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Assessment of Secondary Metabolites from Marine-Derived Fungi as Antioxidant

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ABSTRACT

Marine derived fungi are considered as a promising source of novel drugs due to their biodiversity and consequent chemo-diversity. Although marine microorganisms especially fungi are not well defined taxonomically, making this a promising frontier for the discovery of new medicines. This study focused on marine derived fungi as a model for bio-active exploration for new entities with anti-inflammatory and antioxidant capacity. Three *in-vitro* assays were used to investigate the bioactive antioxidant potentiality of fungal extracts. Thiobarbituric acid (TBARS), *α*,*α*-Diphenyl-*β*-picrylhydrazyl (DPPH) and NO assay are based on their total phenolic and flavonoid content of each extract group. Ch. globosum recorded the highest antioxidant activity (92.82%) in TBARS assay, while *G. dankaliensis* came first by recording 59.28% in DPPH assay in comparison with ascorbic acid (61.83%). In NO inhibition assay, *N. oryzae* showed 49.3% comparing with ascorbic acid (73.12%). From the preliminary result of our extracts, we can consider the marine derived fungi extracts as promising antioxidant and anti-inflammatory drug candidate.

Keywords: DPPH; TBARS; Marine Drugs; Marine Derived Fungi; Anti-Inflammatory; Antioxidant Capacity

1. Introduction

Reactive oxygen species (ROS) or free radicals may be designated as molecular sharks that damage molecules in cell membranes, mitochondria, DNA and are very unstable, tend to strip electrons from the molecules in the immediate surroundings in order to replace their own losses. Reactive oxygen species (ROS) is a collective term, which includes not only the oxygen radicals (O_2•−, and •OH) but also some non-radical derivatives of oxygen. These include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3) [1].

ROS and free radical-initiated reactions are ascertained to play multiple roles in degenerative or pathological events such as aging, cancer, heart dysfunction and Alzheimer's disease [2]. Over about 100 disorders like rheumatoid arthritis, hemorrhagic shock, cardiovascular disorders, cystic fibrosis, metabolic disorders, neurodegenerative diseases, gastrointestinal ulcerogenesis and AIDS have been reported as ROS mediated [3].

The oxidative damage to DNA, proteins and other cellular determinants seems inevitable when the concentration of ROS exceed tolerability of cells.

The free radical nitric oxide (NO) is also a highly reactive free radical capable of mediating a multitude of reactions [4] NO, is an uncharged molecule containing an unpaired electron in its outermost orbital, allowing it to undergo several reactions functioning either as a weak oxidant (electron donor) or as an anti-oxidant (electron acceptor). Also, it is able to react with other inorganic molecules (*i.e.* oxygen, superoxide or transition metals), structures in DNA (pyrimidine bases), prosthetic groups (heme) or with proteins leading to S-nitrosylation of thiol groups, nitration of tyrosine residues or disruption of metal-sulfide clusters such as zinc-finger domains or iron-sulfide complexes [5].

Moreover, free radicals are known to take part in lipid peroxidation in foods, which is responsible for rancid odors and flavors, which decrease the nutritional quality.

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Therefore, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyhydro-quinone (TBHQ) are widely used in the food industry as potential inhibitors of lipid peroxidation. However, previous studies have demonstrated that BHA and BHT accumulate in the body and result in liver damage and carcinogenesis [6-9]. An inflammatory response implicates macrophages and neutrophils, which secrete a number of mediators (eicosinoids, oxidants, cytokine and lytic enzymes) responsible for initiation, progression and persistence of acute or chronic state of inflammation [10]. NO, along with superoxide (O_2•−) and the products of their interaction, initiates a wide range of toxic oxidative reactions causing tissue injury [11].

The role of ROS/RNS in inflammation is clearly demonstrated by the anti-inflammatory effects of the antioxidants. Niric oxide synthase inhibitors are also effective as anti-inflammatory agents in carrageenan-induced rat paw odema method as SOD. These may be due to the removal of O_2•− by SOD, so preventing O_2•− dependent formation of a factor chemotactic for neutrophils [12].

Likewise, the neutrophils also produce oxidants and release granular constituents comprising of lytic enzymes performing important role in inflammatory injury [13]. Reactive oxygen intermediates (ROI) are believed to be mediators of inflammation and responsible for the pathogenesis of tissue destruction in rheumatoid arthritis [14].

The antioxidants could attenuate this oxidative damage of a tissue indirectly by increasing cells' natural defenses [15,16], and/or directly by scavenging the free radical species [17]. In order to reduce the damage of ROS to the human body, antioxidants are commonly used in processed foods. Antioxidants could alleviate the oxidative damage of a tissue indirectly by increasing cells' natural defenses and directly by scavenging the free radical species [9,16,17].

The potent biological activity of many marine natural products is of relevance for their ecological function but is also the basis of their biomedical importance [18]. As a slightly opened reservoir of special bio-resources, some marine microorganisms have demonstrated to be excellent producers of biologically active and/or structurally novel metabolites [19]. Marine organisms, particularly marine fungi, are well-known for their production of unique biologically active metabolites. Among marine fungi, those living in association with algae, represent a rich source of novel antioxidant products. The association between algae and fungi is called "Algicolous".

Antioxidants protect cells against the damaging effects of reactive oxygen species (singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite) and help us to fight diseases like artherosclerosis, dementia and cancer. In addition, they may be useful as therapeutics or food additives [20]. For that, the finding of new antioxidative leads (marine-derived fungi), which are capable of preventing and/or eliminating oxidative stress and the development of suitable screening bioassays merits, consequently, have high priority and would be very welcomed [21-23].

2. Materials and Methods

2.1. Marine Sample

Samples of honeycomb sponge (Hippospongia communis) were collected from the west coast of Alexandria to Libya borders (Sidi Krir area to El-Salloum by dragging ships). While, Ascidians samples (Styela canopus) and seaweeds samples were collected monthly from Anfoshi Bay as shown in **Figure 1**.

2.1.1. Isolation of Sponge-Derived Fungi

To get rid of nonspecific fungal propagules from seawater column on sponge and jellyfish surfaces, animal tissues were rinsed three times with sterile seawater. The surface of the sample was disinfected with 70% ethanol for 2 minutes. The inner tissue was taken out with a scalpel and forceps and then cut into small cubes approx. 0.5 cm^3. A total of 15 - 20 cubes of each sample were placed on isolation media.

All isolation and culture maintaining media for marine taxa were prepared by sea water (SW) and isolation media basically were supplemented with Rose bengal (1/15,000) and chloramphenicol (50 ppm) for suppression of bacterial growth. Five media were adopted for isolation after Atlas (2004) they were: Sea Water Rose bengal Chloramphenicol Agar (SWRCA), Sea Water Czapeks Yeast Extract Agar (SWCYA), Sea Water Oatmeal agar (SWOA), Sea Water Agar (SWA), and Sea Water Potato Dextrose Agar (SWPDA).

For maintaining cultures and for proper identification, pure cultures of isolated fungi were grown on standard media such as Vegetable Agar (V8), Oatmeal Agar (OA), Malt Extract Agar (MEA) Potato Dextrose Agar (PDA) and Potato Carrot Agar (PCA).

2.1.2. Identification of Isolates

Taxonomic identification using morphology characteristics of fungal isolates down to the species level on standard media was mainly based on the following identification keys: [24] Pitt (1980) [25] for *Penicillium* (on Czapek Yeast Extract Agar (CYA) & Malt-Extract Agar (MEA)); Raper & Fennell (1965) [26] for *Aspergillus* (on Czapek Agar (CZ)); Ellis (1971, 1976) [8,27] for dematiaceous hyphomycetes (Potato Carrot Agar (PCA)); Booth (1971) [29] for *Fusarium* (Potato Dextrose Agar (PDA)); Arx (1981) [30], Domsch *et al.* (2007) [31],

Figure 1. The location of sample collection.

