Antibacterial and antibiofilm properties of traditional medicinal plant from Sheikh Buddin range

Amanullah[1], Ghulam Mustafa[1], Muhammad Hanif[2], Muhammad Mohibullah[3], Sattar Bakhsh[4], Sheikh Abdur Rashid[4], Ali Zaman[5], Fazal Rehman[1], Barkat Ali Khan[4]* and Adnan Amin[6]*

[1]Department of Pharmaceutical chemistry, Faculty of Pharmacy, Gomal University D.I. Khan, KPK, Pakistan
[2]Gomal centre of Biochemistry and Biotechnology (GCBB), Gomal University D.I. Khan, KPK, Pakistan
[3]Faculty of Agriculture, Gomal University, KPK, Pakistan
[4]Department of Pharmaceutics, Faculty of Pharmacy, Gomal University D.I. Khan, KPK, Pakistan
[5]Institute of Microbiology, Gomal University, D.I. Khan, KPK, Pakistan
[6]NPRL, Department of Pharmacognosy, Faculty of Pharmacy, Gomal University D.I. Khan, KPK, Pakistan

Abstract: During current project, antibacterial and antibiofilm properties of traditional medicinal plant *Ziziphus nummularia* leaf extract and various fractions was investigated. The plant leaves were dried and extracted using 90% methanol followed by sequential fractionation using liquid-liquid fractionation. The fractions of a diverse polarity including chloroform, *n*-hexane, methanol and ethyl acetate and aqueous extracts were obtained that was further analysed by using HPLC. The phytochemical screening indicated presence of saponins, triterpenes and flavonoids. During DPPH assay, the methanolic fraction presented highest activity (IC_{50} 193.1µg/mL), followed by ethyl acetate (IC_{50} 220µg/mL) and chloroform (IC_{50} 263µg/mL) fractions respectively. During FRAP assay, FRAP value for *Z. nummularia* extract 20.43µM. Among fractions, ethyl acetate fraction presented highest FRAP value (370.2µM), followed by chloroform (204µM) and methanolic (249µM) fractions. The antimicrobial activity of chloroform fraction was significantly high against *P. aureginosa* (6mm), *L. monocytogenes, S. aureus* (5mm), *K. pneumoniae, B. Subtillus* and *E. coli* (4mm). The ethyl acetate part presented significant activity (MIC 4mg/mL) against *S. aureus, B. Subtillus and L. monocytogenes.* The total extract and fractions were further tested for MBC and the MBC for ethyl acetate fractions was 4mg/mL, whereas all other fractions exhibited MBC >10mg/mL. No activity was recorded against *Aspergillus niger*. During antibiofilm assay, *n*-hexane fraction presented highest inhibition (88%) followed by ethyl acetate (69%) chloroform (65%) fractions. It was concluded that *Z. nummularia* possess moderate antimicrobial and antibiofilm activities. Further a synergistic effect is suggested in formulation having *Z. nummularia*.

Keywords: Ayurveda, herbal formulations, *ziziphus*, biofilm, antioxidant.

INTRODUCTION

Medicinal plants are intrinsic part of different traditional medicinal systems (Ayurveda, Tibb, Unani). In such systems, the polyherbal formulations are preferred over single therapy (Elaloui *et al*., 2015). Usually compound isolated from medicinal plants are generally preferred over synthetic molecules on account of safety, effectiveness and patience acceptance. The possessions of various antimicrobials obtained from medicinal plants that aim cellular sustainability of microbes is effectively reported earlier (Savoia, 2012; Negi, 2012). The *Ziziphus nummularia* (Rhamnaceae) is generally used in different local herbal preparations for treatment of various health problems including microbial infections. The plant is used alone and in combination based on certain Ayurvedic and indigenous formulations.

A comprehensive literature review indicates the use of *Z. nummularia* for the treatment of skin diseases (scabies) and as astringent (Khare, 2003). Also, the analgesic, anti-inflammatory and effectiveness of leaf extract (cyclopeptide alkaloid) have been reported (Goyal *et al*., 2013). A few reports have highlighted its uses as tonic, antipyretic, antibacterial, analgesic and antidiabetic (Ray *et al*., 2015; Nair and Chanda *et al*., 2011).

