



Characterization of chickpea (*Cicer arietinum* L.) lectin for biological activity

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Received: 28 April 2017/Revised: 30 December 2017/Accepted: 15 January 2018/Published online: 20 March 2018
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Abstract Lectins are proteins that are subject of intense investigations. Information on lectin from chickpea (*Cicer arietinum* L.) with respect to its biological activities are very limited. In this study, we purified lectin from the seeds of chickpea employing DEAE-cellulose and SP-Sephadex ion exchange chromatography and identified its molecular subunit mass as 35 kDa. The free radical scavenging activity of lectin measured by the DPPH assay has IC₅₀ of 0.88 µg/mL. Lectin exerted antifungal activity against *Candida krusei*, *Fusarium oxysporium oxysporium*, *Saccharomyces cerevisiae* and *Candida albicans*, while antibacterial activity against *E. coli*, *B. subtilis*, *S. marcescens* and *P. aeruginosa*. The minimum inhibitory concentrations were 200, 240, 160 and 140 µg for *C. krusei*, *F. oxysporium*, *S. cerevisiae* and *C. albicans* respectively. Lectin was further examined for its antiproliferative potential against cancerous cell line. The cell viability assay indicated a high inhibition activity on Ishikawa, HepG2, MCF-7 and MDA-MB-231 with IC₅₀ value of 46.67, 44.20, 53.58 and 37.46 µg/mL respectively. These results can provide a background for future research into the benefits of chickpea lectin to pharmacological perspective.

Keywords Chickpea lectin · Sp-Sephadex · SRB assay · Human cancer cell lines

Introduction

Leguminous plants especially food legumes produce variety of structurally different proteins for self-defence. Characterization of such proteins is of utmost importance and lectins are one of them. Lectins are hemagglutinating proteins distributed ubiquitously in different plant species and extracted principally from seeds. The rich hydrophobic amino acid regions of lectins allow interactions with other molecules like host–pathogen interactions, cell targeting and cell–cell communications (Irlanda et al. 2017). Literature perusal confirm that plant lectins show diversity of biological activities including mitogenic (Chan et al. 2011), anti-fungal and anti-HIV-1 reverse transcriptase activities (Ye and Ng 2002) as well as anticancer properties to human cell lines (Liu et al. 2010). The isolation and purification of lectins from seeds of various taxa have been well documented. Examples include *Amaranthus viridis* (Kaur et al. 2006), *Capsicum frutescens* (Ngai and Ng 2007), *Phaseolus vulgaris* (Xia and Ng 2006), *Withania somnifera* (Ghosh 2009), *Zea mays* (Baker et al. 2009) and lima bean (E Lacerda et al. 2017).

Cicer arietinum L., commonly known as chickpea, is an extensively consumed pulse in India and worldwide. Seeds of chickpea provide major source of dietary proteins associated with health promoting benefits in foremost diseases like diabetes, cardiovascular diseases and some cancers (Roy et al. 2010). From chickpea, diverse class of proteins with cyclophilin-like protein (Ye and Ng 2002), chitinase (Vogelsang and Barz 1993) and some peptides displaying antifungal activities have also been identified (Chu et al. 2003). Previous reports of chickpea lectin on various facets include characterization of a clone encoding vegetative lectin from *Cicer arietinum* epicotyls (Esteban et al. 2002), production of stable transgene chickpea

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through expression of insecticidal lectin (Chakraborti et al. 2009) and root lectins (Agrawal et al. 2011). In addition, in silico studies on recombinant chickpea lectin (Wakankar et al. 2013), three dimensional structure determination (Sharma et al. 2015) and α -amylase inhibitory activity against potato beetle larvae (Wang et al. 2017) are also available in literature domain.

