International Journal of Phytomedicine 8 (2016) 202-207
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

The phytochemical investigation and biological activity of *Nepeta clarkei*

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Abstract

The present study is aimed at the isolation and identification of the compounds responsible for the bioactive behavior of *Nepeta clarkei* (Hook. f). The crude extract and its various sub-fractions obtained from *N. clarkei* Hook. f. (chloroform (NCC), n-hexane (NCH), ethyl acetate (NCE), and aqueous (NCA)) along with methanolic extract were screened for anti-cancer activity. Only NCH and NCC fractions suppressed the cancer cell lines (HT29 and HCT116) to less than 20% and were screened for a range of other biological activities (antiglycation, phytotoxicity, antiplattelet, insecticidal and antimicrobial) in vitro. The chloroform fraction exhibited significant (63.31%) antiglycation activity followed by the n-hexane fraction (43.9%). Interestingly n-hexane fraction demonstrated a significant phytotoxic potential (100% inhibition) towards *Lemna minor* at the highest concentration (1000 µg/mL) only, while the chloroform fraction showed moderate activity (33.83%). The n-hexane fraction furthermore demonstrated 100% anti-platelet activity against AA (48 µg/mL) and PAF (15 µg/mL). On the other hand both the chloroform and n-hexane fractions were inactive against fungi and bacteria used in the anti-bacterial and anti-fungal assays. The order of toxicity towards brine shrimps was n-hexane > chloroform fractions. An intensive phytochemical investigation of the chloroform extract of *N. clarkei* resulted in the isolation of eight metabolites including 1,2-benzenedicarboxylic acid bis (2- ethylhexyl) diester (**1**), eupatorin (**2**), achillin (**3**), neoponcirin (**4**), parvifloroside B (**5**), betulinic acid (**6**), β -sitosterol (**7**), and β-sitosterol glucopyranoside (**8**). The structure elucidation was carried out on the basis of 1D (^{1}H- and ^{13}C) and 2D (H-C correlations; HMBC, HSQC) NMR techniques and confirmed by comparison of their physical and spectroscopic data with those reported in literature. All these compounds, to the best of our knowledge, were isolated from *N. clarkei* for the first time.

Keywords: *Nepeta clarkei,* Phytochemical Investigation, Anticancer, Biological Activities.

Introduction

The *Nepeta* (Lamiaceae) comprises of 250 species of which 67 and 58 are present in Iran and Pakistan respectively [1]. Most of the *Nepeta* plants have been used in traditional medicines viz., a selection of *Nepeta* plants from Iran are employed in the treatment of nervous, respiratory, and gastrointestinal related diseases [2]. Moreover *Nepeta* plants have been used as traditional medicines by other countries around the world. For example *Nepeta* plant species are used in combination with other medicinal plants as diuretics, diaphoretics, antispasmodics, sedative agents, antitussive, antiasthmatic, tonic febrifuge, and vulnerary agents [3]. One of the species, viz., *N. clarkei* is presently the least known for its biochemical composition. Compounds isolated from *N. clarkei* (Hook f) have been shown to possess significant antimicrobial and antioxidant activities [1] and as a consequence has prompted us to extend our search for the isolation, characterization and biological activity of the phytochemicals of *Nepeta clarkei.*

Materials and Methods

Plant collection, extraction and isolation

The entire plant of *N. clarkei* was collected at the Parachinar, Kurram Agency, Khyber Pakhtunkhwa Pakistan, in 2005, and was identified by Mr. Muhammad Siraj (plant taxonomist) at the Department of Botany, Govt. Post Graduate College Jehan Zeb, Swat, Pakistan.

