ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACT FROM *Thunbergia laurifolia*

ASMITA SHARMA
5818135

A special project submitted to the School of Biotechnology, Assumption University in part of fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology
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Title	**:**	**Antimicrobial activity of crude extract from *Thunbergia laurifolia***
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Level of study	**:**	**Bachelor of Science**
Department	**:**	**Food Technology**
Faculty	**:**	**Biotechnology**
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(Advisor)

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Asmita Sharma

ABSTRACT

Thunbergia laurifolia (Rang Chuet), a plant native to regions of South Asia, has been used by people since ages in order to treat insecticide, arsenic, alcohol and strychnine poisoning. Leaves of this plant have been found to have many bioactive compounds like phenols, flavonoids, sterols, glycosides etc. Nowadays, research is being carried out to find natural alternatives for preservatives used in food systems. Very few researches have directed their focus to the antimicrobial properties of *T. laurifolia*. In this research, three different parts of the plant – leaf, stem and rhizome – were tested for their ability to inhibit three bacteria – *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*. The different parts of the plant were dried, ground into fine powder and mixed with solvent to obtain crude extract. Extraction conditions were varied in terms of amount of powder used (5, 10 and 15% w/v), concentration of ethanol solvent (0, 25, 50 and 75% ethanol) and time of extraction (24, 48 and 72 hours). Agar disc diffusion method was used to test antimicrobial effect of extract with the three microorganisms by measuring inhibition zone (mm). Crude leaf extract from *T. laurifolia* was found to have the best effect in inhibiting Gram negative microbe *E. coli*, while crude rhizome extract was the most effective in inhibiting Gram positive microorganisms *B. cereus* and *S. aureus*. The highest antimicrobial activity was obtained in case of rhizome extract against *B. cereus* (7.25 ± 0.27 mm), followed by leaf extract with *E. coli* (4.67 ± 0.52 mm) and rhizome extract against *S. aureus* (4.67 ± 0.52 mm). Crude stem extract showed little to no activity against all three microorganisms. These results show that rhizome of *T. laurifolia* has good potential to be used as natural antimicrobial agent. Phenolic compounds were the major compounds responsible for the activity. Further research is essential to determine specific compounds responsible for antimicrobial activity and to improve usage in food systems.

KEYWORDS: *Thunbergia laurifolia* / antimicrobial / phenolic / crude extract

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INTRODUCTION

Thunbergia laurifolia Lindl., commonly known as laurel clock vine and in Thai as Rang Cheut, is a plant native to India and occurs from Indochina to Malaysia. It is a woody climbing hermaphrodite plant which belongs to the botanical family of Acanthaceae and commonly consumed as herbal tea (Oonsivilai et al., 2008). It is widely distributed in the northern parts of Thailand. Many herbal companies in Thailand have started manufacturing and exporting herbal tea made with *T. laurifolia* in the recent years (Chan and Lim, 2006).

Various parts of *T. laurifolia* such as fresh leaves, dried leaves, dried root and bark have been used to treat insecticide, arsenic, alcohol and strychnine poisoning (Tejasen and Thongthapp, 1980). The dried root has been particularly used as anti-inflammatory and antipyretic agents (Thongsaard and Marsden, 2002). One of the main applications of this plant is as an antioxidant. A great variety of toxins and chemicals has the ability to generate free radicles in the body, which can result in grave illnesses such as cancer, stroke, etc. The antioxidant capacity of this plant has the ability to reduce oxidative stress and prevents the occurrence of these diseases. Natural sources of phytochemicals are safer than their synthetic counterparts (Sen et al., 2010).

Rang Cheut's application in traditional medicine has increased the interest of researchers in studying the bioactive compounds in the extracts from leaves, stems, rhizomes and other parts of this plant. Studies by Kanchanapoom et al. (2002) have revealed presence of many kinds of iridoid glucoside compounds isolated from the leaf extracts. Two novel iridoid glucosides of 8-epi- grandifloric acid and 3'-O-β glucopyranosyl-stilbericoside were reported along with seven of known grandifloric acid compounds. The plant was also found to contain phenolics and flavonoids like apegenin, chlorogenic acid etc. (Thongsaard and Marsden, 2002). Extracts of Rang Cheut leaves have been found to have a protective effect on ethanol-induced hepatotoxicity via different mechanisms (Chanawirat et al., 2000). Although there are many research papers exhibiting studies conducted on *T. laurifolia*, most of them are concentrated towards the

antioxidant, anti-diabetic and chelating property (antitoxin) of the extract. There are little to no detailed studies conducted focusing on the anti-microbial properties of the plant extract and the existing ones are about leaf extract/tea. Extract from *T. laurifolia* has the potential to be used as a natural antimicrobial agent in food industry.

Since the variety of bioactive compounds found in *T. laurifolia* is quite large, different solvents used for extraction will result in different chemical composition of the crude extract. The distribution of bioactive compounds also varies with the part of plant used. Different parts of the plant may have different chemical properties, such as antimicrobial activity. Optimization of extraction conditions can help in obtaining crude extract with high antimicrobial activity against specific microbial species.

Therefore, this research is aimed to study the antimicrobial activity of crude extract obtained from leaves, stem and rhizome of *T. laurifolia* and to optimize the extraction condition to obtain the highest possible activity against bacteria.

OBJECTIVES

1. To obtain crude extract from different parts of *Thunbergia laurifolia* by varying extraction conditions
2. To study the effect of extraction conditions on the antimicrobial activity of crude extract from different parts of *T. laurifolia* and determine optimum extraction condition

LITERATURE REVIEW

1. Botanical information of *Thunbergia laurifolia*

Thunbergia laurifolia, commonly known as Laurel Clock Vine or Blue Trumpet Vine, is a flowering plant belonging to the family of Acanthaceae and is native to regions of Africa, Australia and South Asia. It is locally known as "kar tuau" in Malaysia and "Rang Cheut" in Thailand (Chan and Lim, 2006). It is often cultivated as ornamental plants or as twinning climbers. Parts of this plant, both fresh and dry form, are often used in herbal tea and also found to have medicinal applications. In Malaysia, juice from crushed leaves of *T. laurifolia* is used to treat conditions such as menorrhagia. It is placed into ears to reduce deafness and applied on cuts and wounds as paste (Burkill 1966). Barks, leaves and roots are mainly used as antidote for poisoning, anti-inflammatory, anti-pyretic and anti-diabetic properties. (Chan et al., 2011; Sultana et al., 2015, Thongsaard and Marsden, 2002; Oonsavilai et al., 2008)

1.1. Classification of *Thunbergia laurifolia*

Class: Equisetopsida

Subclass: Magnoliidae

Order: Lamiales

Family: Acanthaceae

Genus: *Thunbergia*

Species: *laurifolia*

1.2. Morphology of *Thunbergia laurifolia*

T. laurifolia is a climber with dark green, opposite hearted leaves with pointed tip and serrated leaf margins. The leaves maybe lobed or toothed in shape and occur in many colors, often bright green to yellow when young. As the leaves age, they turn darker shade of green. The leaf blade can grow up to 20 cm in length and 16 cm in width, with petiole growing up to 6 cm in length. Most species in the genus of *Thunbergia* require full-sun and well-drained soil for optimum growth (Sultana et al., 2015). The plant produces unscented trumpet-shaped flowers with pale-purplish blue petals and yellow throat that can grow up to 8cm long and present themselves in pendulous inflorescence (*Figure 1*). The stem of this plant is round (*Figure 3*) and consists of a tuberous root system (*Figure 4*). This plant propagates using root or stem cutting. *T. laurifolia* shows year-long continuous flowering system where flowers open in the morning and abort on the same evening. Pollination usually occurs with the help of carpenter bees. Extracts from these parts of *T. laurifolia* have been found to have many pharmacologically beneficial properties. In traditional medicine in Thailand, tea made using *T. laurifolia* leaves has been long used to treat poisoning, addiction, etc. (Chan et al., 2011).

Figure 1*: *Thunbergia laurifolia

(Source: Sultana et al., 2015)

Figure 2*: Young (left), developing (middle) and mature (right) leaf of *T. laurifolia

(source: Chan et al., 2011)

Figure 3*: Stem of *T. laurifolia

(source: HiHerb, etsy.com)

Figure 4*: Rhizome of *T. laurifolia

(source: http://srikasa.com)

Table 1: **Characteristic features of *Thunbergia laurifolia***

Characteristic	Description
Distribution	Native to India, Thailand and Malaysia
Common Name	Blue Trumpet Vine, Blue-sky vine and Laurel Clock Vine
Height	15 meters in height
Plant type	Perennial climbing, creeper plant
Root system	Tuberous (*Figure 4*)
Leaf arrangement	Opposite
Leaf surface	Pubescent
Leaf margin	Entire or slightly toothed (crenated), hairless (glabrous) and acute or acuminate apex (*Figure 2*)
Leaf shape	Oval (i.e. elliptic) to narrowly egg-shaped in outline (ovate-lanceolate) (*Figure 2*)
Leaf venation	Pinnate
Flower	Blue, violet or purple trumpet shaped flowers having pale yellow or whitish colored throat (*Figure 1*)
Blooming season	Summer and autumn
Fruit	Loculicidal capsule
Soil for cultivation	Moist but well-drained
Water exposure	Moderate
Sun exposure	Full sun to slight shade

(Source: Sultana et al., 2015)

2. Thai herbal market and *T. laurifolia* products

Herbs have been a part of the society for thousands of years. A great portion of Asian history showed that herbal usage was firstly observed in India, which later spread to China, Malacca and Thailand. These places have climate suitable to both natural and cultivated growth of herbal plants. One such herb popular in Thailand is *T. laurifolia*.

Overtime, the herbal product market has seen rapid expansion both domestically in Thailand and internationally, especially in terms of herbal extract. This popularity

coincides with the growing trend of health and beauty consciousness among people. Thai herbal industry still faces some challenges regarding quality of herbal products. A master plan of Thai Herbal Development 2017-2021 was set up to support the Thai herbal industry. This includes research towards ways to improve the efficacy of herbal systems, using nanotechnology. (Nano, 2017).

Rang Chuet has been a part of Thai culture for ages, mainly in traditional medicine. It was often used in fresh or dried form by villagers as a cure for poisoning. However, with time, it has been formulated in modernized forms that provide an easier consumption. Currently, *T. laurfolia* or Rang Chuet is sold to consumers in different commercial product forms such as tea, capsules and powder in the herbal market due to its antipyretic and anti-inflammatory properties and its ability to act as antidote in case of poisoning (*Figures 5 and 6*) (Suwanchaikasem et al., 2013)

***Figure 5*: Asok *Thunbergia laurifolia* Tea**

(source: Bluepea.co.uk)

***Figure 6*: YA Rang Jued capsules**

(source: Thailandstore.org)

3. Phytochemistry of *T. laurifolia*

Many researchers have reported the presence of active compounds in different parts of *T. laurifolia*, mainly leaves. These compounds are responsible for the chemical properties of *T. laurifolia* that make it beneficial for use (*Table 2*)

(1) Phenols: Apigenin, caffeic acid, gallic acid and protocatechuic (Chuthaputti, 2010; Oonsivilai et al. 2007)

(2) Carotenoids: Lutein (Chuthaputti, 2010)

(3) Sterols: Beta sitosterol, stigmasterol, alphaspinasterol

(4) Glycosides: 8-epigrandifloric acid, 3'-O-beta-glucopyranosyl-stilbericoside, grandifloric acid, benzyl beta-glucopyranoside, benzyl beta-(2'-O-beta-glucopyranosyl)-glucopyranoside, etc. (Kanchanapoom et al., 2002; Chuthaputti, 2010)

(5) Flavonoids: Apigenin, casmosiin, chlorogenic acid and delphinidin-3-5-di-O-β-D-glucoside (Thongsaard and Marsden, 2002)

***Table 2*: Phenolic and flavonoid contents of *T. laurifolia* leaf from aqueous extract determined by LC-MS**

Peak No.	Compounds	Retention time (min)	Contents (mg/kg CDE)
1	Catechin	12.54 ± 0.02	69.54 ± 11.55
2	Caffeic acid	13.03 ± 0.04	199.21 ± 20.72
3	Rosmarinic acid	14.67 ± 0.27	90.28 ± 14.51
4	Rutin	5.34 ± 0.01	132.26 ± 11.45
5	Isoquercetin	16.46 ± 0.08	114.54 ± 6.04
6	Hydroquinone	23.80 ± 0.28	ND
7	Eriodictoyl	31.25 ± 0.07	ND
8	Quercetin	34.16 ± 0.28	61.19 ± 8.23
9	Apigenin	43.35 ± 0.03	41.32 ± 4.16
10	Kaempferol	44.33 ± 0.06	ND

Note: ND = Not detected (Source: Junsi et al., 2017)

These bioactive compounds can be affected by many factors such as pH, light, temperature etc., which may alter one or more or their functions. The main bioactive compounds in *T. laurifolia* are flavonoids and phenols. These compounds are sensitive to changes in temperature, especially high temperature (Moura et al., 2017; Przeor and Flaczyk, 2016)

A proximate analysis of leaves (dry weight) in terms of fibre, ash, protein, fat and carbohydrates content showed 16.8, 18.8, 16.7, 1.68 and 46.0 %, respectively (Jaiboon et al., 2011).

