Pharmacognostical Evaluation

1. Plant material and authentication

Fruits of *Pyrus pashia* were collected in month of June year 2015 from the trees located in Dharamsala, district Kangra Himachal Pradesh (32.218°N and 76.320°E). Fruit samples were authenticated by Dr. Brijlal, Senior Principal Scientist and Curator, CSIR-Institute of Himalayan Bioresource and Technology, Palampur, H.P. The sample has been deposited in Institutional herbarium (voucher specimen no: 16583). Fruits were dried, powdered and then were stored in an air tight container for further use.

2. Macroscopic and Microscopic evaluation

Morphological evaluation involves investigation of its shape, size, colour, odour and taste. Macroscopic evaluation is considered as the first step for establishing identity of a crude drug according to (WHO, 2002). For microscopic evaluation the required samples of *P. pashia* fruit were cut and fixed in FAA (Formalin-5 ml + Acetic acid-5 ml + 70% Ethyl alcohol-90 ml). After 24 h of fixing, the specimens were dehydrated with graded series of Tertiary-butyl alcohol (TBA). Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60ºC) until TBA solution attained super saturation. The specimens were then casted into paraffin blocks. The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10-12 µm. Dewaxing of the sections was by customary procedure (Johansen, 1940) the sections were stained with Toluidine blue as per the method published by (Brien et al., 1964), since Toluidine blue is a polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. wherever necessary sections were also stained with

safranin and Fast-green. Powdered materials of different parts were cleared with NaOH and mounted in glycerine medium after staining. Photographs of different magnifications were taken with Nikon labphoto 2 microscopic Unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background.

3. Physicochemical evaluations (WHO, 2002)

Various physicochemical parameters of the powdered fruit samples were evaluated according to methods described in WHO guidelines and Indian Herbal Pharmacopoeia (Anonymous, 2002a) Each study was performed in triplicate; mean values with standard error of mean (SEM) were calculated.

3.1. Foreign matter

The air-dried fruits were evenly spread in a white sheet of paper and carefully monitored using a magnifying glass. Foreign matter like dirt and other particles along with leaves and twigs were separated from the fruits.

3.2. Total ash

About 2–4 g of the air–dried coarsely powdered plant material was taken in a tarred silica dish and incinerated at a temperature not exceeding 450 °C until free from carbon. The silica dish bearing the total ash was cooled and weighed. The percentage (w/w) of ash with reference to air dried drug was then calculated.

3.3. Acid–insoluble ash

Known amount of total ash obtained by incinerating powdered drug material at a temperature not exceeding 450°C was boiled with 25 mL of hydrochloric acid (70 g/L) for 5 min. The solution was filtered through an ash–less filter paper and later washed with hot water to neutral. The filter paper bearing insoluble matter was dried, ignited and later cooled in a desiccator and weighed. The percentage acid insoluble ash (w/w) with reference to air dried drug was calculated.

3.4. Water–soluble ash

Known amount of total ash as obtained from the total ash was boiled with 25 mL of water for 5 min. The insoluble matter was collected on an ash less– filter paper, washed with hot water, and ignited for 15 min at a temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the ash and the difference in weight represents the water–soluble ash. The percentage of ash (w/w) with reference to air dried drug was then calculated.

3.5. Alcohol–soluble extractable matter

About 4 g of the coarsely powdered air–dried drug was macerated with 100 mL of ethanol in a glass stopper conical flask for 24 h. The mixture was shaken frequently during the first 6 h and allowed to stand for 18 h. After 24 h the mixture was filtered and the final volume was made up to the mark. 25 mL of the filtrate was taken in a flat bottom previously weighed dish, evaporate to dryness, dried at 105 °C and finally weighed. The percentage (w/w) of ethanol–soluble extractive with reference to the air–dried drug was calculated.

3.6. Water–soluble extractable matter

For the determination of water soluble extractable matter, the same method as above was followed using water as solvent for extraction. The percentage (w/w) of water–soluble extractive with reference to the air–dried drug was calculated.

3.7. Loss on Drying

The air–dried powdered drug (1g) was taken in a glass–stoppered, shallow weighing bottle that has been previously dried. The powdered drug was evenly distributed by gentle sidewise shaking to a depth not exceeding 10 mm and dried to constant weight for the specified time and temperature. After drying was completed, the bottle was cooled by keeping in desiccators, weighed and the percentage (w/w) loss on drying was determined.

3.8. Pesticide content

The pesticide content analysis of the fruits was performed according to the WHO guideline. 350 mL of acetonitrile: water (65:35) was added to 50 g of grinded powdered fruits and was blended at high speed for 5 min followed by filtration. 250 mL of filtrate was transferred to a separating funnel to which further 100 mL light petroleum, 10 mL of sodium chloride (40%) and 600 mL of water were added with constant shaking up to 35-45 sec. Organic solvent layer was washed twice with 100 mL portions of water to which 15 g of anhydrous sodium sulphate was added with vigorous shaking. The separated extract was then reduced to 5 to 10 mL volume which was subjected to column packed with Florisil R grade 60/100 PR, activated at 650°C maintaining a rate of not more than 5 mL/min. Three different elutes were obtained using three different ratios of ether: light petroleum mixture as mobile phase i.e. Elute 1 contained 6% of ether while elute 2 and 3 contained 15% and 50% of ether. Elutes obtained were transferred to a sample holder, and burned in a suitable combustion flask flushed with oxygen. The gases produced gets absorbed in a suitable

solution in the combustion flask (water for chloride and H_2SO_4 in case of phosphate pesticides). For chloride pesticides, 15 mL of the solution obtained after combustion was mixed with 1 mL of ferric ammonium sulphate (0.25 mol/L) and 3 mL of mercuric thiocyanate followed by swirling it where absorbance was measured at 460 nm. For investigation of phosphate pesticides, 7 mL of the solution obtained after combustion was mixed with 2.2 mL of sulphuric acid (300 g/L), 0.4 mL of ammonium molybdate (40 g/L) and 0.4 mL of amino naphtha sulphonic acid followed by swirling it and heating it at 100°C for 12 min which was then measured at 820 nm.

3.9. Heavy metals analysis

Present study included investigation of four heavy metals *viz.* Lead (Pb), mercury (Hg), zinc (Zn) and cadmium (Cd). 2 g of powdered drug was digested with 10 mL conc. HNO_3 followed by heating in a hot plate for 15 min at 95°C. This process was repeated twice for another 30 min until the red fumes of nitric acid disappeared. The solution was cooled and 2 mL of deionized water and 3 mL hydrogen peroxide (30% v/v) was added to start the peroxide reaction. After the reaction was completed, 5 mL of conc. HCl and 10 mL deionized water was added and the samples were heated for an additional 15 min. The sample was cooled, filtered and volume was made up to 50 ml. The estimation of heavy metals was analyzed by using atomic absorption spectroscopy (Shimadzu–AA6300) (Gomez et al., 2007). The results obtained were expressed in terms of parts per million (PPM) per gram of drug.

3.10. Determination of crude fiber content (Dutch Method)

Crude fiber content of the plant material was determined by treating 2 g of powder with 50 mL of 10% nitric acid, which was heated to boiling with constant stirring. The matter was strained through a muslin cloth and the residue obtained was washed with boiling water and it was further

treated with 50 mL 2.5% w/v sodium hydroxide solution and was heated to boiling. The content was again strained, dried and the percentage (w/w) of crude fiber was calculated.

