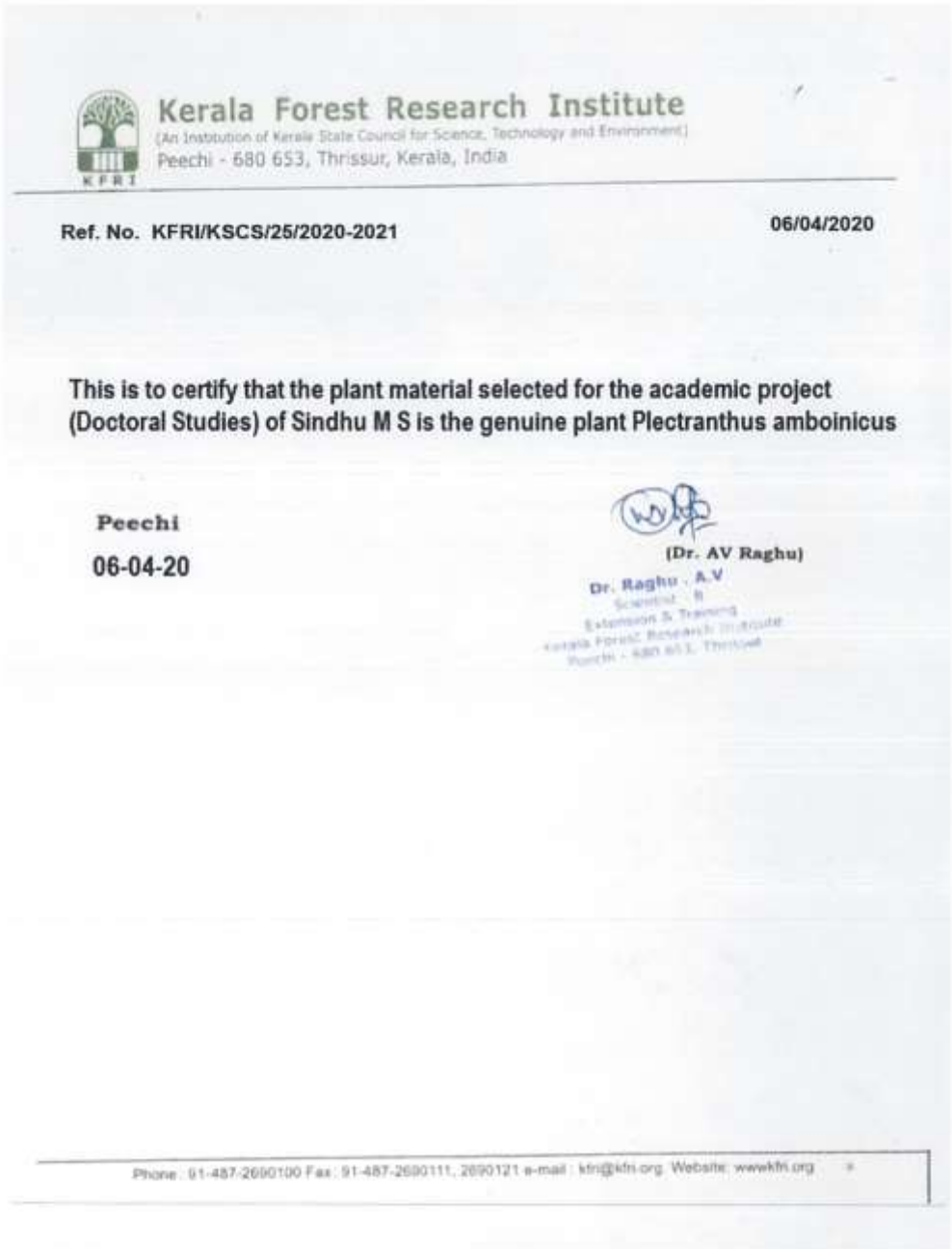
APPENDIX - 1

AUTHENTIFICATION OF PLANT



Kerala Forest Research Institute
(An Institution of Kerala State Council for Science, Technology and Environment)
Peechi - 680 653, Thrissur, Kerala, India

KFRI

Ref. No. KFRI/KSCS/25/2020-2021 06/04/2020

This is to certify that the plant material selected for the academic project (Doctoral Studies) of Sindhu M S is the genuine plant Plectranthus amboinicus

Peechi
06-04-20

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APPENDIX - 2

PRELIMINARY PHYTOCHEMICAL ANALYSES IN THE LEAF EXTRACTS

1. DETECTION OF CARBOHYDRATES

The following tests were performed for the presence of carbohydrates.

a) Molisch test

A few drops of Molisch solution was added to 2 mL of the extracts, thereafter a small volume of concentrated sulphuric acid was allowed to run down the side of the test tube to form a layer without shaking. The interface was observed for a purple colour as a positive indicative for carbohydrates.

b) Benedict's test

Equal volume (1ml) each of Benedict's reagent and the extracts were mixed and heated in boiling water bath for 5 minutes. The reducing sugars were analyzed by the presence of green, yellow or red precipitate depending on the amount of the sugar. Glucose was used as positive control.

