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IN VITRO CYTOTOXICITY AND *IN VIVO* ACUTE ORAL TOXICITY EVALUATION OF *COPTIS CHINENSIS* AQUEOUS EXTRACT

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Abstract – *Objective:* Rhizoma Coptidis *(*Coptis chinensis, *Hunaglian) is most widely used Traditional Chinese Medicine (TCM) in daily life to treat inflammatory diseases and other various clinical conditions in Malaysia and China. Our aim was to evaluate* in vitro *cytotoxicity and* in vivo *acute oral toxicity of aqueous extract of Coptidis Rhizoma (AECR).*

Materials and Methods: *The toxicology profile includes* in vivo *acute oral toxicity study and* in vitro *cytotoxicity against non-cancerous human embryonic hepatic cell lines (WRL68 cells). The* in vivo *study aims to test the acute toxicity in Wistar rats with a fixed oral dose of 2,000 mg/kg for 14 days. Signs of acute oral toxicity in terms of behavior changes and mortality were noted for few hours till 14 days. Concurrently, the* in vitro *cytotoxicity, cell cycle arrest and apoptosis induction assays were performed against WRL68 cells using, MTS cell viability and flow cytometry assays, respectively.*

Results: *In acute oral toxicity study, there was no lethality and acute toxic signs were observed up to 2,000 mg/kg b.w. for the 14 days of observed duration. However, AECR demonstrated low level of* in vitro *cytotoxicity (IC_{50}= 7.167 ± 2.57 µg/ml) and moderate apoptosis inducing effect (p<0.05) against WRL68 cell lines. AECR was found to be nontoxic when acute oral toxicity study was performed. Nevertheless,* in vitro *biological activities suggested that the AECR has potential apoptosis inducing properties.*

Conclusions: *The results of the present study suggest that the* C. chinensis's *aqueous extract may possess the efficacy to develop new drugs for cancer chemotherapy. However, additional molecular studies to understand the genes associated with AECR induced apoptosis and cell cycle arrest are warranted. Furthermore, long-term* in vivo *toxicological evaluations should be undertaken to assess a safe dosage from the plant's potentially toxic effects.*

KEYWORDS: Rhizoma Coptidis*, OECD, Acute oral toxicity, WRL68 cells, Cytotoxicity, Apoptosis, Cell cycle arrest.*

INTRODUCTION

Medical herbs as a natural resource have received remarkable attention in recent years as potential pharmaceutical compounds[1]. One of the approaches practiced in the drug discovery process is phytomedicine as an alternative to chemotherapy due to its low toxicity or damage to healthy cells. Malaysia is endowed with one of the richest biodiversity in the world. However, only about 15% of medicinal plants were claimed to have

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and studied for their medicinal properties and potential bioactivities, but there are still many plants to be investigated for their safety and toxicities[2]. Although traditional herbal medicines are used by an increasing number of Malaysians, little scientific information is available regarding the safety and effectiveness[3]. Most medicinal plants used by the Malaysian have not been tested in terms of their possible toxicity. The abundant use of herbal medicines by numerous Malaysian patients may offer opportunities to ethnopharmacology researchers to evaluate the safety and efficacy of many herbs. It is an essential approach needed early in the drug discovery and development of natural products to determine their likely cytotoxic effects.

Coptidis Rhizoma (CR), a member of the Ranunculaceae family, derives from the dried rhizomes of *Coptis chinensis*. The dried rhizome of *C. chinensis* Franch. (Huanglian) has been widely used in Asian traditional medicine[4]. It was reported that Coptis Rhizomes have anti-inflammatory, immune-modulatory, antidiabetic, cardioprotective and neuroprotective activities[5-9]. Based on current scientific literature, the major pharmacologically active compounds contributing to Coptis's bioactive properties are berberine, coptisine, palmatine, epiberberine, jatorrhizine and magnoflorine and worenine[10]. It also contains antiseptic alkaloids and thus its rhizomes and preparations are widely used for the treatment of fish diseases. Today, CR is still extensively consumed in TCM for the treatment and management of numerous disorders. In China, it is a common component in traditional medicines used to treat cardiovascular associated problems[11].

