depression [30] anti-hyperglycemic [31] and antimicrobial [32-34] agent in plant extracts candidate them for overcoming more health problems. Thymus plant leaves and flower parts has good talent to applied for tonic and herbal tea, antiseptic, antitussive and carminative as well as treating cold [35-37]. Thymus oil and extracts applied in pharmaceutical, cosmetic and perfume industry besides flavoring and food preservation [38]. *Silybum marianum* cancer chemoprevention and hepatoprotection has high and brilliant role in treatment on of Phenolic compounds in aromatic extract and plants are one of the defensive mechanisms against bacterial agents, herbivore and insects [39-46]. Plants metabolites and/or constituent such as tannins, terpenoids, alkaloids, flavonoids, glycosides has more potential to be antimicrobial agent [47-50] and always the antimicrobial property of medicinal extract plants in presence and without of natural silver nanoparticles are significantly differ. In this research, *T. daenensis* and *S. marianum* absence of extracts combination with silver nanoparticles (Ag-NPs) in this media extensively was prepared and subsequently, used against some pathogenic bacteria and fungi to detect new sources of antimicrobial agents.

Materials and Methods

Chemicals, reagents and plant source

All reagents were of analytical grade and obtained from Merck, Dermasdat, Germany. The fresh and healthy plants of *T. daenensis, S. marianum* and *R. officinalis* were collected in June year 2013 from various areas of Yasouj district, Iran and subsequently were identified in photochemistry Lab in Yasouj University.

Extraction

Ultrasound-Assisted Extraction (UAE): Medicinal plants (*Thymus daenensis* and *Silybum marianum*) firstly washed thoroughly to remove impurities, shade dried and then ground to fine powder. Five grams (5.0 g) of Medicinal plants powder were placed in a capper tube and mixed with ethanol. The extraction process was performed with the ultrasonic device (JAC Ultrasonic 2010P, Jinwoo Engineering Co., Ltd., Hwasung, Gyeonggi, Korea) equipped with a digital timer and a temperature controller, the solvent used in the extraction was ethanol solution. The device was operated at a frequency of 40 kHz, an ultrasonic input power of 250 W. After ultrasonic extraction, the sample was centrifuged at 4000 rpm for 15 min, and the supernatant was collected. When the ultrasonic extraction was completed, the extracts were immediately cooled on ice to room temperature, filtered using a 5-mL syringe fitted with a 0.45 μm cellulose syringe filter (Phenomenex Australia Pty. Ltd., Lane Cove, Australia). Ultrasonication is a branch of acoustics that can be applied to solids, liquids and gases at frequencies above the human hearing range [51]. Ultrasonication generates an enormous interfacial area between the oil and alcohol due to micro turbulence leading to the formation of fine emulsions [52].

Preparation of Rosmarinus officinalis extract for synthesis of Ag-NPs

R. officinalis leaves were collected, chopped and dried for two days at room temperature. Dried leaves were washed thoroughly with distilled water and aqueous extract was prepared. Prepared extract was centrifuged and filtered through Whatman 41 filter paper to obtain clear solution. The filtrate was used immediately for Ag-NPs synthesis which served as reducing and stabilizing agent. 50 mL of 1 mM $AgNO_3$ was added to 25 mL of aqueous extract of R. *officinalis* and mixed thoroughly via magnetic stirrer. The reaction mixture was then shaken to mixed completely and allowed to settle at room temperature. The color change to yellow confirm the formation of silver nanoparticles.

Purification of Ag-NPs

To separate unreacted components of reaction mixture was removed from the synthesized Ag-NPs the mixture was centrifuged [53] at 10000 rpm for 25 min and washed for three times using deionized water. Dried powder of the silver nanoparticles was obtained by freeze-drying. Ultra-centrifugation techniques were used to separated nanoparticles based on their size. Apart from centrifugation, chromatographic based separations such as HPLC and ion exchange chromatography was also utilized for separating nanoparticles from reaction mixture [54]. Separation of nanoparticle is a vital step in synthesis of nanoparticles from its reaction [55].

