (OD) absorbance was determined at 570 nm (Feoktistova *et al.,* 2016).

For MTT method, another cell culture set up was prepared. MTT solution was added to each well and the plates were incubated for 4 h at room temperature. Then,

100 µl of DMSO was added and the plates were gently shaken for 5 min until the crystals were fully dissolved. The OD value was measured at 595 nm on a microplate reader (Thirabunyanon *et al.,* 2009). Percentage of cell viability was calculated using the formula [(A-B)/Ax100], where A and B are the OD value of treated and control cells, respectively.

Anti-PRRSV screening by end-point dilution/cytopathic (CPE) assay

Pre-infection

Five mL of MARC-145 cells ($5x10^5$ cells/mL) were seeded in 25-mL flasks at 37°C in 5% CO_2 atmosphere for 16 h. The media were then removed and 4.5 mL of selected concentrations of cinnamon essential oil and benzimidazole derivatives in MEM++ were added to the flasks. Five hundred µL of PRRSV (10^6 $TCID_{50}$/mL) was subsequently added to the flask and the culture was incubated at 37°C in humidified 5% CO_2 atmosphere for 96 h. Supernatant consisting of unknown concentration of virus was collected and kept at -70°C until use. PRRSV titration was performed by the addition of serially 10-fold diluted supernatant in MEM++ and inoculated in 96 well plate containing 100 µL of confluent MARC-145 cells. The cultures were incubated for 96 h at 37°C in a 5 % CO_2 incubator. The concentration of viral titer was calculated by the determination of 50% tissue infection culture dose (TCID50) (Appendix) after CPE were observed under inverted microscope.

Post-infection

Five mL of MARC-145 cells ($5x10^5$ cells/mL) were seeded in 25-mL flasks at 37°C in 5% CO_2 atmosphere for 16 h. The media were then removed and 500 µL of PRRSV (10^6 $TCID_{50}$/mL) was added. The cultures were incubated for 1 h to allow PRRSV infection, then received 4.5 mL of selected concentrations of cinnamon essential oil and benzimidazole derivatives in MEM++. The incubation was carried out for 96 h at 37°C in 5% CO_2 incubator. Supernatant consisting of unknown concentration of virus was collected and kept at -70°C until use. The 10-fold serially diluted supernatant/viral suspension was added to confluent MARC-145 cells in 96-well plate, and the cultures were incubated for 96 h at 37°C in a 5% CO_2 incubator. The viral concentration was

calculated by the determination of $TCID_{50}$/ml values (Appendix) after CPE was observed in each well under inverted microscope.

Percentage virus inhibition analysis

The calculation of virus inhibition percentage was based on the formula (100% x (A-B)/A) where A and B denote to logarithmic number of virus titer in the absence and presence of test sample, respectively.

Determination of viral plaque formation

Plaque assay was performed as previously described (Chuaychu *et al.,* 2013). In brief, $5x10^5$ cells/ml of MARC-145 cell line were sub-cultured in 24-well plate for 16 hours at 37°C in a 5% CO_2 incubator. MEM++ was removed and serially 10-fold diluted supernatant viral suspension from CPE pre-infection or post-infection assay was added. After 1 h, viral titers were pipetted out and 500 µl of medium was added following by the addition of 0.6% agarose. The cell culture was incubated again for 96 hours at 37°C in a 5% CO_2 incubator. Then, the medium was removed and cells were washed by 1X PBS twice. Cell fixation was done by adding cold acetone:methanol (60:40) and incubated at 4°C for 30 min. Cells were dried and stained with 0.25% Coomassie Brilliant Blue R250 (Panreac) in acetic acid and 50% methanol (1:9 v/v). Fixed cells were washed with 1X PBS twice. Plaques were counted and viral titer was expressed as plaque forming units per ml (PFU/ml) using the formula PFU/ml=(A/DxV) where A is number of plaque, D is the dilution factor and V for the volume of viral inoculum added to the wells.

CHAPTER 4

RESULTS AND DISCUSSION

Cinnamon oil yield

The average percentage yield of essential oil from *C. iners* bark obtained from Thailand was shown in Table 1. After extraction of volatile oil by hydrodistillation, the average yield was 0.75 %.

Table 1 Essential oil extraction of cinnamon bark of *C. iners*

	Weight of Cinnamon bark (g)	Volume of essential oil (ml)	% yield
1	1,150	9.13	0.79
2	1,120	8.08	0.72
3	1,130	8.53	0.75
Average	1,130	8.58	0.75

The Table 2 showed the percentage yield of essential oil from the cinnamon bark of *C. burmannii* originated from Indonesia. The average percentage yield of cinnamon oil after hydrodistillation was 0.92%.

Table 2 Essential oil extraction of cinnamon bark of *C. burmannii*

	Weight of Cinnamon bark (g)	Volume of essential oil (mL)	% yield
1	1,060	9.56	0.90
2	1,020	9.84	0.96
3	1,050	9.65	0.91
Average	1,040	9.68	0.92

The yield of cinnamon oil in different species varies (Wang *et al.,* 2009). The essential oil may produce ranging 0.72 to 3.08 % from the bark of different *Cinnamomum* species (Li *et al.,* 2013). Whereas, similar findings to Wang *et al* (2009) were obtained with steam-distilled cinnamon essential oil extracted from two different

species that yields 0.72 to 0.96%. Li *et al* (2013) observed the differences of essential oil harvested in every *Cinnamomum* species and it was due to the environmental factors such as type of weather or climate, geographical distribution, growth conditions and site of cultivation. This implies that results of essential oil isolation from cinnamon bark was correlated with ecological factors mentioned above on which difference between the essential oil percentage yield of *C. iners* and *C. burmannii* was due to species diversity and cultivation site. In addition, Li *et al* (2013) found out that thicker bark has higher cinnamon oil content and the most essential oil was concentrated in the phloem.

Several studies have been conducted about the identification of organic compound composition of cinnamon essential oil. The essential oil from the bark of *C. iners* contained trans-cinnamaldehyde (71.825%) and cinnamyl alcohol (7.52%), other compounds like eugenol, caryophyllene, α-pinene were present. Phenolic compounds were probably responsible for the antioxidative and antimicrobial activity of *C. iners* bark and leaf oils. (Heng, 2008). While, Baruah *et al.,* (2001) identified that the components in the stem bark oil of *C. iners* were 1,8-cineole (40.76%), α-terpineol (15.06%) and terpinen-4-ol (13.85%). Other components identified are β-pinene, γ-terpinolene, and carphyllene oxide. On the other hand, essential oil from the cinnamon stick powder of *C. burmannii* contains 83.6 % trans-cinnamaldehyde as the major component and condensed tannins as minor compounds (Shan *et al.,* 2007). Also, the chemical constituents essential oil of *C. burmannii* demonstrated by Wang *et al.,* 2009 were trans-cinnamaldehyde (60.17%), eugenol (17.62%) and coumarin (13.39%). Other constituents identified in the oil were alcohol, aldehydes and ketones.

Nonetheless, the variances of yield and composition of plant essential oil depends on environmental or climatic conditions (e.g. geographical location, altitude, temperature, wind, rainfall), plant physiology (e.g. parts of the plant, age and ontogeny of plant parts), genetic make-up of the plant (e.g. chemotypes, varieties, morphotypes) agronomic management (e.g. nutrients, pests and diseases, irrigation, pesticide application, harvesting height. harvesting date), post-harvest technology (e.g. drying

biomass, cutting biomass into small pieces), distillation (e.g. duration, method, process parameters), and storage (container, presence of air/water, storage period).

Yield of derived benzimidazole and composition

The appearance of the product after benzimidazole synthesis was orange color, powdered crystal (Appendix figure 2b) The average yield percentages of benzimidazole derivative of cinnamaldehyde from *C. iners* and *C. burmannii* were 36.48% (Table 3) and 34.52%(Table 4), respectively. The melting point for benzimidazole derivative from *C. iners* and *C. burmannii* were 161-165 °C and 163-165 °C, respectively.

Table 3 Yield of synthesized benzimidazole derivative from *C. iners'* cinnamon oil

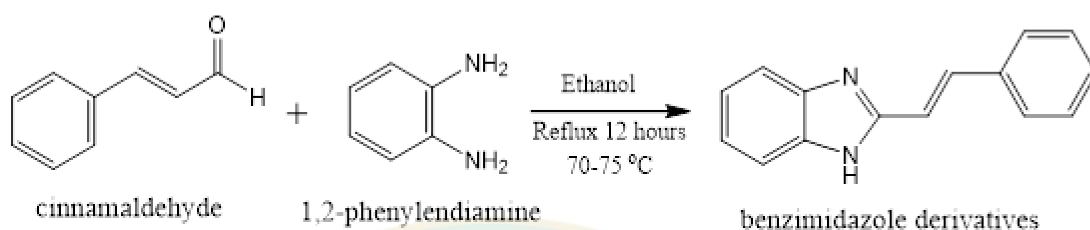
	Weight of benzimidazole product (g)	Yield (%)	Melting temperature (°C)
1	0.5211	36.39	161-165
2	0.5186	36.22	161-165
3	0.5273	36.83	161-165
Average	0.5223	36.48	161-165

Table 4 Yield of synthesized benzimidazole derivative from *C. burmannii*'s cinnamon oil

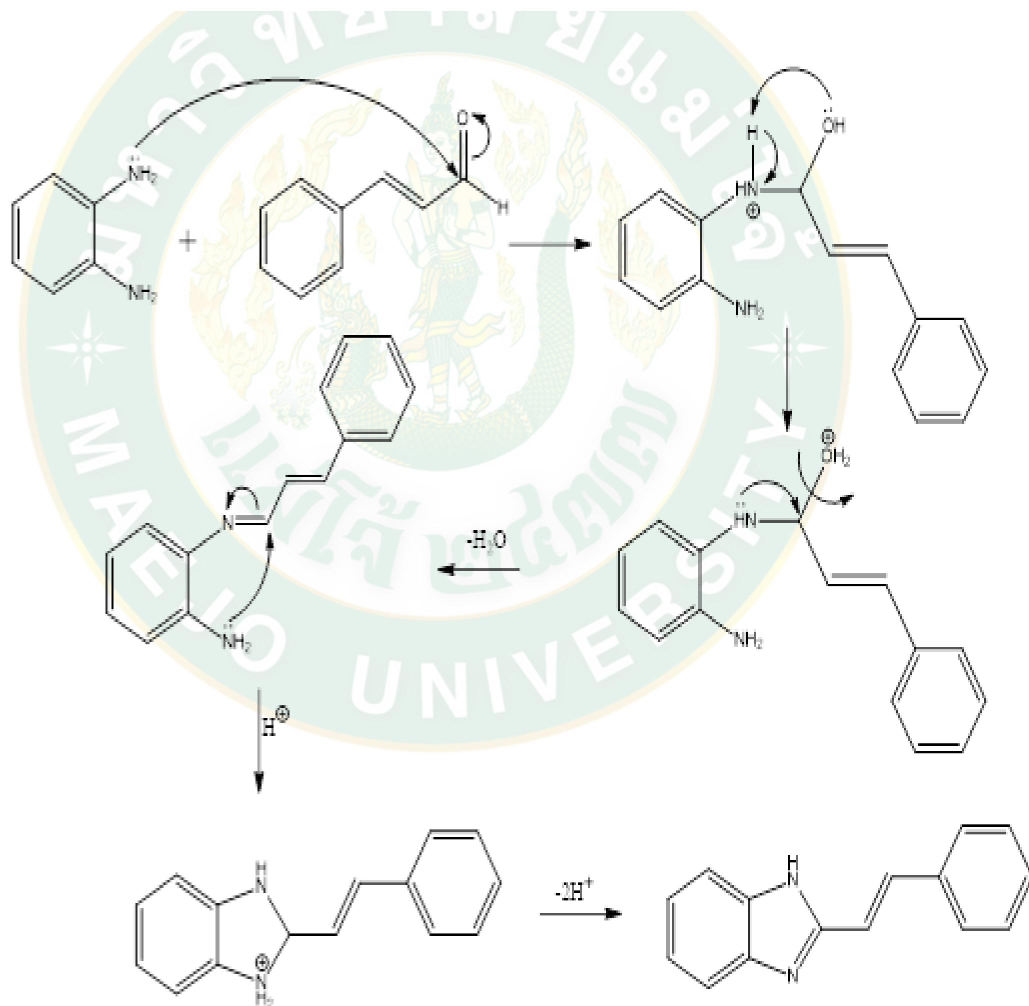
	Weight of benzimidazole product (g)	Yield (%)	Melting temperature (°C)
1	0.4953	34.60	163-165
2	0.4827	33.71	163-165
3	0.5046	35.24	163-165
Average	0.4942	34.52	163-165

The medium yield of benzimidazole derivative in this study is due to the conventional method the absence of catalyst used in the reaction. The proposed

mechanism reaction (Scheme 3) in a step-wise fashion expressed that the lone pair of amino group undertakes as nucleophile attack to the functional carbonyl group of cinnamaldehyde to form benzimidazole derivative.



Scheme 3 Condensation reaction of cinnamaldehdye and *o*-phenylenediamine



Scheme 2 Proposed mechanism for the synthesis of benzimidazole

The retention factor values of each test sample were reported in Table 4. Using the solvent ethyl acetate:methanol (8:2), chromatogram of pure samples was visible in TLC plate (Appendix figure 3).

Table 5 Retention factor of test compound samples

Sample	Rf Value
Cinnamon oil 1 (from C. iners)	0.85
Cinnamon oil 1 (from C. burmannii)	0.77
Benzimidazole 1	0.83
Benzimidazole 2	0.64

Table 6 Spectral data of Cinnamon oil

Functional Group	Cinnamon oil from *C. iners* (cm-1)	Cinnamon oil from *C. burmannii* (cm-1)	Cinnamaldehyde standard
C=O stretching of aldehyde	1677	1679	1671
C-H stretching of aldehyde	2741, 2814	2742, 2814	2742, 2814
C=C stretching of aromatic	1624, 1451	1507, 1452	1624,1449
C-H stretching of aromatic	3058	3064	3060

MAEJO UNIVERSITY

The spectral data of cinnamon oil in comparison with cinnamaldehyde reference was reported in Table 5. This confirms that the essential oil extracted from *C. iners* and *C. burmannii* contain cinnamaldehyde. Only the frequency peak values of interests were presented on Table 5 that corresponds on the cinnamaldehyde structure interrelated with FT-IR simplified correlation chart (Pavia *et al.,* 2014). Peaks such as 1677-1679 cm^{-1} that corresponds to the vibration stretching of a carbonyl aldehyde (C=O), 2741-2742, 2814 cm^{-1} (C-H stretching of aldehyde), 3058-3064 cm^{-1} (C-H stretching of aromatic), 1624-1451 cm^{-1} (C=C stretching of aromatic) were an evident data that extracted cinnamon oil from two *Cinnamomum* species comprise cinnamaldehyde. The spectral data of cinnamaldehyde standard was previously described by Kankeaw and Masong, (2015) whereas FT-IR spectroscopy revealed the functional groups of the cinnamaldehyde chemical structure (Awang *et al.,* 2013; Gende *et al.,* 2008; Kankeaw and Masong, 2015)

Cinnamon oil from tree bark consists of various organic compounds. It has been well studied that cinnamon essential oil's principal volatile compound is cinnamaldehyde (Burlando *et al.,* 2010; Shareef, 2011). Concentration and presence of volatile compounds depends on many factors such as extraction methods, plant part and source. Moreover, Zhang *et al.* (2016) found 92.40% cinnamaldehyde in cinnamon essential oil. Li *et al* (2013) identified 81.97% trans-cinnamaldehyde in *C. loureirii*, *C. verum* was 74.49 % and 74.49% on *C. cassia* essential oil. About 90% cinnamaldehyde was revealed by HPLC (high-performance liquid chromatography) from the steam-distilled essential oil (Wong *et al.,* 2014). Adinew (2014) also identified 87% cinnamaldehyde analyzed by gas chromatography-mass spectrometry (GC-MS) and FT-IR spectroscopy from the essential oil of cinnamon bark. In addition, 85% of cinnamaldehyde in hydro-distilled cinnamon oil from *C. cassia* and *C. verum* was revealed by GC-MS (Ooi *et al.,* 2006). Other than cinnamaldehyde, minute amount of eugenol, benzaldehyde, cinnamyl acetate, limonene di- acetone alcohol, benzylcarboxaldehyde, phenol, trans-cinnamic acid, and octadecadienoic acid can be found in cinnamon tree bark's hydrodistilled essential oil (Li *et al.,* 2013; Unlu *et al.,* 2010; Zhang *et al.,* 2016)

FT-IR spectrum analysis of synthesized benzimidazole derivative

FT-IR spectrum data of the synthesized benzimidazole were compared to benzimidazole derivative reference spectrum. Peaks presented (Table 7) were 3366-3372 (N-H stretching of amine), 3026 (C-H stretching of alkene), 1595-1597 (C=C stretching of alkene), 1495-1450 and 1450-1495 (C=C stretching of aromatic), suggesting the presence of benzimidazole after synthesis.

