MATERIALS AND METHODS

2.1 Chemicals and reagents

Fetal bovine serum (FBS) was purchased from Invitrogen. McCoy’s 5A medium modified, Dulbecco’s modified eagle medium (DMEM), Eagle’s minimum essential medium (MEM), bovine insulin, dimethyl sulfoxide (DMSO), trypsin phosphate versene glucose (TPVG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulforhodamine B (SRB), phosphate buffer saline (PBS), trichloro acetic acid (TCA), acetic acid, dimethyl-benzanthracine (DMBA), 3-methyl cholanthrene (MC), RNase were procured from Sigma-Aldrich, St. Louis, USA. All other chemicals and reagents were of analytical grade and obtained from Merck, India.

2.2 Plant Materials

Plants were collected from their natural habitats. Croton caudatus Geiseler was collected from Saikot Village of Churachandpur District, Manipur. Other three plants, viz., Belamcanda chinensis (Linn.) DC., Magnolia grandiflora Linn. and Nerium indicum Mill. were collected from the campus of Manipur University, Imphal. The plants were identified and herbaria were deposited at Botanical Survey of India, Shillong.
2.2.1 *Belamcanda chinensis* (Linn.) DC

Vernacular name : Black berry lily (English); Kabo-leiteng (Manipuri)
Place of collection : Manipur University campus

Systematic position

Kingdom: Plantae

Division: Magnoliopsida

Class: Liliopsida

Order: Asparagales

Family: Iridaceae

Genus: *Belamcanda*

Species: *chinensis* (Linn.) DC

Parts used : Rhizome

**Description**

*Belamcanda chinensis* is a hardy perennial herb with stout rhizome. Stems are erect, 50-100 cm tall, leafy towards the base. Leaves mostly basal and lower cauline, laterally flattened, ensiform, equitant, 30-50 cm long and 2-4 cm wide, glaucose. Inflorescence loosely corymose at terminals, 1-2 branched, the bracts 4-5 at the apex, narrowly ovate, 1 cm long, the pedicels 4-5 cm long. Flowers are deep orange with red dots, about 5 cm across, star-like, twisting spirally as they fade, perianth segments acute, 6 in numbers, not united, about equal size, filaments are reddish-purple, style with 3 branches, flattened and emarginate at apex.
Fig. 1: *Belamcanda chinensis* (Linn.) DC (a) plants in habitat, (b) flower and (c) rhizomes
Uses and medicinal properties

The whole extract of the *Belamcanda chinensis* has an anti-proliferative effect on prostate cancer (Morrissey *et al.* 2004, Thelen *et al.* 2005). The rhizomes show anti-inflammatory (Shin *et al.* 1999), anti-fungal (Oh *et al.* 2001) and anti-diabetic properties (Jung *et al.* 2002). The dried rhizomes are used in Chinese traditional medicine for treatment of inflammation and asthma as well as throat disorders, such as cough, tonsillitis, and pharyngitis (Chang 1997, Ito *et al.* 1999). In Thai folk medicine, the rhizomes are used for the regulation of menstrual disorders (Pongboonrod 1976). The preliminary study on the crude methanolic extract suggested the high value of this plant as a source of anti-mutagenic and anti-oxidant compounds (Wozniak *et al.* 2006). The major bio-active constituents of this plant, tectoridin and tectorigenin, attribute a number of physiological benefits including anti-inflammatory, anti-angiogenic, and anti-mutagenic properties (Jung *et al.* 2003, 2004, Kim *et al.* 1999, Wozniak *et al.* 2010). *Belamcanda chinensis* extracts containing tectorigenin had a strong hypothalamic and osteotrophic effect without any effect in the uterus or the mammary gland (Seidlová-Wuttke *et al.* 2004).

