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Phytochemical and *in Vitro* and *in Vivo* Evaluation of Anti Diabetic Activity of *Tecomaria capansis* Extract of Leaves in Wistar Albino Rats

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Abstract
Diabetes mellitus is a metabolic issue at first recognized by lost glucose homeostasis, because of disturbances of carbohydrate, fat and protein digestion, coming from imperfections in insulin generation, emission, insulin activity. Diabetes comprises in two types; Type I and Type II. Most hypoglycemic plant constituents, such as alkaloids,phenolic acids and amino acids, inhibit insulinase in vitro and are hypoglycemic in vivo in normal rats. The wide kind of chemical classes indicates that a variety of mechanisms might be involved in the lowering of the blood glucose level. Our results shown that oral administration of *Tecomariacapensis* extract has a beneficial effect on the reducing hyperglycaemia, SGOT & SGPT levels, total cholesterol, triglycerides levels, LDL-cholesterol, VLDL-cholesterol and improving the HDL status. This study suggests that the induction of diabetes mellitus may be prevented by flavonoids of plant constituent's administration. There is a need to continue to explore the mechanisms for anti-diabetes.
Keywords
Diabetes mellitus, medicinal plant, antidiabetic activity.

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INTRODUCTION
Diabetes mellitus is the most common type of metabolic, non-communicable diseases globally. Recent studies states that it is the fourth leading causes of death in the most developed countries [1]. It is characterized by hyperglycemia resulting from defects in either insulin action, insulin secretion, or both. As per World Health Organization (WHO), at least 171 million people (2.8% of worldwide population) have suffered from diabetes alone in year of 2000. It is estimated that by the year 2030, this number of people suffering from diabetes may almost be doubled. The increase in incidence of diabetes in developing countries follows trend of highly urbanization and lifestyle changes, may be because of "Western-style" diet.

Diabetes mellitus is classified into two types, insulin-dependent diabetes mellitus (IDDM, Type I) and non-insulin-dependent diabetes mellitus (NIDDM, Type II). Type I diabetes is an autoimmune disease characterized by a local inflammatory reaction in and around islets of pancreas that is followed by selective destruction of insulin-secreting β cells. Type II diabetes is characterized by peripheral insulin resistance and impaired insulin secretion. [2]

Herbal medicine includes preparation of biologically active natural products that consist of herbal material or herbs, some recipes may contain materials such as fungal and bee products, as well as minerals (kaolin, bentonite), insects, shells, ash, and animal parts, and are used for the maintenance of health and management of various diseases [3].

MATERIALS AND METHODS:

Identification and authentication of selected plant species

The leaves of *Tecomaria capansis* (Thumb.) Spach was collected from Guntur district, A. P and it was authenticated by professor Dr.S.M.Khasim, Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur. The specimen (No: ANU/00129/2009/AP) was deposited in the department of botany and microbiology for future reference.

Collection of plant material: Fresh plant material was collected in bulk, washed under running tap water to remove adhering material, dried under shade and pulverized in a mechanical grinder. The coarse powder was passed through sieve no. 60" #. Care was taken to select healthy plants and normal organs.

Macroscopic study

As per standard procedure matured 25 leaves were taken for the evaluation of morphology of leaves and studied various parameters such as length, width, margin, apex, surface, colour, odour, taste, type, base, midrib and size. By visual method the organoleptic characteristics of leaf was found.

Microscopic study

The required samples of different organs were cut and removed from the plant and fixed in FAA (formalin-5ml+acetic acid-5ml+70%ethyl alcohol-90ml) after 24 hours of fixing the specimen were dehydrated with graded series of tertiary-butyl alcohol. Infiltration of the specimens was carried out by gradual addition of paraffin wax (melting point 58-69˚C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks [5].

