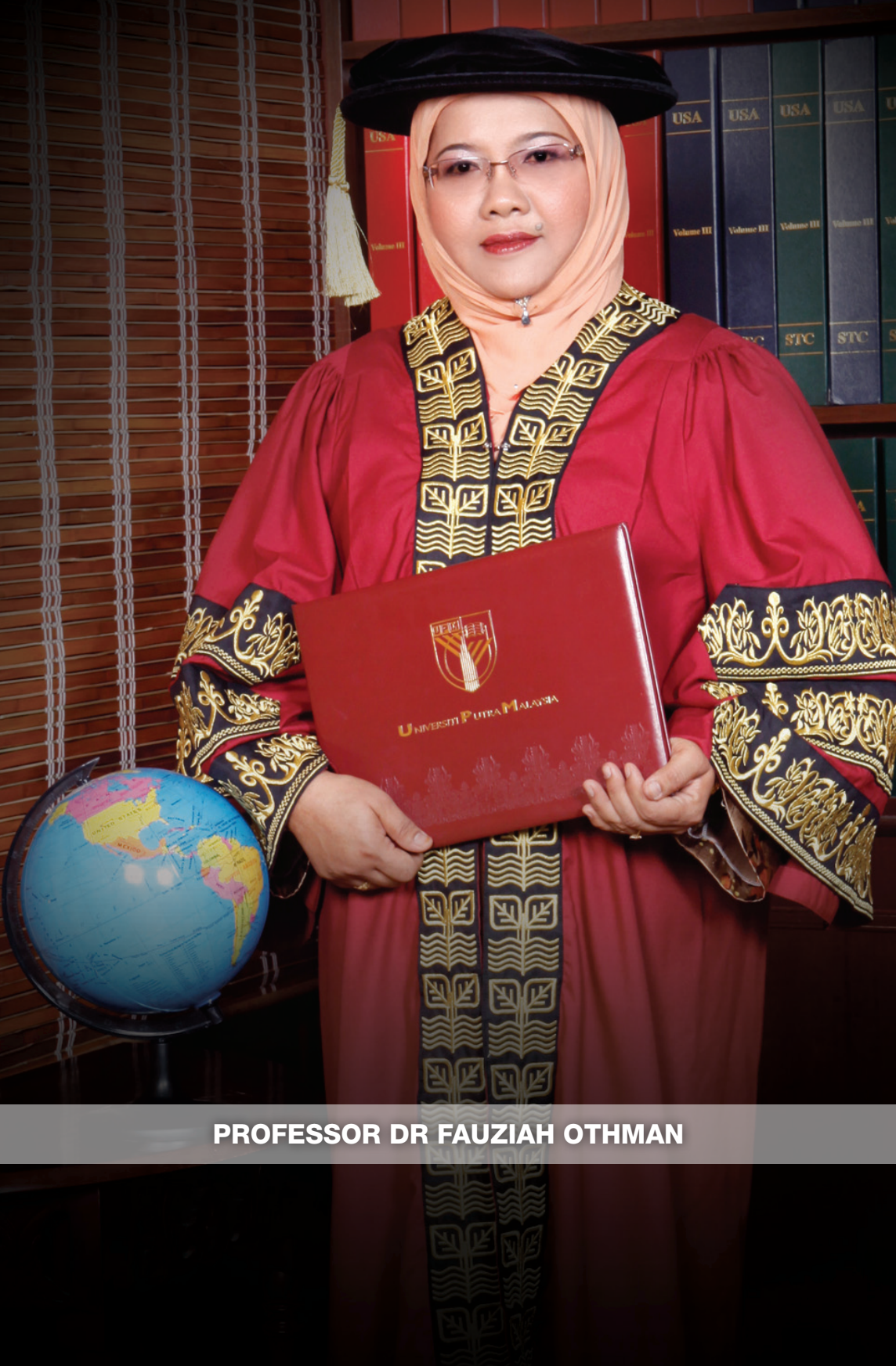
MY Small World

In Biomedical Research



PROFESSOR DR FAUZIAH OTHMAN

MY Small World
In Biomedical Research

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"I don't have much wealth to leave you behind, however,
the education and knowledge that I provide you with,
will take you a long way"

My late father
Haji Othman bin Abdul Samad

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ABSTRACT

Biomedical research in general simply known as medical research, is the basic research, applied research, or translational research conducted to aid and support the body of knowledge in the field of medicine. Cancer is a leading cause of death worldwide representing 13% of all deaths (over 11 million in 2030), breast cancer is the most common diagnosed type of cancer among women accounting for about 28% of all female cancer cases, while liver cancer is the third most common cause of death from cancer worldwide.

Workers exposed to formaldehyde vapour have been well documented to have an association between formaldehyde exposure and several cancers, including nasopharyngeal cancer and leukemia. A study was conducted in hatching chicks which represented a working environment that was exposed to the 10.9 ppm formaldehyde vapour, the result illustrated that there were pathological changes in the respiratory epithelium. And there was also pre-cancerous lesion seen in the epithelium where the normal psuedostratified columnar ciliated epithelium was replaced by stratified squamous epithelium in the trachea.

The existing treatment for cancer such as surgery, chemotherapy and radiotherapy are not always effective and can cause significant side effects. Thus, the author ventured into cancer research via two approaches:

In viro therapy, where, Newcastle Disease Virus was used whilst, in herbal therapy where *Azadirachta Indica* A.Juss (Neem) Extracts, Cola Nut (Cola Nitida) Fruit Aqueous Extract and *Strobilanthes* Crispus Extract were used in *in-vitro* and *in-vivo* study.

The oncolytic effect of Newcastle Disease Virus (NDV) strain AF2240 was investigated on the MCF-7, MDA-MB-231 breast cancer cell lines and 3T3 fibroblast. There were destruction of

the cytoskeletal protein, structural and ultrastructural changes, as well as molecular changes of the oncogenes which enlightened the important biological discoveries of apoptosis in the cancer cells, however, not in the normal cells.

The oncolytic effect of Newcastle Disease Virus AF2240 on Allografted 4T1 Breast Cancer Cells In Balb/c mice was well demonstrated and NDV-AF2240 was detected via *In situ* reverse transcriptase polymerase chain reaction (in situ RT-PCR), polyclonal chicken antibody and goat anti-chicken antibody conjugated with fluorescein isothiocynate (FITC) using confocal laser scanning microscopy (CLSM) and negative staining transmission electron microscopy (NSTEM). The pre-clinical study of this virus is proven to be safe and effective in animal study, however, further study is needed to understand the underlying mechanism in making the NDV strain of AF2240 as an anti-cancer agent in human.

In herbal theraphy research, *Berberis Vulgaris* (L.) Fruit Extract, *Azadirachta Indica* A.Juss (Neem) Extracts, *Strobilanthes Crispus* Extract, and Cola Nut (Cola Nitida) Fruit Aqueous Extract, were found to contain high antioxidant and prevent the formation or viability of cancer cells. *In vitro* and *in-vivo* study showed that they have great potential to be developed either into functional food or further develop into drugs which can be used to either treat liver, breast and cervical cancers.

Tissue and organ failure, resulting from various forms of injury such as traumatic, metabolic, inflammatory and other diseases normally lead to lost of tissue, organ and system function. To overcome these problems, researchers try to implement tissue engineering as a new approach and to assure the proper re-establishment of organ function, the structural and ultrastructural changes and expression

of protein markers during re-modelling of tissue-engineered skin, tissue engineered cornea and tissue engineered bone.

In orthopaedics, administration of antibiotics does not provide good local bone response due to poor vascularisation of bone tissue; low drug penetration and recurrent cases are high due to formation and presence of biofilm. The author again takes the lead to develop a biocompatible material in-cooperated with antibiotic to overcome the problem mentioned above.

As cancer, tissue and organ lost can either be life threatening or compensating quality of life, biomedical research play an important role to support the medical scenario towards human health and wealth creation to the nation.

FORMALDEHYDE AND CANCER RISK

Effect of Formaldehyde Vapour on Respiratory Epithelium of Hatching Chicks

Disinfecting hatching eggs with the use of formaldehyde vapour during the last three days of incubation is a common practice in commercial hatcheries to minimise the presence of potential pathogenic microorganisms and so produce high hatchability and healthy chicks. This study was designed to investigate the use of scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy (LM), the effects of exposure to low levels of formaldehyde vapour (10.9 ppm) on the epithelial lining of the respiratory tract of hatching chicks in a commercial situation. As a prelude to this study, a control study on the development of the respiratory tract was carried out using similar techniques and it was established by the 19th to 20th day of incubation, in which the mucociliated cells (Figure 1) of the entire respiratory tract of chicks were well developed.

Formaldehyde fumigation however, caused destruction to the entire respiratory tract of the chicks, inducing pathological changes including clumping of cilia and microvilli (Figure 2), development of blebs or balloon-like structures on the cilia and microvilli, development of blebs or balloon-like structures on the cilial and microvillial walls, deciliation and desquamation of the epithelium (Figure 3).

Figure 1 Caudal trachea. 3-day-old chick. Dense carpet of cilia interrupted by islands of microvillous cells on the mucosal surface. X2,750

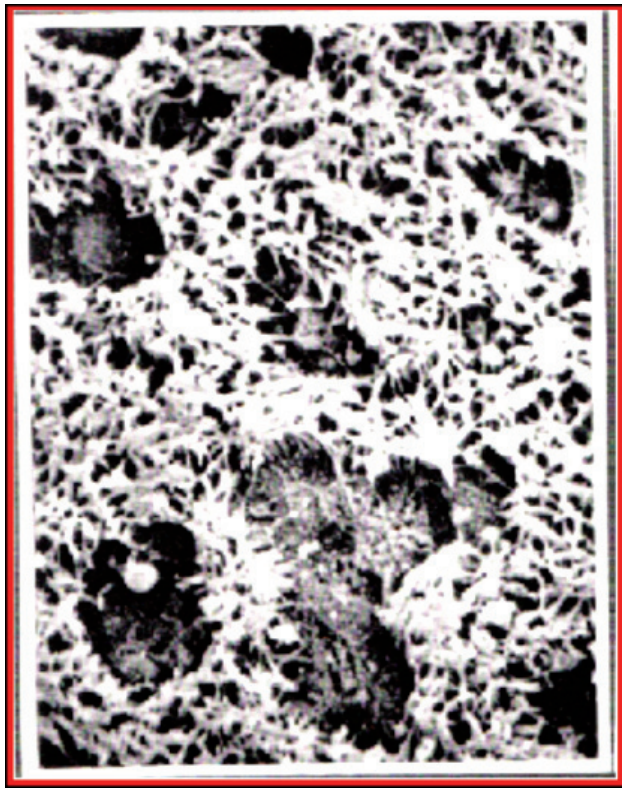


Figure 2 Trachea. 1-day –old chick. Extensive clumping of the cilia. X2,750

Figure 3 Caudal trachea. 11-day old chick. Eroded epithelium (arrow), note basal cell proliferation (open arrow) from the intact epithelial cells. X2,750

In addition, mucus production was also seen to be affected, with increased mucus production and changes in both the nature of the mucosubstances and distribution of the mucous cells and intraepithelial mucous glands. The morphological changes in the lining respiratory tract appeared to last until about the fourth week post-hatching, when regeneration of the lining epithelium appeared to be completed. The effect of the 10.9 ppm on the respiratory epithelium of hatching chicks may also represent the respiratory epithelium of the hatchery workers who are also exopsed to the formaldehyde. Important note here is a pre-cancerous lesion was seen in the epithelium where the normal psuedostratified columnar ciliated epithelium was replaced by obvious squamous metaplasia in the trachea of a few of the chicken. The lesions found in these chicken should be critically reviewed regarding their meaning for humans.

