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Antifungal and Antiproliferative Activities of Lectin from the Rhizomes of *Curcuma amarissima* Roscoe

Norhameemee Kheeree · Polkit Sangvanich · Songchan Puthong · Aphichart Karnchanatat

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Abstract A lectin was purified from the rhizomes of *Curcuma amarissima* Roscoe by aqueous extraction, fractionation with 80% saturated ammonium sulfate, and a combination of affinity and gel chromatography on ConA Sepharose and Superdex G-75, respectively. The molecular mass of the purified lectin was 32.4 kDa, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The lectin showed no significant specificity in its ability to hemagglutinate erythrocytes from human blood groups (A, B, AB, and O), but for other animals, it only agglutinated rabbit and rat, and not mouse, guinea pig, goose, and sheep erythrocytes. The lectin was stable at temperatures below 40°C, but the hemagglutinating activity halved when it was heated to 45–85°C and was completely lost at 95°C. The hemagglutinating activity was more stable at 80°C than at 70°C and was rapidly inactivated at 90°C. It showed a maximum hemagglutination activity within the pH range of 8.0–11.0. The deduced amino acid sequence of an internal tryptic peptide sequence of this purified lectin showed sequence similarity (homology) to other members of the leucoagglutinating phytohemagglutinin precursor family, whilst the complete lectin inhibited the in vitro growth of three plant pathogenic fungi, *Fusarium oxysporum*, *Exserohilum turicicum*, and *Colectrotrichum cassiicola*, at a concentration of 17.5 to 35 μg, and showed in vitro cytotoxicity against the BT474 breast cancer cell line with an IC_{50} of approximately 21.2 μg.

Keywords Lectin · ConA Sepharose · *Curcuma amarissima* · Antifungal · Antiproliferative

N. Kheeree
Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

P. Sangvanich
Research Center for Bioorganic Chemistry, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

P. Sangvanich · S. Puthong · A. Karnchanatat (✉)
The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330, Thailand
e-mail: i_am_top@hotmail.com

Introduction

Lectins are multivalent carbohydrate-binding proteins of non-immune origin that exert biological effects through their ability to specifically bind different carbohydrates structures [1–4]. These proteins are ubiquitous in nature occurring in animals, plants, and microorganisms [5, 6] and present a wide and varied area of applicability including in biotechnological processes and in medical, biological, pharmacological, and biochemical research [7, 8]. They have the unique ability to recognize and bind reversibly to specific carbohydrate ligands without any chemical modification, and this distinguishes lectins from other carbohydrate-binding proteins and enzymes and makes them invaluable tools in biomedical and glycoconjugate research [9]. For example, lectins are widespread throughout the plant kingdom, but are of interest as in addition to their relative ease of purification and high yields from plant tissues, they are diverse enough that different families of plants as well as different tissues within the same plant can contain lectins with different molecular properties and carbohydrate-binding specificities [10]. The functions of plant lectins in nature are extremely diverse and are all based on their ability to recognize and bind the carbohydrate moieties of glycoconjugates, whether in solution or on cells. Because the carbohydrates may be species-, tissue-, or cell-specific, certain lectins are capable of discriminating between self and non-self, this being one strategy of innate immunity and a defense mechanism against foreign enemies [11]. The role of lectins in the defense mechanism of plants may have evolved from the ability to lectins to agglutinate and immobilize microorganisms. The supporting evidence for this proposed role in defense against pathogens falls into two main observed categories, namely (a) the presence of lectins at potential sites of invasion by infectious agents [12] and (b) the binding of lectins to various fungi and their ability to inhibit fungal growth and germination [13].

The rhizomes of the Zingiberaceae (ginger) family are widely used in many Asian countries, and their medicinal functions have been broadly discussed and accepted in many traditional recipes [14]. *Curcuma* is one genus within this family, and it consists of about 80 species in the world. They have been widely cultivated for use as spices [15, 16], food flavoring [17, 18] and coloring [19, 20], and in traditional medical applications [21–25]. Also, some species are commonly used in industrial applications [26]. However, despite the widespread usage of members of this general for medical applications, reports of lectins from *Curcuma* plants are scarce. A mannose-binding lectin from the Zingiberaceae member, *Curcuma zedoaria* Rosc., has been identified and found to have sequence similarity to a mannose-binding lectin from the broad-leaved helleborine orchid, *Epipactis helleborine* (L) Crantz (Asparageles: Orchidaceae) [27]. In the related *Curcuma aromatica*, six putative proteins from nine bands derived from a rhizome preparation showed similarity with other lectin sequences [28], whilst a study on the hemagglutinating activity of *Curcuma* plants found hemagglutinating activity in the species of this study, Khamindum or *Curcuma amarissima* [29].

C. amarissima, a member of the ginger, Zingiberaceae, family is used to treat amoebic dysentery, enteritis, and as a vermicide [30]. However, to the best of our knowledge, despite the widespread use of rhizomes from *C. amarissima* in dietary-based medical applications, there are no reports on the purification lectins from *C. amarissima*. Consequently, the aims of this study were to purify and characterize lectins from the rhizomes of *C. amarissima* and to assay them for bioactivity including antifungal and antiproliferative activities.

Materials and Methods

Biological Material

The fresh rhizomes of *C. amarissima* were purchased from a local market in Bangkok, Thailand. Human blood was obtained from the blood donation office of The Thai Red Cross Society, Bangkok, Thailand. All other non-human animal blood was supplied from the Division of Production and Supply, National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. The three plant pathogenic fungal species (strains) used in the bioassays for lectin antifungal activity, *Collectotrichum cassicola* (DOAC 1196), *Exserohilum turcicum* (DOAC 0549), and *Fusarium oxysporum* (DOAC 1258), were obtained from the Division of Plant Disease and Microbiology, Department of Agriculture, Bangkok, Thailand. The five human tumor cell lines, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), and SW620 (colon), were maintained and obtained from The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand.

Chemicals and Reagents

ConA Sepharose was purchased from Sigma Chemicals Co. (USA), methyl-α-D-glucopyranoside. The reagents used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Plusone Pharmacia Biotech (Sweden), except for the low molecular weight protein calibration kit and Superdex G-75, which were both purchased from Amersham Pharmacia Biotech (UK). All other biochemicals and chemicals used in the investigation were of analytical grade.