4. Pharmacological and Biological properties

The active compounds describes above allow *T. laurifolia* to possess many pharmacological properties. Previous researches have reported these as given below:

4.1. Anti-inflammatory property

Anti-inflammatory activity of water extracted *T. laurifolia* was studied by Nanna et al. (2017). This study used animal models like EPP-induced ear edema, carrageenan or arachidonic acid induced paw edema and cotton pellet-induced granuloma formation to observe the effect. It was observed that the water extract from *T. laurifolia* showed inhibitory effect on ear edema formation. Other models showed the inhibitory effects of extract under different stages of inflammation (Nanna et al., 2017). Anti-inflammatory and antioxidant properties of *T. laurifolia* were also studied in hamsters treated with liver fluke by Wonkchalee et al. (2012). This study reported that fresh or dry aqueous extract from Rang Cheut leaves reduced the number of inflammatory cells treated with *O. viverrini*, a human liver fluke, in Syrian hamsters. Also, rosmarinic acid obtained from ethanolic extract of *T. laurifolia* leaves showed to possess anti-inflammatory behavior against acute and chronic inflammation (Boonyarikpunchai et al., 2014). A study conducted by Pongphasuk et al. (2005) reported that anti-inflammatory efficacy dose of aqueous leaf extract of *T. laurifolia* (2.5 g/kg) is two-fold compared to that of *Garcinia mangostana* (5.5 g/kg). Carageenan-induced paw edema in mice was shown to reduce due to anti-inflammatory effect of alcohol and hexane leaf extracts of *T. laurifolia* (Charumanee et al., 1998)

4.2. Hepatoprotective activity

Many studies have reported the hepatoprotective activity of *T. laurifolia*. Pramyothin et al. (2005) reported hepatoprotective activity of aqueous extract of *T. laurifolia* against ethanol induced liver injury in rat liver cells. The viability of ethanol-treated hepatocyte cultures was increased 2-3 folds with a decrease in alanine transaminase (ALT) and aspartate transaminase (AST), and also promoted rat liver recovery after 14 days of ethanol treatment (Pramyothin et al., 2005; Chan et al., 2011). Fresh and dried *T. laurifolia* extracts reduced inflammatory cells in hepatic tissue in Syrian hamsters administered with NDMA as well as those infected with human liver fluke *Opisthorchis viverrini* (Wonkchalee et al., 2012).

4.3. Anti-diabetic effect

International Diabetes Federation (2014) reported a prevalence of 8.3% for people suffering from diabetes mellitus in 2014. About 60% of diabetic population was expected to be in Asian countries. People have shown concern in the use of anti-hyperglycemic drugs due to possible adverse side effects caused by undesirable pathological conditions. Thus, many researches have focused on finding solution in use of medicinal plants for the treatment of diabetes mellitus. Aritajat et al. (2004) studied the anti-diabetic effect of aqueous leaf extract of *T. laurifolia* using diabetic rats as models. The study showed that treatment with 60 mg/ml/day of extract for 15 days reduces level of blood glucose in diabetic rats, along with observation of some β-cells. It also reported that leaves extract can recover β-cell structure in islet of Langerhans of pancreas and enhance pancreatic secretion of insulin. Hypoglycemic effect of aqueous extract of *T. laurifolia* leaves in alloxan-induced diabetic rats was also reported by Tejasen and Thongtharb (1990). A dose of 500 mg/kg/day of *T. laurifolia* leaves for 28 days in hyperglycemic cats was shown to significantly cause decrease in blood glucose level (Pitoolpong et al., 2014). The pathway used by *T. laurfolia* to possess antidiabetic effect include elevation in insulin production from pancreatic cells (Aritajat et al., 2004), inhibition of alpha amylase activity (Jaiboon et al., 2011) and increased hepatic metabolism (Pramyothin et al, 2005).

4.4. Detoxifying effect

Detoxification effect of *T. laurifolia* was studied by Thonsaard and Marsden (2002) showing effect of hot water extract of the plant leaves on K^+ stimulated dopamine release in rat striatal slices compared to amphetamine, which was found to be similar. Thongsaard et al. (2005) conducted a follow up study showing *T. laurifolia* has the ability to stimulate brain activity similar to addictive drugs like amphetamine. A study conducted by Tangpong et al. (2010) on effects of leave extract in reducing effects of lead poisoning in mice brain showed that the extract can reduce neuronic cell death and memory loss, resulting from lead uptake by mice. Chattaviriya et al. (2010) conducted a study which shows reduced toxicity effects of cadmium in rats fed with aqueous leaf extract of *T. laurifolia*. Usanawarong et al. (2000) reported aqueous leaf extract increased survival rates and lower levels of plasma malonaldehyde in rats subjected to paraquat induced toxicity. Chinacarawat et al. (2012) suggested that orally administered *T. laurifolia* capsule at the dose of 600 mg/day for 2 weeks continuously can reduce organophosphate and carbamate insecticide poisoning and had no side effects in high risk volunteer.

4.5. Wound healing

Wound healing effect is evaluated focusing on different inflammatory phases – inflammatory phase, proliferative phase and remodeling phase (Liu et al., 2008). *T. laurifolia* has been found to have wound healing effects, especially in the case of burn wounds. A study conducted by Kwansang et al. (2015) reports that supercritical CO_2 extract of *T. laurifolia* leaf accelerates burn wound healing by reducing the length of inflammation phase and promoting the proliferation and remodeling phase.

4.6. Non-toxic effects

Chivapat et al. (2009) conducted a chronic toxicity study on *T. laurifolia* aqueous leaf extract on Wistar rats which showed that extract does ranging from 20-2000 mg/kg/day had no effect on body weight, food consumption, behavior and general health. No cumulatve toxic signs or fatal effects were observed. Another toxicity study by Pongphasuk et al. (2005) on aqueous leaf extract of *T. laurifolia* in mice at doses of 1, 2, 4

and 8 g/kg/day reported zero deaths in mice in the first month, suggesting that the extract is non-toxic, effective and safe for consumption.

4.7. Antiproliferative activity

Ethanolic extracts of nine Thai medicinal plants did not antiproliferative activity against SKBR3 human breast adenocarcinoma cells (Moongkarndi et al., 2004). Another study by Oonsavilai et al. (2008) reported that dried leaf powder extract showed weak to no cytotoxic activity against BHK and L929 normal cells and HepG2 and Caco2 cancer cells.

4.8. Antioxidant property

Antioxidants are substances that have the ability to inhibit oxidation of a substrate, even at low concentrations. A large number of studies have been conducted that prove the presence of antioxidant compounds in *T. laurifolia*. A study conducted by Oonsivilai et al. (2008) evaluated the antioxidant activity and phenolic content of *T. laurifolia* extract obtained using water, ethanol and acetone as solvents. It was reported that water extract contains the highest phenolic content, followed by ethanol and acetone extract respectively. It was inferred that polyphenol compounds were responsible for the antioxidant activity in *T. laurifolia* extract. Other studies were conducted studying the optimum time and efficiency of methanol extraction for *T. laurifolia* leaves to obtain the highest antioxidant activity. The highest TPC values were obtained for 1 hour extraction (Chan 2004). This study also reported the variation in TPC in leaves obtained at different time of maturity, collection time and location. Developing leaves had the highest TPC of 513 mg GAE/100 g, followed by young and mature leaves with values of 407 and 290 mg GAE/100 g, respectively. There was also significant difference in TPC in leaves taken from three different locations of the plant. A study conducted by Chan et al. (2010) compared antioxidant properties of different herbal teas, and placed *T. laurifolia* tea among the low antioxidant category. Apart from leaves, flowers of *T. laurifolia* were found to have active antioxidant compounds (Purnima and Gupta, 1978). High TPC and free radical scavenging activity of *T. laurifolia* extract has been stated to be relevant against human breast cancer cells (Jetawattana et al., 2015).

4.9. Anti-microbial compounds

Anti-microbial compounds are active compounds that affect the growth and survival of microorganisms such as bacteria, fungus and virus. The nature of effect of these active compounds can be inhibitory or bactericidal. *Thunbergia* sp. has been found to possess anti-microbial properties which include anti-bacterial, anti-fungal properties and anti-viral properties. Most studies focus on anti-microbial properties of *Thunbergia* genus, but fewer studies have been conducted on *T. laurifolia.* A study conducted by Cheeptham and Towers (2002) analyzed the UV activated anti-microbial properties of ethanolic leaf extract of *T. laurifolia* against *S. aureus, B. subtilis, E. coli, Pseusomonas aeruginosa, Candida albicans* and *Aspergillus fumigatus*. It was reported that *T. laurifolia* extract showed no antibacterial or antifungal activity except in case of *B. subtilis.* Pukumpuang et al. (2012) observed anti-microbial activity of ethanolic and water extracts of *T. laurifolia* against *S. aureus*, MRSA, *S. epidermis* and *S. pyogenes*. This study reported moderate activity on gram positive bacteria. The MIC of *T. laurifolia* against the Gram positive bacteria was found to be ranging from 7.8 to 125 mg/ml while MBC was found to be from 31.3 to 250 mg/ml. The highest activity was observed with *S. pyogenes*, as MIC and MBC for this microbe is the least compared to other test microbes. Ethanolic extract has more antimicrobial activity than water extract (Pukumpuang et al. 2012). Khobjai et al. (2014) reported the protective effect of *T laurifolia* aqueous leaf extract on hemolysis during *Plasmodium berghei* infection in mice. However, not much research has been conducted studying the antimicrobial effects of *T. laurifolia* extracts, and those conducted are limited to leaf extracts.

5. Antimicrobials in food systems

Food producers often depend on chemical substances in order to preserve food from decomposition, fermentation or growth of microorganisms that can cause spoilage and make the food unsafe for consumption. These are called antimicrobial preservatives. There are a wide range of antimicrobials currently employed in food systems such as parabens (methyl, ethyl, propyl and butyl parabens), sorbic acid, sorbates, benzoic acid, sodium metabisulfites, BHT, BHA etc. Among these, parabens are among the most commonly used preservatives due to their ability to act against a broad spectrum of

microorganisms. Methyl parabens is most effective against bacteria and mold while ethyl, propyl and butyl esters are more active against yeast and mold.

Despite the requirement of food additives, their toxicological safety continues to be evaluated and questioned. The approval of a chemical to be used in food system is often a complex process. It is essential to balance the risk against the benefits of the additives, which requires extensive research about its usefulness and toxicological safety. The need for toxiciological safety limits the development of new antimicrobials. It is an expensive process and requires very extensive testing. Other factors to consider include the ability of the antimicrobial to be metabolized and excreted effectively without causing a buildup. The chemicals being used currently have been researched to have no significant risk to humans. However, the industry will always be reluctant to expand its use due to unknown problems that may arise with increased consumption or combined use with other additives.

Many researchers are now investigating a possible total or partial shift to naturally occurring antimicrobials, as they are considered to be less toxic than those manufactured synthetically. This assumption is not always true, as the compounds that are naturally antimicrobial show activity at concentrations that are not usually normal for consumption, even if the natural substance has been a part of human consumption for a long time e.g. spices. Their activity must be tested by either animal testing or continuous consumption by humans over a period of time. In addition, some compounds may not be effectively metabolized or excreted. They also need to be tested for allergenic effects (Antimicrobials in Food, 2005).

MATERIALS AND METHODOLOGY

Plant materials and preparation

The plant material used was dried leaves, stem and rhizome of *Thunbergia laurifolia* (Rang Cheut) obtained from a local farm in Thailand. The different plant parts were sorted for defects, dried at 40°C for 1 hour and ground into fine powder (*Figure* 7-A) with the size of 80-100 mesh and stored in air-tight containers at room temperature.

Microbial culture and media

Three strains of microorganisms- *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*- were used for antimicrobial analysis of the plant extracts. The cultures were obtained from frozen stock stored at -80°C. Plate count agar (PCA) media was used to observe the microbial growth in the presence of test samples.

Methodology

1. **To study the crude extraction conditions from different parts of *T. laurifolia***

The prepared powders of leaf, stem and rhizome of *T. laurifolia* were used for this part. The amount of plant powder varied as 5, 10 and 15% (w/v) was used for extraction at room temperature for 24, 48 and 72 hours on shaker (*Figure 7-B*). The concentrations of solvent were varied as 0 (water), 25, 50 and 75% ethanol. Two replications of each extraction condition were made. Crude extract was obtained by filtering the mixture using Whatman Filter paper No. 4. The obtained crude extracts were stored in Eppendorf tubes at -80°C until further analysis of anti-microbial properties.