Recently the importance of CR has been increased due to new biological and pharmacological investigations about its therapeutic applications. In contrast to its beneficial effect, CR is also found to be toxic in human and animals[12]. Given the intensive therapeutic applications of CR in the TCM, growing concerns have arisen about its unintentional health impacts. There were very few clinical reports of adverse reaction attributable to CR in oral TCM formulation. It has been banned in Singapore for the past three decades due to the implication of berberine in aggravating jaundice and kernicterus in neonates with glucose-6-phosphate dehydrogenase deficiency[13]. The results from a new toxicological study revealed that *C. chinensis* extracts appeared to demonstrate weak embryotoxicity[14]. Even though, the toxicity of this medicinal plant is poorly understood. A review of available literature indicates that there has been a considerably lack of research on its cytotoxic and apoptotic activities. The toxicological studies of AECR were initiated by the *in vitro* evaluation of mammalian cell cytotoxicity performed with rat human embryonic normal liver cells (WRL68 cell line), as hepatic cells are playing a key role in detoxification reactions and general metabolism control. Thus, we conducted *in vivo* acute toxicity, *in vitro* cytotoxic, cell cycle arrest and induction of apoptosis assays in WRL68 cells for the rhizomes of *C. chinensis* to develop an effective natural anticancer drug, which has been reported in the literature to some extent.

MATERIALS AND METHODS

Chemicals and reagents

Gallic acid and Folin-Ciocalteu's reagent were purchased from Merck (Malaysia). Sodium bicarbonate was purchased from Fluka Chemicals. Phosphate buffered saline (PBS) pellet was purchased from MP Biomedicals (MP Biomedicals Inc**,** Santa Ana, CA, USA). Sodium bicarbonate, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), RPMI, penicillin, streptomycin and trypsin were purchased from Sigma-Aldrich (Sigma-Aldrich Co, St Louis, MO, USA). The aqueous solution was obtained from the research laboratory. Annexin-V and propidium iodide (PI) were obtained from Becton Dickinson (Brea, CA, USA). Tetrazolium compound(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt; MTS) was purchased from Promega (Promega, Madison, WI, USA). A dried sample of *Coptidis Rhizoma* was purchased from a local Chinese Medicine supplier. All other reagents were of analytical grade and obtained from common sources.

Preparation of aqueous extract Coptidis Rhizoma (AECR)

Coptidis Rhizoma (50.0 g) was coarsely powdered for the preparation of solvent extraction. The dried Coptidis Rhizoma was extracted with 250.0 ml of aqueous solvent at room temperature. The aqueous extract of Coptidis Rhizoma (AECR) was concentrated in a water bath incubator (Memmert, Buchenbach, Germany) at 60°C-70°C. The extract was obtained and kept in a 4°C refrigerator in the sterile conical flask for further *in vitro* pharmacological studies. The yield of the extracted sample was calculated by the following equation:

$$\text{Extracted} = \frac{\text{weight of Coptidis rhizoma extract (g)}}{\text{weight of dried Coptidis rhizoma (g)}} \times 100\%$$

Cytotoxic assay (MTS)

Cytotoxic activity of AECR against Human Normal Embryonic Liver Cell Line (WRL68) was determined by using standard MTT [(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] viability assay as described previously[15]. Briefly, the WRL-68 cells were counted and seeded ($2x10^4$ cells/mL) in a 96-

well flat-bottom microplate followed by 24-h incubation. Cells were incubated with various concentrations of the AECR (2-500 µg/mL) at 37°C in a CO_2 environment for 72 h. Wells containing DMEM or RPMI 1640 was used as the negative controls. After the incubation periods, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. To dissolve the formazan crystals formed, 100 µL of DMSO was added to each well. The absorbance for each well was measured at 540 nm in a micro-titer plate reader (Bio-Rad, Hercules, CA, USA). The dose-response curve was plotted and the concentration which gave 50% of cell growth (IC_{50}) was calculated. All treatments, including negative control, were carried out in triplicate experiments.

