Cytotoxic Effects of *Quercus Infectoria* Extracts towards Cervical (Hela) and Ovarian (Caov-3) Cancer Cell Lines

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ABSTRACT: *Quercus infectoria* Olivier (manjakani) is a small tree which is known as oak tree. Previous study showed that this plant possessed antioxidant activity which contributes to the anti-carcinogenic effect. Flavanols as one of the major compounds from this plant was reported to inhibit cell proliferation *in vitro*. This present study was carried out to determine the potential of galls of *Q.infectoria a*s an antiproliferative agent towards cervical cancer cells (HeLa) and ovarian cancer cells (Caov-3). The toxicity *in vitro* was evaluated on non malignant cell line (MDCK). HeLa and Caov-3 cancer cell lines and MDCK were incubated in 96-wells plate in the presence and absence of QI galls extracts (methanol, ethanol and aqueous extracts) for 72 hours. The antiproliferative activity of QI galls extracts towards the cell lines were investigated by Methylene Blue Assay and the OD values were read at 660nm. The inhibitory concentrations at 50 % cell population (IC50 values) were determined followed by observation of the morphological features of apoptosis by Hoescht stain. The lowest IC_{50} value for HeLa cell line was 2.82±0.21 µg/ml treated with QI galls ethanol extract and the lowest IC_{50} value for Caov-3 cell line was 6.50±0.24 µg/ml treated with QI galls aqueous extract. The QI galls ethanol extract showed relatively low toxicity to normal cell control (MDCK) with IC_{50} of 74.99±0.09 µg/ml. The cells treated with QI galls ethanol extracts, morphological changes were consistently observed that eventually leads to detachment of cells from monolayer and DNA (chromatin) condensate. From the results, it can be suggested that QI galls extracts have potential as anticancer agent and further study should be carried out to elucidate the active compound of the extracts.

Keywords: cancer cells, *Quercus infectoria* Olivier (QI), antiproliferative assay, apoptosis

Introduction

Cancer is a major health problem and leading cause of death not only in developed countries, but also in developing countries where approximately 72% of all cancer cell death worldwide. Although common methods for cancer treatment have yielded some advantages, there is an ongoing need for both improvement of current therapeutic strategies and need to search for novel agents (Wicaksono et al., 2009). The interest in natural products research has resulted in discovery more efficient drugs for cancer treatment. (Calixto, 2000; Rates, 2001; Phillipson, 2001).

In this research, a white oak, *Quercus infectoria* Olivier (QI) is chosen for antiproliferative activity towards two types of most common women cancer, cervical (HeLa) and ovarian (Caov-3) cells. QI is a small oak, indigenous to Greece and Iran (Umachigi, 2008). The leaves of Q. *infectoria* is ovate-oblong, sinuate-dentatte, very smooth, and deciduous (Daniel, 2005). QI is a small tree or shrub, growing to four to six feet tall. Its stem is crooked and its leaves on short petioles with a few short mucronate teeth on each side (Henrietta, 2009). The galls extract contains Tannic acids and Gallic acids (Dar & Ikram, 1976; Wart & Kumar, 2001; Kokate, 1994) which are powerful astringents (Pin et al., 2006). Khare (2004) reported other phytochemicals such as amentoflavone hexamethyl ether, iso-crytomerin and beta-sitosterol. The antioxidants such as flavanols (Goncalves et al., 2008) from this plant may contribute to the anti-carcinogenic effect (Shahrzard et al., 2001), which inhibits cell proliferation *in vitro* (Scalbert et al., 2005). The presence of flavanols was studied recently as non-timber products from several species of Quercus.

The QI galls aqueous extract was reported to have high potential in skin whitening and antioxidant properties as the extract inhibited the super oxide and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities, and tyrosinase activities (Rohana et al., 2004). Aqueous extract of QI galls was reported shown to have high hydrolysable tannin content which inhibits the lethality of the *Naja kaouthia* (Thai cobra) venom (Pithayanukul et al., 2004). The hydrolysable tannins including

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tannic acid and Gallic acid are powerful astringent that are prescribed in diarrhea. The extract of QI galls also shown high antimicrobial activity against *Escherichia coli* (*E. coli*) O157:H7 (Voravuthikunchai et al., 2004). These studies have revealed its potential to provide an alternative for modern medicinal products as well as cosmetics and skin care products. However, no previous study was done on the antiproliferative activity of *Quercus infectoria* Olivier galls extract.