Table 7 Spectral data of cinnamaldehyde-derived benzimidazole

Functional group	Benzimidazole 1 (derived from *C. iners'* cinnamaldehyde)	Benzimidazole 2 derived from (*C. burmannii's* cinnamaldehyde)
	Wave number (cm^{-1})	
N-H stretching of amine	3372	3366
C-H stretching of alkene	3026	3026
C=C stretching of alkene	1597	1595
C=C stretching of aromatic	1450, 1495	1495, 1450
C=N stretching of amine	2365	2365

Evaluation of least cytotoxic concentration of cinnamon essential oil and benzimidazole derivative

Concentrations of the samples that yielded >95% cell viability based on CV staining and MTT assays were chosen for subsequent antiviral assays. After 96 h of incubation, 3.91 nl/ml of *C. iners'* cinnamon oil and 1.95 nl/ml of *C. burmannii*'s cinnamon oil showed least cytotoxicity to MARC-145 cells (Figure 4). In the succeeding assay, approximately 4 nl/ml of *C. iners'* cinnamon oil and 2 nl/ml of *C. burmannii*'s cinnamon oil were considerably tested. On the other hand, 312.5 ng/ml of synthesized

benzimidazole 1 and 156 ng/ml of synthesized benzimidazole 2 were selected with no detectable cytotoxic effect to the cells (Figure 5) .

Noting that the color of cinnamon oil and benzimidazole derivative afftected the colorimetric assay OD results, data on percentage cell viability using MTT assay are somewhat controversial presenting non-linear correlation to the dilution series treatment and showing discrepancy with CV assay result. MTT assay is an end-point by nature based on the enzyme activity or bioreduction of tetrazolium salts from yellow to the dark blue formazan dye by mitochondrial dehydrogenases (Berridge *et al.,* 2005). MTT is cleaved by metabolically active live cells and the formazan generated is meant to be directly proportional to cell numbers In order to measure the absorbance of the dye, converted formazan has to be released from the cells. However, the metabolic behaviour varies under different cell culture conditions. On the basis, Quent *et al.,* 2010 concluded that such metabolic assays are subject to several variables and are therefore not the finest methods for evaluating cell proliferation, knowing the chemical reliance on the efficiency of metabolic enzymes.

Some of the discrepancies of MTT assay result have been well reviewed by Stockert *et al.,* 2018 stating that in following MTT incubation, and in addition to the cytoplasmic granules, a variable quantity of extracellular needle-shaped formazan crystals can be detected but the foundation of this occurrence has not yet been clarified. The manifestation of extracellular formazan deposits could present a serious error when measuring cell viability, giving rise to incorrect positive values. As such, MTT can also be reduced by the cell culture medium alone to form extracellular formazan (Young *et al.,* 2005).

Another limitation carried out was the effect of the sample compound; its chemical nature of supplement that affected and increased the mitochondrial dehydrogenase activity of cells leading to MTT false positive result, therefore, DNA-based staining methods was used and establishing the measurement of cellular DNA indicates the relative cell number since cellular DNA content is highly regulated (Wang *et al.,* 2010). So, assuming that the cinnamon oil comprising several compounds aside from cinnamaldehyde, and benzimidazole derivatives might contributed to the

increase of enzymatic activity of the cells. But this hypothesis should be clarified in the future,

CV assay is a non-enzymatic and simple assay for the faster analysis of viable adherent cells and colonies, which lacks the restrictions of undermining the accuracy of metabolic enzyme-activity based assay. This kind of assay associated between dye affinities to DNA surface. The amount of absorbed dye depends on the total DNA content in the culture that was correlated to the number of viable cells in the culture (Sliwka *et al.,* 2016). Thus, this study used metabolism independent-CV assay and metabolic activity-dependent MTT assay to evaluate the cell viability due to the possible delimitations of using a single assay linked with the risk of erroneous interpretation.

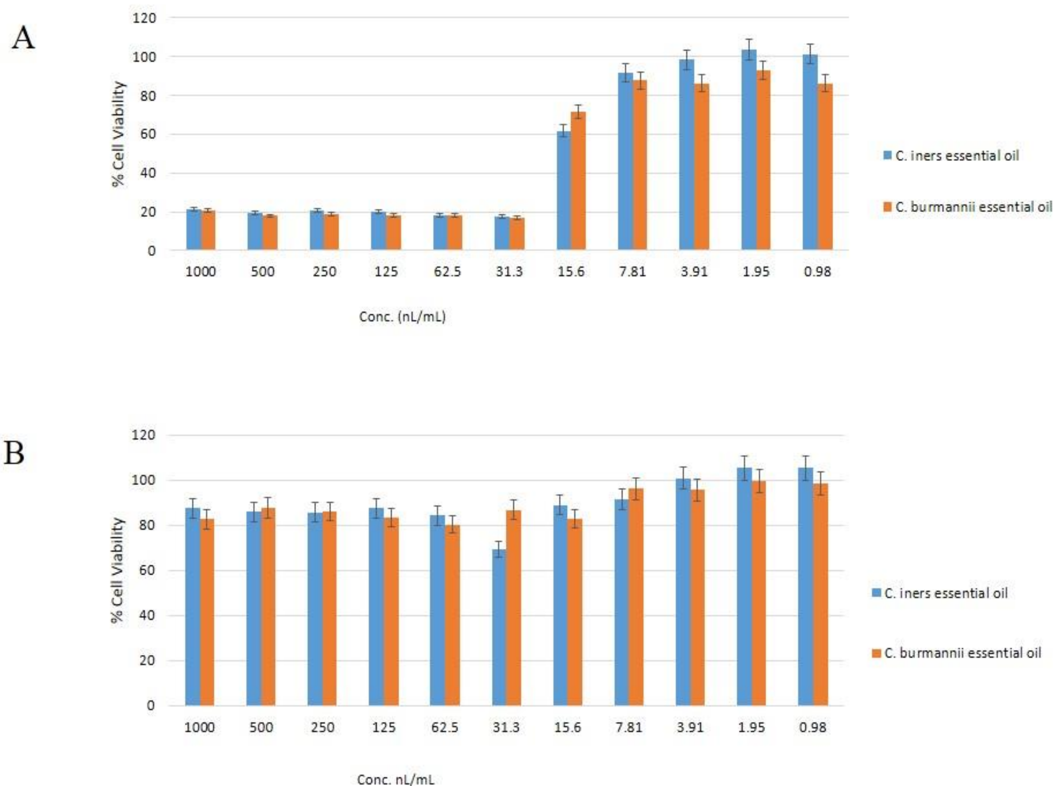


Figure 4 Percent viability of MARC-145 cells treated with various concentrations of cinnamon essential oil as determined by CV assay (A) and MTT assay (B)

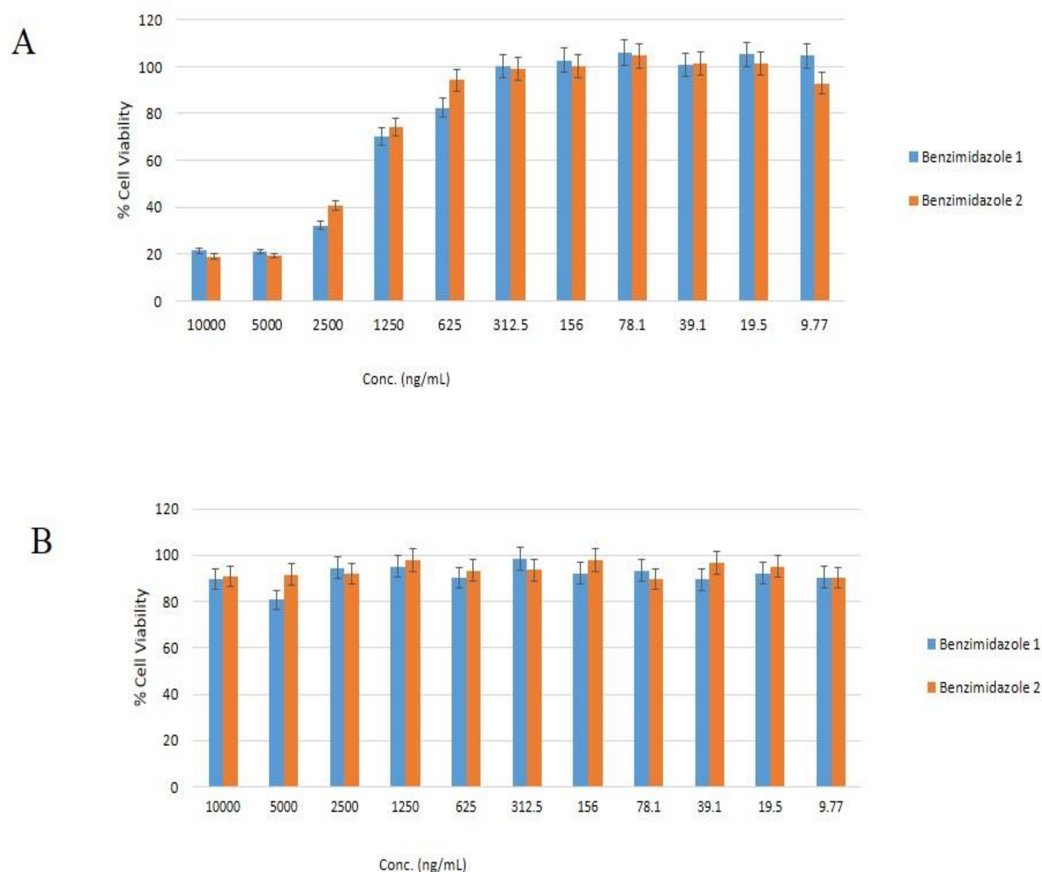


Figure 5 Percent viability of MARC-145 cells treated with various concentrations of benzimidazole derivative from *C. iners* and *C. burmannii* as determined by CV assay (A) and MTT assay (B)

Antiviral assay

The antiviral activity of cinnamon essential oil from two cinnamon bark sources was summarized in Table 8. The selected concentrations of each extract were non-cytotoxic to MARC-145 cells. Three 10-fold dilutions of the non-cytotoxic concentration of the samples were also tested to significantly screen the substantial effect of the decreasing extract concentration on the virus proliferation. The pre-infection entry of virus showed low to moderate anti-PRRSV effect of cinnamon oil 1 (*C. iners*) ranging from 16% to 22% virus inhibition. While 2 nl/ml cinnamon essential oil 2 (*C. burmannii*) showed only 9% inhibition in pre-infection of virus. On the other hand, in post virus

entry, only cinnamon oil 1 reduced PRRSV with 38% to 50% viral reduction .Conversely, cinnamon oil 2 reduced 51 %viral titer at the post-infection treatment.

Table 8 Antiviral activity of cinnamon essential oil and cinnamaldehyde-derived benzimidazole by $TCID_{50}$ endpoint dilution/CPE assay

Extract	Unit	Concentration	% inhibition	
			Pre-virus entry	Post-virus entry
Cinnamon oil 1 (from C. iners)	nl/ml	4	22%	41%
		0.4	16%	50%
		0.04	0%	38%
Cinnamon oil 1 (from C. burmannii)		2	9%	51%
		0.2	3%	-13%
		0.02	3%	23%
Benzimidazole 1	ng/ml	312.5	0 %	0 %
		31.25	4 %	-22 %
		3.125	0 %	-17 %
Benzimidazole 2		156	-62%	20 %
		15.6	-24%	4 %
		1.56	0%	0 %

The antiviral evaluation was of cinnamaldehdye-derived benzimidazole was also summarized in Table 8. In pre-virus entry, benzimidazole 1 presented no PRRSV inhibitory effect with 0 to 4% inhibition. PRRSV titer was increased 62% by benzimidazole 2 on pre-infection treatment. It showed induction of PRRSV replication rather than inhibition.

In post infection treatment, neither benzimidazole 1 nor benzimidazole 2 inhibited PRRSV replication. Instead, benzimidazole 1 did enhance PRRSV replication by up to 22%, while benzimidazole 2 inhibited PRRSV replication by up to 20%.

In plaque titration assay, the last dilution factor of the treatment showing plaques were compared to control set up. Unfortunately, only cinnamon oil of *C.*

burmannii showed 11% and cinnamon oil from *C. burmannii* exhibited 42% plaque reduction in pretreatment infection (Appendix Table 1).

To overcome the difficulties in PRRSV uncountable plaques, cytopathic effect was observed and TCID50 values were calculated. The significant role in TCID50 assay is the quality of the CPE because the infected cells were difficult to visualize. PRRSV produced cytopathic effect on MACR-145 cells characterized by cell rounding, shrinkage, shape deterioration and detachment (Appendix figure 11). But in this case, the result of plaque assay was not parallel to CPE assay as the theoretical relationship between TCID50 and PFU is approximately 1 PFU = 0.69 TCID50. Some of the drawbacks might encountered on its utilization such as counting the different morphologies of a given plaque as negative or the PRRSV strain used in the experiment might not cause adequate level of cellular damage to be visualized as plaque. The ambiguity as to the state of given plaque have been also observed in other positive-sense single stranded RNA virus wherein such effects on the assay utilization, as the native variability of the assay can result to coefficient of variation ranging 5%-44% or higher (Bae, 2003; Shurtleff, 2012).

The moderate antiviral activity of cinnamon essential oil is due to cinnamaldehyde's instability. The bioactivity of essential oil was described to their main component (Burt, 2004; Ojeda-Sana *et al.,* 2013). However, the involvement and synergistic effect of different compounds in essential oil may be attributed to their pharmacological and pharmacokinetic properties (Hussain *et al.,* 2010; Jantan *et al.,* 2008; Ojeda-Sana *et al.,* 2013). Cinnamon essential oil is composed of small quantity of volatile compounds, other than cinnamaldehyde, such as eugenol. Eugenol was also recorded to have antiviral property against Influenza A virus (Dai *et al.,* 2013) and HSV (Dai *et al.,* 2013). Studies on both cinnamaldehyde and hydrodistilled essential oil of cinnamon bark showed significant inhibitory effect on H1N1 influenza virus proliferation. It was concluded that these compounds induced expression of IFN-B in MDCK cells, which is a key antiviral cytokine, by stimulating the TLR-7 and IRAK-4 pathway (Liu *et al.,* 2015). In contrast, the natural occurring trans-cinnamaldehyde from essential oils has low antiviral property alone against viruses but can increase its efficacy when synthesized with derivatives and carrier (Goswami and Rahman, 2010; Li

et al., 2017). Therefore, the moderate anti-PRRSV replication or considerable viral induction may be implied with the principal component cinnamaldehyde or synergistic cascade reactions by both cinnamaldehyde and other minute components of cinnamon essential oil. The results indicate that cinnamaldehyde-derived benzimidazole has anti-PRRSV activity, and rather it enhances PRRSV replication in both pre-virus and post-virus entry studies. Similar results have been reported showing benzimidazole derivatives with no selective antiviral activity against selected RNA and DNA viruses including HIV-1, BVDVM YFV, DENV-2, WNV, HBV, and HCV (Budow *et al.,* 2009). In line with this, various benzimidazole derivatives such as 5-methoxy-l-methylbenzimidazole and 5-methyl-2-n-ribobenzimidazole enhances viral multiplication (Tamm, 1973).

