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Arabinogalactan protein from *Arachis hypogaea*: Role as carrier in drug-formulations

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Abstract

Arabinogalactanl protein (AGP) a highly water-soluble glyco-conjugate from groundnut (*Arachis hypogaea* L.) seedling was isolated and purified by precipitation with β-glucosyl Yariv reagent. Quantification of AGP was done by gel diffusion assay. Purified AGP was conjugated to amphotericin-B (AmB) by Schiff base reaction at pH 11.0, with aim to prepare a water-injectable lesser toxic AGP–AmB conjugate without affecting AmB antifungal potential. The AGP–AmB conjugate antifungal activity was assayed by serial broth dilution and disc method against several *Candida albicans* clinical isolates. Both AmB and AGP–AmB showed similar MICs and MFCs activities, indicating that AGP do not reduced the antifungal activity of AmB. However, the *in vitro* and *in vivo* toxicity assays revealed that AGP–AmB conjugate was lesser toxic than AmB, as high MTD (45 mg/kg body weight) was observed. It is suggested that AGP could be a potent carrier in AmB formulation, which may result in effective treatment of fungal infections.

Keywords: Arabinogalactan protein; Yariv reagent; *Candida albicans*; Amphotericin-B

1. Introduction

In past few years, there has been alarming increase in life threatening mycotic infections in humans caused by various opportunistic fungi, mainly in immunocompromised hosts, such as cancer patients who have gone for surgery and AIDS patients (Bodey, 1977; Denning, 1991; Groll and Walsh, 2001). Chemotherapy has been the common strategy and a large number of antifungal drugs are being used to control the fungal infections. Among them the azoles and its derivatives found to be the most effective and predominant (Johnson and Perfect, 2003; Donnelly and De Pauw, 2004). However, the fungal infection represents a major therapeutic challenge owing to the increasing prevalence of organisms resistant to commonly used azoles (Singh, 2001; Ostrosky-Zeichner et al., 2003). Besides the relatively high cost of azoles and its derivatives, represents a severe limitation in their use. Developing novel drugs and/or treatment strategies to fight these infections is therefore critical and this has led to the development of azoles with enlarged spectrum and to the discovery of other novel, broad-spectrum fungicidal drugs.

Among the non-azoles drugs amphotericin B (AmB) and its derivatives is the drug of choice for the treatment of mycotic infection caused by wide range of fungi (Gallis et al., 1990). In contrast to azoles, amphotericin B (AmB) is inexpensive while being highly fungicidal against most pathogenic fungi, and also found to be free of clinically meaningful resistance so far (Barrett et al., 2003; Ostrosky-Zeichner et al., 2003). The use of AmB, however, is hampered due to two main factors. The first its dose related toxicity, mainly to the kidneys, central nervous system, and liver, the frequency of which may be very high (Sabra and Branch, 1990; Razzaque et al., 2001) and its side effects, such as nausea, fever and shivering (Maddux and Barriere, 1980). Second the lack of solubility in injectable aqueous media due to its highly hydrophobic nature (Hartsel and Bolard, 1996; Lewis and Wiederhold, 2003). Several strategies have been developed and are in practice to circumvent the disadvantage of water insolubility and toxicities, such as AmB administration as a micellar dispersion in sodium deoxycholate (Cleary et al., 2003; Clemons and Stevens, 2004), or as a lipid formulation, such as liposomes (Adler-Moore and Proffitt,

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2002; Manosroi et al., 2004). These strategies, however, also have their limitations that include a narrow therapeutic index for micellar dispersions (Maddux and Barriere, 1980; Harbarth et al., 2002), or high cost for lipid formulations (Cleary et al., 2003). The development of other types of water-stable and well-dispersed aqueous solutions of AmB with low intrinsic toxicity and low manufacturing prices remains therefore highly desirable. A number of attempts have been made to design a water-soluble injectable stable formulation of AmB. These included the synthesis of *N*-acyl derivatives (Kobayashi et al., 1985), *N*-methyl-*N*-D-fructosyl methyl esters (Szlinder-Richert et al., 2004), the entrapment of AmB in amphiphilic micelles (Yu et al., 1998a,b), or its association with microemulsions and monoglyceride–water systems (Moreno et al., 2001). One of the approaches for improving drug performance and reducing toxicity is conjugation to a polymeric carrier (Domb et al., 1996). In recent past, the AmB conjugation with polyethyleneglycol (Conover et al., 2003), or arabinogalactan (Falk et al., 1999), or polyvinylpyrrolidone (Charvalos et al., 2006), respectively, have generated highly water-soluble conjugates that found to be much safer and effective than the commonly used AmB-DOC formulation.

