

Test for saponin glycosides biology essay



**ASSIGN
BUSTER**

Male wistar rats weighing between 150 and 220 gm were used for this study. The animals will be obtained from animal house, IRT, Perundurai medical College, Erode, TamilNadu, India. On arrival, the animals were randomly grouped in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of $24 \pm 2^{\circ}\text{C}$ and relative humidity of 30-70%. A 12:12 light: day cycle was followed. All the animals were allowed to free access to water and fed with standard commercial pelleted rat chaw (M/s. Hindustan Lever Ltd., Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional animal ethics committee (688/2/C-CPCSEA) of NCP and were accordance with the guidelines of the IAEC. Approval was obtained from the IAEC, NCP, Erode (Proposal No: NCP/IAEC/PG/2010-01).

Plant material

The plant material consists of dried powdered bark of vitex negundo Linn. Belonging to the family Verbenaceae.

Plant collection and authentication

The bark of vitex negundo Linn was collected from vaikkalmedu, Erode district in Tamil Nadu, India during the month of June 2010. The plant was identified by Mr. G. V. S. Murthy, Joint Director, Scientist, C-I/C, Botanical survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference number BSI/SRC/5/23/10-11/Tech. 673.

Preparation of plant extract

Fresh bark of vitex negundo linn was collected from koorapalayam, Erode, TamilNadu, India. The bark was dried for one month and latter powdered.

This powder was then macerated with methanol for 72 h with occasional shaking. It was then filtered and the solvent was evaporated under heating mandle. The yield of methanolic extract of bark of vitex negundo linn, (VN) was 34% (w/w).

Drugs and chemicals

Xanthine, DPPH, ascorbic acid, nitro blue tetrazolium, butylated hydroxyl toluene oxidized glutathione, epinephrine and 5 5'Dithiobis-2 nitro benzoic acid were purchased Himedia Labs., Pvt. Ltd., Mumbai. 2-deoxy-2-ribose, Quercetin, O-dianisidine were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai. Trichloroacetic acid and Folin Ciocalteau reagent were purchased from SD Fine Chemicals Ltd., Mumbai. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

Qualitative phytochemical analysis

The extract of bark of Vitex negundo linn subjected to the following chemical tests for the identification of its various active constituents (Kokate 2002).

REAGENTS

Dragendroff's reagent:

Solution (A): Dissolved 0.85 g basic bismuth nitrate in 10 ml glacial acid and 40 ml of distilled Water under heating. If necessary filter.

Solution (B): Dissolved 8 gm of potassium iodide in 30 ml of distilled water.

Stock solution: solution (A) + (B) are mixed 1: 1.

Libermann- Burchard Reagent:

50 ml of acetic anhydride and 5ml concentrated sulphuric acid were added carefully to 50 ml absolute ethanol, while cooling in ice.

Molish's reagent:

Dissolved 10 gm of a-naphthol in 100ml of 95% alcohol.

Legal's Test:

2 ml pyridine, 2ml sodium nitroprusside, 3ml sodium hydroxide solution.

Ninhydrine reagent:

0. 1% ninhydrine in n-butanol.

Fehling's solution:

Dissolved 34. 6 gm of copper sulphate in distilled water and make volume up to 500 ml (solution A).

Dissolved 173 gm of potassium sodium tartarate and 50gm of sodium hydroxide in distilled water and make volume up to 500 ml (solution B). Mix two solutions in equal volume.

Wagner's Reagent:

Dissolved 1. 27 gm Iodine and 2g of potassium iodide in 5 ml of water and make up to

Volume 100 ml with distilled water.

Lead Acetate Reagent:

25 mg lead acetate dissolved in 100 ml sodium hydroxide solution.

Hagner's Reagent:

3 ml of saturated aqueous solution of picric acid.

Braemer's Reagent:

5 ml of 10% ferric chloride solution.

