Butein imparts free radical scavenging, anti-oxidative and pro-apoptotic properties in the flower extracts of *Butea monosperma*

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**ABSTRACT:** The flower of *Butea monosperma* (Lam.) (Fabaceae) has been used in traditional Indian medicine in the treatment of many ailments including liver disorders. To understand the pharmacological basis of its beneficial effects, the extracts of dried flowers in water, methanol, butanol, ethyl acetate and acetone were evaluated for free radical scavenging and pro-apoptotic activities in cell cultures (human hepatoma Huh-7 cell line and immortalized AML-12 mouse hepatocytes). Butrin and butein -the active constituents of flower extracts- were used as reference molecules. The levels of cell injury markers like lactate dehydrogenase, glutathione and lipid peroxidation and primary antioxidant enzymes glutathione S-transferase and catalase were also measured. The aqueous and butanolic extracts exhibited better 2,2-diphenyl-1-picrylhydrazyl scavenging and cytotoxic activities in hepatoma cells than in immortalized hepatocytes. Interestingly, butein inhibited 2,2-diphenyl-1-picrylhydrazyl radical better than butrin. The aqueous and butanolic extracts were further investigated for hepatoprotection against carbon tetrachloride-induced biochemical changes and cell death. Both extracts, just as butrin and butein, significantly reversed the cellular glutathione levels and lipid peroxidation, and glutathione–S-transferase activity. Lactate dehydrogenase leakage and cell death were also prevented. However, only butein revived the catalase activity. Thus, the butein content of *Butea monosperma* flower extracts is important for free radical scavenging activity, apoptotic cell death and protection against oxidative injury in hepatic cells.

**Introduction**

The flowers of *Butea monosperma* (Lam) (Family: Fabaceae) have been traditionally used in India for the treatment of a variety of ailments including liver disorders, microbial and parasitic infections, stress, diarrhea, diuresis, arthritis and sexual disorders (Burlia and Khadeb, 2007), inflammation (Shahavi and Desai, 2008) and diabetes (Bavarva and Narasimhacharya, 2008).

The main chemical constituents of *B. monosperma* flowers include butin, butein and butrin. Isobutrin, palasitrin, coreopsin, isocoreopsin, chalcones and aurones are also present in the flower extracts (Wagner *et al.*, 1986; Chokchaisiri *et al.*, 2009). While butrin and isobutrin are considered to be the hepatoprotective principles of *B. monosperma* flowers (Wagner *et al.*, 1986), butein is reported to confer protection against phorbol ester-induced skin cancer (Aizu *et al.*, 1986) and carbon tetrachloride-induced liver fibrosis (Lee *et al.*, 2003). Besides, butein shows an anti-proliferative effect on a wide range of human tumor cells including breast carcinoma, colon carcinoma, lymphoma, acute myelogenous leukemia, melanoma and hepatic stellate cells (Pandey *et al.*, 2009; Wang *et al.*, 2005). The antiproliferative activity of butein apparently relates to its inhibitory action against various signaling pathways, like mitogen activated protein kinases (Lee *et al.*, 2007; Zhang and Li, 2008), signal transducer and activator of transcription (Pandey *et al.*, 2009), NF-κB (Pandey et
Butein also exhibits antioxidant property against lipid and of low density lipoprotein peroxidation (Cheng et al., 1998). More recently, we and others have shown the chemopreventive effects of the aqueous and methanolic extracts of *B. monosperma* in rodent models of hepatic injury and hepatocellular carcinoma (Choedon et al., 2010; Mathan et al., 2010; Sehrawat et al., 2006; Sehrawat and Sultana, 2006; Sharma and Shukla, 2011). Further, oral administration of the flower powder is also reported to confer protection against paracetamol-induced liver damage in rabbits (Maaz et al., 2010).

In the present study, we have evaluated the protective efficacies of butein, butrin, combination of butein and butrin and extracts of the flowers of *B. monosperma* in five different solvents against free radical accumulation, apoptotic cell death and oxidative stress in liver cells. This study was carried out to evaluate the synergistic effects of two active constituents of *B. monosperma* flowers and compare it with its crude aqueous and alcoholic extracts.

**Materials and Methods**

**Chemicals and biochemicals**

Dulbecco’s modified Eagle’s medium and fetal bovine serum were procured from Invitrogen (California, USA). Ascorbic acid (purity 99%), butein (99.7%), carbon tetrachloride, dimethylsulfoxide. stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPHÉ), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and silibinin (purity 98%) were purchased from Sigma-Aldrich (Missouri, USA). Butrin (98%) was provided by Dr. A.K. Saxena (Indian Institute of Integrative Medicine, Jammu, India). All the solvents used were of analytical grade.