2. **To study anti-microbial property of the crude extract from different parts of *T. laurifolia***

The anti-microbial activity of the crude extracts obtained in part 1 was studied using agar disc diffusion method with three model microorganisms – *E. coli*, *S. aureus*

and *B. cereus*. Overnight culture of each model microorganism was diluted to $O.D._{600}$ of 0.1 (approx. 10^3 to 10^4 cells/ml) and mixed with molten Plate Count Agar (PCA) before plating. Sterile filter paper discs (6 mm) were inoculated with about 10 µl of sterile crude extract and placed on the plate, followed by incubation at 37°C. The diameter of the inhibition zone around the discs was measured after 24 hours incubation. Three duplications of inhibition zone measurement were carried out for each replication of extraction conditions tested (*Figure 7-C*).

***Table 3*: Criteria for classification of crude extract activity**

Activity of crude extract	Inhibition zone range (mm)
Low	≤ 2.50 mm
Moderate	2.51 – 4.99 mm
High	≥ 5.00 mm

3. Experimental design and Statistics analysis

3x3x4 Factorial in Randomized Complete Block Design (ANOVA) with two replications and Duncan's Multiple Range test were used to analyze antimicrobial activity of different plant parts for each microorganism and determine the optimum extraction condition based on analysis (part 1 and 2) using R-Program version R 2.15.3.

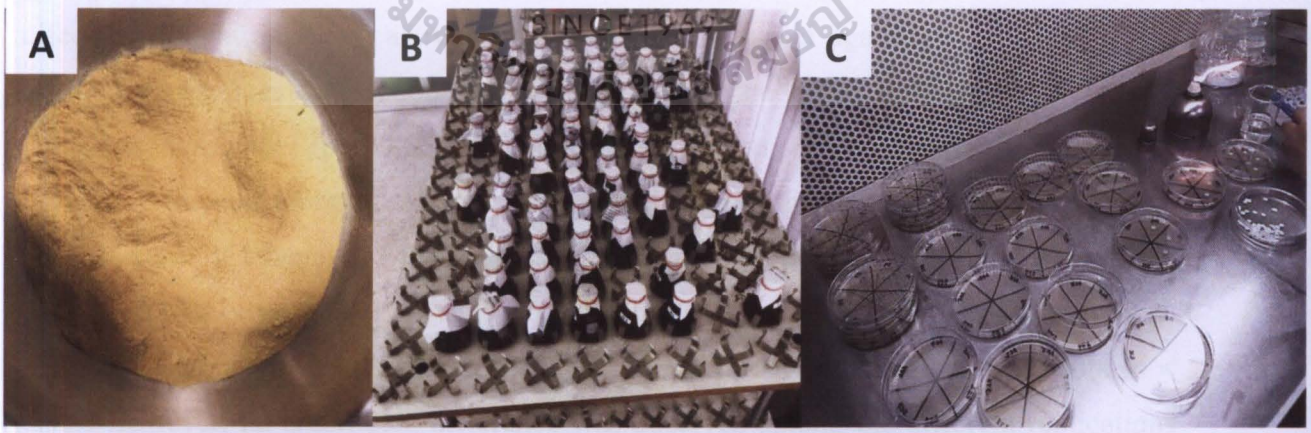

***Figure 7*: Experimental procedure: A - Powdered raw material, B- extraction process and C - laboratory testing of antimicrobial activity**

RESULT AND DISCUSSION

The crude extract from leaf, stem and rhizome of *Thunbergia laurifolia* was tested for antibacterial activity against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*. The crude extract was varied based on three extraction factors – amount of plant powder (5, 10 and 15% (w/v)), concentration of ethanol solvent (0, 25, 50 and 75%) and time of extraction (24, 48 and 72 hours). The inhibition zones were analyzed statistically using Factorial in Randomized Complete Block Design (RCBD) and Duncan's Multiple range test using R-Program version R 2.15.3.

Many researches have been conducted regarding to biochemical activity of leaf extract of *T. laurifolia* identifying main active compounds as phenols and flavonoids such as apigenin, caffeic acid, etc., carotenoids, sterols and glycosides (Oonsavilai et al. 2007, Chuthaputti, 2010, Kanchanapoom et al., 2002, Junsi et al.,2017). The phytochemicals found in *T. laurifolia* include a mixture of polar and non-polar compounds that are responsible for many bioactive properties such as antimicrobial activity.

1. ANTIMICROBIAL ACTIVITY OF CRUDE LEAF EXTRACT

1.1. Activity against *Escherichia coli*

The three different factors/conditions of extraction were found to have significant effect on the antimicrobial activity of the crude leaf extract against *E. coli* ($p < 0.05$). There was significant interaction seen between pairs of the factors ($p < 0.05$) as well as all three factors together ($p < 0.05$). The ratio of powder to solvent and time of extraction influenced the concentration of active compounds present in the crude extract, while the concentration of ethanol solvent influenced the type of active compounds present in the extract – polar or non-polar – group of compounds has antimicrobial activity against *E. coli*. There was significant difference observed in different combinations of factors ($p < 0.05$). However, no significant difference was observed among the different replications of extraction ($p>0.05$).

Table 4: **Antibacterial activity of crude leaf extract produced by using different extraction conditions of *T. laurifolia* against *E. coli***

Amount of leaf powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
5	0	24	0.83 ± 0.41^{no} *
		48	3.00 ± 0.55^{bc}
		72	1.58 ± 0.20^{fghijk}
	25	24	1.00 ± 0.45^{lmn}
		48	3.08 ± 0.38^{bc}
		72	1.67 ± 0.41^{fghij}
	50	24	0.48 ± 0.29^{op}
		48	2.25 ± 0.42^{de}
		72	1.08 ± 0.20^{klmn}
	75	24	0.17 ± 0.26^{p}
		48	3.33 ± 0.52^{b}
		72	1.00 ± 0.00^{lmn}
10	0	24	0.95 ± 0.34^{mno}
		48	3.42 ± 0.49^{b}
		72	1.75 ± 0.42^{fghi}
	25	24	1.00 ± 0.32^{lmn}
		48	3.00 ± 0.32^{bc}
		72	1.42 ± 0.58^{ghijklm}
	50	24	0.25 ± 0.26^{p}
		48	1.92 ± 0.20^{efg}
		72	1.17 ± 0.41^{jklmn}
	75	24	1.08 ± 0.20^{klmn}
		48	2.00 ± 0.00^{ef}
		72	1.00 ± 0.00^{lmn}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

***Table 4*: Antibacterial activity of crude leaf extract produced by using different extraction conditions of *T. laurifolia* against *E. coli* (Cont.)**

Amount of leaf powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
15	0	24	1.25 ± 0.27^{ijklmn} *
		48	3.00 ± 0.45^{bc}
		72	1.75 ± 0.42^{fghi}
	25	24	0.92 ± 0.38^{mno}
		48	2.67 ± 0.98^{cd}
		72	1.33 ± 0.26^{hijklmn}
	50	24	1.50 ± 0.45^{fghijkl}
		48	4.67 ± 0.52^{a}
		72	1.00 ± 0.00^{lmn}
	75	24	1.17 ± 0.26^{jklmn}
		48	1.83 ± 0.26^{efgh}
		72	1.17 ± 0.26^{jklmn}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

The highest inhibition zones in general were observed at 48 hours of extraction. Activity was lower at shorter or longer time of extraction. High inhibition zones were obtained at 0% and 25% ethanol concentration consistently. The group of compounds responsible for high antimicrobial activity at low ethanol concentrations is mainly polar compounds that have higher affinity for water, such as phenolic acids and glycosides. However, as amount of leaf powder used was increased, there was increase in activity in ethanolic extracts at 50% and 75% ethanol. This indicates that some non-polar compounds like some flavonoids that were extracted at higher ethanol concentration showed activity when more powder was used, indicating lower concentration of these non-polar compounds in leaf extract. The antimicrobial activity of the non-polar compounds against *E. coli* was better than polar compounds, as inhibition zone was much larger. The amount of leaf powder and ethanol concentration were important factors for the antimicrobial

activity of extract since extraction of non-polar flavonoids and phenolics was dependent on them. Since ethanol can dissolve both polar and non-polar compounds, the activity in ethanolic extracts was due to combined effect of polar and non-polar phenolics.

Statistical analysis of the inhibition zones obtained under each condition is summarized in *Table 4*. It can be seen that the highest inhibition zone against *E. coli* was obtained with the highest amount of powder (15% w/v), 50% ethanol as solvent and 48 hours extraction (*highlighted in black box*). The highest amount of powder allowed the extraction of some non-polar phenolic compounds, flavonoids and sterols (Widyawati et.al. 2014), which combined effect with polar phenolic acids and glycosides to provide the highest antimicrobial activity against *E. coli*. The interaction of all the factors is synergistic i.e. all increase simultaneously to provide higher activity. The inhibition zone, however, is not very high. The highest obtained value is 4.67 ± 0.52 mm. This indicates that the crude leaf extract from *T. laurifolia* has moderate antimicrobial activity (*Table 3*) against *E. coli*. This result is contrast to previous researches conducted on antimicrobial activity of crude leaf extracts, stating that *E. coli* and other Gram negative bacteria are not inhibited by aqueous or ethanolic extracts (Cheeptham and Towers, 2002; Pukumpuang et al. 2012).

1.2. Activity against *Bacillus cereus*

The three factors of extraction were found to have significant effect on the antimicrobial activity of the leaf extract against *B. cereus* ($p < 0.05$). However, interaction effect was only observed between amount of powder used and time of extraction. The combination of amount of powder used and time of extraction process determined concentration of active antimicrobial compounds present in the leaf extract that showed activity against *B. cereus*. Concentration of ethanol affected the activity independently and was not in interaction with any other factor. There was no intercation effect among all three factors ($p > 0.05$).

Table 5: **Antibacterial activity of crude leaf extract produced by using different extraction conditions of *T. laurifolia* against *B. cereus***

Amount of leaf powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
5	0	24	0.00 ± 0.00^{d} *
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}
	25	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.17 ± 0.26^{abcd}
	50	24	0.33 ± 0.26^{abcd}
		48	0.33 ± 0.41^{abcd}
		72	0.17 ± 0.26^{abcd}
	75	24	0.03 ± 0.08^{cd}
		48	0.08 ± 0.20^{cd}
		72	0.08 ± 0.20^{cd}
10	0	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}
	25	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
	23	72	0.25 ± 0.27^{abcd}
	50	24	0.08 ± 0.20^{cd}
		48	0.25 ± 0.27^{abcd}
		72	0.25 ± 0.27^{abcd}
	75	24	0.12 ± 0.20^{bcd}
		48	0.33 ± 0.26^{abcd}
		72	0.33 ± 0.61^{abcd}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Table 5: **Antibacterial activity of crude leaf extract produced by using different extraction conditions of *T. laurifolia* against *B. cereus* (Cont.)**

Amount of leaf powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
15	0	24	0.03 ± 0.08^{cd} *
		48	0.08 ± 0.20^{cd}
		72	0.00 ± 0.00^{d}
	25	24	0.00 ± 0.00^{d}
		48	0.75 ± 0.88^{a}
		72	0.17 ± 0.26^{abcd}
	50	24	0.17 ± 0.26^{abcd}
		48	0.42 ± 0.38^{abc}
		72	0.33 ± 0.26^{abcd}
	75	24	0.08 ± 0.20^{cd}
		48	0.50 ± 0.77^{ab}
		72	0.33 ± 0.26^{abcd}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

It was seen that most of the antimicrobial activity was observed at higher concentration of ethanol. Very low activity was observed at low ethanol concentration. At low amount of leaf powder used, no antimicrobial activity was observed at 0% ethanol and very low activity at 25% ethanol which was only at 72 hours extraction, indicating that concentration of polar compounds in the extract was not high enough to effectively inhibit *B.cereus.* However, when amount of powder used was increased to 15% (w/v), some activity was seen at 0% ethanol and higher inhibition zone was observed at 25% ethanol. The activity was also higher at 48 hours of extraction. This showed synergistic interaction between amount of powder and time of extraction i.e. when higher amount of powder was used to extract for longer time, more activity was observed at 0% ethanol. This allowed extraction of sufficient concentration of polar compounds to show some activity against *B. cereus*. Overall activity was higher at 48 hours. Most of the

antimicrobial activity of extract against *B.cereus* was due to non-polar phenolic compounds extracted in high ethanol concentration. The inhibition zone increased with increased amount of powder used.

Statistical analysis of inhibition zones is summarized in *Table 5*. It is seen that the highest activity against *B. cereus* was observed at 15% leaf powder (w/v) and 25% ethanol solvent extracted for 48 hours (*highlighted in black box*). Increasing the amount of powder allowed extraction of sufficient concentration of polar compounds at low ethanol concentration. These polar compounds had higher efficiency in inhibiting *B. cereus* compared to non-polar compounds alone, since the inhibition zone obtained was higher. However, at 25% ethanol, some non-polar compounds were also extracted since the solvent consisted of ethanol as well. The antimicrobial activity was a combined effect of polar phenolic acids, non-polar phenolic compounds and flavonoids. The interaction of the factors was synergistic. Since the highest value was 0.75 ± 0.88 mm, the antimicrobial activity of crude leaf extract against *B. cereus* was low (*Table 3*). Previous researches have also reported inhibition of *Bacillus* sp. using leaf extracts (Cheeptham and Towers, 2002).

