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Full Length Research Paper

Platelet aggregation, antiglycation, cytotoxic, phytotoxic and antimicrobial activities of extracts of *Nepeta juncea*

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Various subsequent fractions of *Nepeta juncea* were subjected to test against platelet aggregation, antiglycation, cytoxicity, phytotoxicity and antimicrobial activities. Methanolic extract inhibited arachidonic acid (AA) induced platelet aggregation (IC_{50} 35 ug/ml) and platelet activating factor (PAF)-induced platelet aggregation (IC_{50} 38 µg/ml) in a dose dependant fashion. In case of antiglycation bioassay, n-hexane fraction showed highest inhibitory activity (74.3%), followed by chloroform (72.4%) and water fractions (64.7%). The order of toxicity for brine shrimps was water > chloroform > n-hexane > N-butanol. Except n-hexane fraction (70% inhibition) at higher concentration (1000 ug/ml), n-butanol, chloroform and water fractions exhibited non-significant phytotoxic activity at even highest dose against *Lemna minor*. No significant inhibitory effect of these fractions could be observed against these bacteria and fungi except, n-butanol fraction against *Bacillus subtilis* (27.0%), *Shigella flexneri* (30.7%) and *Staphylococcus aureus* (42.3%). The chloroform fraction showed 25% antibacterial activity against *Salmonella typhi* only. Each fraction might have variable effects in different bioassays.

Key words: *Nepeta juncea*, Lamiaceae, Labiatae, allelopathy, anti-cancer, medicinal plant, biological activity

INTRODUCTION

The multi-regional genus *Nepeta* of Family Lamiaceae (Labiatae) comprises about 250 species. It is widely distributed in temperate Asia, North Africa, and Europe, and most abundant in the Mediterranean region and South-West and Central Asia (Li and Hedge, 1977). In many countries, several *Nepeta* species are used in traditional medicines. They are used as laxative to treat dysentery, kidney and liver diseases and teeth troubles (Baser et al., 2000); used as diuretic, diaphoretic, vulnerary, antispasmodic, antiasthmatic, tonic, febrifuge and sedative agents (Baser et al., 2000; Dabiri and Sefidkon, 2003; Rapisarda et al., 2001; Zargari, 1990). *Nepeta* are also reported to possess biological activities especially reduction of serum lipids and anti-inflammatory effects (Agarwal et al., 1978; Prokopenko and Spiridonov, 1985).

Nepeta juncea Benth. wildly grows in mountainous northern areas and north western mountains of Pakistan. As the member of family Lamiaceae (Labiatae), it also contains essential oils which have been reported by various researchers (Kobaisy et al., 2006; Tripathi et al., 2004, 2008). Essential oils of *N. juncea* have shown antifungal and antibacterial properties (Kobaisy et al., 2006; Tripathi et al., 2004, 2008). Among these essential oils, the major chemical constituent is nepetalactone which has shown antimicrobial activities in *N. juncea* (Inoue, 2000) and insect repellent properties in *N. cataria* (Schultz et al., 2004). The whole plant and essential oils of *N. juncea* have also shown antifungal activity by vapor contact (Inuoe et al., 2001). Ethyl acetate fraction from the flowers and leaves of *N. juncea* from Northern Areas of

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Pakistan, yielded 71.8% nepetalactone using GC-MS (Inoue et al., 2001).

The objective of the present study was to screen the active fractions of *N. juncea* and assay for platelet aggregation, antiglycation, cytotoxicity (Brine Shrimp Bioassay), phytotoxicity (*Lemna* Bioassay), antibacterial and antifungal properties.

MATERIALS AND METHODS

Plant material

Aerial parts of *N. juncea* were collected from the tribal zone, Kurram Agency Pakistan, near Afghanistan border. Herbarium specimens were deposited in the herbaria of Department of Botany, Kohat University of Science and Technology (KUST), Pakistan and Department of Botany, Government Post Graduate Jehanzeb College, Swat Pakistan.

Extraction and fractionation

The whole plant of *N. juncea* was dried in dark, chopped and ground to coarse powder. The powdered plant (4 kg) was initially extracted with methanol (7 days x 3) at room temperature. The combined methanolic extract was evaporated under reduced pressure leaving behind a greenish, syrup residue (150 g). The methanol extract was partitioned in various fractions through separating funnel. It was partitioned into n-hexane (30 g), $CHCl_3$ (50 g), ethyl acetate (20 g), n-butanol (10 g) and water fractions (18 g), successively.

