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A Lectin with Anti-proliferative, Mitogenic and Anti-insect Potential from the Tubers of *Caladium bicolor* Vent

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ABSTRACT

A new lectin with *in vitro* anti-proliferative activity, potent mitogenic potential and anti-insect activity was isolated from the tubers of an ornamental monocotyledonous plant *Caladium bicolor* Vent, from the family Araceae. The lectin was purified by affinity chromatography on asialofetuin-linked amino activated silica beads. The native molecular mass of *C. bicolor* lectin (CBL) was 52 kDa, while subunit molecular mass was 13.1 kDa, indicating the homotetrameric nature of lectin. The purified lectin gave a single band in native PAGE at pH 4.5 while multiple bands were obtained in native PAGE at pH 8.3 and isoelectric focusing revealing the presence of isolectins. The secondary structure analysis by circular dichorism spectroscopy showed the presence of a β-pleated sheet structure. CBL was inhibited by *N*-acetyl-D-lactosamine (LacNAc), which is an important marker in various carcinomas and asialofetuin at 25 mM and 125 μg ml^{-1} respectively. The hemagglutinating activity of the lectin was not affected up to 55°C and in a pH range of 2.5-12.5. The lectin was stable upto 3.0 M of various denaturants tested namely urea, thiourea, guanidine hydrochloride and did not require metal ions for its activity. Out of 13 human cancer cell lines employed in the present study, CBL significantly affect the proliferation of colon and liver cancer cell lines, including HCT-15, HT-29, SW-620 and HOP-62. The lectin also showed a potent mitogenic response towards BALB/c splenocytes and human lymphocytes. Furthermore, CBL significantly prolonged the development period of second instar larvae of melon fruit fly, *Bactrocera cucurbitae* (Coquillett), and reduced the percentage pupation and emergence besides affecting the activity of esterases, an important class of hydrolytic enzymes.

Keywords: Araceae, N-acetyl-D-lactosamine, *Bactrocera*, cancer cell lines

INTRODUCTION

Lectins are the carbohydrate-binding proteins or glycoproteins of non-immune origin capable of specific recognition of, and reversible binding to carbohydrates without altering their covalent structure (Dixon 2003). Lectin-carbohydrate interactions occur in the molecular events underlying the immune response. Lectins are therefore important molecules as polyclonal reagents to investigate the molecular basis and control of lymphocyte activation and proliferation, as histochemical markers and for targeting of drugs, besides playing various important physiological roles (Smart 2004; Lannoo and Van Damme 2010; Gao *et al*. 2011; Hoffmeister 2011; Roth 2011). One of the most dramatic effects of the interaction of lectins with the cells is mitogenicity i.e., triggering of quiescent, non-dividing lymphocytes into a state of growth and proliferation. Mitogenic lectins have been used as invaluable tools to assess the immunocompetence of patients suffering from a variety of diseases and investigate the functioning of the immune system under abnormal conditions (Krickeberg *et al.* 1990; Ashraf and Khan 2005; Valdez-Vega 2011).

Furthermore, it has been reported that cellular protein glycosylation is influenced by several physiological factors, such as the occurrence of a disease and the altered glycoform population of a given glycoprotein might be used for diagnosis of the disease responsible for the alteration itself (Gabius *et al*. 2004; Ambrosi *et al.* 2005). Like anticancer drugs, lectins have also shown cytotoxic effects mediated via apoptosis (Miyoshi *et al.* 2001; Liu *et al.* 2010; Wong *et al.* 2010; Zwierzina *et al.* 2011). During the last decade, there has been a growing interest in lectins, which exhibit anti-cancer activity. For instance, *Agaricus bisporus* lectin possesses anti-cancer activity against human colon cancer cell-line HT-29, breast cancer cell-line MCF-7 while *Tricholoma mongolicum* lectin inhibits mouse mastocytoma P815 cells *in vitro* and sarcoma S-180 cells *in vivo* (Wang *et al.* 1997; Valdez-Vega 2011).

The wide distribution of lectins in all tissues of plants and their ubiquitous presence in the plant kingdom suggest important roles for these proteins. One possible physiological function that has emerged is the defensive role of these carbohydrate-binding proteins against phytopathogenic microorganisms, phytophagous insects and plant-eating animals. Indeed it has been shown that plant lectins possess cytotoxic, fungitoxic, anti-insect and anti-nematode properties either *in vitro* or *in vivo* (Macedo *et al.* 2003; Gaofu *et al.* 2008; Kaur *et al.* 2009; Vandenborre *et al.* 2011). The anti-insect activity of plant lectins can be economically of great potential in pest management because lectins are primary metabolic products and their genes are good candidates for conferring resistance in transgenic crops. Therefore, the purification and characterization of lectins from new sources may reveal genes with potential for use in the genetic improvement of crops. Lectins with a range of specific carbohydrate-binding affinities have been isolated from a variety of plants and study of the toxic effects of these compounds towards insects over a range of orders is one of the current area of interest in integrated pest management (Vasconcelos *et al.* 2004; Kaur *et al.* 2009; Singh *et al.* 2009; Michiels *et al.* 2010).

We report herein a new monocot plant lectin from *Caladium bicolor* (family Araceae) which showed a significant effect on the development of melon fruit fly, *Bactrocera*

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cucurbitae, one of the most important pests of cucurbits, besides affecting the activity of its esterases. In addition to this it significantly inhibited proliferation of human cancer cell lines and was a potent mitogen towards BALB/c splenocytes and human lymphocytes.

