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GC-MS analysis and pharmacological potentials of *Neurada procumbens.*

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Abstract

Objective: *Neurada procumbens* L is a medicinal herb with diverse folk history and is traditionally used to treat diarrhea, dysentery, diabetes, eczema, anticonvulsant, rheumatism, laxative, infestinal and gastrointestinal problems, sexual problems, anthelmintic, infections and respiration function. The aim of this study was to prove the uses of plant *N. procumbens* in gastrointestinal and respiratory disorders scientifically.

Method: The phytoconstituents were identified by GC-MS technique. In this study, twelve (12) different kinds of in vitro biological and enzyme inhibition activities were performed to explore the diverse folkloric use of the plant scientifically.

Results: On phytochemical screening, it was proved that plant *N. procumbens* is rich source of flavonoids as dichloromethane and methanol extracts have 143.45 ± 1.25 and 200.69 ± 1.97 mg QE/g of extract, respectively. By GC-MS analysis, nine (9) compounds in dichloromethane extract and six (06) in methanol extract were detected. The dicloromethane extract of whole plant of *N. procumbens* showed anti-lipooxygenase, anti-chymotripsin, anti- alpha glucosidase, anti-urease and anti-tyrosinase activities, whereas methanol extract showed antioxidant, anti-alpha glucosidase, anti-urease, anti-tyrosinase, anti-carbonic anhydrase II, spasmolytic and bronchorelaxant activities.

Conclusion: The study proofs the folk medicinal use of *N. procumbens* to treat diabetes, diarrhea, and improvement in gastrointestinal and respiration functions which justifies the pharmacological importance of plant. Moreover, this study can be considering a solid back ground for further in vivo studies.

Keywords: Alpha glucosidase, Urease, Glass chromatography-mass spectrometry, *Neurada procumbens*

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Introduction

The *Neurada procumbens* is a desert plant belongs to family Neuradaceae and traditionally used a nerve tonic and cooling agent with the name of herbal preparation "Thadal" in summer by local people. Its folkloric uses are as anthelmintic, anticonvulsant, laxative, infestinal problems, eczema, infections, rheumatism and sexual problems [1]. The leaves and fruits of the plant are used for medicinal purposes and whole plant is a source of fodder for camel [2]. It is an annual prostrate densely tomentose herb. The local name of *Neurada procumbens* is "Chapperi Booti" and "Als'dan". According to Flora of West Pakistan, there is only single species distributed in Pakistan from the genus Neurada [3]. The influence of aqueous extract of *N. procumbens* when administered orally increases the blood pressure of rats. The study provides that elevation of blood pressure was due to vasoconstriction on the aortic strips of rats in vitro [4]. It is also reported that ethanolic extract of *N. procumbens* reduced the blood pressure in rabbits [5]. Quinone oxidoreductase 1 inducer activity of plant is also reported [6]. Recently, some flavonoids are isolated from *N. procumbens* in Egypt [7].

Literature survey revealed that there is too much diverse folkloric use of the plant in residants of Cholistan desert but little work regarding biological screening and enzymatic inhibition studies of *N. procumbens* has been done. Therefore, it was need to explore the therapeutical potential of the plant. The objectives of present study were to investigate the secondary metabolites, chemical constituents and enzymatic or biological activities. Despite the multiple ethanomedicinal uses of *N. procumbens* in diarrhea, dysentery, diabetes, inflammation, ulcer, edema, asthma, oxidative stress and skin problems, no research work is available with respect to its effectiveness in these diseases. The present work on the two crude extracts of the plant *N. procumbens* was undertaken for various biological and enzyme inhibition activities to scientifically rationalize the traditional uses and also includes the phytochemical screening or evaluation of the plant.

Materials and Methods

Collection and extraction of plant

The whole plant of *N. procumbens* was collected freshly from Cholistan desert of Bahawalpur Punjab, Pakistan in April 2013 and identified by research officer of Cholistan Institute of Desert Studies, The Islamia University of Bahawalpur, Pakistan. The plant herbarium sheet was deposited for future reference and voucher specimen number 3477/CIDS/IUB was allocated.

The whole plant was dried under shade for 15 days and ground in fine powder by crushing mill. The plant material powder approximately 850 g was macerated in dichloromethane for 24 hours, the process was repeated for three days and then same in methanol for three days. The both dichloromethane and methanol extracts are concentrated separately in solid residue by using the buchi rotary evaporator under reduce pressure. The dichloromethane extract (15.6 g) and methanol extract (14.2 g) were prepared and there yields were 1.8% and 1.6%, respectively.

Chemicals

Lipooxygenase (EC 1.13.11.12), urease (EC 3.5.1.5), alpha glucosidase (EC 3.2.1.20), chymotrypsin (EC 3.4.21.1), tyrosinase (EC 1.14.18.1), DDPH, Thiourea, urea, acarbose, kojic acid, N-succinyl phenyl-alanine-P-nitroanilide, baicalein, chymostatin, eserine, quercetin and gallic acid were purchased from Sigma-Aldrich Co. St. Louis, Mo. USA, Merck and Fluka companies. The dichloromethane and methanol were of highest purity. Na2CO3, NaNO, Sodium hydroxide, hydrochloric acid, Na2PO4, KH2PO4, Tris-HCl buffer, DTNB [5,5-dithiobis (2-nitrobenzoic acid)], DMSO, Folin Ciocalteu reagent, phenol hypochlorite reagent and alkali reagent were of biochemical/analytical grade.