Procedure for staining

The paraffin embedded specimens were section with the help of rotary microtome. The thickness of the section was 10-12 micrometers. De waxing of the secretion was by customary procedure. The section was stained with Toulidine blue as per the method published by O'Brien.et.al.,1964 since Toulidine blue is a polychromatic stain the staining results were remarkably good and some cyto chemical reactions were also obtained. They 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 saffranin and fast green and iodine (for starch). For studying the stomatal morphology, venation pattern and trichome distribution, para dermal (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffery's maceration fluid were prepared. Glycerin mounted temporary preparation were made for macerated/cleared materials.

Photomicrographs

Microscopic description of tissue or supplemented with micrographs were necessary photographs of different magnifications were taken with Nikon Labophot2 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 bire stringent property, under polarized light they appear bright against dark background, magnification of the figures is indicated by the scale bars [6].

Determination of physicochemical constants [7]

Determination of moisture content (loss on drying)

Place about 10g of drug (without preliminary drying) after accurately weighing (accurately weighed within 0.01g) it in a tarred evaporating dish. For example, for underground or un powdered drug, prepare about 10g of sample by cutting shredding so that the parts are about 3mm in thickness. Seeds and fruits, smaller than 3mm should be cracked. Avoid the use of high-speed mills in preparing the samples, and exercise care that no appreciable amount of moisture is lost during preparation and that the portion taken is representative of the official sample. After placing the above said amount of the drug in the tarred evaporating dish dry at 105˚c for 5 hours, and weigh. Continue the drying and weighing corresponds to not more than 0.25 percent. Constant weight is reached when two consecutive weighing after drying for 30 minutes and cooling for 30 minutes in a desiccator, show not more than 0.01g difference.

Determination of foreign matter

Weigh 100-500g of the drug sample to be examined or the minimum quantity prescribed in the monography and spread it out in a thin layer. The foreign matter should be detected by inspection with the unaided eye or by the use of lens (6xs). Separate and weigh it and calculate the percentage present.

%of foreign matter =Amount of foreign matterx100/Amount of drug taken

Determination of different extractive value

Determination of alcoholic soluble extractive

Macerate 5g of the air dried drug, coarsely powdered, with 100ml of Alcohol of the specified strength in a closed flask for 24 hrs, shaking frequently during six hours and allowing standing for 18hrs. Filter rapidly, taking precautions against loss of solvent, evaporate 25ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105˚C to constant weight and weigh. Calculate the % of alcohol soluble extractive with reference to the air-dried drug.

Determination of water-soluble extractive

Macerate 5g of the air dried drug, coarsely powdered, with 100ml of chloroform water of the specified in a closed flask for 24 hrs, shaking frequently during 6 hrs and allowing to stand for 18 hrs. filter rapidly, taking precautions against loss of solvent, evaporate 25ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105˚C to constant weight and weigh. Calculate the %of chloroform water soluble extractive with reference to the air-dried drug.

Determination of ether soluble extractive

Transfer a suitably weighed quantity (depending on the fixed oil content) of the air dried, crushed drug to an extraction thimble, extract with solvent ether (petroleum ether boiling point 40˚-60˚) in a continuous extraction apparatus (Soxhlet apparatus, Elite, India) for 6 hrs. Filter the extract quantitatively into a tared evaporating dish and evaporate off the solvent on a water bath. Dry the residue at 105˚C to constant weight. Calculate the % of ether soluble extractive with reference to the air-dried drug.

Ash Value Determination

Determination of total ash

Incinerate about 2 to 3 gm accurately weighed, of the ground drug in a tared platinum or silica dish at a temperature not exceeding 450˚C until free from carbon, cool and weigh. If a carbon free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ash less filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness, and ignite at a temperature not exceeding 450˚C. Calculate the % of ash with reference to the air dried drug.

Determination of acid insoluble ash

Boil the ash obtained in total ash for 5 minutes with 25ml of dilute hydrochloric acid, collect the insoluble matter in a Gooch crucible or on a less filter paper, wash with hot water and ignite to constant weight. Calculate the % of acid insoluble ash with reference to the air-dried drug.

Determination of water-soluble ash

Boil the ash for 5 minutes with 25ml of water, collect insoluble matter in a Gooch crucible, or an ash less filter paper, wash with the hot water, and ignite for 15 minutes at a temperature not exceeding 450˚C. Subtract the weight of the insoluble matter from the weight of the ash. And Calculate the % of water-soluble ash with reference the air-dried drug [7].