Bio-active compounds

The anti-inflammatory, anti-angiogenic and anti-mutagenic properties of the *Belamcanda chinensis* is attributed to its major bioactive compounds tectoridin and tectorigenin (Jung *et al.* 2003, 2004, Kim *et al.* 1999, Wozniak *et al.* 2010).
Fig. 2: Known bioactive compounds of *Belamcanda chinensis* (Linn.) DC
Fig. 2: (contd.)
Chemical studies found that a variety of compounds such as isoflavonoids (Ito et al. 2001, Yamaki et al. 1990), stilbenes (Monthakantirat et al. 2005), and iridal-type triterpenoids (Abe et al. 1991, Ito et al. 1999, Seki et al. 1995, Takahashi et al. 2000) are present in this medicinal herb. Isoflavonoids like tectoridin, iridin and their aglycones tectorigenin, irigenin and others like irisflorentin, rhamnocitrin and belamcandin as well as their derivatives are also present in this plant (Ito et al. 2001, Jung et al. 2002, Shin et al. 1999, Woo & Woo 1993, Yamaki et al. 1990). Pharmacological investigations demonstrated that phenols could be major bioactive constituents of Belamcandin (Ahn et al. 2006, Kim et al. 1999, Morrissey et al. 2004, Seidlova-Wuttke et al. 2004, Thelen et al. 2005).

2.2.2 Croton caudatus Geiseler

Vernacular name : Chawilien Damdawei

Place of collection : Saikot village of Churachandpur District, Manipur

Systematic Position

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Angiospermae

Order: Malpighiales

Family: Euphorbiaceae

Genus: Croton
Species: *caudatus* Geiseler

Parts used: Leaves

**Description**

The genus *Croton* belongs to the family of Euphorbiaceae, with more than 700 species, widely distributed throughout tropical and sub-tropical regions. Leaf blades of the plant is about 5-19 cm X 2.5-12 cm and petioles about 1-5.5 cm long. Underside of the leaf blade and leafy twigs are densely clothed in pale stellate hairs. Usually 2 (sometimes 4) stalked glands visible on the underside of the leaf blade close to the junction with the petiole. Oil dots small and numerous, leaf blades 3-veined at the base, stipules about 1 mm long, flowers borne on the basal portion of the raceme and calyx lobes (tepals) about 3-3.5 mm long. Nectary glands opposite the tepals look like much reduced petals except for their position. Staminodes about 10, ovary densely clothed in pale clumped or branched hairs, styles 3, each ending in two linear stigmas, fruits globose, about 15 mm diameter surface densely clothed in short stellate hairs, sometimes interspersed with larger trichomes. Perianth remnants usually persistent at the base while style remnants persistent at the apex. Seeds usually 3 per fruit, each seed about 10 mm X 8-10 mm. Testa clothed in stellate hairs. Embryo is about 8-10.5 mm long. Cotyledons about 6-8 mm X 5 mm. Radicle straight, about 2.5-3 mm long (Geiseler 1807).
Fig. 3: *Croton caudatus* Geiseler plant in habitat and fruit (inset)
Uses and Medicinal Properties

It is well-known as a source of diterpenoids because most of the species of this genus produce a significant variety of such terpenes (clerodane, labdane, kaurane, trachylobane, pimarane, etc.), regarded as the diagnostic ingredients with a wide range of biological activities such as anti-inflammatory (Kuo et al. 2007) and anti-ulcer (Kitazawa et al. 1980). However, only a few flavonoids have been reported from this genus (González-Vázquez et al. 2006, Guerrero et al. 2002, Palmeira et al. 2005). The stems and leaves have been used for the treatment of malaria, ardent fever, convulsions, rheumatic arthritis, numbness (Jiangsu New Medical College 1975) and in the treatment of pain and stomach diseases (Pharmacopoeia Commission of People’s Republic of China 2005).

Bioactive compounds

A new flavone named was isolated from the stems of *Croton caudatus* Geisel. var. tomentosus Hook. together with nine known analogues: 3,5,6,7,8,3',4'-heptamethoxyflavone (Morales et al. 2005), tangeretin (Kuo et al. 2007), nobiletin (Kitazawa et al. 1980), 5,6,7,4'-tetramethoxy-flavone (Guerrero et al. 2002), sinensetin (González-Vázquez et al. 2006), kaempferol (Palmeira et al. 2005), tiliroside (Jiangsu New Medical College 1975), kaempferol-3-Orutinoside (Pharmacopoeia Commission of People’s Republic of China 2005) and rutin (Wang & Zhou 2008).
Fig. 4: Known bioactive active compounds of *Croton caudatus* Geiseler
Fig. 4: (contd.)
2.2.3 *Magnolia grandiflora* Linn.

Vernacular name : U-Thambal Angouba (Manipuri)

Place of collection : Manipur University campus

Systematic position

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Magnoliales

Family: Magnoliaceae

Genus: *Magnolia*

Species: *grandiflora* L.

