Journal of Chemical and Natural Resources Engineering, Special Edition : 99-107

ANTIMICROBIAL SUBSTANCES IN *LAWSONIA INERMIS* AND *STEVIA REBAUDIANA* FOR CONTROLLING SPOILAGE MICRO ORGANISMS

I.I.MUHAMAD[1], N.U. AHMAD[1], N. MOHD NAWI[1] L.C. TIN[1],M. R. SARMIDI[1,2]

ABSTRACT

Lawsonia inermis was extracted by soxhlet apparatus using ethanol and petroleum ether as solvent whereas ethanol and hexane were used in the extract of *Stevia rebaudiana*. All the extract were tested against *B.subtilis* (Gram-positive bacteria), *E.coli* (Gram-negative bacteria), *A. fumigatus* and yeast by disc diffusion method. Both extracts showed good inhibitory effects towards the growth of all the micro organism where the maximum inhibition (3.2±0.1 cm) was recorded for *Lawsonia inermis* against *B. subtilis*. Colony-forming efficiency test was performed to determine the sensitivity of the bacterial strains to the extracts.

Key Words : *Lawsonia inermis, Stevia rebaudiana, B.subtilis, E.coli, A. fumigatus*,disc diffusion

1.0 INTRODUCTION

Antimicrobial activity is defined in Merriam-Webster dictionary as the capability of destroying or inhibiting the growth of microorganism. Antimicrobial also could be explained as a substance that kills or inhibits the growth of microbes such as bacteria (antibacterial activity), fungi (antifungal activity), viruses (antiviral activity), or parasites (antiparasitic activity). *L. inermis* L. or locally known as Daun Inai is a species of plant of family Lythraceae and the sole member of its genus Lawsonia. It is perennial shrub native to North Africa, Asia and Australia, is naturalized and cultivated in the tropics of America, Egypt, India, and parts of the Middle East [1].

Traditionally, *L. inermis* has been used for hands and feet decorative purposes. It also contains natural ingredients, which are vital for hair nourishment. The plant has great healing effects which contain ingredients to be antibacterial, anti-fungal, anti-hemorrhagic, astringent, intestinal anti-neoplastic, cardio-inhibitory, hypotensive, and sedative effects. In addition, it is use in healing athlete's foot, fungal skin infections, and headaches, burning of the soles and palms, and local inflammation. It is also used to heal various ailments, including jaundice, leprosy, smallpox and other afflictions of the skin and body [2]. Depending on where it is grown, the effect and colour given by *L. inermis* varies. The darkest colour given (and most expensive) is that which is grown in Iran, whilst the lightest colour given is grown in Morocco [3].

Stevia rebaudiana is a genus of about 150 species of herbs and shrubs with green leaves that belongs to the aster (Asteraceae) or chrysanthemum family of plants. The

[1]Department of Bioprocess Engineering, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor Bahru, Malaysia.
[2]Chemical Engineering Pilot Plant, Universiti Teknologi Malaysia, 81310 Skudai, Johor Bahru, Malaysia.
Correspondence to : Ida Idayu Muhamad (idayu@fkkksa.utm.my)

species *S. rebaudiana*, commonly known as sweet leaf, or sugar leaf widely grown for its sweet leaves, is a perennial shrub native to subtropical and tropical South America and Paraguay [1]. It has proved to give exceptional benefits when it is being used regularly in skin care which gives healing effect on the blemishes, wounds, cuts and scratches over and above its antimicrobial property. The plant has a negligible effect on blood glucose, even enhancing glucose tolerance, therefore it is attractive as a natural sweetener to diabetics and others on carbohydrate-controlled diets [3-4].

