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Cytotoxic effect of esculetin on myeloma and myelogenous leukemic cells.

Khedhir Hassan Ali Al-Jorany, Noor Al-Huda Ali A.H. Saeed

Dept. of Biology, College of Science, University of AL-Mustansiriya

Abstract:

The cytotoxic effect of Esculetin to mouse myeloma and human acute myelogenous leukemia cells invitro was evaluated. Tumor cells exposed to Esculetin in culture showed an exponential cytotoxic responses. A decreased viabilities of both leukemic cells were noticed with 20, 40 and 80 µg / ml Esculetin. Besides, myeloma cells showed a progressive depletion of viability when exposed continuously for 1,2,3 and 4 days, whereas , pretreatment of myeloma cells with Esculetin for 1,2,4 and 24 h followed by removal of the chemical and culturing the cells for 3 days indicated that the time the cells much effected was 24 h post exposure. At this exposure time , exposure to 10 µg / ml decreased the able cell number to 24.66 × 10-4 cells / ml versus 62.66 × 10-4 cells / ml in the control . t – test = 5.16 , p = 0.007 95 % CI = 17.5 – 58.5. Like wise , a 20 µg / ml concentration of esculetin decreased viable cells number to 23.5 × 10-4 cells / ml versus 62.66 × 10-4 cells / ml of control. T-test 7.39 , P = 0.002. 95 % CI = 24.5 – 53.8.

The efficiency of Esculetin in protecting mice implanted with syngeneic myeloma was studied . The results of invivo experiment demonstrated that the chemical could protect mice from lethal dose of the experimentally implanted tumor . The protection value reached 66.7 % in animals treated with 200 or 400 µg Esculetin for 10 consecutive days. For 200µg drug plus myeloma versus myeloma only , chi – square value was 3.086 p=0.08 and 95% CI = 0-2.4. However , at 400 µg drug plus myeloma versus myeloma only , the chi – square analysis showed a value of 4.17 , P=0.04 95% CI = 0.02 – 1.3. Besides , the survival of the animals was increased . The therapeutic relevance and speculated mechanism of Escletin cytotoxic effects are discussed.

In conclusion , it seems that esculetin has antiproliferative effects to mouse myeloma and to human myelogenous leukemia cells.

Key words: *Esculetin, Anti-tumor, Myeloma, Myelogenous leukemia*

Introduction:

The anti-proliferation and cytotoxic effects of esculetin against human T,B-lymphoid , myeloid , erythroid and mastocytoma cell lines was demonstrated. Esculetin was found to inhibite dose-dependently the proliferation of most cell lines (1).

Cinnamic acid derived chemicals are a natural products that occur widely among plants. They are involved in allelopathic that occur in nature (2). Among these allelopathic chemicals , Esculetin (6,7-dihydroxy-coamarin) is a well known lipoxygenase inhibitor (3,4). Esculetin also inhibits and stimilats cyclooxygenase at a given concentrations (5,6).

Esculetin caused a time and dose-related increase in adipocyte apoptosis and a decrease in this cell viability.

Esculetin-mediated inhibition of adipocyte differentiation occurred during early , intermed and late stages of the differentiation process.(7). Inhibition of 5-lipoxygenase and skin inflammation with esculetin was reported.(8).

Esculetin showed biphasic effect on human mammary MCF-7 cell line. At low concentration, it stimulated cells to grow, while at higher concentration it had anti-proliferative effect. It also has estrogenic effect invivo.(9).

The compound decreases migration and invasion of vascular smooth muscle cells by suppressing matrix metalloproteinase-9 (MMP-9) gene expression, providing an effective therapeutic treatment of atherosclerosis.(10).

Corresponding Address:
Noor Al-Huda Ali A.H. Saeed
Dept. of Biology, College of Science, University of AL-Mustansiriya
Email: nnhuda@yahoo.com

This report describes the cytotoxic effect of Esculetin on mouse myeloma and human acute myelogenous leukemia cultured invitro, in addition the effect of the chemical on invivo tumor model is presented. All indications so far obtained suggest that Escletin has cytotoxic effects on primary cultured and proliferating tumor cells used in this study.

MATERIALS AND METHODS:

Balb/c (H-2d) haplotype mice 6-8 weeks old maintained in our animal facility was used throughout the study .

Sp2 myeloma tumor cells originally derived from NS-1 myeloma of Balb/c mice (11) was provided from flow laboratories (Ayrahire , Scotland). The cells were routinely grown and passed in RPM1-1640 medium supplemented with pyruvate, 5×10-5 M 2-Mercaptoethanol, 10% fetal bovine serum and antibiotics (Flow laboratories).