Characterization of synthesized nanoparticles

UV-Vis spectrometer (Perkin Elmer Lambda 25) was used to record absorbance in the range of 200-800 nm and also monitor the rate of Ag-NPs formation. 250 mL of each sample was diluted with 2 mL deionized water and sonicated for 15 min. The pH of stock solution (1 mg L^{-1}) was adjusted to 11.0 before scanning in quartz cuvettes with deionized water as reference. Factors such as temperature, pH, concentration of leaf extracts and concentration of $AgNO_3$ influences on the formation of Ag-NPs in the reaction mixture were examined by UV-Vis absorbance spectroscopy. The UV-Vis spectra of Ag-NPs which at RT dispersed in distilled water via 30 min of sonication and subsequently centrifuged at speed of 9000 rpm for 30 min was recorded. The pure Ag-NPs was prepared following removal of unbound ligand. X-ray diffraction (XRD) spectra were obtained by an automated Philips X'Pert X-ray diffractometer with Cu Radiation (40 kV and 30 mA) for 2θ values over range of 30°C-80°C. Scanning electron microscope (SEM: Hitachi S-4160) under an acceleration voltage of 30 kV. TEM analysis was recorded using TEM JEOL at 300 kV.

Biological activity

Antimicrobial assays: The well-diffusion method was used to study the antibacterial activity. All the glassware, media, and reagents used were sterilized in an autoclave at 121°C, 103 kPa of pressure for 21 min. Staphylococcus aureus (ATCC 25293), Escherichia coli (ATCC 33218) were used as model test strains for Gram-positive and Gram-negative bacteria, respectively. All the microbial cultures were adjusted to 0.5 McFarland standard, which are visually comparable to a microbial suspension of approximately 1.5 × 108 CFU/mL [56]. 100 μL of fresh bacterial culture was gently spread on the agar surface [57]. The bacterial concentration utilized was of 1.5 × 108 CFU/mL. 6 mm diameter filter paper disc, impregnated with 20 mL dose of each compounds. Using sterile disc (6 mm diameter) were bored into the seeded agar plates and these were loaded with 50 mL volume with concentration of 50, 25 and 12.5 mg L^{-1} of each compound reconstituted in dimethyl sulfoxide (DMSO). All the plates were incubated at 37°C for 24 h. Antibacterial activity of all the complexes was evaluated by measuring the diameter of zone of inhibition in mm (Table 1) and all investigation were undertaken using dimethyl sulfoxide (DMSO) as solvent. Amoxicillin, Cephalexin and Penicillin was used as controlled antibacterial agents and their results for

antibacterial activity was compared with under study material in Table 2.

Compound	Gram-positive			Gram-negative		
	S. aureus			E. coli		
	12.5	25	50	12.5	25	50
T. daenensis	14.00	15.70	17.62	13.40	15.00	15.22
S. marianum	9.70	10.50	12.30	8.50	10.10	11.38
T. daenensis-Ag	14.40	17.40	18.00	13.40	16.00	16.20
S. marianum-Ag	9.90	12.30	19.00	9.70	11.38	11.60
S. marianum	9.70	10.50	12.30	8.50	10.10	11.38

Table 1: Antibacterial activity of compounds in dose of (12.5, 25 and 50 mg L^{-1}). All tests were performed twice and all data are the mean of two measurements.

Antibiotic drugs	*Bacillus subtilis*	*Staphylococcus aureous*	*Pseudomonas aeroginosa*	*Escherichia coli*	*Candida albicans*	*Aspergillus oryzae*
Amphotericin B (0.03 mg/disc)	-	-	-	-	28.10	37.6
Amoxicillin (0.025 mg/disc)	28.10	41.60	-	20.80	-	-
Penicillin (0.01 mg/disc)	30.50	47.60	-	9.70	-	-
Cephalexin (0.03 mg/disc)	37.60	41.60	-	22.56	-	-

Table 2: Diameter of zone of inhibition (mm) of antibiotic drugs.

