PRELIMINARY ANTIMICROBIAL AND PHYTOCHEMICAL ANALYSIS OF *Clinacanthus nutans* AND *Azadirachta indica*

SATHISHA A/P GOONASAKARAN

A dissertation submitted in partial fulfilment of the
requirements for the award of the degree of
Master of Science (Biotechnology)

Faculty of Bioscience and Medical Engineering
Universiti Teknologi Malaysia

JULY 2013

To my beloved father Mr. Goonasakaran and mother Mrs. Chandrika who have given me nothing but love and care.

ACKNOWLEDGEMENT

I would like to extend my heartiest appreciation and thanks to my Supervisor Dr.Goh Kian Mau for guiding me throughout this journey of research project. The supervision and support that he gave truly help the progression and smoothness of my project. Particular acknowledgement goes to Miss Suganthi and Mr Shanmugaprakasham, both of you tirelessly walked with me throughout this work. The brainstorms have helped a lot in improving this project. My special thanks goes to my fiancé Dr. Thava Viknaraj. Thanks for always being there and giving me constant support to complete these studies. I would also like to thank other academicians and practitioners for their guidance and support. Besides, I would like to express my thanks and appreciation to all the members of Special Equipment Laboratory; you were faithful in helping me to motivate myself. Tribute goes to the members of staff in the Faculty of Bioscience and Bioengineering at the University Technology Malaysia for your contributions to see this work a success. Not to forget, I would like to extend my appreciation to those who helped directly or indirectly in my project. Most especially to my beloved family and friends for their cooperation and support through out the project, who made all this possible. Thank you.

ABSTRACT

Clinacanthus nutans which belongs to the family of Acanthaceae and *Azadirachta indica* which belongs to the family of Meliaceae are often used as herbal remedies in complementary and alternative medicine. Extracts from dried leaves of *Clinacanthus nutans* and *Azadirachta indica* was screened for its antimicrobial activity and phytochemical composition. The antimicrobial activity of plant extract was first assessed by using different solvent. Chloroform, absolute ethanol, 70% ethanol and distilled water plant extracts were tested against *Salmonella enterica* serovar Paratyphi B. It was shown that chloroform *Clinacanthus nutans* extract and ethanolic *Azadirachta indica* extract showed a larger inhibition zone upon *Salmonella enterica* serovar Paratyphi B compare to other solvents. The best result producing solvent extracts were then tested upon five strains of *Salmonella* simultaneously. The strains that were subjected to test was respectively *Salmonella enterica* serovar Paratyphi C, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Weltevreden, and *Salmonella enterica* serovar Typhi ATCC and *Salmonella enterica* serovar Paratyphi B. In this test, it is shown that both plant extracts able to form zone of inhibition against the *Salmonella* strains. The enteric fever causing *Salmonella enterica* serovar Typhi ATCC showed the least inhibition with both extracts compared to the *Salmonella enterica* serovar Paratyphi B and *Salmonella enterica* serovar Paratyphi C strains. There was no significant difference in zone of inhibition against gastroenteritis causing *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Weltevreden. The qualitative phytochemical analysis was carried out using powdered leaf samples of *Clinacanthus nutans* and *Azadirachta indica* to determine the major phytoconstituents. The analysed phytochemicals are alkaloids, flavonoids, tannins, saponins and cardiac glycosides. The analysis showed the presences of all five phytoconsituents in *Azadirachta indica* leaf sample. Meanwhile for *Clinacanthus nutan,* a positive result was shown for the presence of alkaloids, tannins, flavonoids and cardiac glycosides but a negative result for the presence of saponin.

