

Full Length Research Paper

The antiviral activity of compounds isolated from Kenyan *Carissa edulis* (Forssk.) Vahl

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Extracts of a Kenyan medicinal plant, *Carissa edulis* (Forssk.) Vahl, were subjected to bioactivity guided phytochemical analysis for identification of biological makers of activity. Pure compounds; lupeol, oleuropein, carissol and β -amyryn were subsequently isolated. *In vitro* evaluations of the compounds against viral strains of *Herpes simplex virus* types 1 revealed a significant activity for lupeol at EC₅₀ of between 2.98 - 4.2 μ g/ml for both sensitive and resistant strains with a high therapeutic index (TI > 38). A concentration of the compound at 10.0 μ g/ml was virucidal, reducing viral yields in Vero E6 cells by 98.3%. On oral administration to mice at 20.0 μ g/ml following a cutaneous viral infection, a delayed onset of infections of slow progression to mild and sever zosteriform lesions were observed ($p \leq 0.05$ test vs. control by repeated measures ANOVA). The mice receiving treatment with the compound also exhibited increased mean survival times as opposed to control ($p \leq 0.05$ test verses control by Student's t-test) with a therapeutic index >5 (LD₅₀ >100.0 μ g/ml). The results indicate promising antiviral activity of lupeol and necessitate further examinations of efficacy and safety in higher mammals.

Key words: Antiviral activity, lupeol, *Carissa edulis*, *Herpes simplex virus*.

INTRODUCTION

Herpes simplex virus (HSV) infections are among the most common viral sexually transmitted diseases (STD) worldwide (Smith and Robinson, 2002; Xu et al., 2006). In 1999, the estimate of the number of sufferers to these infections worldwide was approximated as 86 million people (Halioua and Malkkin, 1999) and since then, the prevalence of HSV infection has been increasing (Smith and Robinson, 2002). In sub-Saharan Africa high seroprevalence rates of between 60 - 80% in young adults have been recorded in population based studies (Wagner et al., 1994; Gwanzura et al., 1998; Obasi et al., 1999; WHO, 2008).

The most widely used drug for prophylaxis and

treatment of HSV infections is acyclovir (Kleymann, 2003). However, the long-term therapy necessary for management of infection has been associated with development of clinically resistant strains of the virus (Morfin and Thouvenot, 2003; Reyes et al., 2003). There is therefore a need to identify new agents for management of HSV infections.

Historically, plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made large contributions to human health and well-being. Artemisinin (Quinghaosu) from *Artemisia annua* is such one example (Hoareau and DaSilva, 1999). In the search for new remedies to treat old and emergent diseases, attention is therefore being given to discovering the active ingredients from plants that have been used routinely and traditionally for disease alleviation. The Kenyan *Carissa edulis* is one such plant. Extracts of the plant had previously shown

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significant *in vitro* and *in vivo* antiviral activity for HSV types 1 and 2 (Tolo et al., 2006). This report is a follow up of the extracts and results are presented of the antiviral activity of the pure compounds isolated from them.

MATERIALS AND METHODS

Plant collection

The *C. edulis* plant was collected from its natural habitat in Gitoro forest of Meru, Eastern Province, Kenya. The roots were collected with acceptable bio-conservation methods. A voucher specimen (Mungai, Rukunga and Tolo; No. 076) was confirmed by a plant taxonomist (Mr. Geoffrey Mungai) and is on deposit at the East African Herbarium, National Museums of Kenya, Nairobi, Kenya.

Extraction and activity guided phytochemical analysis

Extraction and activity guided phytochemical analysis was carried out concurrently. Powdered root bark (150 g) was extracted successively in pure diethylether followed by pure methanol and the resulting extracts dried *in vacuo*. The yields for the diethylether extract was loaded into a column packed with silica gel and eluted gradient wise with increasing concentrations of ethylacetate in hexane (2:1). The fractions of 10 ml each were collected and monitored on pre-coated thin layer chromatography (TLC) plates. Similar fractions were pooled according to the TLC characteristics. One such pooled fraction, coded CEE-3, had reasonable antiviral activity *in vitro* than the others and was a mixture of three compounds.

Further purification of this fraction was carried out using column chromatography packed with reverse phase (RP)-C18 Silica gel and eluted with increasing concentrations of acetonitrile in methanol, starting from 5 - 10% acetonitrile. Fractions were monitored on RP-C18 TLC analytical plates and fractions pooled as appropriate. Three pure compounds coded; CEE-3A, CEE-3B and CEE-3C were subsequently obtained.

