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RESEARCH ARTICLE

Investigating the Antifungal Effect of the Essential Oil of *Thymus Eriocalyx* on Dominant Filamentous Fungal Agents Isolated from Livestock and Poultry Feed

Abdelnasser Mohammadi[1], Sima Shiravand[1], Fatemeh Saleh[2], Mohammad Yarahmadi[3] and Asghar Sepahvand[2,*]

[1]*Department of Biology, Faculty of Science, Lorestan University, Khorramabad, Iran;* [2]*Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran;* [3]*Department of Medical Parasitology and Mycology, Lorestan University of Medical Sciences, Khorramabad, Iran*

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Abstract: ***Background*:** One of the most important principles in disease control is the health of livestock and poultry feed. Given the natural growth of *Th. eriocalyx* in Lorestan province, its essential oil can be added to the livestock and poultry feed and prevent the growth of the dominant filamentous fungi.

***Objective*:** Therefore, this study aimed to identify the dominant moldy fungal agents of livestock and poultry feed, examine phytochemical compounds and analyze antifungal effects, anti-oxidant properties, as well as cytotoxicity against human white blood cells in *Th. eriocalyx*.

***Methods*:** Sixty samples were collected in 2016. The PCR test was used to amplify ITS1 and ASP1 regions. The analysis of essential oil was conducted by gas chromatography and gas chromatography-mass spectrometry devices. MIC and MFC were performed using the broth micro-dilution method. For the analysis of DDPH activity, DDPH was used. Cytotoxicity effect on healthy human lymphocytes was carried out by the MTT method.

***Results*:** In this study, *A. niger*, *F. verticilloides* and *F. circinatum*, *P. oxalicum,* and *P. chrysogenum* were the most resistant species, and *A. oryzae* and *A. fumigatus*, *F. prolifratum* and *F. eqiseti*, *P. janthnellum* were the most susceptible ones. IC_{50} value of *T. daenensis Celak* was 41.33 µg/ml, and 100 µl/ml of the essential oil caused slight cell lysis.

***Conclusion*:** Considering our results, compared with drugs and chemical additives, essential oils can be added to livestock and poultry feed to prevent the growth of filamentous fungi in the livestock and poultry feed.

Keywords: Chromatography, phytochemical, antioxidant, cytotoxicity, PCR, antifungal effect.

1. INTRODUCTION

Livestock, poultry, and their products make up an important part of the national economy of every country and play an important role in preparing high-quality food for humans [1, 2]. The role of the livestock sector is well-known in increasing agricultural productivity with a large contribution to poverty reduction in rural areas [3]. The livestock and poultry feed is at risk of contamination caused by the activity of insects and microbes. The microbes such as bacteria, viruses, yeasts, and molds attack the foodstuffs and produce toxic compounds such as endotoxins or exotoxins and mycotoxins, which are harmful to the health of animals, such as lactating cattle and birds, such as chickens [2]. The fungi are abundant in air, soil, and the environment. If the livestock and poultry feed storage conditions are not suitable, the moisture, air, and proper heat lead to the growth and proliferation of fungi [4]. The most important natural fungal flora in the livestock and poultry food sources include *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria,* and *Cladosporium*, which are grown in appropriate temperatures and moisture on foodstuffs to produce mycotoxins [5, 6].

Mycotoxins are toxic substances and secondary metabolites produced by various fungi. More than 100 fungal species can infect plants and produce mycotoxins. Mycotoxins are mainly produced by *Aspergillus*, *Penicillium,* and *Fusarium* species; Aflatoxin and Fumonisin are among the toxins secreted by these fungi [5, 7, 8]. Aflatoxin is one of the most important mycotoxins produced by some *Aspergillus* species, such as *Aspergillus flavus* and *Aspergillus parasiticus* [9, 10]. These fungi can be found in wheat, maize, barley, bread, and large amounts in moldy food or forage.

*Address correspondence to this author at the Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran; Tel: +98-66-33204005; E-mail: fungimed44@yahoo.com

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Aflatoxins have acute and chronic toxic effects on humans and animals and can produce dangerous diseases such as acute liver disease, liver cirrhosis, and tumor [11, 12]. Fumonisin B1 is mainly produced by *F. verticillioides* and *F. proliferatum* and is found as food contaminants for livestock and corn-based foodstuff in many parts of the world. This toxin is associated with acute and chronic toxicity in animals, congenital diseases, and esophageal cancer in humans [13, 14].

Plants and their essential oils have beneficial resources with antimicrobial properties. Several studies have been conducted on the antimicrobial activity of plant compounds against different types of microbes, including oral pathogens [15-17]. The main components of the essential oils of monoterpenes and sesquiterpene include carbohydrates, phenols, alcohols, ethers, aldehydes, and ketones, which are responsible for the biological activity of plants [18]. *Thymus eriocalyx L.* is one of the most important species of the *Lamiaceae* family; it is one of the most well-known plants containing essential oils [19]. *Th. eriocalyx* are aromatic hairy shrubs with small leaves and pink flowers commonly known as Avishane-Korki in Iran. *Th. eriocalyx* is widespread in Iran and widely used as spices, herbal tea, insecticide, and flavoring agents [20].

