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Full Length Research Paper

Antioxidant and antimicrobial efficacies of *Amaranthus polygonoides* and its impact on L-asparaginase production

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Traditional medicinal herbs play a vital role in primary healthcare throughout the world. The current investigation represents the analysis of antioxidant, antimicrobial activities of *Amaranthus polygonoides* and its application in L-asparaginase (anti-leukemic enzyme) production. The plant leaves were dried and extracted with distilled water to yield their crude extracts. Further, this extract was subjected to screening of antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method and nitric oxide scavenging activity. The extract was found to have the highest DPPH free radical scavenging activity (84%) and nitric oxide scavenging activity (71.8%). Moreover, the extract was proved to be active against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebseilla pneumonia*, *Aspergillus niger* and *Aspergillus fumigatus* through antimicrobial activity test. After the initial assessment, the extract was screened for the production of L-asparaginase by *Streptomyces venezuelae* using rapid plate assay and confirmed the enzyme production by zone formation. The effect of concentration of *A. polygonoides* leaves extract on specific growth rate and specific L-asparaginase production rate was evaluated thorough Monod kinetics and the maximum specific growth rate, μ_m and maximum L-asparaginase production rate, P_m were found to be 0.8683 and 0.7902 h^{-1}, respectively.

Key words:*Amaranthus polygonoides*, antioxidant, L-asparaginase, production kinetics.

INTRODUCTION

Amaranthus polygonoides, a flowering plant commonly known as Amaranth has been extensively used for medicinal purposes. Amaranth seeds consist primarily of starch made up of both glutinous and non-glutinous fractions. The unique aspect of amaranth grain starch is that the size of the starch granules (1 to 3 µM) is much smaller than those found in other cereal grains (Becker, 1989). L-Asparaginase is one of the most effective anti-cancer enzymes whose mode of action is based on the plasma asparagine depletion, an essential amino acid for some cancerous cell lines. This asparagine depletion is caused by the hydrolysis of L-asparagine into L-aspartic acid and ammonia. Thus, cancer cells starve for L-asparagine and are killed by lacking exogenous supply (Lee and Bridges, 1968). However, normal cells are protected from L-asparagine-starvation due to their ability to produce this amino acid (Duval et al., 2002). L-Asparaginase is commercially produced by *Escherichia coli* and *Erwinia crysanthemi* (Amador et al., 2003). Despite the unique mode of action of L-asparaginase from these microbes which show relative selectivity with regard to the metabolism of malignant cells, some patients experience severe immunological reactions during L-asparaginase therapy (Muller and Boos, 1998). The situation becomes worse when L-asparaginase from these microbes is used repeatedly because of the enzyme's relatively short half-life and instability in the process of production and treatment. In order to overcome this constraint, production of L-asparaginase by other bacterium can be explored. The enzyme is produced by a large number of microorganisms that

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include *E. coli* (Kenari et al., 2010), *Enterobacter aerogenes* (Mukherjee et al., 2000), *Bacillus circulans* (Prakasham et al., 2010), *Pseudomonas aeruginosa* (Yasser et al., 2002), *Streptomyces gulbargensis* (Amena et al., 2010) and *Streptomyces albidoflavus* (Narayana et al., 2008) under aerobic condition.

Furthermore, the enzyme is produced in both solid state and submerged fermentation (Bessoumy et al., 2004; Mishra, 2006) using various substrates such as groundnut cake extract (Amena et al., 2010), cotton seed oil cake (Baskar and Renganathan, 2009), palm kernel cake (Sita Kumari and Narasimha Rao, 2010), coconut oil cake, red gram husk, Bengal gram husk (Hymavathi et al., 2009), soy bean meal (Yasser et al., 2002), corn flour extract (Baskar et al., 2010), wheat bran and rice bran (Soniyambi et al., 2011).

However, the cost of the substrate must be low to have a positive impact on the bioprocess of L-asparaginase. Hence for the first time, attempts were made to analyze the antioxidant and antimicrobial effects of *A. polygonoides* and use it for the production of L-asparaginase.

MATERIALS AND METHODS

The leaves of *A. polygonoides* were collected from different places and washed in tap water. After washing, they were shade-dried for ten days and pulverized into fine powder. After sieving, the powder was stored in air tight container and used for further extraction.