1.3. Activity against *Staphylococcus aureus*

None of the three factors had significant effect on the antimicrobial activity of leaf extract against *S. aureus* ($p > 0.05$). Also, there was no interaction observed among any of the factors. This indicated that there might be no antimicrobial activity of leaf extract against *S. aureus*. Different active phenolic compounds or flavonoids, both polar and non-polar, were ineffective in inhibiting the growth of *S. aureus*.

Under most of the extraction conditions, no inhibition zone was obtained in case of *S. aureus*. This indicated that crude leaf extract did not show antimicrobial activity against this microbe. Very low activity was observed in ethanolic extracts at high amount of leaf powder used and long extraction hours. The activity was also found at high percentage of ethanol, indicating that non-polar compounds were responsible for the mild antimicrobial activity. However, since the factors did not have significant effect on the antimicrobial activity of extract against *S. aureus*, the optimum extraction condition could

not be determined through statistical analysis. *S. aureus* showed the resistance to the extract. This result was in contrast with the previous research suggesting antimicrobial activity of ethanolic leaf extract against *S. aureus* (Cheeptham and Towers, 2002; Pukumpuang et al., 2012). This could be due to difference in source of plant part or the specific strain of the microbe used.

Table 6*: Antibacterial activity of crude leaf extract produced by using different extraction conditions of *T. laurifolia* against *S. aureus

Amount of leaf powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
5	0	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	25	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	50	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	75	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Table 6: **Antibacterial activity of crude leaf extract produced by using different extraction conditions of *T. laurifolia* against *S. aureus* (Cont.)**

Amount of leaf powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
10	0	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	25	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	50	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	0	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
15	0	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	25	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	50	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.08 ± 0.20
	75	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Comparing the antimicrobial activity of crude leaf extract against the three test microorganisms, it was seen that the leaf extract was the most effective in inhibiting *E. coli*. The activity against *B. cereus* and *S. aureus* was low or non-existent. This indicated that leaf extract of *T. laurifolia* was more effective in inhibiting Gram-negative bacteria compared to Gram-positive bacteria. The results obtained in case of leaf extract do not agree with previous researches on antimicrobial activity of *T. laurifolia.* Pukumpuang et al. (2012) reported moderate activity of ethanolic extract against Gram positive bacteria, including *S. aureus*. Another study by Cheeptham and Towers (2002) reported antimicrobial activity of UV-induced ethanolic leaf extract against some microbes, except *E. coli,* and *S. aureus*. These studies were in contrast with the present research. This showed that the activity could not be determined with certainty as it can be affected by many factors such as strain and species of test microorganisms, source of the plant and part of plant used. Some variety may occur based on age of leaf or stage of development. All these factors influenced the concentration of active compounds in the extract. Most of the previous researches focused on activity of leaf extract only. It could also be noted that the same group of active compounds had different interaction with different microbial species. Thus, determination of optimum extraction condition to obtain the highest antimicrobial activity is dependent on variety of factors, which require a more extensive research to standardize.

2. ANTIMICROBIAL ACTIVITY OF CRUDE STEM EXTRACT

2.1. Activity against *Escherichia coli*

Among the three factors of extraction, only concentration of ethanol solvent was found to have significant effect on the antimcrobial activity of crude stem extract against *E. coli* ($p < 0.05$). The solvent concentration governs the type of compounds mainly extracted and responsible for any antimicrobial activity. Significant interaction was observed only between concentration of ethanol and time of extraction ($p < 0.05$). This interaction indicated that time of extraction affect the concentration of main active antimicrobial compounds present in the extract, while the solvent determined the nature of compounds.

Table 7*: Antibacterial activity of crude stem extract produced by using different extraction conditions of *T. laurifolia* against *E. coli

Amount of stem powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
5	0	24	0.50 ± 0.32^{ab} *
		48	0.50 ± 0.00^{ab}
		72	0.42 ± 0.20^{abc}
	25	24	0.17 ± 0.26^{cd}
		48	0.08 ± 0.20^{d}
		72	0.08 ± 0.20^{d}
	50	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}
	75	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}
10	0	24	0.58 ± 0.20^{a}
		48	0.50 ± 0.00^{ab}
		72	0.42 ± 0.20^{abc}
	25	24	0.25 ± 0.27^{bcd}
		48	0.08 ± 0.20^{d}
		72	0.17 ± 0.26^{cd}
	50	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}
	75	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

***Table 7*: Antibacterial activity of crude stem extract produced by using different extraction conditions of *T. laurifolia* against *E. coli* (Cont.)**

Amount of stem powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
15	0	24	0.42 ± 0.20^{abc} *
		48	0.50 ± 0.00^{ab}
		72	0.25 ± 0.27^{bcd}
	25	24	0.17 ± 0.26^{cd}
		48	0.00 ± 0.00^{d}
		72	0.17 ± 0.26^{cd}
	50	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}
	75	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

It can be seen that antimicrobial activity was only found at low ethanol concentration i.e. 0 and 25% ethanol. The inhibition zones were higher in case of 0% ethanol, indicating polar phenolic compounds were mainly responsible for the antimicrobial activity. These compounds have higher affinity for water as solvent. There was no significant effect of amount of stem powder used or the time of extraction individually or in interaction with each other, thus increasing the amount of powder also didn't result in activity in ethanolic extracts. Non-polar compounds in the ethanolic stem extracts did not have the ability to inhibit *E. coli*. The only interaction observed between ethanol concentration and time of extraction was synergistic in nature i.e. lower ethanol concentration and lower time of extraction resulted in higher activity.

Table 7 shows the statistically analyzed inhibition zones obtained for *E. coli* using crude stem extract. The overall activity of the extract was quite low. Many conditions gave significantly non-different inhibition zones. However, all the conditions used 0% ethanol as the solvent, indicating polar phenolics such as phenolic acids was the most effective. The best condition was chosen based on economic criteria i.e. the lowest possible amount of stem powder and the lowest possible time of extraction. This could save cost of raw material and operation cost for conducting the extraction process. Thus, the optimum condition chosen for extraction was 5% (w/v) of stem powder with 0% ethanol (water) and 24 hours extraction. The highest inhibition zone value was 0.58 ± 0.20 mm, which was not very high. Thus, the antimicrobial activity of crude stem extract against *E. coli* was low (*Table 3*).

2.2. Activity against *Bacillus cereus*

All the three factors had a significant effect on the antimicrobial activity of crude stem extract against *B. cereus* ($p < 0.05$). There was significant interaction observed among all factors all three together ($p < 0.05$). The amount of stem powder used and the time of extraction influenced the concentration of compounds present in the crude extract, while the ethanol concentration affect the type of compounds predominantly present in the extract – polar or non-polar phenolic compounds.

Table 8*: Antibacterial activity of crude stem extract produced by using different extraction conditions of *T. laurifolia* against *B. cereus

Amount of stem powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition Zone (mm)
5	0	24	0.00 ± 0.00^{f} *
		48	0.00 ± 0.00^{f}
		72	0.42 ± 0.20^{cde}
	25	24	0.00 ± 0.00^{f}
		48	0.00 ± 0.00^{f}
		72	1.00 ± 0.00^{a}
	50	24	0.00 ± 0.00^{f}
		48	0.00 ± 0.00^{f}
		72	0.00 ± 0.00^{f}
	75	24	0.00 ± 0.00^{f}
		48	0.00 ± 0.00^{f}
		72	0.50 ± 0.32^{cd}
10	0	24	0.00 ± 0.00^{f}
		48	0.00 ± 0.00^{f}
		72	0.67 ± 0.26^{bc}
	25	24	0.00 ± 0.00^{f}
		48	0.00 ± 0.00^{f}
		72	1.00 ± 0.00^{a}
	50	24	0.00 ± 0.00^{f}
		48	0.00 ± 0.00^{f}
		72	0.00 ± 0.00^{f}
	75	24	0.17 ± 0.26^{ef}
		48	0.08 ± 0.20^{f}
		72	1.08 ± 0.20^{a}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

***Table 8*: Antibacterial activity of crude stem extract produced by using different extraction conditions of *T. laurifolia* against *B. cereus* (Cont.)**

Amount of stem powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
15	0	24	0.00 ± 0.00^{f} *
		48	0.25 ± 0.27^{def}
		72	0.83 ± 0.26^{ab}
	25	24	0.08 ± 0.20^{f}
		48	0.17 ± 0.26^{ef}
		72	1.08 ± 0.20^{a}
	50	24	0.00 ± 0.00^{f}
		48	0.08 ± 0.20^{f}
		72	0.00 ± 0.00^{f}
	75	24	0.00 ± 0.00^{f}
		48	0.50 ± 0.32^{cd}
		72	0.92 ± 0.20^{ab}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Activity was found mostly at 0, 25 and 75% ethanol. Both polar and non-polar phenolic compounds in the extract showed ability to inhibit *B. cereus*. Interaction between amount of powder used and concentration of ethanol was synergistic i.e. increase in both factors lead to increase in inhibition zones obtained. Also, at low powder amount, inhibition zone was only observed at 72 hours, indicating that longer time was essential to obtain sufficient concentration of polar compounds to show effect. However, at higher amount of powder used, activity was seen at lower extraction time as well, indicating that the interaction between these two factors was also synergistic. Activity at lower ethanol concentration remained mostly high, while that in ethanolic extracts (75% EtOH) increased with increase in amount of powder used. At lower amount of powder used, there was no activity at 50%, but some activity was found at the highest amount of powder.

Statistical analysis of inhibition zones obtained for *B. cereus* showing antimicrobial activity of crude stem extract is shown in *Table 8*. The condition giving the highest inhibition zone was 10% (w/v) stem powder with 75% ethanol as solvent extracted for 72 hours. Non-polar phenolic compounds were mainly responsible for the antimicrobial activity against *B. cereus,* along with some polar compounds also soluble in ethanol. These compounds are the highest in 75% ethanolic extract. Despite being the most effective antimicrobial compounds in the extract, they are low in concentration as it requires 72 hours of extraction to achieve sufficient concentration to show effect. The highest inhibition zone obtained was 1.08 ± 0.20 mm. Thus, the antimicrobial activity of crude stem extract against *B. cereus* was low (*Table 3*).

2.3. Activity against *Staphylococcus aureus*

None of the factors had any significant effect on the antimicrobial activity of the crude stem extract on *S. aureus* ($p > 0.05$). There was no interaction observed between any of the factors ($p > 0.05$). This indicated that compounds in crude stem extract were not able to inhibit the growth of *S. aureus*.

Under all of the extraction conditions, no inhibition zone was obtained in case of *S. aureus*. This indicated that crude stem extract did not show antimicrobial activity against this microbe. The polar and non-polar phenolics present in the crude stem extract were not effective in inhibition of *S. aureus*. Increasing or decreasing the amount of powder, time of extraction or varying the percentage of ethanol used as solvent did not affect the antimicrobial activity of the extract. *S. aureus* showed resistance to the extract.

Table 9: **Antibacterial activity of crude stem extract produced by using different extraction conditions of *T. laurifolia* against *S. aureus***

Amount of stem powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
5	0	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	25	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	50	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	75	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
10	0	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	25	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	50	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	75	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

***Table 9*: Antibacterial activity of crude stem extract produced by using different extraction conditions of *T. laurifolia* against *S. aureus* (Cont.)**

Amount of stem powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
15	0	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	25	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	50	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00
	75	24	0.00 ± 0.00
		48	0.00 ± 0.00
		72	0.00 ± 0.00

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Comparing the antimicrobial activity of crude extract against all three microbes, the highest activity was seen against *B. cereus*. The overall antimicrobial potential of the extract was low, as the highest inhibition zone obtained was also a very low value. The active compounds in stem were not effective antimicrobial agents. The activity was higher against Gram positive rods such as *B. cereus*. No previous researches focused on properties of stem of *T. laurifolia*, though it is believed to have antioxidant and phenolic compounds that have helped in detoxification in ancient times (Thongsaard and Marsden, 2002). No specific study highlighted the antimicrobial ability of the stem extract hence comparisons could not be made. From this experiment, the stem was not suitable for use as natural antimicrobial source due to low activity. However, the results may vary depending on the source of plant, concentration of active compounds in the extract, strain of microbe etc.

3. ANTIMICROBIAL ACTIVITY OF CRUDE RHIZOME EXTRACT

3.1. Activity against *Escherichia coli*

Among the three factors of extraction, only concentration of ethanol solvent significantly affects the antimicrobial activity of the crude rhizome extract against *E. coli* ($p<0.05$). There was no significant interaction among any of the factors ($p > 0.05$). This showed that the concentration of active compound, which was dependent on amount of powder and time of extraction, was not a significant factor. Concentration of ethanol solvent significantly affected the type of compound predominantly present in the extract, which influenced the activity.