The ethanol extract of the galls of *Q. infectoria* have a high potential as antibacterial agent against methicillin-resistant *Staphylococcus aurens* (MRSA) (Voravuthikunchai et al., 2007). Galls of *Q. infectoria* possess pleiotropic therapeutic activities, with particular efficacy against inflammatory disease. Oral administration of gall extract significantly inhibited carrageenan, histamine, serotonin and prostagladin E2 (PGE2) induced paw edemas The extract also inhibit various functions of macrophages and neutrophiles relevant to inflammatory responses (Kaur et al., 2004). Thus, this present study was designed to determine the antiproliferative activity of QI galls extracts towards HeLa and Caov-3 cancer cell lines and its apoptotic cell death mode of action.

Materials and Method

Plant extracts

Q. infectoria extract was supplied with courtesy of Dr. M. Rafiquzzaman from School of Health Sciences, Universiti Sains Malaysia.

Cell culture

Cancer cell lines, HeLa and Caov-3 were cultured in complete medium containing Dulbecco's modified Eagle medium (DMEM) with L-glutamine supplemented with 10% v/v fetal bovine serum (FBS) with supplement of 1% antibiotic.

Antiproliferative assay towards HeLa and Caov-3 cell lines

The assay procedure was slightly modified from Hasmah et al. (2008). Cells were seeded into 96-wells plate at concentration 5 x 10^4 cells/ml and left overnight prior to attachment. QI galls extracts (Methanol, ethanol and aqueous extracts) were added into each well at different concentrations and incubated for 72 hours in 37°C 5 % CO_2. The plate optical density (OD) was read using ELISA microplate reader (GENios with serial no: 12900400903) at wavelength 655nm ($\pm$5nm). The intensity of wavelength absorb is proportional to the above of live cells present in the wells. The IC50 value was determined using graph plotting percentage of viable cells versus $\log_{10}$ concentration (mg/ml) of extract.

Hoescht 33258 staining

Cells were treated with 2.82ug/ml ethanol crude extract and incubated for 24, 48 and 72 hours. Then, the cells were centrifuged at 1000 rpm for 10 minutes and the cell pellets were re-suspended in 2ml of PBS solution for 3 times (Sanchez-Alcazar et al., 2001). The suspended pellet was then spread on a clean poly-prep slide (coated with L-lysine) and left to dry. After completely dry, 4% paraformaldehyde was added to fix the cells. The cells were then washed with PBS for three times to remove the fixatives before deluged with cold (-20°C) 100% methanol and left at room temperature. This step is crucial for cell permeabilization (Blau 2002). The slide is then stained with 0.5 µg/ml Hoescht dye and incubated for 1 h in dark. The slides were then rinsed with PBS for five times and then observed under fluorescent microscope at 340-380 ηm.

Statistical analysis

The absorbance means were calculated using the Excel® software (Microsoft, US) and the standard error of the mean were calculated for 3 wells in triplicated sampling.

Results

Antiproliferative activity

After incubation of cell lines with QI galls extracts for 72 hours, the QI gall extracts obviously showed cytotoxic effects towards Caov-3 and HeLa cell lines in concentration dependent manner (**FIG. 1-3**).

From the results, it can be suggested that the QI galls extracts were more effective towards HeLa cell line compared to Caov-3 cell line as it IC50 was 2.82±0.21 µg/ml when treated with ethanol extract (**TABLE 1**), $p<0.05$.

Effect of QI galls extract towards MDCK

The lowest IC_{50} was proceeded to be tested against MDCK (control cell line) and the result showed that the extract showed very low toxicity towards normal cell.

