ISOLATION OF PIPERINE FROM THE FRUIT OF *Piper retrofractum* Vahl. (PEIK-CHIN-LAY) AND ANTIBACTERIAL SCREENING OF THE CRUDE EXTRACTS AND PIPERINE

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ABSTRACT

The fruit of *Piper retrofractum* Vahl. (Peik-chin-lay) used in the treatment of fever, cough, asthma, diarrhoea and dysentery in traditional Myanmar medicinal system was chosen for present study. The aim of the study is to isolate piperine from the fruit of *Piper retrofractum* Vahl. (Peik-chin-lay) and to screen the antibacterial activity of its crude extracts and piperine. At first, four crude extracts of the fruit sample were prepared by using various solvents: petroleum ether, ethyl acetate, 96 % ethanol and methanol. *In vitro* antibacterial activity of four crude extracts was investigated against 10 bacterial strains by using agar disc diffusion method. Among the four crude extracts, the most active ethyl acetate extract with the inhibition zone diameter (10-28) mm was selected for isolation of active compound by column chromatographic method using the solvent systems (v/v) PE : EtOAc (19:1, 10:1, 9:1, 5:1, 4:1, 3:1 and 2:1) consecutively. The isolated compound, piperine (0.886 %) was identified by melting point determination, TLC examination and modern spectroscopic techniques such as Ultraviolet, Fourier Transform Infrared Spectroscopy compared with authentic piperine. And then, antibacterial activity was tested on 10 bacteria: *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella paratyphi* A, *Salmonella typhi*, *Shigella dysentery*, *Streptococcus pyogens* and *Staphylococcus aureus* by agar disc diffusion method. It was found that piperine showed higher activity (inhibition zone diameter, 20-25 mm) than that of four crude extracts, less than standard ciprofloxacin (25-30) mm.

Keywords: *Piper retrofractum*, Peik-chin-lay, Isolation, Piperine, Antibacterial activity

INTRODUCTION

The study of traditional medicinal plants and their therapeutics plays a very important role in health care system of Myanmar because 70 % of its population lives in the rural area and they have been using traditional medicine for centuries (Dahanukar *et.al*, 2000). In this study, Myanmar medicinal plant, *Piper retrofractum* Vahl. (Peik-chin-lay) was selected to find out of active principle for the treatment of dysentery and diarrhoea. *P. retrofractum* moderately showed anti-oxidant activity whereas it had a prominent antibacterial activity. Therefore, it is very popular for the treatment of related to the gastrointestinal (GI) tract infection. In fact it could be able to be loss of cholesterol in human body because piperine compound mainly contained in it (Wagner and Bladt, 2005). Peik-chin-lay is composed of health benefiting essential oils besides an alkaloid, piperine gives strong spicy pungent character to the fruit of *P. retrofractum*. Therefore, it is medicinally used in arthritis, throat troubles, carminative, colic, gastric aliments, eczema, scabies, alopecia, other skin diseases, asthma, pain, toothache, bronchitis, rheumatism, fever, cough, dysentery, diarrhoea, flatulence, dermatopathy, oedema and obesity. *P. retrofractum* has pharmacological activities such as antioxidant, antibacterial, antifungal, insecticidal, antipyretic and anti-inflammatory (Upadhyaya, 1998). In Myanmar, *P. retrofractum* is used as the house hold remedy in treating diarrhoea, dysentery, fever, cough, indigestion, stomachaches and asthma.

Therefore, antibacterial activity investigation on four crude extracts (PE, EtOAc, 96% EtOH, MeOH) and the isolated phytoconstituent from the fruit of *P. retrofractum* were carried out by using agar disc diffusion method in this study.

Botanical Aspects of *Piper retrofractum* Vahl.

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Scientific name : *Piper retrofractum* Vahl.

English name : Balinese pepper

Myanmar name : Peik-chin-lay

Family : Piperaceae

Fruit (part : Fruits are tiny berries, rod-like structure and merge to a single.

(Kress, *et al*., 2003)

Figure 1 Plant of *Piper retrofractum* Vahl.

Distribution

The *Piper* species are mostly cultivated in Indonesia, Bangladesh, China, Phillippines Thailand and also found in many other parts of the Myanmar as a run wild. The plant of *P. retrofractum* was shown in Figure 1.