3.11. Fluorescence powder drug analysis

Fluorescence analysis plays a pivotal role in differentiation of powdered drug from one another, for examples detection of ergot in flour, cocoa shells in powder cocoa and vice versa. The powdered fruit was subjected to fluorescence analysis according to standard methods published by (Chase and Pratt, 1949) fluorescence pattern of the solution mixture was monitored under day light as well as under ultra violet light (254 nm and 365 nm). The color of the standard fluorescence produced was observed by comparing with the reference standard color available at http://trac.dojotoolkit.org.

4. Preparation of fruit extracts and preliminary phytochemical analysis

Fresh fruits from *P. pashia* tree were plucked. Fruits were cut into quarters and were shade dried for 2 weeks at room temperature. Dried fruits were then coarsely ground to obtain homogenous powder using mechanical grinder. The homogenous powdered drug (1.8 kg) were extracted with 95% ethanol (4l) for three days by cold maceration process, 150 gms of residue was obtained after removal of solvent at reduced pressure. The ethanolic extract of *Pyrus pashia* (EPP) obtained from cold maceration process was subjected to successive fractionation by liquid–liquid partition technique. The dried ethanolic root extract (150 g) was dissolved and suspended in aqueous medium which was later partitioned with solvent of increasing polarity such as hexane, chloroform, ethyl acetate and *n*–butanol. All the successive fractions obtained were filtered and concentrated under reduced pressure. The preliminary phytochemical screenings of the ethanolic extract from *P. pashia* (EPP) as well as its successive fractions were tested following the methods described by (Khandelwal, 2007) and (Harbone, 1984).

4.1. Phytochemical evaluations

4.1.1. Test for alkaloids

Mayer's test

Test solution when treated with Mayer's (Potassium mercuric iodide) reagent results in formation of cream coloured precipitate ensuring the presence of alkaloids.

Dragendroff's test

Required volume of the test solution when treated with Dragendroff's (potassium bismuth iodide) reagent leads to formation of orange to reddish coloured precipitate confirming the presence of alkaloids.

Wagner's test

Test solution (extracts) when treated with Wagner's reagent, (potassium iodide in iodine) formation of reddish brown coloured precipitate ensures the presence of alkaloids

4.1.2. Test for phytosterols/steroids

Libermann Burchard test

To the test solution (extract), 3 mL of acetic anhydride was added. To this solution, two drops of concentrated sulphuric acid was added slowly along the side of the test tube. Appearance of bluish green colour confirms the presence of phytosterols/steroids.

4.1.3. Test for glycosides

4.1.3.1. Test for anthraquinones glycosides:

Borntrager's test

In this test, the extract was first shaken gently with equal volume of chloroform and then the chloroform layer was separated. To this layer dilute ammonium solution was added and if ammonia layer acquires pink colour, it confirms the presence of anthraquinone glycosides.

Modified Borntrager's test

To the extract, 5% ferric chloride solution and dilute hydrochloric acid was added, heated on boiling water bath for 5 min to bring about oxidative hydrolysis and was shaken gently by addition of benzene. Benzene layer was further separated and to this, dilute ammonia solution was added. Formation of pink colour indicates the presence of anthraquinone glycosides.

4.1.3.2. Test for steroidal glycoside

Legal's test

To the test solution, 1 mL of pyridine and a few drops of sodium nitroprusside were added and were made alkaline with NaOH solution. Appearance of pink colour is an indicative of presence of cardiac glycosides.

4.1.3.3. Test for coumarins glycoside

To the test solution (extract), 10% dilute NaOH was added and the mixture was monitored under UV light (366 nm). Appearance of blue colour fluorescence confirms the presence of coumarin glycoside.

4.1.3.4. Test for cynogenetic glycosides

Sodium picrate test

Sodium picrate paper turns brick red in colour when comes in contact with the extract containing cynogenetic glycosides. Sodium picrate paper is prepared by soaking filter paper first with 10% picric acid and then with 10% sodium carbonate.

4.1.4. Test for Phenols

To the test solution (extract dissolved in methanol), few drops of freshly prepared solution of 5% ferric chloride was added. Formation of blue colour indicates the presence of phenol.

4.1.5. Test for tannins

To the test solution (extract dissolved in methanol), 0.5 mL of 10% lead acetate was added. Appearance of white precipitate indicates the presence of tannins.

4.1.6. Test for flavonoids

To the test solution (extract dissolved in methanol), required amount of magnesium turnings and a few drops of concentrated hydrochloric acid were added. Formation of pink colour indicates the presence of flavonoids.

4.1.7. Test for saponins

To the extract, 1 mL of distilled water was added and shaken vigorously. Formation of persistent foam/froth up to 10 min or more indicates the presence of saponins.

4.1.8. Test for carbohydrates

Molisch's test

In this test, the extract was made to react with 0.5 mL of α–naphthol solution. The mixture was vortexed and two drops of concentrated sulphuric acid was added from the side of the test tube. Formation of purple ring at the junction of two liquid showed the presence of carbohydrate.

4.1.9. Test for proteins

Biuret test

Test solution (extract) was treated with 40% sodium hydroxide and dilute copper sulphate solution. Appearance of blue colour indicates presence of proteins.

Xanthoproteic test

The test solution was treated with conc. HNO_3 and boiled for few minutes. Appearance of yellow precipitate indicates presence of proteins.

4.1.10. Test for amino acids

To the known volume of test solution, 2 mL of ninhydrin solution was added and the solution was heated. Formation of violet colour indicates the presence of amino acids.

5. Foaming Index

Determination of foaming index was done as per method prescribe in WHO guidelines. The air–dried powdered drug (1 g) was accurately weighed and transferred into a 500 mL conical flask containing 100 mL of water and was boiled for 30 min. After boiling, the decoction was cooled, filtered and volume was made up to 100 mL with water. The decoctions was then poured into 10 stoppered test–tubes (height 16cm, diameter 16mm) in successive portions of 1 mL, 2 mL, 3 mL, etc. up to 10 mL, and the volume was adjusted with water up to 10 mL. All the test tube were shaken in a lengthwise motion for 15 sec at two shakes per sec and allow to stand for 15 min. The height of the foam was measured following the conditions shown below.

- If the height of the foam in every tube was less than 1 cm, the foaming index is taken to be less than 100.

- If a height of foam of 1 cm was found to be from 1 to 10 in any tube, then the volume of the plant material decoction in this tube is used to determine the index and is marked as 'a' in the formulae given below.
- If this tube was the first or second tube in a series, an intermediate dilution was prepared in a similar manner to obtain a more precise result.
- If the height of the foam was more than 1 cm in every tube, the foaming index is over 1000. In this case the determination was repeated using a new series of dilutions of the decoction in order to obtain the result.

The foaming index was calculated using the following formula:

$$\text{Foaming Index} = 1000/a$$

Here 'a' is the volume in mL of the decoction used for preparing the dilutions in the tube where foaming to a height of 1 cm was observed.

6. Swelling Index

Swelling index is the volume (in mL) taken up by the swelling of 1 g of plant material under specified condition. Accurately 1 gm of the air–dried powdered drug was taken in a 25 mL glass–stoppered measuring cylinder (16 mm internal diameter and 125 mm length). To the powdered drug, 25 mL of water was added and the mixture was thoroughly shaken after every 10 min interval for 1 h and then allowed to stand for 3 h at room temperature. The final volume (in mL) occupied by the plant material, including any sticky mucilage was measured and the mean value of the individual determinations, related to 1 g of plant material was calculated.