Extraction of Lectin from the Rhizomes of *C. amarissima*

One kilogram of crushed and washed *C. amarissima* rhizomes were blended in TBS (20 mM Tris–HCl, pH 7.2, plus 150 mM NaCl) at a 1:5 kg/L ratio and then stirred overnight at 4°C and filtered through cloth. The filtrate was clarified by centrifugation at 15,000×g for 30 min at 4°C, the supernatant harvested, and the protein precipitated by the addition of ammonium sulfate to 80% saturation on ice and centrifuged as above. The supernatant was discarded, and the pelleted proteins were dissolved in TBS, dialyzed against excess water, and then freeze-dried.

Purification of Lectin from the Rhizomes of *C. amarissima*

Affinity Column Chromatography

ConA Sepharose was pre-equilibrated with TBS and transferred to a 1.6×20 cm column. The pre-purified extract was applied to the column at a flow rate of 1.5 ml/min, and the column was then washed with TBS at the same flow rate, collecting 10 ml fractions, until the A_{280} fell to <0.05. The bound proteins were then eluted using TBS supplemented with 0.5 M methyl α-D-glucopyranoside as the competitor, to desorb the lectin from the column, at the same flow rate and collecting 10 ml fractions. The fractions containing lectin, on the basis of A_{280} and a detectable hemagglutination activity were combined, dialyzed against excess water, frozen, and lyophilized.

Gel Filtration Chromatography

The freeze-dried hemagglutination activity positive fraction from the above ConA Sepharose affinity column chromatography was solvated in TBS was applied to the TBS pre-equilibrated Superdex G-75 column (1.6×60 cm) at a flow rate of 0.5 ml/min and then eluted with TBS at the same flow rate collecting 10 ml fractions. Fractions were screened for hemagglutination activity from the column, and contiguous positive fractions were pooled and dialyzed against an excess of TBS prior to further analysis.

Protein Determination

The protein concentration was determined following the Bradford assay [31] with dilutions of a known concentration of bovine serum albumin as the standard. Absorbance was measured at 595 nm. During the column chromatographic separations, the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

Hemagglutination Assay

Serial twofold dilutions of purified lectin in TBS (50 μl) were incubated with 50 μl of a rabbit erythrocyte suspension in each well of a 96-well U-shaped microtiter plate, and the agglutination was scored after 1 h at room temperature. The hemagglutination unit (HU) was expressed as the reciprocal of the highest lectin dilution (lowest lectin concentration) showing detectable visible erythrocyte agglutination, and the specific activity was calculated as HU/mg protein. Hemagglutination activity was assayed separately against erythrocytes from rabbits, rats, mice, guinea pigs, geese, sheep, and the four human ABO blood groups.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Discontinuous reducing SDS-PAGE gels were prepared with 0.1% (*w*/*v*) SDS in 15% and 5% (*w*/*v*) acrylamide separating and stacking gels, respectively, with Tris-glycine buffer pH 8.3 containing 0.1% (*w*/*v*) SDS as the electrode buffer, according to the procedure of Laemmli [32]. Samples to be analyzed were treated with reducing sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in Mini-Gel Electrophoresis unit. Molecular weight standards were co-resolved in adjacent lanes and used to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by standard Coomassie blue R-250 staining.

Effect of Temperature on Lectin Hemagglutinating Activity and Thermostability

The effect of temperature on lectin hemagglutinating activity was determined by incubating lectin samples in TBS at various temperatures (30–100°C in 5°C intervals) for 30 min. The thermostability of the lectin was investigated by incubating lectin samples at 40–90°C in 10°C intervals in TBS for the indicated fixed time intervals (10–120 min), cooling to 4°C and then assaying the residual hemagglutinating activity as described above. In all cases, rabbit erythrocytes were used, and at least three replicates were done for each assay.

pH-Dependence of Agglutination Activity

The effect of pH on the lectin hemagglutinating activity was determined by incubating lectin samples in various buffers ranging from pH 2 to 12 at room temperature for 1 h, then adjusting the mixture to pH 7.2 and assaying the residual hemagglutinating activity as described above. The buffers used for each pH range were 20 mM glycine pH 2–4, 20 mM sodium acetate pH 4–6, 20 mM potassium phosphate pH 6–8, 20 mM Tris–HCl pH 8–10, and 20 mM glycine NaOH pH 10–12. In all cases, rabbit erythrocytes were used, and at least three replicates were done for each assay.

Effect of Metal Ions

For each assay, a 1-ml aliquot of the purified lectin (1 mg/ml) was incubated for 10 h with one of Ca^{2+}, Co^{2+}, EDTA, Hg^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+} at various concentrations (0, 25, 50, 100, and 200 mM) with continuous shaking. After that, 50 μl of a 2–4% (*v*/*v*) suspension of rabbit erythrocytes was added, and the hemagglutination was scored after 1 h as described above using at least three replicates for each assay.

Internal Amino Acid Sequence of Lectin by LC/MS/MS

The internal amino acid sequence of the purified lectin from *C. amarissima* rhizomes was performed by in-gel trypsin digestion of the protein [33] and sequencing of the different tryptic peptides by LC/MS/MS mass spectrometry. Coomassie-stained protein spot was excised from SDS-PAGE gel and washed with 3% hydrogenperoxide. The protein was in-gel reduced, alkalated, and digested with trypsin. After digestion, the peptides were twice extracted from gel with 50% acetronitrile/0.1% TFA and air dried. The trypic peptides were subjected to LC-nano ESI-MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT search of an inhouse NCBI database. The following criteria were used in the Mascot search: trypsin cleavage specificity with up to three missed cleavage, cysteine carbamidomethyl fixed modification, methionine oxidation variable modifications, ±0.2 Da peptide tolerance and MS/MS tolerance, and ESI-TRAP fragmentation scoring.

Assay of Antifungal Activity

Antifungal bioassays, using *Exserohilum turicicum*, *F. oxysporum*, and *Colectrotrichum cassiicola* plant pathogenic isolates, were performed on 90×15 mm petri plates containing 10 ml of standard potato dextrose agar. After the mycelial colony had developed, sterile blank filter paper disks (0.625 cm in diameter) were placed 1 cm away from the rim of the mycelial colony. The suitably diluted lectin or control samples, dissolved in TBS, were added (10 μl) to the disks and the plates then incubated at 25°C until mycelial growth had enveloped the peripheral edges of the control (TBS) disks and had formed crescents of inhibition around the paper disks containing the purified lectin samples in TBS. At this stage, the diameter of the clear zone of inhibition surrounding the sample was taken as a measure of the inhibitory power of the sample against the particular test organism.