MATERIALS AND METHODS

Materials required

Human cancer cell lines i.e. Colo-205 (colon), HT-29 (colon), HCT-15 (colon), SW-620 (colon), HEP-2 (liver), HOP-62 (lung), A-549 (lung), SNB-78 (CNS), IMR-32 (neuroblastoma), SKOV-3 (ovary), MCF-7 (breast), PC-3 (prostate) and SiHa (cervix) were procured from NCCS (National Centre for Cell Sciences, Pune, India). These cell lines were maintained in RPMI-1640 medium (Sigma) supplemented with 10% FCS, glutamine (2 mM), penicillin (100 IU ml^{-1}) and streptomycin (100 µg ml^{-1}) at 37°C, in a humidified atmosphere with 5% CO_2 in a CO_2 incubator (Heraeus, Hanau, Germany). Melon fruit flies were reared by the procedure described by Gupta and coworkers (1978) in wire mesh cages (Rescholar equipment: 45 × 45 × 50 cm). The flies were provided with proteinex (Pfizer, India) and 20% sugar solution as food and with pumpkin fruit *Cucurbitae moschata* Dusch, for oviposition. Cultures of *B. cucurbitae* were maintained at 25 ± 2°C, photoperiod (L10:D14) and 70-80% relative humidity.

All sugars/derivatives, glycoproteins, bovine serum albumin, sulphorhodamine B, 3,4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide (MTT), ^{3}H- thymidine, ConA, blue dextran, histopaque-1077, 5-fluorouracil, mitomycin C and neuraminidase were obtained from Sigma Chemical Co., St. Louis, USA. Amino-activated silica beads used were from Clifmar, Guildford, UK. Biogel P-200 for gel permeation chromatography was purchased from Bio-Rad, California, U.S.A. Standard molecular weight markers, gel filtration markers and ampholines of pI range 3.5-9.5 were from Amersham Pharmacia (New Jersey, USA). RPMI-1640 medium with HEPES modification was from Gibco-BRL (New York, USA). Fetal calf serum (FCS), penicillin, L-glutamine and streptomycin were purchased from Sera Lab (GB), West Sussex, UK. All other chemicals were of analytical grade.

Purification of *C. bicolor* lectin

The tubers of *C. bicolor* Vent were procured from a local nursery. Lectin was extracted from 100 g of tubers by soaking in 0.01 M phosphate-buffered saline (PBS), pH 7.2, over night at 4°C and homogenized in a Waring blender in 1:5 (w/v) of PBS. After filtration, the crude extract was centrifuged at 20,000 × *g* at 4°C for 20 min. Dialyzed crude extract was applied to an affinity column (0.8 × 6.0 cm) of asialofetuin-linked amino activated silica beads. The column was prepared as described elsewhere (Shangary *et al.* 1995). The lectin bound to the matrix was eluted with 0.1 M glycine-HCl buffer, pH 2.5 and neutralized with 2 M Tris-HCl buffer, pH 8.8.

Agglutination and sugar-inhibition assays

Agglutination assays were performed using a battery of normal and neuraminidase-treated erythrocytes from nine different sources, including rabbit, rat, guinea pig, goat, sheep and human ABO blood groups. Besides this, human lymphocytes and murine splenocytes were also used for agglutination assays. The sugar-inhibition assay was carried out by using a spectrum of 40 different sugars, including monosaccharides i.e. D-arabinose, L-arabinose, D-ribose, D-xylose, D-fructose, D-galactose, D-glucose, D-mannose, L-sorbose, L-fucose, L-rhamnose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, methyl-α- and methyl-β-D-glucopyranosides, methyl-α-D-mannopyranoside, sialic acid, methyl-α- and methyl-β-D-galactopyranosides, β-phenyl-D-glucopyranoside, adonitol, *myo*-inositol, β-gentiobiose, D-lactose, D-maltose, D-melibiose, D-trehalose, T-disaccharide, *N*-acetyl-D-lactosamine, D-melizitose, D-raffinose, *N*,*N*',*N*"–triacetylchitotriose, chitin, glycogen, inulin and glycoproteins (fetuin, asialofetuin, porcine stomach mucin and thyroglobulin). Simple sugars and their derivatives were tested at a final concentration of 50 mM while polysaccharides and glycoproteins were tested at a concentration of 2 mg ml^{-1} using rabbit erythrocytes. The assay was performed as described elsewhere (Kaur *et al.* 2002).

Protein and carbohydrate analysis

Protein concentration was determined by the method of Lowry *et al.* (1951) using BSA as a standard. The total neutral carbohydrate content of CBL was estimated by the anthrone method (Spiro 1966) using D-glucose as standard.

Electrophoretic analysis

1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The subunit molecular weight (M_r) of CBL was estimated by discontinuous SDS-PAGE in the presence and absence of 2% 2-mercaptoethanol using 11% (w/v) slab gels at pH 8.3 as described by Laemmli (1970) and protein bands were visualized by Coomassie brilliant blue R-250.

2. Native PAGE

Polyacrylamide tube gel electrophoresis using a discontinuous buffer system was carried out on a 7.5% (w/v) gel at pH 4.5 (Reisfeld 1962) and 10% (w/v) gel at pH 8.3 (Bryan 1977).

3. Isoelectric focusing

Affinity purified CBL was subjected to isoelectric focussing in 5% polyacrylamide tube gel using carrier ampholines having pH range 3.5 to 9.5 (Robertson *et al.* 1987).

Gel filtration chromatography

Native molecular mass of the CBL was determined by gel filtration chromatography on a calibrated Biogel P-200 superfine column (1.6 × 62.0 cm, V_0 = 32.0 ml) using 0.01 M phosphate buffered saline (PBS), pH 7.2, by the method of Andrews (1964).

CD and fluorescence spectra

The circular dichroism measurements were performed using a JASCO J-720 spectropolarimeter, calibrated with ammonium d-10 camphorsulphonate. The spectra were studied over a range of wavelength of 200-250 nm, under constant N_2 purging. CD spectra were analyzed using the K_2D programme. The fluorescence measurements were performed using a Shimadzu RF-540 spectrophotometer in quartz cells of 0.1 mm path length. The samples were excited at 280 and the emission was recorded from 300-500 nm.

Atomic absorption spectrophotometry

The CBL was examined for the presence of various divalent metal cations i.e. Ca^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} by atomic absorption spectrophotometer (Shimadzu AA6200, Japan). Prior to analysis, CBL, which was originally in PBS, extensively dialyzed against deionized water to remove any ions contributed by the buffer. CBL sample was digested in the presence of mixture of concentrated HNO_3 and $HClO_4$ (1: 1) for 30 min. Standards were made by diluting commercial stock solutions of the respective metals.

