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# Production of *Serratia marcescens* L-aspargenase and anticancer activity in vitro

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The study was aimed towards the production of L-asparaginase from spiny cactus peel waste extract (SCPWE) by submerged cultures of *Serratia marcescens*. *S. marcescens* grew well for 120h in SCPWE containing medium, revealed the presence of sufficient amount of carbohydrate source for growing *S. marcescens* and production of L-asparaginase.. Different parameters,such as pH, temperature and incubation period, were optimized for growth and maximum L-asparaginase production. The optimum culture parameters were 30°C, 60h and pH 8.6. Maximum L-asparaginase and biomass were observed at the end of the logarithmic phase (6.4 IU/ml and 2.96g%, respectively). L-asparaginase activity and biomass were increased from 1.4 IU/ml and 0.56g% to 6.4 IU/ml and 2.96 g% respectively at the end of logarithmic phase (60h). Higher yields of L-asparaginase (6.4IU/ml) with a specific activity of 1984IU/ml (89 fold purification with 39 % recovery) were obtained from *S. marcescens* cultures. The purified L-asparaginase was used for the characterization and general properties were used such as effect of pH and temperature as well as stability at pH and temperature on L-asparaginase activity. The optimum pH 8.6 and 50ºC temperature on L-asparaginase showed 100% residual activity. Stability of pH around 8.6 and temperature 70ºC showed 90 and 78 % residual activity at 30 and 60 min respectively. The L-asparaginase showed high stability at alkaline pH (pH 8.6) when incubated for up to 60h.The molecular weight of the produced L-asparaginase was close to 160 kDa. Km and *V*max of the purified L-asparaginase were found to be 6.72mM and 0.16 µM, respectively. Cytotoxic activity of L-asparaginase was examined in vitro using four carcinoma cell lines. Aspargenase has higher effective in growth inhibition against HEPG2 and HCT-116 but lower against HELLA and MCF7 carcinoma cell lines. The data show that aspargenase has a higher cytotoxic activity against HEPG2 and HCT116, revealed higher percentage of cell death, indicating antitumor properties, and demonstrate direct effect on cancer cell proliferation of HEPG2 and HCT116. Therefore, SCPWE was considered to be a suitable carbohydrate source for growing *S. marcescens* and production of L-asparaginase has higher activity and good stability. Purified L-asparaginase obtained from *S. marcescens* could be employed in drug chemotherapy and treatment of cancer.

**Key words:** *S.marcescens*,L-asparaginase, Stability, Cytotoxicity, Agricultural waste.

## 1. INTRODUCTION

Enzymes are extensively used in the manufacturing of food synthesis and analysis of chemicals and become one of the most important branches of different industries (Moharib, 2003 and Moharib and Gad, 2010). Most important enzymes used for industrial purposes and are widely employed in food industry, pharmaceutical industries (Claudi et al. 2001, Eman et al., 2012 and 2018). The need for new enzymes is of great interest in both biotechnology and medicine. L-asparaginase is relatively wide spread enzyme found in many plant tissue, bacteria and fungi. Different microorganisms were used and proved for the production of L-asparaginase enzyme (Subha et al., 1995 and Anuradha et al., 2004). L-asparaginase amidohydrolase enzyme (EC 3.5.1.1) is produced and available by different microbial sources including fungi (Serquis et al. 2004 and Lee et al. 2005), yeast (Kil, et al. 1995 and Ferrara et al.2006) and bacteria (Patil and Sawanth 2007). L-asparaginase an enzyme catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonia, and L-glutamine to L-glutamate (Cedar and Schwartz, 1968 and Malvin et al. 1976). Recently, asparaginase is an enzyme discovery of its ability to inhibit growth of tumors in mouse, rat, and dog (Siddalingeshwara and Kattimani 2010) as well as suppress human leukemias (Umesh et al. 2007). L- asparaginase used as chemotherapeutic agent for a treatment of

human cancer and acts as a catalyst in the breakdown of asparagine, as nutritional requirement for both normal and cancer cells, to aspartic acid and ammonia and effect on nonessential amino acid in vitro and in vivo (Nakamura et al. 1999 and Verma et al. 2007). Microbial L-asparaginase is now known to be a potent antineoplastic agent in animals and has given complete remission in some human leukemia's (Vrooman et al. 2010) and was identified as an effective antitumor agent in human clinical trials, and today it is regarded as one of the useful components of the antitumor therapy. Scientists began systematically examining natural organisms as a source of useful novel drugs and anti-cancer agents (Cragg and Newman 2005). Sehgal (2003) and Vrooman et al. (2010) discovered the natural products were more toxic to cancer cells than normal cells in screening and developed chemotherapy drugs (Cozzi et al.2004, Ando et al 2005, Mishra, 2006 and Raetz and Salzer, 2010). Furthermore, asparaginase has activity in ovarian and prostate cancer cells (Sircar et al, 2012) and protects pancreatic cancer cells from apoptosis induced by glucose deprivation and cisplatin (Cui et al, 2007). L-asparaginase was used to treat hepatocellular cell lines cells with high or low levels in vitro and in vivo to examine the therapeutic efficacy (El-Serag 2011 and Zhang et al. 2013). However, asparaginase from *Escherichia coli* and *Erwinia* sp.and *Serratia marcescens* have been used as anti-tumors agent (Avramis et al. 2009, Vrooman et al.2010 and Siddalingeshwara and Lingappam 2011). It has also been used for treatment of cancer and in other clinical experiments relating to tumor therapy in combination with chemotherapy (Aguayo et al. 1999 and Kucuk 2002). Many enzymes have been used as drugs like wise L-asparaginase attracted much attention because of its use as effective therapeutic agent against different kinds of cancer in man (Ebeid et al. 2008 and Vrooman et al. 2010). Cancer is a leading cause of death worldwide, therefore is a necessity for search of new natural source and compounds with cytotoxic activity as the treatment of cancer. Anticancer drugs available is often unsatisfactory due to their cytotoxicity problem to the normal cells. Recent discoveries have indicated that the L-asparaginase from *Erwinia carotovora* might be more efficient and also to exhibit fewer side effects (Vrooman et al.2010 and Avramis et al. 2009). The need for new therapeutic enzymes is of great interest in both biotechnology and medicine for the treatment of cancer cells. The present study was carried out for production of purified *Serratia marcescens* L- asparaginase. Anti cancer activity of the produced L- asparaginase was done using HCT-116 (colon), HEPG2 (liver), MCF7 (breast) and cervical (HELLA) human cancer cells in vitro.

## 2. MATERIALS AND METHODS

### 2.1. Organism

The strain used in the present study was *Serratia marcescens* obtained from Microbiological Resources Centre (MIRCEN), Ain Shams University,Cairo, Egypt. The stock culture was maintained on agar slants that contained Dextrose, D (1.0%), peptone (1.0%), casein peptone (0.2%), yeast extract (0.2%), NaCl (0.6%), and agar (1.5%). The culture was incubated at 37°C and stored at 4°C.