Preparation of aqueous extract

Briefly, 100 g of the powdered leaf material was macerated with 1000 ml of distilled water for 24 h. The extract was filtered by using muslin cloth and the filtrate was concentrated to dryness by evaporation on water bath at 90°C. Thus, 40% of aqueous extract of leaves was obtained which was used for further studies.

Determination of antioxidant activity

The antioxidant activity of *A. polygonoides* extracts was evaluated by measuring DPPH free radical-scavenging activity (Juan and Chou, 2010) and superoxide anion scavenging activity. A total of 2.5 ml of 75 µM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solutions and 0.5 ml of sample with different concentrations (100, 200, 300, 400 and 500 µg/ml) were mixed.

After incubation at ambient temperature for 90 min, the absorbance was measured at 517 nm by using ascorbic acid as standard. Superoxide anions were chemically generated in a mixture of phenazine methosulphate (PMS) and nicotinamide adenine dinucleotide (NADH). The reaction was quantified by coupling superoxide generation to the reduction of nitro blue tetrazolium (NBT).

In this experiment, the superoxide radicals were generated in 3 ml of Tris HCL buffer (16 mM; pH 8) containing 1 ml of NBT (50 µM), 1 ml of NADH (78 µM) and 1 ml of various concentrations (100, 200, 300, 400 and 500 µg/ml) of aqueous extract of *A. polygonoides* were mixed and finally 1 ml PMS (10 µM) was added. The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm. The DPPH and the superoxide anion free-radical-scavenging activity were calculated using the following equation:

$$\%\ Inhibition = \frac{Absorbance\ of\ Blank - Absorbance\ of\ Sample}{Absorbance\ of\ Blank} \times 100 \quad (1)$$

Nitric oxide scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to form nitrite ions and this was estimated by Griess Illosvoy reaction.

The reaction mixture was prepared by adding 2 ml of 10 mM sodium nitroprusside, 0.5 ml of phosphate saline buffer and 0.5 ml of various concentration of aqueous extract of *A. polygonoides* in methanol. These were incubated at 25°C for 150 min. After incubation, 0.5 ml of reaction mixture containing nitrite was pipetted out and mixed with 1 ml of 0.33% sulfanilic acid reagent in 20% glacial acetic acid and allowed to stand for 5 min for complete diazotization. Subsequently, 1 ml of naphthyl ethylenediamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. The absorbance was measured at 540 nm and the nitric oxide scavenging activity was calculated using the following equation:

$$\%\ Inhibition = \frac{Absorbance\ of\ Blank - Absorbance\ of\ Sample}{Absorbance\ of\ Blank} \times 100 \quad (2)$$

Determination of antimicrobial activity

The antibacterial activity against various Gram negative and Gram positive microorganisms such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus cereus, Bacillus subtilis*, *E. coli*, *P. aeruginosa*, *Klebsiella pneumonia* and antifungal activity of *A. polygonoides* against *Aspergillus niger* and *Aspergillus fumigatus* were determined by disc diffusion method (Gislene and Paulo, 2000).

Screening for the production of L-asparaginase

The screening for the production of L-asparaginase using *A. polygonoides* leaves extract as nitrogen source was done by rapid plate assay (Saxena and Sinha, 1981). Briefly, 0.1 ml of *Streptomyces venezuelae* was inoculated in the medium containing (g/L): starch, 2; *A. polygonoides* leaf extract powder, 1; calcium chloride, 0.2 and agar, 20. The pH was adjusted to 7.0 and supplemented with phenol red (0.009 % final concentration) and incubated at room temperature for 24 h.

