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ORIGINAL ARTICLE

Chemopreventive Potential of Major Flavonoid Compound of Methanolic Bark Extract of *Saraca asoca* (Roxb.) in Benzene-induced Toxicity of Acute Myeloid Leukemia Mice

Manas Kumar Mukhopadhyay, Mithun Shaw, Debjani Nath

Department of Zoology, Cytogenetics and Molecular Biology Laboratory, University of Kalyani, Nadia, West Bengal, India

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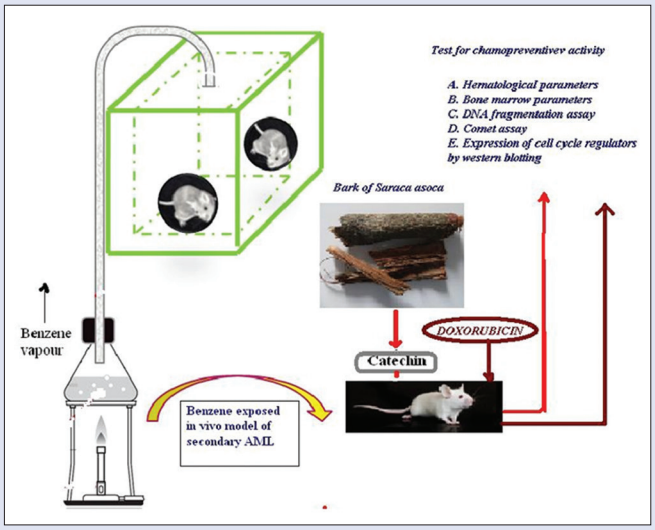
ABSTRACT

Background: *Saraca asoca* (SA) (Roxb.) is one of the folk medicinal plants found in India, Bangladesh, and Sri Lanka. Its major biological activity appears due to the presence of flavonoid group of compounds in its bark extract. **Objective:** In this study, our research aims to analyze the chemopreventive effect of flavonoids, especially a natural phenol catechin present in the bark methanolic extract of SA on acute myeloid leukemia (AML) mice. **Materials and Methods:** The total bark extract was partitioned and analyzed on thin-layer chromatography (TLC) plate. The yellow-brown material of spot 4 was analyzed and identified as catechin. The yellowish brown material (YBM) was tested for their chemopreventive potential. An *in vivo* AML mice model was used to test the efficacy. Hematological parameters (Hb %, red blood cell, and white blood cell count), expression of cell cycle regulatory proteins, and DNA fragmentation analysis were performed. **Results:** After treatment of benzene-exposed mice with the major flavonoid compound, namely catechin, the above parameters increase significantly ($P < 0.05$). There was an upregulation of p53 and p21, caspase 11 myeloperoxidase, bcl2, and CYP2EI in catechin-treated group. DNA was less fragmented in flavonoid-treated group compared to that of control ($P \leq 0.05$). The present study indicates that the secondary metabolites of SA methanolic bark extract, comprising flavonoid catechin as major constituents, have modulatory effect in cell cycle deregulation and hematological abnormalities induced by benzene in mice. **Conclusions:** Our data suggest that catechin from methanolic bark extract of SA effectively attenuates benzene-induced secondary AML in bone marrow, which is likely associated with the anticell cycle deregulation properties of this flavan-3-ol. This study was supported by the observation that catechin (YBM), like doxorubicin, can act as the neutralizer and protector of mortality in cancer cases.

Key words: Catechin, cell cycle regulators, chemoprevention, doxorubicin, flavonoid, secondary acute myeloid leukemia

SUMMARY

- The catechin from methanolic bark extract of *Saraca asoca* has chemoprotective activity in benzene-induced secondary acute myeloid leukemia (AML) in bone marrow
- Hematological parameters, structural analysis of DNA showed that the purified catechin attenuates the conditions responsible for the development of AML
- The purified flavonol, catechin has a modulatory effect on different cell cycle deregulations induced by benzene in AML model.



Correspondence:

Dr. Debjani Nath,
Department of Zoology,
Cytogenetics and Molecular Biology Laboratory,
University of Kalyani, Kalyani, Nadia - 741 235,
West Bengal, India.
E-mail: nath_debjani@yahoo.co.in
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INTRODUCTION

The term "chemoprevention" is based on the concepts of multifocal field carcinogenesis and multistep carcinogenesis.[1,2] A chemopreventive agent should be able to interfere with one or more phases of this multistep carcinogenesis process.[3] It must also be safe, nontoxic, and effective when administered in long-term therapeutic use.[4] In carcinogenesis, normal cells undergo transformation acquiring specific characteristics enabling the development of full malignancy. The loss of functions of cellular repairing system that otherwise repair DNA damage and ensure correct chromosomal segregation during mitosis enables populations of premalignant cells to develop the characteristic hallmarks of cancer cells. These principles represent prime targets for chemoprevention. Chemoprevention activity is often the result of amalgamation of several divergent intracellular dealings, as opposed to

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a single biological retort.[5,6] Some of the more important mechanisms are (1) antioxidant activity, (2) anti-inflammatory activity, and (3) induction of apoptosis through monitoring cell cycle progression at various points.