Apoptosis analysis

Flow cytometry analysis was carried out to investigate the proportion of cancer cells undergoing apoptosis using annexin-V conjugated with fluorescein isothiocyanate (FITC) detection kit according to the manufacturer's instructions. Cells (1×10^6/well) were seeded onto 24-well plates and incubated at 37°C overnight. The next day, cells were treated with various concentrations of AECR and incubated for 72 h. After this, cells were detached using trypsin for 5 min. Cells were collected and centrifuged at 1000 rpm for 5 min. The cell pellet was washed with PBS and resuspended in 100 µL binding buffer. Subsequently, 2.5 µL annexin V-FITC and 3 µL propidium iodide were added and kept in the dark at room temperature for 15 min. Annexin V-FITC/PI stained cells were analyzed using flow cytometry (BD Biosciences LSR II FACS, San Francisco, CA, USA).

Cell Cycle Analysis by flow cytometry

Cells were treated as described in flow cytometry analysis. After treatment and incubation for 72 h, cells were harvested and fixed with ice-cold 70% ethanol (1 mL) at -20°C for 2 h. Ethanol was then removed (1000 rpm, 5 min) and the cells were washed twice with cold PBS. Subsequently, cells were resuspended in 425 µL of PBS, 25 µL propidium iodide (1 mg/mL), and 50 µL RNaseA (1 mg/mL) and incubated for 30 min at room temperature. Distribution of the cell cycle was measured at 585 nm by flow cytometer and data analysis was carried out with ModFitLT software (version 4)[17].

Experimental Animals

Six Male and Six female (nulliparous and nonpregnant) Wistar rats (160–200 g) were housed in a temperature and light-controlled room (24.0 ± 2°C; 12 h light/dark cycle). All the animals were acclimatized in the laboratory for at least 7 days before the toxicity study, with free access to water and food. The experimental protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, University of Malaya, Malaysia.

Ethical approval

The *in vivo* toxicity study and its protocol were approved by the Institutional Animal Ethical Committee, Faculty of Medicine, University of Malaya. The *in vivo* experiments and procedures were performed by adopting international ethical guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals and the Organization for Economic Co-operation and Development (OECD, 2000) guidelines.

The acute oral toxicity study

The acute oral toxicity test was carried out according to the OECD test guideline 425 (Up and Down Procedure), with slight modifications[16]. The limit test was performed at 2000 mg/kg p.o. as a single dose and rats were kept without food for 3-4 h before dosing but had free access to water *ad libitum*. The animals were divided into two groups (Vehicle control and ACER treated), each comprising 6 animals. The dose was administered to a single female rat from the test group according to body weight. The animal was closely observed for the first 30 min, then for 4 h. Food was provided after 1–2 h of dosing. After the survival of the treated rat, 5 more rats were administered with the same dose under similar conditions. The equal procedure was followed for the vehicle-treated (0.1 % carboxymethyl cellulose) control group of 6 rats with the same volume as that of the AECR treated group. Survived animals from both groups were observed closely for any acute toxic effects within the first 4 h and then at regular intervals for a total period of 14 days for other toxic effects. Surviving animals were further visually observed for mortality, behavioral pattern, changes in physical appearance, injury, pain, and other signs of illness were recorded daily for 14 days.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using Dunnett's test and were used to assess differences between means. A significant difference was considered at a level of $p<0.05$. Values are shown as mean ± standard error of the mean (SEM) of three replicates.

