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Comparative efficacy of piperine, curcumin and picroliv against Cd immunotoxicity in mice

Neelima Pathak · Shashi Khandelwal

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Abstract Cadmium (Cd), a well known environmental carcinogen, is a potent immunotoxicant. In rodents, it is primarily characterized by marked thymic atrophy and splenomegaly. Cadmium induces apoptosis in murine lymphocytes and alters the immune functions. Thus, for the amelioration of its effect, three structurally different bioactive herbal extracts such as piperine—alkaloid, picroliv—glycosides and curcumin—polyphenols were evaluated and their efficacy compared. For ascertaining their immunomodulatory role, various biochemical indices of cell damage such as cytotoxicity, oxidative stress (ROS, GSH), apoptosis (mitochondrial membrane potential, caspase-3 activity, phosphatidylserine externalization, apoptotic DNA) along with lymphocyte phenotyping, blastogenesis and cytokine secretion were assessed in thymic and splenic cell suspensions. Of the three herbals examined, piperine displayed maximum efficacy. All the three doses of piperine (1, 10 and 50 μg/ml) increased cell viability in a dose dependent manner, whereas curcumin and picroliv were also effective, but to a lesser degree. Only the two higher doses exhibited cell viability efficacy. The median doses ie 10 μg/ml, were therefore selected, for comparison of their antioxidant, anti-apoptotic and immune function modulation. Restoration of ROS and GSH was most prominent with piperine. The anti-apoptotic potential was directly proportional to their antioxidant nature. In addition, Cd altered blastogenesis, T and B cell phenotypes and cytokine release were also mitigated best with piperine. The ameliorative potential was in order of piperine > curcumin > picroliv and former could be considered the drug of choice under immunocompromised conditions.

Keywords Cadmium · Piperine · Curcumin · Picroliv · Apoptosis · Oxidative stress

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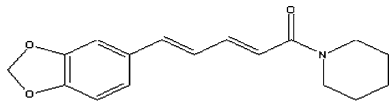
Introduction

Cadmium (Cd), a potent immunotoxicant, causes damage both to humoral immune response and cell mediated immunity (Descotes 1992; Dan et al. 2000). Divergent effects of Cd on the immune system has been demonstrated (Yamada et al. 1981; Ohsawa et al. 1983; Borgman et al. 1986; Cifone et al. 1989). Thymic atrophy in mice on Cd exposure was shown by Yamada et al. (1981). Susceptibility of different thymocyte subpopulations to Cd induced apoptosis revealed a marked decrease in $CD4^+/CD8^+$ ratio (Lafuente et al. 2004). The apoptotic potential of Cd suggested for its B and T cell immune suppression has been shown by Feng et al. (2001). Uptake of metal in thymus and spleen has been reported to be as low as 0.05 and 0.2–0.4% of the injected dose

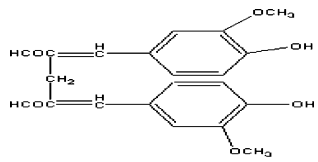
(1.8 mg/kg) respectively, compared to liver (60%) and kidney (6.5–9%) (Suzuki et al. 1981). All these studies do suggest that metal reaches the immune cells in circulation as well as in lymphoid organs.

In our earlier studies, we demonstrated Cd induced mitochondrial-caspase dependent apoptosis in murine splenocytes and thymocytes. The oxidative stress indices i.e. ROS and GSH, acted as intracellular signals preceding mitochondrial membrane depolarization and caspase-3 activation (Pathak and Khandelwal 2006a, b).

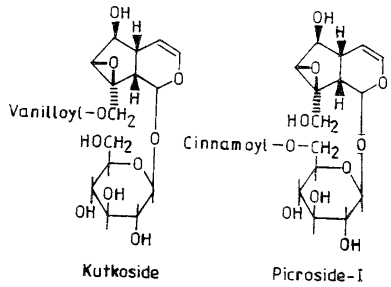
Since Cd is a potent immunotoxicant, studies pertaining to the identification of safe and active plant derived compounds, for its attenuation are warranted. Thus, the three herbals that were tested against Cd were piperine, curcumin and picroliv, having discrete chemical structures.



Piperine



Picroliv



Curcumin

Piperine, main component of *Piper longum* Linn. and *Piper nigrum* Linn., is a plant alkaloid with a long history of medicinal use in Indian medicine. It is known to exhibit a variety of biological activities which include anti-pyretic (Parmar et al. 1997), anti-depressant (Lee et al. 2005), hepatoprotective (Koul and Kapil 1993), anti-metastatic (Pradeep and Kuttan 2002), antithyroid (Panda and Kar 2003), immunomodulatory and antitumor (Sunila and Kuttan 2004). The anti-apoptotic efficacy of piperine has also been demonstrated by Choi et al. (2007) against cisplatin induced apoptosis via heme oxygenase-1 induction in auditory cells. In another study, Li et al. (2007) showed that piperine could reverse the corticosterone induced reduction of brain derived neurotrophic factor (BDNF) mRNA expression in cultured hippocampal neurons. Regarding the pharmacokinetics of this herbal, Suresh and Srinivasan (2008), studied blood levels of piperine upon a single oral dose (170 mg/kg) and found maximum levels within 6 h (11.0 μ/ml at 6 h and 0.93 at 24 h).

Curcumin (diferuloylmethane), the main yellow bioactive component of turmeric is a principal curcuminoid. It has been shown to have a wide spectrum of biological action, including anti-inflammatory (Sandur et al. 2007), antioxidant (Reyes-Gordillo et al. 2007), anti-proliferative, anti-carcinogenic (Xia et al. 2007) and anti-mutagenic activity (Polasa et al. 2004). Regarding its anti-apoptotic nature, Sikora et al. (1997) reported that the inhibition of apoptosis by curcumin in rat thymocytes was accompanied by partial suppression of AP-1 activity. In addition to its biological activities, there are reports that levels of curcumin and their metabolites can be detected in blood of cancer patients (Sharma et al. 2001, 2004).