Effect of temperature, EDTA, pH and denaturants on CBL hemagglutination activity

To examine the thermostability, CBL sample in PBS was incubated at different temperatures ranging from 40 to 100°C for 15 min, with a 5°C increase at each step. After each step of incubation, the hemagglutination assay was performed. The effect of metal ions was checked by prolonged dialysis of purified lectin against 0.1 M EDTA, followed by remetallization with Ca^{2+} and Mn^{2+} (Paulova *et al.* 1971). Hemagglutination activity was as-

sessed before and after the addition of Ca^{2+} and Mn^{2+} ions. The pH stability of the lectin was checked by extensive dialysis of the lectin solution against buffers of different pH values ranging from pH 1.5 to 12.5. The pH of the lectin solution was adjusted to pH 7.0 before hemagglutination activity was examined. Purified lectin was also subjected to increasing concentration of denaturants. An equal volume of urea, thiourea and guanidine-HCl ranging between 0.5 and 8.0 M was incubated with the same volume of CBL. The effect was studied by comparing the hemagglutination titre of treated and untreated samples.

Evaluation of *in vitro* anti-cancer activity against human cancer cell lines

The inhibitory potential of CBL was evaluated against various human cancer cell lines as explained above by the method of Monks *et al.* (1991) The different human cancer cell lines were maintained in RPMI-1640 medium supplemented with 10% FCS at 37°C, 5% carbon dioxide (CO_2) and 90% relative humidity in a CO_2 incubator (Heraeus, Hanau, Germany). The assay was carried out in triplicates of pure cell lines. The cells at a subconfluent stage were harvested by treatment with 0.05% trypsin in 0.01 M PBS and a single cell suspension was made in the above-mentioned medium. An aliquot of 100 µl of cell suspension (10^4 cells ml^{-1}) was added to each well of 96-well plates (Nunc) and the plates were incubated for 24 h in CO_2 incubator maintained at the above-mentioned conditions. Subsequently the lectin solution, which was prepared in PBS, pH 7.2 was added at 10, 30 and 100 µg ml^{-1} and the cultures were incubated for an additional 48 h. The adherent cell cultures were fixed *in situ* by adding 50 µl of 50% (w/v) trichloroacetic acid (final concentration, 10% TCA) and incubated for 1 h at 4°C. The supernatant was discarded and plates were washed five times with deionised water and dried. 100 µl of sulforhodamine B (SRB, 0.4 w/v in 1% acetic acid) was added to each well and the cultures were incubated for 10 min at room temperature. The unbound SRB was removed by washing five times with 1% acetic acid and the plates were dried. The dye bound to basic amino acids of the cell membrane was solubilized with Tris-buffer (10 mM, pH 10.5) and the absorption was measured at 540 nm by ELISA reader to determine the relative cell growth or viability in the treated as well as untreated cells. The anticancer drugs 5-fluorouracil and mytomycin C were used as positive controls at $5 \times l0^{-5}$ M.

Evaluation of mitogenic potential of *C. bicolor* lectin

The mitogenic potential of affinity-purified CBL was checked towards inbred female BALB/c mice splenocytes and human peripheral blood mononuclear cells (PBMC). Splenocytes and human PBMC have been isolated from BALB/c mice spleen and human blood respectively under sterile conditions. The test lectin and standard Concanavalin A (ConA) were filtered through 0.22 µm membrane filters (13 mm diameter, Schleicher and Schull, Germany) under aseptic conditions.

1. [^{3}H] thymidine uptake assay

To check the mitogenic potential of CBL on the proliferation of BALB/c splenocytes, a radioactive assay, known as the methyl-^{3}H thymidine uptake assay, was carried out by the method of Kilpatrick (1998). BALB/c splenocytes at 1.5×10^6 cells ml^{-1} in RPMI-1640 medium supplemented with 10% FCS were cultured with various concentrations of CBL ranging from 0.625 to 10.0 µg ml^{-1} and Con A was used as a positive control. In the control wells the cells were cultured only with medium without lectin. The cultures were set in an atmosphere of 5% CO_2 in a CO_2 incubator for 72 h. 16 h before the termination of cultures, 0.5 µCi of methyl-^{3}H thymidine was added to each well. Emissions were monitored by liquid scintillation counter, Rackbeta (LKB). The CPM (counts per minute) is expressed as mean ± S.D.

2. MTT (3,4,5-dimethylthiazol-2yl-2, 5-diphenyltetrazolium bromide) assay

Human PBMC were separated by the method of Bϕyum (1968) and the cells were set at a final concentration of 1.5×10^6 cells ml^{-1}. The cells were cultured with a different set of concentrations of CBL ranging from 2.5 to 40 µg ml^{-1} using Con A as the positive control. To check the lymphoproliferation, the MTT assay was performed in which the formazan formation was read at 540 nm according to Mosmann (1983).

Evaluation of anti-insect activity of CBL

1. Artificial diet bioassay

The effect of CBL was studied on the development of second instar of melon fruit fly, *B. cucurbitae*, by artificial diet bioassay (Srivastava 1975). Fresh pumpkin (*Cucurbitae moschata* (Dusch.)) pieces were placed in mesh cages, having about 100 pregnant female flies, for 8 h. These charged pumpkin pieces were then dissected in saline water and the harvested larvae were released into sterile culture vials (2.5 D × 100 L mm) containing 5 ml lectin incorporated artificial diet prepared according to the method described by Srivastava (1975). CBL was incorporated in diet at concentrations of 10, 20, 40, 80 and 160 µg ml^{-1}. Seven replicas were used for each concentration as well as for control. Ten second instar larvae were released into each vial. The vials were maintained in a B.O.D. incubator (NSW-152, India) and daily observations were made for the collection of data regarding pupation and adult emergence. The data were calculated for larval period, pupal period, total developmental period, percentage pupation, percentage emergence and lethal dose (LC_{50}).