A specimen of this plant (KUST-375) was deposited in the Herbarium of the College. The whole plant of *N. clarkei* (6.5 kg) was macerated in MeOH at room temperature for two weeks and then filtered. The filtrate was concentrated under vacuum to give a crude extract (180 g). The crude fraction (180 g) was sequentially extracted with n-hexane, chloroform, ethyl acetate and water to give n-hexane (NCH) (45 g), chloroform (NCC) (55 g), ethyl acetate (NCE) (48 g) and aqueous (NCA) (52 g) fractions. After in vitro screening, chloroform fraction (55 g) was subjected to silica gel column 3 (70–230 mesh, Merck, Munich, Germany) using 10% ethyl acetate/n-hexane (2x500 mL) with a 5% gradient of increasing polarity up to 100% ethyl acetate, then by the gradient of methanol (1%, 2%, 5%, 10%, and 20%), and finally washed with 100% methanol as a mobile phase and yielded 10 fractions (NCF-1 to NCF-10). Fraction no. 4 (NCF-4) obtained using 40% ethyl acetate/n-hexane was further applied on a silica gel column and eluted with a gradient solvent systems of ethyl acetate/n-hexane (30:70; 40:60) to afford 1 (7 mg) and 3 (4 mg). Fraction no. 9 (NCF-9) obtained using 5% methanol/ethyl acetate was loaded on silica gel column and eluted with gradients of methanol/ethyl acetate (5:95) to purified compound 2 (5 mg) and 4 (8 mg) (10:90). After taking TLC three fractions (NCF-6 to NCF-8), obtained from 70-90% ethyl acetate/n-hexane system, were combined together and loaded on a silica gel column to get 5 (7 mg) with 75% ethyl acetate/n-hexane, 6 (10 mg) with 60% ethyl acetate/n-hexane, 7 (25 mg) with 40% ethyl acetate /n-hexane and 8 (22 mg) eluted with 60% ethyl acetate/n-hexane.

Anticancer bioassay

Four cancer cell lines viz., colorectal adenocarcinoma (HT29), colorectal adenocarcinoma (HCT116); the human hepatoma derived cell line (HepG2) and the breast cancer cell line (MCF-7) were used for cytotoxicity screening of the medicinal plant extracts. All cell lines were purchased from ATCC, Manassas, VA 20108, USA. Cell lines were cultured in Advanced DMEM supplemented with 10% inactivated NBCS and 5 mM l-glutamine, and grown at 37 ºC in a humidified atmosphere of 5% CO_2 in air. The MTT [3-(4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium bromide] colorimetric assay developed by Mosmann [4] was used with minor modifications to screen for cytotoxic activity of medicinal plant extracts.

Other biological activities

Phytotoxicity, lethality assay for brine shrimp, antiglycation, antiplatelet aggregation, insecticidal and antimicrobial assays was performed according to previously published protocol [5-10].

Results and Discussion

Biological activities of the fractions

Anticancer bioassay

Herbal plants and their phytochemicals have historically been the basis for nearly all medicinal therapies and have, over the ensuing years gained significant recognition in the treatment of various diseases and clinical conditions in humans (including cancer) in ayurvedic medicine [8,9]. For this very reason it remains important to discover more effective herbs and elucidate whatever mechanisms of activity their phytochemicals display in order to develop alternative methods for cancer treatment [4]. Literature has documented that over 3000 plants have been employed to treat different types of cancer [10]. Interestingly, 60% of the current anticancer drugs have their origins from natural sources and still play a major role in the discovery of anticancer drugs [11].

Different fractions (*n*-hexane, chloroform, and methanol) of *N. clarkei* were screened for their biological activities employing hepatoma derived cell (HepG2) lines, and two colorectal adenocarcinoma cell lines viz., HT29 and HCT116. The screening results showed that *n*-hexane (NCH) and chloroform (NCC) fractions suppressed cancer cell growth at 1000 µg /mL, while on the other hand methanol (NCM) fraction displayed moderate activity when compared to the control (Figure 1). Gratifyingly, the *n*-hexane and chloroform fractions suppressed the viability of both the cancer lines HT29 and HCT116 to less than 20% (Figure 1). However, the *n*-hexane, chloroform, and methanol fractions did not show any anticancer activity towards HepG2 cancer cells.

