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Total Phenolic, Flavonoid, Fatty Acid Contents and Cytotoxic, Antioxidant, and Antimicrobial Activities of *Hedysarum aucheri*

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Authors' contributions

This work was carried out in collaboration between all authors. Author ZU designed the study, prepared the plant extracts and wrote the manuscript. Author OK carried out GC-MS analysis and contributed to the design of the work. Author EU performed the antimicrobial activity studies. Author UA extensively participated in the preparation of the plant extracts. Author IK conducted the studies about the total flavonoid and phenolic contents and antioxidant activities. Author AN performed the cytotoxic activity studies. All authors read and approved the final manuscript.

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ABSTRACT

In vitro cytotoxic, antioxidant, and antimicrobial activities along with total phenolic and flavonoid contents of methanol, hexane, dichloromethane, butanol, and aqueous extracts and chemical composition and fatty acids of *n*-hexane extract from endemic *Hedysarum aucheri* were determined. The fatty acid content of the *n*-hexane extract was determined by GC-MS analysis.

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Twenty-four out of 39 compounds identified as fatty acid methyl esters constitute 84.20% of hexane extract content. Five of 6 major components were fatty acids, namely *alpha*-linolenic acid (ALA) (32.37%), palmitic acid (24.69%), linoleic acid (LA) (9.16%), stearic acid (7.11%), Arachidic acid (3.14%), and the other one was an acyclic diterpene alcohol called phytol (12.01%). Butanol extract was found to be the richest in flavonoid (66.3 ±1.3 mg QU/g DW) and phenolic (122.4±1.6 mg GAE/g DW) compounds. Antioxidant activities of the extracts were evaluated by Cupric-ion-reducing antioxidant capacity (CUPRAC), and ABTS radical scavenging capacity. Butanol extract showed the highest antioxidant activity indicating a strong correlation between flavonoid/phenolic content and antioxidant activity. Also, cytotoxic activities of the extracts were evaluated against a panel of cancer cells (PC3, HeLa, CaCo-2, U-87MG, A549, and MCF-7) and normal cell line HEK293. Dichloromethane extract (DCM) exhibited exceptionally high tumor selective cytotoxicity towards cancer cells with IC_{50} values varying between 0.64 and 24 µg/mL while not impairing the normal cells. DCM extract was found to possess higher cytotoxic activity towards the tested cancer cells than some anticancer drugs reported in the literature. Also, antimicrobial activities of the extracts were determined by agar disc diffusion and the micro-dilution methods against a series of microorganisms and hexane extract showed the highest antimicrobial activity. We hereby report both the pharmaceutical potential of *H. aucheri* concerning its various biological activities and the phytochemical composition regarding its total flavonoid, phenolic, and fatty acid content.

Keywords: Essential fatty acids; Hedysarum aucheri; cytotoxicity; antioxidant; antimicrobial; total flavonoid.

1. INTRODUCTION

Fatty acids are involved in the structure of biological components and regulate some metabolic and defense functions of the body [1, 2] apart from their primary role as the fuel source for animal cells. Two fatty acids, alpha-linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid), also known as essential fatty acids (EFAs) are particularly vital in humans as they cannot be produced by humans and need to be taken as food. EFAs have crucial roles in various metabolic processes, but probably the most important one is their role in the life and death of cardiac cells [3-6]. EFA deficiency may cause some illnesses such as osteoporosis [7], dermatitis [8,9], and hair loss. Other secondary metabolites such as flavonoids and phenolic compounds are directly involved in the defense system of the plant and provide protection against diseases. Secondary metabolites of the plants are often associated with the antioxidant activities of the plant and humans use these compounds as medicines and flavorings.

Cancer and oxidative stress-related diseases such as Parkinson's disease, Alzheimer's disease, neural disorders, immune-system decline, brain dysfunction have been and continue to be major global problems claiming millions of lives annually. The treatment of cancer by the conventional synthetic drugs is still not very efficient and comes with some harsh side effects and inherent and acquired resistance to the drug [10-12]. Synthetic antioxidants were also shown to have some genotoxic side effects [13,14]. On the other hand, plant-based therapeutics and antioxidants are usually considered safe due to their traditional use with no documented harmful impact and devoted toxicity studies about them. Plant phenolics and flavonoids are commonly found in both edible and non-edible plants and have been reported to have multiple biological effects including antioxidant activity and preventive role in the development of cancer and heart diseases [15-17]. Thus, there has been recently an upsurge of interest in medicinal plants with the therapeutic potential as anticancer agents and antioxidants that reduce oxidative stress.