Instrumentation

The instruments synergy HT BioTek® USA 96 microplate reader, Buchi rotary evaporator with vacuum pump, Spectrophotometer (Shimadzo, Japan), Gas chromatography-mass spectrometry (GC Agilent system, USA), EZ-Fit Enzyme Kinetics software (Perrella Scientific Inc. Amherst, USA) and Powerlab Data Acquistion System (AD instrument, Sydney, Australia) were used in this study.

Phytochemical screening

Detection of secondary metabolites: The dry powder of whole plant *N. procumbens* was investigated for the presence of secondary metabolites in plant material of whole plant [8]. The flavonoids, glycosides, cardiac glycosides, sterol and terpenes were present.

Total phenol contents determination: Total phenol contents in the both extracts of *N. procumbens* were calculated by folin-ciocalteu reagent (FCR) by using the reported literature method [9]. The 20 µl of plant extract solution (0.5 mg/mL) was added with folin-ciocalteu reagent (90 µl, 1:10 ratio made with water) in micro plate reader. After that added aqueous sodium carbonate (90 µl, w/v) in it. Total phenol contents were determined at wave length 725 nm. The same above mentioned process was repeated for galic acid (GA) standard solution and the calibration curve was developed. On the basis of measured absorbance, the concentration of total phenol contents was quantified (mg/ml) by using calibration curve. The total phenol contents in both extracts were expressed in term of gallic acid equivalent (mg of GA per gram of extract) in Table 1.

Total flavonoid contents determination: Total flavonoid contents in both extracts of *N. procumbens* were analyzed by the reported method [10]. The plant extract solution (20 µl) was mixed with 80 µl de-ionized water in in microplate. Sodium nitrous oxide (5%) solution (6 µl) was added followed by the addition of aluminum chloride (10%) solution (16 µl) and sodium hydroxide (4%) solution (68 µl). After 20 minutes incubation, the absorbance value was read at 510 nm. Above mentioned procedure was repeated for quercetin (QE) standard solution for construction of calibration curve. Total flavonoid contents were calculated (mg/ml) by using calibration curve. The contents of the flavonoids in both extracts were expressed in term of quercetin equivalent (mg QE per gram of extract) in Table 1.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis is used for identification of chemical constituents present in the both extracts of plant *N. procumbens*. It has great importance in drug detection and identification of unknown phytochemical constituents present in the medicinal plants. So, GC-MS analysis included in the study to investigate the chemical constituents of the selected medicinal plant *N. procumbens*. GC-MS analysis [11,12] was performed on GC Agilent system (B 7890) with mass spectrometer detector (MSD-5977A) employing the following condition: Column HP-5 MS, size 30 m × 0.25 mm, 0.25 µ, composed of 100% dimethyl poly siloxane. The source temperature for ionization was set at 250°C. The 2 µl of dichloromethane and methanol extracts of whole plant of *N. procumbens* were used in GC-MS analysis. The GC-MS results revealed the presence of fifteen (15) compounds in two different extracts of *N. procumbens*. The name, molecular formula, molecular weight and structure of the compounds are given in Tables 2 and 3.

Table 1: *Total phenol and flavonoid contents results in dichloromethane and methanol extracts of N. procumbens.*

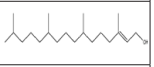
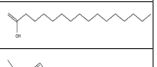
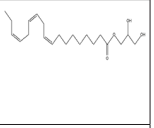
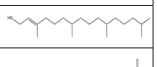
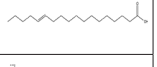
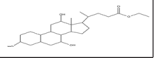
S. No.	Extract code	Total Phenol contents (mg GA/g of extract)	Total Flavonoid contents (mg QE/g of extract)
1	NPD	-------	143.45 ± 1.25
2	NPM	12.37 ± 0.12	200.69 ± 1.97

Biological and enzyme inhibition activities

Antioxidant activity: The DPPH assay is very simple, was performed by reported method [13]. The test solution (10

µl) followed by the addition of 100 µM methanol DPPH solution (90 µl) to make volume of 100 µl in microplate. The mixture was incubated for about half an hour. The absorbance was reduced which was measured at 517 nm using the equipment plate reader. The standard compound is quercetin in this assay. The results are given in Table 4.

