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RESEARCH PAPER

Isolation, Antimicrobial activities, Antioxidant Scavenging activities and Trace Element Investigation of Isolated Actinomycetes spp. in Kalar, Iraqi Kurdistan Region

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A B S T R A C T:

The poor prognosis of the most microbial pathogenic diseases, due to acquiring resistance against the most convenient antimicrobial medication, encourages researchers to find out a new approach to obtain a novel medication. The current study was aimed to obtain new actinomycetes strains or their novel bioactive metabolites. Eleven isolates were found from 45 distinct non-farming soils in Kalar, Iraqi Kurdistan Region. The non-farming soil 2 (NFS2) isolate was demonstrated as the most effective isolate among all other isolates when tested for primary antimicrobial screening. In addition, in case of crude extract, the NFS2 confers great antimicrobial activities particularly against Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Micrococcus luteus* ATTC 9341, as well as, against *Candida albicans* ATCC 10231, with inhibition zones diameters, 25.5, 33.5 and 30.5 mm respectively. However, the isolate has no any antibacterial responses against Gram-negative bacteria, using all types of extracts including crude extract and organic solvent extracts. The crude extract exhibited the powerful antioxidant and H_2O_2 scavenging activities recording 93.46% with 30mg/ml and 43.94% with 1.25 mg/ml respectively. Investigating the trace elements concentration in broth cultures yeast malt extract broth (ISP2) by inductively coupled plasma (ICP), for confirming the value of these elements particularly (Ca, Fe, K, Na, P, Sr, Mg, and Zn). Primarily, these elements were either exhausted or produced during the fermentation process. Furthermore, manipulating the trace elements concentration will play a crucial role in enhancing or diminishing the final secondary metabolites products in actinomycets isolates.

KEY WORDS: Isolation, Actinomycetes, Antimicrobial, Antioxidant, Trace elements
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1.INTRODUCTION:

The screening for obtaining new bioactive metabolites from microbial sources including novel antimicrobial compounds, potentially used in industrial, agricultural and pharmaceutical applications, has become an important field for investigating the impact of the metabolites against currently isolated multi-drug resistance microbial pathogens. The researchers have been deeply focusing in their searching process to achieve novel potent, sustainable, as well as broad-spectrum antimicrobial compounds from different sources especially microbial population and more closely actinomycetes (Berdy, 2005; Praveen *et al.*, 2008; Singh and Tripathi, 2011; Singh *et al.*, 2016).

Actinomycetes are Gram-positive bacteria, spore-forming, free-living and having a filamentous structure with DNA that have high GC content. Also the bacteria spread over all ecological habitat including water, soil and different niche, therefore; the investigators attempt in their searching for obtaining new strains or novel bioactive compounds through playing an extensive role in the pharmaceutical uses as well as in medical application and industry (Janardhan *et al.*, 2014). According to the published and reviewed articles, there are more than 10000 bioactive secondary metabolites which are produced by the group of bacteria called actinomycetes that represent about 45% of all discovered bioactive microbial secondary

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metabolites. Most of these discovered bioactive compounds, isolated and characterized by researchers, have currently been well studied and developed for making novel medications in treating a wide range of common disease in human, animal and agricultural fields (Subathra *et al.,* 2013). In fact, novel metabolites provide antibacterial, anticancer, antifungal, antiviral, anti-infective and anti-parasitic properties, as well as immunosuppressive and enzyme inhibitory (Kekuda *et al.,* 2010). In Iraq, previously, several researches have been attempted to isolate actinomycets and recording some bioactive metabolites (Abbas, 2009, Jaralla, 2014; Burghal, 2015and AL-Mawlah, 2018). Although in the Kalar district Iraqi Kurdistan Region, there were few studies have been documented for exploring biological activities of the metabolites (Risan, *et al.,* 2016). Therefore, the aim of the current study was to screen different soils in the Kalar soil for isolating novel actinomycetes strain for investigating their antimicrobial, antioxidant prosperities as well as evaluating the amount of trace elements concentration consumed or produced during the entire fermentation process.