Human acute mylogenous leukemia cells were obtained from untreated , 13 years old male, first admitted with diagnosed, acute myelogenous leukemia , at Central Medical City, Baghdad. Leukemia cells obtained from buffy coat which was highly enriched with these tumor cells as revealed by differential count of separated cells. These buffy coat predominant cells were used in the chemosensitivity test described below.

Esculetin was provided by Fluka, AG , Switzerland. The chemical was dissolved in deionized sterile water, solubilized with trace quantity of normal sodium hydroxide and mixed 1:1 with 2X RPM1-1640 medium. The substance was frozen at -20C0 until used.

One hundred microliter of Escletin in medium was placed into a 96-wells, flat microtitration plate (Flow) and serially diluted to 160, 80, 40, 20, 10 and 5 µg / ml with RPM1 medium.

Chemosensitivity testing

Myeloma cells at logarithmic growth phase were collected by centrifugation, washed 2 times with medium and dispensed in 100 µl volume at 5×104 cells/well in a 96-wells, flat sterile microtitration plate. The myelogenous leukemic cells were treated as above and were plated at 1×105 cells/well in 100µl RPMI medium.

Both myeloma and myelogenous leukemic cells were treated with the diluted Esculetin to give a final volume of 200 µl. They were kept at 37C0 in a humidified, 5% CO2 incubator for 3 days unless otherwise specified. Cells viability was checked by trypan blue exclusion test (12).

Survival of cells with time calculated from survival fractions which were obtained by dividing the mean viable number of the treated cells over that of untreated cells.

Esculetin pretreated experiment was done by exposing the tumor cells to the chemical for 1,2,4 and 24 h. The chemical was washed out and cells were resuspended in 200 µl medium and cultured for 3 days as mentioned above.

Animal chemotherapy

Groups of Balb c mice histocompatable with SP2- myeloma were injected intraperitoneally with 5×106 cells in 0.5 ml saline using 18 Gauge needle and syringe. Mice implanted with tumor cells were left for 24 hr before given the chemical. Chemical prepared in phosphate buffer saline (PBS) and solubilized, was given at 200 or 400µg / 0.2 ml PBS , every day for 10 consecutive days . Control mice were given myeloma with out chemical or PBS alone. Mice survival was recorded for 31 days post-tumor implantation and cumulative survival was estimated.

Statistical analysis was done by using epidemiological statistics program.t – test and chi- square test were used appropriatly.

RESULTS:

Cytotoxic effects of Esculetin on myeloma cells

Survival of myeloma cells exposed continuously to Esculetin for different times and different concentrations is shown in Fig. 1. As se en in Fig. 1 there is a linear relationship between exposure time at a given Esculetin concentration and surviving myeloma cells. Depletion of viable cells was seen after 1,2,3 and 4, days at 80 and 40 µg /ml Esculetin treated cells. At 20 µg / ml concentration cells survival was increased at days 1,2,3 and 4 continous exposure.

Cytotoxic effect of Esculetin on myelogenous leukemia and myeloma cells

Human acute myelogenous leukemia cells, freshly isolated from patient and exposed to Esculetin continuously for 72 h showed a decrease in viable cell number as seen in fig. 2. The viability of tumor cells decline with increasing concentrations of the chemical. On the other hand , myeloma cells showed more susceptibility than myelogenous leukemia cells fig. 2.

Esculetin pretreatment effects on myeloma

The influence of varying the exposure time of the chemical to proliferating myeloma is presented in table 1. Cells exposed to the chemical for 1,2,4 and 24 h , then the chemical was removed and the cells were resuspended in fresh medium and growth was resumed for 3 days. There were a niglegable effect of esculetin for the cells when they were exposed for 1 and 2 h. At 4 h the cells respond to some extent, however at 24 h there was a pronounced cytotoxic effect compared to control. At 10 µg /ml esculetin treatment, viable cell number was 24.66 ×10-4 cells/ml in test versus 62.66 -4 cells/ml in control . t-test = 5.16 , p = 0.007 , 95% CI = 17.5 – 58.5 . The same significant effect was seen at 20µg / ml esculetin treatment.

Efficiency of esculetin on myeloma in vivo

The effect of esculetin on myeloma implanted into mice was investigated. A dose of 200µg given to mice for 10 consecutive days after myeloma implantation provided 66.7λ protection. Mice imptanted with myeloma only showed 16.7 λ protection p = 0.08 . Mice treated with 400 µg for 10 consecutive days gave about the same protection. Chi – square = 4.2 , p = 0.04, 95% CI = 0.02 – 1.3 . (table 2).