Table 2: *Phytochemical constituents identified by GC-MS in dichloromethane extract of N. procumbens.*

S. No.	Phytoconstituent	Mol. Formula	Mol. Weight	Structure
1	3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	
2	12-methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	280	
3	13-Heptadecyn-1-ol	$C_{17}H_{32}O$	252	
4	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	
5	9,12,15-octadecatrienoic acid, 2,3-dihydroxy propyl ester, (Z,Z,Z)-	$C_{21}H_{36}O_4$	352	
6	Phytol	$C_{20}H_{40}O$	296	
7	Trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282	
8	Oleic acid	$C_{18}H_{34}O_2$	282	
9	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	

Cholinesterase inhibition activity: The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition activities were performed according to the method [14,15]. In both assays 60 µl KH2PO4 buffer (100 mM, pH 7.7) and 10 µl extract solution of plant (0.5 mg/ml) were mixed, followed by the addition of 10 µl enzymes. The contents were pre-incubated and pre-read. The reaction was initiated by the addition of 10 µl of DTNB. The absorbance was measured at 405 nm. Eserine was used as a standard. The results were given in Table 4.

Lipoxygenase (LOXs) inhibition activity: The reported method [16] with little modifications was used. The buffer solution of 100 mM potassium dihydrogen phosphate of pH 8 (140 µl), plant extract solution (20 µl) and 15 µl enzyme lipoxygenase (600 units/well) to made total volume of mixture (200 µl). The mixture was read at 234 nm after incubation for 10 minutes at 25°C. On addition of 25 µl solution of substrate, the reaction was initiated. The absorbance was measured after 10 minutes at 234 nm. The positive control was Baicalein (0.5 mM per well) solution. The results are given in Table 4.

Chymotripsin inhibition activity: The reported method [17] with partial modification was used in this study. The total volume of mixture (100 µl) having 50 mM Tris-HCl buffer of pH 7.6 (60 µL), 10 µL of testing plant extract solution (0.5 mg/ml) and 15 µL of enzyme (0.9 units) enzyme. After incubation for 20 minutes at 37°C, the mixture was read at 410 nm. The reaction was started by adding 15 µL of N-succinyl phenyl-alanine-p-nitroanilide substrate (1.3 mM). After half an hour, the absorbance was measured at 410 nm. The positive control was Chymostatin (0.5 mM per well). The results are given in Table 4.

Alpha glucosidase inhibition activity: The α-glucosidase inhibition activity was performed according to method [18]. The 70 µl phosphate buffer (50 mM, pH 6.8) and 10 µl of extract solution (0.5 mg/ml) were mixed, followed by the addition of 10 µl (0.057 units) enzyme. The contents were pre-incubated and pre-read. The reaction was initiated by the addition of 10 µl of p-nitrophenyl-α-D-glucopyranoside. Acarbose was used as standard. The absorbance was measured at 400 nm. The results are given in Table 4.

Urease inhibition activity: Berthelot assay has been used with partial modification [19]. The 10 µl phosphate buffer solution (pH 7.0), 10 µl of plant extract solution (0.5 mg/ml) and 25 µl of enzyme solution (0.1347 units) were added in well. The total volume of mixture was 85 µl. After incubation for five minutes, 40 µl of 20 mM urea solution was added and again incubated. After ten minutes, phenol hypochlorite reagent (115 µl/well) was added (which is fresh mixture of 45 µl phenol reagent and 70 µl of alkali reagent). On incubation at 37°C for 10 minutes, absorbance was read at 625 nm and after colour development. The results are given in Table 4.

Tyrosinase inhibition activity: In this method [20] 60 µl phosphate buffer (100 mM, pH 6.8), 10 µl plant extract solution (0.5 mg/ml) and 10 µl (5 units) of tyrosinase were mixed. The contents were pre-incubated and pre-read. After incubation, 20 µl of 10 mM L-DOPA was added. The absorbance was measured at 490 nm. Kojic acid was used as a standard. The results were given in Table 4.

Carbonic anhydrase II inhibition activity: The reported method [21] has been used for this activity. The HEPES-tris solution (140 µl) and fresh aqueous solution (20 µl) of purified bovine erythrocyte carbonic anhydrase II (0.1 mg/ml) were mixed. The 20 µl of plant extract solution (0.5 mg/ml) in DMSO solvent and 20 µl solution of 4-nitrophenyl acetate (0.7 mM) substrate were added. After incubation for 15 minutes at 25°C, absorbance value was taken at 400 nm. The positive control was solution of acetazolamide in this activity. The results are given in Table 4.

Phosphodiesterase I inhibition activity: In the assay [22], 97 µl of tris-(hydroxymethyl)-aminomethane buffer (50 mM), 20 µl solution of magnesium acetate (20 mM), 15 µl PDE snake venom (7.42 mg/1500 µl) were mixed. After incubation for half hour at room temperature, added 20 µl of plant extract solution (0.5 mg/ml) and reaction was started by addition of 60 µl of bis (p-nitrophenyl) phosphonate (0.33 mM) dissolved in ammonium acetate (20 mM). The absorbance was measured at 410 nm. The EDTA was used as standard. The results are given in Table 4.

Calculations and statistical analysis:

The percentage enzyme inhibition will be calculated by the following formula:

Inhibition (%)=100 - (Absorbance of test sample/ Absorbance of control) × 100.