Hoescht 33258 staining

Result of staining showed the morphological features of apoptosis when observed under fluorescent microscope. The intensity of

fluorescence increased with time due to the increase of nuclear membrane permeability of apoptotic cells (**FIG. 5**). The increased amount of chromatin condensation which is a characteristic of apoptotic cells, will showed brighter fluorescence (Kuck et al., 2001). The more fluorescent intensity is an indication of increase nuclear pyknosis or karyorrhexis in cells caused by increase permeability to cytosolic molecules in apoptotic cells (Earnshaw, 1995).

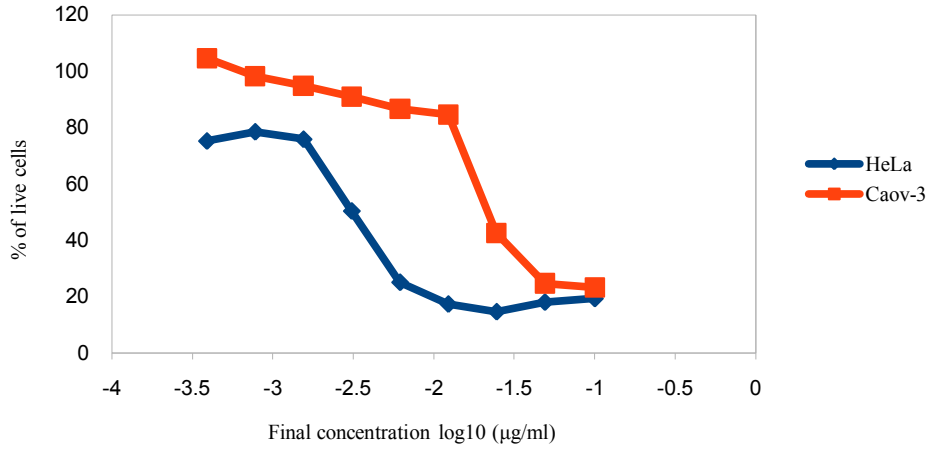

FIG. 1- Cytotoxic effects of QI galls methanol extract towards HeLa and Caov-3 after 72 hours. Each point is mean percentage (%) of live cells with bar show ± standard error mean (SEM).

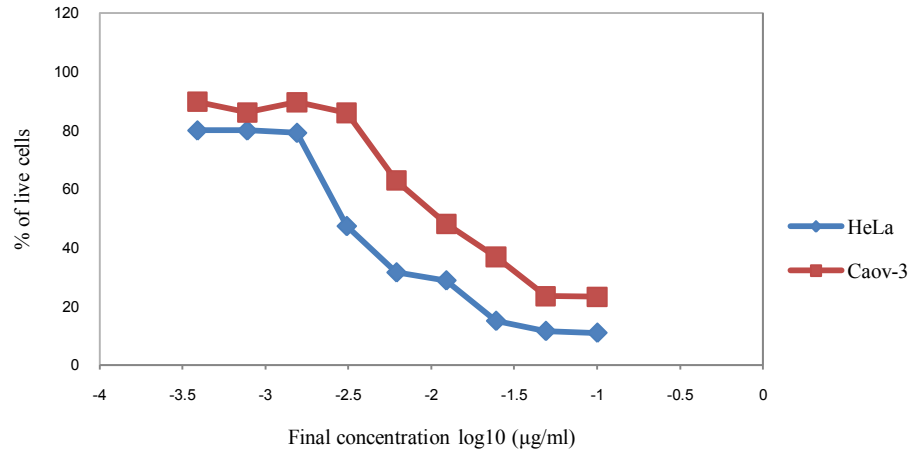

FIG. 2- Cytotoxic effects of QI galls ethanol extract towards HeLa and Caov-3 after 72 hours. Each point is mean percentage (%) of live cells with bar show ± standard error mean (SEM).

