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RESEARCH ARTICLE

Antiproliferative activity of *Humulus lupulus* extracts on human hepatoma (Hep3B), colon (HT-29) cancer cells and proteases, tyrosinase, β-lactamase enzyme inhibition studies

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Abstract

The aims of this study were to examine the antiproliferation of *Humulus lupulus* extracts on human hepatoma carcinoma (Hep3B) and human colon carcinoma (HT-29) cell lines along with enzyme inhibitory effects of the crude extracts. Potential cell cytotoxicity of six different *H. lupulus* extracts were assayed on various cancer cells using MTT assay at 24, 48 and 72 h intervals. Methanol-1 extract has inhibited the cell proliferation with doses of 0.6–1 mg/mL in a time dependent (48 and 72 hours) manner in Hep3B cells with 70% inhibition, while inhibitory effect was not seen in colon cancer cells. Acetone extract has increased the cell proliferation at low doses of 0.1 mg/mL for 72 h in Hep3B cells and 0.1–0.2 mg/mL for 48 and 72 h in HT29 cells. The inhibitory effects of the extracts were compared by relative maximum activity values (V_{max}) using proteases such as α-chymotrypsin, trypsin and papain, tyrosinase and β-lactamase (penicillinase).

Keywords

Antiproliferation, enzyme inhibition, Hep3B, hop female cones, HT-29, *Humulus lupulus*, proteases, tyrosinase, β-lactamase (penicillinase)

History

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Introduction

Until now, *in vitro* assays of bioactive components of *Humulus lupulus* as chemopreventive agents have been studied by many researchers[1–4]. Among main components, xanthohumol (XH) has received the major attention because it appears to inhibit of initiation, promotion and progression stages of carcinogenesis *in vitro*, so appearing as a broad-spectrum chemopreventive agent[5–7].

The best known components of *H. lupulus* extract such as xanthohumol, humulone, isoxanthohumol, dehydrocycloxanthohumol, desmethylxanthohumol, etc. (Figure 1) were investigated against some of the cell lines including MCF-7 (human breast cancer cells)[8], HT-29 (human colon cancer cells)[8], A2780 (human ovarian cancer cells)[8], PC-3[9,10], DU145 (human prostate cancer cells)[9], HUVEC (human umbilical vascular endothelial cells)[11], human colon cancer cells[12], MCF-7[13], T47-D (human breast cancer cells)[13], U937[14] and HL-60[15]. Even though, the screening of such materials can be very complicated due to the fact that they contain a complex mixture of secondary metabolites[16], extracts from natural sources have been a significant source of molecular variety. In fact, several therapeutically very important drugs in drug discovery programs have been isolated from natural sources[16] such as Paclitaxel (Taxol®) from *Taxus brevifolia*, Vinblastine (Velban®) from *Vinca rosea*, etc.

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In the literature, the therapeutic effects have been usually assigned to the pure compounds[17] and the synergism, defined as an effect arising between two or more agents or substances that produces an effect greater than the total of their individual effects, is neglected in many cases. In this work, however, we report that *H. lupulus* extracts articulate their proliferative and antiproliferative effects on the cancerous cell lines in a synergic manner.

In addition, the inhibitory effects of the extracts on the activity of therapeutically important enzymes were also investigated and compared by relative activity values. This is particularly important for finding useful inhibitors as many drug discovery programs incorporate enzyme targets in primary screening assays. For example, proteases or proteinases play a critical role in the normal physiological functions of cells (e.g. protein maturation, digestion, blood coagulation and immune response) and they present potential targets for the possible treatment of a wide range of proteases or proteinases related diseases, due to the excessive activity of proteases, including cancer, pulmonary emphysema, muscular dystrophy, arthritis and pancreatitis. Similarly, tyrosinase is responsible for the biosynthesis of dermal melanin pigment from L-tyrosine and L-DOPA (dihydroxyphenylalanine) in the melanocytes on the melanosomes. The over-production of melanin has been linked with the condition of hyperpigmentation of skin including melasma and ephelides. The inhibition of tyrosinase enzyme is, therefore, a sensible way of managing hyperpigmentation and related conditions[16]. In plants, the physiological substrates contain a variety of phenolics and tyrosinase oxidizes them in the browning pathway, observed when the plant tissues are injured[18].

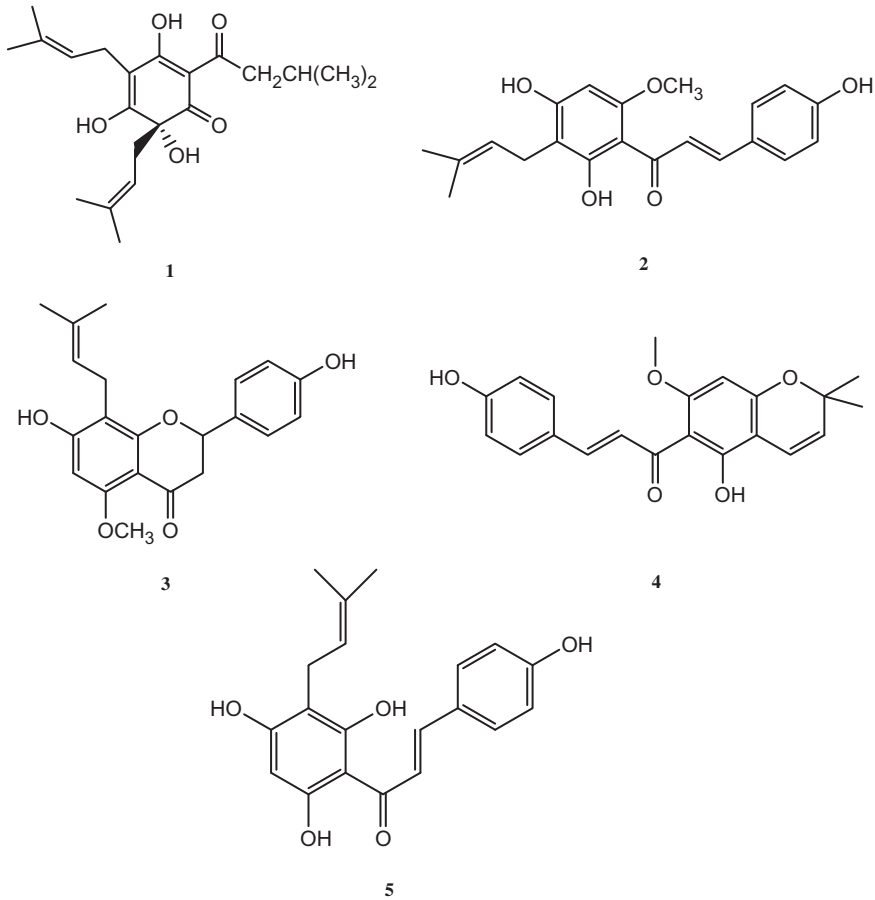

Figure 1. Molecular structures of some of the bioactive components of *H. lupulus* (1) humulone, (2) xanthohumol, (3) isoxanthohumol, (4) dehydroxanthohumol, (5) desmethylxanthohumol.