Bacillus subtilis , E. coli, Aspergillus fumigatus and yeast are several spoilage microbes in food. A member of the genus Bacillus, ***B. subtilis*** is a Gram-positive bacterium commonly found in soil, has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. ***E. coli*** is facultative anaerobic and non-sporulating Gram-negative bacteria that can live on a wide variety of substrates at 37°C up to 49°C. It uses mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate and carbon dioxide. ***Aspergillus fumigatus*** is one of the most common Aspergillus fungus species to cause disease in immunocompromised individuals [5]. *A. fumigatus* has a stable haploid genome, with no known sexual cycle, and reproduces by forming conidiospores that are released into the environment. Capable of growth at 37°C (human body temperature), spores are common inhalation pollutants; typically, however, these are quickly eliminated by the immune system in healthy individuals. Yeasts are single-celled fungi related to the common baker's yeast used to leaven bread, molds that ripen blue cheese and the molds that produce antibiotics for medical and veterinary use [5].

2.0 EXPERIMENTAL

2.1 Material

Fresh *L. .inermis* were collected at the FKKKSA, UTM and S. rebaudiana were supplied by the Green Valley Eco-Village, Kulai. The fresh plant were thoroughly cleaned and dried separately under shade at 25±2°C for about five days and dried at 60°C in oven for about five hours. The dried plant samples were grounded well in a grinder to produce particle size of 1 mm to 5 mm. The ground samples were kept in air tight transparent plastic bags at room temperature before the extraction process.

2.2 Extraction of plant [4]

The extracts were basically extracted out from *L. inermis* and *S. rebaudiana* through soxhlet extraction and being evaporated to remove the solvent. About 10g of the ground leaves was placed in the thimble filter. The thimble filter was then positioned into the central compartment of the soxhlet column. Meanwhile, 150ml of preset solvent (ethanol, petroleum ether or hexane) was placed into the boro silicate glass flask at the lower compartment. The solvent in the boro silicate glass flask was gently heated to boiling. The extraction process was ended according to the pre-set time which is 8 hours. The solution which contained solvent and the extract were subjected to the rotary evaporator to remove the solvent. The extract is stored at chillers at 4°C.

The yield of extraction was calculated as percentage of extract obtained from 10g sample. The yield of extract was calculated as follows:

$$\text{Yield of extract (\%w/w)} = \frac{x \text{ (g) of extract obtained}}{10\text{g of sample}} \times 100 \qquad \text{eqn. 1}$$

2.3 Determination of antimicrobial activity againts *B. subtilis, E. coli, A. fumigatus* and Yeast

Preparation of #0.5 McFarland Standards (1.5 x 108 cfu/ml)

McFarland turbidity standard are used as a reference standard to approximate the number of bacteria in a liquid suspension [5].

Kirby Bauer disc diffusion method

The determination of antimicrobial activity of *L. inemis* and *S. rebaudiana* extracts were investigated by the Kirby-Bauer discs diffusion method. Basically, Kirby-Bauer disc diffusion method was divided into four-stages of procedures which were (i) Inoculums preparation; (ii) Inoculation of Mueller Hinton plate; (iii) Application of the discs and (iv) Inhibition zone measurement [4].

Application of the discs

20µl concentrations of extracts are applied into the Whatman disc (0.6 cm) by using micro-pipette. The amount of 20µl methanol and Streptomycin (1mg/1ml)/ Tricholorocycline hydroxide (1mg/1ml) were also applied into the Whatman disc by using micro-pipette. Methanol and Streptomycin (1mg/1ml)/Tricholorocycline hydroxide (1mg/1ml) were used for negative control and positive control. The disc were placed gently onto the Nutrient agar plate by flamed forceps and positioned in sufficient space between them in order to accommodate resulting zones of inhibition without significant overlap of adjacent zone. Each disc was pressed carefully with the forceps to make the disc stick to the agar since the plate will be inverted while it is incubated. The discs were applied to the surface of the agar within 15 minutes of inoculation. After 10 minutes of plate application, the plate of sample was inverted and incubated at 37°C for 24 hours.

