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Antimicrobial Activity and Cytotoxicity of *Alkanna Tinctoria* (L.) Tausch Root Extracts

Hatice Aysun MERCIMEK TAKCI[1*], Filiz UCAN TURKMEN[2], Fatma Ceren ANLAS[3], Fulya USTUN ALKAN[3], Pemra BAKIRHAN[4], Cihan DEMIR[4], Nazim SEKEROGLU[2]

[1]Arts and Sciences Faculty, Department of Molecular Biology and Genetics, Kilis 7 Aralık University, 79000, Kilis, Turkey. mersimek@hotmail.com
[2]Engineering and Architecture Faculty, Department of Food Engineering, Kilis 7 Aralık University, 79000, Kilis, Turkey. ucanfiliz@gmail.com/nsekeroglu@gmail.com
[3]Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Istanbul University, 34320, Avcilar, Istanbul, Turkey. cerenis@istanbul.edu.tr, fustun@istanbul.edu.tr
[4]The Institute for Graduate Studies in Sciences and Engineering, Kilis 7 Aralık University, Kilis, 79000, Turkey. pemrabakirhan@gmail.com

[*]**Corresponding Author:** mersimek@hotmail.com

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Abstract

Alkanna tinctoria (L.) Tausch produced naphthoquinones known as DNA-topoisomerases inhibitors has been used as the traditional therapeutic agent, especially wound healing in Turkey. The antimicrobial activity against 9 clinical microorganisms and cytotoxicity on canine mammary carcinoma cell line (CMT-U27) of *Alkanna tinctoria* root extracts (methanol, ethanol and acetonitrile) were researched by using MTT analysis. Alkanet root extracts showed antimicrobial activity against 2 (*Proteus* spp. and *S. haemolyticus*) out of 9 microorganisms. *In vitro* study towards CMT-U27 cancer cells, acetonitrile root extract at 100 µg/mL concentration showed strong and significant anti-proliferative effect. Our results are manifested that the Alkanet acetonitrile root extract can be evaluated as potential therapeutic agent, immediately after identification of bio-active metabolits in extract.

Keywords: Alkanet root, *Alkanna tinctoria*, antimicrobial, cytotoxicity

Alkanna Tinctoria (L.) Tausch Kök Ekstraktlarının Antimikrobiyal Aktivitesi ve Sitotoksisitesi

Öz

DNA-topoizomerazları olarak bilinen naftokinonları üreten *Alkanna tinctoria* (L.) Tausch, Türkiye'de özellikle yara iyileştirmede geleneksel terapötik ajan olarak kullanılmaktadır. *Alkanna tinctoria* metanol, etanol ve asetonitril kök ekstraktlarının 9 klinik mikroorganizmaya karşı antimikrobiyal aktivitesi ve köpek meme kanser hücre hattı (CMT-U27) üzerinde sitotoksititesi MTT analizi kullanılarak araştırılmıştır. Alkanat kök ekstraktları 9 mikroorganizmadan sadece ikisine (*Proteus* spp. and *S. haemolyticus*) karşı antimikrobiayl aktivite göstermiştir. CMT-U27 kanser hücrelerine yönelik *in vitro* çalışmada, 100 µg/mL konsantrasyondaki asetonitril kök ekstraktı güçlü ve önemli bir hücre büyümesini engelleyici etki göstermiştir. Sonuçlarımız Alkanat asetonitril kök ekstraktının biyoaktif metabolitlerinin tanımlanmasını takiben potansiyel terapötik ajan olarak değerlendirilebileceğini açıkça ifade etmektedir.

Anahtar Kelimeler: Alkanat kök, *Alkanna tinctoria*, antimikrobiyal, sitotoksisite.

1. Introduction

In ancient civilizations, herbal remedies were widespread used for treatment of the several diseases. Even today, herbal metabolits in medicine are evaluated as therapeutic agents (Hayta et al., 2014). Therefore, the newer researches in pharmaceutical industry and developing medicines are aimed on exploring the medicinal plants and the pharmacological activities of plant metabolits Elsharkawy et al., 2013). In folk medicine, the several therapeutic uses of *Alkanna* genus have attracted attention since ancient times (Elsharkawy et al., 2013; Khan et al., 2015; Alwahibi and Perveen, 2017). The first evidences related to the use of *Alkanna tinctoria* roots for the skin ulcers treatment were found in the work of Hippocrates (Sevimli-Gur et al., 2010; Alwahibi and Perveen, 2017). The similar findings concerning to *Alkanna tinctoria* were also recorded by Theophrastus and Dioscorides (Alwahibi and Perveen, 2017).

