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3. Material and Methods

3.1. Collection and authentication of plant material

Fresh and disease free leaves, stems and roots of the white flowered *N. indicum* plant were collected from the garden of University of North Bengal (26.71°N, 88.35°S), West Bengal, India.

The plant material was identified and authenticated by Prof. Abhaya Prasad Das, senior Taxonomist of Department of Botany, University of North Bengal. A voucher specimen was stored at the herbarium of Department of Botany, University of North Bengal with an Accession no. 09618.

3.2. Preparation of extracts

The plant materials were washed with double distilled water to remove any dirt. The stems and roots were chopped into ½ inch pieces. The plant materials were then shade dried at room temperature (RT) for 2 weeks. The dried parts were grinded to powder using a blender (Lords® Hummer 1100). The powder (100 g) was mixed with 1000 ml of 7:1 methanol: water (v/v) and kept at 37 °C in a shaking incubator (160 rpm) for 18 h. The mixture was then centrifuged at 5000 rpm for 15 min. The supernatants were filtered using a vacuum pump and stored separately. The remaining pellet was again mixed with 1000 ml of 7:1 methanol **:** water (v/v) and again kept in a shaking incubator (160 rpm) for 18 h. The supernatants were again filtered and mixed with the stored filtrate of the previous phase. The final filtrate was concentrated under reduced pressure in a rotary evaporator (Buchi Rotavapour®). The resultant was lyophilized (SJIA-10N) and stored at -20 °C until further use. The hydro-methanolic extracts of *N. indicum* leaf, stem and root was designated as NILE, NISE and NIRE, respectively.

3.3. Maintenance of animals

All the animals were maintained under standard laboratory conditions in the animal house of Department of Zoology, University of North Bengal with food and water ad libitum under a constant 12-h photoperiod (temperature 25±2 °C). All the experiments were approved by the ethical committee of the University of North Bengal (No. 840/ac/04/CPCSEA) and performed in accordance with the legislation for the protection of animals used for scientific purposes.

Swiss albino mice of both sex of 6-8 weeks were used for all the studies. Blood for immunization purpose was collected from sheep by puncturing the jugular vein with a sterile syringe. The blood was diluted with equal volume of Alsever's solution (114 mM dextrose, 27 mM sodium citrate, 71 mM NaCl, pH 6.1) and stored at 4 °C as sheep RBC solution (sRBC) until further use. Blood was collected from a guinea pig by puncturing the heart using a sterile needle and allowed to clot at 4 °C for 30 min to separate the serum. The blood was then centrifuged at 1000 rpm for 5 min and the clear supernatant serum was collected. This pooled serum was used as guinea pig complement and stored at 0 °C to minimize the complement activity. All surgical procedures were performed following standard procedures according to Reeves and Reeves (2001).

3.4. Acute toxicity study

OECD guidelines (test 423: Acute oral toxicity – Acute toxic class method; 2002) were followed to study the acute toxicity of NILE, NISE and NIRE (OECDiLibrary, 2002). Animals were divided into different groups (n=6) and fasted overnight prior to the experiment. The plant extracts were administered orally in an increasing dose upto 2000 mg/kg body weight (BW). Following the dose, all the groups were carefully observed for development of clinical or toxicological symptoms at 30 min and then 2, 4, 8, 24 and 48 h.

No mortality was observed in the experimental animals at 2000 mg/kg dose. Therefore, $1/40^{th}$, $1/20^{th}$ and $1/10^{th}$ of the maximum dose was considered for the *in vivo* studies .

3.5. Immunomodulatory activities

The immunomodulatory activities of leaf, stem and root extracts of *N. indicum* were evaluated through both *in vivo* and *in vitro* experiments:

3.5.1. *In vivo* experiments

3.5.1.1. *Plaque forming cell (PFC) assay*

Previously described methods (Raisuddin, *et al*., 1991; Bondada & Robertson, 2003) were followed with slight modifications to perform the PFC assay. Briefly, Swiss albino mice were divided into 10 groups (n=6). Group I (control) received normal saline; group II, V and VIII received NILE, NISE and NIRE at 50 mg/kg BW respectively; group III, VI and IX received NILE, NISE and NIRE at 100 mg/kg BW respectively; and group IV, VII and X received NILE, NISE and NIRE extract at 200 mg/kg BW respectively, for 10 consecutive days. On day 7, mice were immunized with 0.1 ml of 25% sRBC intravenously through tail vein. On day 4 after immunization with sRBC i.e. 24 h after the last treatment with the extracts, whole blood was collected from the tail vein initially. Mice were sacrificed under proper anesthesia and blood was collected from the heart and used for HA titre assay. Single cell suspension (2×10^6 cells/ml) of the spleen was prepared in phosphate buffer saline (PBS). Guinea pig complement (50 µl) and 50 µl of 25% sRBC (prepared in PBS) were mixed with 100 µl of the cell suspension. The mixture was then charged into the Cuningham chambers, prepared using glass slide and double gum tape. The chambers were then sealed with sealing material prepared by mixing paraffin and petroleum jelly at 1:1 ratio and incubated at 37 °C for 3-4 h. After incubation, the plaques were counted under a binocular microscope and expressed as PFC/10^6 spleen cells.

3.5.1.2. *Estimation of total IgM*

Blood samples from immunized mice of the PFC assay were kept at room temperature for 30 min for separation of serum. The whole blood was then centrifuged at 1000 rpm for 5 min and the straw coloured supernatant serum was collected in fresh vials. The serum was diluted 10,000 fold and then used to estimate the total IgM level using commercially available kit (MyBiosource, USA) according to manufactures instruction. The final quantity was multiplied by the dilution factor.

3.5.1.3. *Hemagglutination (HA) titre assay*

HA titre assay was performed according to a standard method (Karthikumar, *et al*., 2011) with slight modifications. The serum samples collected from the mice of the PFC assay were kept at 56 ^oC for 45 min in a water bath to inactivate the complement activity. Eight clear Khan tubes were taken and double fold diluted serum was added. In the first tube, 0.1 ml serum and

0.9 ml PBS were added. After thorough mixing, 0.5 ml of the mixture was taken to the subsequent tubes. This way, serial dilutions were prepared in the ratio of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, and 1/1280. Hundred microliters of 10% sRBC (prepared in PBS) was added to each tube and mixed thoroughly. The tubes were incubated at 37 ^{o}C for 12 h in a humidified atmosphere. After incubation, visible hemagglutination was observed under the binocular microscope. The highest dilution which gave rise the visible hemagglutination was considered as the optimum concentration of the antibody and the antigen.