Chemical Constituents

In fruits of *P. retrofractum* contained piperine, piperidine, piperlonguminine, sylvatine, guineensine, piperlongumine, filfiline, sistosterol, methyl piperate and a series of piperine-analog retrofractum (Bodiwala, *et.al*, 2007).

MATERIALS AND METHODS

Plant Material

The fruit sample of *P. retrofractum* Vahl. (Peik-chin-lay) was collected from Kyauk-ey Village, Hinthada Township, Ayeyawady Region. It was identified by a botanist from Botany Department, Hinthada University. The fruit of *P. retrofractum* was washed, cleaned and dried at room tempreture. Then the dried sample was powdered and stored in air- tight container.

Instruments: Shimadzu UV-1800 (MeOH) at AMTT (Yangon); Shimazu Perkin Elmer Spectrum GX FT IR (URC) University of Mandalay

Chemicals : CC; Merck Silica gel 60 (70-230) mesh, eluents; Petroleum- ether (PE) - ethyl acetate(EtOAc), TLC; precoated silica gel 60 (F_{254} Aluminium plates, Merck)

Extraction and Isolation of Piperine from Fruit of *Piper retrofractum* Vahl.

Preparation of extracts from fruit of *Piper retrofractum* Vahl. by cold extraction method

The air-dried powder (50 g) was individually cold extracted with (500 mL) of solvents; petroleum ether (60-80 $^{\circ}$C), ethyl acetate, 96 % ethanol and methanol for three weeks and then filtered. The filtrate was evaporated to dryness at normal pressure on a water bath and desiccated. The yield % of petroleum ether (PE), ethyl acetate (EtOAc), 96 % ethanol (EtOH) and methanol (MeOH) extract were determined.

Isolation of phytoconstituent from ethyl acetate extract of fruit of *Piper retrofractum* Vahl. by column chromatographic method

The ethyl acetate (EtOAc) extract was subjected to isolate the active phytoconstituent from Peik-chin-lay fruit by column chromatography. The column was packed with silica gel (50 g) by the wet method using petroleum ether. Then column was eluted consecutively with the solvent system (PE : EtOAc) 19:1, 10:1, 9:1, 5:1, 4:1, 3:1 and 2:1 (v/v) according to their increasing polarity. The column was completely filled with the solvent system and fractionation was started. The fractions were collected by 5 mL per small bottle at the rate of about one drop per second. The collected fractions were monitored by TLC with 5 % sulphuric

acid and Dragendroff's reagent. Total 40 bottles were collected. The fractions that gave similar spots on TLC plates were combined together and the solvent was removed. Finally, isolated compound (yellow crystal, 0.045 g, 0.886 %) was characterized as piperine by TLC method shown in Figure 2 and spectroscopic methods: UV, FT IR compared with authentic sample, piperine obtained from *P. longum* L. (Peik-chin) shown in Figure 3, 3 (a), 4, and 4 (a).

In vitro Studies on the Antibacterial Activity of Fruit of *Piper retrofractum* Vahl. by Agar Disc Diffusion Method

Screening of antibacterial activity of crude extracts against 10 tested bacterial strains

Agar disc diffusion method was used for the detection of antibacterial activity for four crude extracts from *P. retrofractum* fruit. The test procedure was as follows: the extracts (1 g each) were dissolved in 1 mL of their respective solvents; petroleum ether, ethyl acetate, 96 % ethanol and 50 % ethanol, and introduced into sterile petridishes for testing 10 cultural bacterial strains. The discs having 6 mm diameter each with 20 µg extract/disc were allowed to dry at 42 $^{\circ}$ C in incubator. The bacterial suspension from trypticase soy broth was streaked evenly into three places on the surface of the trypticase soy agar plates with sterile cotton swab (Puritan, USA). After the inoculums had dried for 5 min, the dried disc impregnated with extracts were placed on the agar with flamed forceps and gently pressed down to ensure proper contact. A disc impregnated with solvent only was used as control and antibiotics ciprofloxacin was also used as standard for this study. After overnight incubation at 37 $^{\circ}$C, the zones of inhibition diameter including 6 mm discs were measured. Out of four crude extracts, the most active extract (EtOAc) was selected for isolation of active compound and bactericidal action.