Any agent can be recognized as chemopreventive if it is of natural origin or chemically synthesized substances which resist or inhibit the progression of any disease or revert the normal physiological condition of the system.[7-9] However, it is difficult to achieve a good chemopreventive agent. The best evidence is tamoxifen which is approved drug for women at high-risk zone of breast cancer. It is currently approved by the Food and Drug Administration in the United States for the prevention of breast cancer.[10,11] Another modulator, raloxifene, is being considered for breast cancer as a chemopreventive agent. Raloxifene, actually approved for treatment of osteoporosis, was recommended for the patients having a high risk of breast cancer with osteoporosis.[12] It was primarily assumed that raloxifene may reduce the possibility of breast cancer in high-risk individuals under the long-term treatment with tamoxifen. Nearly 19,000 postmenopausal women were assigned to receive either tamoxifen or raloxifene daily for 5 years. The participants have now been followed for a median of 6.8 years.[10] Another study conducted by Alpha-Tocopherol Beta-Carotene (ATBC)-Cancer Prevention Study group including 29,133 male smokers were treated with either alpha tocopherol (50 mg/day) or beta-carotene (20 mg/day) alone, both ATBC or placebo for 5–8 years. It was a shocking result showing a higher percentage of lung cancer among individuals received beta carotene than the control group.[13-15] However, another result of the same study revealed that α-tocopherol supplementation reduced the rate of prostate cancer incidence and mortality to 32% and 41%, respectively.[13] The ATBC cancer prevention study suggested that consumption of large quantities of fruits and vegetables, where beta-carotene was abundantly present, associated with a decreased risk of developing cancer as reviewed by Smith-Warner and Giovannucci.[16,17]

Flavonoids are ubiquitous groups of polyphenolic substances present in most medicinal plants. It can block the initiation or reverse the promotion stage of multistep carcinogenesis.[18] The bark of *Saraca asoca* (SA) (Roxb.), one of the best used medicinal plants, has a stimulating effect on the endometrial and ovarian tissue and has a beneficial effect on uterine fibroids.[19,20] The bark of SA has also been used as a chemopreventive agent in the (7,12 dimethyl benz [a] anthracene) DMBA-induced skin cancer models.[21] The presence of high amount of flavonoids in the methanolic bark extract of SA instigated us for the experimentation on the pharmacological implications of these compounds[22] and to test the bioactivity of the flavonoids as a chemopreventive agent using a benzene-exposed secondary acute myeloid leukemia (AML) model.

The aim and objective of this study are to purify and determine the role of bioactive components of medicinal plant – SA as a chemopreventive agent in comparison with a chemotherapeutic drug, doxorubicin - commonly used for the treatment of AML in benzene-induced secondary mouse AML model developed and reported previously by us.[23-25]

MATERIALS AND METHODS

Plant material collection

SA bark was harvested during the month of mid-February to mid-March from herbal garden, Department of Botany, University of Kalyani, Kalyani. The voucher specimens (PDG/Bot./OB. 36, leaves of OB and PDG/Bot./SA. 42, bark of SA) were deposited and preserved in the Department of Botany, University of Kalyani, West Bengal, India, for reference.

Other materials

The primary antibodies specific for cyclin D1, cyclin E, CDK2, CDK4, CDK6, p21, p53, and β-actin and the secondary antibodies were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA).

Experimental animals

Six to eight-week-old Swiss albino male mice (*Mus musculus*) of maintained strain were purchased from a reputed breeder and were housed in a stainless steel wire cages (Tarsons, India) and maintained on a 12 h light–dark cycle. Pellet diet (West Bengal Dairy and Poultry Development Corp. Ltd., Kalyani, West Bengal, India) was provided *ad libitum* except 6 h daily period of benzene inhalation. Water was supplied automatically throughout the study.

The *in vivo* model for the study of AML was developed according to the method of Saha *et al.* and Akbay *et al.*[25,26] Benzene (MERCK, India) vapor was generated by heating liquid benzene to 16°C and channeled into the inhalation chamber. The groups of experimental mice were exposed 300 ppm, benzene for 6 h/day, 5 days/week for 2 weeks in 1.3 m^3 inhalation chambers (S.B. Equipments, West Bengal, India.), MERCK, India,[26] and the control group was exposed to ambient air for the same time frame. Benzene concentration in the chamber was monitored at regular half an hour interval. The temperature and humidity in the chambers were automatically maintained at 24°C ± 1°C and 55% ±10%, respectively.

Preparation of plant extract

Plant extract was prepared according to the method described earlier.[22] Pieces of bark were air-dried at room temperature until complete drying. They were kept away from direct sunlight to avoid destruction of active compounds. The bark pieces were pounded to powder using metallic motor and pestle. Ten grams of dried crude SA bark powder was used for Soxhlet extraction. Plant material was defatted by petroleum ether before extraction in methanol. Methanol was evaporated from the extract using vacuum evaporator under reduced pressure. Concentrated extract was stored at 4°C in air-tight bottle. The extraction yield was 12% (W/W).

Estimation of total flavonoid content in bark methanolic extract

Total flavonoids content in the bark methanolic extract was determined by aluminum chloride colorimetric method. Quercetin solutions (5–100 μg) were used to build up the calibration curve.[27,28] A calibration curve was made by measuring the absorbance of quercetin at different concentrations at 415 nm with a spectrophotometer.

Concentration of total flavonoids of all plant extracts was calculated by interpolating the X-axis. Total flavonoid content (TFC for 100 g dried plant material) was calculated using the following formula.