Hedysarum, a genus of the Fabaceae family, has around 300 annual and perennial species, and its species are widely used as medicinal plants. Biological activities of some *Hedysarum* species have been extensively investigated mainly in China, and they were found to have potential antitumor [18-20], antioxidant [21,22], anti-aging [23], and antimicrobial [24] activities. Considering the vast potentiality of the biological activities and pharmacological importance of *Hedysarum* genus [25], our study has been focused on the investigation of the endemic *Hedysarum aucheri* plant. This plant is classified as "Vulnerable" (VU) according to IUCN red list criteria and has a high risk of extinction in the wild. *Hedysarum aucheri* used to be brewed as a beverage by the locals near the habitat of the plant and our extensive search yielded no phytochemical, biological or

toxicological study about *Hedysarum aucheri* in the literature. Here, we report fatty acid, total flavonoid, total phenolic contents along with antioxidant, cytotoxic and antimicrobial activities of five extracts obtained from *Hedysarum aucheri* with some potent results.

2. MATERIALS AND METHODS

2.1 Plant Material

Hedysarum aucheri Boiss was collected in June 2013 on roadsides and slopes near the Pertek ferry port (1010 m), Elazig, Turkey. The plant was taxonomically identified by Dr. Murat KURSAT (Department of Biology, Bitlis Eren University, Bitlis-TURKEY).

2.2 Preparation of Plant Extracts

Air-dried and powdered plant material (940 g) was extracted with methanol and concentrated to give the crude methanol extract. The dried residue was extracted with hexane, dichloromethane (DCM), and butanol: water (1:1) solvent system successively. All dried extracts were stored in a fridge until use.

2.3 Determination of Fatty Acid Content of Hexane Extract

After removing hexane using a rotary evaporator, the oily mixtures were derived to their methyl esters by the International Olive Oil Council (IOOC) and International Union of Pure and Applied Chemistry (IUPAC) reports by the trans-esterification process [26,27]. In this process, dried hexane extracts were dissolved in hexane and then extracted with 2 M methanolic KOH at room temperature for 30 s. The upper phases were analyzed by GC-MS systems. Methyl esters of fatty acids were analyzed using Agilent 7890A and Agilent 5975C inert XL MSD (mass selective detector) combined system with HP-5MS column (30 m × 0.25 mm × 0.25 µm). Pure helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min. The oven temperature was programmed as 60℃ for 5 min then 5℃/min to 220℃ and held there for 5 min. The sample (1 µL, in hexane) was injected in the split mode with a split ratio of 20:1. The compounds were identified by NIST–Wiley library data search and only peaks matching similarity index (SI) greater than 70% in NIST library were assigned.

2.4 Determination of Total Flavonoid Content

The total flavonoid content of each extract was estimated by Zhishen method [28]. Each sample (0.5 mL) solution in methanol (100 µg/mL) was mixed with 2 mL of water and 0.15 mL of a $NaNO_2$ solution (15%, w/v). After 6 min, 0.15 mL of $AlCl_3$ solution (10%) was added and allowed to stand for 6 min, then 2 mL of NaOH solution (4%) was added. Immediately, the final volume of the mixture was completed to 5 mL and left for 15 min. The absorbance of the mixture was then determined at 510 nm. Results were expressed as Quercetin equivalents (mg/g, dried extract).

2.5 Determination of Total Phenolic Content

The total phenolic content of each extract was determined by using Folin-Ciocalteu reagent following a slightly modified method of Ainsworth [29]. Briefly, 0.5 mL of the extract (100 µg/mL) was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and 4 mL of aqueous Na_2CO_3 (7.5%, w/v). The mixture was left at room temperature for 60 min. The absorbance was measured at 765 nm by UV-VIS spectrophotometer (Shimadzu UV-1700). The total phenolic contents were determined from the linear equation of a standard curve prepared with Gallic acid. Triplicate measurements were taken, and total phenolic compound contents were expressed as mg/g gallic acid equivalent (GAE) of dry extract.

2.6 Determination of *In vitro* Antioxidant, Cytotoxic and Antimicrobial Activities

Antioxidant activities were determined according to Cupric-Ion-Reducing Antioxidant Capacity (CUPRAC) and ABTS radical scavenging assays. Cytotoxic activity studies were carried out by the MTT assay. Antimicrobial activities were found by the Agar Disc Diffusion Assay and determination of Minimum Inhibitory Concentrations (MIC). All the employed methods along with the cell cultures, microbial strains, reagents, standards, and the data analyses are given in full details in the supplementary material.

