Research Article

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In vitro antioxidant, anti-inflammatory and anti-cancer activities of methanolic extract of *Asparagus horridus* grows in North Cyprus

Kuzey Kıbrıs da yetişen Asparagus horridus metanolik ekstraktının in-vitro antioksidan, anti-enflamatuar ve anti-kanser aktivitesi

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Abstract

Background: *Asparagus horridus* is an edible plant known as "Ayrelli" in North Cyprus. The scientific literature has not yet submitted a report about the antioxidant, anti-inflammatory and anti-cancer activities of *A. horridus* plant from North Cyprus until now. The purpose of the research was to determine the antioxidant, anti-inflammatory and anti-cancer activities of *A. horridus*.

Materials and methods: Soxhlet extraction of *A. horridus* was performed using methanol. Antioxidant activity was determined by DPPH, TFC, FRAP and TPC assays. Protein-denaturation assay was performed to determine the anti-inflammatory effect. The anti-cancer effects of the extract on HepG2 and B-CPAP cell lines were determined with MTT assay.

Results: Antioxidant activity for *A. horridus* extract *was* determined by DPPH (50%), TFC (266.26 µg QUE/mg extract), FRAP (1.27 µg $FeSO_4$/mg extract) and TPC (167.613 µg GAE/mg extract) assays at 25 mg/mL. Inhibition of protein-denaturation activity was found as 29.42% at 25 mg/mL. After 24 h of the extract treatment, cell proliferation of HepG2 and B-CPAP cancer cells were inhibited at IC_{50} values 63.24 µg/mL and 101.24 µg/mL, respectively.

Conclusion: These results have shown that the methanol extract of *A. horridus* grows in North Cyprus has antioxidant, anti-inflammatory and anti-cancer activities.

Keywords: *Asparagus horridus*; North Cyprus; Antioxidant; Anti-inflammatory; Anti-cancer.

Öz

Amaç: *Asparagus horridus* Kuzey Kıbrıs' ta "Ayrelli" olarak bilinen yenilebilir bir bitkidir. Bilimsel literatürde Kuzey Kıbrıs'ta yetişen *A. horridus* bitkisinin antioksidan, anti-enflamatuar ve antikanser aktiviteleri ile ilgili şu ana kadar bir rapor yayınlanmamıştır. Araştırmanın amacı, *A. horridus*' un antioksidan, anti-enflamatuar ve antikanser etkilerini belirlemektir.

Gereç ve Yöntem: *Asparagus horridus* özütünü elde etmek için Soxhlet ekstraksiyon metodu methanol kullanılarak gerçekleştirildi. Antioksidan aktivitesi DPPH, TFC, FRAP ve TPC testleri ile belirlenmiştir. Ekstraktın anti-enflamatuar etkisini belirlemek için protein-denatürasyon analizi yapıldı. Ekstraktın HepG2 ve B-CPAP hücreleri üzerindeki antikanser etkileri ise MTT testi ile belirlendi.

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Bulgular: *Asparagus horridus* ekstraktının antioksidan aktivitesi, DPPH (50%), TFC (266.26 µg QUE/mg ekstrakt), FRAP (1.27 µg $FeSO_4$/mg ekstrakt) ve TPC (167.613 µg GAE/mg ekstrakt) analizlerinden 25 mg/mL' de elde edilen sonuçlara göre belirlendi. Protein denatürasyonu aktivitesinin inhibisyonu, 25 mg/mL' de 29.42% olarak bulundu. 24 saat ekstrakt muamelesinden sonra, HepG2 ve B-CPAP kanser hücrelerinin hücre proliferasyonu, sırasıyla IC_{50} değerlerinde 63.24 µg/mL ve 101.24 µg/mL önemli ölçüde inhibe edildi.
Sonuç: Bu sonuçlar, Kuzey Kıbrıs' da yetişen *A. horridus*' un metanol ekstraktının önemli antioksidan, anti-enflamatuar ve anti-kanser aktiviteye sahip olduğunu göstermiştir.

Anahtar kelimeler: *Asparagus horridus*; Kuzey Kıbrıs; Antioksidan; Anti-enflamatuar; Anti-kanser.