Screening of antibacterial activity of the isolated piperine from active EtOAc extract of fruit (*Piper retrofractum* Vahl.) against 10 tested bacterial strains

The isolated compound piperine (0.045 g, 0.886 %) were subjected to study antibacterial activity against 10 tested bacteria from clinical sources, National Health Laboratory (NHL), Yangon; related to acute diarrhoea (cholera), dysentery, abscess, fever, urinary tract infection (UTI), food poisoning and typhoid.

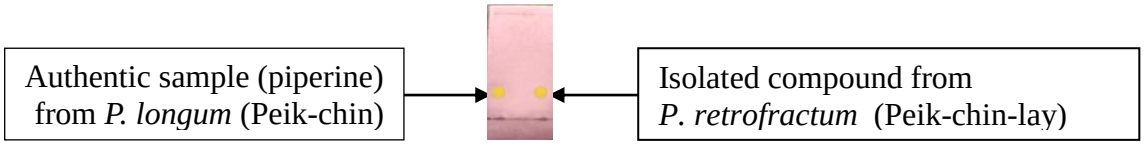
RESULTS AND DISCUSSION

Isolation and Characterization of Piperine from Ethyl Acetate Extract of *Piper retrofractum* Vahl. Fruit

The dried fruit powder collected from Kyauk-ey Village, Hinthada Township, Ayeyawady Region was extracted with various solvents and the yield % of petroleum ether extract (4.20 %), ethyl acetate extract (6.40 %), 96 % ethanol extract (10.60 %) and methanol extract (15.64 %) respectively were obtained. Piperine (yellow crystal, 0.045 g, 0.886 %) was isolated from ethyl acetate extract of *P. retrofractum* by column chromatographic separation method using petroleum ether - ethyl acetate solvent system. The isolated compound as piperine was identified by melting point determination, R_f value with the authentic piperine from *P. longum* (Figure 2) and also some modern spectroscopic techniques such as UV (Figure 3, 3 a), FT IR (Figure 4, 4 a).

Piperine : Yellow crystal (0.045 g, 0.886 % yield); Melting point, 128 °C; R_f value 0.25, PE: EtOAc (2:1), agreeable with authentic piperine (Figure 2); λ_{max} 310, 342 (nm) ($n \rightarrow \pi^*$), λ_{max} 309, 340 (nm) (Ei Ei Khaing, 2005) (Figure 3, 3 a); FT IR /cm^{-1} 3016, (3009, authentic piperine) (aromatic ν_{C-H}), 2935, 2862, (2940, 2920, 2862, authentic piperine) (asymmetric and symmetric ν-CH_2 of aliphatic C-H), 1626, (1634, authentic piperine) ($\nu_{C=O}$), 1581, (1612 authentic piperine) (asymmetric and symmetric $\nu_{C=C}$ diene), 1494, (1584, authentic piperine)

($\nu_{C=C}$ phenyl ring), 1444, 1361, (1449, authentic piperine) (δ -CH_2), 1274, 1195, (1253, 1194, authentic piperine) ($\nu_{asym\ C\text{-}O\text{-}C}$), 1126, (1134, authentic piperine) (δ_{CH} of phenyl), 1026, (1032, 1018, authentic piperine),($\nu_{sym\ C\text{-}O\text{-}C}$), 931, (997, authentic piperine) (δ_{C-H} of trans CH = CH group), 840, 621, (847, 831, 805, authentic piperine) (δ_{C-H} out of plane bending); (Figure 4, 4a) (Silverstein, *et al*., 1991). The FT IR spectrum of isolated compound was comparatively assigned with FT IR spectrum taken from literature (Ei Ei Khaing, 2005).



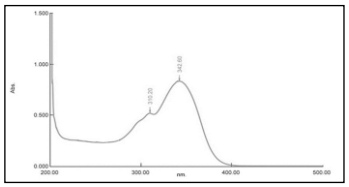
Stationary Phase : Silica gel GF_{254}

Mobile Phase : PE : EtOAc (2:1)

Visualization : Dragendorff's reagent

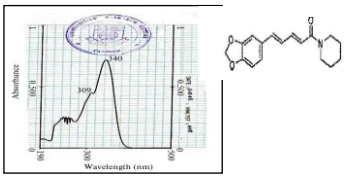
R_f value of isolated compd. & piperine (authentic) : 0.25