What is more the enzyme β-lactamase is produced by bacteria as a part of a resistance mechanism against β-lactam-containing antibiotics including pencillins, cephalosporins, carbapenems and monobactams. β-Lactamase enzyme effectually hydrolyzes the amide bond of the β-lactam moiety, therefore producing products devoid of antibiotic activity[16]. The search for new β-lactamase inhibitors, thus, has a remarkable clinical and industrial potential. In this work, enzyme inhibition studies of the crude extracts were investigated with above mentioned enzymes in search for potential substrates with inhibitory nature[18,19,20].

In our previous study, we have reported the antioxidant activity of *H. lupulus* extracts with solvents including *n*-hexane, acetone, methanol (1, 2 and 3) and 25% aqueous ethanol. Assays of DPPH, TEAC, FRAP and CUPRAC were performed. The amount of total phenolic components determined by the Folin–Ciocalteu reagent was found to be highest in 25% aqueous ethanol (9079 ± 187 mg Ferulic acid equivalent/100 g extract) and methanol-1 (directly) (8343 ± 158 mg Ferulic acid equivalent/100 g extract) extracts[21]. Additionally, comparative screening of antioxidant activities of *H. lupulus* extracts and quantification of some major components by LC−MS/MS, qualitatively analysis of the reported ones which were optimal under negative ion SIM mode and co-injection, are going to be valuable for food and health applications[21]. Consequently, phenolic compounds are likely to contribute to the radical scavenging activity of these plant extracts. Phenolic acids are plant metabolites widely spread throughout the plant kingdom. Recently, interest on phenolic acids increases due to their protective role, through ingestion of fruits and vegetables, against oxidative damage diseases such as coronary heart disease, stroke and cancers[21].

Referring to our previous study, it was clearly shown that kaempherol 3-*O*-glucoside, quercetin, ascorbic acid, ferulic acid, gallic acid, ellagic acid, *p*-coumaric acid, epigallol and pyrogallol are the predominant phenolic compounds identified in the extracts. From methanol-1 extract of *H. lupulus*, for example, ferulic acid, humulone, lupulone, cohumulone, colupulone, adhumulone, adlupulone, xanthohumol, 6-prenylnaringenin, 8-prenylnaringenin, isoxanthohumol, etc. were identified by LC−MS[21] and co-injection methods were performed for several other compounds including kaempferol, epigallol, quercetin, naringenin and naringin. Due to the richness in various phenolic compounds, the hop plant (*H. lupulus* L.) is used for beer-makings and contains a number of potentially bioactive prenylflavonoids.

In this study, the antitumoral effects of different extracts of *H. lupulus* were investigated in human hepatoma (Hep3B) and human colon carcinoma (HT-29) cells. Different concentrations of the extracts were incubated in the cells for 24, 48 and 72 h and then MTT assay was applied to the samples in each time points. We reported that different *H. lupulus* extracts differentially affects cell proliferation of two different cancer cells, namely Hep3B and HT-29 cells. Besides, the extracts were tested for inhibitory effects against selected enzymes including proteases, tyrosinase and β-lactamase.

Materials and methods

Human hepatoma carcinoma (Hep3B) was provided by Dr D. P. Ramji (Cardiff University) and Human colon carcinoma (HT-29) was provided from Animal Cell Culture Collection (HÜKÜK, Ankara, Turkey). Cell culture medium, L-Glutamine and FCS (Fetal Calf Serum) were obtained from Invitrogen (Karlsruhe, Germany). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), PMSF ($\geq$99.0%) (phenylmethanesulfonylchloride), EDTA

(ethylendiamine tetra acetic acid), L-DOPA (3,4-dihydroxy-L-phenylalanin), α-chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4, from bovine pancreas), papain (EC 3.4.22.2, from papaya latex), *N*-Suc-Phe-*p*-nitroanilide, B*z*-*DL*-Arg-*p*-nitroanilide, tyrosinase (EC 1.14.1.8.1, mushroom tyrosinase), penicillinase (from *Bacillus cereus*) and tris(hydroxymethyl)aminomethane (trizma base, minimum 99.9%), sodium chloride, $MgCl_2{\cdot}6H_2O$ (magnesium chloride hexahydrate), KH_2PO_4 (potassium phosphate monobasic), $Na_2HPO_4{\cdot}2H_2O$ (sodium phosphate dibasic dihydrate) were purchased from Sigma. Nitrocefin was purchased from Calbiochem.

Plant extracts

H. lupulus cones were collected from Balıkesir (wayside; altitude, 55 m) and Darıca village district of Manyas in western of Turkey in the month of September 2010 (voucher no: 32808, Uludağ University (Biology Dept. Herbarium)). The extracts were prepared from air-dried clean cones and *H. lupulus*, powdered with an electric blender and the pulverized plant material was divided into four parts for extraction. Firstly, the soxhlet extraction of the plant material (100 g) was carried out sequentially, using *n*-hexane (a), dichloromethane (b), ethylacetate (c) and methanol (d) (methanol-3) (each 1 L). Secondly, hop cones (100 g) of *H. lupulus* were extracted directly with methanol (e) (methanol-1) (1 L). Thirdly, plant material (100 g) was extracted with *n*-hexane, acetone (f) and methanol (g) (methanol-2) (1 L). Finally, plant material (100 g) was extracted with 25% aqueous ethanol (h) (1 L) according to the reported method[21]. Extracts were stored at +4 °C in the refrigerator and detailed information were given in the previous study[21].

Cell culturing

Human hepatoma carcinoma (Hep3B) and human colon carcinoma (HT-29) were cultured in DMEM supplemented with 10% heat inactivated (56 °C for 1 h) Fetal Calf Serum and 2 mM *L*-Glutamine. The cultures were maintained at 37 °C in a humidified incubator containing 5% (v/v) CO_2 in air. The cell lines were grown and routinely passaged twice a week.

Experimental setup for cell cytotoxicity assay

Hep3B and HT-29 cells were seeded into 96-well plates (5000 cells/well) and cultured overnight before the treatment. Different *H. lupulus* extracts (methanol 1, methanol 2 methanol 3, acetone, 25% aqueous ethanol and *n*-hexane) were used for cell cytotoxicity assays. The cells were exposed to 10 different concentrations of the *H. lupulus* extracts ranging from 0.1 to 1 mg/mL. Then the cells were treated with the *H. lupulus* extracts for various time periods such as 24, 48 and 72 h. Methanol, acetone, ether, ethanol and hexane were used as a vehicle control by adding the same volume used to the cells. Each sample was assayed in triplicates.