Watanabe (2002) [32] for miscellaneous fungi (on MEA, PDA, CYA); Arx *et al.* (1986) [33] and Cannon (1986) [34] for *Chaetomium* (Oat Meal Agar + Lupin Stem (OA + LUP)). The systematic arrangement follows the latest system of classification appearing in the 10th edition of Ainsworth and Bisby's Dictionary of the fungi (Kirk *et al*. 2008) [35] and Species Fungorum web site (http://www.speciesfungorum.org/Names/Names.asp).

2.2. Preparation of Marine Fungus Extract

Preparative-scale production (0.5 L) was carried out in 1 L Erlenmeyer flasks contained potato dextrose extract (Difco) for 2 weeks at 28˚C in a shaking incubator at 102 rpm. Pellets were homogenized and centrifuged by using cooling centrifuge at 8000 rpm for 2 min at 4˚C Resultant mixtures were extracted with ethyl acetate (1 × 50 ml), the organic fractions were combined, and the solvent removed at reduced pressure and 35˚C. Residues were redissolved in DMSO for further bioassay. The steps of isolation and extraction of fungi and secondary metabolites are shown in **Figure 2**.

2.3. Biochemical Profiling for Fungal Extract

2.3.1. Determination of Total Phenolic Content in Different Fungal Species Extracts

Total phenolic compounds in the fungal extracts were determined by the method of Taga *et al.* (1984) [36].

2.3.2. Determination of Total Flavonoid Content in Different Fungal Species Extracts

Total flavonoid content was determined by a colorimetric method of Zhishen *et al.* (1999) [37].

2.3.3. Diphenyle *α*-*α*-Picrylhydrazyl (DPPH) Radical Scavenging Effect of Different Fungal Species Extracts

DPPH radical scavenging assay of the total extract was performed by using modified previously established methodology by Blois (1958) [38] and Amarowicz *et al.* (2000) [39].

% scavenging = [(A control − A sample]/A control × 100

2.3.4. Determination of Thiobarbituric Acid Reactive Substance Method Using TBARS Assay for Different Fungal Species Extracts

The method used was adapted from Wallin *et al.* (1993) [40] and modified by Fisch (year).

% scavenging = [(A control − A sample]/A control × 100.

2.3.5. Chromatography

20 µl sample extract analyzed with an eclipsed XDB C18

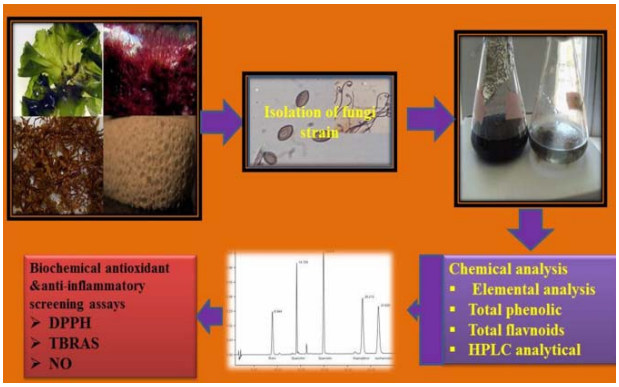


Figure 2. Summarize the production of secondary metabolites from marine derived fungi.

(5 µm, 4.6 × 150 mm) column using a mobile phase consisting 1% (v/v) formic acid in aqueous solution: acetonitrile: 2-propanol (70:22:8), pH 2.5; flow rate: 0.75 ml/min, temperature: 30˚C, UV detection at 320 nm; Agilent technologies 1200 series [41].

3. Result

The total phenolic and flavnoids content in different fungal extracts.

The highest total phenolic content goes to *G. dankaliensis* while total flavnoids content the higher activity recorded belong to *Engyodontium album*.

3.1. Result of Lipid Preoxidation Inhibition Using TBARS Assay in Different Fungal Extracts

The higher activity in the inhibition of lipid preoxidation assay belongs to *Chaetomium globosum* and the lowest activity goes to *G. dankaliensis*.

3.2. Chemical Composition in Different Fungal Extract

The result of chemical copostion showed that *G. dankaliensis* and *Trichurus spiralis* had the highest sulfur ratio than the other three fungal extracts.

3.3. Result of Antioxidant Capacity Using DPPH Assay in Different Fungal Extracts Comparing to Ascorbic Acids

The result of total antioxidant by DPPH assay show that *G. dankaliensis* came first by recording 59.28% in DPPH 49.3% comparing with ascorbic acid (73.12%).

3.4. Result of NO Inhibition Assay in Different Fungal Extracts Comparing to Ascorbic Acids

The result of total anti-inflammatory by NO assay show that *C. globosum* have the highest activity from 1 mg concentration up to 5 mg ,where at 6 mg the highest activity goes to *Nigrospora oryzae*.

3.5. Determination of Total Gallic Acid Content by HPLC

The result of total gallic content in 10 mg show that the highest concentrations belong to *Engyodontium album*. While gallic acid concentration in *C. globosum* at 10 mg recorded zero content.

4. Discussion

In recent decades an increasing tendency towards the use of natural substances instead of synthetic ones has been obtain comparison to natural substances, it will take long time for them to complete their natural cycles and return to nature; thus causing a lot of environmental pollution. Also with the increase in the price of raw materials, the problem of cost benefits for chemical production is becoming more considerable. Natural antioxidants are ginning importance, due to their benefits for human health, decreasing the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecular oxidation [42-44]. Their uses preserving food additives [45].

The antioxidant field receives increased interest and currently about 10,000 articles per year concern antioxidants of various kinds (ISI Web of Knowledge). Oxidation reactions are a major concern for the food industry, and antioxidants are widely needed to prevent oxidative changes in food. But the need for antioxidants for use in other oxidisable goods, such as cosmetics and pharmaceuticals is also on the rise.

Polyphenolic compounds are widely distributed in plant kingdom [46], reports indicate that there is a positive relation between total phenolic content and antioxidant activity in many plant species [47]. Phenolic compounds in plants are viewed as powerful *in vitro* antioxidants due to their ability to donate hydrogen or electrons and to form stable radical intermediates [48]. For small marine antioxidants novel discoveries are increasingly reported [49,50]. Interestingly, antioxidants found in terrestrial plants (catechin, gallic acid, catechol, morin, rutin) were reported present, inhibit reactive oxygen species (ROS) generation through anti-inflammatory effects [51], and that with agreement with our result as in **Figureas 3-7** using DPPH assay as a model for oxidative stress our extracts show antioxidant activity with very low concentration range from 1 mg to 6 mg, as well as scavenge free radicals as in **Figures 8-11** as well as lipid preoxidation inhibition using TBARS inhibition model as in **Figure 12** [51,52].

Our study show that high inhibition activity comparing

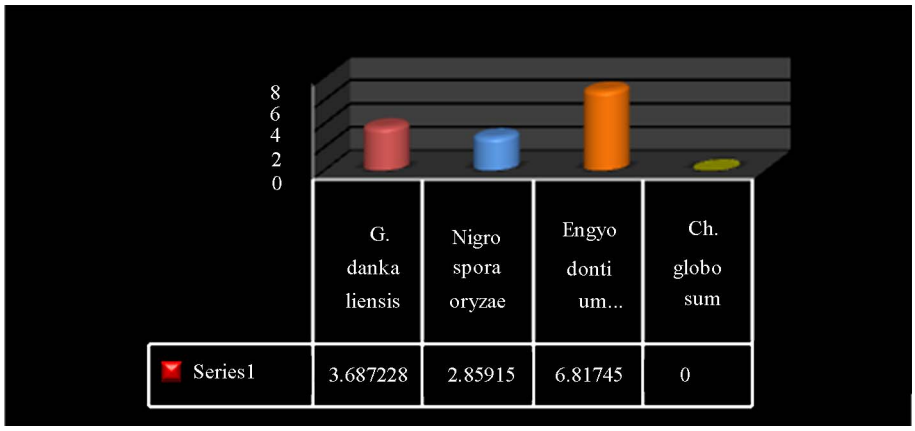


Figure 3. Show that the gallic acid content in different fungal extract by HPLC.