Figure 2 Comparative thin layer chromatogram of isolated compound from *P. retrofractum* fruit and authentic (piperine) from *P. longum* fruit



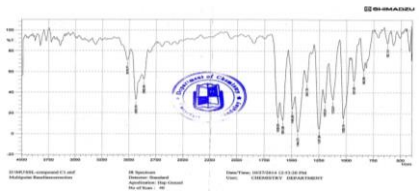
*San San Lae, 2015

Figure 3 UV spectrum of isolated compound from *P. retrofractum* (Peik-chin-lay) fruit*



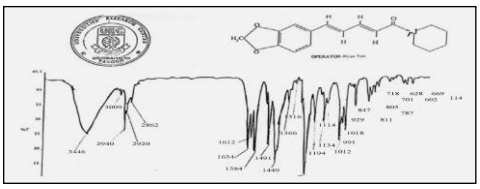
*Ei Ei Khaing, 2005

Figure 3 (a) UV spectrum of authentic (piperine) isolated from *P. longum* (Peik-chin) fruit*



*San San Lae, 2015

Figure 4 FT IR spectrum of isolated compound from *P. retrofractum* (Peik-chin-lay) fruit*



*Ei Ei Khaing, 2005

Figure 4(a) FT IR spectrum of authentic (piperine) isolated from *P. longum* (Peik-chin) fruit*

Screening of Antibacterial Activity of Crude Extracts and Isolated Piperine

Screening of antibacterial activity of the four crude extracts has been done by agar disc diffusion method at NHL. Agar disc diffusion method is based on the zone diameter in millimeter (mm) of agar disc. Larger the zone diameter is the more activity on the tested bacteria. The inhibition zone diameters of extracts tested with 10 species of bacteria took out the patients suffering from fever, diarrhoea, dysentery, UTI, typhoid and food poisoning to know how the selected plant effect on the bacteria from the clinical sources shown in Table 1 and Figure 5. The 10 tested bacteria comprised of the Gram - negative bacteria: E*scherichia*

coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella paratyphi A, *Salmonella typhi, Shigella dysentery* and Gram-positive bacteria: *Bacillus subtilis, Streptococcus pyogen*s, *Staphylococcus aureus*. Out of the four crude extracts of *P. retrofractum* fruit, PE, EtOAc and 96 % EtOH extracts showed against 10 tested bacteria whereas methanol extract did not show activity on *Salmonella typhi*. In general, *P. retrofractum* fruit possess affective bactericidal activity and it could be recommended that the plant's antibacterial substances appear to be more inhibition to Gram-negative bacteria than to Gram-positive type as the results of inhibition zone diameter. Due to the sensitivity of synergistic effect or moderately polar compounds in the most active ethyl acetate extract with the range of inhibition zone diameter (10-28) mm against 10 strains: *Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella paratyphi* A, *Salmonella typhi, Shigella dysentery*, *Streptococcus pyogen*s, and *Staphylococcus aureus* was selected for isolation of active compound. And then antibacterial activity of isolated piperine yielded (0.886 %) from *P. retrofractum* fruit was tested by the comparison with ciprofloxacin (std.) on 10 tested bacteria the same as four crude extracts shown in Table 2 and Figure 6. In Table 2 it was found that the isolated piperine showed remarkable inhibition zone diameters (20-25) mm of all strains as near as ciprofloxacin (std.) resulted range between (25-30) mm. The overall results of throughout the antibacterial screening could be evaluated and assigned that piperine displayed more potent than the four crude extracts of *P. retrofractum* fruit as the structure activity relationship (SAR). The structural features, an aromatic ring with a methylenedioxy bridge, a conjugated dienone system and a piperidine ring constituting an amide bond, possessed by the piperine molecule could be considered important for the molecule to exhibit an array of bactericidal action. Therefore piperine isolated from *P. retrofractum* fruit would be more effective for the treatment of fever, food poisoning, typhoid, dysentery, diarrhoea and UTI. It has antibacterial action against *E. coli* responsible for diarrhoea, *Klebsiella* sp. responsible for fever and UTI, *Salmonella* spp. responsible for food poisoning and *Staphylococcus aureus* responsible for abscess occurred in skin, mouth and nose.