Parts used : Bark and reproductive parts (flower)

Description

*M. grandiflora* L. (Family: Magnoliaceae) also known as balm and lemon balm is a popular beautiful decorative tree. It is as tall as 8-20 m tall. It has large, leathery, shiny leaves which are green on the adaxial surface and light brown on the abaxial surface (Vázquez 1984). Various magnolias are distinguished by their many interesting biological features. The genus Magnolia consists of about 120-130 species and in the tertiary period Magnolias were common in Europe (Azuma et al. 2001, Hunt 1998, Kim et al. 2001). Originally from Mexico (Vázquez 1984), the majority of *Magnolia* (120–130) species grow in the temperate and tropical zones of
Fig. 5: *Magnolia grandiflora* Linn. in habitat with flower (inset)
south-eastern Asia while others are found in the New World. The leaves are alternate, simple and petiolate, with large stipules, which at first surround the stem, but fall off as the leaf expands and leave a characteristic scar around the node. The flowers are bisexual (rarely unisexual), often large and showy, pedunculate, solitary at the ends of branches or in the axils of the leaves (sometimes paired when axillary), the penduncle bears one or more spathaceous bracts which enclose the young flower, but fall off as it expands. The perianth is composed of two or more (usually three) whorls of free tepals which are petaloid, the outer tepals are sometimes reduced and sepal-like. The stamens are numerous, spirally arranged, with stout filaments, the anthers have two lobules opening by longitudinal slits. The carpels are numerous or few (rarely single), spirally arranged, free or partly fused. Each carpel has two or more ventrally placed ovules (Watanabe et al. 2002).

Uses and medicinal properties

*M. grandiflora* has been in used in the treatment of various ailments. It has an inhibitory effect on skin tumor promotion (Ikeda & Nagase 2002). It is used in the treatment of fever, diarrhea, rheumatism, arthritis, high blood pressure, heart disturbances, abdominal discomfort, muscle spasm, infertility and epilepsy (Schuhly et al. 2001). The aqueous extracts of flowers and leaves exhibited cardiovascular effects (Mellado et al. 1980). It is also used as an anti-convulsant (Ramirez et al. 1998), reducing stress (Kennedy et al. 2004), anti-inflammatory (Yip-Schneider et
Fig. 6: Known bioactive compounds of *Magnolia grandiflora* Linn.
Fig. 6: (contd.)
al. 2005), anti-thrombotic (Yang et al. 2001), anti-arrhythmic (Sweeney et al. 2005), anti-oxidant (Battle et al. 2003) and anxiolytic effects (Wiedhopf et al. 1973).

Bio-active compounds

*M. grandiflora* contains a number of sesquiterpene lactones, a class of lactones known to possess anti-inflammatory properties and used for treatment of pain (Feltenstein et al. 2004). A number of biologically active alkaloids (Rao & Davis 1982), glycosides (Rao et al. 1978), sesquiterpenes (Koo et al. 2001), phenolic constituents (Clark et al. 1981) and other compounds (Rao & Davis 1982a) have been isolated from this species. The presence of neolignan compounds magnolol and honokiol in *M. grandiflora* have been reported (Rao & Davis 1982b). Parthenolide, a sesquiterpene lactone isolated from different herbs (including *Magnolia*), is a novel NF-kappa β inhibitor (Wen et al. 2002) with interesting anti-neoplastic properties (Chen et al. 2009, Zhang et al. 2009, Zhao et al. 2009).

2.2.4 *Nerium indicum* Mill.

Vernacular name : Indian Oleander (English), Kabilei Angouba (Manipuri)

Synonym : *Nerium oleander* L.

Place of collection : Manipur University campus
Fig. 7: *Nerium indicum* Mill. (a) plant in habitat, (b) flowers and (c) roots
Systematic position

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: proteals

Family: apocynaceae

Genus: Nerium

Species: indicum Mill.