3.5.1.4. *Counting of peritoneal macrophages*

Different sets of mice were fed orally with NILE, NISE and NIRE for 14 days as described in the PFC assay. A separate group was used as control, which was not treated with any extracts. Twenty four to 36 h prior to the experiment, 0.5 ml of Freund's incomplete adjuvant was injected (Chakraborty & Chakravarty, 1984) in the peritoneum. RPMI-1640 (2 ml) was injected intraperitoneally just prior to the experiment. Under proper anesthesia, a midline incision was made in the abdomen and the peritoneum was carefully washed with RPMI-1640. The peritoneal exudate cells were collected and centrifuged at 1000 rpm at 4 °C, for 5 min (Fortier & Falk, 2001). The pellets were resuspended in RPMI-1640 and incubated for 45 min at 37 °C in petri dish. After incubation, the supernatants were removed and the petri dishes were washed with chilled PBS and centrifuged at 1000 rpm for 5 min. The pellets were resuspended in PBS and the solutions were mixed with equal volume of neutral red and charged on the hemocytometer to count macrophages under a phase contrast microscope.

3.5.1.5. *Stimulation of phagocytic activity of macrophages*

The assay was performed according to a standard method (Fujiki and Yano, 1997) with little modifications. Murine peritoneal macrophages were collected as previously described and 0.1 ml cell suspensions from each group were mixed with 0.1 ml RPMI-1640 medium containing 20% FBS and 100 × 10^6 cells/ml heat-treated (inactivated) yeast cells. The mixtures were incubated at 37 ^{o}C for 1 h with occasional shaking. After incubation, 50 µl of the mixtures were smeared on the glass slide, air dried and stained with Wright-Giemsa stain. The slides were observed under light microscope using oil immersion and around the cells were counted. The phagocytic activity was expressed as phagocytic capacity (PC) and phagocytic index (PI) was calculated using the following formula: PI = A × B, where, A is the percentage of yeast-ingesting phagocytes and B is the number of yeast-ingested per phagocytes. PC is the mean percentage of cells that engulfed ≥4 yeast cells.

3.5.1.6. *Carbon Clearance test*

The test was performed according to a standard method (Gonda, *et al*., 1990) with minor modifications. Different doses (50 and 200 mg/kg) of NILE, NISE and NIRE were administered orally for 14 days to Swiss albino mouse and a control group received water as previously described. On 16^{th} day (48 h after the last dose), 0.1 ml of Indian ink was injected in tail vein and then, 25 µl blood samples were drawn from the orbital vein at 0, 5, 10 and 15 min after injection and mixed with 2 ml of 0.1% Na_2CO_3. The absorbance was read at 650 nm to estimate the extent of carbon clearance or in other words the rate of the elimination of carbon from the blood.

3.5.2. *In vitro* experiments

3.5.2.1. *Cell adhesion assay*

The assay was performed according to the previously described method (Lin, *et al*., 1995) with some modifications. Briefly, 0.5 ml of Freund's incomplete adjuvant was injected intraperitoneally to untreated (not fed with extracts) mouse and the peritoneal exudate macrophages were collected as previously described. The cells were centrifuged at 1000 rpm at 4 °C, for 5 min. The supernatant was discarded and the pellet was resuspended in RPMI-1640. The number of cell was adjusted to 2×10^6 cells/ml. The cells were then seeded in 96 well plate with different concentrations (0-100 µg/ml) of NILE, NISE and NIRE for 60 min. After incubation, the wells were gently washed with RPMI and then 100 µl of 0.5% crystal violet (dissolved in 12% neutral formaldehyde) and 10% ethanol were added to each well and incubated for 4 h at 37 ^oC under humidified condition. After incubation, the wells were washed with RPMI-1640 and air dried for 30 min. To each well 100 µl of 1% SDS (dissolved in RPMI-1640) was added and the absorbance was measured at 570 nm. The increase in adherence property was measured using the following formula: % increase of adherence = $[(A_o - A_1) \div A_o] \times 100$, where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the plant extracts.

3.5.2.2. *Respiratory burst activity*

The assay was performed in accordance with a previously standardized protocol with some modifications (Cook, *et al*., 2001). Murine peritoneal exudate macrophages were collected in RPMI-1640 as previously described and seeded into 96 well plate which was pre-coated with 0.2% poly-L-lysine along with various concentrations (0-100 µg/ml) of *N. indicum* extracts. To this, 0.1% zymosan (in 100 µl RPMI-1640) was added and the plate was incubated for 30 min at 37 °C under humidified condition. The zymosan was discarded and cells were washed thrice with RPMI-1640 followed by staining with 100 µl NBT (0.3%) at RT. After 30 min, NBT solution was discarded and the reaction was stopped by addition of 100 µl absolute

methanol. The formazan which was generated, was dissolved in 120 µl of 2M KOH and 140 µl of DMSO. Absorbance was immediately read at 630 nm. Dose-dependent increase of OD demonstrates increase in respiratory burst activity.

3.5.2.3. *Myeloperoxidase release assay*

The assay was performed according to Sengupta, *et al*., (2011) with minor modifications. Murine peritoneal macrophages (2×10^6 cells/ml) were seeded into 96 well culture plates with 100 ng/ml LPS. To this, varying concentrations (0-100 µg/ml) of *N. indicum* extracts were added and the cells were incubated at 37 °C under humidified condition for 60 min. After incubation, the solutions from each well were centrifuged for 13,000 rpm for 10 min and the supernatants were removed. To the cell-pellet, 0.01% SDS in RPMI-1640 was added to lyse the cells. The solutions were centrifuged at 13,000 rpm for 10 min and supernatant was collected. Supernatants (100 µl) from each group were mixed with 100 µl substrate buffer (orthophenylenediamine) and kept at 37 °C. After 20 min, the reaction was stopped using 100 µl of 2N H_2SO_4. Absorbance was read at 492 nm.

3.5.2.4. *Inhibition of LPS induced nitric oxide production*

Standard Griess reagent method (Hibbs, *et al*., 1988) was followed with some modifications to estimate the change in NO level. Peritoneal macrophage cells were collected as previously described. Cell suspension (2×10^6 cells/ml) was prepared in RPMI-1640 (containing 50 U/ml penicillin, 50 U/ml streptomycin and 50 U/ml nystatin) supplemented with 10% FBS and 200 µl of the cell suspension was added with 100 µl of different concentrations (0-80 µg/ml) of NILE, NISE and NIRE (dissolved in RPMI-1640) to each well of 96-well plate. To each well 20 µg/ml of LPS suspension was added, plates were covered and incubated for 24 hours under 5% CO_2 and humidified atmosphere of 90% air at 37 °C temperature. After incubation, the solutions from each well were centrifuged at 5000 rpm for 5 min. The supernatants were used to determine the NO level.

Briefly, 50 µL of the supernatants were mixed with 200 µL of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in 2.5% H_3PO_4) in each well of 96-well plate. The solution was incubated for 20 min at room temperature and the generated purple azo-dye was detected at 540 nm. The percentage inhibition of NO generated was calculated using the following formula: % of inhibition= $[(A_0 - A_1) \div A_0] \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample.

