Cancer Chemother Pharmacol (2005) 55: 447–452
DOI 10.1007/s00280-004-0917-8

ORIGINAL ARTICLE

Chun-Lin Ye · Jian-Wen Liu · Dong-Zhi Wei
Yan-Hua Lu · Feng Qian

In vivo antitumor activity by 2′,4′-dihydroxy-6′-methoxy-3′, 5′-dimethylchalcone in a solid human carcinoma xenograft model

Received: 11 May 2004 / Accepted: 12 August 2004 / Published online: 22 January 2005

Abstract Previously, we showed that 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC), isolated from the buds of *Cleistocalyx operculatus*, significantly inhibited the growth of human liver cancer SMMC-7721 cells and could induce SMMC-7721 cells apoptosis in vitro. Here, we report the antitumor effects of DMC in vivo, using a solid human tumor xenograft model with a human liver cancer SMMC-7721 cell line. Our results revealed that the average tumor weight in a control group and a 150-mg/kg DMC injection group was 1.42 ± 0.11 g and 0.59 ± 0.12 g, respectively. Flow cytometric analysis of the tumor cell population demonstrated the existence of an aneuploid peak (representing $33.60 \pm 0.80\%$ of the total in the 150-mg/kg DMC injection group). To our knowledge, this is the first time that chalcone compounds were applied to a human tumor xenograft model.

Keywords *Cleistocalyx operculatus* · Flavonoids · Tumor · Acute toxicity · Xenograft

Introduction

Over the past 10 years, research for new drugs to be used in oncology has refocused on natural products. The rediscovery of natural products has yielded promising compounds such as taxanes and camptothecins. In particular, interest has intensified in the class of flavonoids compounds present in a normal human diet and in many folk medicines still in use today. Various pharmacological activities of flavonoids have been studied extensively [1–4].

Cleistocalyx operculatus (Roxb.) Merr. et Perry (Myrtaceae) is a well known medicinal plant whose buds are commonly used as an ingredient for tonic drinks in Southern China. It was reported that the water extract of the buds of *Cleistocalyx operculatus* was shown to increase the contractility and decrease the frequency of contraction in an isolated rat's heart perfusion system [5]. Our previous phytochemical attention to the species has led to the characterization of sterol, flavanone, chalcone, and triterpene acid from its buds [6]. Chalcones, considered as the precursor of flavonoids and isoflavonoids, are abundant in edible plants. A number of chalcones have demonstrated cytotoxic [7, 8] and anticancer properties [9, 10]. To evaluate the effectiveness of 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC), one of the main compounds from the buds of *Cleistocalyx operculatus*, as an anticancer agent, we have performed antitumor screening using six human cell lines. We found that both the MTT assay and the colony-forming assay indicated that SMMC-7721 cells was the most sensitive in one in six of the tested cell lines. Staining with Hoechst 33258 and flow cytometric analysis results indicated that DMC could induce SMMC-7721 cells apoptosis in vitro (Ye CL, Liu JW, Wei DZ, Lu YH, Qian F (2004) In vitro antitumor activity of 2′,4′-dihydroxy-6′-methoxy-3′,5′- dimethylchalcone against six established human cancer cell lines. Pharmacological Research, accepted).

The major objective of this paper is to investigate whether DMC has antitumor activity in vivo by using a solid human tumor xenograft model with a human liver cancer SMMC-7721 cell line. The percentage of hypodiploid cells in the tumors was determined by a flow cytometric analysis and the acute toxicity of DMC was also detected using ICR mice.

C.-L. Ye · J.-W. Liu · D.-Z. Wei (✉) · Y.-H. Lu · F. Qian
State Key Laboratory of Bioreactor Engineering, Institute of Biochemistry, East China University of Science and Technology, Shanghai, 200237, China
E-mail: dzhwei@ecust.edu.cn
Tel.: +86-21-64252981
Fax: +86-21-64250068

Materials and methods

Materials

DMC was isolated from *Cleistocalyx operculatus* in our lab as described by Ye et al. [6]. Previous experiments have shown that DMC purity were above 96%. The structure of the compound is shown in Fig. 1. Mitomycin-C was purchased from Sigma (St. Louis, MO, USA). DMC and mitomycin-C were dissolved in ethanol:PBS (1:20). Control mice received the same amount of vehicle. Rosewell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies Inc. MA, USA.