Antifungal screening by disc diffusion method: *Aspergillus oryzae* (PTCC 5164, *A. oryzae*) and *Candida albicans* (ATCC 10231, *C. albicans*) were used as mode for determinations of anti-fungal activities of extracts of *S. marianum* and *T. daenensis* by the disc diffusion method on the surface of Sabouraud Dextrose Agar inoculated with 1.0 10^5 (CFU/mL) of spore suspension of fungi. The petri plates cultured with Aspergillus oryzae were incubated at 30°C for 24-48 h while the plates cultured with *C. albicans* were incubated at 32°C for 24-48 h. The discs impregnated with compounds solution (solubilized in 5% w/v DMSO) of compounds doses (12.5, 25 and 50 mg/disc) were dispensed at different positions on the agar plate [57]. At end of incubation period, antifungal activities of extracts were determined in term of inhibition zone diameter value. Antifungal activities of control drug Amphotericin B (0.03 mg/disc) is shown in Table 3.

Compound	*Candida albicans*			*Aspergillus oryzae*		
	12.5	25	50	12.5	25	50
T. daenensis	7.50	8.90	11.00	7.20	7.80	10.64
S. marianum	6.90	7.00	7.68	6.94	7.00	7.44
T. daenensis-Ag	8.12	9.38	11.20	8.00	8.10	10.70
S. marianum-Ag	7.20	7.50	8.12	7.10	7.22	7.68

Table 3: Antifungal activity of compounds in dose of (12.5, 25 and 50 µg L^{-1}). All tests were performed twice and all data are the mean of two measurements.

Determination of total phenolic content

The total phenolic content (TPC) of the *T.daenensis* and *S.marianum* extracts was determined by using the Folin-Ciocalteu reagent [58]. 100 µl of the diluted ethanolic extracts containing 500 µg extract was mixed separately with (500 µl) Folin-Ciocalteu reagent and diluted with distilled water and 0.4 ml of (7.5% w/v) sodium carbonate solution (Na_2CO_3). The solution was mixed and allowed to stand for 1 hour at room temperature. Gallic acid solution (from 25 to 300 µg/ml) was used as a standard reagent. Finally, the absorbance was measured at 765 nm using a UV-Vis spectrophotometer. A calibration curve was prepared by using of standard solutions of gallic acid. The results were expressed as mg gallic acid equivalents (GAE)/gr of dried extract.

Determination of total flavonoid

Total flavonoid content of extracts was also determined [59]. 1 mg of extracts were diluted with 1000 µl of distilled water and 100 µl of 5% $NaNO_2$ solution were added. The mixture was kept at room temperature for 5 min and then, 200 µl of 10 % $AlCl_3$ were added to it. This mixture was incubated at room temperature for a 6 min then 1 ml of 1 M NaOH was added to the mixture. The solution absorbance at 510 nm was measured with a UV-Vis spectrophotometer. The concentration of the flavonoid compounds was calculated by using of the equation that obtained from the rutin (50-500 µg/ml) calibration curve.

Scavenging effect on 2, 2-diphenyl-1- picrylhydrazyl (DPPH)

Free radical scavenging activity was estimated by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay by using of Von Gadow method with some modifications60. 2.4 ml of DPPH radical solution (24 µg/ml) prepared in 70% aqueous ethanol. The reaction mixture contained 100 µl of test extracts and 1 ml of methanolic solution of (24 µg/ml) of DPPH radical. The mixture was then shaken vigorously and incubated at 37°C for 10 min. The absorbance was measured at 517 nm by using by trolex solutions (100-1000 µg/ml) as a standard. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity which was calculated using the following equation: DPPH scavenging effects (%) = 100 × (Ac-As)/(Ac) where Ac is the

absorbance of the control reaction and as is the absorbance of reaction mixture containing DPPH and extract at 517 nm.