2.Material and Methods

2.1 Soil Sample collecting and treatment

Forty-five soil samples were collected in Kalar district which located between latitudes 34° 37′ 45″ N and longitudes 45° 19′ 20″ E. Elevation 231 m (758 ft) above the sea level, Iraqi Kurdistan Region (Figure 1). Fifteen gm of soils were collected, each soil sample was collected from 10 to 15 cm depth, and the sample directed placed in polyether bags 15*25 cm. The collected soil samples well crushed, sieved and supplemented with calcium carbonate at a ratio 10:1 (soil: calcium carbonate) in order to reduce the growth of fungi and mold, this was done inside the autoclave for 3 days at 40 °C, the soil sample ready to use for isolation purpose. One gram of dried and pretreatment soil sample was suspended in 99ml of sterilized distal water and making serial dilution up to 10^{-3}. 100µl of each dilution was taken and spread over the surface of yeast malt extract ager (ISP2) medium in triplicate, supplemented with streptomycin and nystatin (50µg/ml from each one). The inoculated plate incubated at 28°C for seven days, after the incubation period. The whitish pin-point colonies, with a clear zone of inhibition around them, indicate the most outstanding characteristic of actinomycetes bacterial population. Via a sterilized needle the colonies with the previous characters transferred to a new plate, which then purified and stored at 4°C for further study (Saadoun *et al.,* 1999; Rahman *et al.,* 2011; Waksman, 1961; Hopwood *et al.,* 1985)

2.2 Primary Screening for Antimicrobial Activity

The purified and isolated colonies of actinomycetes were used to determining the primary antimicrobial screening assay. The protocol was carried out via cross streak method on yeast malt extract agar medium (ISP2) against human microbial pathogens (obtained from media medical center in Irbil, Iraqi Kurdistan Region). The human microbial pathogens were used for conducting the antimicrobial activities include *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATTC27853, *Micrococcus luteus* ATTC 9341 and *Candida albicans* ATCC 10231. The isolated strains were grown on yeast malt extract agar (ISP2) plate by streak it as a parallel line across the entire plate. After 3 days of incubation at 28°C were all of them fully cultured, the tested microbial pathogens streaked at 90° to the originally grown isolate, and then incubated for 24hours at 37°C (Salim *et al.,* 2017).

2.3 Secondary Antimicrobial Screening and Fermentation Condition

The pure isolated actinomycetes used to make bacterial suspension by transferring a loop full of the grown isolate on ISP2 agar to 50ml of yeast malt extract broth and incubating it at 28°C for 4days making stock solution. 1.5ml of the stock solution used to inoculated 150 ml of yeast malt extract broth (ISP2) and incubating it in shaking incubator at 28°C, 150 rpm for seven days. The culture broth was centrifuged at 6000rpm for 10 minutes. The supernatant was filtered through Whatman filter paper No.1, collected and treated as a crude extract. This used for investigating antimicrobial activity against microbial pathogens were grown on Muller Hinton agar plates (20 ml agar medium per plate). The agar wells diffusion techniques was used conduct secondary antimicrobial activates. Sterilized pasture pipette were used for making the wells (5mm); the wells were filled with 75±5 µl of the filtered crude extract supernatant and incubated at 37°C for 24 hours. The diameter of the growth zones of

inhibition was measured in (mm) (Gebreyohannes *et al.,* 2013).

2.4 Extracellular Antimicrobial Metabolites Extraction

In order to extract extracellular antimicrobial metabolites different organic solvents (ethyl acetate, n-butanol and, n- hexane) were used. Equal amounts (1:1 volume) of crude extract supernatant with above solvents were taken, shaking for one hour and were spun at 12000rpm for 12 minutes. After centrifugation, the upper phase was containing the dissolved extracellular antimicrobial metabolites collected in sterilized plates; the plates were kept in the autoclave at 45°C for evaporating the organic solvents. After solvents were completely dried out the sediments (remaining) re-suspended with 500 ul of sterilized distal water; the obtained mixture treated as solvent extracellular extract against tested microbial pathogens, the diameter of the growth zone of inhibition were measured in (mm) (Gurung *et al.,* 2009).