MTT assay

The effects of the plant extracts on the cell viability were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) calorimetric method[22,23]. The assay is now widely used for screening cytotoxicity of the potential anticancer agents[24,25]. MTT solution was added to each well and waited for metabolize at 37 °C for 4 h. Medium was removed and *i*-propanol including 0.004 M HCl was added to each 96-well plate and the plate was vortexed for resolving of formazan crystals and the optical density was measured at a wavelength of 550 nm. Growth inhibition in percent (*I* %) was calculated as given below and the values obtained (Equation (1)) are shown in Table 1 and cytotoxicity of the extracts are shown in Figures 2–4.

$$I\,\% = \left(\frac{A_{blank} - A_{sample}}{A_{blank}}\right) \times 100 \qquad (1)$$

Protease inhibition assay

All of the inhibition activities of the extracts' solutions (0.2 mg/mL) in DMSO were determined by slightly modifying the method developed by Rahman[16]. Assays were conducted in a 96-well microplate and an ELISA microplate reader (Moleculer Devices Spectromax Plus 384) was used to measure absorbance at 410 nm. PMSF (0.0017 mg/mL) was used as an inhibitor (for control). α-Chymotrypsin, trypsin and papain and their specific chromogenic substrates, including *N*-Suc-Phe-*p*-nitroanilide, Nα-Benzoyl-*DL*-arginine-4-nitroanilide hydrochloride, B*z*-*DL*-Arg-*p*-nitroanilide, respectively were used for activity measurements. 10 µL of the extracts were dissolved in DMSO with 130 µL of Tris-HCl buffer solution (pH 7.5), 10 µL of enzyme solution (9.0 Units/mL) was transferred in each well for α-chymotrypsin. Each sample was incubated at 37 °C for 10 min in a microplate reader and 50 µL of *N*-Suc-Phe-*p*-nitroanilide solution (2.6 mM) was dispensed after incubation and the absorbance is monitored continuously in the microplate reader for 15 min with appropriate wavelengths (410 nm). Tris-HCl buffer solution (0.4 M, 130 µL, pH 7.5), trypsin solution (150.0 U/mL, 10 µL) prepared in Tris-HCl buffer solution (50 mM, pH 7.5) and sample solution (10 µL) was added to 96-well plate, respectively. Each sample was incubated at 37 °C for 30 min in a microplate reader. Finally, 50 µL of B*z*-*DL*-Arg-*p*-nitroanilide solution (1.0 mM) prepared in Tris-HCl solution (50 mM, pH 7.5) was added to reader and the absorbance was monitored for 15 min. Similarly, papain (6.0 U/mL, 10 µL) was prepared in Tris-HCl buffer solution (50 mM, 250 µL, pH 7.5) and B*z*-*DL*-Arg-*p*-nitroanilide (1.0 mM, 50 µL) was used as a substrate and monitored in the microplate reader. Control reactions were run without inhibitors. Each sample was assayed in twice.

Tyrosinase inhibition assay

Tyrosinase inhibition assay was determined using the modified dopachrome method with L-DOPA as a substrate. The method applied here was similar to the protease assay. Tyrosinase (3610 Unit/mg) was prepared in phosphate buffer (0.1 M, pH 6.8) and the activity of tyrosinase is proportional to the amount of dopachrome liberated that was measured at 475 nm in a spectrophotometer[16]. Control reactions were run without inhibitors. Each sample was assayed in twice.

β-Lactamase inhibition assay

Penicillinase (final concentration 110 U/mL) and nitrocefin (final concentration 0.19 mM) were used in phosphate buffer (100 mmol, pH 7.0) for this method. Pre-incubate the test sample with the enzyme were performed for 5–10 min. 1.0 mL of nitrocefin solution was added to initiate the reaction and kept at 30 °C. The absorbance was read at 495 nm[16]. Control reactions were run without inhibitors. Each sample was assayed in twice.

Statistical analysis

Standard deviations and *p* values were calculated by using Mini Tab 14 software. One way ANOVA analysis was applied in between pairs for statistical significance. $p < 0.05$ was considered as statistically significant.

Table 1. Effect of the concentrations of *H. lupulus* extracts on Hep3B carcinoma cells.