The prolongation of mice survival was estimated.

In mice treated with 400µg esculetin there were differ-

ences in time of tumor appearance and incidence of animals deaths in treated and non treated animals.

The appearance of tumor was on day 10 in treated animals in contrast non treated animals that showed tumor growth and ascietic fluid accumulation at day 6. Besides, ascietic fluid accumulation differences were evident among treated and untreated animals (date not shown). Treated animals showed less ascietic fluid accumulation compared to untreated animals.

As we can see in fig. 3 , the incidence of death began at day 16 and about 75% of the untreated animals were dead at day 21 copared with those given esculetin which showed 25% of death, whereas animals given esculetin only exhibited no effect.

DISCUSSION:

The cytotoxicity of esculetin, a natural product on tumor cells is presented. The chemical demonstrated a cytotoxic effect on growing myeloma cells. In addition , it killed acute myelogenous leukemic cells also. As seen in fig. 1 there is a continous depletion of proliferating cells. There was no evidence of killing all tumor cells exposed for 1 day to the chemical which might implicate a prerequeste for the cells to reach a metabolic point were the cells can be affected. This conclusion is derived based on the non synchronized nature of myeloma cells used in the present study. This interpretation is supported by the data given in table 1. The chemical started it's greatest influence to kill the cells at 24 h post exposure which might implicate a biochemical basis for it's cytotoxic potential.

Pertaining to the cytotoxicity of esculetin , there was no cell arrest (no change in cell number) , but rather a cell killing was affected as evidenced from viability measurements and total cells count.

Regading it's efficiency in vivo, the chemical gave protection to mice implanted with myeloma cells. The protection reached a valus of 66.7 λ in mice treated wiyh 200 or 400 µg esculetin, besides, the incidence of death was delayed (table 2 fig. 3).

In parallel with our reported effect of esculetin on myeloma and myelogenous leukemic cells, it was shown that esculetin inhibits mammary cell line MCF-7 in vitro. (2).

As to the findings reported here, the mechanism of esculetin cytotoxic effects on proliferating cells is unknown at the present time. Nevertheless, esculetin was shown to inhibit 5 and 12-lipoxygenases of mammalian cells in vitro (3,7,8). The inhibition of lipoxygenases was effected by low concentration of the chemical (10). In addition, the chemical has shown inhibitory effect on cyclooxygenase activity at a concentration more than 10µM. whereas, at lower concentrations, a stimulatory effect on the enzyme was exhibited(9). Apoptosis-induced cell death affected by esculetin might be a mechanism of cell death (3).

If a distinct mechanism(s) that affect DNA replication or other cellular targets or the involvement of cyclooxygenase inhibition in the cytocidal effect of esculetin remain to be elucidated.

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Table 1: Effect of Esculetin pretreatment on total viable myeloma number after three days. (Number × 10^{-4}).

Esculetin Concentration (µg / ml)	Hours			
	1	2	4	24
0	42.00 ± 6.32	44.00 ± 12.11	45.66 ± 16.77	62.66± 9.01
10	56.33 ± 7.50	47.50 ± 4.12	39.25 ± 3.68	24.66 ± 8.96 *
20	42.00 ± 7.25	43.25 ± 10.24	34.50 ± 3.00	23.50 ± 1.29 **

SP-2 myeloma were treated with Esculetin for the times indicated, washed out of chemical and regrown in fresh medium for the rest of the 3 days period then total viable cell number was enumerated.
Values given represent triplicate's means ± SD.
** t = 5.16*
p = 0.007
95 % CI = 17.5 – 58.5
*** t = 7.39*
p = 0.002
95 % CI = 24.5 – 53.87

Table 2: Curing of mice bearing myeloma by esculetin .

Treatment	Esculetin dose (µg)			
	200		400	
	No. dead/total	% protection	No. dead/total	% protection
Drug + Myeloma	2 / 6	66.7 *	4 / 12	66.7 **
Myeloma only	5 / 6	16.7	9 /12	25.0
Drug only	0 / 6	100.0	0 / 12	100.0

Syngeneic BALB / c mice were implanted with SP-2 myeloma I.P, left for one day, treated for 10 consecutive days with esculetin and survival was recorded for 31 days.
** 200*
Drug + myeloma vs myeloma only
Chi – square 3.086
P = 0.079
CI = 0 – 2.4
*** 400*
Chi – square 4.196
P = 0.041
CI = 0.02 – 1.3

Legends to figures

Figure 1: Survival of mouse SP2 myeloma in the presence of Esculetin.