2. Influence on enzyme activity

The effect of CBL was also studied on the activity of enzymes involved in metamorphosis, hydrolysis and detoxification i.e. general esterases (E.C. 3.1.1), acid phosphatases (E.C. 3.1.3.2) and alkaline phosphatases (E.C. 3.1.3.1) in melon fruit fly. The larvae (64-72 h old) were harvested from the charged pumpkin pieces and transferred to an artificial diet containing the LC_{50} concentration of lectin. The larvae were harvested from these vials after three intervals i.e. 24, 48 and 72 h and, enzyme activity was assayed on fresh weight basis (mM g^{-1} fresh weight). Similar experiment was conducted on larvae reared on untreated medium (control). There were six replications for each experiment. The enzyme activity of larvae reared on lectin-incorporated diet was compared with respective controls. The larvae reared on artificial diet without lectin for same time interval as for treatment served as control in all the experiments. Methodology given by Katzenellenbogen and Kafatos (1971) was followed for the estimation of esterases while the method of McIntyre (1971) was used for the measurement of phosphatase activity.

Esterases

The extract (1% w/v) was prepared by homogenizing larvae (25 mg) in 2.5 ml chilled extraction buffer (0.1 M sodium phosphate buffer, pH 6.5). The homogenate was centrifuged at 2,500 × *g* at 4°C for 20 min. The supernatant was used for enzyme assay. The substrate solution was pre-incubated in a water bath at 30°C for 10 min. After adding 0.1 ml of enzyme extract, the tubes were kept in water bath at a constant temperature of 30°C for 30 min. The reaction was stopped by adding 1 ml of post-coupling solution (4% sodium lauryl sulphate and 1% fast red TR salt solutions prepared in extraction buffer, mixed freshly in a 5: 2 ratio) and the absorbance of the mixture was recorded at 540 nm after an interval of 30 minutes. The standard curve was prepared using the serial dilutions of 0.01 M α-napthol (20-200 µM) at 540 nm and the calculated enzyme activity was expressed as M/mg fresh larva weight. In the blank enzyme extract was replaced with extraction buffer.

Acid phosphatases (AcP)

The extract (2% w/v) was prepared by homogenizing larvae in 2.5

Table 1 Affinity purification of the lectin from *Caladium bicolor* tubers.

Step	Total protein (mg)	Total activity (HU)[a,b]	Specific activity (HU mg^{-1})	Purification fold	Recovery (%)	MEAPC (µg ml^{-1})
Crude	429.4	24,532	57.2	1.0	100	17.5
Affinity purification						
PBS fractions	*320.0	-	-	-	-	-
Glycine-HCl fractions	♦58.0	13,382	241.1	4.2	57	4.0

Data are for 100 g of tubers; Volume of crude extract: 500 ml
[a]Total hemagglutination units; [b] 2% rabbit erythrocyte suspension was used for hemagglutination
*Unbound protein; ♦Eluted lectin
MEAPC: Minimal Erythrocyte Agglutinating Protein Concentration

ml of chilled extraction buffer (0.05 M acetate buffer, pH 5.0). The homogenate was centrifuged at 2,500 × *g* at 4°C for 20 min. The supernatant was used for enzyme assay. The substrate solution was pre-incubated at 30°C for 10 min in water bath. 0.2 ml of enzyme extract was added to this and the tubes having this mixture were kept in a water bath at a constant temperature of 30°C for 30 min. The reaction was stopped by adding 2 ml of post-coupling solution (4% sodium lauryl sulphate and 0.2% fast red TR salt solutions prepared in extraction buffer, mixed freshly in a 5: 2 ratio) and the absorbance of the mixture was recorded at 540 nm after an interval of 30 min. The standard curve was prepared using the serial dilutions of 0.01 M α-naphthol (20-200 µM) at 540 nm and the calculated enzyme activity was expressed as M/mg fresh larval weight.

Alkaline phosphatases (AkP)

The extraction and estimation of alkaline phosphatases were done according to the methodology given by MacIntyre (1971). The procedure was similar to that of AcP except that the extraction was done in 0.05 M Tris buffer, pH 8.6 and 1% homogenate of larvae were used instead of 2% used in case of AcP estimations.

Statistical analysis

The results obtained in artificial diet bioassay as well as enzyme activity were expressed as the mean ± SE. One-way analysis of variance (ANOVA) was applied where the multiple variants were to be accessed for their significance in the experiment for development period, percentage pupation and percentage emergence. The Student's *t*-test was applied in order to compare two variables at a particular treatment interval in the case of enzyme studies. LC_{50} values were calculated using probit analysis. All these tests were carried out with the help of SPSS-11 for windows (Landau and Everitt 2004).

RESULTS AND DISCUSSION

Isolation of CBL

CBL was isolated from the tubers of an ornamental plant *Caladium bicolor* Vent, belonging to the Araceae family. This lectin showed potent anti-proliferative, mitogenic, and anti-insect activity. The purification was performed by affinity chromatography on asialofetuin-linked amino activated silica beads. The profile of 0.01 M PBS was devoid of any hemagglutination activity indicating the complete adsorption of CBL to the matrix. The bound lectin was recovered as a sharp peak after elution with 0.1 M glycine-HCl buffer, pH 2.5 (**Fig. 1**). The data of purification are summarized in **Table 1**. The low purification fold of CBL can be attributed to the high lectin content of the total extractable proteins present in the storage tissues (Allen 1995; Kaur *et al.* 2006).

Sugar specificity and Agglutination assay

Out of 40 sugars/derivatives employed in hemagglutination inhibition assay, CBL was specifically inhibited by *N*-acetyl-D-lactosamine (LacNAc) and asialofetuin at a concentration of 25 mM and 125 µg ml^{-1}, respectively. LacNAc is a disaccharide, which is an important marker in malignant carcinomas. LacNAc specific lectins have also been reported from genus *Arisaema* and *Arum maculatum* of family *Araceae* (Shangary *et al.* 1995; Kaur *et al.* 2006, 2009). Asialofetuin, which is a complex desialylated serum glycoprotein, comprises of Thompson-Friedenreich (T) antigen, LacNAc, mannose as its structural components (Ito 1996). When employed individually in hemagglutination assay, all the components, expect LacNAc were found to be non-inhibitory, thus suggesting that the LacNAc structure might be responsible for asialofetuin binding in araceous lectins. Further, CBL was non reactive with any of the other carbohydrates including mono, di, trisaccharide and their derivatives along with three glycoproteins i.e., thyroglobulin, fetuin, mucin. The reactivity of lectin with asialofetuin, but not with fetuin suggests that sialic acid hinders the binding of lectin to the recognition sites in fetuin.

