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Suppression effect of eurycomanone on the growth of HepG2 tumour transplanted in mice

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Eurycoma longifolia Jack, from the family of Simaroubaceae is commonly distributed in South East Asia including Malaysia. Locally it is known as Tongkat Ali. The plant has achieved a considerable attention for its medicinal properties and traditional claims as treatment of ailments including reduces fever, the concoction is used as tonic for wellness, sexual insufficiency and anti ulcer. A lot of research has been carried out on the plant materials for their scientific evidence as claimed. Eurycomanone is a cytotoxic compound found in *Eurycoma longifolia* Jack. Previous studies had noted the cytotoxic effect of eurycomanone against various cancer cell lines. The aim of the study is to investigate the effects of both *Eurycoma longifolia* (crude extract) and eurycomanone, on the growth of xenotransplanted hepatoma cell, HepG2 in nude mice. Nude mice were inoculated with HepG2 cells, subcutaneously in the right flank. When the tumour volume reached 100 mm^3, eurycomanone (6 mg/kg and 17 mg/kg) was applied intraperitoneally once a day, until 30 days. Crude extract (CE) of *Eurycoma longifolia* also administered to the mice bearing tumour to compare the effectiveness between CE and eurycomanone. Tumour size in mice-treated eurycomanone at concentration of 17 mg/kg significantly reduced, compare to control and CE. 39.9% of relative tumour growth ratio value was calculated with relative tumour volume of 1.5±0.09. Half of the tumour growth was suppressed with 50% inhibition rate was calculated. Growth reduction was associated with significantly reduced mitotic index. Eurycomanone has a great effect in suppressing the growth of HepG2 tumour transplanted in nude mice. Thus it is promising for treatment of hepatocellular carcinoma.

Keywords: eurycomanone, hepatoma, liver cancer, xenotransplant

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Introduction

Liver cancer is the fifth most common cancer and third most common cause of death in the world (Parkin, 2000). In developing countries, the survival rate is low. Therefore, high prevalence, high death rate, and ineffective therapy have spurred the search of novel strategies in the prevention rather than treatment of liver cancer (Jia et al., 2005). The present trends in smoking prevalence and the adoption of unhealthy lifestyles, the number of new cases is expected to reach 15 million by 2020. To date, many anticancer drugs have been developed and applied by the clinical doctors. Recently, resistance to anticancer drugs was discovered. Therefore, research and development of new and safe drugs by the pharmaceutical industry have become necessary. *Eurycoma longifolia,* a plant in the family of Simaroubaceae, is one of the most well known folk medicines for antipyretic, antimalarial and restorative activities in Southeast Asia (Perry, 1980) and is known to be a promising natural source of biologically active compounds (Okano et al., 1990). Quassinoids from *Eurycoma longifolia* have been reported to exhibit a wide range of activities in animals, both *in vivo* and *in vitro* (Chan et al., 1986; Okano et al., 1990; Ang, 1992). Eurycomanone was a major compound in the roots of *Eurycoma longifolia.* It is a type of quassinoids which found to have antiulcer activities (Tada et. al 1991) and had cytotoxicity activity against cancer cells (Morite et al., 1993; Kardono et al., 1991). Toxicity–guided chromatographic fractionation of the n-butanol fraction identified eurycomanone as the most toxic component (Chan and Choo, 2002). Previous studies also reported that Eurycomanone have a cytotoxic effects on colon cancer, breast cancer, lung cancer, skin cancer (melanoma), fibrosarcoma and lung cancer (Tada et al., 1991). In order to confirm the potential of eurycomanone as an alternative drug for liver cancer, this experiment towards animal models were carried out.

Materials and Methods

Samples preparation Eurycomanone was dissolved in 1% DMSO (Sigma-Aldrich) and further diluted with distilled water at concentration of 6 mg/kg and 17 mg/kg body weight (BW) of nude mice. *Eurycoma longifolia* crude extract was prepared at concentrations of 50, 100, and 150 mg/kg BW in distilled water.