The methanol extract was loaded into a column packed with sephadex LH-20 and eluted isocratically using pure methanol (MeOH). Ten milliliter (10 ml) fractions were obtained and monitored using TLC. Following bioactivity, one of the fractions was purified using a column packed with RP-C18 and eluted with MeOH : H₂O (35:65). One pure compound coded as GEM-1E was isolated.

These four compounds; CEE-3A, CEE-3B, CEE-3C and GEM-1E were identified by comparison of their spectroscopic profiles with published data and found to be lupeol (Prakash et al., 1968), β -amyryn (Zafar et al., 1985), carissol (Zafar et al., 1985) and oleuropein (Kuwajima et al., 1992; Calis et al., 1993) respectively.

Viruses and cells

The Vero E6 cells were donated by the Virology department, Toyama Medical and Pharmaceutical University, Toyama, Japan. The cells were grown in 5% CO₂ at 37°C in MEM supplemented with 5% FBS for cell growth, and 2% FBS for cell maintenance.

The *Herpes simplex virus* (HSV) strains used in the study were wild-type 7401H HSV-1 (Kurokawa et al., 1993), thymidine kinase deficient (TK⁻) B2006 HSV-1 (Dubbs and Kit, 1964) and acyclovir resistant (AP^r) 7401H HSV-1 (Kurokawa et al., 1995). The viruses were donated by the Virology Department, Toyama Medical and Pharmaceutical University, Toyama, Japan. The virus stocks were prepared from infected Vero E6 cells. The infected cultures were frozen and thawed three times to lyse the cells, and centrifuged at

3000 rpm for 15 min. Their supernatants, containing HSV, were harvested and stored at -80°C until use.

Plaque inhibition assay

The compounds were examined for extent of inhibition of plaques on HSV infected cells as a measure of anti-viral activity *in vitro* using the method described by Kurokawa et al. (1993, 2001). Vero E6 cells were cultured to a confluent monolayer in MEM supplemented with 5% fetal bovine serum (FBS) in 5% CO₂ at 37°C. The cells were infected with 100 plaque forming units (PFU) of HSV and left to adsorb for 1 h at room temperature. The cells were then overlaid with MEM supplemented with 2% FBS and 0.8% Methylcellulose (MC) containing various concentrations of the compounds and incubated at 37°C in 5% CO₂ for 2 days. The infected treated cells were fixed in formalin, washed and stained with 0.03% methylene blue solution. The plaques, appearing as transparent dots against a blue background, were counted using a dissecting microscope and the percent plaque inhibition calculated against control. The effective concentration inhibiting formation of plaques by 50% (EC₅₀) was determined from two independent experiments.

Cell cytotoxicity assay

The cytotoxic concentration causing 50% cell lysis and death (CC₅₀) was determined for the compounds by the method described later in the paper (Tolo et al., 2006). Vero E6 cells were seeded at a concentration of 2.5×10^4 cells/well in 24-well plates and grown in 5% CO₂ at 37°C for 2 days. The culture medium was replaced by fresh medium containing compounds at various concentrations, and cells further grown for 24 h. The cells were treated with trypsin and the number of viable cells determined by the trypan blue exclusion method. The concentration of compound reducing cell viability by 50% (CC₅₀) was determined from a curve relating percent cell viability to the concentration of compounds.

Virus yield reduction assay

Lupeol was compared for its anti-viral activity with acyclovir on the growth of the wild type strain of HSV-1 in the virus yield reduction assay as described elsewhere (Tolo et al., 2006). Briefly, confluent monolayers of Vero E6 cells were infected with HSV-1 at multiplicity of infection of 5 (5 M.O.I). Separate dishes each containing infected cells were treated with lupeol at various concentrations (0 - 10 μ g/ml). The cells were incubated in 5% CO₂ at 37°C for 24 h and lysed. The lysed cultures were collected in 15 ml tubes and centrifuged at 3000 rpm for 10 min and supernatant harvested. A serial dilution of each supernatant was prepared in MEM and the virus titre determined by the plaque inhibition assay.