Cell line and animals

The human liver cancer SMMC-7721 cell line was purchased from the Cell Bank of Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). The cell line was cultured in RPMI 1640 medium with 10% FBS, penicillin (100 units/ml), and streptomycin (100 mg/ml), and was incubated at 37°C with 5% CO_2 in an air atmosphere.

Athymic male (BALB/c nu/nu) nude mice, 4–6 weeks of age, were used for the SMMC-7721 xenograft model. Athymic nude mice were provided with sterilized food and water. ICR male mice, 4–6 weeks of age, were used for the investigation of the acute toxicity of DMC. Both nude mice and ICR mice were purchased from the Shanghai Center of Experimental Animals, Chinese Academy of Sciences (Shanghai, China).

Investigation of the acute toxicity of DMC

The index of the acute toxicity is the LD_{50} value. In this study, the LD_{50} value was detected as described by Lorke [11]. It is proposed that the acute toxicity should be tested in two steps: (1) In the initial investigations on the range of doses producing the toxic effects is established; (2) based on these results, further specific doses are administered to calculate an LD_{50} value. Briefly, in the initial investigations, the ICR mice, 4–6 weeks of age, were randomized into groups of three, and three mice for each group were used in these experiments. The study groups included: (a) animals treated once with DMC (10 mg/kg, intragastric infusion); (b) animals treated once with DMC (100 mg/kg, intragastric infusion); (c) animals treated once with DMC (1,000 mg/kg, intragastric infusion). The mortality of the treated mice was detected within 14 days for each group. Based on the results of the initial investigation, the new dosages are administered to the animals in the second test (as shown in Table 1). In the second test, one animal was used for each group.

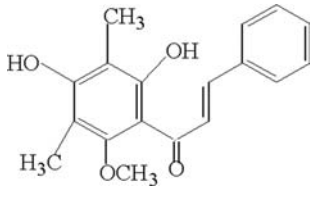

Fig. 1 Structure of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC)

Detection of the plasma levels of DMC

Male nude mice, 4–6 weeks of age, were intraperitoneally (i.p.) injected once with DMC (150 mg/kg). At times 3, 4, 5, 6, 7, 8, 12, and 24 h, the mice were anesthetized with ether and blood samples were taken via cardiac puncture. Firstly, one mouse was tested at each sampling time point, and then two other mice were tested at the time point of maximal value. DMC in the blood sample was extracted by a method as described by Liu et al. [12] with a small modification. Briefly, serum was obtained from the coagulated blood by centrifugation and 400 µl of serum was incubated with 20 µl of 5 N HCl for 30 s and then extracted three times with 500 µl of ethyl acetate. The extract was evaporated to dryness and dissolved in methanol.

Plasma levels of free-form DMC were determined by the high performance liquid chromatography (HPLC) technique with a reversed phase column (ZORBAX, Eclipse XDB-C_{18}, 5 µm, 4.6×250 mm, Agilent, USA). Chromatography was carried out with the isocratic flow program. The flow rate of the mobile phase (A, methanol; B, 0.2% H_3PO_4 aqueous; A:B = 93:7, V/V) was kept constant at 1.0 ml/min and the peaks were detected at 220 nm.