7. Hemolytic index

The haemolytic activity of the plant drug was done as per W.H.O guidelines. The study was done incorporating standard saponin (diosgenin) as the positive control. A suspension of erythrocytes

(Ox blood) was mixed with equal volumes of a serial dilution of the plant material extract. The lowest concentration to affect complete haemolysis was taken as the haemolytic index.

The experiment involves two steps *viz.* preliminary testing and the main haemolytic testing. The erythrocyte suspension was prepared by mixing one–tenth of its volume with sodium citrate (36.5 g/L). 1 mL of this citrated blood was mixed with phosphate buffer (pH 7.4) in a 50 mL volumetric flask to obtain a 2% (v/v) solution. The reference solution was freshly prepared by dissolving 10 mg of diosgenin in phosphate buffer (pH 7.4) and the volume was made up to 100 mL with the buffer. Serial dilution of the plant extract was also prepared in phosphate buffer pH 7.4. The blood suspension and different dilutions of the extract and standard diosgenin were mixed properly and were allowed to stand for 6 hr at room temperature. All the tubes were carefully monitored and examined for haemolysis. The minimum concentration of plant extract and diosgenin that produces heamolysis were noted and the haemolytic activity of the plant material was calculated incorporating the formulae:

$$\text{Heamolytic index} = [(1000) \times (a/b)]$$

Where, 1000 is the defined haemolytic activity of saponin (diosgenin) in relation to ox blood, 'a' is the quantity of saponin (diosgenin) that produces total haemolysis (g), 'b' is the quantity of plant extract that produces total haemolysis (g).

8. Quantitative evaluation

8.1. Estimation of total polyphenols

Total polyphenol content was determined spectrophotometrically with UV-Visible 1700 Shimadzu spectrophotometer, according to a modified method (Sharma et al., 2008) using Folin-Ciocalteu's reagent. 100 µL of ethanolic extract of *Pyrus pashia* (EPP) was taken in a 25 mL volumetric flask followed by addition of 500 µL of Foilin Ciocalteu's reagent (1N) and 1 mL of 30% sodium

carbonate solution in it. Volume was made up to 25 ml using distilled water followed by vigorous shaking. Solution was then left for incubation for 60 min. The appearance of blue colour was measured spectrophotometrically at 730 nm against a sample blank. The total polyphenol content of EPP was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (20, 40, 60, 80, 100 μLaliquots of 0.1% aqueous gallic acid) and expressed as mg gallic acid equivalent (GAE)/gm of the EPP. All the measurements were done in triplicate.

8.2 Determination of total flavonoids

For the determination of total flavonoid content, the samples were measured using a modified colorimetric method (Kosalec et al., 2004) (100 μL) of EPP was taken in 5 mL volumetric flasks followed by addition of 100 μL of 10% aluminium chloride (w/v) and 100 μL of 1 M potassium acetate solution in it. Volume was then adjusted to 5 mL with distil water. Solution then forth was incubated at room temperature (25°C) for 30 min; the absorbance of the reaction mixture relative to blank was measured at 415 nm. The total flavonoid content of samples was expressed as mg quercetin equivalent/gm of EPP. For the preparation of calibration curve 25, 50, 75, 100 and 125 μL of standard quercetin in ratio of (0.5 mg/mL) were mixed with the same reagents as described above and after 30 minutes, the absorbance at 415 nm was measured for determination of total flavonoids. All measurements were done in triplicate.

8.3. Estimation of total alkaloid content

The estimation of total alkaloid content was done as per the gravimetric methods (Wagner and Bladt, 1996). About 5 g of the powdered drug was extracted repeatedly using (3 x 50 mL) 0.1 N H_2SO_4 in an ultrasonic bath. The solution was filtered; the mixed acid solution was washed with 4 successive quantities of 25 mL chloroform (washing each chloroform solution with 20 mL of acid). The chloroform washings was rejected, acid solution was basified with dilute ammonia solution

and was further extracted with (20 mL x 5) diethyl ether. The combined diethyl ether extracts were washed with 5 mL of distilled water and the ether was evaporated to dryness in a weighed beaker on a water bath, which was further dried to constant weight at 105°C and the % w/w of alkaloid was calculated.

8.4. Estimation of total saponin content

The total saponin content was determined as per the methods described by (Baccou et al., 1977). The plant material was first defatted with petroleum ether and 0.5 g of the defatted material was accurately weighed and kept in plastic centrifuge tubes containing 10 mL of absolute methanol. The mixture was kept for overnight in a magnetic stirrer and the content was centrifuged. The supernatant was collected in a 25 mL capacity measuring flask. The remaining residue was again washed out twice with 5 mL of fresh methanol and again centrifuged. The overall supernatant was collected and the final volume was made up to 25 mL with methanol. Standard diosgenin solution was used as reference and was freshly prepared by dissolving 10 mg diosgenin in 20 mL methanol and its standard curve was prepared using different dilutions in concentration ranging between 25–125 µg/mL.

The solution was transferred to the test tubes placed in ice–water bath and vanillin reagent (0.25 mL) was added to all the test tube and was kept on a magnetic stirrer. To this 2.5 mL (75 % v/v) of sulphuric acid with proper stirring was added slowly and the whole mixture was warmed in a water bath at 60°C for 10 min which was later cooled in ice cold water. The absorbance of the solution mixture after cooling was measured at 544 nm. A blank solution was prepared using methanol as solvent. For determination of total saponin in the plant extract 0.25 mL of the extract was used. The results were calculated using the standard regression equation of saponins and the results were expressed as mg/g diosgenin equivalent.

8.5. Estimation of total carbohydrate content

The total carbohydrate content was determined as per the calorimetric method as described by Yemm and Willis (1954) using anthrone reagent which was freshly prepared by dissolving 0.2 g of anthrone in 100 mL of water. The plant material was exhaustively extracted with 70% (v/v) ethanol and was used for this estimation. The anthrone reagent (5 mL) was pipetted out into a thick walled pyrex tube (150 × 25 mm) and which was chilled in ice cold water. The test solution (1 mL) was layered on the acid medium, cooled for further 5 min and was finally mixed thoroughly in cooling condition. The tubes were then loosely fitted with corks, heated in boiling water bath and were later cooled for 5 min. Finally the absorbance of the solution was measured spectrophotometrically at 600 nm, where the blank was prepared and read against water. d-Fructose at different dilutions was used as reference standard for the above estimation.

9. Characterization of polyphenolics, amino acids in *Pyrus pashia* fruits

9.1 Polyphenolic profile of Pyrus pashia

Screening and quantification of major polyphenolics in EPP, was carried out following the method of (Sharma et al., 2007). Analysis was performed on a Waters HPLC system equipped with 600 quaternary gradient pump, 2998-PDA and 717 auto sampler. Separation was achieved at 25˚C on a RP-18 Reverse phase column (250 mm x 4.0 mm, 5 µm), fitted with suitable guard column using 0.05% trifluoroacetic acid in water (A) and acetonitrile (B) as mobile phase. Elution of standards and samples was performed at a flow rate of 1.0 ml/min and detection at 355 nm. Identification of compounds was performed on the basis of the retention time, co-injections, and spectral matching with standards. Three replicates were considered.