Whereas

Absorbance of test sample=Activity in the presence of test extract

Absorbance of control=Total enzyme activity without inhibitor (extract)

IC50 values (concentration at which there is 50% in enzyme catalyzed reaction) compounds were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA). For the determination of IC50 values, test solutions were assayed at various dilutions i.e. 0.5, 0.25, 0.125, 0.0625 mg/ml. All the results (Tables 4 and 5) are mean ± SEM (standard error of mean) of triplicate values (n=3). The statistical parameter applied is the student's t-test with $p<0.05$ consider as significant.

Antibacterial activity: The antibacterial activity was performed in sterile 96-wells microplates under aseptic conditions. The method is based on the principle that microbial cell number increases as the microbial growth proceeds in a log phase of growth which results in increased absorbance of broth medium [17]. Two gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) and three gram-negative (*Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli*) were included in the study. The microorganisms were maintained on stock culture agar medium. The extract solutions were pipette into wells (100 µg/well). Overnight maintained fresh bacterial culture after suitable dilution with fresh nutrient broth was poured into wells (180 µl). The initial absorbance of the culture was strictly maintained between 0.12-0.19 at 540 nm. The absorbance was measured at 540 nm, before and after incubation and the difference was noted as an index of bacterial growth. Ciprofloxacin was used as a standard. The results are given in Table 5.

Spasmolytic activity on isolated tissues: The animals used in the present study were local breed rabbits of both sexes, weighing 1.0-1.5 kg and maintained at 25°C in Animal House, Faculty of Pharmacy, Bahauddin Zakariya University, Multan (Pakistan). These were given fresh green fodder and tap water *ad libitum*. The experiments were performed in accordance with rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (NRC, 1996). The animals were subjected to fasting for 12 hours before experiments but provided free access to drinking water. The rabbits were slaughtered by stunning a blow on the back of head.

The isolated rabbit jejunum preparations were used for screening of the spasmolytic activity of the methanol extracts of *N. procumbens* [23]. The animals were dissected and jejunum was excised and placed in a petri dish having Tyrode's solution at 35 ± 2°C and aerated with carbogen (95% oxygen+5% carbon dioxide). The jejunum was rendered free of attached mesenteries carefully by means of sharp scissor and cut into pieces of 2 cm in length. The segments of isolated rabbit jejunum were mounted in isolated tissue organ bath filled with Tyrode's solution (10 ml) and bubbled with carbogen at 37°C. The isolated rabbit jejunum preparation was allowed to be equilibrated for 30 minutes being attached to isotonic transducer under applied tension of 0.50 mg. The spontaneous periodic contractile and relaxant activity of the mounted isolated rabbit jejunum preparations were recorded isotonically by using Power Lab Data Acquisition System.

Verapamil was used as standard drug possessing relaxant effect on spontaneous contractile activity of isolated rabbit jejunum preparation. The test plant extract (NPM) as well as standard drugs were applied to the isolated rabbit jejunum preparation in different tissue bath concentrations and response observed was used to construct graphs showing concentrations versus responses to determine the respective EC50 by using Graphpad software.

Bronchorelaxant activity on isolated tissues: The crude extract (NPM) had been screened for possible bronchorelaxant activity on isolated rabbit tracheal preparations [24]. The rabbit body was dissected and trachea was excised, cut into rings of about 2-4 mm width in a manner that each ring may contain two cartilages. The rings were opened by longitudinal cut on ventral side opposite to the smooth muscle layer. Thus, forming tracheal strips with smooth muscle sandwiched in between cartilaginous parts on the edges. The preparation was suspended in a 10 ml tissue bath containing Krebs solution at 37°C aerated with carbogen. About 1.0 gram tension was implemented to each of tracheal strip and was permitted to be equilibrated for one hour prior to recording of isometric contractions of tracheal preparation via force displacement transducers connected to power lab. The bronchorelaxant effect of the methanol extract of *N. procumbens* (NPM) was screened on pre-contracted isolated rabbit tracheal preparations with carbachol (1 µM) as well as K+ (80 mM). The NPM exerted relaxant effect on carbachol (1.0 µM) as well as K+(80 mM)-induced contractions in isolated rabbit tracheal preparation when applied in cumulative manner. The experiment was performed in triplicate on each preparation from 5 different animals in order to minimize animal to animal variation in results.

Results and Discussion

The dichloromethane and methanol extracts of *N. procumbens* were evaluated for secondary metabolites, total flavonoids and phenol contents [8-10] and presence of pharmaceutical constituents in crude extracts by using the Gas chromatography-mass spectrometry (GC-MS). Total flavonoid contents were higher in methanol extract (200.69 ± 1.97) with respect to dichloromethane extract (143.45 ± 1.25), whereas total phenol contents were 12.37 ± 0.12 only in methanol extract mg GA/g of *N. procumbens* extract (Table 1). The nine (09) compounds were identified in dichloromethane extract (NPD) and six (06) compounds were identified in methanol extract (NPM) of whole plant of *N. procumbens* (Table 2 and 3).