Table 1 Doses chosen for the initial investigation and the second test

Dose in mg/kg body weight			Doses chosen for the second test (mg/kg)			
Result of the initial investigation						
10	100	1000				
0/3[a]	0/3	0/3		1,600	2,900	5,000
0/3	0/3	1/3	600	1,000	1,600	2,900
0/3	0/3	2/3	200	400	800	1,600
0/3	0/3	3/3	140	225	370	600
0/3	1/3	3/3	50	100	200	400
0/3	2/3	3/3	20	40	80	160
0/3	3/3	3/3	15	25	40	60
1/3	3/3	0/3	5	10	20	40
2/3	3/3	3/3	2	4	8	16
3/3	3/3	3/3	1	2	4	8

[a]Number of animals that died/number of animals used

Human carcinoma xenograft model

The SMMC-7721 cell line was established as a xenograft in athymic male nude mice. Monolayer cultures were harvested with trypsin and resuspended in PBS. About 5×10^6 cells were subcutaneously (s.c.) injected into the right flank of the mouse. After 4 weeks, the tumors were aseptically dissected and tumor slurry was prepared as a single-cell suspension, then s.c. injected (2×10^6 cells in 0.1 ml) into the mice. Animals were used for experiments when the tumors were about 5×5 mm in size.

Tumor bearing mice were randomized into groups of five, and six mice for each group were used in these experiments. The study groups included: (a) control; (b) animals injected with mitomycin-C (2 mg/kg, i.p.) on days 1, 3, 5, 7, 9, 11, 13; (c) animals injected with DMC (150 mg/kg, i.p.) on days 1–14; (d) animals injected with DMC (100 mg/kg, i.p.) on days 1–14; (e) animals injected with DMC (50 mg/kg, i.p.) on days 1–14. We monitored tumor growth starting on the first day of treatment and measured the volume of the xenograft and animals' weights every 4 days. Tumor volumes were measured in two perpendicular diameters (A and B). The tumor volume (V) was estimated according to the following formula [13]:

$$V = \frac{\pi}{6}\left(\frac{\mathrm{A}+\mathrm{B}}{2}\right)^3$$

The curve of the tumor growth was drawn according to tumor volumes and time of implantation. The mice were anaesthetized and killed when the mean tumor weights were over 1 g in the control group. Tumor tissue was excised from the mice and its weight was measured.

Flow cytometry assay

The tumor tissue was rapidly removed, weighed, and placed into 10 ml of ice-cold PBS containing 0.2% bovine serum albumin (BSA) (Sigma), 0.01 mol/l EDTA, and 10 mg/ml deoxyribonuclease I(Sigma). Then, the tissue was disrupted in a glass homogenizer and passed through a 40-μm nylon cell stainer (Becton Dickinson). The suspension was centrifuged at 500×g for 10 min at room temperature. The pellet was resuspended in 500 μl of PBS with BSA and transferred into a fresh tube. The cells obtained by the above method were fixed with ice-cold 70% ethanol in PBS at 4°C for 8 h, then incubated with RNase (20 μg/ml) for 30 min at 37°C and labeled with propidium iodide (50 μg/ml). The DNA contents were measured by a FACSCalibur cytometer (Becton Dickinson). A multicycle software program (CellQuest, Becton Dickinson) was used to produce histograms of DNA content frequency. Sub-diploid DNA peaks were quantified from the DNA content data.

Statistical analysis

Each experimental value is expressed as the mean ± standard deviation (SD). The scientific statistic software package GraphPad Instat version 3.05 was used to evaluate the significance of the differences between groups. Comparisons between groups were done using a one-way ANOVA followed by a Student-Newman-Keul's test, and the criterion of statistical significance was taken as $p < 0.01$ or $p < 0.001$.

Results

Acute toxicity of DMC in ICR mice

In the initial investigations, the mortality of mice was 0/3 in the three treated groups. Based on the results of the initial investigation, the new dosages (1,600 mg/kg, 2,900 mg/kg, and 5,000 mg/kg) are administered to the animals in the second test according to the Table 1. The mortality of mice was 0/1, 0/1, and 1/1 with doses of 1,600 mg/kg, 2,900 mg/kg, and 5,000 mg/kg, respectively. The LD_{50} value was estimated by the geometric mean on the doses of 2,900 mg/kg and 5,000 mg/kg. The LD_{50} value of DMC was 3,800 mg/kg, which meant that DMC was slightly toxic.