Table 1 Results of Antibacterial Activity of Four Extracts of *P. retrofractum* on 10 Species of Bacteria

No.	Type of bacteria	Inhibition zone diameter (mm)				
		PE extract	EtOAc extract	96 % EtOH extract	MeOH extract	Ciprofloxacin (std.)
1	*Bacillus subtilis*	25	27	23	25	28
2	*Escherichia coli*	14	25	15	23	25
3	*Klebsiella pneumonia*	20	10	12	17	28
4	*Proteus vulgaris*	20	16	15	19	25
5	*Pseudomonas aeruginosa*	21	28	22	22	30
6	*Salmonella paratyphi*	20	26	25	25	28
7	*Salmonella typhi*	21	23	27	-	25
8	*Shigella dysentery*	16	24	22	22	25
9	*Streptococcus pyogens*	16	22	20	21	25
10	*Staphylococcus aureus*	16	22	20	22	25

(-) = no activity, Disc diameter = 6 mm

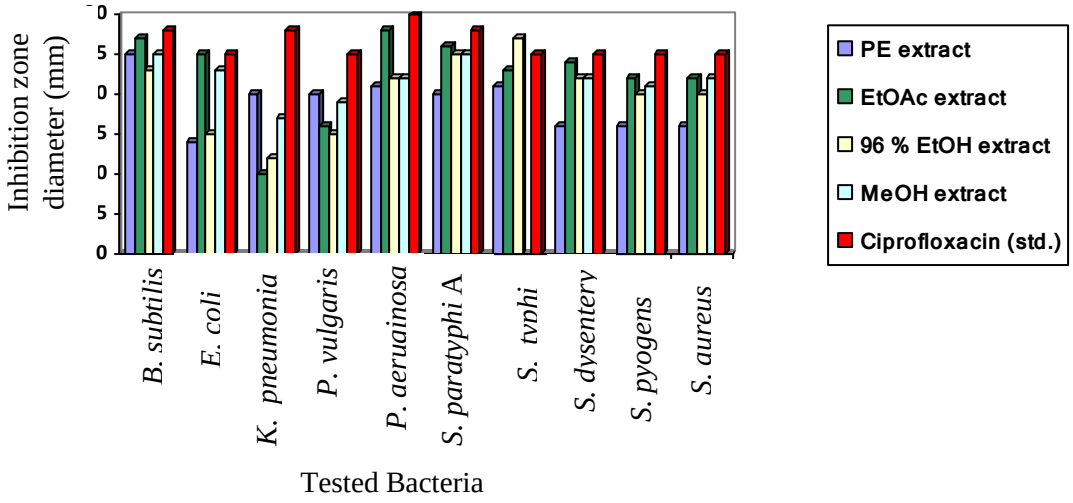


Figure 5 Antibacterial activity of crude extracts of *P. retrofractum* fruit on 10 species of bacteria by using agar disc diffusion method

Table 2 Antibacterial Activity of Piperine Isolated from Fruit of *P. retrofractum*

Sample	Inhibition zone diameter (mm)									
	1	2	3	4	5	6	7	8	9	10
Piperine	25	21	23	22	20	22	20	20	24	24
Blank	-	-	-	-	-	-	-	-	-	-
EtOAc solvent (control)	-	-	-	-	-	-	-	-	-	-
Ciprofloxacin (standard)	25	25	26	30	29	28	25	25	25	25

Tested Bacteria (From Clinical Sources*)

1= *Bacillus subtilis* 6 = *Salmonella paratyphi* A

2 = *Escherichia coli* 7 = *Salmonella typhi*

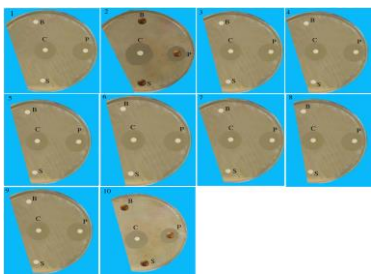
3 = *Klebsiella pneumonia* 8 = *Shigella dysentery*