3. RESULTS AND DISCUSSION

3.1 Analysis of Fatty Acid Content

The chemical composition of *n*-hexane extract of *H. aucheri* is presented in (Table 1). Out of 39 components, 24 compounds were identified as

fatty acids with carbon numbers ranging from C12 to C24. Of these compounds, 11 were unsaturated and 13 were saturated fatty acids with 44.42% and 39.78% abundance, respectively, making up 84.20% of the entire chemical content in total. Five fatty acids, namely *alpha*-linolenic acid (ALA-ω-3) (32.37%), palmitic acid (24.69%), linoleic acid (LA-ω-6) (9.16%), stearic acid (7.11%), arachidic acid (3.14%) and an acyclic diterpene alcohol called phytol (12.01%) were found as the dominant compounds. These major compounds constitute 88.48% of the total chemical content.

Only two fatty acids, ALA and LA, are essential for humans and these essential fatty acids must be ingested from food sources as they cannot be produced by human cells due to the lack of desaturase enzymes. The fats included by EFAs act as not only fuel sources but also essential components for biological processes and they play important roles ranging from the viability of cardiac cells [4,5] to dermatitis [30] and cognitive problems [31]. *Hedysarum aucheri* has high amounts of omega-3 ALA (32.37%) and omega-6 LA (9.16%) and its unsaturated fatty acid content (44.42%) is greater than its saturated fatty acid content (39.78%). Thus, it can be used as a dietary source for EFAs. The only major compound which is not a fatty acid is phytol (12.01%) that is known to have cancer-preventive effects [32,33].

3.2 Extraction Yield, Total Phenolic, and Total Flavonoid Contents

Contents of flavonoid and phenolic compounds and % extraction yields of each extract from *H. aucheri* are presented in (Table 2). Methanol extract is the main fraction extracted from the dry plant material, and the rest of the fractions were extracted from this crude methanol extract. The extraction yields of the fractions varied from 4.3 to 70.0 %. Total phenolic content and total flavonoid content of the extracts were determined from the calibration curves of gallic acid ($y=0.0042x + 0.1895$, $R^2=0.9929$), and quercetin ($y=0.024x + 0.0218$, $R^2=0.9974$), respectively. The results showed that butanol extract had the highest phenolic (122.4 ± 1.6 mg GAE/g of dry material) and flavonoid contents (66.3 ± 1.3 mg QE/g of dry material). Apparently, butanol solvent extracted the majority of these polar secondary metabolites and only highly polar sugar compounds passed into the water during extraction.

3.3 *In vitro* Antioxidant Activity

Antioxidant activities of *H. aucheri* extracts were determined by Cupric-Ion-Reducing Antioxidant Capacity (CUPRAC) and ABTS Radical Scavenging assays, and the results are presented in (Fig. 1a-1b).

In CUPRAC assay, a higher absorbance indicates higher antioxidant activity. All extracts of *H. aucheri* showed an increase in absorbance with the increase in concentration (Fig. 1a). Among all extracts, butanol fraction showed the highest absorbance with 0.823 ± 0.08 at 400 µg/mL concentration. At this concentration, standard, gallic acid, showed slightly higher absorbance, 0.95±0.04. Cu^{2+} ion-reducing power of *H. aucheri* extracts was in the following order: butanol> dichloromethane > hexane > methanol > water. The antioxidant activity results of the extracts by ABTS radical scavenging assay are given in Fig. 1b. All the fractions of *H. aucheri* scavenged ABTS radical in a concentration-dependent way. Butanol extract was fast and the most effective scavenger of the ABTS radical in comparison with the other extracts. Present results showed that the ABTS radical scavenging ability of samples is ranked as butanol >dichloromethane > methanol > n-hexane > water.

Based on the data obtained from these tests, a high correlation has been found between the total phenolic-flavonoid contents and antioxidant activity for butanol extract. Especially CUPRAC assay results showed that the antioxidant capacity of butanol extract is quite close to that of standard, gallic acid. It has been reported in literature that the results obtained from *in vitro* CUPRAC measurements might be more efficiently extended to the possible *in vivo* reactions of antioxidants [34]. For the rest of the extracts, however, no significant correlation could be found between their total phenolic-flavonoid contents and antioxidant activities. For instance, despite having a lower phenolic and flavonoid content, DCM extract showed higher antioxidant activity than methanol extract. It is known that different phenolic compounds have different responses in the Folin-Ciocalteu method. Similarly, the molecular antioxidant response of phenolic compounds in the extracts varies remarkably, depending on their chemical structure [35,36]. Thus, even though phenolic compounds can have a significant contribution to the antioxidant capacity of the extracts, the antioxidant activity of an extract cannot be

predicted solely by its total phenolic or flavonoid content. In other words, the antioxidant activity does not necessarily correlate with high amounts of phenolics, and that is why both phenolic content and antioxidant activity information must be discussed together when evaluating the antioxidant potential of extracts.

