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Research Article Pharmacology

International Journal of Pharma and Bio Sciences

ISSN
0975-6299

NON-MUTAGENIC AND IN VITRO TOXICITY EVALUATION OF EMBELIN ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND MOUSE MACROPHAGES

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ABSTRACT

The development of novel drugs that have chemotherapeutic activity against cancer is a great challenge. The discoveries of these drugs are very difficult, because they must be able to destroy the tumor cells without causing adverse side effects. Significant innovations come forth when human cell lines are used for vetting drugs and other chemicals. Embelin, a benzoquinone is reported to possess anticancer activity on various cancer cell lines. The present investigation was undertaken to determine the toxicity profile of embelin on human peripheral blood lymphocytes and murine macrophages. The effect of embelin on growth and viability of cells was determined by MTT assay. Further embelin was tested for mutagenicity by the Ames test, in *Salmonella typhimurium* strains with and without metabolic activation. Results implicate that embelin was non mutagenic in the tester strains used in the study and did not adversely influence the proliferation of lymphocytes and macrophages and the IC_{50} was found to be 78.7µg/ml and 128.54µg/ml respectively.

KEYWORDS: Embelin, Cytotoxicity, Ames test, lymphocytes

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INTRODUCTION

Embelin, a benzoquinone and XIAP inhibitor is known to possess anticancer activity against the various cell lines. Embelin exhibits antifertility, antitumor, anti-inflammatory, analgesic, antioxidant, hepatoprotective, wound healing and antibacterial[1, 2, 3] activities. It also possess a wide spectrum of biological activities with a strong inhibition of NF-KB and down regulation of variety of gene products involved in tumour cell survival, proliferation, invasion, angiogenesis and inflammation[4]. The chemical structure of embelin has resemblance with that of natural coenzyme Q10 (ubiquinones) and the role of this is well defined in various biochemical protective mechanisms[5]. It is a potent, nonpeptidic molecule because of the presence of both quinone and phenolic groups on the same ring. Recently, several phenolic compounds have demonstrated antimutagenic effect on various mutagens or carcinogens[6]. It has been shown that a procedure called the Ames test is a safe way to assess mutagenicity of various agents. The Ames test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs. The test is also used for submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides. The Ames *Salmonella*/microsome mutagenicity assay (*Salmonella* test; Ames test) is a short-term bacterial reverse mutation assay, specifically designed to detect a wide range of chemical substances that can produce genetic damage and leads to gene mutations. The test employs several histidine dependent *Salmonella* strains, each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagenic agent is added to the plate, the number of revertant colonies per plate increases, usually in a dose-related manner[7]. In this study, mutagenic effect of embelin was assessed using the Ames assay. Further, the antitumor activity of the drug is generally based on the mechanism of action as to how these agents are effective in killing the tumor cells or in preventing the growth of the tumor cells. Tumor cells unlike the normal cells fail to respond to homeostatic control mechanism and there by continuous cell proliferation occurs[8, 9]. The kinetic techniques help us determine the tumor growth in relation to normal cell growth[10]. It is commonly seen that most of the anticancer drugs used produce non selective cell killing of normal as well as cancerous tissues and they act more against tumor than normal cells. Chemotherapeutic drugs kill healthy cells as they pass through the body on their way to the cancerous target. Also, they have adverse side effects and sometimes end up being ineffective of resistant. This may be due to the quantity or dosage of these drugs circulating in the body. It may be true that the effect of chemotherapeutic agents on tumor cells can be more than ten thousand times greater than the normal cells[11]. Normal cell systems can theoretically withstand greater cellular losses to chemotherapy than can tumor cells[12]. Therefore research needs to be done in search for the drugs that can exploit the unique biochemical difference between normal and cancerous cells. Henceforth in the study, embelin is used to check for the selective cytotoxic effects using the normal human peripheral blood lymphocytes and macrophages. Using human peripheral blood lymphocytes as an alternative source of hematopoietic cells is methodological simple and straight forward. It could possibly be used to complement the progenitor models, and serve as a model for a differentiated and nonproliferating peripheral blood cell. So here in the study, we attempt to make a toxicity profiling with normal cells. The test not only determines the toxicity, but also the dosage levels at which these can produce the toxicity. All anticancer drugs are highly cytotoxic agents and may be toxic to normal cells especially the rapidly

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dividing bone marrow cells, fetal cells, germ cells etc. In order to evaluate the cytotoxic effects of embelin MTT assay was performed on both lymphocytes and macrophages in vitro.

