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Scienceline Publication
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ISSN: 2322-4789
Asian Journal of Medical and Pharmaceutical Researches
Asian J. Med. Pharm. Res. 4(4): 156-159, December 25, 2014

AJMPR

Preliminary Investigation on Phytochemical Composition and Anti-Bacterial Activity of the Root of *Cousinia microcarpa* Boiss.

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ABSTRACT: Tribes in the Hezarmasjed protected area of Iran used *Cousinia microcarpa* Boiss. root for the treatment of various disorders like respiratory problems, pulmonary infections, remedy of wounds, mouth ulcers, treatment of toothache, joints pain and rheumatism as a folk medicine. This investigation was conducted to authenticate some aspects of this conventional application scientifically. *Cousinia microcarpa* Boiss. roots were screened phytochemically for the presence of secondary metabolites by using standard methods. The antibacterial activities of the different extracts was also investigated against two Gram-positive and three Gram-negative bacterial strains through disc diffusion assay. The phytochemical analysis of extracts of the roots with different solvents such as methanol, ethanol, chloroform and distilled water demonstrate the presence of active components like tannins, flavonoids, terpenoids etc. All the extracts were found to possess different degrees of antibacterial activity except distilled water extract. Our results indicated that Gram-positive bacteria are more sensitive than the Gram-negative bacteria. Among the microbial strains evaluated *Staphylococcus aureus* was found to be the most susceptible, based on the results demonstrated from the inhibition zones. The findings of this paper confirmed potential antibacterial activities of the plant's root. Thus, *Cousinia microcarpa* Boiss. root can be considered as a candidate for new antibacterial drugs due to its extensive spectrum activity. Further surveys are required to estimate its safety and toxicity.

Keywords: *Cousinia microcarpa*, Phytochemical analysis, Antibacterial activity, Disc diffusion method.

ORIGINAL ARTICLE
PII: S225204301400031-4
Received 11 Oct. 2014
Accepted 05 Dec. 2014

INTRODUCTION

Medicinal plants are the richest bio-resource of drugs of folk medicines, modern medicines, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer et al., 1999). Herbal remedies have significant benefits owning to fewer side effects, better patient tolerance, and better accessibility (Vermani and Garg, 2002). Traditionally used medicinal plants have recently attracted the attention of the pharmaceutical and scientific communities. This has involved the isolation and identification of secondary metabolites produced by plants and their use as active principles in medicinal preparations (Taylor et al., 2001). Plants are rich source of phytochemicals such as, phenols, flavonoids, and essential oils etc. which act as antimicrobials. The most important advantage of using plant extracts is that, the crude extracts contain a mixture of phytochemicals for which it is difficult to develop resistance by bacteria unlike the synthetic antibiotics that contain a single compound (Kiranmayee et al., 2010). Thus, there is an urgent require to explore new antibacterial agents with different chemical structures and novel mode of actions to replace those which have lost their effectiveness. Nowadays, numerous plant species has been investigated for their antimicrobial properties in various regions of the world and Iran. Traditional herbal medicine has played an important role in Iran as antimicrobial agents (Amiri and Joharchi, 2013). Despite the medicinal potential of plants being considerable in our country, knowledge and studies on *Cousinia* species are scarce. The genus *Cousinia* Cass is one of the largest of genera of flowering plants in the sunflower family (Asteraceae). This genus is found worldwide and comprises approximately 672 species, of which about 235 occur in central, western, eastern and southeastern regions of Iran (Sheidai et al., 2006). In recent years, different species of *Cousinia* Cass have been authenticated in Iran. However, data concerning their biological effects remain limited.

Cousinia microcarpa Boiss. is one of the most valuable herbal plants of this genus which is widely used in Hezarmasjed protected area (Razavi Khorasan Province, Iran). Root of this plant (commonly known as shelem in the area) traditionally used for the treatment of various disorders like respiratory problems, pulmonary infections, remedy of wounds, mouth ulcers, treatment of toothache, relief of the pain and rheumatism by indigenous people of this protected area. Although *C. microcarpa* Boiss. has been a popular medicine mainly in the area, few pharmacologic studies have been published. It has been reported that it has hypnotic effect (Amiri et al., 2007). According to our knowledge, there are no published reports on the chemical composition and antimicrobial activity of *Cousinia microcarpa* Boiss. Thus the aim of this survey is

the identification of phytochemical components and to evaluate the antibacterial activity of various extracts of *C. microcarpa* Boiss. against several Gram-positive and Gram-negative bacterial strains in vitro which may be helpful in developing new novel drugs.