9.2. Amino acid profile of Pyrus pashia

AccQ Tag derivatisation

Amino acid derivatisation with Waters AccQ Tag reagents was directed according to the protocol of waters. Briefly, 25 µL of aliquot of Std / Sample + 175µL of borate buffer and vortexed briefly. Added 50 µL of AccQ Tag Ultra reagent and immediately vortexed for several seconds. Allowed to stand for one minute at room temp. The reaction was allowed to proceed for 10 min at 55˚C.

Ultra-pressure liquid chromatography (UPLC) analysis for amino acids

Liquid chromatographic analysis was performed on a Waters Acquity UPLC-H Class system, equipped with a quaternary solvent manager, an autosampler, a column heater and λ PDA detector. Waters AccQ Tag column (3.9 mm i.d. x150 mm, 1.7 µl) was used for the separation of amino acids. The column heater was set at 35˚C and flow rate was 0.4 ml/min. Eluents A and B were 100% AccQ Tag Ultra-pure solvents supplied by Waters. The non-linear separation gradient was 0–3 min (100% A), 7.5 min (52.0% A), 12.5 min (100% B) and 15.0 min (100% A). Waters VanGuard™ column (2.1 mm i.d. x 5 mm, 1.7 lm) was used as the guard column; 1 µl of sample was injected for analysis. The eλ PDA detector was set at 254 nm. Mean area of three replicate injections was considered (Joshi et al., 2013)

10. Isolation of chrysin from ethanolic extract of *Pyrus pashia*

Fruits of *Pyrus pashia* has not been much explored in terms of chemistry till date. The ethanolic extract of *Pyrus pashia* (EPP) was first subjected to phytochemical screening followed by quantification of phytoconstituents. As per literature review it was found that fruits of *Pyrus pashia* are persuasive source of polyphenols (Rana and Singh 2013). Above research further affirmed the richness of fruits in polyphenols. Earlier studies have established that these polyphenols are implicated for treatment of epilepsy eg: luteolin, apigenin, rutin etc (Diniz et al 2015).

Polyphenolic finger printing revealed that chrysin was the major polyphenol in EPP, hence it was selected for isolation. Isolated chrysin was further used for pharmacological studies and quantification of extract with help of UPLC (Sharma et al. 2017).

EPP was partioned with ethylacetate in order to obtain ethyl acetate fraction (PPEA). PPEA was subjected to column chromatography silica gel 60–120 mesh (eluting with a gradient of DCM: Ethyl acetate). DCM: Ethylacetate 50:50 afforded brown residue 40 mg. Purification of brown residue was again re-chromatographed using silica gel column chromatography starting with DCM ethyl acetate gradient elusion, increasing gradient of ethyl acetate up to 70% afforded residue C1 (19.0 mg). This C1 was then washed with ethyl acetate. Extract was then crystallized to give compound 1. C1 was then characterized with the help of U.V, FTIR, HRMS and NMR techniques.

11. Quantification of chrysin in ethanolic extract of *Pyrus pashia* by UPLC

Ultra-performance liquid chromatography (UPLC) analysis was carried out for standardizing EPP. A Waters UPLC system, USA with UV detector was used to perform the following analysis, where separation was carried out with a Cosmosil C_{18} column (150 mm × 4.6 mm, 5 µm particle). A stock solution of EPP (5 mg/ml) and isolated chrysin (0.5 mg/ml) was prepared in methanol. The mobile phase consisted of a gradient mixture prepared from 0.1% formic acid (component A) and acetonitrile (component B).The sample, standard solution and mobile phase were filtered through a 0.45 µm membrane filter prior to use. The flow rate was kept at 0.3 mL/min, with an injection volume of 10 µL. The data was collected at wavelength 264 nm while the peaks were identified by comparing its retention time with that of standard (Class VP series software, Shimadzu, Japan). The UPLC analysis depicted well resolved and single sharp peaks of chrysin confirming its purity. From the standard plot of the chromatogram, the percentage of chrysin in EPP with similar R_t value (4.9min) was reported to be 1.73% (w/w).

Conditions for UPLC

Time	Flow	%A	%B
0.00	0.3	95.0	5.0
0.50	0.3	80.0	20.0
1.00	0.3	75.0	25.0
2.00	0.3	65.0	35.0
2.50	0.3	30.0	70.0
3.50	0.3	5.0	95.0
4.00	0.3	30.0	70.0
4.50	0.3	65.0	35.0
5.00	0.3	75.0	25.0
5.20	0.3	80.0	20.0
5.70	0.3	95.0	5.0
6.00	0.3	95.0	5.0

Table.4 Conditions for UPLC

1. Experimental animals

Inbred Charles Foster albino male rats (180–220 g) were collected from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University, and were housed in polypropylene cages under controlled environmental conditions with a 12 h light/dark cycle. The experimental animals had free access to food and water ad libitum. All the experimental procedures utilized were performed in accordance with the approval of the Institutional Animal Ethics Committee (Protocol No: Dean/2015/ CAEC/1129) under strict compliance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for the experimental studies.

2. Acute toxicity study

An acute oral toxicity study was performed according to the 'Organization for Environmental Control Development' guidelines (OECD: Guidelines 420; Fixed Dose Method) for oral administration of EPP (suspended in 0.5% carboxymethyl cellulose (CMC). Albino male rats (N = 6, 150–200 g), fasted overnight for 18 h were used for the experiment. The EPP was administered

in increasing doses of 5, 50, 300, and a maximum dose up to 2000 mg/kg to the experimental animals. Moreover, the rats did not show any gross behavioral, neurological, or autonomic toxic effects after the first 3 h of EPP administration and for any lethality after 24–72 h till 14 days.

3. Dose selection

A low dose of 100 mg/kg (half of one-tenth of the maximum lethal dose), a median dose of 200 mg/kg (one-tenth of the maximum lethal dose), and a high dose of 400 mg/kg (twice that of one tenth dose) were selected for evaluation of pharmacological activities. Dose for chrysin was selected as per the literature review, 2.5, 5, and 10 mg/kg.

4. Evaluation of anticonvulsant activity of standardized EPP and chrysin

Acute Model

4.1. Maximal electroshock (MES)-induced convulsion model

The animals were divided into eight groups of six each. Animals of negative control (NC), PHT-25, EPP-100, EPP-200 and EPP-400, similarly chrysin-2.5, chrysin-5 and chrysin-10 group received CMC (0.5%; p.o.), Phenytoin (25 mg/kg, i.p.), EPP (100 mg/kg, p.o.), EPP (200 mg/kg, p.o.), EPP (400 mg/kg, p.o.), chrysin (2.5 mg/kg, p.o.), chrysin (5 mg/kg, p.o.), chrysin 10 mg/kg, p.o.) respectively. All the group animals received the respective treatments for 14 days consecutively. At a time lag of 30 min, all the animals received electroshock to induce seizure in the rats on the last day of the experimental schedule. Seizures in terms of hind limb tonic extension (HLTE) were evoked in rats using alternating current stimulus (150 mA, 50 Hz, 0.2 s) via ear clip (Sayyah et al., 2002).The duration of HLTE of the animals was recorded as a parameter to indicate the anticonvulsant activity of the drugs.