Assay of Antiproliferative Activity

The bioassay for in vitro antiproliferative activity towards five human cell lines, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), and SW620 (colon), was performed

in vitro in tissue culture. Cells were routinely maintained in complete media, comprised of RPMI, at 37°C in an atmosphere of 5% (*v*/*v*) CO_2. Cells were seeded at 8×10^4 cells/cm^2 in a total of 1 ml complete media. Prior to the assay, cells at confluence were trypsinized, aspirated, and washed before seeding at a final density of 5×10^3 cells/μl in 200 μl of complete media in 96 well plates and cultured for 24 h as above. Then serial dilutions of the purified lectin were added (0–35 μg/ml final concentration) in a total volume of 200 μl complete media to the cell cultures and incubated for a further 72 h. Next, 10 μl of MTT (3-[5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was then added to each well, incubated for 4 h, the media aspirated off, and the cells gently washed to remove all remaining media prior to the addition of 150 μl DMSO per well for 30 min. The cell remnants and solution were then aspirated to ensure all the cells were lysed and the crystals dissolved, and the absorbance at 540 nm was measured using a microtiter reader. Controls included the absence of cells or the crude lectin extract.

Results and Discussions

The present study describes the purification and characterization of a lectin from the rhizomes of *C. amarissima*. Since the crude extract from the rhizomes of *C. amarissima* contained a lot of soluble components, including carbohydrates and other proteins, ammonium sulfate precipitation was applied as the first step of purification. The lectins were found to precipitate with 80% saturation ammonium sulfate, and so this initial stage was used prior to purification by affinity and then gel filtration chromatography (Table 1). The initial 80% saturation ammonium sulfate precipitation resulted in an affinity chromatography with a ConA Sepharose column resulted in two factions, an un-bound fraction that eluted in the TBS wash through and did not show any detectable hemagglutinating activity, and a bound fraction that eluted in the presence of 0.5 M methyl α-D-glucopyranoside and had hemagglutinating activity with a specific activity of 2.21×10^3 HU/mg protein (Fig. 1a). Affinity chromatography presents advantages in relation to other conventional methods due to its specificity and thus allows a reduced number of steps and gives high yields and purity [34]. As such, it is widely used in the purification of lectins. For example, the mannose-

Table 1 Purification table of the lectin from the rhizomes of *Curcuma amarissima*.

Purification step	Total protein (mg)[a]	Total lectin (titer×ml)[b]	Specific activity (HU/mg)[c]	Yield (%)	Purification (fold)[d]
Crude extract	2,152.98	1.94×10^5	90.29	100.00	1.00
80% $(NH_4)_2SO_4$ precipitation	54.43	1.24×10^5	2.29×10^3	64.00	25.31
ConA Sepharose (bound fraction)	47.77	1.06×10^5	2.21×10^3	54.64	24.52
Superdex G-75 (last peak)	7.37	2.39×10^5	32.41×10^3	123.20	360.09

[a] Crude protein extract from 200 g of rhizomes.

[b] Minimal concentration of protein able to cause visible agglutination of a 2–4% (*v*/*v*) suspension of rabbit erythrocytes.

[c] Specific activity is defined as the hemagglutination unit (HU) divided by the protein concentration (mg/ml) of the assay solution. Rabbit erythrocytes were used for the assay.

[d] Purification index was calculated as the ratio between the minimal concentration of the crude extract able to cause visible agglutination of the rabbit erythrocytes and that of the protein fraction obtained at each purification step.

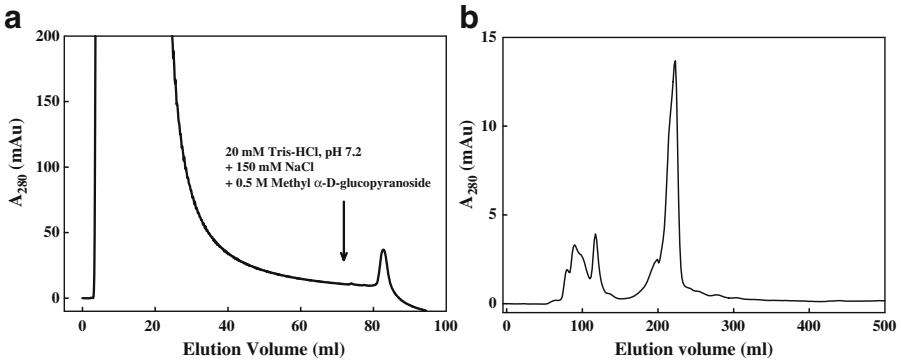

Fig. 1 **a** Affinity chromatogram of *Curcuma amarissima* lectin on a ConA Sepharose column equilibrated and then washed with TBS. Lectin was then eluted with TBS containing 0.2 M Methyl α-D-glucopyranoside as described in the methods section. **b** Elution profile of purified *C. amarissima* lectin on a Superdex G-75 column

glucose specific lectins from the seeds of the tepary bean (*Phaseolus acutifolius*) and mulberry, *Morus* sp. (Rosales: Moraceae), seeds were purified by ConA Sepharose-based affinity chromatography column [35, 36]. However, in some contrast, there have been reports that affinity chromatography could not be applied successfully for the purification of some lectins. For instance, the isolation of *Dolichose biflorus* lectin with N-acetyl-galactosamine (NAG) immobilized to Sepharose was not successful and was caused by the substitution of the binding site at the C-6 hydroxyl of carbohydrate in the matrix. Rather, these lectins were resolved by affinity electrophoresis, a combination of affinity and conventional chromatography [37]. Another example is the lectin from ground elder (*Aegopodium podagraria*) rhizomes which also could not be purified by Gal-NAG-Sepharose, but by an affinity chromatography of erythrocyte membrane protein immobilized on cross-linked agarose [38]. The recovered bound fraction with hemagglutinating activity was dialyzed and concentrated and then resolved by gel filtration chromatography with a Superdex G-75 column, resulting in fractionation into three distinct peaks, only the last one of which showed any hemagglutinating activity and so was harvested. The final homogeneous lectin preparation obtained was purified by 360.09-fold with 123.20% recovery and had a specific activity of 32.41×10^3 HU/mg proteins (Fig. 1b). The purification details of this lectin are summarized in Table 1.