4 = *Proteus vulgaris* 9 = *Streptococcus pyogens*

5 = *Pseudomonas aeruginosa* 10 = *Staphylococcus aureus*

Disc diameter = 6 mm - = no activity

***** National Health Laboratory (NHL), Yangon



Clockwise position

1= *Bacillus subtilis* 6 = *Salmonella paratyphi* A

2 = *Escherichia coli* 7 = *Salmonella typhi*

3 = *Klebsiella pneumonia* 8 = *Shigella dysentery*

4 = *Proteus vulgaris* 9 = *Streptococcus pyogens*

5 = *Pseudomonas aeruginosa* 10 = *Staphylococcus aureus*

P = Piperine S = EtOAc solvent (control)

B = Blank C = Ciprofloxacin (standard)

Figure 6 Antibacterial activity of piperine isolated from *P. retrofractum* on 10 tested bacter

CONCLUSION

From the fruit of *P. retrofractum* Vahl. (Peik-chin-lay) fruit, four crude extracts: PE extract (4.20 %), EtOAc extract (6.40 %), 96 % EtOH extract (10.60 %) and MeOH extract (15.64 %) were obtained and screened the antibacterial activity against 10 tested bacteria by agar disc diffusion method. Among the four crude extracts of Peik-chin-lay, only EtOAc extract showed the most potent antibacterial activity with the related larger zone diameter (10-28) mm on 10 bacterial strains; *Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella paratyphi* A, *Salmonella typhi, Shigella dysentery*, *Streptococcus pyogen*s, and *Staphylococcus aureus.* Using column chromatographic separation, yellow crystal (0.045 g, 0.886 %) was isolated from the most active EtOAc extract of *P. retrofractum* and identified as piperine by melting point determination, R_f value with the authentic piperine from *P. longum* whereas modern techniques: UV and FT IR spectroscopy. *In vitro* antibacterial activity of piperine was also investigated. It was found that piperine showed the range of inhibition zone diameter between (20-25) mm by comparison with inhibition zone diameter of standard ciprofloxacin ranged between (25-30) mm. It may be concluded that the antibacterial activity of piperine, pure compound was more potent than that of the crude EtOAc extract against 10 tested bacteria effective for the treatment of dysentery, urinary tract infection, fever, food poisoning, typhoid abscess and diarrhoea. From these observations, it may be recommended that the ethyl acetate extract of fruit of *P. retrofractum* (Peik-chin-lay) and isolated piperine may be used as main materials for the traditional medicine formulation in the treatment against dysentery, urinary tract infection, fever, food poisoning, typhoid, abscess and diarrhoea.

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REFERENCES

Bodiwala, H. S., Singh, G., Singh, R., Sankar, C. and Shyam, D. (2007). "Antileishmanial amides and lignans from *Piper cubeba* and *Piper retrofractum*", *Journal of Natural Medicines*, vol. 61(4), pp. 418-421

Dahanukar, S. A., Kulkarni, R..A. and Rege, N. N. (2000). "Pharmacology of Medicinal Plants and Natural Products". *Indian Journal of Pharmacology*, vol. 32, pp. 103-104

Ei Ei Khaing. (2005). *Isolation, Identification and Screening of Antibacterial Activities of Some Active Principles of Acorus calamus Linn. (Lin-Ne) and Piper longum Linn. (Peik-chin) for the Treatment of Dysentery and Diarrhoea,* PhD Disseration, Department of Chemistry, University of Yangon

Kress, W.J., Defilipps, R.A., Farr, E. and Daw Yin Yin Kyi. (2003). *A Checklist of the Trees, Shrubs, Herbs and Climbers of Myanmar.* Washington, DC, USA: 1st Ed., Department of Systematic Biology-Botany, National Museum of Natural History, p. 37

San San Lae. (2015). *Some Chemical Analyses of Piper retrofractum Vahl. (Peik-chin-lay) Fruits and Piper betle L. (Kunn) Leaves from Hinthada Area and their Comparative Studies on Antibacterial and Antioxidative Efficiency*, MRes Thesis, Department of Chemistry, Hinthada University, pp. 61-99

Silverstein, R.M., Bassler, G.C. and Terence, C.M. (1991). *Spectrometric Identification of Organic Compounds*, Singapore: 5th Ed., John Wiley and Sons, Inc., pp. 289-315

Upadhyaya, K.N. (1998). *A Text Book of Inorganic Chemistry*. London: 3rd Revised, Vikas Publishing House Private, Limited, pp. 213-217

Wagner, H. and Bladt, S. (2005). *Plant Drug Analysis.* New York: 2nd Ed., Springer, pp. 355-369