Lectins have been the subject of intense investigations. Chickpea lectin demonstrate diversity in molecular weights. In 1983, a 47 kDa lectin from seeds of *Cicer arietinum* was isolated by Kolberg et al. In 2005, our previous studies reported crystallization and preliminary X-ray characterisation of a lectin from chickpea having molecular weight of 43 kDa composed of two identical subunits (Katre et al. 2005). In 2006, Qureshi et al. isolated 120 kDa lectin from seeds of chickpea possessing high affinity for *N*-acetyl-D-galactosamine. Further studies related to its biological activity have not been reported for this lectin and therefore warranted. In the present paper, we describe purification of lectin from the seeds of chickpea and its biological characterization concerning antifungal, antibacterial and anticancerous activity.

Material and methods

Chemicals and reagents

SP-Sephadex and SRB was procured from Sigma-Aldrich, St. Louis, MO, USA. DEAE-cellulose was obtained from Sisco-Research Laboratories (SRL) India. Other chemicals used in the experiment were of analytical grade.

Seed material

Seeds of *Cicer arietinum* L. (cv-Digvijay) was obtained from Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India under MTA understanding.

Isolation and purification of lectin

Defatted 100 g chickpea seed powder was soaked in 500 mL of 10 mM Tris-HCl pH 7.2. The suspension was stirred for 16 h, centrifuged at 10,000 g for 20 min. The obtained supernatant was precipitated with 80% ammonium sulfate and recentrifuged at 10,000 g for 20 min at 4 °C. The precipitate was dissolved in 20 mM Tris-HCl buffer pH 7.2 and dialysed extensively against the same buffer. The dialysate was centrifuged at 10,000 g for 10 min and the clear supernatant was loaded onto a DEAE-cellulose column pre-equilibrated with 20 mM Tris-HCl buffer pH 7.2. The column was washed with the same buffer until the wash showed no absorbance at 280 nm.

The lectin eluted as unadsorbed portion. Fractions (2 mL) of the wash with optical density > 0.2 possessing hemagglutination activities were pooled together and dialysed against 20 mM acetate buffer pH 5.0. Further, sample was loaded onto SP-Sephadex column (4 × 20 cm) pre-equilibrated with 20 mM acetate buffer (pH 5.0) and washed with the same buffer. Finally, the bound protein was eluted with acetate buffer (pH 5.0) containing NaCl in a step gradient of 0.1–0.5 M NaCl in steps of 0.1 M (Katre et al. 2005). The fractions showing hemagglutination activity were pooled and dialysed against deionized water. Protein concentrations at various stages of investigations were determined by Lowry et al. (1951).

Hemagglutination assay

Hemagglutination activity of the lectin was performed using human (A, B, AB and O) and rabbit erythrocytes. The specific activity of lectin is defined as the titre of hemagglutination per mg of protein (Liener 1962).

DPPH radical scavenging assay

The DPPH radical-scavenging capacity of samples was determined as described by Bersuder et al. (1998). A volume of 500 μ L of each sample at different concentrations was added to 375 μ L of 99% ethanol and 125 μ L of DPPH solution (0.02% in ethanol) as free radical source. The mixtures were shaken and then incubated for 60 min in dark at room temperature. Scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. Ascorbic acid was used as positive control. DPPH radical scavenging activity was calculated using the equation:

$$\text{DPPH \%} = (A_{\text{blank}} - A_{\text{sample}}) / (A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the sample) and A_{sample} is the absorbance of sample (with DPPH solution).

Assay of antifungal activity

Antifungal activity of lectin was assessed against various fungal species which includes *Candida krusei* and *Fusarium oxysporum* employing disc diffusion methods. Sterile petri plates (100 × 15 cm) containing 10 mL of potato dextrose agar (PDA) were used. After the mycelia colony had developed, sterile paper discs (6 mm diameter) were laid at a distance of 1 cm from the rim of mycelia colony.

An aliquot of lectin dissolved in milli-Q water (1 mg/mL) was applied to a disk. The petri plates were incubated at 25 °C for 72 h until mycelia growth had enveloped peripheral disks containing control (amphotericin-B) and had produced crescents of inhibition around disks (Ye et al. 2000). The diameter of the clear zone around the well was measured as percent inhibitory influence of lectin using following equation;

$$\% \text{ Inhibition} = (C - T/C) \times 100$$

where C = diameter of the fungal colony in the control petri dish, T = diameter of the fungal colony in the treated petri dish.