Introduction

In recent years, interest in studies involving medicinal plants has increased considerably. Edible plants such as Asparagus have been used in Chinese and Indian medicine as a diuretic, in cancer treatments, remedies for neuritis and rheumatism, to relieve toothache, antitussive and antifebrile [1, 2]. *Asparagus* is a genus (Liliaceae family) that includes 300 species and known to be grown mostly in Mediterranean, Eastern Asia and some parts of Africa [3–5].

Different studies on *Asparagus* species have demonstrated anti-cancer (including breast cancer [6], leukemia [6], lung cancer [6] and liver cancer [7]), anti-inflammatory and antioxidant activities [8–10]. It has been reported that a methanolic extract of different types of *Asparagus* has a strong antioxidant capacity [11]. *Asparagus horridus*, an edible plant known as "Ayrelli", is a common type of *Asparagus* in North Cyprus. The scientific literature has not yet submitted a report about the antioxidant and anti-cancer activities of *A. horridus* plant from North Cyprus until now. Besides that, there has been no pharmacological study performed specifically on *A. horridus* in literature.

The main purpose of this study is to evaluate the antioxidant, anti-inflammatory and anti-cancer activity of *A. horridus* methanolic extract. The present study is the first study to evaluate the potential anti-proliferative activity of *A. horridus* on both HepG2 and B-CPAP cells. In conclusion, significant anticancer activity against both liver and thyroid cancer cells of *A. horridus* methanolic extract indicates that it is a potential therapeutic agent for cancer therapy.

Materials and methods

Chemicals and reagents

Methanol (CN: 24229) used for the soxhlet extraction of asparagus samples was obtained from Sigma-Aldrich (St. Louis, MO, USA). For the antioxidant assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) (CN: D9132), dimethyl sulfoxide (DMSO) (CN: 472301) and gallic acid (CN: 398225) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aluminum chloride (Product code: 10558030) was purchased from Fisher-Scientific and quercetin (CN: Q4951) was obtained from Sigma-Aldrich (St. Louis, MO, USA) for use in total flavonoid content determination. Folin reagent (CN: F9252) and sodium carbonate (CN: 13418) used in total phenolic content determination experiments were also purchased from Sigma-Aldrich (St. Louis, MO, USA). In the use of ferric reducing antioxidant power assay, potassium ferricyanide (CN: 244023), trichloroacetic acid (CN: T6399), iron sulfate (CN: F1543) and iron(III) chloride (CN: 8039451000) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protein denaturation assay was done with the use of bovine serum albumin (BSA) (CN: 232936-2) obtained from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac Sodium (ATC code: M01AB05) purchased from Deva (Kucukcekmece, İstanbul, Turkey) in the form of ampoules.

Plant collection

Plant samples were collected from Famagusta North Cyprus (35°07′55.7″North, 33°51′55.2″East) in March 2019 and identified as *A. horridus* by Prof. Dr. F. Neriman Özhatay, Herbarium Botanist, Faculty of Pharmacy, Eastern Mediterranean University (EMU). A herbarium specimen (voucher number: DE 001) has been deposited and retained in the above herbarium. Plant name was checked with http://www.theplantlist.org.

Methanolic extract preparation

The plant material was washed with distilled water, air-dried at room temperature and grounded to powder form. Soxhlet extraction was used to obtain the extract from 10 g *A. horridus* plant powder using 300 mL methanol. The extraction process was repeated three times at 70°C in which each cycle lasted 8 h. The extract was filtered and the solvent was completely removed using a rotary evaporator.

Antioxidant assays

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The antioxidant activities of the methanolic extract of *A. horridus* was determined using a method based on the reduction of DPPH by Alara et al. [12] with modifications. The extract was dissolved in DMSO and then 5 µL of different doses from each extract was mixed with 195 µL DPPH. The mixture was left in a dark room for 30 min at room temperature. At the end of the incubation, absorbance was measured at 517 nm using a 96-well plate with Varioskan Flash Multimode Microplate Reader (Thermo-Fisher-Scientific, Waltham, MA, USA) and gallic acid was used as a standard with the doses ranging from 0 to 200 µg/mL. The inhibition percentages of the radical scavenging activity were calculated with the following formula, which "control" shows the absorbance of methanol mixed with DPPH solution and a "sample" shows the absorbance of *A. horridus* extract mixed with DPPH solution.