Table 10*: Antibacterial activity of crude rhizome extract produced by using different extraction conditions of *T. laurifolia* against *E. coli

Amount of rhizome powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
5	0	24	0.33 ± 0.26^{abc} *
		48	0.42 ± 0.20^{ab}
		72	0.33 ± 0.26^{abc}
	25	24	0.00 ± 0.00^{d}
		48	0.08 ± 0.20^{cd}
		72	0.08 ± 0.20^{cd}
	50	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}
	75	24	0.00 ± 0.00^{d}
		48	0.00 ± 0.00^{d}
		72	0.00 ± 0.00^{d}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Table 10: **Antibacterial activity of crude rhizome extract produced by using different extraction conditions of *T. laurifolia* against *E. coli* (Cont.)**

Amount of rhizome powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
10	0	24	0.42 ± 0.20 ^{ab} *
		48	0.42 ± 0.20 ^{ab}
		72	0.33 ± 0.26 ^{abc}
	25	24	0.17 ± 0.26 ^{bcd}
		48	0.08 ± 0.20 ^{cd}
		72	0.08 ± 0.20 ^{cd}
	50	24	0.00 ± 0.00 ^{d}
		48	0.00 ± 0.00 ^{d}
		72	0.00 ± 0.00 ^{d}
	75	24	0.00 ± 0.00 ^{d}
		48	0.00 ± 0.00 ^{d}
		72	0.08 ± 0.20 ^{cd}
15	0	24	0.50 ± 0.00 ^{a}
		48	0.42 ± 0.20 ^{ab}
		72	0.25 ± 0.27 ^{abcd}
	25	24	0.08 ± 0.20 ^{cd}
		48	0.00 ± 0.00 ^{d}
		72	0.08 ± 0.20 ^{cd}
	50	24	0.00 ± 0.00 ^{d}
		48	0.00 ± 0.00 ^{d}
		72	0.00 ± 0.00 ^{d}
	75	24	0.00 ± 0.00 ^{d}
		48	0.08 ± 0.20 ^{cd}
		72	0.17 ± 0.26 ^{bcd}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

It was seen that most activity was observed at aqueous extract (0% ethanol) and some activity at 25% ethanol. As amount of rhizome powder was increased, some activity was observed at 75% ethanol as well. However, this effect was not significant. Most of the antimicrobial activity of this extract was due to phenolic compounds or other polar compounds with high affinity for water. Some non-polar compounds also showed mild activity, as inhibition zone was observed in ethanolic extracts. The inhibition zone increased and decreased with time of extraction, indicating that the effect of this factor was not significant. In this case, the concentration of the active compound was not significant for the activity. The type of compound – polar or non-polar was the main factor influencing the activity of the extract against *E.coli*.

Table 10 shows the statistically analyzed inhibition zones obtained for *E. coli* using crude rhizome extract. The overall activity of the extract was quite low. Many conditions gave significantly non-different inhibition zones. However, all the conditions used 0% ethanol as the solvent. The best condition was chosen based on economic criteria i.e. the lowest possible amount of rhizome powder and the lowest possible time of extraction. This could save cost of raw material and operation cost for conducting the extraction process. Thus, the optimum condition was chosen to be extraction using 5% (w/v) of rhizome powder with 0% ethanol (water) extracted for 24 hours. The highest inhibition zone value was 0.50 ± 0.00 mm, which was not very high. Thus, the antimicrobial activity of crude rhizome extract against *E. coli* was low (*Table 3*).

3.2. Activity against *Bacillus cereus*

All three factors had significant effect on antimicrobial activity of crude rhizome extract against *B. cereus* ($p<0.05$). There was significant interaction among all the factors all three together ($p<0.05$). The concentration of ethanol influenced the type of compounds predominant in the crude extract, while the other factors influenced the level of these active compounds in the extract. Time of extraction and amount of powder used interacted with concentration of ethanol to influence the concentration of polar or non-polar compounds in the crude rhizome extract.

Table 11: **Antibacterial activity of crude rhizome extract produced by using different extraction conditions of *T. laurifolia* against *B. cereus***

Amount of rhizome powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
5	0	24	0.00 ± 0.00^{l} *
		48	0.00 ± 0.00^{l}
		72	0.17 ± 0.26^{l}
	25	24	0.08 ± 0.20^{l}
		48	0.00 ± 0.00^{l}
		72	0.33 ± 0.26^{l}
	50	24	5.17 ± 0.26^{hi}
		48	5.42 ± 0.49^{ghi}
		72	4.17 ± 0.26^{k}
	75	24	6.00 ± 0.00^{de}
		48	5.08 ± 0.20^{ij}
		72	5.50 ± 0.32^{fgh}
10	0	24	0.00 ± 0.00^{l}
		48	0.17 ± 0.26^{l}
		72	0.00 ± 0.00^{l}
	25	24	0.08 ± 0.20^{l}
		48	0.17 ± 0.26^{l}
		72	0.25 ± 0.27^{l}
	50	24	5.58 ± 0.38^{fg}
		48	5.83 ± 0.52^{ef}
		72	4.75 ± 0.27^{j}
	75	24	6.58 ± 0.20^{b}
		48	6.17 ± 0.41^{cde}
		72	5.42 ± 0.20^{ghi}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Table 11: **Antibacterial activity of crude rhizome extract produced by using different extraction conditions of *T. laurifolia* against *B. cereus* (Cont.)**

Amount of rhizome powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
15	0	24	0.00 ± 0.00^{l} *
		48	0.00 ± 0.00^{l}
		72	0.00 ± 0.00^{l}
	25	24	0.00 ± 0.00^{l}
		48	0.17 ± 0.26^{l}
		72	0.33 ± 0.26^{l}
	50	24	6.00 ± 0.32^{de}
		48	6.33 ± 0.82^{bcd}
		72	5.08 ± 0.20^{ij}
	75	24	7.25 ± 0.27^{a}
		48	6.42 ± 0.49^{bc}
		72	5.50 ± 0.32^{fgh}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

It was seen that most of the antimicrobial activity was observed in ethanolic extracts i.e. 50% and 75% ethanol. Very low activity was seen at 0% ethanol and 25% ethanol concentration. This indicated that the major compounds responsible for the antimicrobial effect were non-polar compounds, possibly non-polar phenolics predominant in the extract with high ethanol concentration. The interaction between the factors was synergistic i.e. increase in the factors was simultaneous. The inhibition zone at each ethanol concentration increased with increased amount of powder used. However, the relation with the time of extraction was antagonistic i.e. lower time of extraction gave higher inhibition zone. The vast difference between the inhibition zones obtained in ethanolic and aqueous extract indicated that non-polar active compounds in the extracts had more effectiveness in inhibiting *B. cereus*. Polar compounds were present in both ethanolic and aqueous extracts. Since the activity in aqueous extracts where they were

predominant, was low, it indicated that polar phenolic compounds in rhizome extract were not very effective against *B. cereus,* but showed increased activity with higher amount of rhizome powder used. The activity in ethanolic extracts was mainly due to non-polar compounds. Currently, little to no research has been conducted regarding the nature of active compounds found in rhizome of *T. laurifolia*, thus identifying the specific polar or non-polar compounds in rhizome requires more extensive research which is outside the scope of this experiment.

Statistical analysis of inhibition zones obtained for crude rhizome extract against *B. cereus* is shown in *Table 11*. The highest antimicrobial activity of the extract was observed with 15% (w/v) rhizome powder using 75% ethanol and extraction carried out for 24 hours. The highest amount of powder was required to achieve maximum concentration of active compounds that gave highest activity. 75% ethanol extract consisted of highest amount of non-polar compounds that showed the best inhibition of *B. cereus*. Since the interaction effect with time of extraction was found to be antagonistic, the lowest extraction time gave the highest inhibition zone. Overall activity in ethanolic extract was quite high (*Table 3*), even at low amount of rhizome powder used. The highest inhibition zone obtained was 7.25 ± 0.27 mm, which was the highest out of all treatments studied in the whole experiment.

3.3. Activity against *Staphylococcus aureus*

All three factors had significant effect on antimicrobial activity of crude rhizome extract against *B. cereus* ($p<0.05$). There was significant interaction among all the factors ($p<0.05$), except between amount of powder and time of extraction. The concentration of ethanol influenced the type of compounds predominant in the crude extract, while the other factors influenced the level of these active compounds in the extract. Time of extraction and amount of powder used interacted with concentration of ethanol to influence the concentration of polar or non-polar compounds in the crude rhizome extract.

It was seen that only ethanolic extracts (50% and 75% ethanol) had antimicrobial activity against *S. aureus*. The major compounds responsible for the inhibition were non-

polar compounds, such as flavonoids or non-polar phenolic compounds. Inhibition zones increased with increase in amount of powder used, due to increased concentration of active non-polar compounds in the extract. Higher ethanol concentration gave higher inhibition zones as it contained the highest amount of non-polar active compounds. Synergistic interaction was observed between ethanol concentration and time of extraction, as well as with amount of powder used. The interaction between time of extraction and amount of powder was not significant, and thus it did not show a fixed pattern of increase or decrease. No activity was observed in aqueous extracts, indicating the inability of polar active compounds in the crude extract to inhibit *S. aureus*. No effect was observed even at the highest amount of powder used and longest time of extraction.

Table 12*: Antibacterial activity of crude rhizome extract produced by using different extraction conditions of *T. laurifolia* against *S. aureus

Amount of rhizome powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
5	0	24	0.00 ± 0.00^{h} *
		48	0.00 ± 0.00^{h}
		72	0.00 ± 0.00^{h}
	25	24	0.00 ± 0.00^{h}
		48	0.00 ± 0.00^{h}
		72	0.00 ± 0.00^{h}
	50	24	2.17 ± 0.41^{g}
		48	3.17 ± 0.41^{e}
		72	3.00 ± 0.00^{ef}
	75	24	3.67 ± 0.52^{d}
		48	4.00 ± 0.00^{bc}
		72	3.75 ± 0.42^{cd}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Table 12: **Antibacterial activity of crude rhizome extract produced by using different extraction conditions of *T. laurifolia* against *S. aureus* (Cont.)**

Amount of rhizome powder (% w/v)	Concentration of ethanol (%)	Time of extraction (h)	Inhibition zone (mm)
10	0	24	0.00 ± 0.00^{h} *
		48	0.00 ± 0.00^{h}
		72	0.00 ± 0.00^{h}
	25	24	0.00 ± 0.00^{h}
		48	0.00 ± 0.00^{h}
		72	0.00 ± 0.00^{h}
	50	24	2.75 ± 0.27^{f}
		48	3.00 ± 0.00^{ef}
		72	3.08 ± 0.20^{e}
	75	24	4.17 ± 0.75^{b}
		48	4.00 ± 0.00^{bc}
		72	4.00 ± 0.00^{bc}
15	0	24	0.00 ± 0.00^{h}
		48	0.00 ± 0.00^{h}
		72	0.00 ± 0.00^{h}
	25	24	0.00 ± 0.00^{h}
		48	0.00 ± 0.00^{h}
		72	0.00 ± 0.00^{h}
	50	24	2.92 ± 0.20^{ef}
		48	3.17 ± 0.41^{e}
		72	3.58 ± 0.49^{d}
	75	24	4.17 ± 0.41^{b}
		48	4.67 ± 0.52^{a}
		72	4.08 ± 0.20^{b}

Note: *Different superscript in the same column represents significantly different values ($p < 0.05$)

Statistical analysis of inhibition zones obtained for crude rhizome extract against *S. aureus* is shown in *Table 12*. The highest antimicrobial activity of the extract was observed with 15% (w/v) rhizome powder using 75% ethanol and extraction carried out for 48 hours. The highest amount of powder was required to achieve maximum concentration of active compounds that gave the highest activity. 75% ethanol extract consisted of the highest amount of non-polar compounds that showed the best inhibition of *S. aureus*. Extraction time of 48 hours gave the highest inhibition zone. Overall activity in ethanolic extract was quite high, even at low amount of rhizome powder used. The highest inhibition zone obtained was 4.67 ± 0.52 mm. The activity of crude rhizome extract against *S. aureus* was moderate (*Table 3*).

Comparing the activity of crude rhizome extract of *T. laurifolia* against all three microorganisms, it was seen that its effectiveness in inhibitory action was highest in case of *B. cereus*. This activity was mainly due to non-polar active compounds in the crude extract. Also it was seen that the extract was more effective in inhibiting Gram positive microorganisms such as *B. cereus* and *S. aureus*. The low antimicrobial activity observed in case of *E. coli* was due to polar compounds in the aqueous extract. In previous researches and reviews, there was not much information present regarding potential of rhizome as a source of natural antioxidants or antimicrobials. There is no research highlighting the type of active compounds found in rhizome which could be used to understand the activity in detail. However, from the results obtained in this experiment, rhizome extract of *T. laurifolia* showed good potential to be used as a substitute for chemical preservatives.