RESULTS

Effect of AECR on in vitro cytotoxicity

The *in vitro* cytotoxicity was evaluated by MTS assay which can provide firsthand information of the cytotoxicity for AECR. Results from this experiment revealed that AECR and positive control (doxorubicin) exhibited cytotoxic activity towards WRL68 cells with an IC_{50} value of 7.16±2.57 and 1.83±0.38 respectively, after 72 h incubation period as determined by MTS assay. AECR exhibited strong cytotoxicity activity against WRL68 cells as compared to standard, doxorubicin. The results showed that AECR reduces the proliferation activity of WRL-68 with increasing concentration (Figure 1).

Effect of AECR on in vitro apoptosis

To determine if AECR induces apoptosis in WRL68 liver cells, Annexin V/propidium iodide double staining assay and flow cytometry analysis were performed. The flow cytometry data were acquired and analyzed as described earlier, with results presented in Figure 1a and 1b. In the histogram, different quadrants indicate cells at various stages: Lower left, viable cells, lower right; early apoptotic cells, upper left: necrotic cells; upper left: late apoptotic cells. Treatment with 7.167 µg/ml AECR for 72 hours in a small increase in early apoptotic cells (Annexin V positive only) from 3.386% to 7.053. The late apoptotic cells of WRL68 (Annexin V and propidium iodide positive) were increased significantly (p<0.05), from 9.186% to 64.743%. Taken together, we found that the percentage of annexin V positive cells of WRL68 (early and late apoptotic cells) increased significantly compared to the control. Hence, these results showed that AECR inhibited WRL68 cells growth by inducing apoptosis in these cells (Figure 2).

Effect of AECR on in vitro cell cycle

Cell cycle analysis was performed to investigate the inhibitory effect of AECR via induction through apoptosis in the cell cycle progression of WRL68 cells. To examine whether induction of apoptosis in WRL68 cells by AECR might be related to the cell cycle arrest, cell cycle distribution of the AECR treated cells was stained with propidium iodide and the percentage of cells in different stages of the cell cycle was measured using a flow cytometer. The results showed that S-populations in the 7.167 µg/ml AECR treated cells for 72 hrs were moderately increased (25.72 ± 12.94%), and a corresponding decrease in the G1 population (G1=74.11 ± 13.00 %) in the cell cycle compared to vehicle control cells (Figure 3). These results demonstrated that the AECR significantly arrested the cell cycle progression at the G1 phase after 72 hrs of treatment. However, no significant changes in the percentage of the population of cells in other phases were observed. These results reveal AECR affected cellular proliferation, in part by lowering the cells in the G1 phase. Further molecular research is needed to investigate the role of AECR in cell cycle arrest at various stages.