**Plant material and flower extracts**

*B. monosperma* flowers were purchased from Cure Herbs, Delhi and identified by Dr. H.B. Singh, National Institute of Science Communication and Information Resources, New Delhi. A voucher specimen is deposited at the Raw Materials Herbarium and Museum of NISCAIR (Ref. No. NISCAIR/RHMD/Consult/2009-10/1226/30). The flowers were powdered and soaked separately with acetone, ethyl acetate, butanol, methanol and water for 48 h at room temperature with occasional stirring. After filtration through cheese cloth, the filtrates were concentrated using either vacuum evaporator (organic solvents) or lyophilized (aqueous) and stored until further use. The ultraviolet and visible spectra and high performance liquid chromatographic profile of each fraction was recorded.

**High performance liquid chromatography analysis**

Chromatographic analyses of different flower extracts were carried out as reported earlier (Wagner et al., 1986). In brief, the mobile phases were: (A) water with 10 mL 0.1N phosphoric acid, and (B) acetonitrile with 10 mL of 0.1N phosphoric acid. Samples (10 μl) were injected into the column and elution was performed in 20 min using a linear gradient (10 to 30%) of acetonitrile. The presence of butein and butrin was monitored at 380 nm using an A-I detector. Peaks were assigned using pure butrin and butein as reference compounds.

**Cell culture**

The human hepatoma Huh-7 cell line was obtained from Dr. A. Siddiqui (University of Colorado, Denver, USA). AML-12 cells (immortalized mouse hepatocyte, CRL-2 254) were purchased from the American Type Culture Collection (Virginia, USA). Cells were grown in complete growth medium (RPMI-1640 supplemented with fetal bovine serum (100 mL/L), L-glutamine, penicillin and streptomycin, and maintained at 37ºC in a CO₂ incubator. In addition, the medium for AML12 cells contained 5 μg/mL insulin, 5 μg/mL transferrin and 5 ng/mL selenium as supplement.

**Cell viability assay**

The effect of *B. monosperma* extracts and bioactive flavonoids on cell viability was determined by MTT assay (Van de Loosdrecht et al., 1994). Huh7 and AML-12 cells were seeded at a confluency of 60-70% in 12 well culture plates and incubated with the extracts, butein or butrin (in a concentration range of 1 μg to 1 mg/mL) for 24h. After a wash with phosphate-buffered saline, cells were incubated with MTT solution (1 mg/mL in Dulbecco’s modified Eagle’s medium) for 1h at 37ºC. The formazan product was dissolved in 1 mL dimethylsulfoxide and the absorbance was read at 560 nm. A regression analysis was used to calculate the effective concentration 50 (EC₅₀), defined as the concentration of the product needed to produce a 50% decrease of the viability of cells.
Measurement of free radical scavenging activity

The free radical scavenging activity was measured by reducing the free radical scavenger DPPH using the method reported earlier (Sundararajan et al., 2006). Briefly, 0.9 mL solution of DPPH in methanol (0.1 mM) was added to 0.1 mL solution of B. monosperma extracts, butein or butrin in a concentration range of 1 μg/mL - 1 mg/mL. The mixture was shaken and allowed to incubate for 30 min at ambient temperature and absorbance was recorded at 517 nm. The scavenging effect was calculated using the following equation:

\[
\text{DPPHÉ scavenging effect (%) = } \left[ 1 - \left( \frac{A_{\text{sample (517 nm)}}}{A_{\text{control (517 nm)}}} \right) \right] \times 100
\]

A regression analysis was used to calculate the effective concentration 50 (EC\textsubscript{50}), defined as the concentration of the product needed to produce a 50% inhibition of DPPH radical. Ascorbic acid was used as positive control.

Determination of the ameliorative effects of flower extracts

AML-12 cells (4x10^5 cells/60 mm dish) were pretreated with different concentrations of extracts 30 min prior to treatment with carbon tetrachloride (3 mM). Six treatment groups were included for each extract and the experiments were carried out in triplicate. Group I (AML12 cells) served as control. Groups II to VI were subjected to carbon tetrachloride-induced cytotoxicity following treatment with the flower extracts: Group II (control); group III - extract (1 μg/mL); group IV - (10 μg/mL); group V - (100 μg/mL) and group VI - (1 mg/mL).