		24 h		48 h		72 h	
Extract	Conc. (mg/mL)	OD	Growth inhibition (%)	OD	Growth inhibition (%)	OD	Growth inhibition (%)
n-Hexane	Control	0.39 ± 0.01	–	0.59 ± 0.13	–	1.30 ± 0.14	–
	0.1	0.34 ± 0.00	12.82	0.82 ± 0.29	NV	1.16 ± 0.20	10.76
	0.2	0.35 ± 0.00	10.25	0.81 ± 0.13	NV	0.79 ± 0.11	39.23
	0.3	0.34 ± 0.01	12.82	0.58 ± 0.18	1.69	0.75 ± 0.13	42.30
	0.4	0.33 ± 0.01	15.38	0.60 ± 0.10	NV	0.39 ± 0.11	70.00
	0.5	0.33 ± 0.00	15.38	0.54 ± 0.12	8.47	0.40 ± 0.08	69.23
	0.6	0.32 ± 0.01	17.94	0.50 ± 0.07	9.00	0.55 ± 0.10	57.69
	0.7	0.38 ± 0.01	2.56	0.63 ± 0.11	NV	0.49 ± 0.15	62.30
	0.8	0.41 ± 0,02	NV	0.68 ± 0.03	NV	0.56 ± 0.10	56.92
	0.9	0.43 ± 0.01	NV	0.55 ± 0.05	6.78	0.61 ± 0.12	53.07
	1.0	0.53 ± 0.05	NV	0.64 ± 0.09	NV	0.30 ± 0.08	76.92
Acetone	Control	0.45 ± 0.07	–	0.71 ± 0.15	–	0.73 ± 0.07	–
	0.1	0.37 ± 0.00	17.80	1.20 ± 0.17	NV	1.15 ± 0.23	NV
	0.2	0.33 ± 0.00	27.00	0.50 ± 0.01	29.57	0.92 ± 0.19	NV
	0.3	0.32 ± 0.00	29.00	1.03 ± 0.02	NV	0.87 ± 0.12	NV
	0.4	0.33 ± 0.00	27.00	0.94 ± 0.09	NV	0.68 ± 0.02	6.84
	0.5	0.32 ± 0.00	29.00	0.98 ± 0.05	NV	1.09 ± 0.40	NV
	0.6	0.31 ± 0.00	31.11	0.84 ± 0.18	NV	1.02 ± 0.25	NV
	0.7	0.34 ± 0.01	24.44	0.96 ± 0.14	NV	0.62 ± 0.12	15.06
	0.8	0.34 ± 0.00	24.44	0.58 ± 0.06	18.30	0.55 ± 0.00	24.65
	0.9	0.34 ± 0.01	24.44	0.56 ± 0.08	21.12	0.49 ± 0.02	32.87
	1.0	0.43 ± 0.05	4.44	0.47 ± 0.06	33.80	0.53 ± 0.06	27.39
Methanol-1	Control	0.41 ± 0.02	–	0.93 ± 0.01	–	0.78 ± 0.13	–
	0.1	0.37 ± 0.00	9.75	1.48 ± 0.49	NV	1.01 ± 0.14	NV
	0.2	0.34 ± 0.02	17.07	1.22 ± 0.02	NV	1.05 ± 0.07	NV
	0.3	0.33 ± 0.02	19.51	0.92 ± 0.00	1.07	0.95 ± 0.09	NV
	0.4	0.31 ± 0.02	24.39	0.86 ± 0.02	7.52	0.92 ± 0.09	NV
	0.5	0.36 ± 0.02	12.19	0.55 ± 0.21	40.86	0.73 ± 0.00	6.41
	0.6	0.34 ± 0.00	17.07	0.69 ± 0.03	25.80	0.69 ± 0.22	11.53
	0.7	0.36 ± 0.02	12.19	0.71 ± 0.06	23.65	0.57 ± 0.34	26.92
	0.8	0.37 ± 0.00	9.75	0.67 ± 0.16	27.95	0.25 ± 0.02	67.94
	0.9	0.41 ± 0.00	0.00	0.69 ± 0.00	25.80	0.41 ± 0.04	47.43
	1.0	0.59 ± 0.03	NV	0.59 ± 0.07	36.60	0.21 ± 0.02	73.07
Methanol-2	Control	0.49 ± 0.06	–	1.04 ± 0.16	–	0.97 ± 0.49	–
	0.1	0.36 ± 0.00	26.53	1.46 ± 0.12	NV	1.82 ± 0.41	NV
	0.2	0.33 ± 0.00	32.65	0.49 ± 0.05	52.88	2.13 ± 0.23	NV
	0.3	0.34 ± 0.01	30.61	1.34 ± 0.57	NV	2.50 ± 0.71	NV
	0.4	0.32 ± 0.01	34.69	1.22 ± 0.40	NV	2.48 ± 0.84	NV
	0.5	0.34 ± 0.03	30.61	1.09 ± 0.16	NV	2.62 ± 0.58	NV
	0.6	0.35 ± 0.01	28.57	0.90 ± 0.05	13.46	2.99 ± 0.43	NV
	0.7	0.34 ± 0.02	30.61	1.49 ± 0.77	NV	3.01 ± 0.08	NV
	0.8	0.40 ± 0.03	18.36	1.10 ± 0.68	NV	2.90 ± 0.37	NV
	0.9	0.39 ± 0.03	20.41	0.91 ± 0.44	12.50	2.86 ± 0.59	NV
	1.0	0.50 ± 0.04	NV	0.69 ± 0.26	33.65	1.13 ± 0.61	NV
Methanol-3	Control	0.36 ± 0.01	–	0.77 ± 0.13	–	0.98 ± 0.15	–
	0.1	0.35 ± 0.00	2.77	0.92 ± 0.29	NV	1.16 ± 0.28	NV
	0.2	0.38 ± 0.00	NV	0.97 ± 0.13	NV	1.07 ± 0.03	NV
	0.3	0.35 ± 0.01	2.77	1.05 ± 0.18	NV	0.92 ± 0.02	6.12
	0.4	0.34 ± 0.01	5.55	0.99 ± 0.10	NV	1.08 ± 0.19	NV
	0.5	0.36 ± 0.00	0.00	0.72 ± 0.12	6.49	1.33 ± 0.36	NV
	0.6	0.37 ± 0.01	NV	1.06 ± 0.07	NV	1.14 ± 0.18	NV
	0.7	0.36 ± 0.01	0.00	1.03 ± 0.11	NV	0.87 ± 0.07	11.22
	0.8	0.38 ± 0.02	NV	1.00 ± 0.03	NV	1.36 ± 0.43	NV
	0.9	0.39 ± 0.01	NV	0.73 ± 0.05	5.19	1.61 ± 0.63	NV
	1.0	0.51 ± 0.05	NV	0.52 ± 0.09	32.46	0.39 ± 0.03	60.20
25% Aqueous ethanol	Control	0.38 ± 0.05	–	0.42 ± 0.03	–	1.13 ± 0.07	–
	0.1	0.35 ± 0.00	7.89	1.26 ± 0.12	NV	0.98 ± 0.18	13.27
	0.2	0.33 ± 0.00	13.15	1.10 ± 0.06	NV	0.96 ± 0.02	15.04
	0.3	0.33 ± 0.00	13.15	0.98 ± 0.00	NV	0.99 ± 0.25	12.38
	0.4	0.30 ± 0.01	21.05	0.98 ± 0.10	NV	0.81 ± 0.06	28.31
	0.5	0.32 ± 0.00	15.78	0.91 ± 0.09	NV	1.02 ± 0.00	9.73
	0.6	0.31 ± 0.02	18.42	0.57 ± 0.34	NV	0.99 ± 0.03	12.38
	0.7	0.30 ± 0.05	21.05	0.47 ± 0.17	NV	0.90 ± 0.09	20.35
	0.8	0.35 ± 0.04	7.89	0.48 ± 0.14	NV	0.86 ± 0.04	23.89
	0.9	0.31 ± 0.01	18.42	0.37 ± 0.01	11.90	0.67 ± 0.05	40.71
	1.0	0.36 ± 0.02	5.26	0.53 ± 0.26	NV	0.52 ± 0.36	53.98

Results are presented as means ± S.D. of three experiments. NV indicates negative value of growth inhibition.
Reaction conditions: 37 °C, 4 h, 550 nm.