Determination of growth and enzyme production kinetics

0.1 ml of the pure culture was inoculated in two Erlenmeyer flasks containing each 1000 ml of above mentioned sterile fermentation broth with and without *A. polygonoides* leaves extract and incubated at room temperature. Samples were collected at every 3 h interval and subjected for centrifugation at 5000 rpm. The weight of the pellet was measured and the supernatant was used as crude enzyme extract. From the biomass data obtained for various time intervals, the specific growth rate, μ_m h^{-1} was calculated using the following modified Gompertz equations: In Equation 3, X_0, X_{max}, X, t, μ_m and λ denote initial biomass concentration (mg ml^{-1}), maximum

$$\ln\left(\frac{X}{X_o}\right)=\ln\left(\frac{X_{max}}{X_o}\right)\exp\left[-\exp\left(\frac{e.\mu_m}{\ln\left(\frac{X_{max}}{X_o}\right)}(\lambda-t)+1\right)\right] \quad (3)$$

$$P=A.\exp\left[-\exp\left(\frac{e.P_m}{A}(\lambda-t)+1\right)\right] \quad (4)$$

biomass concentration (mg ml^{-1}) and biomass concentration (mg ml^{-1}), incubation time (h), maximum specific growth rate (h^{-1}) and lag time (h), respectively. And in Equation 4, P, A, P_m, t and λ denote enzyme activity (FU/ml), enzyme production potential (FU/ml), specific enzyme production rate (h^{-1}), incubation time (h) and lag time (h), respectively.

Substrate utilization kinetics on cell growth and L-asparaginase production rate

The effect of various *A. polygonoides* leaf extract concentrations on specific growth rate and specific L-asparaginase production rate was analyzed by varying its concentration from 0 to 13 mg/ml in the medium containing component (g/L) Starch, 20; magnesium chloride, 0.5; sodium chloride, 2 and Triton X 100, 0.2 using Monod model as follows:

$$\mu=\frac{\mu_{max}.[S]}{k_s+[S]} \quad (5)$$

$$P=\frac{P_m.[S]}{k_s+[S]} \quad (6)$$

Where, μ and P are the specific growth rate and specific production rate (h^{-1}), respectively; μ_{max} and P_m are the maximum specific growth rate and maximum L-asparaginase production rate (h^{-1}) and k_s is the half velocity constant in mg/ml.

Cell disruption

Cell disruption was done by ultrasonication (Geckil and Gencer, 2004) to increase the enzyme release from cells during fermentation. Hence, all the fermentation broths were subjected to low intensity ultrasonication (20 kHz) at every 12 h for 30 min including 30 s working time and 30 s resting time. Samples were collected at every 3 h interval from the two culture flasks and subjected for centrifugation at 5000 rpm separately. The initial weight of the aluminium foil was taken. The pellet obtained was placed in the foil and kept at 55°C for 10 min until dried, and then the weight of the foil with the dried pellet was measured. From the weight difference, the dry weight was calculated and the supernatant was used as crude enzyme extract. Crude extract samples obtained for every 3 h were collected. The enzyme activity for each sample was measured by enzyme assay.

Enzyme assay

The crude extract obtained for each fermentation broth was assayed both qualitatively and quantitatively. The qualitative assay was performed through fluorometry and the quantitative assay was performed through nesslerization. In the fluorometric assay, 0.5 ml sample of crude enzyme was treated with 100 µL of L-aspartic acid β-(7-amido-4-methylcoumarin) (2 mmol/L) in Tris–HCl buffer (50 mmol/L, pH 7.5) containing 1% ethylene glycol for 30 min at 37°C. The release of 7-amino-4-methylcoumarin was measured fluorometrically at 37°C using a Spectra-Fluor fluorometer. The excitation and emission wavelengths were 360 and 465 nm, respectively (Ylikangas and Mononen, 2000).

The enzyme activity was quantitatively determined by measuring the amount of ammonia released through nesslerization (Wriston and Yellin, 1973), which is the most commonly applied enzyme assay. This was done by adding 0.2 ml of crude extract sample to the pre-warmed 1.8 ml of 0.04 M L-asparagine prepared in 0.05 M Tris HCl buffer, (pH 8.6) and incubated for 30 min at 37°C. The reaction was retarded by adding 1 ml of 15% trichloroacetic acid. Suitable blanks that received trichloroacetic acid before extract addition were included in all assays. The precipitated proteins were removed and to 1.5 ml of the supernatant 3 ml of de-ionized water and 0.5 ml of Nessler's reagent were added. Then all the tubes were incubated at room temperature for 10 min. The absorbance was taken at 480 nm under UV-spectrophotometer. The ammonia concentration produced in there action was determined by ammonium sulfate standard curve. One unit (U) of L-asparaginase activity is defined as the amount of enzyme that liberates 1 µmol of ammonia per minute at 37°C.