4.2 Pentylenetetrazole induced convulsion (PTZ) model

The animals were randomly divided into eight groups of six rats each. Animals of NC, DZ-5, EPP-100, EPP- 200, and EPP-400 similarly chrysin-2.5, chrysin-5 and chrysin-10 group received CMC (0.5%; p.o.), Diazepam (5 mg/kg, i.p.), EPP (100 mg/kg, p.o.), EPP (200 mg/kg, p.o.), EPP (400 mg/kg, p.o.), chrysin (2.5 mg/kg, p.o.), chrysin (5 mg/kg, p.o.), chrysin 10 mg/kg, p.o.) respectively. All the group animals received the respective treatments for 14 days consecutively. At a time lag of 30 min, all the animals received PTZ (60 mg/kg, i.p.) to induce seizure in the rats on the last day of the experimental schedule. The duration of the first onset of convulsion was recorded as an indicator of anticonvulsant activity of the drugs (Patil et al., 2011).

4.3. Evaluation of anticonvulsant activity of EPP (100, 200, and 400mg/kg; p.o.) in PTZ challenged rats

For electroencephalogram (EEG) recording, two stainless steel screw electrodes were implanted stereotaxically (Quintessential stereotaxic injector Stoelting Co., Wood Dale, IL, USA) over visual cortex (6.0 mm posterior to the bregma and 3.5 mm lateral to the midline on both sides) and another electrode (11 mm anterior to the bregma on the midline served as ground) under intraperitoneal thiopentone anesthesia (40 mg/kg, i.p.) to 25 animals (Sahu et al., 2012)Thereafter, electrodes were soldered to a miniature socket and the whole assembly was anchored to the skull with dental acrylate. After 72 h, all animals were randomly divided into five groups of five rats each. Animals of NC, DZ-5, chrysin-2.5, chrysin-5, and chrysin-10 group received CMC (0.5%; p.o.), Diazepam (5 mg/ kg, i.p.), EPP (100 mg/kg, p.o.), EPP (200 mg/kg, p.o.), and EPP (400 mg/kg, p.o.), respectively. All the group animals received the respective treatments for 14 days consecutively. At a time lag of 30 min, all the animals received PTZ (60 mg/kg, i.p.) to induce seizure in the rats. The duration of the first onset of convulsion was recorded as an indicator of anticonvulsant activity

of the drugs on the last day of the experimental schedule subsequently, EEG was recorded using a polygraph MP35 (Biopac System Inc., Santa Barbara, California, USA). The signals were amplified and filtered (0.05–35 Hz), henceforth digitized at a sample rate of 200 Hz and recorded using the data acquisition program BSL Pro. Baseline observations of the animals were recorded 1 h prior to administration of chrysin, Diazepam, or Vehicle. Power spectrum of EEG was computed by Fast Fourier Transformation, using BSL Pro 3.7 (Biopac System Inc.). Digitized values of four major frequency bands, i.e. alpha (8–13 Hz), beta (13–30 Hz), delta (0.5–4 Hz), and theta (4–8 Hz), were recorded. Power spectrum was expressed as the percentage of baseline after PTZ injection. Epoch length of 10 s was recorded in two phases: first during behavioral tonic–clonic seizure and second during maximum EEG activity where tonic–clonic seizure was not observed (between 3 and 10 min after PTZ injection) (Sahu et al., 2012). The animals were sacrificed by decapitation following EEG recording. The brain was excised and microdissected into the hippocampus, cerebellum, and cerebral cortex and were stored at –80°C for future studies. Tissues were homogenized in phosphate buffer (pH 7.4), and thereafter centrifuged for 5 min at 5000×g. (Rodrigues et al., 2011). Supernatants were collected for further biochemical estimation.

4.4. Evaluation of anticonvulsant activity of chrysin (2.5, 5, and 10 mg/kg; p.o.) in PTZ challenged rats.

For electroencephalogram (EEG) recording, two stainless steel screw electrodes were implanted stereotaxically (Quintessential stereotaxic injector Stoelting Co., Wood Dale, IL, USA) over visual cortex (6.0 mm posterior to the bregma and 3.5 mm lateral to the midline on both sides) and another electrode (11 mm anterior to the bregma on the midline served as ground) under intraperitoneal thiopentone anesthesia (40 mg/kg, i.p.) to 25 animals (Sahu et al., 2012). Thereafter, electrodes were soldered to a miniature socket and the whole assembly was anchored to the skull with dental

acrylate. After 72 h, all animals were randomly divided into five groups of five rats each. Animals of NC, DZ-5, chrysin-2.5, chrysin-5, and chrysin-10 group received CMC (0.5%; p.o.), Diazepam (5 mg/ kg, i.p.), chrysin (2.5 mg/kg, p.o.), chrysin (5 mg/kg, p.o.), and chrysin (10 mg/kg, p.o.), respectively. All the group animals received the respective treatments for 14 days consecutively. At a time lag of 30 min, all the animals received PTZ (60 mg/kg, i.p.) to induce seizure in the rats. The duration of the first onset of convulsion was recorded as an indicator of anticonvulsant activity of the drugs on the last day of the experimental schedule (Patil et al., 2011) subsequently, EEG was recorded using a polygraph MP35 (Biopac System Inc., Santa Barbara, California, USA). The signals were amplified and filtered (0.05–35 Hz), henceforth digitized at a sample rate of 200 Hz and recorded using the data acquisition program BSL Pro. Baseline observations of the animals were recorded 1 h prior to administration of chrysin, Diazepam, or vehicle. Power spectrum of EEG was computed by Fast Fourier Transformation, using BSL Pro 3.7 (Biopac System Inc.). Digitized values of four major frequency bands, i.e. alpha (8–13 Hz), beta (13–30 Hz), delta (0.5–4 Hz), and theta (4–8 Hz), were recorded. Power spectrum was expressed as the percentage of baseline after PTZ injection. Epoch length of 10 s was recorded in two phases: first during behavioral tonic–clonic seizure and second during maximum EEG activity where tonic–clonic seizure was not observed (between 3 and 10 min after PTZ injection) (Sahu et al., 2012). The animals were sacrificed by decapitation following EEG recording. The brain was excised and microdissected into the hippocampus, cerebellum, and cerebral cortex and were stored at –80°C for future studies. Tissues were homogenized in phosphate buffer (pH 7.4), and thereafter centrifuged for 5 min at 5000×g. (Rodrigues et al., 2011). Supernatants were collected for further biochemical estimation.

4.5. Effect of EPP (100, 200, and 400mg/kg; p.o.) and chrysin (2.5, 5, and 10 mg/kg) on PTZ induced oxidative stress in different brain regions.

4.5.1. Estimation of Lipid peroxidation (LPO)

Lipid peroxidation in different brain regions was estimated by method described by Ohkawa et al., 1979.

Principle

Malondialdehde (MDA), a secondary product of LPO reacts with thiobarbituric acid in order to form pink chromogen (Thiobarbituric acid-2 malondialdehyde adduct), which is measured spectrophotometrically.

Reagents

a) 8.1 % sodium dodecyl sulphate (Sigma) - 810 mg SDS dissolved in 10 ml distilled water.

b) 20% Acetic acid (Merck) - 20 ml of Glacial acetic acid added to 80 ml of distilled water (freshly prepared). The pH of the solution was adjusted to 3.5 by adding NaOH.

c) 0.8% Thio-barbituric acid (Sigma) - 800 mg TBA dissolved in 100ml of distilled water.

d) 15:1 Butanol and Pyridine mixture (Merck) - 15 ml of n- butanol was mixed with 1 ml of Pyridine.

e) 1, 1, 3, 3-tetramethoxypropane (Sigma) was used as standard for LPO estimation.