TEST FOR STERIODS**Libermann-Burchard Test:**

Treat the extract with few drops of acetic acid anhydride boil and cool. Then add concentrated sulphuric acid from the side of the test tube, brown ring is formed at the junction two layers and upper layer turns green which shows presence of steroids and formation of deep red colour indicates presence of triterpenoids.

Sulphur powder Test

Add a small amount of sulphur powder to the test solution, it sinks at the bottom.

Salkowski test

Treat the extract with few drops of concentrated sulphuric acid red color at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of triterpenoids.

TEST FOR TRITERPENOIDS**Salkowski test**

Treat the extract with few drops of concentrated sulphuric acid , red color at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of triterpenoids.

TEST FOR SAPONIN GLYCOSIDES

Froth formation Test (foam)

Place the 2 ml solution of drug in water in a test tube, shake well, stable froth (foam) is formed.

Haemolysis test

Add 0.2 ml of solution of saponin (prepared in 1% normal saline) to 0.2 ml of blood in normal saline and mix well. Centrifuge and note the red supernatant compare with control tube containing 0.2 ml of 10% blood in normal saline diluted with 0.2 ml of normal saline.

Test for alkaloids:

Small portion of solvent free ethanol extract with few drops of dilute hydrochloric acid and filter. Using the following reagents carried out the tests for alkaloids.

Dragendroff's test:

A drop of extract was spotted on a small piece of precoated TLC plate and the plate was sprayed with modified Dragendroff's reagent. (Orange coloration of the spot indicated presence of alkaloids).

Hager's test:

The extract was treated with a few ml of Hager's reagent (yellow precipitate indicated the presence of alkaloids).

Wagner's test:

The extract was treated with few ml of Wagner's reagent (reddish brown precipitate indicated the presence of alkaloids).

Test for glycosides:

Legal's test: Dissolved the extract (0.1 gm) in pyridine (2ml) and add sodium nitroprusside solution (2ml) and make alkaline with sodium hydroxide solution (pink to red color solution indicated the presence of glycosides).

Baljet test:

To the extract, sodium picrate solution is added. It shows yellow to orange color.

Test for flavonoids:**Shinoda Test:**

2-3ml of extract, a piece of magnesium ribbon and 1ml of concentrated hydrochloric acid was added. (Pink red or red coloration of the solution indicated the presence of flavonoids).

Lead acetate Test:

5ml of extract solution was added with 1ml of lead acetate solution. (Flocculent white precipitate indicated the presence of flavonoids).

Zinc hydrochloride Test:

To the test solution, add a mixture of zinc dust and concentrated hydrochloric acid. It gives red color after few minutes.

Test for tannins:**Ferric chloride Test**

With 1% ferric chloride solution the extract gives blue, green, or brownish green color indicating the presence of tannins.

Gelatin Test

When the extract is treated with 1% solution of gelatin containing 10% sodium chloride, white precipitate is obtained.

In vitro antioxidant activity

1. Reducing power ability

The reducing power was investigated by the Fe^{3+} - Fe^{2+} transformation in the presence of the extract. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. It was measured by mixing 1.0 ml extract of various concentration prepared with distilled water to 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 30 min. After that 2.5 ml of trichloroacetic acid (10%) were added to the mixture and centrifuged for 10 min at 3000 g, 2.5 ml from the upper part were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1%, ferric chloride. The absorbance was measured at 700 nm using UV-spectrophotometer. The reference solution was prepared as above, but contained water instead of the samples. Increased absorbance of the reaction mixture indicates increased reducing power. All experiments were done in triplicate using butylated hydroxytoluene (BHT) as positive control (Yildirim et al., 2001).

2. Superoxide anion scavenging activity

A reaction mixture with a final volume of 3 ml per tube was prepared with 1.4 ml of 50 mM KH_2PO_4 -KOH, pH 7.4 containing 1 mM EDTA, 0.5 ml of 100 μ M hypoxanthine, 0.5 ml of 100 μ M NBT. The reaction was started by adding 0.066 units per tube of xanthine oxidase freshly diluted in 100 μ l of phosphate buffer and 0.5 ml of test extract in saline. The xanthine oxidase

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was added last. The subsequent rate of NBT reduction was determined on the basis of spectrophotometric determinations of absorbance at 560 nm. Ascorbic acid was used as standard. The results are expressed as the percentage inhibition of NBT reduction rate with respect to the reaction mixture without test compound (Guzman et al., 2001).

3. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured by the ability of the extract to scavenge the hydroxyl radicals generated by the Fe³⁺-ascorbate-EDTA-H₂O₂ system (Fenton reaction) (Halliwell, 1994). The reaction mixture in a final volume of 1.0 ml contained 100 µl of 2-deoxy-2-ribose (28 mM in 20 mM KH₂PO₄ buffer, pH 7.4), 500 µl of the fractions at various concentrations (50-800 µg/ml) in buffer, 200 µl of 1.04 mM EDTA and 200 µM FeCl₃ (1:1 v/v), 100 µl of 1.0 mM hydrogen peroxide (H₂O₂) and 100 µl of 1.0 mM ascorbic acid. Test samples were kept at 37°C for 1 h. The free radical damage imposed on the substrate, deoxyribose was measured using the thiobarbituric acid test. One ml of 1% thiobarbituric acid (TBA) and 1.0 ml 2.8% trichloroacetic acid (TCA) were added to the test tubes and were incubated at 100°C for 20 min. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Quercetin (50-800 µg/ml) was used as a positive control.

4. DPPH radical scavenging activity

The hydrogen donating ability of the extract was examined in the presence of DPPH stable radical. One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentrations (10-160 µg) of extract and allowed to react at room temperature. After 30 min, the

absorbance values were measured at 517 nm. Ascorbic acid was used as standard (Guzman et al., 2001).

5. Nitric oxide radical scavenging assay

Various concentrations of the extract and sodium nitroprusside (5mM) in phosphate buffer saline (0.025 M, pH 7.4) in a final volume of 3 ml are incubated at 25°C for 150 min. Control experiments without the test compounds but with equivalent amount of buffer is prepared in the same manner as done for the test. There after, 0.5 ml of incubation solution is removed and diluted with 0.5 ml Griess' reagent (1% sulphanilamide, 2% O-Phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride is read at 546 nm. The percentage inhibition is calculated. The experiment is done in triplicate using curcumin (50-800 µg/ml) as positive control (Sreejayan and Rao, 1997).

6. Ferrous chelating ability

The ferrous level is monitored by measuring the formation of the ferrous ion-ferrozine complex. The reaction mixture containing different concentrations of extracts (50-800µg/ml) were added to 2 mM ferrous chloride (0.1 ml) and 5 mM ferrozine (0.2 ml) to initiate the reaction and the mixture is shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution is measured at 562 nm. The positive control are those using ascorbic acid and all tests and analysis are run in triplicate. The percentage chelating effect of Ferrozine-Fe²⁺ complex formation is calculated (Huang and Kuo, 2000).

Calculation of percentage inhibition (%I)

The concentration (μ g/ml) of the extract required to scavenge the radicals was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated using the formula,

$$I \% = (Ac-As) \times 100$$

Ac

Where Ac is the absorbance of the control and As is the absorbance of the sample.

Calculation of 50% inhibition (IC50)

The concentration of the extract required to scavenge 50% inhibition of radicals was calculated by using the software Graph Pad Instant.

Paracetamol induced nephro and hepatotoxicity.

A total of 30 animals were used and divided into the following 5 groups (n= 6 in each group),

Group I: Normal control group (normal saline 5 ml /kg)

Group II: Paracetamol 750 mg /kg (Negative control)

Group III: Paracetamol 750 mg /kg + standard drug (silymarin 25 mg /kg)

Group IV: Paracetamol 750 mg /kg + plant bark extract (200 mg /kg)

Group V: Paracetamol 750 mg /kg + plant bark extract (400 mg /kg) (S. Palani, 2010).