RESULTS AND DISCUSSION

DPPH and superoxide anion radical-scavenging activity of *A. polygonoides*

DPPH free radical and the superoxide anion scavenging abilities were measured for evaluating the antioxidant activity in a short time. Figure 1A and B shows the dose response curve obtained for both scavenging effects of various concentrations of *A. polygonoides* leaves extract and vitamin C. For each sample, the DPPH and the superoxide anion scavenging activities were increased as the extract concentration increased until a certain point, while compared with Vitamin C, *A. polygonoides* leaves extract showed enhanced activity in both the cases. The highest DPPH scavenging activity of Vitamin C and *A. polygonoides* leaves extract were found to be 66.2 and 84%, respectively. And the enhanced inhibition of superoxide anion formation exhibited by vitamin C and *A. polygonoides* were found to be 83.4 and 93.9%, respectively. These values confirmed that A. polygonoides has remarkable inhibitory effect against superoxide anion formation.

Nitric oxide scavenging activity

The nitric oxide scavenging activity of *A polygonoides* leaves extract was studied at its different concentrations and compared with those of Vitamin C. The results obtained are shown in Figure 1C.

The extract inhibited nitric oxide generation in the reaction mixture to different effect according to its

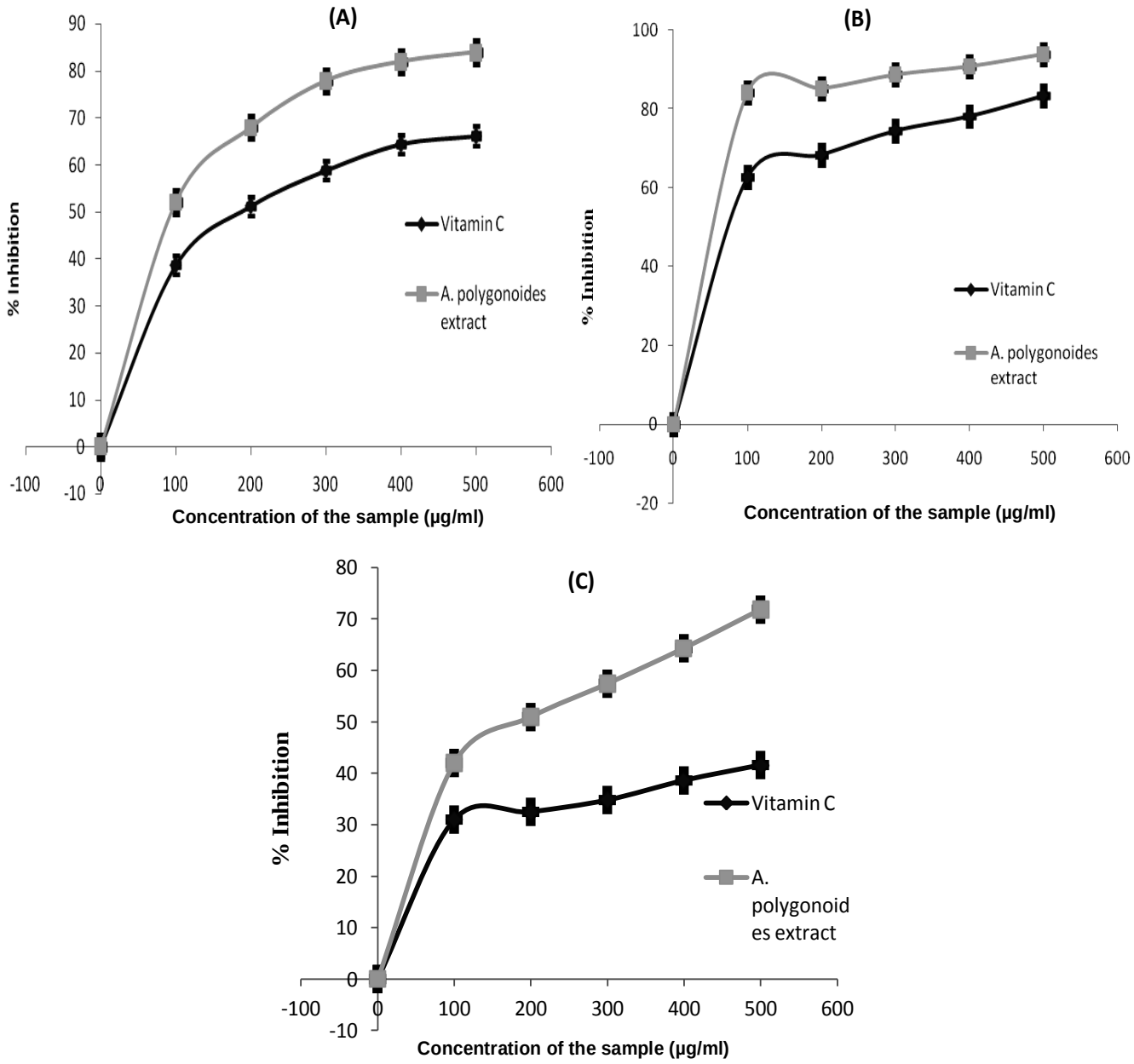

Figure 1. DPPH scavenging activity (A), superoxide anion scavenging activity (B) and nitric oxide scavenging activity (C) of vitamin C and *A. polygonoides* leaves extract at various concentrations.

concentration. A 71.8% of inhibition of nitric oxide generation was observed at the concentration 500 µg/ml which was higher than inhibition caused by vitamin C at the same concentration.

Antimicrobial activity

The plant extract proved to be active against all the Gram-positive such as *S. aureus*, *S. epidermidis*, *M. luteus*, *B. cereus*, *B. subtilis,* the Gram-negative strains, *E. coli*, *P. aeruginosa*, *K. pneumonia* and the fungi *A. niger* and *A. fumigatus* used for this study. The activity of the plant extract was assessed by the formation of zone of inhibition and the results obtained are shown in Figure 2. The antibacterial and the antifungal effect of these extracts were found to be comparable to the antibacterial and antifungal drugs, ciprofloxacin (5 µg/disc) and ketoconazole (50 µg/disc), respectively. Antibacterial and antifungal activity of aqueous extract of *A. polygonoides* at different concen-trations (50, 100 and 150 µg/disc) against Gram-positive, Gram-negative bacteria and the fungi in comparison with ciprofloxacin and ketoconazole are shown in Figure 2A to C and in Table 1.