Fungal PDA plates were prepared for *Saccharomyces cerevisiae* and *Candida albicans* strains and different concentrations (50, 100, 150, 200, 250 and 300 µg) of dissolved lectin was placed on discs. The plates were incubated for 24 h at 28 °C until optimal growth attained and zone of inhibition was measured.

Assay of antibacterial activity

The antibacterial activity of the lectin was tested against *Escherichia coli*, *Bacillus subtilis*, *Serratia marcescens* and *Pseudomonas aeruginosa* employing agar disc diffusion method. The different concentrations (50, 100, 150, 200, 250 and 300 µg/mL) of lectin and a standard antibiotic streptomycin (1 mg/mL) was used for assay. The bacterial cultures were grown on nutrient agar medium at 37 °C and zone of inhibition was recorded.

Cell lines and culture conditions

Human endometrial adenocarcinoma cell line (Ishikawa), human hepatocellular carcinoma cell line (HepG2), Human breast cancer cell line (MCF-7), human breast adenocarcinoma cell line (MDA-MB-231) were procured from National Centre for Cell Science (NCCS), Pune, India. These cell lines were grown and maintained in a high glucose concentration (4.5 g/L) Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin in a constant humidified incubator at 37 °C and in 5% CO₂. The cells were cultured (about 80% of confluent) once a week, followed by change in media one additional time in a week. Experiments were conducted using cells with < 20 passages. Cells were counted using a haemocytometer and plated according to the number of cells for each experiment for 24 h prior to addition of test compound. The test sample was solubilised in sterile filtered DMSO (< 0.5% in the culture medium) prior to addition to the culture media. Control cells were maintained in parallel and subjected to the same changes in media.

Cell viability assay

The effects of chickpea lectin on cell viability were determined by SRB assay (Skehan et al. 1990). Briefly, the cells were fixed with 10% chilled TCA for 1 h at 4 °C. To each well, 100 µl of 0.4% (w/v) SRB (1% in acetic acid) was added and incubated for 30 min at room temperature. Unbound SRB was removed by three washes with 1% acetic acid and the plates air-dried. For extracting the bound stain, 200 µl of unbuffered 10 mM Tris base, pH (10.5) was added and absorbance was read at 560 nm using microplate reader (Biorad, USA). The cell % inhibition was calculated using the following formula:

$$\text{Cell proliferation inhibition}(\%) = [1 - (A_s/A_c)] \times 100$$

where A_s-absorbance of sample; A_c-absorbance of control.

Statistical analysis

The statistical analyzes were performed using the Graph Pad Prism Software (version 5) and data was expressed as mean ± SD. The IC₅₀ concentration was calculated using concentration-effect regression line.

Result

The plants seed contain variety of proteins and lectins are one of them. The lectins studies carried out in legume species are therefore focused in seeds (Damme et al. 1998) due to 15% of lectin accessibility in the total proteins. Therefore, in the present investigation, seeds of *Cicer arietinum* L. were used as a source for lectin isolation. A protein with hemagglutinating activity was purified from the seeds of *Cicer arietinum* L. and designated as CAL. The (NH₄)₂SO₄ precipitated crude extract of seeds gave 546.13 HAU/mg specific activity. The protein was further purified employing sequential ion exchange chromatography i.e. DEAE-cellulose and SP-Sephadex matrices. At every stage of isolation, lectin was agglutinated by trypsin-treated rabbit erythrocytes only.