Determination of *in vivo* efficacy of lupeol in mice

Balb/C mice, reared at the Kenya Medical Research Institute's (KEMRI) animal facility, were used in the experiments. The animals were handled according to the guidelines laid down by the Animal Care and Use Committee of the KEMRI. The *in vivo* evaluation of the efficacy of lupeol was carried out using the method described by Kurokawa et al. (2001). Seven week old female Balb/C mice weighing approximately 20 g were acclimatized for one week in the experimental room. The animals were fed Mice cubes (Unga feeds, Kenya) and water *ad libitum*. The mid flank of each mouse was shaved using an electric hair trimmer (Wahl super taper, England) and hair completely removed by applying a chemical hair remover (Shiseido, Co., Ltd., Tokyo, Japan) on the shaved area. The mice

Table 1. *In vitro* antiviral activity of the compounds.

Compound	EC ₅₀ (µg/ml)*			CC ₅₀	S.I
	7401H HSV-1	AP ^r 7401H HSV-1	TK ⁻ B2006 HSV-1		
Lupeol	2.98	3.66	4.2	>160.0 ^a	>38
Oleuropein	4.83	>5.0 ^a	>5.0 ^a	>160.0 ^a	>32
Carissol	3.76	NE	NE	>10.0 ^a	>2
β -amyirin	>5.0 ^a	NE	NE	>10.0 ^a	ND
Acyclovir	0.45 ± 0.14	>10.0 ^a	>10.0 ^a	>100.0	>200

The letters in parenthesis in the table represent the following; * = Mean of two independent experiments, ^a = Highest tested concentration. The abbreviations represent the following; NE = Not examined ND = Not determined.

were randomly divided into treatment groups each comprising of 5 mice. The shaved mid flank of each mouse was scratched using a bundle of G27 needles and the scarified area infected with 1×10^6 plaque-forming unit (PFU) HSV-1. The groups were administered with the test drug or acyclovir or placebo orally three times a day for seven consecutive days. The development of skin lesions and mortality were continuously monitored every 8 h daily and scored as follows: 0-no lesion; 2-vesicles in local region; 4-erosion and/or ulceration in the local region; 6-mild zosteriform; 8-moderate zosteriform and 10-severe zosteriform and death. The mice were fed and observed for 30 days before the experiment was terminated.

Data analysis

In the *in vitro* assays, the mean number of plaques was used to calculate the percentage of plaques inhibited. A curve relating percent plaque inhibition against concentration (a dose response curve) was used to calculate the inhibitory concentration for 50% (EC₅₀). In the cytotoxicity experiments, the number of viable cells in the untreated control was used to calculate the percent of cells lysed in the treated cells. The cell cytotoxic concentration (CC₅₀) was determined from a plot relating percent cell viability against each tested concentration.

In the *in vivo* experiments, the student's t-test was used to evaluate the significance of differences between control and treated mice in mean survival times and mean times at which skin lesions were initially scored (2 (vesicles in local region) or 6 (zosteriform lesions) after infection. The repeated measure analysis of variance (ANOVA) with Dunn's procedure as a multiple comparison procedure was used to analyze the interactions between treatment and progression of infection in mean lesions scores from day 3 - 10 following HSV-1 infection. A p-value of ≤ 0.05 was defined as statistically significant.

The 1998 StatView computer software by SAS Institute Inc. USA was used for the statistical analysis.

RESULTS

In vitro antiviral activity of the compounds

The *in vitro* antiviral activities of the compounds against sensitive and resistant viral strains of HSV are presented as Table 1. Of the four compounds, lupeol exhibited the most potent antiviral activity; EC₅₀ at 2.98 µg/ml for 7401H HSV-1, 3.66 µg/ml for AP^r 7401H HSV-1 and 4.2 µg/ml for the TK⁻ B2006 HSV-1. The EC₅₀ concentrations were far below the toxic level (CC₅₀ >160.0 µg/ml) giving

lupeol a selectivity index of >38. The 7401H HSV-1 strain was sensitive to oleuropein and carissol; EC₅₀ at 4.83 and 3.76 µg/ml respectively, however, no activity was registered for oleuropein in both the acyclovir resistant viral strains at 5 µg/ml. β-amyirin did not have activity against the 7401H HSV-1 strain at the highest tested concentration of 5.0 µg/ml and was therefore not examined any further. Acyclovir, the reference drug, had activity for 7401H HSV-1; EC₅₀ at 0.45 µg/ml, but did not show any activity for the other strains at the highest tested concentration of 10.0 µg/ml.

Virus yields in Vero E6 cells treated with lupeol or acyclovir *in vitro*

The bar graph Figure 1 compares the virus yields of 7401H HSV-1 in Vero cells treated with varying concentrations of lupeol or acyclovir. Lupeol reduced the virus yields in a dose dependent manner, the highest concentration of 10.0 µg/ml reducing the yields to less than 1×10^5 PFU/ml (~ 98.3% virus yield reduction). At 2.5 µg/ml, lupeol reduced the virus yields by half the amount registered in untreated cells.