Inhibition zone measurement

After 24 hours, the plate was inverted and on the outside of the plate the diameter of the zone of inhibition was measured using a metric ruler in unit of millimetres including the disc. The experiment was done with duplicate and the results were expressed as average value.

2.3 Colony-forming efficiency test

The effects of the *L. inermis* and *S. rebaudiana* extract on bacterial strains were evaluated by the plate count method. Counting viable cells depends on live cell's ability to grow to form colony or develops into a turbid culture. The technique for performing a plate count was spread and pours plate methods of culturing micro organism. A sample of the culture was serially dilute, usually tenfold at each dilution, and then small amount of each dilution was spread on a plate or mixed with melted medium and incubated under appropriate growth condition. The number of colonies on a plate allows calculating the concentration of cells present in the original sample [6].

3.0 RESULTS AND DISCUSSION

3.1 Yield from extraction process

From table 1, extraction using petroleum ether produced slightly higher yield than that in ethanol for both plant, *L. inermis* and *S. rebaudiana* that was 94.3% (w/w) and 88.3% (w/w) respectively.

Table 1 Result of yield of extracts using ethanol and petroleum ether for *L. inermis* and *S. rebaudiana*

Plant	Solvent	of yield
L. inermis	Ethanol	88.6% (w/w)
	petroleum ether	94.3 % (w/w)
S. rebaudiana	Ethanol	87.6 % (w/w)
	petroleum ether	88.3 % (w/w)

3.2 Antimicrobial activity

3.2.1 Disc diffusion method

Principally, a plant was considered actively antimicrobials against both fungi and bacteria when the zone of inhibition was greater than 6mm [7]. Table 2 showed the Control procedure i.e. antimicrobial activity measurement (if any) of ethanol (C_2H_5OH), petroleum ether (Et_2O) and hexane (C_6H_{14}) against *B. subtilis, E. coli, A. fumigatus* and yeast. All these solvent were tested as Control at active concentration of 0.067g/ml. Table 3 showed similar testing of ethanol and petroleum ether extract of *L. inermis* on the micro organisms with comparison to Tetra-chlorocycline and Streptomycin as standard antibiotics. There were two replicates being prepared for each solvent and the activity in both replicates were observed after 24 hours of incubation. From the observation, inhibition zone was identified as clear zone formed surrounding the growth population of the tested micro organisms.

From table 2, it was clearly showed that ethanol has inhibitory effect toward *E. coli* with the size of 0.5±0.1 cm and yeast 0.8±0.1 cm respectively. However, there were no activity shown by ethanol against *B. subtilis* and *A. fumigates* whereby no antimicrobial activity at all being observed on the petroleum ether and hexane at the specified concentration.

Table 2 Antimicrobial Activity of Ethanol, Petroleum Ether and Hexane Solvent (Control)

Micro organism	IZD, diameter of inhibition zone (mm)		
	Ethanol	Petroleum Ether	Hexane
B. subtilis	nil	nil	nil
E. coli	5	nil	nil
A. fumigatus	nil	nil	nil
Yeast	8	nil	nil

Values are means (mm) of duplicate; nil= no inhibition zone
IZD, diameter of inhibition zone (mm) including disc diameter of 6 mm

Table 3 Antimicrobial Activity of *L. inermis* extract and Standard Antibiotic

Micro organism	IZD, diameter of inhibition zone (mm)			
	Ethanol extract	Petroleum ether extract	Tetra-chlorocycline	Streptomycin
B. subtilis	32	27	28	25
E. coli	29	24	25	16
A. fumigatus	20	19	22	18
Yeast	15	16	23	15

Values are means (mm) of duplicate; nil= no inhibition zone
IZD, diameter of inhibition zone (mm) including disc diameter of 6 mm

From table 3, the *L. inermis* extract showed both gram-positive and gram-negative bacteria, *A. fumigatus* and yeast were inhibited. Inhibitory action was greatest against B. subtilis and E. coli but least against yeast. This agrees with previous report that *L. inermis* showed greater inhibition against Gram-positive bacteria [8, 9]. Overall inhibitory effect was showed by ethanol extract and comparable to both standard antibiotics tested. Previous research also confirmed that the antimicrobial activity of raw extract was greater than autoclaved and dried powder but more remarkable in hot water [8]. The antimicrobial substance in henna is highly soluble in water, partially soluble in 70% ethyl alcohol, heat-stable and proved as phenolic compounds [8, 10].