3.6. Anti-inflammatory activities

The anti-inflammatory activity of *N. indicum* extracts were evaluated by the following methods:

3.6.1. Culture of splenocytes

Spleen was removed aseptically from an untreated (not fed with extract) Swiss albino mice under proper anesthesia and splenocyte suspension was prepared in RPMI-1640 using tissue grinder. The splenocytes were washed thrice using RPMI-1640 (1000 rpm) for 10 min and then resuspended in 0.16 M NH_4Cl (in 0.17 M Tris; pH 7.2) to lyse RBCs. After 5 min, ice cold RPMI-1640 was added to stop the reaction. The cells were again centrifuged (1000 rpm) and resuspended in RPMI-1640. Splenocytes were adjusted as 2×10^6 cells/ml with RPMI-1640 (supplemented with 50 U/ml penicillin, 50 U/ml streptomycin, 50 U/ml nystatin and 10% FBS) and seeded into six well culture plates. To this, Concanavalin A (Con A) was added to get a final concentration of 5 µg/ml and 100 µl of different concentrations of extracts (0-80 µg/ml) was then added to the wells. Control did not receive Con A and extracts. The plates were then covered and incubated under 5% CO_2 and humidified atmosphere of 90% air at 37 °C temperature for 48 h.

After incubation, the solutions from each well were centrifuged at 5000 pm for 10 min. The cell-free supernatants and the cell-pellets were used for the following assays.

3.6.2. Measurement of NO inhibition

The inhibition of NO was estimated from the cell-free culture supernatants the Griess reagent method (Hibbs, *et al*., 1988) as described previously (section 3.5.2.4).

3.6.3. Estimation of cytokine expression

The levels of the different pro-inflammatory (IL-2, IFN-γ, TNF-α) and anti-inflammatory (IL-4, IL-10) cytokines were estimated from the cell-free culture supernatants using RayBio ELISA kits according to the manufacturer's instructions. Absorbance was immediately measured using Bio-Rad iMark™ microplate absorbance reader. Standard was run in parallel to the sample.

3.6.4. Measurement of PGE_2 level

PGE_2 was measured by competitive binding between PGE_2 and PGE_2-acetylcholineesterase (PGE_2-AChE) for restricted amount of PGE_2 monoclonal antibody using PGE_2 enzyme immuno assay kit obtained from Cayman Chemicals Company, USA, according to the manufacturer's instructions. Absorbance was read using Bio-Rad iMark™ microplate absorbance reader. Standard curve of PGE_2 was prepared in parallel to the samples.

3.6.5. Measurement of COX activities

The cell pellets after 48 h lymphocyte culture, were homogenized in cold buffer consisting of 0.1 M Tris-HCL in 1 mM EDTA (pH 7.8). The homogenate was centrifuged (10,000 × g for 15 min) and the resultant supernatants were used for the assay. COX-1, COX-2 and total COX activities were measured using COX activity assay kit procured from Cayman Chemicals Company, USA.

3.6.6. Delayed type hypersensitivity (DTH) test

Mice were divided into seven groups (n=3) and treated for 20 days as following**:** Control group received normal saline; NILE low and NILE high group received NILE at 50 and 200 mg/kg respectively; NISE low and NISE high groups received NISE at 50 and 200 mg/kg respectively and NIRE low and NIRE high groups received NIRE at 50 and 200 mg/kg respectively. On day 21 i.e. 24 h after the last treatment, all the, mouse were subcutaneously immunized with 3 mg ovalbumin in 100 µl normal saline emulsified with equal volume of Freund's complete adjuvant. After 14 days of immunization, each mouse were challenged with 50 µg ovalbumin in 50 µl saline in left hind foot-pad. The right hind foot-pad was challenged with 50 µl normal saline. The circumference of the left and right foot-pad were measured using a vernier caliper 24 after the challenge. The degree of DTH reaction was expresses as percentage increase in paw edema.

3.6. 7. Statistical analysis

All data of the immunomodulatory and anti-inflammatory experiments are reported as the mean±SD of six measurements. The comparisons between the control group and the test group were performed by one-way analysis of variance (ANOVA) test using KyPlot version 2.0 beta 15 (32 bit) for windows. $p < 0.05$ was considered significant. Half-maximal inhibitory concentration (IC_{50}) values were calculated using the formula $Y = 100 \times A1/(X + A1)$, where $A1 = IC_{50}$; Y = response (Y = 100% when X = 0); X = inhibitory concentration. The linear correlation analysis was performed by Microsoft excel 2010.

3.7. Antioxidant and free radical scavenging activities

NILE, NISE and NISE were evaluated for their antioxidant and free radical scavenging activities using the following methods:

3.7.1. Trolox equivalent antioxidant capacity (TEAC)

The method was based on the ability of the extracts to scavenge $ABTS^{\bullet+}$ radical cation which was compared to trolox (Re, *et al.*, 1999). The $ABTS^{\bullet+}$ radical cation was pre-generated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubated for 12-16 h in dark at RT until the reaction was completed and absorbance was stable. $ABTS^{\bullet+}$ was diluted with water at RT to equilibrated it's absorbance to 0.70±0.02. Then various concentrations (0.05–10 mg/ml) of the sample (10 µl) was mixed with 1 ml $ABTS^{\bullet+}$ solution and the absorbance was measured at 734 nm after 6 min. The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the TEAC value. TEAC was calculated from dividing the gradient of the plot for the sample by the gradient of the plot for trolox.

3.7.2. DPPH radical scavenging assay

The DPPH scavenging assay was performed according to a standard method (Mahakunakorn, *et al.*, 2004) with slight modifications. Varying concentrations (0-100 µg/ml) of the extracts and standard ascorbic acid were mixed with equal volume of ethanol. Then, 50 µl of DPPH solution (1 mM) was pipetted into the previous mixture and stirred thoroughly. The resulting solutions were incubated for 2 min before the absorbance was taken at 517 nm.

3.7.3. Hydroxyl radical scavenging assay

The assay was performed according to a standard method (Hazra, *et al.*, 2008). Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system, commonly known as the Fenton reaction. At the final volume of 100 µl, the reaction mixture contained 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and various concentrations (0-200 µg/ml) of the plant extracts. The reaction mixtures were kept for incubation for 60 min at 37 °C and after incubation, 0.5 ml of the reaction mixture was mixed with 1 ml 2.8% TCA and 1 ml 1% aqueous TBA was added to it. The solutions were incubated at 90 °C for 15 min to develop the colour. The solution was cooled down and the absorbance was read at 532 nm against an appropriate blank solution. Mannitol which is a classical hydroxyl radical scavenger, was used as a positive control.

3.7.4. Superoxide radical scavenging assay

The experiment was performed based on the reduction of NBT according to a previously reported method (Hazra, *et al.*, 2008). The 100 µl reaction mixture contained phosphate buffer (20 mM, pH7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various concentrations (0-120 µg/ml) of sample solution. The reaction mixture was incubated at RT for 5 min and then absorbance was taken at 562 nm against a blank. Quercetin was used as standard.

3.7.5. Nitric oxide radical scavenging assay

Nitric oxide was generated at physiological pH from aqueous sodium nitroprusside (SNP) solution reacting with oxygen to produce nitrite ions, which was quantified according to the Griess Illosvoy reaction (Garratt, 1964). The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various doses (0-70 µg/ml) of the test solution in a final volume of 3 ml. After incubation for 150 min at 25 °C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 100 µl of napthylethylen-ediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25 °C. Formation of the pink chromophore was measured at 540 nm against a blank sample. Curcumin was used as a standard.