2. DETECTION OF PROTEINS

a) Ninhydrin test

To 3 ml of the extracts 3-4 drops of 5% ninhydrin solution was mixed. The mixture was heated in boiling water bath for 10 minutes and the appearance of purple or bluish colour indicates the presence of proteins and free amino acids.

b) Biuret test

To 3 ml of plant extracts, few drops of 4% NaOH and few drops of 1% $CuSO_4$ were added and the solution was observed for pink or violet colour development which indicates the presence of proteins and free amino acids.

3. DETECTION OF SAPONIN

Froth test

An aliquot of the extracts was shaken vigorously with water and observed for persistent foam for 15 minutes which indicates the presence of saponins.

4. DETECTION OF ALKALOIDS

a) Mayer's test

An aliquot of extracts were treated with Mayer's reagent (1.36g of mercuric chloride and 5g of potassium iodide in 100mL of distilled water) and noted for the formation of cream colored precipitate.

b) Dragendroff's reagent

An aliquot of extracts were treated with Dragendroff's reagent (Solution A : 1.7g of bismuth nitrate, 20mL of glacial acetic acid, 80mL of water. Solution B: Potassium iodide (4g), glacial acetic acid (10ml), 20mL of water. Mix A and B) and observed for the formation of reddish orange precipitate.

c) Wagner's test

To various extracts, 1 ml of Wagner's reagent was added drop by drop. The formation of a reddish-brown precipitate indicated the presence of alkaloids.

5. DETECTION OF TANNINS

a) Braemer's test

To 0.5ml of extracts, 10mL of water was added separately, boiled and filtered. To the filtrate few drops of 10% ferric chloride was added. Appearance of dark green or blue or brown color indicates the presence of tannins.

6. DETECTION OF PHENOLS

a) Ferric chloride test

To an aliquot of the extracts, 5% $FeCl_3$ was added and observed for the formation of deep blue color.

b) Lead acetate test

To an aliquot of the extracts, 10% lead acetate solution was added and observed for the formation of white precipitate.

7. DETECTION OF FLAVONOIDS

a) Aqueous NaOH test

To a small aliquot of the extracts, 1N NaOH solution was added and observed for the formation of yellow-orange color.

b) Schinodo's test

Aliquot of the extracts were treated with a piece of magnesium turnings followed by few drops of concentrated hydrochloric acid, heated slightly and observed for the formation of dark pink color.

8. DETECTION OF GLYCOSIDES

a) Legal's Test

The extracts (1ml) were treated with 2ml of sodium nitroprusside in pyridine and sodium hydroxide solution was added to make it alkaline. Formation of pink to blood red color indicates the presence of glycosides.

b) Keller - Killani test

The extract was treated with a few drops of glacial acetic acid and $FeCl_3$ solution and mixed well. To the mixture, conc. H_2SO_4 was added and observed for the formation of two layers. The lower layer was reddish brown in color. The upper acetic acid layer turned bluish green indicating the presence of glycosides.

c) TEST FOR TERPENOIDS

To the extracts, 2mL of chloroform and few drops of concentrated sulphuric acid were added along the sides of the test tubes. A reddish brown color in the chloroform layer and green fluorescence in the acid layer indicates the presence of terpenoids.

9. DETECTION OF STEROLS

a) Liebermann – Burchard test

To the extracts, 2mL of chloroform was added, followed by 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. The presence of steroids is indicated by the appearance of rose red color, which quickly changes from blue to green.

10. DETECTION OF QUINONES/ ANTHRAQUINONE

a) Borntrager's test

The extracts were hydrolysed with dil. HCl, and then treated with ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture

was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

11. DETECTION OF ANTHOCYANINS

The presence of anthocyanins has been demonstrated by adding 2mL of the extracts with 2mL of 2N HCl. The appearance of a pink-red color that turns purplish blue after addition of ammonia indicates the presence anthocyanins.

12. DETECTION OF LEUCOANTHOCYANIN

Approximately 5 ml of isoamyl alcohol was added to 5 ml of the extracts seperately. The appearance of a red upper layer indicated the presence of leucoanthocyanin.