Picroliv, standardized fraction from the alcoholic extract of root and rhizome of *P. kurroa*, is a mixture of iridoid glycosides containing 55–60% of picroside-I and kutkoside in a ratio of 1:1.5. Its major medicinal properties reported in modern literature are antioxidant (Seth et al. 2003), free radical scavenging (Ray et al. 2002), immunomodulatory (Puri et al. 1992), hepatoprotective (Yadav and Khandelwal 2006), testicular protective (Yadav and Khandelwal 2008), cholagogue and anticholestatic (Saraswat et al. 1993). Some other properties which contribute to its usefulness are anti-inflammatory, cardioprotective and moderate antiviral activity. In vivo detection of Picroside I and Kutkoside (constituents of picroliv) in plasma of rabbits after a iv dose (30 mg/kg) was studied by Vipul et al. (2005).

The experiments were therefore performed, to determine the role of piperine, curcumin and picroliv

in modulating Cd caused oxidative stress, apoptosis and suppressed immune functions. The rationale for evaluating these compounds was based on the fact that they are potent antioxidants, but their immunomodulatory roles remained unexplored. At the same time, their influence on T and B phenotypes and various immune functions such as blastogenesis and cytokine release were also studied to understand their overall cytoprotective ability. The various biochemical reflectors of cell damage such as cytotoxicity (MTT assay), oxidative stress (glutathione, reactive oxygen species), apoptosis (mitochondrial membrane potential, caspase-3 activity, phosphatidylserine externalization, apoptotic DNA, intranucleosomal DNA fragmentation) along with lymphocyte phenotyping, cell proliferative response and cytokine secretion (IL-2 and IFNγ) were assessed in Cd treated thymic and splenic cells.

Materials and methods

Chemicals

Cadmium chloride ($CdCl_2$) of ACS grade, RNase A, RPMI 1640, Antibiotic-antimycotic solution, Dulbecco's phosphate buffered saline (PBS), Fetal bovine serum (FBS), Agarose, 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), Piperine (97% pure), Curcumin(70% pure), Concanavalin A (Con A) and all other chemicals were purchased from Sigma Aldrich, USA. Rhodamine 123 (Rh 123) and 5′-Chloromethylfluorescein diacetate (CMF-DA) from Molecular Probes, Propidium Iodide (PI) from Calbiochem, Annexin V-FITC from Biovision, [^{3}H] Thymidine from BRIT, Bombay, FITC-conjugated anti-CD4 monoclonal antibody, FITC-conjugated anti-CD3 monoclonal antibody, PE-conjugated anti-CD8 monoclonal antibody, PE-conjugated anti-CD19 monoclonal antibody and mouse Interleukin-2 and gamma-Interferon ELISA kits from eBioscience. Cell Death Detection sandwich ELISA kit from Roche, Germany and Caspase-3 fluorometric protease assay kit was purchased from Chemicon, USA.

'Picroliv' the ethanolic extract of *Picrorhiza kurroa* was obtained from Pharmacology Division, Central Drug Research Institute, Lucknow and the activity evaluated by them was more than 55%.

Preparation of single cell suspensions

The BALB/c mice maintained in our animal house, were fed with standard rodent pellet and water ad libitum. Our animal house and breeding facility are registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and CPCSEA guidelines were followed (IAEC approval obtained).

The single cell suspensions of thymus and spleen was prepared under aseptic conditions. Suspensions were filtered through a stainless steel mesh and centrifuged at 200 × g for 10 min at 4°C. Thymocytes were resuspended in complete RPMI medium and splenocytes were suspended in 5.0 ml hypotonic erythrocyte lysing solution (2.42 g Tris and 7.56 g NH_4Cl in 1.0 l deionised water, pH adjusted to 7.2). After 5 min incubation followed by centrifugation, the cells were resuspended in complete medium. The cell density was adjusted to ca. 1.5×10^6 cells/ml for all parameters studied and the viability of freshly isolated cells was always over 95% (trypan blue exclusion test).

For monitoring of various parameters in the present investigation, we have used 25 μM conc of Cd and 1, 10 and 50 μg/ml of piperine, curcuminn and picroliv. The Cd conc selected was based on our earlier work (Pathak and Khandelwal 2006a, b).

Assessment of cell viability

The cell viability was measured by MTT reduction method of Mosmann (1983). Cells in RPMI 1640 at a density of 1.0×10^4 in 96 well plate were treated with Cd along with herbals for 18 h at 37°C in a CO_2 incubator. About 10 μl MTT (5 mg/ml PBS) was added to the wells, 4 h prior to completion of incubation time. The plate was centrifuged at 1200 × g for 10 min and 100 μl of DMSO was added after removing the supernatant, to dissolve the formazan formed. The absorbance was read at 530 nm after 5 min, in a microplate reader (Synergy HT of BIO-TEK International, USA).

Measurement of caspase-3 activity

A population of 3.0×10^6 cells/ml was incubated with Cd and herbals for 1.5, 3 and 6 h at 37°C in a CO_2 incubator. The cells were scraped and lysed on

ice for 10 min using cell lysis buffer. The reaction buffer (10 mM Tris HCl, 1 mM EDTA, 10 mM DTT, 5% glycerol) and DEVD-AFC substrate (50 μM final concentration) was then added and further incubated at 37°C in dark for 1 h. Fluorescence was measured at excitation and emission wavelengths of 400 nm and 505 nm respectively, on a microplate reader. The enzyme activity was expressed as % of control.

DNA fragmentation

The DNA fragmentation pattern (DNA ladder) was carried out by Agarose gel electrophoresis. An aliquot of 2 ml (1. 5 × 10^6 cells/ml) was incubated with Cd and herbals for 6 and 18 h at 37°C in a CO_2 incubator. At the end of incubation, cells were pelleted by centrifugation at 200 × g for 10 min and pellet lysed with 0.5 ml lysis buffer (10 mM Tris HCl, pH 7.5, 20 mM EDTA, 0.5% Triton X-100) on ice for 30 min. The DNA in lysed solution was extracted with phenol/chloroform and precipitated with 3 M sodium acetate (pH 5.2) and cold ethanol. After repeated washings, DNA was dissolved in TE buffer (10 mM Tris HCl, pH 8.0:1 mM EDTA). The purity of DNA at 260 and 280 nm absorbance ratio was between 1.7 and 1.9. DNA (2 μg) was then loaded on 0.7% agarose gel and electrophoresis carried out. The bands were visualized by ethidium bromide staining under UV light.