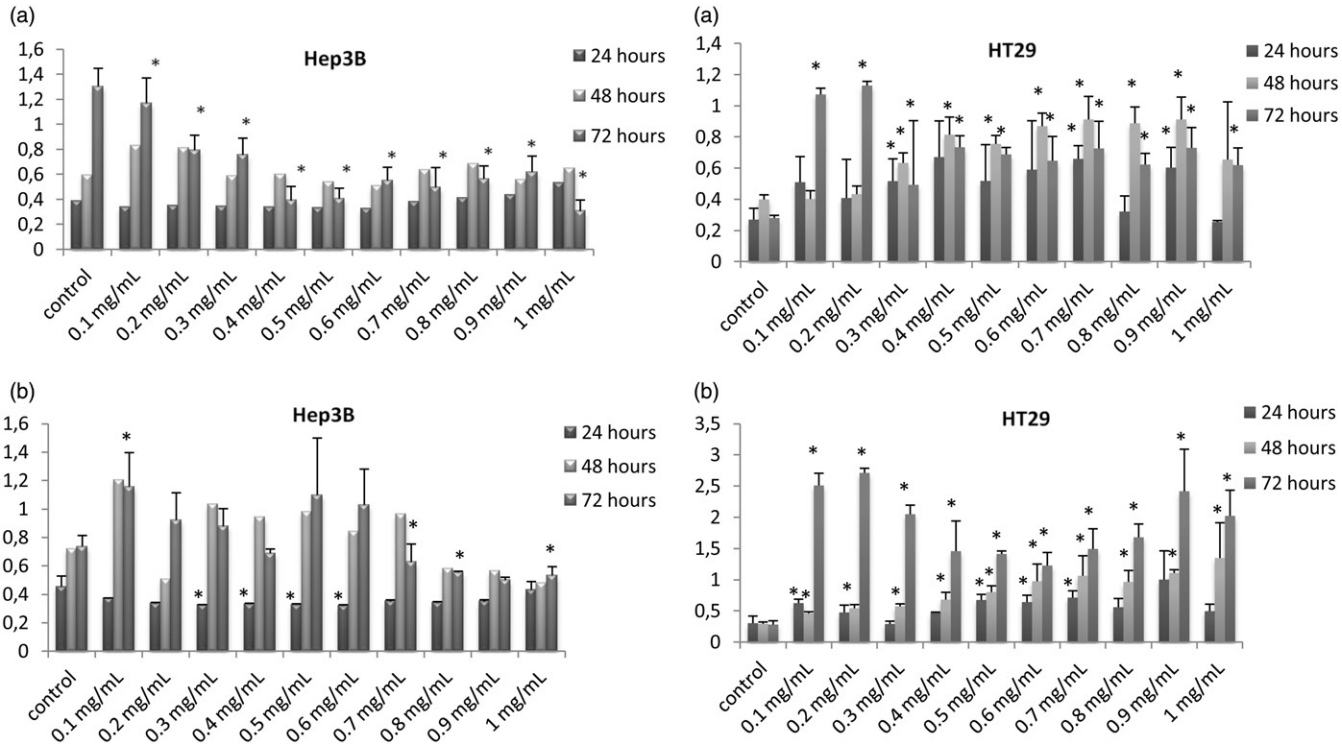

Figure 2. *In vitro* cytotoxicity of *H. lupulus* extracts (a) *n*-hexane, (b) acetone against malignant human hepatoma cells (Hep3B) and human colon cells (HT-29). The cells were treated with DMSO vehicle or the indicated concentrations of *H. lupulus*. Cell viability was determined by using the MTT assay and expressed as means ± S.D. of three separate experiments ($n = 3$). *$p < 0.05$.

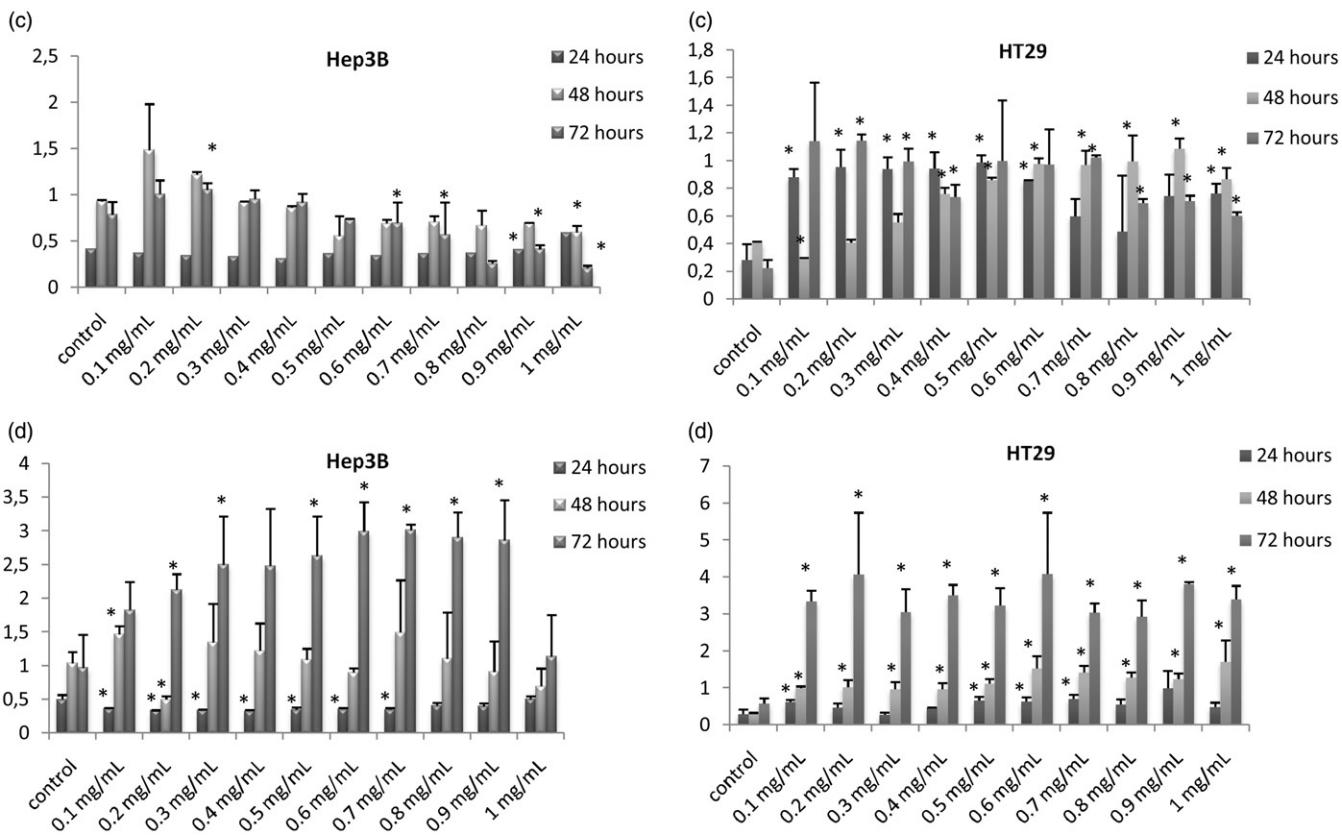

Figure 3. *In vitro* cytotoxicity of *H. lupulus* extracts (c) methanol-1, (d) methanol-2 against malignant human hepatoma cells (Hep3B) and human colon cells (HT-29). The cells were treated with DMSO vehicle or the indicated concentrations of *H. lupulus*. Cell viability was determined using MTT assay and expressed as means ± S.D. of three separate experiments ($n = 3$). *$p < 0.05$.

