Antifungal and Antioxidant Effects of Hops (Humulus lupulus L.) Flower Extracts

1Farhad Niknejad, 2Marzieh Mohammadi, 3Morteza Khomeiri, 4Seyed Hadi Razavi, 5Mehran Alami

1Assistant professor, Laboratory Science Research Center, Golestan University of Medical Sciences, Iran.
2MA in Food Microbiology, Faculty of Food Science and Technology, Gorgan University of Agriculture Sciences and Natural Resources, Iran.
3Associate professor, Faculty of Food Science and Technology, Gorgan University of Agriculture Science and Natural Resources, Iran.
4Associate professor, Department of Food Science, Faculty of Agriculture, Tehran University, Iran.
5Assistant professor, Faculty of Food Science and Technology, Gorgan University of Agriculture Science and Natural Resources, Iran.

ABSTRACT

The aim of study: Medicinal plants in traditional medicine, have been an encouraging factor for researchers to concentrate on plant extracts for medical purposes. Hops (Humulus lupulus L.), one of the plants which have been used in traditional medicine. Material and Methods: In the present study ethanolic extract of Hops was tested for its antifungal and antioxidant effects. Antifungal activity was determined using broth microdilution methods against five strains of the most important food spoilage molds. Total phenolics content test was used for measuring antioxidant compounds. The antioxidant potential was evaluated using following in vitro methods: [Reducing Power, Total Antioxidant Capacity and scavenging of DPPH radicals]. Results

INTRODUCTION

Recently, natural plants have received much attention as sources of biologically active substances including antioxidants, antimicrobials, antimitugens and anticarcinogens nutraceuticals. Numerous studies have been carried out on some plants such as rosemary, sage and oregano, which resulted in the development of natural antioxidant formulations for food, cosmetic and other applications [1].

Phenolic compounds, a large group of natural herbal ingredients include flavonoids, tanins, anthocyanins, etc. are seen usually in fruits, vegetables, leaves, nuts, seeds, roots and other parts of plants. These substances have significant interests in food science, chemistry, pharmaceutical and medicine [21]. Flavonoids and other phenolic compounds have a wide distributed in plants and antioxidant, antimicrobial, anti-inflammatory and vasodilator effects were reported in many research study [24,28,21].

Phenolic compounds with antioxidant, antiradical and antimicrobial activity have a vital role in preservation of food products and maintenance human health [12,15,28,21]. The females’ flowers of hops are also in this group and have a wide variety of volatile oils, flavonoids, tanins, etc [5,11,27].

Humulus lupulus L. commonly has known as hops, from Cannabaceae family, one of the plants which have been used in traditional medicine [26]. Humulus lupulus is reputed a central nervous system (CNS) depressant traditionally used to relief insomnia, anxiety, excitability and restlessness associated with tension headache and gastrointestinal spasms [20]. In addition among female pickers of hops there was evidence of menstrual
disturbances and in Germany hops baths have been used to reduce hot flashes in menopausal women. The plant is also thought to promote healthy digestion and to exert diuretic effects [20].

The radical scavenging activity of hops components (humulones and lupulones) has been evaluated previously using a conventional DPPH assay, employing one of the stable nitrogen-centered free radicals. Xanthohumol was also shown to scavenge super oxidanion radicals, hydroxyl and peroxyl radicals in an Oxygen Radical Absorbance Capacity (ORAC) assay [7].

Hops preservative was recognized in the 12th century and it has been used in beer brewing for its ability taste and prevents spoilage [14]. In the hops flowers there is a group of antimicrobial active ingredients such as lupulone and humulone along with their various isomers and lupulone (also known as beta-acid) is the most antimicrobial constituent [14].

In recent years, it has been demonstrated that the free radicals are the most important factors for food oxidizing, elimination of vitamins and essential fatty acids and produce toxic compounds that lead to inflammatory diseases, cancer, diabetes mellitus. Therefore it seems necessary to use natural antioxidants components to slow down the rate of oxidation in foods [21].

Natural plant extracts are a source of safe and effective substitutes for synthetically produced antimicrobial agents that provided an alternative way to prevent food from fungal contamination. Powders and extracts of various essential oils have been reported to have antimicrobial activity and inhibit toxin formation [23].

The present study was undertaken to examine the antifungal activity of hops extract on the growth of food spoilage molds. Antioxidant effects was also determined by various methods such as Iron reducing power, total antioxidant capacity and scavenging of 2,2-diphenyl-1-picryl hydrazyl (DPPH) radicals.

MATERIALS AND METHODS

Preparation Hops Extract:
The flower extract of hops was produced in Giah-Esans Company in Gorgan, North of Iran by percolation method and sterilized by filtration using a 0.22 µm filter membrane after extracted with ethanol 50%. The sample extract stored in sealed vials at 40°C before examination.