$$\%\ \text{Inhibition} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}}] \times 100$$

Total flavonoid content (TFC)

TFC of the extract was measured using the aluminum chloride ($AlCl_3$) colorimetric method described by Kim et al. [13] with minor modifications. Extract solution with a volume of 50 µL was mixed with 50 µL of 2% $AlCl_3$ solution and incubated for 1 h at room temperature. The absorbance of the supernatant was measured at 420 nm via Varioskan Flash Multimode Microplate Reader (Thermo-Fisher-Scientific, Waltham, MA, USA). Quercetin was used as a standard to draw the calibration curve ($y = 0.0058x + 0.0351$; $r^2 = 0.98811$). The concentration of flavonoid was expressed as µg quercetin equivalent (QE) per mg extract (µg QE/mg extract).

Total phenolic content (TPC)

Total phenolic content of the *A. horridus* extract was measured using Folin-Ciocalteu reagent method with minor modifications [12, 14]. After the transfer of 50 µL from each extract concentration into well plates, 100 µL folin reagent and 100 µL of sodium carbonate (Na_2CO_3) solution was added sequentially to each reaction mixture and then incubated for 30 min at 25°C prior to absorbance measurements at 765 nm using a Varioskan Flash Multimode Microplate Reader (Thermo-Fisher-Scientific, Waltham, MA, USA). Gallic acid (0–250 µg/mL) was used as a standard to draw the calibration curve ($y = 0.0058x - 0.1034$; $r^2 = 0.99174$). The results expressed as µg gallic acid equivalent (GAE) per mg of extract (µg GAE/mg extract).

Ferric reducing antioxidant power (FRAP assay)

The FRAP assay was performed using the Vijayalakshmi and Ruckmani methods with slight modifications [15]. Twenty microliter from the different concentrations of plant extract (1–25 mg/mL) was added to 50 µL phosphate buffer (0.2 M, pH = 6.6) and 50 µL 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution. Mixture was vortexed and incubated at 50°C for 20 min in a water bath. After the incubation, 50 µL 10% trichloroacetic acid (TCA) was added to the reaction mixture and centrifuged for 10 min at 704 *g*. At the end of the centrifugation, 200 µL of the supernatant was mixed with the same proportion of distilled water and 40 µL of 0.1% ferric chloride. Absorbance was measured at 700 nm using a Varioskan Flash Multimode Microplate Reader (Thermo-Fisher-Scientific, Waltham, MA, USA). Different concentrations (100–300 µg/mL) of ferrous sulfate ($FeSO_4$) was used as a standard to draw the calibration curve ($y = 0.0003x - 0.0707$; $r^2 = 0.89526$).

Protein-denaturation assay

Protein denaturation assay was performed based on procedure described by Williams et al. [16]. While preparing the reaction mixture, different concentrations (1–25 mg/mL) of *A. horridus* extract were mixed with 0.5 mL of 1.5 mg/mL BSA and incubated at 37°C for 20 min. Thereafter, reaction mixtures were heated for 3 min at 57°C and 0.5 M phosphate buffer (pH = 6.3) with a volume of 250 µL was added to each reaction mixture and mixed thoroughly. Subsequently after even distribution of molecules in each reaction mixture, 100 µL from each mixture was transferred into separate test tubes followed by the addition of copper-alkaline reagent and 1% (v/v) Folin-Ciocalteu's reagent with the same proportion by volume. Following to the 10 min incubation at 55°C, the tubes were allowed to cool down and absorbance was measured at 650 nm using a Varioskan Flash Multimode Microplate Reader (Thermo-Fisher-Scientific, Waltham, MA, USA). Recorded measurements were evaluated by using different concentrations of diclofenac sodium (100–1000 µg/mL) as a reference drug.