One of the important principles for the control of diseases is the health and control of livestock and poultry feed contaminations because the contamination of livestock and poultry feed can directly provide an appropriate environment for the development of other diseases in the cited animals. By causing contamination in the cycle of human food production, it also causes disease in humans. Therefore, to produce healthy food for humans, the pathogens should be controlled from the beginning of the food cycle production, which is the livestock and poultry feed. The fungal contamination of the livestock and poultry feed and the production of mycotoxins cause economic losses, including the economic losses on the livestock industry, livestock losses, harm to crops, and the prevalence of livestock diseases in cattle and poultry farms. On the other hand, detoxification using chemical methods to prevent fungi growth and neutralize the produced mycotoxins is also harmful [20].

Given the natural growth of *Th. Eriocalyx* in Lorestan province, its essential oil can be added to the livestock and poultry feed and prevent the growth of dominant filamentous fungi such as *Aspergillus* and *Penicillium* in livestock and poultry feed. On the other hand, the appropriate antifungal drugs of natural origin can be achieved by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Therefore, this study aimed to identify the dominant moldy fungal agents in livestock and poultry feed, identify the phytochemical compounds, and examine the antifungal effects of essential oils of *Th. eriocalyx,* and also study this plant's antioxidant and cytotoxic properties against human white blood cells [21].

2. MATERIALS AND METHODS

2.1. Sampling

This study was performed in the summer and autumn of 2016. To follow the aim of the study, 60 samples were collected. Thirty samples of the livestock feed included soybean meal, concentrate, and corn flour, and 30 other samples included poultry farm feed such as corn and soybeans. The samples were randomly collected in sterile plastic bags and transferred to the laboratory after packaging and coding.

2.2. Fungi Isolation

The collected samples were crushed separately in the mill. Then,1 gr of each sample was mixed in the tubes containing 10 ml of distilled sterile water and stored at the laboratory temperature for 1 h. Afterward, 0.1 ccs of the supernatant were removed using sterile swabs and cultured in the plates containing potato dextrose agar (PDA) and rose bengal. The rose bengal agar was used to prevent the growth of *zygomycetes* fungi. In addition, after removing the supernatant, the resulting sediment was also cultured, and all the samples were incubated at 25°C for one week.

To purify the isolates, a fragment of the colony margin with a diameter of 5 mm was added to the test tube containing sterilized distilled water. It was completely mixed to prepare the suspensions of spores. Then, 0.2 ccs of the prepared suspension were cultured on PDA and re-incubated at 25°C for one week, and the pure isolates were obtained. The resulting isolates were identified based on the morphological characteristics and with respect to the identification keys of Barnett and Hunter, Klich, and Dugan [22-24].

2.3. Molecular Identification of Fungi

2.3.1. Molecular Identification of the Fungal Isolates by Sequencing ITS1 and ASP1 Regions DNA Extraction

DNA extraction was conducted using the cetyltrimethylammonium bromide (CTAB) method with some modifications [13]. The Nanodrop spectrophotometer determined the concentration and purity of the DNAs extracted from the fungal isolates, and a concentration of 20 ng/ml was prepared from each sample. The presence of the extracted DNAs was confirmed by electrophoresis on 1% agarose gel. The cultivation of fungal purified isolated in PDB (potato dextrose broth) (Scharlau, Barcelona, Spain) was performed at 25°C for 7 days.

Then, the mycelium was collected and crushed using liquid nitrogen; an even powder was obtained. In the next step, 700 µl of CTAB buffer, including (2% (w/v) of cetyltrimethylammonium bromide, 100 mM Tris-HCl, 1.4 M NaCl, and 20 mM EDTA(pH 8.0) were added to 50-100 g of the crushed powder. After mixing, microtubes were placed at 65°C for 45 min. They were centrifuged for 10 min with 10000 Xg, and 650 µl of the supernatant was mixed with the same volume of chloroform/isoamyl alcohol (24:1 v/v). After 10 min at room temperature, it was re-centrifuged for 10 min with 10000 Xg. The supernatant phase was separated, and the cold isopropanol was added by 0.7% of its volume and stored for 20 min at -20°C. The tubes were centrifuged for 5 min at 10000 Xg; the DNA-containing sediment was rinsed three times with 70% ethanol and centrifuged each time for 5 min at 10000 Xg. Finally, the pellet was air-dried, and the DNA was resuspended in 50 µl of TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA).

Nucleic acid concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and the integrity of each DNA sample was tested on 10 g L-1 agarose. The quality of the extracted DNA was examined on 0.1% agarose gel by electrophoresis, and DNA-safe staining was performed using the gel doc.