Myeloma cells were exposed to 80 µg/ml (●) , 40 µg/ml (○), 20 µg/ml (■) for times indicated, viability was assessed by Trypan blue. Date derived from triplicate readings and means were represented in the figure.

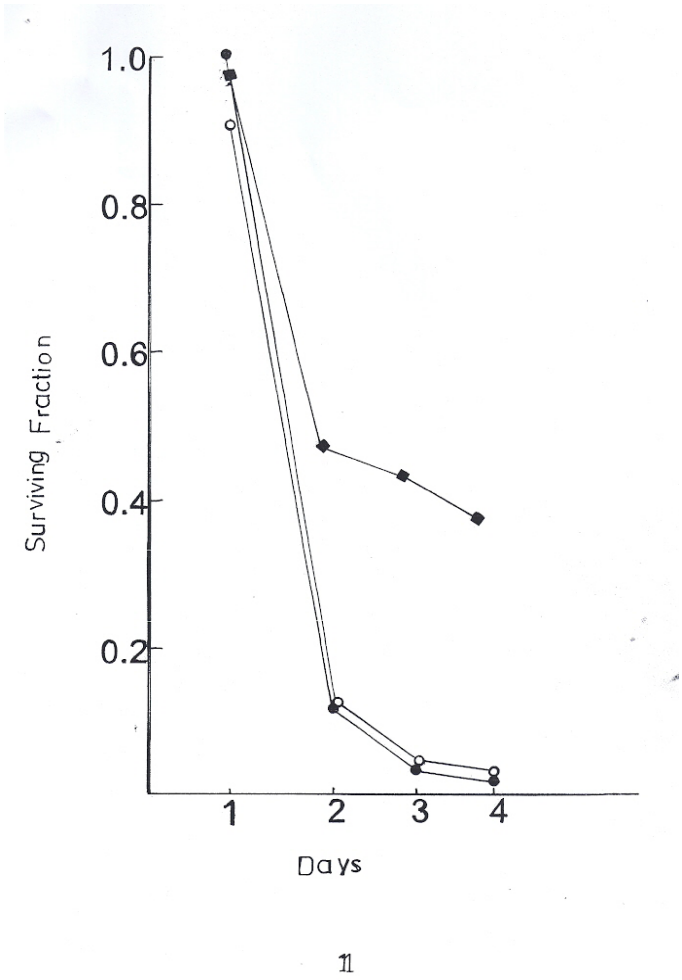
Figure 2 : Survival of mouse myeloma and human myelogenous leukemia cell in the presence of Esculetin.

Survival of mouse SP2 myeloma (○) and human acute myelogenous leukemia cells (●)in the presence of indicated concentration of Esculetin. Tumor cells were exposed for 3 days and viability was assessed by Trypan blue.

Data derived from triplicate readings and means were represented in the figure.

Figure 3 : Cummulative death of mice implanted with myeloma intraperitoneally.

Mice received myeloma were treated one day post-implantation with 400 µg Esculetin daily for 10 consecutive days, control mice received myeloma only .



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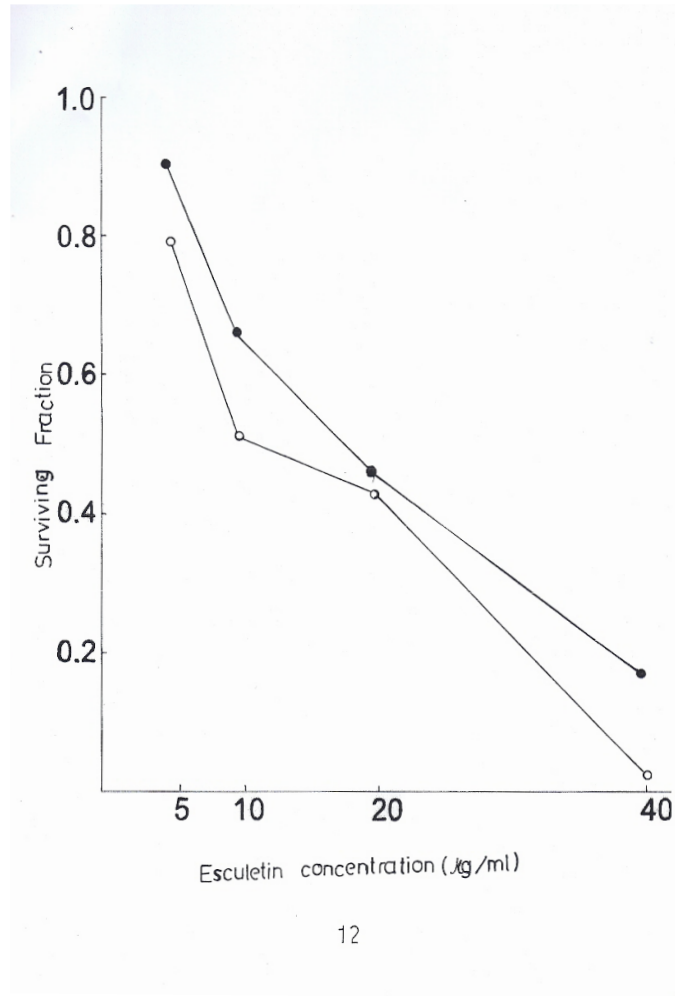