Platelet aggregation

Blood was taken from health human volunteers who have not taken any medication during the last 2 weeks. Blood samples was mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 g for 15 min at 20°C to obtain platelet rich plasma (PRP). The remaining blood sample was centrifuged at 1200 g for 10 min to obtain platelet poor plasma (PPP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37°C with PRP having platelet counts between 2.5 and 3.0 x 10^8 mL^{-1} of plasma (Saeed et al., 2007a, b).

Aggregation was monitored by dual-channel Lumi aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.45 ml aliquots of PRP (Shah et al., 1996). The final volume was made up to 0.5 ml with the aggregating agent, dissolved either in normal saline or appropriate vehicle known to be devoid of any effect on aggregation. Aggregation was induced with arachidonic acid (AA) (1.8 mM) or platelet activating factor (PAF) (0.8 µM). The anti-aggregatory effect of methanolic extract and each fraction of *N. juncea* were studied by addition of aggregation agents (AA and PAF). The resulting aggregation was recorded for 5 min after challenge, by the change in light transmission as a function of time.

Antiglycation

Bovine serum albumin (BSA) was used as 10 mg/ml, dissolved in 67 mM phosphate buffer (pH: 7.4). Glucose as 50 mg/ml, dissolved in 67 mM phosphate buffer (pH 7.4). 3 mM sodium azide was added in required quantity of phosphate buffer to inhibit bacterial growth. 1 mg/1000 µl concentration of each fraction was used to calculate antiglycation activity along with standard inhibitor. The dissolved sample (60 µl in each well of 96-well plate) was incubated for a week at 37°C. After a week, the samples were taken out and cooled at room temperature. Then 6 µl of 100% trichloroacetic acid (TCA) was added to each of the well, supernatants containing unbounded glucose, inhibitor and interfering substances were removed after centrifugation at 14,000 rpm for 4 min, pellets were obtained at the bottom of the wells. Then solvent was removed from each well, and 60 µl of phosphate buffer saline (PBS) pH 10, was added to dissolve the pellets. The comparison of fluorescence intensity at 370 nm excitation and emission at 440 nm was obtained by using a spectrofluorimeter (Matsuda et al., 2003; Matsuura et al., 2002; Nakagawa et al., 2002). Rutin was used as the standard inhibitor (Atta-ur-Rahman et al., 2007). Inhibition percentage was calculated as % inhibition $=100 - [(OD_{sample} / OD_{blank}) \times 100]$.

Brine shrimp lethality bioassay

Brine shrimp (*Artemia salina* larvae) eggs were hatched in a shallow rectangular plastic dish, filled with artificial seawater, which was prepared by mixing a commercial salt mixture (Instant Ocean, Aquarium System, Inc., Mentor, OH, USA) with double distilled water (Meyer et al., 1982).

An unequal partition was made in the plastic dish with the help of a perforated device. 50 mg of eggs were sprinkled into larger compartment, which was placed under the dark condition while the smaller compartment was opened to ordinary light. After two days naupils were collected. A sample of the test fraction was prepared by dissolving 20 mg of each fraction in 2 ml of methanol. From this stock solution, 1000, 100 and 10 µg/mL was transferred to 12 vials; three for each dilution, and three vials were kept as control having 2 ml of methanol only.

The solvent was allowed to evaporate overnight. When shrimp larvae were ready, 1 ml of sea water was added to each vial along with 10 shrimps and the volume was adjusted with sea water to 5 ml per vial. After 24 h, the number of surviving shrimps was counted. Data were analyzed by a Finney computer program (Finney, 1971) to determine the LD_{50} (Meyer et al., 1982). Each experiment was replicated thrice.

Phytotoxicity bioassay

This test was performed according to the modified protocol of McLaughlin (1988). The test fractions were incorporated with sterilized E-medium at different concentrations i.e. 10, 100, 1000 µg/mL in methanol. Sterilized conical flasks were inoculated with fractions of desired concentrations prepared from the stock solution and allowed to evaporate overnight. Each flask was inoculated with 20 ml of sterilized E-medium and then ten *Lemna minor* each containing a rosette of three fronds were placed on media. Other flasks were supplemented with methanol serving as negative control and reference inhibitor i.e. Parquet serving as positive control. Treatment was replicated three times and the flasks incubated at 30°C in Fisons Fi-Totron 600 H growth cabinet for seven days, 9000 lux intensity, 56±10 relative humidity and 12 h day length. Growth of *L. minor* in fraction containing flask was determined by counting the number of fronds per dose and growth inhibition was calculated with reference to negative control (McLaughlin, 1988).