A few compounds have previously been isolated from various parts of *Z. nummularia* including cyclopeptide alkaloids (Amphibine C, Nummularine S, Jabanine O), sugars (D-galactose, L-arabinose, D-galacturonic acid), saponins, (Zizynummin, Manogenin), triterpenes (octadecahydropicene-2,3,14,15-tetraone, nummulari genin), and flavonoids (Taxifolin and its glycoside) (Tuenter *et al*., 2017; Ray *et al*., 2015; Sharma, 1983; Srivastav and Chauhan, 1977, Singh *et al*.,1995; Miana *et al*., 1985).

In Pakistan, *Z. nummularia* (Burm. f.) Wight et Arn has been reported from Himalayas (Abbasi *et al*., 2013), D.I.Khan district (Marwat *et al*., 2011), Soon valley (Khan *et al*., 2008), and Nara desert (Bhatti *et al*., 2001). In general, whole plants including fruits, leaves, bark and

**Corresponding author:* e-mail: barki.gold@gmail.com

seeds (Plastina *et al.*, 2012; Goyal *et al*., 2013) have been reported to possess various biological activities. The *Z. nummularia* is an essential element of various formulations based on traditional herbal medicinal system of subcontinent (India and Pakistan). Despite, scientific data covering its biological activities are limited. Current project mainly focuses the phytochemical and antibiofilm properties of Z. *nummularia* leaves.

MATERIALS AND METHODS

Plant material

Based on detailed literature survey, field visits to different places in Districts of D.I. Khan, Karak were made and finally location near Sheikh Buddin range was decided for plant collection. The herbarium sheets were prepared and sent to Islamabad Herbarium (Islamabad, Pakistan) for identification and voucher no. (175607) was obtained. The plant material was dried using oven in NPRL Lab at temperature below 40°C. After complete drying the plant material was powdered and processed for maceration.

Extraction and fractionation

The plant material was macerated using 90% methanol for 10 days (three times). The extracted plant material was dried using rotary evaporator. The extraction was followed by fractionation using a standard extraction scheme. Various fractions were prepared using solvents of different polarities.

Phytochemical screening

The plant extract and various fractions were subjected to phytochemical screening for different compound classes was performed using previously described methods for alkaloids, saponins, flavonoids, tannins and triterpenes/steroids (Khan *et al*., 2011).

Total phenolic content (TPC)

Total phenolics were determined using Folin-Ciocalteu's methods. Primarily an aliquot of 0.3mL extract was mixed with Folin-Ciocalteu's reagent (2.25 mL) After 5min, equal volume of Na_2CO_3 (6%) was added to the mixture and kept aside untouched for 90min. After wards absorbance was measured at 725nm. The calibration curve (using gallic acid) in the range of 0-300µg/mL was made and outcomes were stated as gram equivalent (GAE) (Chlopicka *et al*., 2012).

Total flavonoid content (TFC)

Total flavonoids was evaluated using the aluminium chloride method. Primarily, 0.5mL of crude extract (various concentrations) was added to aluminium chloride (2%, 1 mL) and sodium acetate (5%, 1 mL). The solution was centrifuged at 3000 rpm for 20min, followed by recording the absorbance at 440 nm. Results were expressed as mg rutin equivalent (RE) (Stankovic, 2011) using the rutin calibration curve (0–150 µg/ mL).

HPLC profiling

HPLC study was made on Perkin Elmer series system. A reverse phase C18 column (220 x 4.6 mm, 5 µm, Perkin Elmer) was used with a gradient mobile phase using acetonitrile: water (1mL/min).

Antioxidant assays

DPPH assay

In this assay a fresh solution of DPPH (0.1 mM) was prepared and added to similar volume of extract (varying concentrations). The mixture was kept untouched in dark for 30 min. Subsequently, the absorbance was measured at 517 nm. Trolox was used as standard. The % inhibition of both standard and samples was determined using following formula

Percent inhibition= (1-Absorbance of sample/ Absorbance of control) x 100

Finally the IC_{50} for tested extracts and fractions was determined (Abdelwahab *et al*., 2011).