2.3.2. ITS1 and ASP1 Gene Sequencing

To perform the PCR for the amplification of the ITS1 region, the following primers were used, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'); for the amplification of the ASP1, the primers of ASAP1 (5'- CAGCGAG-TACATCACCTTGG-3') and ASAP2 (5'- CCATTGTT-GAAAGTTTTAACTGATT-3') were also employed. In the amplification of ITS1 and ASP1 regions, the proposed method by White *et al.* was used [25, 26]. It was performed in a Biorad Thermocycler (S 1000TM). It consisted of an initial denaturation step of 5 min at 94ºC with 40 cycles of denaturation for 1 min at 94ºC, primer annealing for 45 s at 53ºC and primer extension for 90 s at 72ºC, with an initial denaturation at 94ºC for 5 min, and a final extension for 10 min at 72ºC. The reaction was performed in a 25 µL containing 2 µL DNA template, 12.5 µL master mix, and 10 pmol of each primer. PCR products were electrophoresed (1 h at 80 volts) in 0.8% agarose gel in Tris-borate-EDTA buffer at pH = 8. Gels were stained with DNA-safe stain (10 g/mL) and observed in a Gel documentation system (Alpha Innotech, USA). Then, the products obtained from the PCR of the ITS1 and ASP1 regions were sequenced, and after the final confirmation, the fungal species were identified.

2.3.3. Identifying the Constituents of the Essential Oil of Th. eriocalyx Plant Collecting

The flowers and leaves of *Th. eriocalyx* were collected in early June 2016 from the City of Zagheh, Lorestan province. After identifying the collected samples, they were dried in the shade away from direct sunlight, powdered by the mill, and stored in the refrigerator for further use.

2.3.4. Extraction of Essential Oil

The essential oils were extracted by water distillation and a Clevenger apparatus. To this end, 50 g of dry and powdered samples of *Th. eriocalyx* was transferred to a 500 ml flask, and 200 ml of distilled water was added. The extraction of the essential oil continued until 3 h after the time of water boiled. Finally, 300 µl of normal n-hexane was added to the essential oil to separate the organic and water phases. The resulting essential oil was stored in glass-capped containers at -20°C until the device analysis and testing.

2.3.5. Essential Oil Analysis

Essential oil analysis was carried out in the central laboratory of Lorestan University using gas chromatography (GC) and gaschromatography–mass spectrometry (GC/MS) devices made by Shimatzu Co., Japan. GC instrument separated volatile compounds. Also, the MS instrument identified whole separated components based on the mass property with high precision.

After the injection of essential oils, the essential oil compounds were identified using various parameters such as the inhibitory time of compounds (RT), inhibition index (RI), study of mass spectra and their comparison with indexes in reference books and articles, using standard mass spectra and data in the electronic library. The relative percentage of each of the constituent compounds of the essential oil was obtained with respect to the area under the curve in the GC chromatogram with surface normalization while neglecting the coefficients [27].

2.3.6. Examining Antifungal Properties of Essential Oil of Th. eriocalyx Examining Antifungal Activities

After morphological and molecular identification of the studied fungi from the sample of livestock and poultry feed, their antifungal properties were studied. To prepare fresh and active culture of isolated microorganisms, culturing was performed on the Sabouraud Dextrose Agar (SDA); it was incubated at 30°C for one week. From the above colonies, the fungal suspension was prepared in normal saline. Then, a concentration of fungal cells equivalent to 1.5 × 104 spores/ml was obtained.

2.3.7. Determining the MIC and MFC by Micro-Dilution Broth Method

The MIC was performed using the broth microdilution method proposed by CLSI using the M38-A2 method [28]. The serial dilutions (with a volume of 100 µl) were prepared from the essential oil of *Th. eriocalyx* (with dilutions of 64 µl/mL to 0.125 µl/mL) in 96-well microplates using 100 µl of the RPMI-1640 agar (with L-glutamine and without bicarbonate) buffered with MOPS and sterilized by Millipore filters of 0.22 µl. Then, 100 µl of the fungal cell suspension with 1.5 × 104 CFU/mL cells were added to the wells containing the dilutions of essential oils, and the microplates were incubated for 48 h at 37°C in the incubator. The negative control (wells containing broth micro-dilution without fungal suspension) and positive control (wells containing RPMI-1640 and fungal suspension without antifungal agents or essential oil) were also employed. The growth in each well was compared with the growth control well; each experiment was repeated three times.

The results were studied after 48 h, and the last dilution that inhibited fungal growth was considered MIC. To determine the minimum fungicidal concentration of essential oils, 10 µL of the liquid culture medium in wells that showed a lack of eye growth of filamentous fungi agents was passaged on a solid SDA medium and incubated at 35°C. The minimum concentration that prevented the formation of more than 4 colonies was determined as MFC [28].