Preparation of extracts [8]

The technique commonly used in the field of phytochemistry are extraction, isolation, and structural elucidation of natural products, as well as chromatographic techniques. The solvent extraction of any botanical materials may yield very less quantity of volatile oils and large yield of nonvolatile components like resins, pigments, waxes and fatty acids.

Petroleum ether extract

This extract was prepared by using soxhlet apparatus. About 150gm of dried flower powder was taken in a muslin cloth bag. The purified pet ether was passed through the tube where the powder bag was kept. The pet ether will pass through siphon tube and reach the round bottom flask in which the condenser and reach the tube containing powdered bag and the process is repeated. This is continuing for 24hrs. then the round bottom flask containing extract is transferred to a beaker and is allowed to evaporate in a water bath. This concentrated pet ether extract is used for further studies.

N-hexane extract

This extract was prepared by using soxhlet apparatus. About 150gm of dried flower powder was taken in a muslin cloth bag. The purified N-Hexane was passed through the tube where the powder bag was kept. The N-Hexane will pass through siphon tube under the reach the round bottom flask in which porcelain chips are provided. The vapors containing the constituents pass through the condenser and reach the tube containing powder bag and the process is repeated. This is continued for 24hrs. Then the round bottom flask containing is transferred to a beaker and is allowed to evaporate in a water bath. This concentrated N-Hexane extract is used for further studies.

ETHYL ACETATE EXTRACT

This extract was prepared by using soxhlet apparatus. About 150gm of dried flower powder was taken in a muslin cloth bag. The purified Ethyl acetate was passed through the tube where the powder bag was kept. The Ethyl acetate will pass through siphon tube and reach the round bottom flask in which porcelain chips are provided. The vapors containing the constituents pass through the condenser and reach the tube containing powder bag and the process is repeated. This is continued for 24hrs. Then the round bottom flask containing extract is transferred to a beaker and is allowed to evaporate in a water bath. This concentrated Ethyl acetate extract is used for further studies.

Chloroform extract

This extract was prepared by using soxhlet apparatus. About 150gm of dried flower powder was taken in a muslin cloth bag. The purified chloroform was passed through the tube where the powder bag was kept. The chloroform will pass through siphon tube and reach the round bottom flask in which porcelain chips are provided. The vapors containing the constituents pass through the condenser and reach the tube containing powder bag and the process is repeated. This is continued for 24hrs. Then the round bottom flask containing extract is transferred to a beaker and is allowed to evaporate in a water bath. This concentrated chloroform extract is used for further studies.

Ethanol extract

This extract was prepared by using soxhlet apparatus. About 150gm of dried flower powder was taken in a muslin cloth bag. The purified ethanol was passed through the powder bag was kept. The ethanol will pass through siphon tube and reach the round bottom flask in which porcelain chips are provided. The vapors containing the constituents pass through the condenser and reach the tube containing powder bag and the process is repeated. This is continued for 24hrs. then the round bottom flask containing extract is transferred to a beaker and is allowed to evaporate in a water bath. This concentrated ethanol extract is used for further studies.

Aqueous extract

This extract was prepared by using soxhlet apparatus. About 150gm of dried powder was taken in a muslin cloth bag. The purified water passed through the tube where the powder bag was kept. The water will pass through the siphon tube and reach the round bottom flask in which porcelain chips are provided. The vapors containing the constituents pass through the condenser and reach the tube containing powder bag and the process is repeated. This is continued for 24hrs.Then the round bottom flask containing extract is transferred to a beaker and is allowed to evaporate in a water bath. This concentrated aqueous extract is used for further studies.

Recovery of solvent

For purification of solvents the distillation apparatus was first set.40%of solvents were added to the

bottom flask kept in electrical mantle. The respective solvents were allowed to distill, and pure solvents were collected in a vessel from the adapter attached to the condenser.

