

Production of L-Asparaginase (L-ASN) from endophytic *Lasiodiplodia theobromae* hosted *Teucrium polium* in Egypt

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Abstract

Endophytic fungi produce important bioactive primary and secondary metabolites. Researchers used L-Asparaginase for the treatment of Acute Lymphoblastic leukemia (ALL). Endophytic taxon, *Lasiodiplodia theobromae* (Pat.) Griff. and Maubl. has been isolated from *Teucrium polium* plant tissues collected from Saint Katherine Protectorate and was screened for its ability to produce L-Asparaginase qualitatively and quantitatively. The crude enzyme has been partially purified using gel-filtration Sephadex G-100 with an activity of 21.00 IMmL⁻¹ min⁻¹. The results revealed that *L. theobromae* (SCUF-TP2016) represents a rich source of bioactive metabolites that can be manipulated to produce L-Asparaginase which is a desirable novel analog for chemotherapy.

Key words – Anti-leukemic, enzymes, Saint Katherine Protectorate, gel filtration.

Introduction

Endophytic fungi are group of endobionts that live asymptotically inside plant tissues for some time (Strobel and Daisy 2003). Such mutualistic relation between both of the host plant and endophytes lead to a benefits for both sides (Zhao *et al.* 2010), where endophytes would create such defense mechanism for the host plant by the production of several biologically active compounds that would used in the field of industry, medicine and agriculture (Chow and Ting 2015).

Several studies have been carried to reveal the relation between the active compounds produced by some plants and the associated endophytic fungi and it came out with a conclusion that they are very closely related, where the endophytic fungi would produce the same active compound produced by the host plant and likely it was found to be sometimes much more active than the host plant itself (Strobel and Daisy 2003; Gunatilaka 2006; Suryanarayanan *et al.* 2009; Pandey *et al.* 2014).

Acute lymphoblastic leukemia (ALL) considered one of the most common cancer diseases among children; it's characterized by the over production of immature white blood cells (Neoplastic), such cells prevent the bone marrow from the formation of normal white blood cells. It cannot function properly toward any infections. ALL would occur at any age but much more common the age of (0-14 years) representing around 60% of the infected cases. Neoplastic

cells lack L-asparagine synthetase which leads to prevention of asparagine synthesis, in the mean neoplastic cells require high amounts of L-asparagine to proliferate. Thus they depend on external sources for asparagine, preventing the neoplastic of the asparagine will lead to the starving of the neoplastic cells and die off (El-Nagga *et al.* 2014; Sanjotha and Manawadi 2017).

L-Asparaginase belongs to the amidase family (EC 3.5.1.1) which is responsible for the hydrolysis of L-asparagine into ammonia and aspartic acid (Dias *et al.* 2016), such enzyme is widely distributed among several organisms of Prokaryotic and Eukaryotic sources (Kumar and Sobha 2012; Kumar *et al.* 2013)

Due to the mode of enzyme action previously described, it would have an important role in the treatment of ALL. Different taxa of fungi have been screened for the production of L-Asparaginase as a safe alternative source for e.g. *Aspergillus*, *Penicillium* and *Fusarium* (Sarquis *et al.* 2004; Baskar and Renganathan 2009).

Unlikely, few studies carried by Egyptian researchers on endophytic fungi producing L-asparaginase in Egypt such as Moharram *et al.* (2016), continuing search for endophytic fungi hosted medicinal plants in Saint Katherine, Egypt. During the presented study *Lasiodiplodia theobromae* (SCUF-TP2016) which is recovered from *Teucrium polium* showed a remarkable potentiality for the production of extracellular L-Asparaginase by an endophytic native isolate of.

Materials and Methods

Sampling

Vegetative shoots of *Teucrium polium* L. were collected from 10 sites representing different elevation wadis (1290 meters above sea level (m.a.s.l.) up to 1800 m.a.s.l.) in Saint Katherine Protectorate, Sinai, Egypt, following the ethical rules of the protectorates. Samples were collected in sterile polyethylene bags, closed using rubber bands, and transferred to the laboratory until plating.