Procedure

LPO level was estimated in terms of malondialdehyde (MDA). To 0.2 ml of 100mg/mL tissue homogenate, 0.1 ml of 8.1% SDS, 0.75 mL of 20% acetic acid solution (pH 3.5) and 0.75 mL of 0.8% aqueous solution of TBA was added in stoppered tubes. The mixture was made up to 2mL with distilled water and 2.5 mL of mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 3000 rpm for 10 min the organic layer was taken and its absorbance at 532 nm was measured against blank containing 0.2 mL of distilled water in place of

sample. 1, 1, 3, 3-tetramethoxypropane (Sigma) was used as external standard and the level of LPO was expressed as nmol MDA/g wet tissue.

4.5.2. Estimation of Catalase (CAT)

CAT activity in discrete brain regions was evaluated by the method described by (Beers and Sizer 1952).

Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in presence of hydrogen peroxide (H_2O_2), resulting in formation of perchromic acid as an unstable intermediate. The chromic acetate, thus produced is measured spectrophotometrically.

Reagents

a) Phosphate buffer (0.01M; pH 7.0)

b) Disodium hydrogen phosphate

c) Potassium dihydrogen orthophosphate

d) Potassium dichromate ($K_2Cr_2O_7.7H_2O$-5%)

e) Glacial acetic acid

f) Hydrogen peroxide (H_2O_2-0.2M)

Procedure

The tissue homogenate was mixed with phosphate buffer (1.0mL) followed by addition of H_2O_2 for initiation of reaction. The reaction was arrested immediately by addition of 2.0 mL dichromate-acetic acid reagent at 0, 30, and 60 seconds interval. The reagent blank was prepared by addition of 1.6 mL of buffer 2.0 mL of dichromate acetic acid reagent taken in separate tubes. The test and blank tubes were heated in boiling water bath for 10 min to develop green colour. The tubes were

cooled to room temperature and their intensity was measured at 570 nm using spectrophotometer against the blank. Results obtained were expressed as µmol H_2O_2 consumed /min/mg of protein.

4.5.3. Estimation of Reduced Glutathione (GSH)

(Aksenov and Markesbery 2001) method was followed to estimate GSH in different brain regions.

Principle

DTNB is a disulphide compound which is reduced by sulphydryl group of reduced glutathione (GSH) and can form yellow colour. The yellow colour is measured by spectrophotometer.

Reagents

a) 50% Trichoroacetic acid- 10 gm of TCA added to 20 mL distilled water.

b) 0.4M Tris buffer (Sigma) – Prepared by dissolving 4.84 g of Tris in small amount of distilled water and by adding 10 mL of 0.2 M EDTA and make up to 100 mL with distilled water. pH was adjusted to 8.9 with 1N HCl.

c) 0.2 M Ethylene diamine tetra acetic acid (Merck) 7.4 g of sodium salt of EDTA was dissolved in distilled water to make it a volume of 100 mL.

d) 0.02M EDTA- 10 mL of 0.2M EDTA was made up to volume of 100 mL with distilled water.

e) 0.1M Di-thio bis nitro benzoic acid (Ellman's reagent) (Hi media) – 9.9 mg of DTNB ($C_{14}H_{17}N_3O_6S$) was dissolved in 25 mL of absolute methanol.

f) Reduced glutathione ($C_{10}H_{17}N_3O_6S$) was used as standard for GSH estimation.

Procedure

1ml of tissue homogenate (100mg/mL) was mixed in 15 mL test tubes with 0.8 mL of distilled water and 0.2 mL of 50 % TCA. The tubes were shaken intermittently for 10–15 min and centrifuged for 15 min at 3000 rpm. 0.6 ml of supernatant was mixed with 0.8 ml of 0.4 M Tris

buffer (pH 8.9) and 20 µL 0.1 mL of DTNB in absolute alcohol and the sample was shaken. The absorbance was read within 5 min of the addition 40 µL of DTNB at 412 nm against a reagent blank with no homogenate. The results were expressed as mcg/g wet tissue and were calculated from the standard curve prepared by using standard glutathione.

4.5.4. Estimation of Superoxide dismutase (SOD)

Superoxide dismutase (SOD) was estimated by following the procedure of Kakkar and associates (1984).

Principle

The assay of SOD is based on inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction is extracted in butanol and measured at 560 nm.

Reagents

a) 0.52 M sodium pyrophosphate (Sigma) – 1.16 gm of SPP was in 50 mL of distilled water and pH was adjusted to 8.3

b) 186µM Phenazine methosulphate (Sigma) – 18 µL of 100mM of PMS added to 10 mL of distilled water. 100mM of PMS prepared by adding 306 mg in 10 mL of distilled water.

c) 300µM Nitroblue Tetrazolium (Sigma) – 2.754 mg of NBT dissolved in 10 mL of distilled water.

d) 780µM Nicotienamide adenine dineucleotide (Sigma) – 11.1 mg of NADH dissolved in 20 mL of distilled water.

e) Glacial acetic acid.

f) n-butanol.

Procedure

The inhibition of reduction of nitro blue tetrazolium (NBT) to blue colored formozan in presence of phenazine metha sulphate (PMS) and NADH was measured at 560 nm using n-butanol as blank. To 0.2 ml of the homogenate was added 0.6 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml of 186µM of phenazine methosulphate, 0.3 ml 300 µM nitroblue tetrazolium and 0.4ml of distilled water to make up the volume up to 1.5 including with 0.8 ml of NADH (780 µM). Reaction was started by the addition of NADH. After incubation at 30° C for 60 sec, the reaction was stopped by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml of *n*-butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was taken out. Colour intensity of the chromogen in the butanol was measured at 560 nm in spectrophotometer against butanol. A system devoid of enzyme was served as control. One unit of enzyme activity is defined as enzyme concentration required for inhibiting the optical density at 560 nm of chromogen protection by 50% in one min under the assay conditions, and the results have been expressed as units (U) of SOD activity/mg wet tissue.

4.5.5. Estimation of Protein Carbonyl Content

Principle

Protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer 1994).

Reagents

a) Dinitrophenylhydrazine (DNPH),prepared in 2 M HCl.

b) 20% trichloroacetic acid.

c) Guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3).

d) Ethanol

e) Ethyl acetate.

Procedure

Briefly, 100 µl of homogenate were added to plastic tubes containing 400 µl of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 h and vortexed each 15 minutes. After that, 500 µl of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 20.000 g for 3 minutes. The supernatant obtained was discarded. The pellet was washed with 1 ml ethanol/ethyl acetate (1:1 v/v), vortexed, and centrifuged at 20.000 g for 3 minutes. The supernatant was discarded and the pellet resuspended in 600 µl of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60 ºC for 15 minutes. After that, it was centrifuged at 20.000 g for 3 minutes and the absorbance was measured at 370 nm (UV) in a quartz cuvette. Results were represented as protein carbonyl content (nmol/mg protein).

5. Evaluation of motor dysfunction test of EPP (100,200 and 400 mg/kg; p.o.) and chrysin (2.5, 5, and 10 mg/kg; p.o.) in rats

Animals were randomly divided into 8 groups of five rats each. Animals of control, DZ-5, EPP-100, EPP-200, and EPP-400, chrysin-2.5, chrysin-5 and chrysin-10 group received CMC (0.5%; p.o.), Diazepam (5 mg/kg, i.p.), EPP (100 mg/kg, p.o.), EPP (200 mg/kg, p.o.), EPP (400 mg/kg, p.o.), chrysin (2.5 mg/kg, p.o.), chrysin (5 mg/kg, p.o.), and chrysin (10 mg/kg, p.o.), respectively. All the group animals received the respective treatments for 14 days consecutively. After 30 min to last treatment, all the animals were subjected to assess neurotoxicity in terms of duration of movement (spontaneous locomotor activity) using a standard protocol in a photoactometer. (Kulkarni et al., 1997). Subsequently, all the animals were subjected to rotarod test apparatus at a

time lag of 30 min to evaluate the extent in loss in motor function in terms of running time duration (Goyal et al., 2009).