Discontinuous SDS-PAGE has been shown to be a relatively sensitive technique for lectin separation [34], and here, in the presence of 2-merceptoethanol under reducing conditions, revealed a single strong band lectin corresponding to an apparent molecular weight of 34.2 kDa after Coomassie blue R250 staining (Fig. 2). In addition, since only a single band of the same apparent size was seen under non-reducing conditions (not shown), this suggests that the purified lectin could be a monomeric protein, or at least if a multimeric one that dissociates into subunits under these conditions, then this subunit has hemagglutination activity alone. The apparent size of approximately 34.2 kDa is in agreement with the previously published sizes of the other plant lectins which ranged from 30 to 35 kDa [39–41].

The carbohydrates specificities of many lectins have been grouped by the ability of monosaccharides or their glycosides to inhibit the lectin-induced hemagglutination [42]. However, lectins of the same apparent monosaccharide specificity were found to demonstrate different reactivity's towards different oligosaccharide chains, and differential affinities to animal cells and glycoproteins, which implies that they have their own binding specificity determinants that extend beyond the monosaccharide unit [43]. The lectin from *C. amarissima* rhizomes purified here showed the strongest affinity of all the tested animal

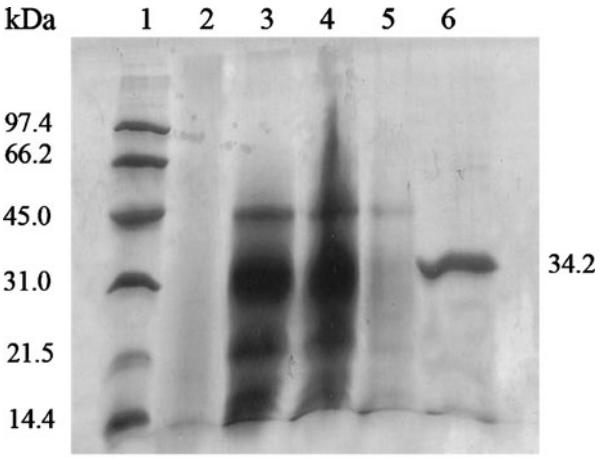

Fig. 2 Reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of *Curcuma amarissima* lectin. *Lane 1*, molecular weight standards; *lane 2*, the crude extract (homogenate); *lane 3*, the 80% ammonium sulfate precipitated and kept fraction; *lane 4*, the non bound ConA Sepharose fraction discarded; *lane 5*, the bound fraction kept; and *lane 6*, the peak obtained hemagglutinating activity from the gel filtration

erythrocytes to rabbits followed by human blood groups A and AB, then to human blood groups B and O and finally to rat erythrocytes, suggesting that *C. amarissima* lectin recognizes the surface of erythrocyte membranes (Table 2). In contrast, no detectable hemagglutination of *C. amarissima* lectin with goose, guinea pig, mouse, and sheep was observed. Several lectins have been reported previously to demonstrate a preference in agglutinating one more types of human blood groups or animal erythrocytes, such as the lectins from *Sphenostyles stenocarpa* for human blood type O [44], *Hevea brasiliensis* for rabbit erythrocytes [45], and *Talisia esculenta* lectin for human blood type AB [46]. However, this lectin purified here from *C. amarissima* rhizomes showed only partial specificity with respect to either human blood groups or animals in general (Table 2).

The *C. amarissima* lectin was stable at temperatures below 40°C. The hemagglutinating activity decreased to half when it was heated to 45–85°C. Moreover, its activity was completely lost at 95°C (Fig. 3a). Similarly, the rhizomatic lectin isolated from *Aspidistra elatior* Blume was reported to at 90°C [47], whilst the lectin from *Arundo donax* was stable up to 55°C for 15 min and showed only 80% activity at 60°C and no activity at 85°C and higher temperatures [48]. The thermal stability of this *C. amarissima* lectin at various exposure times revealed that no loss of hemagglutination activity was noted at temperatures up to 60°C for 10 min, but that the activity decreased as the duration of the exposure

Table 2 Hemagglutinating activity of the purified lectin from the rhizomes of *Curcuma amarissima* against human and animal erythrocytes.

Erythrocyte source	Agglutination (titer)[a]
Mouse	0
Rat	2^2
Guinea pig	0
Goose	0
Sheep	0
Rabbit	2^6
Human Type A	2^4
Human Type B	2^3
Human Type O	2^3
Human Type AB	2^4

[a] Titer is defined as the reciprocal of the end point dilution causing detectable agglutination of erythrocytes. The concentration of *C. amarissima* lectin used in this assays was 0.045 mg/ml and was serially 1:1 (*v*/*v*) diluted. Data shown are the mean±1 SD and are derived from three repeats.

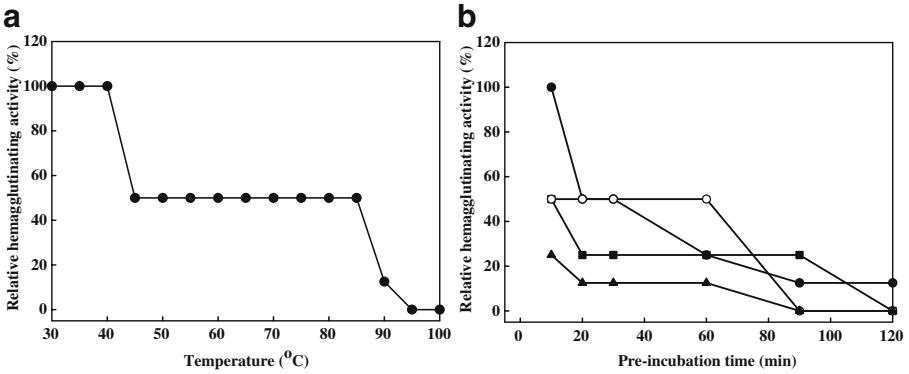

Fig. 3 **a** Effect of temperature on the agglutinating activity of the purified *Curcuma amarissima* lectin towards a rabbit erythrocyte suspension in TBS. **b** Thermostability of the same purified lectin towards a rabbit erythrocyte suspension in TBS at (*open circle*) 40°C, (*closed circle*) 50°C, (*square*) 60°C, and (*triangle*) 70°C. For both panels, the data are shown as the mean±1 SD and are derived from three repeats. Full activity (100%) corresponds to a titer of 2^4

increased (Fig. 3b). The thermal stability observed for this *C. amarissima* lectin is comparable to that already reported for some other thermostable lectins treated under similar conditions [49, 50]. Indeed, the only thermophilic lectin isolated to date is from *Momordica charantia*, which has a maximal activity at 55°C [51].

