

Endophytic fungi: A gold mine of antioxidants

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Abstract

Endophytic fungi residing in medicinal plants have the ability to produce the same pharmacologic bioactive secondary metabolites as their host medicinal plants, which have been used for thousands of years in traditional medicine and still are used for their health benefits. Nowadays, medicinal plants are quarrying for isolation of plant-derived drugs as they are very effective and have reasonably less or no side effects. However, the natural resources of ethnomedicinal plants are gradually exhausted and access to plant bioactive compounds is challenged by the low levels at which these products accumulate in native medicinal plants. For example, to meet the market demands of 3 Kg per year of *Vinca* alkaloids, powerful plant-derived anticancer drugs, 1.5×10^6 Kg dry leaves are required. In this regard, this review articles aims to highlight the fact that endophytic fungi residing in medicinal plants are capable to biosynthesize pharmacologically active secondary metabolites as antioxidant identical to those produced by their host medicinal plant. Furthermore, the evolutionary origin of the genes involved in these metabolic pathways as well as the approaches designed to enhance the production of these secondary metabolites by the isolated endophytic fungi medicinal plant have a lot of type of antioxidant mostly polyphenols, flavonoids which exhibit high antioxidants bioactivity. In addition to aforementioned reasons, this article also will shed the light on the efforts of Abdel-Azeem and his co-workers at Botany department, Faculty of Science, Suez Canal University and their continuing search for biologically active natural products from Egyptian endophytic fungi hosted medicinal plants in Saint Katherine Protectorate, Arid Sinai, Egypt.

Keywords – Arid Sinai, DPPH, Egyptian ethnomedicinal plants, Saint Katherine Protectorate, in vivo.

Introduction

Endophytes are the group of microorganism (bacteria, Actinobacteria, or fungi) that reside in the internal tissues of the plants in a symbiotic association without causing any disease symptoms. Among the endophytes, endophytic fungi are one of the important ingredients of plant micro-ecosystems having significant effect on the growth and development of host plant (Singh 2019). Endophytic fungi have been well documented and showed beneficial effects to the host plant either by preventing pathogenic organisms from colonization or enriching the rhizospheric soil by enhancing the nutrients for the plants to uptake (Singh 2019). Majorly, the endophytic fungi have been categorized into two main groups (clavicipitaceous and non-clavicipitaceous) based on the differences in the evolution, taxonomy, their host, and ecological roles (Rodriguez *et al.* 2009).

They are important constituent of plant micro-ecosystems (Tan and Zhou 2001; Rodriguez *et al.* 2009). Plant endophytic fungi have been found in each plant species examined, and it is estimated that there are over one million fungal endophytes dwelled in the nature (Petrini 1991).

They have been recognized as an important and novel resource of natural bioactive compounds with potential application in agriculture, medicine and food industry.

Research data suggested that during the last two decades, extensive research has been carried out of endophytic fungi and several biologically active compounds have been isolated from endophytic fungi (Singh 2019). Natural products from medicinal plants and their hosted microbiota are the most consistent and productive source for the 'first-in-class' drugs (Newman and Cragg 2007; Abdel-Azeem *et al.* 2016). Recently, a great deal of interest has been generated by discovery of remarkable pharmacological agents from endophytic fungi (Strobel and Daisy 2003). As reviewed by Schulz *et al.* (2002), 51% of the biologically active substances isolated from endophytes were previously unknown. Although a number of bio-pharmacological compounds with antimicrobial, antitumor, antiinflammatory, and antiviral activities have been previously isolated from endophytes (Aly *et al.* 2008; Liu *et al.* 2008; Souza *et al.* 2008; Salem and Abdel-Azeem 2014; Abdel-Azeem *et al.* 2016, 2018, 2019).

Bioprospecting is generally described as the search for naturally occurring chemical compounds and biological material, especially in extreme or biodiversity-rich environments (Abdel-Azeem *et al.* 2012). Biologically active metabolites are produced by a great number of *Fungi* and most bioprospecting programs have been limited to certain ecological groups of Egyptian fungi (Abdel-Azeem 2010). Pupo (2006) mentioned that endophytic fungi have been shown to be a promising source of novel natural bioactive agents. Several crude extracts from different fungal culture broths showed that plants growing in unique environmental setting and have ethanobotanical uses with endemic location produce novel endophytic micro fungi of which the secondary metabolite are usually unique and may have applicability in medicine (Salem and Abdel-Azeem 2014 ; Abdel-Azeem *et al.* 2016, 2018). Many investigations focused on antioxidant activities of the endophytic fungi isolated from ethnomedicinal plants (Ranjan and Joshi 2012; Abdel-Azeem *et al.* 2018; Abo Nahas 2018).

Reactive Oxygen Species (ROS)

During the process of oxygen utilization in a normal physiological and catabolic process, approximately 5% of oxygen gets univalently reduced to reactive oxygen species (ROS) like superoxide anions (O_2^-), nitric oxide (NO), hydroxyl (OH), which damage cellular constituents causing tissue injury through covalent binding (Yu 1994; Katsube *et al.* 2006). Free radicals have been implicated in causation of diseases such as diabetes, inflammation, cancer, neurodegenerative disorders, atherosclerosis, liver cirrhosis, ephrotoxicity, etc. (Lachance *et al.* 2001). It has been suggested that fruits, vegetables, plants are the main source of antioxidant in the diet. Natural antioxidants may have free-radical scavengers, reducing agents, complexes of pro-oxidant metals, quenchers of singlet oxygen etc.

Free radicals which have one or more unpaired electrons (superoxide, peroxy, hydroxyl) are produced in normal or pathological cell metabolism and the compounds that can scavenge free radicals have great potential in ameliorating the diseases and pathological cells (Halliwell 1995; Squadriato and Peyor 1998; Gulcin *et al.* 2002). Antioxidants thus play an important role to protect the human body against damage by ROS. Free radicals or Reactive Oxygen Species are produced *in vivo* from various biochemical reactions and also from the respiratory chain reactions as a result occasional challenges. These free radicals are the main perpetrator in lipid peroxidation. Plants congaing bioactive compounds have been reported to possess potential antioxidant properties. In many inflammatory disorders there is extravagant activation of phagocytes, production of O_2^- , OH radicals as well as non free radicals species (H_2O_2) (Gilham *et al.* 1997) which can harm severely tissues either by powerful direct oxidizing action or indirect with hydrogen peroxide and OH radical formed from O_2^- which initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors (Lewis 1989). The reactive oxygen species are also known to activate matrix metalloproteinase (MMPs) damage seen in various arthritic tissues (Cotran *et al.* 1994). The

literature survey is giving the importance of plants and their antioxidant properties (Essawi and Srouf 2000) of plant active compounds especially phenolic and flavonoid compounds have proved as potent antioxidant and free radical scavenger (Silva *et al.* 2005).