Flow cytometry analysis

The following assays were carried out on thymocytes and splenocytes treated with Cd and herbals for different time intervals (60 min, 1.5, 3, 6 and 18 h) at 37°C in a CO_2 incubator. The flow cytometric analysis were done on BD-LSR flow cytometer. Cell debris, characterized by a low FSC/SSC was excluded from analysis. The data was analysed by Cell Quest software and mean fluorescence intensity was obtained by histogram statistics.

Apoptotic DNA analysis

The cells with hypodiploid DNA were determined by cell cycle studies. After the treatment period, harvested cells were washed with PBS and fixed by drop-by-drop addition of ice cold 70% ethanol and stored at 4°C overnight. The fixed cells were harvested, washed with PBS and suspended in 1 ml PBS. Phosphate citrate buffer (200 μl, pH 7.8) was added and the cells incubated for 60 min at room temp. After centrifugation, cells were resuspended in 0.5 ml of PI stain (10 mg PI, 0.1 ml Triton X-100 and 3.7 mg EDTA in 100 ml PBS) and 0.5 ml of RNase A (50 μg/ml) and further incubated for 30 min in dark. The PI fluorescence was measured through a FL-2 filter (585 nm) and 10,000 events were acquired (Darzynkiewicz et al. 1992).

Assessment of apoptotic and necrotic cells

The apoptotic and necrotic cell distribution was analysed by Annexin V binding and PI uptake. Positioning of quadrants on Annexin V/PI dot plots was performed and living cells (Annexin V^-/PI^-), early apoptotic/primary apoptotic cells (Annexin V^+/PI^-), late apoptotic/secondary apoptotic cells (Annexin V^+/PI^+) and necrotic cells (Annexin V^-/PI^+) were distinguished (Vermes et al. 1995). Therefore, the total apoptotic proportion included the percentage of cells with fluorescence Annexin V^+/PI^- and Annexin V^+/PI^+. Briefly, after the treatment period (6 and 18 h), harvested cells were suspended in 1 ml binding buffer (1 X). An aliquot of 100 μl was incubated with 5 μl Annexin V-FITC and 10 μl PI for 15 min in dark at room temperature and 400 μl binding buffer (1X) was added to each sample. The FITC and PI fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired.

Assesssment of T and B lymphocyte population

T-cell phenotyping was conducted on the basis of CD4 and CD8 surface molecules. Positioning of quadrants on FITC/PE dot plots was performed and $CD4^+$, $CD8^+$, $CD4^+CD8^+$ (Double positive; DP) and $CD4^-CD8^-$ (Double negative; DN) subpopulations were distinguished. The lymphocyte population of splenocytes was based on CD3 (for T cells) and CD19 (for B cells) surface molecules. Briefly, the cells were suspended in 1 ml PBS. An aliquot of 100 μl was incubated with 5 μl FITC-conjugated anti-CD4 monoclonal antibody and 5 μl PE-conjugated anti-CD8 monoclonal antibody for thymocytes. In the case of splenocytes, they were incubated with 5 μl FITC-conjugated anti-CD3 monoclonal antibody

and 5 μl PE-conjugated anti-CD19 monoclonal antibody seperately for 30 min in dark at room temperature. After incubation, 400 μl PBS was added to each sample. The FITC and PE fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, on flow cytometer and 10,000 events were acquired.

Mitochondrial membrane potential

For the detection of mitochondrial membrane potential, cells were incubated with Rh 123 (5 μg/ml final conc) for 60 min in dark at 37°C, harvested and suspended in PBS. The mitochondrial membrane potential was measured by fluorescence intensity (FL-1, 530 nm) of 10,000 cells (Bai et al. 1999).

Reactive Oxygen Species (ROS) measurement

The generation of reactive oxygen species was detected by DCF fluorescence. Lymphocytes were incubated with Cd and herbals and DCFH-DA (100 μM final conc) was added simultaneously to the medium. Cells were harvested, suspended in PBS and ROS was measured by DCF fluorescence intensity (FL-1, 530 nm) of 10,000 cells (Wang et al. 1996).

Glutathione (GSH) measurement

Intracellular glutathione was monitored by CMF-DA. After treatment, the cells were incubated with CMF-DA (1 μM final conc) for 30 min in dark at 37°C. After harvesting, cells were suspended in PBS and GSH was measured by CMF fluorescence intensity (FL-1, 530 nm) of 10,000 cells (Okada et al. 1996).

[^{3}H] Thymidine incorporation measurement

To measure lymphocyte proliferation, 1.0×10^4 cells were seeded in 96-well plates in 200 μl of complete medium with or without one or more reagents {2.5 μg/ml Concanavalin A, 5 μg/ml Lipopolysaccharide (LPS), Cd, piperine, curcumin and picroliv} and incubated for 72 h at 37°C in a CO_2 incubator. [^{3}H] Thymidine (2 μCi) was added to the wells, 18 h prior to completion of incubation time. The cells were collected with cell harvester and incorporated radioactivity was measured in a liquid scintillation counter (Hewett Packard).

Release of cytokines

The release of Interleukin-2 (IL-2) and gamma-Interferon (IFN-γ) in lymphocytes were measured by using Mouse IL-2 and IFN-γ ELISA kits, respectively. Briefly, 1.0×10^4 cells in 200 μl of complete medium were seeded in 96-well plate with or without one or more reagents (2.5 μg/ml Concanavalin A, Cd and herbals) and incubated for 72 h at 37°C in a CO_2 incubator. The plate was centrifuged and supernatant was used for measuring cytokine release by ELISA. The ELISA plates were incubated overnight with 100 μl/well of capture antibody in coating buffer under cold conditions. After aspirating coating solution, wells were washed 3 times with 300 μl wash buffer and after removing the buffer carefully, 200 μl of blocking solution was added for 1.0 hr at 15–25°C. The plates were washed and standard/sample solution (100 μl) were added into each well and incubated for 2.0 h at 15–25°C. After washing the plates 5 times, 100 μl of detection antibody solution was added to each well and the plates were further incubated for 1.0 hr at 15–25°C, followed by washing. About 100 μl of Avidin-HRP was then added and incubated for 30 min and plates were again washed 7 times. The plates were further incubated with 100 μl of substrate solution for 15 min, 50 μl of stop solution was added to each well and the absorbance measured at 450 nm.