Isolation and identification of endophytes

The aerial parts of collected plant samples were washed in running water, cut into small pieces, immersed in 75% ethanol for 1 minute, dipped in sterile distilled water twice then 0.05 g/ml of a sodium hypochlorite solution for 3-5 minutes, and rinsed three times in sterile distilled water (Abdel-Azeem and Salem 2012). The surface-sterilized segments were cultured on potato dextrose agar medium (PDA, Difco) amended with 0.05 g/L rose Bengal and 150 mg/L chloramphenicol. Petri dishes were sealed using parafilm and incubated at 28±1°C for 7 to 21 days. Growing fungi were purified for identification.

Phenotypic identification

Phenotypic identification of endophytic fungal isolates were primarily based on the relevant identification keys for *Penicillium* (Pitt 1979), *Aspergillus* (Klich 2002), dematiaceous hyphomycetes (Ellis 1971, 1976), *Fusarium* (Leslie and Summerell 2006), miscellaneous fungi (Domsch *et al.* 2007), soil ascomycetes (Guarro *et al.* 2012), and *Alternaria* (Simmons 2007). The names of fungal taxa have been shortened in accordance to Kirk and Ansell (1992), and their systematic arrangement followed Ainsworth and Bisby's Dictionary of the Fungi (Kirk *et al.* 2008). All reported taxa were checked against Index Fungorum website database (Kirk 2018) for name corrections, authorities, and taxonomic assignments. All isolated taxa were deposited in Suez Canal University Fungarium (SCUF-http://www.wfcc.info/ccinfo/index.php/collection/by_id/1180/).

Qualitative analysis

All of the isolated species have been tested for the ability to produce L-Asparaginase. A modified technique used for the qualitative screening of L-asparaginase as follows; 5 mm fungal

plug transferred into falcon tubes (50 ml) contains 30 ml of Modified Capek Dox medium (MCD). The tubes incubated for 5 days at 30 C, the color change recorded from yellow to blue taking in consideration the degree of color change, which gave a prediction on the amount of ammonia released (Mahajan *et al.* 2013).

Quantitative assay

To clean test tube 0.1 ml of L-asparagine (40 mM), dissolved by shaking gently, 1 ml of the crude sample extract (media after filtration), 0.5 ml of 0.5 M phosphate buffer (pH 7.0) and 0.4 ml of dH₂O were added. Enzyme sample was handled very carefully and kept on ice. The samples tubes were incubated at room temperature for 30 minutes. After 30 minutes, the reaction was stopped by the addition of 0.5ml trichloroacetic acid 1.5M (TCA), followed by centrifugation at 10,000 rpm for 5 min. Pellet obtained was discarded. 3.7 ml of distilled water was added to 0.1 ml of supernatant solution. To this solution, Nessler's reagent was added (1 ml); followed by vortex and incubation for 10 min. Control was prepared containing all the other reagents except enzyme. Absorbance measured at 450 nm, using standard curve of ammonia the amount of ammonia liberated from protein sample of interest calculated (Kumar *et al.* 2010).

Partial purification of L-Asparaginase produced by *Lasiodiplodia theobromae*

Large scale media cultivation

The most potent L-Asparaginase-producing taxon; *Lasiodiplodia theobromae* was selected for further investigation. To get a large amount of crude extract of the L-asparaginase purified *Lasiodiplodia theobromae* inoculated into MCD media (4 L) without any indicators and incubated in shaking incubator at 120 rpm for average 5 days at 30°C. The obtained media filtered through sterile filter papers and then centrifuged for 10 minutes at 10,000 rpm (4°C) to remove cell debris and mycelia.

Protein precipitation

The filtrate subjected to protein precipitation by using cold acetone technique in which acetone was kept at -80°C prior to precipitation (Mahajan *et al.* 2014). Cold acetone was added to the culture filtrate of selected sample in ratio of 2X and it was incubated at -20°C overnight. The next day, the samples were centrifuged at 13,000 rpm for 15 minutes. Supernatant was discarded and pellet obtained was weighed and dissolved in suitable volume of 0.5 M phosphate buffer (pH 7.0) and stored at -80°C till further use.