3.7.6. Hydrogen peroxide scavenging assay

The assay was performed according to a previously described method (Long, *et al.*, 1999). with minor modifications. An aliquot of 50 mM H_2O_2 and various concentrations (0-2 mg/ml) of samples were mixed (1**:**1 v/v) and incubated for 30 min at room temperature. After incubation, 90 µl of the H_2O_2-sample solution was mixed with 10 µl HPLC-grade methanol and 0.9 ml FOX reagent was added (9 volumes of 4.4 mM BHT in HPLC-grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 MH_2SO_4). The reaction mixture was vortexed and incubated at room temperature for 30 min. The absorbance was measured at 560 nm. Sodium pyruvate was used as the reference compound.

3.7.7. Peroxynitrite scavenging activity

A previously described standard method (Beckman, *et al.*, 1994) was followed to synthesize peroxynitrite ($ONOO^-$). In brief, 5 ml 0.6 M KNO_2 was mixed with an acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) on ice bath and 5 ml of ice-cold 1.2 M NaOH was added to it. The solution was subjected to treatment with granular MnO_2 prewashed with 1.2 M NaOH to remove the excess H_2O_2. The reaction mixture was left overnight at -20 °C. Peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$).

Evans blue bleaching assay was used to measure the peroxynitrite scavenging activity. (Bailly, *et al.*, 2000). The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM Evans Blue, various doses of plant extract (0-200 μg/ml) and 1 mM peroxynitrite in a final volume of 1 ml. The mixture was incubated at 25 °C for 30 min and the absorbance was measured at 611 nm. Gallic acid was used as the reference compound.

3.7.8. Singlet oxygen scavenging assay

The assay was performed according to previously reported spectrophotometric method with minor modifications (Pedraza-Chaverri, *et al.*, 2004). Singlet oxygen was generated by a reaction between NaOCl and H_2O_2 and the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO) was read at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50-mM NaOCl, 50-mM H_2O_2, 50-mM L-histidine, 10 μM RNO and various concentrations (0-200 μg/ml) of sample in a final volume 2 ml. The solution mixtures were incubated at 30 °C for 40 min and decrease in the absorbance of RNO was measured at 440 nm. Lipoic acid was used as a reference compound.

3.7.9. Hypochlorous acid scavenging assay

Hypochlorous acid (HOCl) was prepared freshly just before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4, and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 per M/cm. The assay was carried out according to a previously described method with slight modifications (Hazra, *et al.*, 2008). The reaction mixtures contained, in a final volume of 100 μl, 50-mM phosphate buffer (pH 6.8), catalase (7.2 μM), HOCl (8.4 mM) and increasing concentrations (0-100 μg/ml) of plant extracts. The assay mixture was incubated at 25 °C for 20 min and the absorbance was measured at 404 nm. Ascorbic acid was used as the reference compound.

3.7.10. Iron chelation assay

The ferrous ion chelating activity was evaluated by a standard spectrophotometric method (Haro-Vicente, *et al.*, 2006) with minor changes. The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Various concentrations of plant extracts (0-120 μg/ml) were mixed with 12.5 μM ferrous sulfate solution. The reaction was initiated by the addition of 75 μM ferrozine. The mixture was shaken vigorously and incubated for 20 min at room temperature and the absorbance was measured at 562 nm. EDTA was used as a standard.

3.7.11. Total reducing power

The method described by Oyaizu (1986) was followed with slight modification to evaluate the total reducing power of the *N. indicum* extracts. Different concentrations (0-1 mg/ml) of extracts (0.5 ml) were mixed with 0.5 ml phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexacyanoferrate. The solutions were incubated at 50 °C in a water bath for 20 min. Then, 0.5 ml of 10% TCA was added in each tube to terminate the reactions. The upper portion of the solutions (1 ml) were mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.01%) was added. The reaction mixtures were left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power. Butylated hydroxytoluene (BHT) was used as a positive control.

3.7.12. Inhibition of lipid peroxidation

The assay was carried out according to a previously described method (Kizil, *et al.*, 2008) with slight modifications. Brain homogenate was prepared by centrifuging (3000 rpm for 10 min) Swiss albino mice brain (20±2 g) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl. Then 100 µl of the supernatant homogenates were mixed with various concentrations (0-25 µg/ml) of the plant extracts, followed by addition of 0.1 mM Fe_SO_4 and 0.1 mM ascorbic acid, and incubated for 60 min at 37 °C. Then 500 µl 28% TCA was added to stop the reaction and 380 µl 2% TBA was added with heating at 95 °C for 30 min. The solutions were cooled on ice, centrifuged at 8000 rpm for 2 min and the absorbance was measured at 532 nm. Trolox was used as the standard.

3.7.13. Quantification of total phenolic content

A standard method was followed to determine the total phenolic contents using Folin-Ciocalteu (FC) reagent (Singleton & Rossi, 1965). Briefly, 0.1 ml of extracts were mixed with 0.75 mL of FC reagent (previously diluted 1000 fold with distilled water). The reaction mixtures were incubated for 5 min at 22 °C and then 0.06% Na_2CO_3 solution was added to the mixtures. After incubation at 22 °C for 90 min, the absorbance was measured at 725 nm. The phenolic contents were evaluated from a gallic acid standard curve.

3.7.14. Quantification of total flavonoid content

Total flavonoid contents were quantified according to a standard method using quercetin as a standard (Zhishen, *et al.*, 1999). The plant extracts (0.1 ml) were mixed with 0.3 ml distilled water followed by 0.03 ml 5% $NaNO_2$. After 5 min incubation at 25 °C, 0.03 ml 10% $AlCl_3$ was added. After another 5 min, the reaction mixtures were treated with 0.2 ml 1 mM NaOH. Finally the reaction mixtures were diluted to volume (1 ml) with distilled water. Then the

absorbance was measured at 510 nm. The flavonoid contents were calculated from a quercetin standard curve.

3.7.15. Statistical analysis

All data are represented as the mean±SD of six measurements. Statistical analysis were performed using KyPlot version 2.0 beta 15 (32 bit). The percentage of inhibition/scavenging were calculated by the formula: $[(A_0 - A_1) \div A_0] \times 100$, where A_0 was the absorbance of control and A_1 was the absorbance of samples and standard. The IC_{50} values were calculated by the formula $Y = 100 \times A_x/(X + A_x)$, where $A_x = IC_{50}$, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired t tests. $p < 0.05$ was considered significant.