Cinnamaldehdye itself has been recorded to have antiviral activity on positively stranded RNA virus. It increases the survival rate of mice with coxsackievirus B3 (CVB3)-induced viral myocarditis (VMC) because viral titer reduction (Ding *et al.,* 2010). When applied in-vivo, cinnamaldehyde decreased CVB3 mRNA levels in virally infected cardiomyocytes. In terms of pro-inflammatory cytokine expression having an essential role on the susceptible environment for positively RNA replication, cinnamaldehyde inhibits TNF-α and IL-1β (Zhang *et al.,* 2012). On the other hand, cinnamaldehyde inhibited RNA virus (influenza A/PR/8) in madin darby canine kidney cells revealed by RT-PCR and SDS-PAGE analyses. It showed that cinnamaldehyde inhibited viral protein synthesis at the post-transcriptional level of influenza virus A/PR/8 (Hayashi *et al.,* 2007. However, cinnamon oil and cinnamaldehyde significantly increased the IFN-B secretion of virus infected MDCK cells. Cinnamaldehyde and cinnamon essential oil could significantly improve the expression levels of TLR7 and IRAK-4 in RNA virus infected cell, while showed no impacts on the expression level of TLR3 and TRAF-3 mRNA. This indicated that the mechanism against RNA virus was related to the high expression of IFN-B, which stimulated the TLR7 signal pathway and improving the expression of essential protein IRAK-4. IRAK-4 expression was one of the key ways to increase IFN-B expression induced by cinnamaldehyde and volatile oil. Since there were many subtypes of TLRS, further researches were still needed on whether cinnamaldehyde and volatile oil affected the adjustments of other pathways, whether essential oil

reduced the TLR3 mRNA expression of infected MDCK cells, and whether other chemical components in volatile played the function of negative feedback (Li *et al.,* 2015). These existing records may suggest that cinnamaldehyde and cinnamon oil targeted the RNA virus like PRRSV directly, enhanced the antiviral cytokine expression or obstructed the pro-PRRSV cytokines.

Condensation of *o*-phenylenediamine and 3-phenyl-2-propenal (cinnamaldehyde) yields cinnamaldehyde-derived benzimidazole (Scheme 2). There is no recorded anti-PRRSV evaluation report of 2-(2-phenylethenyl)-1H-benzimidazole on MARC-145 cells. However, many antiviral reports of 2-substituted benzimidazole products from the condensation reaction of *o*-phenelenediamine and aldehydes were recorded (Tonelli *et al.,* 2010; Tonelli *et al.,* 2014). The chemical structure activity relationship (SAR) study between the synthesized compound and its pharmacological/pharmacokinetic activity is substantial. Numerous structures of benzimidazole derivatives resulted to their various therapeutic functionalities and their potent modulation that may imply to antiviral specificity (Tonelli *et al.,* 2014). Depending on the functional group position, the antiviral mechanism can be varied, such as the synthesized benzimidazole by Boido *et al.,* (2009), acting against RSV and HCV. Another instance of study on series of non-halogenated, brominated, chlorinated benzimidazole derivatives have no significant antiviral activity against selected positive-sensed RNA viruses, i.e. HIV-1, BVDV, YFV, and WNV (Budow *et al.,* 2009). Contrary to PRRSV enhancement result of 2-(2-phenylethenyl)-1H-benzimidazole, Pan *et al.* (2015) found that 2-substituted benzimidazole with *N,N*-dialkyl amine, *m*-methoxy and *p*-hydroxyl derivatives showed significant anti-HIV-1 replication whereas derivative *N,N*-dialkyl amine is required for anti-HIV-1. A set of derived benzimidazole compounds were tested against HCV and inclusion of methyl group in the ring system showed significant anti-HCV property (Tsay *et al.,* 2013). It was also reported that pyridine at the C2 of benzimidazole ring have the most observed antiviral activity against coxsackie virus and echo virus (Starčević *et al.,* 2007). These evidences recognized that the structural position or functional group substituted to the benzimidazole ring system may attribute to the enhancement or suppression of virus mechanism. In this case, benzimidazole derivative synthesized from *o*-phenelenediamine and cinnamon oil's

cinnamaldehyde that affords the proposed compound (2-(2-phenylethenyl)-1H-benzimidazole) induced the viral replication and its 2-substituted structure may imply the enhancement of PRRSV proliferation in MARC-145 cells. Aside from this, the enhancing activity of derived benzimidazole may activated the overexpression of proviral cytokine interleukin (e.g. IL-10), may suppressed the type IFN 1 network system or activate other significant signaling pathway to permit PRRSV to replicate well than expected. These assumptions were drawn but needs to be clarified in the future research.

CHAPTER 5

SUMMARY, CONCLUSION AND RECOMMENDATION

This study evaluated the antiviral potential of cinnamon essential oil from two *Cinnamomum* species, i.e. *C. iners* and *C. burmannii* and cinnamaldehyde-derived benzimidazole (2-(2-phenylethenyl)-1H-benzimidazole) against PRRSV grown in MARC-145 cells. Essential oil was obtained by hydrodistillation while benzimidazole derivative was synthesized by the condensation of *o*-phenylenediamine and cinnamon essential oil mainly comprised with 3-phenyl-2-propenal (cinnamaldehyde). The test compounds were subjected to TLC, FT-IR, cell viability tests. Assessment of anti-PRRSV activity was conducted by CPE and PFU assay with concentration of samples that are non-cytotoxic to the cell line. Results showed that cinnamon essential oil from *C. iners* have moderate to low antiviral property with 0% to 22% viral titer reduction on pre-treatment infection while cinnamon oil from *C. burmannii* also exhibited modest PRRSV titer reduction with 51% inhibition. Contrasting result was obtained with the two synthesized benzimidazole derivatives. The cinnamaldehyde- derived benzimidazole (2-(2- phenylethenyl) - 1H- benzimidazole) enhanced the PRRSV replication on MARC-145 cells in which viral titer was increased up to 62 % after pre-treatment infection and 17% after post treatment infection. Formation of plaques by the virus was also not reduced by the derived-benzimidazole while cinnamon essential oil reduced the plaque formation up to 42%.

In conclusion, cinnamon essential oil has average anti-PRRSV replication. The low to moderate potency of cinnamon essential oil may be interrelated with its chief compound cinnamaldehyde, and/or the synergistic or cascade effect of its various chemical compounds. On the other hand, cinnamaldehyde-derived benzimidazole (2-(2-phenylethenyl)-1H-benzimidazole) induced the in vitro viral replication mechanism of PRRSV on MARC-145 cells. Such enhancing property was decreased when tested in serially 10-fold diluted benzimidazole derivative. The enhancing property was replicable in three independent experiments. Further investigation is required to elucidate the PRRSV enhancing effect of this synthesized derivative. Studies on

synthesizing cinnamaldehyde structure have to be carried out to enhance the efficacy of this organic compound on its anti-PRRSV properties. Nonetheless, our findings suggest that cinnamaldehyde-derived benzimidazole has limited potential for anti-PRRSV purpose.

REFERENCES

Adinew, B. 2014. GC-MS and FT-IR analysis of constituents of essential oil from Cinnamon bark growing in South-west of Ethiopia. **International Journal of Herbal Medicine**, 1(6), 22-31.

Alaqeel, S. I. 2017. Synthetic approaches to benzimidazoles from o-phenylenediamine: A literature review. **Journal of Saudi Chemical Society**, 21(2), 229-237.

Allan, G., McNeilly, F., Kennedy, S., Meehan, B., Ellis, J. & Krakowka, S. 2000. Immunostimulation, PCV-2 [porcine circovirus] and PMWS [porcine wasting syndrome]. **Veterinary Record**, 147(6), 170-171.

Allende, R., Lewis, T., Lu, Z., Rock, D., Kutish, G., Ali, A., Doster, A. & Osorio, F. 1999. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. **Journal of General Virology**, 80(2), 307-315.

Amari, M., Fodili, M., Nedjar‐Kolli, B., Hoffmann, A. P. & PÉriÉ, J. 2002. Reactivity studies on 4‐aminopyrones: Access to benzimidazole and benzimidazolone derivatives. **Journal of heterocyclic chemistry**, 39(4), 811-816.

Ansari, K. & Lal, C. 2009. Synthesis, physicochemical properties and antimicrobial activity of some new benzimidazole derivatives. **European Journal of Medicinal Chemistry**, 44(10), 4028-4033.

Asadipour, A., Edraki, N., Nakhjiri, M., Yahya-Meymandi, A., Alipour, E., Saniee, P., Siavoshi, F., Shafiee, A. & Foroumadi, A. 2013. Anti-Helicobacter pylori activity and Structure-Activity Relationship study of 2-Alkylthio-5-(nitroaryl)-1, 3, 4-thiadiazole Derivatives. **Iranian journal of pharmaceutical research: IJPR**, 12(3), 281-287.

Awang, A. F. I. B., Susanti, D. & Taher, M. 2013. Antimicrobial activity and synergic effect of Cinnamomum burmannii's essential oil & its isolated compound

(cinnamaldehyde). p. 26-29. In **International Conference on Chemical, Agricultural and Medical Sciences (CAMS-2013)**. Kuala Lumpur, Malaysia.

Bansal, Y. & Silakari, O. 2012. The therapeutic journey of benzimidazoles: a review. **Bioorganic & medicinal chemistry**, 20(21), 6208-6236.

Baruah, A., Nath, S. C. & Hazarika, A. K. 2001. Stem bark oil of Cinnamomum iners Reinw. **Indian Perfumer**, 45(4), 261-263.

Beaulieu, P. L., Bös, M., Bousquet, Y., Fazal, G., Gauthier, J., Gillard, J., Goulet, S., LaPlante, S., Poupart, M.-A. & Lefebvre, S. 2004. Non-nucleoside inhibitors of the hepatitis C virus NS5B polymerase: discovery and preliminary SAR of benzimidazole derivatives. **Bioorganic & medicinal chemistry letters**, 14(1), 119-124.

Benfield, D. A., Nelson, E., Collins, J. E., Harris, L., Goyal, S. M., Robison, D., Christianson, W. T., Morrison, R. B., Gorcyca, D. & Chladek, D. 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). **Journal of Veterinary Diagnostic Investigation**, 4(2), 127-133.

Berridge, M. V., Herst, P. M. & Tan, A. S. 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. **Biotechnology annual review**, 11(127-152.

Bloemraad, M., de Kluijver, E. P., Petersen, A., Burkhardt, G. E. & Wensvoort, G. 1994. Porcine reproductive and respiratory syndrome: temperature and pH stability of Lelystad virus and its survival in tissue specimens from viraemic pigs. **Veterinary Microbiology**, 42(4), 361-371.

Boido, V., Paglietti, G., Tonelli, M. & Vitale, G. 2009. Non-nucleoside benzimidazoles as antiviral drugs against HCV and RSV infections. p. 41-93. In **RNA-Viruses. Enzymatic and Receptoral Inhibitors (Ed. A. Carta)**. Kerala: Research Signpost.

Bousbia, N., Vian, M. A., Ferhat, M. A., Petitcolas, E., Meklati, B. Y. & Chemat, F. 2009. Comparison of two isolation methods for essential oil from rosemary leaves: Hydrodistillation and microwave hydrodiffusion and gravity. **Food Chemistry**, 114(1), 355-362.

Brar, M. S., Shi, M., Murtaugh, M. P. & Leung, F. C.-C. 2015. Evolutionary diversification of type 2 porcine reproductive and respiratory syndrome virus. **Journal of General Virology**, 96(7), 1570-1580.

Brockmeier, S. L., Halbur, P. G. & Thacker, E. L. (2002). **Porcine respiratory disease complex, In: Polymicrobial Diseases** (pp. 231–258.). Washington, DC: ASM Press.

Brockmeier, S. L., Palmer, M. V. & Bolin, S. R. 2000. Effects of intranasal inoculation of porcine reproductive and respiratory syndrome virus, Bordetella bronchiseptica, or a combination of both organisms in pigs. **American journal of veterinary research**, 61(8), 892-899.

Budow, S., Kozlowska, M., Gorska, A., Kazimierczuk, Z., Eickmeier, H., La Colla, P., Gosselin, G. & Seela, F. 2009. Substituted benzimidazoles: antiviral activity and synthesis of nucleosides. **ARKIVOC: Online Journal of Organic Chemistry**, 3), 225-220.

Burlando, B., Verotta, L., Cornara, L. & Bottini-Massa, E. 2010. **Herbal principles in cosmetics: Properties and mechanisms of action**. CRC Press.

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. **International journal of food microbiology**, 94(3), 223-253.

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G. & Welch, S.-K. W. 2007. CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. **Journal of virology**, 81(14), 7371-7379.

Camacho, S., Michlig, S., de Senarclens-Bezençon, C., Meylan, J., Meystre, J., Pezzoli, M., Markram, H. & Le Coutre, J. 2015. Anti-obesity and anti-hyperglycemic effects of cinnamaldehyde via altered ghrelin secretion and functional impact on food intake and gastric emptying. **Scientific reports**, 5(7919), 1-10.

Cavanagh, D. 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. **Arch. Virol.**, 142(3), 629-633.

Cha, S.-H., Choi, E.-J., Park, J.-H., Yoon, S.-R., Song, J.-Y., Kwon, J.-H., Song, H.-J. & Yoon, K.-J. 2006. Molecular characterization of recent Korean porcine reproductive

and respiratory syndrome (PRRS) viruses and comparison to other Asian PRRS viruses. **Veterinary microbiology**, 117(2-4), 248-257.

Chakrabarty, M., Karmakar, S., Mukherji, A., Arima, S. & Harigaya, Y. 2006. Application of Sulfamic Acid as an Eco-Friendly Catalyst in an Expedient Synthesis of Benzimidazoles. **ChemInform**, 37(40).

Chand, R. J., Trible, B. R. & Rowland, R. R. 2012. Pathogenesis of porcine reproductive and respiratory syndrome virus. **Current opinion in virology**, 2(3), 256-263.

Cho, J. G., Dee, S. A., Deen, J., Trincado, C., Fano, E., Jiang, Y., Faaberg, K., Murtaugh, M. P., Guedes, A. & Collins, J. E. 2006. The impact of animal age, bacterial coinfection, and isolate pathogenicity on the shedding of porcine reproductive and respiratory syndrome virus in aerosols from experimentally infected pigs. **Canadian journal of veterinary research**, 70(4), 297-301.

Cho, J. G., Deen, J. & Dee, S. A. 2007. Influence of isolate pathogenicity on the aerosol transmission of Porcine reproductive and respiratory syndrome virus. **Canadian journal of veterinary research**, 71(1), 23-27.

Christianson, W. T., Choi, C.-S., Collins, J. E., Molitor, T. W., Morrison, R. B. & Joo, H.-S. 1993. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. **Canadian Journal of Veterinary Research**, 57(4), 262-268.

Christopher-Hennings, J., Nelson, E. A., Althouse, G. C. & Lunney, J. 2008. Comparative antiviral and proviral factors in semen and vaccines for preventing viral dissemination from the male reproductive tract and semen. **Animal health research reviews**, 9(1), 59-69.

Christopher-Hennings, J., Nelson, E. A., Hines, R. J., Nelson, J. K., Swenson, S. L., Zimmerman, J. J., Chase, C. C., Yaeger, M. J. & Benfield, D. A. 1995a. Persistence of porcine reproductive and respiratory syndrome virus in serum and semen of adult boars. **Journal of Veterinary Diagnostic Investigation**, 7(4), 456-464.

Christopher-Hennings, J., Nelson, E. A., Nelson, J. K., Hines, R. J., Swenson, S. L., Hill, H. T., Zimmerman, J. J., Katz, J. B., Yaeger, M. J. & Chase, C. 1995b. Detection of

porcine reproductive and respiratory syndrome virus in boar semen by PCR. **Journal of clinical microbiology**, 33(7), 1730-1734.

