ASIAN JOURNAL OF PHARMACOGNOSY
Asian J. Pharmacogn 3(4):27 - 35

eISSN-0128-1119

Research Article

In Vivo evaluation of *Catharanthus roseus* L. (pink) extracts against *Rigidoporus microporus* on rubber (*Hevea brasiliensis*) seedlings

Rozihawati Zahari[1], Razak Terhem[1], Normala Halimoon[2], Mohd Farid Ahmad[3] and Nor Hayati Abdullah[4]

[1]*Laboratory of Forest Pathology, Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia.*
[2]*Department of Environmental Sciences, Faculty of Environmental Studies, Universiti Putra Malaysia, Serdang, Malaysia.*
[3]*Biodiversity Division, Forest Research Institute Malaysia, 52109 Kepong, Selangor, Malaysia.*
[4]*Herbal Product Development Program, Natural Product Division, Forest Research Institute Malaysia, 52109 Kepong, Selangor, Malaysia.*

*For correspondence: razakterhem@upm.edu.my

Abstract: *Hevea brasiliensis* (rubber) is an important species in rubber industries. The species is often attacked by *Rigidoporus microporus* in the plantations and chemical fungicides have been widely used to control this fungus. However, fungicides contribute to human health problems and environmental pollutions. To address the issues, bio-fungicide from the plant sources is an innovative alternative to replace the chemical fungicide. Thus, the objectives of the study were to investigate the effect of *Catharanthus roseus* extract in different concentrations against *R. microporus* on rubber seedlings. The efficacy of *C. roseus* extracts against *R. microporus* was assessed based on healthy effects percentage of the rubber seedlings, by assessing the symptoms on leaves and roots. The assessment was based on disease index (DI) and disease suppression (DS%). The DI results showed, at 2000 µg/mL was the most effective against *R. microporus* on rubber seedlings with level disease index of level 0 (0%) compared to 1500 and 1000 µg/mL with level 2 (26 - 50%), and level 4 (76 - 100%), respectively. The DS% results also showed that the extract was most effective at 2000 µg/mL in controlling the growth of *R. microporus* on rubber seedlings with a value of 100% compared to 1500 and 1000 µg/mL with a value of 0.0%. Hence, *C. roseus* extract might be suitable use as a biofungicide for controlling *R. microporus* on rubber crops.

Keywords: Root rot disease, extract concentration, Pathogenicity test and Application of plant extract

ASIAN JOURNAL OF PHARMACOGNOSY *Research Article*
Asian J. Pharmacogn 3(4):27 - 35

eISSN-0128-1119

INTRODUCTION

Hevea brasiliensis is an important species that receive high demand throughout the world and thus, contributes to our country's economy (Van Beilen & Poirier, 2007; Tey et al., 2008). The latex from rubber trees is important especially in rubber industries (Van Beilen & Poirier, 2007). The rubber is also being widely used in pharmaceuticals, pesticides, and immune allergens resources (Ravi, 2011). However, mortality in rubber plantations is nowadays owed to root rot disease. According to Mohd Farid (2010), white root rot disease caused by *R. microporus,* is one of the major root diseases in the rubber plantations. It causes considerable damage to rubber plantations in Malaysia involving 18% of over 700 hectares (Holiday, 1980). The white root rot fungus (*R. microporus*) is also the most aggressive in killing *Acacia mangium, Azadiractha excelsa,* and *Tectona grandis* in Peninsular Malaysia (Mohd Farid et al., 2006). The disease is currently being kept under control with fungicides such as bayfidan and bayleton (Chan et al., 1991). These chemical fungicides are effective for controlling the root rot disease. However, these fungicides result in various problems in environmental ecosystems (Brent & Hollomon, 1998).