Recently interest has been increased considerably in finding natural occurring antioxidants for use in foods or medicinal products to replace synthetic antioxidants, which are being restricted due to their adverse reaction such as carcinogenicity. Antioxidant constituents from natural resources possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance (Kumaran and Karunakaran 2007; Abdel-Azeem *et al.* 2018; Abo Nahas 2018).

Antioxidants: Elixir of life

Antioxidants are a broad group of compounds which constitute the first line of defense against free radical damage thus are essential for maintaining optimum health and being as protective agents able to deactivate or stabilizing free radical before attacking cells (Fig. 1) (Kalam *et al.* 2012). Free radical exposure elevate the need for intake of antioxidants, free radical elevate due to pollution, cigarette smoke, illness, therapeutics agents, stress and even exercise. Diet supplemented with antioxidants are nowadays recognized as demanding way of protecting cells from harmful effect of free radical. Identification of pharmacologically potential antioxidant-compounds increased staggeringly as they exhibit no side effect for us in preventive medicine and food industry. Antioxidant compounds are present in fruits, vegetables and many natural beverage, balanced diets are naturally rich in antioxidants display an array of like they support renal function, improve reproductive function and maintain dental health, improve nervous system communications and functioning, have antiaging effect, protect hepatic cell from damage, improve defense mechanism of the body, offer protection against digestive system disorders, reduce obesity. Antioxidants are a family of compounds considered the best strive against a number of age related problems such as Alzheimer (Hajieva *et al.* 2006). Considered as wonder element, antioxidants are essential to good health and well-being as the concept of health improvement has become a legitimate part of health care. The ability to utilize oxygen has provided humans with the benefit of metabolizing fats, proteins and carbohydrates for energy (Percival 1998).

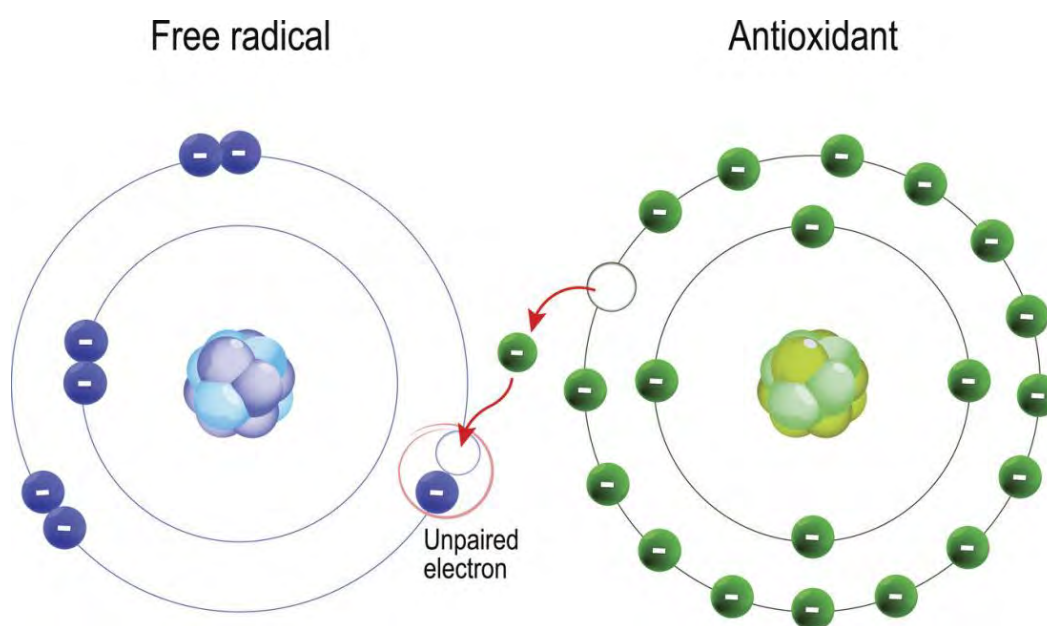


Fig 1- Reaction between free radicals as unstable atoms and antioxidants.

Synthetic antioxidants Vs natural antioxidants

Global antioxidants market was valued at \$2,923 million in 2015 and is expected to reach \$4,531 million by 2022, registering a compound annual growth rate (CAGR) of 6.42% during the forecast period. Antioxidants are used as vitamin supplements in pharmaceutical industry and help in preserving food products (alliedmarketresearch.com). The global antioxidants market is poised to witness significant growth during the forecast period owing to decrease in their costs, increase in investment & product approval by regulatory authorities, and increase in their demand in the food & beverage industry. In addition, developed economies have discovered new growth opportunities by shifting their focus on natural antioxidants such as rosemary extract. Conversely, Asia-Pacific is expected to show growth in the demand for synthetic antioxidants during the forecast period (alliedmarketresearch.com).

Edible antioxidant is the major way of acquiring antioxidants in stressful environment and studies suggest addition of antioxidants with food have diverse positive effects (Willis *et al.* 2009). Synthetic phenolic compound as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT) and propylgallate are used as food additives to prevent oxidation of food material. While use of synthetic antioxidants such as butylatedhydroxytoluene and butylatedhydroxyanisole to maintain the quality of ready-to-eat food products has become common place. Moreover, people are consuming synthetic antioxidant as vitamins, colorants, flavoring agents, spice, herbs, long-term antioxidants (Kukreja *et al.* 2015).

Polyphenolic compounds are structural classes which are characterized by multiple structural units which are characterized by multiple structural units of phenols which are usually found in our daily dietary products as fruits, vegetables and vitamin E found in nuts. Some vitamins (ascorbic acid and α -tocopherol), many herbs and spices (rosemary, oregano, sage, basil, pepper, clove, cinnamon and nutmeg), also with associated phytochemicals which are often produced between plants as a response to light, stress, injury.....etc (Valentine *et al.* 2003).

Study of polyphenolic compounds and total antioxidant potentiality extracted from 21 endophytic fungi recovered from five Sudanese ethnomedicinal plants namely: *Calotropis procera*, *Catharanthus roseus*, *Euphorbia prostrata*, *Trigonella faenum-graecum* and *Vernonia amygdalina* were carried out by Khiralla *et al.* (2015). The isolated fungal strains were classified into 12 different taxa (Table 1). Ten strains belong to Ascomycetes, whereas seven strains belong to fungal class Deuteromycetes, four strains were failed to sporulate and were grouped as mycelia sterilia. Total phenolic content (TPC) of ethyl acetate crude extracts of 21 endophytes and different parts of their host plants were estimated using the classical Folin–Ciocalteu colorimetric method. All the recovered 21 endophytic fungi showed positive results of production of natural phenolic antioxidants, act as powerful reducing agents, as they absorb light in the ultraviolet region (100–400 nm) and effective metal chelators. So in vivo and in vitro activities of antioxidant extraction from endophytic fungi are demonstrated in many studies to clarify their potentiality as powerful compounds can rely on as substitute to synthetic antioxidant (Gautam *et al.* 2018).