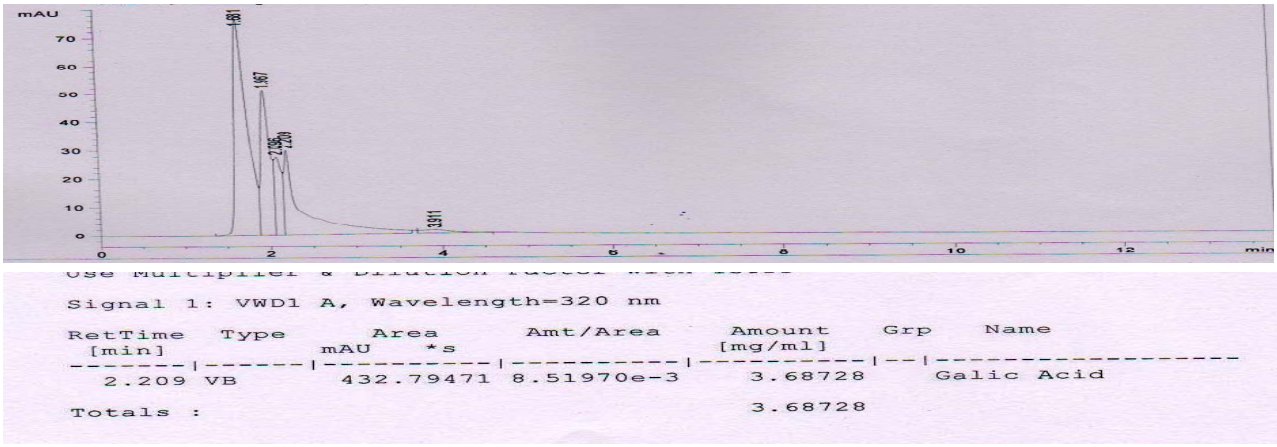


Figure 4. HPLC chromatogram shows the result of gallic acid in *G. dankaliensis*.

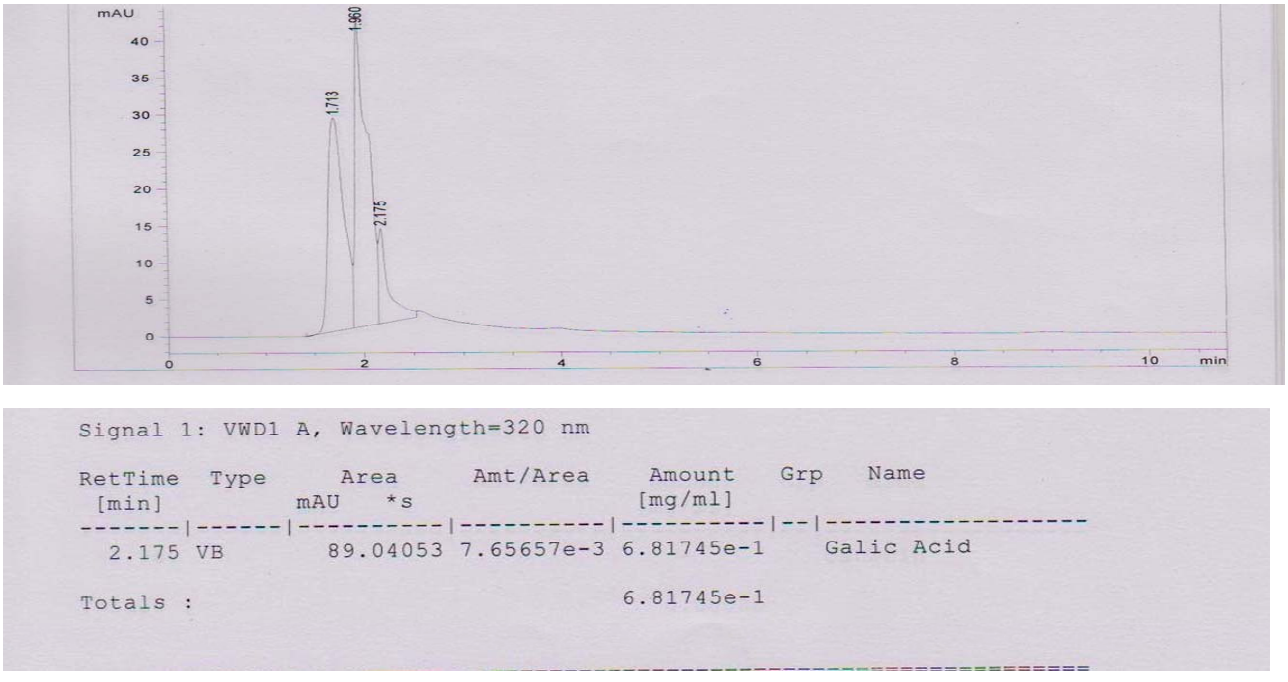
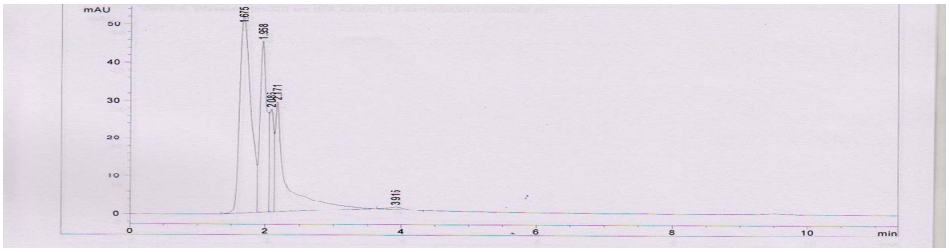


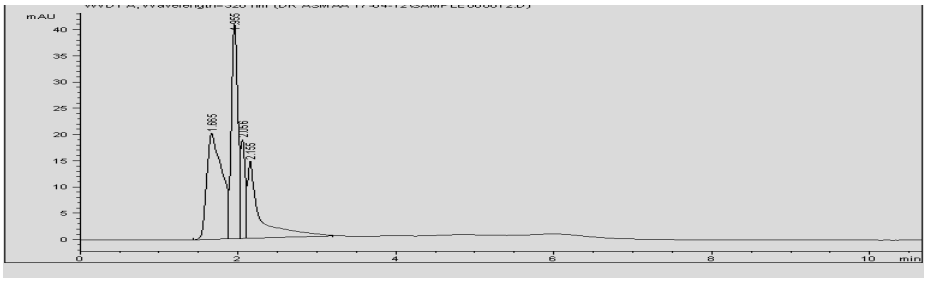
Figure 5. HPLC chromatogram shows the result of gallic acid in *Nigrospora oryzae*.



Signal 1: VWD1 A, Wavelength=320 nm

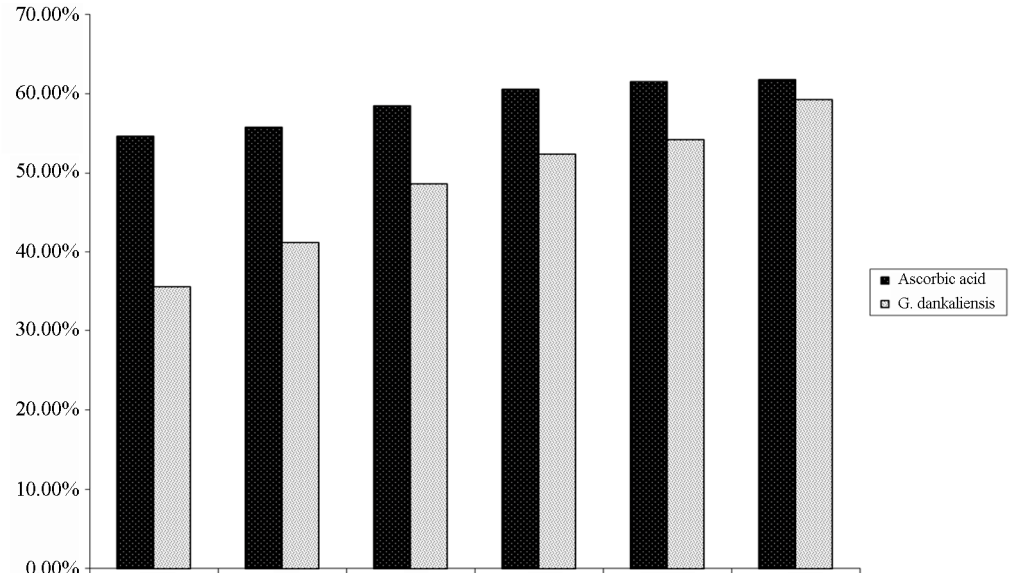
RetTime [min]	Type	Area mAU *s	Amt/Area	Amount [mg/ml]	Grp	Name
2.171	VB	338.07870	8.45706e-3	2.85915		Galic Acid
Totals :				2.85915		

Figure 6. HPLC chromatogram shows the result of gallic acid in *Engyodontium album*.