ABSTRAK

Clinacanthus nutans yang tergolong dalam famili Acanthaceae dan *Azadirachta indica* yang tergolong dalam famili Meliaceae sering digunakan sebagai ubatan herbal. Dedaun kering daripada *Clinacanthus nutans* dan *Azadirachta indica* telah dikaji untuk aktiviti antimikrob dan komposisi fitokimia. Sebagai langah pertama, aktiviti antimikrob ekstrak tumbuhan dinilai dengan menggunakan pelarut yang berbeza. Ekstrak daripada kloroform, etanol mutlak, 70% etanol dan air suling telah diuji terhadap *Salmonella enterica* serovar Paratyphi B. Kajian ini menunjukkan bahawa ekstrak kloroform *Clinacanthus nutans* dan ekstrak etanol mutlak *Azadirachta indica* telah menghasilkan zon perencatan yang baik terhadap *Salmonella enterica* serovar Paratyphi B berbanding dengan pelarut lain. Ekstrak tersebut kemudiannya diuji ke atas lima spesies *Salmonella* iaitu *Salmonella enterica* serovar Paratyphi C, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Weltevreden, dan *Salmonella enterica* serovar Typhi dan *Salmonella enterica* serovar Paratyphi B. Ujian ini telah menunjukkan bahawa kedua-dua ekstrak tumbuhan dapat membentuk zon perencatan terhadap spesies *Salmonella* walaupun terdapat sedikit perbezaan dalam ukuran zon perencatan. Antara spesies yang menyebabkan demam enterik, *Salmonella enterica* serovar Typhi ATCC menunjukkan zon perencatan yang kecil berbading dengan spesies paratyphi. Spesies yang menyebabkan penyakit gastroenteritis, *Salmonella enterica* serovar Typhimurium dan *Salmonella enterica* serovar Weltevreden tidak menunjukkan perbezaan yang signifikan dalam zon perencatan. Analisis kualitatif fitokimia telah dijalankan dengan menggunakan sampel serbuk dedaun *Clinacanthus nutans* dan *Azadirachta indica* untuk menentukan kehadiran fitokimia yang tertentu. Fitokimia yang dianalisis adalah alkaloid, flavonoid, tanin, saponin dan glikosida kardiak. Keputusan yang positif telah dipemerhati untuk kehadiran kesemua lima fitokima dalam sampel dedaun *Azadirachta indica*. Manakala, bagi *Clinacanthus nutan,* keputusan positif telah dilihat untuk kehadiran alkaloid, tanin, flavonoid dan glikosida tetapi keputusan negatif bagi kehadiran saponin.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS / SYMBOLS

ATCC	-	American type culture collection
CAM	-	Complementary and alternative medicine
DNA	-	Deoxyribonucleic acid
DMSO	-	Dimethyl sulfoxide
et al.,	-	and friends
$FeCl_3$	-	Iron(III) chloride
GCMS,	-	Gas chromatography–mass spectrometry
H_2SO_4	-	Sulfuric acid
HCl	-	Hydrocloric acid
HPLC	-	High performance liquid chromatography
NCCAM	-	National Centre for Complementary and Alternative Medicine
MHA	-	Muller Hinton agar
UV-B	-	Ultra Violet B
WHO	-	World Health Organization
°C	-	Degree celcius
µL	-	Microliter
mg	-	Miligram
ml	-	Mililiter
min	-	Minute
g	-	gram

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Salmonella is a human pathogen that known to cause infection when a person ingests foods that contain a high concentration of this bacteria. Young children are much more prone to infection even with a small number of *Salmonella*. This bacteria causes gastroenteritis, enteric fever, bacteremia with or without metastatic disease, and asymptomatic carriage in host (Lennan *et al.,* 2008). In Malaysia, most of the food poisoning cases that occur at National Service Camps, school canteens, public events and etc are caused by Enterobacteriaceae such as *Samonella* (Mushaireen *et al.,* 2010). *Salmonella* infections are one of concerned cases in developing country like Malaysia.

Instead of depending on commercial antibiotics, plant extracts can be used to treat *Salmonella* infection as an alternative treatment. This is because, plants are well known for their healing activity against both biotic and abiotic stress in their natural environment. It was found that the defence mechanism of plants against these threats derived from its own secondary metabolites (Rios *et al.,* 2005). The secondary metabolites synthesized by plants include alkaloid, phenols, quinones, glycosides flavones, flavonoids, flavonols, tannins and coumarins (Malkhan *et al.,* 2012). Plant secondary metabolites are extensively exploited as an alternative antimicrobial agent

against both human and plant pathogen. The urge to have alternate antibiotic source from other resources such as plants is high now due to the lack of resource from microorganism and also the resistance of pathogen towards the existing antibiotic (Abreu *et al.,* 2012).

