Investigation the effects of used solvent components proportions for extraction the antimicrobial compounds of *Cichorium intybus* L. on their antibacterial and antifungal activities

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**Key words**: Chicory herb, antibacterial and antifungal effects, dilution, Disk diffusion method, MIC, MBC, *Pasteurella multocida*, *Bacillus subtilis*.

http://dx.doi.org/10.12692/ijb/6.7.73-81 Article published on April 10, 2015

**Abstract**

Bacterial infections are some of the major problems of human life and the drugs used to treat these kinds of disease to be prepared naturally or in synthetic way. Herbs are the most valuable resources for antimicrobial therapies that their medicinal properties, especially in recent decades are considered. The aim of this study was to evaluate the effect of the solvent used to extract the chicory plant on antimicrobial properties. According to Mixture design method, ethanol, acetone and hexane solvents were used alone or in combination of two or three to extract plant. Antibacterial and antifungal tests were carried out by disk diffusion, MIC and MBC methods. The results showed that however, extracts from the leaves of chicory had no antibacterial effect, but most of the seed extracts of this herb had Inhibitory and bactericidal effects in First to third dilutions on *Pasteurella multocida* and *Bacillus subtilis*. There was no Antifungal activity in any of the chicory plant extracts. Result, in addition to proving weak antibacterial effect of chicory seed, shows the Importance of the used solvent nature and subsequently their polarity.

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Introduction

According to a report of world Health Organization three fourth of world population cannot afford modern medicine and rely on traditional medicine of plant origin (Rai et al. 2000). Also the growing concern about food safety has recently led to the development of natural antimicrobials to control food borne pathogens and spoilage bacteria (Snyder 1997). Many medicinal plants are being extracted for drugs by pharmaceutical industry (Nishmura et al. 1999). Plant products have been shown to have side effect free, good therapeutic potential, due to the presence of active pharmacologically important substances, such as terpenes, alkaloids, flavonoids and glycosides (Yusuf et al. 2002, Farrukh and Ahmed 2003).

*Cichorium intybus* L is a member of the family Asteraceae It is an as an important medicinal herb has been used Ayurveda, Unani and Siddha system of medicine for diseases of hepatobiliary system and renal system (Zaman et al. 2013, Zargari 1996). Chicory is one of the earliest known and most widely used raw materials for manufacturing of coffee substitutes (Pazola. 1987). The leaves of chicory plant can be used as salad as they are rich source of vitamin A & C. and also micronutrients (Bremness 1998). Chicory is the major of inulin; Inulin can be used as an alternative to antibiotics in human and animal diet (Park and Park 2012). Heimler et al. (Heimler et al. 2009) reported the conventionally and biodynamically grown chicory (*Cichorium intybus*) for its polyphenol content and antiradical activity. HPLC/DAD/Ms analysis identified five hydroxycinnamic acids and eight flavonoids (quercetin, kaempferol, luteolin and apigenin glycosides) in *c.intybus* plant. Norbeak et al. (Norbaek et al. 2002) reported the anthocyanins from flowers of *Cichorium intybus*. The aim of this research is study on effects of used solvent percentage composition for chicory leaf extraction on antibacterial and antifungal properties.

Material and methods

Extraction

Seeds of the selected medicinal plant *Cichorium intybus* were purchased from the local market of Arasbaran and identified from the Department of botany and Agricultural, Islamic Azad University, Tabriz branch, Iran. After preparation of the Seeds, they were dried by ambient temperature. Then the Seeds were crushed using a ball mill and Leaf powder was obtained. In this study, ethanol, acetone and hexane were used for extraction (table 1-1). After mixing the solvent and chicory seeds (18gr leaf powder and 100 ml solvent), it made 48 hours in the darkroom, and then extracted with rotary device. After extraction, extract was filtered by filter paper (125 mm, produced by Whatman Company) then they leaved in 80 degree Celsius about 1 hour a day for 3 days.