2.5 Comparing Antibacterial Activities between Crude Extract and Synthetic Antibiotic Discs

Different synthetic antibiotic discs were used to compare the antibacterial activities with the crude extract of NFS2 against human microbial pathogens *S aureus* ATCC 25923, *E coli* ATCC 25922, *P aeruginosa* ATTC ATTC27853, *M luteus* ATTC 9341. The microbial pathogens were grown on Muller Hinton agar. Five different types of the antibiotic susceptibility discs from Bioanalyse were used include (Tobramycin 10 µg; ToB 10, Gentamicin 10 µg; CN 10, Cefriaxono 10 µg; CRO 10, Amoxicillin/Clavulanic acid 30 µg; AMC 30 and Ciprofloxacin 10 µg; Cip 10). Inhibition zones were measured after 24 hours after incubation on 37°C.

2.6 Evaluating Antioxidant Properties of Crude Extract

The standard solution was prepared by dissolving 50 mg of ascorbic acid in 50ml methanol. Different concentrations of ascorbic acid (1, 0.5, 0.25, 0.125.0.64 0.32 mg/ml) were prepared, then mixed with Diphenyl Picryl Hydrazil solution (DPPH) (0.002mg/100ml), and then kept for 30 minutes at room temperature in dark. Absorbance was read by spectrophotometer at 517nm after 30minutes of reaction, to obtain a standard curve for calculating DPPH radical scavenging activity.

1,1-diphenyl-2-picryl hydrazyl free radical scavenging assay was performed to evaluate radical scavenging activities of the NFS2 crude extract. Exactly 0.002gm of DPPH dissolved in 100ml methanol and kept in darkness. The crude extract concentration mixed with methanol to obtain final dilution concentration as (0.1, 0.5, 1.0, 3.0, 5.0, 10.0, 20.0 and 30.0 mg/ml). A mixture of 2.5ml of DPPH solution and 0.5ml of the crude extract of NFS2 were mixed, and then kept at room temperature in a dark place for 30 minutes. The absorbance was read at 517 nm by using visible spectrophotometer after 30min of reaction. The ability to scavenge the DPPH radicals (antioxidant activity) was calculated as follow:

DPPH radical scavenging activity% = (A_0–A1) A_0*100; Where A_0 is the absorbance of the control after 30 minutes and A1 is the absorbance of the sample after 30 minutes. All concentration was analyzed in duplicates (Khalaf *et al.,* 2008).

2.7 Hydrogen Peroxide Radical Scavenging Activity

According to (Patel *et al.,* 2016) and (Dholakiya *et al.,* 2017) hydrogen peroxide solution (40mmol/L) was prepared in phosphate buffer (pH 7.4). Different concentrations of NFS2 crude extract after drying it was prepared in the same phosphate buffer ranging from (5 to 0.08 mg/ml). 4ml of the NFS2 extract was mixed with 0.6 ml of H_2O_2 solution, and incubated at room temperature for 10 minutes. The optical density (OD) after 10 minutes was calculated at 230nm by the UV-visible spectrometer. All concentration was analyzed in duplicates. The percentage of H_2O_2 scavenging activity was determined as follow;

H_2O_2 Scavenging activity (%) = [OD320 of control−OD320 of Extract/OD320 of control] ×100

2.8 Trace Elements Contain Analyzing by Inductively Coupled Plasma (ICP)

According to (Hsiung *et al*., 1997) protocol with little modification; several elements in the yeast malt extract broth (ISP2) contained were detected using Inductively Coupled Plasma (ICP). The assay was conducted by comparing 18 trace elements concentrations (mg/l) include (Ba, Ca, Co, Cu, Fe, Hg, K, Li, Mn, Mo, Na, Ni, P, Se, Sr, Ti, Zn and Mg). The comparing carried out between only broth (negative control) at the zero points without the NFS2 isolate and the supernatant of crude extract inoculated with NFS2