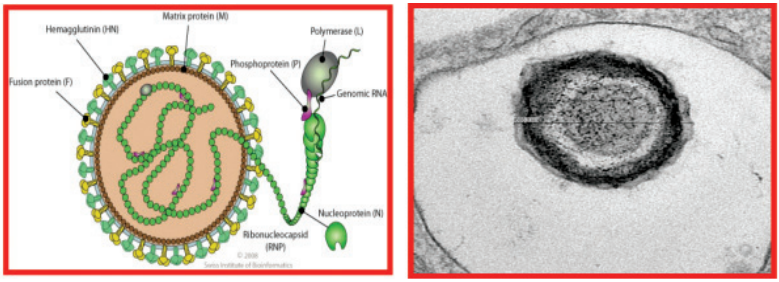
ANTICANCER RESEARCH

Virotheraphy

Cancer is a group of disease characterised by uncontrolled growth and spread of abnormal cells. Uncontrolled spreading of the abnormal cells eventually will lead to death. Cancer is caused by external factors (carcinogenic chemicals, radiation, environment and infectious organisms) and internal factors (inherited genetics and hormones) (Sainsbury *et al.*, 2000). Despite recent advances in treatment, an estimated of 192, 370 new cases of invasive breast cancer were expected to occur among women in the US during 2009; about 1,910 new cases were expected in men (Anonymous, 2009). In Malaysia, breast cancer is the most commonest cancer regardless of ethnicity and age. Existing treatments such as chemotherapy and radiotherapy are not always effective and can cause significant side effects. With the development of advanced biology techniques, viruses of animal origin have been tested for virus therapy of human cancers. There has been active interest in the potential use of replication-competent oncolytic viruses as therapeutic agents in the treatment of cancer (Schirrmacher *et al.*,1998). In the current virus taxonomy Newcastle Disease Virus (NDV), or avian paramyxovirus type 1, is classified, with the other avian paramyxoviruses,in the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales* (Dennis *et al.,* 2006; Khadijah and Tan, 2007).

NDV contains a non segmented single stranded RNA genome which coded six proteins including Nucleocapsid Protein (NP), Phosphoprotein (P), large protein (L), envelope Matrix protein (M), hemaglutinin-neuraminidase (HN), and Fusion protein (F) (Figure 4). Generally, NDV is harmless to human. It can cause mild flu or conjunctivitis or laryngitis. Innumerable studies have been

conducted in several different human tumor cell lines and tumor models worldwide (Washburn and Schirrmacher,2002; Phuangsab *et al.*,2001; Csatary *et al.*,1993; Mallman,1993; Liebrich *et al.*,1991; Bohleet *al.*,1990; Schild *et al.,*1988; Cassel and Garret, 1965). The NDV possesses several unique properties, it binds specifically to tumor cells, it replicates selectively in tumour cell cytoplasm, it is relatively safe and it can act as an adjuvant (Schirrmacher *et al.*, 1998).



(a) **(b)**

Figure 4 a. Schematic representation of the virion structure of NDV. Note the carboxy-terminal end of HN and the amino-terminal end of F that are exposed on the surface of the virion. b. Negative staining electron micrograph of AF 2240 NDV

In the present study, a Malaysian local strain of NDV-AF2240 was tested as an oncolytic agent on 4T1 breast cancer cell allografted on Balb/c mice. Although the concept of using viruses as an anti-cancer agent is still new in Malaysia, recent advances in molecular biology and virology enable researchers to manipulate and enhance the possibility and the ability of NDV as an oncolytic agent and possible future agents in combating breast cancer. Several *in vitro* studies have demonstrated that NDV AF2240 has an oncolytic effect towards several types of cancer cells (Fauziah *et al*., 2002) and studies using fluorescent antibody and electron microscopy revealed

that AF2240 replicated in the cytoplasm (Zolkapli, 2006). The ability of Newcastle disease virus AF2240 to replicate efficiently in cancer cells has been demonstrated in both *in vivo* and *in vitro* (Zolkapli, 2006 and Hadiyatul-Hanim, 2009).

Virotherapy holds great promises as a treatment platform for cancer. Advantages include the potential lack of cross-resistance with standard therapies and their ability to cause tumor destruction by numerous mechanisms. However, hurdles such as immune response, systemic distribution and intratumoral spread are major potential limitations and must be addressed (David *et al*.,2001).

Molecular and Cytoskeletal Changes in Breast Cancer Cell Lines Treated with Velogenic NDV Strain AF2240

The oncolytic effect of Newcastle Disease Virus (NDV) strain AF2240 on the MCF-7, MDA-MB-231 breast cancer cell lines and 3T3 fibroblast was carried out to investigate the cytoskeletal protein (Figure 5), NDV structure and the molecular changes of the oncogenes. The AF2240 strainwas propagated in 11 days old embryonated eggs for 72 hours. The virus in the allantoic fluid was harvested, purified and stored at -80°C. The haemagglutination (HA) test was conducted on the purified virus to determine the HA titre of the NDV strain AF2240 which was 16384 HA units. The inhibition concentration of AF2240 towards several types of breast cancer cell lines was carried out using microculture tetrazolium (MTT) assay via two methods; monolayer and co-culture techniques to determine the inhibition concentration (IC_{50}) value. The IC_{50} values for MDA-MB-231 breast cancer cell lines treated with NDV strain AF2240 were 8 and 2 HA units for the monolayer and co-culture techniques respectively, whereas the IC_{50} value for MCF-7 was 2 HA units for both techniques. For detection of the virus, polyclonal antibody and anti-chicken conjugated with fluorescein isothiocyanate (FITC)

were used. The virus particles were detected in the cytoplasm of both breast cancer cell lines after 24 and 48 hours post treatment. By using independent t-test, the analysis revealed that NDV strain AF2240 works better towards MDA-MB-231 cells compared to MCF-7 ($p \leq 0.05$). These methods confirmed that NDV causes cell death to the breast cancer cells via apoptosis. Moreover, these findings also suggested that NDV reacts better towards MDA-MB-231 cells compared to MCF-7 cell ($p \leq 0.05$).

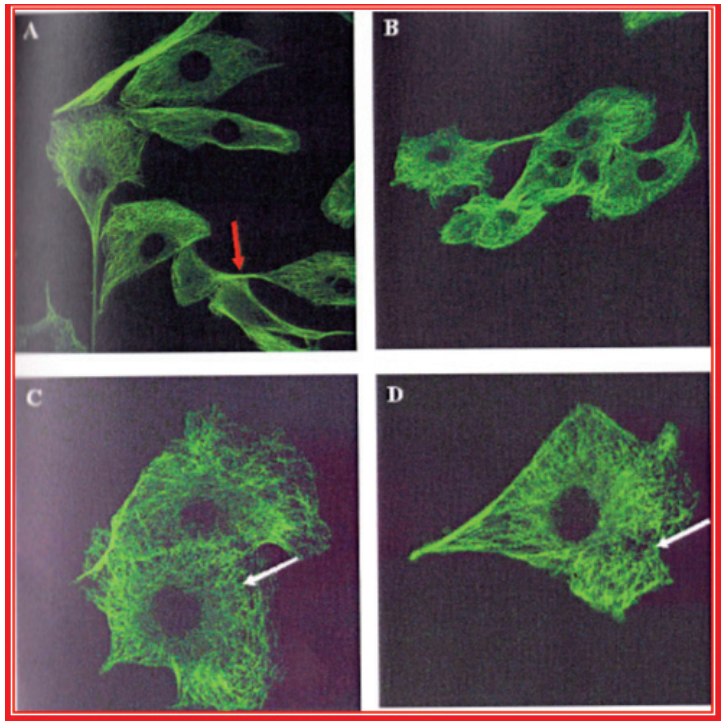


Figure 5 Confocal micrograph of α-tubulin of MCF-7 breast cancer cell lines stained with monoclonal anti-α-tubulin conjugated with FITC for untreated (A) and treated for 24, 48 and 72 hours (B, C and D) respectively. Disruption of α-tubulin (white arrows) was noted after 48 and 72 hours post treatment (C-D). Magnification: (A-B) 60X, (C-D) X 120

The study of oncogenes was conducted by using reverse transriptase polymerase chain reaction (RT-PCR) method. The expressions of c-myc, c-erb-2 and c-fos oncogenes were detected at pre and post-treatment in the MCF-7 and MDA-MB-231 breast cancer cell lines. These results proved that cells which had undergone apoptosis due to NDV strain AF2240 treatment did not suppress the oncogenes (Figure 6). It can be concluded that even though AF2240 NDV strain has significant cytotoxic effect towards MCF-7 breast cancer cell lines, the number of apoptotic cells are higher in MDA-MB-231 cell line. Therefore, further study is needed to understand the underlying mechanism in making the NDV strain of AF2240 as an anti-cancer agent.

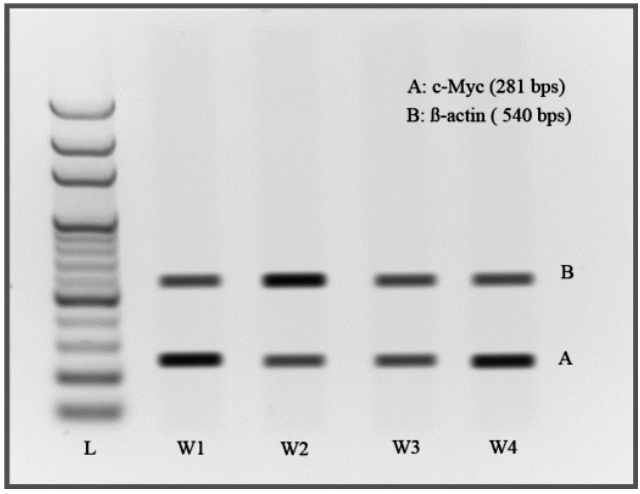


Figure 6 RT-PCR amplification of c-myc oncogene with 281bps (B) and β-actin housekeeping gene with 540bps (A) of breast cancer model treated with tamoxifen and separated by gel electrophoresis at week 1, 2, 3 and 4 (W1, W2, W3 and W4 respectively). The amplification of c-myc along with β-actin was detected throughout the experimental period

Localisation of (NDV-AF2240) in 4T1 Xenotransplant Breast Cancer Balb/c Mice

In situ reverse transcriptase polymerase chain reaction (in situ RT-PCR), polyclonal chicken antibody and goat anti-chicken antibody conjugated with fluorescein isothiocynate (FITC) using confocal laser scanning microscopy (CLSM) and negative staining transmission electron microscopy (NSTEM) were carried out to detect the NDV-AF2240 (Figure 7, 8 and 9) in tumor, liver, brain and lung during intratumural injection in 4T1 xenotransplant breast tumor in female Balb/c mice. Balb/c mice were divided into cancerous and non cancerous groups. To localise HN gene expression of NDV-AF2240 in tissues, *in situ* RT-PCR was applied on formalin fixed paraffin-embedded (FFPE) sections that were positive by negative staining transmission electron microscopy. The HN gene expression was detected in all the breast tumor cells. However, it was found mainly in the blood vessels of the brain, liver and lung. There was no significant difference ($p > 0.05$) in the HN gene intensity of CT/NDV8 and Ct/NDV32 and CT/NDV64 groups. *In situ* RT-PCR showed similar constant strong intensity of β actin gene expression in all mentioned tissues. Immunofluoresence and CLSM successfully detected the virus particles in tumor and all the organs of the cancerous groups during intratumoral injection. In tumor tissue, virus were found in the cells, whereas, in the lung, brain and liver were found mainly in the blood vessels. Negative staining with transmission electron microscopy as a gold standard, method was successfully used to detect the NDV-AF2240 at breast tumor, lung, liver and brain tissues during intratumoral injection in 4T1 xenotransplant breast cancer induced in mice. The results illustrated the presence of NDV-AF2240 in all organs of cancerous groups. The morphology of NDV was seen pleomorphic, spherical and ranging from 60-320 nm. The findings showed that

NDV-AF2240 suppressed the growth of breast cancer and it was disseminated in blood vessels of the brain, lung and liver, however, found in the cells of the breast cancer.