MATERIALS AND METHODS

i. Preparation of stock solution of embelin

Embelin was procured from Sigma-Aldrich Co. (St. Louis, MO, USA). It was solubilised in DMSO (final concentration of DMSO is 0.1%) and various aliquots from stock solutions were used throughout the assays indicated.

ii.Test to confirm the non-mutagenic effects of embelin- Ames test [13]

The non-mutagenic property of embelin was not reported previously. As embelin exhibits potent anticancer activity, it becomes further necessary to check the anti-mutagenic effects of embelin. This test was performed using standard plate incorporation procedures as escribedpreviously[13]. The test was carried in the presence and absence of liver S9 mix. Two histidine deficient (his-) tester strains of *Salmonella typhimurium* namely TA-98 and TA-100 were procured from Microbial Type Culture Collection, MTCC, Chandigarh- India and were used to detect the frame shift and base pair substitution mutagens respectively. Prior to the use in the assay, strains were checked for the presence of strain specific markers. The S9 fraction of the liver was prepared following standard methods reported earlier [12]. The fraction was aliquoted in 1-2ml portions and stored at -80 C. The S9 mix was freshly prepared with 8mM MgCl2, 33mM KCl, 5mM Glucose 6 Phosphate, 4mM NADP, 100mM Sodium Phosphate and the S9 fraction at a concentration of 0.1ml/ml of mix. For the test, Embelin was used at concentration of 25 µg, 50 µg and 100 µg per plate. NPD, 4-nitro-o-phenylenediamine (200µg/plate) and SA, Sodium Azide (1µg/plate) were used as positive mutagens for TA-98 and TA-100, respectively. In the presence of S9 mix, 2-AF, 2-Amino fluorine (20µg/plate) was used as a positive mutagen for both the strains as it required a metabolic activator. The number of revertants was counted and tabulated.

iii. Blood Withdrawal

Fresh human peripheral blood was drawn by venipuncture from healthy volunteers aging between 18-20 sticking on to the regulations laid down by the Helsinki protocol. Post collection, the blood was aseptically transferred to sterile disposable centrifuge tubes containing EDTA. This was used for the isolation of lymphocytes within two hours of collection.

iv. Isolation and culture of lymphocytes

Lymphocytes were separated from the freshly collected blood following the protocol described previously[14]. Lymphocyte separation of whole blood was based on density gradient centrifugation using Separation media (HiSep TM-LSM 1001) procured from Himedia Laboratories Private Ltd, Mumbai, India. Lymphocytes having >90% of viability (as ascertained by trypan blue staining) were suspended in 1ml of complete DMEM containing 10% FBS. Cell count was adjusted to 5×10^6 cells /ml and cells were plated onto 96 well microtiter plates and incubated at 37˚C with 5% CO2 and 95% humidity in a CO2 incubator (Forma Scientific, USA).

v. Procurement of Mouse macrophage cell line RAW 264.7 and culture

The murine macrophage cell line RAW 264.7 was procured from National Center for Cell Science, Pune, India. The cells were maintained in DMEM medium supplemented with 10% Fetal Bovine Serum and antibiotic/antimycotic solution. On confluency, the cells were detached with Trypsin- EDTA and subcultured in $25cm^2$ tissue culture flasks at a split ratio of 1: 3 and for further arriving at confluency were plated onto 96 well microtiter plates for the assays or cryopreserved in appropriate medium in liquid nitrogen for further use.

vi. MTT assay [15]

This was performed following the method described previously[15]. The assay was optimized for the experiment. Briefly, 5×10^6 cells per well was cultured in 96 well plate overnight and pretreated with various concentrations of Embelin and allowed to proliferate for 72 hours. After 72 hours of proliferation 25 µl of MTT

(10mg/ml in PBS) was added to the wells and plates were incubated for 5 to 6 hours and the formazan crystals formed were solubilised by adding 75 µl of DMSO and the plate was read at 570nm.The concentrations that inhibited cell growth by 50% (IC_{50}) after 72hours of treatment were calculated based on the survival rate compared with the untreated cells.

vii. Data Analysis and Statistical methods

In vitro experiments were carried out using three cultures, each time for each treatment and then repeated twice. Results were given as means of SD. Statistical differences were assessed by Student's t-test, comparisons were made with untreated control and embelin treated cells and values were expressed as follows $^{***}P< 0.001$; $^{**}P< 0.01$; $^{*}P< 0.05$.