APPENDIX - 3

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

DPPH (1, 1 – diphenyl-2-picrylhydrazyl) radical scavenging ability of the methanolic leaf extract of *Plectranthus amboinicus* was assessed following the method of Mensor *et al*. (2001).

Principle

DPPH (2,2-diphenyl-2-picryl hydrazyl), a stable free radical, when acted upon by an antioxidant, is converted into diphenyl-picryl hydrazine with a colour change from deep violet to light yellow colour. This can be quantified spectrophotometrically at 515 nm to indicate the extent of DPPH Scavenging activity by the extracts.

Reagents

1. DPPH (0.3mM in methanol)
2. Methanol

Procedure

An aliquot of the methanolic extract (0.01ml) at different concentrations was added to 0.5ml of methanolic solution of 0.3mM DPPH and made upto 1ml with

methanol. The reaction mixture was incubated for 30 minutes at room temperature in dark. DPPH solution with methanol served as a positive control and methanol alone acted as blank. After incubation, the discoloration from deep violet to yellow color was noted at 515nm and the percent radical scavenging activity was calculated and compared with ascorbic acid which served as a standard.

$$\text{Scavenging activity (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

APPENDIX - 4

DETERMINATION OF ABTS RADICAL SCAVENGING ACTIVITY

The radical scavenging effect of methanolic extract of *Plectranthus amboinicus* leaves was assessed by ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation de-colourisation assay (Shirwaikar *et al*., 2006).

Principle

ABTS, a chromogen, changes into $ABTS^+$, a coloured mono-cation radical form in the presence of an oxidative agent, and has absorption peak at 750nm. Antioxidants will reduce $ABTS^+$ into its colourless form and the extent of decolourisation corresponds to the per cent reduction of $ABTS^+$

Reagents

1. Ethanol
2. ABTS solution (7mM ABTS with 2.45mM ammonium persulfate)

   The solution was incubated at room temperature for 12-16 hours before use.

Procedure

The ABTS radical cations ($ABTS^+$) were produced by adding 7mM ABTS solution with 2.45mM ammonium persulphate. The reaction mixture was incubated for 12-16 hours in dark at room temperature. To different concentrations

of the leaf extract (0.01ml), ABTS solution (0.3ml) was added and the volume was made up to 1ml with ethanol finally. ABTS in ethanol without the test samples and ethanol alone served as the positive control and blank respectively. Ascorbic acid (20- 100µg/ml) was used as a reference compound. The absorbance was read at 745nm and the per cent inhibition was calculated using the formula,

$$\text{Inhibition (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

APPENDIX - 5

DETERMINATION OF HYDROGEN PEROXIDE SCAVENGING ACTIVITY

The scavenging of H_2O_2 by the methanolic extract of *Plectranthus amboinicus* leaves was determined by the method of Ruch *et al*. (1989).

Principle

H_2O_2 scavenging activity can be measured in terms of a decrease in absorbance at 230 nm spectrophotometrically.

Reagents

1. Phosphate buffer (0.1M, pH 7.4)
2. H_2O_2 (40mM in phosphate buffer)

Procedure

The ability of the leaf extract to scavenge hydrogen peroxide was assessed by adding varying concentrations of the leaf extract and ascorbic acid acid (0.01ml) to 0.6ml of H_2O_2. The volume was made upto 3ml with phosphate buffer solution. After 10 minutes of incubation, the absorbance of the reaction mixture was measured spectrophotometrically at 230nm against blank containing phosphate buffer without H_2O_2. The per cent inhibition was calculated and compared with the standard ascorbic acid.

$$H_2O_2 \text{ scavenging activity (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

APPENDIX - 6

DETERMINATION OF INHIBITION OF SUPER OXIDE RADICAL GENERATION

The potential of the methanolic extract of *Plectranthus amboinicus* leaves to inhibit the *in vitro* generation of superoxide was tested following the method of Winterbourne *et al*. (1975).

Principle

The assay is based on the inhibition of the production of nitroblue tetrazolium formazon of the superoxide ion by the extracts and measured spectrophotometrically at 560 nm.

Reagents

1. EDTA (0.1M containing 1.5mg NaCN/10ml)
2. NBT (1.5mM)
3. Riboflavin (0.12mM in distilled H_2O)
4. Phosphate buffer (0.067M, pH 7.6)

Procedure

To the different concentrations of methanolic extract (0.01ml), 0.2ml of EDTA, 0.1ml of nitrobluetetrazolium and 0.05ml of riboflavin was added and the volume was made upto 3ml with phosphate buffer solution. The tubes were illuminated consistently by means of a fluorescent lamp for 30 minutes, and the absorbance was measured again at 560 nm. The disparity in optical density before and after illumination was taken as the measure of superoxide generation and compared with the standard ascorbic acid. The results were expressed in per cent inhibition and compared with the standard

$$\text{Superoxide Scavenged (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

APPENDIX - 7

DETERMINATION OF INHIBITION OF NITRIC OXIDE RADICAL GENERATION

The method developed by Green *et al*. (1982) was employed to test the inhibition of *in vitro* generated nitric oxide radicals by the methanolic extract of *Plectranthus amboinicus* leaves.