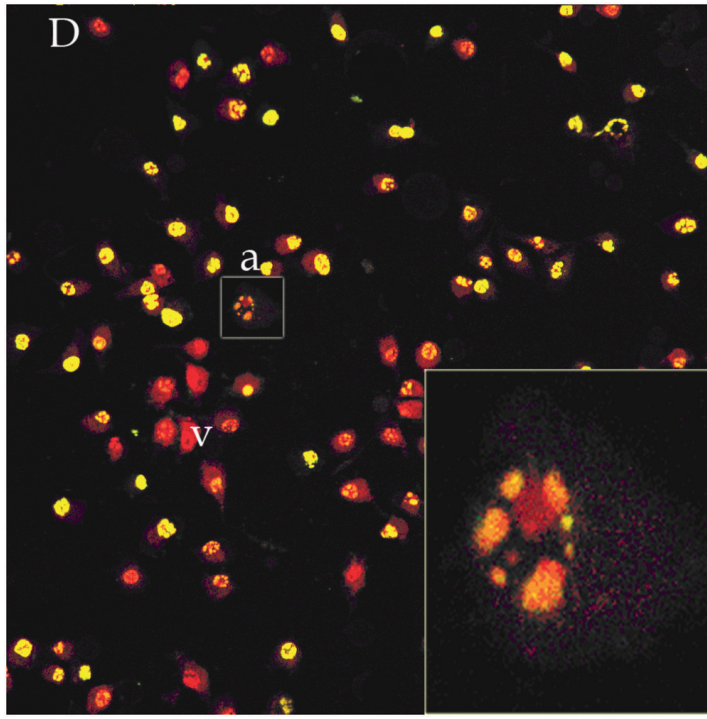


Figure 7 Apoptotic features of cells treated with NDV

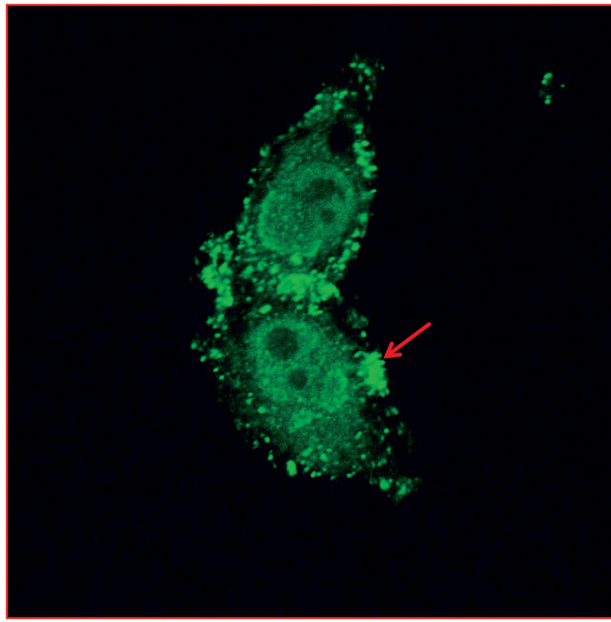


Figure 8 Replication of NDV in cells. Confocal micrograph of NDV in the cytoplasm of MCF-7 cells (arrow)

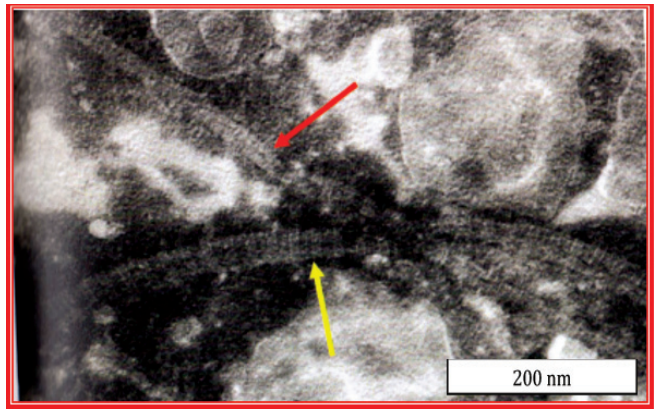


Figure 9 Transmission electron micrograph of NDV-AF2240 isolated from the brain of CT/NDV16 group by NSEM. Arrows show the filamentous nucleocapsids

Effect of Newcastle Disease Virus AF2240 on Allografted 4T1 Breast Cancer Cells in Balb/c Mice

This study was carried out to investigate the antitumor effect of NDV AF2240 *in vivo* using mouse 4T1 breast cancer cell line. 120 female mice were assigned randomly into ten groups; negative control (CC), cancer treated with 0.5µg/mL tamoxifen citrate (CT), cancer treated with NDV titre 8HA (CNDV8), NDV 16HA (CNDV16), NDV 32HA (CNDV32), NDV 64HA (CNDV64), combination of NDV 8HA+tamoxifen (CNDV8+T), NDV 64HA+tamoxifen (CNDV16+T), NDV 32HA+tamoxifen (CNDV32+T) and NDV 64HA+tamoxifen (CNDV64+T). 48 mice with tumour growth were euthanised weekly to remove tumour samples. At the end of the experiment, microscopic examinations were done on the cross-sections of tumour samples of these mice. Tumour growth was observed in groups; CC, CT, CNDV32+T and CNDB64+T, whereas, the rest of the groups had no tumour growth. CND32+T and CNDV64+T groups did not show any tumour regression (Figure 10) having a very low apoptotic index (AI) and a high mitotic index (MI) throughout the one month treatment indicating that these treatments were not therapeutic. Tamoxifen alone was able to regress the tumour but not with a significant difference.

In groups CNDV32+T and CNDV64+T, there was evidence that NDV caused cytoplasmic sequestration of p53 protein from the nucleus (Figure 11) to the cytoplasm, indicating the enhancement by the virus to induce apoptosis on these cells. The findings of this study suggested that NDV titres 8, 16, 32 and 64HA inhibited the growth of 4T1 cells, preventing tumour formation. Not all the combinations of NDV and tamoxifen were effective, the higher NDV titres combined with tamoxifen were neither able to inhibit nor regress tumour growth.

Figure 10 Clinical signs seen in the treated mice

In summary, NDV AF2240 alone can inhibit growth of 4T1 cancer cells and, thus, can be used as a potential oncolytic agent for breast cancer treatments. NDV is significantly more effective than tamoxifen and can be a very useful alternative anticancer agent for breast tumours.

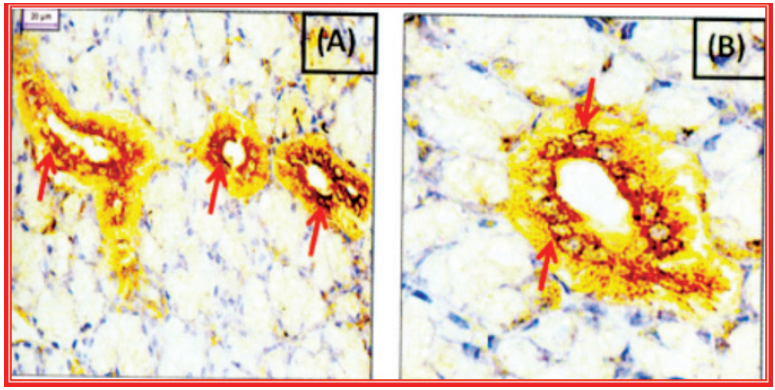


Figure 11 Light micrographs showing the expression of p53 protein representing treatment groups without tumour mass; CNDV8, CNDV16, CNDV32, CNDV64, CNDV8+T and CNDV16+T. A: p53 protein is highly expressed in the cytoplasm of ductal epithelium cell in perinuclear fashion. B: Quiescent mammary gland of a normal mouse breast tissue also expressing p53 protein (arrow) in the cytoplasm. Surrounding the ductal epithelium are fat cells. Mag x20 and B is zoomed

Newcastle Disease Virus Strain AF2240 on Xenotransplant Breast Cancer Cells In Balb/c Mice and Its Effects on Cytokines and Liver Enzymes

The study was carried out to investigate the effects of very virulent Newcastle disease virus (VVNDV) strain AF2240 on Balb/c mice induced with 4T1 breast cancer cells. The effects were determined by the evaluation of cytokines such as Interleukin-6 (IL-6), Interleukin-10 (IL-10), Monocytes Chemo-Attractant Protein 1(MCP-1), Tumor Necrosis Factor-alpha (TNF-α), Interferon gamma (IFN-γ) and Interleukin-12 (IL-12) in Balb/c mice since cytokines can be down regulated or up regulated in the mice induced by breast cancer cells. The present study also monitored the effects of liver enzymes such as total bilirubin (TBIL), Alanine

Aminotransferase (ALT) and Aspartate Aminotransferase (AST) in order to confirm that the viruses were safe and inert. All the inoculation process of 1X 10^4 4T1 breast cancer cells to all the fifteen (15) target groups were conducted via co-culture technique on the first week of the study. The treatment with NDV AF2240 was also given on the first day of week one and given daily until the end of study at week four. On day one of week one, blood samples from Balb/c mice were collected via cardiac puncture and spleen tissues were dissected from the mice were then kept for future analysis. The blood samples were processed to get the serum for the analysis of Cytometric Bead Array (CBA) with flow cytometer and to conduct biochemical tests for liver enzymes. Spleen tissue were processed immediately in order to ensure that the lymphocytes in the spleen were still fresh during ELISPOT analysis. The results showed that from all fifteen groups, there were no significant adverse changes in the body weight over time due to the rapid progress of the tumor cell. The ANOVA statistical analysis showed the tumor weight profile, Cancer Control (CC) and Cancer Tamoxifen (CT) groups had a significant heavier tumor weight compared to Normal Control (NC) and groups that were treated with VVNDV AF2240. The Liver Function Test (LFT) analysis which indicated damage to the hepatocytes had shown total bilirubin (TBIL) concentration for CC group was significantly different ($p \leq 0.05$) compared to the other groups especially NC. While for concentration of AST and ALT, CC group, Balb/c treated with NDV AF2240 32 HA and Tamoxifen (CTNDV32) group and Balb/c treated with NDV AF2240 64 HA and Tamoxifen (CTNDV64) group had shown significant difference compared to NC group. Two methods were used to observe the cytokine elevation secreted in the serum which is through CBA method and the ELISPOT method for the cytokine produced by the lymphocytes. The studies showed no significant correlation between

these two methods, since CBA cannot distinguish whether few or more cells had been given the cytokine which can be detected separately by the ELISPOT analysis. On the overall, for IL- 6, it showed some cytokine elevation between the two methods when IL-6 had elevated significantly at CC, CT, Balb/c treated with NDV AF2240 32 HA (CNDV32), Balb/c treated with NDV AF2240 64 HA (CNDV64), Balb/c treated with NDV AF2240 8 HA and Tamoxifen (CTNDV8), Balb/c treated with NDV AF2240 16 HA and Tamoxifen (CTNDV16) and CTNDV 32 groups. For IFN-γ it followed the same trend and groups treated with NDV had high expression of IFN-γ compared to CC group, which was believed to promote tumor growth. Ironically in this study, IL-10 was observed to be up regulated significantly in CTNDV8 until CTNDV64 groups compared to untreated groups and groups treated with NDV only. While for IL-12 cytokine, in the groups which had been given the treatment with NDV as the anti cancer agent, it had shown some regulation mechanism to it. For TNF-α cytokine which can promote breast cancer metastasis had shown significant elevation on breast cancer groups treated with high titer of NDVs such as CTNDV32 and CTNDV64. As for MCP-1 cytokine, which is always involved in recruiting and migration of inflammatory cells, it also showed significant elevation ($p \leq 0.05$) and was detected in the groups bearing cancer cells and CC group. In conclusion, elevation of pro and anti inflammatory cytokine such as IL-6, IL-10, IFN-γ, IL-12, TNF-α and MCP-1 had contributed to the progression or regression of the tumor.

Effect of Velogenic Newcastle Disease Virus Strain AFF2240 Towards 4T1 Breast Cancer Cell Allografted on Balb/c Mice

The present study was conducted to find a new anti-cancer agent for the treatment of breast cancer. The AF-2240 strain of NDV was propagated in the allantoic fluid of 11-days-old embryonated eggs for 72 hours. The virus was harvested, purified and stored at -80^{0}C. The haemagglutination (HA) test conducted on the purified virus showed that the virus obtained was at 64 HA unit. The induction of breast cancer was done on the auxiliary region of female inbred Balb/c mice by using 1 X 10^4 4T1 breast cancer cells. The treatment given and the condition of the animals had no effect in term of bodyweight as there was no significant difference noticed between tumor bearing mice and tumor-free mice ($p>0.05$). The effectiveness of the treatments was later translated by observing the number of apoptotic cells. All tumor samples exhibited apoptotic features analysed by using apoptotic peroxidase staining and comet assay. The analysis showed that combination treatments using NDV and tamoxifen have no significant effect toward the breast cancer cells. Only CT group which were treated with tamoxifen showed significant ($p<0.05$) higher number of apoptotic cells compared to the rest of the groups. Like any other types of *paramyxovirus*, NDV-AF2240 was found to be localised in the cytoplasm of the breast cancer cells observed by using transmission electron microscope. Further analysis on the oncogenes (c-myc, c-cerbB2 and c-fos) revealed that the presences of the oncogenes in all tumor bearing mice group regardless treatment given. In conclusion, NDV-AF2240 has the potential as an anti-cancer agent if it is used alone or at low HA titre if in combination with tamoxifen. The used of the virus at high HA titre and in combination with tamoxifen has to be monitored with cautious as it has an antagonist effect.