On the other hand, acyclovir reduced the viral yields more sharply, the 2.5 µg/ml concentration reducing the yields significantly (to less than 1×10^2 PFU/ml) and completely at 10.0 µg/ml. The mean virus yields at each tested concentration for both lupeol and acyclovir were statistically significant ($p \leq 0.05$ test verses control by Student's t-test).

Efficacy of lupeol in a cutaneous HSV-1 infection model

Table 2 presents the efficacy of lupeol on 7401H HSV-1 cutaneous infection in mice. Three doses (5, 10 and 20 mg/kg) were orally administered to mice over a period of 7 days and observations for onset of infection, progression, mean survival times and mortality recorded. Vesicles in local region (score 2) were observed on the 4th, 5th and 6th days for 5, 10 and 20 mg/kg treatments respectively. Vesicles appeared on controls by the 4th day while the group on acyclovir at 5 mg/kg on the 7th day.

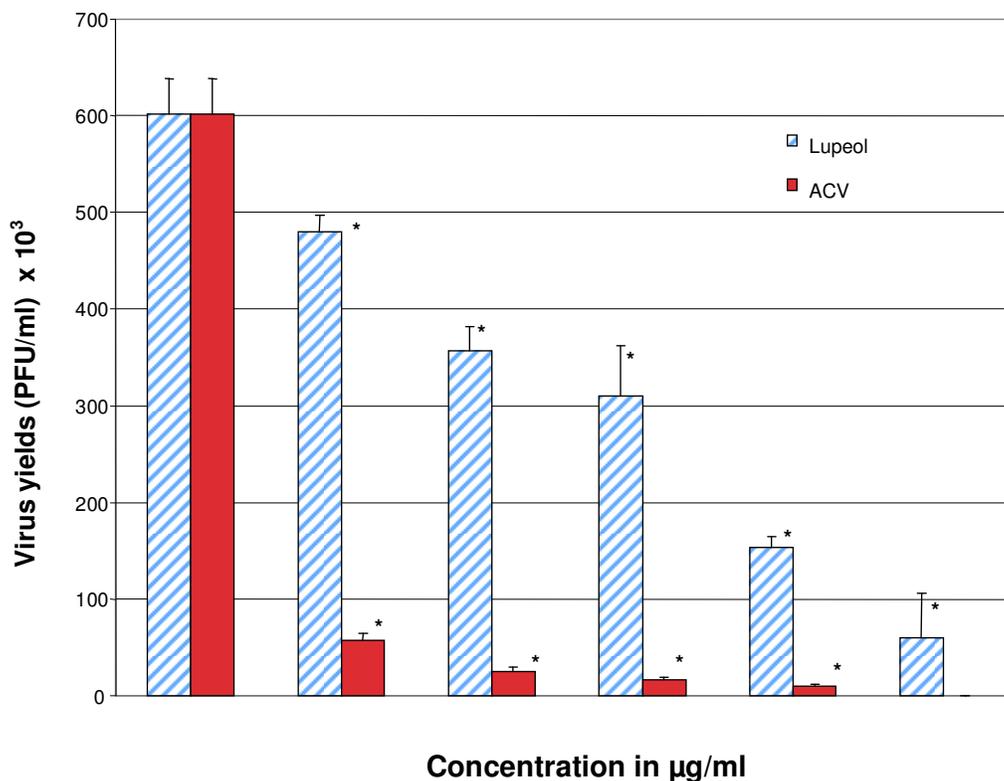


Figure 1. The virus yields in 7401H HSV-1 infected Vero E6 cells treated with lupeol or acyclovir *in vitro*. The error bars indicate the levels of deviation within the mean of three independent experiments at each tested concentration. * = $p \leq 0.05$ (Test versus control by student's t-test).

Table 2. The efficacy of lupeol in a cutaneous 7410H HSV-1 infection in mice.