Cell culturing and preparation for transplantation The human hepatoma cell line, HepG2 from American Type Culture Collection (ATCC) was cultured in complete DMEM medium in an atmosphere containing 5% carbon dioxide (CO_2). Prior to injection, HepG2 cells were harvested using 0.25% trypsin-EDTA (Sigma), washed three times in PBS. Cell viability was determined by trypan blue exclusion and cultures with more than 90% viability were used for *in vivo* experiments.

Human Tumour xenotransplant All animal experiments were performed according to the guidelines and with approval of the IMR animal care committee. The prepared HepG2 cells were inoculated by subcutaneous flank into nude mice using 28-gauge needle. Before injection, the flank of athymic nude mice was prepped with 70% alcohol. This method was based on research carried out by Randy and Robert (2001), with some modifications. When Tumours were palpable, which corresponded to a mice bearing solid Tumour that had grown to 90-100 mm^3 in volume, mice were randomly distributed into one control group which received the vehicle alone and five treatment groups. The first treatment group received 6 mg/kg BW of eurycomanone and the second group received 17 mg/kg BW of eurycomanone. The rest of the treatment groups received *Eurycoma longifolia* crude extract. One group of clean nude mice was also used as a control. The compound or extract was administered by intraperitoneal injection using 28-gauge needle once a day for 30 days. After 30 days of treatment, mice were killed by euthanized with overdose of ether. The tumour was surgically removed. The final Tumour size or volume and weights were registered.

Body weight and Tumour measurement Body weights, tumour growth and secondary tumour incidences were recorded every 3 day intervals throughout the experimental period. Tumour volume was calculated using the formula, $V=width^2 \times length \times 0.52$ (Randy and Robert, 2001). Relative tumour volume (RTV) was calculated as follow; RTV=Vt/V0: Vt=tumour volume at day n and V_0=initial tumour volume (day 0).

Histology study Tumours were fixed in 10% neutral buffered formalin and embedded in paraffin. Four micrometre Tumour sections adhered to poly L-lysine coated slides were sent to Haematology Department at IMR for Hematoxylin and Eosin (H&E) staining. Mitotic index was calculated by microscopic examination in 5 fields of view. The percentage of mitotic figures was evaluated by counting the number of cells at the mitotic stage within a view and divided by the number of cells per view. This was done for 5 random views (van Diest et al., 1982).

Results and Discussions

Human carcinoma cell lines, HepG2 was implanted into the right flank of nude mice by subcutaneous injection of 2.5×10^6 viable cells. Mice were observed everyday and at day 7, which is one week after cells injection, solid Tumour started to appear at the back of nude mice. At day 14, Tumour volume reached at 100 mm^3 and the treatment was started. The treatment was stopped at day 30 and solid Tumour was isolated from the nude mice. Tumour volume was seen to be more vigorous than mice treated with crude extract and, eurycomanone. In mice treated with eurycomanone, the palpable Tumour size was seen to be suppressed. Tumour size in eurycomanone-treated mice significantly reduced at concentration of 17 mg/kg. However, treatment with 6 mg/kg of eurycomanone, was proved to be less effective than 17 mg/kg. Tumour size treated with crude extract of *Eurycoma longifolia* (CE) at concentration of 50 mg/kg did not show a significant reduction when compared to control. As for concentration 150 mg/kg, a slight reduction of Tumour size was observed. At concentration of 100 mg/kg, crude extract of *Eurycoma longifolia* showed an effect in inhibition of Tumour development compared to the other concentrations used.

Tumour weight was also recorded at the end of experiment. Mice injected with crude extract at the dose of 150 mg/kg (0.62±0.052 g) and 50 mg/kg (0.547±0.045 g) showed a slight decrease at Tumour weight, compared with control group or untreated mice (0.76±0.127 g). Surprisingly, at the dose of 100 mg/kg of crude extract, Tumour weight was reduced more than the highest concentration of crude extract used in this experiment, with 0.46±0.06 g was recorded. For the mice treated with eurycomanone, great decrease of Tumour weight was observed for the mice treated with 17 mg/kg, with 3.6 fold (0.21±0.046 g) of decrease compared to control. However, eurycomanone at the dose of 6 mg/kg did not show a decrease in Tumour weight (0.55±0.10 g) as great as 17 mg/kg. It was showed that, eurycomanone at the lowest concentration did not show a good suppression of tumour growth *in vivo*.