Ammonium sulfate precipitated dialysed seed extract was then chromatographed on DEAE-cellulose and protein eluted in flow-through giving hemagglutinating activity. This impure DEAE-cellulose unbound lectin was further subjected to strong cation exchange SP-Sephadex chromatography. The protein was allowed to adsorb on the cation exchange matrix containing sulfopropyl groups with 30 mL/h flow rate and elution was achieved with acetate buffer employing NaCl gradient (0.1–0.5 M). The 0.2 M eluted fractions showed highest A₂₈₀ and yielded two peaks (Fig. 1) i.e. P-1 and P-2. The fractions of P-1 didn't show any hemagglutination and hence was not taken into

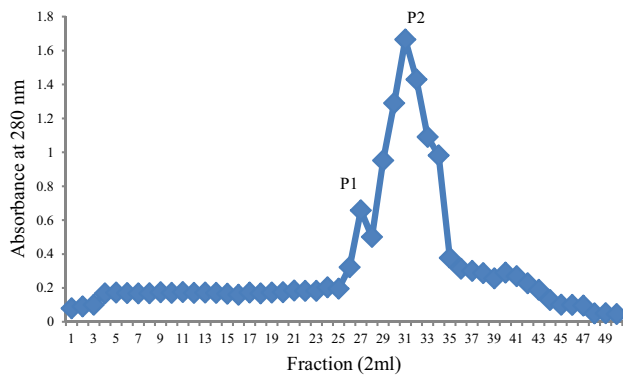


Fig. 1 The cation exchanger SP-Sephadex chromatography of lectin eluted with 0.2 M NaCl in acetate buffer. The fractions of P-2 gave hemagglutination activity and hence pooled

account for further analysis. The fractions of P-2 gave hemagglutination activity and hence pooled together. The purification chart entailing protein concentration and hemagglutinating activity values for each step are shown in Table 1. At the end of purification process, increased specific activity was found to be 2155.78 HAU/mg proteins. The purification of lectin was confirmed by the presence of single band (Fig. 2), having subunit molecular mass of 35 kDa. Our previous studies reported a 43 kDa chickpea lectin demonstrating molecular mass variation may be due to cultivars differences. The CAL mass is identical to phytohemagglutinin (PHA) of red kidney bean (Nowell 1960), soyabean (SBA) (Rizzi et al. 2003), jack bean (concanavalin-A) (Summer and Howell 1936) and a peanut lectin (PNA) (Pueppke 1979).

Antioxidants can donate electrons or hydrogen to radicals based on their reduction potential, power to chelate metals involved in oxidative processes and other traits (Manach et al. 2004). Legumes show the presence of bioactive proteins and phenolic compounds and are therefore rich in antioxidant compounds (Cardador-Martínez et al. 2002; Carrasco-Castilla et al. 2012). Therefore, in the present investigation in vitro antioxidant activity of lectin was evaluated employing reduction of DPPH that participates in radicle scavenging (Llorach et al. 2003). Figure 3 shows the significant differences ($P < 0.05$) in DPPH

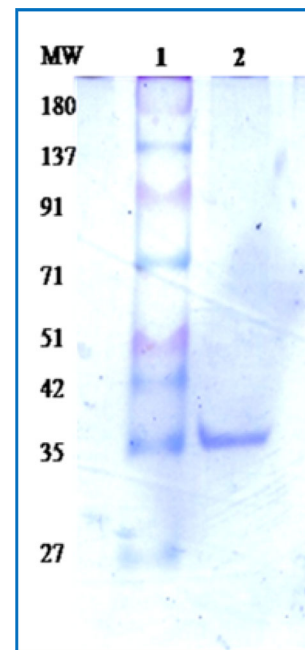


Fig. 2 Size fractionation of purified lectin employing SDS-PAGE. Lane-1: Molecular marker, Lane-2: purified lectin

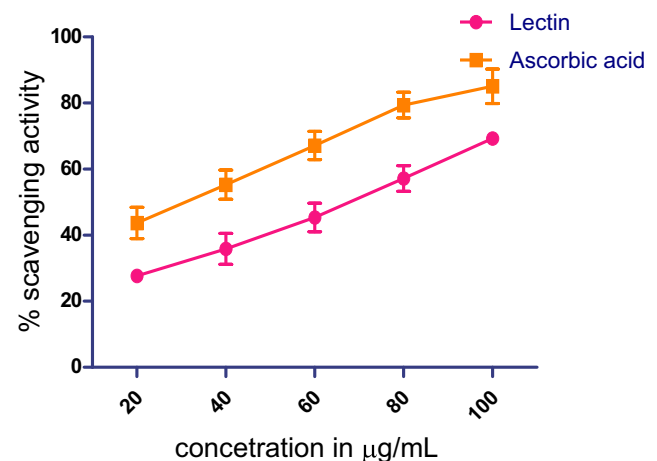


Fig. 3 Antioxidant activity displayed by lectin. Ascorbic acid was used as a standard