The glycoconjugates mainly the polysaccharides and polysaccharide–protein complexes could be a suitable alternative due to their high water solubility. Arabinogalactan proteins (AGPs) are a family of plant derived glyco-conjugate with established history of pharmaceutical and other industrial applications, such as emulsifier, etc. (Egert and Beuscher, 1992; Hauer and Anderer, 1993; Yu et al., 1998a,b; Classen et al., 2000). AGPs from a numbers of crop and medicinal plants have been purified and its structure determined (Gasper et al., 2001; Showalter, 2001). AGPs are typically hydroxyproline rich heavily glycosylated protein and the carbohydrate chain is arabinogalactan (AG) type which primarily consists of gatactose and arabinose. The arabinogalactan chain is usually a branched polysaccharide consisting of a (1-3)-β-D-galactan backbone having (1-6)-β-galactan side chains, which are terminally modified by arabinose with some exception where other less-abundant sugar may present (Gasper et al., 2001; Showalter, 2001). Both the intact AGP and its purified polysaccharide fraction arabinogalactan, are found to be highly water-soluble and posses a high degree of biocompatibility and used in a number of pharmaceutical and neutraceutical preparations (Egert and Beuscher, 1992; Yu et al., 1998a,b; Kelly, 1999; Classen et al., 2000). In one of the earlier study, a water-soluble injectable conjugate of amphoptericin B–arabinogalactan (AmB–AG) was formed using commercial preparation of AmB and AG (Falk et al., 1999). The conjugate found to be stable and shown to increase the solubility and stability of AmB in aqueous solution and significantly reduces its toxicity and posses a high degree of biocompatibility (Ehrenfreund-Kleinman et al., 2002; Folk et al., 2004).

Although the simple two-step Schiff base method has been established for conjugation of AmB with arabinogalactan, yet no attempt had been made so for to conjugate the AmB with AGP. The aim of the present study was to reduce the toxicity of AmB by preparing a water-soluble injectable AGP–AmB conjugate without affecting its antifungal activity. The present paper describes the isolation and purification of AGP (glycoconjugate) from peanut (*Arachis hypogaea*) and its conjugation with AmB. The water solubility, toxicity of the conjugate was evaluated and its antifungal activity was tested against clinical isolate of *Candida albicans*. The findings are presented and discuss.

2. Materials and methods

2.1. Growth and maintenance of C. albicans clinical isolates

Seven clinical isolate were obtain from AIIMS, New Delhi, India. The clinical isolates were routinely grown and maintained in YPD (0.5% yeast extract, 1% peptone, 2% dextrose) medium in laboratory unless otherwise mention.

2.2. Isolation and purification of arabinogalactan proteins from A. hypogaea

Seeds of *A. hypogaea* large variety were surface sterilized and washed thrice with ultra pure water. The seeds were soaked in sterilized water for 24 h, and then kept in dark for sprouting. The germinated seeds were further grown in Hogland's medium, in a plant growth chamber under controlled condition of light and temperature. Seven days old seedlings were collected for AGP preparation. The isolation and purification of AGP was carried out as described (Schultz et al., 2000) with slight modification. To extract AGP, 10 g (fresh weight) of seedling tissues were cut in to small pieces and ground to a fine powder in liquid nitrogen. To the ground tissues 10 ml of extraction buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.1% β mercaptoethanol and 1% (w/v) Triton X-100) was added and incubated at 4 °C for 3 h. Samples were centrifuge for 10 min at 14000 × *g*. The supernatant was precipitated with 5 volume of ethanol at 4 °C, overnight. The pellet was resuspended in a 5 ml of 50 mM Tris–HCl pH 8.0. The insoluble material was removed by centrifugation and supernatant was collected. The pellet was resuspended in additional 5 ml of 50 mM Tris–HCl pH 8.0. The supernatant were pooled together and freeze dry overnight to concentrate the sample. The dried sample was dissolved in 500 μl of 1% (w/v) NaCl and transfer to a 1.5 ml microcentrifuge tube. AGPs were precipitated with the β-glucosyl Yariv reagent (β-GlcY) by mixing the resuspended sample with equal volume of β-GlcY (2 mg/ml) in 1% NaCl and incubated at 4 °C, overnight. The insoluble AGP-Yariv complex was collected by centrifugation at 14000 × *g* in a microcentrifuge for 1 h. The pellet was washed with 0.1 M NaCl and deionized water. Sodium hydrosulphide ($Na_2S_2O_4$) was added to final concentration of 10% (w/v) to decompose β-GLcY. The solution was heated to 50 °C till the red color disappears. The samples were dialyzed extensively against water at 4 °C and freeze dried.