Phytochemical screening [9, 10]

The following chemical tests were performed to identify the photochemical constituents present in chloroform of *Tecomaria capensis*.

Test for alkaloids

Dragendroff's reagent:

The alcoholic extract was shaken with dilute HCL to 2-3ml of filtrate add few drops of Dragendroff's reagent. Orange brown precipitate is formed.

Mayer's test:

The alcoholic extract was shaken with dilute Hcl and filtered. 2-3 ml of the filtrate add Mayer's reagent. Cream precipitate is formed.

Hager's test:

The alcoholic extract was shaken with dilute Hcl and filtered. To 2-3 ml filtrate add Hager's reagent. Yellow precipitate is formed**.**

Wagner's test:

The alcoholic extract was shaken with dilute Hcl and filtered. And to the 2-3 ml of filtrate add Wagner's reagent. Reddish brown precipitate is formed.

Test for flavonoids

Schinoda test:

To the extract add 5ml 95%ethanol few drops of conc.HCL and 0.5g magnesium turnings. Pink color is observed.

Alkaline test:

10mg of extract was dissolved in 2 ml of water and treated with 1ml of 10% ammonium hydroxide and observe for coloration. 2 drops of dilute Hcl was added and again observe for discoloration. The formation of intense yellow colour which turns to colorless on addition of dilute acid indicates the presence of flavanoids.

Test for cardiac glycosides

Baljet test:

A thick section shows yellow to orange colour with sodium picrate.

Legal's test:

To the alcoholic extract add 1 ml of pyridine and 1ml of sodium nitroprusside. Pink to red color appears.

Liebermann's test:

Mix 3 ml extract with 3 ml acetic anhydride. Heat and cool. Add few drops of conc. Sulphuric acid. Blue colour appears.

Test for anthraquinolone glycosides

Borntrager's test:

To 3ml of extract add dilute sulphuric acid. Boil and filter. To cold filtrate add equal volumes benzene and chloroform. Shake well. Separate the organic solvent. Add ammonia. Ammonical layer turns pink or red.

Modified brontrager's test:

To 5ml extract add 5ml of 5% ferric chloride and 5ml dilute Hcl. Heat for 5mins in boiling water bath. Add equal volume of chloroform or benzene. Shake well. Separate the organic solvent. Add ammonia. Ammonical layer shows pinkish red colour.

Test for saponin glycosides

Foam test:

Shake the drug extract or dry the powder vigorously with water. Persistent foam is produced.

Hemolytic test:

Add drug or dry powder to one drop of blood placed on a glass slide. Hemolytic zone appears.

Test for tannins and phenolic compounds

Lead acetate solution:

To 2-3ml of alcoholic extract add few drops of lead acetate solution. A white precipitate is formed.

5% ferric chloride solution:

To 2-3ml of the alcoholic extract add few drops of 5% ferric chloride solution. A deep blue colour appears.

Test for steroids

Salkowski reaction:

To 2ml of extract add 2ml chloroform and 2ml conc. Sulphuric acid. Shake well. chloroform layer appears red and acid layer shows greenish yellow fluorescence.

Liebermann's test:

Mix 3ml extract with 3 ml acetic anhydride. Heat and cool. Add few drops of conc. Sulphuric acid blue colour appear.

Test for carbohydrates

Molishs's test: To 2-3 ml of extract add, few drops of alpha naphthol solution in alcohol, shake and add concentrated sulphuric acid from sides of the test tube. Violet ring is formed at the junction of 2 liquids.

Fehling's test:

About 50gms of extract was hydrolyzed with 10ml of dilute hydrochloric acid and neutralized with alkali. The mixture was heated with 1 ml of Fehling's solution. A and B and observes for precipitate. Formation of red precipitate indicates the presence of reducing sugars.

Benedict's test:

To 0.5ml of filtrate add 0.5ml of Benedicts reagent. The mixture was heated on water bath for 2 minutes and observe for precipitate. Formation of orange red precipitate indicates the presence of reducing sugars.