The inhibitory effect of eurycomanone towards liver cancer in xenotransplanted nude mice, compared with crude extract is shown in Figure 1. The effect was determined by accessing Tumour volume. Results of this study demonstrated that of all drugs tested, 17 mg/kg of eurycomanone showed the greatest inhibitory effect in Tumour suppression. The Tumour growth rates and the Tumour weight were reduced in the eurycomanone-treated groups at the time of death. For the crude extract, at the higher concentration used, 150 mg/kg BW, did not show as good inhibitory effect as the lower concentration, 100 mg/kg. As for mice treated with 50 mg/kg of crude extract, the Tumour volume curve was almost similar with mice treated with 150 mg/kg crude extract in the beginning of experiment but, however the Tumour volume was raised towards the end of the experiment. At the end of experiment, the Tumour volume for both concentration of CE (50 mg/kg and 150 mg/kg), shared the same value. As previously mentioned, the body weights of mice were measured every 3 days for weight loss, as an indication of toxicity. However, no significant changes in body weight were observed in mice bearing Tumour for all groups of treatment (Figure 2). Animals generally gained weight as they aged towards the end of treatment duration thus proving the 17 mg/kg to be non-toxic. None of the mice were died in the end of the experiment including mice in untreated group.

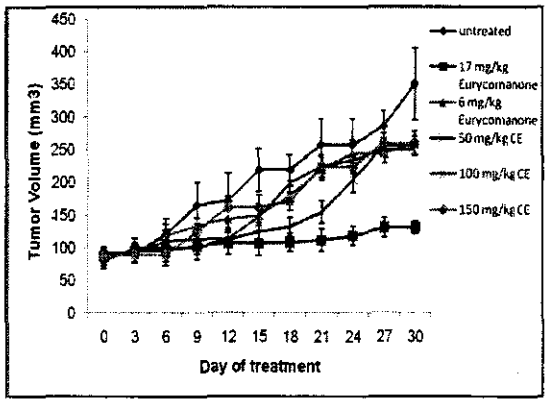


Figure 1 The effect of eurycomanone and CE against tumour volume of nude mice

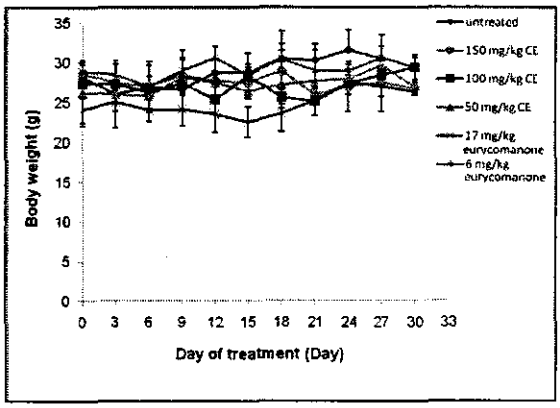
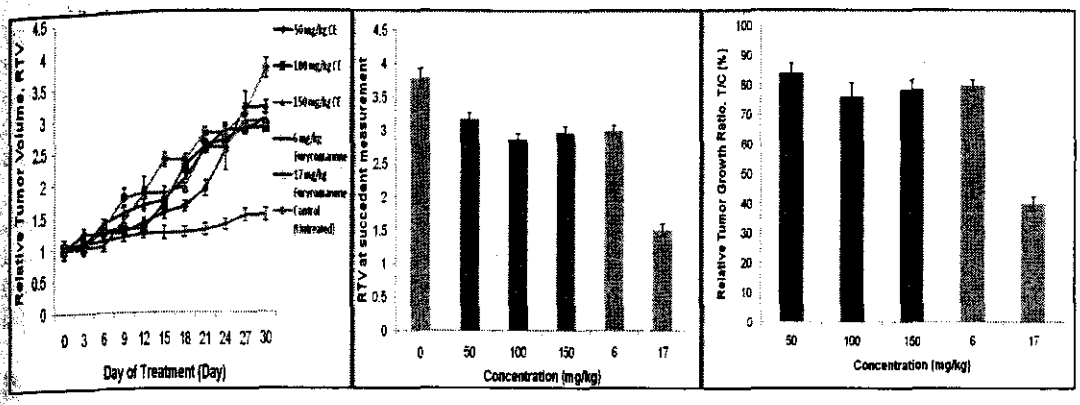