Table 13: **Summary of the extraction condition of crude extract from different parts of *T. laurifolia* showing the best antimicrobial activity for each test microorganism along with respective Total Phenolic Content (mg/ml)**

Microorganism	Part Of Plant	Best Extraction Condition			Inhibition zone (mm)	Total Phenolic Content (mg/ml)
		Amount of powder (% w/v)	Concentration of ethanol solvent (%)	Time of extraction (h)		
Escherichia coli	**Leaf**	**15**	**50**	**48**	**4.67 ± 0.52 [a]**	**235.41 ± 149.44**
	Stem	5	0	24	0.50 ± 0.32 [b]	182.08 ± 104.72
	Rhizome	5	0	24	0.33 ± 0.26 [b]	27.85 ± 23.23
Bacillus cereus	Leaf	15	25	48	0.75 ± 0.88 [b]	186.44 ± 17.77
	Stem	10	75	72	1.08 ± 0.20 [b]	109.66 ± 34.40
	Rhizome	**15**	**75**	**24**	**7.25 ± 0.27 [a]**	**699.46 ± 108.33**
Staphylococcus aureus	Leaf	-	-	-	-	-
	Stem	-	-	-	-	-
	Rhizome	**15**	**75**	**48**	**4.67 ± 0.52**	**887.54 ± 39.91**

Note: - Different superscript in the same column represent significantly different values ($p < 0.05$).

- Statistical analysis for each microbe is performed separately

(TPC Source: Catherine Nabbala 2018)

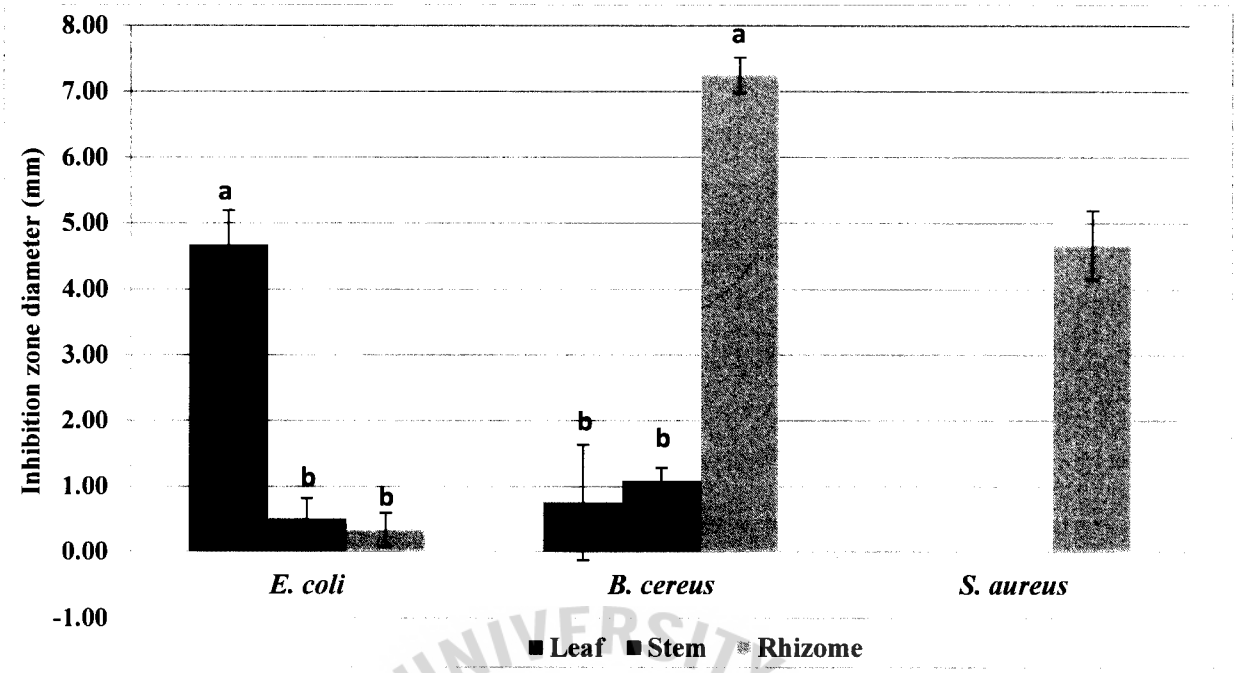

Figure 8: **Comparison of antimicrobial activity of crude extract produced from the best extraction condition of three parts of *T. laurifolia* for each microorganism**

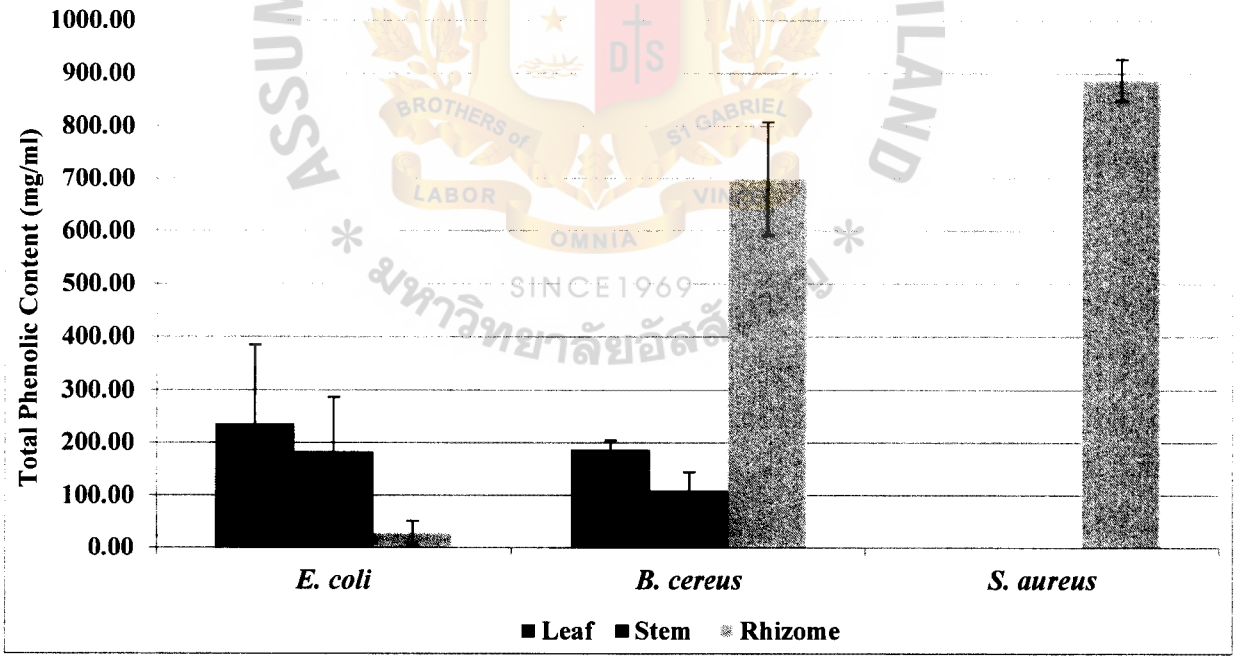

Figure 9: **Comparison of Total Phenolic Content (mg/ml) of crude extract produced from the best extraction condition of three parts of *T. laurifolia* for each microorganism**

The best extraction condition for each part of *T. laurifolia* showing the highest antimicrobial activity against all three microorganisms is summarized in *Table 13*. The best conditions of each microbe were compared to determine the part of plant that showed the highest effectiveness in inhibiting the respective microorganism (*Figure 8*). In case of the Gram negative rod *E. coli*, leaf extract of *T. laurifolia* was the most effective in inhibition, giving an inhibition zone of 4.67 ± 0.52 mm. The activity of leaf extract against *E. coli* was significantly better than that of stem and rhizome extract ($p<0.05$). The extraction carried out to obtain the highest activity must include use of 15% (w/v) of leaf powder, 50% ethanol as solvent and the mixture extracted for 48 hours. In case of Gram positive rod *B. cereus*, rhizome extract of *T. laurifolia* showed the best antimicrobial activity. This activity was the highest compared to all other conditions studied in this experiment. The inhibition zone obtained was 7.25 ± 0.27 mm. The best extraction condition included preparation of ethanolic extract using 75% ethanol as solvent, 15% (w/v) of rhizome powder and 24 hours of extraction. Finally, in case of Gram positive coccus *S. aureus*, the part of plant showing the highest activity was also found to be the rhizome. The extract with the highest activity was also ethanolic, using 75% ethanol as solvent, 15% (w/v) of rhizome powder and 48 hours of extraction. This condition provided the highest inhibition zone for *S. aureus* as 4.67 ± 0.52 mm. Among all the three microorganisms, *B. cereus* showed the highest sensitivity to the active compounds in *T. laurifolia* in all three parts of plant overall. This microbe was significantly inhibited by all the three parts of the plant.

It was also noted that the parts of plant showing the highest antimicrobial activity for each microbe also showed the highest Total Phenolic Content (TPC) (mg/ml) for that particular extraction condition (*Figure 9*). This indicated that the group of compounds responsible for the antimicrobial activity of the extract could be phenolic compounds, both polar and non-polar. However, since TPC represents a big group of a wide variety of compounds, identification of the specific compound responsible for the activity in each case requires further detailed research. The specific phenolic compounds present in different parts of the plant may be different, which was observed as varied response to the same microorganism by the same group of compounds. Comparing the activity of different parts of *T. laurifolia*, it was concluded that leaf extract had higher ability to

inhibit Gram negative microorganisms such as *E. coli* compared to stem and rhizome. Similarly, rhizome extract showed better ability to inhibit Gram positive microorganisms, especially Gram positive rods like *Bacillus* sp. which were more sensitive.

The mechanism of action of the phenolic compounds on microorganisms could not be determined with certainty unless the specific compound responsible for the antimicrobial action was identified. However, an estimation of the mechanism can be made based on general action of cell disruption by phenolic compounds. Theoretically, Gram positive cells are more sensitive to antimicrobial agents due to the presence of thick peptidoglycan layer in their cell wall in contrast to Gram negative cells, which contain thin peptidoglycan layer and an outer lipopolysaccharide membrane. The single peptidoglycan layer in Gram positive cells makes it easier for the antimicrobial agents to weaken it. Phenolic compounds function effectively as antibacterial agents due to their partial hydrophobic nature. They interact with the lip-water interface of the bacterial membrane, causing it to lose its plasticity. This leads to destabilization and subsequent disruption of cell membrane and transport system (Resende et al., 2015). The polar and non-polar polyphenols interact with the membrane in different way and cause instability of the bacterial system. The action also depends on number of hydroxyl groups (-OH) in the specific compound. Some compounds are more successful than the others depending on their specific structure, which is seen as varied activity for different microorganisms. Other studies showed specific phenolic compounds like *p*-coumaric acid killing bacteria by disrupting membrane and binding to its genomic DNA to impair cellular functions (Lou, Z. et al, 2012). Chlorogenic acid was found to damage *S. aureus* cells by membrane hyperpolarization due to pH changes or increased ion movement across the membrane (Li, G. et al., 2014; Bot and Prodan, 2009). Another study conducted by Wu, Y. et al. (2016) showed the action of 3-*p*-*trans*-Coumaroyl-2-hydroxyquinic acid (CHQA) on *S. aureus* causing increase in membrane fluidity due to interaction with membrane lipids and proteins and changing the conformation. Since this experiment only considers the possibility of phenolic compounds being the main antimicrobial agents in *T. laurifolia*, the mechanism of action cannot be determined for sure. Further research in identification of these specific compounds can provide a better picture of the antimicrobial action.

CONCLUSION

T. laurifolia, a plant native to parts of Asia including Thailand, has potential to be used as a natural antimicrobial agent. Crude extract from leaves, stem and rhizome of the plant showed antimicrobial activity differently for different microorganisms. Crude leaf extract showed good inhibitory activity against Gram negative microorganisms such as *E. coli*, while crude rhizome extract was more effective against Gram positive microorganisms such as *B. cereus* and *S. aureus*. Stem showed little to no antimicrobial effect against all three microorganisms. The highest sensitivity against natural polyphenols was observed in case of *B. cereus*. Polyphenols were found to be possible compounds responsible for the antimicrobial activity of extract from *T. laurifolia* and the possible mechanism involved cell disruption due to impairment of membrane fluidity, transport system and hyperpolarization. Out of all the parts of the plant, rhizome showed the best potential to be used as natural substitute for antimicrobial agents used in food industry. However, further research is necessary in order to determine the specific compounds responsible for the activity and to obtain proper delivery systems of these compounds to be used in food systems.