Antifungal Activity Assay:
Tests Organisms:
The antifungal activity of the hops extract was evaluated against Aspergillus niger (ATCC 9029), Aspergillus flavus (PTCC5006), Penicillium citrinum (ATCC 38065), Penicillium. chrysogenum (ATCC 11709) and Rhizopus oryza (ATCC 9363) that were obtained from Iranian Research Organization for Science.

Determination of Minimum Inhibitory Concentration (MIC):
Minimal inhibitory concentrations of the hops extract were determined by Broth Microdilution Methods as recommended by the Clinical and Laboratory Standards Institute (CLSI), with some modifications [4,8]. YMB media (0.3g yeast extract, 0.3g malt extract, 0.5g peptone and 1g glucose per litter) purchased from Sigma Aldrich USA. Fungi were sub cultured on to Sabouraud Dextrose Agar at 28°C for 48h. The inoculums were suspended in distilled water containing 0.5% (v/v) tween 80 and adjusted to a final concentration (5×10^5 spores/ml). Serial dilution of hops extract (0.9 to 6 mg/ml) was prepared in sterile microtubes using YMB media and incubated at 28°C for 48h after addition 100µl of hops extract. Negative controls (Blank) and Positive Growth controls (medium with inoculums without the extract) were prepared and growth in each tube was compared with negative control tube. MIC were visually determined and defined as the lowest concentration of the hops extract that produced no visible growth. Each experiment was performed in triplicate.

In order to determine the Minimum Fungicidal Concentration (MFC), 5µl of all tubes with no visible growth were cultured on SDA plates and incubated at 28°C for 3.

Antioxidant Activity Assay:
Antioxidant property of the hops extract was evaluated using three In vitro assays: Reducing power capacity, total antioxidant capacity and scavenging of DPPH radicals.the assays were carried out in three sample replication and value were represented as the average of the three replication.

Chemical Material:
DPPH, BHT and Gallic acid were obtained from Sigma chemical company (St. Luis, USA). SDA Culture media were obtained from Merck Company (Darmstadt, Germany) and all the reagents were analytical grade.

Determination of totals phenolic content:
Totals phenolic content was determined with the Folin Ciocalteu (FC) reagent as previously described [10]. Each test tube containing 100µl ethanolic hops extract, 2.8 ml distilled water and 100µl Folin-Ciocalteu as a reagent was mixed and followed by addition of 2ml Na₂CO₃ solution (%2). All tubes allowed standing at room
temperature in the dark for 30 min and absorbance was measured at 750 nm. Gallic acid was used as a standard for calibration curve. The phenolic content was expressed as Gallic acid equivalents using the following linear equation based on the calibration curve:

$$A = 0.0663C + 0.0142$$

A: Absorbance
C: Concentration of Gallic acid (µg/ml)

The experiment was performed in triplicate and the average reported.

**Total antioxidant capacity:**

This assay is based on the reduction of Molybdenum (Mo VI to V) by the sample and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH [17]. An aliquot of 0.1 ml of ethanolic extract of Hops (containing 100–1000 µg of dried extract in corresponding solvent) was combined in an eppendorf tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were incubated at 95 °C for 90 min and absorbance was measured after cooling samples at 695 nm. Tube with 1 ml of reagent solution and the appropriate volume of the same solvent used as blank sample. EC50 (concentration of the extract is absorbed approximately 0.5 at 695nm) was calculated using diagram and synthetic antioxidant BHT was used as a positive control.

**Reducing power assay:**

The reducing power of samples was determined according to the method of Yildirim et al., [25]. The dried extract (100–1000 µg) in 1 ml of the ethanol 50%, was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [K3Fe (CN)6; 10 g/l], and incubated at 50 °C for 30 min. Then 2.5 ml of tri chloro acetic acid (100 g/l) were added and centrifuged at 1650g for 10 min. Finally 2.5 ml of the supernatant solution were mixed with 2.5 ml of distilled water and 0.5 ml of FeCl3 (1 g /l) and absorbance was measured at 700 nm. For comparison, the assay was conducted in the same manner and BHT was added instead of sample solution.

**DPPH radical scavenging activity:**

The ability of extracts to scavenge DPPH radicals was determined according to the method of Blois, [3]. Briefly, 1 ml of a 1mM methanolic solution of DPPH was mixed with 3 ml of extract solution in methanol (containing 100-1000 µg of dried extract/ml). The mixture was then shacked vigorously and left for 30 min at room temperature in dark place. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging relative to control using the following equation:

$$\text{DPPH scavenging activity (%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

EC50 (concentration of the extract causing 50% scavenging) compared to the BHT.