Preparation of cell lysates

All procedures were carried out at 0-4°C on ice. For measuring cellular glutathione level, AML-12 cells were washed twice with ice cold phosphate-buffered saline (pH 7.4) and aliquots (500 μl) of sulfosalicylic acid (4%) were added. After 30 min, cells were centrifuged at 1200g for 20 min and the supernatant was collected. Protein concentrations in cell lysates were determined using a Bio-Rad protein assay kit.

For measuring the level of lipid peroxidation and activities of lactate dehydrogenase, glutathione-S-transferase and catalase, cells were pelleted at 5,000g for 5 min and washed with ice-cold phosphate-buffered saline. Cells were lysed in phosphate-buffered saline containing 1% (v/v) Triton X-100 for 20 min, centrifuged at 300g for 15 min and the supernatant was collected for measuring different enzymatic activities.

Determination of hepatocyte injury in vitro

The percentage of lactate dehydrogenase release was measured as an index of hepatoprotection and plasma membrane integrity. The lactate dehydrogenase activity in the cell lysates and medium was measured according to Haïdara et al. (1999).

### TABLE 1.

| Extract/bioactive flavonoid | Scavenging effect (%) |
|-----------------------------|-----------------------|
|                             | 1mg/mL | 100mg/mL | 10mg/mL | 1mg/mL |
| Aqueous extract             | 72.8 ± 1.9 | 8.97 ± 0.27 | 1.55 ± 0.18 | 0.21 ± 0.03 |
| Methanolic extract          | 68.9 ± 1.4 | 12.4 ± 0.21 | 2.07 ± 0.19 | 1.25 ± 0.13 |
| Butanolic extract           | 24.5 ± 1.7 | 4.54 ± 0.12 | -        | -        |
| Acetone extract             | 6.74 ± 1.09 | -        | -        | -        |
| Ethyl acetate extract       | 65.3 ± 0.94 | 11.7 ± 1.2 | -        | -        |
| Butrin                      | 21.8 ± 0.50 | -        | -        | -        |
| Butein                      | 87.3 ± 0.48 | 74.1 ± 0.22 | 15.8 ± 1.58 | 1.53 ± 0.16 |
| Ascorbic acid               | - | 91.1 ± 1.20 | 18.6 ± 0.97 | 2.40 ± 0.09 |

Values are expressed as Mean ± SE (n=3). -, not used. Ascorbic acid was used as a reference control.
Biochemical assays

The glutathione levels in cell lysates were determined according to the method of Jollow et al. (1974). Glutathione S-transferase and catalase activities, and lipid peroxidation, were measured according the methods of Habig et al. (1974), Aebi (1984) and Wright et al. (1981) respectively.

Statistical analysis

The data were expressed as the mean ± standard error of mean (SEM) calculated from three independent observations. The level of significance between different groups was based on analysis of variance test followed by the Dunnett’s t-test. The values p<0.05 were considered as significant.

Results

The free radical scavenging activity of the B. monosperma extracts and its active constituents was studied over a concentration range of 1 μg/mL to 1 mg/mL. Ascorbic acid was used as a positive control. We observed that aqueous and methanol extracts had a strong antiradical activity (73% and 69%, respectively at 1 mg/mL) with the EC₅₀ values of 0.63 and 0.71 mg/mL respectively (Table 1). Under these conditions, the EC₅₀ value of ascorbic acid was 51.7 μg/mL. However, ethyl acetate, acetone and butanol extracts showed a significant inhibition only at highest concentration (1 mg/mL). Butein appeared to be a more potent inhibitor (87% at 1 mg/mL) with EC₅₀ = 0.36 mg/mL as compared to butrin which showed only 22% inhibition under these conditions. At lower concentrations, butrin was ineffective.
The effect of flower extracts and flavonoids on cell viability was also studied over a range of concentrations (1 μg/mL to 1 mg/mL). Silibinin—a principal hepatoprotective and anti-neoplastic component of the herbal drug formulation of Silymarin—was used as a positive control (Lah et al., 2007). As shown in Fig. 1A, maximum death of Huh-7 cells was observed at 1 mg/mL of different extracts. The maximum cell death (70%) was induced by butanolic extract followed by aqueous (68%), methanol (59%), ethyl acetate (39%) and acetone (32%) extracts. As expected, silibinin induced significant cell death (~59% at 100 μg/mL) in Huh7 cells (Lah et al., 2007). Interestingly, no appreciable cell death was observed with either aqueous or butanolic extracts on AML-12 cells (Fig. 1B) suggesting their differential effect on transformed and non-transformed cells. Nevertheless, methanolic (8-53%), acetone (23-76%) and ethyl acetate (99%) extracts induced significant cell death in AML-12 cell populations. Under these conditions, silibinin recorded minimal cell death (6–14%) even at 100 μg/mL (data not shown) while butein induced more cell death (79% at 100 μg/mL) and butrin which showed 47% death at 1 mg/mL (Fig. 1C). There was no co-operativity between butein and butrin in cell killing. The EC_{50} values for butein, butrin and their combination were 53.34 μg/mL, 1.24 mg/mL and 55.27 μg/mL respectively. In case of AML-12 cells also, butein alone or in combination with butrin induced a dramatic cell mortality (~70%), at 1 mg/mL as compared to butrin (28%) (Fig. 1D). Thus, butein seems to be a potent inducer of cell death irrespective of the cell types used.

