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Cell growth inhibitory potential of *Craterellus cornucopioides (L.)* Pers. together with antioxidant and antimicrobial properties

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Antioksidan ve antimiktobiyal özellikleri ile birlikte *Craterellus cornucopioides (L.)* Pers. türünün hücre büyümesi baskılama potansiyeli

Abstract: *Craterellus cornucopioides (L.)* Pers which is also known as trumpet of death or horn of plenty, is a wild edible macrofungus. This study was conducted to elucidate the potential health beneficial properties of *C. cornucopioides*. Bioactive ingredients (phenolics, flavonoids, β-carotene and lycopene) and DPPH radical scavenging activities were determined. Additionally, cell growth inhibitory effects on HepG2 cells together with some bacteria were evaluated. Accordingly, water and methanol extracts contains 37.71±1.42 µg/mg and 13.78±1.60 µg/mg phenolic contents, respectively. Similarly, methanolic extracts have higher β-caroten and lycopene content as compared to aqueous extracts. In parallel with these antioxidants, methanolic extracts have also higher DPPH scavenging activity (IC_{50}: 5.26±0.67 mg/ml). Besides, water extracts have higher flavonoid contents (2.13±0.06 µg/mg) then the methanolic extracts. *C. cornucopioides* has also an important cell growth inhibitory effects on HepG2 cell (IC_{50}: 18.41±1.10 mg/ml for aqueous extracts and IC_{50}: 3.14±1.07 mg/ml for methanolic extracts). Moreover, both extracts were effective on six different bacteria tested. As a result, this study indicates that *C. cornucopioides* could reduce the cellular oxidative stress because of its high antioxidant ingredients, inhibit the growth of pathogen microrganisms and have some degree of cell growth inhibitory potential at least to the HepG2 cells.

Key words: *Craterellus cornucopioides*, antioxidant, antibacterial, cytotoxicity, HepG2

Özet: Ölüm trompeti veya bolluk boynuzu olarak da bilinen *Craterellus cornucopioides (L.)* Pers, yenilebilir bir makrofungustur. Bu çalışma *Craterellus cornucopioides (L.)* Pers mantarının sağlık açısından yararlı özelliklerini açığa çıkarmak amacıyla yapılmıştır. Çalışmada ilgili mantarın biyoaktif içerikleri (fenolikler, flavonoidler, β-karoten ve likopen) ve DPPH radikal süpürücü aktiviteleri belirlenmiştir. Ek olarak, HepG2 hücreleri ve bazı bakteri türleri üzrine hücre büyümesini baskılayıcı etkileri değerlendirilmiştir. Buna göre, su ve metanol ekstraktları sırasıyla 37.71±1.42 µg/mg ve 13.78±1.60 µg/mg fenolik içeriğe sahiptir. Benzer şekilde, metanol ekstraktları sulu ekstrelere kıyasla daha yüksek β-karoten ve likopen içeriğine sahiptir. Bu antioksidanlara paralel olarak, metanol ekstraktları da DPPH süpürme aktivitesine daha fazla sahiptir (ICso: 5.26±0.67 mg/ml). Ayrıca su ekstraktları, metanol ekstraklarına göre daha yüksek flavonoid içeriğine (2.13±0.06 µg/mg) sahiptir. *C. cornucopioides*, HepG2 hücresi üzerinde de önemli bir hücre büyümesi engelleyici etkiye sahiptir (IC_{50}: su ekstratları için 18.41±1.10 mg/ml ve metanol ektraktları için IC_{50}: 3.14±1.07 mg/ml). Buna ek olarak, her iki ekstrakt da test edilen altı farklı bakteri türü üzeine etkili olmuştur. Sonuç olarak, bu çalışma *C. cornucopioides*'in yüksek antioksidan bileşenleri nedeniyle hücresel oksidatif stresi azaltabildiğini, patojen mikroorganizmalarının büyümesini inhibe edebileceğini ve en azından HepG2 hücrelerine bir miktar hücre büyümesi inhibitör potansiyeline sahip olduğunu göstermektedir.