Cell culture and cell viability

Cell culture experiment was performed on both human liver cancer cell line HepG2 (ATCC, HB-8065) obtained from ATCC (Manassas, VA, USA) and papillary thyroid carcinoma cell line B-CPAP (DSMZ, ACC 27) obtained from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI-1640) (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), 1% L-glutamine, 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, M2003) assay described by Heijden et al. [17]. MTT is a quantitative colorimetric assay for measuring cellular growth, cell survival and cell proliferation based on the quantity of living cells. *Asparagus horridus* extract stock solution (200 mg/mL) was prepared in DMSO and stored at –20°C. Before being used in MTT assay, the stock solution was diluted with RPMI-1640 medium to keep final concentration of DMSO below 0.1% in extract solution. For MTT assay, 10×10^3 cells/well were seeded in 96 well plates in 100 µL of medium and incubated at 37°C for 24 h. Cells were then treated with different concentrations of *A. horridus* extract and incubated for 24 h. Afterward, 10 µL of 5 mg/mL solution of MTT in PBS was added to each well plates. After 4 h of incubation with MTT at 37°C, supernatant was carefully removed and then 100 µL DMSO was added to each well. Plates were then placed in a microplate shaker for 5 min. Finally, cell viability assay was determined by measuring the absorbance at 595 nm with a Varioskan Flash Multimode Microplate Reader (Thermo-Fisher-Scientific, Waltham, MA, USA). The percentage of cell viability was calculated manually using the formula:

$$\%\text{Viable cells} = (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})/(\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}) * 100$$

The mean of triplicate experiments for each dose was used to calculate the concentration of extract required for 50% inhibition of cell viability (IC_{50}) as determined using the Biosoft CalcuSyn program.

Statistical analysis

The experiments were performed in triplicates and results were expressed as mean ± standard deviation (SD). Test results were calculated in Microsoft Excel 2015 software (Microsoft, Redmond, WA, USA). For statistical comparisons, results were analyzed using ANOVA/Dunnet's Multiple Comparisons test and GraphPad Prism Version 8 software to carry out statistical tests. A statistical significance of $p < 0.05$ was considered as significant.

Results

Determination of antioxidant activities

The DPPH assay was used to determine the antioxidant potential of plant extracts by measuring their ability to act as free radical scavengers. Evaluation of antioxidant activities for standard gallic acid concentrations at 10 and 200 µg/mL indicated 9.37% and 60.92%, respectively (data was not shown). Increase in *A. horridus* extract concentration resulted in a simultaneous increase in DPPH radical scavenging activities significantly at extract concentration of 10 mg/mL ($p < 0.05$) and 15, 20, 25 mg/mL ($p < 0.0001$) compared to 1 mg/mL concentration of extract. No difference (ns#) was observed between 1 mg/mL (4%) and 5 mg/mL (4%) concentration of extract. The highest DPPH scavenging activity of methanolic extract of *A. horridus* was 50% at 25 mg/mL (Figure 1).

Total flavonoid content (TFC) of extract was measured via colorimetric method using aluminium chloride. The results were derived from the calibration curve ($y = 0.0058x + 0.0351$; $r^2 = 0.98811$) of quercetin (0–200 µg/

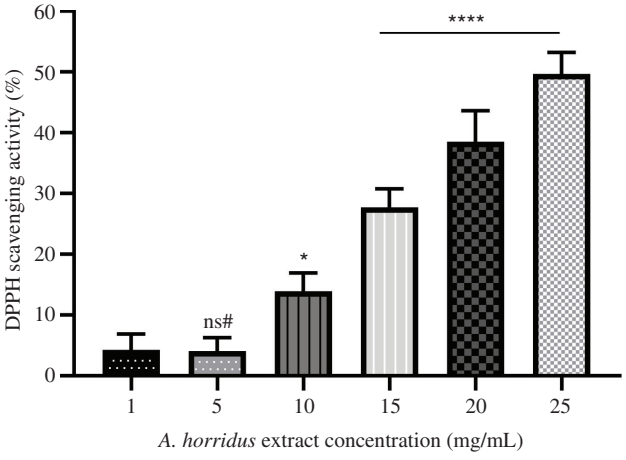

Figure 1: DPPH radical scavenging activity of the different concentrations of methanolic extract of *A. horridus*.
Data results were expressed as % radical scavenging activity relative to 100% radical scavenging activity of gallic acid as a reference. Values were expressed as the mean ± standard deviation (n = 3). Statistical results were given as (ns#, not significant; $p < 0.5$*, $p < 0.0001$****).

mL) and expressed in µg quercetin equivalents (QE)/mg extract (data was not shown). Our results showed that flavonoid content of *A. horridus* was 51.23 µg/mL at 5 mg/mL compared to 1 mg/mL concentration of extract (ns#). Total flavonoid content of 10, 15, 20, and 25 mg/mL extracts was determined as 115.19, 161.37, 200.08 and 266.26 µg/mL, respectively compared to 1 mg/mL concentration of extract ($p < 0.0001$) (Figure 2).