IN VITRO HEPATOPROTECTIVE ACTIVITY

Composition of Krebs, s Ringer HEPES medium (KRH)

2. 5 mM HEPES PH 7. 4

118 mM Sodium chloride

2. 85 mM Potassium chloride

2. 5 mM Calcium chloride

1. 15 mM Potassium dihydrogen phosphate

1. 18 mM Magnesium sulphate

4. 0 mM Glucose

Double distilled water

Liver slice culture in vitro

Liver slice culture was maintained following the protocol developed by (Wormser et al., 1990). Wistar albino rats weighing about 150-200 g was dissected after cervical dislocation and liver lobes were removed and transferred to pre-warmed KRH medium. Liver was then cut into thin slices using sharp scalpel blades. The liver slices were weighed and those weighing between 20 and 25 mg were used for the experiment. Each experimental system contained 20-22 slices weighing about 500-600 mg. The slices were washed with 10 ml KRH medium, every 10 min for a period of 1 h. They were

then pre-incubated for 60 min in small plugged beakers containing 2 ml KRH medium on a shaker water bath at 37°C. At the end of the pre-incubation, the medium was replaced by fresh 2 ml KRH and incubated for 2 h at 37°C with either paracetamol or plant extracts or both. Two different experimental conditions were used for treatment with plant extract; i. e. 1) Plant extract was present for 30 min during pre-incubation and also for next 2 h along with paracetamol. 2) Plant was present only for 2 h along with paracetamol. A portion of liver tissue in each group after respective drug treatment was preserved in 10% formalin for histopathological studies. At the end of incubation, each group of slices was homogenized in appropriate volume of chilled potassium phosphate buffer (100 mM, pH 7.8) in an ice bath to give a tissue concentration of 100 mg/ml. The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C and the supernatants assayed for lactate dehydrogenase (LDH), catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and reduced glutathione.

Preparation of homogenate

Liver and kidney were removed from the animal and kept in the 0.2M, pH 6.6, phosphate buffer, by using the homogenizer liver, and kidney were crushed and the obtained homogenate was subjected to centrifugation process and centrifuged at 3000 rpm for 10 min. The obtained supernatant was used for following tests.

Estimation of protein content

Protein content of the tissue homogenate was assayed by the method of Lowry et al., (1951). The blue copper developed by the reduction of phosphomolybdic phosphotungstic components in the Folin-Ciocalteu

reagent by the amino acid tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured by the Lowry's method. About 50 mg of bovine serum albumin was weighed accurately and dissolved in distilled water and made up to 50 ml in a standard flask. About 10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 μg proteins. About 0.2 to 1.0 ml of the working standard was pipette out into series of test tube. About 0.1 ml of supernatant was pipetted out in other test tubes. The volume was made up to 1 ml in all the test tubes with distilled water. A tube with 1 ml of water served as the blank. Five ml of alkaline copper solution was added to the test tubes and allowed to stand for 5 min. Then 0.5 ml of Folin reagent was added and incubated at room temperature in dark for 30 min. The absorbance was measured at 660 nm. Protein content was expressed as $\mu\text{g}/\text{mg}$ of protein (Lowry et al., 1951).

Estimation of malondialdehyde (MDA)

Lipid per oxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) and hydroperoxides (HP) were measured by the method of Nieshus and Samuelsson, 1986. About 0.1 ml of lens homogenate (Tris HCl buffer, PH 7.4) was treated with 2 ml (1:1:1 ratio) of TBA -TCA-HCL reagent (Thiobarburic acid 0.37%, 0.25N HCl and 15% TCA) and placed in a water bath for 15 min, cooled and centrifuged at 1000 g at room temperature for 10 min. the absorbance of the clear supernatant was measured against a reference blank at 535 nm. The values are expressed as nmoles of MDA/min/mg protein.

Estimation of lipid hydroperoxides

About 0.1 ml of homogenate was treated with 0.9 ml of Fox reagent (188 mg Butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium ion sulphate were added to 90 ml of methanol and 10 ml 250mM sulphuric acid) and incubated for 30 min. the colour developed was read at 560 nm using a colorimeter. The values are expressed as nmoles/mg protein (Nieshus and Samuelsson, 1986).