Table 1 Table depicting yield of lectin during different purification steps

Purification steps	Total protein (mg/100 g seeds)	Hemagglutination unit (HAU)	Specific activity (HAU/mg protein)	Purification (fold)	Yield (%)
Crude extract	1600	8192	512.10	1	100
20–80% ammonium sulfate fraction	750	4096	546.13	1.06	50
DEAE-cellulose fraction	455	4096	900.21	1.75	25
SP-sephadex fraction	95	2048	2155.78	4.20	25

At every stage of purification, lectin was agglutinated by trypsin-treated rabbit erythrocytes only

Table 2 Antifungal activity of chickpea lectin

S. N.	Organism	Concentration (μg)	Growth inhibition	Positive control
1	<i>Candida krusei</i>	200	85 \pm 0.2%	88.9 \pm 0.12%
2	<i>Fusarium oxysporium</i>	240	81 \pm 0.1%	86.2 \pm 0.18%
3	<i>Saccharomyces cerevisiae</i>	160	14 \pm 0.3 mm	23 \pm 0.51 mm
4	<i>Candida albicans</i>	140	8 \pm 0.5 mm	30 \pm 0.34 mm

Amphotericin B is used as a positive control

scavenging activities of lectin in a concentration-dependent manner (20–100 $\mu\text{g}/\text{mL}$). Chickpea lectin exhibited an IC_{50} of 0.88 $\mu\text{g}/\text{mL}$, compared to the antioxidant activity of ascorbic acid with IC_{50} of 0.52 $\mu\text{g}/\text{mL}$, which was used as a positive control.

Literature perusal suggests that lectins are useful reagents for fungal inhibition (Jebali et al. 2014). The chitin and glucans are the major constituents of fungal cell wall through which an interaction of lectin antifungal activity occurs (Adams 2004). Lectins validate exploitable antifungal and biological activities. CAL demonstrated an antifungal activity to a growth reduction of 85% against *C. krusei* while 81% for *Fusarium oxysporium* (Table 2). Amphotericin B, a positive control showed 88.9% of growth inhibition. The zone of inhibition was measured against *S. cerevisiae* at 14 mm while *C. albicans* at 8 mm distance (Fig. 4).

Lectins inhibit the synthesis and/or deposition of chitin in the cell wall and thus, detrimental to spore germination

(Lis and Sharon 1981; Selitrennikoff 2001; Trindade et al. 2006). The cell wall morphogenesis can be inhibited by small molecular weight lectins. For example hevein (4.7 kDa) and poutein (14 kDa) reach the plasma membrane of fungal cell wall by penetration and blocks the catalytic domain of enzymes (Boleti et al. 2007; Gomes et al. 2012). Recent studies have demonstrated the potential of lectins from different origin and carbohydrate specificities as antifungal agents. Plant lectins against phytopathogenic species have reported antifungal effects binding to hyphae, causing inhibition of growth and prevention of spore germination. The antifungal activity reported from plant lectins are restricted including those from *Phaseolus vulgaris* (Ang et al. 2014), wheat germ (Pusztai 1993), *Pisum sativum* (Sitohy et al. 2007) and *Archidendron jiringa* (Charungchitrak et al. 2011). Lectins from *Phaseolus vulgaris* seeds inhibited the growth of *Coprinus comatus* and *Rhizoctonia solani* (Ye et al. 2001). Lectins of many plant species for example red kidney bean and wheat germ lectin specifically binds with sugar *N*-acetylglucosamine and possessed antifungal activity (Sitohy et al. 2007).