3.8. Hepatoprotective activities

The hepatoprotective activity of *N. indicum* was evaluated by the following methods:

3.8.1. Experimental design: in vivo

Swiss albino mice were randomly divided into different groups (n=6) and following treatments were done once per day for 10 consecutive days: Control group received normal saline; CCl_4 group received 1**:**1 (v/v) CCl_4 in olive oil; Silymarin group received 1**:**1 (v/v) CCl_4 in olive oil and 100 mg/kg bw silymarin; Three low dose extract groups received 1**:**1 (v/v) CCl_4 in olive oil and 50 mg/kg bw NILE, NISE and NIRE, respectively; Three high dose extract groups received 1**:**1 (v/v) CCl_4 in olive oil and 200 mg/kg bw NILE, NISE and NIRE, respectively.

On 11^{th} day i.e. 24 h after the last dose, blood was collected by cardiac puncture under anesthesia and finally the animals were sacrificed. Blood was allowed to clot for 60 min at room temperature (20 °C) and then serum was separated by centrifuging at 1000 rpm for 5 min. The straw coloured serum was used to study liver marker enzymes. Liver from the animals were isolated by separating the liver from diaphragm by cutting the falciform and coronary ligaments. The livers were wash with double distilled water to remove blood and used for antioxidant enzymatic assays. Liver tissue required for histological study were collected in Bouin's solution.

3.8.2. Liver function test: in vivo

The serum samples from each group were used to study ACP, albumin, globulin, glucose, ALP, bilirubin, cholesterol, LDH, GGT, AST, ALT, total protein, urea and urea N_2 levels using commercially available kits (Crest Biosystems, India).

3.8.3. Estimation of peroxidase activity

Peroxidase activity in liver of the experimental mice were estimated by measuring the oxidation of guiacol according to a standard method (Sadasivam & Manickam, 2008). In brief, 50 mg tissue samples were homogenized in 1 ml ice-cold 0.1 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 3000 rpm for 15 min and the supernatant was separated. Then, 100 µl supernatant was mixed with 20 mM guiacol. In presence of 300 µl H_2SO_4 (12.3 mM), the time was recorded for the increase of absorbance by 0.1 at 436 nm.

3.8.4. Estimation of catalase activity

Catalase activity was measured by degradation of substrate H_2O_2 by catalase in the liver tissue samples following the standard method described by Luck (1963) with some modifications. Briefly, 50 mg tissue samples were homogenized in 0.05 M of 1 ml Tris-HCl buffer (pH 7.0)

and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatants were collected. Next, in a spectrophotometric cuvette, 500 µl of 0.34 mM H_2O_2 and 2.5 mL H_2O were added and absorbance was read at 240 nm. Then, 40 µl supernatant was added into the cuvette and change in absorbance was noted six times at 30 sec intervals.

3.8.5. Experimental design: in vitro

The *in vitro* hepatoprotective capacity of *N. indicum* extracts were studied according to previously standardized methods with minor modifications (Freshney, 2005; Mishra, *et al*., 2011). Briefly, different groups of primary explant culture of mice hepatocytes were prepared in RPMI-1640 medium (containing 50 U/ml penicillin, 50 U/ml streptomycin and 50 U/ml nystatin) supplemented with 10% fetal bovine serum (FBS). After 48 h of the culture the following treatments were done**:** Control had no separate treatment; CCl_4 group received 25 µl/ml CCl_4; Silymarin group received 25 µl/ml CCl_4 and 100 µg/ml silymarin; Low dose extract groups received 25 µl/ml CCl_4 and 25 µg/ml NILE, NISE and NIRE, respectively; High dose extract groups received 25 µl/ml CCl_4 and 100 µg/ml NILE, NISE and NIRE, respectively. The plates were incubated for 2 h and then culture supernatants were collected by centrifugation (5000 rpm for 10 min).

3.8.6. Liver function test: in vitro

Culture supernatants from each group were analysed for ACP, ALP, bilirubin, LDH, AST, ALT and total protein levels using commercially available kits (Crest Biosystems, India).

3.8.7. Measurement of lipid peroxidation

The MDA content was determined using TBARS assay kit (Cayman, USA) according to the manufacturer's instructions. The absorbance of the supernatant was measured at 340 nm.

3.8.8. Measurement of TNF-α

The amount of TNF-α released in culture supernatants were measured using TNF-α ELISA kit (Ray Bio, USA) according to the manufacturer's instructions. Absorbance was immediately measured after the assay at 450 nm using Bio-Rad iMark™ microplate absorbance reader. Standard was run in parallel to the samples.

3.8.9. Measurement of inhibition of NO

Culture supernatants were used to determine the NO level using the Griess reagent method (Hibbs, *et al*., 1988) with some modifications. Briefly, 60 µl culture supernatant from each group was mixed with 240 µl of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in 2.5% H_3PO_4) in a 96-well plate and incubated for 20 min at room temperature. The purple azo-dye formed, was detected at 540 nm.

3.8.10. MTT cytotoxicity assay

Hepatocyte necrosis results due to CCl_4 toxicity. Therefore, MTT cytotoxicity assay was performed to examine the protection rendered by NILE against CCl_4 mediated toxicity. Hepatocytes were cultured as described previously. The cytotoxicity assay was performed in six sets using EZcount^{TM} MTT Cell Assay Kit (HiMedia) according to the manufacturer's instructions.

3.8.11. Histological studies

Livers were removed from the animals of the *in vivo* experiments after the collection of blood and were fixed overnight in 10% buffered formalin. The samples were subjected to dehydration and the embedded in paraffin. Thick sections (4 µm) of the paraffin embedded livers were cut by microtome and then dewaxed in xylene, rehydrated in a series of different grades of alcohol and then washed with distilled water for 5 min. Subsequently, the sections were stained with haematoxylin for 40 sec and counterstained with eosin for 20 sec. The sections were dehydrated in graded alcohol series and washed in xylene. The slides were observed using Magnus trinocular microscope MLX-TR (Olympus microscopes) for signs of necrosis, portal inflammation, vascular congestion, fatty infiltration, vacoular degeneration, , leukocyte infiltration, loss of structure of hepatic nodules and so forth (Knodell, *et al*., 1981; Ruwart, *et al*., 1989).

3.8.12. Statistical analysis

All data are reported as the mean±SD of six measurements. Statistical analysis was performed and graphs were prepared using KyPlot version 2.0 beta 15 (32 bit). Comparison between groups were performed using one-way analysis of variance (ANOVA) with Dunnett's test. $p<0.05$ was considered significant.

3.9. Anti-diabetic activities

NILE, NISE and NIRE were evaluated for their anti-diabetic activities by the following methods:

3.9.1. Alpha-amylase inhibitory activity

The assay was carried out according to the standard method of Hansawasdi, *et al.*, (2000) with some modifications. Briefly, 1% starch azure was suspended in 0.5 M Tris-HCl (pH 6.9) containing 0.01 M $CaCl_2$ and boiled on water bath for 5 min. The tubes were cooled down to room temperature and different concentrations (0-200 µg/ml) of *N. indicum* extracts and amylase (2 U/ml) in Tris-HCl buffer was added to it. The solution were mixed properly and incubated for 5 min at 37 °C. The reactions were stopped by addition of 250 µl of 1 M HCl followed by centrifuge at 3000 rpm for 10 min. The supernatants were collected and absorbance were read at 595 nm. The percentage inhibition was calculated from the following formula**:** % of inhibition [α-amylase] = $\frac{A0 - A1}{A0} \times 100$, where A0 was the absorbance of the blank and A1 was the absorbance in the presence of the varying concentrations of extracts.