Statistical analysis

Significance of mean of different parameters between the treatment groups were analysed using one way analysis of variance (ANOVA) after ascertaining the homogeneity of variance between treatments. Pair wise comparisons were done by calculating the least significant difference.

Results

Cell viability

Cadmium causes a conc. dependent loss in cell viability in thymic and splenic cells at 18 h. The 25 μM Cd conc caused ~50% cell death in lymphocytes. The viability of cells was increased by piperine, curcumin and picroliv treatment. All the three doses of piperine could substantially enhance

percentage of viable cells, while with curcumin and picroliv, only the two higher doses exhibited significant change (Fig. 1). Median dose (10 μg/ml) was effective in improving cell viability in the order of piperine 84.3% > curcumin 79.2% > picroliv 69.4% in thymocytes. A similar trend was also observed in splenocytes. Herbals alone did not exhibit any cytotoxicity.

All subsequent experiments were carried out with multiple doses of herbals but for comparative efficacy, only the data of their median doses were considered in this study. Herbals per se did not elicit any effect on any of the parameters. Another reason for the selection of median dose, was based on literature reports (piperine: Pradeep and Kuttan 2004; curcumin: Meghana et al. 2007; picroliv: Visen et al. 1998).

Oxidative stress

ROS and GSH are oxidative stress markers and act as early intracellular signals for lymphocytes to undergo programmed cell death (Pathak and Khandelwal 2006a, b). DCF fluorescence proportionate to the ROS levels in cells, was monitored on flow cytometer. Cadmium caused significant ROS generation at all time points (1.5–18 h). The earliest discernible effect was observed with piperine at 3 h and in splenocytes, it exhibited significant lowering at all time points. At 6 and 18 h, all the three displayed restoration of ROS (Fig. 2), piperine taking the lead.

The CMF fluorescence which indicates intracellular glutathione predominantly, was substantially decreased by Cd from 3 h onwards. Suppressed GSH levels were significantly elevated by piperine and curcumin at 3 h, but with advancing time, the former exhibited higher efficacy as shown in Fig. 3. ROS and GSH data suggest the antioxidant characteristics of piperine > curcumin > picroliv.

Once establishing the antioxidative efficacy, our next aim was to evaluate their anti-apoptotic potential.

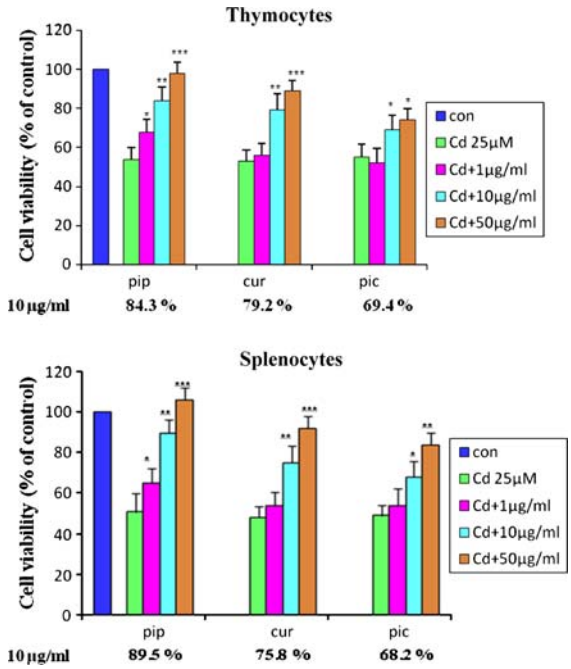


Fig. 1 Effect of piperine, curcumin and picroliv on cell viability. Freshly isolated lymphocytes (1.5×10^4) were treated with Cd (25 μM) and herbals (1, 10 & 50 μg/ml) for 18 h. Absorbance was measured at 530 nm. Each bar represents mean ± SD ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ as compared to Cd group, using one-way ANOVA

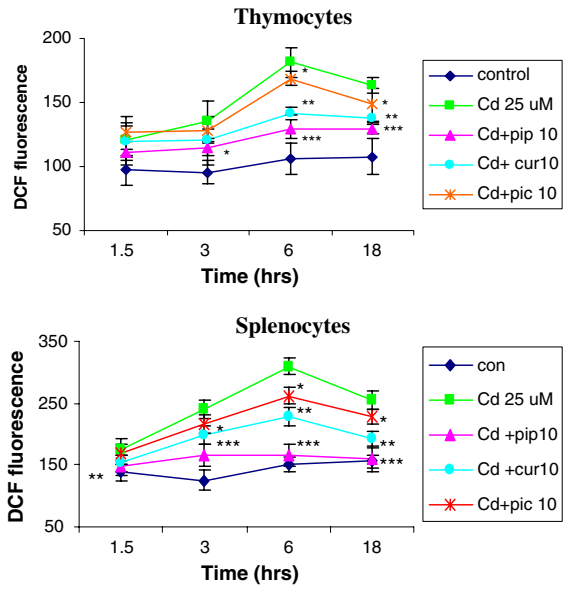


Fig. 2 Effect of piperine, curcumin and picroliv on ROS. Freshly isolated lymphocytes (1.5×10^6) were treated with Cd (25 μM), herbals (10 μg/ml) and DCFH-DA (100 μM) for 1.5, 3, 6 and 18 h at 37°C. DCF fluorescence was measured using flow cytometer with FL-1 filter. Results were expressed as a mean fluorescence obtained from the histogram statistics. Each bar represents mean ± SD ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ as compared to Cd group, using one-way ANOVA

DNA damage

The number of apoptotic thymocytes and splenocytes were monitored by cell cycle studies on flow cytometer. Fraction of hypodiploid cells were gradually increased by Cd at 6 and 18 h. Sub-G1 population was suppressed by piperine ($P < 0.001$), curcumin ($P < 0.01$) and picroliv ($P < 0.05$) in both the cells at 18 h (Table 1). At 6 h, only piperine could effectively minimise the apoptotic cell population.