Chuaychu, S., Banchonglikitkul, C., Kajsongkram, T., Kawaree, R. & W., C. 2013. Anti-viral potential of Rhinacanthus nasutus (L.) Kurz crude extracts on porcine reproductive and respiratory syndrome virus infection in MARC-145 cells. **Journal of Agricultural Research & Extension**, 30(1), 33-43.

Conzelmann, K.-K., Visser, N., Van Woensel, P. & Thiel, H.-J. 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. **Virology**, 193(1), 329-339.

Dai, J.-P., Zhao, X.-F., Zeng, J., Wan, Q.-Y., Yang, J.-C., Li, W.-Z., Chen, X.-X., Wang, G.-F. & Li, K.-S. 2013. Drug screening for autophagy inhibitors based on the dissociation of Beclin1-Bcl2 complex using BiFC technique and mechanism of eugenol on anti-influenza A virus activity. **PLoS One**, 8(4), e61026.

Das, P. B., Dinh, P. X., Ansari, I. H., De Lima, M., Osorio, F. A. & Pattnaik, A. K. 2010. The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact with the receptor CD163. **Journal of virology**, 84(4), 1731-1740.

Dee, S., Otake, S. & Deen, J. 2011. An evaluation of ultraviolet light (UV254) as a means to inactivate porcine reproductive and respiratory syndrome virus on common farm surfaces and materials. **Veterinary microbiology**, 150(1-2), 96-99.

Delputte, P., Costers, S. & Nauwynck, H. 2005. Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. **Journal of general virology**, 86(5), 1441-1445.

Ding, Y., Wang, S., Qiu, L., Zhao, G. & Xu, J. 2010. Influence of cinnamaldehyde on viral myocarditis in mice. **The American journal of the medical sciences**, 340(2), 114-120.

Du, T., Nan, Y., Xiao, S., Zhao, Q. & Zhou, E.-M. 2017. Antiviral strategies against PRRSV infection. **Trends in microbiology**, 25(12), 968-979.

Duan, X., Nauwynck, H. & Pensaert, M. 1997. Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). **Veterinary microbiology**, 56(1-2), 9-19.

Faaberg, K., Balasuriya, U., Brinton, M., Gorbalenya, A., Leung, F., Nauwynck, H., Snijder, E., Stadejek, T., Yang, H. & Yoo, D. (2012). Family arteriviridae. In **Virus taxonomy. Ninth report of the international committee on taxonomy of viruses** (pp. 796-805). Amsterdam: Elsevier Academic Press.

Fabra, M., Castro-Mayorga, J., Randazzo, W., Lagarón, J., López-Rubio, A., Aznar, R. & Sánchez, G. 2016. Efficacy of cinnamaldehyde against enteric viruses and its activity after incorporation into biodegradable multilayer systems of interest in food packaging. **Food and environmental virology**, 8(2), 125-132.

Fang, Y. & Snijder, E. J. 2010. The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. **Virus research**, 154(1-2), 61-76.

Fang, Y., Treffers, E. E., Li, Y., Tas, A., Sun, Z., Van Der Meer, Y., De Ru, A. H., Van Veelen, P. A., Atkins, J. F. & Snijder, E. J. 2012. Efficient– 2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. **Proceedings of the National Academy of Sciences of the United States of America**, 109(43), E2920-E2928.

Feoktistova, M., Geserick, P. & Leverkus, M. 2016. Crystal violet assay for determining viability of cultured cells. **Cold Spring Harbor Protocols**, 2016(4), 0873-0879.

Firth, A. E., Zevenhoven-Dobbe, J. C., Wills, N. M., Go, Y. Y., Balasuriya, U. B., Atkins, J. F., Snijder, E. J. & Posthuma, C. C. 2011. Discovery of a small arterivirus gene that overlaps the GP5 coding sequence and is important for virus production. **Journal of General Virology**, 92(5), 1097-1106.

Food and Drug Administration. 2015 Department of Health and Human Services. Code of Federal Regulations part 182: Substances Generally Recognized as Safe, Sec. 182.20 Essential oils, oleoresins (solvent-free), and natural extractives (including distillates),

Forsberg, R. 2005. Divergence time of porcine reproductive and respiratory syndrome virus subtypes. **Molecular biology and evolution**, 22(11), 2131-2134.

Forsberg, R., Storgaard, T., Nielsen, H. S., Oleksiewicz, M. B., Cordioli, P., Sala, G., Hein, J. & Bøtner, A. 2002. The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. **Virology**, 299(1), 38-47.

Friedman, M., Kozukue, N. & Harden, L. A. 2000. Cinnamaldehyde content in foods determined by gas chromatography– mass spectrometry. **Journal of Agricultural and Food Chemistry**, 48(11), 5702-5709.

Frydas, I. S., Trus, I., Kvisgaard, L. K., Bonckaert, C., Reddy, V. R., Li, Y., Larsen, L. E. & Nauwynck, H. J. 2015. Different clinical, virological, serological and tissue tropism outcomes of two new and one old Belgian type 1 subtype 1 porcine reproductive and respiratory virus (PRRSV) isolates. **Veterinary research**, 46(1), 37-54.

Frydas, I. S., Verbeeck, M., Cao, J. & Nauwynck, H. J. 2013. Replication characteristics of porcine reproductive and respiratory syndrome virus (PRRSV) European subtype 1 (Lelystad) and subtype 3 (Lena) strains in nasal mucosa and cells of the monocytic lineage: indications for the use of new receptors of PRRSV (Lena). **Veterinary research**, 44(1), 73-86.

Frydman-Marom, A., Levin, A., Farfara, D., Benromano, T., Scherzer-Attali, R., Peled, S., Vassar, R., Segal, D., Gazit, E. & Frenkel, D. 2011. Orally administrated cinnamon extract reduces β-amyloid oligomerization and corrects cognitive impairment in Alzheimer's disease animal models. **PloS one**, 6(1), e16564.

Gaba, M., Singh, D., Singh, S., Sharma, V. & Gaba, P. 2010. Synthesis and pharmacological evaluation of novel 5-substituted-1-(phenylsulfonyl)-2-methylbenzimidazole derivatives as anti-inflammatory and analgesic agents. **European journal of medicinal chemistry**, 45(6), 2245-2249.

Gende, L. B., Floris, I., Fritz, R. & Eguaras, M. J. 2008. Antimicrobial activity of cinnamon (Cinnamomum zeylanicum) essential oil and its main components against Paenibacillus larvae from Argentine. **Bulletin of insectology**, 61(1), 1-4.

Geng, S., Cui, Z., Huang, X., Chen, Y., Xu, D. & Xiong, P. 2011. Variations in essential oil yield and composition during Cinnamomum cassia bark growth. **Industrial crops and products**, 33(1), 248-252.

Gilbert, S., Larochelle, R., Magar, R., Cho, H. & Deregt, D. 1997. Typing of porcine reproductive and respiratory syndrome viruses by a multiplex PCR assay. **Journal of clinical microbiology**, 35(1), 264-267.

Gogoi, P. & Konwar, D. 2006. An efficient and one-pot synthesis of imidazolines and benzimidazoles via anaerobic oxidation of carbon–nitrogen bonds in water. **Tetrahedron Letters**, 47(1), 79-82.

Goswami, A. & Rahman, A. 2010. Antiviral activity of (E)-cinnamaldehyde revisited with nanoscience tools. **Nature Precedings**, Available from http://hdl.handle.net/10101/npre.2010.5043.1(

Goyal, S. M. 1993. Porcine reproductive and respiratory syndrome. **Journal of Veterinary Diagnostic Investigation**, 5(4), 656-664.

Grivennikov, S. I., Greten, F. R. & Karin, M. 2010. Immunity, inflammation, and cancer. **Cell**, 140(6), 883-899.

Halbur, P., Miller, L., Paul, P., Meng, X.-J., Huffman, E. & Andrews, J. 1995a. Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. **Veterinary pathology**, 32(2), 200-204.

Halbur, P., Paul, P., Frey, M., Landgraf, J., Eernisse, K., Meng, X.-J., Lum, M., Andrews, J. & Rathje, J. 1995b. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. **Veterinary pathology**, 32(6), 648-660.

Halbur, P. G., Paul, P. S., Meng, X.-J., Lum, M. A., Andrews, J. J. & Rathje, J. A. 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. **Journal of Veterinary Diagnostic Investigation**, 8(1), 11-20.

Han, J., Wang, Y. & Faaberg, K. S. 2006. Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. **Virus research**, 122(1-2), 175-182.

Han, M. & Yoo, D. 2014. Engineering the PRRS virus genome: Updates and perspectives. **Veterinary microbiology**, 174(3-4), 279-295.

Heng, P. F. (2008). Characterization of essential oils from Cinnamomum Iners. Universiti Malaysia Sabah.

Hussain, A. I., Anwar, F., Chatha, S. A. S., Jabbar, A., Mahboob, S. & Nigam, P. S. 2010. Rosmarinus officinalis essential oil: antiproliferative, antioxidant and antibacterial activities. **Brazilian Journal of Microbiology**, 41(4), 1070-1078.

Jaafarpour, M., Hatefi, M., Khani, A. & Khajavikhan, J. 2015. Comparative effect of cinnamon and Ibuprofen for treatment of primary dysmenorrhea: a randomized double-blind clinical trial. **Journal of clinical and diagnostic research: JCDR**, 9(4), QC04.

Jantan, I. b., Karim Moharam, B. A., Santhanam, J. & Jamal, J. A. 2008. Correlation between chemical composition and antifungal activity of the essential oils of eight Cinnamomum. species. **Pharmaceutical Biology**, 46(6), 406-412.

Johnson, C. R., Griggs, T. F., Gnanandarajah, J. & Murtaugh, M. P. 2011. Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. **Journal of General Virology**, 92(5), 1107-1116.

Jumbo, L. O. V., Faroni, L. R., Oliveira, E. E., Pimentel, M. A. & Silva, G. N. 2014. Potential use of clove and cinnamon essential oils to control the bean weevil, Acanthoscelides obtectus Say, in small storage units. **Industrial Crops and Products**, 56(0), 27-34.

Kankeaw, U. & Masong, E. 2015. The Antioxidant Activity from Hydroquinone Derivatives by the Synthesis of Cinnamomium Verum J. Presl Bark's Extracted. **International Journal of Chemical Engineering and Applications**, 6(2), 91.

Kankeaw, U. & Rawanna, R. 2015. The Study of Antibacterial Activity of Benzimidazole Derivative Synthesized from Citronellal. **International Journal of Bioscience, Biochemistry and Bioinformatics**, 5(5), 280.

Karniychuk, U. U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T. A. & Nauwynck, H. J. 2010. Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. **BMC veterinary research**, 6(1), 30-40.

Kaskatepe, B., Kiymaci, M. E., Suzuk, S., Erdem, S. A., Cesur, S. & Yildiz, S. 2016. Antibacterial effects of cinnamon oil against carbapenem resistant nosocomial Acinetobacter baumannii and Pseudomonas aeruginosa isolates. **Industrial Crops and Products**, 81(0), 191-194.

Keffaber, K. 1989. Reproductive failure of unknown etiol-ogy. **Am. Assoc. Swine Pract. Newsl**, 1(0), 1-9.

Kim, Y.-G., Lee, J.-H., Kim, S.-I., Baek, K.-H. & Lee, J. 2015. Cinnamon bark oil and its components inhibit biofilm formation and toxin production. **International journal of food microbiology**, 195(0), 30-39.

Kittawornrat, A., Prickett, J., Chittick, W., Wang, C., Engle, M., Johnson, J., Patnayak, D., Schwartz, T., Whitney, D. & Olsen, C. 2010. Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: will oral fluid replace serum for PRRSV surveillance? **Virus research**, 154(1-2), 170-176.

Ko, J. H., Nguyen, P.-L., Ahn, J.-Y., Yoon, H., Min, J., Lee, L., Cho, S.-J., Sekhon, S. S. & Kim, Y.-H. 2015. The global research trend of porcine reproductive and respiratory syndrome virus (PRRSV): A mini review. **Toxicology and Environmental Health Sciences**, 7(5), 241-250.

Koh, W., Yoon, S., Kwon, B., Jeong, T., Nam, K. & Han, M. 1998. Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation. **International journal of immunopharmacology**, 20(11), 643-660.

Köhler, P. 2001. The biochemical basis of anthelmintic action and resistance. 31(4), 336-345.

Kong, J.-O., Lee, S.-M., Moon, Y.-S., Lee, S.-G. & Ahn, Y.-J. 2007. Nematicidal activity of cassia and cinnamon oil compounds and related compounds toward Bursaphelenchus xylophilus (Nematoda: Parasitaphelenchidae). **Journal of nematology**, 39(1), 31-36.

Kuhn, J. H., Lauck, M., Bailey, A. L., Shchetinin, A. M., Vishnevskaya, T. V., Bào, Y., Ng, T. F. F., LeBreton, M., Schneider, B. S. & Gillis, A. 2016. Reorganization and expansion of the nidoviral family Arteriviridae. **Archives of virology**, 161(3), 755-768.

Larochelle, R. & Magar, R. 1997. Detection of porcine reproductive and respiratory syndrome virus in paraffin-embedded tissues: comparison of immunohistochemistry and in situ hybridization. **Journal of virological methods**, 63(1-2), 227-235.

Lawson, S. R., Rossow, K. D., Collins, J. E., Benfield, D. A. & Rowland, R. R. 1997. Porcine reproductive and respiratory syndrome virus infection of gnotobiotic pigs: sites of virus replication and co-localization with MAC-387 staining at 21 days post-infection. **Virus research**, 51(2), 105-113.

Le Gall, A., Legeay, O., Bourhy, H., Arnauld, C., Albina, E. & Jestin, A. 1998. Molecular variation in the nucleoprotein gene (ORF7) of the porcine reproductive and respiratory syndrome virus (PRRSV). **Virus research**, 54(1), 9-21.

Lee, S. H., Lee, S. Y., Son, D. J., Lee, H., Yoo, H. S., Song, S., Oh, K. W., Han, D. C., Kwon, B. M. & Hong, J. T. 2005. Inhibitory effect of 2′-hydroxycinnamaldehyde on nitric oxide production through inhibition of NF-**K**B activation in RAW 264.7 cells. **Biochemical pharmacology**, 69(5), 791-799.

Li, X.-Q., Liu, X.-X., Wang, X.-Y., Xie, Y.-H., Yang, Q., Liu, X.-X., Ding, Y.-Y., Cao, W. & Wang, S.-W. 2017. Cinnamaldehyde Derivatives Inhibit Coxsackievirus B3-Induced Viral Myocarditis. **Biomolecules & therapeutics**, 25(3), 279-287.

Li, Y.-q., Kong, D.-x. & Wu, H. 2013. Analysis and evaluation of essential oil components of cinnamon barks using GC–MS and FTIR spectroscopy. **Industrial Crops and Products**, 41(0), 269-278.

Li, Y., Treffers, E. E., Napthine, S., Tas, A., Zhu, L., Sun, Z., Bell, S., Mark, B. L., van Veelen, P. A. & van Hemert, M. J. 2014. Transactivation of programmed ribosomal frameshifting by a viral protein. **Proceedings of the National Academy of Sciences**, 111(21), 2172-2181.

Liu, R., He, T., Zeng, N., Chen, T., Gou, L. & Liu, J. 2015. Mechanism of anti-influenza virus of cinnamaldehyde and volatile oils from Ramulus cinnamomi. **Medicinal Plant**, 6(1/2), 4-8.

Lunney, J. K., Fang, Y., Ladinig, A., Chen, N., Li, Y., Rowland, B. & Renukaradhya, G. J. 2016. Porcine reproductive and respiratory syndrome virus (PRRSV): pathogenesis and interaction with the immune system. **Annual review of animal biosciences**, 4(0), 129-154.

Mengeling, W., Lager, K. & Vorwald, A. 1998. Clinical consequences of exposing pregnant gilts to strains of porcine reproductive and respiratory syndrome (PRRS) virus isolated from field cases of" atypical" PRRS. **American journal of veterinary research**, 59(12), 1540-1544.

Mengeling, W., Vorwald, A., Lager, K. & Brockmeier, S. 1996. Comparison among strains of porcine reproductive and respiratory syndrome virus for their ability to cause reproductive failure. **American journal of veterinary research**, 57(6), 834-839.