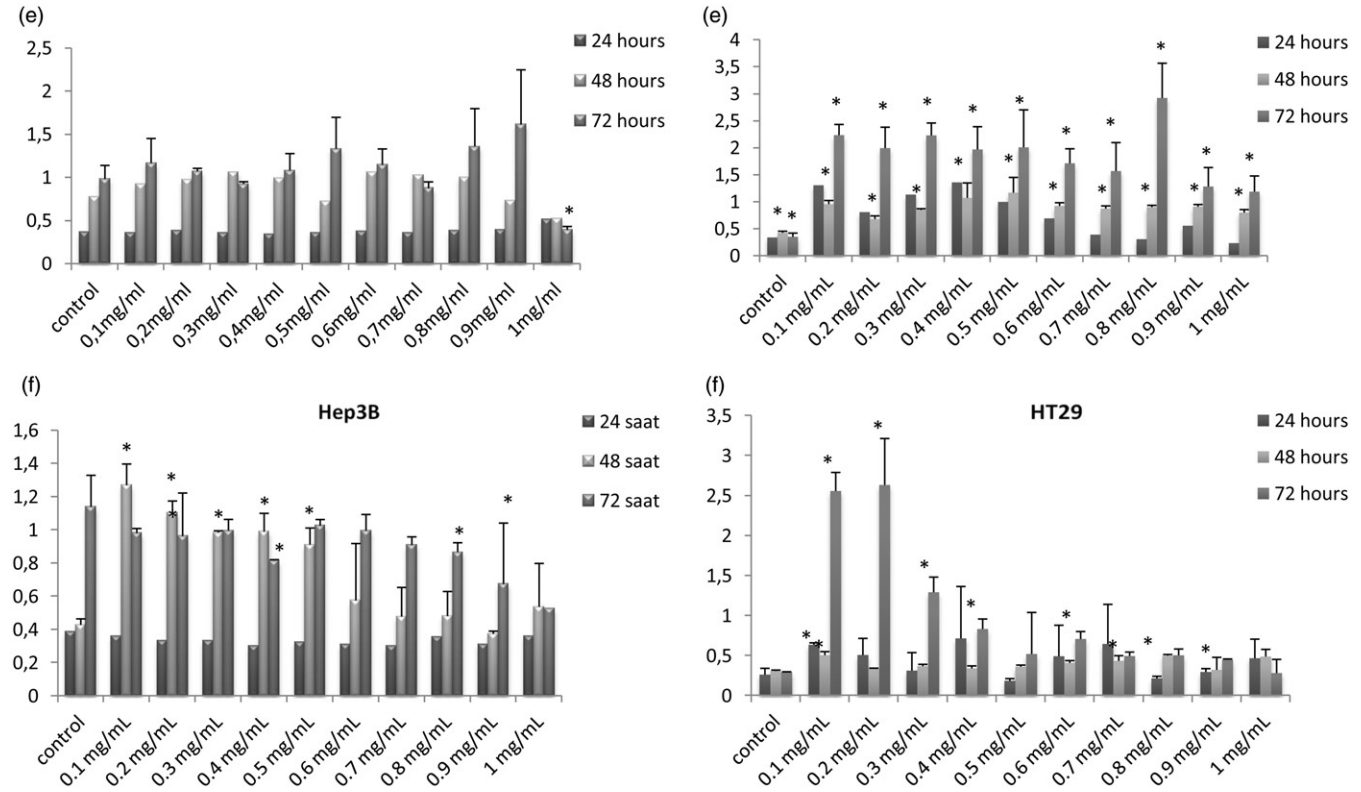

Figure 4. *In vitro* cytotoxicity of *H. lupulus* extracts (e) methanol-3, (f) 25% aqueous ethanol against malignant human hepatoma cells (Hep3B) and human colon cells (HT-29). The cells were treated with DMSO vehicle or the indicated concentrations of *H. lupulus*. Cell viability was determined using MTT assay and expressed as means ± S.D. of three separate experiments ($n = 3$). *$p < 0.05$.

Results

Cell cytotoxicity assay of *H. lupulus* extracts

The effect of various *H. lupulus* extracts, namely *n*-hexane, acetone, methanol (1, 2 and 3) and 25% aqueous ethanol on the viability of human colon cells, HT-29 and human Hep3B cells was evaluated *in vitro*. The MTT assay adopted for this study is based on measurements of *in vitro* growth of the cells by human cell-mediated reduction of tetrazolium. The assay is widely used for screening the cytotoxicity of potential anticancer agents[17]. The concentration of formazan produced is directly proportional to the number of viable cells. Figures 2–4 show the effect of 10 different concentrations (0.1–1 mg/mL) of each extraction of HT-29 cell line for 24, 48 and 72 h. Viability of colon cancer line was not inhibited by all extracts, namely *n*-hexane, acetone, methanol-1, methanol-2 and methanol-3 and 25% aqueous ethanol extracts and, interestingly, we noticed that all the doses of methanol-1, methanol-2 and methanol-3 extracts increase the proliferation of colon cancer cell lines for treatment of 24, 48 and 72 h (Figures 2–4). Most strikingly, 25% aqueous ethanol extract also increased the cell proliferation even at low doses (0.1 and 0.2 mg/mL) for 72 h.

On the other hand, all extracts show the differential effects on Hep3B cells. Acetone extract increased the cell proliferation at low doses for 48 and 72 h. The antiproliferative effect of all the doses of *n*-hexane extract on cancer cell viability has been observed at 72 h in Hep3B cell line (Table 1 and Figure 2). Additionally, methanol-1 extract inhibited the cell proliferation dose-dependent and time-dependent manner so that 1 mg/mL concentration of this extract was statistically significant for antiproliferation at 72 h in Hep3B cells with 70% inhibition effect (Table 1, Figure 3). Methanol-2 extract shows antiproliferative effect at early time points, 24 h, while all doses of methanol-2 extract were increased the cell viability for 48 and 72 h. Methanol-3 extract did not show any statistically significant effect on the human hepatoma cancer, Hep3B cell line (Table 1, Figure 4). Lastly, 25% aqueous ethanol extract, which displayed a major antioxidant effect with the antioxidant capacity methods[21], was indicated the proliferative effect at 48 h at low doses of this extract for Hep3B cell line.