Since butein displayed a major role in free radical scavenging and cell killing activities, the butein and butrin contents of different flower extracts of B. monosperma were monitored by high performance liquid chromatography and recorded at 380 nm. Pure butein and butrin were used as reference molecules. As shown in Fig. 2, all the extracts invariably showed two major peaks with retention times and ultra violet spectra similar to that of butein and butrin. Some minor components were also present. The butein and butrin contents of the aqueous and butanolic extracts were higher as compared to other extracts and thus, selected for further studies.

FIGURE 2. High performance liquid chromatogram showing elution profiles of different extracts of flowers of B. monosperma monitored at 380 nm. The inset shows elution profiles of standard butrin and butein along with their structural formula.
To understand the biochemical basis of ameliorating effects of the flower extracts, the levels of specific markers of cell injury such as lactate dehydrogenase, glutathione and lipid peroxidation and primary antioxidant enzymes, like glutathione S-transferase and catalase were measured against carbon tetrachloride-induced injury in AML-12 cells. A significant increase in cell viability was observed in the presence of both aqueous and butanolic extracts (63% and 81% viability at 1 mg/mL respectively) (Fig. 3A). Among bioactive flavonoids, butein conferred best protection (91% cell viability at 100 μg/mL) as compared to the extracts. Butrin was inef-

![FIGURE 3. Effects of B. monosperma flower extracts, butein and butrin on cell viability and lactate dehydrogenase leakage. AML-12 cells were incubated with indicated concentrations of aqueous and butanolic extracts, butrin or butein for 30 min and followed by carbon tetrachloride treatment for 3h. Finally either cell viability was measured by MTT assay (A) or the cell extracts were used to determine lactate dehydrogenase leakage (B). Data are expressed as Mean ± standard error of mean of four independent observations. *, p<0.001, as compared to control; †, p<0.01; ‡, p<0.001; and o, p = not significant, as compared with carbon tetrachloride-treated group.](image-url)
effective. Analysis of lactate dehydrogenase leakage into culture media revealed that the carbon tetrachloride-treated cells had more leakage of lactate dehydrogenase (~50%) as compared to control cells (7%) (Fig. 3B). Both aqueous and butanolic extracts showed dose dependent protection against lactate dehydrogenase leakage. Treatment with butrin and butein also led to the significant reversal of damage induced by carbon tetrachloride.

Treatment of AML12 cells with carbon tetrachloride also caused a significant decrease (>50%) in glutathione content and glutathione S-transferase activity (Fig. 4) as compared to respective controls. Pretreatment with aqueous extract showed a more pronounced protective effect (up to 20%) as compared to butanolic extracts (up to 40%) and butrin (~52%) at 1 mg/mL concentration (Fig. 4A). Further, treatment with butanolic extract showed a much better recovery in glutathione S-transferase activity (>40%) as compared to the aqueous extract (up to 8%) at 1 mg/mL (Fig. 4B). The percent recovery of glutathione S-transferase in the presence of butein was found to be more effective (up to 29%) than butrin (up to 17%) at 1 mg/mL. Changes of the catalase activity of AML12 cells in response to treatment with test extracts and compounds are shown in Fig. 4C. There was a significant reduction (20%) in catalase activity in carbon tetrachloride-treated cells as compared to control. However, pre-treatment with both aqueous or butanolic extracts and butrin showed no major recovery in the catalase activity. Butein, on the other hand, displayed a significant recovery at higher concentrations. Results of lipid peroxidation as an index of oxidative stress are shown in Fig. 4D. Treatment of AML-12 cells with carbon tetrachloride significantly increased (~56%) the peroxidation of lipid membranes as compared to control cells (~19%). Pretreatment with aqueous and butanolic extracts significantly reduced malondialdehyde accumulation (14% and 25%, respectively, at 1 mg/mL). Butein displayed a better protection (up to 15%) against lipid peroxidation than butrin (7% at 100 μg/mL).