Due to these problems, the search for an effective and environmentally safe compound to control this fungus is highly warranted. Recent studies show that biofungicides of plant origin are not only effective on the target pathogens, but they are also environmentally safe and have gained a wide public acceptance (Lee et al., 2007). As reported by Alam (2009), most of the researchers focused on medicinal plants which have been used as important sources of antifungal agents and development of bio-fungicides production. One of the locally available plants with abundant active compounds present in the extract is *C. roseus*. This plant has an antifungal activity (Balaabirami & Patharajan, 2012). For example, the leaves of *C. roseus* extract was active against *Aspergillus fumigatus, Candida albicans, Penicillium chrysogenum,* and *Aspergillus niger* (Balaabirami & Patharajan, 2012).

Flower extracts of the plant also effective against *A. niger, C. albicans,* and *Candida lipolytica* (Eufrocinio et al., 2002). According to Moreno et al. (1994a), the leaves of *C. roseus* contains phenolic compound such as 2,3-dihydroxybenzoic acid which acts as antifungal against *Phytium aphaniderma*. Leaves of *C. roseus* extracts also showed antifungal activity against *Fusarium moniliforme* (Kratika & Sharmita, 2013). The stems of *C. roseus* extract had also a potential effect in inhibiting the growth of *R. microporus* (Rozihawati et al., 2014). Therefore, the plant might be suitable to be used as organic fungicides. Thus, the study aims to study the application of *C. roseus* extract to control *R. microporus* on rubber seedlings. Besides, the effectiveness of plant extract in controlling this fungus on the seedling stage is mainly important to ensure that the planting stock production is adequate.

MATERIALS AND METHODS

Preparation of Plant Extract and Fungus: Stems of *C. roseus* with pink flower species were collected from Terengganu, Malaysia. These plants were extracted with dichloromethane (DCM) as described by Rozihawati et al. (2014). About 200 g of *C. roseus* crude extract was used in the study. This extract was dissolved in 5% dimethyl sulfoxide (DMSO) as per Sharif et al. (2010). Then, the extract was mixed with Tween 20 (200 μg/mL) to reduce contamination. The extract was dissolved in distilled water at 1000, 1500, and 2000 μg/mL concentrations. The plant extracts were stirred at 35°C for one hour using a hotplate magnetic stirrer. *R. microporus* (FRIM646) was obtained from the Pathology Laboratory at FRIM, Kepong. This fungus was cultured on potato dextrose agar (PDA) and incubated in a culture room at 25±2°C for six days.

ASIAN JOURNAL OF PHARMACOGNOSY *Research Article*
Asian J. Pharmacogn 3(4):27 - 35

eISSN-0128-1119

Planting Stock of Rubber Seedlings: A total of 55 of healthy seedlings for each rubber seedling were used in the experiment. Five months old rubber seedlings were obtained from a nursery at Sungai Buloh, Selangor. Seedlings were planted in polybag containing sterilized mixed soil (topsoil:sand: peat soil at a ratio of (3: 2: 1 v/v/v). The potting size was 20 x 30 cm. Potted seedlings were kept in a greenhouse condition with relative humidity at 75%, the air temperature around 31ºC and 75% light intensity.

Inoculum Blocks Preparation of Fungus: Inoculums blocks using young rubber branches were prepared according to the method as described by Mohd Farid (2010). For the pathogenicity test, the rubber branches were debarked and cut into woodblocks of approximately 1.5 – 2.0 cm diameter by 8 cm length. A total of 6 pieces of the branch cuttings were placed in autoclave plastic bags filled with 50 mL of 2% Malt Extract (ME). Then, the mouth of the bags was sealed with PVC rings and cotton plugged. The bags were autoclaved at 121°C, 1 kgf/cm2 for 20 minutes. One week old of five fungal discs actively growing cultures were inoculated into the bags using a sterilized needle. The bags were then plugged with cotton and incubated in the dark at room temperature (28 ± 2°C) for one month.