3.9.2. Induction of experimental diabetes

Diabetes was induced in experimental mice by a single intraperitoneal injection (0.2 ml) of freshly dissolved alloxan monohydrate in saline (154 mM NaCl) with a dose of 150 mg/kg BW. Mice with 12 h fasting blood glucose level >200 mg/dl on the third day of alloxan administration were considered diabetic and selected for the following treatments.

3.9.3. Drug administration in diabetic animals

Swiss albino mice of either sex were randomly divided into groups (n=6). Control (non-diabetic mice) received normal saline; T1D group (diabetic mice) received normal saline; Glibenclamide group (diabetic mice) received glibenclamide at 5 mg/kg BW per day; Three low dose group (diabetic mice) received NILE, NISE and NIRE, respectively (in distilled water) at 50 mg/kg BW per day. Three high dose group (diabetic mice) received NILE, NISE and NIRE, respectively (in distilled water) at 200 mg/kg BW per day. The treatments were done for 20 consecutive days.

3.9.4. Estimation of body weight and blood glucose level

Total body weight of all the experimental animals were recorded on day 1, day 10 and day 20 of the treatments and 12 h fasting blood glucose levels were estimated using Bayer glucometer (contour TS meter) from the tail vein on the aforementioned days.

3.9.5. Collection of serum and tissue

On day 21, i.e. 24 h after the last treatment, (12 h fasting) mice were sacrificed under mild ether anesthesia and blood was collected by puncturing the heart. Whole blood (50 µl) was used to estimated glycated haemoglobin (HbA1c) level by ion-exchange high-performance liquid chromatography (HPLC) using Bio-Rad D-10^{TM} Dual HbA1c program 220-0201 according to manufactures instructions. The remaining blood was allowed to clot for 60 min at room temperature and then serum was separated by centrifuging at 1000 rpm for 10 min. Separated serum was kept at -20 ^{o}C until further use. Skeletal muscle collected from the thigh muscle and tissue samples of liver and kidney were separated and washed thoroughly with phosphate buffer saline (PBS) and stored at -20 ^{o}C for future use.

3.9.6. Study of serum biochemical parameters

Serum insulin level was estimated by ELISA method using AccuBind Universal ELISA kit (Monobind Inc., USA) according to manufactures instructions. The serum samples from all the groups were used to study ACP, ALP, cholesterol, creatinine, triglyceride, AST, ALT, uric acid, urea and urea N_2 levels using commercially available kits of Crest Biosystems (India).

3.9.7. Measurement of lipid peroxidation

The extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) content in serum. The assay was performed in six sets by the thiobarbituric acid reactive substances (TBARS) assay kit according to the manufacturer's (Cayman, USA) instructions.

3.9.8. Estimation of hepatic glycogen

Glycogen level was estimated in liver samples by the standard anthrone reagent method previously described by Carroll, *et al.*, (1956) with some modifications. In brief, liver samples were mixed with trichloroacetic acid (TCA) and homogenized properly followed by centrifugation at 3000 rpm for 15 min. Supernatants were decanted separately and the pellets were re-homogenized using TCA, followed by centrifugation. The process was repeated twice and then the supernatants from all the phases were mixed together and the volume was made upto the mark using 5% TCA. The mixture (200 µl) was mixed with 1 ml 95% ethanol, mixed properly and incubated at RT for 12 h. The tubes were then centrifuges at 3000 rpm for 15 min. The supernatants were removed and the pellet was mixed with 2 ml H_2O followed by addition of 10 ml anthrone reagent into each tube. The tubes were then subjected to boiling water bath for 15 min, cooled down and the OD was meadured at 620 nm. Glycogen content was measured from a glucose standard curve prepared in parallel to the samples.

3.9.9. Estimation of anti-oxidative enzymes: Peroxidase and Catalase

Estimation of peroxidase and catalase activities in liver, kidney and skeletal muscle were estimated in the experimental groups by the standard methods of Sadasivam and Manickam (2008) and Luck (1963), respectively, as described previously (section 3.8.3 and 3.8.4).

3.9.10. Oral glucose tolerance test (OGTT)

A different set of diabetic and non-diabetic mice were divided into 6 groups (n=6) as previously described and treated with glibenclamide and NOLE for 20 days. After the last dose, the mice were fasted for 12 h and subsequently, a dose of glucose (2.5 g/kg body weight) were orally administered. Blood glucose levels were estimated from the blood samples collected from the tail vein just prior to glucose administration and 60, 120 and 180 min post-glucose administration.

3.9.11. Statistical analysis

Statistical analysis was performed and graphs were prepared using KyPlot version 2.0 beta 15 (32 bit). Data were statistically analyzed using one-way analysis of variance (ANOVA) with Dunnett's test. Data of OGTT was analyzed using two-way ANOVA. $p < 0.05$ was considered significant. The IC_{50} value was calculated by the formula $Y = \frac{100 \times A1}{X \times A1}$ where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. All data are reported as the mean±SD of six measurements.

3.10. Phytochemical analysis

The phytochemical constituents in *N. indicum* leaf, stem and root were studied by the following methods**:**

3.10.1. Sample preparation for preliminary phytochemical analysis

Disease free fresh leaves, stem and root were collected from a mature *N. indicum* plant. The parts were washed properly first with tap water and then with double distilled water to remove dirt. The parts were then shade dried at room temperature for 14 days and grinded to powder using a blender **(**Lords® Hummer 1100**)**. The powder was then passed through a 0.5 mm metallic mesh. Aqueous and methanolic fractions was prepared to perform the qualitative test.

Aqueous extract: The crude dried powder (10 g) was taken in a 250 ml conical flask and 100 ml of double distilled water was added to it. The mixture was stirred on a magnetic stirrer for 10 h and then filtered through Whatman filter paper number 1 (150 mm). The filtrate was used for the following phytochemical tests.

Methanolic extract: 10 g of crude dried plant powder was taken with 100 ml of 70% methanol in a 250 ml conical flask. The mixture was mixed in a magnetic stirrer for 10 h in room temperature and filtered through Whatman filter paper number 1.

The resultant extracts were used for the preliminary phytochemical investigations according to the standard chemical tests (Brain & Turner, 1975; The Indian Pharmacopoeia, 1996; Khandelwal, 2008; Gokhale & Kokate, 2008).

3.10.2. Qualitative tests

3.10.2.1. Tannin

The aqueous extract (10 ml) was mixed with few drops of 0.1% $FeCl_3$ solution. Formation of blue-black precipitate indicated the presence of tannin.

3.10.2.2. Phlobatannin

The aqueous extract (10 ml) was taken in a boiling tube and 2 ml of concentrated HCl was added to it. The mixture was boiled for 1 minute. Deposition of red precipitate indicated the presence of phlobatannins.