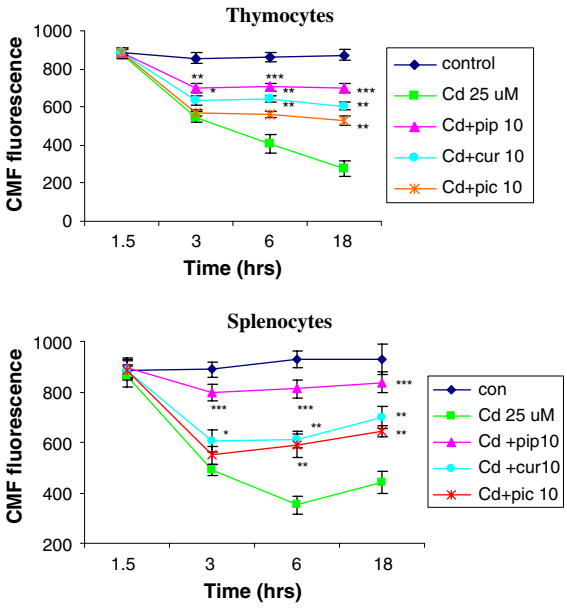


Fig. 3 Effect of piperine, curcumin and picroliv on GSH. Freshly isolated lymphocytes (1.5×10^6) were treated with Cd (25 μM) and herbals (10 μg/ml) for 1.5, 3, 6 and 18 h at 37°C. CMF-DA was added and incubated for 30 min. CMF fluorescence was measured using flow cytometer with FL-1 filter. Results were expressed as mean fluorescence obtained from the histogram statistics. Each bar represents mean ± SD ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ as compared to Cd group, using one-way ANOVA

We have previously reported the progressive increase in apoptotic and necrotic cell death in thymocytes and only apoptosis in splenocytes by Cd. At 18 h, from 20.6% apoptotic (early and late) thymic cells, it declined to 10.4% by piperine, 13.2 by curcumin and 16.1 by picroliv, in comparison to control (4.4%). The 5.8% necrotic cells were reduced to 3.8% by piperine.

Similarly in splenocytes, apoptotic cells declined to 12.0, 16.7 and 18.9% from 26.9% (Fig. 4). With piperine, the cells were maximally restored. Results of Annexin V binding assay by flow cytometry corroborated with the hypodiploid DNA data.

The intranucleosomal DNA damage in lymphocytes was shown by DNA ladder on agarose gel. DNA ladder induced by Cd was completely diminished by piperine (Fig. 5). With curcumin and picroliv, the bands were of low intensity.

DNA damage observed by different techniques confirmed anti-apoptotic potential of the herbals and amongst them, piperine appeared superior. Subsequent to establishing significant protection by these herbals, we further investigated whether these herbals can modulate the mitochondrial—caspase pathway of Cd.

Mitochondrial membrane potential and caspase-3 activity

Mitochondrial membrane potential, proportionate to the uptake of lipophilic cationic dye Rhodamine 123,

Table 1 Effect of piperine, curcumin and picroliv on Cd induced apoptotic DNA

Sub-G1 population (% of apoptotic cells)						
Groups	Thymocytes			Splenocytes		
	3 h	6 h	18 h	3 h	6 h	18 h
Control	2.1 ± 0.5	2.6 ± 0.4	4.2 ± 1.8	2.1 ± 0.7	3.2 ± 0.8	4.1 ± 0.4
Cd 25 μM	2.6 ± 0.8	7.2 ± 0.8	20.2 ± 3.7	1.4 ± 0.3	16.4 ± 0.8	25.6 ± 1.8
Cd + pip10	2.3 ± 0.9	4.8 ± 1.2*	7.6 ± 3.8***	1.9 ± 0.5	13.5 ± 1.4*	11.9 ± 2.1***
Cd + cur10	3.2 ± 0.8	8.1 ± 1.8	12.4 ± 2.8**	1.4 ± 0.6	16.2 ± 1.4	18.4 ± 2.7**
Cd + pic10	3.6 ± 0.6	8.2 ± 2.4	15.4 ± 4.6*	1.8 ± 0.6	16.8 ± 1.6	20.2 ± 3.8*

Freshly isolated thymocyte and splenocytes (1.5×10^6) were treated with Cd (25 μM), piperine, curcumin and picrolic (10 μg/ml) for 3, 6 and 18 h. The propidium iodide fluorescence was measured using a flow cytometer with FL-2 filter. Results were expressed as the percentage of apoptotic cells obtained from the histogram statistics. Each value represents mean ± SD ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ as compared to Cd treated group, using one-way ANOVA