**Statistical Analysis:**

Data were analyzed using means ± standard deviation of triplicate measurements. Analyses of variance were performed by ANOVA test and differences between the means were determined by Duncan’s test and p ≤ 0.05 considered as significant.

**Results:**

**MICs determination:**

The antifungal activities of hops extract against selected fungi are shown in Table 1. The hops extract inhibited the growth of all molds at concentrations 1.875 to 3.25 mg/ml. Furthermore the extract exhibited fungicidal activity (MFC) at concentrations ranging from 3.25 to 5.25 mg/ml. MIC and MFC values were similar at 3.25 mg/ml for A. flavus.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MICs</th>
<th>MFCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger</td>
<td>3.25 mg/ml</td>
<td>3.75 mg/ml</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>3.25 mg/ml</td>
<td>3.75 mg/ml</td>
</tr>
<tr>
<td>Rhizopus oryza</td>
<td>3 mg/ml</td>
<td>5.25 mg/ml</td>
</tr>
<tr>
<td>Penicillium citrinum</td>
<td>2.25 mg/ml</td>
<td>3.75 mg/ml</td>
</tr>
<tr>
<td>Penicillium chrysogenum</td>
<td>1.875 mg/ml</td>
<td>3.75 mg/ml</td>
</tr>
</tbody>
</table>

**Total phenolic content:**

The yield and totals phenolic content for ethanolic extract from hops flowers are shown in Table 2. The amount of phenolic content, using the equation for Gallic acid and according to grams of Gallic acid per 100 g (dry weight) of extract was showed.
Table 2: Antioxidant activity and total phenolic content of hops flower extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TAOC IC50*µg/ml</th>
<th>Reducing power IC50*µg/ml</th>
<th>DPPH IC50†µg/ml</th>
<th>Totals phenolic content↕ grams of Gallic acid per 100 g (dry weight) of extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolic extract</td>
<td>624.40</td>
<td>Nd</td>
<td>284.68</td>
<td>0.134</td>
</tr>
<tr>
<td>BHT</td>
<td>141.59</td>
<td>Nd</td>
<td>112.66</td>
<td>-</td>
</tr>
</tbody>
</table>

* µg/ml
† The effective concentration at which formation of a green phosphate/Mo (V) complex by 50% (phosphomolybdenum assay)
† The effective concentration at which DPPH radicals were scavenged by 50%
↕ Grams of Gallic acid per 100 g (dry weight) of extract (Totals Phenolic)

Values followed by different letters (a, b) within each column are significantly different (p ≤ 0.05)

**Total antioxidant capacity:**

In the phosphomolybdenum assay, which is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity), the extract exhibited some degree of activity in a dose-dependent manner; however, the activities was less than of BHT (Fig. 1, Table 2).

![Fig. 1: Total antioxidant activities of ethanolic extract of hops flower, BHT was used as positive control. Columns followed by different letters for each concentration are significantly different (p ≤ 0.05)](image)

**Reducing power:**

Different studies have indicated that the electron donation capacity (reflecting the reducing power) of bioactive compounds is associated with antioxidant activity [22]. During the reducing power assay, the presence of reductions in the tested samples result in reducing Fe3+ ferricyanide complex to the ferrous form (Fe2+). The Fe2+ can therefore be monitored by measuring the formation of Perl’s Prussian blue at 700 nm [25]. Reducing power of extract increased with the high concentration of reducing agents in extract (Fig. 2), it reached to 0.377 of absorbance at 700 nm with concentration of 0.7 mg/ml, but increased slightly afterwards. This result revealed that hops extract could function as electron donors, but its reducing power was inferior to that of BHT (Fig. 2, Table 2).

**DPPH radical scavenging activity:**

DPPH radical-scavenging activity increased with concentration of hops extract. This activity increased drastically at the low concentration of hops extract but it nearly reached a plateau (Fig. 3, Table 2). DPPH radical was scavenged by hops extract with donation of hydrogen to form a stable DPPH-H molecule. The colour change from purple to yellow by acceptance of hydrogen radical from hops extract and it become a stable diamagnetic molecule [3]. Either hops extract can function as hydrogen donors, as can be seen from Table 2, the DPPH radical-scavenging activity of hops extract was less strong than BHT.
Fig. 2: Reducing powers of ethanolic extract of hops flower, BHT was used as positive control. Columns followed by different letters for each concentration are significantly different (p ≤ 0.05)

Fig. 3: DPPH radical scavenging activities of ethanolic extract of Hops flower. BHT was used as positive controls.
*Percentage radical scavenging capacity relative to control
Columns followed by different letters for each concentration are significantly different (p ≤ 0.05)

Discussion:
In present study, the hops extract exhibited fungistatic and fungicidal activities against of the tested fungi, which can be considered as a good result in compared to the previous investigation [9,16]. MFCs of the extract were almost similar or two times greater than those of their corresponding MICs (table 1).