Morgan, S., Graham, S., Salguero, F., Cordon, P. S., Mokhtar, H., Rebel, J., Weesendorp, E., Bodman-Smith, K., Steinbach, F. & Frossard, J. 2013. Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. **Veterinary microbiology**, 163(1-2), 13-22.

Moussa, Z., El-Sharief, M. A. S. & Abbas, S. Y. 2016. New imidazolidineiminothione derivatives: Synthesis, spectral characterization and evaluation of antitumor, antiviral, antibacterial and antifungal activities. **European journal of medicinal chemistry**, 122(0), 419-428.

Muhammad, J. S., Zaidi, S. F., Shaharyar, S., Refaat, A., Usmanghani, K., Saiki, I. & Sugiyama, T. 2015. Anti-inflammatory effect of cinnamaldehyde in Helicobacter pylori induced gastric inflammation. **Biological and Pharmaceutical Bulletin**, 38(1), 109-115.

Murtaugh, M. P., Stadejek, T., Abrahante, J. E., Lam, T. T. & Leung, F. C.-C. 2010. The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. **Virus research**, 154(1-2), 18-30.

Naik, P., Murumkar, P., Giridhar, R. & Yadav, M. R. 2010. Angiotensin II receptor type 1 (AT 1) selective nonpeptidic antagonists—A perspective. **Bioorganic & medicinal chemistry**, 18(24), 8418-8456.

Nauwynck, H., Duan, X., Favoreel, H., Van Oostveldt, P. & Pensaert, M. 1999. Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis. **Journal of General Virology**, 80(2), 297-305.

Nauwynck, H., Van Gorp, H., Vanhee, M., Karniychuk, U., Geldhof, M., Cao, A., Verbeeck, M. & Van Breedam, W. 2012. Micro-Dissecting the Pathogenesis and Immune Response of PRRSV Infection Paves the Way for More Efficient PRRSV Vaccines. **Transboundary and emerging diseases**, 59(s1), 50-54.

Naveena, B., Muthukumar, M., Sen, A., Praveen Kumar, Y. & Kiran, M. 2014. Use of cinnamaldehyde as a potential antioxidant in ground spent hen meat. **Journal of food processing and preservation**, 38(4), 1911-1917.

Nielsen, T. L., Nielsen, J., Have, P., Bækbo, P., Hoff-Jørgensen, R. & Bøtner, A. 1997. Examination of virus shedding in semen from vaccinated and from previously infected boars after experimental challenge with porcine reproductive and respiratory syndrome virus. **Veterinary microbiology**, 54(2), 101-112.

O'Neill, M., Smith, A., Heckelman, P. & Budavari, S. (Eds.). (2001). **The Merck Index** (Vol. 1257). Whitehouse Station, NJ: Merck& Co, US.

Oh, M. 2011. The acaricidal and repellent effect of cinnamon essential oil against house dust mite. **World Acad Sci Eng Technol**, 60(0), 710-714.

Ojeda-Sana, A. M., van Baren, C. M., Elechosa, M. A., Juárez, M. A. & Moreno, S. 2013. New insights into antibacterial and antioxidant activities of rosemary essential oils and their main components. **Food Control**, 31(1), 189-195.

Ooi, L. S., Li, Y., Kam, S.-L., Wang, H., Wong, E. Y. & Ooi, V. E. 2006. Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb Cinnamomum cassia Blume. **The American journal of Chinese medicine**, 34(03), 511-522.

Ouattara, B., Simard, R. E., Holley, R. A., Piette, G. J.-P. & Bégin, A. 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. **International journal of food microbiology**, 37(2-3), 155-162.

Pan, T., He, X., Chen, B., Chen, H., Geng, G., Luo, H., Zhang, H. & Bai, C. 2015. Development of benzimidazole derivatives to inhibit HIV-1 replication through protecting APOBEC3G protein. **European journal of medicinal chemistry**, 95(0), 500-513.

Park, J. Y., Kim, H. S. & Seo, S. H. 2008. Characterization of interaction between porcine reproductive and respiratory syndrome virus and porcine dendritic cells. **J Microbiol Biotechnol**, 18(10), 1709-1716.

Pasternak, A. O., Spaan, W. J. & Snijder, E. J. 2006. Nidovirus transcription: how to make sense...? **Journal of general virology**, 87(6), 1403-1421.

Pavia, D. L., Lampman, G. M., Kriz, G. S. & Vyvyan, J. A. 2014. **Introduction to spectroscopy**. Cengage Learning.

Pol, J., Van Dijk, J., Wensvoort, G. & Terpstra, C. 1991. Pathological, ultrastructural, and immunohistochemical changes caused by Lelystad virus in experimentally induced infections of mystery swine disease (synonym: porcine epidemic abortion and respiratory syndrome (PEARS)). **Veterinary Quarterly**, 13(3), 137-143.

Prickett, J., Simer, R., Christopher-Hennings, J., Yoon, K.-J., Evans, R. B. & Zimmerman, J. J. 2008. Detection of Porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: a longitudinal study under experimental conditions. **Journal of Veterinary Diagnostic Investigation**, 20(2), 156-163.

Quent, V. M., Loessner, D., Friis, T., Reichert, J. C. & Hutmacher, D. W. 2010. Discrepancies between metabolic activity and DNA content as tool to assess cell proliferation in cancer research. **Journal of cellular and molecular medicine**, 14(4), 1003-1013.

Rana, V. S., Devi, C. B., Verdeguer, M. & Blázquez, M. A. 2009. Variation of Terpenoids Constituents in Natural Population of Cinnamomum tamala (L.) Leaves. **Journal of Essential Oil Research**, 21(6), 531-534.

Rathod, C., Rajurkar, R. & Thonte, S. 2013. Benzimidazole synthesis and biological evaluation: A review. **Indo Am. J. Pharm. Res**, 3(2), 2323-2329.

Rossow, K. 1998. Porcine reproductive and respiratory syndrome. **Veterinary pathology**, 35(1), 1-20.

Rossow, K., Collins, J., Goyal, S., Nelson, E., Christopher-Hennings, J. & Benfield, D. 1995. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. **Veterinary pathology**, 32(4), 361-373.

Rossow, K. D., Bautista, E. M., Goyal, S. M., Molitor, T. W., Murtaugh, M. P., Morrison, R. B., Benfield, D. A. & Collins, J. E. 1994. Experimental porcine reproductive and respiratory syndrome virus infection in one-, four-, and 10-week-old pigs. **Journal of Veterinary Diagnostic Investigation**, 6(1), 3-12.

Rowland, R. R., Lawson, S., Rossow, K. & Benfield, D. A. 2003. Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. **Veterinary microbiology**, 96(3), 219-235.

Saberi, A. 2015. Efficient synthesis of Benzimidazoles using zeolite, alumina and silica gel under microwave irradiation. **Iranian Journal of Science and Technology (Sciences)**, 39(1), 7-10.

Shan, B., Cai, Y.-Z., Brooks, J. D. & Corke, H. 2007. The in vitro antibacterial activity of dietary spice and medicinal herb extracts. **International Journal of food microbiology**, 117(1), 112-119.

Shareef, A. A. 2011. Evaluation of antibacterial activity of essential oils of Cinnamomum sp. and Boswellia sp. **J. Basrah Researches (Sciences)**, 37(5), 60-71.

Shirai, J., Kanno, T., Tsuchiya, Y., Mitsubayashi, S. & Seki, R. 2000. Effects of chlorine, iodine, and quaternary ammonium compound disinfectants on several exotic disease viruses. **Journal of Veterinary Medical Science**, 62(1), 85-92.

Singh, G., Maurya, S. & Catalan, C. A. 2007. A comparison of chemical, antioxidant and antimicrobial studies of cinnamon leaf and bark volatile oils, oleoresins and their constituents. **Food and chemical toxicology**, 45(9), 1650-1661.

Śliwka, L., Wiktorska, K., Suchocki, P., Milczarek, M., Mielczarek, S., Lubelska, K., Cierpiał, T., Łyżwa, P., Kiełbasiński, P. & Jaromin, A. 2016. The comparison of MTT and CVS assays for the assessment of anticancer agent interactions. **PloS one**, 11(5), e0155772.

Snijder, E. J., Kikkert, M. & Fang, Y. 2013. Arterivirus molecular biology and pathogenesis. **Journal of General Virology**, 94(10), 2141-2163.

Snijder, E. J. & Meulenberg, J. 1998. The molecular biology of arteriviruses. **Journal of general virology**, 79(5), 961-979.

Solano, G. I., Segalés, J., Collins, J. E., Molitor, T. W. & Pijoan, C. 1997. Porcine reproductive and respiratory syndrome virus (PRRSv) interaction with Haemophilus parasuis. **Veterinary microbiology**, 55(1-4), 247-257.

Sondhi, S. M., Rani, R., Singh, J., Roy, P., Agrawal, S. & Saxena, A. 2010. Solvent free synthesis, anti-inflammatory and anticancer activity evaluation of tricyclic and tetracyclic benzimidazole derivatives. **Bioorganic & medicinal chemistry letters**, 20(7), 2306-2310.

Spilman, M. S., Welbon, C., Nelson, E. & Dokland, T. 2009. Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. **Journal of general virology**, 90(3), 527-535.

Srisukh, V., Tribuddharat, C., Nukoolkarn, V., Bunyapraphatsara, N., Chokephaibulkit, K., Phoomniyom, S., Chuanphung, S. & Srifuenfung, S. 2012. Antibacterial activity of essential oils from Citrus hystrix (makrut lime) against respiratory tract pathogens. **Science Asia**, 38(2), 212-217.

Stadejek, T., Larsen, L. E., Podgórska, K., Bøtner, A., Botti, S., Dolka, I., Fabisiak, M., Heegaard, P. M., Hjulsager, C. K. & Huć, T. 2017. Pathogenicity of three genetically diverse strains of PRRSV Type 1 in specific pathogen free pigs. **Veterinary microbiology**, 209(0), 13-19.

Stadejek, T., Oleksiewicz, M., Potapchuk, D. & Podgorska, K. 2006. Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes. **Journal of general virology**, 87(7), 1835-1841.

Stadejek, T., Oleksiewicz, M. B., Scherbakov, A. V., Timina, A. M., Krabbe, J. S., Chabros, K. & Potapchuk, D. 2008. Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. **Archives of virology**, 153(8), 1479-1488.

Stadejek, T., Stankevicius, A., Murtaugh, M. P. & Oleksiewicz, M. B. 2013. Molecular evolution of PRRSV in Europe: current state of play. **Veterinary microbiology**, 165(1-2), 21-28.

Starčević, K., Kralj, M., Ester, K., Sabol, I., Grce, M., Pavelić, K. & Karminski-Zamola, G. 2007. Synthesis, antiviral and antitumor activity of 2-substituted-5-amidino-benzimidazoles. **Bioorganic & medicinal chemistry**, 15(13), 4419-4426.

Stevenson, G. & Torremorell, M. 2012. Porcine reproductive and respiratory syndrome virus (porcine arterivirus). **Diseases of Swine, 10th ed.; Zimmerman, JJ, Karriker, LA, Ramirez, A., Schwartz, KJ, Stevenson, GW, Eds**, 461-486.

Stockert, J. C., Horobin, R. W., Colombo, L. L. & Blázquez-Castro, A. 2018. Tetrazolium salts and formazan products in cell biology: viability assessment, fluorescence imaging, and labeling perspectives. **Acta histochemica**, 120(0), 159-167.

Suárez, P., Zardoya, R., Martín, M. J., Prieto, C., Dopazo, J., Solana, A. & Castro, J. 1996. Phylogenetic relationships of European strains of porcine reproductive and respiratory syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. **Virus research**, 42(1-2), 159-165.

Sun, N., Zhao, X., Bai, X.-Y., Niu, L., Song, M.-Q., Sun, Y.-G., Jiang, J.-B. & Li, H.-Q. 2012. Anti-PRRSV effect and mechanism of sodium tanshinone IIA sulfonate in vitro. **Journal of Asian natural products research**, 14(8), 721-728.

Swenson, S. L., Hill, H. T., Zimmerman, J., Evans, L. E., Landgraf, J. G., Wills, R., Sanderson, T., McGinley, M., Brevik, A. & Ciszewski, D. 1994. Excretion of porcine reproductive and respiratory syndrome (PRRS) virus in semen following experimental infection of boars. **Porcine reproductive and respiratory syndrome virus and the experimentally infected boar**, 22.

Tamm, I. 1973. Benzimidazole derivatives: new enhancers of influenza virus multiplication. **Journal of Experimental Medicine**, 138(4), 858-874.

Terpstra, C., Wensvoort, G. & Pol, J. 1991. Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad vims: Koch's postulates fulfilled. **Veterinary Quarterly**, 13(3), 131-136.

Thacker, E. L., Halbur, P. G., Ross, R. F., Thanawongnuwech, R. & Thacker, B. J. 1999. Mycoplasma hyopneumoniae potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. **Journal of Clinical Microbiology**, 37(3), 620-627.

Thirabunyanon, M., Boonprasom, P. & Niamsup, P. 2009. Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells. **Biotechnology letters**, 31(4), 571-576.

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D. & Tian, X. 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. **PloS one**, 2(6), e526.

Tonelli, M., Novelli, F., Tasso, B., Vazzana, I., Sparatore, A., Boido, V., Sparatore, F., La Colla, P., Sanna, G. & Giliberti, G. 2014. Antiviral activity of benzimidazole derivatives. III. Novel anti-CVB-5, anti-RSV and anti-Sb-1 agents. **Bioorganic & medicinal chemistry**, 22(17), 4893-4909.

Tonelli, M., Simone, M., Tasso, B., Novelli, F., Boido, V., Sparatore, F., Paglietti, G., Pricl, S., Giliberti, G. & Blois, S. 2010. Antiviral activity of benzimidazole derivatives. II. Antiviral activity of 2-phenylbenzimidazole derivatives. **Bioorganic & medicinal chemistry**, 18(8), 2937-2953.

Tsay, S.-C., Hwu, J. R., Singha, R., Huang, W.-C., Chang, Y. H., Hsu, M.-H., Shieh, F.-k., Lin, C.-C., Hwang, K. C. & Horng, J.-C. 2013. Coumarins hinged directly on benzimidazoles and their ribofuranosides to inhibit hepatitis C virus. **European journal of medicinal chemistry**, 63(0), 290-298.

Tung, Y.-T., Chua, M.-T., Wang, S.-Y. & Chang, S.-T. 2008. Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (Cinnamomum osmophloeum) twigs. **Bioresource technology**, 99(9), 3908-3913.

Unlu, M., Ergene, E., Unlu, G. V., Zeytinoglu, H. S. & Vural, N. 2010. Composition, antimicrobial activity and in vitro cytotoxicity of essential oil from Cinnamomum zeylanicum Blume (Lauraceae). **Food and Chemical Toxicology**, 48(11), 3274-3280.

Valdez-Padilla, D., Rodríguez-Morales, S., Hernández-Campos, A., Hernández-Luis, F., Yépez-Mulia, L., Tapia-Contreras, A. & Castillo, R. 2009. Synthesis and antiprotozoal activity of novel 1-methylbenzimidazole derivatives. **Bioorganic & Medicinal Chemistry**, 17(4), 1724-1730.

Van Alstine, W., Kanitz, C. & Stevenson, G. 1993. Time and temperature survivability of PRRS virus in serum and tissues. **Journal of Veterinary Diagnostic Investigation**, 5(0), 621-621.

Van Breedam, W., Delputte, P. L., Van Gorp, H., Misinzo, G., Vanderheijden, N., Duan, X. & Nauwynck, H. J. 2010a. Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. **Journal of General Virology**, 91(7), 1659-1667.

Van Breedam, W., Van Gorp, H., Zhang, J. Q., Crocker, P. R., Delputte, P. L. & Nauwynck, H. J. 2010b. The M/GP5 glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. **PLoS pathogens**, 6(1), e1000730.