Stress may result in decline of immunoresponse and developments of asthma, allergies, obesity which become serious concerns for public health. In a study involving seven individuals with reactions to BHA and BHT reported with symptoms included vasomotor rhinitis, headache, flushing, asthma, conjunctival suffusion, dull retrosternal (behind the breastbone) pain radiating to the back, diaphoresis (excessive sweating), or somnolence (sleepiness) (Fisherman and Cohen 1973). Therefore the quest for more safety supplements that are destitute of negative effect as motivated the researchers to search for friendly antioxidants derived from plants and other natural sources (Gautam *et al.* 2018).

Plants and endophytic fungi as natural source of antioxidants

Currently, the food industry is focused in replacing the use of synthetic by natural antioxidants. Caleja *et al.* (2017) studied the use of fennel and chamomile extracts, rich in phenolic compounds, as natural antioxidants in biscuits and compared their performance with a synthetic

antioxidant widely used, the butylated hydroxyl anisole (BHA). The complete nutritional profile, free sugars, fatty acids and antioxidant activity were determined immediately after baking and also after 15, 30, 45 and 60 days of storage. The results showed that the incorporation of natural and synthetic additives did not cause significant changes in colour or in nutritional value of biscuits when compared with control samples. Both natural and synthetic additives conferred similar antioxidant activity to the biscuits. Therefore, natural additives are a more convenient solution for consumers who prefer foods “free” from synthetic additives (Fig. 2). Additionally, natural additives were obtained by aqueous extraction, an environment friendly and safe process (Caleja *et al.* 2017).

Secondary metabolites were isolated from endophytic *Pseudocercospora* sp. Anitha and Mythili (2017) investigated the antioxidant properties of secondary metabolites isolated from *Achaetomium* sp., an endophytic fungus associated with *Euphorbia hirta*. A variety of metabolic compounds were found and estimated using a standard protocol. The presence of total phenolic compounds was determined as $44.02 \pm 1.57 \mu\text{g}$, total flavonoid content was $54.540 \pm 1.820 \mu\text{g}$, and total tannin content was observed to be $18.790 \pm 1.018 \mu\text{g}$. Based on the phytochemical profile of the endophytic fungus, ethyl acetate crude extract was assayed for antioxidant, antimicrobial, and antitumor activity. Antioxidant activity was measured and found to be $66.890 \pm 1.385\%$ to $87.340 \pm 0.289\%$ in the presence of total phenolic, total flavonoid, and total tannin acid. Antimicrobial activity against gram-positive and gram-negative bacteria was measured and shown to be significant for the inhibition of microorganisms.



Fig 2- Synthetics and natural antioxidants.

It is noteworthy that the antioxidant activities of extracellular polysaccharides (EPSs) from endophytic mangrove fungi are most significantly analyzed among the numerous biological functions of EPS. Sun *et al.* (2004) reported free-radical-scavenging activities of an EPS (EPS2) isolated from the marine filamentous fungus *Keissleriella* sp. YS 4108. Radical elimination and other antioxidant actions of EPS2 (glycan) were evidenced in various in vitro systems showing that EPS2 demonstrated first-rate superoxide radical scavenging activity (Sun *et al.* 2004). EPS antioxidant activity was estimated by several in vitro assays, including by its metal chelating capability (Yang *et al.* 2005), reducing power, lipid peroxidation inhibition assay (Chen *et al.* 2011), and hydroxyl, superoxide, and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (Sun *et al.* 2009).

Classification of antioxidants

Antioxidants can be categorized into two types: **Enzymatic** and **non-enzymatic antioxidants** (Gupta 2015).

Non-enzymatic antioxidants work by interrupting free radical chain reactions. For example, vitamin E may interrupt a chain of free radical activity after only five reactions. Non-enzymatic antioxidants include vitamin C, vitamin E, plant polyphenols, carotenoids, Selenium and glutathione (GSH).

Enzymatic antioxidants work by breaking down and removing free radicals. In general, these antioxidant enzymes flush out dangerous oxidative products by converting them into hydrogen peroxide, then into water, in a multi-step process that requires a number of trace metal cofactors (copper, zinc, manganese and iron). These enzymatic antioxidants cannot be supplemented orally but must be produced in our body.

The principle enzymatic antioxidants are the following: **Superoxide dismutase (SOD)**: Assisted by copper, zinc, manganese and iron, SOD breaks down superoxide (which plays a major role in lipid peroxidation) into oxygen and hydrogen peroxide. SOD is present in nearly all aerobic cells and extracellular fluids. **Catalase (CAT)**: Converts hydrogen peroxide into water and oxygen (using iron and manganese cofactors), hence finishing up the detoxification process that SOD started. **Glutathione peroxidase (GSHpx) and glutathione reductase**: These selenium-containing enzymes help break down hydrogen peroxide and organic peroxides into alcohols, and are particularly abundant in your liver. **Selenium** is an essential trace element having fundamental importance to human health as it is a constituent of the small group of selenocysteine containing selenoproteins (over 25 different proteins) which is important for structural and enzymatic functions. Selenoproteins include several forms of the enzymes glutathione peroxidase (GPx), thioredoxin reductase and iodothyronine deiodinase. Selenium glutathione peroxidases catalyze the elimination of hydrogen peroxide as well as organic peroxides (R-O-OH) by the oxidation of GSH (Papp *et al.* 2007).

Water-soluble (hydrophilic) and lipid-soluble (lipophilic) antioxidants. Another categorization of antioxidants is based on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). The interior of our cells and the fluid between them are composed mainly of water but cell membranes are made largely made of lipids. The lipid-soluble antioxidants (such as vitamins E and A, carotenoids, and lipoic acid) are primarily located in the cell membranes, whereas the water-soluble antioxidants (such as vitamin C, polyphenols and glutathione) are present in aqueous body fluids, such as blood and the fluids within and around the cells (the cytosol, or cytoplasmic matrix). Free radicals can strike the watery cell contents or the fatty cellular membrane, so the cell needs defenses for both. The lipid-soluble antioxidants are the ones that protect the cell membranes from lipid peroxidation (Davies and Dean 1997).

Various antioxidant capacity Assays

A number of protocols have been proposed to determine the antioxidant capacity. Few use radicals and some use metal ions as the oxidizing agents. The wavelength at which measurement is done in the various protocols is tabulated in Table1 after Gupta (2015).