isolate after 7 days of incubation in shaking incubator. Taking 1ml of both broth and diluted with 4 ml of the sterilized distal water to make final dilution 5 times. 18 trace elements included in the analysis through using ICP (Spectro Arcos, Germany). The instrument conditions were used: Spray chamber is Scott spray; Nebulizer: cross-flow; RF power/W: 1400; pump speed: 30 RPM; Coolant flow (L/min): 14; Auxiliary flow (L/min): 0.9; nebulizer gas flow (L/min): 0.8; Preflush (s): 40; Measure time (s): 28; replicate measurement: 3; multi-elements stock solutions containing 1000 mg/L were obtained from Bernd Kraft (Bernd Kraft GmbH, Duisburg, Germany); standard solutions were diluted by several dilution into 0.1, 0.5, 2 ppm in 0.5% nitric acid.

3.Results and Discussions

Eleven different isolates were obtained from non-farming soil (NFS), (NFS 1,2,3,4,5,6, 7,14,19,34 and 36) out of 45 collected samples in Kalar district, by plate diluting techniques on yeast malt extract agar ISP2 depending on the most general characters of actinomycetes isolation guidelines. The result was in agreement with that described by (Portillo *et al.,* 2009) that the colony number may represent from two to four per each plate and the number will be reduced when similar habitat or soils are present. At the first step, all obtained colonies were screened using Gram stain method (Figure2 A), and the results showed that all isolates are Gram positive filamentous bacteria. The primary screening assay via cross streak method was carried out for determining the most powerful antimicrobial isolate as shown in (Figure 2 B and C). Among all eleven isolated actinomycetes, the isolate NFS2 was presented on ISP2 medium before adding the microbial pathogens after 3 days (Figure 2 B) and seven days (Figure 2D) of incubations. Following incubation of the actinomycets microbial pathogens, *E coli* ATCC 25922 (E), *S aureus* ATCC 25923 (S), *Micrococcus luteus* ATTC 9341 (M), *C albicans* ATCC 10231 (C) and *P aeruginosa* ATTC27853 (P) were added, the results showed that the NFS2 isolate presented powerful antimicrobial activities against the most used microbial pathogens especially *S aureus, M luteus* and *C albicans* (Figure 2 C).

The results for secondary metabolites activities against all microbial pathogens as crude extract and extracellular antimicrobial metabolites such (n-hexan, n- butanol and ethyl acetate) are presented in (Table 1). The crude extract exhibits powerful antimicrobial activity against both Gram-positive bacteria (*S aureus* and *M luteus*) and *C albicans*. The inhibition zones were 25.5 and 33.5 mm for *S aureus* ATCC 25923 and *M luteus* ATCC 9341 respectively, while 30.5 mm was recorded for *Candida albicans* ATCC 10231. On the other side, the crude extract does not exhibit any inhibition zone against the Gram-negative bacteria used including *E coli* ATCC 25922 and *P aeruginosa* ATCC 27853. These results indicate that the crude extract from the bacterial fermentation only has an effective antimicrobial against both Gram-positive bacteria and *C albicans*. The extracellular secondary metabolites extracted from all organic solvents also showed antimicrobial activities (Table 1). For instance, the result for n-hexane extract was 28 mm zones of inhibition against *S aureus* ATCC 25923. In the case of *M luteus* ATCC 9341, both n-hexane and n-butanol have the same antimicrobial activities which are 21 mm zones of inhibitions. In addition, the difference in the antibacterial powerful activity by using different organic solvent extracts may be due to their difference in the solubility of bioactive compounds in various solvents and variation in the cell wall structure of tested bacteria (Aali *et al.,* 2018). Finally the result for *C albicans* ATCC 10231 for n-hexane extract was 29 mm zones of inhibition. The results for Gram-positive bacteria were in agreement with that obtained and described by (Kim *et al.,* 1994; Singh *et al.,* 2016), as they demonstrate that the Gram negative bacterial cell wall structure is mainly composed of lipopolysaccharide (LPS). However, the Gram-positive bacteria lack this structure, and this causes the bacteria to become more susceptible to our extracted metabolites.