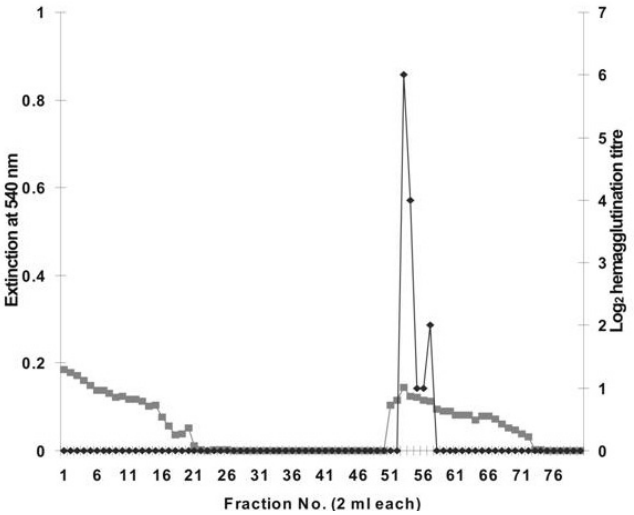

Fig. 1 Affinity purification of *C. bicolor* lectin from crude extract of tubers on asailofetuin-linked amino activated silica beads (0.8x6.0 cm) Dialyzed crude extract of CBL (22 mg) was applied to the affinity column, pre-equilibrated with 0.01 M PBS, pH 7.2. Bound lectins were eluted with glycine-HC1, pH 2.5. Flow rate, 50 ml/h; Absorbance at 540 nm.

Table 2 Biological activity of purified CBL.

	MEAPC (µg/ml)	
	Untreated	Neuraminidase treated
Rabbit	4.0	4.0
Rat	40.9	20.4
Guinea pig	163.5	10.2
Goat	0	163.5
Sheep	327.0	20.4
Human ABO blood groups	0	0

CBL was also employed for agglutination assays using a spectrum of erythrocytes. CBL was unable to agglutinate normal and neuraminidase-treated human ABO blood group erythrocytes. However, this lectin reacted with rabbit, guinea pig, rat and sheep red blood cells but agglutinated goat erythrocytes after neuraminidase treatment. The MEAPC for rabbit erythrocytes showed no change after neuraminidase treatment, but a marked decrease was observed in case of rat, sheep and guinea pig (**Table 2**), thus indicating that

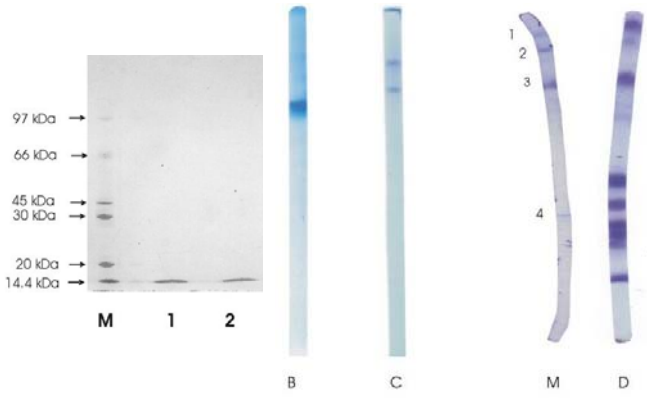

Fig. 2 (**A**) SDS-PAGE, pH 8.3, patterns of purified CBL using 11% gel in the presence of 2% 2-mercaptoethanol (lane l) and without 2-mercaptoethanol (lane 2). Running time 3 h at a constant 100 V. The amount of purified lectin loaded is 40 µg. Lane M represents M_r markers (from top to bottom): Phosphorylase b (97 kDa); albumin bovine (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20 kDa); and α-lactalbumin (14.4 kDa). (**B**) Discontinuous-PAGE, pH 4.5, using 7.5% gel (running time 6 h at a constant 100 V); protein load, 75 µg. (**C**) Discontinuous-PAGE, pH 8.3, using 10% gel (running time 6 h at a constant 100 V); amount of protein loaded, 45 µg. (**D**) Isoelectric focusing of non-denatured CBL on 5% polyacrylamide gel using carrier ampholines of pH range 3.5-10.0 (running time 12 h at a constant 200 V); protein load, 30 µg; lane M, position of pI marker proteins; (1) Myoglobulin a1(pI 7.2) (2) Myoglobulin a2 (pI 6.8) (3) carbonic anhydrase II, bovine erythrocytes (pI 5.9); (3) trypsin inhibitor, soybean (pI 4.6). The gels were stained with Coomassie brilliant blue.

sialic acid hinders agglutination in these cases. Removal of sialic acid from the cell surface increases the cell agglutination, an observation that correlates with the binding of lectin to asialofetuin, but not to fetuin.

Biochemical and biophysical characterization

The affinity purified CBL exhibited an apparent molecular mass of 52 kDa, as determined by gel filtration chromatography, In SDS-PAGE, pH 8.3, CBL gave a single band with subunit molecular mass of 13.1 kDa. Furthermore, a single band in SDS-PAGE, both under reducing and non-reducing conditions suggested that lectin subunits are not linked by disulphide linkage (**Fig. 2A**).

Thus, these results collectively indicated that native lectin is composed of four identical subunits, which may be associated by hydrophobic interactions and/or hydrogen bonds.

In native PAGE under acidic conditions, CBL gave a single band while under basic conditions it gave two distinct bands (**Fig. 2B, 2C**). In isoelectric focusing, CBL existed as a complex mixture of isolectins mostly in acidic range (**Fig. 2D**). A similar type of charge microheterogeneity has been reported from various other lectins (Allen 1995; Kaur *et al.* 2006). It has been reported that isolectins are encoded by nearly identical genes thus producing polypeptides with few altered amino acids resulting into isolectins (Van Damme *et al.* 1991).