3.10.2.3. Carbohydrate

The aqueous extract (2 ml) was mixed with 2 ml of Molish's reagent (5% α-napthol in absolute ethanol) and shaken vigorously. Concentrated H_2SO_4 (2 ml) was slowly added along the wall of the test tube. Formation of reddish-violet ring at the junction of two liquids indicated the presence of carbohydrates.

3.10.2.4. Proteins

The aqueous solution (2 ml) was mixed with 1 ml of 40% NaOH solution. Few drops of $CuSO_4$ solution was added to it. Change of colour of the solution into violet indicated the presence of proteins.

3.10.2.5. Terpenoid

The methanolic extract (5 ml) was mixed with 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was added slowly along the wall of the test tube. Development of reddish-brown colour at the junction of two liquid phases indicated the presence of terpenoids.

3.10.2.6. Glycoside

The methanol extract (5 ml) was mixed with 2 ml of glacial acetic acid containing 2% $FeCl_3$ solution. Concentrated H_2SO_4 (1 ml) was added slowly along the walls of the test tube. Formation of a brown ring at the interphase of two liquid notified the presence of glycoside.

3.10.2.7. Steroid

The methanol extract (5 ml) was treated with 0.5 ml of anhydrous CH_3COOH and was cooled on an ice bath for 15 min. Then chloroform (0.5 ml) was added to the solution and 1 ml of concentrated H_2SO_4 was poured along the walls of test tube. Formation of a reddish-brown ring at the separation level of two liquids was an indication of the presence of steroids.

3.10.2.8. Cholesterol

The methanolic extract (2 ml) was mixed with 2 ml of chloroform followed by addition of 10-12 drops of acetic acid anhydride. Then, few drops of concentrated $H_2SO_4$was added to it. Change of reddish-brown color to blue-green on addition of H_2SO_4 indicated the presence of cholesterol.

3.10.2.9. Alkaloid

The methanolic extract (2 ml) was taken in a test tube and 2 ml of 2N HCl was added to it. The solution was shaken vigorously to mix and incubated for 5 min at room temperature. The aqueous phase formed was separated from the two liquid phases and few drops of Mayer's reagent ($HgCl_2$ + KI in water) was added to it and shaken. Generation of creamy coloured precipitate indicate the presence of alkaloids..

3.10.2.10. Phenolics

The methanolic extract (10 ml) was treated with 4-5 drops of 2% $FeCl_3$ solution. Change of coloration of the solution indicates presence of phenolics.

3.10.2.11. Flavonoid

The crude powdered plant sample (2 g) was heated with 10 ml of ethyl acetate over a water bath for 5 minutes. The solution was filtered through Whatman filter paper number 1. The filtrate (2 ml) was mixed with dilute ammonia solution (10%) and shaken vigorously. Yellow coloration of the solution indicates the presence of flavonoids.

3.10.2.12. Anthraquinone

The crude plant powder (500 mg) was mixed with 20 ml of benzene and stirred in a magnetic stirrer for 4 h and filtered. The filtrate (10 ml) was mixed with 0.5 ml ammonia solution and mixed properly. Presence of violet colour at the layer phase indicates presence of anthraquinones.

3.10.2.13. Saponin

The powdered plant material (500 mg) was boiled with 15 ml of double distilled water in a boiling water bath. Formation of intensive froth is the indication of the presence of saponin.

3.10.3. Quantitative tests

The quantitative estimation of different phytochemicals were performed according to various standard methods with minor modifications.

3.10.3.1. Alkaloid

The total alkaloid content was estimated according to the methods with minor modifications (Obadoni & Ochuko, 2001; Harborne, 1983). In brief, 5 g of powdered sample was mixed with 20% CH_3COOH in ethanol. The mixture was shaken on a magnetic stirrer for 10 h and filtered. The filtrate was placed on a hot water bath (60 °C) until the volume turns $¼^{th}$ of its initial volume. Concentrated NH_4OH was added drop wise till the saturation point which gave rise thick precipitate. The whole solution was allowed to settle down. The precipitate was collected by filtration, dried in an oven and weighed.

3.10.3.2. Flavonoid

A standard method (Boham & Kocipai DC, 1994) was followed with slight modifications to quantify the total flavonoid content. The powered sample was mixed with 100 ml of 70% methanol and was stirred using a magnetic stirrer for 3 hours and filtered. The remaining powdered material was re-extracted once again with 70% methanol and filtered as previous. Filtrates of both the phases were mixed and transferred into a crucible and evaporated to dryness over a water bath of 60 °C and weighed.

3.10.3.3. Saponin

Total saponin content was estimated according to a slightly modified standard method (Edeoga, *et al*., 2005). The powdered plant material (10 g) was mixed with 100 ml of 20% ethanol and heated over a hot water bath of 55 °C for a period of 5 h with stirring. The mixture was filtered and the supernatant liquid was separated. The residue was again re-extracted with 20% ethanol as previous. The supernatant liquids of both phases were mixed and placed on a hot water bath of 90 °C and heated till the volume of the extract was reduced to 20% of its initial volume. Then 10 ml of diethyl ether was added to it and shaken vigorously. After the solution settles down the aqueous layer was separated carefully into

another flask and the ether layer was discarded. The purification process was repeated. Then 60 ml n-butanol extracts were washed twice with 10 ml of 5% aqueous NaCl solution. The remaining solution was heated in a water bath at 50 °C until the solvent evaporates and the solution turns to semi dried form. The sample was then dried in an oven into a constant weight. The saponin content was calculated by the following equation: Percentage of saponin = $(W_{EP} / W_S) \times 100$, where, W_{EP} = weight of oven dried end product and W_S = weight of powdered sample taken for test.

3.10.3.4. Tannin

The assay was performed according to a previously described standard method with slight modifications (Van-Burden & Robinton, 1969). The crude powder sample (1 g) was mixed with 50 ml of double distilled water and shaken on a magnetic stirrer for 10 h at room temperature. The mixture was filtered and made up to 50 ml using distilled water. The solution (5 ml) was pipetted out in a test tube and 0.008 M $K_4[Fe(CN)_6]$ and 0.1 M $FeCl_3$ in 0.1 N HCl was added to it. The absorbance was measured in spectrophotometer at 605 nm within 10 minutes. A blank was prepared and read at the same wavelength. Tannic acid was used to prepare standard curve.

3.10.3.5. Riboflavin

The test was performed according to a standard method (Abe & Yamauchi, 1992) with slight modifications. The dry powder (10 g) was mixed with 100 ml 50% ethanol and stirred for 10 h on a magnetic stirrer at room temperature. The solution was filtered and 25 ml of 5% $KMnO_4$ solution was added to it. The mixture was stirred continuously while 25 ml of 30% H_2O_2 was added to it. This was placed on a 80 °C water bath for 30 min. Then, 5 ml 40% Na_2SO_4 was added to it and the absorbance was measured at 510 nm against a suitable blank. The riboflavin content was calculated from a riboflavin standard curve.