6 Mechanistic studies

As assessed in our earlier research EPP 200 and 400 mg/kg, along with chrysin 5 and 10 mg/kg showed significant anticonvulsant effects. Hence minimum effective doses of EPP (200 mg/kg) and chrysin (5mg/kg) were selected for further investigation.

6.1. Pentylenetetrazole-Induced Seizures

Pentylenetetrazole (60 mg kg^{-1}, s.c.) was used to induce clonic convulsions (Patil et al., 2011). Mice were divided into 4 groups (n=6) and received EPP (200 mg/kg, *p.o.)* and chrysin (5 mg/kg, *p.o.*), diazepam (1, mg/ kg, s.c.) or vehicle (normal saline; 10 mL/kg i.p.) 30 min prior the injection of PTZ, respectively. After PTZ injection, animals were placed in testing chambers (made of Perspex of dimensions 15 cm x15 cm x15 cm). Animals were observed for behavioral parameter including, latency, of clonic convulsions and percentage protection. The observed clonic seizures were characterized for the appearance of facial myoclonus, forepaw myoclonus and forelimb clonus. The ability of a drug to prevent the seizures or delay/prolong the latency or onset of the clonic convulsions was considered as an indication of anticonvulsant activity.

6.2. Picrotoxin-Induced Seizures

Anticonvulsant testing method of (Singh et al 2012) was used. Briefly, mice were divided into 4 groups (n=6) and received EPP (200 mg/kg, *p.o.)* and chrysin (5 mg/kg, *p.o.*), diazepam (1 mg/kg, i.p.) or vehicle (normal saline; 10 mL kg-1 i.p.) 30 min before the injection of picrotoxin (PTX) (3.5 mg kg-1 i.p.) respectively. Latency to convulsions, and percentage protection of animals were recorded for 30 min.

6.3. Isoniazid-Induced Seizures

Mice were divided into four groups (n=6) and received EPP (200 mg/kg, *p.o.)* and chrysin (5 mg/kg, *p.o.*), vehicle (normal saline; 10 mL kg/ i.p.) or the standard drug diazepam (1.0 mg kg/, i.p.),30 min after administration of test compounds, animals were injected with isoniazid (300 mg/kg, s.c.). Thereafter, mice were observed for 120 min for characteristic behavioural signs, such as intermittent forelimb extension, clonic seizures, tonic seizures and death. The latencies to the onset of the convulsive episode (clonic or tonic) and death were recorded as indicators of pro- or anticonvulsive effect of compounds (Madhu et al., 2009).

6.4 Strychnine-Induced Seizures

Method as described by (Amabeoku et al 2007) was employed. Mice were divided into 4 groups (n=6) and received EPP (200 mg/kg, *p.o.)* and chrysin (5 mg/kg, *p.o.*), diazepam (1 mg/kg, i.p.) and vehicle (normal saline; 10 mL/kg i.p.) 30 min before the injection of strychnine STN (2 mg/kg, i.p.), respectively. Latency, of clonic convulsions were assessed for 30 min.

6.5. 4-Aminopyridine-Induced Seizures:

The method adopted for this study was as described by Rogawski and Porter (1990) with modifications. Mice were divided into 4 groups (n=6) and received EPP (200 mg/kg, *p.o.)* and chrysin (5 mg/kg, *p.o.*), vehicle and the standard drug carbamazepine (30 mg/kg, *p.o.*). Thirty minutes after administration of test compounds, animals were treated with a single injection of 4-AP (12 mg/kg, i.p.). Thereafter, mice were observed 30 min for both clonic and tonic seizures. Clonic seizures were characterized as appearance of facial myoclonus, forepaw myoclonus and forelimb clonus and tonic seizures were characterized as explosive clonic seizures with wild running and tonic forelimb and hind limb extension. Latencies for the onset of convulsive episodes

(clonic or tonic) and death were recorded as indicators of pro or anticonvulsive effect of compounds.

6.6. Maximal Electroshock Seizure Test:

Electroconvulsions were produced by means of an electric current (50 Hz, 150 mA, 0.2 s) delivered via ear-clip electrodes with an Electroconvulsometer Unit 100-3 (Ambala, India). This current intensity elicited complete tonic extension of the hind limbs in control mice. Mice received EPP (200 mg/kg, *p.o.)* and chrysin (5 mg/kg, *p.o.*), phenytoin (25 mg/kg, *i.p.*) and vehicle (normal saline; 10 mL/kg, *i.p)* 30 min before tonic hind-limb convulsions were induced. Protection against tonic hind limb seizures was determined. The duration of HLTE of the animals was recorded as a parameter to indicate the anticonvulsant activity of the drugs. An animal was considered to be protected if the characteristic electroshock convulsive seizure pattern was absent (Sayyah et al., 2002).

6.7. N-methyl D-aspartate test

Mice were divided into four groups (n=6) and received EPP (200 mg/kg, *p.o.)* and chrysin (5 mg/kg, *p.o.*), vehicle and the standard drug, D-2-amino-7-phosphonoheptanoate (D-AP7; 33 nmol/kg, i.p.). Turning behavior was induced in mice by subcutaneous injection of N-Methyl-D-aspartate 75 mg/kg, 30 min after treatment. Mice were observed for30 min. The latency time to turning behavior was recorded. Animals that did not exhibit turning behavior within the 30 min were declared protected (Taiwe et al., 2009).

6.8. Effect on $GABA_A$

To investigate the possible involvement of $GABA_A$ receptors in the anticonvulsant action of chrysin, mice were pre-treated with flumazenil (2 mg/kg, i.p.), a selective benzodiazepine receptor antagonist or vehicle 15 min before EPP (200 mg/kg, *p.o.)* and chrysin (5 mg/kg, *p.o.*)and DZP (1

mg/kg, i.p.) administration. After 45 min, mice were challenged subcutaneously with PTZ (65 mg/kg) and assessed 30 min for latency, frequency and duration of clonic convulsions (Adongo et al., 2017)

7. Chronic studies

7.1. Animals

Male Swiss albino mice of around 3 months, weighing 20–30 g, were used in this study were collected from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University, and were housed in polypropylene cages under controlled environmental conditions with a 12 h light/dark cycle. The experimental animals had free access to food and water ad libitum. All the experimental procedures utilized were performed in accordance with the approval of the Institutional Animal Ethics Committee (Protocol No: Dean/2015/ CAEC/1129) under strict compliance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for the experimental studies.

7.2. Kindling induction

Pentylenetetrazole, dissolved in normal saline was injected intraperitoneally (i.p.) at subconvulsive dose of (35mg/kg) at 48±2hrs interval for a total of 14 times (Dhir 2012). After each injection, the mice were placed individually into plexiglass cages (20x20x30cm) and were observed for 30 min. The intensity of convulsions was recorded according to Racine's scale. Stage 0: no response; stage 1: hyperactivity, restlessness and vibrissae twitching; stage 2: head nodding, head clonus and myoclonic jerks; stage 3: continuous myoclonic jerk with tail rigidity; stage 4: generalized limbic seizures with kangaroo posture or violent convulsions; stage 5: generalized tonic–clonic seizures with falling and stage 6: hind limb extensor and stage 7: death or recovery. The animals were

considered epileptic after appearance of stage 5 (tonic–clonic convulsions) seizures on consecutive pentylenetetrazole administration.