TFC = R × D.F × V × 100/W (When R = Result obtained from the standard curve, D.F. is Dilution factor, V is Volume of stock Solution, and W is Weight of plant used in the experiment).

Analysis and purification of flavonoids from methanolic extract of *Saraca asoca*

Gas chromatography–mass spectrometry spectrometric analysis of bark methanolic extract

The gas chromatography–mass spectrometry (GC-MS) analysis of the extracts was performed using a GC-MS (QP 2010 series, Shimadzu, Tokyo, Japan) attached with a ZV 5 silica capillary column (30 m in length, 0.25 mm in diameter, and 0.25 μm of film). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.99%) was used as carrier gas at a flow rate of

1.51 ml/min injector and mass transfer temperature was set at 200°C and 240°C, respectively. The oven temperature was programed from 70°C to 250°C at 10°C/min held isothermal for 1 min and finally raised to 300°C at 10°C/min. Two microliters of the diluted samples was manually injected in the split, with split ratio of 1:40 and mass scan of 50–600 amu. Total running time of GC-MS was 30 min. The relative percentage of the extract constituents was expressed as a percentage with peak area normalization.

The identity of the compounds in the extract was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with published literature. NIST08.LIB, WILEY8.LIB library sources were used for matching the identified components from the plant material.

Partitioning, thin-layer chromatography, and nuclear magnetic resonance analysis

The total crude methanolic extract of SA bark was evaporated in vacuum to yield a residue (130 g) that was then suspended in water (1 L) and successively partitioned with ethyl acetate (1 L × 3 times) and *n*-butanol (1 L × 3 times), yielding EA, BuOH, and water dissolved fractions. Each fraction was evaporated on a rotary evaporator to remove organic solvent and then lyophilized until dry. The yields of the EA, BuOH, and water fractions were 12.1, 13.2, and 79.0%, respectively, of the total crude extract in dry weight. The aqueous fraction was chromatographed over a RP-18 silica gel column (Cosmosil 75C18-OPN) and thin-layer chromatography (TLC) analysis of plant extracts was performed on RP-18 F254S TLC aluminum sheets (Merck), with compounds visualized by spraying with 10% (v/v) H_2SO_4. Each spots on TLC plate were cut mechanically, collected, and stored in amber-colored vessel separately until further purified. The collected material was dissolved in methanol and filtered through Whatman no. 1 filter paper. Filtrate was then evaporated to dryness and weighed. Each residue was then tested for preliminary chemical nature. Spot 4 was identified as flavonoid. The yellowish brown material (YBM) from spot 4 was further run through RP-18 silica gel column, and the collected YBM (20 mg) was analyzed for H^1 and C^{13} NMR (BRUKER 600 mHz) spectrum.[29]

Subacute toxicity study of the yellowish brown material

The YBM from TLC was evaluated for their toxicity in male Swiss albino mice aged 6–8 weeks and weighing 22–28 g. According to Saha *et al.*,[26] the test of toxicity of the YBM, 30 mice were randomly divided into three groups of ten animals (five males and five females) per cage. Before oral administration, the mice were fasted for 1 to 2 h. Then, the extract was dissolved in distilled water and administered daily by oral gavage to the mice of Groups B and C. The control group (Group A) received a respective volume of distilled water (vehicle) for 29 days. The mice in Group B were given orally the YBM at a dose of 50 mg/kg, and the mice in Group C were given the extract at a dose of 1500 mg/kg of body weight. The mice were then observed continuously for 1 h after the treatment, intermittently for 4 h, and for 29 days. The mice were observed for gross behavioral changes, condition of coat, discharge, movement, body weight changes, serum biochemistry, hematological parameters, and mortality for 30 days to test subacute toxicity.

Treatment of benzene-exposed mice with yellowish brown material of (*Saraca asoca*)

From the result of toxicity study of YBM, it was found that the plant extract is nontoxic. Hence, the benzene exposed group of mice was treated with the YBM at a dose of 50 mg/kg body weight orally from the date of commencement of benzene exposure for 4 weeks daily. Control group of mice was treated with the respective volume of distilled water.

Treatment schedule

Groups	Treatment
I	Not received any treatment
II	Benzene exposed, received only vehicle and distilled water
III	Benzene exposed, received yellowish brown material suspended in distilled water, at a dose of 50 mg/kg body weight for 4 weeks
IV	Benzene exposed, received doxorubicin at a dose of 2 mg/kg body weight

Hematological parameters

After completion of the treatment with plant extract, the animals were kept in fasting overnight and sacrificed after 30 days by decapitation and blood was collected. Blood hemoglobin concentration (g %) was determined using Sahil's hemoglobinometer, and total white blood cell (WBC) and red blood cell (RBC) were counted using Neuber's hemocytometer.[30] Other blood parameters included platelet numbers, differential count of monocyte, lymphocyte, neutrophil, and different RBC indices such as hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were performed.

Bone marrow sampling

The femur bone of the sacrificed experimental mice was dissected out, and both the proximal and distal ends of the bone were removed. Phosphate buffer (pH 7.2) was injected gently into one end of the shaft. This process was repeated to flush out the bone marrow through the opposite end into a collection vessel.