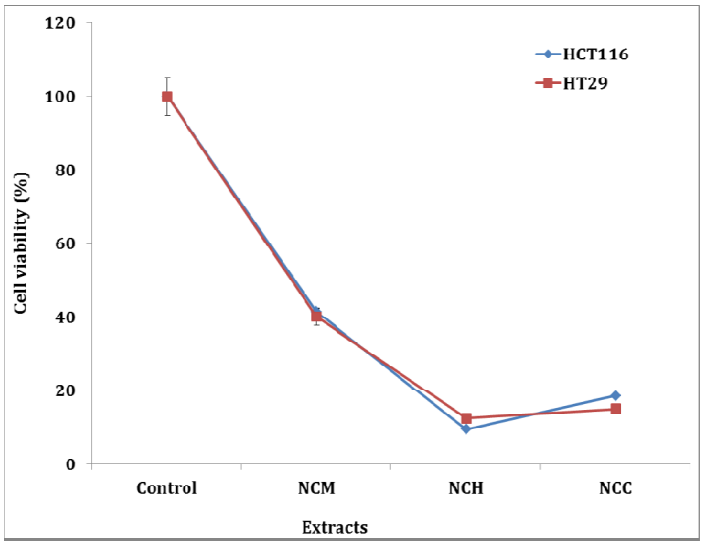

Figure 1- Anticancer activity of different extracts of *N. clarkei* plant; NCH: *n*-hexane; NCC: Chloroform; Methanol (NCM)

Phytotoxic bioassay

It has been reported that new herbicides derived from natural sources are receiving greater attention as compared to synthetic agrochemicals because natural agrochemicals are effective and biodegradable and consequently will present less harmful effects to

the environment and health [12]. Interestingly; the *n*-hexane fraction demonstrated a significant phytotoxic potential (100% inhibition) towards *Lemna minor* at the highest concentration (1000 µg/mL) (Table 1; Figure 2). On the other hand the *n*-hexane fraction demonstrated a modest activity of 33.8% at 100 µg/mL concentration and was almost inactive at 10 µg/mL concentration. The above results lead credence to *N. clarkei* extracts being employed in the development as potential herbicides. The same findings were also observed in the *n*-hexane fraction of *Nepeta juncea* (70%) [5], *Ajuga bracteosa* (100%)[6], and *Nepeta distance* (100%)[7].

Table.1.Phytotoxic studies of various fractions of *N. clarkei* against *Lemna minor* L.

Name of plant	Conc. (µg/mL)	No. of fronds		% Growth regulation	Conc. of std. drug (µg/mL)
		Sample	Control		
NCC[a]					
Lemna minor L	1000	13	19.66	33.87	0.015
	100	19		3.35	
	10	20		1.72	
NCH[b]					
Lemna minor L	1000	0	19.66	100	0.015
	100	18		8.44	
	10	20		1.72	

[a]NCC: Chloroform;[b]NCH: *n*-Hexane

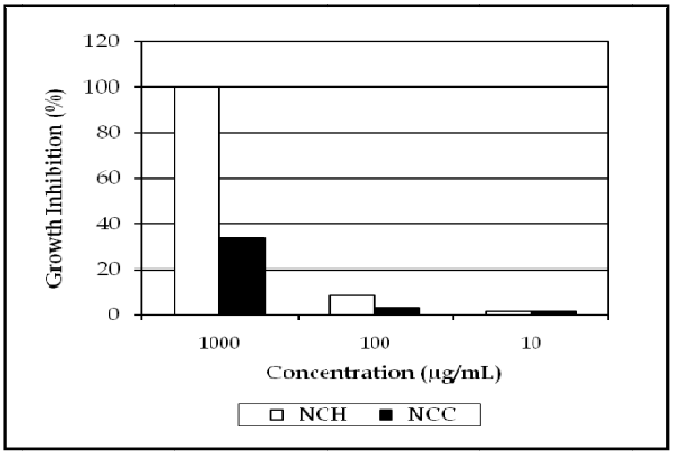

Figure 2. Phytotoxic activity of chloroform (NCC) and hexane (NCH) extracts of *N. clarkei* against *Lemna minor*.

Brine shrimp lethality

Brine shrimp lethality results indicated that *n*-hexane and chloroform extracts demonstrated a very low toxicity with LD_{50} greater than 300 µg/mL (*n*-hexane LD_{50}: 521.4 and chloroform LD_{50}: 311.4 µg/mL) (Table 2).

Table 2. Mortality rates (%) of brine shrimps lethality caused by treatments of *N. clarkei* Hook. f. fractions.