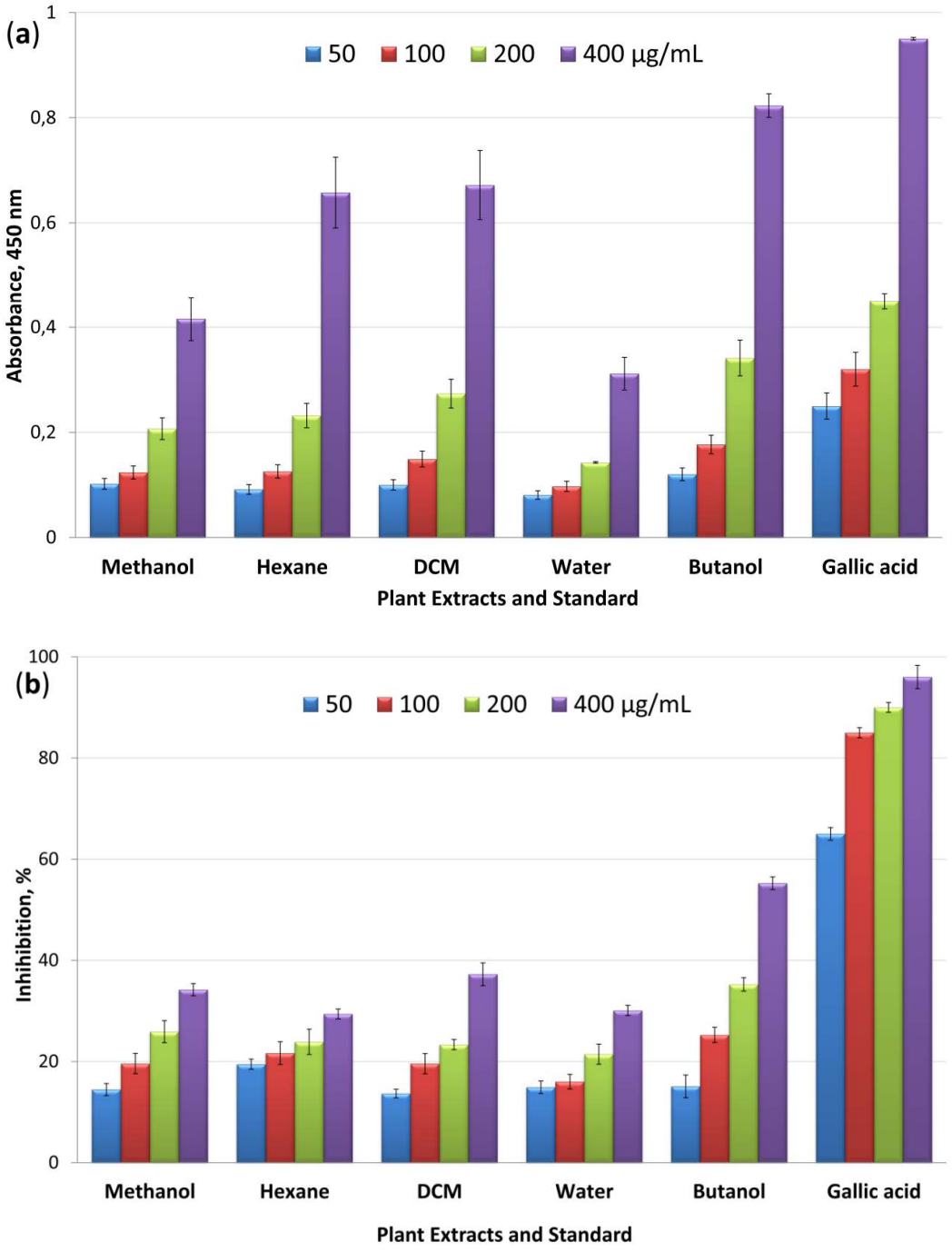

Fig. 1. Antioxidant activities of *H. aucheri* extracts and standard (gallic acid) by CUPRAC (a) and ABTS radical scavenging (b) assays