### 2.2. Materials

1-Samples of spiny cactus peel wastes (agriculture wastes) were collected and obtained from the Egyptian local market, Cairo, Egypt. Spiny cactus peel wastes were cut into small pieces then they were dried in an oven at 60ºC till constant weight. Finally, the dried materials were ground in a food grinder to a very fine powder, sifted through a 16 mesh sieve, packed in bags, and stored at room temperature till used.
2-D-glucose, D-galactose, D-mannose, Dxylose, L-arabinose, L-fucose and L-rhamnose used as standards as well as DEAE-Sepharose were purchased from Sigma Chemical Co. (USA).
3-Sephacryl S-200 and standard proteins were purchased from Pharmacia, Uppsala, Sweden.

#### 2.2.1. Preparation of spiny cactus peel extract

A known weight of spiny cactus peel waste (Total carbohydrates, 65.2 %, nitrogen, 5.8 %, moisture, 19.2 % and ash, 9.8 %). A known weight was suspended in a known volume of 0.05 N HCl in a conical flask, shaken on a rotary shaker for 2 h at 30 °C and 200 rpm, then autoclaved at 121±1°C for 20 min (1Moharib and Gad 2010). The spiny cactus peel suspension was filtered and the filtrate was designated as the spiny cactus peel waste extract (SCPWE). Chromatographic analysis of SCPWE (Wilson1959), indicated that the major components were sucrose > glucose > fructose > arabinose > mannose. Sucrose content was determined according to the method described by Santos et al. (2005). The monosaccharide composition of SCPWE was also determined (Wilson1959). The SCPWE was used as a cheap source of carbohydrate for growing *S. marcescens*.

### 2.2.2. Inoculum

Growth from an agar slant was transferred to the sterile liquid media using 250-ml Erlenmeyer flask, containing 50ml cultures and was incubated (18-20h) at 29±1°C and used as inoculums.

### 2.3. Fermentation procedure

Fermentation was accomplished in 250-ml Erlenmeyer flasks, each containing 100 ml of the modified Czapek Dox liquid medium (g/L) : Lasparagine, 10.0; $K_2HPO_4$,1.52; KCl,0.52; $MgSO_4.7H_2O$,0.52; $CuNO_3.3H_2O$,0.003; $ZnSO_4.7H_2O$, 0.005 and $FeSO_4$. $7H_2O$, 0.003, (Saxena and Sinha,1981 and Lapmak et al. 2010 ) and 100 ml of spiny cactus peel extract as carbohydrate source (Moharib and Gad 2010). The pH values (pH 7.2) of all fermentation media were adjusted before sterilization. A 24-36h liquid culture was used as inoculums (1:10, v/v) and added aseptically. Erlenmeyer flasks (250ml) each contain 100 ml of the modified Czapek Dox liquid medium were inoculated with *S. marcescens* (1:10 v/v). The flasks were incubated at 30ºC for 120h in a controlled incubator shaker at 150rpm. The *S. marcescens* cells were harvested at different intervals (12h) of growth over the fermentation period (120h) by centrifugation at 15000 rpm for 10 min (Sigma 2K15 cooling centrifuge) and the enzyme activities were estimated in the culture cell free supernatant.

### 2.4. Analytical procedures

Protein concentration was estimated according to the methods of Bradford (1976). Total carbohydrates were estimated using phenol-sulphuric acid test (Dubois et al. 1956). Total lipid was removed and estimated according to the method described by Pantis et al. (1987). Ashe was quantified gravimetrically after incineration in a muffle oven at 550°C. The pH values were estimated using a two-point automatic pH-meter (Pharmacia Co.).

### 2.5. Purification and characterization of L-asparaginase

At the end of each interval of the fermentation period, asparaginase produced by *S. marcescens* was separated by filtration, centrifugation and precipitated from three liters (3000ml) of filtered broth by addition of solid (NH4)2 SO4 at 0-80% saturation and kept overnight at 4ºC. The solutions were allowed to stand for 3h at room temperature and centrifuged at 15,000 x g for 20 min. L-aspargenase produced by *S. marcescens* was purified and used for characterization studies according to the methods of Palmieri et al. (2001). The precipitates were re-suspended in 25mM phosphate buffer (pH 8.6) and extensively dialyzed against the same buffer (pH 8.6) for 48h. The dialyzed samples were centrifuged using Sigma 2K15cooling centrifuge. The supernatants were collected and used for enzyme characterization studies.

### 2.5.1. Anion exchange chromatography

The (NH4)2 SO4 precipitated samples were concentrated and applied to a DEAE-Sepharose column (1.6x15 cm) previously equilibrated with 25mMTris-HCl buffer (pH 8.6). The (NH4)2 SO4 precipitated samples were washed with three column volumes of the same buffer, and eluted with 25mM Tris-HCl buffer (pH 8.6). The column was washed at a flow rate of 60ml/h with 150ml of the same buffer, and a 0-0.5 M NaCL linear gradient was applied. Two column volumes of eluted fractions containing high L-aspargenase activity were collected and concentrated.

### 2.5.2. Gel filtration chromatography

Gel filtration was performed according to Aworh and Nakai (1988). The concentrated eluted fractions were applied onto a Sephacryl S-200 column (1.8x85 cm) and eluted with 25mM Tris- HCl buffer (pH 8.6) containing 0.15M NaCL The eluted fractions were assayed for L-aspargenase activities as described by Dharmsthiti and Luechai (2010). The active samples were pooled and dialyzed against 20mM Tris HCL buffer (pH 8.6) containing 0.1M NaCl. After concentration, the solutions were applied onto a Sephacryl S-200 column and eluted with the same buffer as above to obtain purified products.

### 2.5.3. Determination of molecular weight

The average molecular weight of L-aspargenase isolated from *S. marcescens* culture was determined by gel filtration chromatography (Andrews 1964 and Ramble et al. 2006) using Sephacryl S-200 column (1x100). The sample was applied through a glass column packed with pre calibrated Sephacryl S-200 column (1x100) equilbrated and developed with 25mM standard buffer. Fractions of 0.5 ml were collected at a flow-rate of 0.05 ml/min. The following known molecular weight standards were used for the molecular weight determination under optimum conditions. The known

molecular weight markers were used for calibration of the column of Dextran (2,000kDa), $\beta$-amylase (200kDa), dehydrogenase (150kDa), phosphorylase (97kDa), bovine serum albumin (67kDa), ovalbumin (42kDa), pepsin (35.5kDa), carbonic anhydrase (29kDa), trypsin (24kDa), myoglobin (17.8kDa), lysozyme (14kDa) and cytochrome C (12,4kDa) as reported by Prakasham et al. (2010).