Alkanna Tausch belongs to Boraginaceae family of the Tubiflorae order, is a genus represented by 34 (about endemic) species and 40 taxa in the flora of Turkey (Kayabası et al., 2000; Akcin et al., 2004; Gumus and Ozlu, 2007; Ozer et al., 2010; Sevimli-Gur et al., 2010). *Alkanna tinctoria* (L.) Tausch with the blue-blooming is grown in Europe, the Mediterranean region, and Western Asia, subtropical areas of the world (Gumus and Ozlu, 2007; Ozer et al., 2010; Mohammed et al., 2016). *Alkanna tinctoria* name originates from the Spanish word alcana and Arabic al-hena. It is also known as Alkanet or dyers, bugloss, orchanet, Spanish bugloss or Languedoc bugloss (Ozer et al., 2010; Elsharkawy et al., 2013). Besides, it has diverse local names such as Havaciva, Egnik, Kızılenik, Tuyluboya and Yerinegi are for *A. tinctoria,* in Turkey (Akcin et al., 2004).

The appearance of Alkanet root is described as dark red root of blackish externally but blue-red inside, with a whitish core (Ozer et al., 2010; Elsharkawy et al., 2013). Since antiquity, a fine red colouring compound produced by Alkanet roots has been used for dying cloth, giving color to medicines, varnishes, food, wines etc. (Akcin et al., 2004; Ozer et al., 2010; Amal and Abdel-Hamid, 2011; Elsharkawy et al., 2013; Kheiri et al., 2017). The Alkanet roots as a dye, are insoluble in water but soluble in solvents such as ethanol, methanol, acetonitrile, ether etc (Elsharkawy et al., 2013).

As well as its pigmentation activity, Alkanet root extracts has been traditionally performed for wound healing, anti-nociceptive, anti-inflammatory and pain-related ailments (Gumus and Ozlu, 2007; Alwahibi and Perveen, 2017). The existence of alkannin and its derivates that have cytotoxic, antimicrobial, antioxidant, anti-leishmanial and anti-inflammatory activities as active compunds found in these extracts were indicated by Papageorgiou and his co-workers in 1976 (Assimopoulou et al., 2005; Sevimli-Gur et al., 2010; Tsermentseli et al., 2013; Tung et al., 2013; Yazdinezhad et al., 2013; Alwahibi and Perveen, 2017; Kheiri et al., 2017). Alkannin and its enantiomer shikonin in

Alkanet root extracts are isohexenylnaphthazarine derivatives of hydroxynaphthoquinones (Assimopoulou et al., 2005; Sevimli-Gur et al., 2010). Nowadays, the wound healing ointments such as the trademark helixderm and histoplastin red include alkannin and related derivates as active component (Tsermentseli et al., 2013; Alwahibi and Perveen, 2017).

Thus, present study was undertaken to determine antimicrobial and cytotoxic effects the of *Alkanna tinctoria* (L.) Tausch root extracts grown in Mus, Turkey.

2. Materials and Methods

2.1. Collection and preparation root extracts of Alkanna tinctoria

Alkanna tinctoria (L.) Tausch was collected from Malazgirt mountain, Mus, Turkey in July and August 2016. For the preparation of root extracts, the surface and the root parts of samples were separated, then dried at room temperature. The chopped roots were then grinded at porcelain mortar. 20 g of the powdered roots in 200 mL of each selected solvent (methanol, ethanol and acetonitrile) were extracted on shaking incubator for 48 h at room temperature. The brown-deep red root extracts were filtered by filter paper and then evaporated at 70°C. The dryness residues were scraped and dissolved by methanol at the 20 mg/mL final volume and stored at 4°C until further use.

2.2. Antimicrobial effect

Antimicrobial activity of the root extracts was evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Proteus* spp, *Klebsiella* spp, *Enterococcus faecalis*, *Staphylococcus haemolyticus*, *Bacillus subtilis* and *Candida albicans* by Kirby Bauer disc difussion method. Microbial strains were provided from Kilis state hospital. As positive and negative control, chloramphenicol standard disc and pure methanol were used, respectively. These strains were spread on Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) for each four extracts. 50 µL of the root extracts were embrued to blank sterile discs and then applied on the agar plates. After incubation at 37°C and room temperature for PDA 24-48 h, inhibition zone around the disc on agar plate were measured as millimetres (mm). PDA plates were used for *C. albicans*.