Table 1. Chemical composition of hexane extract of *H. aucheri*

No	RT (min)	C:D	n-x	Compound name (Special name)	M^+	%
1	12.675	-		Benzoic acid, methyl ester	136	0.08
2	24.687	**12:0**		**Dodecanoic acid, methyl ester (Lauric acid ME)**	214	**0.36**
3	24.947	-		4,4,7A-trimethyl-5,6,7,7A-tetrahydro-1-benzofuran-2(4H)-one	180	0.13
4	25.263	**-**		**Nonanedioic acid, dimethyl ester (Azelaic acid, diME)**	217	**0.10**
5	28.573	-		Bicyclo[4.3.1]decan-10- one	152	0.17
6	28.693	-		(E)-7-Dodecen-1-ol acetate	226	0.18
7	29.040	-		Hexadecanal	240	0.07
8	29.263	**14:0**		**Tetradecanoic acid, methyl ester (Myristic acid, ME)**	242	**0.96**
9	30.779	**15:0**		**12-methyl-tetradecanoic acid, methyl ester (Sarcinic acid, ME)**	256	**0.08**
10	31.043	**12:1**	*n-7*	***cis*-5- Dodecenoic acid, methyl ester**	212	**0.19**
11	31.380	**15:0**		**Pentadecanoic acid, methyl ester**	256	**0.38**
12	31.790	-		6,10,14-trimethyl-2-pentadecanone	268	0.42
13	32.595	-		3-(3,4-dimethoxyphenyl)-2-Propenoic acid, methyl ester	222	0.41
14	32.885	**16:1**	*n-7*	**(E)-9-Hexadecenoic acid, methyl ester (E-Palmitoleic acid, ME)**	268	**0.08**
15	32.932	**16:1**	*n-9*	**7-Hexadecenoic acid, methyl ester**	268	**0.10**
16	32.984	**16:1**	*n-7*	**(Z)-9-Hexadecenoic acid, methyl ester (Z-Palmitoleic acid, ME)**	268	**0.73**
17	33.326	**16:1**	*n-5*	**11-Hexadecenoic acid, methyl ester (Palmitvaccenic acid, ME)**	268	**0.98**
18	33.477	**16:0**		**Hexadecanoic Acid, methyl ester (Palmitic acid, ME)**	270	**24.69**
19	34.063	-		*cis*-9-Tetradecen-1-ol	212	0.18
20	34.213	-		(Z)-1,6-Tridecadiene	180	0.17
21	34.390	**16:1**	*n-14*	**2-Hexadecenoic acid, methyl ester**	268	**0.09**
22	34.883	**17:1**	*n-7*	***cis*-10-Heptadecenoic acid, methyl ester**	282	**0.26**
23	35.329	**17:0**		**Heptadecanoic acid, methyl ester (Margaric acid, ME)**	284	**0.82**
24	36.403	-		1-Octadecene	252	0.12
25	36.631	**18:2**	*n-6,9*	**(Z, Z)-9,12-Octadecadienoic acid, methyl ester (Linoleic Acid, ME)**	294	**9.16**
26	36.818	**18:3**	*n-3,6,9*	**(Z, Z, Z)-9,12,15-Octadecatrienoic acid, methyl ester (ALA, ME)**	292	**32.37**
27	36.989	-		Phytol	296	12.01
28	37.207	**18:0**		**Octadecanoic acid, methyl ester (Stearic Acid, ME)**	298	**7.11**
29	37.306	**24:0**		**Tetracosanoic acid, methyl ester (Lignoceric acid,ME)**	382	**0.24**
30	37.628	**23:0**		**Tricosanoic acid, methyl ester**	368	**0.58**
31	38.328	-		2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-Tetracosahexaene	410	0.67
32	38.868	-		Eicosane	282	0.43

No	RT (min)	C:D	n-x	Compound name (Special name)	M^+	%
33	39.236	**19:0**		**Nonadecanoic acid, methyl ester**	312	**0.20**
34	40.476	**22:0**		**Docosanoic acid, methyl ester (Behenic acid, ME)**	354	**1.12**
35	40.684	-		*cis*-9-Tricosene	322	0.30
36	41.031	-		Tricosane	324	0.42
37	41.125	**20:1**	*n-9*	**cis-11-Eicosenoic acid, methyl ester**	324	**0.26**
38	41.177	**20:3**	*n-3,6,9*	**11,14,17-Eicosatrienoic acid, methyl ester (Dihomolinolenic Acid)**	320	**0.20**
39	41.861	**20:0**		**Eicosanoic acid, methyl ester (Arachidic Acid, ME)**	326	**3.14**
				Total Saturated Fatty Acid %		**39.78**
				Total Unsaturated Fatty Acid %		**44.42**
				Total Fatty Acid%		**84.20**
				Other Components %		**15.76**