Comparing antibacterial activities between NFS2 crude extract and synthetic antibiotic discs were presented in (Figure 3). Panel (A) represents the activity of NFS2 crude extract and synthetic antibiotic discs against *S aureus* ATCC 25923. The results indicated that the NFS2 crude extract records higher inhibition zone overall used synthetic except Gentamicin 10 µg (CN 10), while in case of *M luteus* ATCC 9341, Panel (B), NFS2 recorded the best inhibition zone overall used synthetic zone except Ciprofloxacin 10 µg (Cip

10). In contrast, in the case of both Gram-negative bacteria *E coli* ATCC 25922, *P aeruginosa* ATCC 27853 as presented in Panel (C and D) respectively, there were no any results recorded, which point out the NFS2 extract only effects on Gram-positive bacteria.

The antioxidant scavenge activity of NFS2 isolate crude extract was summarized in (Figure 4). The result was gradually increased depending on the crude extract concentration for scavenging free radical presented in the solution till recording the maximum activity with 30mg/ml which record 93.46% scavenging activity with the standard deviation (SD±8.43). The presented result was in agreement with that obtained and recorded by (Mbwambo *et al.*, 2007; Mohammad-Amin *et al.*, 2016; Maikhan *et al.*, 2018). On the other hands, the result for H_2O_2 scavenging activities presented in (Figure 5), indicate that the crude extract of NFS2 isolate has the potent for scavenging the great amount of H_2O_2 in the solution, the highest scavenging activity result was recorded 43.94% scavenging activity which obtained by 1.25 mg/ml of crude extract with standard deviation (SD±1.8). Hydrogen peroxide easily penetrates across the cell membrane, producing the ROS (reactive oxygen species) after reacting with (Fe^{2+} and Cu^{2+}) which having more toxic affects such hydroxyl free radical (Sawant *et al.*, 2009). The results for both antioxidant activity and H_2O_2 scavenging activity indicate the presence of active compounds in the crude extract with the competence of reducing Fe^{+3} ions as described by (Kekuda *et al.*, 2010). As well as, perform the preventing the progress of various oxidative stress-related disorders (Poongodi *et al.*, 2012).

The results for comparing trace elements concentration between the non-inoculated ISP2 broth as a negative control, with inoculated ISP2 broth by NFS2 after 7 days of fermentation was presented in (Table 2), the results indicate that were trace elements in the culture broth highly changed, some of them decreased while a little of them increased, this fluctuation of the trace elements concentration (especially decreased elements) due to their consumed during the fermentation process by the bacteria, and other increased trace elements concentration due to their production by the bacterial cell in response to the fermentation process. Some of the tested trace elements play a vital role in the bacterial life cycle such (Ca, Fe, K, Na, P, Sr, Mg, and Zn) for enhancing or inhibiting the bacterial secondary metabolites production as in the study of (Chesters and Rolinson, 1951), they investigate that some trace elements are essential for growth such zinc and copper, as well as, other required for production of antibiotics as for grown alone such iron, therefore, manipulating these trace elements in the broth culture during fermentation process will give the new results differ widely from steadily running standard or internationally pre-described medium.

Table1: Secondary antimicrobial activities of crude extract alone and extracellular metabolites activities against microbial pathogens (mm zones of inhibition)

Microbial pathogens	**Inhibition zone (mm)**			
	Crude extract	**n-hexan extract**	**n- butanol extract**	**Ethyl acetate extract**
Escherichia coli (ATCC 25922)	**0**	**0**	**0**	**0**
Staphylococcus aureus (ATCC 25923)	**25.5**	**28**	**25**	**18**
Pseudomonas aeruginosa (ATTC27853)	**0**	**0**	**0**	**0**
Micrococcus luteus (ATTC 9341)	**33.5**	**21**	**21**	**14**
Candida albicans (ATCC 10231)	**30.5**	**29**	**25**	**20**

Table 2: trace elements concentration comparison (mg/l) of ISP2 broth media, between negative control starting point and inoculating broth with NFS2 after 7 days incubation