The total fifteen (15) compounds; (1) 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, (2) 12-methyl-E,E-2,13-octadecadien-1-ol, (3) 13-heptadecyn-1-ol, (4) n-hexadecanoic acid, (5)

9,12,15-octadecatrienoic acid, 2,3-dihydroxy propyl ester, (Z,Z,Z)-, (6) Phytol, (7) Trans-13-octadecenoic acid, (8) Oleic acid, (9) Ethyl iso-allocholate, (10) Pterin-6-carboxylic acid, (11) Ergosta-5,22-dien-3-ol, acetate, (3β,22E)-, (12) 5H-cyclopropa [3,4] benz [1, 2-e]azulen-5-one, 9, 9a-bis (acetyloxy)-1, 1a, 1b, 2, 4a, 7a, 7b, 8, 9, 9a-decahydro-2, 4a, 7b-trihydroxy-3-(hydroxyl methyl)-1, 1, 6, 8-tetra methyl-, [1aR-(1aα,1bβ,2β,4aβ, 7aα, 7bα, 8α, 9β, 9aα]-, (13) Hexadecanoic acid, methyl ester, (14) 7,10-Octadecadienoic acid and (15) 10-Octadecenoic acid, methyl ester were identified in whole plant material extracts (NPD and NPM) of *Neurada procumbens* by performing the GC-MS analysis.

The twelve (12) different kinds of in vitro biological and enzyme inhibition activities antioxidant, anticholinesterase, antilipooxygenase, antichymotripsin, antialpha glucosidase, antiurease, antityrosinase, anticarbonic anhydrase II, antiphosphodiesterase I, antibacterial, spasmolytic and brochorelaxant activities were performed including the two activities (spasmolytic and bronchorelaxant activities) on isolated tissues to evaluate the diverse folkloric use of the plant *N. procumbens* scientifically (Table 4). The dichloromethane extracts (NPD) of *N. procumbens* showed antilipooxygenase, antichymotripsin, antialpha glucosidase, antiurease and antityrosinase activities. The methanol extracts (NPM) of *N. procumbens* showed antioxidant, antichymotripsin, antialpha glucosidase, antiurease, antityrosinase, anticarbonic anhydrase II, spasmolytic and brochorelaxant activities. Spasmolytic activity of methanol extract (NPM) on rabbit jejunum was demonstrated (Figures 1A and 1B) caused concentration dependant inhibition of spontaneous contractions (0.01-1.0 mg/ml) with EC50 value 0.157 µM.

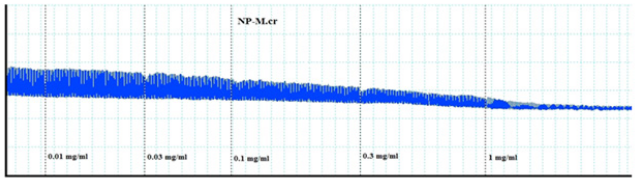


***Figure 1(A)**. Tracing showing influence of methanol extract of N. procumbens (NPM) on spontaneous rhythmic contractions in isolated rabbit jejunum preparations.*

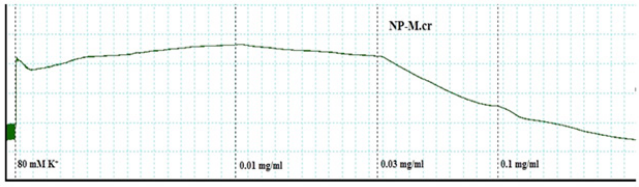


***Figure 1(B)**. Tracing showing influence of methanol extract of N. procumbens (NPM) on K+(80 mM)-induced contractions in isolated rabbit jejunum preparations.*

Spontaneous and K+(80 mM) induced contractions (0.01-0.1 mg/ml) with EC50 value 0.024 µM. Spontaneous and K+(80 mM) induced contractions were completely inhibited by standard drug verapamil (Figures 2 and 3).

Bronchorelaxant activity of methanol extract (NPM) on rabbit trachea, caused relaxation for carbachol (1.0 µM) between 0.011-0.3 mg/ml with EC50 value 0.624 µM and high K+ (80 mM) induced contractions (0.01-0.1 mg/ml) with EC50

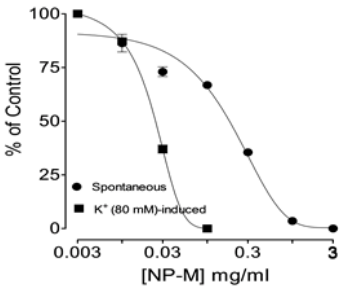


***Figure 2.** Effect of methanol extract of N. procumbens (NPM) on spontaneous rhythmic and K+(80 mM)-induced contractions in isolated rabbit jejunum preparations.*

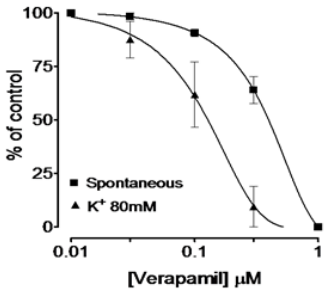


***Figure 3.** Effect of verapamil on spontaneous rhythmic and K+(80 mM)-induced contractions in isolated rabbit jejunum preparations.*

value 0.096 µM (Figures 4 and 5). The isometric contractile response was recorded through Power lab Data Acquisition system (AD instruments, Sydney, Australia) attached to a computer system having software Lab Chart (version 7). The standard drug Verapamil having Ca++ channel blocking effect was tested on carbachol (1.0 µM) and K+(80 mM) induced spastic contractions to evaluate the spasmolytic and bronchorelaxant effect of methanol extract (NPM) of *N. procumbens*. *N. procumbens* has folkloric value in the management of multiple diseases pertaining to hyperglycemia (diabetes), gastrointestinal (diarrhea, dysentery, ulcer), cardiac (antioxidant use) and respiratory (asthma, inflammation) systems. The present work was a scientific validation of folkloric claims of the plant.