Saint Katherine Protectorate (SKP)

One of the most unique habitats in Egypt is Saint Katherine Protectorate (Abdel-Azeem *et al.* 2018). Saint Katherine is located at an elevation of 1500 to 2624 meters above sea level (m a. s. l.) which includes the main mountains in the area. The Saint Katherine area is situated in the southern part of Sinai between 33°57' to 34°00' South, 28°33' North and 28°26' to 28°34' East (Danin 1983). The area is composed of igneous and metamorphic rocks; chiefly granites are intensely dissected and rugged (Said 1990). The Protectorate of Saint Katherine covers about 4350 km² of Southern Sinai.

Table 1 Type of assay with operating principle.

| Assay | Radical/Chromophore | Wavelength of Measurement | pH of measurement | Mode of assay | HAT / ET based |
|---|---|---|--|--------------------------------|-------------------------------|
| ORAC | AAPH [•] (Fluoresein) | $\lambda_{ex}=485$ nm and $\lambda_{em}=538$ nm | pH 7.4 | Fluorescence decay measurement | HAT based assay |
| Total Peroxyl Radical-Trapping Antioxidant Parameter (TRAP) | AAPH [•] (R-phycoerythrin/Luminol) | $\lambda_{ex}=495$ nm and $\lambda_{em}=575$ nm | pH 7.5 | Fluorescence decay measurement | HAT based assay |
| B-Carotene Bleaching Assay | Peroxyl radicals, ROO [•] | 470 nm | pH 5.5 – 7.5 | Absorbance measurement | HAT based assay |
| Crocin Bleaching Assay | Peroxyl radicals, ROO [•] | 440 nm | pH 7.0-7.5 | Absorbance measurement | HAT based assay |
| Total Phenolic Content | Mo ⁶⁺ (yellow) → Mo ⁵⁺ (blue) | 765 nm | pH 10 | Absorbance measurement | ET based assay |
| Ferric ion Reducing Antioxidant Power assay (FRAP) | Chelated Fe ³⁺ ions | 595 nm | pH 3.6 | Absorbance measurement | ET based assay |
| DPPH | DPPH [•] | 515 nm | pH 7.0-7.4 | Absorbance measurement | ET based assay |
| Trolox equivalent Antioxidant capacity (TEAC) | ABTS ^{•+} | 734 nm | pH 7.4 (using PBS) | Absorbance measurement | ET based assay |
| CUPRAC | Cu ²⁺ → Cu ⁺ (complexed with neocuproine) | 450 nm | Acidic/ Neutral/ alkaline | Absorbance measurement | ET based assay |
| CERAC | Ce ⁴⁺ → Ce ³⁺ | $\lambda_{ex}=256$ nm and $\lambda_{em}=360$ nm | Acidic (0.3 M H ₂ SO ₄) | Fluorescence decay measurement | ET based assay |
| Lipid Peroxidation Inhibition Assay | N-methyl-2-phenylindole | 586 nm | pH 7.4 | Absorbance measurement | HAT based assay |
| Hydroxyl radical averting Capacity (HORAC assay) | HO [•] (p-hydroxybenzoic acid) fluorescein | $\lambda_{ex}=488$ nm and $\lambda_{em}=515$ nm | Phosphate buffer | Fluorescence decay measurement | HAT based assay |
| Fe ²⁺ ions chelating Assay | Ferrozine-Fe ²⁺ complex | 562 nm | pH 4-10 | Absorbance measurement | ET based assay |
| Nitric oxide free radical scavenging activity | Griess reagent | 546 nm | pH 7.2 | Absorbance measurement | ET based assay |
| Potassium Ferricyanide Reducing Power | Fe ³⁺ → Fe ²⁺ | 700 nm | pH 6.6 | Absorbance measurement | ET based assay |
| Thiobarbituric acid reactive substances (TBARS) | MDA-TBA Adduct | 532 nm | pH 2 | Absorbance measurement | ET based assay |
| N,N-dimethyl-p-phenylenediamine DMPD | DMPD ^{•+} (Purple) | 505 nm | pH 5.25 | Absorbance measurement | Fenton type ET based reaction |
| Photochemiluminescence Assay | O ₂ ^{-•} (Using Luminol) | 360 nm (blue luminescence) | pH 10.5 | Chemiluminescence | HAT reaction |

The Saint Katherine Protectorate covers the mountainous region of Southern Sinai, and the site as a protected area declared by Prime Ministerial Decrees 613/1988 and 940/1996 due to its immense biological and cultural interest (Abdel-Azeem 2009). Within this Protectorate, UNESCO at its 26th session in Budapest in June 2002 declared as a World Heritage Site (WHS No. 954), an area that coincides with the Saint Katherine ring dyke. The nomination is of an area of 601 km²

located in the high mountainous region of southern Sinai (average 1500-2000 m above mean sea level), containing the Monastery of Saint Katherine at its centre. This region is composed mainly of granite rocks (Eyal and Hezkiyahu 1980).

The Saint Katherine area has an extremely arid climate with long, hot and rainless summers and cool winters. The mean annual precipitation in this area over 25 years is 45 mm per year, the high mountains receive more precipitation (100 mm/year) as rain and snow. In some parts of this place, floods sometimes resulting from connective rains have been observed during the winter and spring. The maximum air temperature ranged from 20.2 °C to 32.7 °C and the minimum temperature ranged from 1.9 °C to 20.2 °C with the lowest temperature in December and January and the highest temperature in July and August (Mosallam 2007; Abdel-Azeem 2009).

The unique environments, substantial diversity, ethnobotanical history, and endemism of endophyte host plants was already considered during the studies from Abdel-Azeem and co-workers (Fig. 3). The mountainous region of southern Sinai exhibits greater biodiversity than the rest of Egypt, and 4350 km² of this area was declared a Protectorate in 1996 (Zalat *et al.* 2008; Abdel-Azeem *et al.* 2019). Approximately 170 plant species that inhabit south Sinai are used traditionally in folk medicine (Fayed and Shaltout 2004; Abdel-Azeem *et al.* 2019).

Different elevation Wadis in Saint Katherine surveyed by Abdel-Azeem and his co-workers

Low Elevation Wadis

Wadi Zaghra

This wadi is located as north-east of Saint Katherine at 28°39'45"N and 34°19'44"E, as revealed in location map. Its surface consists of stones and rocky substrates. The mountains of this Wadi are dark colour. *Moringa peregrina* trees grow on the foot-hills of mountains. Wadi Zaghra is rich in *Capparis* species (Fig. 4).

Wadi Itlah

Wadi Itlah (28°58'72.3" N, 33°92'01.7" E) is low elevation Wadi (1385 m.a.s.l.). Wadi heavily used for gardening by Bedouin. It consists of sporadic large boulders scattered throughout Wadi bed. It is approximately 30 meters wide, with pink granite geology. There is also a high amount of litter in the Wadi. There is high plant diversity despite the disturbances found. This is possibly because of a higher moisture regime. Dominating plant species include: *Chiliadenus montanus* (Vahl) Brullo. (Fig. 5).