Anahtar Kelimeler: *Craterellus cornucopioides*, antioksidan, antibakteriyel, sitotoksisite, HepG2

1. Introduction

Fungi are eukaryotic and heterotrofic organisms that are composed of tubular filamentous cells, free chlorophyll and create spores. They could not produce their own foods thus which live as saprophyts, mycorrhizal and parasite. Edible mushrooms contain macro-molecules, which are normally 19 to 35% protein, and all protein content almost comprises essential amino-acids. They also include polyunsaturated fatty acids (72-85%) and carbohydrates (51-88%) according to dry or fresh weight (Chang et al., 1996). Medicinal mushrooms have important therapeutic features and, due to these features, they have been used against many kinds of disease for treatment in traditional medicine. For example, many genre mushroom such as *Agaricus, Aleurodiscus, Clitocybe, Coprinus, Daedalea, Ganoderma, Lentinula, Merulius, Pleurotus, Polyporus, Poria, Psathyrella* and *Tricholoma* rise in value due to their major properties such as anti-microbial (Chang et al., 2012), anti-viral (Pan et al. 2013), anti-oxidant (Palacios et al., *2011*), anti-cancer (Mattila et al., 2000).

Free radicals have been generated by many biological pathways or infections in organisms, damaging the cellular components such as organelles (Yıldız et al., 2015, Manivannan et al., 2011) and they are quenched by antioxidant molecules or related antioxidant enzymes. Mushrooms gain importance according to rich bioactive compounds such as polyphenols, polysaccharides, vitamins, carotenoids and minerals (Kozarski *et al*. 2015, Cheung et al., 2002). Studies have shown that antioxidant rich foods might play an important role in reducing the risk of disease, such as cardiovascular diseases, stroke and cancer (Gan et al., 2013, Barros et al., 2007; Jagadish et al. 2009). Therefore, antioxidants in edible mushrooms might act against reactive oxygen species and contribute to create antioxidant responses. Macrofungi also gain importance in cancer inhibition and treatment with the secondary metabolites found in their structure. Moreover, they also could be used against microbial infections. Some metabolites might prevent the growth of certain bacterial and fungal pathogens (Alves et al., 2012).

For example, applanoxidic acid A isolated from *Ganoderma annulare* (Fr.) Gilbn. has been showed to be effective against *Trichophyton mentagrophytes*. Moreover, 5a-ergosta- 7,22-dien-3b-ol 5,8-epidioxy-5a,8a-ergosta-

6,22-dien-3b-ol isolated from *Ganoderma applanatum* (Pers.) Pat., have been shown to affect several gram-positive and gram-negative microorganisms (Smania et al., 1999, Smania et al., 2003). In this study, *C. cornucopioides* was investigated for its antimicrobial, antioxidant, and cytotoxic properties to contribute the studies that are done in pharmaceutical area.

2. Materials and Method

2.1. Preparation of Fungal Extracts

Craterellus cornucopioides (L.) Pers that was used in this study was collected from Trabzon province with a voucher number of Yuzun 1852. The samples are kept at Karamanoglu Mehmetbey University, Kamil Özdağ Science Faculty, Department of Biology. Water and methanol extracts were prepared to examine cytotoxic, antioxidant and antimicrobial effects. Ten grams of entire mushrooms were homogenized by using liquid nitrogen, mortar and pestle and exposed to extraction in 300 ml methanol or distilled water with Soxhlet extraction apparatus. Then, extracts were concentrated in a rotary evaporator, lyophilized and stored at +4 ° C for further use.

2.2. Determination of Total Phenolic Content

Folin-Ciocalteu method was used for the determination of total phenolic content (Taga et al., 1984). Gallic acid (0.02 to 1.00 mM) was used as standard. Fungal extracts (10 mg/ml) and standards (0.02-1.00 mM) were placed in 20 µl microplate wells. Afterwards, 20 µl of Folin reagent (2N) was added and mixed by pipetting. After incubation for 3 min in the dark, 20 µl of 35% (w/v) sodium carbonate and 140 µl of dH_2O were added to the plate and incubated for 10 min in the dark. The absorbance values were recorded against the blank tube at 725 nm and the amount of total phenolic content in one mg extract was calculated using a standard calibration curve generated with gallic acid.