As a summary, *H. lupulus* extracts have diverse effects in different concentrations and two different cell lines, HT-29 and Hep3B cells. In this study, the cell proliferation and antiproliferation simultaneously took place so that antitumoral effect is determined for only a few doses of some of the extracts and it is shown that bioactive components of *H. lupulus* are dose dependent and cell type dependent.

Based on the literature, at least two mechanisms can be proposed to explain this result. First mechanism is that the protective effect of drug could be attributed to an anti-apoptotic mechanism and secondly, through an antioxidant mechanism by enhancing the activity of the intracellular antioxidant enzymatic system. As a result, we suggest that *H. lupulus* extracts at high concentration might have an effect on the viability of human colon cancer cells with a similar anti-apoptotic and/or an antioxidant mechanisms. To explain certain mechanism(s) of this potential dual role of *H. lupulus* on survival and growth of the cancer cells, and to precise cut off value and concentration limits of *H. lupulus* extracts before its use as an anti-neoplastic agent will need some further studies[26].

Proteases, tyrosinase and β-lactamase inhibitory activity

n-Hexane, acetone, methanol-1, methanol-2, methanol-3 and 25% aqueous ethanol extracts of *H. lupulus* were examined for their enzyme inhibitory activity against proteases, tyrosinase and

Table 2. V_{max} (average) and % inhibition values of the extracts during enzyme inhibition assay.

		Proteases									
		α-Chymotripsin		Trypsin		Papain		Tyrosinase		β-Lactamase	
	Extracts	Average	% inh V_{max}	Average	% inh V_{max}	Average	% inh V_{max}	Average	% inh V_{max}	Average	% inh V_{max}
A	Control	28.212	–	80.957	–	27.306	–	39.445	–	35.509	–
B	*n*-Hexane	27.566	2.32	106.973	NV	22.273	18.43	49.966	NV	21.961	38.00
C	Acetone	28.695	NV	90.445	NV	28.383	NV	70.153	NV	28.043	21.05
D	Methanol-2	29.297	NV	96.339	NV	34.080	NV	58.643	NV	32.894	07.36
E	25% Aqueous ethanol	27.193	3.61	96.218	NV	32.780	NV	55.635	NV	36.867	NV
F	Methanol-3	29.026	NV	95.514	NV	27.059	0.90	63.633	NV	30.728	15.67
G	Methanol-1	27.834	1.34	110.394	NV	27.784	NV	50.938	NV	29.387	17.24
H	PMSF inhibitory	0.004	–	0.580	–	–*	–	–*	–	0.002	0.002

– (dashed line) indicates control for samples. *indicates without inhibitory as PMSF. NV indicates negative value.
Reaction conditions: The assay mixture is incubated at 37 °C for 30 min. 410 nm for proteases. The absorbance is monitored at 475 nm for tyrosinase.
The test sample is preincubate with the enzyme for 5–10 min and the absorbance is read at 495 nm for β-lactamase enzymes.
Control includes (enzyme+substrate+DMSO+buffer solution).
Extract includes (enzyme+sample solution+substrate+buffer solution).

β-lactamase. None of the extracts efficiently showed inhibitory effect against the selected enzymes while known inhibitor of PMSF was showing inhibitory effect against the tested enzymes during the reaction. The reaction rate graphs showing the enzyme activities are given in Figures S1–S10. Additionally, V_{max} values of the extracts were determined as milli-units per min from the reaction rate graphs. These values are shown in Table 2 and Figures S1–S10. Maximal velocity (V_{max}) which is used first time by Michaelis and Menten in 1913, is a mathematical description of enzyme action. This graphical method transforms the Michaelis–Menten equation to a linear line by taking the reciprocal of both sides of the Michaelis–Menton equation[16]. Inhibition (%) values of the tested samples against to control were calculated from average V_{max} by using Equation (1) in order to compare with one another. V_{max} values determined with microplate reader and % inhibition values calculated by using Equation (1) of the extracts are shown in Table 2.

According to Table 2, in protease inhibition assay, the highest % inhibition value with α-chymotrypsin enzyme was observed for 25% aqueous ethanol (3.61%), and following as *n*-hexane (2.32%), methanol-1 (1.34%) extracts, while the others (acetone, methanol-2 and methanol-3) have negative values and do not show any inhibition against α-chymotrypsin enzyme.

According to the reaction rate graph and V_{max} values of the sample solutions with trypsin enzyme, none of these samples did inhibit trypsin enzyme. Papain inhibition assay was carried out without PMSF inhibitor and there were no differences in the trypsin enzyme results. *n*-Hexane (18.43%) and methanol-3 (0.9%) extracts may be a little active for papain enzyme.

In the tyrosinase enzyme assay, V_{max} values are similar to controls so the extracts did not inhibit the tyrosinase. The V_{max} values of extracts with the penicillinase (β-lactamase) enzyme which ranged from 21.9 to 32.9 as average V_{max}, were measured as *n*-hexane > acetone > methanol-1 > methanol-3 and % inhibition values varied from 38.0% to 15.7%, respectively. Thus, the highest V_{max} value was seen in *n*-hexane extract as 38% and 40.0 milli-units per min and the lowest % inhibition value of methanol-2 extract was calculated as 7.36% with the penicillinase enzyme.

As known, the pure/bioactive compounds of *H. lupulus* widely inhibit the enzymes[21,27,28], but in this study the extracts were observed in low inhibitory effects with selected enzymes. According to the reaction rates (V_{max}) in the graphs, *n*-hexane extract was determined as the mostly active extract. The inhibition activities of methanol-1 extract are observed as 1.34% and 17.24% for α-chymotrypsin and β-lactamase, respectively. Besides this, *n*-hexane extract inhibits α-chymotrypsin (2.32%), papain (18.43%) and β-lactamase (38%). Generally, strong inhibition activities of the extracts were observed with penicillinase enzyme, and *n*-hexane extract is more active than the others so bitter acids or organic acids of *H. lupulus* such as humulones and lupulones may be a kind of β-lactamase inhibitors. Furthermore, antibiotic effects of these compounds are known[21,28].

We know that the highest antioxidant capacity was seen for the *n*-hexane extract of *H. lupulus* with DPPH (IC_{50}: 08.67 ± 0.07 µg/mL)[21]. Gallic acid (185 µg/g) and kaempherol-3-*O*-glucoside (163 µg/g) were abundantly found and quantitatively identified compounds of *H. lupulus* by LC–MS/MS[21] besides the known active components such as humulones, lupulones[29,30], etc. of *n*-hexane extract. The synergic effect of several compounds that present in hop plant extracts has not been tested yet and this is the aim of this work. The antioxidant activities of the *H. lupulus* extracts were better than that of the individual components, while the cytotoxicity and enzyme inhibition studies were almost contrary to[21,27]. Furthermore, the inhibition assays for protease, tyrosinase and β-lactamase which were compiled by Rahman[16] are run the first time for the hop extracts in this paper.