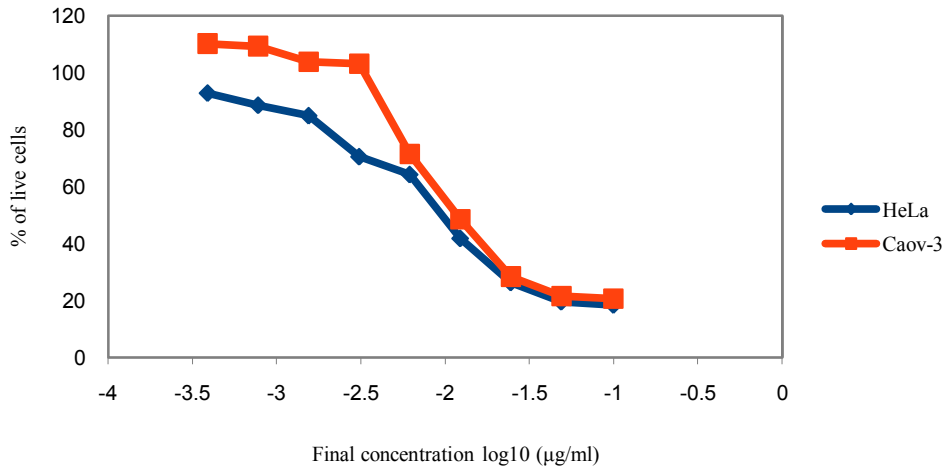

FIG. 3- Cytotoxic effects of QI galls aqueous extract towards HeLa and Caov-3 after 72 hours. Each point is mean percentage (%) of live cells with bar show ± standard error mean (SEM)

TABLE 1- IC_{50} values of HeLa and Caov-3 cell lines treated with QI galls extracts for 72 hours

Extract	IC_{50} for cell lines (µg/ml)	
	HeLa	Caov-3
Aqueous-Mixed group-soxlet	4.47±0.48	10.00±0.018
Aqueous-Mixed group-soaking	8.91±0.48	12.02±0.18
Ethanol-Mixed group-soaking	2.82±0.21	15.40±0.27
Methanol-soaking	3.16±0.08	23.17±0.33
Aqueous-soaking	6.49±0.24	6.50±0.29

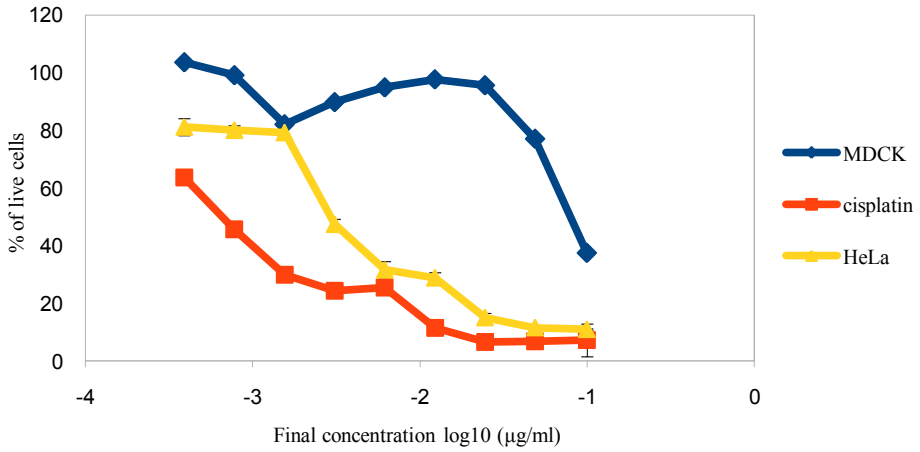

FIG. 4- Cytotoxic effects of QI galls ethanol extract towards HeLa and MDCK after 72 hours. Cisplatin was used as drug control for HeLa cell line. Each point is mean percentage (%) of live cells with bar show ± standard error mean (SEM)

Discussion

The cancer remains one of the leading causes of death worldwide even today. Various cancer therapies have currently been tried, including the use of natural products from higher plants (Wicaksono et al., 2009). Therefore, the need to discover an effective, novel and scientifically reliable natural compound is urgent. Natural products provide a fertile ground for seeking out treatments with fewer side effects and equal or better results (Subhuti, 1999).