The *C.nutans* which locally known as Sabah Snake plant or Belalai Gajah is well known for its anti-inflammatory, anti-viral property and also as a snake bite remedy (Yoosook *et al.* 1999). The *A.indica* plant, locally known as Neem plant or pokok mambu is used due to its multiple medicinal values. Different parts of *A.indica* was proven to have active substances that has medicinal, spermicidal, antiviral, antibacterial, insecticidal, antiprotozoal, insect-repellent, and antifungal properties. They are also believed to remove toxins from the body, neutralize free radicals, and purify the blood. *A.indica* is been extensively studied for anticancer treatment, hepato-renal protective activity, and hypolipidemic effects (Shyamapada, 2011).

In order to absorb the bioactive compounds from the plant extracts, solvents play an important role. Solvent selection will determine the absorptions of hydrophilic or lipophilic compounds from the plants. Polar solvent able to absorb hydrophilic compounds, semi polar solvent able to absorb both hydrophilic and lipophilic compounds and non polar solvent absorbs lipophilic compounds. Assessing plant extract with different solvent increase the possibilities to absorb a wide range of compounds (Charmi *et al.,* 2011).

This experiment will be conducted in order to study the antimicrobial activity of *C.nutans* and *A.indica* using four different solvent against *Salmonella* strains and to identify the possible secondary metabolites involve in the antimicrobial activity. The outcome from this study will be valuable information in drug designing using natural product in future.

1.2 Problem Statement

Due to the decreasing number of new antibiotics and the increasing number of drug-resistance pathogens, an urge to find alternative antibiotics has formed (Abreu *et al.,* 2012). Naturally existing plant secondary metabolites can be a good source for antimicrobial. In fact, the usage of plant extracts to treat various diseases has been in practice among different societies since ancient times. New technologies and research are now used to validate these practices. *In-vitro* antimicrobial activity of many plant extracts such as *Ocimum sactum (*Joshi *et al.,* 2011*), Orthosiphon stamineus* (Chun *et al.,* 2010) has given a positive outcome. Malaysia is a country with rich diversity of plants. Although many works have been conducted, yet only a fraction of total plant has been explored for the secondary metabolites and its unique usage such as antimicrobial activity (Huda *et al.,* 2007).

1.3 Scope of Study

To the best of knowledge, little study was done on *C.nutans* and *A.indica* leaves extract against different species *Salmonella*. Hence the plants will be extracted using absolute ethanol, 70% ethanol, chloroform and distilled water and will be tested against *Salmonella enterica* serovar Paratyphi B. The larger zone of inhibition forming extracts will be tested against five different strains of *Salmonella* which are respectively *Salmonella enterica* serovar Typhi ATCC, *Salmonella enterica* serovar Weltevreden, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Paratyphi C and *Salmonella enterica* serovar Paratyphi B simultaneously to analyse the antimicrobial activity. Besides, in this experiment, the powdered plant leaves will be subjected to qualitative phytochemical analysis for the presence of tannins, alkaloids, saponins, flavonoids, and cardiac glycosides.

1.4 Significant of Study

This study which is done on *C.nutans* and *A.indica* can provide valuable information on its capability to prevent growth of human pathogens such as *Salmonella*. This study can be a stepping stone for further, detail studies on the plant's antimicrobial characteristic against other pathogens. The screening for basic phytochemicals that is done in this experiment can also provide an insight of the plants bioactive compounds and other possible activities such as antiviral, antioxidant, and anti-cancerous apart from antimicrobial activity. The outcome of a detail research from these preliminary studies can also be commercialised in future.

1.5 Objectives of Study

i. To evaluate the effect of *C.nutans* and *A.indica* extracts using four different solvent against *Salmonella enterica* serovar Paratyphi B.
ii. To evaluate the antimicrobial activity of *C.nutans* and *A.indica* extract against five different strains of *Salmonella.*
iii. To qualitatively analyse the presence of selective secondary metabolites of *C.nutans* and *A.indica.*

REFERENCES

Abreu, A.C., McBain, A.J., and Simoes M. (2012). Plants as Sources of New Antimicrobials and Resistance-modifying Agents. *Natural Product Reports*. 29(9):1007-21.

Akira, N., Hiromichi, M., and Hidehiko, K. (2011). A Bacterial Platform for Fermentative Production of Plant Alkaloids. *Nature Communications*. 2(1):326-329.

Amalesh, S., Gouranga, D., and Sanjoy, K.D. (2011). Roles of Flavonoids in Plants. *The International Journal of Pharmaceutical Science and Technology.* 6(1): 12-35.

Arvind, S., and Samuel, K. K. (2008). Plant-derived Compounds in Clinical Trials. *Drug Discovery Today*. 13(4):161-171.