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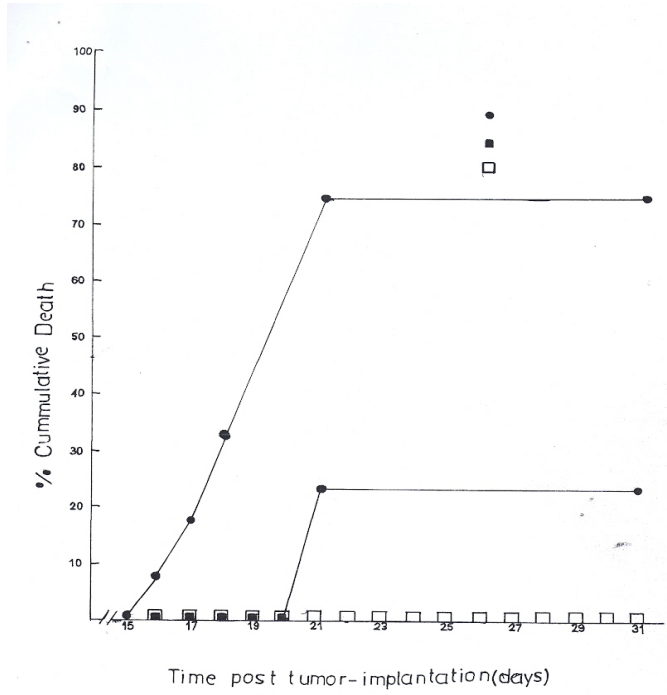

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التأثير السمي للأسكولتين لخلايا المايلوما والخلايا السرطانية النقوية

خضرحسن علي الجوراني، نورالهدى علي عبدالحسين سعيد

قسم علوم الحياة/ كلية العلوم/ الجامعة المستنصرية

الخلاصة:

تم تقدير التأثير المسمم لخلايا الاسكولتين تجاه خلايا الفأر المايلومية والخلايا السرطانية النقوية العائدة للانسان. اظهرت الخلايا المعرضة للاسكولتين استجابة تسممية تصاعدية. اظهرت الجرع 20 و 40 و 80 مايكروغرام / سم3 تأثيرا في انخفاض الحيوية لكلا النوعين من الخلايا السرطانية. اضافة الى ذلك فان خلايا المايلوما اظهرت نقصانا مستمرا في الحيوية عند التعرض ليوم او ثلاثة او اربعة ايام. وقد كان بدء التاثير في الاربع والعشرين ساعة بعد التعرض. اذ كان في هذا الوقت وبتعرض 10 مايكروغرام/سم3 عددا يقدر بـ 104 × 24.66 خلية / سم3 للخلايا المعرضة مقارنة ب 104 × 62.66 للخلايا غير المعرضة لمادة الاسكولتين.

كما ان التعرض لجرعة 20 مايكروغرام / سم3 ادى الى نتائج مقاربة (t = 5.16 , P = 0.007 , 95 % CI = 17.5 – 85.5).

تمت دراسة تاثير الاسكولتين في حفظ الفئران المستلمة لخلايا المايلوما . اشارت النتائج الى ان الاسكولتين له تاثير حافظ لتلك الفئران.اذ بلغت نسبة الحفظ 66.7 % للحيوانات المعاملة بجرع 200 او 400 اسكولتين لمدة 10 ايام متتالية واشارت نتائج التحليل الاحصائي ان النتائج ذات شأن للجرعتين. تم مناقشة امكانية المادة العلاجية والية عمل المادة المسممة