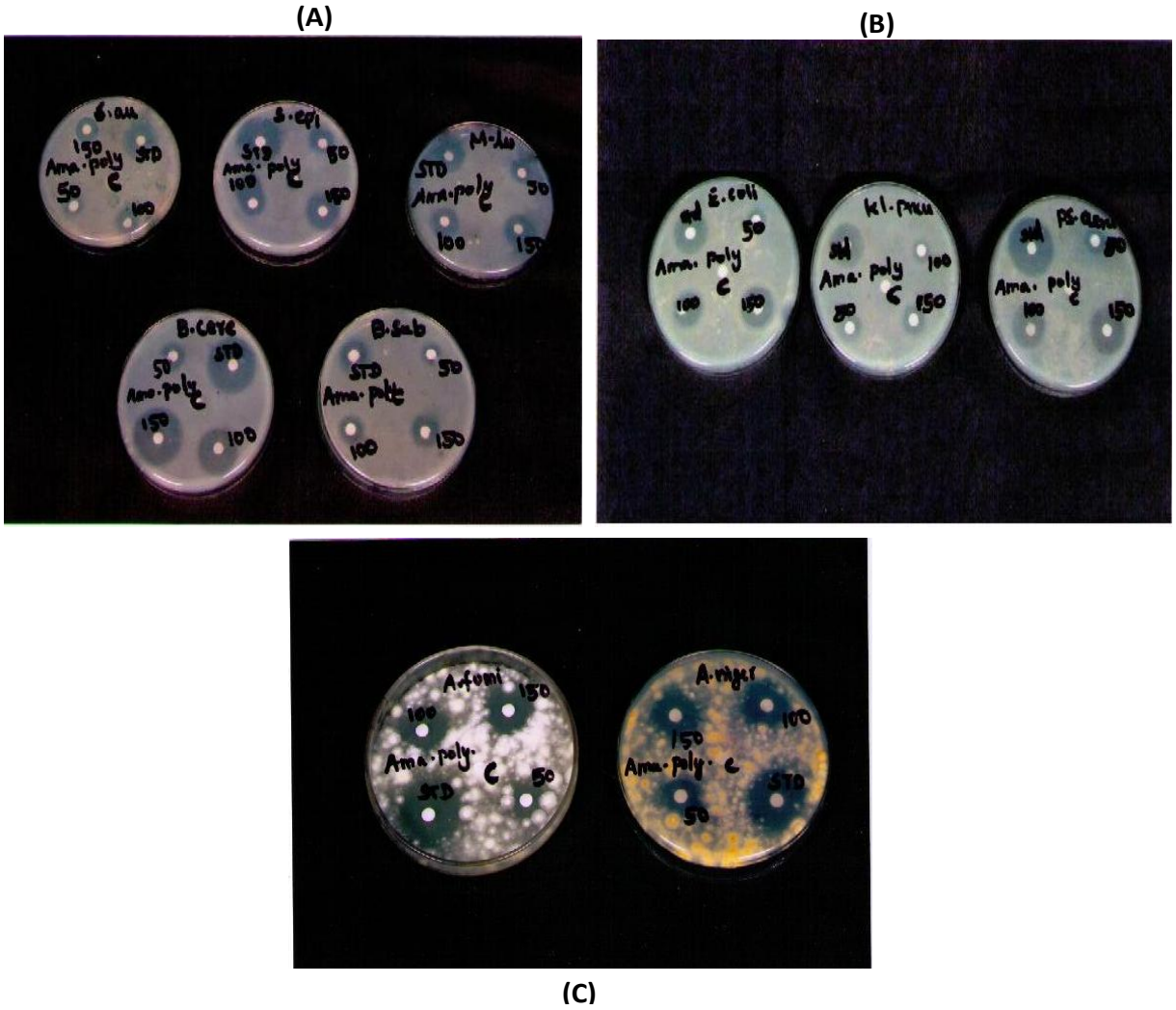

Figure 2. Antibacterial and antifungal effect of *A*. *polygonoides* leaves extract. (A) Antibacterial effect against Gram positive microorganisms, *Staphylococcus aureus*, *S*. *epidermidis*, *Micrococcus luteus*, *Bacillus cereus* and *Bacillus subtilis*; (B) antibacterial effect against Gram negative microorganisms, *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*; (C) antifungal effect against *Aspergillus fumigatus* and *Aspergillus niger*

Evaluation of specific growth rate and specific production rate

The colonies which showed pink zone on the medium containing *A. polygonoides* leaves extract powder were isolated and subjected for further analyses. The time profile of cell growth and L-asparaginase production was analyzed using modified Gompertz Equations 3 and 4, respectively with the effect of *A*. *polygonoides* leaves extract as nitrogen source. The experimental data obtained were fitted through Matlab 7.0 and represented in Figure 3. Though both the fermentation media -with and without leaves extract- showed maximum growth and L-asparaginase production at the same period of time, *S*. *venezuelae* showed higher growth (Figure 3A) and enzyme activity (Figure 3B) only in the presence of the leaves extract. The specific growth rate and specific production rate obtained for both these conditions are given in Table 2. It was inferred from these results that the L-asparaginase production is growth-associated as reported by Shah et al. (2010). Moreover, it was also found that the amino acid, L-asparagine in *A. polygonoides* leaves extract act as a nitrogen source for the increased of cell concentration and enzyme production.

Effect of *A. polygonoides* leaves extract concentration

The growth and L-asparaginase production kinetics of *S. venezuelae* was analyzed using Monod model and this

Table 1. Antimicrobial activity of *Amaranthus polygonoides*.