Pathogenicity Test and Application of Catharanthus roseus Extract for Controlling Rigidoporus microporus on Rubber Seedling: A total of 30 rubber seedlings were used for pathogenicity and disease index (DI) tests. The stocks of *R. microporus* were prepared from seven days cultured in PDA at 28ºC. Preparations of inoculums blocks using young rubber branches were done according to Mohd Farid (2010). Three inoculums were placed on main root into planting a poly bag measuring 20 cm radius × 30 cm height, as described by Mohd Farid (2010). Each seedling was placed the inoculum blocks in contact with the main root approximately 10 cm below the soil surface (Mohd Farid, 2010). After inoculation, 500 mL of plant extract at 1000, 1500, and 2000 µg/mL were poured in the poly bag for each seedling. The application of *C. roseus* extract was conducted for three days. Five hundered mL of plant extract was also poured into each seedling every 2 weeks. The seedlings not treated with plant extract were used as the control. The inoculated rubber seedlings were observed for disease infection (Mohd Farid, 2010). The above-inoculated rubber seedlings were examined at two weekly intervals for four months of rubber seedlings. Disease index (DI) based on leaf symptoms as shown in Table 1. Disease index (DI%) = [(Yellowing of leaves number/total leaves number)] × 100 (Kaewchai & Soytong, 2010). The root fungal infection assessment symptoms were done as described by Mohd Farid (2010). A total of 25 seedlings of each rubber were used for the roots assess based on observation of the root surface. The root and leaf symptoms from rubber seedling were assessed every two weeks for 4 months. The mortality of seedlings was also recorded. The antifungal efficacy of the infected plant samples was also evaluated by the percentage of disease suppression efficacy as described by Sharif et al. (2010).

Table 1. Scale of disease index rating based on leaf number and symptoms of white root rot disease (*Source: Kaewchai and Soytong, 2010*).

Level	Percentage (%) based on leaf number	Number of seedling (N)	Symptoms
0	0	5	Healthy – Plant grows vigorously with leaves green and well-formed canopy
1	1 – 25	5	Yellowing of leaves
2	26 – 50	5	Yellowing of leaves
3	51 – 75	5	Yellowing of leaves
4	76 – 99	5	Yellowing of leaves
5	100	5	No leaves or dead

ASIAN JOURNAL OF PHARMACOGNOSY
Asian J. Pharmacogn 3(4):27 - 35

eISSN-0128-1119

Research Article

Statistical Analysis: The experiments used Complete Randomised Design (CRD) with five replicates. Kruskal-Wallis and Tukey's range tests were used to compare the means of seedling and concentration treatments in the experiment.

RESULTS

Mortality of Rubber Seedling: Mortality percentages of rubber (*H. brasiliensis*) seedlings were tested against *R. microporus* and observed every two weeks for four months (Figure 1). The mortality of rubber seedling began to occur at week 6 and ended at week 16 with a record of death 2 % and 100 %, respectively. The mortality of the seedlings increased in every two weeks. However, treatment control (water + DMSO) did not show any mortality effect until week 16.