Principle

At physiological pH, nitric oxide generated from the aqueous solution of sodium nitroprusside interacts with oxygen to produce nitrite ions, which are quantified spectrophotometrically at 546 nm by the Griess Innosvoy reaction.

Reagents

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (PBS) (pH 7.4)
3. Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diaminedihydrochloride)

Procedure

The capability of the leaf extract to inhibit nitric oxide generation was initiated by the addition of 2ml of sodium nitroprusside and 0.5ml phosphate buffer saline to 0.5ml of various concentrations of leaf extract. The reaction mixture was mixed well and incubated at 25ºC for 150 minutes. The control tube was prepared without the leaf extract. Griess reagent (0.5ml) was added to the reaction mixture and allowed to stand for 30 minutes. The absorbance of the pink colored chromogen was measured at 546 nm against a reagent blank and the per cent inhibition was calculated and compared with ascorbic acid, the reference compound

$$\text{Nitric oxide scavenging activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

APPENDIX - 8

ESTIMATION OF TOTAL PHENOLS

(Mallick and Singh, 1980)

Principle

The hydroxyl (-OH) groups of phenolic compounds reduce the phosphomolybdic acid to molybdenum blue in the presence of an alkaline medium(present in Folin's reagent). The blue coloured complex was then spectrophotometrically measured at λ 760 nm.

Regents

1. Stock standard solution : Dissolve 100mg of gallic acid in 100ml of distilled water(1ml=1µg).
2. Working standard solution : Makeup 10ml of the stock to 50ml with distilled water (1ml=200µg).
3. Folin-Ciocalteu reagent: Mix the Folin's reagent with distilled water in the ratio of 1:1. This reagent should be prepared freshly.
4. Sodium carbonate (7%)

Procedure

Pipette out 0.1, 0.2, 0.3, 0.4,and 0.5ml(concenteration varying from 20 to 100µg) of the working standard solution into a series of test tubes. Pipette out 0.125ml of the unknowm sample into a test tubes. To all the tubes, including the blank, add distilled water to make up to 3.5ml. To all the test tubes, add 0.125ml of Folin's reagent.Incubate the test tubes at room temperature for 6 minutes. Add 1.25ml of 7% sodium carbonate in all the test tubes. Incubate the test tubes at room temperature for 9ominutes. Note the absorbance spectrophotometrically at λ 760nm. Draw the graph by plotting the concenteration of total phenolics along the X-axis and the optical density reading along Y-axis. From the satandard curve on the graph, calculate the unknown sample concenteration. The values were expressed as mg/g of dry weight.

APPENDIX - 9

ETHICAL CLEARANCE FORM

Avinashilingam Institute for Home Science and Higher Education for Women
(Deemed to be University under Category 'A' by MHRD, Estd. u/s 3 of UGC Act, 1956)
Re-accredited with 'A' grade by NAAC, Recognised by UGC under Section 12 B
Coimbatore – 641 043, Tamil Nadu, India
(Reg. No. *623/PO/ReBi/S/02/CPCSEA*)

Certificate

This is certify that the project title Role of Syringic acid in benzo(a)pyrene induced lung cancer in Mice

has been approved by the IAEC.

Proposal Number: IAEC/17-18/05

Approval date: 02-12-2017, Approval No.: AIW:IAEC-2017:Z00:03

No. of animals approved: 40 Mice **Gender:** Male/Female

Expiry date (Termination of the project):

	Name	Signature with date
IAEC Chairperson/ ~~Member Secretary~~:	Dr. P. R. Padma	2/12/17
CPCSEA nominee:	Dr. Ganesan Anbara Sivakumar	2/12/17

APPENDIX - 10

ESTIMATION OF HAEMOGLOBIN
(Drabkin and Austin, 1932)

Principle

Haemoglobin is converted into acid haematin committee by the action of HCl. The acid haematin solution is further diluted with distilled water until its colour matches with exactly that of permanent standard of comparator block. The Hb concentration is read directly from the calibration tube.