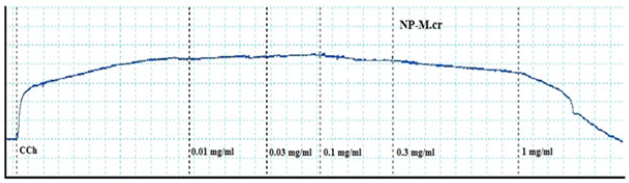


***Figure 4(A)**. Tracing showing influence of methanol extract of N. procumbens (NPM) on cabachol (1 µM)-induced contractions in isolated rabbit tracheal preparations.*

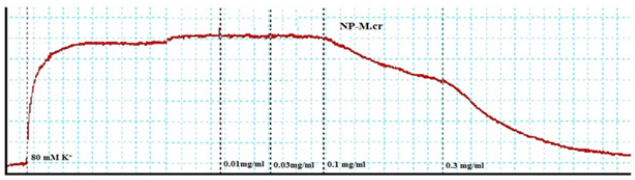


***Figure 4(B)**. Tracing showing influence of methanol extract of N. procumbens (NPM) on K+(80 mM)-induced contractions in isolated rabbit tracheal preparations.*

Table 3: *Phytochemical constituents identified by GC-MS in methanol extract of N. procumbens.*

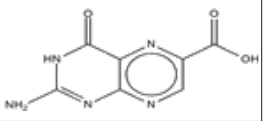
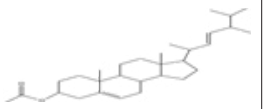
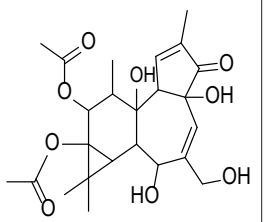
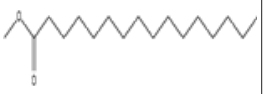
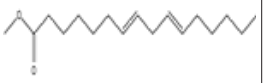
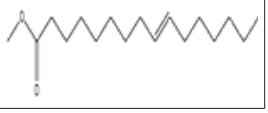
S. No.	Phytoconstituent	Mol. Formula	Mol. Weight	Structure
1	Pterin-6-carboxylic acid	C7H5N5O3	207	
2	Ergosta-5,22-dien-3-ol,acetate, (3β,22E)-	C30H48O2	440	
3	5H- cyclopropa [3, 4] benz [1,2–e] azulen–5- one, 9, 9a-bis (acetyloxy)-1, 1a, 1b, 2, 4a, 7a, 7b, 8, 9, 9a-decahydro-2, 4a, 7b-trihydroxy-3- (hydroxyl methyl)-1,1,6,8- tetra methyl-, [1aR -(1aα,1bβ, 2β,4aβ,7aα,7bα,8α,9β,9aα]-	C24H32O9	464	
4	Hexadecanoic acid, methyl ester	C17H34O2	270	
5	7,10-octadecadienoic acid	C19 H34O2	294	
6	10-octadecenoic acid, methyl ester	C19 H36O2	296	

Table 4: *Results of biological and enzyme inhibition activities of dichloromethane and methanol extracts of whole plant of N. procumbens.*

S. No.	Biological and enzyme inhibition activities	Standard / control		DCM extract		Methanol extract	
		Inhibition (%)	IC50 (µMoles)	Inhibition (%)	IC50 (µMoles)	Inhibition (%)	IC50 (µMoles)
1	Antioxidant activity	83.68 ± 3.76 Quercetin	16.96 ± 0.14 Quercetin	1.28 ± 1.66	Inactive	75.38 ± 3.16	88.30 ± 1.21
2	Acetylcholin-esterase inhibition activity	91.27 ± 0.17 Eserine	0.04 ± 0.001 Eserine	-38.27 ± 0.48	Inactive	23.88 ± 0.01	Inactive
3	Butyrylcholi-nesterase inhibition activity	91.27 ± 0.17Eserine	0.04 ± 0.001 Eserine	-38.27 ± 0.48	Inactive	23.88 ± 0.01	Inactive
4	Lipoxygenase inhibition activity	93.79 ± 1.27 Baicalein	22.47 ± 0.04 Baicalein	44.60 ± 0.23	524.60 ± 1.02	31.70 ± 0.76	Inactive
5	Chymotripsin inhibition activity	93.50 ± 0.91 Chymostatin	8.24 ± 0.11 Chymostatin	84.38 ± 0.05	115.71 ± 0.03	18.60 ± 0.03	Inactive
6	Alpha glucosidase inhibition activity	92.23 ± 0.14 Acarbose	38.25 ± 0.12 Acarbose	95.49 ± 0.65	24.32 ± 0.62	99.45 ± 0.58	8.43 ± 0.56
7	Urease inhibition activity	82.11 ± 0.14 Thiourea	22.04 ± 0.12 Thiourea	57.23 ± 0.50	375.60 ± 0.58	61.90 ± 0.41	315.00 ± 2.91
8	Tyrosinase inhibition activity	93.50 ± 0.14 Kojic acid	6.04 ± 0.11 Kojic acid	57.23 ± 0.50	321.60 ± 0.45	63.92 ± 0.41	305.00 ± 6.91
9	Carbonic Anhydrase II inhibition activity	89.03 ± 0.03 Acetazolamide	0.10 ± 0.04 Acetazolamide	37.43 ± 0.41	Inactive	73.92 ± 0.41	102.31 ± 0.85
10	Phosphodiester-ase I inhibition activity	69.02 ± 0.02 EDTA	277.69 ± 2.52 EDTA	1.93 ± 0.41	Inactive	30.92 ± 0.33	Inactive