The pH sensitivity profile of the purified *C. amarissima* lectin is summarized in Fig. 4, where it can be seen that this lectin exhibited a broad pH optima between pH 8.0–11.0, with a 50% loss of agglutination activity at one pH unit either side, that is at pH 12 or 7. This is in contrast to the other reported lectins, which are stable over a broader pH range such as the lectins from *Koelreuteria paniculata* seeds, *T. esculenta*, and *S. stenocarpa* which were stable over a pH range of 5–9, 3–9, and 2–10, respectively [44, 46, 52]. The hemagglutinating ability of the purified *C. amarissima* lectin reported here was totally inactive at a pH of 4 or less, which might be due to the α and β subunits of the lectin dissociating at an acidic condition [53]. Regardless, the optimum activity of this lectin was at a basic pH (8–11), although higher pH levels induced denaturation.

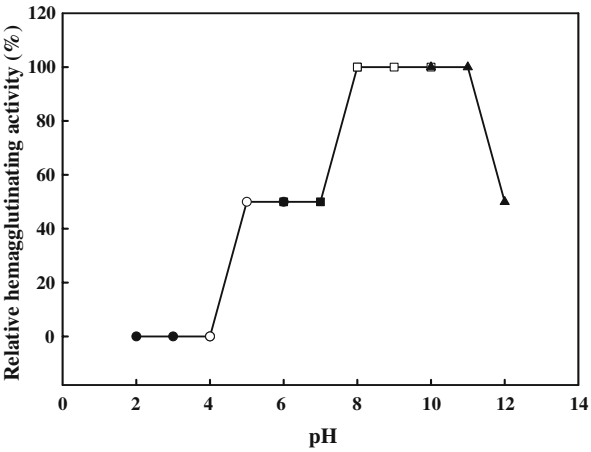

Fig. 4 Effect of pH pretreatment on the agglutinating activity of the purified *Curcuma amarissima* lectin towards rabbit erythrocytes in TBS. Pretreatment was with (*closed circle*) 20 mM glycine-HCl (pH 2–4), (*open circle*) 20 mM sodium acetate (pH 4–6), (*open square*) 20 mM potassium phosphate (pH 6–8), (*closed square*) 20 mM Tris–HCl (pH 8–10), and (*triangle*) 20 mM glycine-NaOH (pH 10–12). Data are shown as the mean±1 SD and are derived from three repeats. Full activity (100%) corresponds to a titer of 2^5

The effect of divalent cations on *C. amarissima* lectins was evaluated with six different divalent metal ions. Ca^{2+}, Mg^{2+}, and Mn^{2+} were found to satisfy the requirements for hemagglutination activity, whereas Co^{2+} and Hg^{2+} were not able to support agglutination, and Fe^{2+} was only able to support it at high concentrations (Table 3). Similar results have been reported for the *Dioclea altissima* lectin [54]. Many lectins have been reported to be metalloproteins, and these metal cation cofactors are required for such activities as hemagglutination [42, 55, 56], and indeed, the requirement for divalent metal ions like Ca^{2+}, Mg^{2+}, and Mn^{2+} is a general physico-chemical property of most legume lectins [42, 56], suggesting that they are essential for the hemagglutination activity.

The sequence analysis of a partial internal fragment of the purified lectin from *C. amarissima* rhizomes, obtained by in gel digestion with trypsin and subsequent sequence analysis with LC-MS/MS, revealed a peptide fragment with the likely sequence GNVET NDVLS WSFAS KL (Fig. 5a). Comparisons to all protein sequences in the SwissProt database using BLASTP searching identified this fragment as a likely homolog of parts of a lectin precursor from the common bean, *Phaseolus vulgaris* L. (Fabales: Fabaceae) [57]. The high degree of internal amino acid sequence identity between the peptide fragment of this lectin, from *C. amarissima* rhizomes, with those of other members of the leucoagglutinating phytohemagglutinin precursor family (Fig. 5b) suggests that this protein could be a member of this lectin family as well.

The purified *C. amarissima* lectin showed in vitro antifungal activity against three plant pathogenic fungal species, *C. cassiicola*, *E. turicicum*, and *F. oxysporum*. It strongly inhibited the growth of *C. cassiicola* at 17.5 μg/ml for *F. oxysporum* and *E. turicicum*, which were strongly inhibited at the higher concentration of 35 μg/ml (Fig. 6). Antifungal activity has been observed in other lectins where, for example, *Astragalus mongholicus* root lectin revealed antifungal activity against various species of phytopathogenic fungi [58]. Similarly, the lectin from *T. esculenta* seeds inhibited the growth of *F. oxysporum*, *Colletotrichum lindemuthianum*, and *Saccharomyces cerevisiae* [46]. In vitro studies demonstrated that two novel chitin-binding lectins from the seeds of *Artocarpus integrifolia* inhibited the growth of *Fusarium moniliforme* and *S. cerevisiae* [59]. Many studies of plant lectins have assumed that they are implicated in the host defense mechanism as antifungal proteins. However, to date, only a small number of lectins have been reported to have actual antifungal activity, including those from potato tubers, *Amaranthus caudatus* seeds, stinging nettle rhizomes, wheat germ, and *P. vulgaris* seeds [60–63].

Lectins, and in particular, plant lectins, have recently become of great interest since they have been reported as potential anticancer reagents that can seek out and stop multiplication of cancer cells. In this study, the in vitro antiproliferative and cytotoxic

Table 3 Effect of selected divalent metal cations on the hemagglutination ability of the purified lectin from *Curcuma amarissima*.