Procedure

0.1N HCl was added in the haemoglobinometer upto the lowest marking. 20µl of blood was drawn up to 20µl in the Sahli's pipette. Adjusted the blood column carefully without bubbles. Wiped the excess of blood on the sides of the pipette by using a dry piece of cotton. Blown the blood into the acid solution in the graduated tube, rinsed the pipette well. Mixed the reaction and allowed the mixture to stand at room temperature for 10 minutes. Diluted the solution with distilled water by adding a few drops of water carefully and by mixing the reaction mixture until the colour matches the colour in the comparator. The lower miniscus of the fluid was noted and reading was noted in g/100ml

APPENDIX - 11

ENUMERATION OF RED BLOOD CORPUSCULES
Diluting fluid method
(Sanderson and Phillips, 1981)

Principle

The blood specimen is diluted to 1:200 dilutions with RBC diluting fluid which does not remove the white cells but allows the red cells to be counted under high power (40x) objective using a Neubauer's counting chamber. The number of cells in undiluted blood is calculated as the number of red cells per cubic mm of whole blood.

Reagents

RBC diluting fluid (Hayem's fluid) - 5g of sodium sulphate, 1g of sodium chloride, 0.5g of mercuric chloride were dissolved in 200ml of distilled water.

Procedure

Blood was sucked exactly up to the 20µl mark in the RBC pipette and the diluting fluid was drawn immediately up to the mark and the blood was mixed thoroughly with the diluting fluid. It was left for 2-3 minutes for proper mixing. The Neubauer counting chamber was placed along with the cover glass in position. The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding first 3-5 drops.

Charging of the counting chamber

One drop of diluted blood was released into the groove of the Neubauer counting chamber. It was left for cells to settle for 2-3 minutes, the counting chamber was put under the microscope and the ruled area was located. Erythrocytes were counted in the 5 squares of the counting area of 1mm square. The number of cells in the 4-corner square was counted.

Calculation

The total number of cells found in 16 squares is multiplied by 10,000 to give the number of cells in millions/mm^3 of blood.

APPENDIX - 12

DETERMINATION OF PLATELET COUNT
(Sanderson and Phillips, 1981)

Reagents

Dacies fluid: This was prepared by dissolving 5.0g of sodium citrate and 1 ml of 40% formaldehyde and made upto 100 ml with distilled water. To 19 ml of this solution 1 ml of 0.2% brilliant cresyl blue solution was added just before use. The solution was filtered and used.

Procedure

Venous blood collected with EDTA was used for platelet count. 0.05 ml of sample was diluted with 0.95 ml of Dacies fluid and mixed well, using a narrow bore Pasteur pipette and the counting chamber was filled with the diluted blood. The cells were allowed to settle to the bottom of the chamber for 15 min to prevent from drying, the chamber was placed in a petridish, which contained a pieces of wet filter paper. Using the 40X objective with reduced condenser aperture the platelets were counted in 1/5 sq.mm-5 of the small squares of the large center square. From this the number of platelets in cu.mm of blood was calculated and expressed as number of cells/mm^3.

APPENDIX - 13

ENUMERATION OF WHITE BLOOD CORPUSCULES

Truck's fluid method

(Sanderson and Phillips, 1981)

Principle

Blood is diluted with weak acid solution which removes the red cells by hemolysis and also accentuates the nuclei of the white cells thus making the counting of the white cells easy. The number of white cells per cubic mm in undiluted whole blood is calculated by knowing the volume of fluid examined and the dilution of the blood.

Reagents

WBC diluting fluid - Glacial acetic acid (1ml), Gentian violet – 2ml (1%), Water - 95ml

Procedurez

Blood was sucked exactly up to the 20µl mark in the RBC pipette and the diluting fluid was drawn immediately up to the mark and the blood mixed thoroughly with the diluting fluid. It was left for 2-3 minutes for proper mixing. The Neubauer counting chamber was placed along with the cover glass in position.

The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding first 3-5 drops.

Charging of the counting chamber

One drop of diluted blood was released into the groove of the Neubauer counting chamber. It was left for cells to settle for 2-3 minutes, the counting chamber was put under the microscope and the ruled area was located, the count is made in 4 large(1mm) cover squares of the Neubauer counting chamber.

Calculation

The total number of cells in 4 squares is multiplied by a factor of 2500 to give the count/mm^3 of blood.

APPENDIX - 14

DETERMINATION OF DIFFERENTIAL LEUKOCYTE COUNT (Sanderson and Phillips, 1981)

Principle

A differential white blood cell count is performed to determine the percentage of each of the various types of white blood cells present in a blood sample. The test is useful because the relative proportions of white blood cells may change in particular diseases.