Gel-filtration purification (Sephadex G-100)

The precipitated protein samples were loaded on column pre-equilibrated with sodium sulphate buffer 0.5M (pH7.0) Sephadex G 100. It was eluted with the same buffer, and fractions were collected at the flow rate of 1ml/min. Fraction viewing high activity were pooled and asparaginase activity was assayed by previously described assay method (Asha and Pallavi 2012).

Results

General description and taxonomy of isolated endophytes

During our study a total of 424 endophytic colony forming units (CFUs) isolated (Table 1) which represented by twelve genera, 16 species. Taxonomically the 16 species have been assigned to one phylum, two classes, five orders and five families. Order Pleosporales came first by recorded five species, followed by order Sordariales by four species, order Eurotiales by 3 species, and other orders recorded only one to two species. The species to genus ratio (S/G) per family showed that family Nectriaceae was the most diverse with a ratio of 2, followed by family

Aspergillaceae with a ratio of 1.5, Pleosporaceae and Chaetomiaceae with 1.3, and other families showed S/G ratio of one (Table 2).

Regarding species richness during seasons; spring showed 185 CFUs, Summer 121 CFUs, autumn 71 CFUs and Winter 47 CFUs (Fig. 1). While, regarding the species total count variations among the seasons it showed a remarkable variation over seasons for *Alternaria alternata*, *Aspergillus flavus*, *Chaetomium nigricolor*, *Thielavia terricola* and *Aspergillus niger* they showed no growth at winter, while for *Lasiodiplodia theobromae*, *Alternaria phragmospora*, *Curvularia lunata*, *Preussia minimoides* and *Nigrospora oryzae* they showed no growth in autumn (Fig. 2).

Table 1 Fungi recorded, total counts (TC, calculated per 120 plates), percentage counts (% C, calculated per total fungal catch), number of cases of isolation (NCI, per season) and occurrence remarks (OR) of all fungi recovered.

Species	TC	%C	NCI	OR
<i>Alternaria alternata</i> (Fr.) Keissl	83	19.6	3	M
<i>Alternaria phragmospora</i> Emden	10	2.4	3	M
<i>Curvularia lunata</i> (Wakker) Boedijn	40	9.4	3	M
<i>Lasiodiplodia theobromae</i> (Pat.) Griffon and Maubl.	62	14.6	3	M
<i>Preussia minimoides</i> (S.I. Ahmed and Cain) Valldos. and Guarro	20	4.7	4	H
<i>Ulocladium tuberculatum</i> E.G. Simmons	20	4.7	4	H
<i>Aspergillus flavus</i> Link	10	2.4	3	M
<i>Aspergillus niger</i> Tiegh.	10	2.4	3	M
<i>Penicillium notatum</i> Westling	28	6.6	4	H
<i>Chaetomium brasiliense</i> Bat. and Pontual	15	3.5	4	H
<i>Chaetomium globosum</i> Kunze	15	3.5	3	M
<i>Chaetomium nigricolor</i> L.M. Ames	11	2.6	3	M
<i>Fusarium oxysporum sensu</i> Smith and Swingle	50	11.8	4	H
<i>Fusarium solani</i> (Mart.) Sacc.	10	2.4	3	M
<i>Nigrospora oryzae</i> (Berk. and Broome) Petch	10	2.4	3	M
<i>Thielavia terricola</i> (J.C. Gilman and E.V. Abbott) C.W. Emmons	30	7.1	4	H
Total	424	100.0	54	

Occurrence remarks (OR): H: high, 3-4 of the seasons; M: moderate, 2-3; L: low, 1-2, R: rare, 0-1.