In this method, the non-drug control medium (fungal culture sample in the non-drug medium) was used for the comparison. After the culture of the non-drug control sample turned out to be positive, the minimum concentration of the drug caused death, and as a result, the lack of growth of fungus on the SDA medium was determined. All the above steps were performed with the standard medication Amphotericin B for comparison with the effect of essential oil [28].

2.3.8. Antioxidant Properties of Essential Oils of Th. eriocalyx

This method used 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable lipophile compound with a maximum absorption of 518 nm. DPPH and methanol solvent without essential oil were used as antioxidant control. The ability to transfer hydrogen atoms or electrons by chemical compounds and different extracts was measured from the bleaching value of DPPH violet solution in the methanol 8. The concentrations of 100, 50, 25, and 12.5 µg of essential oil of *Th. eriocalyx* in 900 µl of distilled water was prepared in separate tubes; in the next step, 500 µl of DPPH solution (0.0006 g per 25 mL of methanol), which is the free radical source of production, and 450 µl of methanol were added and mixed by the shaker and stored for 30 min in darkness. The microplate reader read the absorbance of the mixtures. Finally, the absorbance of the DPPH free radicals was measured using the following formula:

$$\text{Radical scavenging activity (RSC)\%} = [(A0-A1)/A0] \times 100$$

In this formula, A0 and A1 are the optical absorption of negative control (without essential oil) and oil (sample), respectively. RSC% shows the inhibition percentage of DPPH free radicals.

2.3.9. Studying Cytotoxicity of Essential Oil of Th. eriocalyx

In order to evaluate the essential oil of *Th. eriocalyx,* its cytotoxicity effect was evaluated on the growth and proliferation of lymphocyte cells using MTT colorimetric method. As an explanation for the use of lymphocytes, it should be said that due to lack of access to a cell bank, a human peripheral blood sample was used as a source of white blood cells, which were used to access the cell cytotoxicity.

The MTT method is a competitive mitochondrial metabolic test based on the breakdown of tetrazolium salt by mitochondrial succinate dehydrogenase of living cells. Three ml of blood from a healthy donor was poured into a test tube containing 4% sodium citrate and mixed with 5 ml of phosphate-buffered saline (PBS). Then, 3 ml of ficoll was carefully added and centrifuged for 30 min at 2000 rpm. After centrifugation, three layers of plasma, white blood cells, and red blood cells were formed; the white layer (opaque) consisting of white blood cells (mononuclear cells) was collected with the sampler, mixed with 5 ml of PBS and centrifuged for 10 min at 1500 rpm. This was repeated three times for lymphocyte rinsing. Then, it was added to the plate containing 10 ml cells of RPMI-1640 medium supplemented with 10% of inactive fetal bovine serum (the fetal bovine serum was inactivated at 56°C in the water bath for half an hour), and the cell count was carried out using the Hemacytometer slide.

Concentrations of 100 µl of the suspension containing 1×105 cells/ml were cultivated in three rows of wells from 96-well flat microplates and incubated for 24 h in the incubator at 37°C and humidity of 5% CO_2. After 24 h of incubation, the concentrations of 100, 50, 25, and 12.5 µl of the essential oil were added to the cells. After 24 h of incubation, 20 µl of MTT (at the concentration of 5 mg/ml) was added, slowly mixed, and incubated for 4 h. After the required time was passed, an amount of supernatant solution in each well was carefully removed, and 100 µl of DMSO solution was added to each well to solve the purple formazan. After 2 hours of incubation, the optical absorption was read using a Biotec microplate reader at 570 nm. The inhibitory percentage of the growth of cells exposed to the essential oil solution of *Th. eriocalyx* was calculated using the following formula.

$$\textbf{Growth inhibition percentage} = \mathbf{100} - \left(\frac{\text{Average absorption of cells exposed to the sample}}{\text{Average absorption of control cells}}\right) \times 100$$

The concentration of the sample, which inhibited the growth of the cells by 50%, was used as a parameter for cytotoxicity. Normal lymphocytes were used as cell cytotoxicity control.

3. RESULTS

3.1. Isolation and Identification of Fungal Species

In this research, 60 samples of frequently used livestock and poultry feed were collected, 80% of which had fungal contamination. Fifty-eight fungal species were isolated. After identification based on morphological characteristics (macroscopic and microscopic characteristics) and their comparison with fungal sources, the isolates with the described properties were matched with *Aspergillus* 43.1%, *Fusarium* 29.3%, *Penicillium* 22.4%, and *Fuma* 1.7%. Two samples (3.4%) were also identified as Fungal spp.

In this study, *Fusarium* and *Penicillium* species underwent molecular identification using molecular methods based on the ITS region of ribosomal DNA (ITS-rDNA); also, *Aspergillus* species were identified based on the sequencing of the ASP1 region. The ITS1 and ASP1 regions of the isolates were amplified using PCR. Sequences of the ITS1 and ASP1 regions of the isolates were compared with the sequences in the NCBI database using the Blast (basic logical alignment search tool), and the results showed that the sequence of the isolates had the maximum similarity of 99-100% with *Aspergillus*, *Fusarium* and *Penicillium* species. Among the *Aspergillus* species, *Aspergillus flavus* (40%), and *Aspergillus niger* (32%), among the *Fusarium* species, *Fusarium verticilloides* (50%), *Fusarium saccharin* (18.75%), and *Fusarium proliferatum* (12.5%), and among the *Penicillium* species, *Phenicillium oxalicum* (38.46%) and *Penicillium chrysogenum* (23%) had the highest frequency (Table **1**).