Reagents

1. Leishman's stain powder – 0.15g

2. Methylalcohol, acetone free – 100ml

Procedure

The blood film was placed in a level position and the dry blood film was covered with the Leishman's stain, which should be evenly distributed over the entire slide. At the end of one minute, the quantity of distilled water was doubled carefully and mixed with the stain by means of the clean pipette. The film was

allowed to stain for 7 to 8 minutes and the excess stain was removed by washing with the distilled water for 2 minutes. The water was then washed off with fresh distilled water. The film is dried in air. When the film was dried, it was examined microscopically.

APPENDIX - 15

ESTIMATION OF ASPARTATE TRANSAMINASE (AST)
(Reitman and Frankel, 1957)

Principle

Aspartate Transaminase (AST) or Serum glutamine oxaloacetate transaminase (SGOT) catalyses the reversible transfer of an amino group from aspartate to α-keto glutarate forming glutamate and oxaloacetate. SGOT catalyses the following reaction:

L – Aspartate + α – Keto glutarate ⟶ Oxaloacetate + L– Glutamate (pH 7.4)

Oxaloacetate + 2,4 DNPH ⟶ 2,4 dinitrophenyl hydrazine (brown colored)

(Alkaline medium)

The liberated oxaloacetate is allowed to react with 2, 4 dinitrophenylhydrazine (DNPH) and sodium hydroxide. The brown color developed is read at 520 nm.

Reagents

1. 0.1M Phosphate buffer (pH 7.5)
2. Substrate: 1.78 g of L-aspartic acid and 30 mg of a-ketoglutarate were dissolved in 20 ml of phosphate buffer. 0.5 ml of 1 N NaOH was added and made upto 100ml with buffer.
3. 0.2% 2, 4-Dinitrophenyl hydrazine in 1N HCl
4. 0.4 N Sodium hydroxide
5. Standard pyruvate: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contains one mole of pyruvate/ml.

Procedure

0.5 ml of the buffered substrate was incubated for 10 minutes at 37°C. Then 0.1 ml of serum was added and incubation was continued for an hour. To the control tubes alone serum was added after arresting the reaction with ml of DNPH and the tubes were kept at room temperature for 20 minutes. Then 5 ml of 0.4 N sodium hydroxide was added to all the tubes. A set of standard pyruvate were also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activity is expressed as Units/L in serum.

APPENDIX - 16

ESTIMATION OF ALANINE TRANSAMINASE
(Reitman and Frankel, 1957)

Principle

Alanine Transaminase (ALT) or Serum glutamine pyruvate transaminase (SGPT) catalyses the reversible transfer of amino group from L-alanine to alpha ketoglutarate with the formation of pyruvate and glutamate. The enzyme catalyses the following reaction:

L-Alanine + α-Ketoglutarte ⟶ Pyruvate + L-Glutamate (pH 7.4)

Pyruvate + 2,4 DNPH ⟶ 2,4 dinitrophenyl hydrazine (Brown Colored) (Alkaline Medium)

The pyruvate so formed is allowed to react with 2-4 dinitrophenylhydrazine (DNPH) to produce 2, 4- dinitrophenyl hydrazone derivative, which is measured spectrophotometrically at 520 nm.

Reagents

1. 0.1M Phosphate buffer (pH 7.5)
2. Substrate: Dissolved 146 mg of a-ketoglutarate and 17.8 g of L-alanine in 1 N Sodium hydroxide with constant stirring. The volume was made up to 100 ml with phosphate buffer (pH 7.4).

3. 0.4 N Sodium hydroxide
4. 0.2% 2, 4-Dinitro phenyl hydrazine in 1N HCI
5. Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contains 1pmole of pyruvate/ ml.

Procedure

0.5 ml of the buffered substrate was incubated for 10 minutes at 37°C. Then 0.1 ml of serum was added and incubation was continued for an hour. To the control tubes alone serum was added after arresting the reaction with 0.5ml of DNPH and the tubes were kept at room temperature for 20 minutes. Then 5 ml of 0.4 N sodium hydroxide was added to all the tubes. A set of standard pyruvate were also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activity is expressed as Units/L in serum.

APPENDIX - 17

ESTIMATION OF ALKALINE PHOSPHATASE
(King and Armstrong, 1934)

Principle

Alkaline phosphatase (ALP) is an enzyme which catalyses the splitting of phosphoric acid from certain monophosphoric esters. In this method disodium phenyl phosphate was hydrolyzed by alkaline phosphatase with the liberation of phenol which reacts under alkaline conditions with Folin's phenol reagent to form blue colour. The amount of phenol formed was estimated in a spectrophotometer at 680nm.

Reagents

1. Disodium phenyl phosphate (0.01M) – 1.09g of disodium phenyl phosphate was dissolved in water and made up to 500ml. It was then boiled, cooled and little chloroform was added and kept in refrigerator (Solution A).