MIC, MBC and Disk Diffusion

For this study we were used MIC, MBC and Disk Diffusion (fig 1-1) methods. We were used *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas Alcaligenes*, *Pasteurella multocida*, *Bacillus subtilis*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Penicillium*.

For Disk diffusion method, nine experimental tubes were selected and 0.5 ml of Dimethyl sulfoxide (DMSO-Merck©) was poured for all tubes. 0.5 ml of extract was added to the first tube, and then by 0.5 ml transferring, serial dilutions were prepared. At the end 0.5 ml was poured out from last tube. This method used for each extract individually (67% ethanol, 67% acetone and 67% hexane). After all, three Blank discs (6.4mm diameter, produced by Padtanteb Company) placed in each tube and after half an hour, disks were removed from the tubes and placed in an incubator with 37±2 degrees Celsius for 20 minutes, for drying. After drying, disks were ready to use. For control group we were used Blank discs with solvents that they were dried in same method.

For Antibiogram method we were used Mueller-Hinton agar culture that produced by Merck Company. 24 hour BHI-broth culture was used for this research. For Mueller-Hinton agar culture, we were used McFarland half. We were designed 4 disks per culture plate for each dilution that one of them was control disk (dried extract-free solvent). Cultures
were placed in an incubator at 37 degrees Celsius for 18 hours. We were used these levels for each solvents and each bacteria individually.

The MIC (fig1-2) value of the extract was determined as the lowest concentration that completely inhibited bacterial growth after 48 hr of incubation at 37°C. For the determination of MBC, a portion of liquid (5 μl) from each plates well that exhibited no growth were taken and then incubating 37°C for 24 hr. The lowest concentration that revealed no visible bacterial growth after sub-culturing was taken as MBC. Positive and negative cultures were also prepared.

**Statistical Analysis**

**Table 1-1. Solvent Combination.**

<table>
<thead>
<tr>
<th>Solvent Combination</th>
<th>%</th>
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<tbody>
<tr>
<td>S1 Ethanol</td>
<td>100</td>
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<tr>
<td>S2 Acetone</td>
<td>100</td>
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<tr>
<td>S3 Hexane</td>
<td>100</td>
</tr>
<tr>
<td>S4 Ethanol and acetone</td>
<td>50</td>
</tr>
<tr>
<td>S5 Ethanol and hexane</td>
<td>50</td>
</tr>
<tr>
<td>S6 Acetone and hexane</td>
<td>50</td>
</tr>
<tr>
<td>S7 Ethanol</td>
<td>67</td>
</tr>
<tr>
<td>S8 Acetone and hexane</td>
<td>67</td>
</tr>
<tr>
<td>S9 Hexane, Acetone</td>
<td>67</td>
</tr>
<tr>
<td>S10 Each</td>
<td>33</td>
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</tbody>
</table>

According to table 1-2 results there were significant differences between groups on Inhibition zone. In Acetone-Ethanol50%, Ethanol 100% and Each33% was maximum inhibition zone around disks. According to MIC results, Bacillus subtilis maximum effect was on ethanol 100% with 0.125 mg/ml value. In MBC results, there was Bactericidal activity except Ethanol 67%, Hexane 67, Ethanol 100% and each 33%.

According to Fig1-4 hexane extract has maximum inhibitory activity In contrast to Ethanol and Acetone solvents. In Fig 1-3 there is maximum antibacterial activity in the center of the triangle which mean maximum antibacterial activity can be observed in combination of Ethanol, Acetone and Hexane. But whatever, we away from center of triangle to Ethanol, we observe lesser anti bacterial activity. According to Fig 1-5 in Ethanol-Acetone or in combination of all solvents, we observe maximum inhibitory zone and Ethanol-Hexane minimum inhibitory zone that corresponded with the MIC results.

According to table 1-2 results, there was significant difference on Antibacterial effects of Acetone-Ethanol 50%.