Table 2. Total flavonoid and phenolic contents in different *Hedysarum aucheri* extracts and their extraction yields (%)

Extracts	Total flavonoid cont. (mg QU/g DW)	Total phenolic cont. (mg GAE/g DW)	Extraction yield (%)
Methanol	42.6±1.4	56.9± 1.3	14.2[a]
Hexane	20.8 ±0.6	51.7±1.6	9.5[b]
Dichloromethane	22.7 ±2.5	25.4 ±0.8	4.3[b]
Water	9.4 ±1.1	15.7±0.4	70.0[b]
Butanol	66.3 ±1.3	122.4±1.6	16.0[b]

[a] *The extraction yield as the percentage of the weight of the crude extract to the plant material (940 g)*
[b] *The extraction yields as the percentage of the weight of the extract to the crude MeOH extract (133 g)*
Green color indicates high content

3.4 *In vitro* Cytotoxic Activity

The cytotoxic activities and IC_{50} values of the plant extracts against a panel of human cancer and normal cell lines were determined by using different concentrations of the plant extracts up to 50 µg/mL. The estimated IC_{50} values of the plant extracts after 48 h following the extract treatment are given in (Table 3) along with the IC_{50} values of some widely used chemotherapeutic drugs reported in the literature against both cancer and normal cell lines.

DCM extract showed remarkable cytotoxic activity against all the tested cancer cells except for PC3, whereas the other extracts did not have a noteworthy effect on any cell type with no detection of IC_{50} value within the tested concentration range (0-50 µg/mL). The morphological changes of cancer cells were observed all over the post-treatment with DCM extract for 48 h exposure. Increasing DCM extract concentration resulted in increased growth inhibition and the incidence of various morphological abnormalities such as cell shrinkage, loss of surface contact when compared with the untreated control cells. DCM extract showed exceptional cell growth inhibition on cancer cells and had better IC_{50} values in comparison to reported IC_{50} values of some popular chemotherapeutic agents such as A0, cisplatin and DHA [37-40]. Its most potent cytotoxicity was observed towards CaCo-2, MCF-7 and U87MG cells with IC_{50} values of 0.64, 2.6 and 5.02 µg/mL, respectively. They were followed by A549 and HeLa cells with IC_{50} values of 15.6 and 24 µg/mL. IC_{50} values for both PC3 and HEK293 cell lines were above 50 µg/mL. The percentage of cell viability of the tested cell lines following the exposure to DCM extract at 0.5, 5, and 50 µg/mL concentrations are presented in (Fig. 2.)

Based on these results, DCM extract exhibited cytotoxicity against all tested cancer cells in a dose-dependent manner. CaCo-2, MCF-7, and U87MG were the most sensitive cells against the DCM extract, respectively while A549 and HeLa cells had relatively higher viability. Even though viability of U87MG, A549, and HeLa cells was high at low concentrations, their viability dropped substantially at higher concentrations. Healthy HEK293 cells, on the other hand, were quite resistant to the extract exposure at all concentrations and no considerable cell death was observed even when the concentration was increased 100 times from 0.5 µg/mL to 50 µg/mL. This selective tumor cytotoxicity of the DCM extract in HEK293 cells is a significant feature because a chemotherapeutic is wanted to target only infected cells while not harming the healthy cells regardless of the concentration. Thus, selectivity and good cytotoxic efficacy are some of the basic requirements of an anticancer agent. The unmanageable side effects caused by the unselectivity of the majority of the current chemotherapy drugs to treat cancer are a challenging and continuing problem. For instance, cisplatin has good cytotoxicity but poor selectivity. It induces death of cancerous cells along with the healthy cells of the host. In contrast, DCM extract of *H. aucheri* showed both good cytotoxicity and selectivity. No detrimental effects on non-cancerous HEK293 cells were observed as opposed to the tested cancer cells.

When these results are compared with some reported data about the cytotoxicity of other members of *Hedysarum* genus reported in the literature, it is clear that *H. aucheri* is probably one of the most promising members of this family. A study showed that MTT assays revealed a polysaccharide from the roots of *H. polybotrys* inhibited the proliferation of human hepatocellular carcinoma HEP-G2 cells and human gastric cancer MGC-803 cells *in vitro* with a cell viability ratio of 90% and 95% respectively after 72 h at 50 µg/mL concentration [18]. Another study reported that a compound obtained from *H. polybotrys* showed inhibitory activity on HEP-G2 cell line with IC_{50} values of 10.69 µmol/L [20].