Plasma pharmacokinetics of DMC in nude mice

Figure 2 shows the plasma levels of free-form DMC in samples taken at various times from the nude mice treated with a single i.p. injection of DMC (150 mg/kg). During a period of 24 h, the plasma levels of free-form DMC rose to a peak at about 3 h, then declined rapidly to a low level at 12 h, and up to 24 h, the level was 2.749 μg/ml. This result suggests that DMC was quite stable in the blood of mice, and continuous administration of DMC should be beneficial to maximizing the

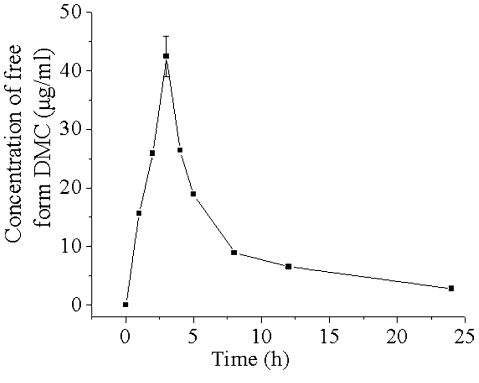

Fig. 2 Plasma levels of DMC in nude mice. Mice were i.p. injected once with DMC with a dose of 150 mg/kg and samples were taken at various times. Plasma levels of free-form DMC were determined by HPLC. The maximal value at 3 h after injection is presented as the mean ± SD of the three mice

effect of DMC in vivo, because at 24 h, the DMC level in blood dropped to a relatively low level.

In vivo antitumor activity of DMC in a human tumor xenograft model

The antitumor activity of DMC in vivo was evaluated by using a xenograft of human liver carcinoma. When tumors were treated with mitomycin-C and DMC of different doses, tumor growth suppression was observed. Figure 3a shows the curves of tumor growth in the nude xenografts of the liver cancer cells. The effects of mitomycin-C and DMC on the weights of the SMMC-7721 solid tumors are shown in Figure 3b. The average tumor weight was 1.42 ± 0.11 g, 1.12 ± 0.18 g, 0.74 ± 0.15 g, and 0.59 ± 0.12 g in the control group, 50-mg/kg, 100-mg/kg, and 150-mg/kg DMC injection groups, respectively. Furthermore, there was no substantial decrease in body weights in the mice treated with DMC compared with those treated with the control level (data not shown). This finding suggested that DMC had low toxic side effects.