Discussion

We have herein explained the active extracts of *H. lupulus* of Turkish origin for their enzyme inhibitory potentials as well as antioxidant activity of each extracts by four different methods, due to its medicinal applications. Inhibition of cyclooxygenase enzymes such as COX1 and COX2 was studied *in vitro via* bioactive components (XN, 8-PN) of *H. lupulus* and inhibition of nitric oxide synthase (iNos) was studied *in vitro via* bioactive components (XN, IXN) of *H. lupulus*[27] and IC_{50} values ranges of these components were determined as 16–27 and 41.5 µM for COX1 and COX2 enzymes, respectively[27]. As far as we know that it is the first time, proteases (α-chymotrypsin, trypsin, papain), tyrosinase and β-lactamase enzyme inhibition assays were applied to *H. lupulus* extracts. Although several studies regarding *H. lupulus* extracts have been reported on biological activities, these studies generally examined single component, for instance, xanthohumol, 8-prenylnaringenin, etc. As a result, *in vitro* biological studies with combination of bioactive components of *H. lupulus* have attracted more interest in their usage as food supplementary recently.

Although several polyphenols, including flavonoids or stilbenoid, substrate analogues, free radical scavengers and copper

chelators, have been known to inhibit tyrosinase[19], *H. lupulus* extracts which have several polyphenols did not show any significant inhibitory effects. According to a study[19], some of the experimental observations of the inhibition of tyrosinase activity are indicated that ascorbic acid which is used as melanogenesis inhibitor and some phenolic compounds is known as alternative enzyme substrates, but there were no efficiently tyrosinase inhibition for *H. lupulus* extracts rich in phenolics. These results confirm that pure compounds of *H. lupulus in vitro* studies are more active than the extracts. It should be stated here again that tyrosinase activity is very important. That is because, if uncontrolled during melanoma, it results in increased melanin synthesis. A mutation in the tyrosinase gene resulting in impaired tyrosinase production leads to type I oculocutaneous albinism, a hereditary disorder that affects one in every 17 000 people[31].

In our previous study, we reported the first time the quantitative analysis of some of the major components of extracts by LC−MS/MS and the first comparative study emphasize the antioxidant activities of the extracts of naturally growing plant in Turkey with DPPH, TEAC, FRAP and CUPRAC assays and given IC_{50} values of active extracts of *H. lupulus*. According to these results, we investigated the antiproliferation and enzyme inhibition of the active extracts of *H. lupulus* with MTT assay and proteases, tyrosinase and β-lactamase enzymes in this paper, respectively. Significance values of the extracts are shown in Tables 1 and 2, Figures 2–4 and Figures S1–S10.

In summary, *H. lupulus* extracts differentially effect on hepatoma and colon cancer cells, also the extracts were not cytotoxic to the cells. Actually, antiproliferative effect, antioxidant activity, phenolics and the other bioactive components in the *H. lupulus* extracts did not have a correlation in both HT-29 and Hep3B cell lines, even so, cell proliferation indicated meaningful differences among extract treatments and the control (Figures 2–4 and Table 1). Strong antiproliferative effects in different cancer models to determine the effects are important, for this purpose, the antitumor effects of the active extracts of *H. lupulus* in different concentrations were examined *in vitro* on Hep3B and HT-29 cell lines in this paper.

This paper proposes that synergism of varied bioactive components (kaempferol-3-*O*-glucoside, ellagic acid, pyrogallol, ascorbic acid, *p*-coumaric acid, gallic acid) were determined by LC–MS/MS as well as identified by LC–MS (SIM mode) major compounds such as humulone, lupulone, etc.[21] that may be significant to inhibit cell proliferation. Finally, researchers suggested that the major chalcone and the others such as XN, 6-PN and 8-PN show strong antioxidant activity *in vitro* in the micromolar range and these are weakly cytotoxic to certain of the cancer lines[32]. Prenylated flavonoids, bitter acids and polyphenolic content of *H. lupulus* increase its influence contrary to some of the diseases, so it may be cured on some of the illnesses. After all, there are many studies about these effects of *H. lupulus* constituents for a long time, but it was clear that these studies generally have examined the impression of the pure compounds of the plant. Otherwise, although enhancement properties of *H. lupulus* are known, we decided to illustrate the synergy of all the components by the time period and different concentrations in each extracts, so this study may be a guide for next studies.

In previous studies (see references), the cytotoxity of pure compounds of *H. lupulus* were examined and the results were generally found highly effective at low concentrations by the researchers, but the effects of the components change adversely if they used as an extract, so it is a very important factor for pharmacological studies or trials. Similarly, according to the literature[3], as xanthohumol is known to be an effective inhibitor of cytochrome P450 enzymes, our results display that synergism of all of the components in an extract have different influence with different enzymes. Up to this time, proteases, tyrosinase and β-lactamase enzymes have not studied yet for *H. lupulus* plant, whereas, applications of these enzymes are widely used and new inhibitors need to be find out. For instance, tyrosinase inhibitors are widely used in dermatological treatments and also applied in cosmetics[18]. For infectious organisms such as bacteria, viruses and parasites, generally need protease inhibitors and HIV has been successfully targeted by protease inhibitors and these are used clinically (Amprenavir, Atazanavir, etc.)[20].

Conclusions

Results obtained from the plant extracts provided some evidences on the reactivity of bioactive components of *H. lupulus* by using the instrumental techniques such as cyclic voltammetry, UV–VIS, FT-IR and GC–MS analysis[33]. Some alternative ways need to be considered, because of the extracts might not show beneficial effects as far as the pure compounds in the pharmaceutical studies. However, *n*-hexane and methanol-1 extracts, bitter acids and main prenylated chalcones of female hop cones have significant effects and these components need to be investigated in many treatments such as new therapeutics and/or new serin/threonin inhibitors. Because, nature has been a source of therapeutic agents for thousands of years, and an impressive number of modern drugs have been derived from natural sources, many based on their use in traditional medicine[34]. Also, antiproliferation, cytotoxicity and enzyme activity[35] studies have been developed in recent years and these studies lead to strategy of drug development, other processes associated with usage of the plant bioactive components, etc. The contents of plant bioactive components generally depend on various conditions such as region, climate and type of soil. Similarly, due to the differences of the preparation methods of the extracts and the applied assay procedures to the extracts, it is open to new approaches for phytochemical studies.

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Declaration of interest

The authors declare no competing financial interest.

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Supplementary material available online
Supplementary Figures S1–10