Herbal Theraphy

From ancient to modern times, herbs and other plants have been used as medicinal agents. Rediscovery of the connection between plant biotechnology and health is a new generation of botanical therapeutics that included plant derivate pharmaceuticals (Raskin *et al.,* 2002). In the recent years, there has been growing interest in alternative therapies and therapeutic use of natural products, especially from plant derivative (Vuito and Snet, 1998) and trend towards the use of natural substances which is also believed to have potential value as cancer chemo-preventive or therapeutic agents. The potential use of higher plants as a source of new drugs is still being explored. From the estimated 250,000 – 500,000 plants species, only 5,000 species have been studied for medicinal use (Payne et.al, 1991). Up to 1992, the NCI had only found 3 out 33,000 plant extracts tested to have anti-tumour activity (Williamson et.al, 1996). Traditional medicine is well-known for its nutritional value, as well as, its ability to cure various ailments. In recent years various constituents have been found to provide protection against any disease including cancer (Hakama et.al, 1997; Sporn and Suh, 2000). Any significant role by dietary intervention is encouraging and emerging as an acceptable approach for controlling the cancer incidence worldwide (Kellof, 2000).

Since the beginning of live, plants have played a major role in influencing human life. Since a few thousand years ago until today, plants ae widely used as food and medicine. To obtain plants for medicinal purposes, it is better if only healthy plants are used. This is possible by obtaining plants from its natural habitat. The constituent of a plant may also change according to the environment and seasons. These are important considerations when collecting plants. Other factors affecting the medicinal capabilities of a plant are: the time when it is picked, season, age of the plant and also

its stage in the plant live cycle. These are important medicinal considerations to herbs collectors (Muhammad and Mustafa, 1994)

In traditional medicine, these ingredients are eaten directly, as in the raw form, whereas in modern medicine, the extract is reprocessed to obtain the active chemical compound in concentrate form (National Research Council, 1992). Plants produce primary metabolites and secondary metabolite. Primary metabolite is used by the plant itself for its growth and also stored as reserve food. Besides that, plants produce secondary metabolites which also called secondary compounds. Plant secondary metabolite has potent to obtain the needed bioactive chemical. Due to their large biological activities, secondary metabolite of plants has been used for century in traditional medicine (Hammerschmidt, 2004). Natural product is an attractive source of new therapeutic candidate compound of sources of new drugs as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms (Da Rocha *et.al,* 2001).

About 25% of the drugs prescribed worldwide come from plants and 121 active compounds being in current use. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001). Examples of important drugs obtained from plants are degoxin from *Digitalis* spp., quinine and quinidine from *Chinchona* spp., vincristine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*. However, the potential use of higher plants as a source of new drugs is still poorly explored. Of the estimated 250,000 plants species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of the pharmacological properties;

in most case, only a pharmacological screening or perliminary studies are carried out. It is estimated that 5000 species have been studied for the medicinal use (Payne *et.al*, 1991).

In the methods to isolate active compound, the plant extracts were first qualitatively analysed by thin layer chromatography (TLC) and/or other chromatographic methods. Screening should be continued to determine the biological activity or to obtain general evaluation of biological activities (Figure 12). For purification and isolation, the active plant extracts were sequentially fractioned (Rates, 2001; Vepoorte, 1989). Each extract or fraction or pure compound was subjected to bioassay which can be performed using microorganisms, insects, cellular system, cell culture, or *in vivo* (Souza Brito, 1996).

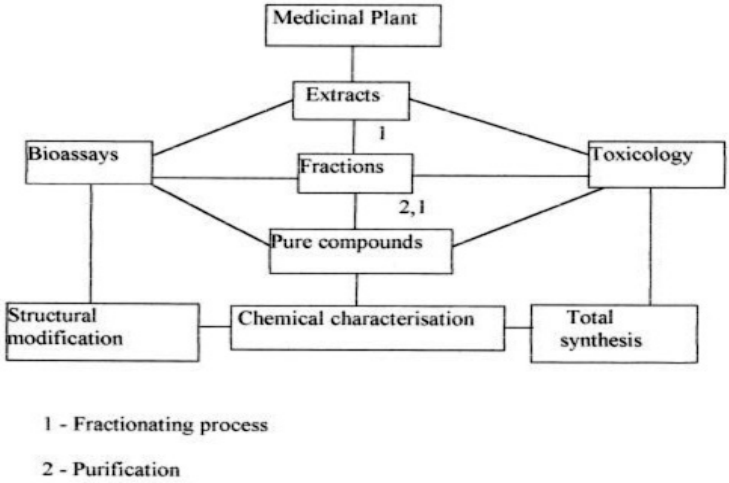


Figure 12 Methods for Obtaining Active Substance from Plants (Rates, 2001)

Cancer is the second highest cause of death (12.8%) worldwide after cardiovascular disease (29.3%) (WHO, 2004). There were 10.1 million new cases, 6.2 million deaths and 22 million people living with cancer in 2000 (Parkin, 2001). Cancer is the fourth leading cause of death in Malaysia, nearly 30 000 cases in each year (Lim, 2002). Liver cancer is the fifth most important cancer worldwide (5.6% incidences), but, because of the very poor prognosis, the number of deaths is almost the same as incidence and is the third most common cause of death of cancer (Parkin, 2001). Survival with liver cancer (HCC) often differed significantly according to the tumor stage or size of the tumor (Markovic *et al.,* 1999). Cancer is a cellular phenomemnon (Brock &Madigan, 1991). The development of cancer cells in many organs and tissues have altered growth requirements and continue to grow, piling up to form a small 'focus of growth' or 'foci' (Brock & Madigan, 1991). Hepatic chemical carcinogenesis is a multistep process in experimental animals (Sarveswaram *et al.,* 2006).

Carcinogens initiate the process, which is followed by regeneration, growth and clonal proliferation, eventually leading to cancer. N-Nitrosodiethylamine (DEN) is a representative chemical of a family of carcinogenic N-nitroso compounds. Administration of DEN to animals has been shown to cause cancer in liver and at low incidence in other organs also. Initiation during or after DEN exposure is thought to be a rapid metabolism of DEN to ractive metabolites that interact with DNA, forming various DNA adducts that can lead to mutations. There is extensive evidence that the free radicals participate in DEN-induced hepatocarcinogenesis, which is confirmed by overexpression of 8-hydroxyguanine in DEN administered rat liver.

Generally, oxygen free radicals are natural physicological products, but also extremely reactive oxygen species, (ROS). They

have been proven to cause numerous cellular anomalies, including but not limited to protein damage, deactivation of enzymatic activity, alteration of DNA and lipid peroxidation of membranes. Continuous interaction of the animal with these free radicals causes damage of proteins, lipid, DNA, carbohydrates and membrane, resulting in oxidative stress. In order to maintain cellular health, it is essential to have a specific and effective chemical scavenger to target multiple types of radicals. Most of the commercially based antioxidant supplements are single oxidant. It was also observed that majority of the antioxidants originate from natural sources.

It has been noticed that many of the plants, rich in phenolic compunds, are widely used as antioxidant and antimutagenic (Sarveswaram *et al.,* 2006). Barbery (*Berberis vulgaris* L., Var. asperma Don., family Berberidaceaae) grows in Asia and Europe. Barberry is a well known medicinal plant in Iran and the fruits have been used as food (Zargari, 1983; Amin, 1991). Medicinal properties for all parts of the plant have been reported, including tonic, antimicrobial, antiemetic, antipyretic, antipruritic and cholagogue actions, and has been used in some cases like cholecystitis, jaundice, dysentery, leishmaniasis, malaria and gallstones (Zargari, 1983; Aynehchi, 1986; Nafissi, 1990). In the present investigation, we studied the anticarcinogenic effect of *B.vulgaris* fruit extract (BFE) on DEN induced hepatocarcinogenesis in rats.

Effects of *Berberis Vulgaris* (L.) Fruit Extraction on Antioxidant Enzyme Activities, α-Fetoprotein Content and Histology of Hepatocarcinogenic Rats

The chemopreventive agent of *Berberis vulgaris* fruit extract in hepatocarcinogenesis female Sprague Dawley rats was studied to investigate the possible cancer preventive effect of the plant. Total antioxidant activity and phenolic content of BFE

extracts were measured. Total phenolic content of BFE in 80% methanol was (28000±500 mg/100g), followed by BFE in water (10000±400mg/100g). There was an inverse relationship between antioxidant activity and phenolic content of *Berberis vulgaris* fruit extract. The severity of neoplasia was studied by histological evaluations, body and relative liver weight profile and liver tumour marker. Histological evaluations showed loss of normal cell organisation when carcinogens were introduced into the body. Microscopic observations of the lesion score have shown significant difference ($p< 0.05$) between DEN/AAF and normal control group. In liver cancer rats treated with *Berberis vulgaris*, the activities of GST and GGT were significantly lower ($p<0.05$) compared with the DEN/AAF group. The findings showed that BFE could reduce the activity of liver enzymes of rats during hepatocarcinogenesis. Meanwhile, the RT-PCR analysis of hepatocytes illustrated the AFP gene expression in DEN/AAF group only.

Antioxidant Analysis and In Vitro Anticancer Activities of Azadirachta Indica A. Juss (Neem) Extracts

Azadirachta indica, A.Juss (neem) is one of medical plant which is found throughout India, Pakistan and Southeast Asia which have many wonderous properties. In this study, neuraceutical analysis, antioxidant properties, cytotoxic activities and expression of causing-cancer genes after exposed to *A.indica* were carried out. Macro and micro mineral content were determined using the atomic absorption spectrophotometer (AAS) and energy dispersive X-ray microanalysis (EDX). The analysis of antioxidant vitamins A, C and E was carried out using high performance liquid chromatography (HPLC), where as vitamin C was the highest vitamin content. The antioxidant properties of *Azadirachta indica* was assayed by diphenyl-1-picrylhydrazyle (DPPH), β-carotene and total

phenolic content. The cytotoxic property was determined using the microculture tetrazolium salt (MTT) assay on MCF-7, MDA-MB-231 breast cancer, cervical cancer (HeLa), Chang Liver, ovarian cancer (Caov-3) and normal human breast (MCF-10A) cells. The ethanolic extract has the strongest act to inhibit HeLa cells growth with IC_{50} value of 28 µg/mL, followed with IC_{50} at 50 µg/mL for MDA-MB 231 and IC_{50} at 55 µg/mL for MCF-7 cells. All the solvent extractions of Azadirachta indica used exhibited cytotoxic effect on hela, cervical cancer, MDA-MB 231 and MCF-7, breast cancer cells in range 28 µg/mL-69 µg/mL but not in MCF-10A, Chang liver and Caov-3 cells. The study showed that ethanolic extract of neem significantly reduce level of c-fos and c-myc expression on HeLa cells. Contrary, none of level c-myc, c-erb and c-fos expression was reduced by ethanolic extract on MCF-7 and MDA-MB 231 cells. This present study revealed that although apoptosis was induced (Figure 13), the reducing of the oncogene expression is regulated by the type of extract and type of cell lines.