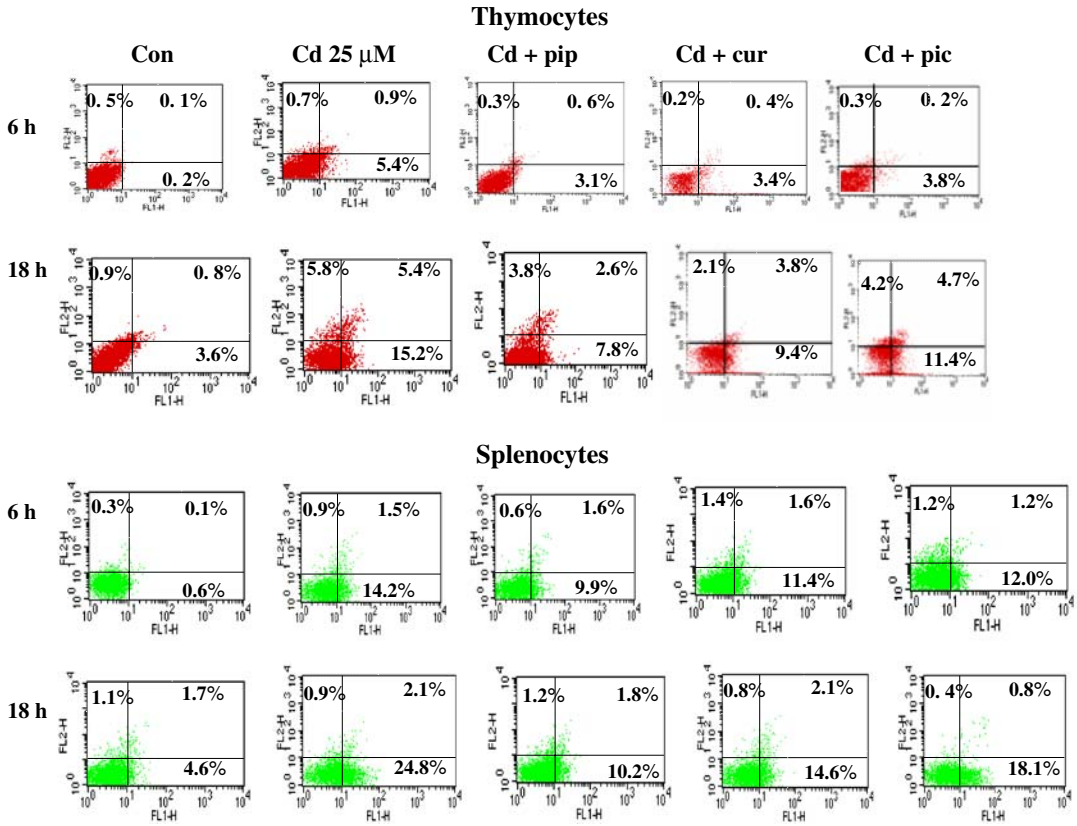


Fig. 4 Effect of piperine, curcumin and picroliv on apoptotic and necrotic cell distribution. Freshly isolated lymphocytes (1.5×10^6) were treated with Cd (25 μM) and herbals (10 μg/ml) for 6 and 18 h and the cell distribution was analysed using Annexin V binding and PI uptake. The FITC and PI fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results were expressed as dot plot representing one of the three independent experiments. LL, living cells (Annexin V^-/PI^-), LR, early/primary apoptotic cells (Annexin V^+/PI^-), UR, late/secondary apoptotic cells (Annexin V^+/PI^+) and UL, necrotic cells (Annexin V^-/PI^+). AC: Apoptotic cells, NC: Necrotic cells

was monitored as an early marker of apoptosis. The depolarizing effect of Cd at 3 and 6 h, was markedly abolished by herbals (Fig. 6) and the intensity of fluorescence in presence of piperine was maximum. Concurrently, Cd induced caspase activity was also ameliorated by herbals (Table 2). At 3 h, 2.8 fold increase in enzyme activity by Cd in thymic cells was reduced by 1.3 folds by piperine, 1.7 by curcumin and 2.0 by picroliv. Similarly in splenocytes, the restorative ability was highest with piperine.

From the above data, it appears that these herbals have both anti-oxidative and anti-apoptogenic potential, modulating cell death machinery following mitochondrial-caspase pathway. Our next step was to study the influence of these herbals on cell phenotypes.

T- and B-cell phenotypes

The effect of herbals on Cd altered thymocyte differentiation and development was measured by cell surface marker expression using flow cytometry. Cadmium altered the relative frequency of each subpopulation, a substantial increase in $CD8^+$ cells (from 6.8 to 24.1%) and a simultaneous fall in $CD4^+$ cells (from 10.5 to 4.0%) as depicted in Table 3. These distinct phenotypic changes were abolished by herbals. With piperine, both $CD8^+$ and $CD4^+$ populations were 10.1 and 8.6%, respectively. Regarding splenic population, B- and T-cells lowered by Cd (30.8 & 25.3%) were elevated by piperine (44.6 & 34.8%), curcumin (38.3 & 28.4%) and picroliv (34.2 & 26.8%) as evident in Table 3.

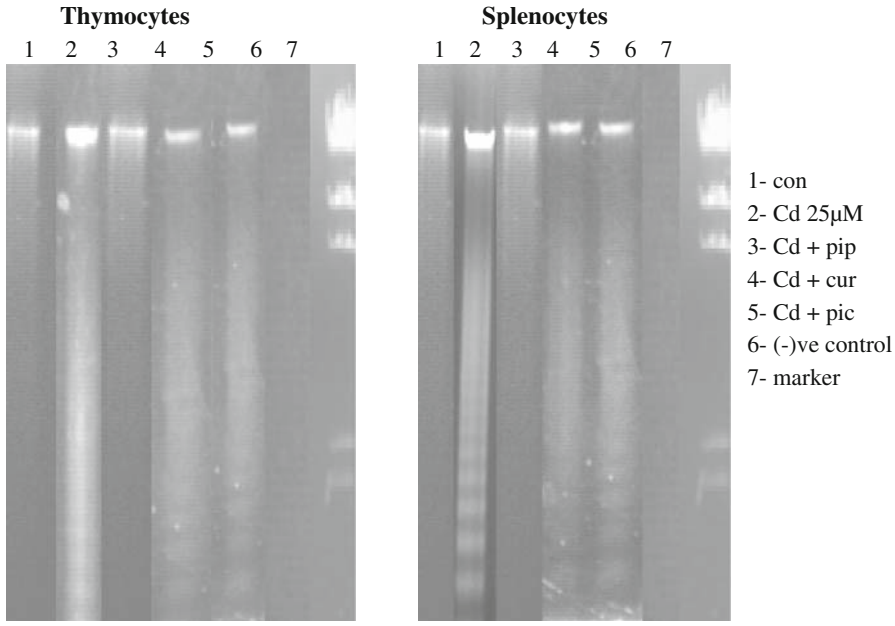


Fig. 5 Effect of piperine, curcumin and picroliv on intranucleosomal DNA fragmentation by agarose (0.7%) gel electrophoresis. The lymphocytes were isolated from mice and treated with Cd (25 μM) and herbals (10 μg/ml) for 18 h