2.3. Quantification of AGP

AGP determination was done by glucosyl Yariv reagent binding in a radial gel diffusion assay. Agarose gel 1% containing

0.02% Yariv reagent, 0.15 M NaCl, 0.02% sodium azide were poured into petri dishes and solidified. Wells were made (4 mm diameter) in the gel using cork borer. Fifteen microliter and 30 μl of AGP solution (4 mg/ml) were loaded in to the wells. Buffer alone without AGP was used as control, and gum arabic and larch AG (Sigma, USA) were used as positive and negative test control, respectively. The petri dishes were sealed with parafilm and kept in dark at room temperature for 2 days to allow the color to develop.

2.4. Conjugation of AGP with AmB

The conjugation of AGP to AmB was carried out by a Schiff base reaction as per protocol as described (Falk et al., 1999) with little modification. The AGP was first converted to an oxidized dialdehyde form (DAAGP), which was then conjugated to AmB by amine bond with the amino group ($-NH_2$) of the AmB. To the AGP (1%) solution in water, potassium periodate (0.05 M) was mixed and the mixture was stirred at room temperature, until it was dissolved completely. This resulted in dialdehyde AGP formation (DAAGP), which was purified from excess perisodate using Dowex-1-acetate (Sigma, USA) column (6 mm × 8 mm). The purified DAAGP formed was resuspended in 0.2 M borate buffer pH 11.0 (12.5 mg/ml) and mixed with AmB (MERK, Germany) final concentration of 6.25 mg/ml. The conjugation reaction was carried out at 37 °C for 48 h. After incubation 1.2 M sodium borohydrate was added to the conjugation mixture at 4 °C for 60 min with stirring.

The final conjugate was purified by dialysis against deionized water for 48 h at 4 °C. The dialysate was centrifuged at 2000 × *g* for 10 min and lyophilized. The conjugate was stored in dry powered form. The conjugate was filter sterilized through 0.2 μm pore size membrane prior to use.

2.5. Susceptibility method by broth method

The drug susceptibility testing was done against seven *C. albicans* clinical isolates. MICs were determined by microdilution broth method as per standard norms. A 10 mg/ml stock solution of AmB and AGP–AmB were prepared in dimethyl sulphoxide (DMSO) and water, respectively. Various serial dilutions of both stocks (ranging from 120 to 0.01 μg/ml) were prepared using filter sterilized RPMI 1640 broth medium pH 7.0 (Hi Media India). In 96 well round bottom microtiter plate 0.1 ml of various serial dilution of drugs were taken in triplicate. Pre-inoculum of *Candida* isolate were prepared in YPD medium. From the pre-inoculum, the final inoculum of each isolate was prepared using sterile RPMI 1640 broth to give 10^5 cells/ml, as was determined by counting using hematocytometer. To the microtiter plate wells containing 0.1 ml of serially diluted drugs, 0.1 ml of each inoculum was added. For control 0.1 ml of drug free medium and 0.1 ml of inoculum were taken. The experiment was carried in triplicate. The microtiter plates were incubated at 35 °C for 24 h. The growth in each well was determined visually. The MIC was defined as lowest drug concentration in complete inhibition of visible growth.

The minimum fungicidal concentration (MFC) was determined by measuring the number of CFU on YPD plate. Just after determination of the MIC, 50 μl of sample from wells that show no growth were spread on YPD plate and incubated at 35 °C for 24 h. The MFC was established at lowest concentration of drug at which CFU found to be negative.

2.6. Susceptibility using disc method

Drugs susceptibility was also tested against various *C. albicans* isolate. The *Candida* cells (10^4 ml^{-1}) were mix with sterile YPD medium and pour into petri dishes. The plate were allowed to solidify, after solidification sterile filtered disc were put on plate and loaded with different concentration of AmB and AGP–AmB conjugate. The plates were incubated for 24 h at 30 °C formation of hallo zone around disc were monitored.

2.7. In vitro toxicity assay

In vitro toxicity was studied using sheep erythrocytes (SRBCs). SRBCs were suspended in phosphate buffer saline and washed twice by centrifugation at 3000 × *g* for 10 min. 0.1 ml of serially diluted drugs were taken in glass tubes and mix with 0.9 ml of SRBCs. The tubes were incubated for 1 h at 37 °C. The extent of hemolysis was measured visually.