Now a days antimicrobial resistant are the major problems in medical cure however, probing compounds from plant sources are still interesting. Plant lectins are projected to plug this void due to their bioactive principles. The antimicrobial activities of chickpea lectin was therefore evaluated against four species of bacteria *B. subtilis*, *E. coli*, *S. marcescens* and *P. aeruginosa*. Chickpea lectin showed antimicrobial activity with MICs in the range of 80–180 $\mu\text{g}/\text{mL}$ (Table 3). Lectin performance tested against *B. subtilis*, *E. coli*, *S. marcescens* and *P. aeruginosa* showed zone of inhibition at 16, 15, 4 and 5 mm respectively. The zones of inhibition displayed by the standard drug were 30, 28, 40 and 30 mm respectively (Fig. 5). The lectin exert antimicrobial action in coherence with peptidoglycan layer and/or lipopolysaccharide of bacterial cell wall (Dziarski et al. 2000). The muramic acid and glycogen-binding sites of lectin forms hydrogen bond manifests antibacterial activity as demonstrated by lectin from *Lathyrus ochrus* (Bourne et al. 1994). The rhizome lectin hinder microbial growth in the four species of *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* at MICs values of ≥ 0.011 , 0.005, 0.092 and 0.002 mg/mL

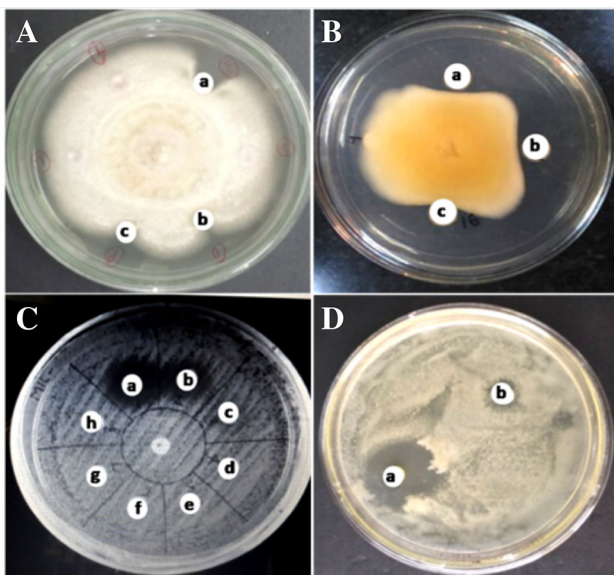


Fig. 4 Lectin exhibiting antifungal activity towards. **A** *C. krusei* (a) control-amphotericin B (20 μg), (b) 200 μg lectin and (c) 250 μg lectin, **B** *F. oxysporium* (a) control-amphotericin B (20 μg), (b) 150 μg lectin and (c) 200 μg lectin, **C** *S. cerevisiae* (a) control-amphotericin B (20 μg) and (b) 160 μg lectin, **D** *C. albicans* (a) control amphotericin B (20 μg) and (b) 140 μg lectin

Table 3 Antibacterial activity of lectin

S. N.	Organism	Concentration of lectin ($\mu\text{g/mL}$)	Zone of inhibition (mm)	Positive control (mm)
1	<i>Bacillus subtilis</i>	140 μg	16 ± 0.14	30 ± 0.4
2	<i>Escherichia coli</i>	180 μg	15 ± 0.09	28 ± 0.2
3	<i>Serratia marcescens</i>	80 μg	4 ± 0.30	40 ± 0.4
4	<i>Pseudomonas aeruginosa</i>	120 μg	5 ± 0.19	30 ± 0.6