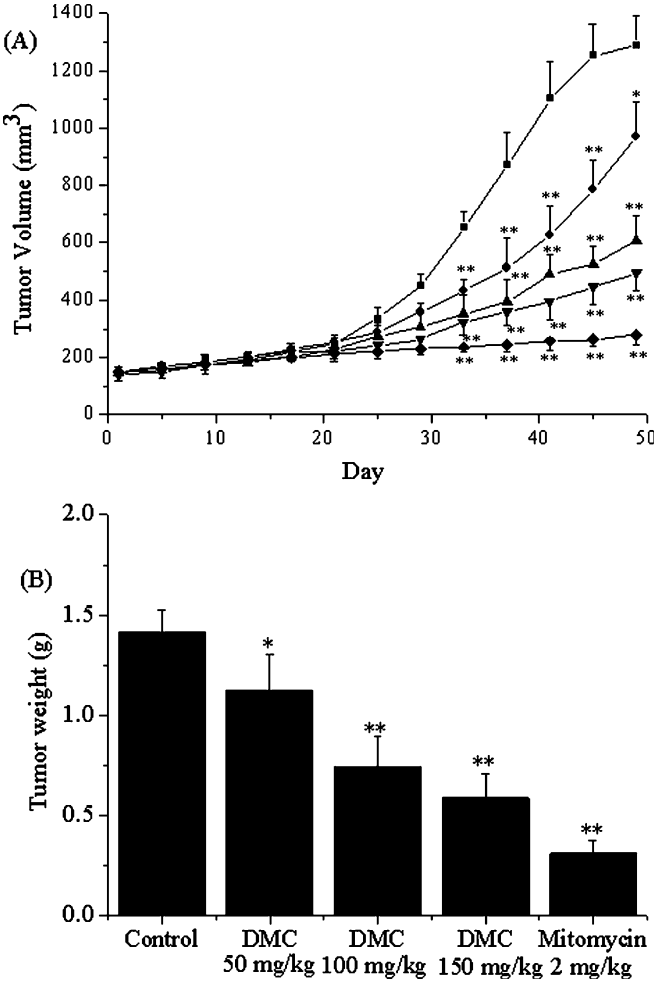

Fig. 3a, b Antitumor activity of DMC in vivo. **a** Effect of DMC in tumor volume. Groups were: control (*squares*); animals injected with DMC (50 mg/kg, i.p.) on days 1–14 (*circles*); animals injected with DMC (100 mg/kg, i.p.) on days 1–14 (*triangles*); animals injected with DMC (150 mg/kg, i.p.) on days 1–14 (*stars*); and animals injected with mitomycin (2 mg/kg, i.p.) on days 1, 3, 5, 7, 9, 11, 13 (*diamonds*). Data are expressed as mean ± SD of 6 samples. Significant difference from control: $*p < 0.01$; $**p < 0.001$. **b** Effect of DMC in tumor weight. Each column represents the mean ± SD for 6 mice. Significant difference from control: $*p < 0.01$; $**p < 0.001$

Effects of DMC on the population of hypodiploid cells in the tumors in mice

We examined the fact that DMC induces the appearance of a hypodiploid peak in the tumors in mice. As shown in Fig. 4, the sub-$G_{o/1}$ population in drug-treated groups increased with the dose of the drug increment. $4.80 \pm 0.61\%$, $16.42 \pm 0.91\%$, $20.80 \pm 1.45\%$, and $33.60 \pm 0.80\%$ of the cells in tumors were hypodiploid in the control group, 50-mg/kg, 100-mg/kg, and 150-mg/kg DMC injection groups, respectively. The treatment resulted in the appearance of a hypodiploid peak (A_0region) [14], probably due to the presence of apoptosing cells and/or apoptotic bodies with a DNA content less than 2n.

Discussion

Among the number of substances identified from plants, flavonoids represent one of the most important and interesting classes of biologically active compounds. The common synthone of the flavonoid family is that of chalcones, which, in the cyclized form, generates flavanones, flavones, isoflavones, and flavonols. Chalcones

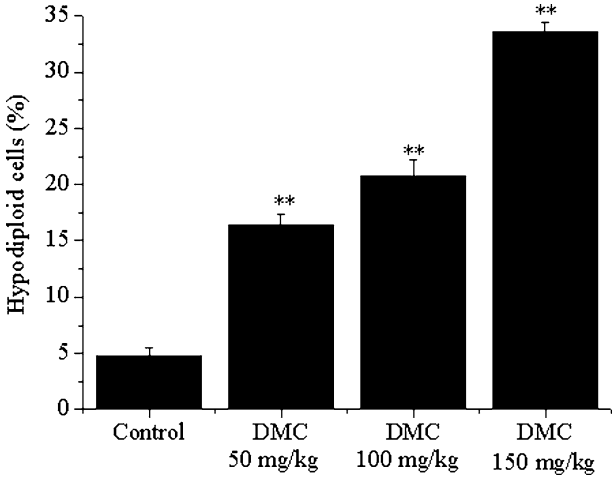

Fig. 4 The percentage of hypodiploid cells after staining with propidium iodide. Tumor tissues were processed to isolate cells, and then the cells obtained were stained with propidium iodide, and analyzed in a FACScan flow cytometer. Groups were: control; animals injected with DMC (50 mg/kg, i.p.) on days 1–14; animals injected with DMC (100 mg/kg, i.p.) on days 1–14; and animals injected with DMC (150 mg/kg, i.p.) on days 1–14. Data are presented as the mean ± SD of three tumors per group. Significant difference from control: $**p < 0.001$