Animals treatment	Mean time (Days \pm S.D)				Mortality ^c
	mg/kg	Score 2 ^a	Score 6 ^a	Survival ^b	
Control	0	4.2 \pm 0.45	6.6 \pm 0.89	8.0 \pm 0.71	5/5
Acyclovir	5	7.0 \pm 2.16	8.0 \pm 0.0	9.0 \pm 0.0	1/5
Lupeol	5	4.8 \pm 0.84	7.4 \pm 0.89	8.2 \pm 0.45	5/5
Lupeol	10	5.2 \pm 1.10	7.8 \pm 0.84	8.8 \pm 0.84	5/5
Lupeol	20	6.0 \pm 1.23	8.0 \pm 0.82*	9.5 \pm 1.00*	4/5

The letters and symbols in parenthesis represent the following; * = $p \leq 0.05$ (Test Vs control by Student's t-test) ^a = Mean times at which score 2 or 6 was first observed, ^b = Surviving mice were not included for the calculation of mean survival times and ^c = Number of dead mice against total number surviving in the group. Mortality was calculated on day 10.

Mild zosteriform lesions (score 6) were observed on 7th day for groups receiving treatment at 5 and 10 mg/kg while for the 20 mg/kg and acyclovir groups, lesions were noted on 8th day. The mean survival times for lupeol treatments at 5 and 10 mg/kg were similar to the control group (approximately 8 days) while the group on 20 mg/kg was 9 days ($p \leq 0.05$ test Vs control by Student's t-test). A high mortality was observed in all the groups that received lupeol treatment. No toxicity was observed in uninfected mice treated with the highest dose of lupeol

(results not shown).

Figure 2 outlines the interaction line plots for progression of 7401H HSV-1 infection in mice following oral treatments with lupeol at 5, 10 and 20 mg/kg. The progression of infection in mice treated with 5 mg/kg was similar to that of control, where once the infection was on progresses, it continued uninterrupted until the final lethal stages. The 10 mg/ml treatment delayed the progression of infection in the treatment phase, however on withdrawal of treatment on the 7th day, the infection