Parts used : Root bark

Description

Nerium indicum Mill. also known as Nerium oleander Linn. is a common fast-growing evergreen shrub or tree, native to the Mediterranean origin and naturalize and distributed to southern Europe and South-west Asia (Aksoy & Ozturk 1997, Hardin and Arena 1974, Kingsbury 1964, Seaward & Mashhour 1991). It can reach up to 4 m in height. Leaves are short-stalked, green or grey-green in color and range from about 10 to 20 cm long. Some have white or yellow variegated leaves. Flowers are produced in terminal heads and their colors vary from deep to pale pink, lilac, carmine, purple, salmon, apricot, copper, orange, yellow and white (Huxley 1992).
Uses and medicinal properties

The leaves and the flowers are cardio tonic, diaphoretic, diuretic, anti-cancer, antibacterial (Chopra et al. 1986), anti-fungal (Wang et al. 2000) and expectorant. A decoction of the leaves has been applied externally in the treatment of scabies and to reduce swellings. This is a very poisonous plant, containing a powerful cardiac toxin and is used externally only and with extreme caution. The root is powerfully resolvent, is used in the form of plasters and is applied to tumors. It is beaten into a paste with water and applied to lesion and ulcers on the penis (Marchioni & Distefano 1989). Bark is bitter and is used as cathartic, febrifuge and in the treatment of intermittent fever. Plants have an extensive root system and are often used to stabilize soil in warmer areas. Oil prepared from the root bark is used in the treatment of leprosy and skin diseases of a scaly nature. Seeds are poisonous, abortifacient and alternative, and used as purgative in dropsy and rheumatism. The whole plant has anti-cancer properties and has been used in the treatment of cancer (Abe et al. 1992, Valnet 1976). The flowers, leaves, leaf juice or latex, bark and roots have been used against corns, warts, cancerous ulcers, carcinoma, ulcerating and hard tumors. The aqueous and ethanol extracts of oleander leaves possess significant antinociceptive activity, but ethanolic extract is more pronounced. However, extract has been shown to induce gastric and ulcerogenicity in mice. Flowers, either dried or fresh, also exhibit potent antinociceptive activity (Zia et al. 1995). Hussain and Gors (2004) reported the anti-microbial activity of leaves and roots of Nerium oleander against Bacillus pumilis, B. subtilis, Staphylococcus aureus, Escherichia
Fig. 8: Known bioactive compounds of *Nerium indicum* Mill.
Fig. 8: (contd.)
coli and Aspergillus niger. Zia et al. (1995) reported that purified fractions obtained from the methanol extract of fresh oleander leaves possess a CNS depressant and significant analgesic activities. The chief active principle oleandrin was found to stimulate the heart function and also had a diuretic effect. The effect of odorin on the heart of rabbits and dogs is identical to that of Digitalis group whereas neriodin is twice as active as digitoxin in Digitalis like action similar to that of oleandrin (Pathak 2000). The leaf extracts also showed significant effects on Diabetes mellitus (Sikarwar et al. 2009).

Bio-active compounds

Oleander is one of the most poisonous plants in the world and contains numerous toxic compounds, many of which can be deadly to people, especially young children. The toxicity of Oleander is considered extremely high and it has been reported that in some cases only a small amount had lethal or near lethal effects. The most significant of these toxins are oleandrin and nerine, which are cardiac glycosides (Begum et al. 1999). Investigations on different parts of the plant have revealed the presence of steroidal glycosides, triterpenes and straight-chain compounds (Begum et al. 1997, Siddiqui et al. 1986, Siddiqui et al. 1987a & b). The leaves of N. oleander contain two new steroids, dehydroadynerizoside and neristigmol (Siddiqui et al. 2006). Cardioactive glycosides – nerodorin, neridorein and karabin are also found to be present in this plant. Alcoholic extracts of root bark showed the presence of α-amyrin, β-sitosterol, ether fraction showed the presence of kaempferol and the
β-sitosterol, ether fraction showed the presence of kaempferol and the chloroform extract showed the presence of odoroside (Randhawa & Mukhopadhyaya 1986).

2.3 Plant collection and preparation for test

Plants and the parts used in the experiment were collected during suitable seasons. Inventory of plants guided by locations of their occurrence mentioned in the Medicinal Plants of Manipur (Sinha 1996) had been done. Identification of the plants was done at Botanical Survey of India (BSI), Shillong and herbaria of the collected plants were deposited. The name of the plants collected, parts used in the experiment and their Accession number given by the BSI are shown in Table 1. After collection, the parts were washed in tap water and shade-dried at 28±2°C. The dried parts were kept inside an oven at 55-60°C for a week and ground to coarse powder. The coarse powdered parts of the plants were extracted using Soxhlet apparatus with 50% ethanol as solvent. The extracts were concentrated using rotary vacuum evaporator, lyophilized and were kept at 4°C.
Table 1: Name of the plants, accession number (Acc. No.) given by Botanical Survey of India (BSI), Shillong, plant parts and solvents used