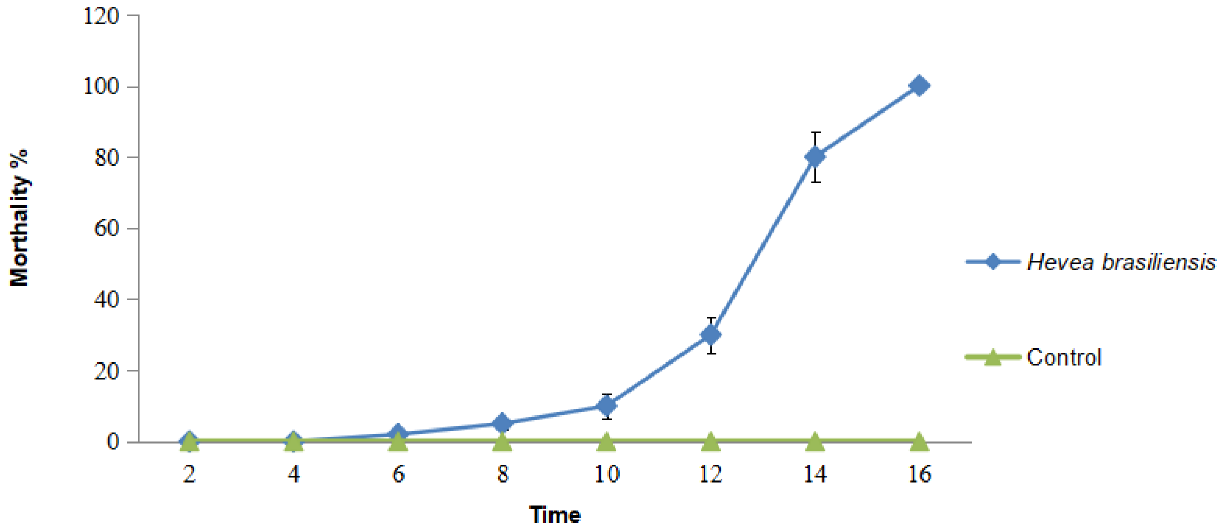

Fig 1: Mortality of rubber (*Hevea brasiliensis*) seedlings with treated by *Rigidoporus microporus.* Values of mortality are average of five replicates ± SEM.

Level Disease Index of Rubber Seedling: Level disease index (DI) of rubber seedlings based on the effect of *C. roseus* extract against *R. microporus* for 4 months is shown in Figure 2. The results of this treatment after 16 weeks was significant ($p \leq 0.05$) by Kruskal-Wallis test analysis (Table 2). Two thousand µg/mL was the most effective concentration to control the fungus on rubber seedlings with level disease index of level 0 (0%) compared to 1500 and 1000 µg/mL with level 2 (26 - 50%) and level 4 (76 - 100%), respectively. The leaves of rubber seedlings inoculated with *R. microporus* showed yellow symptoms at levels 2 and 4 on week 16. Meanwhile, those in 2000 µg/mL and the controls (water + DMSO) remained healthy with no symptoms of disease infection.