### 2.5.4. L-asparaginase assay

Assay of asparaginase activity was carried out according to the method described by Dharmsthiti and Luechai (2010). The dialyzed concentrated supernatants were used for determination of asparaginase activity. The reaction mixture contained 0.2ml of 0.05M Tris-HCl buffer, pH8.6, 1.7 ml of 0.01M asparagine prepared in the same buffer and 0.1ml of diluted enzyme. The reaction was carried out at 37ºC for 30 min. The reaction was stopped by adding 0.1ml of 1.5M trichloroacetic acid (TCA). The mixture was centrifuged to obtain clear solution. Then, 0.5ml of the clear solution was transferred to another tube containing 6.5ml of distilled water and mixed with 1.0 ml of Nessler's reagent. The mixture was left at room temperature for 10 min. The blank was run by adding enzyme after the addition of TCA. The developed color was measured at 450nm. The asparaginase activity was expressed as International Unit (IU). One unit of asparaginase activity was defined as the amount of enzyme which liberates one µmole of ammonia per min under optimal assay conditions.

#### 2.5.4.1. Effect of fermentation period on L-aspargenase activity

The fermentation process of *S. marcescens* was carried out at different time intervals (0-120h) and each aspargenase activity was assayed. Activity of L-aspargenase produced by *S. marcescens* cultures was determined at different intervals over the fermentation period (0-120 h) under optimum values of pH and temperature.

#### 2.5.4.2. Effect of pH values on L-aspargenase activity

The optimum pH of the purified asparaginase activity produced by *S. marcescens* was determined by assaying the enzyme activity at different pH values (3-11) as described by Siddalingeshwara and Lingappa (2011).

#### 2.5.4.3. Effect of temperature on L-aspargenase activity

The optimum incubation temperature of the purified aspargenase activity produced by *S. marcescens* was determined by assaying the enzyme activity at different temperatures (10-80°C).

#### 2.5.4.4. Effect of physiological parameters on L-aspargenase stability

The pH stability of L-asparaginase was determined by pre-incubating the enzyme for 30 and 60 min for 45ºC in buffers of various pH values. The residual activity was measured as described by Gaffar (2005). The stability of enzyme to temperature was determined (Gaffar2005). The reaction mixture (without substrate) containing enzyme and buffer was pre-incubated for 30 and 60 min with different temperatures ranging 30-75ºC and cooled. The residual activity was measured (Gaffar 2005).

#### 2.5.4.5. Kinetic Parameters.

The values of Michaelis constants (Km) and maximum velocity (Vmax) were determined using L-asparagine as substrate. A Lineweaver-Burk plot was used to detect the dependence of L-asparaginase activity on the L-asparagine concentration (Ramble et al. 2006).

### 2.5.5. Cytotoxicity in vitro studies

The purified aspargenase obtained was sterile and sent to Bioassay Laboratory, Cancer Institute, Cairo, University, Egypt. Cytotoxicity test of the purified aspargenase was done in vitro using four different human carcinoma cell lines particularly those of colon (HCT 116), liver (HEPG2), breast (MCF7) and cervical (HELLA) carcinoma cell lines. The potential cytotoxicity of L-aspargenase was measured and assayed by sulforhodamine B (SRB) according to the method described by Skehan et al. (1990).

## 3. RESULTS AND DISCUSSION

### 3.1. Production of L-asparginase

Production of L-asparginases from different microorganisms was reported in several studies. In the present study, an attempt is made to utilize the hydrolyzed extract of spiny cactus peel waste (SCPWE) as a substrate containing medium for growing *S. marcescens* for 120h and production of aspargenase as inexpensive carbohydrate source. The SCPWE have nitrogen and carbohydrate contents (3.2% and 65.4% respectively), revealed the suitable C/N ratio for growing microorganisms. The chromatographic analysis of SCPWE revealed the major components were sucrose, 18.4% glucose 8.8% fructose, 7.6%, mannose, 3.4% and arabinose, 2.4%. Anuradha et al. (2004) and Ferrara et al. (2006) reported the carbon source such as sucrose, glucose, maltose and galactose was stimulated L-asparaginase production. SCPWE was considered as suitable carbohydrate source for growing *S. marcescens*. Fermentation was made in which the concentration of SCPWE containing medium was 10%. The SCPWE containing medium was observed to be suitable for growing *S. marcescens* using submerged culture and production of L-aspargenase and other useful products. *S. marcescens* showed maximum biomass and L-asparaginase enzyme production (2.96g% and 6.4U respectively). Different strains of *S. marcescens* previous examined synthesized L-asparaginase (Malvin et al. 1976). The differences in L-asparaginase activity may be attributed to the presence of SCPWE, C: N ratio (1:20), and degradability of SCPWE (containing different carbohydrates) as a carbon source (Bessoumy et al. 2004 and Moharib and Gad 2010). The present results suggested that L-asparaginase production was regulated by carbon and nitrogen ratio as investigated by Sarquis et al. (2004). Higher yield of L-asparginases in the present results can be attributed to the presence of SCPWE as carbon source in the culture medium stimulated L-asparaginase production. Production of L-asparaginase starts from 10-12h reaching maximum at 60h of fermentation period and then decline (Fig. 1).

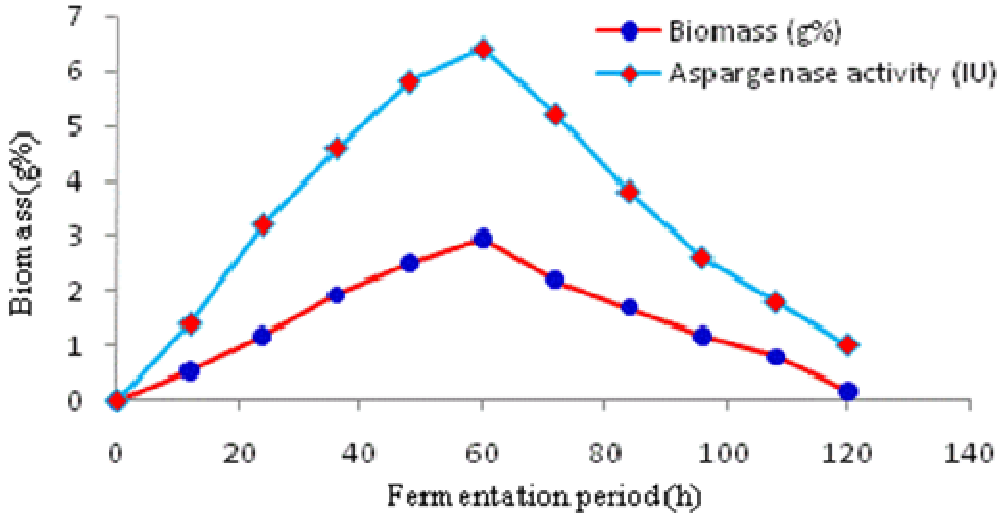



Figure (1) : Effect of fermention period on S. marcscens biomass and aspargenase activity.