<table>
<thead>
<tr>
<th>Plants</th>
<th>BSI Acc. No.</th>
<th>Parts used</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Belamcanda chinensis</em> DC</td>
<td>47956</td>
<td>rhizome</td>
<td>50% ethanol</td>
</tr>
<tr>
<td>2. <em>Croton caudatus</em> Geiseler</td>
<td>44111</td>
<td>leaves</td>
<td>water</td>
</tr>
<tr>
<td>3. <em>Magnolia grandiflora</em> Linn.</td>
<td>18972</td>
<td>bark, flower</td>
<td>50% ethanol</td>
</tr>
<tr>
<td>4. <em>Nerium indicum</em> Mill.</td>
<td>15141</td>
<td>root bark</td>
<td>50% ethanol</td>
</tr>
</tbody>
</table>

2.4 Cancer cell lines and their maintenance

Five different types of human cancer cell lines were used in the present work for *in vitro* cytotoxic activity test. Two of the cell lines viz., Bu25Tk- (human cervical cancer cell) and Sp2/01 Ag-14 (mouse myeloma cancer cell) were procured from the National Centre for Cell Science, Pune. MCF-7 (human breast cancer cell) and M6180 (human cervical cancer cell) were obtained from Advanced Centre for Training, Education & Research on Cancer (ACTREC) and K562 (human chronic myeloid leukaemia cell) from the Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong. The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C, medium was changed at 48 to 72 hours intervals according to the cell types. Cells at the exponentially growing phase were used for *in*
vitro cytotoxic test. The different types of the cell lines with their complete media are given in Table 2.

Table 2: Name of the cells, types of culture, source organ, type of cancer and media used

<table>
<thead>
<tr>
<th>Name</th>
<th>Type of culture</th>
<th>Source organ</th>
<th>Type of cancer</th>
<th>Media used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bu25Tk</td>
<td>adherent</td>
<td>cervix</td>
<td>adenocarcinoma</td>
<td>DMEM-90%, FBS-10%</td>
</tr>
<tr>
<td>MCF-7</td>
<td>adherent</td>
<td>breast</td>
<td>adenocarcinoma</td>
<td>DMEM-90%, FBS-10%</td>
</tr>
<tr>
<td>ME180</td>
<td>adherent</td>
<td>cervix</td>
<td>epidermoid carcinoma</td>
<td>McCoy's 5A-90%, FBS-10%</td>
</tr>
<tr>
<td>K562</td>
<td>suspension</td>
<td>bone marrow</td>
<td>CML</td>
<td>DMEM-90%, FBS-10%</td>
</tr>
<tr>
<td>Sp2/01Ag-14</td>
<td>suspension</td>
<td>spleen</td>
<td>myeloma</td>
<td>DMEM-90%, FBS-10%</td>
</tr>
</tbody>
</table>

2.5 In vivo tumor growth inhibition

Swiss albino mice (Mus musculus), both male and female, were reared in the animal house and were used for the induction of tumor. The mice were housed in animal cages (Tarsons 29 x 22 x 14 cm$^3$) bedded with rice husk in a hot and cold controlled air-conditioned room (temperature 22° ± 1° C, humidity, 85% and 12 hrs dark/light cycle) with standard laboratory diet and water ad libitum. The study was conducted after obtaining Institutional Ethics Committee approval and guidelines of CPCSEA were followed. The maximum tolerated dose (MTD) was determined by administration of the test system with various concentrations of the extract less than LD$_{50}$ until 100% survival of the treated system was achieved. Mice, having an
average weight of about 20±2 gm and 10-12 weeks old were used. Tumors were
induced in both male and female (females being nulliparous and non-pregnant) mice
through oral administration of 1mg DMBA (Sigma) dissolved in 0.5 ml olive oil,
given once a week for the first six consecutive weeks. Three months after the
administration of DMBA the mice were again administered a single dose of 3-methyl
cholanthrene (MC) at a dose of 1 mg of MC dissolved in 0.5 ml of olive oil and
separated into three groups (n=15). The animals of the first group were administered
21µg plant extract (833 µg/Kg body weight) dissolved in 0.5 ml PBS while those of
the second group were given 42µg plant extracts (1.66 mg/Kg body weight)
dissolved in 0.5 ml PBS. The animals of the third group were given 0.5ml of PBS
daily. The administration of the plant extracts for the tested groups and PBS for the
carcinogen-treated control group was continued till the end of the experiment. The
animals receiving neither DMBA nor MC nor plant extracts were used as control
group.