3.5 *In vitro* Antimicrobial Activity

Disc diffusion method and broth micro-dilution technique were employed to determine *in vitro* antimicrobial and antifungal activities against representative Gram-negative and Gram-positive bacterial strains and the yeast, respectively. Disc diffusion method was used for qualitative determination of activity and broth micro-dilution was utilized to determine MIC to provide quantitative information. Antimicrobial activities of the plant extracts were determined by agar disc diffusion method by measuring the diameter of the zone inhibition around the discs infused with the plant extracts over the bacterial and fungal culture plates, and the results are presented in (Table 4).

Table 3. IC_{50} values for the plant extracts in this study and some commonly used chemotherapeutics in the literature towards cancer and normal cell lines

Cell lines		IC_{50} values of the plant extracts from *Hedysarum aucheri* (µg/ml)					IC_{50} values of some widely used drugs (µg/ml)		
		MeOH	BuOH	Hexane	H_2O	DCM	A0	Cisplatin	DHA
Normal Cells	**HEK293**	-	-	-	-	>**50**±0.054	-	**1.7** [38]	-
Malicious Cells	**CaCo-2**	-	-	-	-	**0.64**±0.022	**13** [37]	**17** [37]	-
	MCF-7	-	-	-	-	**2.6**±0.019	**50** [37]	**15** [37] **33** [40]	-
	U-87MG	-	-	-	-	**5.02**±0.027	-	**8.2** [39]	**15.8** [39]
	A549	-	-	-	-	**15.6**±0.023	**40** [37]	**36** [37]	**10.1** [39]
	HeLa	-	-	-	-	**24**±0.031	**16** [37]	**13** [37]	-
	PC3	-	-	-	-	>**50**±0.043	**34** [37]	**12** [37]	-

- IC_{50} values not detected within the tested concentrations
Green color indicates better cytotoxic activity
[37-40]: References

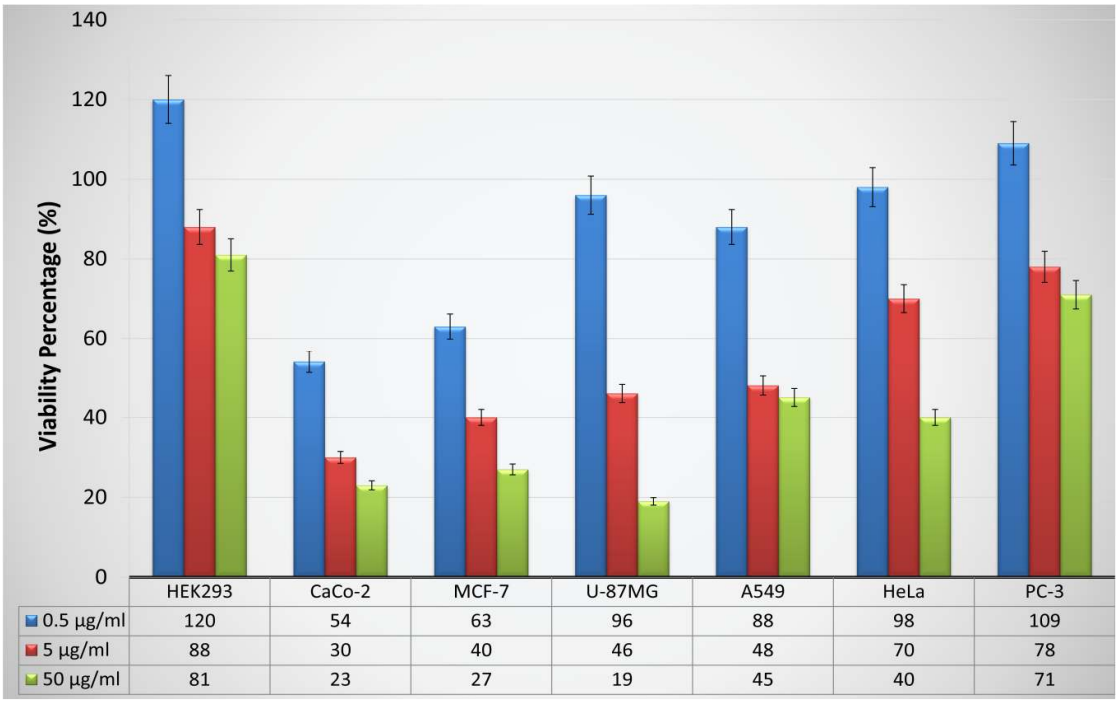

Fig. 2. Effect of DCM extract of *Hedysarum aucheri* on cancer and normal cells viability after 48 h of exposure to different concentrations
Data are expressed as mean ± SD