The fermentation process indicated that the L-asparaginase production was increase with increase the fermentation period at optimum pH and temperature (Fig.1). *S. marcescen*s was synthesized L-asparaginase with yields ranging from 2.2 to 6.4 U/ml (Fig. 1). L-asparaginase synthesis was start after 10h of cultivation and its velocity was higher at48h and the highest level of accumulation was attained at 60h of cultivation (6.4U/ml). The present results are in accordance with results obtained previously by several workers (Krasotkina et al. 2004**,** Kotzia and Labrou 2007 and Prakasham et al. 2010) illustrated the extra-cellular L-asparaginase production during the growth of different bacteria. However, the rate of aspargenase synthesis is directly correlated to cell growth rate. Thus, *S. marcescens* can utilize SCPWE efficiently as carbon source for production of high yields of L-aspargenase activity. In present study, L-asparaginase activity was 6.4 IU/ml by *S. marcescens* which is higher as compared to previously produced by *S. marcescens* sp. (Malvin et al. 1976). On contrast, highest enzyme activity was produced by *Staphylococcus* sp and *Bacillus circulans* (Prakasham et al. 2007 and Prakasham et al. 2010 respectively) as will as *Leuconostoc mesenteroides* grown on medium containing different carbon and nitrogen sources (Moharib and Gad 2010). Consider the substrate containing medium, *S. marcescen*s showed maximum enzyme production using SCPWE (2.96 g %) at pH 8.6 (Baysal et al. 2003).

The fermentation study was indicated that the L-asparaginase production was maximum (6.4 IU) at 60h of fermentation period and then decreased till 120h (Fig. 1). Similar results were obtained by other investigators (Kil et al.,1995 and Mukherjee et al., 2000).The present results are in the range with those reported by Sarquis et al. (2004) who reported highest L asparaginase activity of *A. terreus* in liquid medium at 48h (Mishra 2006 and Lee et al. 2005). The production of enzyme appeared growth dependent and a 60h of fermentation time supported maximal yield (6.4IU) under optimum at pH 8.6 and at 30°C (Fig.1). Sutthinan et al. (2009) and Siddalingeshwara and Lingappa (2011) reported that the maximum L-asparaginase production was observed at pH 8.6 and temperature 30°C at 72h of fermentation period.

### 3.2. Purification and characterization of L-asparaginase

The present research was carried out to produce L-asparaginase enzyme using SCPWE for growing *S. marcescens*. The purification of crude L-asparaginase of the fermented *S. marcescens* cultures was carried out by ammonium sulfate precipitation (0-80%) and showed more than 80% of the enzyme activity was remained (Table 1). The enzyme resulting from 0-80% $(NH_4)_2$ $SO_4$ precipitation of *S. marcescens* cultures was applied to a DEAE-Sepharose column and the L-asparaginase were collected in the 0.15M NaCL eluted fractions. The active fractions were eluted and concentrated and then applied to a Sephacryl S-200 gel filtration column from which the activity eluted fraction was 25-50 respectively.

Table (1): Partial purification steps of L-asparaginase produced by *S. marcescens* grown on SCPWE containing media.

| Ingredients | Total volume | Total protein (mg ) | Total activity units (IU/ml) | Specific activity (IU/mg) | Purification fold | Recovery % |
|---|---|---|---|---|---|---|
| **Cultures broth** | 3000 | 108000 | 2401920 | 22.24 | 0 | 100 |
| **(NH3)4SO4 precpitation** | 1800 | 22244 | 1921920 | 86.4 | 4 | 80.02 |
| **Sepharose CL6B** | 630 | 2616 | 1345344 | 514.2 | 23 | 56 |
| **Sephacryl S-200** | 160 | 475 | 941740.8 | 1984 | 89.2 | 39.2 |

Mean of three samples

The results of purification steps are summarized in Table (1). The specific activity of L-asparaginase was increased to 514.2 and 1984 U/mg after the Sepharose CL6B and Sephacryl S-200 gel filtration column chromatography respectively. Chromatography on DEAE-Sepharose elution fractions have higher L-asparaginase activity were concentrated and applied onto a Sephacryl S-200 column to be pure (Dharmsthiti and Luechai 2010). One active peak was resulted in *S. marcescens* at pH 8.6 (Fig. 2), with L-asparaginase activity (514.2 IU/mg) and 23 purification folds.