Affinity purified CBL was found to be a glycoprotein having neutral sugar content of 2.2%. The results of heat denaturation showed that CBL was stable up to 55°C and its hemagglutination activity started declining afterwards, indicating that activity depends on the native conformation. However, CBL retained 25% of its residual activity even after boiling for 15 min. The examination of CBL activity towards different pH showed that CBL was stable from pH 2.5-12.5. With respect to its pH stability, this lectin resembles mannose-binding lectins from the Amaryllidaceae and Alliaceae families which possess only one disulfide bridge per polypeptide chain, are resistant to protease and stable in the pH range between 2 and 13 (Barre *et al.* 1996). Furthermore, after treatment with denaturing agents such as urea, thiourea and guanidine-HC1, the hemagglutination activity of CBL started declining after 3.0 M concentration. This decrease in lectin activity may be the effect of these denaturants on hydrogen bonding and hydrophobic interactions in lectin molecule which stabilize their three dimensional structure (Nelson and Cox 2001). CBL do not require metal ions for the hemagglutination activity. Atomic absorption spectroscopy was performed to examine the presence/absence of various divalent metal ions. The results showed the presence of Cr^{2+}, Co^{2+}, Zn^{2+} and Ca^{2+} at a concentration ranging between 0.006-0.05 mole per mole of lectin subunits thus representing a very small fraction of divalent metal ions in CBL, which might suggest adventitious metal ion binding. The other ions tested i.e. Cu^{2+}, Fe^{2+}, Mn^{2+} and Ni^{2+} were found to be absent.

Biological characterization

1. In vitro anti-cancer activity

The *in vitro* anti-cancer activity of CBL was evaluated against 13 human cancer cell lines representing different organs and tissues as described in Materials and methods. Out of 4 colon cancer cell lines employed in SRB assay, CBL inhibited proliferation of HCT-15 and HT-29 by 80%, while in case of SW-620 and Colo-205 inhibition was approximately 50% at 100 µg ml^{-1} of CBL. HOP-62, a liver cancer cell line was also inhibited by 82%. Four cancer cell lines of reproductive system were also employed, out of which MCF-7 and SKOV-3 showed 31% and 55% inhibition respectively, while other cell lines tested were virtually non-inhibitory (**Fig. 3**). The anti-proliferative effect of CBL towards HCT-15, HT-29, SW-620 and HOP-62 was comparable to earlier reported araceous lectins from *Arisaema helleborifolium and A. jacquemontii* (Kaur *et al.* 2006). However, unlike *Arisaema helleborifolium* lectin, CBL also inhibited the proliferation of MCF-7, SKOV-3, Colo-205. This suggests that the anti-proliferative effect of CBL has selectivity to different tumor cell lines. The variation of proliferation inhibition on different cell-lines may be due to the presence of glycoconjugates varying slightly in their activity, thus leading to different signalling action of lectins (Brooks *et al.* 2002). The exact molecular mechanism(s) of the anti-proliferative effect of plant lectins is not clear at present, although several hypotheses have been put forward which suggests that this effect is associated with the ability of lectins to modulate the growth, differentiation, proliferation, and apoptosis of premature cells *in vivo* and *in vitro* (Yu *et al.* 1999). Additional studies are required to understand the exact mechanisms of the anti-proliferative effect of plant lectins and future studies should be focused on the examination of these possibilities in appropriate models of human diseases and it is also possible that what other agents (e.g. synthetic compounds) should interact with plant lectins in a synergistic manner to prevent cancer and make a better prediction to which type of protocol (e.g., chemopreventive or chemotherapeutic) for the anti-proliferative effect of plant lectins is most likely to be successful, in animal models and ultimately in human diseases. Thus, the anti-proliferative ability of CBL seems to be an interesting line of investigation as this novel araceous lectin can prove to be a useful tool in cancer research, as well as for diagnostic and lectin therapy in modern medicine.

2. Mitogenic potential

CBL showed potent mitogenic response towards BALB/c splenocytes and human peripheral blood mononuclear cells (PBMC). The relative mitogenic stimulation of CBL towards human lymphocytes was almost double than that of Con A, a well-known standard plant mitogen. The optimum dose of CBL was 1.25 and 5 µg/ml respectively in case of BALB/c and human lymphocytes. The mitogenic lectins can be of significant use in increased understanding of the relationship between chromosomal abnormality and human