Table 2 Taxonomic assignment of the isolated taxa according to Kirk *et al.* (2008)

Phylum	Class	Order	Family	Genus	Species	S/G
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	3	4	1.3
			Sporormiaceae	1	1	1.0
		Botryosphaeriales	Botryosphaeriaceae	1	1	1.0
		Eurotiales	Aspergillaceae	2	3	1.5
	Eurotiomycetes	Sordariales	Chaetomiaceae	3	4	1.3
		Hypocreales	Nectriaceae	1	2	2.0
		Incertae sedis	Incertae sedis	1	1	1.0
Total	2	5	5	12	16	

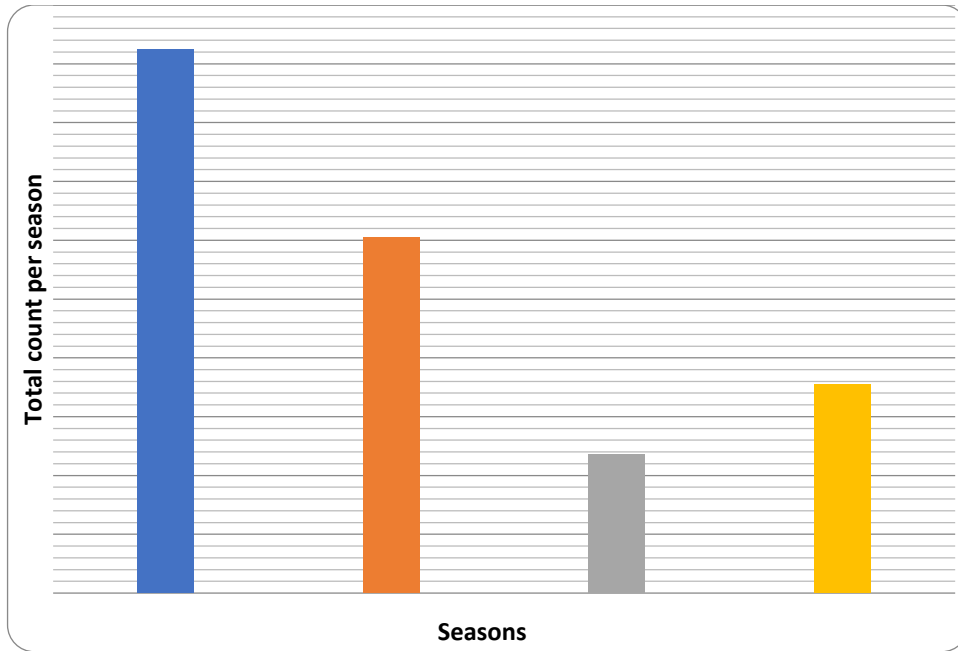


Fig. 1 -The total count of the isolated species among four seasons represented as CFU.