Van Dinten, L., Wassenaar, A., Gorbalenya, A. E., Spaan, W. & Snijder, E. J. 1996. Processing of the equine arteritis virus replicase ORF1b protein: identification of cleavage products containing the putative viral polymerase and helicase domains. **Journal of virology**, 70(10), 6625-6633.

Van Gorp, H., Van Breedam, W., Delputte, P. & Nauwynck, H. 2009. The porcine reproductive and respiratory syndrome virus requires trafficking through CD163-positive early endosomes, but not late endosomes, for productive infection. **Archives of virology**, 154(12), 1939-1943.

Van Gorp, H., Van Breedam, W., Delputte, P. L. & Nauwynck, H. J. 2008. Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. **Journal of General Virology**, 89(12), 2943-2953.

Van Reeth, K., Nauwynck, H. & Pensaert, M. 1996. Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study. **Veterinary microbiology**, 48(3-4), 325-335.

Vanderheijden, N., Delputte, P. L., Favoreel, H. W., Vandekerckhove, J., Van Damme, J., van Woensel, P. A. & Nauwynck, H. J. 2003. Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. **Journal of virology**, 77(15), 8207-8215.

Vangalapati, M. & Prakash, D. S. 2013. In-vitro anti-cancer studies of Cinnamaldehyde on breast cancer cell line (MCF-7). **BioTechnology: An Indian Journal**, 7(3), 81-84.

Verheije, M., Welting, T., Jansen, H., Rottier, P. & Meulenberg, J. 2002. Chimeric arteriviruses generated by swapping of the M protein ectodomain rule out a role of this domain in viral targeting. **Virology**, 303(2), 364-373.

Wagstrom, E. A., Chang, C.-C., Yoon, K.-J. & Zimmerman, J. J. 2001. Shedding of porcine reproductive and respiratory syndrome virus in mammary gland secretions of sows. **American journal of veterinary research**, 62(12), 1876-1880.

Wang, P., Henning, S. M. & Heber, D. 2010. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. **PloS one**, 5(4), e10202.

Wang, R., Wang, R. & Yang, B. 2009. Extraction of essential oils from five cinnamon leaves and identification of their volatile compound compositions. **Innovative Food Science & Emerging Technologies**, 10(2), 289-292.

Weesendorp, E., Morgan, S., Stockhofe-Zurwieden, N., Popma-De Graaf, D. J., Graham, S. P. & Rebel, J. M. 2013. Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. **Veterinary microbiology**, 163(1-2), 1-12.

Weesendorp, E., Rebel, J. M., Popma-De Graaf, D. J., Fijten, H. P. & Stockhofe-Zurwieden, N. 2014. Lung pathogenicity of European genotype 3 strain porcine

reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains. **Veterinary microbiology**, 174(1-2), 127-138.

Wensvoort, G., de Kluyver, E. P., Luijtze, E. A., den Besten, A., Harris, L., Collins, J. E., Christianson, W. T. & Chladek, D. 1992. Antigenic comparison of Lelystad virus and swine infertility and respiratory syndrome (SIRS) virus. **Journal of Veterinary Diagnostic Investigation**, 4(2), 134-138.

Wensvoort, G., Terpstra, C., Pol, J., Ter Laak, E., Bloemraad, M., De Kluyver, E., Kragten, C., Van Buiten, L. d., Den Besten, A. & Wagenaar, F. 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. **Veterinary Quarterly**, 13(3), 121-130.

Wills, R., Zimmerman, J., Yoon, K.-J., Swenson, S., McGinley, M., Hill, H., Platt, K., Christopher-Hennings, J. & Nelson, E. 1997a. Porcine reproductive and respiratory syndrome virus: a persistent infection. **Veterinary microbiology**, 55(1-4), 231-240.

Wills, R. W., Zimmerman, J. J., Yoon, K.-J., Swenson, S. L., Hoffman, L. J., McGinley, M. J., Hill, H. T. & Platt, K. B. 1997b. Porcine reproductive and respiratory syndrome virus: routes of excretion. **Veterinary microbiology**, 57(1), 69-81.

Wissink, E., Kroese, M., Van Wijk, H., Rijsewijk, F., Meulenberg, J. & Rottier, P. 2005. Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. **Journal of virology**, 79(19), 12495-12506.

Wong, S. Y., Grant, I. R., Friedman, M., Elliott, C. T. & Situ, C. 2008. Antibacterial activities of naturally occurring compounds against Mycobacterium avium subsp. paratuberculosis. **Applied and environmental microbiology**, 74(19), 5986-5990.

Wong, Y., Ahmad-Mudzaqqir, M. & Wan-Nurdiyana, W. 2014. Extraction of essential oil from cinnamon (Cinnamomum zeylanicum). **Oriental journal of chemistry**, 30(1), 37-47.

Wuu-Kuang, S. 2011. Taxonomic revision of Cinnamomum (Lauraceae) in Borneo. **Blumea-Biodiversity, Evolution and Biogeography of Plants**, 56(3), 241-264.

Xing, F., Hua, H., Selvaraj, J. N., Zhao, Y., Zhou, L., Liu, X. & Liu, Y. 2014. Growth inhibition and morphological alterations of Fusarium verticillioides by cinnamon oil and cinnamaldehyde. **Food Control**, 46(0), 343-350.

Yaeger, M. J., Prieve, T., Collins, J., Christopher-Hennings, J., Nelson, E. & Benfield, D. 1993. Evidence for the transmission of porcine reproductive and respiratory syndrome (PRRS) virus in boar semen. **Swine Health Prod**, 1(5), 7-9.

Yossa, N., Patel, J., Macarisin, D., Millner, P., Murphy, C., Bauchan, G. & Lo, Y. M. 2014. Antibacterial activity of cinnamaldehyde and sporan against Escherichia coli O157: H7 and Salmonella. **Journal of food processing and preservation**, 38(3), 749-757.

Yuan, J., Dieter, M., Bucher, J. & Jameson, C. 1992. Toxicokinetics of cinnamaldehyde in F344 rats. **Food and chemical toxicology**, 30(12), 997-1004.

Zhang, Y., Cao, W., Xie, Y.-H., Yang, Q., Li, X.-Q., Liu, X.-X. & Wang, S.-W. 2012. The comparison of α-bromo-4-chlorocinnamaldehyde and cinnamaldehyde on coxsackie virus B3-induced myocarditis and their mechanisms. **International immunopharmacology**, 14(1), 107-113.

Zhang, Y., Liu, X., Wang, Y., Jiang, P. & Quek, S. 2016. Antibacterial activity and mechanism of cinnamon essential oil against Escherichia coli and Staphylococcus aureus. **Food Control**, 59(0), 282-289.

Zhao, L., Lee, J. Y. & Hwang, D. H. 2011. Inhibition of pattern recognition receptor-mediated inflammation by bioactive phytochemicals. **Nutrition reviews**, 69(6), 310-320.

Zhou, Y., Yang, X., Wang, H.-N., Zhang, A., Zhang, Z., Kang, R., Zeng, F. & Li, H. 2012. Molecular characterization of a complete genome and 12 Nsp2 genes of PRRSV of southwestern China. **Food and environmental virology**, 4(3), 102-114.

APPENDICES

Appendix A. Tables of data

Appendix Table 1 Plaque reduction of cinnamon essential oils on MARC-145 cells

Extract	Conc. (nL/mL)	Virus Entry	# Plaques	last Dilution factor	Virus added (ml)	pfu/ml	# of plaques of control (corresponding dilution factor)	% Inhibition
Cinnamon oil 1 (from *C. iners*)	4	PRE	16.5	1.00E-03	0.5	8.25E+03	15	-9.0909091
		POST	11	1.00E-04	0.5	5.50E+04	3	-72.727273
	0.4	PRE	6.5	1.00E-04	0.5	3.25E+04	3	-53.846154
		POST	17	1.00E-03	0.5	8.50E+03	10	-41.176471
	0.04	PRE	10.5	1.00E-03	0.5	5.25E+03	15	42.857143
		POST	21.5	1.00E-03	0.5	1.08E+04	10	-53.488372
Cinnamon oil 2 (from *C. burmannii*)	2	PRE	13.5	1.00E-03	0.5	6.75E+03	15	11.111111
		POST	10	1.00E-04	0.5	5.00E+04	3	-70
	0.2	PRE	15.5	1.00E-03	0.5	7.75E+03	15	-3.2258065
		POST	18.5	1.00E-03	0.5	9.25E+03	10	-45.945946
	0.02	PRE	11.5	1.00E-04	0.5	5.75E+04	3	-73.913043
		POST	10	1.00E-04	0.5	5.00E+04	3	-70

Appendix Table 2 Plaque reduction of cinnamaldehyde-derived benzimidazoles on MARC-145 cells

Extract	Conc. (ng/ml)	Virus Entry	# Plaques	last Dilution factor	Virus added (ml)	pfu/ml	# of plaques of control (corresponding dilution factor)	% inhibition
Benzimidaole 1	312.5	PRE	28.5	1.00E-03	0.5	1.43E+04	10	-64.912281
		POST	17.5	1.00E-03	0.5	8.75E+03	3	-82.857143
	31.25	PRE	10	1.00E-04	0.5	5.00E+04	3	-70
		POST	17	1.00E-04	0.5	8.50E+04	3	-82.352941
	3.125	PRE	16	1.00E-04	0.5	8.00E+04	3	-81.25
		POST	4.5	1.00E-04	0.5	2.25E+04	3	-33.333333
Benzimidazole 2	156	PRE	12	1.00E-04	0.5	6.00E+04	3	-75
		POST	14	1.00E-03	0.5	7.00E+03	3	-78.571429
	15.6	PRE	12.5	1.00E-05	0.5	6.25E+05	0	-100

Appendix Table 3 Tissue culture infection dose on MARC-145 cells treated with cinnamon essential oil

Extract	Concentration (nL/ml)	Virus Entry	%CPE above 50%	%CPE below 50%	50%	Proportionate Distance (PD)	neg log of last dilution above 50 %	per 0.1 ml infection dose	10 Log $TCID_{50}$/ml
Cinnamon oil 1 (from *C. iners*)	4	PRE	75	0	50	0.333333	3	3.333333	4.333333
		POST	75	0	50	0.333333	3	3.333333	4.333333
	0.4	PRE	100	25	50	0.666667	3	3.666667	4.666667
		POST	100	25	50	0.666667	2	2.666667	3.666667
	0.04	PRE	100	0	50	0.5	4	4.5	5.5
		POST	75	25	50	0.5	3	3.5	4.5
Cinnamon oil 2 (from *C. burmannii*)	0.2	PRE	75	0	50	0.333333	3	3.333333	4.333333
		POST	100	25	50	0.666667	2	2.666667	3.666667
	0.02	PRE	75	0	50	0.333333	4	4.333333	5.333333
		POST	75	0	50	0.333333	7	7.333333	8.333333
		PRE	75	0	50	0.333333	4	4.333333	5.333333
		POST	100	25	50	0.666667	4	4.666667	5.666667

Appendix Table 4 Tissue culture infection dose on MARC-145 cells treated with cinnamaldehyde-derived benzimidazole

Extract	Concentration (nL/ml)	Virus Entry	%CPE above 50%	%CPE below 50%	50%	Proportionate Distance (PD)	neg log of last dilution above 50 %	per 0.1 ml infection dose	10 Log $TCID_{50}$/ml
Benzimidazole 1	312.5 nl/ml	PRE	100	0	50	0.5	3	3.5	**4.5**
		POST	100	0	50	0.5	3	3.5	**4.5**
	31.25 nl/ml	PRE	75	0	50	0.333333	3	3.333333	**4.333333**
		POST	75	25	50	0.5	4	4.5	**5.5**
	3.125 nl/ml	PRE	100	0	50	0.5	3	3.5	**4.5**
		POST	75	0	50	0.333333	4	4.333333	**5.333333**
Benzimidazole 2	156 nl/ml	PRE	75	0	50	0.333333	6	6.333333	**7.333333**
		POST	100	25	50	0.666667	2	2.666667	**3.666667**
	15.6 nl/ml	PRE	100	25	50	0.666667	4	4.666667	**5.666667**
		POST	75	0	50	0.333333	3	3.333333	**4.333333**
	1.56 nl/ml	PRE	100	0	50	0.5	3	3.5	**4.5**
		POST	100	25	50	0.666667	2	2.666667	3.666667

Appendix Table 6 Percentage inhibition of cinnamon essential oil

EXTRACT	Conc. (nl/ml)	Pre-infection		Post-infection	
		log TCID/ml	% Inhibition	log TCID/ml	% Inhibition
Cinnamon oil 1 (from *C. iners*)	4	4.3	21.81818	4.3	41.09589
	0.4	4.6	16.36364	3.6	50.68493
	0.04	5.5	0	4.5	38.35616
Cinnamon oil 2 (from *C. burmannii*)	2	5	9.090909	3.6	50.68493
	0.2	5.3	3.636364	8.3	-13.6986
	2	5.3	3.636364	5.6	23.28767
Control		5.5		7.3	

Appendix Table 5 Percentage inhibition of cinnamaldehyde derived benzimidazole

EXTRACT	Conc. (nl/ml)	Pre-infection		Post-infection	
		log TCID/ml	% Inhibition	log TCID/ml	% Inhibition
Benzimidazole 1	312.5	4.5	0	4.5	0
	31.25	4.3	4.444444	5.5	-22.2222
	3.125	4.5	0	5.3	-17.7778
Benzimidazole 2	156	7.3	-62.2222	3.6	20
	15.6	5.6	-24.4444	4.3	4.444444
	1.56	4.5	0	4.5	0
Control		4.5		7.3	

Appendix Table 7 OD absorbance raw data of cinnamon essential oil by CV assay

Sample	Conc. (ul/ml)											
	1	**0.5**	**0.25**	**0.125**	**0.0625**	**0.03125**	**0.015625**	**0.007813**	**0.003906**	**0.001953**	**0.000977**	**negative control**
Cinnamon oil 1 (from *C. iners*)	0.165	0.130	0.146	0.133	0.131	0.129	0.333	0.688	0.730	0.772	0.821	0.890
	0.151	0.144	0.150	0.149	0.153	0.135	0.495	0.725	0.747	0.807	0.760	0.818
	0.153	0.161	0.155	0.181	0.122	0.143	0.501	0.682	0.749	0.742	0.753	0.768
	0.168	0.150	0.171	0.147	0.141	0.161	0.530	0.665	0.690	0.763	0.718	0.792
Cinnamon oil 2 (from *C. burmannii*)	0.127	0.114	0.139	0.152	0.124	0.131	0.465	0.597	0.501	0.733	0.593	0.690
	0.145	0.136	0.132	0.132	0.132	0.132	0.593	0.618	0.632	0.702	0.669	0.727
	0.163	0.138	0.147	0.125	0.142	0.133	0.554	0.725	0.731	0.653	0.770	0.580
	0.183	0.151	0.148	0.140	0.150	0.133	0.546	0.703	0.734	0.666	0.569	0.778

Appendix Table 8 OD absorbance raw data of cinnamaldehyde-derived benzimidazole by CV assay

Sample	Conc. (ug/ml)											
	10	**5**	**2.5**	**1.25**	**0.625**	**0.3125**	**0.15625**	**0.078125**	**0.039063**	**0.019531**	**0.009766**	**negative control**
Benzimidazole 1	0.165	0.130	0.146	0.133	0.131	0.129	0.333	0.688	0.730	0.772	0.821	0.890
	0.151	0.144	0.150	0.149	0.153	0.135	0.495	0.725	0.747	0.807	0.760	0.818
	0.153	0.161	0.155	0.181	0.122	0.143	0.501	0.682	0.749	0.742	0.753	0.768
	0.168	0.150	0.171	0.147	0.141	0.161	0.530	0.665	0.690	0.763	0.718	0.792
Benzimidazole 2	0.127	0.114	0.139	0.152	0.124	0.131	0.465	0.597	0.501	0.733	0.593	0.690
	0.145	0.136	0.132	0.132	0.132	0.132	0.593	0.618	0.632	0.702	0.669	0.727
	0.163	0.138	0.147	0.125	0.142	0.133	0.554	0.725	0.731	0.653	0.770	0.580
	0.183	0.151	0.148	0.140	0.150	0.133	0.546	0.703	0.734	0.666	0.569	0.778