FRAP assay

During FRAP experiments fresh FRAP reagent was primed by using 10 mM TPTZ solution (10 mL), 300 mM acetate buffer (100 mL), and 20 mM $FeCl_3.6H_2O$ (10 mL) solution and placed at 37°C. The FRAP solution (2850 µl) was added to test sample (150µl extract or fractions) and placed undisturbed for 30 min dark cabinet. After 30 min, the absorbance was noted at 593nm. The results were stated as trolox equivalent (TE)/g of extract (Gan *et al*., 2010).

Antimicrobial assays

The antibacterial assay including disc diffusion assay (Jeong *et al*., 2014), Broth dilution assay for MIC and MBC (Andrews, 2001) and antifungal assay (Jeong *et al*., 2014) performed as mentioned earlier. The test strains used during investigation included, *Staphylococcus aureus* (ATCC 33862), *Bacillus subtillus* (ATCC *3366*), *L. monocytogenes* (ATCC BAA839) and gram negative (*Klebsiella pneumoniae* (ATCC 1705), *E-coli* (ATCC 29050) *and Pseudomonas aureginosa* (ATCC 27853). The fungal strain used was *Aspergillus niger* (wild).

Biofilm assay

The biofilm formation assay was accomplished using 12 well polystyrene plates with slight modified method. Briefly, bacterial strains were inoculated in LB medium (280µl) at an initial turbidity of was mixed with nutrient broth to have turbidity of 0.05 at 600 nm (0.5 McFarland). Afterwards 100µl of tested extract (various concentrations) was added to bacterial culture followed by incubation at 37°C for 24 hrs. The cell growths in plates were measured at 620 and 592nm. For quantification, the biofilms in 12 well plates was stained using crystal violet. Afterwards 95% ethanol was added in stained cells and absorbance was recorded at 592 nm to quantify total biofilm formation (Fletcher, 1998).

Table 1: Phytochemical analysis of the *Z. nummularia* leaves

Extract fraction	Compound classes				
	Flavonoids	Steroids/triterpenoids	Alkaloids	Tannins	Saponins
MeOH 90%	++	+++	+	-	-
Chloroform	++	++	+	-	-
Ethyl acetate	++	+	-	-	-
n-Hexane	-	-	-	-	-
Aqueous	-	-	-	++	+++

Highly present: dark coloration moderately present: medium colouration slightly present: very little colouration absent: no change in colour (Senguttuvan *et al*., 2014) Phytochemicals: +++ profoundly present, ++ moderately present, + slightly present, - absent.

Table 2: Antioxidant profile of *Z. nummularia*

Sample	TPC	TFC	DPPH	FRAP value
	(mg/gGAE[a])	(mg/gRUE[b])	(IC_{50}µg/mL)	(µM)
Total extract	212.2	10.2	89.9	20.4
Ethyl acetate	260.7	27.6	220.7	370.2
Chloroform	212.5	30.0	263.2	204.3
Aquous	182.3	16.3	114.2	120.5
Methanol	212.2	28.1	193.1	249.6
Standard			15[c]	

[a]mg equivalents per gram of gallic acid, [b]mg equivalents per gram of rutin, [c] Trolox (µM),

Table 3: Antimicrobial activity of various fraction of *Z. nummularia* (4mg/mL).

Fraction	Gram positive bacteria			Gram negative bacteria		
	S. aureus	*B. subtillus*	*L. monocytogenes*	*E. coli*	*K. Pneumoniae*	*P. aureginosa*
Total extract	0	0	0	3	0	3
Chloroform	5	4	5	4	4	6
Ethyl acetate	4	3	5	0	0	6
n-hexane	0	3	0	0	0	3
Aqueous	0	0	0	0	0	0
Standard*	9[a]	11[b]	12	14[a]	9[a]	10[a]

Table 4: Antimicrobial activity of various fraction of *Z. nummularia* (2mg/mL).