FT-IR Spectrum analysis of extracts

FT-IR (JASCO FT/IR-460 System in the 400-4000 cm^{-1}, Japan) relies on the fact that the most molecules absorb light in the infra-red region of electromagnetic spectrum. This absorption corresponds specifically to the bonds present in the molecule. The frequency ranges are measured as wave numbers typically over the range 4000-400 cm^{-1}. Figure 1 shows the FT-IR peaks of dried extracts. Hydroxyl group in alcoholic and phenolic compound which is supported by the presence of a strong peaks. The absorbance bands are associated with the stretch vibrations of alkyl C-C, conjugated C-C with a benzene ring, bending in plate of C-O-H, C-O stretch and bending out of plate C-H in saturated tertiary or secondary highly symmetric alcohol in extracts, respectively.

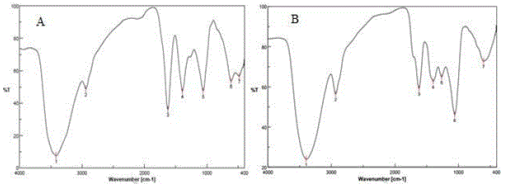

Figure 1: Fourier transform infrared absorption spectra of dried extracts. A) *T. daenensis* B) *S. marianum.*

Results and Discussion

Characterization of Ag-NPs

Figure 2 shows the FT-IR peaks of dried *R. officinalis* leaf extract. Typical peak of hydroxyl groups of phenolic compounds indicated by at 3397 cm^{-1}. The peaks at 2933 cm^{-1}, 1621 cm^{-1}, 1400 cm^{-1}, 1265 cm^{-1}, 1060 cm^{-1} and 605 cm^{-1} are associated with stretching vibrations of alkyl C-C, conjugated C-C of benzenoid rings, bending in plate of C-O-H, C-O stretch and bending out of plane C-H, respectively of saturated, tertiary and secondary symmetric hydroxylic groups of *R. officinalis* leaf extract.

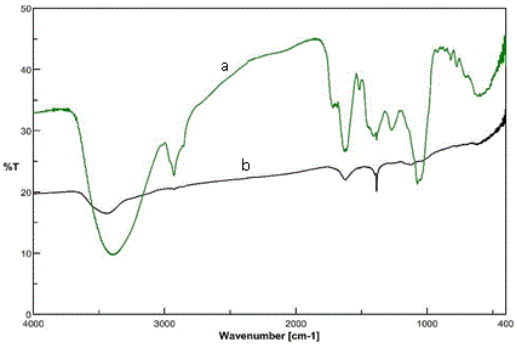

Figure 2: FT-IR spectra of (a) dried Rosmarinus officinalis leaf extract and (b) synthesized Ag-NP's.

The XRD pattern of Ag-NPs Figure 3a represent distinguished diffraction peaks at 2θ of 38.5° (111), 44.1°C (200), 64.5 °C (2 2 0) and 77.3°C (311) which strongly indicates the face-centered cubic (FCC) crystalline structure of Ag-NPs. According to Debye sheerer and half width of XRD patterns, average particle size of Ag-NPs was calculated around 33 nm and intense reflection at (111) compare to other peaks support the growth direction of nanocrystals. SEM image of drop-coated film of the Ag-NPs synthesized with R. officinalis leaf extract (Figure 3b) reveal spherical and uniformly nano-size Ag-NPs. TEM image (Figure 3c) also confirms the spherical shape of AgNPs which are of FCC oriented with aggregation, while its size range of 10-33 nm and average value is around 29 nm with good agreement with XRD data.