3.2. Chemical Composition of the Essential Oil *Th. eriocalyx*

Table **2** shows the GC–MS results, which proved that 10 constituents represent 83.39% of the essential oil. The main components of essential oil were Carvacrol, (63.81%), m-cymene (8.29%), gamma Terpinene (7.33%), and Myrcene (3.01). About 4% of essential oil compounds were not identified.

Table 1. Molecular and morphological identification, MIC, and MFC determination of fungi isolated from livestock and poultry feed.

Sample No	Isolate Number	Source	Molecular Identification	Accession Number	MIC µg/ml	MBC µg/ml
1	80	Soybean meal	*A. flavus*	Jq899451.1	4	8
2	81	Soybean	*A. Fumigatus*	KF152942.1	1	2
3	82	Corn	*P. janthinellum*	EF550979.1	1	2
4	83	Concentrate	*A. niger*	AM270052.1	32	64
5	84	Corn flour	*P. oxalicum*	KY400098.1	2	4
6	85	Corn	*P. oxalicum*	KY400080.1	2	4
7	86	Soybean	*A. fiuvus*	JQ89945.1	2	4
8	87	Corn	*A. fiuvus*	JQ89945.1	2	3
9	88	Concentrate	*A.oryzae*	DQ15587.1	0.5	1
10	89	Soybean	*A. oryzae*	JQ899451.1	2	4
11	90	Corn	*A. flavus*	JQ899451.1	1	4
12	92	Corn	*F. verticillioides*	KY426419.1	0.25	1
13	101	Soybean meal	*P. chrysogenum*	KY524460	4	8
14	102	Corn flour	*F. verticillioides*	KY426419.1	0.25	1
15	103	Corn	*F.verticillioides*	KY436182.1	0.25	1
16	104	Corn	*Fungal sp*	KY776210.1	0.125	0.5
17	105	Soybean	*A. niger*	AM270051.1	0.25	0.5
18	106	Soybean	*A. flavus*	AM270052.1	0.5	1
19	107	Corn	*Fungal sp*	KY776210.1	0.5	1
20	108	Concentrate	*A.niger*	AM270052.1	0.25	0.5
21	109	Corn flour	*A. flavus*	JQ899451.1	2	4
22	110	Soybean	*A. oryzae*	DQ155287.1	0.5	1
23	111	Corn flour	*F.verticillioides*	KF494135.1	0.5	1
24	112	Soybean meal	*A. oryzae*	DQ155287.1	1	2
25	113	Corn	*P. oxalicum*	KY400080.1	0.5	2
26	114	Soybean	*A. flavus*	JQ899451.1	1	2
27	115	Concentrate	*A. niger*	AM270052.1	0.5	1
28	116	Corn flour	*A. flavus*	Jq899451.1	0.5	1
29	117	Soybean	*Penicillium SP*	KP760061.1	1	2
30	118	Soybean meal	*A. niger*	AM270052.1	1	4
31	119	Corn	*A. oryzae*	DQ155287.1	0.5	1
32	120	Corn flour	*F. verticillioides*	Ky426419.1	0.5	1
33	121	Soybean	*P. funiculosum*	JX045839.1	1	2
34	200	Soybean meal	*A.niger*	Ky204007.1	0.5	1
35	201	Corn	*F. proilratum*	MG437416.1	1	2

(Table 1) contd….

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Sample No	Isolate Number	Source	Molecular Identification	Accession Number	MIC µg/ml	MBC µg/ml
36	202	Soybean	*A. niger*	AM270052.1	1	2
37	203	Soybean	*F. proilratum*	MG437416.1	0.5	1
38	220	Soybean meal	*F. sacchari*	Ky36198.1	1	2
39	221	Soybean	*P. oxalicum*	MF061780.1	0.5	1
40	222	Soybean	*A. niger*	MG65969.1	0.5	1
41	223	Concentrate	*A. oryzae*	7173.1	0.25	0.5
42	224	Corn	*F. ubglutinans*	MG274315.1	1	2
43	225	Soybean meal	*P. janthinellum*	EF550979.1	0.25	0.5
44	226	Corn	*F. verticillioides*	KY436182.1	0.5	1
45	227	Corn	*P. oxalicum*	KY400098.1	0.25	0.5
46	228	Corn	*A. flavus*	MF324887.1	0.5	2
47	229	Soybean	*F. sacchari*	KY436198.1	2	4
48	230	Corn	*Phoma Sp*	KU508291.1	2	4
49	231	Corn flour	*F. subglotinans*	KX681580.1	2	8
50	232	Soybean meal	*F. sacchari*	KY436198.1	2	4
51	233	Soybean meal	*P. crustosum*	MG202157.1	1	2
52	234	Soybean meal	*F. verticillioides*	KT587649.1	4	8
53	236	Corn	*F. verticillioides*	KY436182.1	1	2
54	237	Corn flour	*A. flavus*	MF319893.1	4	8
55	238	Soybean	*F. eqiseti*	KM979505.1	1	2
56	239	Corn	*P. chrysogenum*	KY524460.1	4	8
57	240	Corn	*F. circinatum*	KY524460.1	4	8
58	241	Soybean	*P. chrysogenum*	KY524460.1	2	4