RESULTS AND DISCUSSION

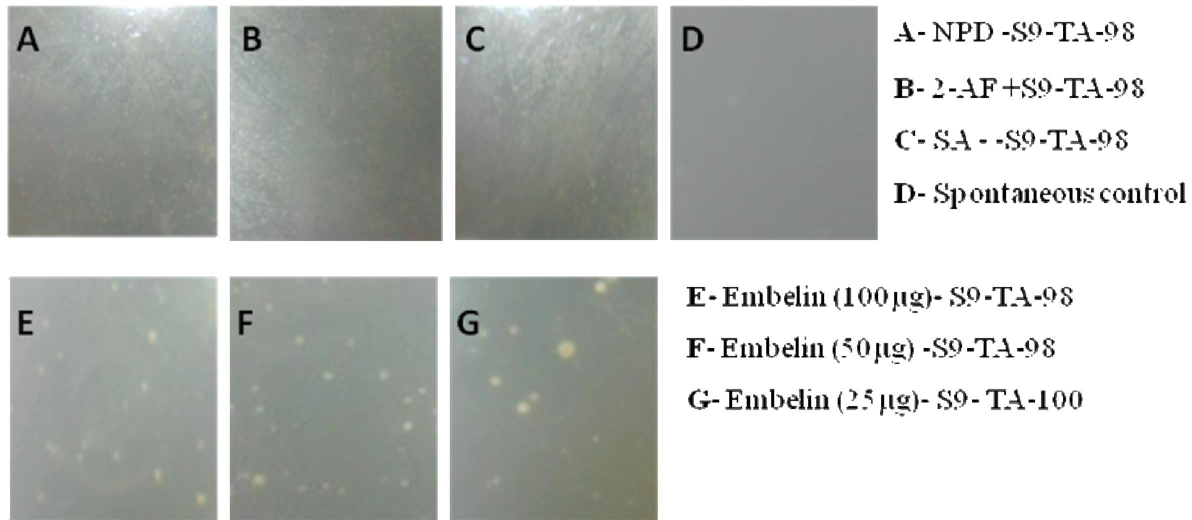
It is essential to evaluate the biohazard nature of chemicals having potential as chemotherapeutic agents in order to work out the risk-benefit situation for the use of these compounds. The *S.typhimurium* mutagenicity system provides an efficient pre-screen to detect potentially hazardous chemicals[16]. The standard plate incorporation methods for the Ames test using *Salmonella typhimurium* standard tester strains TA-98 and TA-100 exposed to embelin with and without S9 metabolic activation were performed. The Ames test without S9 metabolic activation can only detect direct mutagens while with S9 metabolic activation allows the detection of indirect mutagens, often caused by conjugation reactions of metabolic oxidation systems. Table 1 present the reversion response of the *Salmonella typhimurium* tester strain to the different dilutions of the embelin (100 µg/plate, 50 µg/plate, 25 µg/plate).

Table 1
Mutagenic effects of Embelin on S.typhimurium TA-98 and TA-100 strains

Test Substances	Concentration (µg/plate)	TA-98		TA-100	
		-S9	+S9	-S9	+S9
NPD	200 µg/plate	5300±371***	_	_	_
2-AF	20 µg/plate	_	4774±334.18*	_	1326±92.82**
SA	1 µg/plate	_	_	432±29.37*	_
Spontaneous control	_	9±0.63	33±2.24	146±9.92	79±5.53
Embelin (100µg)	100 µg/plate	17±1.19*	16±1.08***	54±3.67*	49±3.43*
Embelin (50 µg)	50 µg/plate	22±1.54*	16±1.11*	15±1.08*	52±3.64*
Embelin (25 µg)	25 µg/plate	22±1.50*	12±0.84*	44±3.08*	75±5.25*
DMSO (1%)	200 µl/plate	41±2.87*	12±0.97	73±5.12**	71±4.97*

Values are expressed as mean ±SD, Students 't' test - Comparisons are made with the control
P< 0.001; **P< 0.01; P< 0.05; NS: Non significant.