2.8. Toxicity in animal model

In vivo toxicity was tested using albino BALB/C mice as described (Falk et al., 1999). Male BALB/C mice approximately 25 g weight was injected via tail vain with various doses of AmB and AGP–AmB conjugate. Each dose was given as a single bolus injection of 0.1 ml intra-venously for each dose 10 mice were injected after every 10 min until death was observed. The survival of the mice that receive the maximum tolerance dose (MTD) was monitored for 8 days.

3. Results

3.1. Purification and quantification of AGPs

Purification of AGP was carried out by using Yariv reagent that is specific for AGP, as described in Section 2. The quantification of AGP and its derivatives was done using gel diffusion assay containing Yariv reagent (Fig. 1). It is clear from result that Yariv reagent reacted with all AGP preparation and form colored zone (Fig. 1, A, AGP 1, 2, 3, AGP–AmB 1, 2, 3), while no colored zone develop with non-AGP preparations (Fig. 1, B, C and AmB).

3.2. Conjugation of AGP and AmB

Conjugation of AGP and AmB was carried out as described in Section 2. Since the yield of conjugation depend on both concentration and pH of reaction as indicated in earlier studies. The conjugation of AGP and AmB was done at pH 11.0 at which maximum yield has obtained. The general scheme of

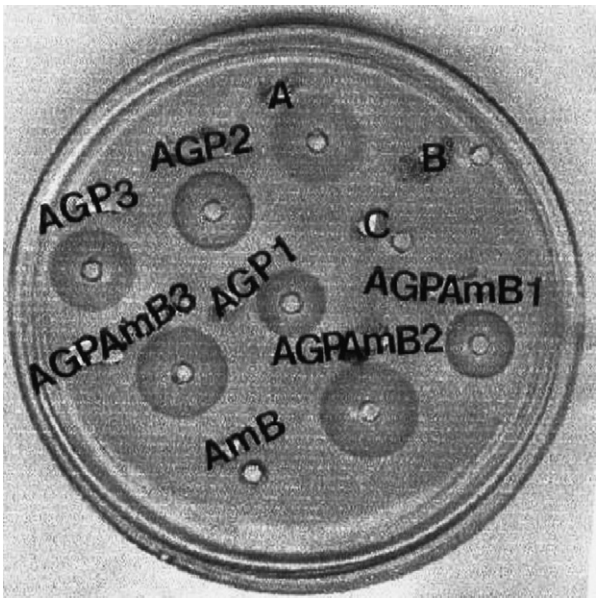

Fig. 1. Radial gel diffusion assay of AGP using Yariv reagent. Agarose gel (1%) containing 0.02% Yariv reagent, 0.15 M NaCl, 0.02% sodium azide was poured into petri dish. After solidification, wells (4 mm diameter) were made and loaded with respective samples. AGP 1, AGP 2 and AGP 3 are 15 μl, 30 μl (4 mg/ml) and 30 μl (4 mg/ml) after 6-month storage, of AGP, respectively. AGP–AmB1, AGP–AmB2 and AGP–AmB3, are 15 μl, 30 μl (4 mg/ml) and 30 μl (4 mg/ml) after 6-month storage, of AGP–AmB conjugate, respectively. A is 30 μl (4 mg/ml) of gum arabic as positive control, AmB, B and C are, amphotericin B, buffer alone and 30 μl (4 mg/ml) of larch arabinogalactan as negative controls, respectively.

conjugation reaction is explained in Fig. 2. In general, the conjugation is two-step process, firstly the AGP is converted to an oxidized dialdehyde form, which was purified and then reacted with AmB, and finally the AGP–AmB conjugate formed by reaction of $-NH_2$ group of AmB with dialdehyde AGP. Conjugation product was confirmed by presence of AmB in the final product by measuring absorbance at 250–500 nm and its reaction with Yariv reagent (Fig. 1).

3.3. Evaluation of antifungal activity of the AGP–AmB conjugate

The antifungal activity of the AGP–AmB conjugate against several *C. albicans* clinical isolates were determine as described in materials and methods section. The MICs and MFCs are given in Table 1. The AGP–AmB conjugate showed almost similar antifungal activity as free AmB. Thus, it is clear that AGP binding does not reduce the antifungal activity of AmB similar results were also observed in disc method assay (Fig. 3). The AGP–AmB conjugate and AmB showed almost similar antifungal activity against different *C. albicans* clinical isolates, except the one which seems to be AmB resistance isolate no. 7 (Fig. 3C). Relatively higher MIC and MFC were observed in this isolate compare to others (Table 1 isolate no.7).