Concentration of divalent metal cations (mM)	$CaCl_2$	$MgCl_2$	$MnCl_2$	$FeCl_2$	$CoCl_2$	$HgCl_2$	EDTA
25	+	+	+	+	−	−	−
50	+	+	+	−	−	−	−
100	+	+	+	−	−	−	−
200	−	−	−	−	−	−	−

+ hemagglutinating activity, − no hemagglutinating activity.

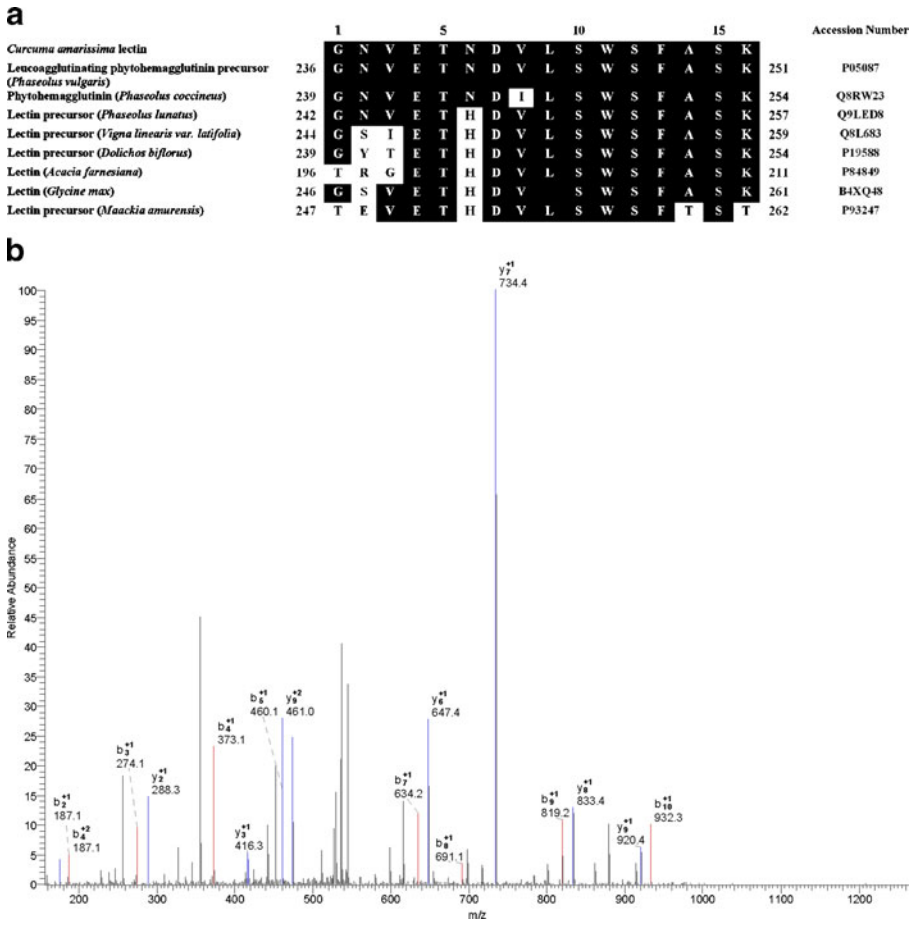

Fig. 5 **a** Amino acid sequence from the tryptic fragments of the purified *Curcuma amarissima* lectin. Comparisons are made with other lectins from the lectin family that showed the highest sequence homology in BLASTP searches of the NCBI and SwissProt databases. Shaded regions represent regions of identity. **b** LC/MS/MS spectra of the tryptic digest of the purified lectin used to derive the data in **a** above

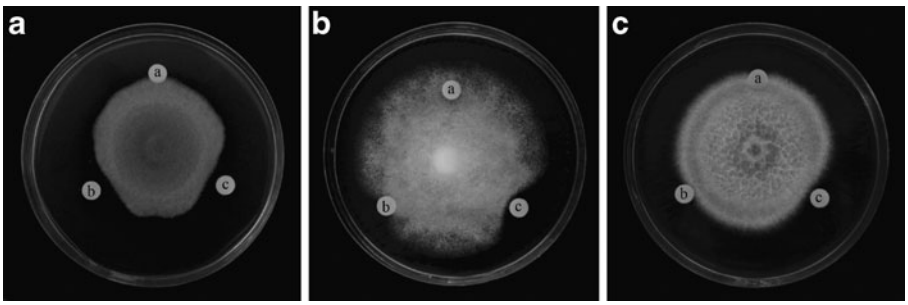

Fig. 6 Inhibitory effect of purified *Curcuma amarissima* lectin on the in vitro growth on potato dextrose agar plates (as an antifungal activity bioassay) of (**a**) *Colectrotrichum cassiicola*, (**b**) *Fusarium oxysporum*, and (**c**) *Exserohilum turicicum*. For each plate, 0.625 cm diameter disks were seeded with 10 μl of TBS (**a**) alone as the negative control, or containing either (**b**) 17.5 or (**c**) 35 μg/ml purified *C. amarissima* lectin

activity of the lectin isolated from the rhizomes of *C. amarissima* was evaluated against five human cancer cell lines, representing different organs and tissues, in tissue culture. This lectin was found to be active against the breast cancer cell line (BT474) at all the concentrations studied and showed the highest antiproliferation activity with an IC_{50} of approximately 21.2 μg (Fig. 7). These data are in agreement with the reported variation in antiproliferative potentials of a variety of lectins with cancer cell lines [64] and may be related to the sugar-binding activity of lectins where each specific carbohydrate chain, which is limited to the surface of tumor cells, acts as the receptor for one or more of the lectins [65]. Several kinds of plant lectins have been reported to have antiproliferative effects upon tumor cell lines, such as the lectins from *Cratylia mollis* [66] and *Viscum album* [67].

Conclusion

In this study, a lectin from the rhizomes of *C. amarissima* was purified by affinity and gel filtration chromatography to apparent homogeneity. The molecular mass of the purified lectin was 32.4 kDa, as estimated by SDS-PAGE, and although not non-specific, it showed no single target or distinctive specificity in its ability to hemagglutinate erythrocytes from either different human blood groups (A, B, AB, and O), or from different animals where it agglutinated erythrocytes from rabbits, humans and, weakly, rats, but not mice, guinea pigs, geese, and sheep. The lectin was thermostable up to 40°C and showed an optimum activity within the pH range of 8.0–11.0. Divalent cations appear to be essential for the hemagglutination activity of this lectin. The lectin was able to inhibit the in vitro growth of the plant pathogenic fungi, *E. turcicum, F. oxysporum*, and *C. cassicola*, within the range of 17.5–35 μg/ml. Furthermore, this *C. amarissima* rhizome-derived lectin showed in vitro cytotoxicity against the BT474 human breast cancer cell line with an IC_{50} of approximately 21.2 μg/ml. The deduced internal amino acid sequence of the lectin showed similarity (homology) to the sequences of the leucoagglutinating phytohemagglutinin precursor lectin family.