Figure 2 Animal body weight curve

In order to evaluate the potential of eurycomanone in tumour growth inhibition, the efficacy and the index of anti-tumour activity of eurycomanone, in comparison with crude extract are presented in Figure 3 and Figure 4 respectively. The RTV, was used as a value to show the efficacy of eurycomanone in inhibition of tumour growth. The results can therefore be expressed by TC (%). The results obtained confirmed that eurycomanone at concentration of 17 mg/kg highly inhibited the growth of subcutaneous xenograft of human hepatoma in nude mice with RTV of succeeding measurement of 1.5±0.09 and 39.9% of T/C. Evaluation as effective was based on T/C (%) of 50% or less (Inaba et al. 1989). But, at concentration of 6 mg/kg, eurycomanone was unable to suppress cancer cell proliferation efficiently. The value of RTV observed was 3.0±0.06 and the percentage of T/C was 79.57%. The inhibitory effect of crude extract at concentration of 150 mg/kg (78.5%) and 50 mg/kg (83.87%) were not significantly different from 6 mg/kg of eurycomanone. However, there was a slight inhibition of tumour growth in mice treated with crude extract at concentration of 100 mg/kg with 75.9% of T/C calculated and RTV value of 2.7±0.09 (Figure 5). Histopathological examination of tumours (Figure 6) depicts the effect of eurycomanone in reducing mitosis in tumour in order to inhibit tumour development. The number of mitoses in tumour treated with eurycomanone was reduced, compared to the untreated tumour. Significant decrease in mitotic index was noticed and evaluated as percentage of mitotic cells which was found to be 5.98±0.19 at concentration of 6 mg/kg and 3.35±0.24 at concentration of 17 mg/kg in eurycomanone treated group in comparison to 9.85±1.56 of untreated control group (Table 1). A decrease in mitotic index was also observed in CE treated group. At concentration of 100 mg/kg, CE resulted in significant decrease in mitotic index by 4.08±1.0. However, at the highest concentration used (150 mg/kg), the mitotic index was 6.15±1.12 while at the lowest concentration of CE (50 mg/kg), the mitotic index recorded was 6.35±0.89. Eurycomanone at concentration of 17 mg/kg was greatly arrested cells from underwent mitosis process. These findings suggested that treatment of tumour-bearing mice with CE and eurycomanone caused mitotic arrest.



(a) (b)

Figure 3 Relative tumour volume. (a) RTV (b) The efficacy of eurycomanone and CE in tumour suppression was shown by the calculation of RTV at the succeeding measurement, day 30.

Figure 4 Percentage of relative tumour growth ratio of eurycomanone (yellow) and CE (blue) towards nude mice bearing tumor, HepG2.

Table 1 Mitotic index of CE and eurycomanone treatment on subcutaneous xenotransplanted tumour, HepG2 in nude mice

Groups (treatment)	Mitotic Inde (%)
Untreated (control)	9.85±1.56*
E. longifolia crude extract (CE)	
▪ 50 mg/kg	6.35±0.89**
▪ 100 mg/kg	4.08±1.04*
▪ 150 mg/kg	6.15±1.12*
Eurycomanone	
▪ 6 mg/kg	5.98±0.19**
▪ 17 mg/kg	3.35±0.24*

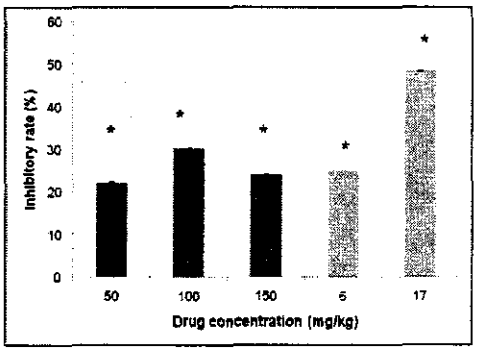


Figure 5 Inhibitory effects of eurycomanone (yellow) and CE (blue) on implantation tumour HepG2 in nude mice.