ASIAN JOURNAL OF PHARMACOGNOSY
Asian J. Pharmacogn 3(4):27 - 35

eISSN-0128-1119

Research Article

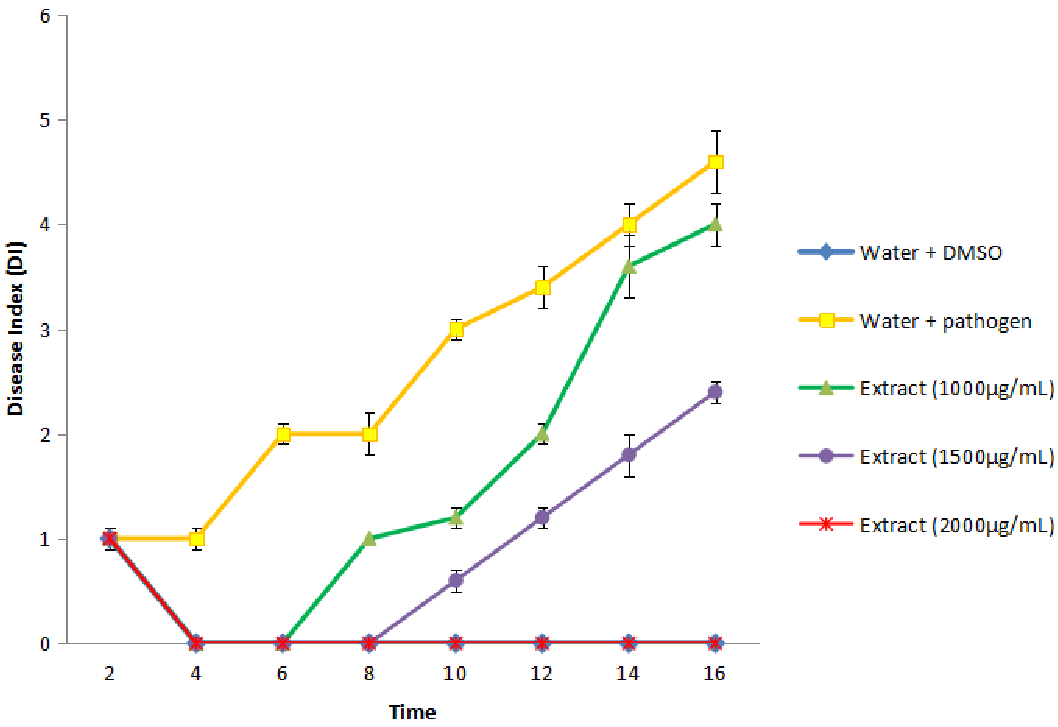

Fig. 2: Level disease index (DI) of rubber (*Hevea brasiliensis*) seedlings for comparison between different concentrations of *Catharanthus roseus* extract against *Rigidoporus microporus* for 16 weeks. Values of disease index (DI) are average of five replicates ± SEM.

Table 2. Kruskal-Wallis test of level disease index (DI) of rubber (*Hevea brasiliensis*) seedlings for comparison between different concentrations of *Catharanthus roseus* extract against *Rigidoporus microporus* after 16 weeks.

Seedlings	Category	Mean	K-Wallis value	R value
	Water + DMSO (control)	0 ± 0.0	33.1	0.000*
	Water + Pathogen (control)	5 ± 0.1		
Rubber	Extract (1000µg/mL)	5 ± 0.1		
	Extract (1500µg/mL)	4 ± 0.4		
	Extract (2000µg/mL)	0 ± 0.0		

R values of the column are significantly different at p ≤ 0.05.

Percentage of Disease Suppression on Rubber Seedling: Percentage for disease suppression (% DS) of rubber seedlings were observed based on healthy effects percentage of the seedlings by assessing the symptoms of leaves and roots after inoculating *R. microporus* of each two weeks interval for 16 weeks (Figure 3). The results of this treatment after 16 weeks was significant ($p \leq 0.05$) by Kruskal - Wallis test analysis (Table 3). The % DS results shows that the extract at concentration of 2000

ASIAN JOURNAL OF PHARMACOGNOSY
Asian J. Pharmacogn 3(4):27 - 35

eISSN-0128-1119

µg/mL with a value of 100 % was the most effective to control fungal pathogen on rubber seedlings compared to 1500 and 1000 µg/mL with value 0.0 %. The fungal pathogen treated on the seedlings also showed % DS with value 0.0 %. This value indicates that the fungus was damaging the main root of the seedlings.