Immunoblotting

Protein extracts of the bone marrow from four groups of experimental animals were prepared by sonication of bone marrow cells in a cell lysis buffer containing 20% sodium dodecyl sulfate (SDS), 2 mM phenyl methyl sulfonyl fluoride, and a protease inhibitor cocktail.[31] Protein concentration of bone marrow extract was quantified using Bradford method.[32] Protein extracts from bone marrow (15 μg for p21 and 10 μg for other) were denatured, subjected to 12% (W/V) SDS-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked by incubating the membranes with 5% nonfat dried milk and 0.1% Tween 20 in Tris-buffered saline (TTBS, pH 7.4) for 1 h at room temperature; the membranes were incubated with diluted primary antibodies overnight at 4°C. The membranes were then washed with TTBS and incubated with 1:2000 horseradish peroxidase-conjugated secondary antibodies for 50 min at room temperature. To detect the bands, the membranes were treated with a detection reagent, and band densities were measured using an image analyzer (ImageJ, NIH).

DNA fragmentation assay

DNA was extracted from bone marrow cells of four groups of animals using reagents and protocol suggested by Bangalore Genei (PI.NO: KT23). DNA concentration was measured at 260 nm using a spectrophotometer (Varian, Germany). DNA extracts were then subjected to 0.8% agarose gel electrophoresis using 1X

TAE buffer as running buffer (composition: 40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid [EDTA]), and ethidium bromide (Et Br) (final concentration into gel 0.5 µg/ml) as staining agent. Five microliters of 100 bp DNA ladder was loaded into one well in each assay. The gel was visualized and photographed using a gel documentation system (Genei™ image system).

Comet assay

The method of Singh *et al.*[33] was followed with minor modification. The lymphocyte cells were isolated from blood of four groups of mice using reagents and protocol provided by HiSep LSM (Hi Media, Germany). The cells were suspended in 110 µl of low melting point agarose (0.65% LMPA – W/V in phosphate-buffered saline [PBS], pH = 7.4) and smeared on a frosted glass microscope slide precoated with 140 ml of 1% normal melting point agarose (in PBS, pH 7.4). The agarose was allowed to set for 10 min at 4°C and thereafter the coverslip was removed. The slides were exposed to lysis solution overnight (2.5M NaCl; 0.1M Na_2HPO_4; 10 mM Tris-Cl; 0.3M NaOH; 1% Triton X100; 10% dimethyl sulfoxide pH 10). The solution was kept at 4 C for 1 h before use. On next day, slides were transferred into a horizontal electrophoresis chamber containing electrophoresis buffer (300 mM NaOH, 1 mM Na_2-EDTA; pH 13.0) and presoaked for 20 min to unwind DNA. Electrophoresis was carried out for 20 min (30 mA, 20 V). Slides were then washed thoroughly with neutralizing buffer (Tris 0.4M, pH 7.5), stained with Et Br (final concentration of 40 µg/ml), and examined under a Leica fluorescence microscope. The DNA breakage percentage was determined by measuring the COMET tail length using the software Motic Image China.

Ethical clearance

The protocol used in this study was approved by the Animal Ethical Committee of University of Kalyani (under Committee for the Purpose of Control and Supervision of Experiments on Animals).

Statistical analysis

The two-tailed Student's *t*-test was done to determine the significance of differences between benzene-exposed and plant leaf extract-treated group to control group. Significance level was considered at $P < 0.05$ level.

RESULTS

The analysis of total flavonoid using quercetin as standard flavonoid (y = 0.0003x + 0.07, R^2 = 0.969) of methanolic bark extract shows average flavonoids content is 28% throughout the year.

In the GC-MS analysis [Figure 1 and Table 1], five bioactive phytochemical compounds were identified in the methanolic bark extract of SA. The identification of phytochemical compounds is based on the peak area, molecular weight, and molecular formula. Catechin ($C_{15}H_{14}O_6$) with retention time (RT) 12.817 has peak area 36.25%, 2,5-ditritylpyrrole 1H-pyrrole, 2,5-bis (triphenyl methyl)-(67370-39-2) ($C_{42}H_{33}N$) with RT 13.117 has peak area 7.76%, procyanidin B2 ($C_{30}H_{26}O_{12}$) with RT 13.167 ranks next having peak area 25.84%. 2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-(36555-25-6) with RT 13.233 ranks with peak area 12.17% and n-Decanoic acid ($C_{10}H_{20}O_2$) has RT 13.288 with peak area 18.06% with minor compounds.

Thin-layer chromatography and nuclear magnetic resonance analysis of the plant (*Saraca asoca*)

Extract

The TLC profile study of the aqueous fraction of the methanolic extract of the bark of SA with details has been described in Table 2 and Figure 2. R_f values[34] for different spots were calculated accordingly.