Fraction	Gram positive bacteria			Gram negative bacteria		
	S. aureus	*B. subtillus*	*L. monocytogenes*	*E. coli*	*K. pneumoniae*	*P. aureginosa*
Total extract	0	0	0	2	0	3
Chloroform	3	2	3	3	1	4
Ethyl acetate	3	2	4	0	0	4
n-hexane	0	3	0	0	0	2
Aqueous	0	0	0	0	0	0
Standard*	9[a]	11[b]	12	14[a]	9[a]	10[a]

[a]Ciprofloxacin (100µg/mL), [b]amoxicillin (100µg/mL),

Table 5: MIC values of various fractions of *Z. nummularia*

Fraction	MIC mg/mL					
	S. aureus	*B. subtillus*	*L. monocytogenes*	*E. coli*	*K. pneumoniae*	*P. aureginosa*
Total extract	>10	>10	>10	>10	>10	>10
Chloroform	4	4	4	>10	>10	>10
Ethyl acetate	8	>10	>10	>10	>10	>10
n-hexane	8	>10	>10	>10	>10	>10
Aqueous	>10	>10	>10	>10	>10	>10
Standard*	0.5	0.5	0.5	1	1	1

* Ciprofloxacin

Table 6: MBC values of various fractions of *Z. nummularia*

Fraction	MBC mg/mL					
	S. aureus	*B. subtillus*	*L. monocytogenes*	*E. coli*	*K. pneumoniae*	*P. aureginosa*
Total extract	>10	>10	>10	>10	>10	>10
Chloroform	8	>10	>10	>10	>10	>10
Ethyl acetate	>10	>10	>10	>10	>10	>10
n-hexane	>10	>10	>10	>10	>10	>10
Aqueous	>10	>10	>10	>10	>10	>10
Standard*	0.5	0.5	0.5	1	1	1