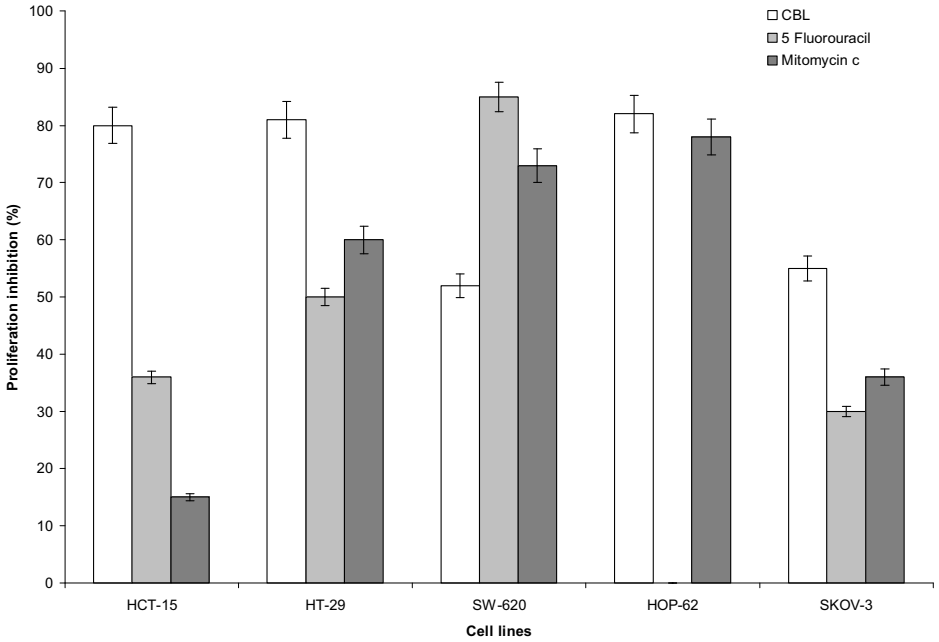

Fig. 3 ***In vitro*** **anti-cancer potential of lectins was checked on 13 human cancer cell lines using SRB assay.** The cells were seeded at a concentration of 10^4 cells ml^{-1} in complete RPMI-1640 medium containing 50 µg ml^{-1} of gentamycin. The absorbance was measured at 540 nm using ELISA reader. Anticancer drugs (1×10^{-4} M) 5-fluorouracil and mitomycin C were used as positive controls, while cells cultured with medium alone (no lectin) constitute negative control. Bars indicate percentage inhibition at 100 µg/ml.

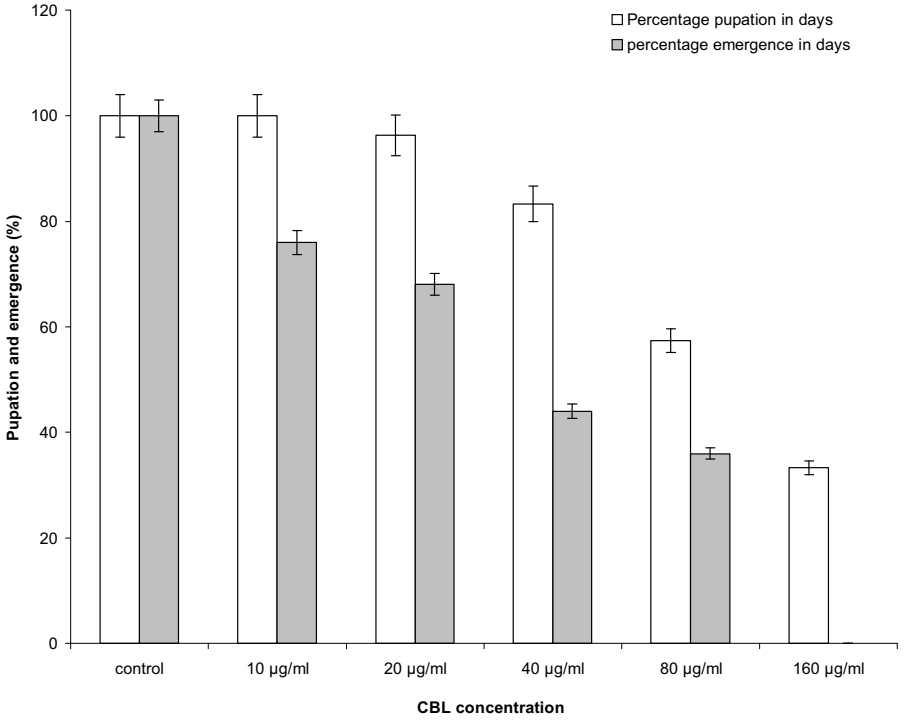

Fig. 4 Histogram showing the effect of lectins on percentage pupation and emergence of ***Bactrocera cucurbitae*****.** The second instar larvae were reared on artificial diet containing affinity-purified lectins at various concentrations. There were seven replications for each treatment as well as control.

diseases, which can tremendously help the diagnosis. Besides other cells, lymphocytes have been the usual target cells for mitogenic assays, and the study of lectin-lymphocyte interaction can result in substantial contribution of elucidating the mechanism of lymphocyte activation and its control, thereby contributing to our understanding of cell growth and development.

3. Antiinsect potential

The melon fruit fly is a serious pest of Cucurbitaceae worldwide besides affecting various other plants. The pest has so far defied most of the conventional control methods. Furthermore, mannose-binding monocot lectins having tetrameric structure have shown more potent anti-insect property and anti-fungal activity than the dimeric or trimeric lectins because of their ability to interact strongly with complex glycoconjugates due to their multivalency (Barre *et al.* 1996). CEA having a similar structure but different sugar specificity needs to be investigated in this regard.

CBL significantly affected the development period of melon fruit fly in artificial diet bioassay. Incorporation of *C. bicolor* lectin in diet showed a significant ($P < 0.01$) prolongation in larval period from 6.14 ± 0.13 days in control to 10.97 ± 0.29 days i.e. 4.83 days at the highest concentration investigated, while pupal period showed a non-significant increase. The total development period delayed significantly up to 5.93 days at 160 µg ml^{-1}. Percentage pupation and percentage emergence was only 33 and 36%, respectively at

160 µg ml^{-1} relative to larvae reared on control diet (**Fig. 4**). Ultrastructural studies have shown that insecticidal lectins bound to midgut epithelial cells in a number of insect pests (Habibi *et al.* 1998). Alternatively, evidence for the delivery