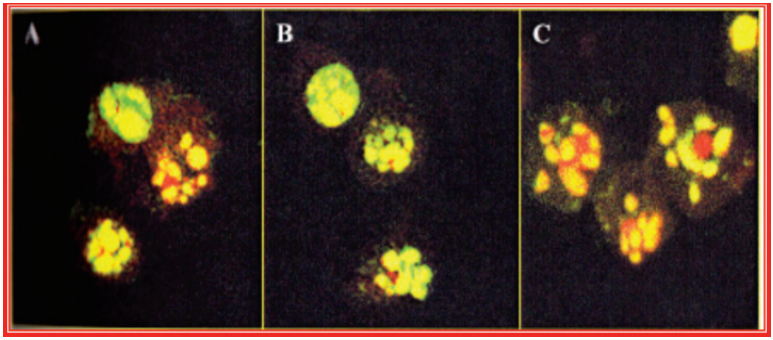


Figure 13 Confocal micrograph of treated MCF-7 (A), MDA-MB 2431 (B) and HeLa cells (C) after exposed to ethanolic extract of *A. Indica* (neem) shows appearance of nucleus fragmentation. Mag 1000x

Effect of *Strobilanthes Crispus* Extract Enzymes Activities and Liver Cell during Hepatocarcinogenesis

This study was conducted to determine the effect of aqueous extract of *Strobilanthes Crispus* (SC) with four different doses on experimental male albino rats species with induced diethylnitrosamine as initiator and 2-acethylaminofluorene as promoter agent. This study was also conducted through observing liver cell morphology by using light microscope and transmission electron microscope. The exposure of animals to carcinogen showed the increased of all specific enzymes used. Based on the result it showed that *S.crispus* may inhibit the development of hepatocarcinogenesis before the cells undergo to cirrhosis. Transmission electron micrograph showed the ultrastructural features of cell such as nucleus, mitochondria and rough endoplasmic reticulum (RER). Shrunken nucleus and disarrangement of mitochondria and rough endoplasmic reticulum (RER) were observed in DEN/AAF induced groups. However, the shape of the nucleus and the arrangement of rough endoplasmic reticulum (RER) and mitochondria appeared normal in DEN/AAF induced rat treated SC group. As a conclusion, this study revealed that SC aqueous extract has a potential as an inhibition agent during hepatocarcinogenesis without interfering the normal growth of cells.

Effects of Cola Nut (*Cola Nitida* (Vent.) Schott & Endl.) Aqueous Extract on Rat Liver during Hepatocarcinogenesis

The use of herbs as medicines has played an important role in nearly every culture on earth, including Africa, Asia, Americas and the Europe. Herbal therapies are unconventional treatments and are widely used for many diseases. Approximately 70 to 90% of health care worldwide is delivered by alternative medicine (Lewis and Elvin-Lewis, 2003). Among patients with cancer, the

use of unconventional medicines, including herbal therapies, has been reported to be as low as 5 percent and as high as 60 percent (Eisenberg *et.al*,1993; Risberg *et al*., 1998). Herbal products are taken specifically to prevent diseases or tone down the effects of risk for certain diseases. Examples include the consumption of green tea and other flavonoid-rich botanicals to take advantage of the natural antioxidants in them and the use of garlic because of the rich organosulfur compounds that have been shown, experimentally at least, to prevent cancer in animals (Wargovich, 1987; Wargivich *et al.,* 1988).

Liver cancer is the sixth most common cancer in terms of numbers of cases (626,000 or 5.7% of new cancer cases) worldwide, but because of the substandard prognosis; the number of deaths is almost the same (598,000). Liver cancer is therefore the third most common cause of death related to cancer worldwide (Parkin, Bray, Ferlay and Pizani, 2005). Liver cancer is the second of deaths due to cancer among medically certified deaths in Malaysia (Lim, 2002).

It has been estimated that close to about 80-90% of the forms of most human cancers are caused by exposure of individuals to environmental factors (Figure 14) (Oliveira *et al.*, 2007; Jensen and Madsen, 1988). Several agents including viruses, chemicals and radiation have been realised to induce cancer in both experimental animals and humans (Cooper, 2000).

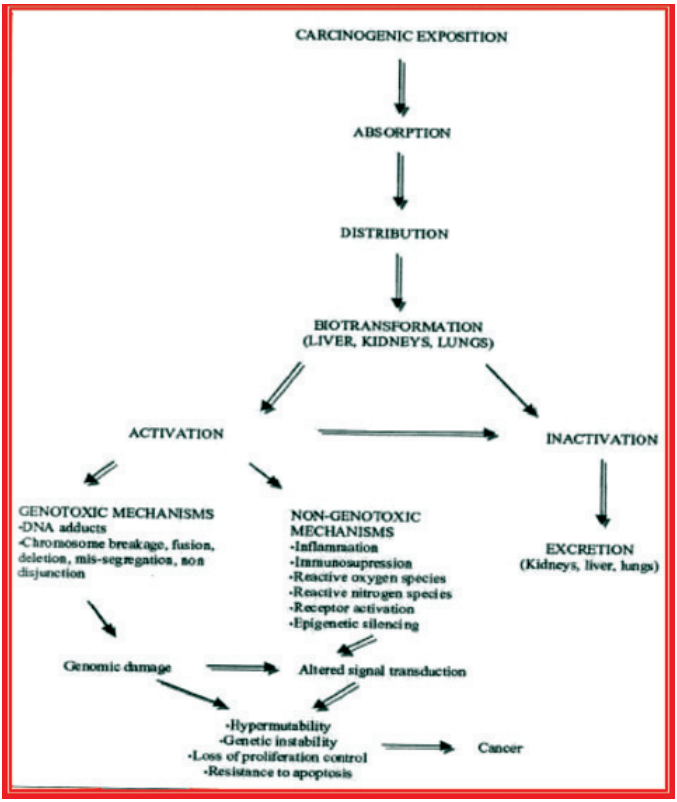


Figure 14 Metabolic activation of chemical carcinogens and genotoxic and non-genotoxic effects of carcinogens (Oliveira *et al.*, 2007)

Cola nitida is the source of a stimulant and contains methylxanthine alkaloids that are also found in coffee, cocoa and tea. The cola nuts are used as an herbal medicine worldwide, especially in West Africa; partly due to the fact that the cola is a precious commodity (Jayeola, 2001; Morton, 1992; Trindall, 1997). It has been stated that *Cola nitida* possesses antioxidant activity and other medicinal properties (Duke, 2001); as well as its anti-proliferation effect in breast cancer cell line (Fontenot, Naragoni, Claville and Gray, 2007).

The effect of *Cola nitida* aqueous extract in hepatocarcinogenesis induced male Sprague Dawley rat livers, and elemental analysis of the cola nut were studied to investigate the possible anticancer activity. The unprocessed cola nuts were observed for their surface morphological structure under the scanning electron microscope (SEM). SEM study of cola nut illustrated numerous crystals packed in clusters within the cell wall. The elemental analysis results revealed that the cola nut contained high amount of oxygen and carbon, in addition to potassium, phosphorus and magnesium. Potassium, magnesium and phosphorus have been well reported as co-factors of antioxidant enzymes to protect the body from oxygen free radicals. Additionally, these elements play important roles in metabolic mechanisms in the body. Hepatocarcinogenesis was induced in rat livers according to the modified Solt and Farber method. Diethylnitrosamine (DEN) was injected into the rats at 200 mg/kg body weight to initiate hepatocarcinogenesis and after two weeks this was followed by feeding 0.02% 2-Acetylaminofluorene (AAF) to promote the hepatocarcinogenesis. The DEN/AAF induced rats were treated with 1, 2.5, and 5% (w/v) concentrations of cola nut extract or 0.001, 0.0025, and 0.005% w/v dilutions of glycyrrhizin as a drug control. The supplementation of cola nut extract decreased the level of plasma and microsomal GGT and GST tumor marker enzymes significantly in DENA/AAF induced liver tissues even better than glycyrrhizin. Additionally, it was revealed that cola nut extract has no effect on the level of GST and GGT enzymes in normal cells. The histological and ultrastructural examination as well as the lesions scoring results demonstrated that the cola nut extract reduced neoplastic stage of the hepatocarcinogenenic liver cells more than glycyrrhizin based on their abnormal morphology, inflammation, necrosis and fibrosis. Moreover, rat's normal hepatocytes treated with cola nut extract

illustrated normal features. These findings suggested that cola nut might act as a promising anticancer against hepatocarcinogenesis with even higher efficacy compared to glycyrrhizin, without any side effects in normal liver cells.

TISSUE ENGINEERING

Engineered Organs

Tissue and organ failure, resulting from various form of injuries either traumatic, metabolic, inflammatory and other disease, accounts for about half the total annual expenditure in the world health care (Middelkoop *et al*., 2004). Various treatment modalities are employed to overcome the problems which include organ transplantation, surgical repair, plastic surgery, artificial prostheses, drug theraphy and the use of mechanical devices. However, organ and tissue damage cannot be repaired and healed by fibrous repair which result in permanent loss of functional tissue. In organ transplant, rejection may occur and frequent monitoring is needed. The presence of tissue engineering technology provides an alternative choice to solve this problem of tissue loss and it has been reported to be safe and side effects are minimal (Robert and Vacanti, 1993).

Cell based therapies hold the promise of becoming the major therapeutic modalities in the 21st century. A basic understanding of cell and developmental biology, bioengineering analysis and design, and clinical implementation must be met, in order to implement these therapies (Palson and Bhatia, 2004). To overcome these problems, researchers try to implement tissue engineering as a new approach. Tissue engineering is an interdisciplinary research field that grows rapidly, merging the principles of engineering and life sciences (Stock and Vacanti, 2001; Vacanti and Langer, 1999).

This emerging field is very promising for the future of medicine that aims to develop biological substitutes that restore, maintain or improve tissue functions.

At present, there are increasing numbers of tissue types being explored and engineered where stem cell technology was employed. Stem cells are used because they are undifferentiated cells which are pluripotent in nature and can be induce to differentiate into desired cell type. More and more tissue types are being explored and engineered. Among other examples of tissue engineered human substitutes that are developed includes tissue-engineered bone, blood vessels, liver, muscle and nerve conduits (Anon, 2002).

Electron microscopy has become a standard tool to assess the quality of the fabricated tissue constructs as light microscopy may not be able to achieve the resolution obtained by electron microscopy. Electron microscopy has been used by many researchers, for instance, to assess the biodegradation and bioresorption of calcium phosphate ceramics (Damein *et al.,* 1994; LeGeros, 1993), cell attachment (Baxter *et al.,* 2002), cell morphology (Trentz *et al.,* 2003), cell proliferation (Chou *et al.,* 2005), tracing of tissue specific proteins (Bianco *et al.,* 1991), calcification of biomaterials (Declercq *et al.,* 2005) and bone formation (Hing *et al.,* 1999; Gatti *et al.,* 1990).

Morphological Changes and Expression of Protein Markers during Remodeling of Tissue-Engineered Skin

This study was carried out to evaluate the skin remodelling and skin development after bilayered fibrin-fibroblast/fibrin-keratinocytes skin equivalent (B FF/FK SE) and fibrin without seeded cell (FWC) were transplanted into eight weeks old athymic mice. During skin modelling, the structural, ultrastructural features and protein expression were investigated. Light microscopy revealed that B FF/

FK SE has good skin remodelling capacity with 6-12 cells thick after 60 days post-transplantation whereas FWC was only 3-4 cells thick. Futher studies were done using the structural features of B FF/FK SE and FWC *in vitro* and *in vivo*. Scanning electron microscopy (SEM) revealed that keratinocytes and fibroblasts in B FF/FK SE showed an excellent adherence in fibrin matrix and changes in their morphology after 1 to 14 days *in vitro* (Figure 15). It ranged from rounded to elongated and stellate shape, whereas, for FWC, no cells were detected. Transmission electron microscopy (TEM) showed that the ultrastructural features during epidermal differentiation and regeneration as well as basement membrane formation were well developed after B FF/FK SE and FWC transplanted onto athymic mice. Confocal microscopy revealed that immunolabellingof desmoglein 3 and plakophilin 1 at stratified layer, type IV collagen, integrin α6 and type VII collagen at basement membrane zone and type I collagen at dermal margin were present after 60 days B FF/Fk SE post-transplantation which was similar to native human skin (Figure 16). In conclusion, B FF/FK SE showed better skin regeneration similar to native human skin and required a shorter period of time during wound healing without any contraction.