Note: Accession number: Molecular code of fungi in NCBI and FASTA website.

Table 2. Identification of the essential oil of the *Th. eriocalyx* species.

Row	Compound Name	%	KI
1	Octane	0.7	800
2	alpha pinene	0.89	939
3	Myrcene	3.01	991
4	Terpinene (alpha)	2.52	1014
5	m-cymene	8.29	1028
6	gamma Terpinene	7.33	1062
7	Linalool	2.19	1098
8	alpha terpineol	1.23	1205
9	Thymol methyl ether	0.74	1235
10	Carvacrol	63.81	1309

Abbreviation: (KI): Kovats index.

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3.3. Evaluation of Antifungal Properties of the Essential Oil *Th. eriocalyx*

In this study, the antifungal effects of essential oil of *Th. eriocalyx* were evaluated against *Aspergillus*, *Fusarium,* and *Penicillium* species by micro-dilution to determine the MIC and MFC. Results of MIC and MFC examinations of essential oils of *Th. eriocalyx* against fungal species are presented in Table **3**.

Aspergillus species with the MIC range of 0.25-8 µg/ml had higher resistance than *Penicillium* and *Fusarium* species (MIC = 0.25-4 µg/ml). Among *Aspergillus* species, *A. niger* (8 isolates) with MIC Range = 0.25-8 µg/ml was the most resistant, and *A. oryzae* (6 isolates) with MIC Range = 0.25-2 µg/ml and *A. fumigatus* (1 isolate) with MIC = 1 µg/ml were the most susceptible species. Among the *Fusarium* species, *F. verticillidus* (8 isolates) with MIC Range = 0.25-4 µg/ml and *F. circinatum* with MIC = 4 (1 isolate) were the most resistant, and *F. prolifratum* with MIC Range = 0. 5-1 µg/ml (2 isolates) and *F. eqiseti* (1 isolate) with MIC = 1 µg/ml were the most susceptible species. Among the *Penicillium* species, *P. oxalicum* with MIC Range = 0.25-4 µg/ml and *P. chrysogenum* with MIC Range = 2-4 µg/ml were the most resistant, and *P. janthnellum* with MIC Range = 0.25-1 µg/ml was the most susceptible ones.

3.4. Evaluating the Antioxidant Activity of the Essential Oil *Th. eriocalyx*

Evaluating the trapping activity of DPPH free radicals is one of the methods to determine antioxidant activity. In this method, the purple DPPH free radical at the wavelength of 518 nm is reduced by antioxidant compounds and converted into yellow. The strength of essential oil of *Th. eriocalyx* in inhibiting DPPH free radicals is shown in Table **4**. There is a direct correlation between the concentration of essential oil and the radical inhibiting effect; as the concentration of essential oil increases, its radical inhibitory effect increases. The highest and the lowest percentage of free radical damage belongs to 100 µl and 12.5 µl concentrations, respectively.

In this research, the IC_{50} value of *Thymus daenensis* was determined to be equal to 41.33 µg/ml. The data is given in Table **4**; it was deleted to avoid repetition of the data. IC_{50}

Table 3. Antifungal activity of *T. capitatus* extracts against *A. niger*, *A. oryzae*, *P. digitatum*, and *F. solani* a.

Fungal Species	Number of Isolates	Essential Oil		AMB	
		µg/mL		µg/mL	
	-	Range MIC	Range MFC	MIC	MFC
Aspergillus	25	0.25-8	0.5-16	0.5-16	1-32
A. flavus	10	0.5-4	0.5-16	0.5-8	2-16
A. niger	8	0.25-8	0.5-16	0.5-16	1-32
A. oryzae	6	0.25-2	0.5-4	0.5-4	1-8
A .fumigatus	1	1	2	4	8
Fusarium	17	0.25-4	0.5-8	0.5-8	1-16
F. verticilliodes	8	0.25-4	0.5-8	0.5-8	1-16
F. prolifratum	2	0.5-1	1-2	2-4	4-8
F. sacchari	3	1-2	2-4	2-4	4-8
F. subglotinans	2	1-2	2-4	1-4	2-8
F. eqiseti	1	1	2	2	`4
F. circinatum	1	4	8	4	8
Penicillium	13	0.25-4	0.5-8	0.5-4	1-16
P.oxalicum	5	0.25-4	0.5-8	0.25-4	0.5-8
P. chrysogenum	3	2-4	4-8	2-4	4-8
P. janthinellum	2	0.25-1	0.5-2	1-2	2-4
P.crustusum	1	1	2	1	2
P. funiculosum	1	1	2	4	8

Abbreviation: AMB: Amphotricin B.