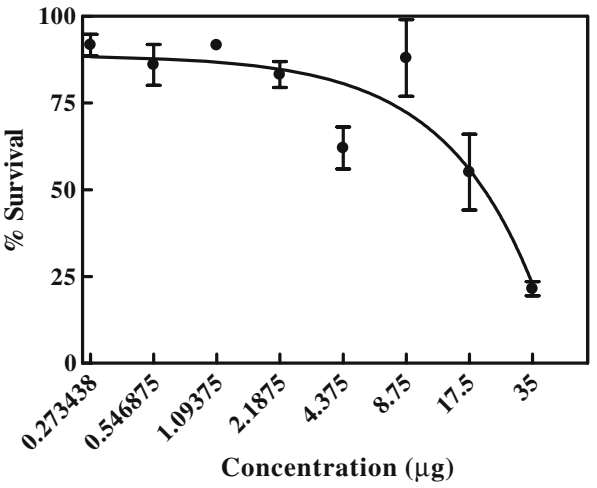

Fig. 7 **a** Inhibitory activity of *Curcuma amarissima* lectin towards the breast cancer cell line (BT474). Each data point represents the mean±1 SD of triplicate determinations

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References

1. Rini, J. M. (1995). *Annual Review of Biophysics and Biomolecular Structure, 24*, 551–577.
2. Vijayan, M., & Chandra, N. R. (1999). *Current Opinion in Structural Biology, 9*, 707–714.
3. Chandra, N. R., Kumar, N., Jeyakani, J., Singh, D. D., Gowda, S. B., & Prathima, M. (2006). *Glycobiology, 16*, 938–946.
4. Bies, C., Lehr, C. M., & Woodley, J. F. (2004). *Advanced Drug Delivery Reviews, 56*, 425–435.
5. Lis, H., & Sharon, N. (1998). *Chemical Reviews, 98*, 637–674.
6. Mo, H., Winter, H. C., & Goldstein, I. J. (2000). *Journal of Biological Chemistry, 275*, 10623–10629.
7. Bonneil, E., Young, N. M., Lis, H., Sharon, N., & Thibault, P. (2004). *Archives of Biochemistry and Biophysics, 426*, 241–249.
8. Wong, J. H., Wong, C. C. T., & Ng, T. B. (1998). *Plant Physiology & Biochemistry, 36*, 889–905.
9. Sharon, N., & Lis, N. (2001). *Advances in Experimental Medicine and Biology, 49*, 11–16.
10. Van Damme, E. J. M., Peumans, W. J., Barre, A., & Rougé, P. (1998). *Critical Reviews in Plant Sciences, 17*, 575–692.
11. Fujita, T. (2002). *Nature Reviews. Immunology, 2*, 346–353.
12. Fountain, D. W., Foaed, D. E., Replogle, W. D., & Yuong, W. K. (1977). *Science, 197*, 1185–1187.
13. Mirelman, D., Galan, E., Sharon, N., & Lotan, R. (1975). *Nature, 256*, 414–416.
14. Sririrugsa, P. (1998). *Pure and Applied Chemistry, 70*, 2111–2118.
15. Hermann, P. T. A., & Martin, A. W. (1991). *Planta Medica, 57*, 1–7.
16. Chaudhary, A. S., Sachan, S. K., & Singh, R. L. (2006). *Indian Journal of Crop Science, 1*, 189–190.
17. Govindarajan, V. S. (1980). *CRC Critical Reviews in Food Science and Nutrition, 12*, 199–300.
18. Khanna, N. M. (1999). *Current Science, 76*, 1351–1356.
19. Lin, J. K., Pan, M. H., & Lin-Shiau, S. Y. (2000). *BioFactors, 13*, 153–158.
20. Aggarwal, B. B., Sundaram, C., Malani, N., & Ichikawa, H. (2007). *Advances in Experimental Medicine and Biology, 595*, 1–75.
21. Miquel, J., Bernd, A., Sempere, J. M., Díaz-Alperi, J., & Ramírez, A. (2002). *Archives of Gerontology and Geriatrics, 34*, 37–46.
22. Wu, W. Y., Xu, Q., Shi, L. C., & Zhang, W. B. (2000). *World Journal of Gastroenterology, 6*, 216–219.
23. Brinkhaus, B., Hentschel, C., Von Keudell, C., Schindler, G., Lindner, M., Stützer, H., et al. (2005). *Scandinavian Journal of Gastroenterology, 40*, 936–943.
24. Chearwae, W., Wu, C. P., Chu, H. Y., Lee, T. R., Ambudkar, S. V., & Limtrakul, P. (2006). *Cancer Chemotherapy and Pharmacology, 57*, 376–388.
25. Baatout, S., Derraji, H., Jacquet, P., Ooms, D., Michaux, A., & Mergeay, M. (2004). *International Journal of Oncology, 24*, 321–329.
26. Kim, J. K., Jo, C., Hwang, H. J., Park, H. J., Kim, Y. J., & Byun, M. W. (2006). *Radiation Physics and Chemistry, 75*, 449–452.
27. Tipthara, P., Sangvanich, P., Macth, M., & Petsom, A. (2007). *Journal of Plant Biology, 50*, 167–173.
28. Tiptara, P., Petsom, A., Roengsumran, S., & Sangvanich, P. (2008). *Journal of the Science of Food and Agriculture, 88*, 1025–1034.
29. Sangvanich, P., Kaeothip, S., Srisomsap, C., Thiptara, P., Petsom, A., Boonmee, A., et al. (2007). *Fitoterapia, 78*, 29–31.
30. Larsen, K. (1996). *Thai Forest Bulletin (Botany), 24*, 35–49.
31. Bradford, M. M. (1976). *Analytical Biochemistry, 7*, 248–257.
32. Laemmli, U. K. (1970). *Nature, 227*, 680–685.
33. Mortz, E., Vorm, O., Mann, M., & Roepstorff, P. (1994). *Biological Mass Spectrometry, 23*, 249–261.
34. Goldenberg, D. P. (1989). In T. Creighton (Ed.), *Protein structure: a practical approach* (pp. 225–250). Oxford: IRL Press.
35. Richard, C., Narendra, P., Singh, K., Shade, R. E., Murdock, L. L., & Bressan, R. A. (1990). *Plant Physiology, 93*, 1453–1459.
36. Absar, N., Yeasmin, T., Raza, M. S., Sarkar, S. K., & Arisaka, F. (2005). *Protein Journal, 24*, 369–377.