Atlas, R.M. (2004). *Handbook of Microbiological Media*. London CRC Press.

Ballhorn, D.J., Kautz, S. H. M., and Hegeman, A.D. (2009). Cyanogenesis of Wild Lima Bean (*Phaseolus lunatus* L.) is an Efficient Direct Defence in Nature. *Plant Signaling and Behavior*. 4(8):735-745.

Benbott, A., Yahyia, A., and Belaidi, A. (2012). Assessment of the Antibacterial Activity Crude Alkaloids Extracted from Seeds and Roots of the Plant *Peganum harmala*. *Journal of Natural Products and Plant Resources*. 2(5):568-573.

Bhan, M.K., Bahl, R., and Bhatnagar, S. (2005). Typhoid and Paratyphoid Fever. *Lancet*. 366 (94):49–62.

Bronze, M.S., and Greenfield, R.A. (2005). Biodefence Principles and Pathogens. *Horizon Bioscience*. 1:377-389.

Charmi, P.S., Vachhani, U.D., Trivedi, M.N., Joshi, V.J., and Santani, D.D. (2011). Evaluation of Antibacterial Potential of *Terminalia chebula* against Pathogenic Organisms of Lacrimal system. *Pharmacologyonline* 1:506-509.

Chun, H. H., Ismail, N., Shaida, F. S., and Ahmad, R. (2010). *In-vitro* Antibacterial and Antioxidant Activities of *Orthosiphon stamineus* Extracts against Food-borne Bacteria. *Food Chemistry*. 122(4):1168-1172.

Christopher, J.F. (2008). Classification of Complementary and Alternative Medical Practices. *Canadian Family Physician*. 54(11):1570–1571.

Cynthia, W., Zabta, K.S., Imran, A., and Riffat, N.M. (2011). Antibacterial Activity in Herbal Products Used in Pakistan. *Special Issue Pakistan Journal of Botany*. 43(1):155-162.

Davies, P. R., Scott, H. H., Funk, J. A., Fedorka, P. J., and Jones, F. T. (2004). The Role of Contaminated Feed in the Epidemiology and Control of *Salmonella enterica* in Pork Production. *Foodborne Pathogens and Disease*. 1(4):202-215.

Daniyan, S. Y., and Muhammad, H. B. (2008). Evaluation of the Antimicrobial Activities and Phytochemical Properties of Extracts of *Tamaridus indica* against Some Diseases Causing Bacteria. *African Journal of Biotechnology*. 7(14):2451–2453.

Edwards, R. A., Olsen, G. J., and Maloy, S. R. (2002). Comparative Genomics of Closely Related *Salmonella*. *Trends in Microbiology*. 10(1):94-99.

Farland, M. B., Bigelow, D., Zani, B., Newsom, J., and Kaplan, M. (2002). Complementary and Alternative Medicine Use in Canada and the United States. *America Journal of Public Health*. 92(1):1616–1618.

Fluit, A. C. (2005). Towards More Virulent and Antibiotic-resistant *Salmonella*. *Immunology and Medical Microbiology*. 43(1):1-11.

Freeman, B.A. (1985). *Burrows Textbook of Microbiology.* (22nd ed.) Saunders Company.

Gurinder, J.K., and Daljit, S.A. (2009). Complementary and Alternative Medicine. *Biomedicine Central*. 9(1):30-33.

Harold, M. (2004). *On food and Cooking: The Science and Lore of the Kitchen.* (1st ed.) New York: Scribner.

Ho, K.Y., Tsai, C.C., Huang, J.S., Chen, C.P., Lin T.C., and Lin, C.C. (2001). Antimicrobial Activity of Tannin Components from *Vaccinium vitis-idaea* L. *Journal of Pharmacy and Pharmacology*. 53(2):87-91.

Hostettmann, K., and Marston, A. (1995). *Saponins.* (2nd ed.) Cambridge University Press.

Huda, F. N., Noriham, A., Norrakiah, A.S., and Babji, A.S. (2007). Antioxidative Activities of Water Extracts of Some Malaysian Herbs. *ASEAN Food Journal*. 14(1):61-68.

Jeu M. P. Y., Shiuan W., Hong L. J., Chin C. L., and Ji, Y.L. (2012). Effects of *Clinacanthus nutans* Leaf Extracts on Protection of Plasmid DNA from Riboflavin Photoreaction. *MC-Transaction on Biotechnology*. 4(1):13-15.