Trace elements	Negative control starting point mg/l	ISP2 after fermentation mg/l	Notes
Ba	1.17	1.015	-
Ca	24.785	20.49	-
Co	0.01	0.005	-
Cu	ND	ND	
Fe	0.045	0.02	-
Hg	0.145	0.135	-
K	121.85	95.09	-
Li	0.095	0.075	-
Mn	0.005	0	-
Mo	ND	ND	
Na	184.695	127.85	-
Ni	0.025	0.03	+
P	14.125	6.285	-
Se	0.165	0.25	+
Sr	3.95	3.555	-
Ti	0.07	0.055	-
Zn	0.12	0.085	-
Mg	9.945	7.96	-

ND: note detected; -: Decreased; +: increased

Figure 1: Kalar district location and their border on Iraqi map, 45 soil samples were collected from non-farming soil. The picture was get from the Google map

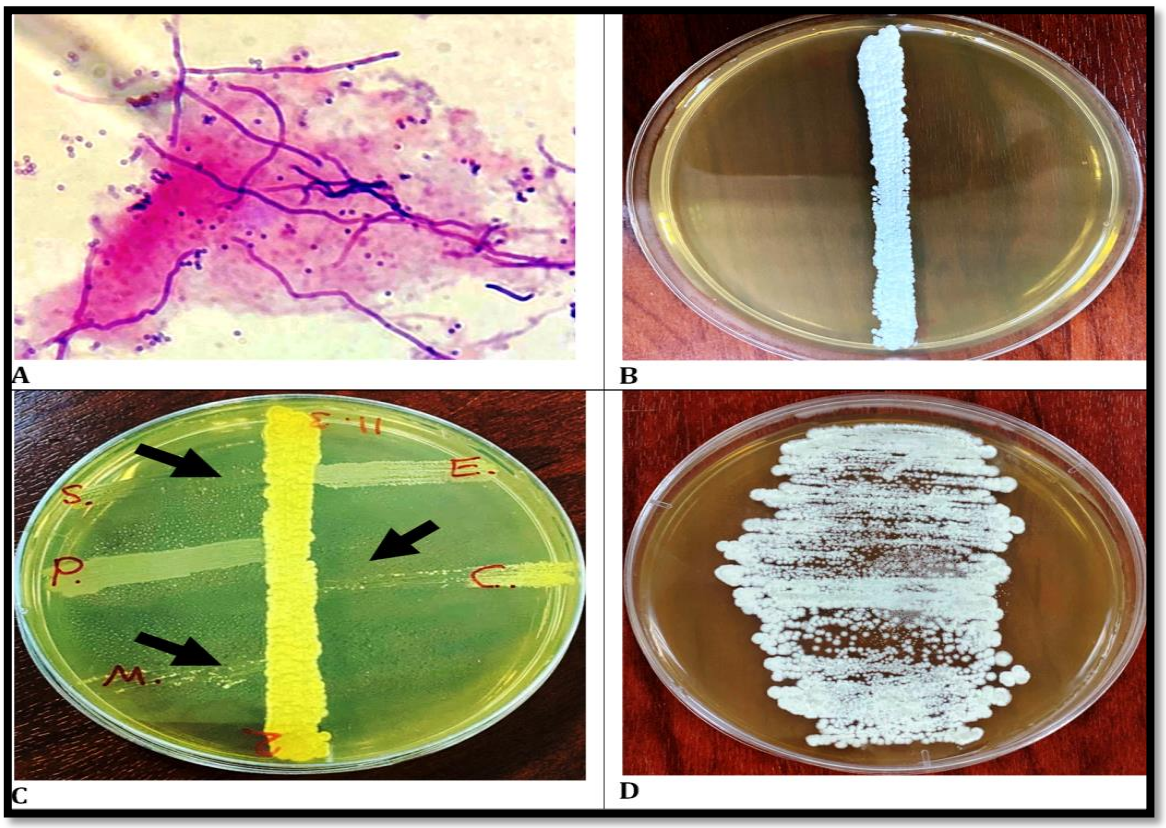

Figure 2: **A** Gram stain for all isolates (positive results; NFS2); **B** primary screening assay, cross streak assay after 3 days of incubations without the microbial pathogens; **C**, Primary screening assay results for NFS2 on all microbial pathogens; **D**, pure culture colonies of NFS2 on ISP2 after 7 days of incubation.