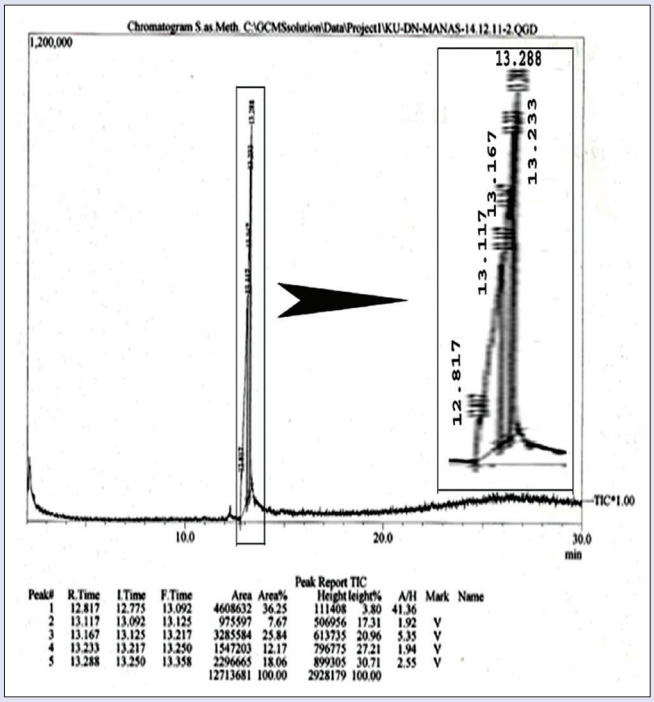

Figure 1: Gas chromatography–mass spectrometry spectrophotometric analysis of *Saraca asoca* bark methanolic extract

YBM: Ultraviolet λ, max = 275 nm;

^{1}H NMR: 6.741 (6, 1H, dd, J = 8.499, J = 1.047), 7.001 (7, 1H, dd, J = 8.499, J = 2.585), 6.103 (8, 1H, d, J = 0.000), 6.722 (9, 1H, dd, J = 2.585, J = 1.047), 6.103 (10, 1H, d, J = 0.000), 2.737 (11, 1H, dd, J = 15.632, J = 1.520), 2.744 (11, 1H, dd, J = 15.632, J = 4.780), 4.181 (20, 1H, ddd, J = 4.780, J = 1.920, J = 1.520), 5.003 (21, 1H, d, J = 1.920).

Comparing all these spectral data with that stored in library using the software program MestReNova, the closely matched flavonol compound was catechin.

Evaluation on toxicity of yellowish brown material

Physicochemical observations were recorded to determine potential toxic effects of infused plant material in the animals. No adverse effects such as behavioral changes, coat conditions' alterations, discharge, and movement abnormality were seen for both Group B and Group C animals. No mice were found to be died during the study and no significant changes of body weight, serum biochemical parameters, and hematological parameters [Table 3] were due to normal growth of the animals. Hence, the selected dose of plant extract is nontoxic.

Plant material, yellowish brown material (Saraca asoca) arrest benzene-induced cell cycle deregulation

Expression of key cell cycle regulatory proteins [Figure 3] such as p53 and p21 has been reduced in benzene-induced hematotoxicity in mice (Lane 2). Based on our results, we hypothesized that the plant extract might have modulatory effects on cell cycle regulation. As evident from Figure 3, level of caspase 11 has been upregulated in benzene-exposed group and downregulated in catechin-treated (Group III) as well as in doxorubicin-treated group (Group IV) compared to the control group of animal. Myeloperoxidase (MPO), bcl2, and CYP2EI follow a similar trend as indicated by panel 2, 3, and 4 in the adjoining figure. In case of caspase 12, the level is lowered in benzene-exposed animals, and increased

Table 1: List of phytoconstituents in methanolic bark extract of *Saraca asoca*

RT (min)	Name of the compound	RI	Molecular weight	Molecular formula	RA (percentage of compounds)
12.817	3,3',4',5,7–flavanpentol, catechin	4,608,632	290	$C_{15}H_{14}O_6$	36.25
13.117	1H-pyrrole, 2,5-bis (triphenyl methyl)	975,597	551	$C_{42}H_{33}N$	7.67
13.167	Procyanidin B2	3,285,584	578	$C_{30}H_{26}O_{12}$	25.84
13.233	2H-Pyran-2-one, tetrahydro-4-hydroxy-6-pentyl-(36555-25-6)	1,547,203	186	$C_{10}H_{18}O_3$	12.17
13.288	n-decanoic acid	2,296,665	172	$C_{10}H_{20}O_2$	18.06

RI: Retention indices; RT: Retention time; RA: Peak area ratio

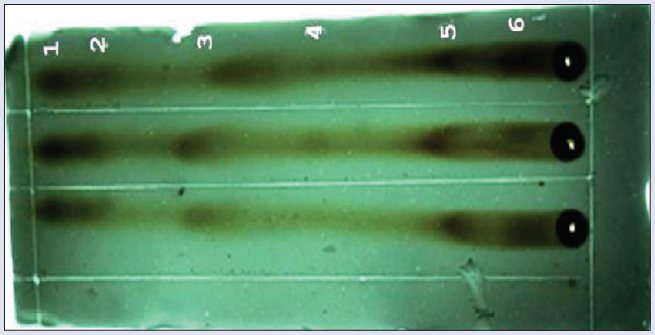

Figure 2: Thin-layer chromatography profile of the aqueous fraction of methanolic bark extract of *Saraca asoca*. Among six different spots visualized under ultraviolet light, the spot number 4, i.e., R_f (4) =0.451 was identified by chemical analysis as flavonoid and the purified compound appeared as yellowish brown powder

expression of this protein was found in another two groups. Treatment of both YBM from SA and doxorubicin resulted in the significant upregulation of the level of p21 and p53 in benzene-exposed mice compared with nontreated benzene-exposed mice.