Determination of total phenolic content (TPC) of *A. horridus* extracts with respect to standard calibration curve of gallic acid was done using Folin-Ciocalteu reagent. The standard calibration curve was obtained from different concentrations of gallic acid (0–200 mg/mL) (data was not shown). The highest TPC of the extract was found as 169.713 (µg GAE)/mg at 20 mg/mL concentration. At the lowest concentration of 5 mg/mL, TPC was found as 42.61 (µg GAE)/mg and it was demonstrated that there is no statically significant difference between the TPC of 5 mg/mL and 10 mg/mL ($p > 0.05$). However, TPC of the higher extract concentrations at 15, 20 and 25 mg/mL was determined as 140.683, 169.713 and 167.613 (µg GAE)/mg extract, respectively ($p < 0.01$) (Figure 3).

In the ferric ion reducing antioxidant potential (FRAP) assay, the reducing power capability of extract concentrations between 1 and 25 mg/mL were illustrated using standard calibration curve of iron sulfate ($FeSO_4$) (Figure 4). For the FRAP assay, varying iron sulfate ($FeSO_4$) concentrations (200–1000 µg/mL) were used as a calibration curve (data was not shown). Reducing power capability of the extract was found to be 0.04, 0.09, 0.36, 0.86, 1.2 and 1.27 (µg $FeSO_4$)/mg FRAP values for 1, 5, 10 ($p < 0.05$), 15 ($p < 0.0001$), 20 ($p < 0.0001$) and 25 mg/mL ($p < 0.0001$) concentrations of extract, respectively. Statistical evaluations showed that although there was no significant difference ($p < 0.05$) between the concentrations of 5 mg/mL and 1 mg/mL extracts, FRAP values for the rest of the concentrations were considered as statistically significant.

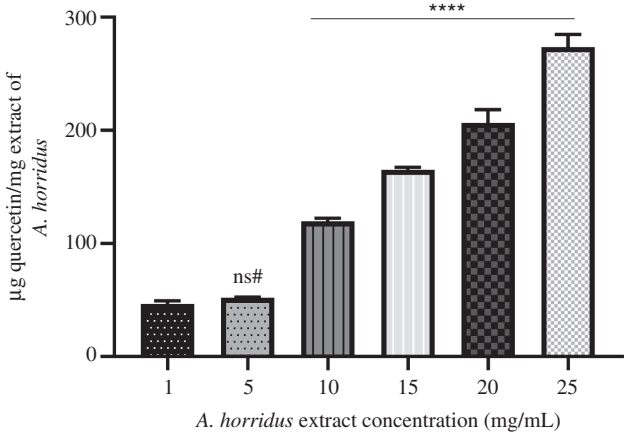

Figure 2: Bar chart representation of total flavonoid content of methanolic extract of *A. horridus*.
Graph shows the total phenolic content expressed as µg quercetin/mg of extract. Values were expressed as the mean ± standard deviation (n = 3). Results were given as (ns#, not significant; $p < 0.0001$****).

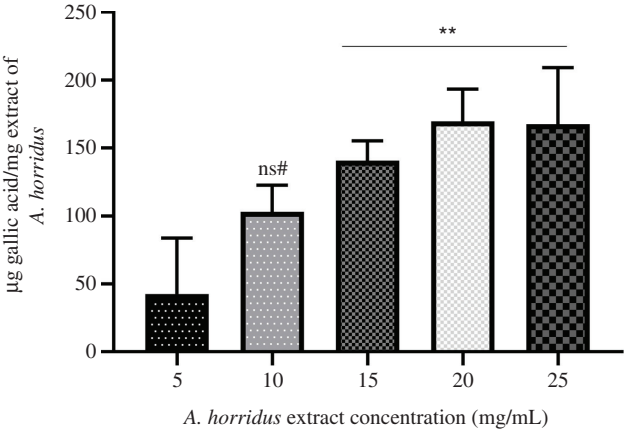

Figure 3: Total phenolic content of *A. horridus* extract.
Graph shows the total phenolic content expressed as µg GAE/mg of extract. Values are expressed as the mean standard deviation (n = 3). Results were given as (ns#, not significant; $p < 0.01$**).