The present research describes the antiproliferative properties of *Quercus infectoria* galls crude extracts. The *in vitro* cytotoxicity activity against two human cancer cell lines, cervical cancer and ovarian cancer showed that the most potent extract was QI ethanolic extracts produced by soaking method with a percentage of inhibition of 50% at 2.82 ±0.21 µg/ml against the HeLa. All the crude extracts inhibited cells growth in a concentration-dependent manner. It was reported that types of solvent used in the extract preparation greatly influenced the bioactive compound extraction (Pinelo et al., 2005). Therefore we found the different effect with different extraction solvents and methods.

In this study, all the crude extracts of QI galls having IC_{50} less than 100 µg/ml towards both HeLa and Caov-3 cells. The differences between both values are small explaining the hydrophilic compounds extracted might have same effects on the cell lines. These findings revealed the potential of *Quercus infectoria* galls extract as an anticancer agent. Established cancer drug, cisplatin treatment demonstrated inhibitory effect on HeLa cells multiplication at lower concentration but shown toxicity at higher concentration and longer treatment duration (Majumdar, 2001). QI galls active compound that inhibited the cancer cell growth can be achieved by purification of QI galls extracts via bio-assay guided fractionation. The phytochemical work carried by Umachigi et al. (2008) revealed that ethanolic extracts of the galls of QI contains high amount of tannins, presence of gallic acid, ellagic acid, syringic acid, -sitosterol and amenotoflavone, implied that tannin is one of the active compounds which may be responsible for the antiproliferative activity. Several antioxidants in plants have been suggested to contribute to the anti carcinogenic effects and other such as flavanols have been able to inhibit cancer cell proliferation *in vitro* (Scalbert et al., 2005). It was suggested by Fellows (1992) that ethanol extracts more polar compounds compare to other

solvent. The focus of discussion was usually given to methanol extracted compound as it is polar and lipophilic (Mans et al. 2000) which might contain important secondary metabolite such as tannins, ellagitannins and other important metabolites.

Other researches done using different *Quercus* sp. also exhibited good IC_{50} against HeLa cell lines. The research done by Rocha-Guzman et al. (2009) using *Quercus resinosa* leaves shown 50% of inhibition after 24 hours treatment with 69.34 ± 8.1 µg/mL of the aqueous extract. Antiproliferative assay done by Goncalves (2008) on *Quercus suber* L., showed the IC_{50} value for aqueous extract on HeLa cells was 298.80 ug/ml. However, the ethanol extract was not included in the assay. As compared to the others, QI galls ethanol extract shown a better $IC_{50,}$ which is 4.47±0.48 µg/ml.

Established cancer drug, cisplatin treatment demonstrated inhibitory effect on HeLa cells multiplication at lower concentration but shown toxicity at higher concentration and longer treatment duration (Majumdar, 2001). The antioxidants present in plants may contribute to the anti-carcinogenic effect and other such as flavanols have been able to inhibit cell proliferation *in vitro* (Scalbert et al., 2005). The presence of flavanols was studied recently as non-timber products from several species of *Quercus* (Gonclaves et al. 2008).

The extract probably can be subjected to purification in order to get better IC_{50}. This proved by fractionation of *Hydnophytum formicarium* crude extract exerted better IC_{50} value towards HeLa and Caov-3 cell lines after methanol fractionation (Hasmah et al., 2008). However, further investigations in needed to elucidate the active compound of the extracts and its mechanism of action towards cancer cells for future therapeutic application as anticancer agent.