S/N	Microorganism	Zone of inhibition (mm)			
		Standard	50 µL	100 µL	150 µL
1	*Staphylococcus aureus*	30	15	17	20
2	*Staphylococcus epidermidis*	31	17	22	25
3	*Micrococcus luteus*	31	16	24	30
4	*Bacillus cereus*	33	16	24	30
5	*Bacillus subtilis*	29	14	18	20
6	*Escherichia coli*	30	13	18	22
7	*Pseudomonas aeruginosa*	32	15	21	24
8	*Klebsiella pneumonia*	30	14	16	19
9	*Aspergillus niger*	32	14	19	22
10	*Aspergillus fumigatus*	31	16	21	24

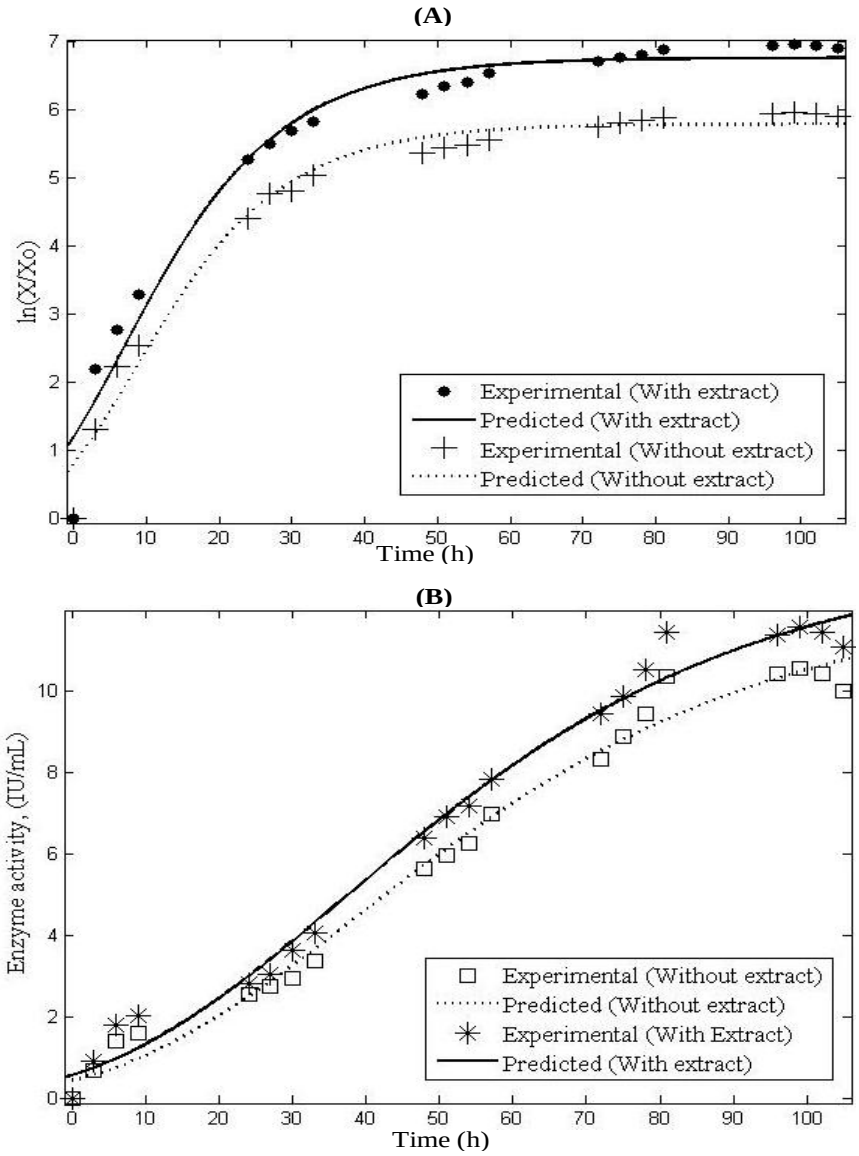

Figure 3. Growth kinetic model of *Streptomyces venezuelae* with the effect of *A. polygonoides* leaves extract.

Table 2. Bio-kinetic parameters evaluated for Monod model.