MATERIAL AND METHODS

The Plant Material

Cousinia microcarpa Boiss root were collected in the blooming stage in June 2013 from the Tirgan mountains region located in Hezarmasjed protected area (Razavi Khorasan Province, Iran), at altitude 1470 m above sea level. The voucher specimen was prepared and deposited in the Herbarium of the Department of Biology, Payame Noor University, Daregaz (No.: 377).

Preparation of the extracts

The roots were chopped into small pieces, air dried under shade and powdered by using a pulverizor. 100g of root powder of *C. microcarpa* Boiss. was subjected to successive extraction with various solvents like methanol, ethanol, chloroform and distilled water by using soxhlet apparatus. Then they were evaporated under low pressure to remove excess solvent and stored in desiccators at 4°C. Then, they were used for preliminary phytochemical screening and antibacterial activity.

Preliminary phytochemical screening

The qualitative chemical analysis of different extracts were performed for the presence of alkaloids, flavanoids, saponins, tannins, glycosides and phenols using the standard protocols adopted in similar surveys (Harbone, 1973).

Test for Alkaloids

To the test solution add diluted HCL and filter, filters were treated with saturated picric acids and formation of brown precipitate indicates the presence of alkaloids.

Test for Tannins

Extract mixed with 2 ml of 2% solution of FeCl3. Black color indicated the presence of tannins.

Test for Phenols

To the test solution add 2 ml of 2% solution of FeCl3. Blue/green color indicated the presence of phenols.

Test for Saponins

Extract was taken in a test tube and shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponines.

Test for Terpenoids

Extract was mixed with 2 ml of chloroform. Then 2 ml of concentrated Sulfuric acid was added carefully and shaken gently. Reddish brown colors observed in the interphase indicate the presence of terpinoids.

Test for Flavonoids

To the test solution add few drops of sodium hydroxide solution, then formation of intense yellow color. Which becomes colorless on addition of dilute acid indicate the presence of flavonoids.

Test for Glycosides

Extract was mixed with 2 ml of glacial acetic acid containing few drops of 2% $FeCl^3$, mixture poured into another tube containing 2 ml of concentrated sulfuric acids. A brown ring at the inter phase indicates the presence of glycosides.

Antimicrobial activity

Microbial strains: In this study five pathogenic bacterial strains were used as the test organisms for antibacterial screening of the plant extract. These microorganisms were obtained from Persian Type Culture Collection, PTCC, Tehran, Iran**.** Among them *Staphylococcus aureus* (PTCC 1337) and *Bacillus subtilis* (PTCC 1023) were Gram-positive and *Escherichia coli* (PTCC 1330), *Pseudomonas aeruginosa* (PTCC 1047) and *Klebsiella pneumoniae* (PTCC 1053) were Gram-negative. In this assay standard antibiotic (Gentamycine) and DMSO were served as positive and negative controls for the sensitivity of the tested bacteria respectively**.**

Antimicrobial assay: The antibacterial activity screening was carried out by disc diffusion technique for different extracts. The Mueller Hinton Agar (MHA) was used as bacteriological medium. Mueller Hinton Agar plates were prepared by pouring 15ml of molten media into the sterile petriplates. The plates were allowed to solidify for 15 minutes and 0.1% inoculums suspension was swabbed uniformly and inoculums was allowed to dry for 5 minutes. Under aseptic conditions, Whatman paper discs (6 mm in diameter) filter paper disc were impregnated with 10µl (contains 5mg/ disc) of various extracts of *Cousinia microcarpa* Boiss. dissolved in dimethylsulfoxide (DMSO). The discs were overlaid on MHA plates and incubated at 37°C for 24 hours. The diameter of growth inhibition zones produced by different extracts was compared with standard drugs (10µg/ disc Gentamycin). For each bacterial strain controls were maintained, where DMSO is used instead of extracts. All the experiments were set in triplicate to minimize the error and the mean values are presented and reported (Basser et al., 1966).

RESULTS

The study of chemical constituents of the medicinal plants has acquired a lot of importance all over the world. In the present study plant sample collected from the area and were authentified. Then they were dried, powdered and

subjected to phytochemical screening. Powders were subjected to extraction with various solvents like methanol, ethanol, chloroform and distilled water respectively. The qualitative tests for four different solvents were performed. The investigation shown that positive (+) and negative (-) indicates the presence or absence of active components in roots extract with different solvents.