Reduction of Ag^{+} ions to Ag (in 1 mM solution of $AgNO_3$) was traced by *R. officinalis* leaf extract absorption spectra as function of time reveal. (Figure 4a) shows the clear solution of *R. officinalis* leaf extract and Figure 4b shows the yellowish brown coloured Ag-NP's colloidal solution. Maximum absorbance was observed at 450 nm which is related to formation of Ag-NPs (Figure 3d) reveal that Plasmon resonance (SPR) of conducting electrons of Ag-NPs. This unique peak is due to surface R. officinalis. The nucleation process is acceleration slowly and reaction took about 4.5 h to complete, while no significant Ag-NPs formation was seen at 30 min. The rate of formation increases rapidly toward t=270 min and confirmed by adsorption platue at further time. The Ag-NPs synthesis was completed in 4.5 h and was very stable during further experiments. The XRD analysis was used to study the crystalline nature of green synthesized Ag-NPs.

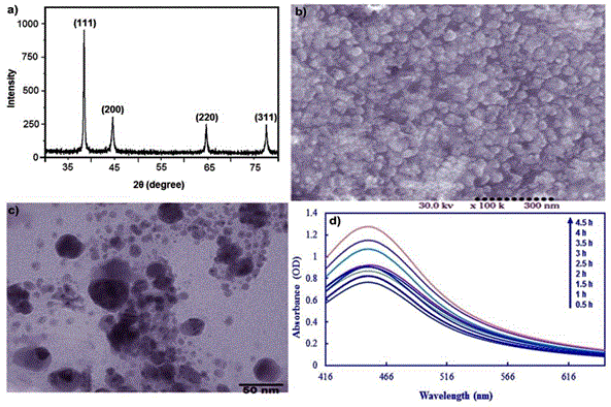

Figure 3: a) XRD pattern of green-synthesized silver nanoparticles by using leaf of *R.officinalis*, b) SEM image of Ag nanoparticles formed by *R.officinalis*, c) TEM image and d) Ultraviolet-visible spectra for formation of Ag-NP are as a function of time.

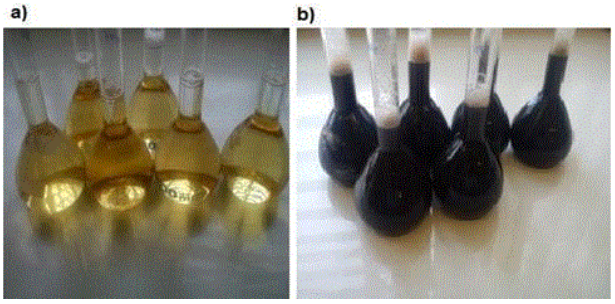

Figure 4: (a) Clear solution of *R.officinalis* leaf extract, (b) Ag-NP's after reaction of $AgNO_3$ with *R.officinalis* leaf extract.

Biological activity

Antimicrobial assays (*in vitro*): The *in vitro* antibacterial activity of Ag-NPs/extracts and *T. daenensis* and *S. marianum* extracts were evaluated based on the growth inhibition zone [mm; Figures 5-13; Tables 1-3]. Maximum zone of inhibition in concentrations (12.5, 25 and 50 mg/disc) was found to be (14.40, 17.4, 19) mm in Staphylococcus aureus (Table 1). The images of zone of inhibition in discs are shown in Figure 14. The solvent used for the preparation of compound solutions (DMSO) did not show inhibition against the tested organisms [as a negative control]. Extracts of *T. daenensis* and *S. marianum* exhibited significant activity against *S. aureus* and moderate activity against *E. coli* against. Finally, inhibitory effects of Ag-NPs/ extracts confirm that the best effects and significant activity against *S. aureus* and *E. coli* even at low concentration [12.5 and 25 mg/mL]. Increasing the concentration of Ag-NPs /extracts solution to 50 mg/mL is associated with significant activity against *S. aureus* and *E. coli*, Silybum-Ag showed strong activity against *T. daenensis*-Ag at 50 mg/mL [Figures 6 and 3]. The Ag-NPs/ *T. daenensis* had the best effects and showed significant activity against pathogen bacteria even at low concentration [12.5-25 mg/mL] [Figures 5,6,8-11]. Compounds *T. daenensis*, *S. marianum* and Ag-NPs/extracts was active against *C. albicans* at 12.50, 25, 50 mg/mL concentration that have better antifungal activity against than *A.oryzae*, *T.daenensis* and Ag-NPs/ Silybum, better activity against *Silybum marianum* extract but was weak against Ag-NPs/ *Thymus* and *T.daenensis* extract [Figures 5-14].