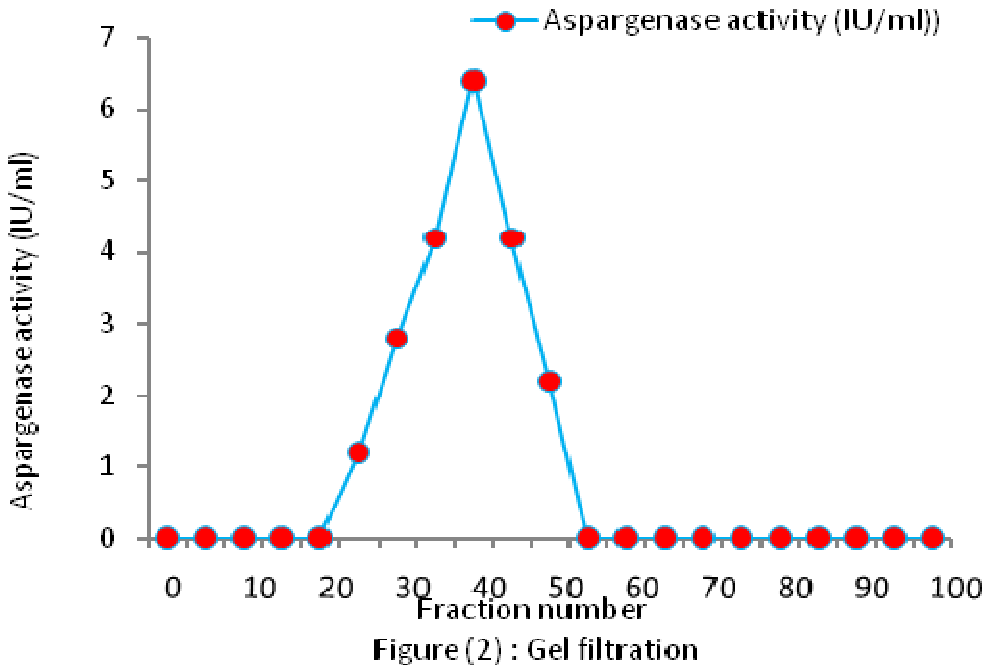



Figure (2) : Gel filtration

The yield was 56% (1465mg) of total proteins (2616mg) and 514.2 units (753393IU) of the total activity units (1345344IU) as shown in Table (1). This indicates that these enzymes were recovered with the yields 56% from the previous step. The peak has higher levels of total protein and total activity units (2616mg and 1345344IU respectively). These results are higher than those reported by other investigators (Cedar and Schwartz, 1968, Kozak and Jurgab, 2002 and Sarquis et al. 2004).For further purification, it is reasonable to use Sephacryl S-200 column chromatography. Results shows, that *S. marcescens* gave one active L-asparaginase have higher specific activities (1984IU/mg), accounting 39% of their activity units (941740.8).The present results are in accordance to those reported by Kamble et al. (2006). Ramble et al. (2006) indicates that the purification steps were suitable to obtain purified products as the present results. From these results, it can be observed that the *S. marcescens* L-asparaginase have higher levels in total proteins, total activity units as well as specific activities over the purification steps (Table 1). The molecular weight of the *S. marcescens* asparaginase enzymes was estimated by comparing their elution volume from a column of a Sephacryl S-200 (1.8x85cm) with those of a series of marker protein with known molecular weights : dehydrogenase (150kDa), phosphorylase (97kDa), bovine serum albumin (67 kDa), ovalbumin (42 kDa), carbonic anhydrase (31 kDa), trypsin (24 kDa) and myoglobin (17.8kDa).The apparent molecular weight of L-aspargenase produced by *S. marcescens* in the present study was 160kDa. This finding is in agreement with molecular weights of bacterial L-asparaginases (Mesas et al., 1990 and Manna et al. 1995), reported that some species of bacteria were produced L-asparaginase with variable molecular weights ranged from 140 to 160 kDa. The biochemical properties of L-asparaginase determined in the present study tend to supplement the hypothesis reported previously by other investigators (Kozak and Jurga, 2002, Anuradha et al. 2004 and Prakasham et al. 2010), who indicated that some L-asparaginases have similar molecular weights (140-160kDa) and the optimum pH was 8.5. Malvin et al. (1976) found average molecular weight of *S. marcescens* ATCC 60 was 171-180kDa. The present results are in the range with those reported by other investigators (Heler 1979, Anuradha et al. 2004). Low molecular weight (80kDa) was obtained from *Corynebateriun glutamicum* (Mesas et al., 1990). Similar results were obtained from plant origin L-asparaginase (Sieciechowicz and Ireland, 1989, Ali, 2009 and Makky et al. 2014). Low molecular weight was detected for L-asparaginase from plant origin (Shanmugaprakash et al. 2015). A very low molecular weight (11.2 kDa) was detected for *Streptobacillus* L-asparaginase (Makky et al. 2014). Other bacterial species produced L-asparaginase with variable molecular weights (Rozalska, 1989). Hence, it can be concluded that *S. marcescens* was consider strong potential for production of L-asparaginase using SCPWE containing medium. Results in Fig. (3) showed Michaelis constants (Km) of 7.02 mM

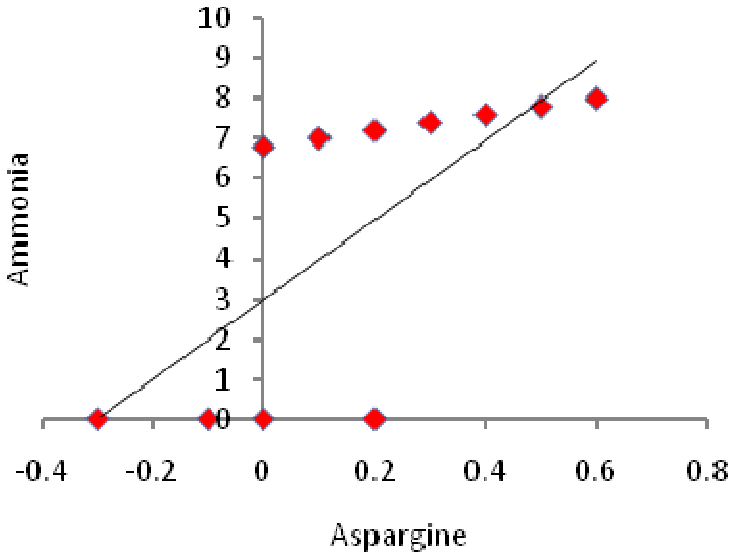



Figure (3) : Km and Vmax of aspargenase enzyme from S.marcscens.A Lineweaver-Burk plot was used to detect the dependence of aspargenase activity on asparginе concentration.

asparagine and a maximum velocity (Vmax) value of 0.2 $\mu$M ammonia/ml (Fig.3). These results indicated high affinity of the enzyme to substrate. However, L-Asparaginase of different microorganisms has different affinities and may be have different physiological effects in the enzyme activity (Mannan et al. 1995 and Kamble et al. 2006). The obtained results of Km value are higher than those reported for L-asparaginase (2.5 and 3.5mM) from *C. glueamicum* and *E. coli* (Sobis and Mikucki,. 1991 and Kozak and Jurga, 2002 respectively). On the other hand, a lower Km values were obtained for L-

asparaginase by other workers (Willis and Woolfolk, 1974 and Qian et al. 1996). A Lineweaver-Burk plot was used to detect the dependence of L-asparaginase activity on the L-asparagine concentration (Ramble et al. 2006).

### 3.3. Effect of incubation time on L-asparaginase activity

L-asparaginase activity was studied in the ranges of zero to 60 min (Fig.4). L-asparaginase activity was increased as the

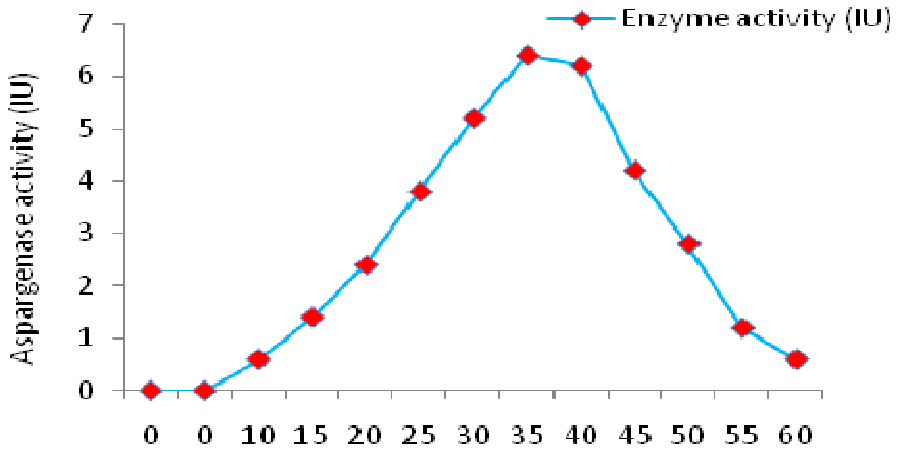



Figure (4) : Effect of incubation time on aspargenase activity of S. marcescens.

incubation time increased. Incubation of L-asparaginase at 37°C for different times showed that the activity was reached its maximum at 30 min and then decreased as the time was increased. These results are in agreement with the results obtained by other investigators (Manna et al., 1995, Gaffar, 2005, Avramis et al. 2009 and Vrooman et al. 2010).

### 3.4. Effect of temperature on L-aspargenase activity

Results obtained (Fig. 5) indicate that optimum temperature for L-asparaginase activity was between 35°C and 40°C.