The results of the photochemical screening of the roots of *Cousinia microcarpa* Boiss. are illustrated in Table 1. The phytochemical analysis revealed the presence of flavanoids, tannins, saponins, terpenoids and phenols. The antimicrobial susceptibility test (AST) is an essential technique in many disciplines of science. AST standard tests can be conveniently divided into diffusion and dilution methods. Common diffusion tests include agar well diffusion, agar disk diffusion and bioautography, while dilution methods include agar dilution and broth micro/macrodilution. The broth and agar based methods are the conventional reference methods for AST (Tenover et al., 1995). In this assay, disk diffusion method was applied for the evaluation of the antibacterial activity. The results of antibacterial activity of different extract of *C. microcarpa* Boiss. roots against 5 bacterial strains are explained in Table 2.

The results of antimicrobial tests indicated that this medicinal plant has potent antibacterial activities against Gram-positive bacteria (*Staphylococcus aureus* & *Bacillus subtilis*) but low antibacterial effect against Gram-negative (*Escherichia coli, Pseudomonas aeroginosa* & *Klebsiella pneumoniae*).

Table 1. Shows the preliminary phytochemical screening of various extracts of the root of *Cousinia microcarpa* Boiss.

Extracts	Alkaloids	Tannins	Flavonoids	Glycosides	Terpenoids	Phenols	Saponins
Methanol	-	+	+	-	+	+	+
Ethanol	-	+	+	-	+	-	+
Chloroform	-	+	+	-	-	-	-
Distilled Water	-	-	-	-	-	-	-

Positive (+) show presence of constituents; whenever negative (-) show absence of constituents in the root extract

Table 2. Antibacterial activity of various extracts of root of *Cousinia microcarpa* Boiss. against various bacterial strains by disc diffusion method

Microorganisms		Methanol	Ethanol	Chloroform	Distilled Water	Gentamycine	DMSO
Gram-positive	***Staphylococcus aureus***	19	15	8	-	21	-
	Bacillus subtilis	17	11	12	-	18	-
Gram-negative	***Escherichia coli***	11	10	8	-	19	-
	Klebsiella pneumonia	12	-	9	-	23	-
	Pseudomonas aeruginosa	14	11	-	-	20	-

Mean values of the growth inhibition zones (in mm); Values include cup border diameter (6mm); Values are mean of three replicates; DMSO: dimethylsulfoxide

DISCUSSION

Medicinal plants have been used for centuries in traditional medicines as remedies for human diseases and many active antimicrobial agents isolated form it include alkaloids, phenolic acids, quinones, tannins, coumarins, flavonoids, and terpenoids (Ahmed et al., 2006). In this preliminary phytochemical analysis of roots of *Cousinia microcarpa* Boiss tannins, flavanoids, phenols, terpenoids and saponins were detected. These results indicate that these compounds may possibly be responsible for the biological properties. The results of antimicrobial activity showed that all the extracts were found to possess various degrees of antibacterial activities except distilled water extract. Ethanol extract showed antibacterial activity to all microorganisms except *Klebsiella pneumoniae*. Chloroform extract showed antibacterial activity except to *Pseudomonas aeroginosa*. Methanolic extract showed the maximum zone of inhibition against Gram-positive bacteria. Methanolic extract showed a broad spectrum antibacterial activities may be due to the presence of secondary metabolites such as tannins and other phenolic groups. Tannins are well known factor responsible for antimicrobial activities (Murugan et al., 2011). Tannins are quite resistant to microbial attack and are known to inhibit the growth of some microorganisms. Among the microbial strains evaluated *Staphylococcus aureus* was found to be the most susceptible, based on the results demonstrated from the inhibition zones. Different workers have already shown that Gram-positive bacteria are more sensitive towards plant extracts as compared to Gram-negative bacteria (Lin et al., 1999; Parekh and Chanda, 2006). These differences may be related to fact that the cell wall in Gram-positive bacteria is of a single layer whereas the Gram-negative cell wall is multilayered structure (Yao and Moellering, 1995). In this assay all tested microorganisms were completely non-sensitive to control disks imbued with DMSO. In conclusion, it can be stated that the antibacterial activities in vitro of the various extracts of *C. microcarpa* Boiss provides a primary platform for further photochemical and pharmacological surveys. This paper therefore provides the scientific basis for traditional application of *C. microcarpa* Boiss as ethnomedicine with antibacterial activities which can be used as antimicrobial

agents in new drugs for the therapy of infectious disease caused by pathogens. According to the obtained results from this investigation and increasing usage limitations of synthetic antimicrobial agents due to side effects and microbial resistances, it is necessary to replace these substances with natural ones.

Acknowledgments

This work is supported by Payame Noor University and it is appreciated.

Conflict of Interest

There is not any conflict of interest in this study.

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