2.3. Determination of Total Flavonoid Content

Total flavonoids of water and methanol extracts were determined according to method (Pal et al., 2010) with slight modifications. A volume of 50 µl of extracts (10 mg/ml) were mixed with 215 µl of ethyl alcohol (80% v/v), 5 µl of aluminum nitrate (10% w/v) and 5 µl potassium acetate (1 M) in microtiter plates and incubated for 40 min at room temperature. After reading at 415 nm, total flavonoid contents were calculated according to following equation:

Total flavonoid contents (µg/mg extract) = (A_{415} + 0.01089)/0.002108

2.4. Determination of β-carotene and Lycopene Contents

Different extracts with water and methanol that were obtained from *C. cornucopioides* were reextracted with 10 ml of acetone:hexane (4:6) mixture and filtered through Whatman No. 4 filter paper to determine β-carotene and lycopene contents. After filtration, absorbance of the filtrates was measured at 453, 505 and 663 nm. β-carotene and lycopene contents were calculated according to following equations.

β-carotene content (mg/100 mg) =0.216 A_{663} - 0.304 A_{505} + 0.452 A_{453}

Lycopene content (mg/100 mg) = −0.0458 A_{663} + 0.372 A_{505} - 0.0806 A_{453}

2.5. Identification and Quantification of Main Phenolic Compounds by HPLC

Phenolic substance identification was carried out with HPLC (Shimadzu LC-20AD system, Japan). Data was processed using LC Solution software (Shimadzu, Japan). As mobile phase; (A) 0.1% (v/v) formic acid and (B) acetonitrile mixtures were used as gradient. Gradient elution conditions are as follows: Starting 20% B; 0-10 min 20% -30% B; 10-40 min 30-40% B; 40-60 min 40% -560% B; 60-80 min 60-80% B; and finally, 90 min from 80% -20% B. The flow rate was set to 1 ml/min, the column temperature was fixed at 30°C. Phenolic compounds were identified by using standards that were in known concentration. Gallic acid, catechin, epicatechin, epigallocatechin gallate, syringic acid, p-coumaric acid, rosmarinic acid, t-resveratrol and quercetin standard curves were constructed and the amount of these phenolics in *C. cornucopioides* extracts were quantitatively determined. At least three applications were performed for each sample (Standard or sample) and sample absorbances were monitored at 271 nm, 280 nm and 309 nm.

2.6. Determination of Reducing Power

After the slight modifications that were made to adopt the method to microplate measurement, reducing powers of different extracts were determined according to the previously prescribed protocol (Sadi et al., 2015). Gallic acid (0.01–0.10 mM) was used as a standard antioxidant. Briefly, in a total volume of 200 µl, various concentrations of 50 µl mushroom extracts (2, 4, 6, 8, 10 mg/ml) were mixed with 75 µl phosphate buffer (0.2 M pH: 6.6),75 µl potassium ferricyanide (1% w/v) and incubated at 50°C for 20 min. After adding 75 µl trichloroacetic acid (10% w/v), samples were centrifuged for 10 min at 1000 *g* and supernatants (75 µl) were transferred to another microtiter plate. Then, they were mixed with 75 µl distilled water and 15 µl iron (III) chloride (0.1% w/v). Spectrophotometric measurements were employed at 700 nm and the effective concentrations (EC_{50}) at which the absorbance was 0.500 for the reducing power was calculated.