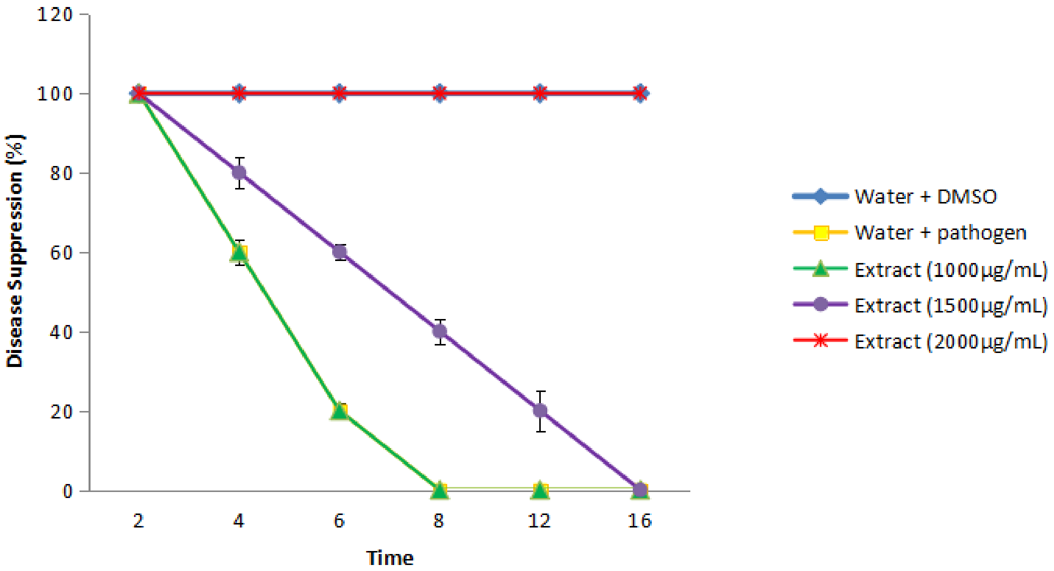

Fig. 3: Percentage of disease suppression of rubber (*Hevea brasiliensis*) seedlings for comparison between different concentrations of *Catharanthus roseus* extract against *Rigidoporus microporus* for 16 weeks. Values of disease suppression percentage are average of five replicates ± SEM.

Table 3. Kruskal-wallis test of disease suppression percentage efficacy of rubber seedlings for comparison between different concentrations of *Catharanthus roseus* extract against *Rigidoporus microporus* after 16 weeks.

Seedlings	Category	Mean	K-Wallis value	R value
Rubber	Water + DMSO (control)	100 ± 0.0	31.09	0.000*
	Water + Pathogen (control)	0 ± 0.0		
	Extract (1000 µg/mL)	0 ± 0.0		
	Extract (1500 µg/mL)	0 ± 0.0		
	Extract (2000 µg/mL)	100 ± 0.0		

R values of the column are significantly different at $p \leq 0.05$.

ASIAN JOURNAL OF PHARMACOGNOSY *Research Article*
Asian J. Pharmacogn 3(4):27 - 35

eISSN-0128-1119

DISCUSSION

In this study, at week 16, it was found that 100% mortality of rubber seedling aged five months old was caused by *R. microporus* attack. The fungus also has caused 80% mortality of one year old rubber seedling within four months in nursery (Mohd Farid, 2010). The fungus also damaged rubber plantations in Malaysia by 18% over 700 hectares (Holiday, 1980). According to Lee (1996), several types of root or woody root rot diseases have been recognized in *Acacia mangium* plantations and they are characterized as white *R. microporus* based on colour of the fungal tissue infection. These symptoms are caused by the fungal hyphae damage to the root cells; the rapid progress of the infection causes mortality (Ismail & Azaldin, 1985).

The study also showed that the leaves of rubber seedlings became yellowish on week 16. According to Mohd Farid (2010), rubber inoculated with *R. microporus* was the fastest to develop the yellow symptom on the leaves. Moreover, the fungus only took 12 weeks in turning the leaves into yellowish colour to the majority of the inoculated seedlings (93.33%). The symptom indicated that the invasion of fungus into root systems was relatively rapid. As reported by Nicole et al. (1986), rubber seedlings inoculated with *R. microporus* developed a fast above ground symptoms toward the infection. When the fungus seriously attacks the root cells, it can also cause the leaves to wilt and fall. At this stage, fungicide treatment on the crops for controlling the fungal pathogens will be not effective (Ismail & Azaldin, 1985).

Therefore, early treatment was proposed in the study to ascertain the plant extract will effectively control *R. microporus* on rubber seedlings. Effectiveness of *C. roseus* extract on the fungus was determined by disease suppression (%DS). %DS results showed that the extract concentration of 2000 µg/mL with a value of 100 % was effective to control *R. microporus.* However, it was found from the DS % results that the concentration of extract at 1500 and 1000 µg/mL was not effective against the fungus (0.0%). It indicated that the fungus was damaging the main root of the rubber seedlings. As reported by Mohd Farid (2010), the fungus affects the lateral roots first before attacking the main root. When the rhizomorphs get in contact with healthy roots of the rubber tree, penetration and death of the cambium occur.