Traditional balm prepared from Alkanet roots is used as wound healing agent. One spoonful butter and olive oil are melted on medium heat, then added Alkanet root pieces to mixture until the red color is achieved (Figure 1).

Figure 1. The preparation of traditional balm from Alkanet roots

Antimicrobial effect of this balm by dissolving in chloroform, was searched against same microbial strains. Chloroform as negative control was used.

2.3. Cytotoxicity

Cell culture: Cytotoxic effect of the root extracts were tested on canine mammary carcinoma cell line (CMT-U27). CMT-U27 cells were supplied from Upsala University, Prof. Dr. Eva Héllmen. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-F12 supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37ºC with medium changed every 2 to 3 days. The culture was continued until reaching 80% confluency. Cells were detached from the culture surface using 0.25% trypsin-EDTA solution.

Cell viability assay:Plant extracts were dissolved in DMSO and further serial dilutions made with DMEM F-12. The final DMSO concentration did not exceed 0.25% and this condition was used as negative control. The cytotoxic effects of the root extracts on the proliferation of CMT-U27 cells were detected by using MTT cell proliferation kit (Roche, Germany). This colorimetric assay is based on reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) by metabolically active cells. NAD(P)H-dependent oxidoreductase enzymes in viable cells reduce the MTT reagent to formazan and occur a deep purple color crystalline product. This insoluble product is dissolved and then, quantified by spectrophotometrically.

MTT analysis was applied according to method described by Ustun Alkan et al (2014). Cancer cells were transferred into a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h. After incubation, the medium was changed to fresh medium and cell line was treated by different concentrations (6.25, 12.5, 25, 50 100 µg/mL) of Alkanet root extracts and DMSO (as control). Cell viability by following 24-48 h, the absorbance of each well, including the blanks was measured by multimode microplate reader (FilterMax F5, Molecular Devices, USA) at 595 nm.

The average values were obtained from triplicate readings. The viable cell rate in vehicle treated control was defined as 100% viable and % viability was measured by the formula:

Viability (%) = (sample absorbance/control absorbance) x 100

The statistical significance of results was evaluated by one-way ANOVA with Tukey post hoc test.

3. Findings and Discussion

These extracts were detected to show antimicrobial activity against 2 out of 9 microorganisms. *Proteus* spp. and *S. haemolyticus* were sensitive to all of extracts, but resistance against methanol as negative control (Table 1).

Table 1. Antimicrobial activity of Alkanet root extracts (mm inhibition zone)

	Ethanol extract	***Methanol extract***	***Acetonitrile extract***	***Methanol***	***Chloramphenicol***
E. coli	-*	-*	-*	-*	23
S. aureus	-*	-*	-*	-*	20
C. albicans	-*	-*	-*	-*	-*
B. subtilis	-*	-*	-*	-*	35
Enteric bacteria	-*	-*	-*	-*	10
***Proteus* spp.**	**15**	**12**	**14**	-*	25
***Klebsiella* spp.**	-*	-*	-*	-*	35
S. haemolyticus	**10**	**16**	**10**	-*	35
E. faecalis	-*	-*	-*	-*	-*

*: Inhibition zone was not observed.

The highest inhibiton zones of the ethanol and acetonitrile root extracts were detected against *Proteus* spp (Figure 2). The methanol root extract appeared the highest inhibiton zone (16 mm) against *S. haemolyticus*.