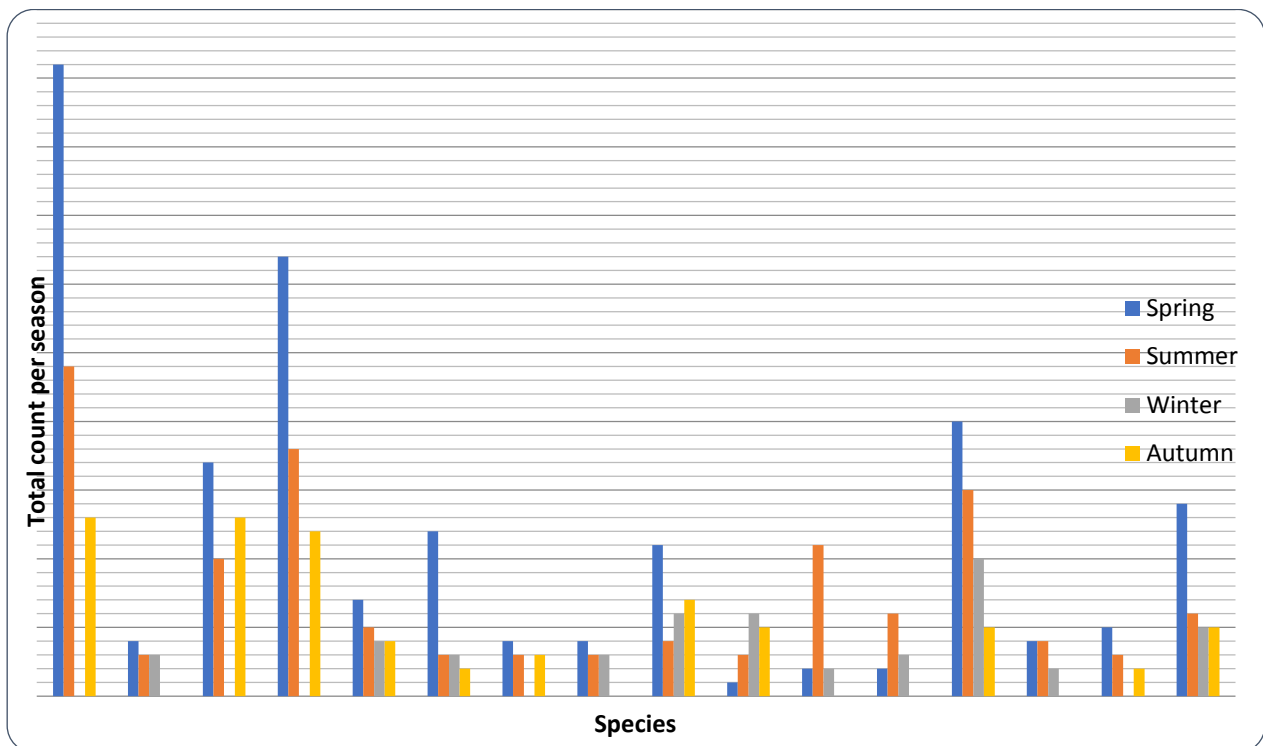


Fig. 2 -The total count of the isolated taxa among four seasons.

Regarding the isolates total count over the four seasons *Alternaria alternata* showed the highest count with 83 colonies, while *Alternaria phragmospora*, *Chaetomium nigricolor*, *Curvularia lunata*, *Preussia minimoides* and *Thielavia terricola* showed the lowest count with 10 colonies, others showed a moderate count (Fig.3).

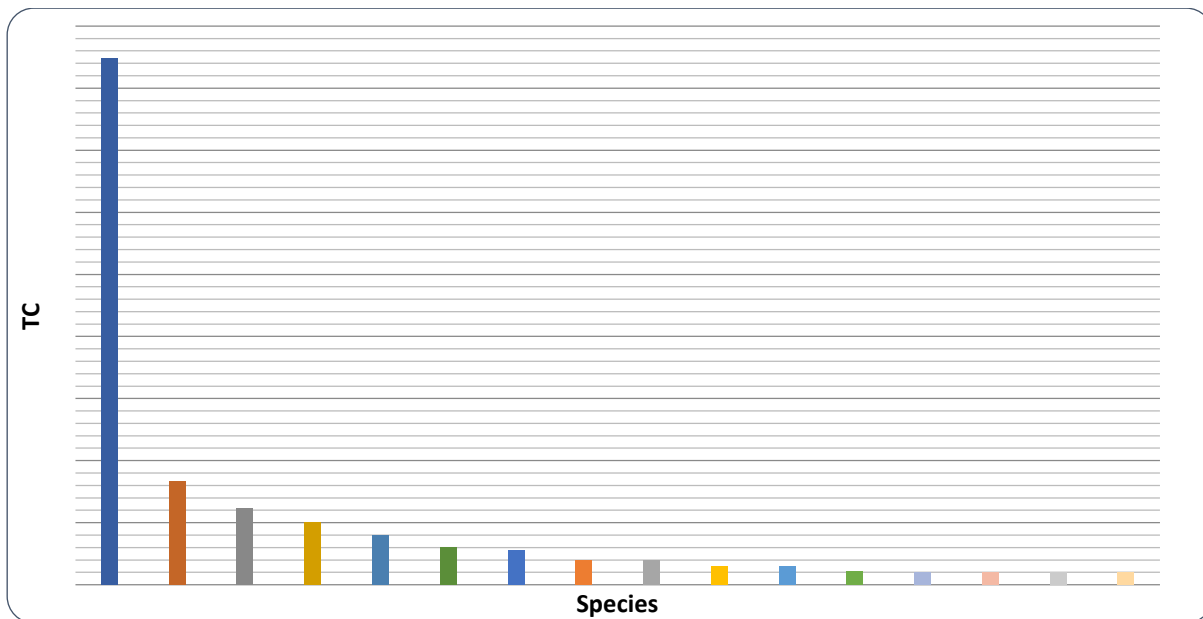


Fig. 3 -The total count of the sixteen isolated taxa during the study.