* Ciprofloxacin

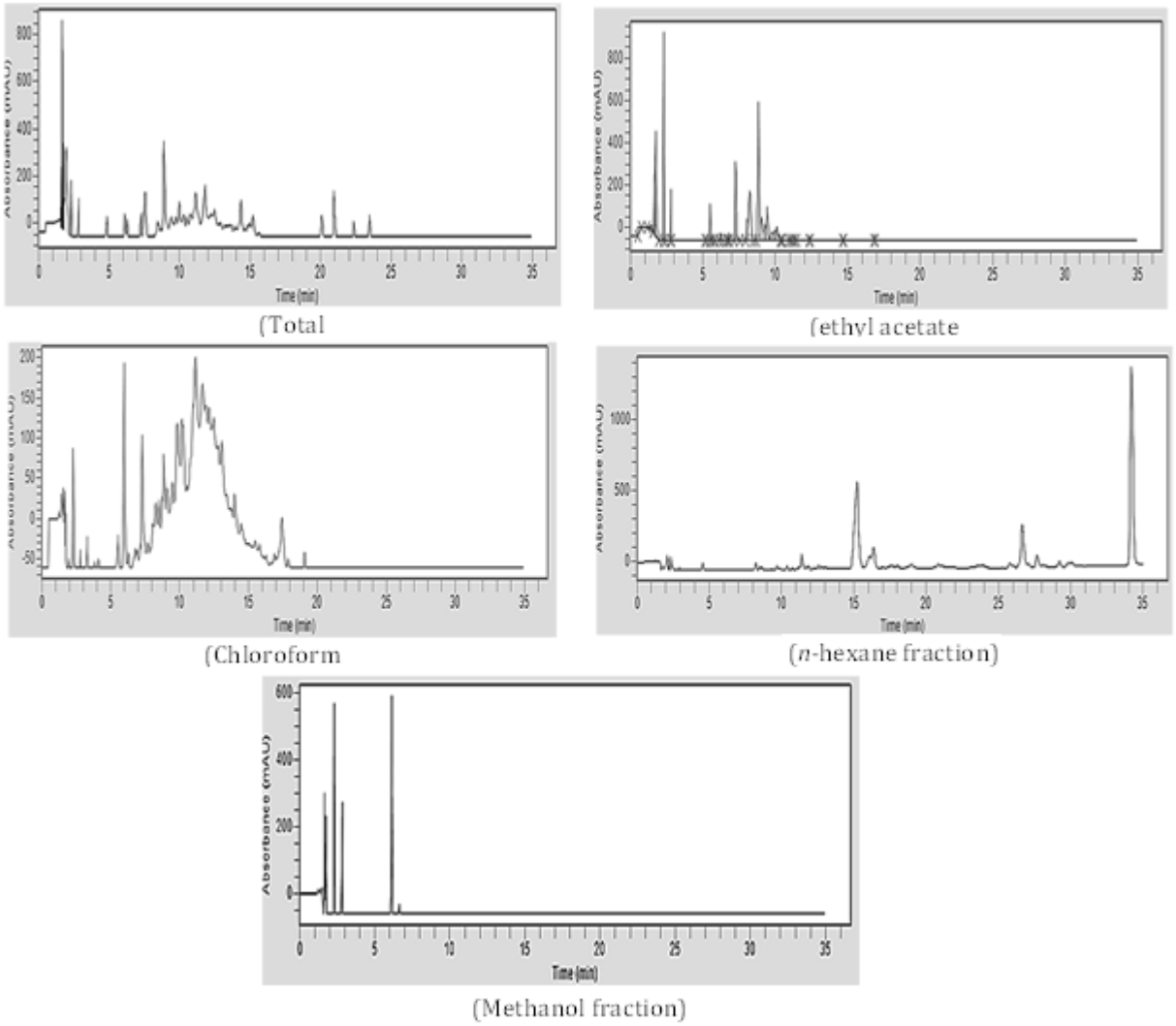


Fig. 1: HPLC profiling of *Z. nummularia* leaves

RESULTS

During this project initially, phytochemical investigation was performed. The fractions of middle polarity possessed terpenes and flavonoids, that were indicated by change in colour (table 1). Weekly positive results were recorded for alkaloids using cerium sulphate (cyclopeptide alkaloids) and Mayer's and Hagers' tests. Whereas the Dragendorf's test was negative. The saponins and tannins were positive in aqueous fractions that are mainly responsible for soap formations. The HPLC profiling of total extract and various fractions indicated a detailed overview of compounds in various fractions. Distribution of compounds in fractions of diverse polarity is clear from HPLC chromatograms seen at different retention time (fig. 1).

The antioxidant potential of *Z.nummularia* was invetsigated using 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), and IC_{50} were calculated. It was noted that

among fractions, methanolic fraction presented highest activity (IC_{50} 193.1µg/mL), followed by ethyl acetate (IC_{50} 220µg/mL) and chloroform (IC_{50} 263 µg/mL) fractions respectively. Interestingly, the total extract however presented highest activity (IC_{50} 89.9 µg/mL), that is indicative of synergistic effect (table 2). Likewise the ferrous reducing power of *Z. nummularia* was invetsigated using FRAP assay. In the first instance, an upright linearity of Ferrous sulfate ($FeSO_4$) and was obtained within the set range of 0.0625 -0.003 mM (R^2 = 0.998) (fig. 2). Afterwards the FRAP value of given samples was calculated using previously described method (materials and method section). Overall FRAP value of *Z. nummularia* calculated was 20.43µM. Among fractions, ethyl acetate fraction presented FRAP value of 370.2µM, followed by chloroform (204 µM) and methanolic (249µM) fractions (table 2). The ferrous reducing power of the fractions of medium polarity (ethyl acetate and chloroform fractions) was significantly high (table 2).