3.10.3.6. Thiamine

A previously standard method was followed with slight modifications (Poornima & Rai, 2009) to quantify the thiamine content. The dried plant powder (50 g) was dispersed in 50 ml ethanolic NaOH (20%) and stirred over a magnetic stirrer for 3 h at room temperature and then filtered. Then, 10 ml of the filtrate was mixed with 10 ml of 2% potassium dichromate solution. The absorbance was read at 360 nm against a suitable blank. The thiamine content was calculated from a thiamine standard curve.

3.10.3.7. Ascorbic acid

Slightly modified method of Barakat, *et al*., (1993) was followed to estimate the quantity of ascorbic acid. The dried powder sample (5 g) was mixed with 100 ml extraction mixture

(TCA **:** EDTA at 2**:**1) and stirred on a magnetic stirrer for 3 h at room temperature. This was centrifuged at 2000 rpm for 30 min. After centrifugation the supernatant liquid was filtered and 2-3 drops of 1% starch indicator was added to it and was titrated against 20% $CuSO_4$ solution until a dark end point is reached.

3.10.3.8. Phenols

To estimate total phenol (Obadoni & Ochuko, 2001) content the test sample needed to be fat free. The crude plant powder (5 g) was mixed with 100 ml n-hexane and defatted using a soxlet apparatus for 2 h. The resultant was used for determination of total phenols.

The fat free sample was boiled with 50 ml ether for 15 min. The resultant was filtered and 5 ml of the filtrate was mixed with 10 ml of double distilled water. Then, 2 ml of NH_4OH solution and 5 ml of concentrated amyl alcohol was added to the solution with constant stirring. The solution was incubated at room temperature for 30 min for colour development and the absorbance was read at 550 nm against a suitable blank. The phenolic content was evaluated from a gallic acid standard curve.

3.10.3.9. Protein

Total protein was estimated according to the method proposed by Lowry, *et al*.(1951) with slight modifications. Known concentrations of bovine serum albumin was taken as standard and the OD was read at 750 nm using a suitable blank.

3.10.3.10. Lipid content

The assay was performed according to a standard method (Jayaraman, 2011) with slight modifications. The dried sample (1 g) was macerated with 10 ml distilled water. To this, 30 ml of chloroform-methanol (2**:**1 v/v) was mixed thoroughly and the mixture was left overnight at room temperature. Then, 20 ml chloroform and equal volume of distilled water was added and centrifuged at 1000 rpm for 10 min. After centrifugation three layers were formed, out of which the lower layer was collected which contained chloroform containing lipid. The mixture was kept in an oven for 60 min at 50 °C to evaporate the chloroform. Weight of the remaining was calculated.

3.10.3.11. Total sugar

The total sugar content was determined according to DuBois, *et al*. (1951) with slight modifications. The powdered sample (50 g) was macerated in a pestle and mortar with 20 ml of ethanol and kept for incubation at 30 °C for 10 h. The mixture was centrifuged at 1500 rpm for 20 min and the supernatant was collected separately. To the resultant 1 ml of alcoholic extract, 1 ml 5% phenol solution was added and mixed thoroughly. Then, 5 ml of

concentrated H_2SO_4 was added rapidly with constant stirring. This was allowed to stand for 30 min at room temperature. Absorbance was measured at 490 nm against a blank.

3.10.3.12. Moisture and ash content

Moisture and ash content of different parts of the plant was estimated by subjecting specific amount of sample to 90 °C for 12 h in an oven and at 400° - 450 °C in a furnace for 5 min, respectively. The resultant weight was calculated for moisture and ash content estimation respectively.

3.10.4. Fourier Transform Infrared Spectroscopy analysis

FTIR spectrophotometry was used to identify the characteristic functional groups in NILE, NISE and NIRE. Small quantity (<10 mg) of the extracts were taken in CaF_2 vessel and placed in a sample cup of a diffuse reflectance accessory. The IR spectrum was obtained using Shimadzu 8300 FT-IR spectrophotometer at ambient temperature. Background correction was made by taking IR spectrum of de-ionized water as the reference in identical condition. The sample was scanned from 400 to 4000 cm^{-1} for 16 times to increase the signal to noise ratio.

3.10.5. Gas chromatography-mass spectrometry analysis

NILE, NISE and NIRE were separately dissolved in dichloromethane and n-hexane and the mixtures was centrifuged thrice at 12,000 rpm for 15 min. The clear supernatant was used for GC-MS analysis. Agilent 5975C GC-MS system (Agilent Technologies, USA) attached with HP-5ms Capillary Column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) and equipped with inert MSD triple axis mass detector conditioned at ion trap 200 °C, transfer line 280 °C, electron energy 70 eV (vacuum pressure- 2.21e - 0.5 torr) was used for analysis. The carrier gas was helium at a flow rate of 1 ml/min. The sample (2 µl) was injected in a splitless mode. The column temperature was set at 60 ºC for 1 min. followed by 5 ºC/ min upto 250 °C. The major and essential compounds in NOLE were identified by their retention times and mass fragmentation patterns using Agilent Chem Station integrator and the database of National Institute Standard and Technology (NIST) with a MS library version 2010.

3.10.6. High Performance Liquid Chromatography analysis

NILE, NISE and NIRE were subjected to Bligh and Dyer method (1959) to remove the lipid contents. The methanolic fractions were separated and mixed with 4 volumes of chilled acetone and incubated for 60 min at -20 °C. The solution was then centrifuged at 15,000 × *g* for 15 min at 4 °C. The pellet containing protein was discarded and the supernatant was subjected to thin layer chromatography (TLC) on a silica gel plate using 10% acetic acid in chloroform as solvent. The corresponding bands of secondary metabolites were eluted by

acetonitrile after detection with 20% w/v Na_2CO_3 and diluted Folin-Ciocaltaeu reagent (1:3). The solution was then analysed using HPLC (Agilent, USA) having Zorbax SB-C18 column (4.6×150 mm, 3.5 micron) and equipped with Diod Array Detector. Gradient concentration of mobile phase A - methanol (M) and B - water (W) with 0.02% H_3PO_4 were as follows: 25% A + 75% B for 5 min, 30% A + 70% for 10 min, 45% A + 55% for 30 min and 80% A + 20% B for 45 min. The injection volume was 20 µl and the flow rate was kept at 0.4 ml/min. Analytes were scanned in four wave length of 254 nm, 275 nm, 280 nm and 320 nm. The peaks were identified by comparing the relative retention time (RRT) against standard phenolic acids (Sigma, USA; ChromaDex, USA), flavonoids and methylphenols (ChromaDex, USA); co-chromatography with the authentic compounds and considering their respective spectral patterns. Identified compounds were estimated using external method after calibration with response factor of authentic compounds with specific concentration, considering proper validation criteria by the following formula: Response factor = area of standard ÷ amount of standard. Amount of the analyte = peak area of the analyte ÷ response factor.

The concentration of the analytes were expressed in µg/mg as calculated from the dry weight (DW) of NILE, NISE and NIRE initially measured for the extraction process.