Antimicrobial activities

The antibacterial activity was determined by agar well diffusion method. A loopful of a 10^4-10^6 suspension of 24 h old broth of each bacterium was streaked on the surface of Mueller- Hinton agar (BBl-USA) plates. Wells were dug in the agar with the help of sterile dimethyl sulfoxide (DMSO). Dilutions of the stock solution containing 50,100, 150 and 200 µg were prepared in DMSO and 100 µl of each dilution was added in the respective wells. The plates were then

incubated at 37°C for 24 h and zone of inhibitions were measured in millimeters (mm) and compared with the control (Atta-ur-Rahman, 1991). Antibacterial activity of fractions of *N. juncea* was studied against *Eschericha coli, Bicillus subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi*. Ampicillin, Tobramycin and Amoxacilline were used as standard drugs.

RESULTS AND DISCUSSION

Platelet aggregation

Crude extract and subsequent fractions of *N. juncea* (methanol (NJM), n-hexane (NJH), chloroform (NJC) ethyl acetate (NJE), n-butanol (NJB), and water (NJW)) were investigated for activity against human platelet aggregation. Platelet aggregation was induced by a number of platelet agonists (PAF and AA). However, methanol extract (NJM) inhibited AA induced platelet aggregation in a dose dependant fashion (Figure 1). Concentration of methanol extract (NJM) required to inhibit AA induced human platelet aggregation by 100% (IC_{50}) was found to be AA = 35 µg/ml (Figure 1). Similarly methanol extract (NJM) was also effective in inhibiting PAF-induced platelet aggregation in a dose related manner with IC_{50} of 38 µg/ml (Figure 1). Methanol extract (NJM) appears to inhibit platelet COX as this enzyme is responsible for the production of TXA_2 through which AA mediates its aggregatory effect. Ethyl acetate fraction (NJE) inhibited AA induced platelet aggregation in a dose dependant fashion (Figure 2). Concentration of ethyl acetate fraction (NJE) required to inhibit AA induced human platelet aggregation by 100% (IC_{50}) was found to be AA = 45 µg/ml. n-butanol fraction (NJB) was also found effective against AA (Figure 3) and the (IC_{50}) AA= 53 µg/ml. Similarly, water fraction (NJW) and n-hexane (NJH) were effective against AA and (IC_{50}) AA= 13 µg/ml, (IC_{50} value= AA=48 µg/ml) (Figures 4 and 5) respectively. However chloroform (NJC) fraction did not show any effects on PAF induced platelet aggregation. Contrary findings were observed by Shehnaz et al. (1999) when they studied the effect of extracts of *Cestrum parqui* on human platelet aggregation. They observed that PAF induced platelet aggregation was blocked by all the concentrations but the extract exhibited no effect on AA induced platelet aggregation. Our results showed that the inhibition of AA induced platelet aggregation by n-hexane, ethylacetate, n-butanol and water fractions was selective for AA but not for PAF (Shehnaz et al., 1999). Aqueous extracts of Tibetan medicinal plant, *Lamiophlomis rotata* showed platelet aggregation inhibition (Li et al., 2008). Similarly aqueous extracts of 8 from the total of 17 Guatemalan medicinal plants have shown antiplatelet aggregation (Villar et al., 1997).