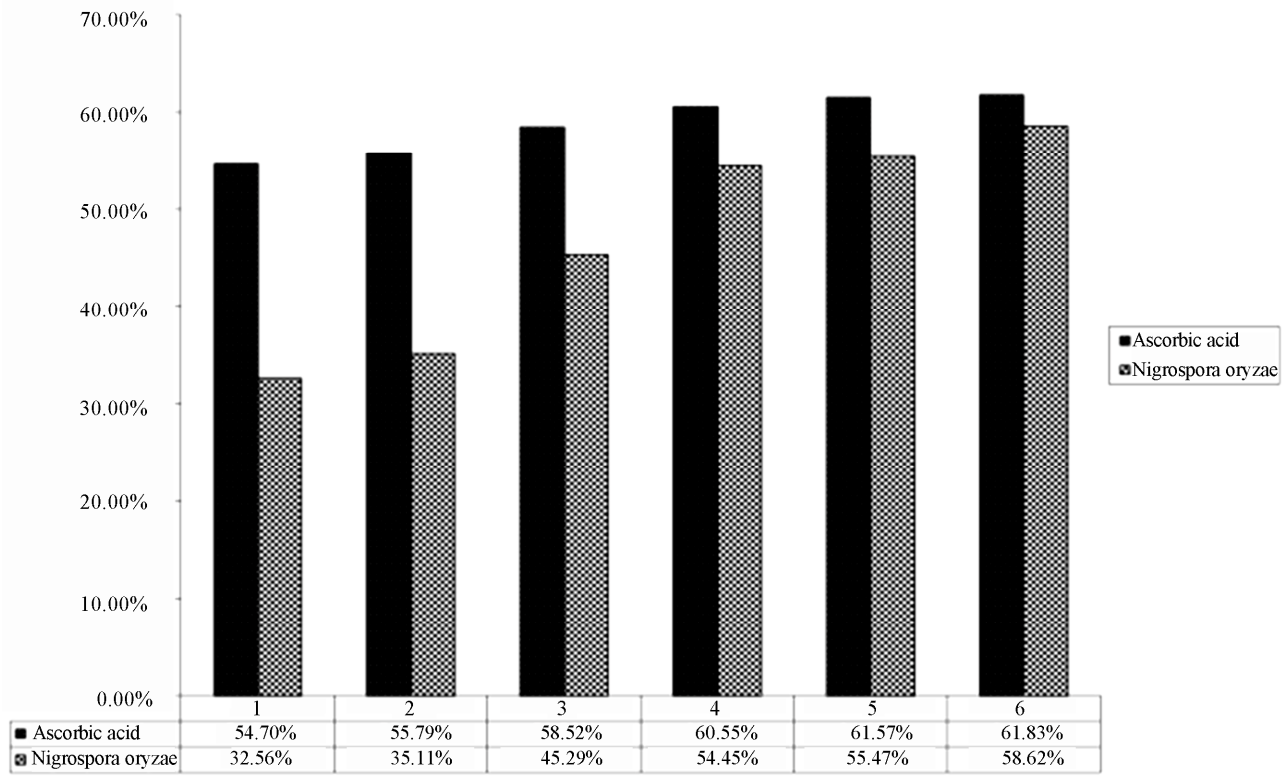
RetTime [min]	Type	Area mAU *s	Amt/Area	Amount [mg/ml]	Grp	Name
2.220		-	-	-		Galic Acid
Totals :				0.00000		

Figure 7. HPLC chromatogram shows the result of Gallic acid in *Chaetomium globosum*.



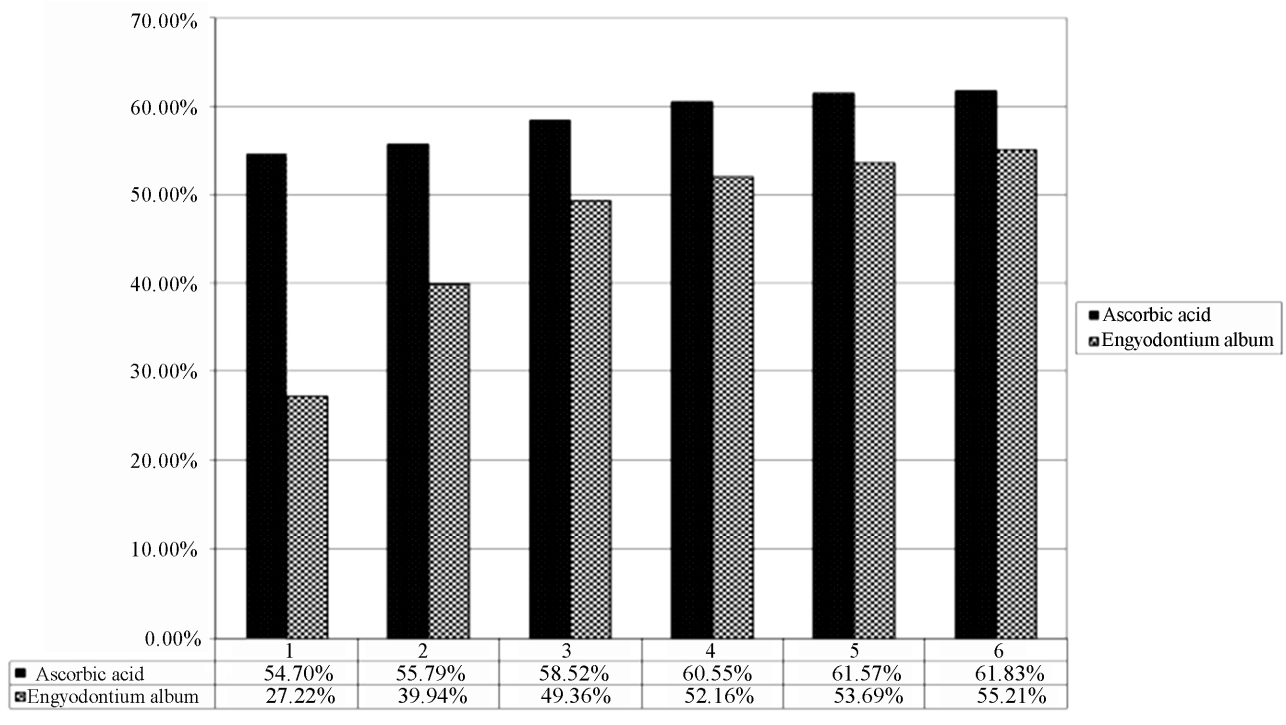
	1	2	3	4	5	6
Ascorbic acid	54.70%	55.79%	58.52%	60.55%	61.57%	61.83%
G. dankaliensis	35.62%	41.22%	48.60%	52.41%	54.19%	59.28%

Figure 8. Show the DPPH inhibition assay by *G. dankaliensis*.