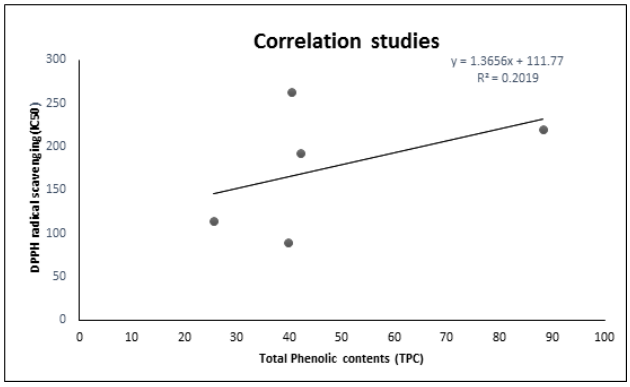
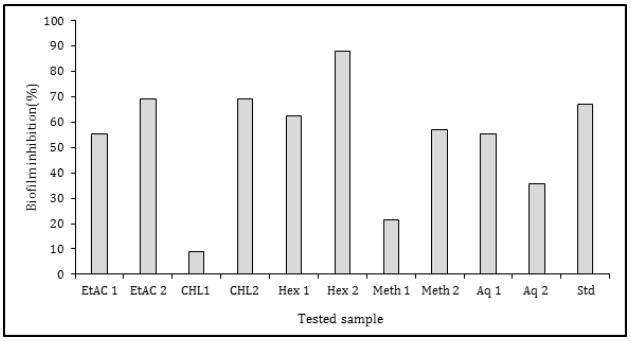


Fig. 2: Correlation studies between DPPH and TPC

Since the antioxidant properties of extracts and isolated compunds is due to presence of phenolic contents, it was therefore considrered necessary to determine the total phenolic contents of *Z. nummularia* using gallic acid callibration curve and GAE were determined. Overall the total phenolic contents of crude extract recorded were 212.2 mg/g GAE. Among fractions ethyl acetate fraction (260.7 mg/gGAE) posessed highest phenolic contents followed by methanol (212.2 mg/gGAE) and chloroform (212.5 mg/g GAE). The aquous fraction posessed least phenolic contents(182.3 mg/gGAE) (table 2). The results are clear indicative of moderate levels of phenolic compounds in the all fractions including total extract. as determined in previous studies (Singh *et al*., 2015). Also a correlation study was performed (fig. 2) that indicated a weak relationship between TPC and antioxidant activites as we already know that *Z. nummularia* conatin phenolic compounds in minor concentration. The total flavonoid contents of *Z. nummularia* were assayed by using rutin calibration curve to determine the extent of antioxidant activity and RUE (rutin equivalents) were determined. The total flavonoid contents of crude extract calculated were 10.2mg/g RUE. Among fractions ethyl acetate fraction (27.6mg/g RUE) posessed highest phenolic contents followed by methanol (28.1 mg/g RUE), chloroform (30.0mg/g RUE) and aquous fraction (16.7mg/g RUE) (table 2).



(n=3; ETAC= ethyl acetate fraction; CHL= chloroform fraction, Hex = Hexane fraction, Meth= methanol fraction, Aq= aquous fraction, 1=1mg/mL; 2= 2mg/mL, Std (Ciprofloxacin =0.5mg/mL)

Fig. 3: Antibiofilm activities of *Z.nummularia* fractions

The total extract and fractions of *Z. nummularia* were screened for preliminary antimicrobial activity and various leafs extract showed interesting antimicrobial activity in different concentrations. At the concentration of 4 mg/mL (maximum tested concentration) chloroform and ethyl acetate fraction of *Z. nummularia* was found to be most effective against both gram negative and positive bacteria (table 3). The methanol, and aqueous fraction did not present any activity towards tested microorganism. The ciprofloxacin and amoxicillin were used as a standard. Similarly, at the concentration of 2 mg/mL (maximum tested concentration), both chloroform and ethyl acetate fraction of *Z. nummularia* presented highest activity against both gram negative and positive bacteria (table 4). The methanol and aqueous fraction did not present any activity towards tested microorganism. The trend clearly indicated a dose dependent inhibition. The ciprofloxacin and amoxicillin were used as a standard. Overall a mild to moderate antimicrobial activity was seen against tested strains. The Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) were determined using broth macrodilution assay as explained in methodology section. Ciprofloxacin was used as standard. The antimicrobial activity demonstrated by fractions were chiefly dependent on dose and duration of exposure. Moreover, the antimicrobial activities shown by all extracts were typically related to pathogen since they presented different antimicrobial activities. The ethyl acetate fraction showed the highest activity (MIC 4 mg/mL) against *S. aureus, B. Subtillus* and *L. monocytogenes*. Likewise, chloroform and *n*-hexane fraction showed better results with all exhibiting poor activities (MIC values 8 mg/mL) against *S. aureus* (table 5). The rest of all extracts exhibited MIC > 10.0 mg/ mL.