Antiglycation bioassay

All three fractions have shown good inhibitory potential *in*

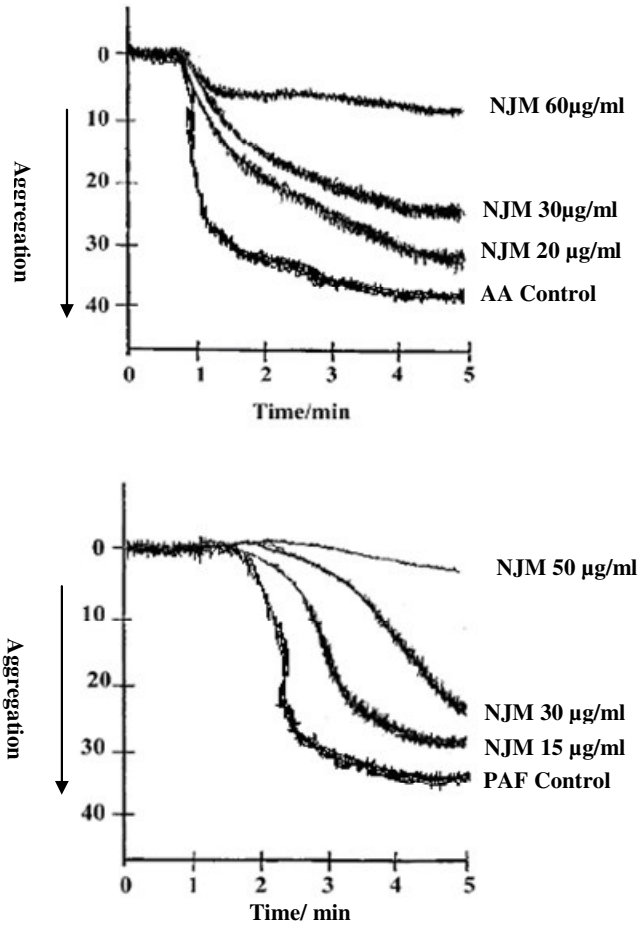

Figure 1. Tracings of inhibition of the platelet aggregation by NJM-Methanol extract, against PAF and AA. Control is aggregation curve obtained by adding AA (1.8 mM) or PAF (0.8 µM) and was taken as 100% aggregation. (IC_{50} value= AA=35 µg/ml, PAF=38 µg/ml).

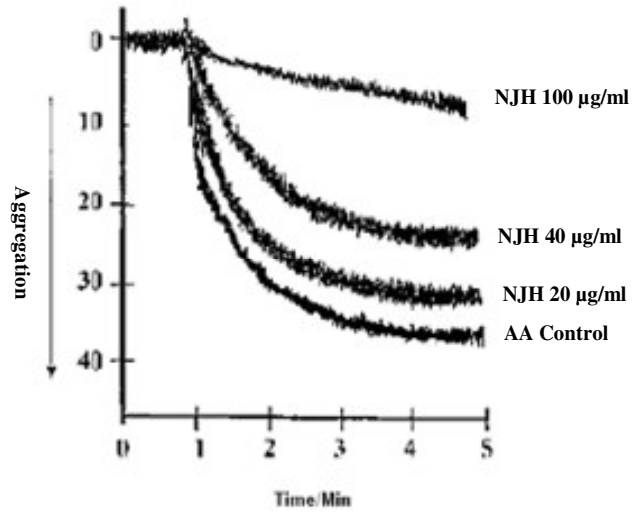

Figure 2. Tracings of inhibition of the platelet aggregation by N-hexane extract (NJH) against AA. Control is aggregation curve obtained by adding AA (1.8mM) and was taken as 100% aggregation. (IC_{50} value= AA=48 µg/ml).

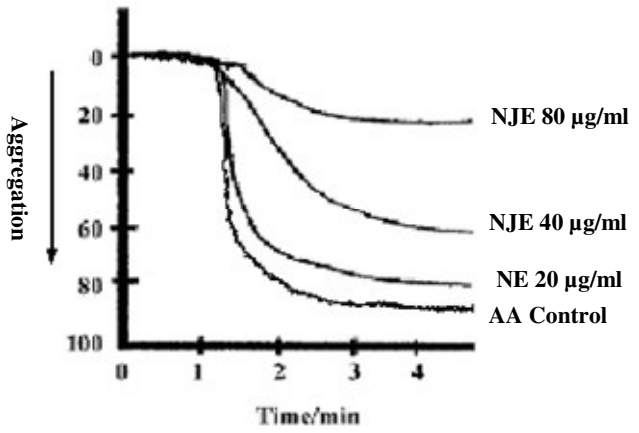

Figure 3. Tracings of inhibition of the platelet aggregation by NJE (Ethyl acetate) against AA. Control is aggregation curve obtained by adding AA (1.8mM) and was taken as 100% aggregation. (IC_{50} value= AA=45 µg/ml).