Wadi Tala'

Wadi Tala' (28°34'02.3" N, 33°55'55.8" E) is low elevation Wadi (1481-1670 m.a.s.l.) is a U-shaped rocky Wadi running north south for approximately 2.5 km west of Saint Katherine city. Granitic geology, Wadi has high species richness and high plant coverage. Area used heavily for Bedouin gardens. Dominating plant species include: *Achillea fragrantissima* (Forssk.) Schultz Bip. and *Origanum syriacum* L. (Fig. 6).

Wadi Abu Sayla

Abu Sayla (28°53'82.6" N, 33°55'26.5" E) is low elevation Wadi (1484 m.a.s.l.). Wadi is located near the small village of Abu Sayla and is in close proximity to Wadi Itlah. Vegetation (6%) cover is low with low plant diversity.

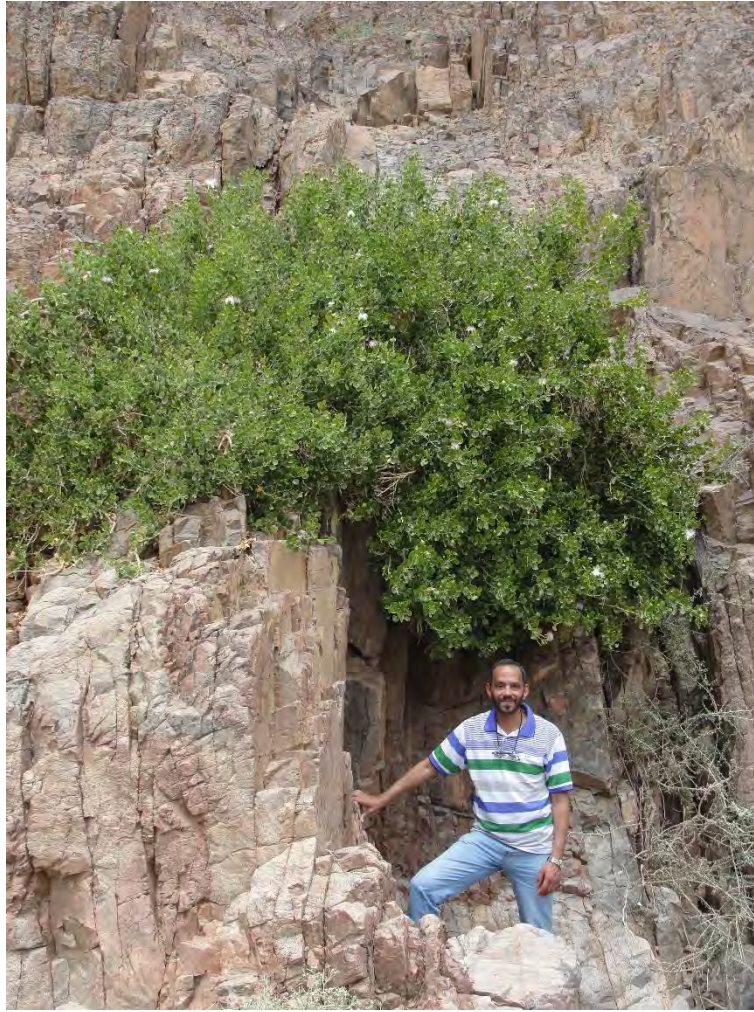


Fig 4 – Abdel-Azeem in Wadi Zaghra (Courtesy of Prof. Ahmed M. Abdel-Azeem).



Fig 5 – Abdel-Azeem's Team in Wadi Itlah (Courtesy of Prof. Ahmed M. Abdel-Azeem).



Fig 6 – Abdel-Azeem's Team in Wadi Tala' (Courtesy of Prof. Ahmed M. Abdel-Azeem).

Mid Elevation Wadis

Wadi El-Arbaein

Wadi El-Arbaein (28°54'54" N, 33°55'36" E) is mid elevation Wadi (1385-1859 m.a.s.l.), very narrow, shaded and subjected to severe tourist's actions and considered as the most floristically richest Wadi in the whole area (Fig. 6). Vegetation richness and percentage cover is high, perhaps because of moderately high moisture availability. Dominating plant species include *Artemisia herba-alba* Asso.

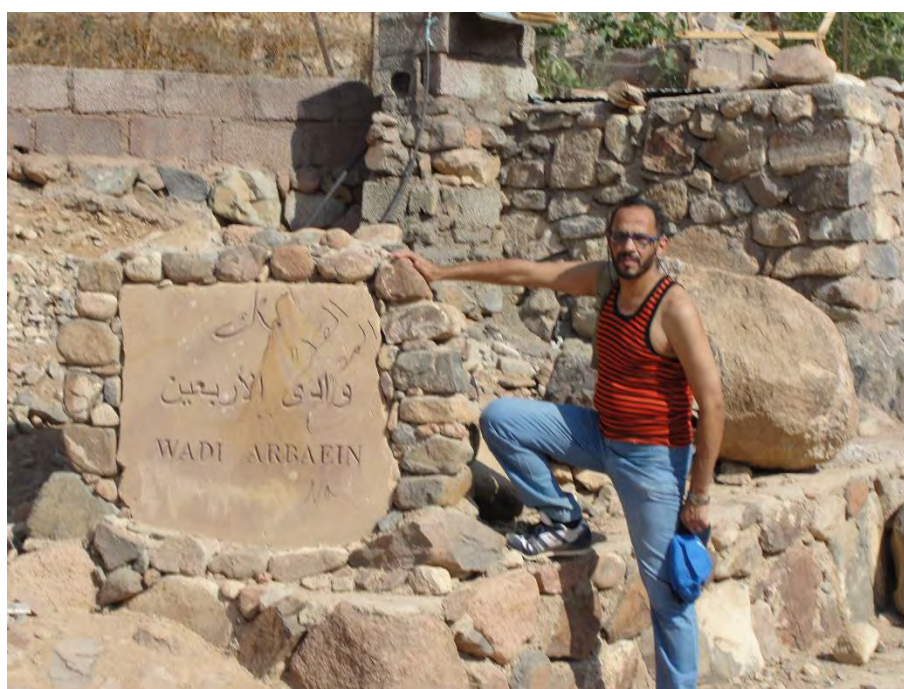


Fig 6 – Abdel-Azeem in Wadi El-Arbaein (Courtesy of Prof. Ahmed M. Abdel-Azeem).

Wadi Shyraj

Wadi Shyraj (28°55'14.7" N, 33°56'25.0" E) is mid elevation Wadi (1731 m.a.s.l.). Wadi located behind Fox Camp, with a steep climb up rocky terrain to access Wadi (Fig. 5). However, the Wadi is relatively flat at the top. A small foot trail runs through Wadi, with low impacts to the surrounding vegetation. Wadi width is approximately 50 meters. However, the water channel is only about 10 meters in width. Slopes of Wadi consist of large boulders with pink granite geology. High plant diversity due to increased water regime. Dominating plant species include *Artemisia herba-alba* Asso. and *Phlomis aurea* Decne (Fig. 7).