7.3. Experimental protocol

Forty animals in the different groups were subjected to kindling with sub-effective dose of PTZ (35mg/kg, i.p.) on every alternating days. Animals in group I (veh/ptz) received daily 10 ml/kg, p.o. vehicle treatment, whereas group II Diazepam (DZP1/ptz) received standard, DZP 1mg/kg, i.p. group III (EPP200/ptz) and group IV (chrysin5/ptz) were administered with 200 and 5 mg/kg doses of EPP and chrysin p.o. dissolved in distilled water respectively. All the animals in groups I-IV were injected with 35 mg/kg, i.p. PTZ (dissolved in normal saline), at 48±2-hr intervals, 30 min. after their respective vehicle/DZP/EPP/chrysin treatment. Immediately after PTZ administration, the animals were individually placed in isolated transparent chambers for behavioral observations for 1 hr. The severity of induced convulsions as a consequence of PTZ administration was scored on a seven-point scale according to Racine's scale. Seizure severity score of the day the animals achieved kindling in the veh/ptz group, 14th PTZ injection was compared with (DZP1/ptz), (EPP200/ptz) and (chrysin5/ptz). In the present study, 14 injections of PTZ were required to acquire kindling in mice (27 days). Tests for cognitive functions were performed for the next 6 days that is day 28 to day 33, after kindling procedure (Figure.38).

8. Cognitive function test

8.1. Morris water maze

To determine whether EPP/chrysin affects PTZ-induced cognitive impairment, mice were tested in the MWM starting 24 h after the last PTZ injection (Vorhees and Williams 2006). The apparatus consisted of a water pool (120-cm diameter) that was virtually distributed into four quadrants and filled with water (22±1°C). A colorless escape platform (10 cm in diameter) was submerged 2 cm

below the water surface in a labelled target quadrant. The pool was placed in a noiseless room. The experiment involved two parts: learning trials (existed platform) and probe trials (non-existed platform). Prior to the experiment, mice were trained for 5 consecutive days. Mice underwent 2 sessions daily, having intersession interval of 2hrs. Each session comprised of 4 trials, with inter-trial interval of 30 seconds. In each trial, the rat was placed in a random quadrant with its head toward the wall and was required to find the hidden platform. Mice were allotted 60 seconds to trace the hidden platform; those that were unable to locate the platform within 60 seconds were directed and allowed to sit on the platform for 15 seconds. The escape latency – the time taken by each mouse to identify the platform was recorded. On the sixth day, the platform was removed from the target quadrant and the time spent by the mice in the target quadrant looking for the platform was recorded.

9. Detection of cell death in brain tissue using propidium iodide staining:

Propidium iodide (PI) staining was used to detect cell apoptosis. PI is a polar compound that can only enter into dead and dying neurons and binds to nucleic acid resulting in a red fluorescence emission at 630 nm upon excitation at 495 nm with an intensity linearly related to the number of dead cells (Naseer et al. 2009). Mice were anaesthetized with sodium pentobarbital (50 mg/kg BW) and fixed on stereotaxy apparatus (Stoelting Co., USA). The hair was removed from the head and skin was sterilized. The skin was breached from the skull after making an incision using scalpel. The bregma and lambda points were located and a small burr hole was made at the following coordinates: -4.0 mm posterior to bregma, -1.6 mm lateral to the midline (right side) (Medel-Matus et al., 2014). The hole was drilled to a depth 3.5 mm through the skull slowly at the injection site using 25-gauge needle. The mice were administered with 1µl PI at the rate of 0.5 µl/min. The mice were transcardially perfused with PBS followed by 4% paraformaldehyde.

Further, the brain from different groups of mice were dissected out and fixed in 4 % paraformaldehyde at 4 °C for 12 hrs. Following fixation, the tissue was immersed in 30% sucrose solution and embedded in cryomounting medium (O.C.T solution, Europe). Thereafter, 20 µm coronal sections were cut by a cryotome and mounted on poly-L-lysine-coated slides (0.1 % v/v). The sections were mounted with Vectashield mountant containing DAPI (Vector Laboratories Inc, USA) and PI-positive neurons in the hippocampus region was detected under fluorescence microscope.

10. Expression analysis of apoptotic biomarkers and BDNF levels by western blotting: For western blot studies, mice (n=6) were sacrificed by cervical dislocation, following behavioral testing. Hippocampus of mice was dissected and the tissue was lysed in buffer containing complete protease inhibitor cocktail. Protein concentrations were determined according to (Bradford and Ward 1976). A standard plot was generated using bovine serum albumin. An aliquot of each sample was electrophoresed in 10% SDS-PAGE gel. Thereafter, the membrane was incubated separately with primary antibodies, anti-cytochrome c (ab 13575, Abcam UK), 1:10,000; anti caspase 9 (ab 184786, Abcam UK), 1:10,000; anti caspase 3 (ab 179517, Abcam UK), 1:10,000; anti BDNF(ab 205067, Abcam UK),1:10,000; anti CREB(ab 32515, Abcam UK) , 1:10,000; mouse anti CREB [(phospho S133) (ab 32096),]1:10,000 . After detection with the desired antibodies against the proteins of interest, the membrane was stripped with stripping buffer (25 mM Glycine pH 2.0, 2% SDS) for 30 min at room temperature and reprobed overnight with rabbit anti β- actin polyclonal primary antibody (8226, Abcam, UK) at a dilution of 1 : 500 to confirm equal loading of protein. Further, membrane was probed with corresponding secondary antibodies. Immunoreactive band of proteins were detected by chemiluminescence using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). Quantification of the results

was performed by densitometric scan of films. The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software.

11. Expression analysis of gephyrin levels in hippocampus region of kindled animals

Total protein was isolated from the hippocampi of mice of different experimental groups using lysis buffer containing 20 mM Tris–HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, and 1 mM protease inhibitor cocktail (Sigma-Aldrich, USA) at 4 °C and centrifugation at 10, 000Xg for 10 min (Bradford and Ward 1976). The supernatant was used for western blotting. 40 µg protein samples were estimated, denatured and resolved by 10% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein was transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) skimmed milk in tris-buffered saline (TBS) for 2 hr. The membrane was incubated overnight with mouse anti-gephyrin antibody (610584, BD Biosciences, 1:2000) and washed three times with 1X TBS. The membranes were incubated with HRP conjugated goat anti-mouse secondary antibodies (62114068001A, Bangalore Genei, 1:2000) for 2 hr, washed thrice with 1X TBS and subjected to enhanced chemiluminescent (ECL) detection on X-ray films. The membranes were reprobed with β actin (1:3000, A3854; Sigma-Aldrich, USA) as endogenous control for 4 h and detected by ECL on X-ray films.

Immunoreactive band of proteins were detected by chemiluminescence using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). Quantification of the results was performed by densitometric scan of films. The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software.

12. Data analysis

All values were expressed as mean ± standard error of mean (SEM). All the statistical analysis of data was done using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc test to monitor significance among groups. $P < 0.05$ was considered as significant.