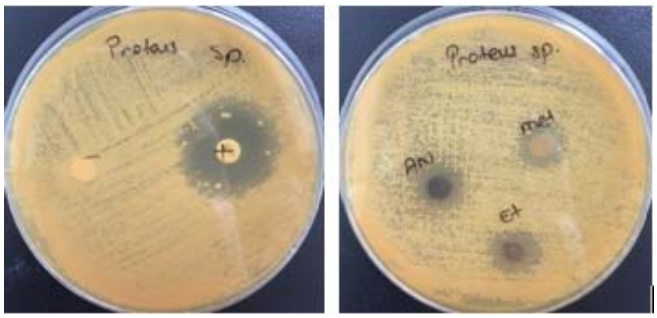

Figure 2. The antimicrobial activity of Alkanet root extracts on *Proteus* spp. (-:negative control; +:positive control)

Antimicrobial effects of the ethanol and acetonitrile root extracts on *S. haemolyticus* were same level of inhibition (10 mm) (Figure 3). The inhibiton was not encountered against *S. aureus*, the important bacterial agent of wound infections. By comparison with inhibitory zone, the inhibition of standard antibiotic against all test microorganisms was better effective than Alkanet root extracts.

Figure 3. The antimicrobial activity of Alkanet root extracts on *S. haemolyticus* (-:negative control; +:positive control

The microbial inhibition mechanism of extracts associates hydroxynaphthoquinones in Alkanet root. This agent is a effective chelator of divalent metal ions. As suggested in literature, it binds to a zinc finger domain of topoisomeras proteins and may inhibit them (Plyta et al., 1998). The dissociation rate of the bioactive compounds is different based on by using solvent. So, our antimicrobial results can be stated to the presence of chelator agents at different concentration in root extracts.

Sengul et al. (2009) determined the highest inhibiton zone (10 mm) against *Alcaligenes feacalis* for methanol extract, similarly our result. Alwahibi and Perween depicted that the ethanol extract of *A. tinctoria* had maximum zone of inhibition against *B. subtilis* (28 mm) (Alwahibi and Perveen, 2017). Khan et. al (2015) reported anti-multi-drug resistant bacterial activity of extracts of *Alkanna tinctoria* leaves and the best activity against test microorganisms detected in aqueous extract. Compared to literature, antimicrobial activity results related to ethanol and methanol extracts had potential in inhibiting of the bacterial growth. But the traditional root cream against test microorganisms was not effective.

The in vitro cytotoxicities of the prepared Alkanet root extracts against canine mammary carcinoma cell line (CMT-U27) were tested by the standard MTT assay. 6.25 µg/mL and 12.5 µg/mL concentrations for each root extracts on cell viability was not statistically observed a meaningful effective (Figure 4).

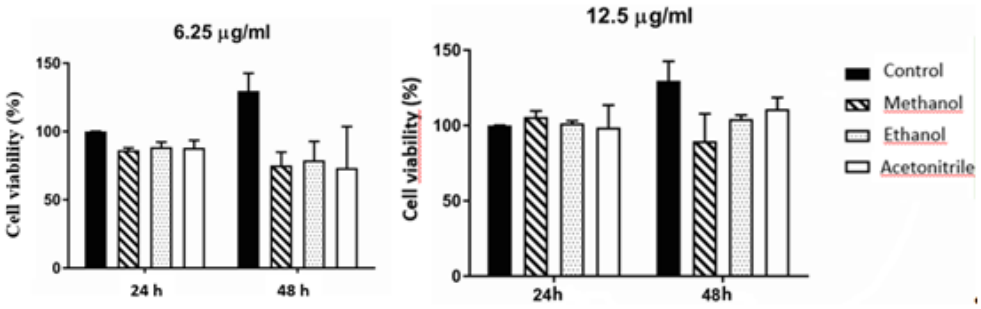

Figure 4. Cytotoxic effect of Alkanet root extracts (6.25-12.5 µg/mL) on CMT-U27, time-dependent.

After treatment with 25 µg/mL Alkanet root extracts for 24 h showed no cytotoxicity to the cancer cells viability. The cell viability of CMT-U27 cells was decreased dose and time-dependently. After 48 h, the cancer cells viability was inhibited by average 10 and 40% in culture applied ethanol and acetonitrile, respectively (Figure 5).