2. Sodium carbonate-sodium bicarbonate buffer (0.1M) - 3.18g of anhydrous sodium carbonate and 1.68g of sodium bicarbonate was dissolved in water and made up to 500ml (Solution B).
3. Buffered substrate for use - Equal volume of solution A and solution B was mixed which has pH of 10.
4. Trichloro acetic acid (20%) – Acid molybdate reagent - 5g of ammonium molybdate dissolved in 5N sulphuric acid.
5. 1, 2, 4 – ANSA : 0.25% of 1,2,4 – ANSA was prepared by adding 0.5g of dry powder ANSA to 190 ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite, stoppered the bottle and shaken until it dissolved.
6. Stock phosphate solution – 2.194g of pure potassium dihydrogen phosphate was dissolved in water and made up to 500ml. A few drops of chloroform was added to it (1mg/1ml of phosphate).
7. Working standard: Two ml of stock standard was diluted to 500ml.

Procedure

In a test tube 6ml of buffered substrate was pipetted out and placed in water bath at 37^0C for few minutes. Then, 0.3ml of serum was added, mixed well and incubated for 15 minutes. At the same time control and blank were also kept. For blank, 0.3ml of water was added to 6ml buffered substrate. For control, 0.3ml of serum was added to 6ml of distilled water. Later, 1.2ml of 20% TCA was added and shaken well. Five ml of the filtrate was taken in separate test tubes. To the blank and control, 0.8ml of acid molybdate was added followed by 0.2ml of ANSA. It was then mixed well and allowed to stand for 10 minutes at 37^0C and the colour developed was read at 650nm. Pipetted out 1.0 to 4.0 ml of standard solution and made up to 5ml with distilled water. Acid molybdate (0.8ml) was added followed by 0.2ml of ANSA. Standards were also read at 650nm. Alkaline phosphatase activity in serum was expressed as U/L.

APPENDIX - 18

ESTIMATION OF UREA

(Netelson, 1957)

Principle

Urea is hydrolysed to ammonia and carbondioxide in the presence of urease. Ammonia reacts with 2 - oxoglutarate in the presence of NADH, which is oxidised and measured at 340nm.

Reagents

1. 10% TCA
2. Reagent A - 50mg of ferric chloride, 0.2ml of water, 1ml of O-phosphoric acid and 2.5ml of water
3. Reagent B - 50ml of concentrated sulphuric acid and 450ml of water
4. Reagent C - 1g of diacetyl monoxime in 50ml water
5. Reagent D - 250mg of thiosemicarbazide in 50ml of water
6. Reagent I - 0.25ml of Reagent A was mixed with 500ml of Reagent B
7. Reagent II - 33.5ml of Reagent C was mixed with 33.5ml of Reagent D and diluted to 500ml.

Procedure

To 0.2ml of blood, 1.8ml of 10% TCA was added, mixed well and centrifuged after 10 minutes. The supernatant (0.5ml) was taken and the volume was made upto 3ml with water. Then 2ml of reagent I was mixed with 2ml of freshly prepared reagent II. It was then mixed well, stoppered with marbles and heated vigorously in a boiling water bath for 20 minutes. Blank and standards (10- 50µg) were treated similarly. The tubes were removed, cooled and read against the blank at 340nm.

APPENDIX - 19

ESTIMATION OF URIC ACID

(Caraway, 1955)

Principle

Uric acid in the sample is oxidized by uricase to allantoin. In this reaction 1 mole of hydrogen peroxide is formed for every mole of uric acid oxidized. Hydrogen peroxide reacts with 3, 5-dichloro-2-hydroxybenzene sulfonic acid and 4-amino antipyrine to give quinoneimine dye. Intensity of the colour of this dye was proportional to the concentration of uric acid in the sample.

Reagents

1. Enzyme reagent: 4-Aminoantipyrine (4mM), 3,5-dichloro-2-hydroxybenzene sulfonate (2mM), microbial uricase (150U/L), horseradish peroxidase (10,000 U/L).
2. Standard uric acid: 5mg/100ml

Procedure

To 1ml of the enzyme reagent, 25µl of plasma was added and mixed by inversion. 25µl of standard and 25µl of distilled water (blank) also processed simultaneously. The tubes were incubated at 37°C for 5 minutes and the colour developed was read at 510nm. The values were expressed as mg/dl of blood.

APPENDIX - 20

ESTIMATION OF CREATININE

(Owen *et al*., 1954)

Principle

Creatinine in alkaline medium react with picrate ions to form yellow orange complex whose color intensity is measured at 492nm.