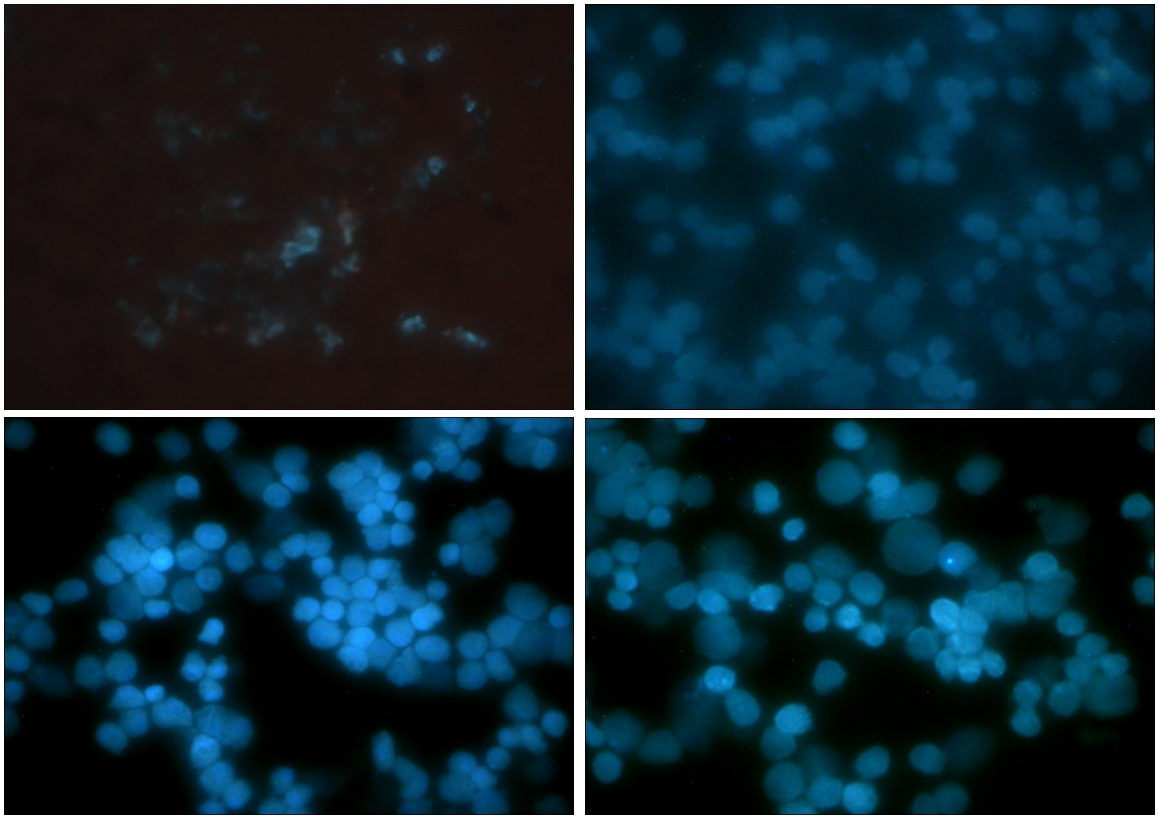

Fig. 5- Morphology changes of HeLa cell line of when stained with Hoescht stain 33258 under microscope at magnification x40, a) no treatment, minimum fluorescence shown as the nuclear membrane is intact; b) treatment with ethanol extract of QI galls at concentration 2.82±0.21 µg/ml for 24 hours. A low intensity of fluorescence can be seen; c) treatment with ethanol extract of QI galls at concentration 2.82±0.21 µg/ml for 48 hours with moderate fluorescence intensity indicated that nuclear membrane is moderately permeable to the stain; d) treatment with ethanol extract of QI galls at concentration 2.82±0.21 µg/ml for 72 hours with higher intensity of fluorescence which indicates its nuclear membrane is highly permeable to Hoechst stain.

Nuclear staining has distinguished apoptosis and necrosis (Eric & Huseyin 2003). The cells treated with ethanol extracts of *Quercus infectoria g*alls, exhibited morphological changes that eventually leads to detachment of cells from monolayer. Cells that undergo apoptosis with condensation and DNA fragmentation were detected by Hoescht 33258 staining with the principle of increased cell and nuclear membrane permeability apoptotic cells caused by increase mobilization of stain into the cells. The increased amount of apoptotic cells characterized by chromatin condensation will resulted brighter fluorescence (Kuck et al. 2001). Present study showed DNA (chromatin) condensation was observed in treated cells, suggesting the presence in apoptosis in these cells (Searle et al. 1974).