Hematological parameters

The results shown in Table 4 indicate that number of RBC, WBC, and Hb (g%) has been decreased in benzene-exposed group compared to control group of animals. Hb (g%), RBC, and WBC have been decreased to 9.86 ± 1.6, 2.11 ± 0.19, and 3.28 ± 0.45, respectively [Table 4]. After treatment of benzene-exposed mice with the YBM, the above parameters increase significantly ($P < 0.05$) and the value reaches to 11.23 ± 1.01, 2.91 ± 0.18, and 6.21 ± 0.27, respectively, whereas after treatment with doxorubicin, the values reach to 10.21 ± 0.97, 2.23 ± 0.16, and 5.87 ± 0.35, respectively. This result is also in accordance with overexpression of p21 in YBM (Group III) and doxorubicin-treated (Group IV) group of animals as shown in immunoblot analysis [Figure 3]. In total WBC count, number of lymphocytes increases significantly ($P < 0.05$) after treatment with the YBM as well as doxorubicin. Hct as RBC index also increased significantly ($P < 0.05$) in both the groups after treatment [Table 5]. The bone marrow cellularity showed the same pattern of changes as showed by Mukhopadhyay and Nath[25] in benzene-exposed model. The gross cellularity was changed toward the control system after treatment with catechin as well as doxorubicin (data not shown).

DNA fragmentation analysis

DNA fragmentation characteristic of apoptosis was clearly detected after benzene exposure to mice as shown in Figure 4a and it is being less fragmented in treated group with catechin and lesser fragmented in doxorubicin-treated group of animals. The comet tail length [Figure 4b] showed by 3.2-fold reduction in comet tail length in blood

Table 2: Respective R_f values of six different spots on total leukocyte count plates

Name of fraction	Solvent system	R_f values
Aqueous fraction	Ethyl acetate:methanol (7:3)	R_f (1)=14.5/15.5=0.935
-Do-	-Do-	R_f (2)=13.5/15.5=0.870
-Do-	-Do-	R_f (3)=11/15.5=0.709
-Do-	-Do-	R_f (4)=7/15.5=0.451
-Do-	-Do-	R_f (5)=3.5/15.5=0.225
-Do-	-Do-	R_f (6)=2/15.5=0.129

Table 3: Evaluation of nontoxicity of the plant material

Parameters	Control (Group A)	Lower dose (50 mg/kg body weight) (Group B)	Higher dose (1500 mg/kg body weight) (Group C)
SGOT (IU/dL)	40.75±1.12	40.08±1.19	40.96±1.15
SGPT (IU/dL)	34.22±1.19	33.98±1.16	32.11±1.13
ALP (IU/dL)	80.12±1.78	81.56±0.76	79.39±1.72
Bilirubin (mg/dL)	0.86±0.13	0.85±0.11	0.86±0.12
Cholesterol (mg/dL)	149.25±9.3	150.33±8.7	150.69±8.9
Urea (mg/dL)	40.64±1.32	41.09±1.21	40.67±1.31
Uric acid (mg/dL)	4.48±1.77	4.12±1.56	4.19±1.67
Creatinine (mg/mL)	1.00±0.12	0.91±0.21	1.00±0.18
RBC ($\times 10^{12}$/L)	6.6±0.12	6.6±0.16	6.7±0.13
Hb (g/dL)	13.2±0.22	13.10±0.28	12.54±0.31
WBC ($\times 10^9$/L)	29.3±0.42	28.2±0.38	32.1±0.26
Platelet ($\times 10^9$/L)	756±41.2	736±46.1	750±42.4
Body weight (g)	22.3±1.2	21.9±1.5	22.95±1.02

Yellowish brown material on serum biochemical, hematological parameters, and body weight after subacute administration. Data are represented as mean±SEM. RBC: Red blood cell; WBC: White blood cell; Hb: Hemoglobin; SEM: Standard error of mean; ALP: Alkaline phosphatase

Table 4: Variation of blood parameters among benzene-exposed mice and treated with plant extract (*Saraca asoca*) and doxorubicin-treated group

Group	Hb (g%)	RBC ($\times 10^{12}$/L)	WBC ($\times 10^9$/L)
Control	12.27±1.9	4.08±0.38	8.21±1.2
BE	9.86±1.6	2.11±0.19	3.28±0.45
Treated with PE (SA)	11.23±1.01*	2.91±0.18*	6.21±0.27*
Treated with doxorubicin	10.21±0.97*	2.23±0.16*	5.87±0.35*

Data are expressed as mean±SEM. *Significant increase ($P<0.05$) in parameters. BE: Benzene exposed; PE: Plant extract; RBC: Red blood cell; WBC: White blood cell; SA: *Saraca asoca*; Hb: Hemoglobin; SEM: Standard error of mean

lymphocytes ($P < 0.01$) of bark catechin-treated AML mice (Plate III) compared to that (Plate II) of benzene-exposed mice whereas decreased to 2.2 fold in doxorubicin-treated AML mice (Plate IV) under the same exposure schedule. The data were coincident with the results of the cell cycle regulating caspases and other regulatory proteins

Table 5: Variation of different blood parameters among benzene-exposed mice and treated with plant extract (*Saraca asoca*) and doxorubicin group