Table 4. Antimicrobial activity of *Hedysarum aucheri* extracts by disc diffusion assay

	Microorganisms	Inhibition zone of the extracts (mm)*						
		MeOH	BuOH	Hexane	H_2O	DCM	CF	NYS
Gram Negative	*Salmonella thyphimurium CCM 5445*	10	10	10	8	8	14	-
	Escherichia coli ATCC 25922	8	10	9	8	8	14	-
	Escherichia coli 0157H7	8	10	9	8	8	13	-
Gram Positive	*Staphylococcus aureus ATCC 25923*	10	10	10	8	8	20	-
	Staphylococcus epidermidis ATCC 12228	8	8	10	8	8	12	-
	Enterococcus faecalis ATCC 29212	9	8	10	9	8	17	-
	Enterococcus faecium DSM 13590	8	8	10	8	8	15	-
Yst	*Candida albicans ATCC 10239*	10	11	10	10	8	-	18

*CF: Ceftazidime (30 µg/disc); NYS: Nystatin (20 µg/disc), * Includes filter paper disc diameter (6 mm). - Not detected. Green color indicates better antimicrobial activity*

Table 5. Minimum inhibitory concentration of *Hedysarum aucheri* extracts

Microorganisms		MIC values (µg/ml) of extracts and reference antimicrobials						
		MeOH	BuOH	Hexane	H_2O	DCM	AMP	FC
Gram Negative	*Salmonella thyphimurium CCM 5445*	31.2	31.2	15.6	62.5	125	3.9	-
	Escherichia coli ATCC 25922	125	62.5	31.2	125	250	1.9	-
	Escherichia coli 0157H7	125	31.2	31.2	125	250	3.9	-
Gram Positive	*Staphylococcus aureus ATCC 25923*	62.5	31.2	15.6	62.5	125	3.9	-
	Staphylococcus epidermidis ATCC 12228	15.6	31.2	15.6	62.5	125	1.9	-
	Enterococcus faecalis ATCC 29212	62.5	31.2	31.2	62.5	250	7.8	-
	Enterococcus faecium DSM 13590	31.2	31.2	31.2	62.5	250	3.9	-
Yst	*Candida albicans ATCC 10239*	15.6	31.2	15.6	31.2	62.5	-	7.8

AMP: Ampicillin, FC: Flucytosine, - Not detected, Green color indicates better antimicrobial activity

All extracts showed antimicrobial activity against all tested strains. The inhibition zones were varied from 8 to 11 mm. Antimicrobial activity of the extracts was observed roughly in order of hexane ≥ butanol > methanol > water > dichloromethane. *C. albicans* was the most vulnerable microorganism tested. Furthermore, minimum inhibition concentrations of the extracts were determined by MIC method, and the results are presented in (Table 5).

Disc diffusion and MIC results are compatible with each other. Hexane extract showed higher antimicrobial activity overall with lower MIC values compared to other extracts. MIC values obtained for hexane extract are ranged from 15.6 to 32.1 µg/mL.

4. CONCLUSION

Determination of the fatty acid profile of *Hedysarum aucheri* by GC-MS revealed that 24 of 39 compounds found in Hexane extract were fatty acids which constituted 84.20% of the total content. Both ALA and LA which are the only fatty acids known to be essential for humans were detected in high abundance, 32.37% and 9.16%, respectively. Each extract excelled over the others in different biological activities. DCM extract with the best cytotoxic activity, butanol extract with the best antioxidant activity and hexane extract with the best antimicrobial activity come into prominence. DCM extract of *H. aucheri* showed exceptional inhibition of cell proliferation on tested cancer cells with quite remarkable IC_{50} values which are even better than some reported values for widely used chemotherapy drugs while not impairing healthy normal cells (HEK293). Butanol extract had the highest total flavonoid-phenolic contents and showed the highest antioxidant activity. For the rest of the extracts, no strong correlation was discovered between the total flavonoid-phenolic contents and the antioxidant activity. Although all extracts have displayed considerable potency on tested microorganisms, hexane extract showed the highest antimicrobial activity.

Hedysarum aucheri is an endemic member of biologically active *Hedysarum* genus and considered to be a vulnerable plant that has a high risk of extinction in the wild. This study serves as a basis for further research on this precious plant to investigate its other biological activities along with finding out the bioactive compounds in the extracts.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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