have demonstrated cytotoxic [7, 8] and anticancer properties [9, 10]. In the last decade, various pharmacological activities of chalcone, such as anticancer and antioxidant activity [15], anti-invasive activity inhibitor of aromatase [16], 17β-hydroxysteroid dehydrogenase activity [17], cytotoxic and inhibitory effects on proliferation of leukemic [18], and antiangiogenic activity [19], have been described. However, there have been few reports on in vivo antitumor activity by chalcone compounds so far. In this paper, we attempted to determine whether 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC) could inhibit the growth of tumors in vivo.

Human tumor xenografts are now well established tools for the preclinical screening of anticancer drugs and are an integral part of the current NCI and EORTC disease-orientated strategies for drug screening [20]. Our results reveal that the average tumor weight was 1.42 ± 0.11 g, 1.12 ± 0.18 g, 0.74 ± 0.15 g, and 0.59 ± 0.12 g in the control group, 50-mg/kg, 100-mg/kg, and 150-mg/kg DMC injection groups, respectively. Furthermore, there was no substantial decrease in body weights in the mice treated with DMC compared with those treated with the vehicle. These results suggested that DMC, at a dose of 150 mg/kg, could remarkably inhibit the growth of tumor, with low side effects, in a solid human carcinoma xenograft model.

Previously, we showed that DMC could induce SMMC-7721 cell apoptosis in vitro (Ye CL, Liu JW, Wei DZ, Lu YH, Qian F (2004) In vitro antitumor activity of 2′,4′-dihydroxy-6′-methoxy-3′,5′- dimethylchalcone against six established human cancer cell lines. Pharmacological Research, accepted). In the present study, flow cytometry results indicated that the sub-$G_{o/1}$ population in the drug-treated groups increased with the dose of drug increment and $33.60 \pm 0.80\%$ of the cells in tumors are hypodiploid in the 150-mg/kg DMC injection group. These findings demonstrate that DMC can induce SMMC-7721 cell apoptosis both in vitro and in vivo. The mechanism of the antitumor of chalcones remains to be fully clarified. Further studies are now in progress in our laboratory to clarify the mechanisms of induction of apoptosis by DMC, such as bcl-2 gene expression, myc gene expression, and decreased activation of the NF-κB transcription factor.

The results of plasma pharmacokinetics of DMC in our study indicates that, at 3 h after DMC administration, the plasma level of free-form DMC was 42.47 ± 3.47 μg/ml, which was relatively high. Consecutive administrations of DMC in the following 14 days possibly caused the remain of a relatively high level of DMC.

Investigation of the acute toxicity is the first step in the toxicological investigations of a substance. Our result reveals that the LD_{50} value of DMC is 3,800 mg/kg, which means that DMC is slightly toxic. This result is consistent with the report of Havsteen [21]. It is not possible for humans to suffer acute toxic effects from the consumption of DMC, with the exception of a rare occurrence of an allergy. The margin of safety for the therapeutic use of DMC in humans, therefore, is very large and is probably not surpassed by any other drug in current use.

In conclusion, DMC could remarkably inhibit the growth of tumors in a solid human carcinoma xenograft model. To our knowledge, this is the first time that chalcone compounds were applied to a human tumor xenograft model. Our results suggest that DMC is a promising chemotherapeutic agent with low toxicity and high efficacy, and holds great promise for further studies.

Acknowledgements This work was supported by the Key Disciplinary Foundation of Shanghai, China.