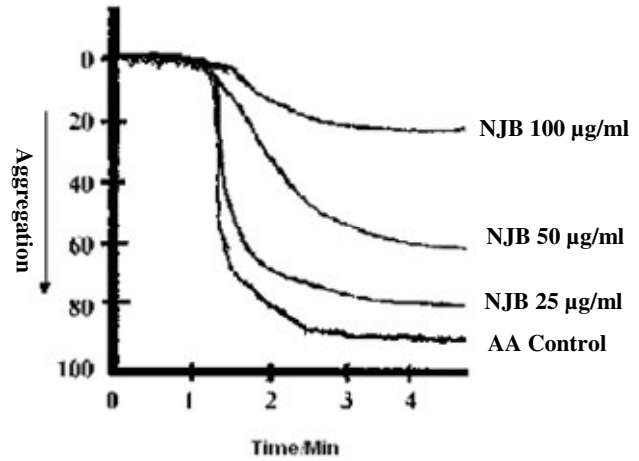

Figure 4. Tracings of inhibition of the platelet aggregation by NJB (N-butanol fraction) against AA. Control is aggregation curve obtained by adding AA (1.8mM) and was taken as 100% aggregation. (IC_{50} value= AA=53 µg/ml).

Table 1. Summary of results of antiglycation bioassay of *Nepeta juncea* fractions.

Sample	Inhibition (%)
N-hexane extract (NJH)	74.3
Chloroform extract (NJC)	72.4
Ethylacetate fraction (NJE)	No Activity
N-butanol fraction (NJB)	No Activity
Water fraction (NJW)	64.7

I mg/1000 µL of each sample was used in the assay.

vitro at given concentration of 1 mg/1000 µL (Table 1). N-hexane fraction showed highest inhibitory activity (74.3%), followed by chloroform (72.4%) and water fractions (64.7%). N-butanolic and ethylacetate fractions did not show any antiglycation activity. We could not conduct IC_{50} studies due to unavailability of samples. However the current study showed that n-hexane fraction might contain our potential chemical compounds respon- sible for higher antiglycation activity. Our assumption is supported by one of the studies in which n-butanolic fraction of *Plantago asiatica* had shown higher antiglycation activity (75%) which was actually due to the presence of a new compound, plantamajoside (Choi et al., 2008).

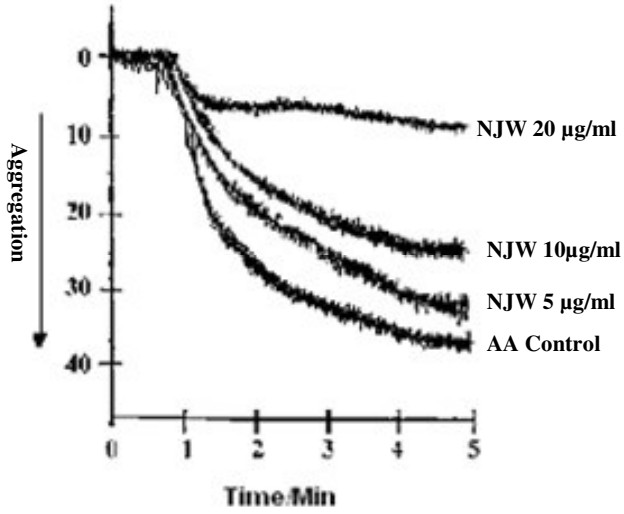

Figure 5. Tracings of inhibition of the platelet aggregation by NJW (water) against AA. Control is aggregation curve obtained by adding AA (1.8mM) and was taken as 100% aggregation. (IC_{50} value= AA=13 µg/ml).

Brine-shrimp lethality bioassay

The order of toxicity for brine shrimps was water > chloroform > n-hexane > n-butanol in all the three concentrations (Table 2). N-butanol and n-hexane fractions did not show any significant cytotoxic activity. However, water fraction exhibited lower LD_{50} values (88.1253 ug/ml) as compared to LD_{50} value of the chloroform (771.46 ug/ml) fraction. Water fraction could be the potential one which might contain potential cytotoxic compound/s. The extracts with LC_{50} values higher than 200 mg/l in the brine shrimp test can be considered inactive (Anderson et al., 1991). Water (0.08 mg/ml) and chloroform (0.77 mg/l) fractions of *N. juncea* were active having less than 200 mg/l. Brine shrimp lethality test for ethanolic extracts of *Paeonia emodi* did not show significant toxic effects at 1000, 100 and 10 ug/ml while the IC_{50} value was also higher than 1000 ug/ml (Khan et al., 2005).