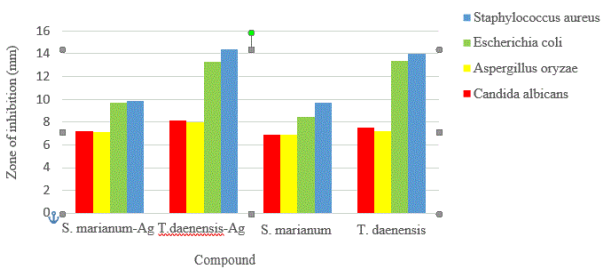

Figure 5: Antimicrobial activity of extracts in presence and without of Ag-NPs [12.5 mg/mL].

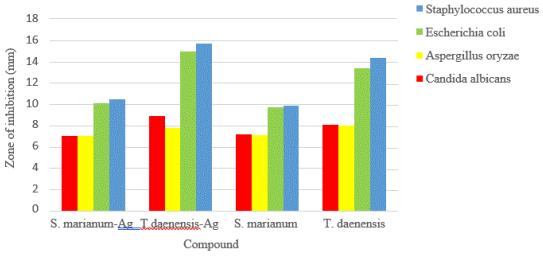

Figure 6: Antimicrobial activity of extracts in presence and without of Ag-NPs. [25 mg/mL].

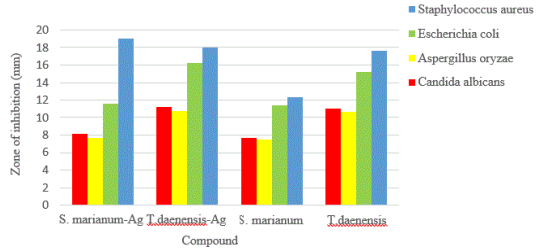

Figure 7: Antimicrobial activity of extracts in presence and without of Ag-NPs [50 mg/mL].

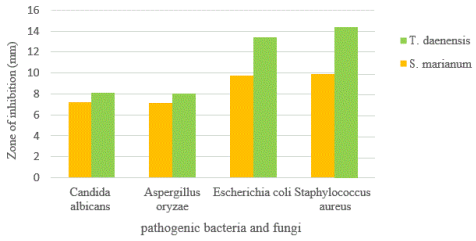

Figure 8: Antimicrobial activity of extracts in presence of natural silver nanoparticles [12.5 mg/mL].

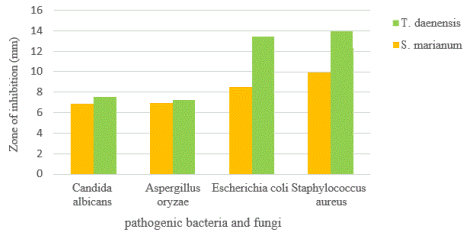

Figure 9: Antimicrobial activity of extracts [12.5 mg/mL].

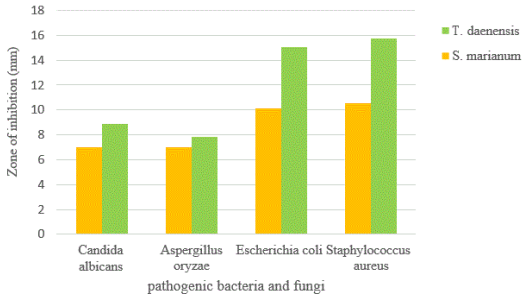

Figure 10: Antimicrobial activity of extracts [25 mg/mL].