Appropriate use of medicinal plants, requires accurate information and knowledge about chemical compounds in them because these compounds causes health effects in plants [21]. The antimicrobial activity of herbal extracts and their inhibitory effects varies widely and depends on the type of extract, extraction method and testing methods used to evaluate of the antimicrobial activity [19]. Phenolic compounds and bitter acids in hops female flowers extract consist of humulone, lupulone, xanthohomol and 8-prenyl narengenin can be major antimicrobial agents in the extract [13]. Atei [2] detected active ingredients in ethanolic extract of dried hops flower by GC-MS analyse and showed that the extract contains α-pinene, α-Humulen, β-myrcene and α – Limonene. α-Humulen is the most important antifungal composition of the hops extracts.
In contrast, Niknejad [16] showed that ethanolic extract of hops flower could inhibit the growth of Trichophyton mentagrophytes, Microsporum canis, Epidermophyton floccosum at 3 mg/ml and complete growth inhibition of Microsporum gypseum was showed at 2mg/ml. The growth inhibition of Trichophyton rubrum and Trichophyton verrucosum was observed at 5 mg/ml and 7mg/ml of the extract mostly prevented Aspergillus niger sporulation but had no activity against Candida albicans. Kasra-Kermanshahi, et al., [9] found that ethanolic extract of hops flower could inhibit the growth of Gram-positive bacteria included: Bacillus subtilis at 125mg/ml and Staphylococcus aureus at 62.5 mg/ml. However there were no reports about effects of other parts of hops plant, such as stem or leaf.

The key role of phenolic compounds as free radicals eliminating has been reported in several studies [18,6,21]. It should be noted that these compounds effectively act as a hydrogen donor and therefore are identified an active antioxidant [21].

Many factors may affect the amount of phenolic compounds such as genetic, species, varieties, amount of sunlight, soil conditions, degree of maturity and climate condition at harvesting time, climate, and post-harvest operations for storage.

The solubility of phenolic compounds varies and depending on the type of solvent, degree of polymerization and the interaction with other plant chemicals [6].

The total antioxidant activity of plant foods is the result of individual activities of each of the antioxidant compounds present such as vitamin C, tocopherols, carotenoids and phenolic compounds, the latter being the major phytochemicals responsible for antioxidant activity of plant materials. Moreover, these compounds render their effects via different mechanisms such as radical scavenging, metal chelation, inhibition of lipid peroxidation, quenching of singlet oxygen and so on to act as antioxidants. Even if a sample exhibits high activity with one of these methods, it does not always show similar good results with all other methods. Therefore, it is essential to evaluate samples accurately by several methods [1].

Generally reduction features are associated with the presence of electron donor compounds. In other words, with the increasing phenolic compounds in the extract, rises the reduction activity and as a result the extract will be able to donate more electrons, broken the chain reaction of free radicals formation and would delay the oxidation [6].

In DPPH radical-scavenging activity, along with increasing concentration of phenolic compounds, due to increase hydroxyl groups in the reaction medium, probability to donate hydrogen to free radicals and consequently increase the power of scavenging effect of the extract [6].

Probably due to a state of saturation at very high concentration of the extract, increasing the concentration has no significant effect on the amount free radicals were scavenged by the extract and a critical concentration of the phenolic compounds is sufficient to scavenge DPPH radicals [6].

Usually, IC50 is used to compare the anti-radical activity that refers to a concentration of the extract which is scavenged 50% of DPPH radicals. Thus whatever this value is lower shows the more anti-radical effect [18].

Ethanolic extract from hops flower showed antioxidant activity in different test systems in a dose-dependent manner. The antioxidant activity was correlated with the amount of total phenolic present in the extract in each assay. Based on the reducing power, total antioxidant activity and radical-scavenging activity of hops extract, it could possibly be used as antioxidant in food products.

As the food industry tend to reduce the use of chemical preservatives and synthetic antioxidants in food formulations, hops extract with active potential of antimicrobial and antioxidant properties might be considered as a natural source for the maintenance or extension of the shelf-life of products. As these tests have all been in vitro, the next step may be further investigations in food products to see if spoilage can be delay by hops extract.

ACKNOWLEDGMENT

The results described in this paper were part of MS Thesis. The Authors extend their appreciation to Dr Soleymani chief of Giah-Essence Pharmaceutical Company in Gorgan, North of Iran for prepare hops extract.

REFERENCES