According to figure 1-6, Ethanol-Acetone has maximum inhibitory effects compared with other solvents. Maximum impact factor solvent was on Hexane 100% and Hexane 67%, and Minimum
impact factor solvent was on Acetone-Ethanol 50%. Hexane solvents have minimum effect. In Figure 1-7, Maximum bactericidal effects seen in Ethanol. Also minimum bactericidal effect observed in Acetone. According to figure 1-8 maximum Inhibition zone can bee seen on Ethanol, Acetone-Ethanol and Acetone-Ethanol-Hexane solvents. This property may be lower with increasing hexane.

Table 1-2. Results and P value.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Sol</th>
<th>Dilution (mg/ml)</th>
<th>MIC Regression</th>
<th>MBC Regression</th>
<th>Disk Regression</th>
<th>P</th>
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<th>P</th>
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Discussion

Nontyphoidal salmonellosis is one of the most important bacterial zoonotic diseases, yearly resulting in an estimated 155,000 deaths worldwide (Majowicz et al. 2010). A great number of animal species, including humans, can be infected by different Salmonella serotypes. Some of them are highly host-adapted for example, Salmonella typhi, Salmonella gallinarum and Salmonella typhi-suis, which specifically infect humans, poultry and pigs, respectively. In contrast, other serotypes, like Salmonella enteritidis, can infect a broad range of hosts. The type of disease caused by Salmonella depends on the serotype, the infected species and the immunological status of the host. The clinical manifestations of salmonellosis range from a mild gastroenteritis to a severe systemic infection (Finlay 1994). Salmonella is member of Enterobacteriaceae family; Members of the Enterobacteriaceae are Gram negative, straight rods, some of which are motile. Most species grow well at 37°C, although some species grow better at 25-30°C. They are facultatively anaerobic, oxidase negative and catalase positive (except Shigella dysenteriae type 1). They are distributed worldwide and may be found in soil, water, plants and animals (Quinn et al. 1994). Bacillus subtilis is a ubiquitous naturally occurring saprophytic bacterium that is commonly recovered from soil, water, air, and decomposing plant material (Quinn et al. 1994). Under most conditions, however, it is not biologically active and is present in the spore form (Tadjbakhsh 1997). Different strains of B. subtilis can be used as biological control agents under different situations (Quinn et al. 1994). There are two
general categories of B. subtilis strains; those that are applied to the foliage of a plant, and those applied to the soil or transplant mix when seeding (Tabatabayi et al. 2011). B. subtilis bacteria produce a class of lipopeptide antibiotics including iturins. Iturins help B. subtilis bacteria out-compete other microorganisms by either killing them or reducing their growth rate (Tadjbakhsh 1997).

Fig. 1-1. Disk diffusion method.

Fig. 1-2. Results of MIC method. B (Bacillus subtilis) P (Pasteurella multocida).

Ethanol (ethyl alcohol, grain alcohol) is a clear, colorless liquid with a characteristic, agreeable odor (Mohagheghi et al. 2011, Reichard 2011). In dilute aqueous solution, it has a somewhat sweet flavor, but in more concentrated solutions it has a burning taste (Reichard 2011). Ethanol, CH₃CH₂OH, is an alcohol, a group of chemical compounds whose molecules contain a hydroxyl group, −OH, bonded to a carbon atom (Reichard 2011). The selection of hexane as the solvent of choice for extraction of oils, other nonpolar constituents of plant foods, or removal of undesirable constituents from plant foods is one of the most common practices in the food industry (Reichard 2011, Liauw et al. 2008, Sepidar et al. 2009). The solvent extraction process generally involves solubilization and partitioning in hexane, separation of the extract, and solvent volatilization and removal to recover the extracted constituents (Liauw et al. 2008, Sotillo et al. 1994, Mohagheghi 2011). Acetone is the organic compound with the formula (CH₃)₂CO. It is a colorless, volatile, flammable liquid, and is the simplest ketone (Mohagheghi et al. 2011, Reichard 2011).

Fig. 1-3. Mixture contour plot of MBC of Bacillus subtilis.

In our research we conducted that different solvents can change the antibacterial properties of extract. Solvents as Ethanol, Hexane and Acetone have different ability to extract from plant parts. Some of our results.