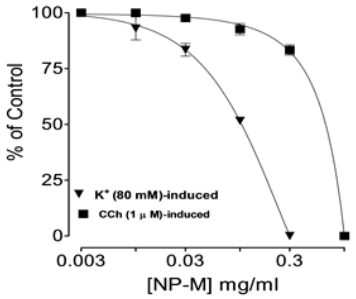


***Figure 5**. Effect of methanol extract of N. procumbens (NPM) on carbachol (1 μM)- and K+(80 mM)-induced contractions in isolated rabbit tracheal preparations.*

On phytochemical screening, it was found that *N. procumbens* is rich source of flavonoid contents (Table 1). The fifteen (15) compounds were identified in the plant first time by using the GC-MS technique (Tables 2 and 3). The only methanol extract (NPM) possess very good antioxidant activity with inhibition 75.38 ± 3.16% with IC50 88.30 ± 1.21 μMoles which is significant comparable to standard quercetin (83.68 ± 3.76% with IC50 16.96 ± 0.14 μMoles) proving the antioxidant potential of the plant in oxidative stress and cardiac problems [25-27]. The products of lipoxygenase play a vital role in many medical disorders such as inflammation, bronchial asthma [29] and tumor angiogenesis [30]. The only dichloromethane extract NPD possess moderate lipoxygenase inhibition activity with value 44.60 ± 0.23% with IC50 524.60 ± 1.02 μMoles in comparison to standard Baicalein (93.79 ± 1.27% with IC50 22.47 ± 0.04 μMoles), which linking the importance of the plant in bronchial problems. The dichloromethane extract exhibited 84.38 ± 0.05% inhibition of chymotrypsin with IC50 value 115.71 ± 0.03 μM which is significant and close to value of standard chymostatin (93.50 ± 0.91%) which indicates that non-polar or less polar compounds present in this extract are responsible for this activity and proving the folkloric importance of plant in the treatment of edema and inflammation [31-33].

The alpha glucosidase enzyme catalysis the hydrolysis of disaccharides into glucose, inhibition of this enzyme can suppress the post prandial hyperglycemia and this inhibition will be useful invention for management of diabetes type II [36]. The alpha glucosidase inhibition activity of dichloromethane and methanol extracts of *N. procumbens* was significant and higher than acarbose. The dichloromethane extract possess percentage inhibition 95.49 ± 0.65 with IC50 24.32 ± 0.62 μMoles which was more potent than standard inhibitor acarbose (92.23 ± 0.14% with IC50 38.25 ± 0.12 μMoles) whereas the percentage inhibition and IC50 values of methanol extract (99.45 ± 0.58 with IC50 8.43 ± 0.56 μMoles) was found five times more potent than the standard acarbose. Urease is of medical importance because it is involved for establishing disease state like peptic ulcer, kidney stones, pyelonephritis and other diseases [30]. So due to the role of urease in the production of certain diseases it is of much importance in pharmaceutical research [28]. Urease enzyme is found in most of the organisms. It breaks down the urea to ammonium carbonate and is mostly used for determination of urea [33]. Urea catalysis is carried out by urease forming carbonate anions and ammonium cations. Research is going on urea in many laboratories as it has gained much worth in biotechnology and medicine [34]. The both NPD and NPM extracts possess urease inhibition activity with values 57.23 ± 0.50% with IC50 375.60 ± 0.58 μMoles and 61.90 ± 0.41% with IC50 315.00 ± 2.91 μMoles respectively, whereas the standard thiourea (82.11 ± 0.14% with IC50 22.04 ± 0.12 μMoles) was used which indicates the non-polar or less polar compounds present in the plant producing this activity.