	1	2	3	4	5	6
Ascorbic acid	54.70%	55.79%	58.52%	60.55%	61.57%	61.83%
Nigrospora oryzae	32.56%	35.11%	45.29%	54.45%	55.47%	58.62%

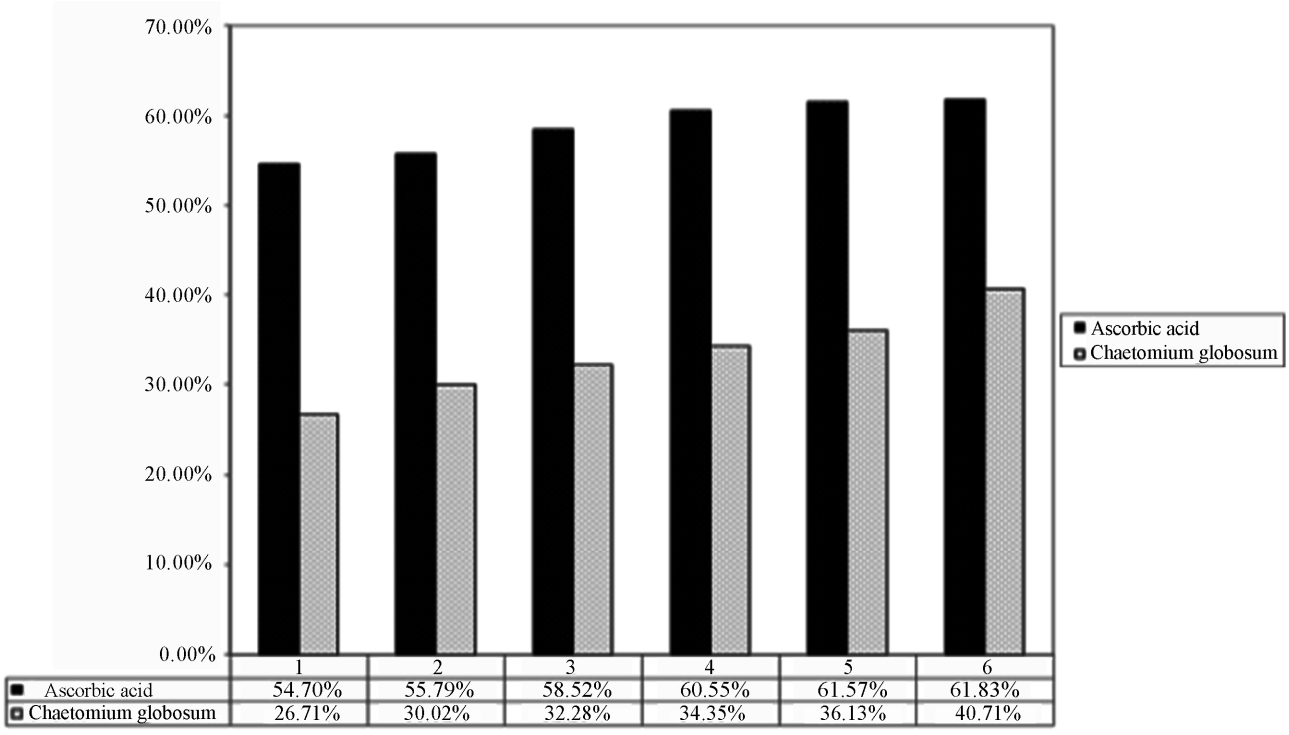
Figure 9. Show the DPPH inhibition assay by *Nigrospora oryzae*.



	1	2	3	4	5	6
Ascorbic acid	54.70%	55.79%	58.52%	60.55%	61.57%	61.83%
Engyodontium album	27.22%	39.94%	49.36%	52.16%	53.69%	55.21%

Figure 10. Show the DPPH inhibition assay by *Engyodontium album*.

with ascorbic acids and regard to low concentration used in our study, this activity may be attributed to the high content of total phenolic and flavnoids which act as excellent antioxidant, as shown in **Figures 13** and **14**. where the best-described property of almost every group of flavonoids is their capacity to act as antioxidants. Flavonoids



	1	2	3	4	5	6
Ascorbic acid	54.70%	55.79%	58.52%	60.55%	61.57%	61.83%
Chaetomium globosum	26.71%	30.02%	32.28%	34.35%	36.13%	40.71%

Figure 11. Show the DPPH inhibition assay by *C. globosum*.

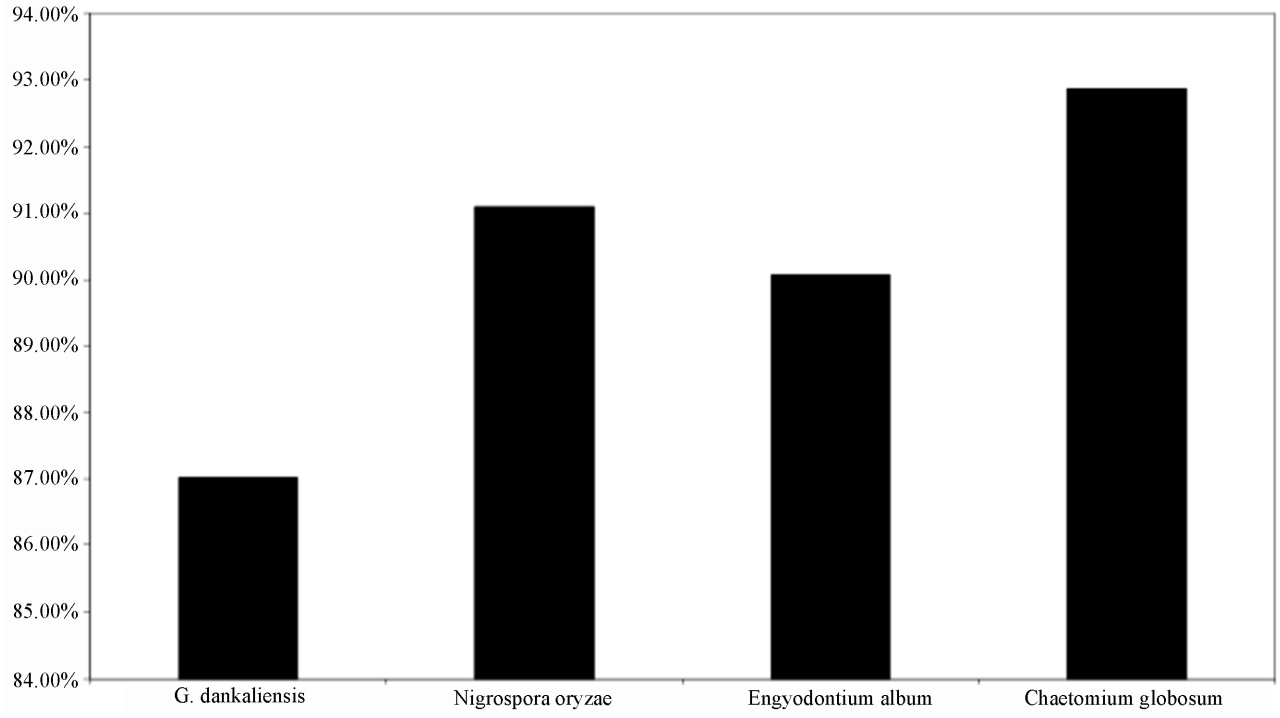


Figure 12. The inhibition of lipid preoxidation of different fungal extracts.

are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive, according to the following equation; Flavonoid (OH) + R• > flavonoid (O•) + RH [53], and that can explain the antioxidant capacity of our extracts, due