Discussion

Oxidative stress together with decreased antioxidant defense plays an important role in promoting the
initiation and progression of various diseases including cancer. Antioxidants on the other hand, interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and scavenging reactive oxygen species. Medicinal plants have been considered as a major source of bioactive molecules for developing novel agents for prevention and treatment of various diseases including cancer (Mukherjee, 2002). In the present study, we have evaluated the protective efficacies of butein, butrin and five different solvents extracts of the flower of B. monosperma against free radical generation, cell death and oxidative stress.

We observed that the extracts of B. monosperma flowers exhibited the free radical scavenging activity in following order: aqueous > methanol > ethyl acetate > butanol > acetone (Table 1) which is similar to an earlier report (Lavhale and Mishra, 2007). However, among active constituents, butein showed a much better scavenging activity as compared to butrin. Therefore, the butein content of a B. monosperma preparation could be an important determinant in protection against free radical-associated damage in the human body. Evaluation of the cytotoxic effects of different flower extracts and bioactive flavonoids on human hepatoma (Huh-7) and non-cancerous (AML-12) cell lines presented a rather different picture. We believe that this difference in cytotoxic effect may relate to the nature of cell lines used. For example, bioactive flavonoids, butein alone or in combination with butrin induced dramatic cell mortality in both hepatoma and immortalized cell lines. However, butrin showed a high degree of selectivity for hepatoma cells.

Carbon tetrachloride is a well-documented hepato-toxic agent associated with cell membrane malfunctions, mitochondria swelling and cell death in liver in a free radical mediated fashion (Recknagel et al., 1989). We observed that the viability of carbon tetrachloride–treated AML-12 cells improved significantly in the presence of both aqueous and butanol extracts as well as butein but not butrin. On the contrary, treatment with aqueous and butanolic extracts as well as both flavonoids significantly reduced the lactate dehydrogenase release suggesting the protective effects of B. monosperma on plasma membranes (Fig. 3). Since butrin is a known hepatoprotective agent, the cytoprotective effect of aqueous and butanolic extracts of the flowers may relate to the presence of both butrin and butein. In fact the high performance liquid chromatographic analysis confirmed the presence of relatively higher amounts of butein and butrin in both these extracts (Fig. 2).

The protective effects of the flower extracts also appeared to relate to depletion in the reduced glutathione content and antioxidant enzymes, and increase in lipid peroxidation. Glutathione is well known to play a central role in protecting the cell against oxidative injury, directly by reacting with oxidant species and indirectly through the intermediacy of enzyme catalyzed reactions (Wu et al., 2004). Elevated levels of malondialdehyde could reflect the degrees of lipid peroxidation induced injury in hepatocytes which might contribute to decreased glutathione content and increased cell death (Manna et al., 2006). Accordingly, we observed that carbon tetrachloride-exposed AML-12 hepatocytes exhibited increased lipid peroxidation and low glutathione content (Fig. 4). Our data clearly demonstrated the protective effects of extracts (aqueous and butanolic), butein and butrin on glutathione level, lipid peroxidation as well as glutathione S-transferase activity in tune with of high levels of endogenous glutathione and glutathione S-transferase (Fig. 4). It is well known that glutathione S-transferase can catalyze the conjugation of xenobiotics with reduced glutathione to form glucuronyl-conjugates that are less toxic and easily excretable (Wattengberg, 1993). Further, glutathione S-transferase and catalase constitute a good combination of defense against reactive oxygen species (Bandhopadhy et al., 1999). It may be noted that the catalase activity of carbon-tetrachloride-treated cells could be partially revived only in the presence of butein but not the flower extracts or butrin.

Thus, the present study shows that butein is an important ingredient of the flower extract of Butea monosperma which contributes towards its free radical scavenging, antioxidant and anti-apoptotic properties. Therefore, the butein content of B. monosperma preparations seems to be crucial for its beneficial effects against-hepatic disorders.

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