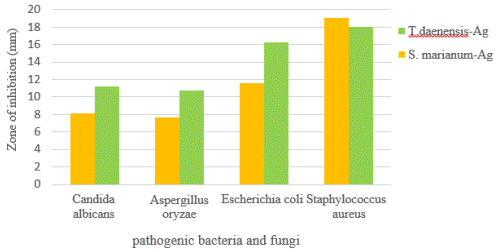

Figure 11: Antimicrobial activity of extracts in presence of natural silver nanoparticles [25 mg/mL].

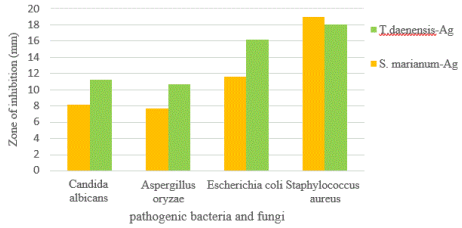

Figure 12: Antimicrobial activity of extracts [50 mg/mL].

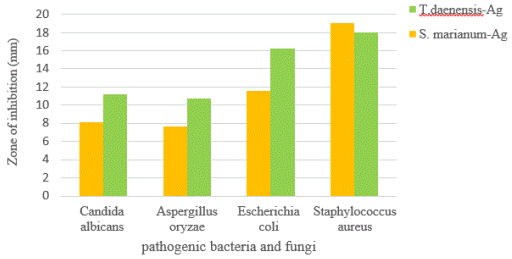

Figure 13: Antimicrobial activity of extracts in presence of natural silver nanoparticles [50 mg/mL].

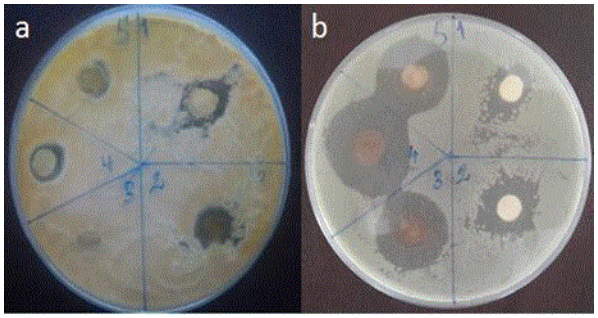

Figure 14: The inhibition halos for antifungal and (a) antibacterial activity (b) (50 mg L^{-1}).

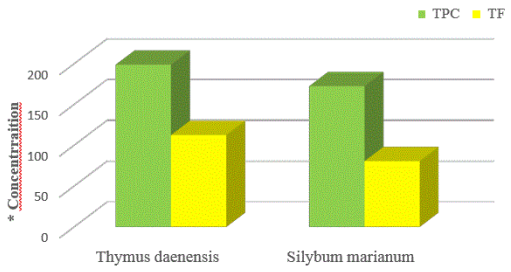

Figure 15: *TPC: mg gallic acid equivalent/g of dried extract and TF: mg rutin equivalents (RuE)/g of dried extract.

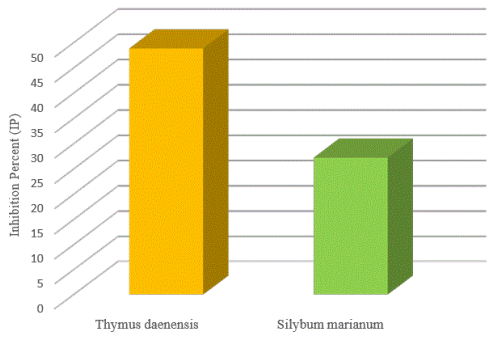

Figure 16: Comparison of inhibition percent.

Total flavonoids and phenolic content: Flavonoids are polyphenolic compounds which play an important role in stabilizing lipid oxidation and are also associated with antioxidative action [61]. Flavonoids found ubiquitously in plants and are the most common group of phytophenolics. Flavonoid content of the extracts in terms of (mg/g) rutin equivalents was recorded.