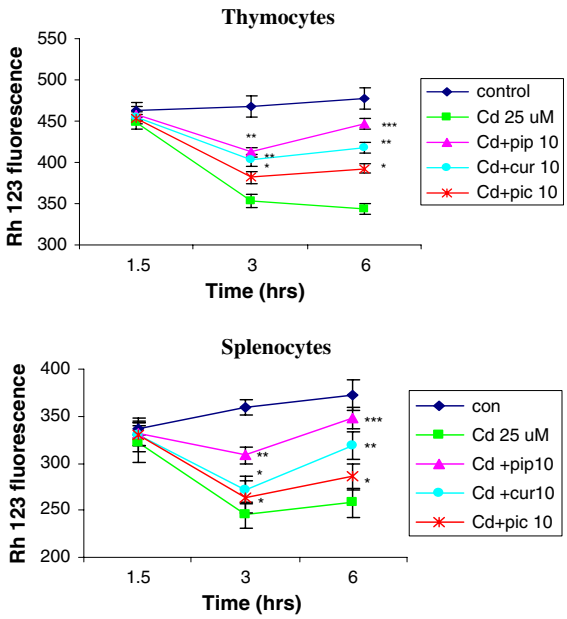


Fig. 6 Effect of piperine, curcumin and picroliv on mitochondrial membrane potential. Freshly isolated lymphocytes (1.5×10^6) were treated with Cd (25 μM) and herbals (10 μg/ml) for 1.5, 3 and 6 h at 37°C. Rh 123 was added and incubated for 60 min. The fluorescence was measured using flow cytometer with FL-1 filter. Results were expressed as mean fluorescence obtained from the histogram statistics. Each bar represents mean ± SD ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ as compared to Cd group, using one-way ANOVA

Table 2 Effect of piperine on Cd induced Caspase-3 activity

Fluorescence (% of control)						
Groups	Thymocytes			Splenocytes		
	1.5 h	3.0 h	6.0 h	1.5 h	3.0 h	6.0 h
Cd 25 μM	231	277	186	178	196	190
Cd + pip 10	120	127	98	112	123	110
Cd + cur 10	136	173	110	143	149	122
Cd + pic 10	153	201	118	146	172	144

Freshly isolated thymocytes and splenocytes (3.0×10^6) were treated with Cd (25 μM) and piperine, curcumin and picroliv (10 μg/ml) for 1.5, 3 and 6 h at 37°C. The enzyme activity was determined by Fluorometric Protease assay kit. The fluorescence was measured at Ex: 400 nm and Em: 505 nm. The value represents mean of two independent experiments

Blastogenesis

The proliferative capacity of cells towards antigenic response was measured by (^{3}H) thymidine incorporation into cellular DNA. Cadmium inhibited the (^{3}H) thymidine uptake in ConA stimulated thymocytes and ConA and LPS stimulated splenocytes. All these herbals effectively abrogated the inhibitory effect of Cd in both stimulated as well as non-stimulated T and B cells (Fig. 7), highest efficacy was demonstrated by piperine.

Cytokine release

Cadmium caused a significant suppression of IL-2 and IFN-γ in ConA stimulated thymic cells (Fig. 8). The cytokine levels were enhanced by piperine and curcumin in thymocytes, while picroliv could elevate IFN-γ only in splenocytes. In non stimulated cells, herbals had no influence. The results of B and T cell phenotypes, blastogenesis and cytokine data appear related to their anti-apoptotic nature. Since immune functions are directly dependent on viable cells, mitigation of apoptosis by herbals, would certainly augment the lymphocytes' performance.

Discussion

Earlier, we have demonstrated the induction of apoptosis by Cd in murine lymphocytes via mitochondrial—caspase pathway (Pathak and Khandelwal 2006a, b). Alterations in ROS and GSH, which served as intracellular early signals, affected the degree of apoptosis. This, in turn altered blastogenesis and cytokine release and could also relate to lymphoid organ damage (Pathak and Khandelwal 2007).

In this investigation, we explored various cellular checkpoints to understand the immunomodulatory efficacy of piperine (alkaloid), curcumin (curcuminoid) and picroliv (irridoid glycoside). These compounds restored not only thymic and splenic

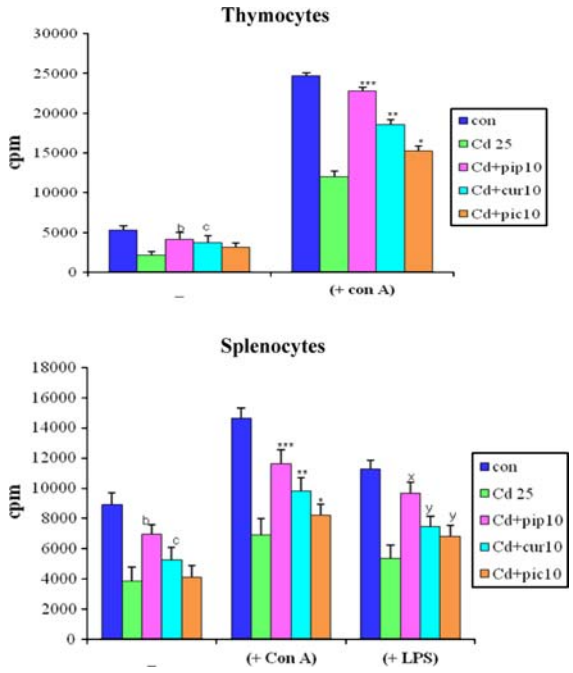


Fig. 7 Effect of piperine, curcum in and picroliv on blastogenesis. Freshly isolated lymphocytes (1.5×10^4) were treated with Cd (25 μM), herbals (10 μg/ml), Con A (2.5 μg/ml) and LPS (5 μg/ml) for 72 h. [^{3}H] Thymidine (2 μCi) was added, 18 h prior to completion of incubation time. The radioactivity was measured in liquid scintillation counter and the results were expressed as cpm. Each bar represents mean ± SD ($n = 6$). ^{b} $P < 0.01$, ^{c} $P < 0.05$ as compared to Cd treated group, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ as compared to Cd + Con A treated group and ^{x} $P < 0.001$, ^{y} $P < 0.01$ as compared to Cd + LPS treated group using one-way ANOVA

Table 3 Effect of piperine, curcumin and picroliv on T- and B-cell phenotypic changes

% of subpopulations					
Groups	Thymocytes			Splenocytes	
	$CD4^+$	$CD8^+$	$CD4^+CD8^+$	CD3	CD19
Control	10.5 ± 1.3	6.8 ± 1.2	80.6 ± 4.8	39.8 ± 5.6	56.2 ± 4.3
Cd 25 μM	4.0 ± .0.9	24.1 ± 5.2	44.2 ± 4.3	25.3 ± 2.1	30.8 ± 3.1
Cd + pip10	8.6 ± 0.6**	10.1 ± 1.8**	78.4 ± 5.2**	34.8 ± 2.8**	44.6 ± 2.2**
Cd + cur10	6.8 ± 0.8*	15.6 ± 2.1*	64.6 ± 3.4*	28.4 ± 1.6	38.3 ± 2.7*
Cd + pic10	3.9 ± 0.6	20.8 ± 2.4	51.8 ± 3.6	26.8 ± 1.4	34.2 ± 3.8