Thus, it was found that the *C. roseus* extract at 2000 µg/mL concentration was the most effective to control the growth of *R. microporus* as compared to 1500 and 1000 µg/mL concentration. *C. roseus* extract contained ursolic acid and the compound was terpenoids (Lili et al., 2012). According to Gurjar et al. (2012), terpenoids show antimicrobial effect which can destroy the membrane cells of fungi. The *C. roseus* extract also contained various phenolics and alkaloids. The compounds are actively acting as antifungal (Moreno et al., 1994a; Natali and Robert, 2007; Singh et al., 2010). In addition, *C. roseus* extract contained flavonoids, alkaloids and carbohydrates (Phani Deepthi Yadav et al., 2013). The phenolics and alkaloids can destroy the cell wall and nucleus of microbial (Gurjar et al., 2012). These compounds from this plant are environmental friendly and thus will provide benefits to the organic fungicide manufacturers. This caused, the organic fungicide demand will increase due to the awareness of people on the impact of fungicide to human health and environment ecosystem (Moss, 2002).

CONCLUSION

Based on Disease index (DI) and disease suppression (%DS), *C. roseus* extract at concentration of 2000 µg/ml, showed the most effective effect to control *R. microporus* on rubber

eISSN-0128-1119

seedlings compared to 1000 and 1500 μg/mL. Therefore, it can be concluded that the *C. roseus* extract is effective against *R. microporus* on rubber seedlings. *C. roseus* extract, might be suitable to be used as a biofungicide for controlling rubber diseases caused by *R. microporus*.

ACKNOWLEDGEMENTS

We sincerely thank Universiti Putra Malaysia for the financial assistance through the Research University Grant Schemes (RUGS).

DECLARATION OF CONFLICT OF INTEREST

No conflict of interest to declare.

REFERENCES

Alam, S. (2009). Antimicrobial activity of natural products from medicinal plants. *Gomal Journal of medical sciences,* 7: 111 – 118.

Balaabirami, S. and Patharajan, S. (2012). *In vitro* antimicrobial and antifungal activity of *Catharanthus roseus* leaves extract against important pathogenic organisms. International Journal of Pharmacy and Pharmaceutical Sciences, 4 (3): 487-490.

Brent, K.J. and Hollomon, D.W. (1998). Fungicide Resistance: The Assessment of Risk, FRAC, Global Crop Protection, pp. 1–48. Federation, Brussels.

Chan, W.H., Wong, C.P. and Wong, C.C. (1991). Control of white root disease in immature rubber with three systemic fungicides. The planter, 61: 251 – 256.

Eufrocinio, C.M., Shin'ichiro, K., Ei-ichiro, F. and Akio, K. Trichosetin, (2001). A Novel Tetramic Acid Antibiotic Produced in Dual Culture of *Trichoderma harzianum* and *Catharanthus roseus* Callus. Assessed on November 21, 2001. Natural forsch,1: 465-470.

Gurja, M. S., Shahid, A., Masood, A., Kangabam, S.S. (2012). Efficacy of plant extracts in plant disease management. Agricultural Sciences, 3: 425-433.

Holiday, P. (1980). Fungus diseases of Tropical crops. Cambridge University Press, 1980. Pp. 607. United Kingdom.

Ismail, H. and Azaldin, M.Y. (1985). Interaction of Sulphur with soil pH and root disease of rubber. Journal Rubber Research Institute Malaysia, 33:59-69.

Kaewchai, S. and Soytong, K. (2010). Application of biofungicides against *Rigidoporus microporus* causing white root disease of rubber trees. Journal of Agricultural Technology, 6: 346 – 363.

Kratika, K and Shamita, G. (2013). Antifungal properties of leaf extract of Catharanthus roseus l (g.) Don. American Journal of Phytomedicine and Clinical Therapeutics, 3: 101 -105.

Lee, S.O., Choi, G.J., Jang, K.S. and Kim, J.C. (2007). Antifungal Activity of five plant essential oils as fumigant against postharvest and soil borne plant pathogenic fungi. Plant Pathology Journal, 23: 97-102.