Fig 7– Abdel-Azeem’s team in Wadi Shyraj (Courtesy of Prof. Ahmed M. Abdel-Azeem).

High Elevation Wadis

Gebel Ahmar

Gebel Ahmar (28°52'83" N, 33°61'83" E) is high elevation area (1892 m a s l), steep and deeply cut gorge habitat and with extremely high plant diversity with a high percentage of cover. Dominating plant species include *Origanum syriacum* L., *Phlomis aurea* Decne. and *Thymus decussatus* Benth. Black granite geological features (Fig. 8).

Farsh Ras Sefsafa

Sefsafa mountain (28°55'13.6" N, 33°96'46.5" E) is high elevation area (1981 m.a.s.l.). Farsh type habitat at the base of Sefsafa Mountain and substrate composed of volcanic rocks. Area has granitic and sandstone geological features. Foot trail near transect which creates grazing and trampling impacts on vegetation. Number of plant species increases away from the trail. Transect done in open, flat area. Dominating plant species include *Tanacetum sinaicum* Fresen.) Delile ex Bremer & Humphries and *Artemisia herba-alba* Asso. (Fig. 9).



Fig 8– Abdel-Azeem in Gebel Ahmar (Courtesy of Prof. Ahmed M. Abdel-Azeem).



Fig 9 – Abdel-Azeem's team in Farsh Ras Sefsafa (Courtesy of Prof. Ahmed M. Abdel-Azeem).

Egyptian endophytic fungi as promising source of antioxidants

In this section we will discuss some of the results obtained by Abdel-Azeem and his co-workers during the period from 2016 till 2018 on the endophytic taxa as new antioxidants producers. Chávez *et al.* in 2015 discussed why fungi isolated from extreme environments are an excellent potential source of new natural products with novel and/or unusual chemical structures, and proposed some genome and metagenome mining methodologies which can be successfully applied to them.

How to study antioxidant's producing endophytic fungi?

Sampling

Plant species characterized by antioxidant properties will be collected from different Wadis in SKP. Aerial parts from each plant species will be collected in sterilized polyethylene bags, closed by rubber band, and transferred to the laboratory until plating out. Samples located in national parks or protectorates as in SKP will be collected under the permission and no endangered species will be involved in this study.

Isolation of endophytic mycobiota

The foliage parts (leaf and stem) will be washed in running water, and cut into small pieces, surface-sterilized by immersing in 75% ethanol (EtOH) (v/v) for 1 min. dipped in sterile distilled water, and then 0.05 g/ml sodium hypochlorite (NaOCl) solution (v/v) for 3-5 min. according to the tissue thickness, followed by two rinses in sterile distilled water. The sterilized samples will be plated on different isolation media (Abdel-Azeem and Salem 2012). Plates will be incubated at different temperature degrees based on the collection site and assessing each plate for hyphal growth every three days for 21 days. Growing fungi will be purified for identification and transferred into slants for preservation.

Media of isolation

Different isolation media will be used for primary isolation, maintaining cultures and for induction of teleomorphs after Atlas (2004) namely: Czapek's yeast extract agar (CYA), Malt Extract Agar (MEA), Oatmeal Agar (OA), Potato Carrot Agar (PCA), Eight Vegetables Agar (V8), Potato Dextrose Agar (PDA) and Hardy Modified Cellulose Agar (HMCA). All isolation media are basically will be amended with antibiotics e.g. rose bengal (1/15000) and chloramphenicol (50 ppm) and sterilized by autoclaving at 121°C for 15-20 minutes.

Identification of isolated taxa

Phenotypic identification

Taxonomic identification using phenotypic characteristics of fungal isolates down to the species level on standard media will be mainly based on the relevant identification keys e.g. Raper & Thom (1949), Pitt (1980) for *Penicillium*; Raper & Fennell (1965), Klich (2002) for *Aspergillus*; Ellis (1971, 1976) for dematiaceous hyphomycetes; Booth (1971), Leslie & Summerell (2006) for *Fusarium*; Arx (1981), Domsch *et al.* (2007) for miscellaneous fungi; Guarro *et al.* (2012) for ascomycetes; Arx *et al.* (1986), Cannon (1986), Asgari & Zare (2011) and Doveri (2013) for *Chaetomium*; and Simmons (2007) for *Alternaria*.

The names of authors of fungal taxa will be abbreviated according to Kirk and Ansell (1992). The systematic arrangement follows the latest system of classification appearing in the 10th edition of Anisworth & Bisby's Dictionary of the fungi (Kirk *et al.* 2008). Name corrections, authorities, and taxonomic assignments of all taxa reported, will be checked against the Index Fungorum website database (www.indexfungorum.org).

Molecular identification and phylogenetic analysis

DNA will be extracted from fungal culture using an adapted chloroform procedure (Arenz and Blanchette 2011). The internal transcribed spacer (ITS) region of ribosomal DNA will be targeted for PCR amplification with the primers ITS1 and ITS4 for large subunit amplification (White *et al.* 1990). PCR amplifications will be done using and 1 ml of template DNA using the following parameters: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final extension step of 5 min at 72 °C. PCR amplicons were visualized on a 1 % agarose gel using SYBR green 1 pre-stain and a transilluminator. Consensus sequences will be assembled using relevant software e.g. Geneious 9.0 (Kearse *et al.* 2012) and compared to those in GenBank using BLASTn for identification. The sequences of isolated taxa will be compared with reference ITS sequences from the GenBank database at the National Center for Biotechnology Information (NCBI), using the basic local alignment search tool (BLAST). Evolutionary distance matrices based on the neighbor-joining algorithm (with max sequence difference of 0.75) will be calculated using Kimura's two-parameter model (Kimura 1980). Tree topology will be inferred by the neighbor-joining method in the program MEGA7 (Kumar *et al.* 2016), with bootstrap values based on 1,000 replications.

Preparation of fungal fermentation broth

Isolated endophytic fungi chosen to survey their ability to produce potential anti-rheumatoid metabolites will be grown on Potato Dextrose Agar (PDA) medium at 28°C for 5 days, and then will be cultured in potato dextrose broth (PDB) for 14 days at 25°C on a shaker at 180 rpm. Crude fermentation broth will be blended thoroughly and cultures (2L) for each species will be separated by filtration. Liquid filtrate will be extracted with an equal volume (v/v) of ethyl acetate (EtOAc) twice, and then both of aqueous and solvent layers will be collected separately. The frozen mycelia will be smashed and extracted by grinding with an equal volume of EtOAc. The extracts will be then evaporated under reduced pressure to obtain a crude broth extract. The extracts were reconstituted in 5% dimethylsulfoxide (DMSO, Merck) in ethanol (v/v) to a final suitable concentration for toxicity evaluation and antirheumatoid activity screening (Salem and Abdel-Azeem 2014). After evaporation, the dried extract will be stored in away from light in a refrigerator until further use. For injection of rats, fresh prepared solution of solid metabolites will be applied through re-suspension in sterile 10% Tween-80 in saline solution.