3.4. In vitro and in vivo toxicity studies

In vitro toxicity was determined visually by hemolysis of SRBCs by AmB and AGP–AmB conjugate, respectively. No hemolytic activities were monitored up to 1.2 mg/ml of AGP–AmB conjugate. However, a very little hemolytic activity was observed at concentration >1.5 mg/ml, the highest concentration tested. On the other hand, hemolysis was observed at much lower concentration (10 μg/ml) of free AmB.

In vivo toxicity was carried out in BALB/C mice. The result is shown in Fig. 4. It is clear that AGP–AmB conjugate showed much higher MTD (45 mg/kg) compare to AmB, which has 5 mg/kg. This indicated that AGP–AmB conjugate is less toxic than AmB.

4. Discussion

In present study, an attempt has been made to develop a new formulation of AmB that is AGP–AmB conjugate, which could be highly water-soluble and safer than the other commonly used

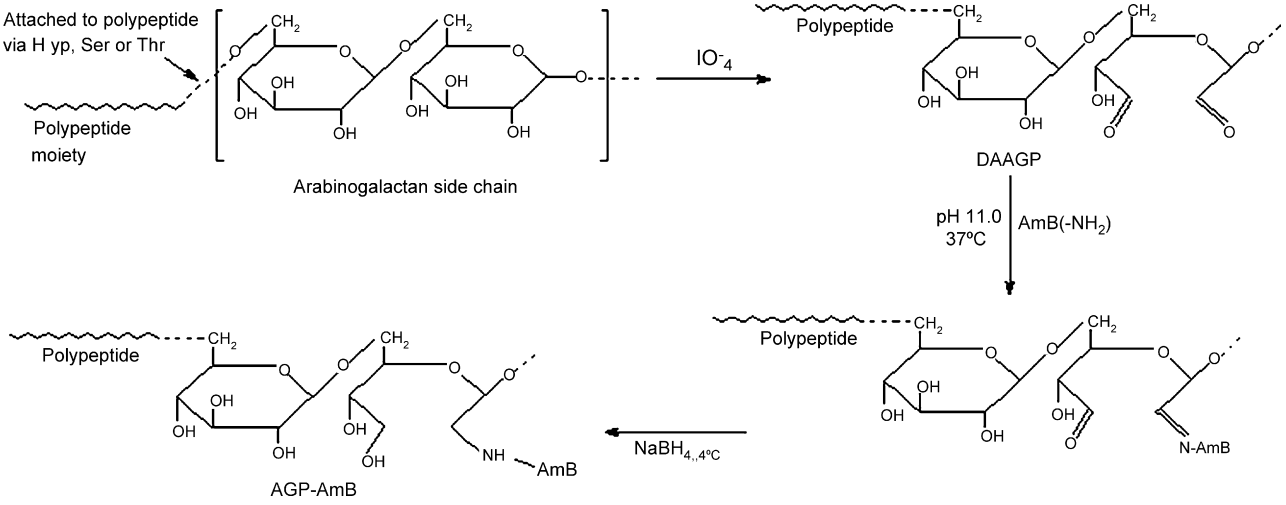

Fig. 2. General scheme of AGP and AmB conjugate preparation. This is a two-step process where in AGP is first converted to dialdehyde form (DAAGP) by treatment with periodate. The DAAGP after purification, is then mixed with AmB and AGP–AmB conjugate formed via amide linkage using $-NH_2$ of AmB (AGP—a hypothetical general structure of arabinogalactan protein, where arabinogalactan side chain is attached to polypeptide through Hyp, Ser or Thr). DAAGP—dialdehyde form of AGP; AGP–AmB—arabinogalactan protein–amphotericin conjugate).