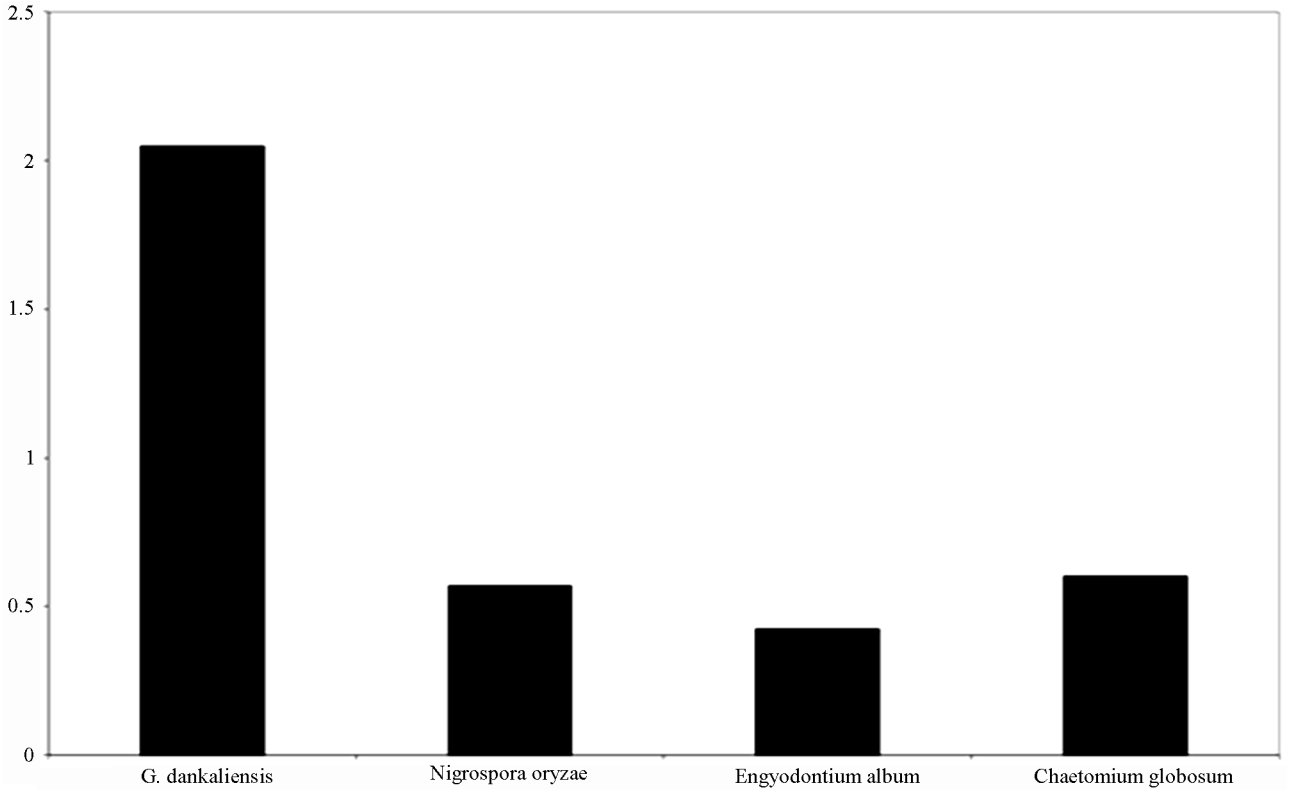


Figure 13. Total phenolic content in different fungal extract.

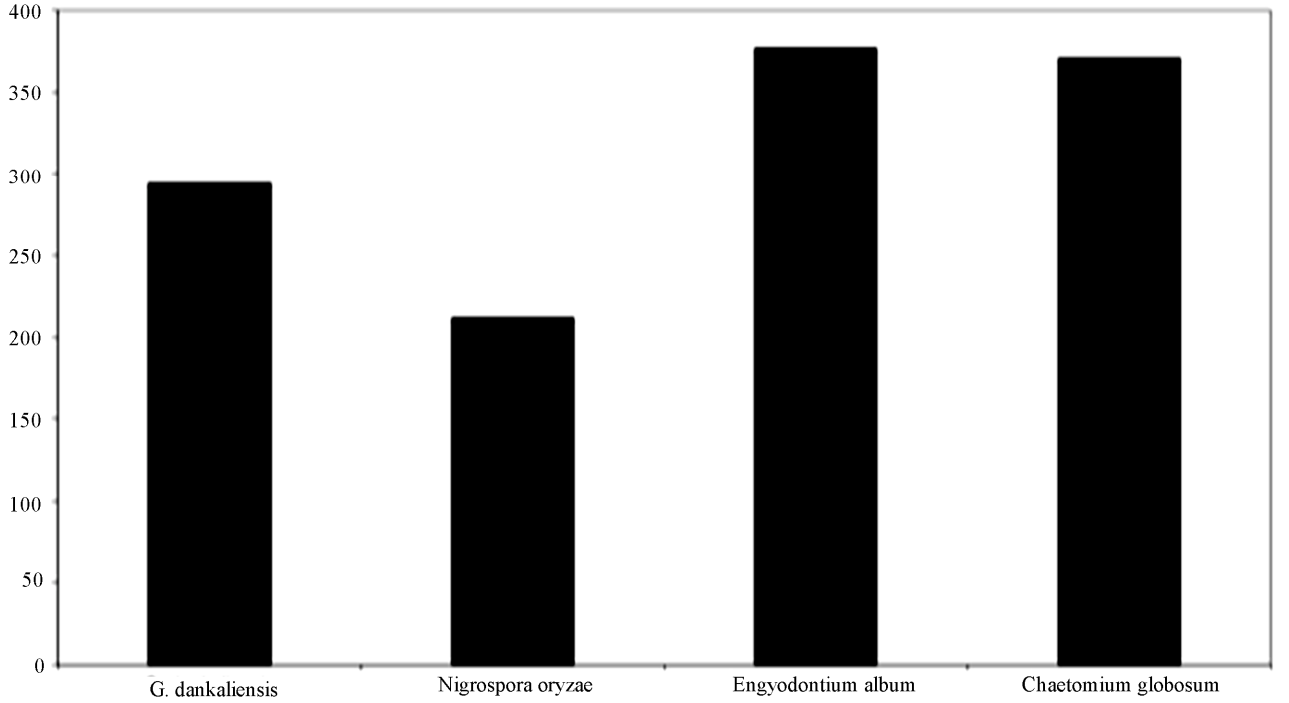
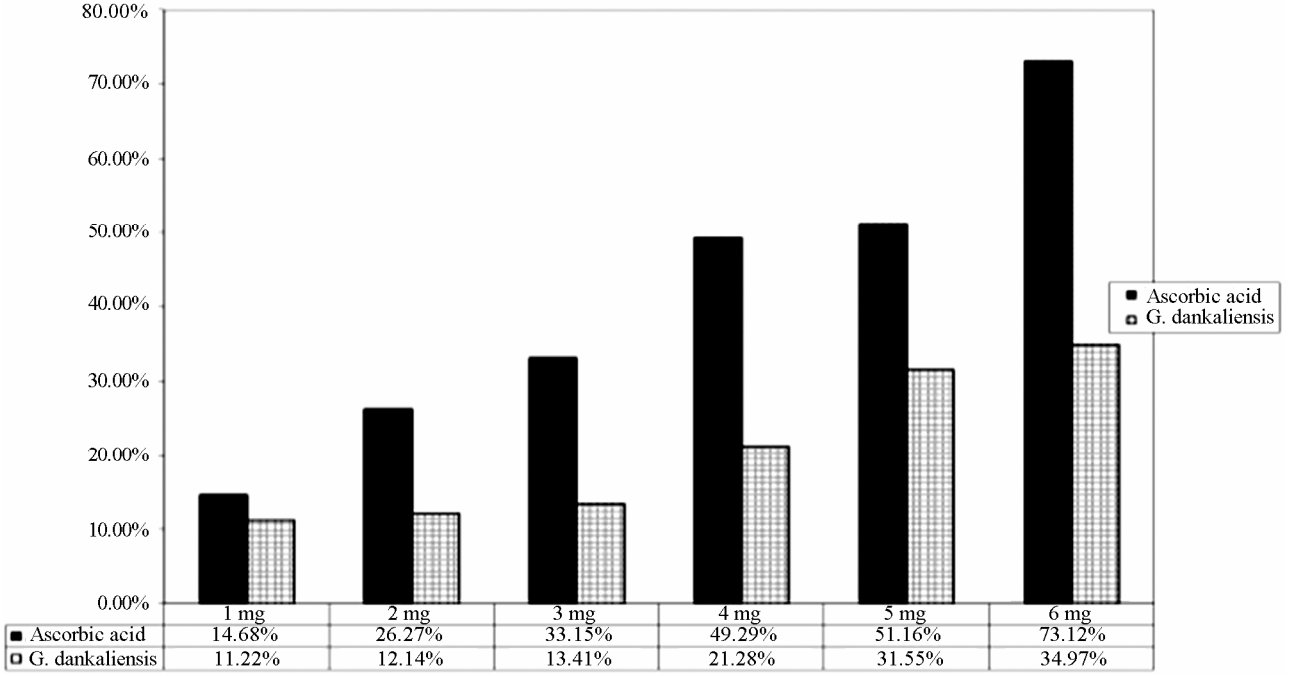