Tyrosinase over production causes hyperpigmentation and ocular retinitis pigmentosa. Hyperpigmentation in human skin makes the researcher to findout a potent tyrosinase inhibitor as skin whitening agent [37]. This enzyme is also related to the formation of neuromelanin in human brain which leads to neurodegeneration [25]. Keeping in view this importance, tyrosinase inhibition activity was evaluated and found 57.23 ± 0.50% and 63.92 ± 0.41% for dichloromethane extract (NPD) and methanol extract (NPM), respectively against the standard Kojic acid (93.50 ± 0.14%). The results are given in Table 4. The antibacterial activity of both dichloromethane and methanol extracts was evaluated against two gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) and three gram-negative (*Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli*). The results are given in Table 5 shows that bacterial growth inhibition is ranging from 39.62 ± 2.45 to 48.83 ± 3.83%, which is not significant and inhibition was less than 50% against all strains.

The folkloric uses in gastrointestinal and respiratory systems of the *N. procumbens* was evaluated by searching spasmolytic and bronchorelaxant activities by observing the effect of methanol extract of the plant (NPM) on isolated tissues of rabbit jejunum and trachea. Spasmolytic activity on rabbit jejunum was established using methanol extract of *N. procumbens*. The concentration-dependent inhibition of spontaneous contractions (0.01-1 mg/ml) with EC50 value 0.157 μM and K+ (80 mM) induced contractions (0.01-0.1 mg/ml) with EC50 value 0.024 μM were observed. Spontaneous K+ (80 mM) induced contractions were completely inhibited by standard drug verapamil (Figures 1A and 1B).

Inhibitory effect of crude methanol extract of *N. procumbens* was observed on spontaneous contraction of rabbit jejunum that suggests a spasmolytic activity similar to verapamil (Figures 2 and 3). Verapamil was used as standard exhibited relaxation effect on spontaneous contracting jejunum in dose range of 0.03-1.0 μM with EC50 value 0.35 μM and K+ (80 mM) in dose range 0.03-0.3 μM with EC50 value 0.13 μM (Figure 3). The possible mechanism was investigated by using a high K+ (80 mM) was used to cause smooth muscles contractions through opening of calcium channels which results in increase of intra cellular calcium and contractile effect [7]. This result supports the traditional use of *N. procumbens* in hyperactive gut or in disease diarrhea as calcium antagonist [35-38].

Justification for the folk use of *N. procumbens* in respiratory disorders was obtained by experiments on the isolated rabbit trachea. The Figures 4A, 4B and 5 show that methanol extract of *N. procumbens* relaxed tracheal preparation pre contracted with carbachol (1.0 μM) or K+ 80 mM in a concentration dependent fashion similar to standard verapamil. It indicates

Table 5: *Results of antibacterial activity of dichloromethane and methanol extracts of whole plant of N. procumbens.*

Extract code	Type	Bacillus subtilis (G+ve)	Staphylococcus aureus (G+ve)	Pseudomonas aeruginosa, (G-ve)	Salmonella typhi (G-ve)	Escherichia coli (G-ve)
DCM Extract (NPD)	Inhibition (%)	40.28 ± 0.60	42.89 ± 1.93	39.62 ± 2.45	40.48 ± 0.10	45.45 ± 3.64
	MIC50	Inactive	Inactive	Inactive	Inactive	Inactive
Methanol Extract (NPM)	Inhibition (%)	48.83 ± 3.83	46.86 ± 2.94	43.68 ± 0.19	45.19 ± 1.00	45.91 ± 3.73
	MIC50	Inactive	Inactive	Inactive	Inactive	Inactive
Ciprofloxacin (standard)	Inhibition (%)	91.23 ± 1.07	91.23 ± 1.07	90.88 ± 0.16	92.65 ± 1.10	91.45 ± 2.19
	MIC50	7.52 ± 0.67	7.03 ± 0.53	7.58 ± 0.19	7.23 ± 0.71	8.21 ± 0.11
Note: All extracts are soluble in methanol.						

non-specific bronchodilation of trachea mediated through calcium channel blockage. When methanol extract of *N. procumbens* was applied against carbachol (1.0 μM) and high K+ (80 mM), it produced bronchodilator effect on rabbit trachea and concentration dependent inhibition with respective EC50 value of 0.624 mg/ml (0.01-0.3 mg/ml) and value of EC50 was 0.096 mg/ml (0.01-0.1 mg/ml) (Figures 4A and 4B). In our previous work, we have evaluated and proved the spasmolytic effect of another medicinal plant *Farsetia hamiltonii* Royle from the same source Cholistan desert of Pakistan [38].

Conclusion

The results of antioxidant, antilipoxygenase, antichymotrypsin, anti-alpha glucosidase, antiurease, antityrosinase, anticarbonic anhydrase II, spasmolytic and brochorelaxant activities obtained from this study may provide the scientific evidence for folkloric uses of *Neurada procumbens* in treating oxidative stress, asthma, inflammation, edema, diabetes, skin, gastrointestinal and respiratory disorders. So, this study offers rational hypotheses for antidiarrheal, antiasthmatic and antidiabetic medicinal uses of *N. procumbens* L. Further, work is required regarding evaluation of medicinal effects of fifteen (15) identified compounds from this study to find out the molecule, which may make basis of the development of a new drug in gastrointestinal, respiratory ad hyperglycemic disorders.

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Author's contribution

It is declared that this work was done by first author under the supervision of second author named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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