Reagents

1.Picric acid - 35mmol/l

2.Sodium hydroxide - 0.32mmol

3.Creatinine standard -2 mg/dl Sodium tungstate

4.Sulphuric acid

Procedure

To 0.2ml of serum, 3ml of water, 1ml of 10% sodium tungstate and 2ml of 2/3N sulphuric acid were added. It was then kept for 10 minutes and centrifuged. To 3ml of supernatant, 1ml of 0.04M picric acid solution and 1ml of 0.75N sodium hydroxide was added and allowed to stand for 20 minutes. Blank and standards (10-50µg) were treated similarly. The colour developed was read at 492nm.

APPENDIX - 21

ESTIMATION OF GAMMA GLUTAMYL TRANSFERASE

(Persijn and van der Slik, 1978)

Principle

GGT catalyzes the transfer of amino group between L-g-glutamyl-3-carboxy-4- nitroanilide and glycylglycine to form L-g-glutamyl glycylglycine and 5-amino-2- nitrobenzoate, which is measured as an increase in absorbance proportional to the GGT activity in the sample.

Reagents

1. Buffer
2. Substrate (L-γ-glutamyl-3-carboxy-4-nitroanilide)
3. Working reagent : The substrate tablet was dissolved in 2.2ml of buffer

Procedure

Working reagent (1.0ml) was incubated at assay temperature (37°C) for one minute and 0.1ml of sample was added. The contents were mixed well and

the initial absorbance was read at 405nm in a spectrophotometer (Genesys 10-S, USA) after one minute and the absorbance reading was repeated after every 1, 2 and 3 minutes. The mean absorbance change per minute was calculated (DA/minute) and enzyme activity is expressed as IU/L.

APPENDIX - 22

ESTIMATION OF LACTATE DEHYDROGENASE

(King, 1965)

Principle

The enzyme lactate dehydrogenase catalyzes the conversion of lactate to pyruvate in the presence of NAD. The pyruvate formed reacts with 2, 4-dinitrophenyl hydrazine in alkaline medium to yield a brown colour that is measured spectrophotometrically at 520nm.

Reagents

1. 0.1M glycine buffer: 7.505 g of glycine and 5.85 of sodium chloride were dissolved in 1 litre of water.
2. Buffered substrate: 2.78 g of lithium lactate was dissolved in 125 ml of glycine buffer containing 75 ml of 0.1 N sodium hydroxide solution.
3. Nicotinamide adenine dinucleotide: 10 mg of NAD was dissolved in 2 ml of water.
4. 2, 4-dinitro phenyl hydrazine reagent (DNPH): 200 mg of DNPH was dissolved in 1 litre of 0.1N hydrochloric acid.
5. Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of buffer.

Procedure

To 1.0 ml of buffered substrate with 0.05 ml of serum was mixed and the tubes were incubated at 37 °C for 15 minutes. 0.2 ml of coenzyme solution (NAD) was then added and again incubated for 15 minutes. The reaction was stopped by the addition of 1.0 ml of 2, 4-dinitrophenylhydrazine followed by 0.05 ml of serum by the control. The tubes were incubated at 37 °C for another 15 minutes and 5 ml

of 0.4 N sodium hydroxide was added to each tube and mixed well. The intensity of the colour was measured at 520 nm. The enzyme activity is expressed as Units/L in serum.

APPENDIX - 23

ESTIMATION OF ADENINE DEAMINASE
(Giusti and Galanti's, 1994)

Principle

Adenosine deaminase catalyses the deamination of adenosine to inosine and ammonia.

Reagents

- Adenosine, ADA, 0.2 U/mL xanthine oxidase (XOD), 0.1 U/mL purine nucleoside phosphorylase (PNP), Hypoxanthine (HX), Xanthine (XAN), 2,6-dichiorophenolindophenol(DCIP)

Procedure

The molar absorbance of the DCIP dye was determined in 0.2 mol/L phosphate buffer (pH 7.2). Xanthene or hypoxanthene was added to give a concentration of 0.025 mmol/L in the final reaction volume. The concentration of DCIP in both solutions was 0.075mmol/L. The decrease in absorbance (endpoint reaction) was measured at 606nm and the amount of DCIP reduced was calculated using the absorptivity determined for oxidized DCIP. The reaction temperature was kept at 37ºC and the examination of various buffers at various pH values. The effects of variation in sample volumes, amount of secondary enzymes (PNP; XOD) added, and concentration of DCIP was evaluated experimentally. After the optimized conditions were determined manually, the method was transferred to the Cobas Mira analyzer. The automated version of the method was used in all studies done to evaluate the performance of the method. The samples were incubated with adenosine and the released ammonium ions were determined. ADA activity was defined as the concentration of ammonium ions (mol/l) formed in 1min and expressed as units per litre.