Determination of enzymatic anti oxidants

Estimation of superoxide dismutase (SOD)

SOD activity was determined by the inhibition of auto catalyzed adrenochrome formation in the presence of tissue homogenate at 480 nm. The reaction mixture contained 150 µl of homogenate, 1.8 ml of 30 mM carbonate buffer (pH 10.2), and 0.7 ml of distilled water and 400 µl of epinephrine (45 mM). Auto oxidation of epinephrine to adrenochrome was performed in a control tube without the homogenate. The activity was expressed as units/mg tissue protein (Misra and Fridovich, 1972).

Estimation of catalase (CAT)

The catalysis of H₂O₂ to H₂O in an incubation mixture adjusted to pH 7.0 was recorded at 254 nm. The reaction mixture contained 2.6 ml of 25 mM potassium phosphate buffer pH 7.0 and 0.1 ml of tissue homogenate and was incubated at 37°C for 15 min and the reaction was started with the addition of 0.1 ml of 10 mM H₂O₂. The time required for the decrease in absorbance from 0.45 to 0.4 representing the linear portion of the curve was used for the calculation of enzymatic activity. One unit of catalase

activity was defined as the amount of enzymes causing the decomposition of $\mu\text{mol H}_2\text{O}_2/\text{mg protein}/\text{min}$ at pH, 7.0 at 25°C (Aebi, 1984).

Estimation of peroxides (Px)

Peroxidase activity was measured spectrophotometrically by following the change in absorbance at 460 nm due to o-dianisidine oxidation in the presence of H_2O_2 and enzyme. Reaction mixture contained 0.2 ml of 15 mM o-dianisidine, 0.1 ml of homogenate and 2.5 ml of 0.1 M potassium phosphate buffer pH 5.0 and were incubated at 37°C for 15 min and the reaction was started with the addition of 0.2 ml of hydrogen peroxide and the absorbance at 460 nm was followed against a blank, spectrophotometrically for about 3-5 min at 37°C. Unit of enzyme activity defined as micro moles of o-dianisidine /min/ at 37°C (Lobarzewski and Ginalska, 1995).

Estimation of glutathione peroxidase (GPx)

Glutathione peroxidase activity was measured by the method described by Paglia and Valentine (1967). The reaction mixture consisted of 0.2 ml of 0.4 M phosphate buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.1 ml of 0.2 mM hydrogen peroxide, 0.2 ml of glutathione and 0.2 ml of supernatant. The contents were incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.4 ml 10% TCA and the absorbance was measured at 340 nm.

Estimation of glutathione reductase (GSSH)

The enzyme activity was determined spectrophotometrically by the decrease in absorbance of NADPH at 340 nm. The reaction mixture contained 2.1 ml

of 0.25 mM, potassium phosphate buffer pH 7.6, 0.1 ml of 0.001 M NADPH, 0.2 ml of 0.0165 M oxidized glutathione and 0.1 ml (10 mg/ml) of bovine serum albumin (BSA). The reaction was started by addition of 0.02 ml of liver and kidney homogenate with mixing and the decrease in the absorbance at 340 nm was measured for 3 min against a blank. Glutathione reductase activity was expressed as moles NADPH oxidized/min/mg at 30°C (Racker, 1955).

Determination of non enzymatic antioxidant

Estimation of reduced glutathione (GSH)

The method was based on the reaction of reduced glutathione with dithionitrobenzoic acid (DTNB) to give a compound that absorbs at 412 nm. Briefly after centrifugation, 0.5 ml of supernatant was taken and mixed with 2.0 ml of 0.3 mol/L di-sodium hydrogen phosphate (Na_2HPO_4) solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml, 1% sodium citrate) was added and the absorbance was measured immediately after mixing. Results were expressed in $\mu\text{mol GSH}/\text{min}/\text{mg protein}$ (Sener et al., 2007).

Statistical analysis

Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. P values <0.05 were considered significant.