Jeremy, P. E. S. (2008). Flavonoids: Modulators of Brain Function? *British Journal of Nutrition.* 99(2):60–77.

Joshi, B., Govind, P.S., Buddha, B.B., Megh, R.B., Dinita, S., Krishna, S., Janardhan, P., and Rajani, M. (2011). Phytochemical Extraction and Antimicrobial Properties of Different Medicinal Plants: *Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan) and *Azadirachta indica* (Neem). *Journal of Microbiology and Antimicrobials*. 3(1):1-7.

Kausik, B., Ishita, C., Ranajit, K. B., and Uday, B. (2002). Biological Activities and Medicinal Properties of Neem (*Azadirachta indica*). *Current Science*. 82(11):7-10.

Katarzyna, W., Krzysztof, B., and Anna, B. (2006). Cardiac Glycosides in Cancer Research and Cancer Therapy. *Acta Poloniae Pharmaceutica ñ Drug Research*. 63(2):109-115.

Khemani, L.D., Srivastava, M.M., and Shalini S.S. (2012). *Chemistry of Phytopotentials: Health, Energy and Environmental Perspectives*. (1st ed.) Springer Heidelberg Dordrecht London New York.

Kusnieczyk, A., Winge, P., Midelfart, H., Armbruster, W.S., Rossiter, J.T., and Bones, A.M. (2007). Transcriptional Responses of *Arabidopsis thaliana* Ecotypes with Different Glucosinolate Profiles after Attack by *Polyphagous myzus persicae* and *Oligophagous brevicoryne brassicae*. *Journal of Experimental Botany*. 58(10):2537-2552.

Lennan, C.A., Gondwe, E.N., and Msefula, C.L. (2008). The Neglected Role of Antibody in Protection against Bacteremia Caused by Nontyphoidal Strains of *Salmonella* in African Children. *Journal of Clinical Investigation*. 118(4): 53–62.

Lambert, E., Faizal, A., and Geelen, D. (2011). Modulation of Triterpene Saponin Production: *In-Vitro* Cultures, Elicitation, and Metabolic Engineering. *Applied biochemistry and biotechnology*.164(2):220-237.

Leland, J. C. (2006). *Natural Products from Plants*. (2nd Ed.) CRC Press.

Lim, S.K., Migma D.T., Hyang, M. N., Tae, S.K., Geum C.J., and Suk C. J. (2011). Emergence of Extended-Spectrum β-Lactamase (CTX-M-15 and CTX-M-14)-Producing Nontyphoid *Salmonella* with Reduced Susceptibility to Ciprofloxacin among Food Animals and Humans in Korea. *Journal of Clinical Microbiolgy*. 49(7): 2671-2675.

Malkhan, S.G., Shahid, A., Masood, A., and Kangabam, S.S. (2012). Efficacy of Plant Extracts in Plant Disease Management. *Agricultural Sciences.* 3 (1):425-433.

Mazid, M., Khan, T.A., and Mohammad, F. (2011). Role of Secondary Metabolites in Defence Mechanisms of Plants. *Biology and Medicine.* 3(2):232-249.

Miller, S.I., and Pegues, D.A. (2000). *In Principles and Practice of Infectious Diseases.* (2nd Ed.) Philadelphia: Churchill Livingstone.

Mullika, T.C., Suvimol, S., Veena, S., and Nukoolkarn, W.G. (2005). Antimicrobial Effects of Thai Medicinal Plants against Acne-inducing Bacteria. *Journal of Ethnopharmacology*. 101(3):330-333.

Mushaireen, M., Kamaruzaman, J., Khazainah, K., Rashdi A. P., Jazira, A., and Harnizam, Z. (2010). Food Borne Illness Risk Factors Assessment in UiTM Shah Alam, Malaysia. *World Applied Sciences Journal.* 8(7):864-870.

Nissen, N., and Manderson, L. (2013). Researching Alternative and Complementary Therapies: Mapping the Field. *Medical Anthropology*. 32 (1):1–7.

Nayak, A., Nayak, R.N., Soumya, G., Bhat, K., and Kudalkar, M. (2011). Evaluation of Antimicrobial and Anticandidial Efficacy of Aqueous and Alcoholic Extract of Neem (*Azadirachta indica*). *International Journal of Research in Ayurveda and Pharmacy.* 2(1):230-235.