Phytotoxicity bioassay

The phytotoxicity of all fractions obtained from the crude

Table 2. Mortality rates (%) of brine shrimps caused by treatments of *Nepeta juncea* fractions.

Dose (µg/mL)	N-hexane (NJH)	Chloroform (NJC)	N-butanol (NJB)	Water (NJW)
1000	30.0	50.0	13.3	73.3
100	20.0	46.7	10.0	50.0
10	10.0	40.0	03.3	30.0
LD_{50} (ug/ml)	-	771.5	-	88.1

Table 3. Phytotoxic studies of various fractions of *Nepeta juncea* against *Lemna minor.*

Conc. (µg/mL)	No. of Fonds					Growth regulation (%)				
	NJH	NJC	NJW	NJB	Control	NJH	NJC	NJW	NJB	Control
1000	4	12	10	14	13.3	70	9.8	24.9	70	0.015
100	8	13	13	15		39.9	2.3	2.3	39.9	
10	14	16	20	17.7		-5.2	-20.3	-50.3	-5.2	

methanolic extract was carried out against *L. minor*. This assay was performed at three different concentrations i.e. 1000,100 and 10 µg/ml. N-Butanol (NJB), chloroform (NJC-5) and water (NJW) fractions showed non-significant activity at even highest dose while n-hexane fraction (NJH) showed significant activity at the highest dose (Table 3). Phytotoxic properties of genus *Nepeta* have not been studied in detail. However, essential oils of *N. pannonica* [60 components with 1,8-cineole (28.9%), and 4α,7ß,7α-nepetalactone (14.3%) as the major constituents] exhibited more phytotoxicity to bentgrass as compared to lettuce seeds, with 100% growth inhibition observed at 0.3, and 1.0 mg/ml, respectively (Kobaisy et al., 2005).

Antimicrobial activity

We tested all the fractions of *N. juncea* against 6 bacteria (*B. subtilis, E. coli, S. flexneri, S. aureus, P. aeruginosa,* and *S. typhi*) and 6 fungi (*Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis,* and *Trichphyton longifusus*). No significant inhibitory effects of these fractions could be observed against these bacteria and fungi. However, the n-butanol fraction showed weak antibacterial activity against *B. subtilis* (27.0%), *S. flexneri* (30.7%) and *S. aureus* (42.3%). The chloroform fraction showed 25% antibacterial activity against *S. typhi* only. Khan et al. (2005) had used the same bacterial and fungal strains as test against crude extracts of *P. emodi* but they also found no significant antibacterial or antifungal activities against these strains. However, essential oils of *N. juncea*, with major constituent of nepetalactone, have shown antifungal and antibacterial properties (Inoue et al., 2001; Tripathi et al., 2004, 2008; Kobaisy et al., 2006). Similarly essential oils (major constituent nepetalactone) of *N. cadmia* (Celick et al., 2008) and *Nepeta rtanjensis* were active against five bacterial strains but not against a fungal strain, while *Aspergillus niger* (Stojanovic et al., 2005), *Nepeta crispa* (Sonboli et al., 2004), *Nepeta sibthorpii* (Galati et al., 2006), *N. cataria* (Billerbeck, 2007), and *Nepeta ispahanica* (Salehi et al., 2007) showed antimicrobial and antifungal activities. But in our case, the fractions did not show effective inhibitory measures against bacterial and fungal strains. It shows that the essential oils of *N. juncea* (but not the various extracts) contain the chemical compound(s) responsible for antimicrobial activity. In this case, each minor to major chemical constituent of essential oils needs to be bio-assayed in future to identify the targeted compound.

Conclusion

The results suggest that each fraction has variable effects in different bioassays. The water fraction showed highest inhibition in platelet aggregation as well as in brine shrimp lethality bioassay which may be the potential fraction for characterization and isolation of targeted compounds. While chloroform fraction showed significant antiglycation activity, only n-hexane fraction at higher concentration (1000 ug/ml) showed inhibitory effect (70%) against *L. minor* while rest of the extracts and concentrations did not show any signification inhibitory effect. In case of antimicrobial activities, the fractions did not exhibit any inhibitory effect against bacterial and fungal strains while essential oils of *N. juncea* have already shown significant inhibitory effects. Thus essential oils may contain the targeted compound for antimicrobial activity.

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