At the first and last day of the experiment, the body weight of each mouse was
measured. The changes in the dietary habit of the animals during the experiment
were observed. At the end of the experiment, the mice were sacrificed and necropsies
were performed. Weights of the vital organs as well as tumor were measured. The
tumor volume was estimated on the basis of two dimensional tumor measurements
Tumor volume = 0.5A \times B^2

where A is the longest diameter and B, the shortest diameter of the tumor.

The tumor inhibitory rate was calculated using the formula:

Tumor Inhibitory Rate (%) = 100 \times (\text{mean tumor weight in control mice} - \text{mean tumor weight in treated mice}) / \text{mean tumor weight in control mice}.

2.6 Histopathological examination

Lungs, liver and tumors developed in the lungs were taken and fixed at 10% paraformaldehyde (pH 7.5) and processed for paraffin embedding according to the standard histological procedures. Five micrometer (5μm) thick sections were prepared and stained with HE stain. Histopathological examinations were completed using Leica light microscopy.

2.7 Cytogenetic test

The plant extract at MTD was administered intraperitoneally to the mice. Negative control animals received PBS equivalent to that of the plant extract while the positive control animals received DMBA at the dose of 40mg/kg body weight. Each treatment and control group consisted of 5 animals. The treatment protocol is shown in Table 3. The animals were sacrificed after 24 hrs treatment and cytological slide preparations
control animals received DMBA at the dose of 40mg/kg body weight. Each treatment and control group consisted of 5 animals. The treatment protocol is shown in Table 3. The animals were sacrificed after 24 hrs treatment and cytological slide preparations were made. Three slides each were prepared from treated, positive control and negative control animals as follows: metaphase chromosomes were prepared from the bone marrow cells using standard colchicines hypotonic-spreading air drying technique. One millilitre of 1% colchicine is administered intraperitoneally to the animals and kept for 1 hrs. The animals were sacrificed after 1 hrs, removed the femoral bone and flushed out the bone marrow cells using 0.56% KCl, suspended the cells and kept for 30 mins at 37°C. The cell suspension was centrifuged at 1200 rpm for 8 min and pellets were again suspended in carnoy's fixative, kept for 30 min and centrifuged. The pellets were again collected and suspended in fixative. The cell suspension was spread on a methanol chilled slide and flame dried. Synaptosomal complexes (SCs) were prepared from spermatocytes following the method of Westergaard et al. (1972) with slight modification. One drop of the testicular cell suspension in RPMI-1640 was spread over a large drop of 0.2M sucrose on a clean slide for 30 sec., slides were fixed in 4% paraformaldehyde for 5 minutes, washed in 0.2 photoflow for 30 seconds and dried. SC damages were scored according to Allen et al. (1988). The result from the treated animals was compared with those from the control animals using t-test. The metaphase chromosome preparations were stained with 10% giemsa and that of synaptosomal complex preparations with 70% silver nitrate (AgNO₃). The slides were mounted with DPX and observed under 100X
Table 3: Experimental protocol for the treatment of animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Treatment period</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Croton caudatus</em></td>
<td>8.25 mg/kg body weight</td>
<td>24 hours</td>
<td>5</td>
</tr>
<tr>
<td>DMBA</td>
<td>40 mg/kg body weight</td>
<td>24 hours</td>
<td>5</td>
</tr>
<tr>
<td>Water</td>
<td>40 ml/kg body weight</td>
<td>24 hours</td>
<td>5</td>
</tr>
</tbody>
</table>