Tumour xenografts in several animal models, such as the one used in the present study (i.e. the immunodepressed nude mice lacking thymus and hence T cells), have been used in many relevant investigations aimed at gaining insight into the clinical activity of new anticancer drugs because the implanted cells retain the characteristics of the parental ones (Maria et al., 2001; Moss, 1998). Our *in vivo* study indicated that eurycomanone significantly inhibited the growth of hepatoma HepG2 cells in nude mice. The proliferation ratio of HepG2 was reduced and the inhibitory ratio was increased. It is reported that eurycomanone had significant anti-tumour activities and it was different from common chemotherapeutical drugs. Based on cytotoxicity study, eurycomanone can inhibit liver cancer cells more selectively. So, it provides prospects in finding new anti-tumour drugs. However, there was a little evidence about the anti-tumour action and mechanism of eurycomanone in animal study especially towards liver cancer.

Anti-tumour action refers to the ability of a compound to elicit regression of established tumours or delay progression of cancers in an experimental model (Moss, 1998). The current study demonstrates that eurycomanone inhibition also significantly reduced the growth of xenotransplant HepG2 *in vivo*. Growth reduction was associated with significantly reduced mitogenenis. These results suggest eurycomanone as a potential preventive as well as therapeutic agent against human HCC. However, no previous studies have reported about the activity of eurycomanone *in vivo*. Moreover, a few research of eurycomanone as anti-cancer agent *in vitro* has been published. According to Nurkhasanah and Azimahtol, (2008) eurycomanone was cytotoxic on cancerous cells (CaOv-3, HeLa, HepG2, HM3KO, MCF-7) *in vitro*.

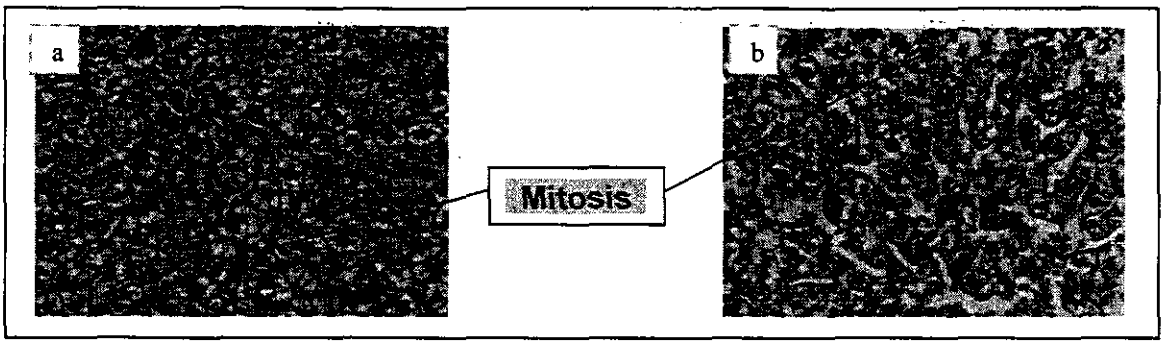


Figure 6 Histological study of isolated tumour from nude mice. The number of mitoses in the tumour with eurycomanone (b) was reduced compared to that of the PBS control (a). Magnification x 100.