References

1. Mori A, Nishino C, Enoki N, Tawata S (1988) Cytotoxicity of plant flavonoids against Hela cells. Phytochemistry 27:1017
2. Hirano T, Oka K, Akiba M (1989) Antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast carcinoma cell ZR-75-1. Res Commun Chem Pathol Pharmacol 64:69
3. Lin YM, Chen FC, Lee KH (1989) Hinokiflavone, a cytotoxic principle from Rhus succedanea and the cytotoxicity of the related biflavonoids. Planta Medica 55:166
4. Cushman M, Nagarathnam D (1991) Cytotoxicities of some flavonoid analogues. J Nat Prod 54:1556
5. Woo AYH, Waye MMY, Kwan HS, Chan MCY, Chau CF, Cheng CHK (2002) Inhibition of ATPases by *Cleistocalyx operculatus*: a possible mechanism for the cardiotonic actions of the herb. Vascular Pharmacology 38:163
6. Ye CL, Lu YH, Wei DZ (2004) Flavonoids from *Cleistocalyx operculatus*. Phytochemistry 65:445
7. Yit CC, Das NP (1994) Cytotoxic effect of butein on human colon adenocarcinoma cell proliferation. Cancer Lett 82:65
8. Satomi Y (1993) Inhibitory effects of 3-methyl-3hydroxychalcone on proliferation of human malignant tumor cells and on skin carcinogenesis. Int J Cancer 55:506
9. Wattenberg LW, Coccia JB, Galhaith AR (1994) Inhibition of carcinogen-induced pulmonary and mammary carcinogenesis by chalcone administered after carcinogen exposure. Cancer Lett 83:165
10. Edwards ML, Stemerick DM, Sunkara SP (1988) Chalcone derivatives useful in controlling growth of tumor tissue and their preparation. Eur Pat 288:794
11. Lorke D (1983) A new approach to practical acute toxicity testing. Arch Toxicol 54:275
12. Liu JD, Chen SH, Lin CL, Tsai SH, Liang YC (2001) Inhibition of melanoma growth and metastasis by combination with (-)-epigallocatechin-3-gallate and dacarbazine in mice. J Cell Biochem 83:631
13. Fua LW, Zhang YM, Liang YJ, Yang XP, Pan QC (2002) The multidrug resistance of tumour cells was reversed by tetrandrine in vitro and in xenografts derived from human breast adenocarcinoma MCF-7/adr cells. Eur J Cancer 38:418
14. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 139:271
15. Anto RJ, Sukumara K, Kuttan G, Rao MNA, Subbaraju V, Kuttan R (1995) Anticancer and antioxidant of synthetic chalcones and related compounds. Cancer Lett 97:33
16. Parmar VS, Sharma NK, Husain M, Watterson AC, Kumar J, Samuelson LA, Cholli AL, Prasad AK, Kumar A, Malhotra S, Kumar N, Jha A, Singh A, Singh I, Himanshu, Vats A, Shakil NA, Trikha S, Mukherjee S, Sharma SK, Singh SK, Kumar A, Jha HN, Olsen CE, Stove CP, Brackef ME, Mareelf MM (2003)

Synthesis, characterization and in vitro anti-invasive activity screening of polyphenolic and heterocyclic compounds. Bioorganic Medicinal Chemistry 11:913
17. Le Bail JC, Pouget C, Fagnere C, Basly JP, Chulia AJ, Habrioux G (2001) Chalcones are potent inhibitors of aromatase and 17β-hydroxysteroid dehydrogenase activities. Life Sciences 68:751
18. Saydam G, Hakan Aydin H, Sahin F, Kucukoglu O, Ericiyas E, Terzioglu E, Buyukkececi F, Bedii Omay S (2003) Cytotoxic and inhibitory effects of 4′,4′-dihydroxy chalcone (RVC-588) on proliferation of human leukemic HL-60 cells. Leukemia Res 27:57
19. Nam N, Kim Y, You Y, Hong D, Kim H, Ahn B (2003) Cytotoxic 2′,4′-dihydroxychalcones with unexpected antiangiogenic activity. Eur J Med Chem 38:179
20. Winograd B, Boven E, Lobbegoo MW, Pinedo HM (1987) Human tumor xenografts in the nude mouse and their value as test models in anti-cancer drug development. In Vivo 1:1
21. Havsteen BH (2002) The biochemistry and medical significance of the flavonoids. Pharmacol Therapeut 96:67