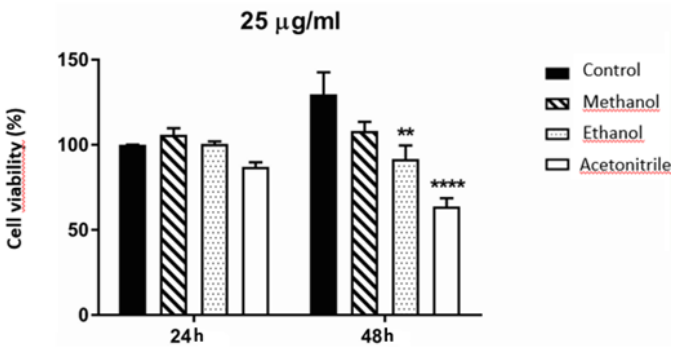

Figure 5. Cytotoxic effect of Alkanet root extracts (25 µg/mL) on CMT-U27, time-dependent. ***** (P <0.01): **** (P <0.0001) as statistically significant at each time point, according to control.***

The acetonitrile root extracts showed the stronger anti-proliferative and cytotoxic effect against the cancer cell line than other extracts. Compared to the viability of cancer cells, the cell proliferation was increased in the control group. This increase was average at 30%. For methanol root extract, cytotoxic activity was not recorded as time-dependently.

The acetonitrile extract decreased viability of cancer cells at 50 µg/mL concentrations for 24 and 48 h (Figure 6).

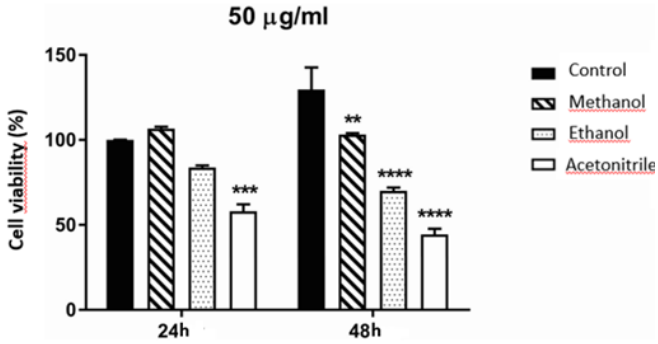

Figure 6. Cytotoxic effect of Alkanet root extracts (50 µg/mL) on CMT-U27, time-dependent ***** (P <0.01): *** (P <0.001): **** (P <0.0001) as statistically significant at each time point, according to control.***

The viabilities of CMT-U27 cells were about 58% and 44%, respectively. Compared with the ethanol and methanol extract, it can be mentioned from a significant cytotoxic effect of acetonitrile extract. The second best inhibitory effect in the viability of CMT-U27 cells, the distribution was to be average 30% after treatment with ethanol extract for 48 h. The considering to viability percentages of cancer cells treated by 100 µg/mL root extracts, the better cytotoxic activity showed acetonitrile extract (average 80%) (Figure 7).

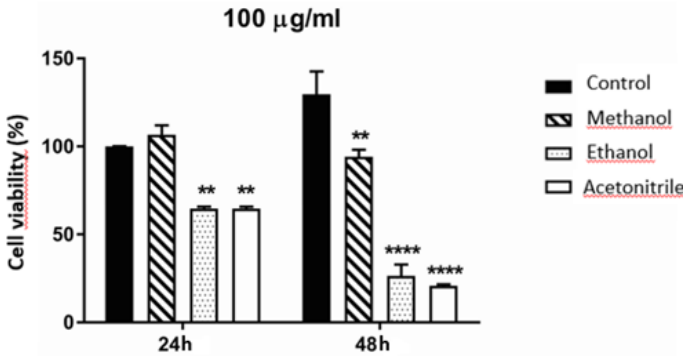

Figure 7. Cytotoxic effect of Alkanet root extracts (100 µg/mL) on CMT-U27, time-dependent ***** (P <0.01): *** (P <0.001): **** (P <0.0001) as statistically significant at each time point, according to control.***

In summary, at 25, 50 and 100 µg/mL test concentrations of alkanet extracts, can be said to show cytotoxic effects and acetonitrile be the most effective solvent. For acetonitrile root extract with the lowest antioxidant activity, the cytotoxic activity on the cells correlated with the amount of phenolic and flavonoid substance. On treating CMT-U27 cells with extracts for 48 h, 25, 50 and 100 µg/mL acetonitrile extracts exhibited maximum cytotoxic effect with ~40, 60 and 80% inhibition of viability, respectively.

4. Conclusions and Recommendations

This investigation is the first study that intended to evaluate the cytotoxic potential of the *A. tinctoria* root extracts on CMT-U27 cell line. According to our results, a selective cytotoxic activity of acetonitrile root extract can be referred. So, this extract can be evaluated as potential substance for anticancer drug therapies. But at first, identification of biologically active compounds in extracts are needed.

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