Ncube, N., Afolayan, S.A.J., and Okoh, A.I. (2008). Assessment Techniques of Antimicrobial Properties of Natural Compounds of Plant Origin: Current Methods and Future Trends. *African Journal of Biotechnology*. 7(2):1797-1806.

Nostro, A., Germano, M.P., Angelo, V. D., Marino, A., and Cannatelli, M.A. (2000). Extraction Methods and Bioautography for Evaluation of Medicinal Plant Antimicrobial Activity. *Letters in Applied Microbiology.* 30(5):379–384.

Parry, C. M. (2006). *Epidemiological and Clinical Aspects of Human Typhoid Fever. Salmonella Infections: Clinical, Immunological and Molecular Aspects.* (3rd ed.) New York: Cambridge University Press.

Peter, N.D., and John, P.R. (2010). Plant Immunity: Towards an Integrated View of Plant–pathogen Interactions. *Nature Reviews Genetics.* 11(3):539–548.

Philippe, R., Francois, X.W., Christiane, D., Stephen, B., Sylvain, B., Nguyen, T.C., Thi A.H.L., Camilo, J.A., Jeremy, F., Gordon, D., and Mark, A. (2006). Evolutionary History of *Salmonella Typhi. Science*. 314(5803):1301-1304.

Ponmurugan, K., and Shyamkumar, R. (2012). Antibacterial Effect of *Allium sativum* Cloves and *Zingiber officinale* Rhizomes against Multiple-drug Resistant Clinical Pathogens. *Asian Pacific Journal of Tropical Biomedicine*. 7:597-601.

Pui, C. F., Wong, W. C., Chai, L. C., Tunung, R., Jeyaletchumi, P., Noor H.M.S., Ubong, A., Farinazleen, M. G., Cheah, Y.K., and Son, R. (2011). *Salmonella*: A Foodborne Pathogen. *International Food Research Journal*. 18:465-473.

Rios, J.L., and Recio, M.C. (2005). Medicinal Plants and Antimicrobial Activity. *Journal of Ethnopharmacology.* 100(4):1–12.

Raymond, V., Barbehenna, B.C., and Peter C. (2011). Tannins in Plant–herbivore Interactions. *Phytochemistry*. 72:13-15.

Robert, A.N., Peiying, Y., Alison, D.P., and Keith, I.B. (2008). Cardiac Glycosides as Novel Cancer Therapeutic Agents. *Molecular Invention*. 8(5):36-49.

Rajkumar, P., Murari, P., and Nand, K. S. (2011). Anticancer Biology of *Azadirachta indica* L (Neem): A Mini Review. *Cancer Biology and Therapy*. 12:467 – 476.

Santi, S., Aussavashai, S., Thaweephol, D. N. A., Peter, G. W., and Gloria, K. (2006). Chemical Composition Investigation of the *Clinacanthus nutans* Leaves. *Thai Journal of Phytopharmacy.* 13(2): 15-16.

Sarfaraj, M.H., Sheeba, F., and Mohd, S. (2012). Current Approaches Towards Production of Secondary Metabolites. *Journal of Pharmacy Bioallied Science*. 4(1):10-20.

Schafer, H. and Wink, M. (2009). Medicinally Important Secondary Metabolites in Recombinant Microorganisms or Plants: Progress in Alkaloid Biosynthesis. *Biotechnology Journal*. 4(12):1684-1703.

Sejali S.N., and Anuar, M.S. (2011). Effect of Drying Methods on Phenolic Contents of Neem (*Azadirachta indica*) Leaf Powder. *Journal of Herb, Spices and Medicinal Plant*. 17:119-131.

Shahbudin, S., Muhammad, T., Deny, S., Haitham, Q., and Anis, F.I. (2012). *In-vitro* Antimicrobial Activity of Mangrove Plant *Sonneratia alba*. *Asian Pacific Journal of Tropical Biomedicine.* 8(5):427-429.

Sharad, V., and Swati, C. (2012). Isolation of Natural Products. *Current Pharma Research.* 2(3): 584-599.

Shelobolina, E.S., Sullivan, S.A., Neil, K.R., Nervin, K.P., and Lovely, D.R. (2004). Isolation, Characterization, and U(VI)-reducing Potential of a Facultative Anaerobic, Acid-resistant Bacterium from Low-pH, Nitrate and

U(VI) Contaminated Subsurface Sediment and Description of *Salmonella subterranea sp. Applied Enviromental Microbiology*. 70(7):2959–2965.