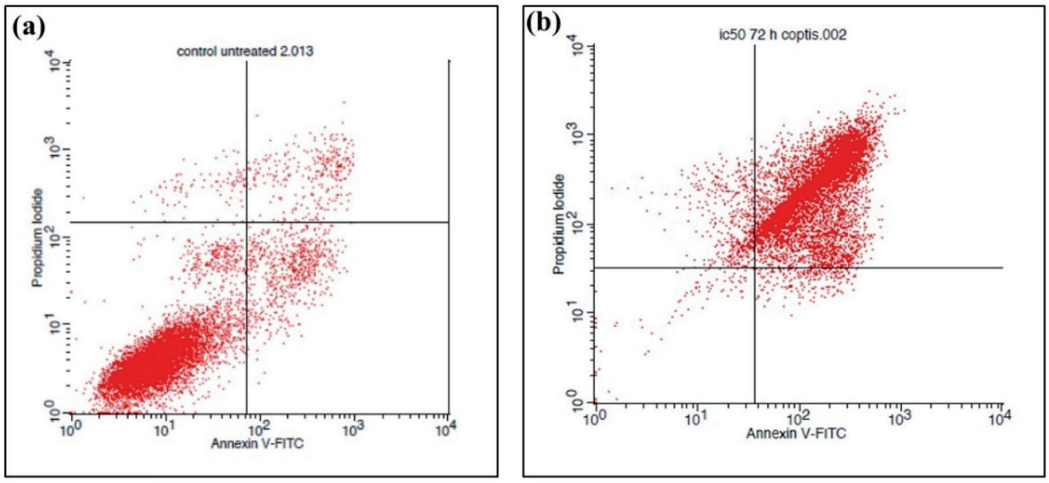

Fig. 1. Refiguration of the applied Mathematical model. Figure 1. Flow cytometric analysis of Annexin V and FITC/propidium iodide staining of WRL68 cells exposed to AECR. WRL68 cells (1×10^6 /well) were incubated with 7.16 µg/ml of AECR or (a) control and (b) 72 h at 37˚C. Lower left quadrant in each panel signifies viable WRL68 cells which excluded propidium iodide and were negative for annexin V-FITC binding. Lower right, upper right and upper left quadrant contains early apoptotic cells, late apoptotic cells, necrotic cells positive respectively, and positive for annexin V-FITC binding. Three independent experiments are performed in triplicate.

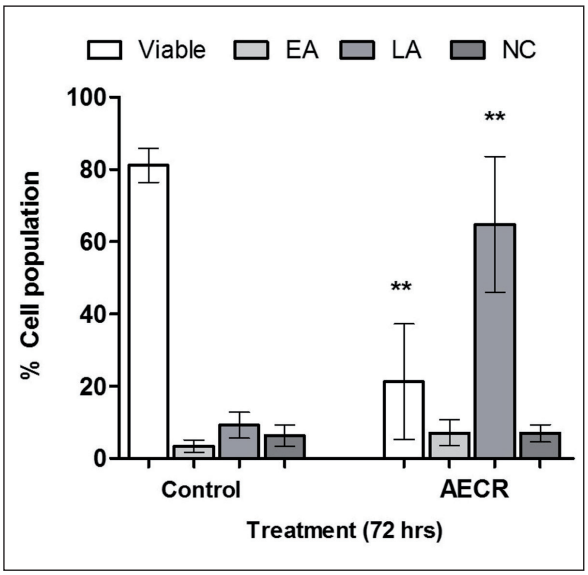

Fig. 2. Flow cytometric analysis of Annexin V and FITC/ propidium iodide staining of WRL68 cells exposed to AECR. Three independent experiments are performed in triplicate. ** $p<0.05$ significantly different from control cells (one ANOVA and Bonferroni test); when not indicated, the differences were not statistically significant.

Effect of AECR on in vivo acute oral toxicity

The acute toxicity test using the Up and Down method at an oral limit doses of 2000 mg/kg of AECR caused no death in the rats. No significant changes were observed in wellness parameters used for the evaluation of acute oral toxicity (Table 1). Furthermore, no deaths were observed in Wistar rats treated with AECR at a dose of 2000 mg/kg thus establishing its safety in use. All the behavioral and other visual observations were found to be normal for the AECR (2000 mg/kg) and vehicle-treated groups.

DISCUSSION

The rhizomes of *C. chinensis* is traditionally used in Chinese folk medicine for treating various ailments and reported to have numerous pharmacological activities[18,19]. The best known biologically active compounds are the alkaloids identified in the rhizome of the plant[20]. Despite the various traditional claim, phytochemical and pharmacological activities, little is known about its effect on cell survival, growth, and apoptosis to define its cytotoxic nature. Safety assessment is a part of experimental research on the extensive therapeutic effects besides the major biological activity. On the other hand, the issue of the safety of traditional herbal preparations worldwide has been constantly questioned. Moreover, the utilization of herbal drugs is recently improved and thus the safety evidence is considered a key issue for new drug development, traditional herbal prescription, and medication counseling[21]. The majority of *C. chinensis in vitro* studies were designed to look for its beneficial cytotoxic effects against different cancers, with only a few studies focusing on its possible toxicity[22-24]. There are still no systematic investigations on the general toxicities of *C. chinensis* and its alkaloids. According to the chemical labelling and classification of acute systemic toxicity recommended by OECD, AECR was assigned with the lowest toxicity class (LD_{50}> 2000 mg/kg). Our results are following previously published works and the LD_{50} value of AECR by