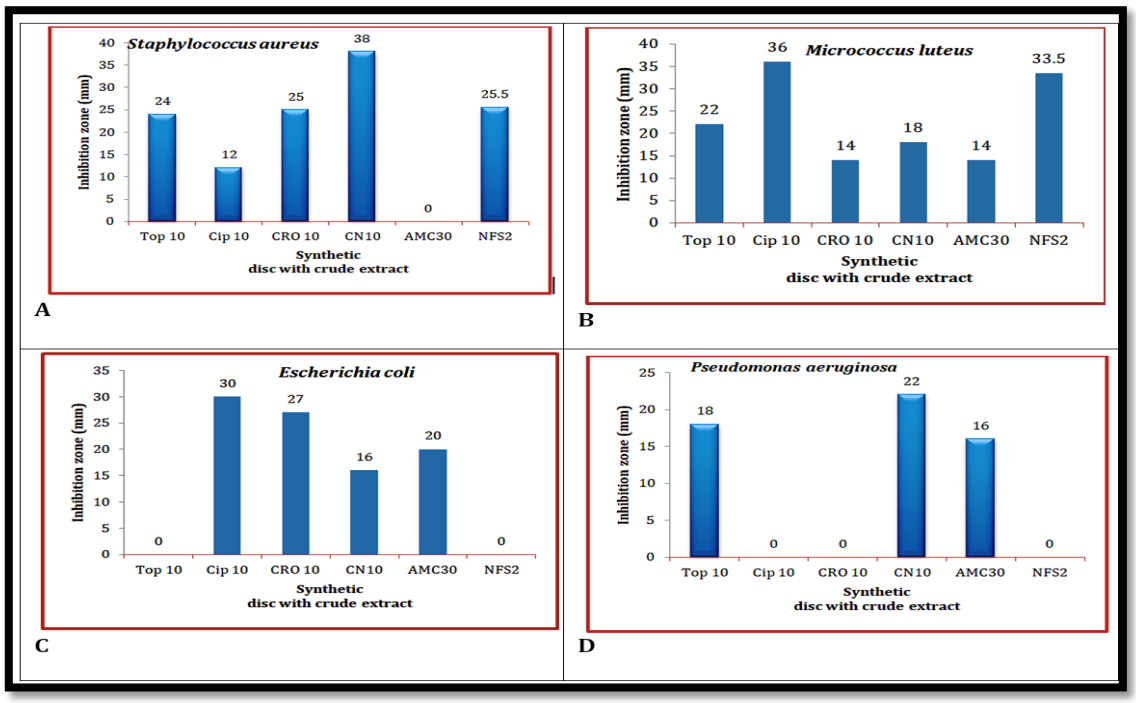

Figure 3: Comparing antibacterial activity of different synthetic antibiotic discs with NFS2 crude extract on microbial pathogen; **A:** *Staphylococcus aureus*, **B:** *Micrococcus luteus*, **C:** *Escherichia coli*, **D:** *Pseudomonas aeruginosa*. n=1