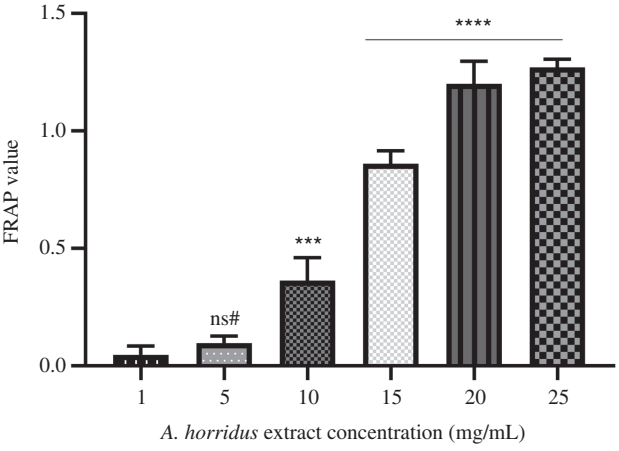

Figure 4: Ferric reducing antioxidant power assay of *A. horridus* extract.
Values were expressed as the mean ± standard deviation (n = 3). Results were given as (ns#, not significant; $p < 0.001$***, $p < 0.0001$****).

Effect of *A. horridus* on in vitro anti-inflammatory activity

The protein denaturation assay was performed to determine the in vitro anti-inflammatory activity and

Table 1: Effect at increased concentrations of *A. horridus* and diclofenac sodium against protein denaturation.

Concentration (mg/mL)	% Inhibition of denaturation of BSA
1	0.28 ± 0.08
5	2.72 ± 0.34
10	9.47 ± 0.43
15	16.33 ± 0.61
20	22.67 ± 0.61
25	29.42 ± 0.34

concentration-dependent inhibition of protein-denaturation activity was observed for *A. horridus*. For protein-denaturation assay, diclofenac sodium was used as a positive control from 100 to 1000 µg/mL concentrations to represent the percentage inhibition of protein denaturation activity ranging between 32.95% and 83.53%, respectively (data was not shown). As shown in Table 1, percent inhibitions attained by varying extract concentrations exhibited less activity compared to the standard agent and the maximum percent inhibition was observed by the highest extract concentration (25 mg/mL) as 29.42 ± 0.34.

Anti-cancer effect of *A. horridus* on HepG2 and B-CPAP cells

The MTT assay was performed to evaluate the effects of *A. horridus* extract on HepG2 and B-CPAP cell proliferation. As shown in Figure 5A, treatment with 1 ($p < 0.001$), 5, 10, 20, 50, and 100 µg/mL ($p < 0.001$) concentrations of *A. horridus* extract for 24 h, caused a significant reduction in the HepG2 cell viability to 88.76%, 75.72%, 69.5%, 62.23%, 55.31% and 44.64%, respectively. As shown in Figure 5B, treatment with 10 ($p < 0.05$), 20, 50, 70, 100, 150, 200 and 300 µg/mL ($p < 0.001$) concentrations of *A. horridus* extract for 24 h decreased the B-CPAP cell viability to 82.24%, 68.74%, 62.80%, 58.55%, 54.03%, 44.57%, 20% and 5.69%, respectively. The IC_{50} values of *A. horridus* on HepG2 and B-CPAP cells were 63.24 µg/mL and 101.24 µg/mL after 24 h, respectively.