Streptomycin is used as a positive control

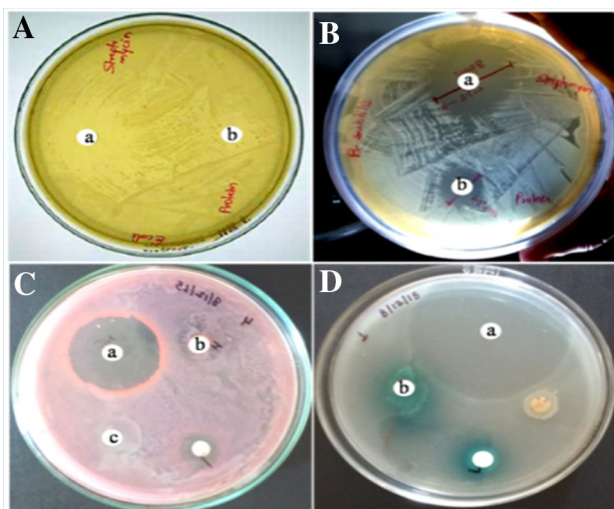


Fig. 5 Lectin exhibiting antibacterial activity towards. **A** *E. coli* (a) control-streptomycin (20 μg), (b) 180 μg lectin, **B** *B. Subtilis* (a) control-streptomycin (20 μg) and (b) 140 μg lectin, **C** *S. marcescens* (a) control-streptomycin (20 μg) and (b) 120 μg lectin, **D** *P. aeruginosa* (a) control-streptomycin (20 μg) and (b) 80 μg lectin

respectively (Petnual et al. 2010). Our results are in line with earlier reports signifying lectins role against bacteria.

The potential of lectins displaying anticancer activity are well documented. Many studies are underway exploring different cell surface sugar moieties of cancerous cells. Literature perusal revealed different modes of cytotoxic effect by plant lectins (Lin et al. 2008; Li et al. 2010). Earlier studies revealed ethanol/acetone extract from *Cicer arietinum* L. inhibited Caco-2 cells (Giron-Calle et al. 2004) while C-25, an antifungal protein exerted reduction in human oral carcinoma cell proliferation (Kumar et al. 2014). The other legume lectin family examples include concanavalin A (Schwarz et al. 1999), *Canavalia ensiformis* (Faheina-Martins et al. 2012), *Typhonium divaricatum* L. (Luo et al. 2007), phytohaemagglutinin-L (Schwarz et al. 1999), *Griffonia simplicifolia* agglutinin and wheat germ agglutinin (Kiss et al. 1997).

The growth inhibitory effects of CAL on human cancer cell lines were tested. This included Ishikawa, HepG2, MCF-7 and MDA-MB-231 employing SRB assay. In this assay, the fixed cells having basic amino acid binds electrostatically to a dye sulforhodamine-B. This assay is