Appendix Table 9 OD absorbance raw data of cinnamon essential oil by MTT assay

Sample	Conc. (ul/ml)											
	1	**0.5**	**0.25**	**0.125**	**0.0625**	**0.03125**	**0.015625**	**0.007813**	**0.003906**	**0.001953**	**0.000977**	**negative control**
Cinnamon oil 1 (from *C. iners*)	0.431	0.415	0.355	0.355	0.367	0.342	0.360	0.468	0.410	0.526	0.530	0.459
	0.370	0.387	0.356	0.393	0.338	0.397	0.396	0.468	0.453	0.356	0.376	0.429
	0.406	0.617	0.389	0.367	0.379	0.396	0.393	0.438	0.432	0.442	0.431	0.437
	0.358	0.312	0.382	0.398	0.374	0.450	0.389	0.352	0.449	0.483	0.404	0.481
Cinnamon oil 2 (from *C. burmannii*)	0.378	0.401	0.399	0.405	0.407	0.416	0.402	0.480	0.480	0.475	0.457	0.486
	0.389	0.403	0.373	0.353	0.342	0.356	0.373	0.438	0.461	0.459	0.463	0.497
	0.386	0.424	0.429	0.386	0.322	0.419	0.351	0.439	0.439	0.471	0.451	0.470
	0.401	0.423	0.419	0.426	0.442	0.442	0.435	0.492	0.517	0.500	0.483	0.510

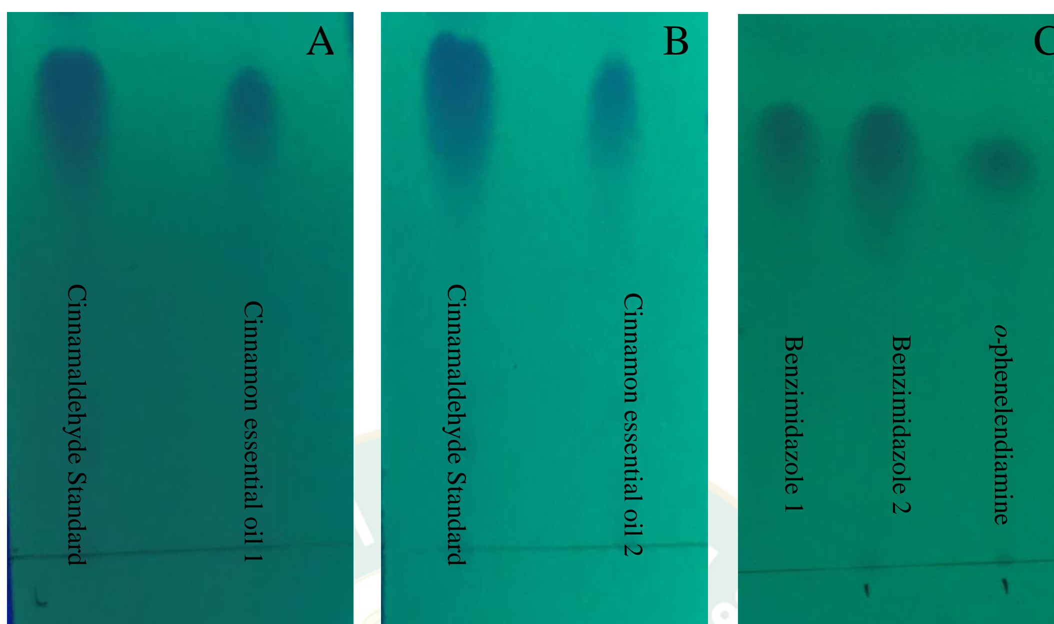
Appendix Table 10 OD absorbance raw data of cinnamaldehyde-derived benzimidazole by MTT assay

Sample	Conc. (ug/ml)											
	10	**5**	**2.5**	**1.25**	**0.625**	**0.3125**	**0.15625**	**0.078125**	**0.039063**	**0.019531**	**0.009766**	**negative control**
Benzimidazole 1	0.489	0.363	0.460	0.469	0.433	0.487	0.460	0.492	0.462	0.486	0.462	0.495
	0.397	0.390	0.433	0.452	0.448	0.458	0.466	0.469	0.448	0.425	0.415	0.468
	0.423	0.405	0.461	0.455	0.426	0.467	0.427	0.449	0.408	0.437	0.411	0.478
	0.377	0.363	0.443	0.417	0.391	0.418	0.382	0.429	0.367	0.441	0.416	0.431
Benzimidazole 2	0.408	0.410	0.415	0.405	0.386	0.374	0.375	0.394	0.410	0.359	0.401	0.443
	0.395	0.354	0.378	0.399	0.412	0.412	0.429	0.391	0.406	0.396	0.354	0.410
	0.381	0.381	0.407	0.409	0.421	0.436	0.424	0.385	0.410	0.413	0.360	0.392
	0.483	0.449	0.451	0.489	0.439	0.469	0.512	0.466	0.457	0.485	0.454	0.465

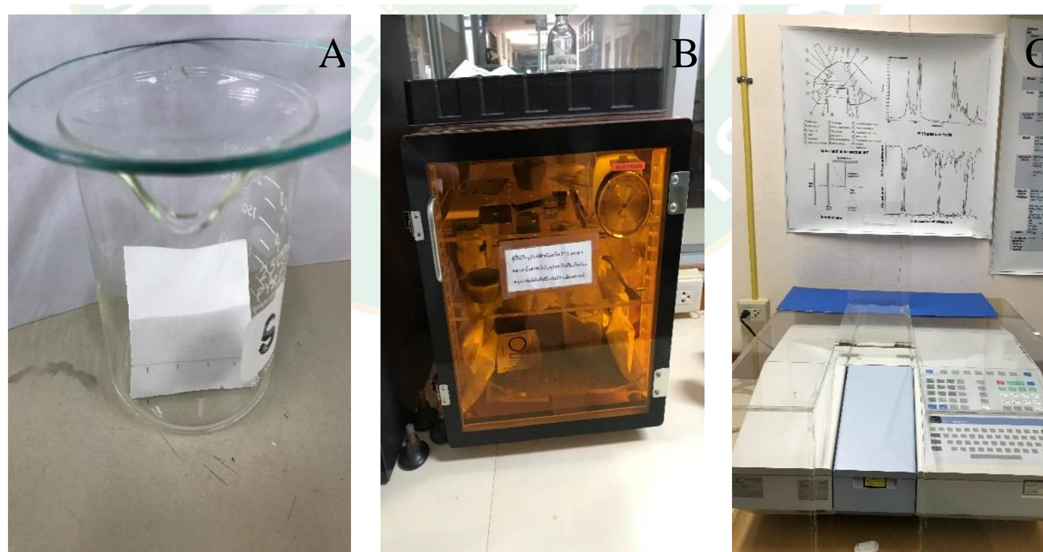
Appendix B. Figures

Appendix figure 2 Chemical Compound Isolation: A. Sample cinnamon bark: B. Simple hydrodistillation setup; C. Distillate product containing cinnamaldehyde; D. Separation of Cinnamaldehyde; E. Rotary evaporation of excess solvent; F. Reflux set up

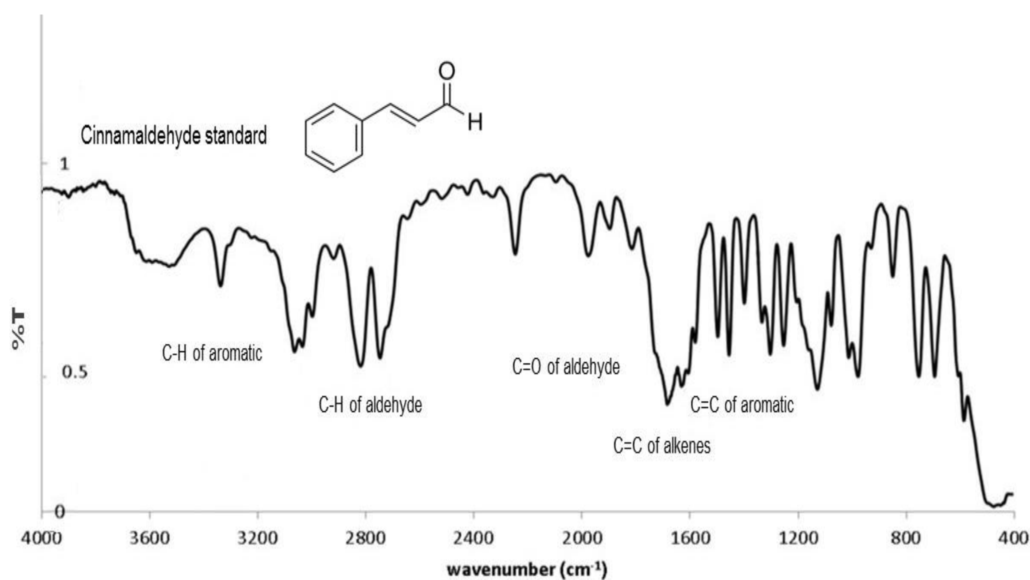
Appendix figure 1 Product Sample A. Cinnamon essential oil; B. Cinnamaldehyde-derived benzimidazole



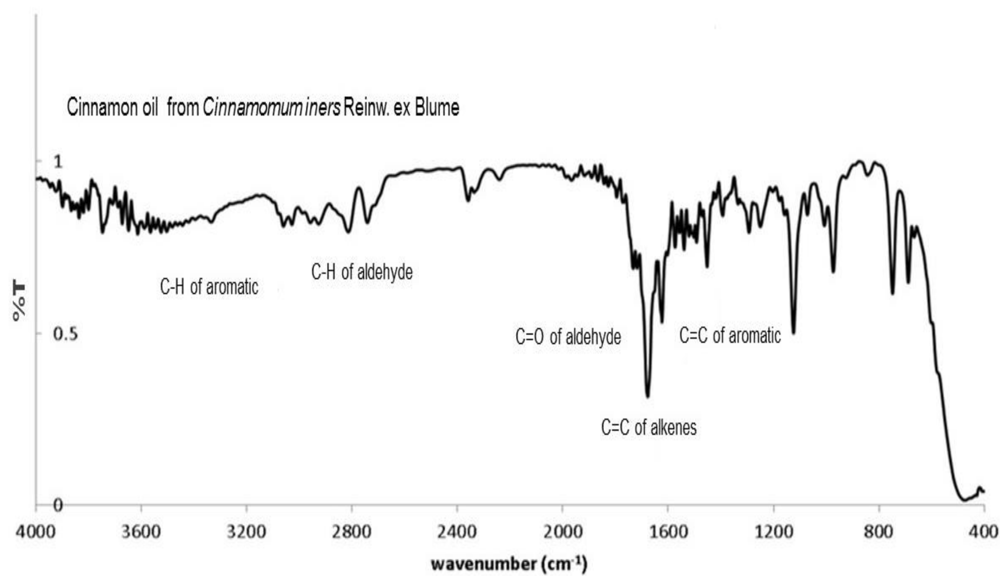
Appendix figure 4 Visible chromatogram of test samples on TLC plate under the UV light. A. Cinnamon essential oil chromatogram from *C. iners*; B. Cinnamon essential oil chromatogram from *C. burmannii*; C. Chromatogram of benzimidazole derivatives synthesized from *C. iners* (Benzimidazole 1) and *C. burmannii* (Benzimidazole 2)



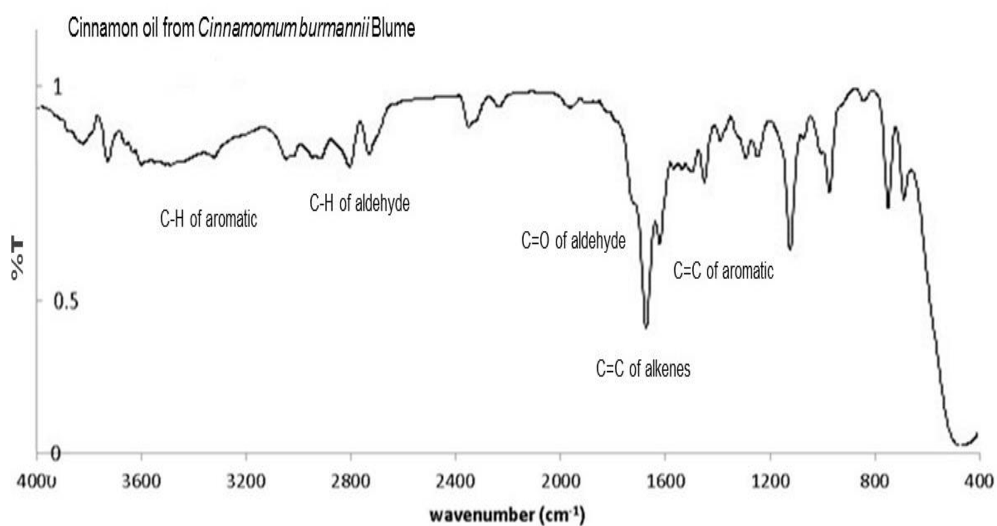
Appendix figure 3 TLC and FT-IR. A. TLC chamber; B. Laboratory equipment in desiccator chamber used for FT-IR spectroscopy C. Actual FT-IR machine used in the study



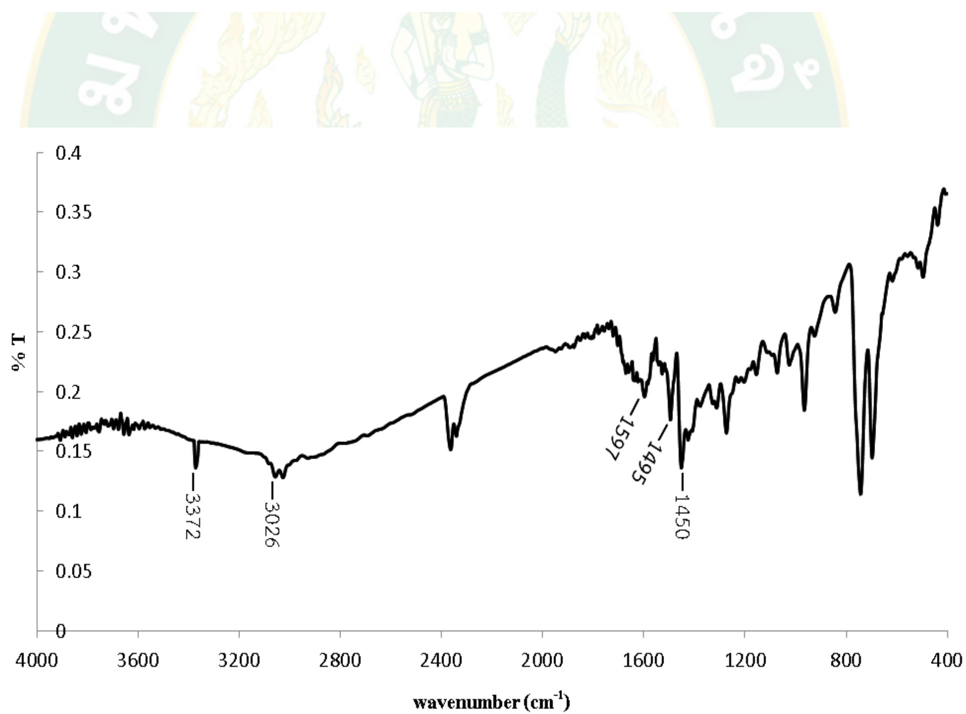
Appendix figure 5 FT-IR spectrum of cinnamaldehyde reference