37. Borrebaeck, C., & Etzler, M. E. (1980). *FEBS Letters, 117*, 237–240.
38. Peuman, W. J., Lubaki, M. N., Peeters, B., & Broekaert, W. J. (1985). *Planta, 164*, 75–82.
39. Kamemura, K., Furuichi, Y., Umekawa, H., & Takahashi, T. (1996). *Biochimica et Biophysica Acta, 1289*, 87–94.
40. Van Damme, E. J., Barre, A., Rougé, P., Van Leuven, F., & Peumans, W. J. (1995). *Plant Molecular Biology, 29*, 1197–1210.
41. Franz, H., Ziska, P., & Kindt, A. (1981). *Biochemical Journal, 195*, 481–484.
42. Goldstein, I. J., & Poretz, R. D. (1986). In I. E. Liener, R. Sharon & I. J. Goldstein (Eds.), *The lectins, properties, functions and application in biology and medicine* (pp. 33–247). Orlando, Florida: Academic.
43. Gallagher, J. T. (1984). *Bioscience Reports, 4*, 621–632.
44. Machuka, J. S., Okeola, O. G., Van Damme, E. J. M., Chrispeels, M. J., Van Leuven, F., & Peumans, W. J. (1999). *Phytochemistry, 51*, 721–728.
45. Wittsuwannakul, R., Wititsuwannakul, D., & Sakulborirug, C. (1998). *Phytochemistry, 47*, 183–187.
46. Freire, M. G., Gomes, V. M., Corsini, R. E., Machado, O. L. T., De Simone, S. G., Novello, J. C., et al. (2002). *Plant Physiology & Biochemistry, 40*, 61–68.
47. Xu, X., Wu, C., Liu, C., Luo, Y., Li, J., Zhao, X., et al. (2007). *Acta Biochimica et Biophysica Sinica, 39*, 507–519.
48. Kaur, A., Singh, J., Kamboj, S. S., Sexana, A. K., Pandita, R. M., & Shamnugavel, M. (2005). *Phytochemistry, 66*, 1933–1940.
49. Oliveira, J. T. A., Melo, V. M. M., Câmara, M. F. L., Vasconcelos, I. M., Beltramini, L. M., Machado, O. L. T., et al. (2002). *Phytochemistry, 61*, 301–310.
50. Konozy, E. H. E., Bernardes, E. S., Rosa, C., Faca, V., Greene, L. J., & Ward, R. J. (2003). *Archives of Biochemistry and Biophysics, 410*, 222–229.
51. Toyama, J., Tanaka, H., Horie, A., Uchiyama, T., & Akashi, R. (2008). *Asian Journal of Plant Science, 7*, 647–653.
52. Macedo, M. L. R., Damico, D. C. S., Freire, M. G. D. M., Toyama, M. H., Marangoni, S., & Novello, J. C. (2003). *Journal of Agricultural and Food Chemistry, 51*, 2980–2986.
53. Schwarz, F. P., Puri, K. D., Bhat, R. G., & Surolia, A. (1993). *Journal of Biological Chemistry, 268*, 7668–7677.
54. Moreira, R. A., & Perrone, J. C. (1977). *Plant Physiology, 59*, 783–787.
55. Itoh, M., Kondo, K., Komada, H., Izutsu, K., Shimabayashi, Y., & Takahashi, T. (1980). *Agricultural and Biological Chemistry, 44*, 125–133.
56. Sharon, N., & Lis, H. (1990). *FASEB Journal, 4*, 3198–3208.
57. Hoffman, L. M., & Donaldson, D. D. (1985). *EMBO Journal, 4*, 883–889.
58. Yan, Q., Jiang, Z., Yang, S., Deng, W., & Han, L. (2005). *Archives of Biochemistry and Biophysics, 442*, 72–81.
59. Trindade, M. B., Lopes, J. L., Soares-Costa, A., Monteiro-Moreira, A. C., Moreira, R. A., Oliva, M. L., et al. (2006). *Biochimica et Biophysica Acta, 1764*, 146–152.
60. Gozia, O., Ciopraga, J., Bentia, T., Lungu, M., Zamfirescu, I., Tudor, R., et al. (1993). *FEBS Letters, 370*, 245–249.
61. Verheyden, R., Pletinckx, J., Pepermans, D., Wyns, H., Willem, I., & Martin, J. (1995). *FEBS Letters, 370*, 245–249.
62. Broekaert, W. F., & Peumans, W. J. (1986). In T. C. Bog-Hansen & E. Van Driessche (Ed.), *Lectin, biology, biochemistry, clinical biochemistry* (pp. 57–65). Berlin: Walter de Gruyter.
63. Yu, L. G., Milton, J. D., Fernig, D. G., & Rhodes, J. M. (2001). *Journal of Cellular Physiology, 186*, 282–287.
64. Wang, H., Ng, T. B., Ooi, V. E. C., & Liu, W. K. (2000). *International Journal of Biochemistry & Cell Biology, 32*, 365–372.
65. Mody, R., Joshi, S., & Chaney, W. (1995). *Journal of Pharmacological and Toxicological Methods, 33*, 1–10.
66. Andrade, C. A. S., Correia, M. T. S., Coelho, L. C. B. B., Nascimento, S. C., & Santos-Magalhães, N. S. (2004). *International Journal of Pharmaceutics, 278*, 435–445.
67. Yoon, T. J., Yoo, Y. C., Kang, T. B., Song, S. K., Lee, K. B., Her, E., et al. (2003). *Archives of Pharmacal Research, 26*, 861–867.