Figure 1
Revertant colonies as observed in AMES test



The results revealed that embelin was non-mutagenic towards the *Salmonella typhimurium* strains used for the assay with and without metabolic activation. The average His+ revertants observed for all the tester strains caused by the embelin at all the concentrations with and without metabolic activation did not satisfy the criteria for mutagenicity. There was no notable dose-dependent increase in the number of revertants (Fig.1) and the numbers of revertants were all either not equal to or greater than twice that of the negative control. There was also no decrease in the number of revertant colonies to levels far below the negative control (spontaneous reversion) which could also be classified as toxic. Results of the MTT assay indicate that the lymphocytes treated with embelin (10 µg/ml to 100 µg/ml) did not show any significant decrease in proliferation at low doses but showed a marked decrease in proliferation at high doses (Fig.2) as compared to control. The decrease in proliferation observed at high doses was found to be statistically significant ($P<0.001$). IC_{50} value of embelin were found to be 78.7µg/ml on human peripheral blood lymphocytes. Further the effect of embelin on the proliferation of macrophages was determined and IC_{50} value was found to be 128.54µg/ml (Fig.3).

Figure 2
Effect of embelin on proliferation of human peripheral blood lymphocytes

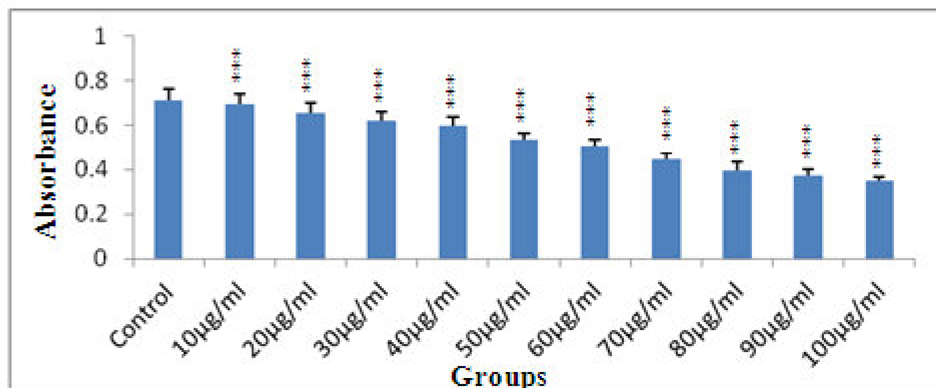
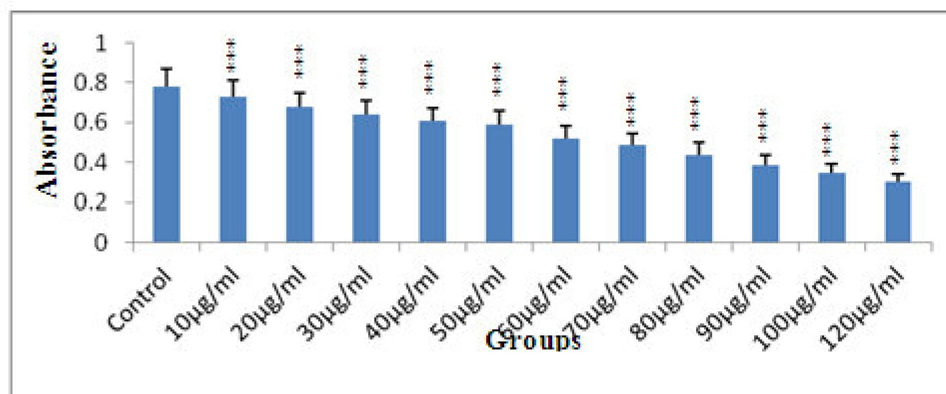


Figure 3
Effect of embelin on proliferation of murine macrophages



CONCLUSION

We report for the first time, the non-mutagenic potential of benzoquinone and apoptosis activator embelin on *Salmonella typhimurium* tester strains TA-98 and TA-100. The results also indicate that the IC_{50} values of embelin are quite high on lymphocytes and macrophages implicating its less toxic effects on normal cells as against cancer cells. This could be because of the fact that embelin has affinity for cancer cells that express more levels of XIAP (X-linked inhibitor for apoptosis) than the normal cells. The high IC_{50} in normal cells also justifies the traditional use of *Embelia ribes* (the plant which is a rich source of embelin) which was perceived both as food and medicine in ancient Indian systems of medicine. The results indicate the potential use of embelin against cancer and oxidative stress-related diseases.

ACKNOWLEDGEMENT

The financial assistance provided to this work by the Department of Science and Technology-Science and Engineering Research Board (DST-SERB), Government of India under the Fast track scheme for young scientists is gratefully acknowledged.

CONFLICT OF INTEREST
Declared none

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