Researchers in America and Japan reported that some plant chemicals in the group of quassinoids and alkaloids found in *Eurycoma longifolia* have the effect of inhibiting the growth of cancer cells (breast cancer cells, colon cancer cells, leukemia) in animal laboratory experiments (Kinghorn et al., 1999). However, our present study is the first report of the anti-hepatoma effect of eurycomanone *in vivo*. Although there was no study report on eurycomanone *in vivo*, but a lot of researches have been done *in vitro* against several cancer cell lines. Besides, other than eurycomanone, a few natural compounds are confirmed to have anticancer or hepatoprotective effect towards HepG2 cells. Curcumin which has been mentioned in previous chapter, in a high dose might be an effective anti-angiogenic drug in the treatment against tumour (Yoysungnoen et al., 2005). Niuchangchih or also known as Ganoderma, is a fungus that only grows on the brown heartwood of *Cinnamomum kanehirae* Hayata (Lauraceae) in Taiwan (Wu et al., 1997). *In vivo* study of this plant showed that Niuchangchih exhibited the activities of preventing and ameliorating liver diseases, such as preventing ethanol and CCl4-induced liver injury, inhibiting the hepatitis B virus, ameliorating fatty liver and liver fibrosis, and inhibiting liver cancer cell growth (Wu et al., 1997). Similar with eurycomanone, nowadays, in Malaysia, Ganoderma is popular as a nutrient supporting health.

A recent study also indicated that the consumption of rooibos (*Aspalathus linearis*) and honeybush herbal tea (*Cyclopia intermedia*), enhanced the activity of phase II detoxifying enzymes, as well as altering the oxidative status in the liver of rats (Marnewick et al., 2005). The aqueous extracts of both plants are likely to alter the carcinogenic potency of hepatocarcinogens *in vivo* (Marnewick at al., 2009). Another compound which has shown *in vivo* hepatoprotective is tocotrienol. It is a natural vitamin E and has been reported to have higher biological activities (He et al., 1997). Wada and his colleagues (2005), demonstrated that oral administration of tocotrienol mixture could prevent liver carcinogenesis *in vivo*. Their results suggested that tocotrienol could be promising agent to the cancer prevention in liver. Eurycomanone, as presently show a suppression effect on the growth of subcutaneous HepG2 may also potential to be a future hepatoprotective drug, but further study on this field need to be done. Much progress has been made in the understanding of the pathogenesis of liver diseases, resulting in improved prevention and therapy with promising prospects for even more effective treatments. In view of the severe undesirable side effects of synthetic agents, there is a growing focus on systematic research methodology and evaluating the scientific basis of traditional herbal medicines that claim to possess hepatoprotective activity (Shahani, 1999; Achliya et al., 2004).

Currently, the method of inoculated athymic nude mice with a human cancer cell lines subcutaneously, was also established. Many researches on cancer study were done using this approach. A quite similar experimental design with our studies was done by Cho and his colleagues, (2008). However, they used nude mice xenograft model in the study of sulforaphane (SFN) against squamous carcinoma. Human squamous carcinoma cells, KB cells, were also subcutaneously injected into the flank of nude mice. Subcutaneous implantation route allows access to the blood compartment (Radinsky, 1995; Furukawa et al., 1993). They found that SFN inhibited tumour growth in the xenograft model appeared to occur without any detrimental side effects (Cho et al., 2008). As compared to our present studies, eurycomanone was also suppressed tumour growth in nude mice without affected their body weights, and no animal was recorded dying during a period of experiment. Interestingly, Loisel et al., (2005) compared a method of subcutaneous and intravenous inoculated of leukemia cell lines in nude mice. Based on their results, intravenous route lead to a systemic disease in comparison with subcutaneous route. This result was also supported by other studies conducted by Hummel et al., (1996) and Hernandez et al., (2003). Our present data, and in agreement with other researches, suggest that the nude mouse model of human cancer cell line appears to be very useful for evaluating the efficacy of new therapeutic agents.

In spite of many advances in cancer treatment, chemotherapy for solid tumour is still greatly limited by a lack of selective anticancer drugs and by the recurrences of drug-resistant tumours. Finding a source of novel chemotherapeutics continues to be a focus of effort. Eurycomanone could be potentially useful as an anticancer chemotherapeutic drug from our data in *in vivo* studies. However, the exact mechanism of eurycomanone is still unclear.

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