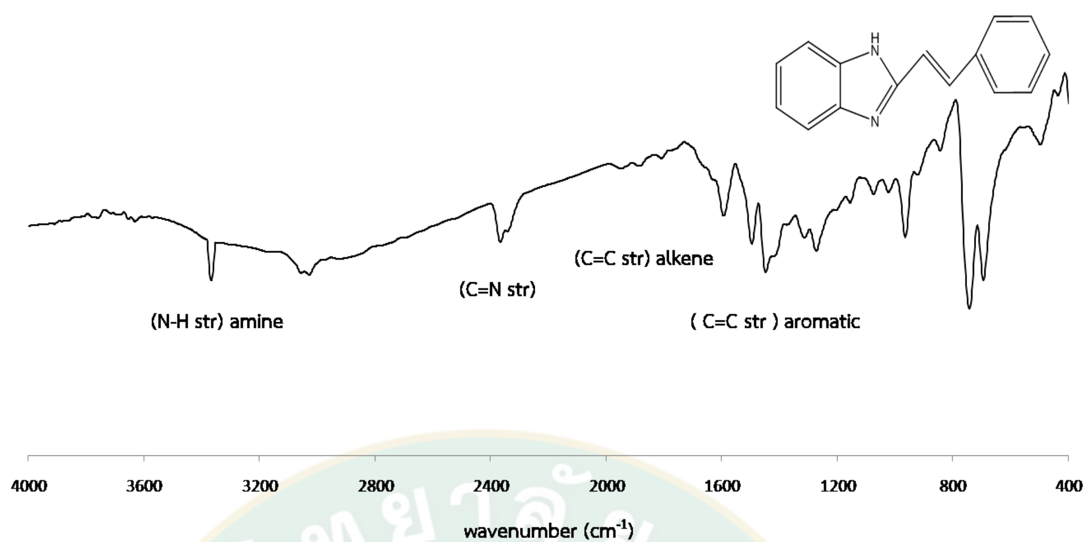
Appendix figure 6 FT-IR spectrum of cinnamon essential oil from *C. iners*



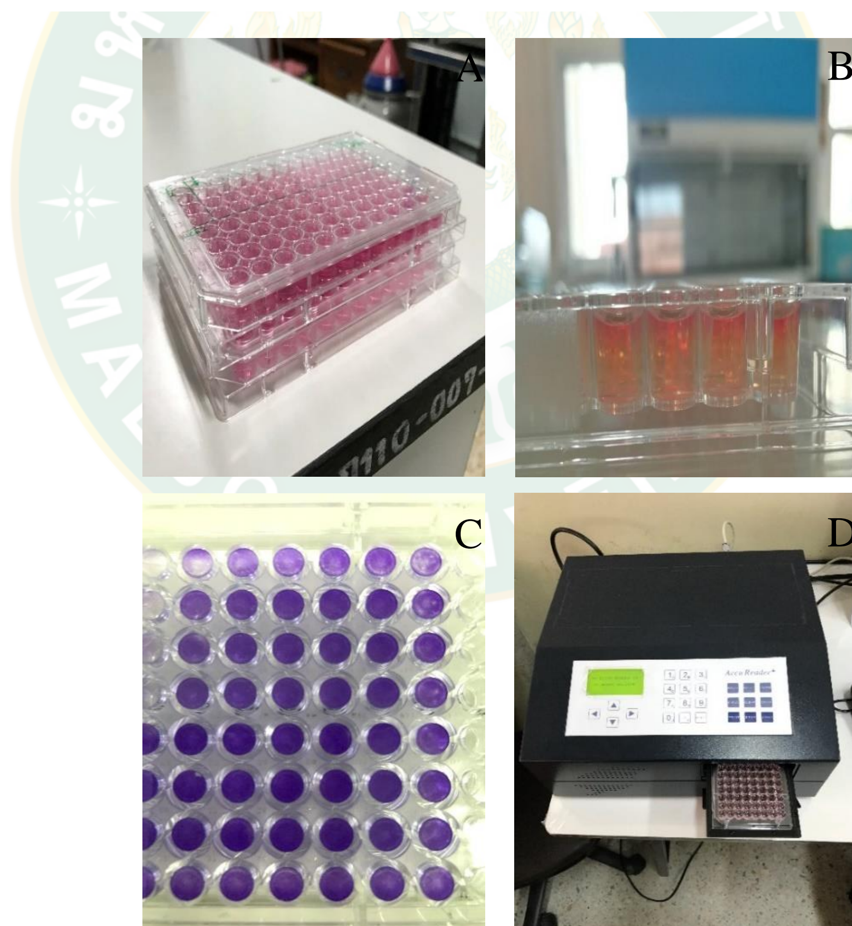
Appendix figure 7 FT-IR spectrum of essential oil from *C. burmannii*



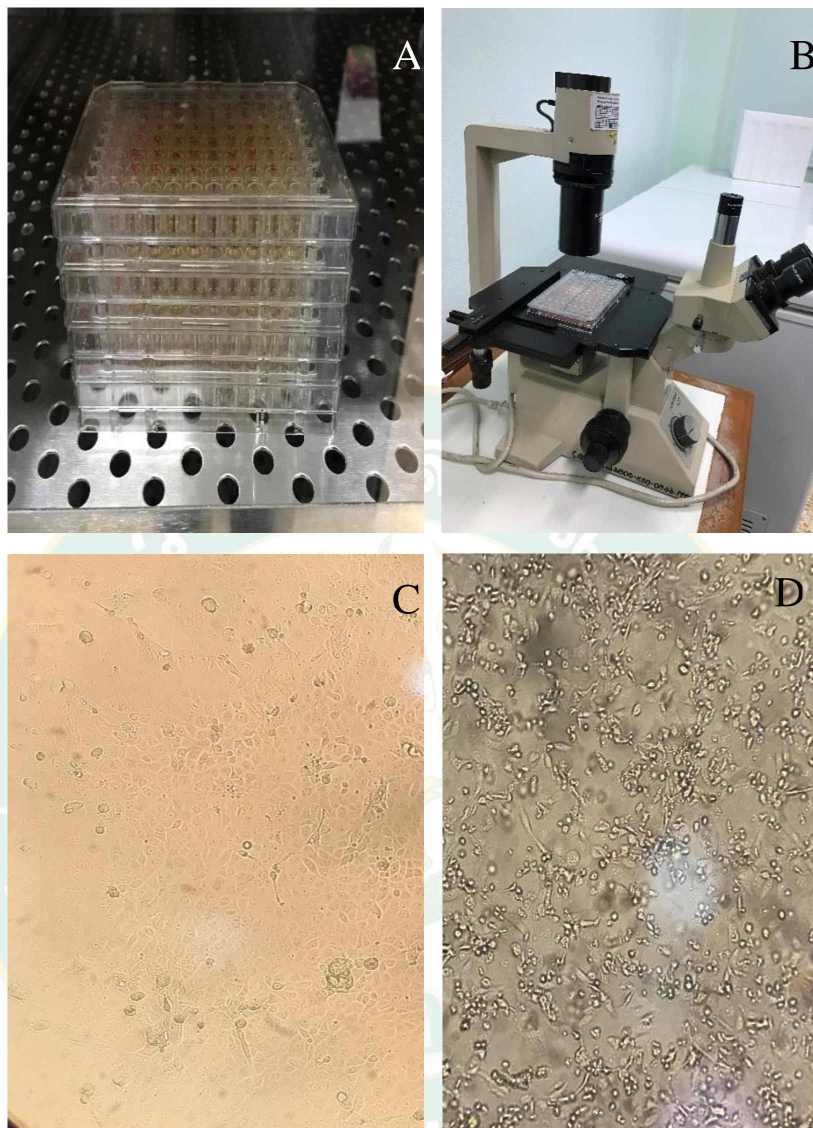
Appendix figure 8 FT-IR spectrum of cinnamaldehyde-derived benzimidazole from *C. iners*



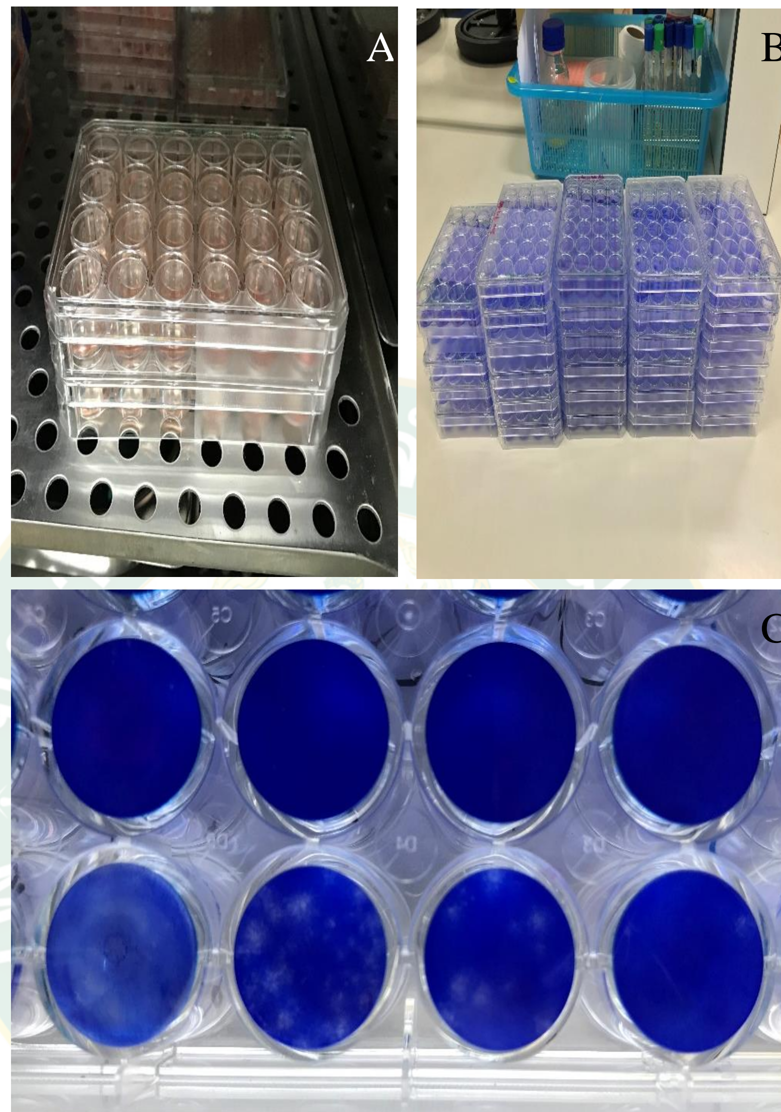
Appendix figure 10 FT-IR spectrum of cinnamaldehyde-derived benzimidazole from *C. burmannii*



Appendix figure 9 Cytotoxicity assay setup. A. Monolayer of MARC-145 cells treated with samples; B. MTT assay setup C. CV assay setup; D. Actual microplate reader used in the study



Appendix figure 11 Cytopathic effect assay A. CPE assay set up in CO_2 incubator; B. Observation of MARC-145 cells under inverted microscope; C. Uninfected or mock MARC-145 cell line; D. Infected MARC-145 cell line



Appendix figure 12 Plaque forming unit assay A. PFU assay setup in 24-well plate inside CO2 incubator; B. PFU assay stained with coomassie brilliant; C. Plaques formed in wells

Appendix C. Calculations

1. Calculating viral concentration using Reed and Muench $TCID_{50}$

The 50% tissue culture infectious dose (TCID50) method allows to simply add up the total number of positive wells from the plate and convert it to a titer that represents an endpoint. The procedure was performed to determine the infectious titer of PRRSV which caused cytopathic effects (CPE) in MARC-145 cell culture over a reasonable period of 4 days while cells in culture remain viable. Using the following formula, $TCID_{50}$/ml was determined.

A. **P**roportionate **D**istance (PD)=(% CPE at dilution above 50%) – (50%)
(% CPE at dilution above 50%) – (% CPE at dilution below 50%)

B. -Log = dilution above 50% CPE ratio (i.e. 10^{-3} would be -3)

C. ((PD)+(-log(dilution interval))

D. $TCID_{50} = 10^{(B+C)}$

E. This will give the dilution of the original suspension that would be equal to the $TCID_{50}$. The reciprocal would give you the # of $TCID_{50}$ in the original suspension applied to the wells (usually 0.1 or 0.2 ml). To determine the titer per ml, multiply by the reciprocal of the volume of the inoculum, then convert to $\log_{10}$.

2. Calculating viral titer based on the plaque assay method

To calculate the plaque forfing unites (pful) per ml, count the number of isolated plaques in each wells. Then use the following formula to determine the titer (pfu/ml) of your viral stock.

$$\text{pfu/ml} = \frac{\text{Average number of plaques}}{\text{A X B}}$$

Where,

A = dilution factor

B = Volume of diluted virus added to the well

3. Calculation of Essential oil Percentage Yield

Assume we are converting between gram (water) and milliliter. The SI derived unit for volume is the cubic meter where 1 cubic meter is equal to 1000000 g, or 1000000 ml, therefore 1 mL = 1 g of the sample. The following formula was used to determine the essential oil percentage yield.

$$\text{\% essential oil yield} = \frac{\text{mass of cinnamon bark}}{\text{mass of essential oil}} \times 100$$

4. Benzimidazole derivative percentage yield

First, check to see if the reaction is balanced. Identify the moles of each reagent used in the reaction to determine the limiting reactant (1,2 phenylenediamine) which has the lesser amount of moles.

$$\textbf{Moles of reagent} = \frac{\text{mass (g)}}{\text{molecular weight (g/mol)}}$$

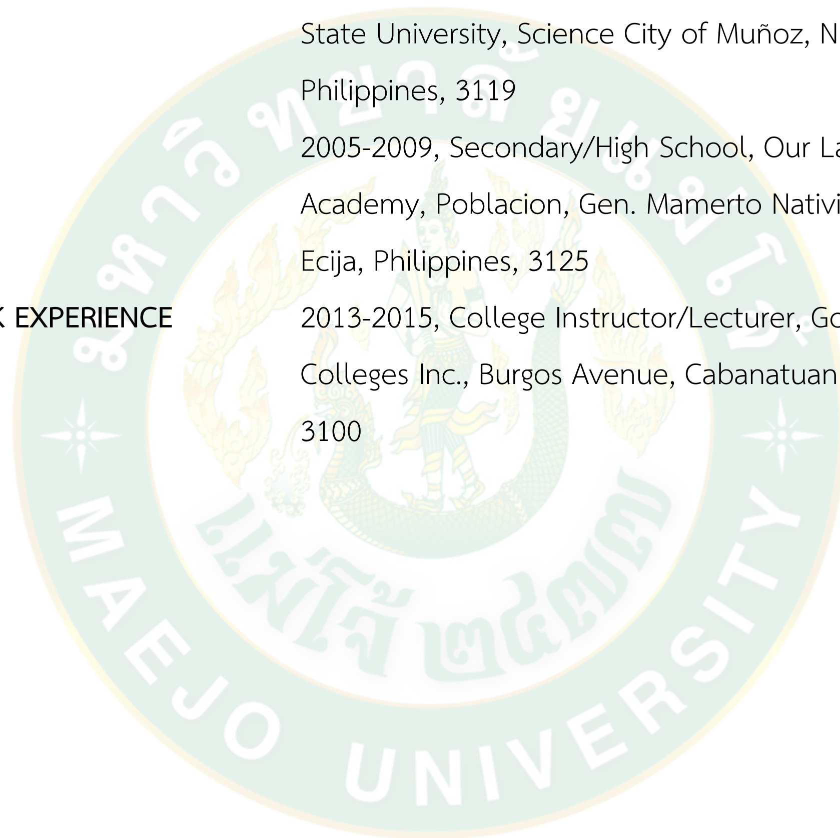
Then, calculate the theoretical yield of the chemical reaction using the following formula.

Theoretical yield (g) = mole of limiting reactant (mol) X molecular weight of product (benzimidazole derivative) (g/mol)

The following was used to calculate the percent yield of product in the chemical reaction.

$$\textbf{\% yield of benzimidazole derivative} = \frac{\text{actual yield}}{\text{theoretical yield}} \times 100$$

CURRICULUM VITAE



NAME	Dante Mendillo Fabros Jr.
DATE OF BIRTH	12 August 1993
EDUCATION	2009-2013, Tertiary/College Education, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines, 3119 2005-2009, Secondary/High School, Our Lady of Fatima Academy, Poblacion, Gen. Mamerto Natividad, Nueva Ecija, Philippines, 3125
WORK EXPERIENCE	2013-2015, College Instructor/Lecturer, Good Samaritan Colleges Inc., Burgos Avenue, Cabanatuan City, Philippines, 3100