Qualitative screening of L-asparaginase

Out of the 16 isolated endophytes only three showed L-asparaginase activity on MCD broth, which have been detected by the change of the yellow color of media (acidic) to blue color (basic) due to the production of ammonia from the hydrolysis process of asparagine to aspartic acid and ammonia, where ammonia lead to the increase of pH toward basic status, which in return change the Bromothymol blue indicator color from yellow to blue, which indicated the presence of L-Asparaginase.

Quantitative screening of L-asparaginase

Positive isolates gave activity range from 0.55 to 31.5 $\text{IMmL}^{-1} \text{min}^{-1}$, individually *Fusarium oxysporum* (0.55), *Fusarium solani* (0.89), where *Lasiodiplodia theobromae* recorded the highest activity (31.5).

Phenotypic description of *Lasiodiplodia theobromae* (SCUF-TP2016)

Colony characterized by the presence of abundant woolly mycelium and colonies from olive-grey to dark grey. The colonies were initially white to pale grey becoming dark grey with age and with fluffy aerial mycelia and black pigment on the reverse side of plate. Pycnidia formed with septated paraphyses between the conidiogenous cells. Conidia size of *L. theobromae* isolates (Figs 4) were $21\text{-}31 \times 13\text{-}15 \mu\text{m}$ ($x = 26.2 \times 14.2 \mu\text{m}$). They were initially hyaline, thin-walled and one septated, cylindrical to sub ovoid in shape. They later turned dark brown, formed a single medium septum and became thick-walled with longitudinal striations on the inner surface. Mature conidia were ovoid with a broad and rounded apex and tapered at the base (Fig. 4).

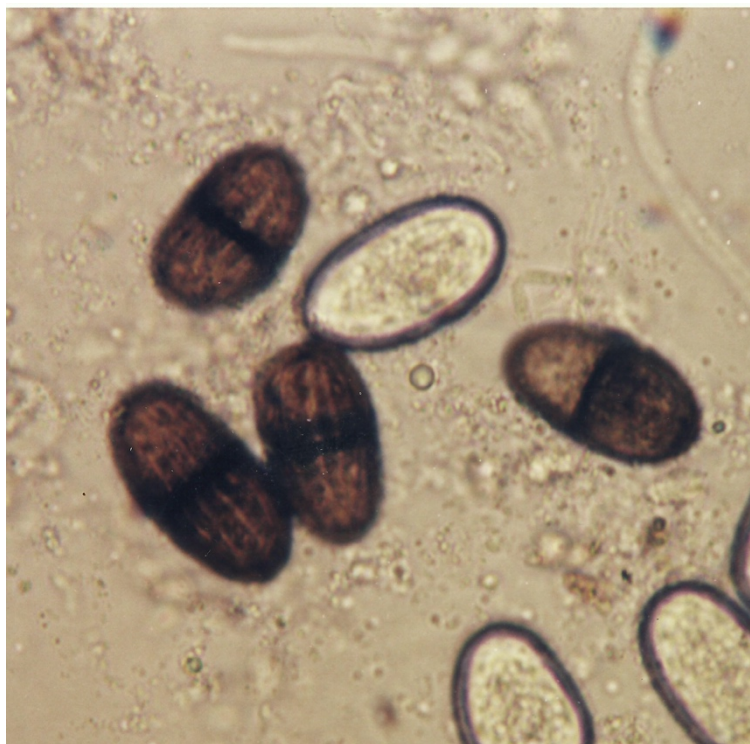


Fig. 4 - Conidia of *L. theobromae* (SCUF-TP2016).

Partial purification

Highly active fraction could be noticed at figure (4), where fraction number 18 recorded activity of $8.17 \text{ IMmL}^{-1} \text{ min}^{-1}$ using Nessler's reaction and after pooling the positive fractions together we got an activity of $21.00 \text{ IMmL}^{-1} \text{ min}^{-1}$. The one unit of enzyme is the amount of it catalyzed the formation of $1 \mu\text{mol}$ ammonia per minute at 37°C .