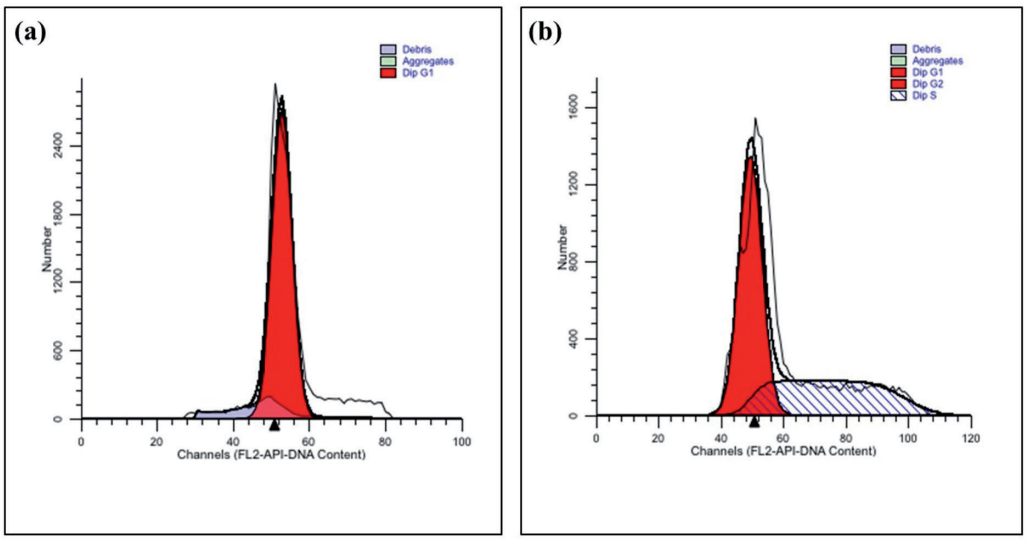

Fig. 3. Effect of AECR on cell cycle progression in WRL68 cells. (a) Control cells (b) Cells (1×10^6 /well) were treated with 7.16 µg/ml of AECR for 72 h at 37˚C. Data are representative of three independent experiments.

TABLE 1. Effect of oral administration of AECR (2000 mg/kg) on behavioral response and general appearance.

Parameters	*Control 2 ml/kg*	*AECR 2000 mg/kg*
Alertness	Normal	Normal
Touch and Pain Response	Normal	Normal
Food and water Intake	Normal	Normal
Tremors/Epilepsy/Sedation/Coma	Not observed	Not observed
Gripping/Corneal/Righting reflex	Normal	Normal
Salivation, Urination	Normal	Normal
Skin color and pupils	Normal	Normal
General physique	Normal	Normal
Temperature	Normal	Normal
Faeces consistency	Normal	Normal
Mortality	Not observed	Not observed

oral route was more than 2000 mg/kg, thereby suggesting that AECR is a non-toxic drug[25]. It is indicated that the AECR can be used for further investigation of any preclinical and other pharmacological effects on the above-mentioned dose of extract. However, a detailed subacute and chronic experimental analysis of its long-term toxicity is essential for further clinical support of this drug.