Fig. 1-4. Mixture contour plot of MIC of Bacillus subtilis.
In research conducted by Khakzadihe et al. (2014) antimicrobial effect of chicory leaf extract with different solvents on Staphylococcus aureus and Escherichia coli were studied. Staph-aureus and E-coli antibiograms with different solvents had no significant difference between groups and no inhibition zone observed around different extract disks and control disks on Mueller-Hinton agar culture. In their research they couldn’t find any chicory leaves extract antibacterial effect against S.aureus and E. coli. This research accepts our study that chicory planet has no antibacterial effects against E.coli and S.aureus (Khakzadihe et al. 2014).

According to Zaman et al. (2013) study, analysis of the seeds gave the following values: Oil. 4.7%, Fatty acid composition, Saturated 21.7%, Unsaturated 78.3% (Zaman et al. 2013).

Koner et al. (2011) reported that active chemical compounds present in chicory (Cichorium intybus) should certainly find place in treatment of the various bacterial infections. In Koner et al study, the ethyl acetate extract of chicory root was tested for antibacterial and anti-fungal properties. Fractionation by column chromatography of ethyl acetate extracted root powder contains the compound, inhibiting both Gram positive and Gram negative bacteria and was found to be bacteriostatic rather than bacteriocidal. The effect of chicory root extract has more bacteriostatic effect on Gram Positive bacteria than Gram negative bacteria as MIC value is more in case of Gram negative bacteria than Gram positive bacteria. The ethyl acetate fraction of ethyl acetate root which is obtained by silica gel column chromatography has also antifungal activity as it inhibits growth of yeast and moulds. Koner et al could find antibacterial activity from chicory root (Koner et al. 2011).

In a research designed by Mehmood et al (2012) antioxidant, antimicrobial and phytochemical analysis of Cichorium intybus seeds extract and various organic fractions studied. In Mehmood et al study, the methanolic extract and ethylacetate fraction of seeds exhibited good antioxidant activity. The various fractions of C. intybus showed moderate activity as antibacterial agent while antifungal activity of C. intybus extract/fractions was very low against A. flavus and A. niger while mild against R. solani. They reported constituents of this plant extract may serve as a source of drugs useful in the chemotherapy of some infections caused by bacteria and also as an antioxidant agent.
According to the study of Aqil and Ahmad (2007), the extract of Cichorium intybus L. had mild antibacterial effect against Escherichia coli and Staphylococcus aureus. They also showed that there is a synergistic antibacterial effect between the respective medicinal plants with tetracycline, chloramphenicol and ciprofloxacin (Aqil and Ahmad 2007).

Antiviral effects of 20 medicinal plants which are traditionally used against infectious diseases were evaluated by zyaei et al. (2007). They revealed that Aristolochia, Terminalia chebula Retz and Cichorium intybus indicated antiviral effect on adenoviruses. The root of Cichorium intybus also inhibited replication of Herpes type ones (zyaei et al. 2007).

In a research conducted by Ghaderi et al (2012) Cichorium Intybus L. had no antibacterial effect on Streptococcus pyogen, Staphylococcus aureus and Enterococcus. They studied Comparison of Antibacterial Effect of Cichorium Intybus L. with Vancomycin, Ceftriaxone, Ciprofloxacin and Penicillin (Ghaderi et al. 2012).

In a research conducted by Verna et al (2013) In vitro Antibacterial Activity of Cichorium intybus against some Pathogenic Bacteria studied. They reported that plant fractions under study have great potential as antibacterial compound against E. coli and P. aeruginosa and they can be used in the treatment of infectious diseases caused by above resistant microorganisms (Verna et al. 2013).

In present study, there was no significant antibacterial effect against Staphylococcus aureus, Escherichia coli, Pseudomonas Alcaligenes, Listeria monocytogenes and Salmonella typhimurium in all solvents with different combinations. But there was significant antibacterial effect against Pasteurella multocida and Bacillus subtilis. We observed different antibacterial effects from different solvents.

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