Table 1
MICs and MFCs of AmB and AGP–AmB conjugate against various *Candida albicans* clinical isolates

Name of isolate	AmB[a]		AGP–AmB[b]	
	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
Candida albicans isolate no. 1	0.14–0.30	0.35–0.50	0.14–0.30	0.35–0.50
Candida albicans isolate no. 2	0.14–0.30	0.35–0.50	0.14–0.30	0.35–0.50
Candida albicans isolate no. 3	0.14–0.35	0.35–0.50	0.14–0.35	0.35–0.50
Candida albicans isolate no. 4	0.14–0.35	0.35–0.50	0.14–0.35	0.35–0.50
Candida albicans isolate no. 5	0.14–0.30	0.30–0.50	0.14–0.35	0.30–0.50
Candida albicans isolate no. 6	0.12–0.30	0.30–0.50	0.12–0.35	0.30–0.50
Candida albicans isolate no. 7	0.20–0.50	0.50–1.20	0.20–0.50	0.50–1.20

[a] AmB, free amphotericin B.
[b] AGP–AmB, arabinogalactan protein–amphotericin B conjugate.

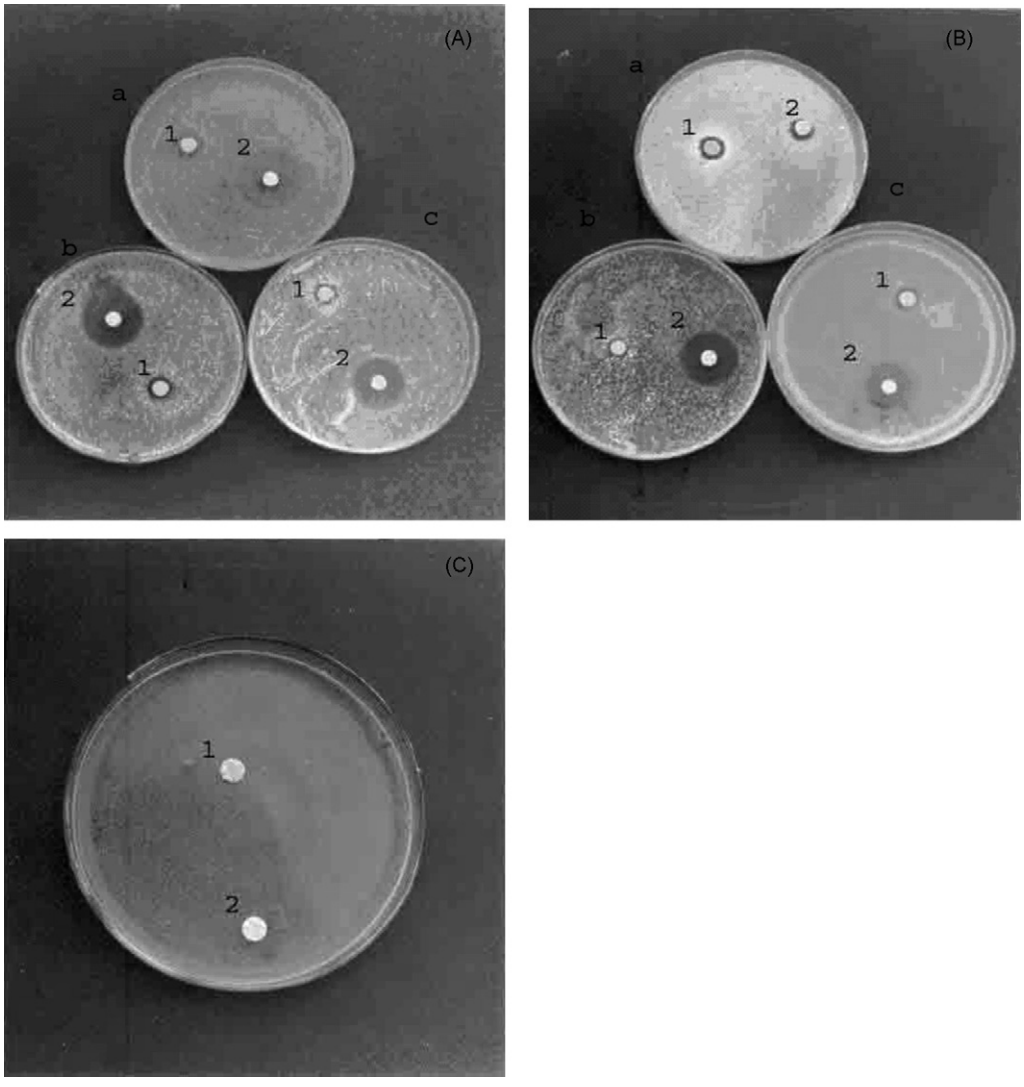

Fig. 3. Antifungal activities of free AmB and AGP–AmB conjugate were tested against different *Candida albicans* clinical isolates by disc method. In total seven isolated were used however, results of only few are shown here. (A) Antifungal activity of AmB against clinical isolate no. 1 (a), isolate no. 2 (b) and isolate no. 3 (c), respectively. 1 and 2 are two different concentrations 10 and 20 μl of AmB (1 mg/ml), respectively. (B) Antifungal activity of AGP–AmB conjugate against clinical isolate no. 1 (a), isolate no. 2 (b) and isolate no. 3 (c), respectively. 1 and 2 are two different concentrations 10 and 20 μl of AGP–AmB (1 mg/ml), respectively. (C) Antifungal activity against an amphotericin B resistance isolate (isolated no. 7). 1, AmB and 2, AGP–AmB, respectively.