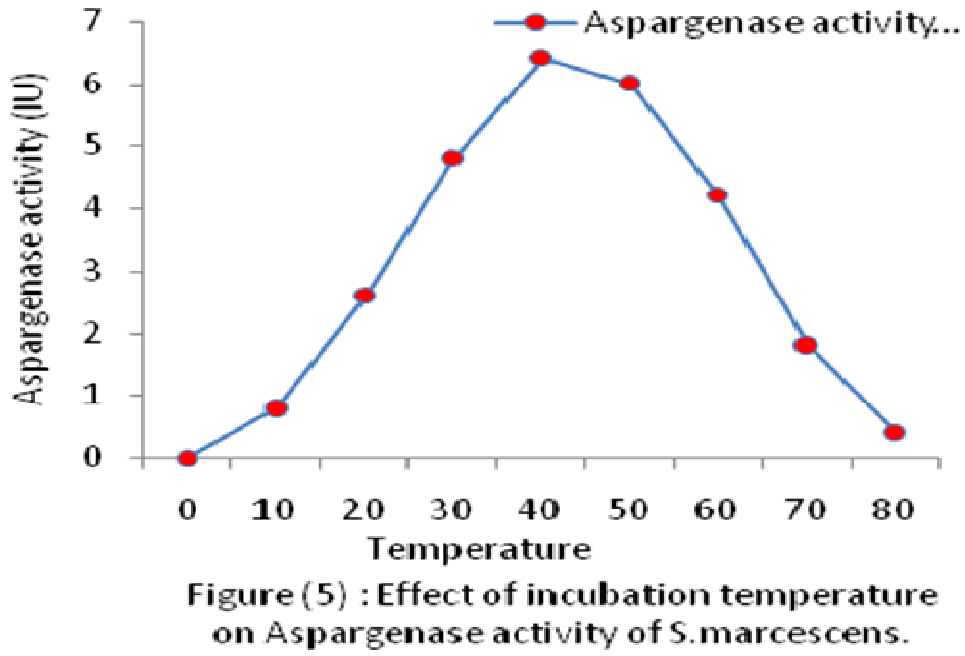



Figure (5) : Effect of incubation temperature on Aspargenase activity of S.marcescens.

Maximum activity was obtained at 37ºC. Mannan et al. (1995) reported the optimum temperature for L-asparaginase activity was 37°C**.** The reaction rate of L-asparaginase activity of *S. marcescens* was measured at various temperatures, concluding that the enzyme activity was stable at wide temperature range (10ºC to 70ºC). Results obtained (Fig. 5), showed their activities were increases gradually as temperature increase, till reach 40°C then decreased markedly. These results are consistent with those reported by other investigators (Maladkar et al., 1993 and Manna et al., 1995) found that the activity of different bacterial L-asparaginase are stable at 45°C-50°C. Qian et al., (1996) proved that *E. coli* L-asparaginase lost its activity more rapidly at higher temperatures. L-asparaginase from *chrombacteriaceae* had maximum activity at 20°C (Roberts et al. 1972).

### 3.5. Effect of pH on L-aspargenase activity

Effect of pH on the activity of L-asparaginase produced from *S. marcescens* was studied in the range of ph values (3-11) under assay conditions (Fig. 6). Results showed the obtained purified L-asparaginase was active at different pH values. Enzyme activity increased with increase in pH value reaches optimum at pH 8.6 then decreased as shown in Fig. (6). Maximum L-asparaginase activity was observed at pH 8.6. Similar results were obtained by Shanmugaprakash et al. (2015). A similar pH value was obtained by several investigators (Heler, 1979, Anuradha et al. 2004 and Ramble et al. 2006) and many other microbial L-asparaginase activities (Balcao et al., 2001 and Patil and Sawanth, 2007). Similar observation reported by other investigators (Ali. 2009 and Khalaf et al. 2012), indicated that many L-asparaginase from

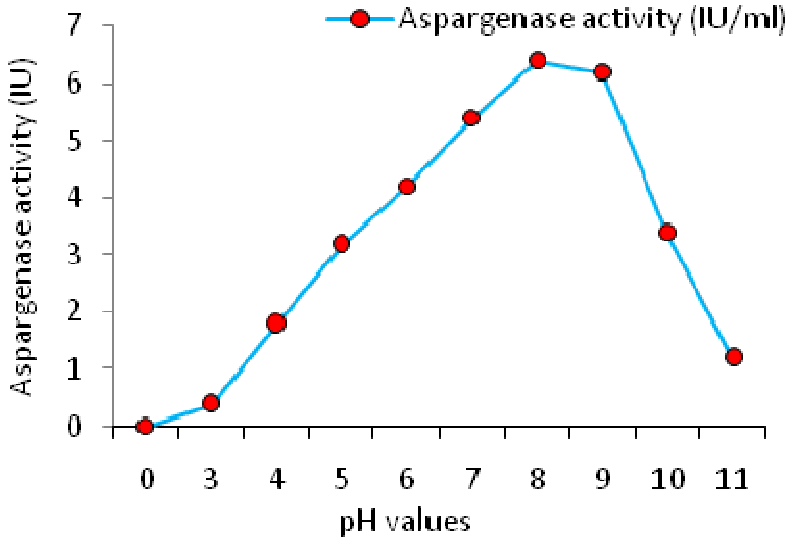



Figure (6) : Effect of pH on aspargenase activity of *S.marcescens.*

plant origin had higher activities at pH ranged from 8.0 to 8.5. Mesas et al. (1990) have found the optimal L-asparaginase activity at pH 7.0. The enzyme activity was slightly lowered at pH values of 7.0 or 8.0. Similarly, the pH 9.2 is optimum was reported by Pritsa and Kyriakidis **(**2001), Gaffar, (2005) and Siddalingeshwara and Lingappam (2011). Moreover, alkaline pH (8.0–10) showed optimum pH for most bacterial L-asparaginases activity (Kamble et al. 2006 and Makky et al. 2014). The present study revealed that the selected parameters examined, showed a considerable impact on L-asparaginase production by *S. marcescens* its greater activity at pH and temperature also its stability over a wide range of pH and temperature along with anticancer activity (Siddalingeshwara and Lingappam 2011).