Determination of total phenolic content

Total phenolic content will be done on extracts of the most prominent two fungi based on their H₂O₂ scavenging activity (*Chaetomium globosum* and *Curvularia lunata*). The amount of total polyphenolic compounds will be measured according to the method described by Taga *et al.*, (1984). 15 mg of each extract will be dissolved in 1ml of 90% ethanol. A 10µl aliquot of the resulting solution will be added to 2ml of 2% Na₂CO₃ and after 2 minutes 100µl of Folin-ciocalteu reagent (diluted with water 1:1) will be added. After a further 30 minutes, the absorbance will be measured at 750nm using UV/Vis Spectrophotometer. The concentration will be calculated using gallic acid as standard (0–1 mg/ml), and the results will be expressed as mg gallic acid equivalents per gm extract.

Determination of total flavonoids

Measurement of total flavonoid content in the investigated extracts (*Chaetomium globosum* and *Curvularia lunata*) was determined spectrophotometrically according to Zhishen *et al.* (1999), using a method based on the formation of complex flavonoid-aluminium with the absorption maxima at 510 nm. The reaction mixture contained 0.5 ml of extract in DMSO or standard solutions of (+)-catechin, diluted with 2 ml distilled water and 0.15 ml of 5% NaNO₂. After 5 min, 0.3 ml of 10% AlCl₃ was added. After 6 min, 1 ml of 1 M NaOH was added and the total volume was made up to 5 ml with water. The solution was mixed well and the absorbance

was measured against a prepared reagent blank at 510 nm using PG T90+ UV/Vis Spectrophotometer.

Abdel-Azeem and co-workers in 2016 investigated the antiinflammatory and antirheumatoid activity of secondary metabolites produced by endophytic mycobiota in Egypt. In their study, a total of 27 endophytic fungi were isolated from 10 dominant medicinal plant host species in Wadi Tala, Saint Katherine Protectorate, and arid Sinai, Egypt. Of those taxa, seven isolates of *Chaetomium globosum* (CG1-CG7), being the most frequent taxon, were recovered from seven different host plants and screened for production of active anti-inflammatory metabolites. Isolates were cultivated on half-strength potato dextrose broth for 21 days at 28°C on a rotatory shaker at 180 rpm, and extracted in ethyl acetate and methanol, respectively. The probable inhibitory effects of both extracts against an adjuvant-induced arthritis (AIA) rat model were examined and compared with the effects of methotrexate (MTX) as a standard disease-modifying antirheumatoid drug. Disease activity and mobility scoring of AIA, histopathology, and transmission electron microscopy (TEM) were used to evaluate probable inhibitory roles. A significant reduction ($P < 0.05$) in the severity of arthritis was observed in both the methanolic extract of CG6 (MCG6) and MTX treatment groups six days after treatment commenced. The average arthritis score of the MCG6 treatment group was (10.7 ± 0.82) compared to (13.8 ± 0.98) in the positive control group. The mobility score of the MCG6 treatment group (1.50 ± 0.55) was significantly lower than that of the positive control group (3.33 ± 0.82). In contrast, the ethyl acetate extract of the CG6 (EACG6) treatment group showed no improvements in arthritis and mobility scores in AIA model rats. Histopathology and TEM findings confirmed the observation. Arulselvan *et al.* (2016) confirmed the role of antioxidants and natural products in inflammation and this is already proved by Abdel-Azeem *et al.* (2016) that *Chaetomium globosum* KC811080 is a native fungus with promising anti-inflammatory and anti-rheumatoid secondary metabolites.

In 2018 Abdel-Azeem *et al.* studied the hepato-curative effects of endophytic fungi hosted medicinal plants in SKP. During their study, 36 species belonging to 21 genera were isolated from 7 medicinal plants. Ascomycota was represented by 35 species and only 1 for Zygomycota. The dominant and most frequently isolated taxa were *Aspergillus flavus* and *A. niger* (they were omitted during our study due to their ability to produce mycotoxins), followed by *Alternaria alternata*, *Curvularia lunata*, *Penicillium chrysogenum*, *Chaetomium globosum* and *Trichoderma viride*.

Seven plant species inhabit different elevation Wadis in SKP based on their medicinal uses by local bedouins. The plant species under investigation were: *Adiantum capillus-veneris* L. (Adiantaceae), *Capparis spinosa* L., *C. cartilaginea* Decne. (Capparaceae), *Thymus decussates* Benth. (Lamiaceae), *Echinops spinosissimus* Turra. (Asteraceae), *Verbascum sinaiticum* Benth. (Scrophulariaceae) and *Calotropis procera* (Aiton) W. T. Aiton (Asclepiadaceae) (Fig. 10).

The previously mentioned five species were surveyed for their H₂O₂ scavenging activity. The results showed that, among the five species, *Chaetomium globosum* recovered from *Adiantum capillus-veneris* and *Curvularia lunata* isolated from *Verbascum sinaiticum* have the higher radical scavenging activity as recorded 75.31% and 73.44% respectively. So, both taxa were chosen for determination of their flavonoid and phenolic content. *Chaetomium globosum* and *C. lunata* recorded 92.5 and 106 µg/ml of total flavenoid and 3.594 and 3.172 mg/ml of total phenolic content respectively.

Depending on the results of antioxidant and biochemical studies, *Chaetomium globosum* (CG) and *Curvularia lunata* (CL) were selected to survey their metabolites curative potentiality against paracetamol induced-liver injury in mice. Both taxa were cultivated on potato dextrose broth (PDB) medium for 15 days at 28°C, followed by extraction with ethyl acetate (EtOAc). For each species two extracted were examined watery (W) and ethyl acetate (E).

An acute toxicity study has been carried out to estimate approximately LD₅₀ and in turn determine the appropriate dose for the study. The results indicated that approximately LD₅₀ of

CGE, CGW, CLE and CLW is equal to 274.16, 2285.6, 1127.2 and 2728.98 mg/kg B.W. respectively.