The total extract and fractions were further tested for MBC and the MBC for ethyl acetate fractions was 4 mg/mL, whereas all other fractions exhibited MBC > 10mg/mL (table 6). The crude extract and fractions were tested against *Aspergillus niger* for potential antifungal potential. Our results for antifungal assay indicated that none of fractions including crude methanolic extract did not inhibited fungal growth at the maximum tested concentration (10mg/mL).

The crude extracts were tested for antibiofilm activity using a known biofilm producer strain *Pseudomonas aureginosa* (ATCC 15442), using previously described method. A dose dependent assay was performed (1-2mg/mL). Among all tested fractions, there was a dose dependent inhibition of biofilm. The *n*-hexane, chloroform and ethyl acetate fractions presented reportable inhibition of biofilm in all tested concentrations (fig. 3). Overall the *n*-hexane fraction presented highest inhibition (88%) followed by ethyl acetate (69%) chloroform (65%) fractions at concentration of 2mg/mL. A notable decrease in inhibition was recorded with a fifty percent concentration.

DISCUSSION

Bacterial biofilm formation is an emerging health concern these days especially in developing countries since various modern antibiotics are becoming resistant. As the medicinal plants do offer an effective treatment option, we therefore investigated *Ziziphus nummularia* for antibiofilm potential. During detailed phytochemical investigation flavonoids, saponins and terpenes were detected as reported earlier (Srivastav and Chauhan, 1977, Sharma, 1983; Ray *et al*., 2015). In case of alkaloids, weekly positive results were recorded that could be due to presence of certain cyclopeptide alkaloids (Goyal *et al*., 2013) in leaves and week reaction is mainly indicative of presence of such alkaloidal types in much low concentrations.

During antioxidant assays, a week inhibition of DPPH was recorded. The antioxidant potential of extracts is primarily due to occurrence of flavonoids and phenolic compounds (Zhang *et al*., 2010). In our findings, the fractions of medium polarity (ethyl acetate and chloroform) fractions presented comparable results. This is because of fact that flavonoids and terpenes are mainly present in these fractions (Farasat *et al*., 2014). However generally such fractions possess strong DPPH inhibition that was not obvious in this case. The possible reason the fact is presence of flavonoids in less concentrations (Srivastava and Chauhuan, 1977; Harborne *et al*., 1982) and thus showed week proton donor activity (Chanda *et al*., 2011). During our investigation, the ferrous reducing power (FRAP) of the fractions of medium polarity (ethyl acetate and chloroform fractions) was significantly high due to presence of phenolic compounds as discussed earlier. Overall a high FRAP value is indicative of antioxidant activity as we have noticed in case of DPPH radical scavenging effect. This property was due to high flavonoid and phenolic contents as recorded earlier (Singh *et al*., 2015). This can be further explained by the notable point that a few flavonoids have been isolated from *Z. nummularia* leaves and they are from lesser flavonoids as discussed earlier (Harborne *et al*., 1982; Singh *et al*., 2015).

The extracts and fraction presented interesting antimicrobial activity. The trend clearly indicated a dose dependent inhibition. Overall a mild to moderate antimicrobial activity was seen against tested strains. This is due to less concentrations of phenolic and alkaloidal compounds. As it has been reported previously that phenolic compounds and alkaloids are major contributors towards antimicrobial activity (Tsuchiya *et al*., 1996; Shah *et al.*, 1990, Shahat *et al*., 2017). Finally during antibiofilm assays, a dose dependent inhibition of biofilm was recorded. This is first report showing the antibiofilm properties of leaves fractions of *Z. nummularia*.

CONCLUSION

It was concluded that *Z. nummularia* possess moderate antimicrobial and antibiofilm activities. Thus, the folklore usage of *Z. nummularia* is confirmed. Since the plant is mainly used in combination with other medicinal plants, a synergism is proposed in herbal formulations using this plant.

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