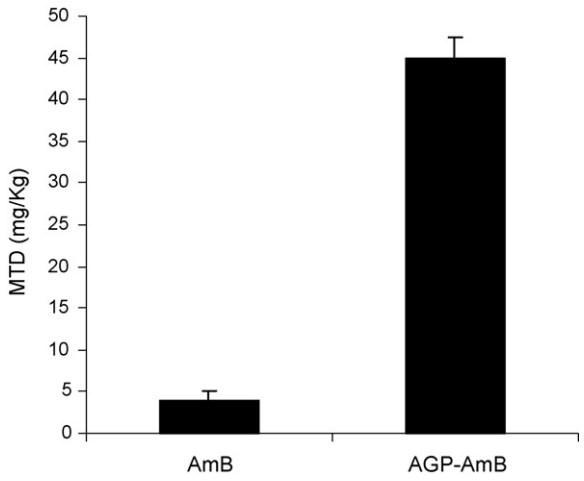

Fig. 4. *In vivo* toxicity assay of, AmB and AGP–AmB in mice. Maximum tolerance doses (MTD) were determined. AmB, amphotericin B; AGP–AmB, arabinogalactan protein–amphotericin conjugates (data shown are mean triplicate ± S.E.).

water insoluble and liposomal formulations. The AGP was chosen to form water-soluble AGP–AmB conjugate due to two main reasons. The firstly, it is closely related to arabinogalactan and posses its all important properties, such as high water solubility, biodegradability, biocompatibility and ease of conjugation in aqueous media and, secondly, the relatively easy purification of AGP compared to AG which is more tedious. AGP from *A. hypogaea* was isolated and purified using Yariv reagent which is specific for AGP. The purity and quantitative estimation was done using Yariv reagent (Fig. 1). Color zone was observed with AGP preparation but not with non-AGP.

The purified AGP was then conjugated with AmB under conditions, which result in maximum yield. The AGP–AmB conjugation was carried out in two steps. Firstly, the AGP was oxidized by reaction with the oxidizing agent potassium periodate. Since the AmB being a polyene is sensitive to oxidation, therefore, it was essential to prevent the oxidation and degradation of AmB by oxidizing agent. Thus, the excess iodate and periodate ions were removed from the oxidized AGP using ion exchange column before conjugation to AmB otherwise, it will leads to poor yield. In the second step, the oxidized AGP molecules were conjugated to AmB under optimum conditions. As it has been observed in one of the earlier study that conjugation of AG (the polysaccharide fraction) with AmB, was optimal at pH 11.0, in borate buffer (Falk et al., 1999). Therefore, in order to achieve high yield in present study AGP–AmB conjugation was carried out in borate buffer, pH 11.0, at which maximum yield expected. The conjugation under these conditions resulted in high yield (up to 90%). The high yield could be due to the fact that boric acid forms complexes with AmB, which increases its water solubility and minimize aggregate formation, which is one of the major problem of AmB (Ernst et al., 1981; Straus and Kral, 1982). The solubility of AmB-borate complexes found to be highest at pH 11 or above, while lesser at lower pH and minimum in neutral solution. The formation of such complexes facilitates the AmB molecules to dissolve into reaction solution, and makes them available for conjugation with oxidized AGP.