Phenols are the simplest bioactive phytochemicals having free radical scavenging ability due to the presence of hydroxyl groups. The sites and the numbers of hydroxyl groups are related to their relative toxicity for microorganisms, recently is shown that increasing in hydroxylation of these compounds cause to increasing in their toxicity properties [62]. The phenolic contents of hydroalcoholic extracts of

plants were tested using the diluted Folin-Ciocalteu reagent (FCR). Phenolic compounds react with FCR only under basic conditions (adjusted by a sodium carbonate solution to pH=10). Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR. The reaction occurs through electron transfer mechanism. The blue compounds formed between phenolate and FCR are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds. It is believed that FCR contains hetero polyphosphotunstates-molybdates [63]. The highest contents of total flavonoid and phenolics were observed for Shoot *L. usitatissimum* results clearly. (Table 4 and Figure 15).

Extracts	TPC[a]	TF[b]	(DPPH) inhibition %
T. daenensis	198.71 ± 1.50	172.42 ± 1.43	48.80 ± 0.59
S. marianum	112.44 ± 1.38	80.50 ± 1.41	27.20 ± 0.32
[a]TPC: total phenolic content, mg gallic acid equivalent/g of dried extract			
[b]TF: total flavonoid content, mg rutin equivalents (RuE)/g of dried extract			

Table 4: Total phenolic, flavonoid content and antioxidant activity of hydroalcoholy extracts.

Antioxidant capacity

Recently, using of antioxidants is proposed to protect people from oxidative stress damages. This study indicated that higher concentration of phenolic compounds in hydroalcoholic extracts improved antioxidant activity. Then these plants can be a use as a source of natural antioxidants to remove harmful effects of free radicals. The in-vitro antioxidant activity of test extracts were estimated by using of DPPH assay. DPPH radical-scavenging activity test measures the capacity of the extracts to scavenge the stable radical 2,2-diphenyl-1-picrylhydrazyl. If the extracts have this capacity, the initial blue/purple solution will change to a yellow color due to the formation of diphenyl picrylhydrazine. The antioxidants reacted with DPPH, a purple coloured stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The amount of DPPH reduced was estimated by measuring the decrease in absorbance at 517 nm. The highest DPPH radical scavenging was obtained by aqueous/ethanolic extract of *T. daenensis* (Bois leaves 48.80 ± 0.59%) Figure 15 results was showed in Table 4. The different types of functional groups of extracts were identified from Figure 1 and Table 5.

S. No	Average Peaks value	Stretching	Interpretation
1	423.37	C-H bending outside of page	Alkanes
2	592.72	C-O Stretching	Alcohols
3	1067.22	O-H bending outside of page	Alcohols
4	1404.07	C-H Bending	Alkanes
5	1625.78	C=C Stretching	Alkenes
6	2932.12	C-H Stretching	Alkanes
7	3398.62	O-H Stretching	Alcohols

Table 5: Infrared spectrum analysis of extracts.

Conclusion

The antimicrobial activities of *T. daenensis* and *S. marianum* extracts and Ag-NPs/extracts assessed against pathogenic bacteria and fungi. Information of Tables of both samples was showed that biological Ag nanoparticle (Ag-NPs/*T. daenensis* and Ag-NPs/*S. marianum*) has more antimicrobial effects to extracts (*T. daenensis* and *S. marianum*). Therefore, by completion of these experiments and the use of metal nanoparticles with plant extract in sensitive environments such as hospital, etc., suggested. Among the most promising nanomaterials with antibacterial properties are metallic nanoparticles, which exhibit increasing chemical activity due to their large surface to volume ratios and crystallographic surface structure. using of medicinal plant extracts with metal nanoparticles can be effective to eliminate the bacterial infections, as an alternative to antibiotics. *T. daenensis* and *S. marianum* are an abundant source of phenol and flavonoids, which have antioxidant properties and significantly reduce the effects of free radicals.

Acknowledgment

This work was supported by a grant from Research Council of Yasouj University.

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