Lee, S.S. (1996). Diseases of some tropical plantation Acacias in Peninsular Malaysia. In Proceeding of an international Workshop on Disease of Tropical Acacias held at Subanjerjii (South Sumatra) 28 April – 3 May 1996. Eds. K.M. Old, S.S. Lee and J.K. Sharma, CIFOR special Publication, Indonesia Press.

Lili, H., Jia, Li., Hechun, Y., Changfu, L., Hong, W., Benye, L., Yansheng, Z. (2012). Molecular characterization of the pentacyclic triterpenoid biosynthetic pathway in Catharanthus roseus. Planta 236:1571-1581.

ASIAN JOURNAL OF PHARMACOGNOSY *Research Article*
Asian J. Pharmacogn 3(4):27 - 35

eISSN-0128-1119

Mohd Farid, A. (2010). Disease survey, identification and pathogenicity of root fungi in forest plantation of Peninsular Malaysia. Ph.D thesis. Universiti Sains Malaysia

Mohd Farid, A., Lee, S.S., Maziah, Z., Mohd Rosli, H. and Norwati, M. (2006). Root rot in tree species other than *A. mangium.* In Potter, K. *et al. (eds) Heart Rot and Root rot in Tropical Acacia Plantations.* ACIAR Proceedings No.124 Australia, Pp.60-66.

Moreno, P.R.H., Van der Heijden R. and Verpoorte R. (1994a). Elicitor-mediated induction of isochorismate synthase and accumulation of 2,3-dihydroxybenzoic acid in *Catharanthus roseus* cell suspension and shoot cultures. Plant Cell Reports*,* 14: 188-191.

Moss, M.O. (2002). Mycotoxins review 1: Aspargillus and Penicillium. Mycologist, 16: 116-119

Natali, R.M. and Robert, V. (2007). Phenolic compounds in *Catharanthus roseus.* Phytochemical Review, 6:243–258.

Nicole, M., Geiger, J.P. and Nandris, D. (1986). Penetration and degradation of suberized cells of *Hevea brasilliensis* infected with root rot fungi. Physiological and Molecular Plant Pathology, 28: 181 – 185.

Phani Deepthi Yadav, CH.S.D, Bharadwaj, N.S.P., Yedukondalu, M. Methushala, C. H. and Ravi Kumar, A. (2013) Phytochemical evaluation of *Nyctanthes arbortristis, Nerium oleander* and *Catharanthus roseus.* Indian Journal of Research in Pharmacy and Biotechnology, 1 (2): 333-336.

Ravi, K. U. (2011). Plant latex: A natural source of pharmaceuticals and pesticides. Review article*,* 5: 169 – 180.

Rozihawati, Z., Normala, H., Sajap, A.S., Mohd Farid, A. and Mohd Roslan, M. (2014). Bioantifungal Activity of Selected Medicinal Plant Extracts against Root Rot of Fungal Disease*. Journal of Plant Sciences,* 2 (1): 31-36.

Sharif, M.A., Atiqur, R., Yunus, A. and Sun, C.K. (2010). Inhibition of plant pathogens in vitro and in vivo with essential oil and organic extracts of *Cestrum nocturnum* L. Pesticide Biochemistry and Physiology*,* 96: 86 – 92.

Singh, S., Srivastava, R. and Choudhary, S. (2010). Antifungal and HPLC analysis of the crude extracts of *Acorus calamus*, *Tinospora cordifolia* and *Celestrus paniculatu*. Journal of Agricultural Technology, 6:149-158.

Tey, Y. S., Mad Nasir, S.,Zainalabidin, M., Amin, M.A. and Alias, R. (2008). Analysis of demand for vegetables in Malaysia. http//www.com.Analysis_of_demand for vegetables in Malaysia.pdf. (accessed May 2008).

Van Beilen, J.B. and Poirier, Y. (2007). Establishment of new crops for the production of natural rubber. Trends Biotechnology, 25: 522-529.