NCC[a]				
Dose(µg/mL)	No. of shrimps	No. of survivors	LD_{50} (µg/mL)	LD_{50}(µg/mL)[c]
1000	30	11	311.493	7.642
100	30	19		
10	30	25		
NCH[b]				
1000	30	13	521.416	7.642
100	30	20		
10	30	26		

[a]NCC: Chloroform;[b]NCH: *n*-Hexane; LD_{50} of standard drug

Antiglycation activity

The chloroform and n-hexane extracts were evaluated for their inhibitory potential against protein glycation in vitro. Among these fractions, chloroform fraction proved to have significant antiglycation activity with 63.31% inhibition against protein glycation at a concentration of 0.5 mg/1000 µL, while the n-hexane fraction demonstrated moderate inhibition with 43.90% at the same concentration (Figure 3). Chloroform fraction of *N. clarkei* showed similar inhibition to that of n-hexane (64.06%) fraction of *Rhynchosia reniformis* [13], water (64.7%) fraction of *Nepeta juncea* [6] and ethyl acetate fraction (65.60%) of *Nepeta suavis* [14]; while less than ethyl acetate (74.0%) and n-hexane (71.2%) fractions of *Nepeta laevigata* [15]; n-hexane fraction of *Nepeta kurramensis* (67%) [15]; n-hexane (74.3%) and chloroform (72.4%) fractions of *Nepeta juncea* [6]; chloroform (76.02%) and ethyl acetate (70.27%) fraction of *Rhynchosia reniformis*.

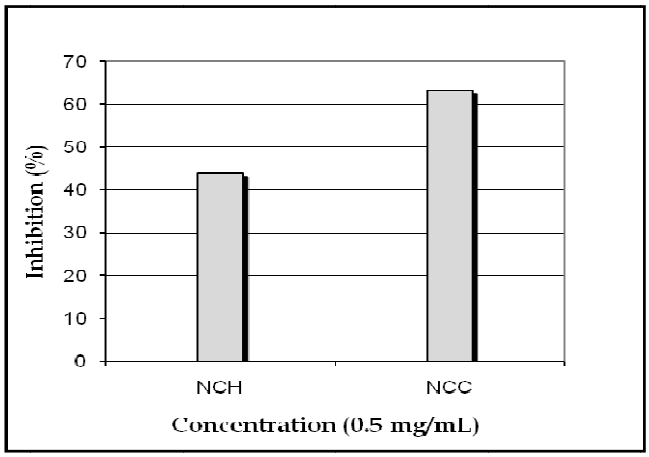

Figure 3. Antiglycation activity of chloroform (NCC) and *n*-hexane (NCH) fractions of *N. clarkei*.

Antiplatelet aggregation

Both fractions of *N. clarkei* were screened for antiplatelet aggregation activity in which n-hexane fraction demonstrated strong inhibition (100%) against AA (arachidonic acid) with IC_{50} value = 48 µg/mL and PAF (platelet activating factor) with IC_{50} value = 15 µg/mL. The aggregation activity shown by n-hexane fraction (NCH) was confirmed by dose-dependent studies, which inhibited AA induced platelet aggregation in a dose dependent fashion. Hexane (NCH) fraction seems to be independent of its COX inhibitory activity. According to Ahmad et al (2009), n-hexane fraction of *R. reniformis* was effective against AA, and PAF, while n-butanol and ethyl acetate fractions showed no action against AA, PAF [13]. Similarly, n-hexane fraction of *N. distance* showed significant activity against AA with IC_{50} of 53 µg/mL and chloroform fraction displayed against PAF (IC_{50} of 53 µg/mL) [7], while n-hexane fraction of *N. juncae* (IC_{50} value = 48 µg/mL) showed significant activity against AA and PAF, while chloroform was found inactive [6] which further strengthen our present findings.

Other biological activities

Insecticidal activity of the chloroform and n-hexane fractions was performed using different insects including *Rhyzopertha dominica*, *Tribolium castaneum* and *Callosbruchus analis* but did not show any promising results against these insects. Moreover, the above mentioned fractions did not show any antibacterial and antifungal activities against the following bacteria and fungi viz., *Bacillus subtilis, Shigella flexneri, Eschericha coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, Fusarium solani, Salmonella typhi, Aspergillus flavus, Candida glabrata, Microsporum canis,* and *Trichphyton longifusus*.

Phytochemical investigations

The chloroform fraction was subjected to column chromatographic techniques which resulted in the isolation of eight pure chemical constituents from the plant and reported here for the first time. Their structures were determined by NMR and mass spectroscopic techniques to allow for the assignment of 1, 2-benzenedicarboxylic acid, bis (2-ethylhexyl) diester (1) [16], eupatorin (2) [17], achillin (3) [18], neoponcirin (4) [19], parvifloroside B (5) [20], betulinic acid (6) [21], β-sitosterol (7) [22], and β- sitosterol glucopyranoside (8) [23]. Spectral information obtained was compared with that previously published [16–23] and structures of these compounds are given in Figure 4.