2.7. Determination of DPPH Radical Scavenging Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of *C. cornucopioides* was measured (Türkoglu et al., 2007) to determine antioxidant power. Accordingly, different concentration *C. cornucopioides* extracts (0.25 to 10 mg/ml) and gallic acid (0.005 to 0.2 mM) were prepared for measuring the elimination activity of DPPH radical. From *C. cornucopioides* extracts and standards, 20 µl was added to each microplate well and 180 µl DPPH (0.06 mM in methanol) was added. After incubation for 60 min in dark, the reduction of DPPH radical was followed by the absorbance values that were measured at 517 nm. Free radical capturing activities were calculated according to the following formula. The DPPH radical scavenging activity (RSA) was calculated as IC_{50} values for each sample.

$$\text{RSA (\%)} = \frac{\text{DPPH absorbance} - \text{DPPH and extract absorbance}}{\text{DPPH absorbance}}$$

2.8. Determination of cell growth inhibitory potential

2.8.1. Antimicrobial properties

Six test bacteria; *Bacillus subtilis, Enterococcus faecalis, Bacillus licheniformis, Staphylococcus aureus* (ATCC 2921) as Gram Positive; *Agrobacterium tumefaciens* and *Escherichia coli* (0157: H7 ATCC 43895) as Gram negative were grown in liquid Mueller Hinton Broth overnight in a shaker incubator. The concentrations of the microorganisms were adjusted to be equal to 0.5 McFarland standard ($1.5x10^{+8}$ CFU/ml). The empty discs were loaded with 20 µl of stock 200 mg/ml *C. cornucopioides* extracts and placed on petri plates for disk diffusion method. Gentamicin was used as the standard antibiotic. Then *A. tumefaciens* (28°C) and other microorganisms (35°C) were incubated overnight to examine the antimicrobial activity in the petri dishes. At the end of this period, the zones of inhibition around the discs were measured by means of a digital ruler.

2.8.2. Cytotoxic effects on HepG2 cells

HepG2 (human hepatocellular cancer) cells were used to investigate of cytotoxic effect of aqueous and methanol extracts of *C. cornucopioides.* Growth medium (RPMI with L-glutamine) was heated to 37°C and cells were added. Then, they were grown in a 5% CO_2 incubator (Sanyo, USA) at 37 °C with 95% humidity. One day after, the cells were washed with PBS and detached by trypsinization when 80-90% saturation was reached. Passaged cells were grown again at 37 °C in 5% CO_2 (Sanyo MCO 17AIC, USA) until confluency of 90%. The cytotoxic effects of *C. cornucopioides* extracts were determined in accordance with the manufacturer's protocol with the XTT cell proliferation assay kit (Biological Industries, Israel). For this, 50 µl of activated XTT (Cell Proliferation Assay Kit) was added to the cells which were preincubated with different concentration of *C. cornucopioides* extracts for 48 hours at 37 ° C, 5% CO_2. At the end of the 5 h XTT incubation, absorbance values at 450 nm was measured with a microplate reader (Multiskan^{TM} GO, Thermo Scientific, USA) and IC_{50} values were calculated.

2.9. Statistical Analyses

All the assays were carried out at least in triplicate measurements. The results are expressed as mean values and standard error of mean (SEM). Antioxidant, antibacterial and cytotoxicity activities were analyzed using Student t-test and values with $P < 0.05$ were considered as statistically significant. IC_{50} and EC_{50} values were calculated with non-lineer regression analysis. For all statistical calculations Statistical Package for Social Sciences (SPSS®, version 21.0) were utilized.

3. Results

Health and nutrition problems are getting increase due to world population which is irregularly growing. Nowadays, unconscious consumption of natural resources and economic difficulties obligates the use of natural resources and this increases the importance of macrofungi in diet. In addition to the nutritional properties, their biologically active substances have gained reputation in the pharmaceutical area.

In studies of antioxidant, antimicrobial and cytotoxic activities of macrofungi and other medical effects, important data have been obtained. In this study, antioxidant, antimicrobial and cytotoxic activities of *Craterellus cornucopioides* (L.) Pers. is researched.