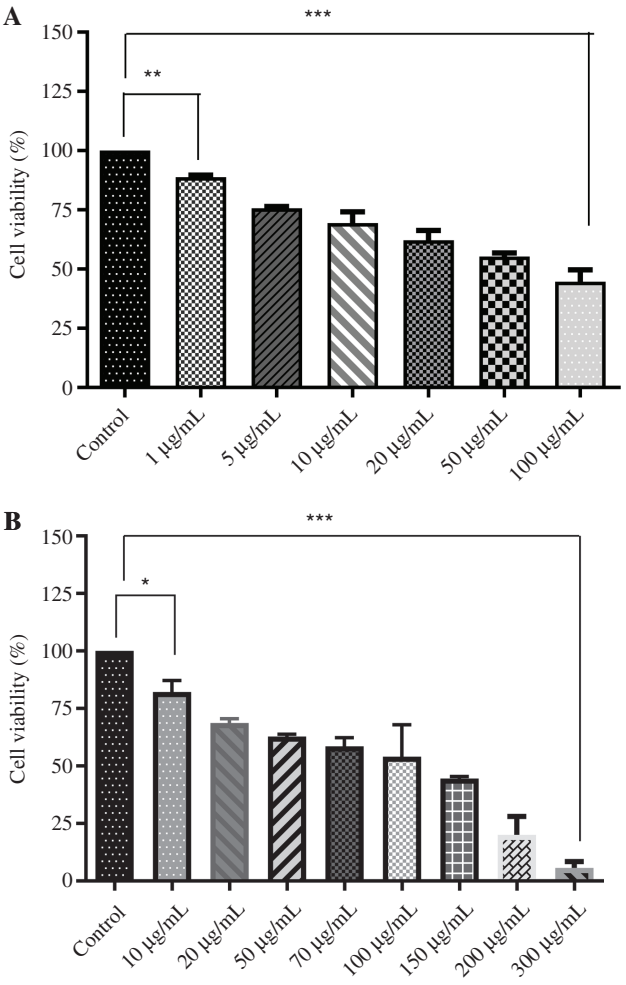

Figure 5: Effects of *A. horridus* on HepG2 and B-CPAP cell viability. Cells were treated with indicated concentrations of the extract on HepG2 (Figure 5A) and B-CPAP (Figure 5B) cancer cells for 24 h. Values were expressed as the mean ± standard deviation (n = 3). Results were given as ($p < 0.05$*, $p < 0.01$**, $p < 0.001$***).

Discussion

The aim of the present study was to evaluate the in vitro antioxidant, anti-inflammatory and anti-cancer activities of methanol extract of *A. horridus* on both HepG2 and B-CPAP cancer cells. Extracts derived from nature origin possess phenolic compounds, such as flavonoids, coumarins, phenolic acids. These antioxidant compounds have anti-inflammatory, anti-cancer, anti-mutagenic, anti-bacterial and apoptotic effects of phenolic compounds [18]. In this study, four different antioxidant methods (DPPH, TPC, TFC and FRAP) were used to determine the antioxidant activity of *A. horridus*. Based on the results of antioxidant assays, it was found that *A. horridus* has potential antioxidant capability. Apart from antioxidant tests, anti-inflammatory activity of *A. horridus* extract was analysed via the inhibition studies on albumin degradation.

This study is the first report which focuses on anti-inflammatory response of *A. horridus* against denaturation of proteins. In a previous study, % inhibition of protein denaturation of rhizophora mucronata leaves was observed as 33%, at 100 mg/mL extract concentration compared to the standard agent, diclofenac sodium [19].

This signifies the importance of 29% inhibition of protein denaturation achieved by 25 mg/mL *A. horridus* extract concentration in our study.

Phenolic compounds such as flavonoids have long been reported as chemopreventive agents in cancer treatment [20–22]. Quercetin is a major flavonoid which has an anti-cancer effect against prostate and breast cancers [23]. Gliricidin7-*O*-hexoside and Quercetin 7-*O*-rutinoside are known as flavonoids and they have anticancer effect on human hepatoma HepG2 and human carcinoma HeLa cells [24].

The effect of quercetin concentration on the B-CPAP cells proliferation was previously studied and our previous results showed that specifically 50 μM and 75 μM quercetin concentrations were desired to inhibit the B-CPAP cells proliferation [25]. In our current study, anti-proliferative effect of *A. horridus* on B-CPAP cells was observed and it was found that *A. horridus* extract with 101.24 μg/mL is required to reduce the B-CPAP cell proliferation by half. Based on the vast amount of cytotoxicity and anticancer studies, it has been confirmed that *Asparagus* species can be used as natural anticancer agent against various types of cancer [6, 11, 26–31]. In the light of literature sources and our studies, it is concluded that methanol extract of *A. horridus* could be a potential anticancer agent for both liver and thyroid cancer treatments.

Conflict of interest statement: Authors have no conflict of interest regarding this study.

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