Freshly isolated lymphocytes (1.5×10^6) were treated with Cd (25 μM) and herbals (10 μg/ml) for 18 h and the cells were stained with FITC-conjugated anti-CD4 monoclonal antibody, FITC-conjugated anti-CD3 monoclonal antibody, PE-conjugated anti-CD8 monoclonal antibody and PE-conjugated anti-CD19 monoclonal antibody. The FITC and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results were expressed as the percentage of subpopulations obtained from the histogram statistics. Each value represents mean ± SD ($n = 3$). ** $P < 0.01$, * $P < 0.05$ as compared to Cd treated group, using one-way ANOVA

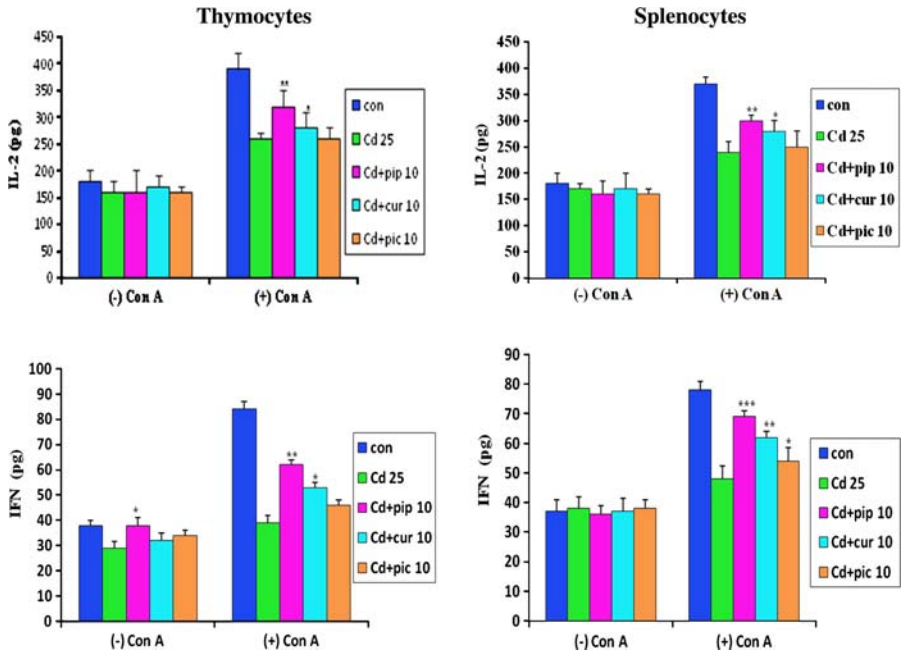


Fig. 8 Effect of piperine, curcumin and picroliv on cytokine levels. Freshly isolated lymphocytes (1.5×10^4) were treated with Cd (25 μM), herbals (10 μg/ml) and Con A (2.5 μg/ml) for 72 h at 37°C. The cytokines (IL-2 and IFNγ) were measured by ELISA kits. Each bar represents mean ± SD ($n = 3$). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ as compared to control, using one-way ANOVA

apoptotic cells, but the necrotic cells of thymus as well, to a varying degree. This anti-apoptotic effect of herbals could be responsible for modulation in immune functions (blastogenesis and cytokine release). Maximum efficacy was shown by piperine > curcumin > picroliv.

Literature reports reveal that all these herbals act as antioxidants. Simultaneous supplementation with black pepper or piperine in rats fed high fat diet lowered TBARS and conjugated dienes levels and maintained SOD, CAT, GPX, GST and GSH levels close to controls in rats (Vijayakumar et al. 2004). Curcumin treatment also prevented the GSH oxidation and maintained GSH/GSSH ratio in rats after oral administration of CCl_4 (Reyes-Gordillo et al. 2007). Yadav et al. (2005) and Yadav and Khandelwal (2008) have reported the antioxidative potential of picroliv in liver and testis of rats against cadmium. Cadmium induced enhanced lipid peroxidation, membrane fluidity and reduced levels of nonprotein sulphydryls and Na^+K^+ATPase were significantly restored by picroliv.

Regarding their structures, piperine contains pentacyclic oxindole group which is effective for immunomodulation (Reinhart and Uncaria 1999). Curcumin contains phenolic group which is important for its antioxidant activity (Venkatesan and Rao 2000) while the antioxidant potential of picroliv could be related to electrophilic free radical scavenging nature of its iridoid glycosides (Chander et al. 1992). As such, all the herbals are reported as powerful antioxidants, but our data suggests that pentacyclic oxindole group of piperine may be responsible for its higher efficacy than others.

Thiol modulators such as N-acetyl cysteine (NAC), buthionine sulfoximine (BSO) and pyrrolidine dithiocarbamate (PDTC) play a significant role in prevention of Cd induced apoptosis. Protection against acute and chronic Cd toxicity by reducing oxidative stress has been reported by many groups (Sunitha et al. 2001; Khandelwal et al. 2002; Shaikh and Tang 2002; Ray et al. 2002; Yadav et al. 2005). Similarly in our studies, antioxidant property could be one of the main triggering factors, responsible for mitigation of all downstream events initiating with ROS and GSH preceding mitochondrial membrane potential, caspase-3 activation followed by DNA damage and phenotypic alterations at 18 h and blastogenesis and cytokine release at a much later stage. Among the three herbals, piperine displayed maximum restoration of all cellular events and since anti-apoptotic potential appears directly proportional to antioxidant nature, piperine proved to be the most potent of all.

We therefore, conclude that immunomodulation by piperine, curcumin and picroliv may be clearly attributed to their multi-faceted activities such as anti-

oxidative, anti-apoptotic and restorative ability against cell proliferative mitogenic response, thymic and splenic cell population and cytokine release. Ameliorative response was in the sequence of piperine > curcumin > picroliv and former could be the drug of choice in immuno-compromised conditions.

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