Conclusion

Results indicated that *Quercus infectoria* holds promise of being a novel antiproliferative agent deserving further investigation. Further purification of the extract from QI galls and broadens the screening to other cancer cell lines should be done in future.

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References

1. Calixto, J.B. (2000). Efficacy, safety, quality control, marketing and regulatory guildlines for herbal medicines (phytotherapeutic agents). *Brazilian Journal and biological research*, 33, 179-189.
2. Daniel M. (2005). *Medicinal plants chemistry and properties*. Science Publishers.
3. Dar, M.S., Ikram, M., & Fakouchi, T. (1976). Pharmacology of *Quercus infectoria*. *Journal Pharmaceutical Science,* 65, 1791-1794.
4. Earnshaw, W.C. (1995). Nuclear changes in apoptosis. *Current Biology*, 7, 337-343.
5. Eric, H.B., & Huseyin, M. (2003). *Nuclear changes in dying cells*. In Hughes, D and Huseyin, M(eds). Cell proliferation & apoptosis. *Bios Scientific Publishers Limited*, 295-327.
6. Fellows, L.E. (1992). *Pharmaceuticals from traditional medicinal plants and others*: *Future prospects. In: Coombes, JD (eds) New drugs from natural sources*. London, IBC: Technical Services.
7. Goncalves, S., Xavier, C., Alberício, F., & Romano, A. (2008). Methanolic and aqueous extracts from *Quercus suber* Leaves Induce Apoptosis in HeLa Cells, pp. P20
8. Hasmah, A. Azimahtol Hawariah L.P., Hohmann, J., & Molnar, J. (2008). Antiproliferative compounds from *Hydnophytum formicarium*. *Journal of Tropical Medicinal Plants*, 9(2), 366.
9. Henritte's Herbal Homepage. (2009). 111. *Quercus infectoria,* Olivier-the gall, or Dyer's Oak. Henriette Kress. Retrieved July 20, 2009 from http://www.henriettesherbal.com/eclectic/pereira/quercus-infe.html
10. Kaur, G., Hamid, H., Ali, A., Alam, M.S., & Athar, M. (2004). Anti-inflammatory evaluation of alcoholic extract of galls of *Quercus infectoria*. *Journal Ethnopharmacology*, 90, 285-292.
11. Khare, C.P. (2004). *Indian herbal remedies, rational western therapy in ayurvedic and other traditional usage*. Botany: Springer.
12. Kokate, C.K. (1994). *Practical Phermacognosy*. New Delhi: Vallabh Prakashan, pp. 107-111
13. Kuck, D., Kolmerer, B., Iking-Konert, C., Krammer, P.H., Stremmel, W., & Rudi, J. (2001). Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis in human gastric epithelial cell line ABS. *Infection and Immunity*, 69(8), 5080-5087.
14. Majumdar, S.K., Valdellon, J.A., & Brown, K.A. (2001). *In vitro* investigations on the toxicity and cell death induced by tamoxifen on two non-breast cancer cell types. *Journal of Biomedicine and Biotechnology,* 1(3), 99-107.
15. Mans, D.R.A, Rocha, A.B. and Schwartsmann, G. (2000). Anti-Cancer Drug Discovery and Development in Brazil: Targeted Plant Collection. *Oncologist*. 5: 185-198 http://www.cancer.gov/cancertopics/factsheet/Therapy
16. Philipson, J.D. (2001). Phytochemistry and medicinal plants. *Phytochemistry*, 56(3), 237-243.
17. Pin, K.Y., Chuah, T.G., Abdull Rashih, A., Rasadah, M.A., Choong, T.S.Y., & Law, C.L. (2006). Effects of the concentrations of *Quercus infectoria* galls (Manjakani) extracts on moisture contents and quality of its freeze-dried product. *International Journal Engineering and Technology*, 3(2), 167-174.
18. Pinelo, M., Fabbro, P.D., Marzocco, L., Nunez, M.J., & Vicoli, M.C. (2005). Optimization of continuous phenol extraction from *Vitis vinifera* by products. *Journal Food Chemistry,* 92(1), 109-117.
19. Pithayanukul, P., Ruenraroengsak, P., Bavovada, R., Pakmanee, N., Suttisri, R., &

Saen-oon, S. (2004). Inhibition of *Naja kaouthia* venom activities by plant polyphenols. *Journal of Ethnopharmacology*, 97.