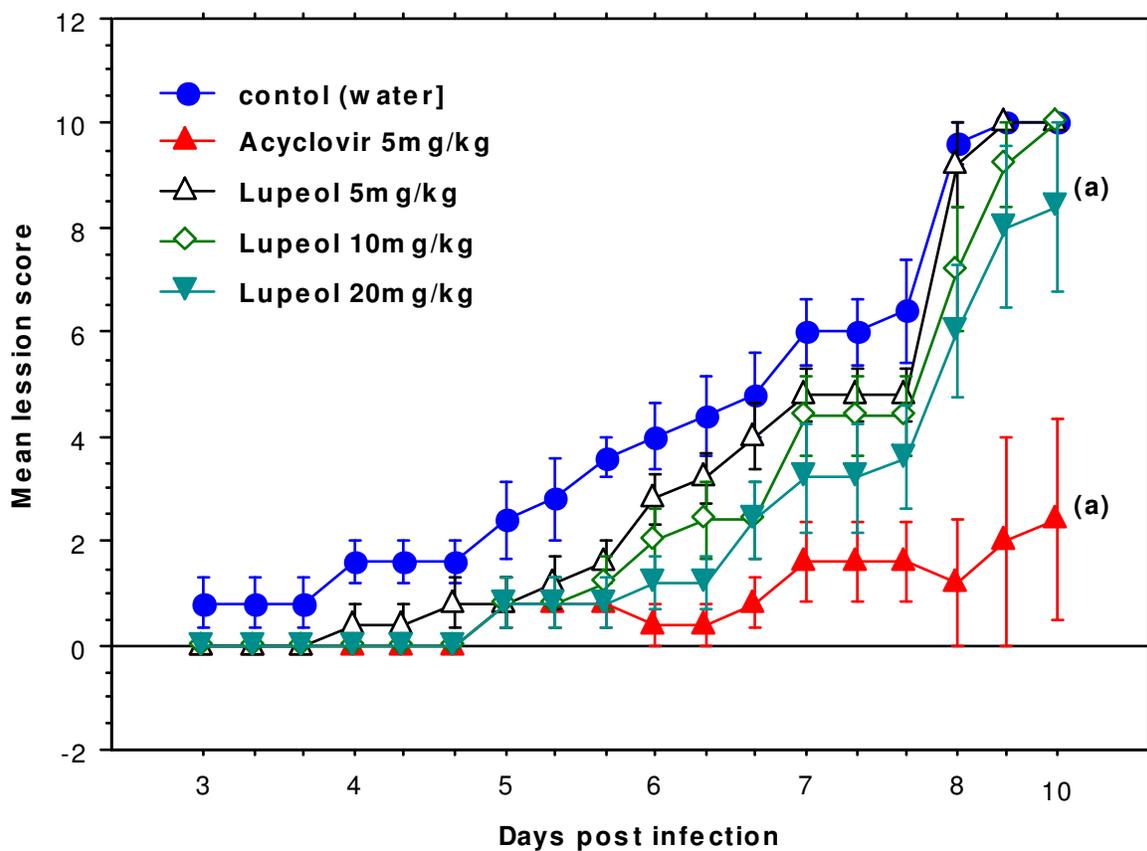


Figure 2. The interaction line plots for progression of 7401H HSV-1 infection in Balb/C mice following oral treatments with increasing doses of lupeol. In the figure, ^a = $p \leq 0.05$ Test Vs control by repeated measures ANOVA (Benferroni/Dunn). Error bars are deviations within the mean of 5 mice in each group.

progressed faster to the lethal stages. The mice on 20 mg/kg had a much slower progression of infection than the 10 mg/kg treatment. The influence of treatment on progression of infection at 10 and 20 mg/kg were statistically significant ($p \leq 0.05$ test Vs control by repeated measures ANOVA (Benferroni/Dunn)). Acyclovir treatment contained progression of infection to below mild zosteriform lesions, with a statistical significance ($p \leq 0.05$ test vs. control by repeated measures ANOVA (Benferroni/Dunn)).

DISCUSSION

Three of the four compounds isolated from the root bark of *C. edulis*; lupeol, oleuropein and carissol demonstrated anti-HSV activity *in vitro*. However, it was only lupeol that had activity for both the sensitive and resistant viral strains. Lupeol, a triterpene, is found in various edible plants, fruits and medicinal plants used in many parts of the world (Beveridge et al., 2002; Kakunda et al., 2002; Saleem et al., 2005). The compound has been shown to exhibit strong anti-inflammatory, anti-arthritic, anti- mutagenic,

anti-malarial and anti-viral activity in *in vitro* and *in vivo* systems (Tanaka et al., 2004; Saleem et al., 2005). The *in vitro* anti-HSV activity of lupeol has previously been demonstrated by other researchers. Tanaka et al. (2004), reported that lupeol isolated from the roots of *Strobilanthes cusia* BREMEK (Acanthaceae) had anti-HSV activity for the KOS strain HSV-1 (EC_{50} : 11.7 μ M) and showed 100% inhibition of virus plaque formation at 25 μ g/ml in Vero cells. The results of this study confirm these findings and move a step further by demonstrating the sensitivity of the acyclovir resistant strains to the compound. The virucidal effect of lupeol, as witnessed in the virus yield reduction assay, was another pointer of the antiviral potential of the compound. Since the acyclovir resistant strains were sensitive to lupeol, this indicated possibilities of a difference in the mechanism of antiviral action to that of acyclovir that needs to be explored. The cytotoxic level of lupeol in Vero cells was well above EC_{50} giving it a good selectivity index. A similar level of cytotoxicity (CC_{50} ; 196 μ g/ml) has been reported by other researchers in Vero cells (Badami et al., 2003).

The *in vivo* efficacy of lupeol for cutaneous HSV infection in mice was however weak. Even though the

progression of infection was comparatively slow (against control), leading to improved mean survival times, there was high mortality in all the treatment groups. It is difficult to provide explanations for these observations at this stage other than being speculative. For instance, the observed low activity could be attributed to biological factors in play in an *in vivo* environment. Since efficacy was investigated following an oral route, there are possibilities that once in the alimentary canal, lupeol could have been bio-transformed into other inactive or less active forms by the digestive system and thereby reducing its potency. Alternatively, perhaps due to poor bioavailability, the drug was not readily absorbed from the alimentary canal so as to reach the target sites of infection and thus the low effect. However, as already mentioned, these are just assumptions since this line of thought needs to be verified with data. The LD₅₀ for lupeol was well above the therapeutic level (>100 mg/kg (results not shown)) and therefore toxicity could not have contributed to the high mortality.

Conclusion

Lupeol is a promising candidate for development into an anti-HSV agent. However, much still needs to be done before a meaningful comparison between its therapeutic values are comparable to that of acyclovir. It would be worthwhile though to investigate the activity of a combination of lupeol and acyclovir for a possible synergistic effect. In addition, it would also be necessary to determine the mechanism of anti-HSV action of lupeol since this would help explain the activity on the acyclovir resistant viral strains. The bioavailability and pharmacokinetic profiles of the drug also needs to be examined to determine the best dosage regimen and administration route for further investigations. The results show a great potential and warrants further work, possibly in higher mammals.

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