Table 4 showed the antimicrobial activity of ethanol and hexane extract of *S. Rebaudiana* against all micro organism. The ethanol extract of *S. rebaudiana* showed remarkable zone of inhibition against *A. fumigatus*, followed by *E. coli* and *B. subtilis*. In the meantime, the hexane extract of *S. rebaudiana* also showed maximum zone of inhibition against *E. coli*, followed by *B. subtilis* and *A. fumigates*. However, both extract showed no inhibition for yeast growth within the range of concentration used. This findings contradict with previous study which reported higher antifungal inhibition observed in ethanol extract on Aspergillus strains than hexane but quite similar strength in both bacterial inhibitions [11].

Table 4 Antimicrobial Activity of *S. rebaudiana* and Standard Antibiotic

Micro organism	IZD, Inhibition zone (mm)			
	Ethanol extract	Hexane	Tetra chlorocy	Streptomycin
B. subtilis	13	12	28	25
E .coli	18	25	25	16
A. fumigatus	21	11	22	18
Yeast	nil	nil	23	15

Values are means (mm) of duplicate; nil= no inhibition zone
IZD, diameter of inhibition zone (mm) including disc diameter of 6 mm

The results of present study indicated that *S. rebaudiana* leaf extracts showed inhibitory effects against micro organisms, even though slightly lower than *L. Inermis* extracts at that concentration used. This phenomena might be due to the presence of more antimicrobial substances in *L. Inermis* such as alkaloids, anthocyanin, phenols, xanthoproteins, flavanoids, carboxylic acids, coumarins and sterols [12-14]. However, it was reported that petroleum ether extracts of Stevia rebaudiana is found to be potent enough in exhibiting substantial antimicrobial activity against dreaded animal pathogens [11].

3.2.2 Colony-forming efficiency test

In order to compare the sensitivity of the *B. subtilis* and *E. coli* strains to the extracts, the extracts that exhibited antimicrobial activity in disc diffusion assay were submitted to the colony-forming efficiency test using plate count agar (also known as viable cell count).

Figures.1 and 2 were plotted to determine which extract was the most efficient to inhibit bacterial. Figure 1 showed that ethanol extract of *L. inermis* was the best extract to combat gram-positive bacteria (*B. subtilis*). In contrast, petroleum ether extract of *L. inermis* was the best extract against the gram-negative bacteria (*E. coli*).