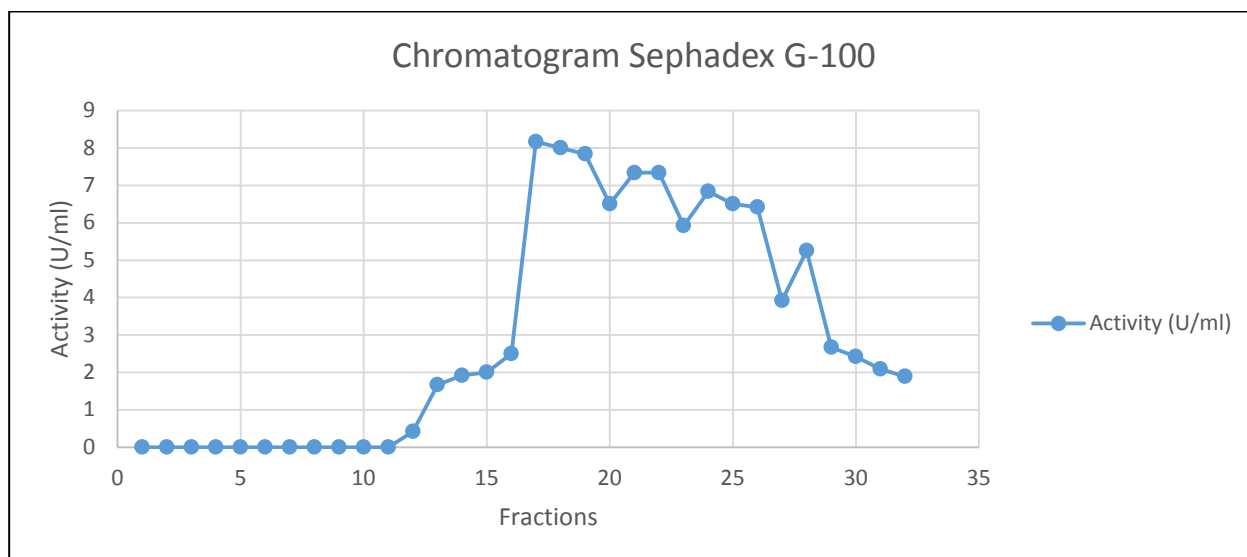


Fig. 4 - Asparaginase activity of *Lasiodiplodia theobromae* after Sephadex column G-100 purification.

Discussion

Endophytic fungi considered as one of the most important bio-factories to produce several important chemical bio-active compound, which as; anticancer, antiviral, anti-inflammatory and antimicrobial (Salem and Abdel-Azeem 2014; Abdel-Azeem et al. 2016, 2018).

Due to the high biodiversity showed at Saint Katherine protectorate, several studies have been carried to study the diversity of endophytic fungi associated with endemic and medicinal plants, our study recorded moderate counts of fungal populations and species diversity associated with *Teucrium polium* plant, which was similar to other studies carried out by several investigators at Saint Katherine protectorate (Abdel-Azeem 2009; Selim et al. 2011; Abdel-Azeem and Salem 2012; Salem and Abdel-Azeem 2014; Abdel-Azeem et al. 2016).

Acute Lymphoblastic Leukemia (ALL) is widely distributed among children: 60% at the age of (0-14), 40% to other ages, several studies have been carried out to obtain a safe form of the L-Asparaginase out of fungal sources and they already succeed to get a safe forms of L-Asparaginase (Asha and Pallavi 2012; Abbas Ahmed 2015; Kumar and Sobha 2012). Our results revealed that L-Asparaginase activity were recorded in three native species namely: *Fusarium oxysporum*, *Fusarium solani* and *Lasiodiplodia theobromae*. Our promising taxon is *Lasiodiplodia theobromae* which showed high production of L-Asparaginase with a remarkable activity ($31.5 \text{ IMmL}^{-1} \text{ min}^{-1}$). Our results in agreement with Nagarajan et al.(2014) who recorded quite similar activity from the same species.

Conflict of Interest

The authors do not have any conflicts of interest.

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