20. Rates, S.M.K. (2001). Plants as source of drugs. *Toxicon*, 39, 603-613.
21. Rocha-Guzmána, N.E., Gallegos-Infantea, J.A., González-Laredoa, R.F.,Reynoso-Camachob, R., Ramos-Gómezb, M., Garcia-Gascac, T., Rodríguez-Muñozd, M.E., Guzmán-Maldonadoe, S.H., Medina-Torresf, L., & Lujan-García, B.A. (2009). Antioxidant activity and genotoxic effect on HeLa cells of phenolic compounds from infusions of *Quercus resinosa* leaves. *Journal of Food Chemistry*, 115, 1320-1325.
22. Rohana, S., Vimala, S., Abdull Rashih, A., & Mohd Ilham, A. (2004). *Skin whitening and antioxidant properties of Quercus infectoria galls*. In Proceedings of the Seminar on Medicinal plant, Forest Research Institute Malaysia (FRIM), Selangor. pp. 188-191.
23. Sanchez-Alcazar, J.A., Khodjakov, A.K., & Schneider, E. (2001). Anticancer drugs induced increase mitochondrial cytochrome c expression that proceeds cell death. *Cancer Researches*, 61, 4006-4010.
24. Scalbet, A.,Johnson, I.T., & Saltmarsh, M. (2005). Antioxidants and beyond. *American Journal of Clinical Nutrition*, 81, 215S-217S.
25. Shahrzard, S., Aoyagi, K., Winter, A., Koyama, A., & Bitsch, I. (2001). Pharmacokinetics of gallic acid and its relative bioavailability from tea in healthy humans. *The Journal of Nutrition*, 131, 1207-1210.
26. Searle, J., Lawson, T.A., Abott, P.J., Harmon, B., & Kerr, J.F.R. (1974). An electron microscope study of the mode of cell death induced by cancer chemotherapeutic agents in populations of proliferating normal and neoplastic cells. *Journal Pathology*, 116, 129-138.
27. Subhuti, D. (1999). Prognosis after diagnosis of liver cancer: The Influence of Chinese Herbs. Retrieved from August 19, 2009 from http://www.itmonline.org/arts/livercancr.htm
28. Umachigi, S.P., Jayaveera, K.N., Ashok Kumar, C.K., Kumar, G.S., Vrushabendra swamy B.M., & Kishore Kumar D.V. (2008). Studied on wound healing properties of *Quercus infectoria*. *Tropical Journal of Pharmaceutical Research*, 7(1), 913-919.
29. Voravuthikunchai, S., Lortheeanuwat, A., Jeeju, W., Sririrak, T., Phongpaichit, S., & Supawita, T. (2004). Effective medicinal plants against enterohaemorrhagic *Escherichia coli* O 157:H7. *Journal of Ethnopharmacology*, 94, 49-50.
30. Voravuthikunchai, S., Chusri, S., & Kleiner, P. (2007). Inhibitory activity and killing activity of extracts from the gall of *Quercus infectoria* against methicillin-resistant *Staphylococcus aureus*. ESCMID, NR1980.
31. Wart, C., & Kumar, A. (2001). *Practical handbook of pharmacognosy*. Malaysia: Pearson Education Malaysian Sdn Bhd.
32. Wicaksono, B.D., Handoko, Y.A., Arung, E.T., Kasuma, I.W., Yulia, D., Pancaoutra, A.N., & Sandra F. (2009). Antiproliferative effect of methanol extract of *Piper Crocatum*. *Tropical Journal of Pharmaceutical Research*, 8(4), 345-352.