REFERENCES

Aritajat, S., Wutteerapol, S. and Saenphet, K. (2004). Antidiabetic effect of *Thunbergia laurifolia* Linn. aqueous extract. *The Southeast Asian Journal of Tropical Medicine and Public Health* 35(2), 53-57

Boonyarikpunchai, W., Sukrong, S.. and Towiwat, P. (2014) Antinociceptive and anti-inflammatory effects of rosmarinic acid isolated from *Thunbergia laurifolia* Lindl. *Pharmacol Biochem Behav* 124, 67-73.

Bot, C.; Prodan, C. Probing the membrane potential of living cells by dielectric spectroscopy. Eur. Biophys. J. 2009, 38, 1049–1059

Burkill I.H (1966) A dictionary of the economic products of the Malay Peninsula. Volume II (I–Z). Ministry of Agriculture and Cooperatives, Kuala Lumpur

Chan, E.W.C. and Lim, Y.Y. (2006), Antioxidant Activity of *Thunbergia laurifolia* tea, *Journal of Tropical Forest Science*, 18(2), 130-136

Chan, E.W.C., Eng, S.Y., Tan, Y.P. and Wong, Z.C. (2011), Phytochemistry and Pharmacological Properties of *Thumbergia laurifolia*: A Review, *Pharmacology Journal*, 3(24)

Chanawirat, A., Toshulkao, C., Temcharoen, P. and Glinsukon, T. (2000), Protective effect of *Thunbergia laurifolia* extract on ethanol-induced hepatotoxicity in mice, (Unpublished thesis), Faculty of Graduate Studies, Mahidol University, Bangkok, Thailand

Charumanee S, Vejabhikul S, Taesotikul T, Netsingha W, Sirisaard P, Leelapornpisit P. Development of topical anti-inflammatory preparations from *Thunbergia laurifolia* Lindl. Research Report, Faculty of Pharmacy, Chiangmai University, Chiangmai, Thailand. (1998).

Chattaviriya, P., Morkmek, N., Lertprasertsuke, N. and Ruangyuttikarn, W. (2010), Drinking *Thunbergia laurifolia* Lindl. leaf extract helps prevent renal toxicity induced by cadmium in rats, *Thai J Toxicol* , 25(2), 124-32

Cheeptham, N. and Towers, G. H. N. (2002), Light-mediated activities of some Thai medicinal plant teas, *Fitoterapia* 73(7-8), 651-662

Chinacarawat, N., Kiettinun, S., Amatayakul, C., Jaiaree, N., Itharat, A. and Chinsoi, P. 2012. Study on the efficacy and side effects of Thunbergia laurifolia Lindl. extract on reducing chemical toxicity among agricultural workers receiving organophosphate and carbamate insecticide poisoning (clinical trial phase II). Thammasat Medical Journal 12(2): 496-505.

Chivapat S, Chavalittumrong P, Attawish A, Bansiddhi J, Padungpat S. Chronic toxicity of *Thunbergia laurifolia* Lindl. extract. J Thai Tradit AlternMed 2009; 7(1):17-24.

Chuthaputti, A. (2010), Rang Jerd: Laurel Clock Vine (*Thunbergia laurifolia* Lindl.): a detoxifying herb, *Journal of Thai Traditional & Alternative Medicine,* 8(2-3), 211-220

Jaiboon V, Boonyanupahap J, Suwansri S, Ratanatraiwong P, Hansawasdi C. Alpha amylase inhibition and roasting time of local vegetables and herbs prepared for diabetes risk reduction chili paste. Asian J Food Ag-Ind 2011; 3(1):1-12

Jetawattana S., Boonsirichai K., Charoen S. and Martin S.M. (2015), Radical intermediate generation and cell cycle arrest by aqueous extract of *Thunbergia laurifolia* Linn. In human breast cancer cells, *Asian Pac J Cancer Prev*, 16(10), 4357-4361

Junsi, M., Siripongvutikorn, S., Yupanqui C.T. and Usawakesmanee W. (2017), Phenolic and flavonoid compounds in aqueous extracts of *Thunbergia laurifolia* leaves and their effect on the toxicity of the carbamate insecticide methomyl to murine macrophage cells, *Functional Foods in Health and Disease*, 7(7), 529-544

Kanchanapoom, T., Kasai, R. and Yamasaki, K. (2002), Iridoid glucosides from *Thunbergia laurifolia*, *Phytochemistry*, 60, 769-771

Khobjai, W., Jaihan U., Watcharasamphankul, W. and Somsak, V. (2014), Protective effect of *Thunbergia laurifolia* extract on hemolysis during *Plasmodium berghei* infection, *parasitology Research*, 113(5), 1843-1846

Kwansang, J., Itthipanichpong, C. and Limpanasithikul, W. (2015), Evaluation of wound healing activity of *Thunbergia laurifolia* supercritical carbon dioxide extract in

rats with second-degree burn wounds, *Journal of Advanced Pharmacological Technology & Research,* 6(3):103-7

Li, G.; Wang, X.; Xu, Y.; Zhang, B.; Xia, X. Antimicrobial effect and mode of action of chlorogenic acid on Staphylococcus aureus. Eur. Food Res. Technol. 2014, 238, 589–596.

Liu, M., Dai, Y., Li, Y., Luo, Y., Huang, F. and Gong, Z. (2008), Madecassoside isolated from *Centella asiatica* herbs facilitates burn wound healing in mice, *Planta Med* 74(8), 809-815

Lou, Z.; Wang, H.; Rao, S.; Sun, J.; Ma, C.; Li, J. p-Coumaric acid kills bacteria through dual damage mechanisms. Food Control 2012, 25, 550–554.

Moongkarndi P, Kosem N, Luanratana O, Jongsomboonkusol S, Pongpan N. Anti-proliferative activity of Thai medicinal plant extracts on human breast adenocarcinoma cell line. Fitoterapia 2004; 75:375-7.

Nanna, U., Chiruntanat, N., Jaijoy, K., Rojsanga, P. and Sireeratawong, S. (2017), Effect of *Thunbergia laurifolia* Lindl. Extract on Anti-Inflammatory, Analgesic and Antipyretic Activity, *J Med Assoc Thai*, 100(Suppl. 5), S98-S106

Oonsivilai, R., Cheng, C., Bomser, J., Ferruzzi, M. G. and Ningsanond, S. (2007), Phytochemical profiling and phase II enzyme-inducing properties of *Thunbergia laurifolia* Lindl. (RC) extracts. *Journal of Ethnopharmacology* 114(3), 300-306

Oonsivilai, R., Ferruzi, M.G. and Ningsanond, S. (2008), Antioxidant activity and cytotoxicity of Rang Cheut (*Thunbergia laurifolia* Lindl.) extracts, *Asian Journal of Food and Agro-Industry*, 1(02), 116-128

Pitoolpong N, Kanthawat S, Thaipradist S, Singh R. Effect of Thunbergia laurifolia Linn. Extract in hyperglycemic cats. The Veterinary Practitioner Association of Thailand (VPAT), The Processing of The 8th VPAT Regional Veterinary Congress, Thailand, 18-21 May 2014, pp.23-26

Pongphasuk N, Khunkitti W, Chitcharoenthum M. Anti-inflammatory and analgesic activities of the extract from *Garcinia mangostana* Linn. Acta Hortic 2005; 680:125-30.

Pramyothin, P., Chirdchupunsare, H., Rungsipipat, A. and Chaichantipyuth, C. (2005). Hepatoprotective activity of *Thunbergia laurifolia* Linn extract in rats treated with ethanol: in vitro and in vivo studies. *Journal of Ethnopharmacol* 102(3), 408-411

Przeor, M. and Flaczyk, E. (2016), Antioxidant properties of Paratha type flat bread enriched with white mulberry leaf extract, *Indian Journal of Traditional Knowledge*, 15(2), 237-244

Pukumpuang, W., Thongwai, N. and Tragoolpua, Y. (2012). Total phenolic contents, antibacterial and antioxidant activities of some Thai medicinal plant extracts, *Journal of Medicinal Plants Research*, 6(35), 4953- 4960

Purnima, M. and Gupta, P.C. (1978), Colouring matters from the flowers of *Thunbergia laurifolia*, *J Indian Chem Soc,* 55(6), 622-3

Resende F.A., Nogueira, L.G., Bauab, T.M., Vilegas, W. and Varanda, E.A. (2015), Antibacterial potential of flavonoids with different hydroxylation patterns, *Eclética Química*, 40, 173-179

Sen, S., Chakraborty, R., Sridhar, C. and Reddy, Y.S.R. (2010), Free radicals, antioxidants, diseases and phytomedicines: Current status and future prospect, *International Journal of Pharmaceutical Sciences and Research*, 3, 91-100

Sultana K.H, Chatterjee S, Roy A and Chandra I. (2015) Ethnopharmacological and Phytochemical Review on *Thunbergia sp.*. *Medicinal & Aromat Plants* 4(5), 217

Suwanchaikasem, P., Phadungcharoen, T. and Sukrong, S. (2013), Authentication of Thai medicinal plants shariong common name 'Rang Chuet': *Thunbergia laurifolia*, *Crotalaria spectabilis* and *Curcuma* aff. *amada* by combined techniques of TLC, PCR-RFLP fingerprints and antioxidant activities, *ScienceAsia39*, 124-133

Tangpong, J. and Satarug, S. (2010), Alleviation of lead poisoning in the brain with aqueous leaf extract of *Thunbergia laurifolia* (Lindl.), *Toxicol Lett*,198, 83-88

Tejasen, P. and Thonthapp, C. (1980), The study of the insecticide antitoxicity of *Thunbergia laurifolia* Linn., *Chiang Mai Med. Bull.*, 19, 105-114

Thongsaard, W. and Marsden, C.A. (2002), A herbal medicine used in the treatment of addiction mimics the action of amphetamine on in vitro rats trial dopamine release, *Neuroscience Letters*, 329 (2), 129-132

Thongsaard, W., Marsden, C.A., Morris, P., Prior, M. and Shah, Y.B. (2005), Effect of *Thunbergia laurifolia*, a Thai natural product used to treat drug addiction, on cerebral activity detected by functional magnetic resonance imaging in the rat, *Psychopharmacology* 180: 752- 760

Usanawarong, S., Thesiri, T., Mahakunakorn, P. and Parasupattana, S. (2000), Effect of *Thubergia laurifolia* Linn. on Detoxication of Paraquat, *Department of Pharmacognosy and Toxicology, Khonkaen University.*

Widyawati, P.S., Budianta, T.D.W., Kusuma, F.A. and Wijaya, E.L. (2014), Difference of Solvent Polarity to Phytochemical content and Antioxidant Activity of *Pluchea indica Less* leaves extracts, *International Journal of Pharmacology and Phytochemical Research,* 6(4), 850-855

Wonkchalee, O., Boonmars, T., Aromdee, C., Laummaunwai, P., Khunkitti, W., Vaeteewoottacharn, K., Sriraj, P., Aukkanimart, R., Loilome, W., Chamgramol, Y., Pairojkul, C., Wu, Z., Juasook, A. and Sudsarn, P. (2012), Anti-inflammatory, antioxidant and hepatoprotective effects of *Thunbergia laurifolia* Linn. on experimental opisthorchiasis. *Parasitology Research* 111(1), 353-359

Wu, Y., Bai, J., Zhong, K., Huang, Y., Qi, H., Jiang, Y. and Gao, H. (2016), Antibacterial activity and Membrane-disruptive Mechanism of 3-*p-trans*-Coumaroyl-2-hydroxyquinic acid, a Novel Phenolic Compound from Pine Needles of *Cedrus deodara*, against *Staphylococcus aureus*, *Molecules*, 21, 1084

APPENDIX

R-Program Code and Output

CODE:

```
attach(Dataset)
Dataset
Fact.RCBD<-aov(y~a+b+c+rep+dup+a:b+b:c+a:c+a:b:c)
summary(Fact.RCBD)
attach(Dataset)
RCBD<-aov(y~trt+rep+dup,data=Dataset)
summary(RCBD)
library(agricolae)
attach(Dataset)
model<-aov(y~trt, data=Dataset)
comparison<-duncan.test(model,"trt",main="y dealt with different trt")
duncan.test(model,"trt",alpha=0.05,console=TRUE)
```

OUTPUT:

Crude leaf extract:

1. ***Escherichia coli***

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	3.03	1.52	11.479	2.05e-05 ***
b	3	8.58	2.86	21.636	5.51e-12 ***
c	2	151.99	75.99	575.031	< 2e-16 ***
rep	1	0.31	0.31	2.356	0.1266
dup	2	2.97	1.48	11.227	2.57e-05 ***
a:b	6	15.39	2.57	19.409	< 2e-16 ***
b:c	6	2.96	0.49	3.733	0.0016 **
a:c	4	3.89	0.97	7.367	1.64e-05 ***
a:b:c	12	23.88	1.99	15.056	< 2e-16 ***
Residuals	177	23.39	0.13		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	209.72	5.992	45.341	< 2e-16 ***
rep	1	0.31	0.311	2.356	0.127
dup	2	2.97	1.484	11.227	2.57e-05 ***
Residuals	177	23.39	0.132		
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Mean Square Error: 0.1481667

Means with the same letter are not significantly different.