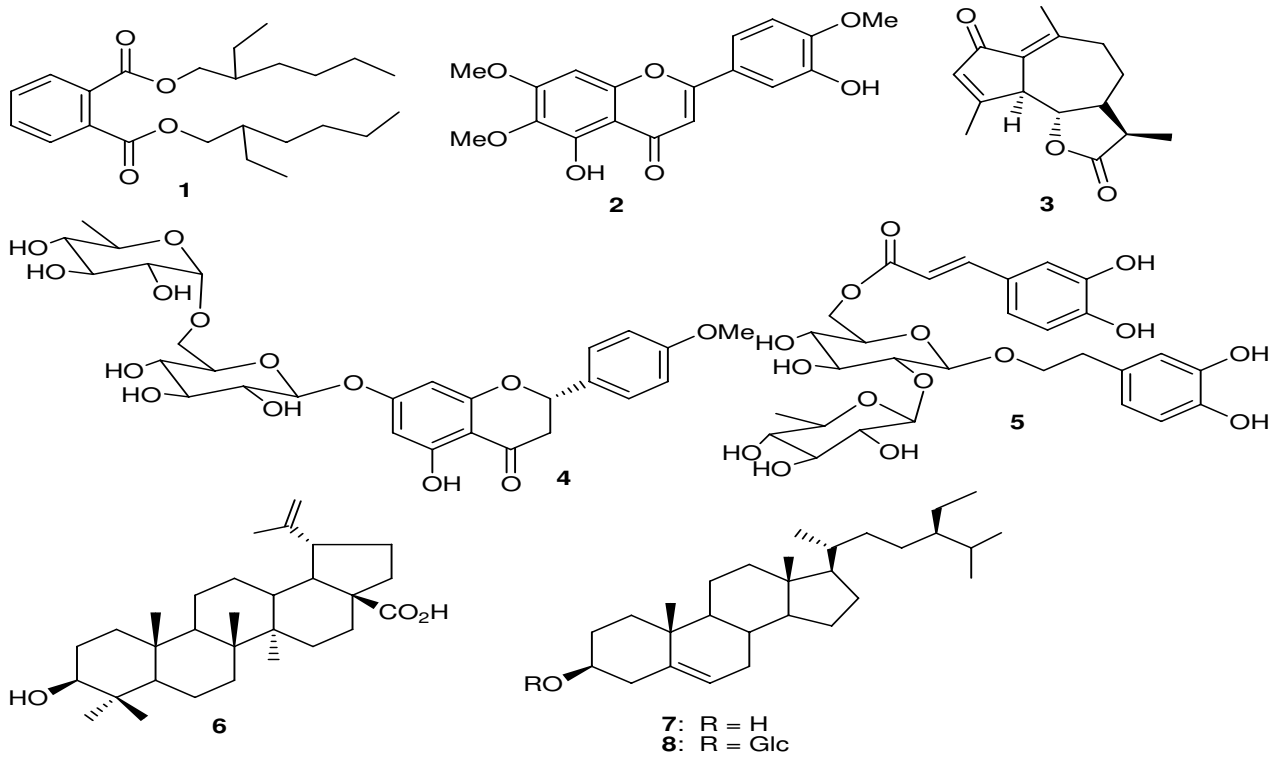

Figure4. Compounds 1–8 isolated from *N. Clarkei*.

Conclusion

On basis of the results obtained in the present study, it is concluded that chloroform and n-hexane fractions of *N. clarkei* have shown potent antiglycation, anticancer, antiplatelet, phytotoxic, and cytotoxic effect. Both fractions were found inactive against tested insects and microbes. Looking for antiglycating agents from natural source, chloroform fraction was applied on silica gel column and eight metabolites were isolated as a result of bio-assay guided isolation. Moreover, further investigation is needed to isolate and identify the active compounds responsible for different. pharmacological activities present in the plant.

Acknowledgments

This study was supported by National Research Program for Universities of HEC (Pakistan).

Conflicts of Interest

State any potential conflicts of interest here or "The authors declare no conflict of interest".

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