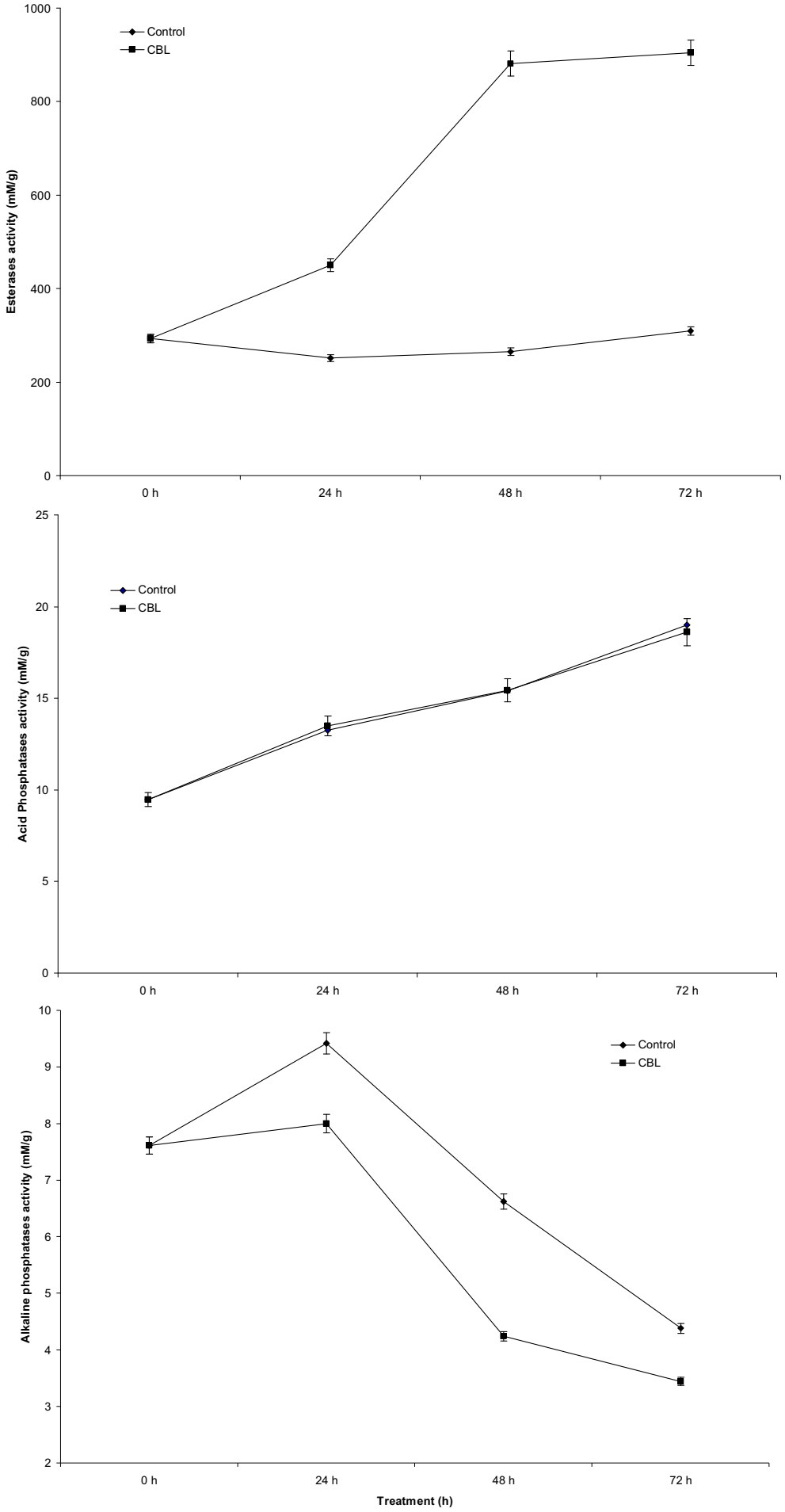

Fig. 5 (previous page) Effect of LC_{50} of lectins on activity of various enzymes at various time intervals. The larvae (64-72 h old) were transferred to artificial diet containing LC_{50} concentration of respective lectins. The larvae were harvested from these vials after three time intervals i.e. 24, 48 and 72 h i.e. at the age of 96, 120 and 144 h respectively. Similar experiment was conducted with untreated medium (control). The enzyme activity of larvae reared on lectin-incorporated diet was compared with respective controls. (**A**) Effect of LC_{50} of lectins on activity of esterases; (**B**) Effect of LC_{50} of lectins on activity of acid phosphatases; (**C**) Effect of LC_{50} of lectins on activity of alkaline phosphatases.

of ingested lectins to the haemolymph in Homoptera and Lepidoptera species has highlighted the possibility for systemic mode of lectin action (Bandyopadhyay *et al.* 2001; Fitches *et al.* 2010). To ascertain the mechanism of action, the effect of CBL was investigated on various hydrolytic enzymes generally involved in digestion, development and metabolism i.e. Esterases, acid phosphatases (AcP) and alkaline phosphates (AkP) in the second instar larvae (64-72 h) of *B. cucurbitae*, for three time intervals (24, 48 and 72 h).

Esterases are the enzymes involved in the metamorphosis of insects. Their levels rise during the late larval stages (Hammock *et al.* 1990; Shanmugavelu *et al.* 2000). A perusual of literature indicates that their levels also increase under stress due to anti-metabolic agents (Kaur *et al.* 2009; Singh *et al.* 2009). They have been shown to play a role in the development of resistance and sequestration of xenobiotics (Wu *et al.* 2011; Zou *et al.* 2011). The larvae were reared on diet containing lectin showed a significant ($P < 0.01$) increase in esterase activity when compared to respective controls i.e. approximately three times that of control was observed (**Fig. 5A**). The increase in the plateau of esterase activity in the lectin treated larvae suggest that esterases might be playing a significant role in detoxification of these lectins and the increase in activity could be attributed to positive feedback response (Devorshak and Roe 1999; Rup *et al.* 1999). Phosphatases are reported to be the biosensors as they are inhibited by organophosphorus compounds (Luskova *et al.* 2002). They have been reported to be the detoxifying enzymes of insects (Oppenoorth 1985). The alkaline phosphatases are involved in transport across free cell borders. These hydrolytic enzymes have their roles in recycling of phosphate and energy transfer. Because of these roles, their expressions are expected to increase during high metabolic activity. In case of larvae reared on diet containing *C. bicolor* lectin there was no significant change in acid phosphatase activity (**Fig. 5B**). Alkaline phosphatase (AkP) activity increased up to 96 h of age and then decreases significantly as the age advanced to 144 h in normal course of development (**Fig. 5C**). The treatment resulted in the suppression of AkP activity. The suppression of other two hydrolases (acid and alkaline Phosphatases) indicated that phosphatases play no role in the detoxification of these lectins in *B. cucurbitae* and that lectin might be interfering in the feedback biomechanism of these enzymes during their synthesis.

CONCLUSION

In conclusion, this was a basic approach to isolate *C. bicolor* lectin and still more research is needed at the molecular and biochemical levels as CBL, due to its properties, can be a useful tool in cancer research, immunomodulation, glycobiology and integrated pest management.

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