2.8 Colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

For testing the cytotoxic or proliferative activity of the plants, the protocol of the MTT assay by Carnichael *et al.* (1987) with slight modification was used. Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Medium was changed at 48 to 72 hrs intervals according to the cell types. Cells at the logarithmic growth phase were used for the assay. Single cell suspensions were obtained by mechanical disaggregation of the floating cell and trypsinization of the monolayer cells of the adhesive cultures. Counting of the cells was done with haemocytometer. One hundred microlitre (μl) of cell culture containing 400–1000 cells were dispensed in 96-well culture plates. Following 24 hrs incubation at 37°C, 5% CO₂, 100% RH (Shellab 2123 CO₂ incubator), 100 μl of plant extract at different concentrations or culture medium or drug carrier (PBS) were dispensed and incubated for 48 hours. After 48 hrs, 50μl MTT working solution (1mg/ ml PBS) were added to each well, incubated for 4 hours and centrifuged at
1700 rpm for 10 minutes. Supernatant was removed from the wells using 18 gauge needle leaving around 20–30 μl in each well. 150 μl of DMSO (Sigma) were added to each well. The plate was thoroughly mixed using a plate shaker and measured the absorbance at 546 nm with Huma Reader HS human microwell plate reader. All results represented the average of nine (9) wells. Percent inhibition of growth of the cells was calculated by using the formula:

\[
\text{Inhibition of growth } \% = \left\{ 1 - \left( \frac{\text{Test absorbance at 546 nm}}{\text{control absorbance}} \right) \right\} \times 100.
\]

2.9 Estimation of relative cell growth by sulforhodamine B (SRB) assay

Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Medium was changed at 48 to 72 hrs intervals according to the cell types. The Assay was done following the method of Skehan et al. (1990) with slight modification. Single cell suspensions were obtained by mechanical disaggregation of the floating cell and by trypsinization of monolayer cells of the adhesive cultures. Cell counts were performed using haemocytometer and experimental cultures were plated in microtitre plates containing 100μl of cell suspension per well at densities of 400 to 1,000 cells. Following 24 hours incubation, 100 μl of plant extract at different concentrations or culture medium or carrier (PBS) were dispensed and incubated for two days. On the 4th day, cultures were fixed with trichloroacetic acid (TCA). Cells attached to the plastic substratum were fixed by gently layering 50μl of 50% TCA
(4°C) at top of the growth medium in each well to produce a final TCA concentration of 10%. The cultures were incubated at 4°C for 1 hr and then washed five times with tap water to remove TCA, growth medium, low molecular weight protein and serum proteins and plates were air-dried overnight. TCA fixed cells were stained for 30 minutes with 50 μl of 0.4% (w/v) SRB dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed four times with 1% acetic acid to remove unbound dye, and the cultures were allowed to dry. The bound dye was solubilised with 10 mM unbuffered Tris base (pH 10.5) for 5 min on a gyratory shaker and the optical densities were read at Huma Reader HS human microwell plate reader at 515 nm. Besides the optical density of the test wells (T), the optical density of the control wells (C) as well as optical density of the wells at time zero (optical densities of the cells on the day of drug addition, (T₀) were also taken. Using these measurements, cellular responses can be calculated for growth stimulation, no drug effect and growth inhibition. If the optical density (OD) of the test wells (T) is greater than or equal to T₀, the calculation is 100 X \{(T-T₀)/(C- T₀)\}. If T is less than T₀, there is net reduction in the number of cells and can be calculated from 100 X \{(T-T₀)/ T₀\} (Skehan et al. 1990).

2.10 Cell cycle analysis

The effect of the plant extracts on various phases of the cell cycle in human chronic myelogenous leukemia cancer cell K562 were analyzed by ethanol fixation and staining with propidium iodide using the method described by Crissman & Steincamp
(1982) with slight modifications. Cells in exponential proliferation were dissociated, counted and seeded at $10^6$ cells in 5 ml. of complete medium per 25 cm$^2$ flask (Tarsons) for 24 hours. Optimal seeding densities were determined to measure exponential growth. Following incubation of the cells at 37°C, 5% CO$_2$ and 100% RH, plant extracts at different concentrations were added and incubated for 24 and 48 hrs. Single cell suspension of the cells was then made by mechanical dissociation. The cells were fixed at chilled ethanol and kept for 24 hours at 4°C. The fixed cells were again resuspended, incubated with RNase A for 45 min at 37°C and stained with propidium iodide. Stained cells, suspended in the staining solution, were analyzed using Becton Dickinson (BD) FACS Caliber flow cytometer as described by Crissman & Steinkamp (1982). Data were collected from 10,000 viable cells per sample. Histogram plots were created using Cell Quest Program (Beckon Dickinson, San Jose, CA). Percentage of cells existing within various phases of the cell cycle were calculated using Cell Quest by gating on G$_0$-G$_1$, G$_1$, S, G$_2$ cell populations visualized using the scatter plot.