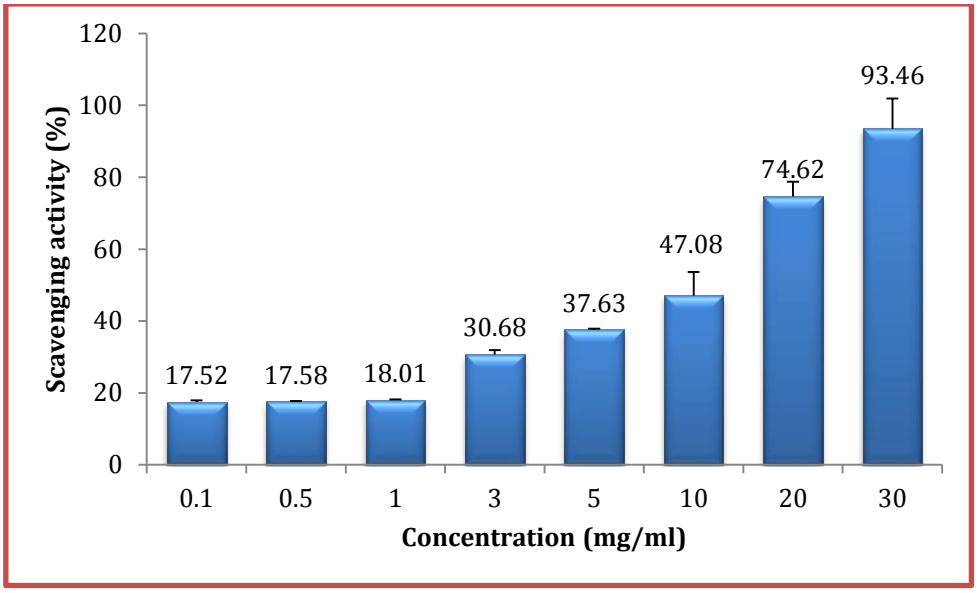

Figure 4: Antioxidant scavenging activities of NFS2 crude extract by DPPH radical scavenging activity assay, mean of the antioxidant scavenging activity with standard deviation, n=2

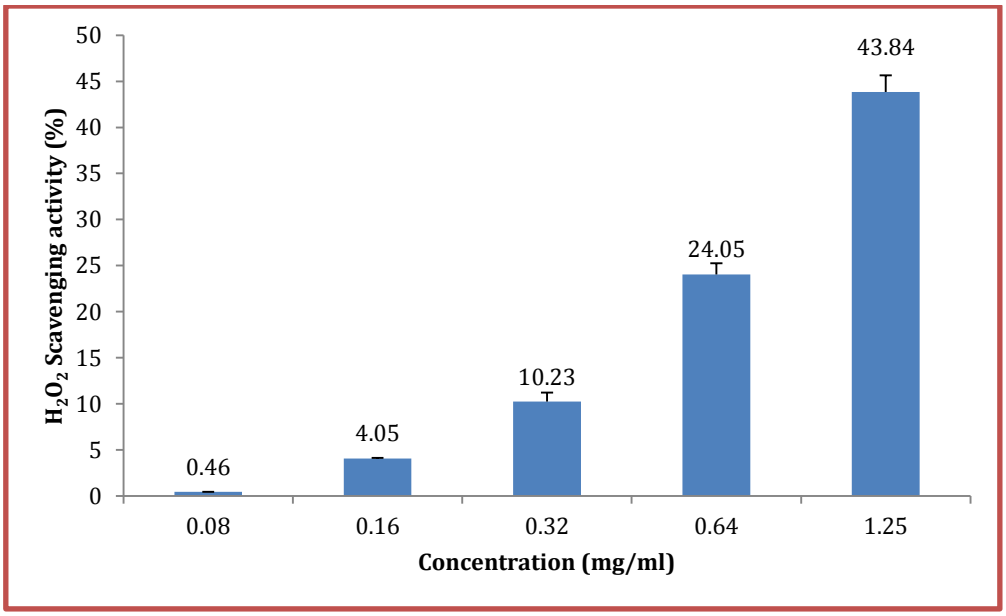

Figure 5: H_2O_2 scavenged activity of NFS2 crude extract with different concentration, mean of the scavenging activity with standard deviation, n=2

4.Conclusion

Eleven isolates of actinomycetes were obtained from forty five soil samples in Kalar district. NFS2 isolate had the most potent antimicrobial metabolites against Gram positive and Candida. Furthermore, this isolate showed antioxidant activity through scavenging the free radicals. Different trace elements concentrations in ISP2 were detected throughout the fermentation process by ICP. This suggests that the NFS2 isolate may use some of these trace elements in their growth process.

Recommendation

The screening protocol for isolating novel isolates from different habitats needs more soil types with different depth. Manipulating the trace element concentrations in the ISP2 broth media will exhibit different results especially relating with antagonistic or antioxidant activities. Future study should focus on separating the bioactive compounds which can be used for discovering new antibiotics.

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