Amount of bioactive compounds present in aqueous and methanolic extracts of *C. cornucopioides* are summarized in Table 1. Results demonstrated that water extracts of *C. cornucopioides* has very high amount of total phenolics (37.71±1.42 µg/mg) which is also in parallel with the main phenolics; gallic acid and p-coumaric acid determined with HPLC analysis. Similarly, total flavonoid contents (2.13±0.06 µg/mg) were also higher as compared with methanolic extracts (1.83±0.02 µg/mg). On the other hand, methanolic extracts have higher content of the β-carotene and lycopene. Additionally, the phenolic compounds such as gallic acid, catechin, epicatechin, epigallocatechin gallate, syringic acid, p-coumaric acid, rosmarinic acid, t-resveratrol and quercetin in *C. cornucopioides* extracts were quantitatively determined by HPLC but, the amount of other phenolic compounds, except p-coumaric acid and gallic acid was either under HPLC limit or had no in *C. cornucopioides* extracts.

Table 1: Antioxidant activity, reducing power and bioactive ingredients of *C. cornucopioides* extracts.

Bioactive Ingredients	*C. cornucopioides* extract	Content
DPPH scavenging (IC_{50} : mg/ml)	Water	12.01±1.72
	MeOH	5.26±0.67
Reducing power (EC_{50}: mg/ml)	Water	4.54±0.61
	MeOH	6.52±1.53
Phenolics (µg//mg)	Water	37.71±1.42
	MeOH	13.78±1.59
ß-carotene (µg/mg)	Water	3.89±0.06
	MeOH	6.34±0.08
Lycopene (µg/mg)	Water	2.49±0.01
	MeOH	5.55±0.08
Flavonoid (µg/mg)	Water	2.13± 0.06
	MeOH	1.83±0.02
Gallic Acid (µg/mg)	Water	0.55 ± 0.02
	MeOH	0.29 ± 0.02
p-coumaric acid (µg/mg)	Water	3.73 ± 0.01
	MeOH	1.76 ± 0.01

DPPH radical scavenging activities were measured with different concentrations of *C. cornucopioides* up to 10 mg/ml of extracts and scavenging activities enhanced with elevated concentrations (Fig. 1). The best radical scavenging was obtained with the methanolic extracts of *C. cornucopioides* in 10 mg/ml concentration, as over than 75% DPPH reduction takes place. Aqueous extracts of the same mushrooms also possessed 40% inhibition at highest concentration tested. According to IC_{50} values, methanolic extracts of *C. cornucopioides* (IC_{50}: 5.26±0.67 mg/ml) are more efficient than its aqueous extracts (IC_{50}: 12.01±1.72 mg/ml).

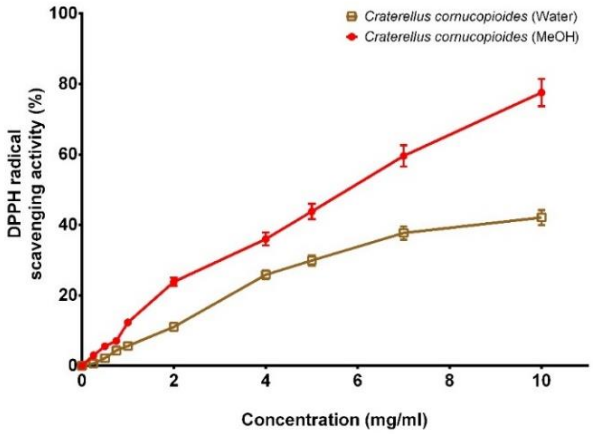

Figure 1: DPPH radical scavenging activities of aqueous and methanolic extracts of *C. cornucopioides.*

Reducing power of a pharmaceutical generally strongly correlates well with the antioxidant capacity. Therefore, EC_{50} values were determined to describe the extract concentration yielding an absorbance value of 0.500. According to the results, aqueous extracts showed higher reducing activity than methanolic extracts in general view (Fig. 2). Aqueous extract of *C. cornucopioides* had the highest reducing activity with the lowest EC_{50} value (4.54±0.61 mg/ml).