The synthesis of AGP–AmB conjugate was confirmed by measuring absorbance of the final product at wavelength range 250–500 nm using UV–vis spectrophotometer (Perkin-Elmer, Lambda Bio 40, USA) and also it reaction with Yariv reagent (Fig. 1). A number of study showed that the free AmB solution has typical peaks at 408, 385, 365 and 348 nm, which is concentration dependant and linked to aggregate formation (Lamy-Freund et al., 1993; Balakrishan and Easwaran, 1993). The ratio A_{348}/A_{408} is indicative of the aggregated/monomeric ratio and a value of ∼0.25 being predominated by monomeric form, while the value >2.0 is predominated by aggregate form. Similar to the previous studied, free AmB solution has showed its characteristic typical peaks at mention wavelengths range. On the other hand, free AGP solution has peak at 250 nm, while the AGP–AmB conjugates solution showed a composite spectrum with typical peaks of both AmB and AGP (spectroscopic data not shown here). Unlike the free AmB, AGP–AmB conjugate has constant absorbance at all concentrations, which indicates that it stabilized the drug and reduced aggregate formation. However, in present work we have not performed any further study to confirm the aggregate formation. The final product which showed composite spectra of both AmB and AGP reacted with Yariv reagent. Thus, both the spectroscopy and Yariv reagent confirmed the AGP–AmB conjugate formation.

The AGP–AmB conjugate showed high water solubility (up to range of 85 mg/ml) and poorly soluble in DMSO (1 mg/ml). On the other hand, AmB is poorly soluble in water (0.1 mg/ml at pH 2 and 11), and highly soluble in DMSO (up to 40 mg/ml) as observed in present study and also in earlier report (Budavari et al., 1989). Thus, AGP–AmB conjugates to showed improved quality compared to free AmB.

The antifungal activity and toxicity of the AGP–AmB conjugate were tested in *in vitro* and *in vivo* conditions. It was clear from the antifungal activity assays both by broth serial dilution (Table 1) as well as disc method (Fig. 3), against several *C. albicans* clinical isolates that AmB and AGP–AmB conjugate have almost similar antifungal activity. Our results show, quite unambiguously, that the activity of AmB was not impaired by its complexation with AGP (based on MIC, MFC). Being a member of polyene antifungal, AmB involves the formation of complexes with ergosterol leading to the creation of transmembrane channels that disrupt the membrane permeability properties of the fungal cells (Baginski et al., 2002). This interaction relies on the availability of free amphotericin. Thus, it is clear that conjugation to AGP does not affect the interaction AmB with ergosterol present in the fungal membrane. A similar observation is, nevertheless, made for the liposomal form of amphotericin (Adler-Moore and Proffitt, 2002) and AmB–polyvinylpyrrolidone (PVP) conjugate (Charvalos et al., 2006).

On the other hand, the *in vitro* and *in vivo* toxicity assays showed reduction in toxicity. It has been reported that besides its interaction with ergosterol, amphotericin may, however, also interact with cholesterol, which is the most likely explanation for its toxicity towards eukaryotic cells (Brajtburg and

Bolard, 1996). Therefore, measurement of hemolytic index of SRBC is one of the parameter and is one of the methods commonly used for *in vitro* toxicity of AmB (Foster et al., 1988). It was observed that hemolytic index of AGP–AmB conjugate was significantly higher than that of AmB (>1 mg/ml). The toxicity of the amphotericin–AGP complexes appeared markedly decreased compared with free amphotericin, based on the measurement of its lytic activity towards red blood cells. Amphotericin–cholesterol interaction occurs more easily when the drug is in an aggregated form as compared with monomers (Barwicz and Tancrede, 1997; Huang et al., 2002). A reduction of aggregation by complexation with AGP could, therefore, have been critical to explain the lesser cytotoxicity of AGP–AmB compared with AmB. *In vivo* toxicity study in mice model also confirmed that AGP–AmB conjugate significantly lesser toxic than free AmB, as MTD were 45 and 4 mg/kg, respectively (Fig. 4). This could be due to the improved water solubility which leads lower cellular accumulation of the drug as suggested in earlier studied (Folk et al., 2004; Charvalos et al., 2006). However, in present work, we have not done any study regarding cellular accumulation of drug. Thus, it is clear from the present work that conjugation of AGP with AmB significantly reduces its toxicity and does not have any adverse effect on its antifungal activity. Therefore, it is suggested that AGP could serve as novel potent carrier for AmB drug formulation. Study regarding the testing of the efficacy of conjugate in murine model of candidosis need to be done to further confirm its potential. Since the AGPs from various sources found to have immuno-stimulatory activity and the fungal infections are more common in immuno compromised host. It is speculated that conjugation of AmB to AGP (with proven immuno-stimulatory activity) could be advantageous by having both antifungal as well as immuno-stimulatory properties. However, this is merely a speculation unless AmB is conjugated to AGP (with proven immuo-stimulatory activity) and the final conjugate tested for its antifungal and immuno-stimulatory activity. Neither we have tested nor do we claim regarding immuno-stimulatory properties of AGP used in present study.

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