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Table 4. Inhibitory ability of free radicals of essential oil of *Th. Eriocalyx.*

Essential Oil Concentration	100 µl/mL	50 µl/mL	25 µl/mL	12.5 µl/mL
% Free radical scavenging	83	58	41	31
standard (Ascorbic acid)	93	83	78	77

Table 5. The results of cytotoxic effects of essential oil *Th. eriocalyx.*

Essential Oil Concentration (µl/ml)	100	50	25	12.5	IC_{50}
% Cytotoxicity	21	7	4	1	245(µl/ml)

was inversely related to the anti-radical activity of the compounds; the lower the IC_{50}, the higher the antioxidant activity. The results were compared with ascorbic acid as a positive control (standard).

3.5. Evaluating Cytotoxicity of *Th. eriocalyx*

The cytotoxicity at the concentrations of 100, 50, 25, and 12.5 µm was estimated after 24 h on healthy human lymphocytes by the MTT method. As shown in Table **5**, the essential oil caused slight cell lysis at 100 µl/ml concentration.

4. DISCUSSION

The present study showed the presence of fungi in livestock and poultry feed and a high level of contamination (80% of the total samples). The growth of fungi reduces the quality of livestock and poultry feed and contributes to the fungal contamination of these materials.

Aspergillus, *Fusarium,* and *Penicillium* species are the dominant species of livestock and poultry feed. Baliukoniene *et al.* (2003) reported that *Aspergillus* and *Fusarium* were dominant species isolated from barley seeds [29]. In the study by Orsi *et al.*, the major fungi isolated from corn were *Aspergillus*, *Fusarium,* and *Penicillium* [30]. Atehnkeng *et al.* showed that the corn grains in the warehouses were contaminated with *Aspergillus* and *Fusarium* fungi [31]. In the study by Dawlal *et al.*, the contaminating genera of corn in South Africa were *Aspergillus*, *Fusarium,* and *Penicillium* [32]. Also, the results of Rosa *et al.*'s investigation showed the poultry feed had the highest levels of contamination with *Aspergillus* and *Penicillium* fungi [33]. In this study, among different species of *Aspergillus*, *A. flavus* (40%) and *A. Niger* (32%), and *Fusarium* species, *F. verticilloides* (50%) were the dominant species. Among the *Phenicillium* species, the *P. oxalicum* was also dominant.

Most of the isolated species in this study produced mycotoxins, such as *A. flavus*, which is among the important toxin-causing species that could be grown in livestock and poultry diets and produce aflatoxins [34, 35]. *A. niger* and *F. verticilloides* are also among species that could produce mycotoxin and fumonisin and contaminate livestock diet [36-38]. Like other foodstuffs, livestock, and poultry feed are susceptible to fungi growth, especially the *Aspergillus*, *Fusarium*, and *Penicillium* species. Therefore, the presence of fungi in livestock and poultry feed can lead to the production of mycotoxins; finally, it affects the function of foodstuffs.

Consumption of these materials contaminated with mycotoxin by livestock causes diseases and reduces animal growth; consuming the contaminated milk and meat would cause human disease [35, 39]. In the present study, the compounds in the essential oil of *Th. eriocalyx* were identified by GC and GC–MS spectrometry, and the inhibitory effect of essential oil of *Th. eriocalyx* was defined by determining the MIC and MFC of *Aspergillus*, *Fusarium,* and *Penicillium* species using the broth microdilution method proposed by CLSI using the M38-A2 method.

In this study, the major components of the essential oil of *Th. eriocalyx* were Carvacrol (63.81%), m-cymene (8.29%), gamma Terpinene (7.33%), Myrcene (3%), Linalool (2.19%), and Terpinene alpha (2.5%). Thymol and carvacrol are among the most important constituents of the thymus species and conventionally account for the highest percentage. Carvacrol and thymol have a wide range of antimicrobial and antifungal effects, which inhibit ATPase activity and increase the nonspecific permeability of fungal cell membranes [40]. In previous studies, *Thymus* species have shown a wide range of antifungal activity, such that *T. eriocalyx* and *T. x-porlock* containing 64.3 and 30.7% thymol, respectively; they demonstrated strong antifungal activities against *A. parasiticus*. Moreover, *T. spathulifolius* with thymol (36.5%) has inhibited the growth of *Trichophyton* spp., *Fusarium* spp., *Penicillium* spp., *Rhizopus* spp., *Alternaria* spp. and *Aspergillus* spp. with MICs variable between 31 and 250 µg/ml [41].