Group	Differential leukocyte count			Differential of RBC				Platelet count (×10^9/L)
	Neutrophil (%)	Monocyte (%)	Lymphocyte (%)	Hct (%)	MCV (μm^3)	MCH (pg)	MCHC (g/dL)	
Control	22.3±1.98	1.92±0.06	68.34±5.12	38.09±2.11	52.78±3.46	16.22±0.12	26.44±0.35	750±38.21
BE	21.68±1.32	1.88±0.05	48.49±3.51	10.23±0.96	47.55±2.98	14.11±0.14	24.33±0.38	798±40.48
Treated with PE	18.91±1.23	1.86±0.06	56.12±3.92*	21.28±1.05*	38.29±0.65	12.78±1.20	18.25±0.05	705±55.26
Treated with doxorubicin	19.62±1.22	1.91±0.07	58.34±4.03	23.33±2.31	42.33±0.79	12.76±1.21	18.42±0.06	723±42.76

Data are expressed as mean±SEM. *Significant increase (P<0.05) in parameters. BE: Benzene exposed; PE: Plant extract; RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; Hct: Hematocrit; SEM: Standard error of mean

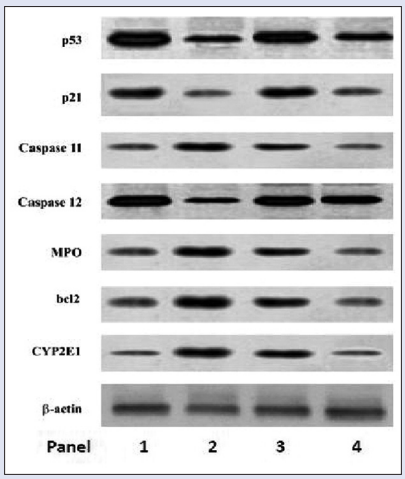

Figure 3: Immunoblotting of p53, p21, and caspase 11 and 12; myeloperoxidase; bcl$_2$ and CYP2E1 panel 1-proteins from control animal; panel 2-proteins from benzene-exposed animal; Panel 3 proteins from acute myeloid leukemia animal treated with plant extract (*Saraca asoca*); Panel 4 proteins from doxorubicin-treated acute myeloid leukemia group

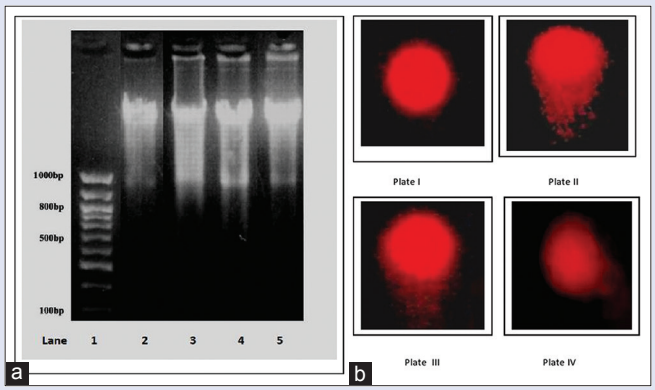

Figure 4: (a) DNA fragmentation analysis (Lane 1, M = Marker; Lane 2, control DNA; Lane 3 = DNA from benzene-exposed group; Lane 4 = DNA from yellowish brown material treated (*Saraca asoca*) group and Lane 5 = DNA from Dox-treated group. (b) Comet assay from blood lymphocytes of control (Plate I); benzene-exposed mice (Plate II), acute myeloid leukemia mice treated with yellowish brown material (Catechin) (Plate III) and treated with doxorubicin (Plate IV) showing comet tails

Survival of benzene-exposed animals treated with yellowish brown material and doxorubicin

As shown in Figure 5, benzene-exposed (Group II) animals survive for a period of 12.5 ± 1.2 weeks.[25] Due to the treatment of the benzene-exposed animals with the YBM from SA and doxorubicin, mean survival time of the animal increases to 15.5 ± 1.4 and 16.3 ± 1.5 weeks, respectively.

DISCUSSION

Benzene vapor induces inflammatory responses, oxidative stress, alterations in cell cycle progression, and DNA damage in mice. All these changes have been associated with the development of various blood diseases including AML.[35,36] Hence, benzene-exposed animal model[25,26] can act as a significant template for the study of secondary cancers with respect to different parameters measuring clinical manifestations.

Among many other dietary chemicals, the flavonoids are major components of fruits and vegetables. This is known that flavonoids have antimutagenic activity. Quercetin was shown to inhibit the mutagenic activity of benzo[a] pyrene (BP), a representative polycyclic aromatic hydrocarbons carcinogen, in studies of bacterial mutagenicity.[37] It was also shown to inhibit BP-induced nuclear damage in colonic epithelial cells of mice.[38] The investigators also found that most of 13 other studied flavonoids were anticlastogenic when administered orally before and after intraperitoneal exposure to BP. It was also noteworthy that several hydroxylated flavonoids were found to inhibit mutagenic activity of bay-region diol-epoxides (putative ultimate mutagens/carcinogens) of BP.[39] Catechin is a flavan-3-ol, a type of natural phenol and antioxidant. It is a plant secondary metabolite. It belongs to the group of flavan-3-ols (or simply flavanols), part of the chemical family of flavonoids. Catechin, epicatechin, quercetin, and resveratrol, which account for more than 70% of polyphenolic compounds in red wine, were shown to inhibit the growth of human breast cancer cells at picomolar concentrations.[40] The same compounds were also shown to potentially inhibit human prostate cancer cells.[41,42] The tea polyphenols, (2)-epicatechin gallate, and epigallocatechin gallate inhibited the adhesion of mouse lung carcinoma 3 LL cells to the monola.