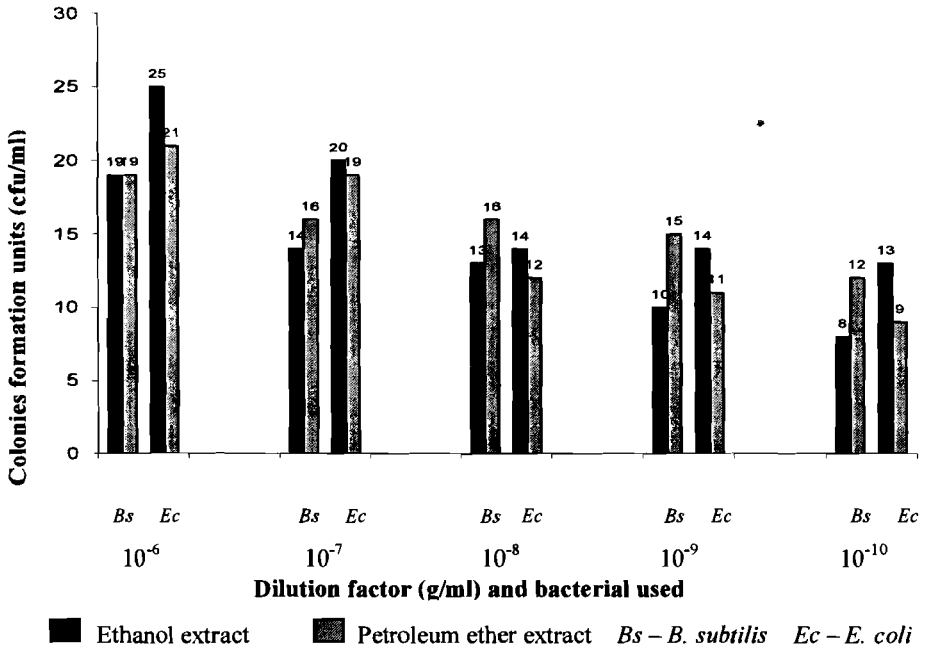

Figure 1 Number of Colonies Formation Units (cfu/ml) versus Dilution Factor (g/ml) of *L. inermis* extracts towards *B. subtilis* and *E. coli* strains

Similar result was obtained in Figure 2 where ethanol extract of *S. rebaudiana* was the best extract against gram-positive bacteria (*B. subtilis*) whereas hexane extract was the strongest against gram-negative bacteria (*E. coli*).

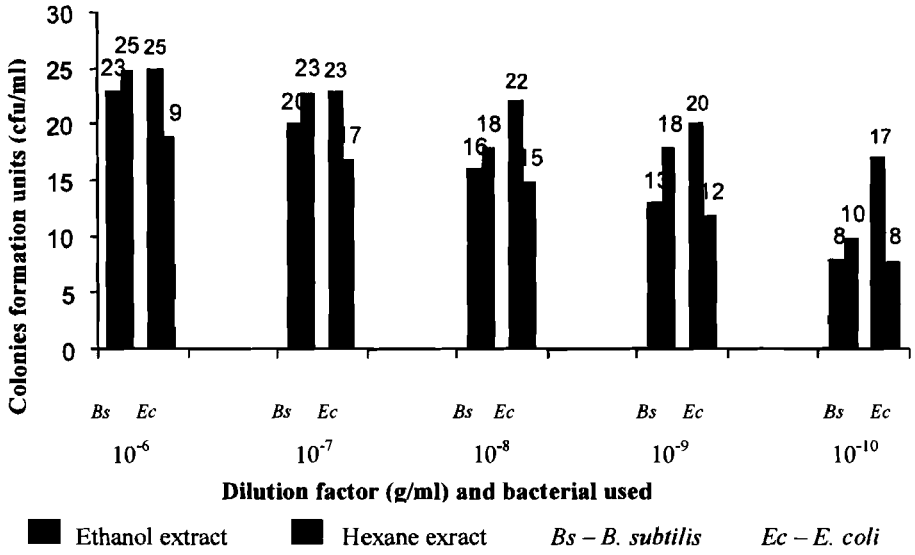

Figure 2 Number of Colonies Formation Units (cfu/ml) versus Dilution Factor (g/ml) of *S. Rebaudiana* using *B. subtilis* and *E. coli* strains

The *S. Rebaudiana* extract contains flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls, hydroxycynnamic acids (caffeic, chlorogenic, etc.), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils and trace elements [15].

4.0 CONCLUSIONS

The *L. inermis* extract exhibited antimicrobial activity towards Gram-positive bacteria, Gram-negative bacteria, *A. fumigatus* and yeast. Similar effects were observed in *S. rebaudiana* extract however no inhibition effect on yeast at the applied concentration. *L inermis* is more effective plant compared to *S. rebaudiana* as it exhibited greater antimicrobial activity against all micro organisms. compared to *S. Rebaudiana.*, The ethanol extract of both plant showed 20% inhibition capacity to combat Gram-positive bacteria whereas petroleum ether extract of *L. inermis* and hexane extract of *S. rebaudiana* are 20% better inhibition extract against the Gram-negative pathogen.