In another study conducted in 2015, the antifungal effects of the essential oils of 15 plant species, including *Th. vulgaris* were evaluated, and the results showed that the essential oils of these plants had a wide range of antifungal activity against different *Penicillium* species [42]. Varga, E., *et al*., reported that the essential oil of *Th. vulgaris* (Carvacrol 13.38%), *Th. serpyllum* (Carvacrol 25.8%), and *Th. pulegiodes* (Carvacrol 26.7%) prevented the growth of *C. albicans* and *Saccharomyces cerevisiae* [43]. In 2014, Stevic *et al.* studied the essential oil of a number of plants, in-

cluding *thymus* against the *Aspergillus*, *Fusarium,* and *Penicillium* species, and the value of MIC was in the range of 0.04 and 28.7 µg/ml. While in the present study, the value of MIC was obtained in the range of 0.25-8 µg/ml, which indicated the high anti-fungal potential of the essential oil of *T. eriocalyx* [44].

According to the results of this study, *A. fumigatus* had MIC = 1 µl/ml, and *A. niger* had MIC Range = 0.25-8 µl/ml. In the study carried out by Tabti L on *Th. capitatus*, the highest activity observed against *A. niger* with the minimum concentration of 0.1 µg/ml, led to a 100% increase in inhibition [45]. In another research conducted in 2015 by Nikan J on the antifungal properties of *Th. eriocalyx* on the *A. flavus* fungi, the highest inhibitory level was 0.14, and the lowest inhibitory level was 0.02 µg/ml. In the study by KohiYama, MIC, and MFC were determined in *A. flavus* as 250 µg/ml, which indicated the consistency of the results of this research with previous studies [46].

In the present study, the anti-oxidant properties of *Th. eriocalyx* were also investigated. The colorimetric method was used to examine the inhibitory potential of DPPH free radicals. Given the direct relationship between the concentration of essential oil and DPPH free radical inhibitory potential, the highest degradation percentage of free radicals for the concentration of 100 µl was 83%, and the lowest percentage for 12.5 µl was 31 µg/ml. In the study carried out by Tabti *et al.* (2014), the inhibitory value of the essential oil in 200 µl was 78 µg/ml, which was considerably compared to the concentration of 100 µl of essential oil in the present study 45. S. bounatirou *et al.* conducted a study in 2010 that used Th's essential oil. *capitatus*, at the concentration of 100 mg of essential oil, and consequently, the inhibitory level was determined to equal 38.5 µg/ml; it was significantly comparable to the results of this study [47].

The cytotoxicity was estimated after 24 h on healthy human lymphocytes by the MTT method. The essential oil at 100 µl/ml concentration caused slight cell lysis. In a research carried out in 2016 by Ana KarenthLopez-Meneses *et al.*, the cytotoxicity level of *Th. Capitatus* was reported on the calf *thymus* with concentrations of 1000 and 2500 µl L-1 [48]. In the present study, the cytotoxicity of essential oil against human lymphocytes was slight at the concentration of 100 µl/ml, and its IC_{50} was obtained equal to 231 µg/ml (Table **4**).

Given the effect of livestock and poultry feed contamination, particularly the contamination with toxigenic fungi such as *A. flavus* and *F. verticillioides* on the health of livestock and poultry, their products, and the secondary effect on the human health, the control for fungal contamination of livestock and poultry feed is the best way to prevent the livestock and poultry contamination with aflatoxin and fumonisin. It is also the best way to promote public health. It seems that the major source of contamination should be found in the raw materials for livestock feed. Besides, the factors increasing contamination should be identified in the particular areas of livestock feed factories and transportation systems. The causes of the increase in heat, moisture, and dust should be identified and tested. These agents are critical factors for contaminant control.

CONCLUSION

Given the favorable results of MIC, antioxidant properties, and slight cytotoxicity of essential oil of *Th. eriocalyx*, and antifungal properties obtained in this study against pathogenic fungi such as *Fusarium*, *Aspergillus,* and *Penicillium*, compared to drugs and chemical additives and with regard to the natural growth of this plant in Lorestan province, its essential oil can be added to livestock and poultry feed to prevent the growth of dominant filamentous fungi such as *Aspergillus* and *Fusarium* in the livestock and poultry feed. Moreover, due to the favorable antifungal effects and the point that these fungi are common pathogens for humans and animals, the essential oil of this plant can also be used to treat human diseases.

LIST OF ABBREVIATIONS

CTAB	=	Cetyltrimethylammonium Bromide
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
GC	=	Gas Chromatography
GC/MS	=	Gaschromatography–Mass Spectrometry
MFC	=	Minimum Fungicidal Concentration
MIC	=	Minimum Inhibitory Concentration
PBS	=	Phosphate-buffered Saline
PDA	=	Potato Dextrose Agar
PDB	=	Potato Dextrose Broth
SDA	=	Sabouraud Dextrose Agar

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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