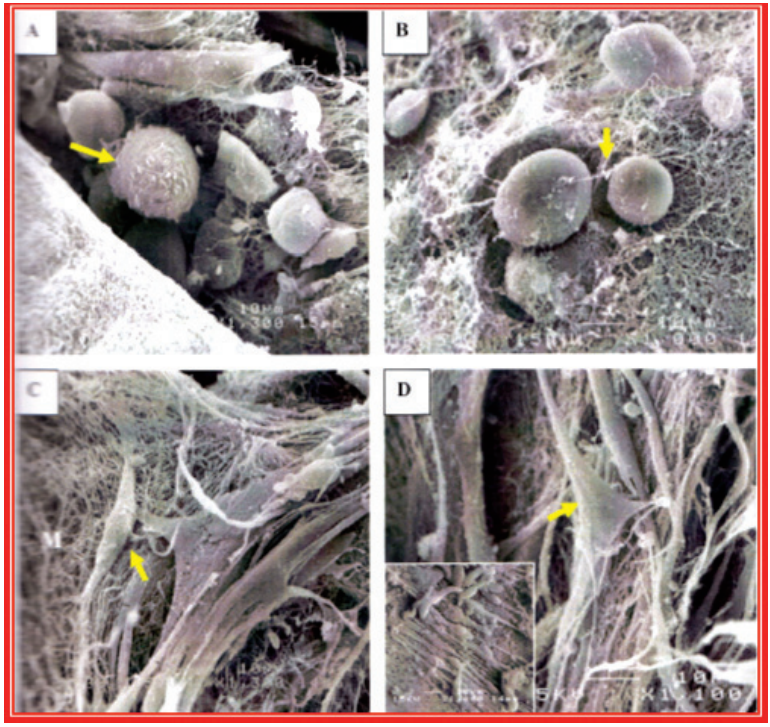


Figure 15 Scanning electron micrograph of in vitro of B FF/FK SE (A-D). (A) Note, presence of round keratinocytes (arrow), 1300X. (B) Note, keratinocytes secreting the extracellular matrix (arrow), 1000X. (C) Note, keratinocyte with presence of filopodia which adhere and spread over fibrin matrix (m) 7 days post-polymerization (arrow), 1300X. (D, insert) Increased in the number of keratinocytes 14 days post-polymerisation, occasionally with stellate shape keratinocytes (arrow), 1000X and 1100X

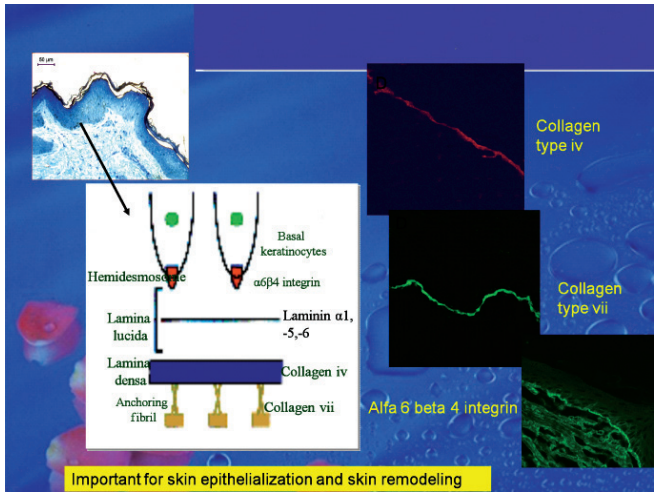


Figure 16 Skin basement membrane zone (BMZ) indicating the relative location of known BMZ components

Structural and Ultrastructural Studies of Tissue Engineered Cornea

Evaluation of corneal organisation and regeneration after transplantation of bilayer *in vitro* cornea construct (BICC) into the New Zealand White Strain rabbit's eye was carried in this study. A study was conducted to investigate the structural and ultrastructural features after corneal regeneration 90 days post-transplantation. Slit lamp microscopic analysis revealed that engineered cornea (EC) showed good corneal regeneration with no significant difference in cornea transparency to normal cornea (NC). Scanning electron microscope (SEM) analysis demonstrated that epithelial surface of EC showed significantly similar features to NC compared to fibrin cornea (FC) and defect cornea (DC) ($p < 0.05$) (Figure 17). Transmission electron microscope (TEM) analysis showed that the basal lamina development of EC was similar to NC with the establishment of cell junction compared to FC and DC.

Furthermore, the EC showed a compact stromal organisation with homogenous collagen fibrils diameter similar to NC ($p<0.05$). However, FC and DC showed a loose stromal organization with heterogenous fibrils diameter, with FC fibrils diameter were bigger than that of NC; while for DC, the fibrils diameter was smaller than NC ($p<0.05$). Confocal microscopy analysis confirmed that the regenerated epithelial cells in all groups were corneal epithelial cells by using corneal differentiation marker, cytokeratin 3 (CK3). As a conclusion, the EC demonstrated excellent regenerative ability of cornea and better wound healing.

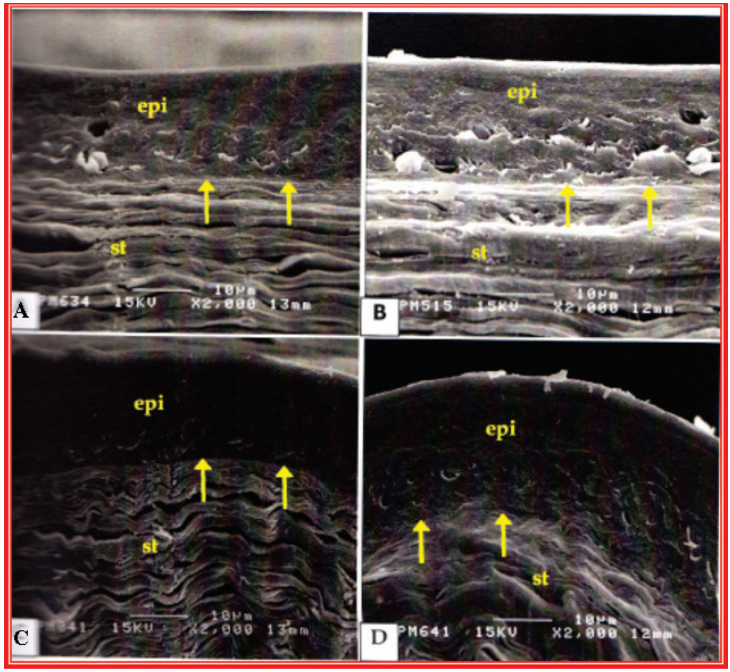


Figure 17 Scanning electron micrographs of epithelial thickness of central cornea (A-D) at different groups. Normal cornea (A), engineered cornea (B), fibrin cornea (C) and defect cornea (D) groups. Note, all the group appeared to have differentiated epithelial layer laying on top of the basal lamina (arrow). Epi=epithelium, st=stroma. X2000

An electron Microscopic Analysis of Tricalcium Phosphate Hydroxyapatite and Synthetic Hydroxyapatite Bioceramics for Bone Tissue Engineering

Tricalcium phosphate hydroxyapatite, TCP/HA (resorbable) and synthetic hydroxyapatite, HA (slow-resorbable) scaffold biomaterials, both composed of calcium and phosphate in varying compositions, were assessed as possible potential scaffold materials in bone tissue engineering. The present study used correlative light and electron microscopic analysis to investigate the surface morphology of human osteoprogenitor cells and its proliferation rate in response to alpha medium and differentiation medium at 1 week post-culture, effect of fibrin matrix inclusion into scaffold on cells and physio-chemical characteristic of the scaffolds (TCP/HA and HA) studied in a three-week *in vitro* model. An *in vivo* study of a three months post-implantation tissue-engineered bone constructs in nude mice included the detection of fibroblast and inherent proteins of bone tissue, such a s extracellular matrix protein (collagen type I) and non-collagenous proteins (osteopontin and bone sialoprotein) by scanning electron microscopic immunogold-silver labelling, elemental analysis and calcium phosphate elemental mapping determined by energy-dispersive X-ray (EDX) microanalysis and assessment of bone formation via light and electron microscopy, namely scanning electron microscopy (SEM) and transmission electron microscopy (TEM). *In vivo* study, TEM observation illustrated new bone formed interdigitally in the micropores of TCP/HA and HA grains (Figure 18); however, no bone formation was observed at the periphery of the TCP/HA and HA granules as revealed by light microscopy. Elemental mapping and structural imaging using BSE grey-level and EDX analysis ascertain the similar bone formation patterns in the two composites. The expressions of

bone matrix proteins, bone sialoprotein and osteopontin and ECM deposition, which are predominantly collagen type I as determined by SEM immunolabelling clearly verified that the tissue-engineered bone constructs possess inherent characterictics of a bone tissue. Hence, the correlative microscopy study indicated that TCP/HA is much favourable than HA as bone substitute as it promotes new bone replacement in places (Figure 19) where degradation can be observed and moreover, the bone regeneration rate befitting to the resorption rate of the TCP/HA.

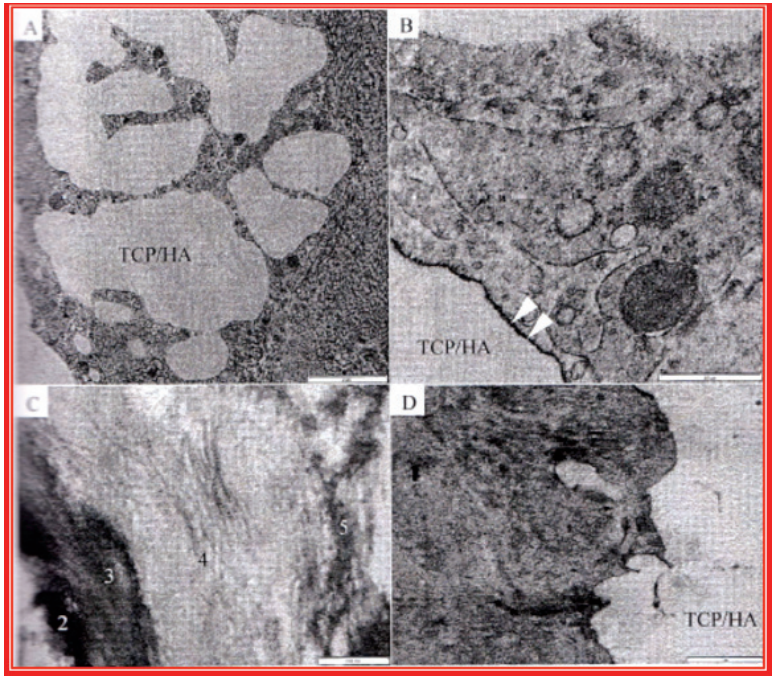


Figure 18 TEM image illustrating (A) the granul shape of TCP/HA surrounded by dense collagenous matrix. (Bar: 2μm). (B) An electron-dense interfacial layer seen at tissue-TCP/HA interface (Bar: 0.5μm). (C) Several stratified layers were formed on the TCP/HA substrates. The first layer in direct contact of the (1) TCP/HA grain appeared electron dense (2),overlying by a moderate dense flocculent material (3), followed by amorphous substances with few fibrillar-like strands (4) and the outmost layer contained irregular patches of electron dense substances (5). (100,000x, bar: 200nm/0.2μm). (D) Maturation of osteoid as indicated by condensed collagen matrix and loss of periodic banding in collagen fibers due to mineral deposition and extracelllar matrix organic components within the fibrils (21,500x, bar: 1μm).