ACKNOWLEDGEMENT

The authors are grateful to the Green Valley Eco-Village, Kulai for supply of raw materials, the Ministry of Higher Education (vot FRGS 78099: MOHE) and the Research Management Centre UTM for support funding of this study.

REFERENCES

[1] Anonymous, 2007. Url:http://wikipedia.org/lawsonia. Retrieved date: 1 August 2007

[2] Roy, P. K., M. Singh, & P. Tewari. 2005. Composition of Henna Powder, Quality Parameters and Changing Trends in its Usage. *Henna, Cultivation, Improvement and Trade*: 39 – 40. Jodhpur, India, Central Arid Zone Research Institute

[3] Anonymous, 2007. Url:http://wikipedia.org/stevia. Retrieved date: 1 August 2007

[4] Rosnani Hisyam. 2007. The Effects of Processing Parameter on The Antibacterial Activity on *Zingiber Zerumbet* Oleoresin. Msc Thesis, Universiti Teknologi Malaysia.

[5] Kerr, J. Thomas and Barbara McHale. 2001 Applications in General Microbiology, A Laboratory Manual. 6th Edition. Melbourne. Hunter Textbook, Inc., Publ.

[6] Sauriasari R., Da-Hong Wang, Yoko Takemura, Ken Tsutsui, Noriyoshi Masuokac, Kuniaki Sano, Masako Horita, Bing-Ling Wang, Keiki Ogino. 2007. Cytotoxicity of Lawsone and Cytoprotective Activity of Antioxidants in Catalase Mutant Escherichia Coli. *Toxicology*. 235: 103–111

[7] Muhammad H. S. and Muhammad S, (2005). The use of *Lawsonia Inermis* linn. (Henna) in the management of burn wound infections. Biotechnology, Vol. 4, 934-937

[8] Malekzadeh F. 1968. Antimicrobial Activity of *L. inermis* L. *Appl. Microbiol.* 663-664.

[9] Karuppusamy, S. and N. Karmegam. 2001. Antibacterial activity of Tribal Medicinal Plants against Locally Isolated Microbes. *J. Ecotoxicol. Environ. Monit.* 11 (1) : 47 – 51.

[10] Ali Awadh N.A, W.-D. Ju¨ lich, C. Kusnick, U. Lindequist. 2001. Screening of Yemeni Medicinal Plants for Antibacterial and Cytotoxic Activities. *Journal of Ethnopharmacology*. 74, 173–179

[11] Sumit Ghosh, Enketeswara Subudhi, Sanghamitra Nayak. 2008. Antimicrobial essay of *Stevia rebaudiana* Bertoni leaf extracts against 10 pathogens. *International Journal of Integrative Biology*. 2(1): 27-31.

[12] Sudharameshwari K. and Radhika J., (2007). Antibacterial Screening of *Aegle Marmelos, lawsonia Inermis* and *Albizzia Libbeck. Complementary and Alternative Medicine*, 4 (2): 199-204

[13] Komissarenko, N.F., Derkach, A.I., Kovalyov, I.P., Bublik, N.P., (1994). Diterpene glycosides and phenylpropanoids of Stevia rebaudiana Bertoni: Rast. Research 1 (2), 53–64

[14] Thitilertdecha N., Aphiwat Teerawutgulrag, Nuansri Rakariyatham (2008). Antioxidant and antibacterial activities of Nephelium lappaceum L. extracts. Food Science and Technology, 1-7

[15] Komissarenko, N.F., A.I. Derkach, I.P. Kovalyov, N.P. Bublik. 1994. Diterpene glycosides and phenylpropanoids of *Stevia rebaudiana Bertoni*: *Rast. Research* 1 (2), 53–64