Shemsu, U., Alemu, T., and Nigatu, K. (2013). Antidiarrhoeal and Antimicrobial Activity of *Calpurnia aurea* Leaf Extract. *Biomedicine Central.* 13:21–22.

Shyamapada, M. (2011). Effect of *Azadirachta indica* A. Juss (Meliaceae) Seed Oil and Extract Against *Culex quinquefasciatus* Larval Susceptibility of Indian Subcontinent. *Macedonian Journal of Medical Sciences*. 4(1): 5-11.

Siti, Z.M., Tahir, A., Ida F.A., Ami, F.S. M., Sondi, S., Azman, A. H., Maimunah, A. H., Haniza, M. A., Siti, H.M. D., Zulkarnain, A. K., Zakiah, I., and Wan, Z.W.C. (2009). Use of Traditional and Complementary Medicine in Malaysia: A Baseline Study. *Complementary Therapies in Medicine*. 17:292-299.

Skene, F., Caroline D., and Philip, S. (2006). Saponin-adjuvanted Particulate Vaccines for Clinical Use. *Methods*. 40(1):9-53.

Sparg, S.G., Light, M.E., and Van, S. J. (2004). Biological Activities and Distribution of Plant Saponins. *Journal of Ethnopharmacology*. 94(14): 219–243.

Syed, S.H., Syed, I.A., Nadeem, I.B., and William C.W.L. (2009). Use of Complementary and Alternative Medicine among Patients with Chronic Diseases at Outpatient Clinics. *Complementary Therapies in Clinical Practice.* 15(3):152-157.

Tindall, B.J., Grimont, P.A., Garrity, G.M., and Euzeby J.P. (2005). Nomenclature and Taxonomy of the Genus *Salmonella*. *International Journal of Systematic and Evolutionary Microbiology*. 55: 521–4.

Tambekar, D.H., and Dahikar, S.B. (2011). Antibacterial Activity of Some Indian Ayurvedic Preparations against Enteric Bacterial Pathogens. *Journal of Advance Pharmaceutical Technology and Research.* 2(13):24-29.

Van, E. H., Temporini, E., and Wasmann, C. (2001). Phytoalexin (and Phytoanticipin) Tolerance as a Virulence Trait: Why Is It Not Required by All Pathogens? *Physiological and Molecular Plant Pathology*. 59(8):83-93.

Vergas, F.D., and Lopez, O.P. (2003). Natural Colourants for Food and Nutraceutical Uses. *Food Chemistry*. 4: 93-109.

Watson, R., Ronald, R.W., and Victor, R.P. (2008). *Botanical Medicine in Clinical Practice.* (3rd ed.) CAB International Cambridge.

Wain, J., House, D., Zafar, A., Baker, S., and Nair, S. (2005). Vi Antigen Expression in *Salmonella enterica* serovar Typhi Clinical Isolates from Pakistan. *Journal of Clinical Microbiology*. 43: 1158–1165.

Yecheng, D., Yanzhen, Y., Haiyu, L., Ming, Z., Xu, Q., and Lifeng, L. (2011). Antimicrobial Activity of Extract and Two Alkaloids from Traditional Chinese Medicinal Plant *Stephania dielsiana. Food Chemistry.* 124(4): 1556–1560.

Yoosook, C., Panpisutchai, Y., Chaichana, S., Santisuk, T., and Reutrakul, V. (1999). Evaluation of Anti-HSV-2 Activities of Barleria lupulina and *Clinacanthus nutans*. *Journal of Ethnopharmacology.* 67(2):179–187.

Yoke, K.Y., Jun, J.T., Soek, S.T., Siau, H.M., Gwendoline, C.L.E., Hoe, S.C., and Zuraini, A. (2013). *Clinacanthus nutans* Extracts Are Antioxidant with Antiproliferative Effect on Cultured Human Cancer Cell Lines. *Evidence-Based Complementary and Alternative Medicine.* 1 (1):8-10.

Zhang, X.L., Jeza, V.T., and Pan, Q. (2008). *Salmonella typhi*: From a Human Pathogen to a Vaccine Vector. *Cell Molecular Immunology.* 5(17): 91–97.