### 3.6. Effect of physiological parameters on L-asparaginase stability

Stability of enzyme at different temperature and pH ranges making it suitable for wide pharmaceutical applications. The activity of L-asparaginase was evaluated at different values of pH and temperature under assay conditions and the amount of ammonia liberated was determined. However, L-asparaginase activity was increased with increase in pH and thereafter it decreased. Fig. (6) shows *S. marcescens* L-asparaginase have its maximum activities at pH 8.6 and the gradually losses activities was observed before and after pH value (8.6). It is commonly observed that optimum pH value of the obtained L-asparaginase was in alkaline range and have maximum activities only at pH 8.6. The enzyme activity gradually increased until pH 9, at which time the maximum activity was observed. The enzyme was maximally stable at

pH range from 8.0 to 9.0. At higher pH, the enzyme activity was decreased (Fig.6).The enzyme was stable at alkaline pH 8.6 and nearly remains 100% activity after incubation for 30 and 60 min at 37ºC (Fig. 7). The enzyme appeared pH stable, because no appreciable loss in activity was observed over a wide range of pH under refrigerated condition. The enzyme remains 55% and 40% activity at pH 11 for 30 and 60 min respectively. The L-aspargenase enzyme obtained

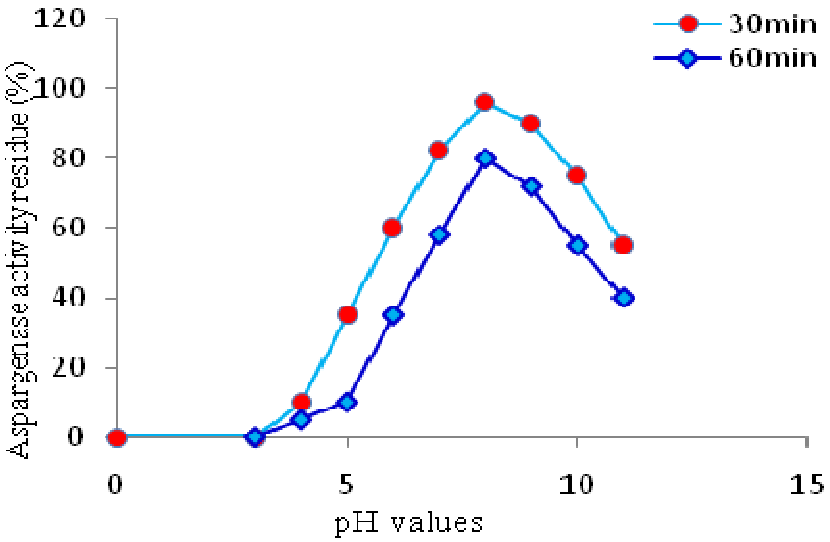



Figure (7) : Effect of pH values on S. marcescens aspargenase stability.

from *S. marcescens* was more stable at alkaline pH than at the acidic one. These finding are consistent with those reported by several investigators (Kil et al. 1995, Kamble et al. 2006 and Makky et al. 2014) found the enzyme activity obtained was more stable at alkaline pH than at the acidic one. The effect of temperature on enzyme stability are present in Fig. (8). Results indicated that the enzyme activity was not lost when incubated at 70ºC for 30 and 60 min. The residual activity is nearly100% at this temperature. The present results showed at 80°C, 69% and 60% L-aspargenase activity was remained at 30 and 60min respectively. Pritsa and Kyriakidis (2001) and Ramble et al. (2006) found 100% activity of enzyme at 77ºC.

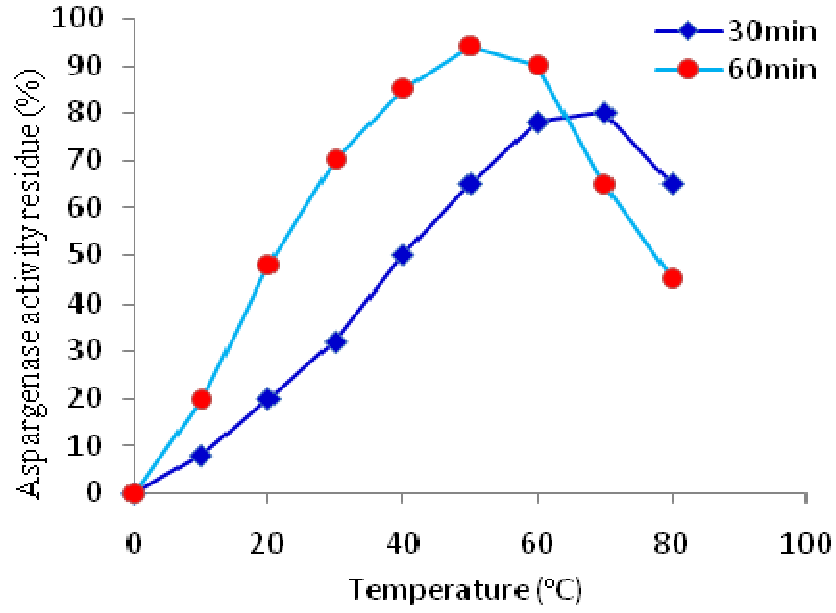
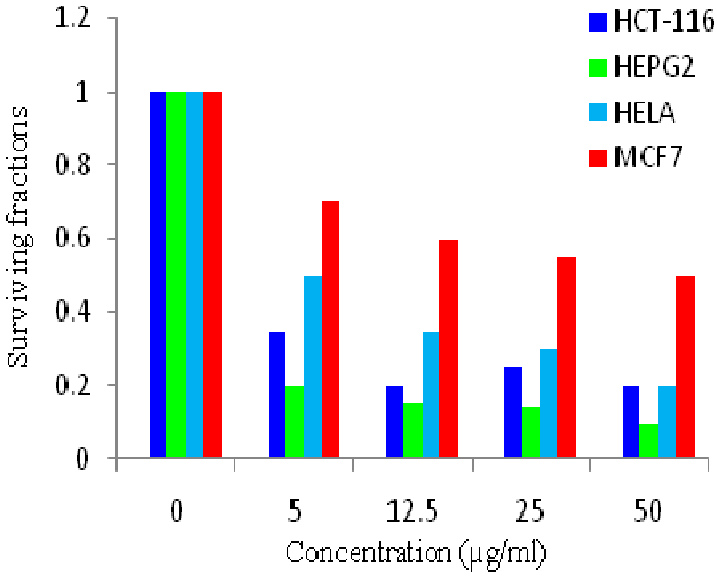



Figure (8) : Effect of temperature on S. marcescens aspargenase avtivity.

The reaction rate of L-asparaginase appeared optimal at 37°C. Loss activity was observed over this temperature, although 50% activity retained even at 60°C (Fig.6). Similar results were reported by Maladkar et al. (1993) and Manna et al. (1995) found that the activity of different bacterial L-asparaginase are stable at 45°C-60°C. Maximum activity of L-asparaginase from *S. marcescens* at near physiological pH and temperature makes this enzyme superior to that of bacterial origin as a chemotherapeutic agent in the treatment of leukemia and other diseases.

### 3.7. In vitro L-aspargenase cytotoxicity

The present study was carried out to evaluate the potential efficacy of L-aspargenase against four different carcinoma cell lines in vitro (Figs. 9, 10). The effect of L-aspargenase used in vitro cytotoxicity test was done to identify activity of the L-aspargenase in growth inhibition of four different carcinoma cell lines, colon (HCT-116), liver (HEPG2), cervical (HELA) and Brest (MCF7). Lavi et al. 2006, Shengtao et al. 2012, Abd el Monem et al. 2013 and Moharib et al. 2014) studied the cytotoxicity test of the different materials to identify their activity in growth inhibition of different carcinoma cell lines (HEPG2, HCT 116, MCF7 and HELA) in vitro. The cytotoxic activity of L-asparaginase was observed against




Fifure (9): Cytotoxic effects of aspargenase on carcinoma cell lines.