In the case of flavonoids of SA bark, GC-MS study [Figure 1 and Table 1] showed the presence of catechin with RT 12.817 having peak area 36.25%. TLC [Figure 2 and Table 2] and NMR analysis showed that the 4th spot of YBM is closely related to flavonol compound "Catechin." As shown in Figure 3, Catechin treatment in benzene-exposed mice resulted in the upregulation of p53 and p21 and control over the cell cycle deregulation. MPO is an enzyme that is expressed in neutrophil granulocytes. Simultaneous exposure of the benzene-induced AML animal to the bark flavonol enhanced the expression of MPO which was downregulated in the Group II animals showing a reduced level of immunodeficiency. Caspase 11 and 12 are closely associated with the pro-apoptotic signals[43] and once activated they cleave and activate the downstream effector caspases such as 3, 6, and 9. Exposure to benzene was observed to cause the expression of lower level of both caspase 11 and 12, but significant upregulation of caspase 12 was observed without any significant effect on expression of caspase 11. Overexpression of bcl2, a well-known anti-apoptotic protein, was found in the control animal AML model in contrast with catechin-treated animal where expression of bcl2 indicated the prevention of deregulation of the

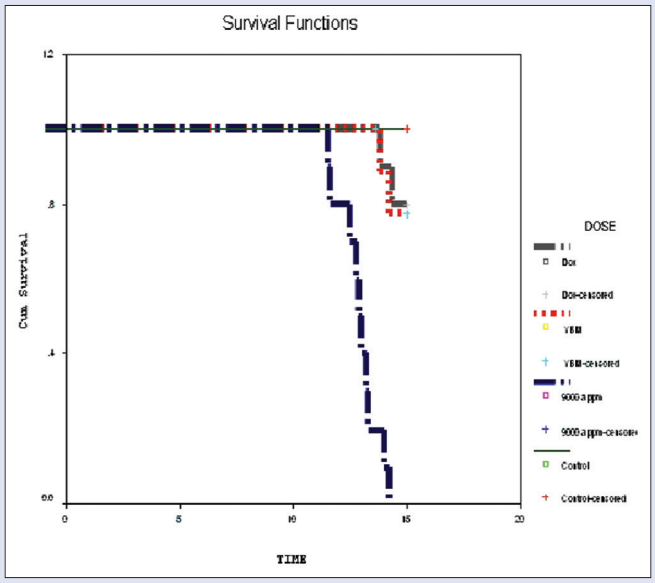

Figure 5: Survival of mice exposed to 9000a ppm of benzene by inhalation and treatment with yellowish brown material (*Saraca asoca*) and doxorubicin. The data are presented in Kaplan–Meier format showing the cumulative survival of mice at various time (weeks) interval after exposure and treatment

cellular cycles that proceed to the development of cancer. This event is also supported by the lowering of expression of CYP2E1, a member of cytochrome p450 mixed oxidation system related to the xenobiotic metabolism[44] [Figure 3]. Doxorubicin-treated secondary AML animal model showed the same profile of recovery in the deregulated cell cycle progression in cancer cases. This study was supported by the observation that YBM, like doxorubicin, can act as the neutralizer and protector of mortality in cancer cases [Figure 5].

Analysis of blood parameters is relevant to risk evaluation as the hematological system has a higher predictive value for toxicity in humans (91%) when assay involve rodents and nonrodents.[26,45] Blood is an important index of physiological and pathological status in man and animals and the parameters usually measured are hemoglobin, total RBC, and leukocyte (WBC) counts.[46] The hematotoxicity of benzene is characterized by suppression of erythromyelopoiesis, resulting in the depression of leukocyte (lymphocyte count mainly) and erythrocyte levels in peripheral blood.[47,48] This result is also in accordance with overexpression of p21 as shown in immunoblot analysis [Figure 3]. However, after treatment with bark catechin, it was found that it can be preventive in control of blood cell number if peripheral circulation in benzene-exposed AML mouse (T) supported by the observation of chemoprevention of DNA damage [Figure 4a]. This analysis is supported by the comet assay test showing declined DNA damage in the bone marrow cells [Figure 4b]. In conclusion, protection by plant secondary metabolites is currently an important strategy for controlling the process of various diseases including hematotoxicity. Therefore, there is a need to explore medicinal plants or other natural agents that can work as protective agents against hematotoxicity. In the present work, our results show the significant activity of purified catechin from SA bark against benzene-induced hematotoxicity in mice. On the basis of above evidences, it is possible that catechin present in the extract may be responsible for the chemopreventive activity in mice.

However, this claim demands extensive studies on synergistic pharmacodynamic interactions of the phytochemicals as well as the effects on signal modulation which are essential for the development of multiactive natural drugs from SA for cancer chemoprevention in the near future. It is also hoped that tailored supplementation with designer foods that consists of chemopreventive phytochemicals – each having their own distinct anticancer mechanisms – will be available in the near future. These should be developed in line with advances in the genetic and molecular epidemiology of carcinogenesis.

CONCLUSIONS

Our data suggest that catechin from methanolic bark extract of SA effectively attenuate benzene-induced secondary AML in bone marrow, which is likely associated with the anticell cycle deregulation properties of this flavan-3-ol. This study was supported by the observation that catechin (YBM), like doxorubicin, can act as the neutralizer and protector of mortality in cancer cases.

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Conflicts of interest

There are no conflicts of interest.

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