Variable	R^2	K_s , (mg/ml)	μ_m / P_m (h^{-1})
Specific growth rate, (μ)	0.9684	6.008	0.7902
Specific L-asparaginase production rate, (P)	0.9708	5.516	0.8683

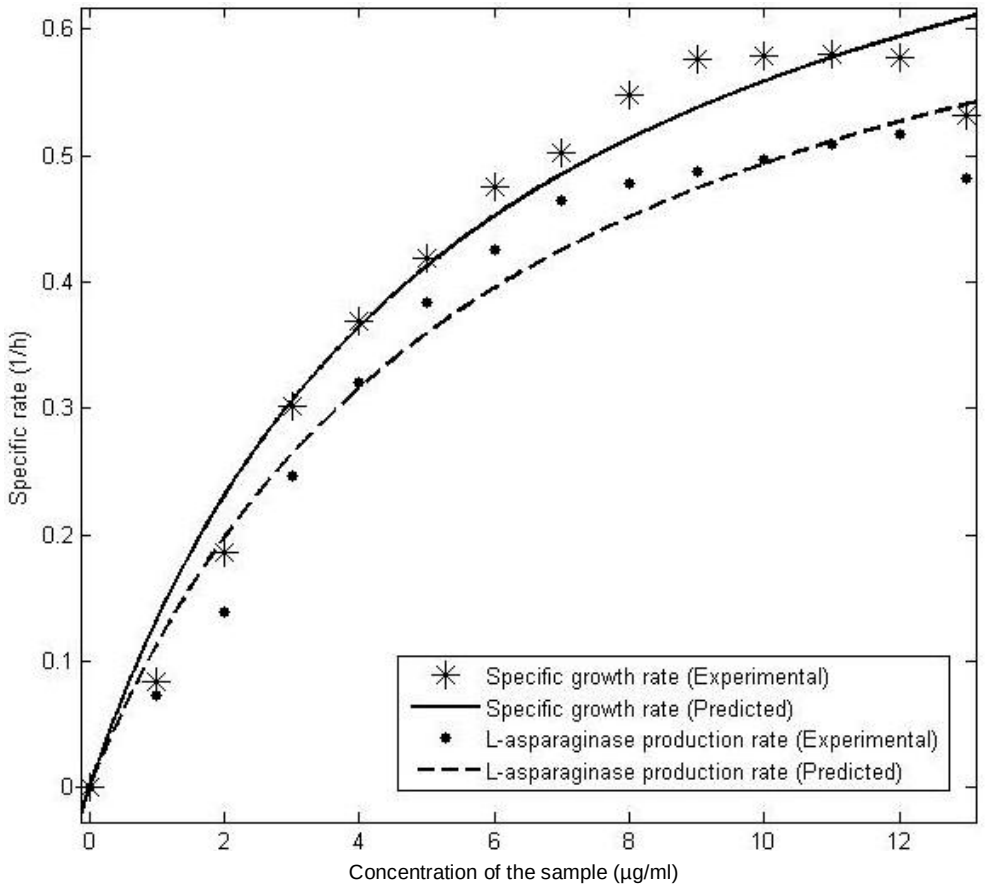

Figure 4. Effect of *A*. *polygonoide* concentration specific growth rate and L-asparaginase production rate.

was performed by analyzing the cell growth and L-asparaginase activity at different substrate concentration as shown in Figure 4. And the kinetic parameters obtained for this model are given in Table 2. The specific growth rate and the L-asparaginase production rate were found to increase initially and their maximum values were obtained at 12 mg/ml of *A. polygonoides* leaves extract, but remained constant beyond this level. The decrease in biomass at higher substrate concentrations was due to the osmotic effects. The increase in substrate concentration may have increased the osmotic pressure and thus affected the cell growth by either removing water from the cell or preventing the diffusion of water into the cells (Ghaly and Taweel, 1995). Moreover, it has also been reported that the rate of fermentation and substrate utilization decreases above critical substrate concentration. This is due to the combined effect of reduced water activity and plasmolysis (Roukas et al., 1991).

Conclusion

In the present investigation, the antioxidant activity of *A*. *polygonoides* was found to be higher than that of Vitamin C and the plant extract was proved to be active against pathogens such as *S. aureus*, *S. epidermidis*, *M. luteus*, *B. cereus*, *B*. *subtilis*, *E. coli*, *P. aeruginosa*, *K. pneumonia*, *A. niger* and *A. fumigatus.* The plant leaves extract is known to possess some antibacterial and antifungal effect that can inhibit the contamination of pathogens during bioprocess of L-asparaginase, and the enzyme produced from this may also contain some of

these effects against the pathogens which are suitable for human consumption. Moreover, as the concentration of the plant leaves extract increased, the specific growth rate increased thereby increasing the L-asparaginase production rate.

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