The primary aim of cancer chemotherapy is to kill cancer cells selectively while limiting toxicity in healthy cells. This is a downside to the use of many chemotherapeutic agents; as a result, selective toxicity must be factored when exploring cancer treatment leads. Cytotoxicity screening models provide important preliminary data to help select plant extracts with potential cytotoxic or bioactivities for future work[26]. The criteria of cytotoxicity activity for the crude extracts, as established by the American National Cancer Institute is an IC_{50} < 30 µg/ml in the preliminary assay following incubation time between 48 and 72 h[27]. An important point is that AECR shows cytotoxic response on WRL68 cells for 72 hrs with an IC_{50} value of 7.167 µg/ml. The IC_{50} value was found to be lower than that specified by the American National Cancer Institute for categorization of developing as a promising chemotherapeutic agent. The reduction of cell viability as compared with untreated control cell may be contributed by its apoptosis-inducing or proliferation inhibiting effects. This investigation provides confirmation for cytotoxicity in WRL68 which may be due to existing phytochemicals in the aqueous extract *C. chinensis* as mentioned previously[28].

Apoptosis also seems to be a reliable biomarker for the preliminary study of cytotoxic potential of natural compounds[29]. Our current study concerning AECR's ability to induce apoptosis may provide valuable information for the possible toxic effect in normal human embryonic liver cells. The cytotoxic activity was further confirmed by measuring the different phases of cell cycle progression of AECR treated WRL68 cells using flow cytometry assay. It appears that AECR at 7.167 µg/ml concentrations caused an increase in the proportion of cells at the S phase and a decrease in the proportion of cells at the G1 phase of the cell cycle. For WRL68 cells, after 72 hrs treatment, a significant decrease in the G1 phase coupled with the accumulation of cells in the S phase was observed. This effect suggests that the reduction in cell viability and proliferation of WRL68 caused by the induction of apoptosis.

In this study, we showed that AECR efficiently lowered the proliferation of a WRL 68 cell line, by signifying the roles of *C. rhizome's* phytochemical in decreasing the number of cells, in the induction of apoptosis, and in interfering with cell cycle arrest at the S and G1 phases of the cell cycle. As experimental noted above, alterations in cell growth measured in AECR treated cells may be due to the induction of the apoptosis process in corresponding to cell cycle arrest. This outcome showed that the AECR might be capable of inducing DNA fragmentation which is the hallmark for programmed cell death. Taken together, our results suggest that there are potentially multiple mechanisms by which CR can inhibit the growth of WRL68 cells. Our preliminary study potentially offers valuable information that may be used to expand the safety application and chemotherapeutic investigation of CR to the field of TCM. Remarkably, several recent investigations reveal that CR induces apoptosis in various cancer cells, suggesting that tumour cells may be more susceptible to CR than normal healthy cells[30-33]. The limitation of the current study is that detection of key molecules related to apoptosis such as BCL2, BAX, PRPP and caspase cascades was not performed to confirm the cell apoptosis as additional confounding information due to economic constraint. Our current study also recommends that care should be taken in terms of overdose and sustained treatments, which causes accumulation of the CR in the normal organism[34]. Long-term *in vivo* and *in vitro* studies of cytotoxic, cell cycle arrest and apoptotic effects of AECR on other normal and cancerous cells are now in progress.

CONCLUSIONS

Acute *in vivo* oral administration of aqueous extract of *C. chinensis* rhizome is non-toxic nature in healthy animals. However, the *in vitro* cytotoxicity and apoptosis analysis revealed the cytotoxic potential of the rhizome extracts on WRL-68 cells. Additional research into subacute toxicity in healthy animals and chemoprevention mechanisms in various cancer cells is strongly needed.

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Authors' contributions:
KC primarily designed the whole investigation and protocols, conducted the *in vivo* toxicological experiments, performed Behavioural analysis, measured acute toxicity signs, and secured funding. KC had a chief role in the scientific writing of the manuscript. KC and CMJ involved an active role in the arrangement of research facilities and served as the project's supervisor. CMJ completely carried out the *in vitro* cytotoxicity, apoptosis analysis and wrote the comments on *in vitro* part. KC actively involved in the scientific analysis of the results, assisting to draft the manuscript, statistical study. Finally, both authors reviewed the whole manuscript and agreed to submit the paper for scientific communication.

Conflict of interest:
The author declares that there are no conflicts of interest.

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