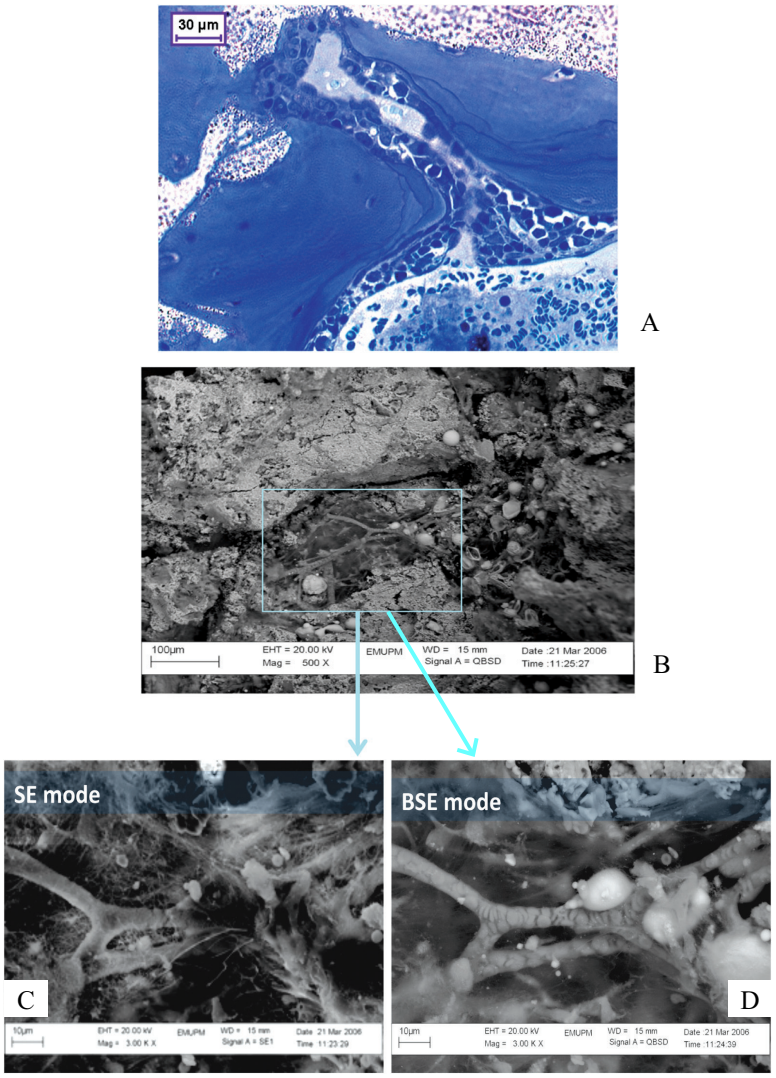


Figure 19 Vascularisation was observed in the TCP/HA. A) Light micrograph. B) Scanning electron micrograph. C) Secondary scanning electron micrograph of blood vesssel. D) Backscattered scanning electron micrograph of blood vessel. Note RBC seen through the blood vessel wall

ANTIBIOTIC SLOW RELEASE

Biomaterials play an important role in treating diseases and improving healthcare. They are commonly used in dental, surgery and drug delivery applications. Biomaterials can be used for a benign function for example as a heart valve or may be bioactive and used for a more interactive purpose such as hydroxyapatite-coated hip implants.

Biofilm is the accumulation of adherent microorganism attached onto a solid surface by the βexcretion of protective and adhesive extracellular polymeric substances (EPS) to form a structured community (Costerton et.al, 1999). Biofilms are often characterised by surface attachment, structural heterogeneity, genetic diversity, complex community interactions and extracellular matrix of polymeric substances (Hall-Sttodley *et al.,* 2004).

Common microorganisms capable of forming biofilm to infect prosthetic devices are the gram-positive *Staphylococcus aureus* and gram-negative Pseudomonas aeruginosa (Hatch and Schiller, 1998). *S.aureus* in pasticular is associated with biofilm-related diseases for example infectious arthritis, endicarditis and cystic fibrosis (Raad, 1998). It has the ability to adhere to specofoc host substrate, evade host defense, resist antibiotic therapy and is well adapted to the human host (Fedtke *et al.,* 2004).

The delivery of antibiotics in the treatment of bone infection at a local site was evaluated in various biodegradable system (Schmidt *et al.,*1995), such as HAP/TCP (hydroxyapatite and β-tricalcium phosphate) (Laurent *et al.,*2008) and GR-HA (glass-reinforced hydroxyapatite) (Queiroz *et al.,*2001). Hydroxyapatite (HA) is a commonly used biomaterial for medical implants. It is a biodegradable polymer with three-dimensional porous polymeric matrixes to enhance bone regeneration and facilities cell migration and proliferation (Putnam and Mooney, 1996). Hydroxyapatite (HA)

is also frequently used in orthopaedic and dental applications owing to its biocompatibility, osteoconductivity and bioactivity (Damien and Parsons,1991).

In orthopaedics, parenteral administration of antibiotics does not provide good local bone response due to poor vascularisation of bone tissue and low drug penetration. Local administration of antibiotics would increase drug penetration and also reduce the toxicity associated with systemic treatment with antibiotics (Le Ray *et al.,* 2005). Furthermore, medical implants remain in close contact with biological tissue for prolonged periods and the host tissue response should be tested before clinical use.

Gentamicin-coated Hydroxyapatite in Prevention of Biofilm Formation in Bone Tissue

Biofilm is a multilayered complex microorganism and is typically more resistant to the host immune response and routine antibiotic therapy. In order to limit biofilm formation, biomaterials loaded with suitable antibiotics can be used as a preventive measure. Biomaterial hydroxyapatite (HA) is an osteoconductive space filler and is produced locally at Malaysia Nuclear Agency. In this study, HA coated with the antibiotic gentamicin was explored to examine whether it could be reduce or remove biofilm formation. To assess IC50 values of gentamicin-coated HA, 10^8 CFU/ml of *Staphylococcus aureus* (ATCC 12600) and *Pseudomonas aeruginosa* were cultured for 48 hours in a 96-well plate for biofilm formation. It was demonstrated that IC50 values of gentamicin-coated HA were 0.1mg/ml *S.aureus* and 5mg/ml for *P.aeruginosa* biofilm. Fluorescence staining with acridine orange and propidium iodide (AOPI) was also conducted to visualise viability of the biofilm. The efficacy of gentamicin-coated HA was also tested *in vivo*. A Teflon catheter was used to create catheter-associated biofilm

segments for *in vivo* implantation. Catheter-associated biofilm was examined with scanning electron microscope (SEM) to confirm *S.aureus* biofilm formation (Figure 20). This study showed that the gentamicin-coated HA significantly reduced *S.aureus* bacteria count from 14.12 ± 1.09 $\log_{10}$ CFU/ml to 4.61± 0.49 $\log_{10}$ CFU/ml ($p \leq 0.05$). Therefore, to investigate the structure of biofilm formation in vivo post-implantation, tissues immediately surrounding the implanted catheter was histologically assessed using haematoxylin and eosin (H&E) staining. Thus, this study showed that gentamicin-coated HA is effective in reducing biofilm viability without causing overt toxicity to human osteoblasts *in vitro* or inflammation when implanted in skin.

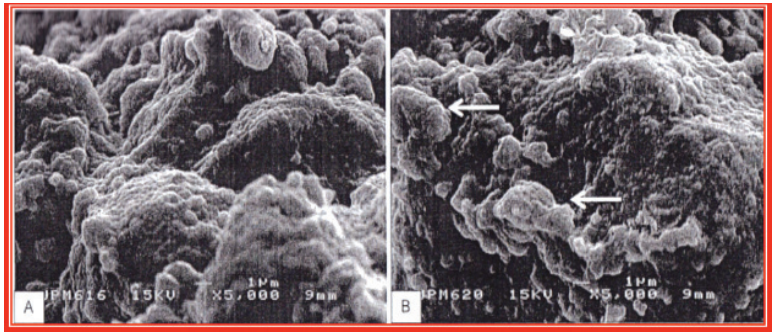


Figure 20 Scanning electron micrographs of gentamicin-coated HA with osteoblasts. (A) The osteoblasts attached onto the HA surface coated with 0.1m/ml gentamicin (5000x). (B) The osteoblasts shrinked and some cells sloughed off (arrows) from the HA surface coated with 10.0 mg/ml gentamicin (5000x)

Tobramycin and Gentamicin-Incorporated Calcium Phosphate Delivery System in Preventing Biofilm Formation

A biofilm is a thick community of bacteria that is attached to a substratum, interface or to each other and embedded in a matrix of

extracellular polymeric substances (EPS). In this present study, the live event for the development of *S. aureus* biofilm was viewed under live cell imaging system and the morphology of biofilm was viewed under scanning electron microscopy (SEM). These microscopic studies of *S. aureus* biofilm are useful for morphological identifiers for classifying bacteria biofilms. In order to prevent biofilm infection localised to bone and bone tissue (osteomyelitis), calcium phosphate was incorporated with either tobramycin or gentamicin to form 2 types of antibiotic beads. In this study, 3(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess the efficacy of tobramycin and gentamicin-incorporated calcium phosphate against *S. aureus* biofilm. There was a significant different between tobramycin-incorporated calcium phosphate and gentamicin-incorporated calcium phosphate on cell viability of *S. aureus* biofilm ($p<0.05$). Ninhydrin assay was used to investigate the elution of tobramycin and gentamicin from the calcium phosphate carrier. Gentamicin was released from calcium phosphate higher than tobramycin. Tobramycin-incorporated calcium phosphate was more cytotoxic on osteoblast than gentamicin-incorporated calcium phosphate. Moreover, investigation on the cell morphology and cell adherence by using SEM and CLSM showed that seeded cells were well attached to the tobramycin and gentamicin-incorporated calcium phosphate and continue to grow throughout the 5-days period (Figure 21 and Figure 22). In conclusion, tobramycin and gentamicin-incorporated calcium phosphate have the potential to be used as a new local drug delivery system in the prevention and treatment of bone infections. Furthermore, tobramycin and gentamicin-incorporated calcium phosphate scaffold could serve as a promising platform for the regeneration of osteoid tissues because of their slow release of antibiotic, biocompatibility and biodegradability.

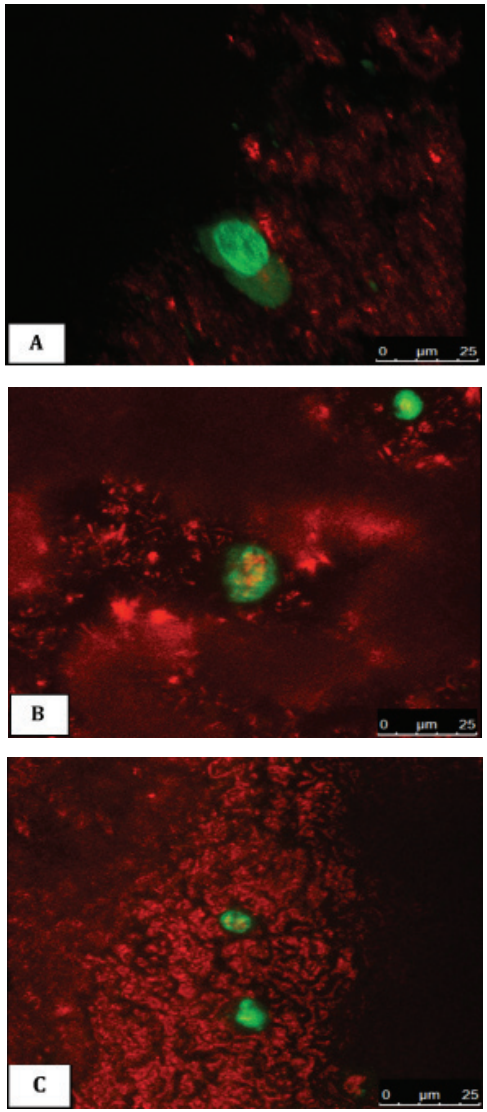


Figure 21 Confocal micrographs of human osteoblasts cultures on tobramycin-incorporated calcium phosphate at (A) day 1 , (B) day 3 and (C) day 5. Note the live osteoblast (green) attached on the surface of material (red) (630x)