Groups, Treatments and means		
a	32	4.667
b	14	3.417
b	11	3.333
bc	5	3.083
bc	17	3
bc	2	3
bc	26	3
cd	29	2.667
de	8	2.25
ef	23	2
efg	20	1.917
efgh	35	1.833
fghi	15	1.75
fghi	27	1.75
fghij	6	1.667
fghijk	3	1.583
fghijkl	31	1.5
ghijklm	18	1.417
hijklmn	30	1.333
ijklmn	25	1.25
jklmn	21	1.167
jklmn	34	1.167
jklmn	36	1.167
klmn	22	1.083
klmn	9	1.083
lmn	12	1
lmn	16	1
lmn	24	1
lmn	33	1
lmn	4	1
mno	13	0.95
mno	28	0.9167
no	1	0.8333
op	7	0.4833
p	19	0.3333
p	10	0.1667

2. *Bacillus cereus*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	0.752	0.3762	4.239	0.015909 *
b	3	1.847	0.6157	6.939	0.000192 ***
c	2	0.929	0.4646	5.236	0.006175 **
rep	1	0.013	0.0134	0.151	0.698250
dup	2	0.640	0.3198	3.604	0.029236 *
a:b	6	0.643	0.1072	1.208	0.304120
b:c	6	0.382	0.0636	0.717	0.636182
a:c	4	1.025	0.2564	2.889	0.023812 *
a:b:c	12	1.049	0.0874	0.985	0.464862
Residuals	177	15.705	0.0887		

Signif. codes:	0	'***' 0.001	'**' 0.01	'*' 0.05	'.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	6.628	0.1894	2.134	0.000717 ***
rep	1	0.013	0.0134	0.151	0.698250
dup	2	0.640	0.3198	3.604	0.029236 *
Residuals	177	15.705	0.0887		

Signif. codes:	0	'***' 0.001	'**' 0.01	'*' 0.05	'.' 0.1 ' ' 1

Mean Square Error: 0.09087963

Means with the same letter are not significantly different.

Groups, Treatments and means		
a	29	0.75
ab	35	0.5
abc	32	0.4167
abcd	24	0.3333
abcd	33	0.3333
abcd	36	0.3333
abcd	7	0.3333
abcd	8	0.3333
abcd	23	0.3333
abcd	18	0.25
abcd	20	0.25
abcd	21	0.25
abcd	6	0.1667
abcd	30	0.1667
abcd	31	0.1667
abcd	9	0.1667
bcd	22	0.1167
cd	26	0.08
cd	34	0.08
cd	11	0.08
cd	12	0.08
cd	19	0.08
cd	10	0.03
cd	25	0.03
d	1	0
d	2	0
d	3	0
d	4	0
d	5	0
d	13	0
d	14	0
d	15	0
d	16	0
d	17	0
d	27	0
d	28	0

> duncan.test(model,"trt",alpha=0.0

3. *Staphylococcus aureus*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	0.00454	0.0022685	1.690	0.188
b	3	0.00310	0.0010340	0.770	0.512
c	2	0.00176	0.0008796	0.655	0.521
rep	1	0.00227	0.0022685	1.690	0.195
dup	2	0.00176	0.0008796	0.655	0.521
a:b	6	0.00620	0.0010340	0.770	0.594
b:c	6	0.00898	0.0014969	1.115	0.355
a:c	4	0.00352	0.0008796	0.655	0.624
a:b:c	12	0.01796	0.0014969	1.115	0.351
Residuals	177	0.23764	0.0013426		
Signif. codes:	0	'***' 0.001	'**' 0.01	'*' 0.05	'.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	0.04606	0.0013161	0.980	0.507
rep	1	0.00227	0.0022685	1.690	0.195
dup	2	0.00176	0.0008796	0.655	0.521
Residuals	177	0.23764	0.0013426		

Signif. codes:	0	'***' 0.001	'**' 0.01	'*' 0.05	'.' 0.1 ' ' 1

Mean Square Error: 0.001342593

Crude stem extract:

1. ***Escherichia coli***

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	0.062	0.0312	1.541	0.21700
b	3	7.429	2.4765	122.129	< 2e-16 ***
c	2	0.090	0.0451	2.226	0.11097
rep	1	0.196	0.1956	9.646	0.00221 **
dup	2	0.257	0.1285	6.336	0.00220 **
a:b	6	0.095	0.0158	0.780	0.58658
b:c	6	0.317	0.0529	2.607	0.01909 *
a:c	4	0.014	0.0035	0.171	0.95287
a:b:c	12	0.106	0.0089	0.438	0.94644
Residuals	177	3.589	0.0203		

Signif. codes:	0	'***' 0.001	'**' 0.01	'*' 0.05	'.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	8.115	0.23185	11.434	< 2e-16 ***
rep	1	0.196	0.19560	9.646	0.00221 **
dup	2	0.257	0.12847	6.336	0.00220 **
Residuals	177	3.589	0.02028		

Signif. codes:	0	'***' 0.001	'**' 0.01	'*' 0.05	'.' 0.1 ' ' 1

Mean Square Error: 0.0224537

Means with the same letter are not significantly different.

Groups, Treatments and means		
a	7	0.5833
ab	1	0.5
ab	14	0.5
ab	2	0.5
ab	8	0.5
abc	13	0.4167
abc	3	0.4167
abc	9	0.4167
bcd	10	0.25
bcd	15	0.25
cd	12	0.1667
cd	16	0.1667
cd	18	0.1667
cd	4	0.1667
d	11	0.08333
d	5	0.08333
d	6	0.08333
d	17	0
d	7	0
d	8	0
d	9	0
d	10	0
d	11	0
d	12	0
d	19	0
d	20	0
d	21	0
d	22	0
d	23	0
d	24	0
d	31	0
d	32	0
d	33	0
d	34	0
d	35	0
d	36	0

duncan.test(model,"trt",alpha=0.05,console=TRUE)

2. *Bacillus cereus*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	1.002	0.501	22.215	2.47e-09 ***
b	3	4.579	1.526	67.653	< 2e-16 ***
c	2	15.738	7.869	348.818	< 2e-16 ***
rep	1	0.074	0.074	3.283	0.071675 .
dup	2	0.100	0.050	2.206	0.113150
a:b	6	0.637	0.106	4.703	0.000182 ***
b:c	6	6.484	1.081	47.901	< 2e-16 ***
a:c	4	0.713	0.178	7.901	6.99e-06 ***
a:b:c	12	0.676	0.056	2.497	0.004757 **
Residuals	177	3.993	0.023		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	29.829	0.8522	37.778	<2e-16 ***
rep	1	0.074	0.0741	3.283	0.0717 .
dup	2	0.100	0.0498	2.206	0.1132
Residuals	177	3.993	0.0226		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Mean Square Error: 0.02314815

Means with the same letter are not significantly different.

Groups, Treatments and means		
a	30	1.083
a	24	1.083
a	6	1
a	18	1
ab	36	0.9167
ab	27	0.8333
bc	15	0.6667
cd	35	0.5
cd	12	0.5
cde	3	0.4167
def	26	0.25
ef	29	0.1667
ef	22	0.1667
f	28	0.08333
f	32	0.08333
f	23	0.08333
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0
f	1	0

```
duncan.test(model,"trt",alpha=0.05,console=T
```

3. *Staphylococcus aureus*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	0	0		
b	3	0	0		
c	2	0	0		
rep	1	0	0		
dup	2	0	0		
a:b	6	0	0		
b:c	6	0	0		
a:c	4	0	0		
a:b:c	12	0	0		
Residuals	177	0	0		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	0	0		
rep	1	0	0		
dup	2	0	0		
Residuals	177	0	0		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Mean Square Error: 0

Crude rhizome extract:

1. *Escherichia coli*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	0.037	0.0185	0.795	0.453060
b	3	4.902	1.6339	70.167	< 2e-16 ***
c	2	0.002	0.0012	0.050	0.951523
rep	1	0.057	0.0567	2.436	0.120398
dup	2	0.363	0.1817	7.804	0.000565 ***
a:b	6	0.074	0.0123	0.530	0.784867
b:c	6	0.220	0.0367	1.574	0.157201
a:c	4	0.032	0.0081	0.348	0.845219
a:b:c	12	0.190	0.0158	0.679	0.769925
Residuals	177	4.122	0.0233		

Signif. codes:	0	'***' 0.001	'**' 0.01	'*' 0.05	'.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	5.457	0.15592	6.696	< 2e-16 ***
rep	1	0.057	0.05671	2.436	0.120398
dup	2	0.363	0.18171	7.804	0.000565 ***
Residuals	177	4.122	0.02329		

Signif. codes:	0	'***' 0.001	'**' 0.01	'*' 0.05	'.' 0.1 ' ' 1

Mean Square Error: 0.02523148

Means with the same letter are not significantly different.

Groups, Treatments and means		
a	25	0.5
ab	26	0.4167
ab	2	0.4167
ab	13	0.4167
ab	14	0.4167
abc	1	0.3333
abc	3	0.3333
abc	15	0.3333
abcd	27	0.25
bcd	16	0.1667
bcd	36	0.1667
cd	17	0.08333
cd	18	0.08333
cd	24	0.08333
cd	28	0.08333
cd	30	0.08333
cd	35	0.08333
cd	5	0.08333
cd	6	0.08333
d	22	0
d	23	0
d	29	0
d	34	0
d	4	0
d	7	0
d	8	0
d	9	0
d	10	0
d	11	0
d	12	0
d	19	0
d	20	0
d	21	0
d	31	0
d	32	0
d	33	0

```
>
duncan.test(model,"trt",alpha=0.05,console=T
```

2. *Bacillus cereus*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	6.8	3.4	40.008	4.61e-15 ***
b	3	1694.2	564.7	6687.422	< 2e-16 ***
c	2	7.8	3.9	46.011	< 2e-16 ***
rep	1	0.0	0.0	0.055	0.81514
dup	2	0.2	0.1	1.275	0.28208
a:b	6	7.2	1.2	14.204	3.50e-13 ***
b:c	6	18.9	3.2	37.312	< 2e-16 ***
a:c	4	1.5	0.4	4.297	0.00242 **
a:b:c	12	2.9	0.2	2.890	0.00114 **
Residuals	177	14.9	0.1		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	1739.2	49.69	588.436	<2e-16 ***
rep	1	0.0	0.00	0.055	0.815
dup	2	0.2	0.11	1.275	0.282
Residuals	177	14.9	0.08		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Mean Square Error: 0.08425926

Means with the same letter are not significantly different.

Groups, Treatments and means		
a	34	7.25
b	22	6.583
bc	35	6.417
bcd	32	6.333
cde	23	6.167
de	10	6
de	31	6
ef	20	5.833
fg	19	5.583
fgh	12	5.5
fgh	36	5.5
ghi	24	5.417
ghi	8	5.417
hi	7	5.167
ij	11	5.083
ij	33	5.083
j	21	4.75
k	9	4.167
l	30	0.3333
l	6	0.3333
l	18	0.25
l	14	0.1667
l	17	0.1667
l	29	0.1667
l	3	0.1667
l	16	0.08333
l	4	0.08333
l	1	0
l	13	0
l	15	0
l	2	0
l	25	0
l	26	0
l	27	0
l	28	0
l	5	0

```
>
duncan.test(model,"trt",alpha=0.05,console=T
```

3. *Staphylococcus aureus*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
a	2	2.0	1.01	15.576	5.88e-07 ***
b	3	699.7	233.22	3603.375	< 2e-16 ***
c	2	1.3	0.64	9.943	8.09e-05 ***
rep	1	0.2	0.17	2.575	0.1103
dup	2	0.3	0.15	2.271	0.1062
a:b	6	2.0	0.34	5.275	5.01e-05 ***
b:c	6	3.3	0.55	8.512	3.96e-08 ***
a:c	4	0.6	0.15	2.298	0.0608 .
a:b:c	12	1.6	0.14	2.119	0.0178 *
Residuals	177	11.5	0.06		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	35	710.6	20.302	313.672	<2e-16 ***
rep	1	0.2	0.167	2.575	0.110
dup	2	0.3	0.147	2.271	0.106
Residuals	177	11.5	0.065		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Mean Square Error: 0.0662037

Means with the same letter are not significantly different.

Groups, Treatments and means		
a	35	4.667
b	22	4.167
b	34	4.167
b	36	4.083
bc	11	4
bc	23	4
bc	24	4
cd	12	3.75
d	10	3.667
d	33	3.583
e	32	3.167
e	8	3.167
e	21	3.083
ef	20	3
ef	9	3
ef	31	2.917
f	19	2.75
g	7	2.167
h	1	0
h	13	0
h	14	0
h	15	0
h	16	0
h	17	0
h	18	0
h	2	0
h	25	0
h	26	0
h	27	0
h	28	0
h	29	0
h	3	0
h	30	0
h	4	0
h	5	0
h	6	0

```
>
duncan.test(model,"trt",alpha=0.05,console=T
```