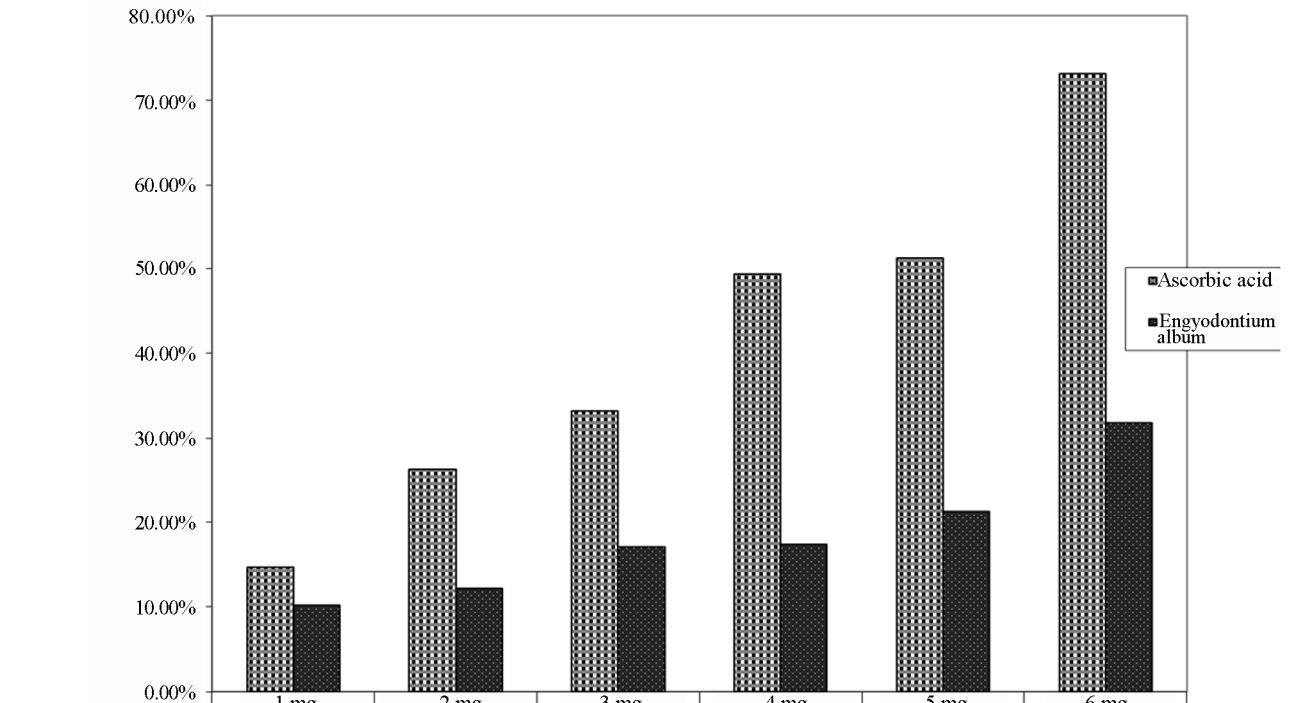
Figure 14. Total flavnoids content different fungal extracts.

to their flavnoids content. also the scavenger of nitric oxide (anti-inflammatory effect) show in **Figures 15-18** can be attributed to the total phenolic and flavnoids content, as The same in case of scavenger of nitric oxide as nitric oxide reacts with free radicals, thereby producing the highly damaging peroxynitrite. Nitric oxide injury



	1 mg	2 mg	3 mg	4 mg	5 mg	6 mg
Ascorbic acid	14.68%	26.27%	33.15%	49.29%	51.16%	73.12%
G. dankaliensis	11.22%	12.14%	13.41%	21.28%	31.55%	34.97%

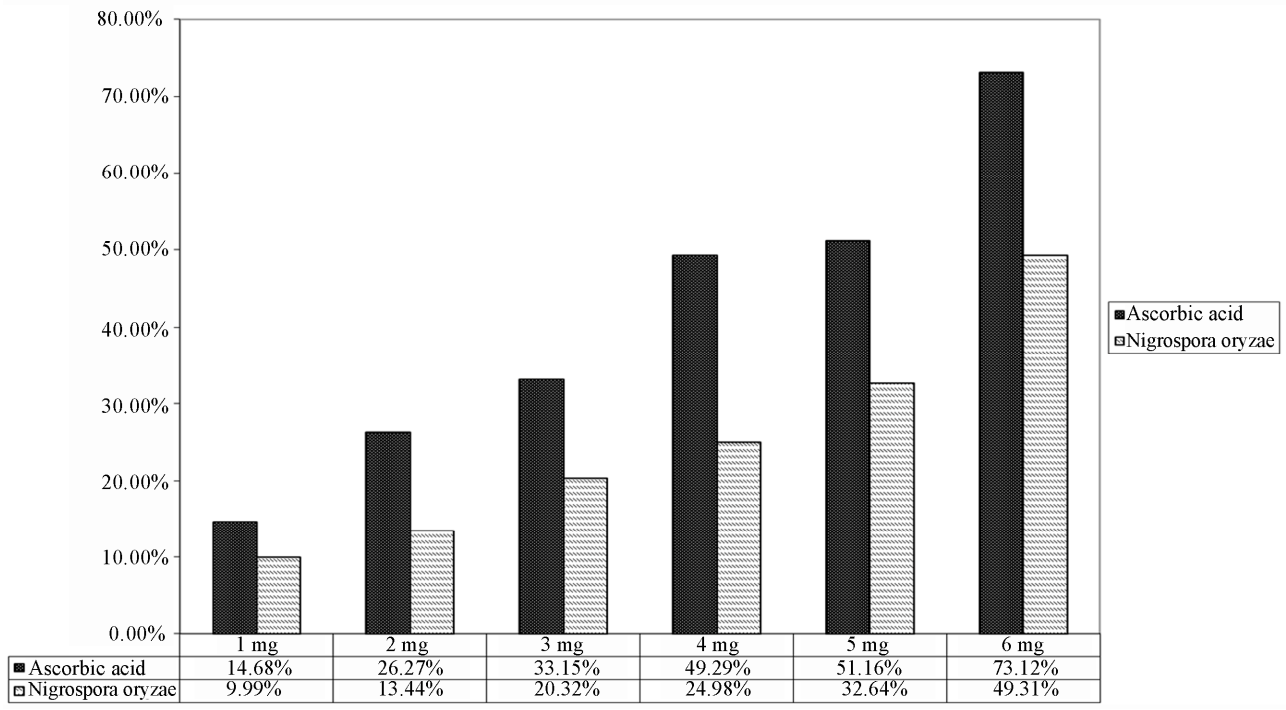
Figure 15. Show the inhibition of NO by *G. dankaliensis* fungal extract.