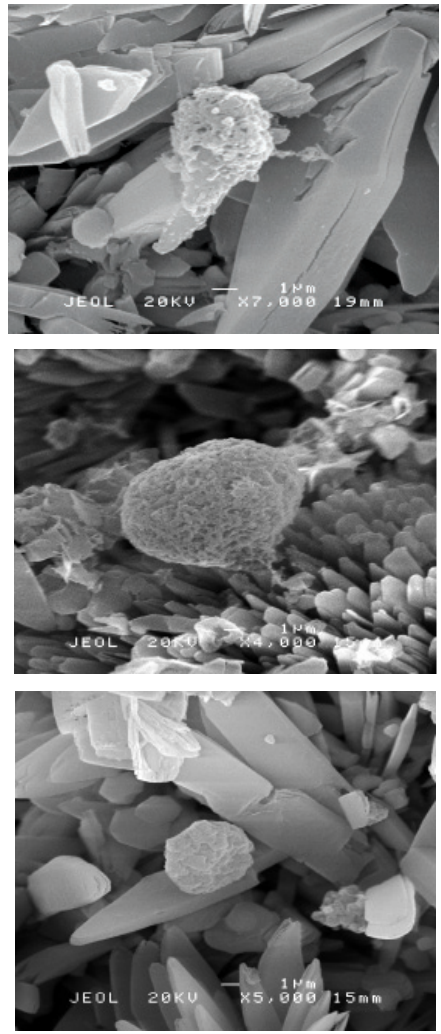


Figure 22 Scanning electron micrographs of human osteoblasts morphologies on tobramycin-incorporated calcium phosphate. A) Spherical cell began to attach on the long plate like-crystals surface at day 1 (4,000x). B) Spherical cell found were still attached to the intervene plate like-crystal at day 3 (5,000x). C) Osteoblast found to spread with pseudopodia (arrow) at day 5 (7,000x)

CONCLUSION

A major breakthrough or discovery is a finding or process, often preceded by numerous small advances, which lead to a new way of thinking about a problem. This new way of thinking is highly useful to numerous scientists in addressing problems in diverse fields of science. A major breakthrough in biomedical science is a radical or new idea, the development of a new methodology, or a new instrument or invention. It usually does not occur all at once, but involved a process of investigation taking place over a substantial period of time and required a great deal of local knowledge, if not both. High engagement in research activities by enthusiastic scientists need to be facilitated with the practical application of scientific discoveries to the development and implementation of new ways to prevent, diagnose, and treat disease also known as *translational medicine.* Before clinical trials in human can begin, pre-clinical research have to be conducted during which important feasibility, iterative testing and drug safety data are collected. Finally, small and large firms in learning how to integrate strategic entrepreneurship and collaborative innovation have to be well positioned so as they will lead small biomedical research to explore bigger horizon before being used commercially towards wealth creation.

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PATENTS

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Filed Year	:	2010-03-31
Application No.	:	PCT/MY2010/000044
Country Filing	:	PCT
Apllicant	:	Universiti Putra Malaysia; Malaysian Nuklear Agency (Nuklear Malaysia)

ABSTRACT

The hydroxyapatite (HA) biomaterial has been examined for its surface morphology. It is irregular shape chips of 2-3 mm. However, the pore size is between 150-350μm, which imcluded microspores and microspores, while the porosity is between 65-70%. When placed in contact with viable bone, new bone forms on and between the pores of HA. Therefore, the microspores have been covered when loaded in gentamycin solution through the images of gentamycin-loaded HA under scanning electron microscope (SEM). HA was biocompatible with osteoblast by testing with MTT assay. There was no significance of IC50 gentamycin at the percentage of viability cell was high. In the words, there was significance of IC50 of gentamycin which was 0.3mg/ml after treatment. The percentage of viability of biofilm dropped. The higher concentration of gentamycin had been loaded, the lower of viable biofilm percentage. Thus, the results indicated that the gentamycin-loaded HA had the ability to reduce or eradicate the biofilm formation which formed on the implant devices.

Technology	:	Drug delivery system
Inventor	:	Fauziah Othman, Asmah Rahmat, Idris Besar, Rusnah Mustaffa

TITLE	**:**	**An Anticancer Agent**
Filed Year	:	2009-12-09
Application No.	:	PCT/MY2009/000204
Country Filing	:	PCT
Apllicant	:	Universiti Putra Malaysia

ABSTRACT

The present invention relates to Cola nitida, an anticancer agent, the anticancer properties include antioxidant activities: FTC, TBA, and DPPH; antioxidant minerals: oxygen, carbon, potassium, phosphorus and magnesium; total phenolic and total flavonoid content.

Inventor : Fauziah Othman, Asmah Rahmat, Susi Endrini, Suherman Jakfa, Wan Nor Izzah Wan Mohamad Zain

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BIOGRAPHY

Professor Dr. Fauziah Othman, born in Kota Bharu, Kelantan was a student at Datin Khadijah Primary Girls School, Kuala Kangsar, Perak (1967-1972), Raja Muda Musa Secondary School, Kuala Kangsar, Perak (1973-1975) and Anglo Chinese Secondary School, Kampar, Perak (1975-1977). In 1985, she obtained her Doctor of Veterinary Medicine and in 1992, her Master of Science (Virology) from Universiti Putra Malaysia under the supervision of Professor Emeritus Dato' Dr. Abdul Latif Ibrahim and Professor Datin Paduka Dr. Aini Ideris . She got the opportunity to work under the supervision of Professor Dr. Peter Spradbrow at University of Queensland, Brisbane, Australia in the Department of Virology under ACIAR project in 1986. In 1989, she was awarded a British Council- Chiche Scholarship, which gave her an opportunity to work with Professor Emeritus Dr. Anthony King, an anatomist in the Department of Anatomy, Faculty of Veterinary Medicine, University of Liverpool, United Kingdom. She was awarded a PhD in histopathology from University of Glasgow, Scotland, United Kingdom in 1996 under the supervision of Professor Sally Solomon and Dr. Michael Purton. In 2007, she did her sabbatical training in University of Monash, Melbourne, Australia particularly in live cell imaging and anatomy teaching in the Department of Anatomy, Faculty of Medicine.

Prof. Fauziah has been involved in research and teaching of Anatomy, Medical Biotechnology and Cancer Research. She was appointed as an academician at the Department of Biomedical Science, Faculty of Biomedical and Health Science, Universiti Putra Malaysia in 1992 and was then appointed as an Associate Professor in 2001 and promoted to full Professor in 2006 in the Department of Human Anatomy, Faculty of Medicine and Health Sciences, UPM. She teaches courses in gross anatomy, micro anatomy, embryology,

microscopy and ultrastructure to medical, biomedical, nursing and veterinary both undergraduate and post graduate students. Her research interest includes virotheraphy and herbal theraphy in cancer and tissue engineering. She was the project leader for 14 projects under the MOSTI Priority Research Scheme, Ministry of Higher Education (FRGS), IRPA, MAKNA, E Science Fund, and RUGS. Ultimately, she also published 2 international patents with the title of An Anticancer Agent and A Drug Delivery System in Bone Tissue. During her 26 years at Universiti Putra Malaysia, she supervised 100 postgraduate and undergraduate research students. She has been appointed as Chairman, Internal and External Examiner for Examination Committee for PhD and Masters Thesesas well as External Examiner, for Bachelor of Medicine and Bachelor of Surgery, in Mansoura University, Egypt and International Medical University (IMU), Malaysia.

Prof Dr Fauziah Othman held many important administrative posts at the university and national NGO level such as, Head of Department of Human Anatomy, Faculty of Medicine and Health Science, UPM, Head of Immunotherapeutics and Vaccine Laboratory, Institute of Bioscience, and Head of Centre for Electron Microscopy and Imaging System, Institute of Bioscience, UPM where she was actively involved in the setting up, commissioning and running of this centre towards excellence in research. She is also the package 7 coordinator and reproductive module coordinator for medical programmes. She is actively involved in electron microscopy where she was the protem and first secretary for Electron Microscopy Society Malaysia (EMSM), and was appointed President from 2004 to 2008. She was also recognised internationally whereby she was also a member of the Executive Committee of the Asia-Pacific Electron Microscopy of the Asia Pacific Societies of Electron Microscopy (CAPSEM) and the

ASEAN Microscopy Society and Advisory Committee, Regional Biomaterials Scientific Meeting 2010 and Advisor for 20th scientific meeting for EMSM. She is currently the President for Tissue Engineering Society of Malaysia (TESMA), Advisor for Persatuan Seni Silat Cekak Malaysia-UPM (PSSCM-UPM), Exco Member of Biomaterial Society of Malaysia and was the President for PERMATA ladies Association of UPM, as well as held other positions such as advisor, vice president, secretary, treasurer, auditor and member of various societies locally and internationally.

Prof Dr Fauziah Othman has an excellent record in publications where she has published 271 papers, 116 in peer reviewed scientific journals, impact factor journals, 150 in proceedings, 4 in books, and 1 in monograph. She is well known internationally in her field of expertise and has been recognised and as either a plenary speaker or an invited speaker at International meetings such as in United States of America, Singapore, Australia, Thailand, Yemen, Iran, Indonesia, India and Egypt. She had been invited by Iranian Government in the International Collaborative Research Programme (ICRP) to enhance research and supervision of post graduate students between Malaysia and Iran. She was the Editor-In-Chief for Proceedings of Herbal Symposium, Pagoh, Johor, 2003. She is currently the Editor-In-Chief for Malaysian Journal of Microscopy (2005-to date) as well as the editor, and a member of the Editorial Committee for a few seminars, conferences, proceedings and newsletters.

Prof. Fauziah Othman contributes and excels in teaching, research, professional services, administration and leadership nationally and internationally. She has great potential and confidence for more challenging academic roles towards wealth creation nationally and globally.

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- **E-Science Fund**
  Combating Biofilm: Antibiotic Drug Delivery System in Bone Tissue (2006 –2009)

II. MINISTRY OF HIGHER EDUCATION: FRGS, MALAYSIA

- Effect of Ethanol Neem Extract on Cervical Cancer-Induced Balb/C Mice Model(2010 – to date)
- Anticancer Study and Mechanism of Cola Nut (Cola Nitida) Extract. (2007– 2009)

III. UPM RESEARCH

- Tobramycin-incorporated Biomaterial Delivery System in Combating Biofilm (2008 – to date)
- Confocal Microscopy of Breast Cancer Cell Lines Inoculated with Newcastle Disease Virus (1999-2000)
- Elemental Analysis of *Strobilanthus crispus* during Hepatocarcinogenesis (2000-2001)
- An ultrastructural Study of Apoptosis in Human Breast Cancer Cell Induced by chicken anemia Virus (VP3). (2000-2001)
- Ultrastructure and elemental analysis of breast cancer (1998)
- Respiratory epithelium, production performance and behavior of formaldehyde-exposed broiler chicks in Malaysian hatchery (1997-1998)
- The effects of formaldehyde vapour on the morphology of the respiratory epithelium of the pre and post-hatched chick. (1998)

IV. MAJLIS KANSER NASIONAL (MAKNA)

- Newcastle Disease virus as an anticancer agent. (2001 – 2011)

IV. INTENSIFICATION OF RESEARCH IN PRIORITY AREA (IRPA). MOSTI, MALAYSIA

- Tissue engineering for future clinical application. (2003 – 2008)
- Neem leaf extract as an anticancer agent in breast cancer (2002- 2007)
- Molecular Study of Apoptosis in Human Breast Cancer Cell Induced by Chicken Anaemia Virus (VP3). (2000-2001).

LIST OF INAUGURAL LECTURES

1. Prof. Dr. Sulaiman M. Yassin
   The Challenge to Communication Research in Extension
   22 July 1989

2. Prof. Ir. Abang Abdullah Abang Ali
   Indigenous Materials and Technology for Low Cost Housing
   30 August 1990

3. Prof. Dr. Abdul Rahman Abdul Razak
   Plant Parasitic Nematodes, Lesser Known Pests of Agricultural Crops
   30 January 1993

4. Prof. Dr. Mohamed Suleiman
   Numerical Solution of Ordinary Differential Equations: A Historical Perspective
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   Changing Roles of Agricultural Economics
   5 March 1994

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   Marketing Management: Prospects and Challenges for Agriculture
   6 April 1994

7. Prof. Dr. Mohamed Mahyuddin Mohd. Dahan
   The Changing Demand for Livestock Products
   20 April 1994

8. Prof. Dr. Ruth Kiew
   Plant Taxonomy, Biodiversity and Conservation
   11 May 1994

9. Prof. Ir. Dr. Mohd. Zohadie Bardaie
   Engineering Technological Developments Propelling Agriculture into the 21st Century
   28 May 1994

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    Rock, Mineral and Soil
    18 June 1994

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29 June 1994

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23 July 1994

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6 Ogos 1994

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