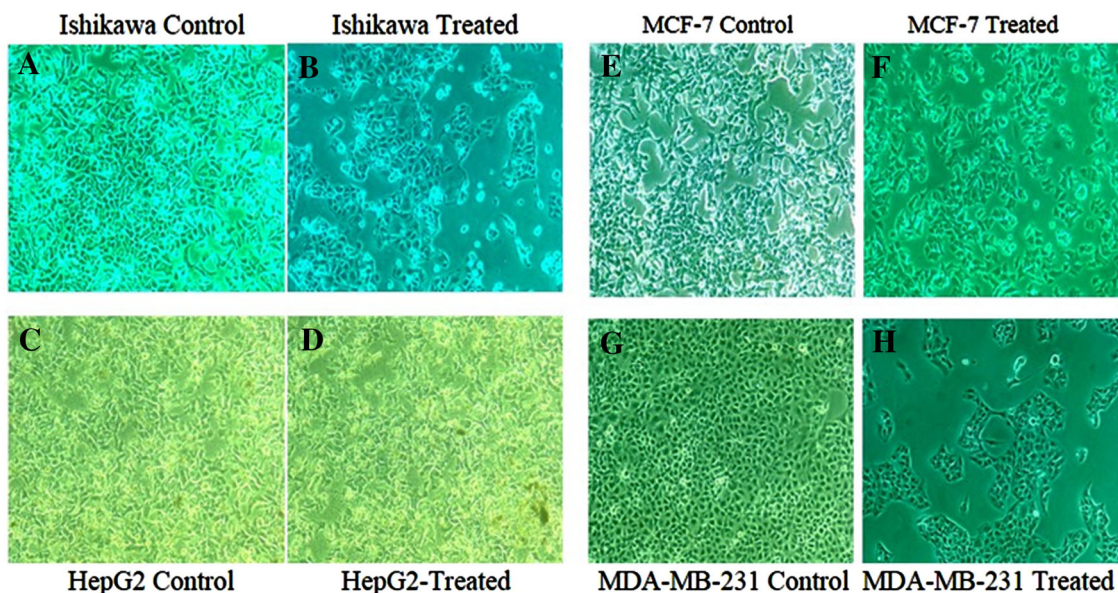


Fig. 6 Effect of chickpea lectin on the viability of human cancer cell lines

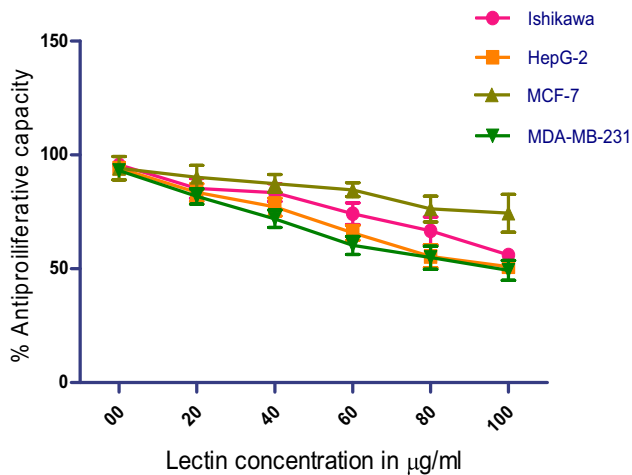


Fig. 7 Cell viability of cancer cells treated with different concentration of lectin

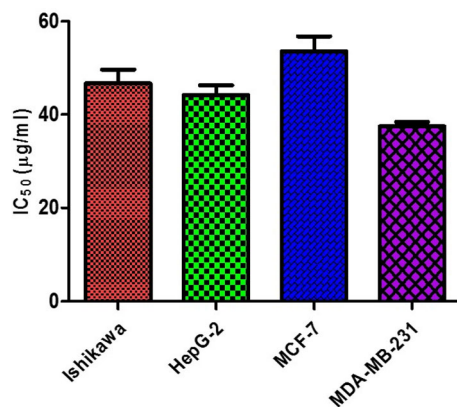


Fig. 8 The inhibitory concentration of lectin on Ishikawa, HepG2, MCF-7 and MDA-MB-231 cancerous cell line

sensitive at large-scale application evaluating cytotoxicity. The MTT assay requires cellular metabolic activity to convert the colorless tetrazolium to the purple colored formazan dye. Therefore, it detects only viable cells, whereas the SRB method does not distinguish between viable and dead cells. This difference however does not compromise the ability of the SRB assay to detect cytotoxic effects of a drug. The morphological changes due to different concentrations of lectin revealed (Fig. 6) marked inhibition on cell viability in a dose-dependent manner. A spectrum of lectin concentration 20, 40, 60, 80 100 µg/mL was employed to test its efficacy on these cell lines (Fig. 7). After 24 h treatment, Ishikawa, HepG2, MCF-7 and MDA-MB-231 cell line possess the IC₅₀ value of 46.67, 44.20, 53.58 and 37.46 µg/mL respectively (Fig. 8). The previous report on other plant hemagglutinins finds similar observation validating anticancer activity (Ye and Ng 2011). Lectins induce apoptosis and regulate the

expression of apoptotic genes in cancer cells (Lam and Ng 2010; Ramnath et al. 2009). The present study manifest chickpea lectins as bactericide and fungicide in addition to an antiproliferative agent against cancer cell lines. These results can provide a background for future research into the benefits of chickpea lectin to pharmacological perspective.

Acknowledgement The authors are thankful to University Grants Commission (UGC), New Delhi for funding and specially Mr. Ajay Gautam, for providing research fellowship. The authors are indebted to Dr. M. Yasin, Senior Breeder, RAK Agriculture College, Sehore for providing chickpea seed material.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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