	1 mg	2 mg	3 mg	4 mg	5 mg	6 mg
Ascorbic acid	14.68%	26.27%	33.15%	49.29%	51.16%	73.12%
Engyodontium album	10.13%	12.11%	17.07%	17.38%	21.28%	31.75%

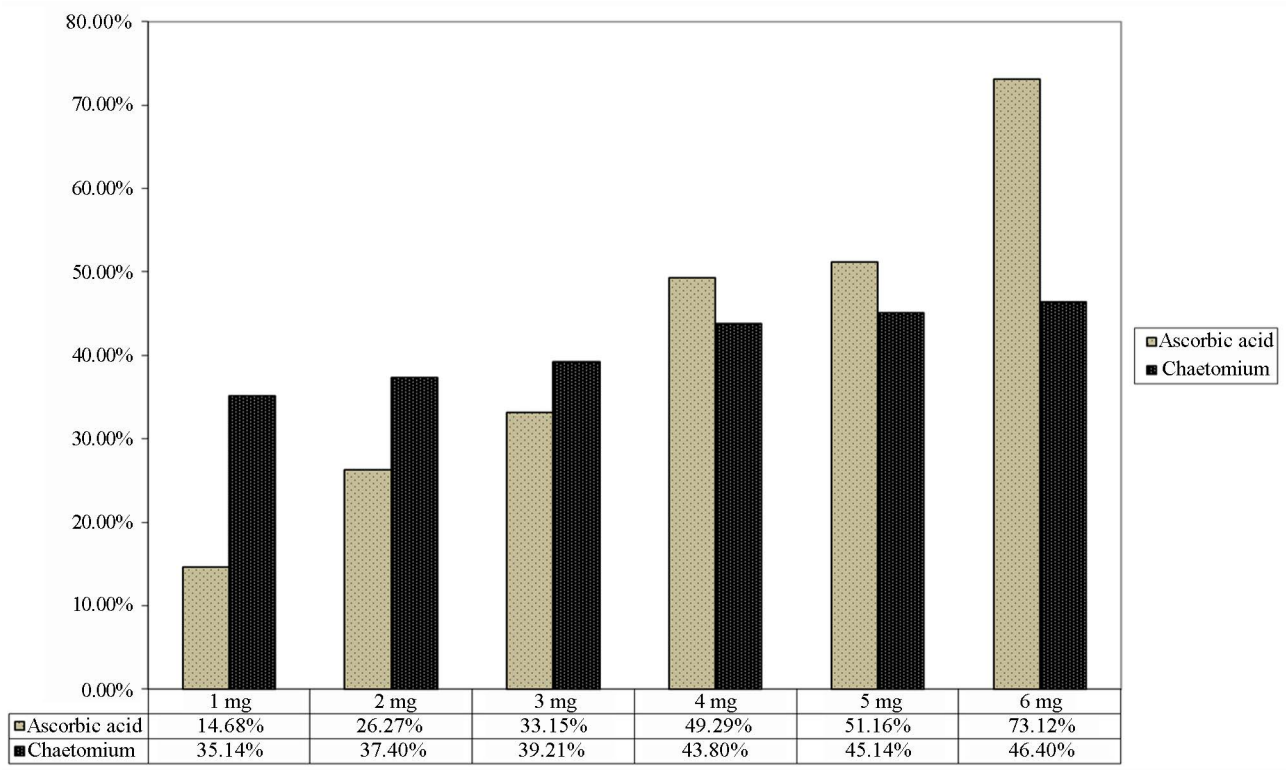
Figure 16. Show the inhibition of NO by *Nigrospora oryzae* fungal extract.

takes place for the most part through the peroxynitrite route because peroxynitrite can directly oxidize LDLs, resulting in irreversible damage to the cell membrane. When flavonoids are used as antioxidants, free radicals are scavenged and therefore can no longer react with nitric oxide, resulting in less damage [54]. Interestingly, nitric oxide can be viewed as a radical itself, and it is was reported that nitric oxide molecules are directly scav-



	1 mg	2 mg	3 mg	4 mg	5 mg	6 mg
Ascorbic acid	14.68%	26.27%	33.15%	49.29%	51.16%	73.12%
Nigrospora oryzae	9.99%	13.44%	20.32%	24.98%	32.64%	49.31%

Figure 17. Show the inhibition of NO by *Engyodontium album* fungal extract.

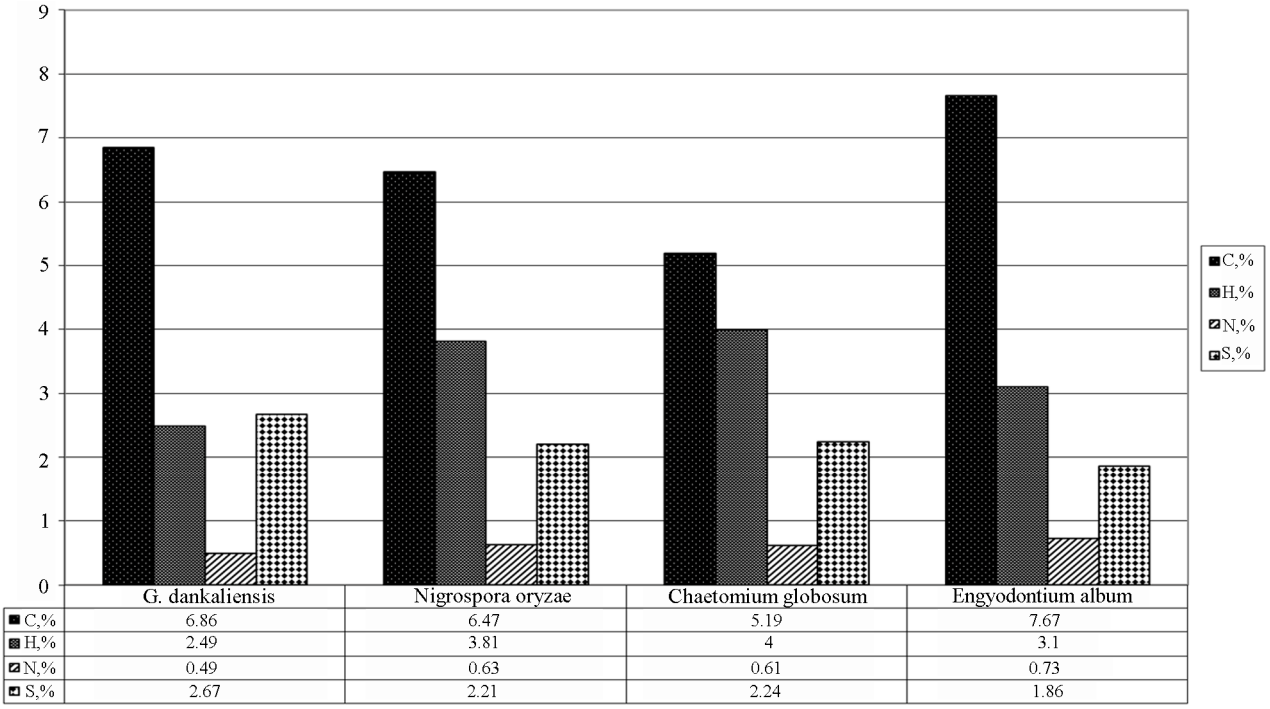


	1 mg	2 mg	3 mg	4 mg	5 mg	6 mg
Ascorbic acid	14.68%	26.27%	33.15%	49.29%	51.16%	73.12%
Chaetomium	35.14%	37.40%	39.21%	43.80%	45.14%	46.40%

Figure 18. Show the inhibition of NO by *C. globosum* fungal extract.

enged by flavonoids [55].

Although we can attributed the antioxidant activity and anti-inflammatory effect of different fungal extracts, this may be due to the marine chemodiversty, which is also heightened by their composition of sea water itself where, concentration of halides in sea water of 1900 mg/L



	G. dankaliensis	Nigrospora oryzae	Chaetomium globosum	Engyodontium album
C,%	6.86	6.47	5.19	7.67
H,%	2.49	3.81	4	3.1
N,%	0.49	0.63	0.61	0.73
S,%	2.67	2.21	2.24	1.86

Figure 19. Show % of C, N, H, S in different fungal extract where C: carbon, H: hydrogen, N: nitrogen, S: sulphur.

Cl^-, 65 mg/L Br^-, 5×10^{-4} and I/IO^{3-} are reflected by the number of compounds incorporating these elements and sulfated compounds can accounted for by the relatively high concentration of sulfur, 2700 mg /L seawater, and that confirmed in our study as shown in **Figure 19** [56] .

5. Conclusion

In conclusion, we observed beneficial influence of investigated fungal extracts as antioxidants and anti-inflammatory. We could assume that this extracts possess anti-inflammatory property due to their proven antioxidant, free radical scavenging properties, and their high sulfur content. However, further investigations on the structures, of the bioactivity, in the four different fungal extracts, as well as elucidation of compounds responsible for such activities and their effect on different antioxidant application should be carryied on.

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