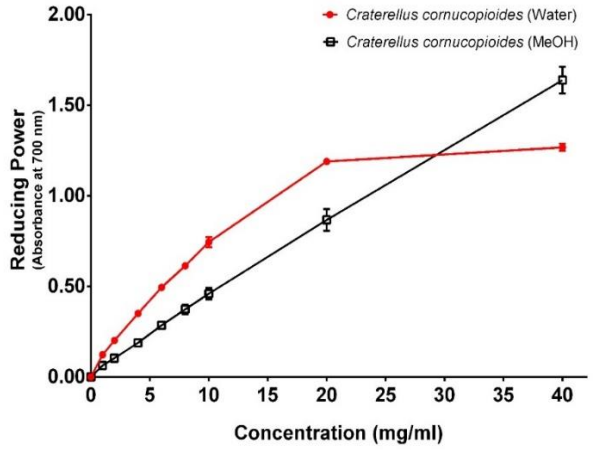

Figure 2: Reducing powers of aqueous and methanolic extracts from *C. cornucopioides*.

Cell growth inhibitory potentials of *C. cornucopioides* extracts on HepG2 cells were also inspected in this study, which was not reported previously. Results indicated that extracts have some degree of cytotoxicity over HepG2 cells and cytotoxic effects of the extracts increased with elevated concentrations (Fig. 3). Methanolic extracts of *C. cornucopioides* had the lowest IC_{50} values (3.14±1.07 mg/ml). Considering the water extracts, IC_{50} values of 18.41±1.10 mg/ml were obtained showing that methanolic extracts has higher cell growth inhibitory potential. As a result, *C. cornucopioides* might play a role in cancer and related researches in cytotoxic effect studies.

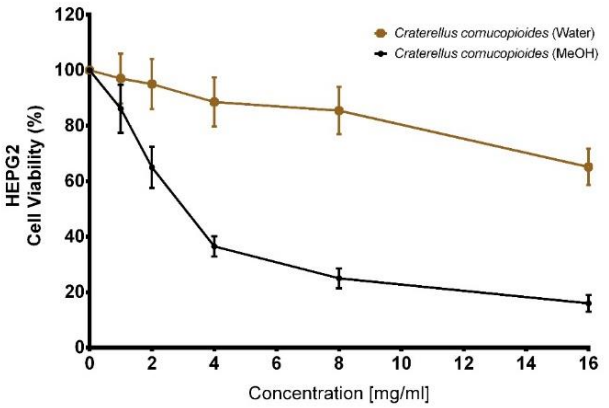

Figure 3: Cytotoxicity of aqueous and methanolic extracts from *C. cornucopioides* over HepG2 cells after 48 hours exposure time.

In antibacterial studies, it was revealed that extracts of *C. cornucopioides* have high antimicrobial potential. As it can be seen clearly in Table 2, *C. cornucopioides* exerted antibacterial activity on all tested microorganisms; *B. subtilis*, *E. faecalis*, *B. licheniformis*, *A tumefaciens*, *E. coli* and *S. aureus*. With its both water and methanolic extracts, *C. cornucopioides* inhibited microbial growth with inhibitory zone (IZ) values ranging 6-8 mm length.

Table 2: Antibacterial effects of *C. cornucopioides* on six different tested microorganisms.

Tested Bacteria	Extract	*C. cornucopioides* IZ: mm	Gentamicin IZ: cm
E. coli	Water	6	2.3
	MeOH	7	
S. aureus	Water	6	2.4
	MeOH	6	
B. subtilis	Water	6	2.5
	MeOH	8	
B. licheniformis	Water	6	2.4
	MeOH	7	
A. tumefaciens	Water	6	2.7
	MeOH	7	
E. faecalis	Water	6	2.5
	MeOH	7	

In conclusion, in the near feature mashrooms having nutritional and economical values will be more used in medicine, pharmacy and industrial area due to their high antioxidant, antimicrobial, and cytotoxic features. *C. cornucopioides* shows noticeable activities with its antioxidant, antibacterial and cell growth inhibitory potential together with high gallic acid and p-coumaric acid content. It might be utilized as a promising source of therapeutics since it might provide an appropriate source of antioxidant, antibacterial and cytotoxic natural compounds and also could be searched as potent antibacterial drugs against infectious diseases.

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