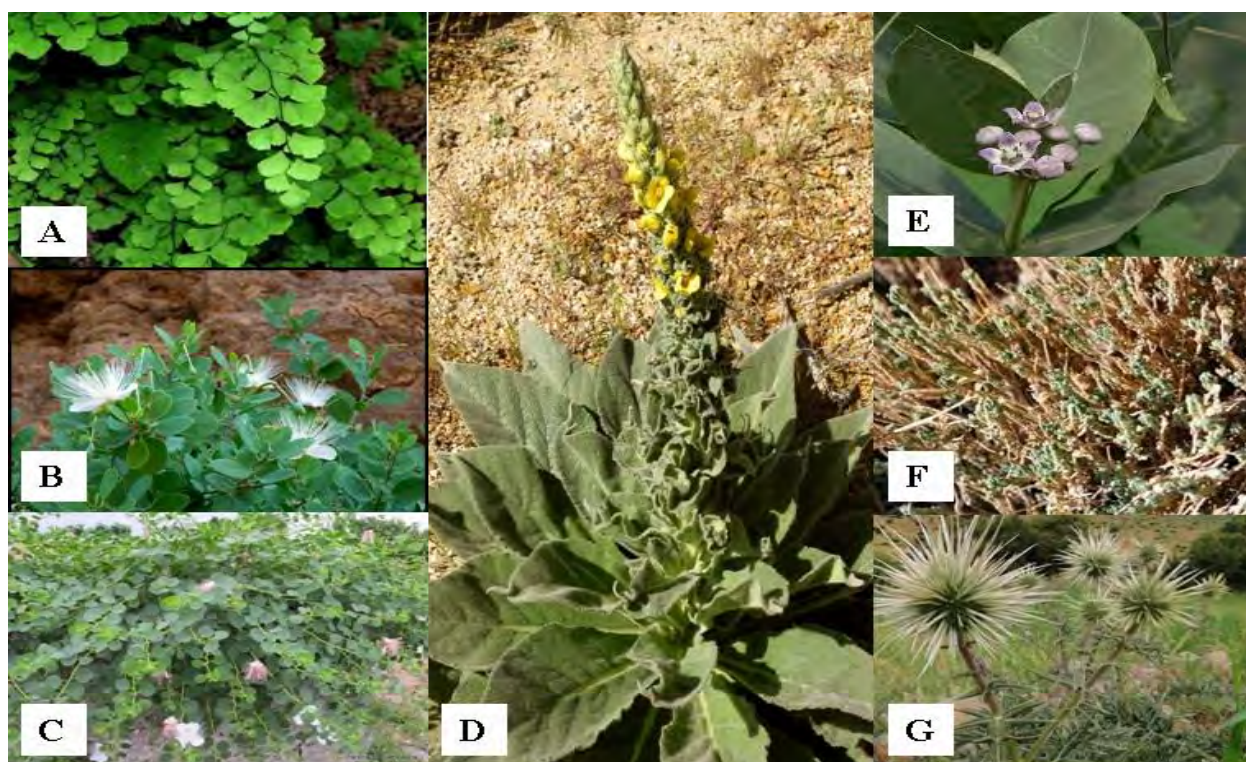


Fig 10 – Plant taxa collected by Abdel-Azeem *et al.* (2018) from five Wadis of SKP. A= *Adiantum capillus-verenis*, B= *Capparis spinosa*, C= *Capparis cartilaginea*, D= *Verbascum sinaiticum*, E= *Calotropis procera*, F= *Thymus decussatus* and G= *Echinops spinosissimus*.

To evaluate the effect of the four extracts on liver biochemical parameters, 1/10 of LD₅₀ of CGE, CGW, CLE and CLW were used in 27, 228, 113 and 273 mg/kg B.W. respectively and the results revealed that CGE showed significant improvement than others. CGE decreased ALT, AST, GGT, ALP and T. bilirubin while increased albumin in comparison with positive control group.

Liver biomarker parameters have been measured after five days of treatment with three different doses of CGE, 1/10 LD₅₀ (27 mg /kg B.W), 1/20 LD₅₀ (13.5 mg /kg B.W) and 1/40 LD₅₀ (6.75 mg /kg B.W). The highest dose of CGE showed the most significant hepato-curative effect when compared with positive control group while the lowest dose remains the safest dose with non-significant effect.

To evaluate the blood antioxidant, 1/10 LD₅₀ (27 mg /kg B.W) of CGE was used and the results revealed that CGE significantly decreased MDA while significantly increased GSH and SOD compared to positive control group.

To evaluate the liver antioxidant, 1/10 LD₅₀ (27 mg /kg B.W) of CGE was used and the results revealed that CGE significantly decreased malondialdehywde (MDA) while significantly increased glutathione (GSH), SOD and catalase (CAT) in comparison with control positive group. Total protein was non-significantly increased compared to positive control group.

Regarding the hepatic histopathological inspection, injection of a single high dose of paracetamol (500 mg/kg B.W) in the positive control group induced vacuolar degeneration in hepatocytes with pyknotic nuclei, high incidence of hyperplasia Kupffer cells and mononuclear leukocyte infiltration. Dilated central vein and blood vessels in portal area were also seen. These lesions were greatly improved after silymarin (100 mg/kg B.wt) and CGE (27 mg/kg B.wt) treatments for 10 days. Only eosinophilic cytoplasm with central spherical vesicular nuclei were

observed in hepatocytes of silymarin-treated group, while few vacuolated hepatocytes, hyperplasia Kupffer cells and mild dilatation of hepatic sinusoids were verified in CGE-treated mice.

Concerning the other treatments, groups received CGW (228 mg/kg B. wt), CLE (113 mg/kg B.wt) or CLW (273 mg/kg B.wt) for 10 days showed variable limited improvements in hepatic histopathology when compared to silymarin or CGE-treated groups.

The study carried by Abdel-Azeem *et al.* 2018 revealed that the ethyl acetate extract of *Chaetomium globosum* (CGE) from *Adiantum capillus-verenis* shown prominent hepato-therapeutic activities in paracetamol induced hepatic damage in mice. The results indicate that the antioxidants effects of CGE may be responsible for their hepato-therapeutic activity. Subsequently, further investigations should be carried out on *Chaetomium globosum* ethyl acetate extract to elucidate the compound that are responsible for such therapeutic activities and their antioxidants capacities.

Conclusion

In addition to find the novel species, exploration of endophytes opens the way to find many potential bioactive for medicinal purpose and to cope more understanding the complex mechanism of interaction between plants and microorganism. Earth is the home of nearly 300,000 species of plants, where each individual plant is host to one or more endophytes. With approximately 11% of the globe's plant species. Endophytes inhabiting these hosts are poorly studied in Egypt, which leaves a promising but also challenging research for the future. Egyptian mycologist should directed their research towards exploring the antioxidant potentiality of our native taxa. Our taxa and their genetic resources will be used for generating high potential safe natural products. Understanding the knowledge of relationship between the endophytes and their host will facilitate the ideal production of better drugs. Traditional way to produce natural product based drugs is extraction directly from the ethanomedicinal plants should be stopped to conserve rare plants all over the world.

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