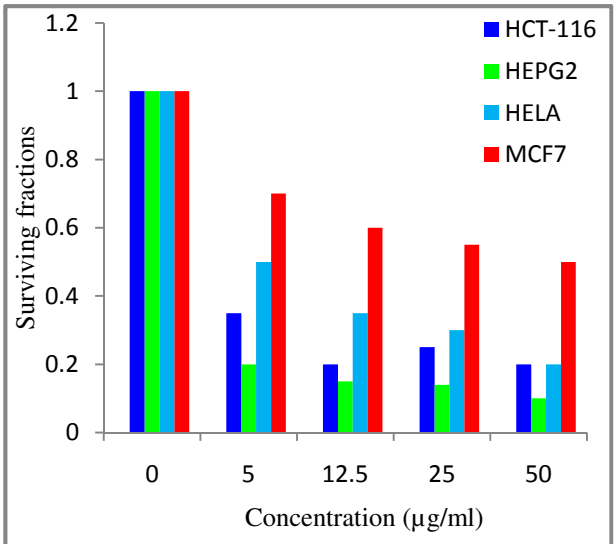



Figure (9): Cytotoxic effects of aspargenase on carcinoma cell lines.

all carcinoma cell lines in different percentages (Cozzi et al. 2004 and Itharat et al. 2004). Results showed that L-aspargenase has higher effective in inhibition against HEPG2 and HCT-116 but lower effective against HELLA and MCF7 cancer cell lines. Similar results were found using carcinoma cell lines in vitro (Prasanna et al. 2009 and Li et al. 2010). L-aspargenase exhibited more effectiveness on growth inhibition of HEPG2 but less percentage on HCT-116 cancer cell lines (Fig. 9). Results also showed that L-aspargenase was effective in inhibition of HELLA but slightly effective against MCF7 cancer cell line (Fig. 9). The effect of L-aspargenase on HEPG2 and HCT 116 cancer cell line in vitro revealed that L-aspargenase capable on inhibit cell proliferation of human HEPG2 and HCT-116. Results in Fig. (10), illustrate the dose response (IC50) of L-aspargenase on HEPG2, HCT-116, HELA and MCF7. The present results showed the growth inhibitory effect of L-aspargenase on HEPG2, HCT-116 and HELLA. The data show that L-aspargenase have a higher cytotoxic activity against HEPG2 and HCT116 than the other cell lines (Figs. 9, 10). These results indicated that L-aspargenase have more anticancer effect against HEPG2 and HCT-116. The L-aspargenase reduced the survival fraction to 50% where less than 10µg of L-aspargenase killed 50 % of cancer cells, particularly HEPG2, HCT116 and HELLA respectively (Fig. 10). Similar results were found by other workers (Abd el Monem et al. 2013 and Moharib et al. 2014). It can be observed that asparaginase inhibits cell proliferation of HEPG2 and HCT-116 (human cancer cell lines) that could arrest the cell cycle and generate apoptosis, which explain the in vitro anti-proliferative effect of asparaginase. Asparaginase reduce the survival fraction to 50%, it means that L-asparaginase kill 50% of the carcinoma cells lines (Ando et al. 2005). Anticancer activity by enzyme L-asparaginase was confirmed on HELLA cell line. It is now known that L-asparaginase produced from *S. marcescens* possesses antitumor activity. Asparaginase from *E. coli* and *Erwinia* (Avramis et al. 2009), however, has been shown to be effective as an antitumor

agent. The produced L-asparaginase exhibited insignificant loss of enzyme activity after lyophilization for two weeks at 4ºC with high specific activity. Some materials from microbial (Ando et al. 2005 and Ebeid et al. 2008) and plant origins (Shanmugaprakash et al. 2015) have L-asparaginase activities at different pH values (3.0-9.0), and used in therapeutic and drug chemotherapy (Kucuk 2002, Verma et al. 2007 and Raetz and Salzer 2010). However, around pH 8.6 could

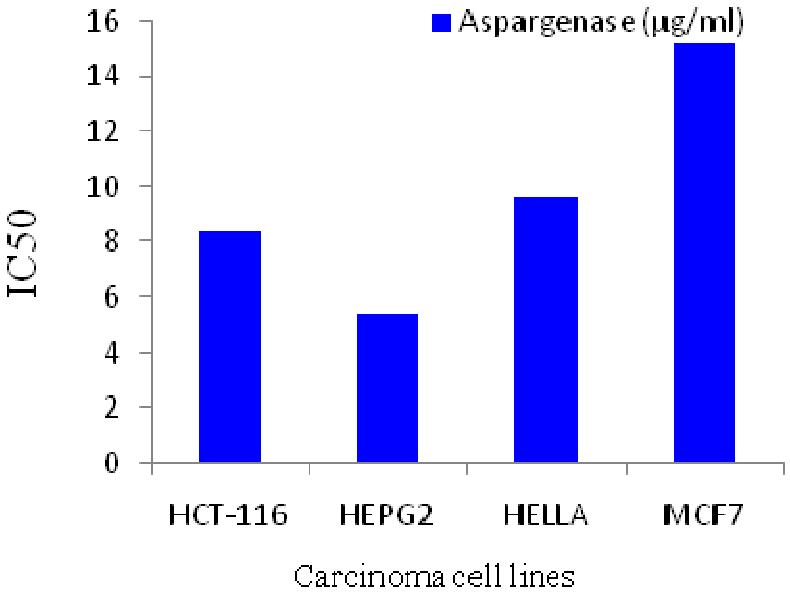



Figure (10) : IC50 of Aspargenase against carcinoma cell lines.

represents an optimum pH value, the obtained *S. marcescens* L-asparaginase could perform chemotherapy drug. These finding may be related to the biological role of microbial origin L-asparaginase. L-asparaginase produced in the present study are differ greatly than those of animal and plant origin which able to used in medicine at pH 8.6. The properties of the obtained L-aspargenase enzyme activity at the alkaline pH range and at 40°C make it suitable in chemotherapeutic treatment of different diseases.

## CONCLUSION

Spiny cactus peel waste extract (SCPWE) used as agricultural waste residues was considered to be a suitable substrate for growing *S. marcescens* and production of L-asparaginase. L-asparaginase was produced economically attractive as it is a cheap and readily available agriculture waste. Purification procedure was simple due to low number of separations, stages and yields suitable amounts of L-asparaginase with high specific activities and good stability over a wide range of physiological conditions. The obtained purified L-aspargenase has higher effective in growth inhibition and cytotoxic activity against different carcinoma cell lines. L-aspargenase showed higher percentage of cell death, indicating antitumor properties and demonstrate direct effect on colon and liver cancer cell proliferation. Finally, it can be concluded the purified L-asparaginase produced from *S. marcescens* could be employed in drug chemotherapy and inexpensive treatment of different kinds of cancer.

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