Test for proteins

Xanthoprotein test:

Mix 3ml test solution with 1ml conc. Sulphuric acid. White precipitate is formed. Boil. Precipitate turns to

yellow. Add ammonium hydroxide, precipitate turns orange.

Biuret test:

To 3 ml test solution add 4% ammonium hydroxide and few drops of 1% copper sulphate solution. Violet or pink color appears.

Test for fats and oils

Solubility test:

Oils are soluble in ether, benzene and chloroform, but insoluble in 90% ethanolic and water.

Sudan red 3 test:

Place a thin section of drug on glass slide. Add a drop of Sudan red 3 reagent. After 2 minutes wash with 50% alcohol. Mount in glycerin. Observe under microscope. oil globules appear as red.

Test for gums

Test solution is hydrolyzed and treated with Benedict's reagent. Red colour is developed.

Pharmacological screening

Alpha-amylase inhibition assay [11, 12, 13]

Importance alpha amylase enzyme in the body in humans, the digestion of starch involves several stages. Initially, partial digestion by the salivary amylase results in the degradation of polymeric substrates into shorter oligomers. Later on in to the gut these are further hydrolyzed by pancreatic alpha amylase into maltose, malto triose and small malto oligosaccharides. The digestive enzyme is responsible for hydrolyzing dietary starch, which breaks down into glucose prior to absorption. Inhibition of alpha amylase can lead to reduction in post prandial hyper glycaemia in diabetic condition. Importance of alpha glucosidase enzyme in the body alpha glucosidase is a membrane bound enzyme located on the epithelium of the small intestine, catalyzing the cleavage of disaccharides to form glucose. Inhibitors can retard the uptake of dietary carbohydrates and suppress post prandial hyperglycemia. Therefore, inhibition of alpha glucosidase could be one of the most effective approaches to control diaetes. Glucosidases are not only essential to carbohydrate digestion, but also vital for the processing of glycoprotein and glycolipids. The enzyme is a target for antiviral agents that interfere with the formation of essential glycoprotein required in viral assembly, secretion and infection *Glucosidases are also involved in a variety of metabolic disorders and carcinogenesis.*

MATERIALS AND METHODS

The selected methanol and petroleum ether extract of *Ipomoea sepiaria* were serially diluted to get a required concentration to perform alpha amylase assay.

Alpha-amylase inhibition assay principle and procedure [14, 15]

Alpha-amylase activity was measured in-vitro by hydrolysis of starch in presence of α-amylase enzyme. This process was quantified by using iodine, which gives blue colour with starch. The reduced intensity of blue colour indicated the enzyme-included hydrolysis of starch into monosaccharides. If the substance/extract possesses α-amylase inhibitory activity, the intensity of blue colour will be more. In other words, the intensity of the blue colour in test sample is directly proportional to α-amylase inhibitory activity. Enzyme: (Type VI B: From porcine pancreas, 5,00,000U, [otto-S2366] [15.8U/mg solid at pH 6.9] – stored at 2-8°C Substrate: Starch 1% Positive Control: Acarbose-stored at RT-Glucobay (Bayer pharma, India).

Sodium dihydrogen orthophosphate (NaHat RT)

Disodium hydrogen phosphate (Na Indicator: Iodine solution 1% instrument -UV-visible spectrometer.

Preparation of working solution:

Phosphate buffer (40mM, pH 7, 25°C)

Solution A: 6.24 g of NaH_2PO_4-1L

Solution B: 7.12 g of $NaHPO_4$-1L

Enzyme (0.5128U/ml) – 3.246 mg α-amylase-100ml of 40 mM Phosphate Buffer

NaCl solution (0.006M)

Positive control:

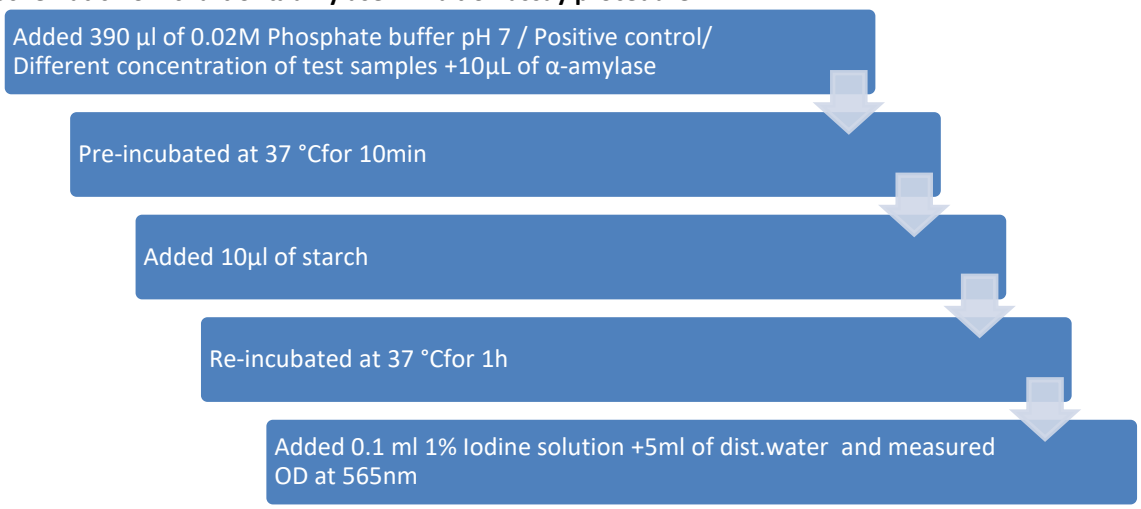
Stock- 50mg of Acarbose in 50ml of 40m Phosphate buffer

Working stock: Take 25 µl stock, made upto 10ml (2.5 µg/ml) with 40 mM phosphate buffer.

Procedure:

α-amylase activity was carried out by starch-iodine method. 10µL of α-amylase solution (0.025mg/mL) was mixed with 390µl of phosphate buffer (0.02M containing 0.006 M NaCl, pH 7.0) containing different concentration of extracts. After incubation at 37°C for 10min, 100µL of starch solution (1%) was added, and the mixture was re-incubated for 1hr. Next, 0.1 ml of 1% iodine solution was added, and after adding 5ml distilled water, the absorbance was taken at 565nm, Sample, substrate and α-amylase blank determinations were carried out under the same reaction conditions. Inhibition of enzyme activity was calculated as (%) = (A-C) × 100/(B-C), where, A=absorbance of sample, B=absorbance of blank (without α-amylase) and C= absorbance of control (without starch).

Schematic flow chart of α-amylase inhibition assay procedure



Experimental animals

White male Wister rats weighing about 150-180 gm were used. They were purchased form the Mahaveer enterprises Hyderabad. They were kept under observation for about 15 days before the onset of the experiment to exclude any intercurrent infection. The chosen animals were housed in plastic well aerated cages at normal atmospheric temperature (25±5 °C) and normal 12-hr light/dark cycle. Moreover, they had free access to water and were supplied daily with standard diet of known composition *ad libitum.* All animal procedures were in accordance with the recommendation of the ethical committee guidelines for Care and Use of Animals.

Induction and treatment of diabetes

Diabetes was induced by a single injection fasting for at least 16hr, in freshly prepared 1% sodium carboxy methyl cellulose, blood glucose levels were measured 48hr development of diabetes mellitus was proven by sustained hyper-glycemia (diabetic rats had glycaemia> 200mg/dL). The diabetes developed rats were selected for the study and treated with the Ethanolic extract of *Tecomariacapensis* 200 mg/Kg per oral (*p.o).,* Ethanolic extract of 400mg/Kg (*p.o).,* and Metformin 100 mg/Kg (*p.o)*for 8 consecutive days.

Chemical agents:

Cleome Gynandra plant : Chittoor district of A.P, India

Metformin : Natco. Pharma, Hyderabad, India

Glucose Kit : Excel diagnostics Pvt Ltd. Mumbai

Lipid profile kit : Excel diagnostics Pvt Ltd. Mumbai

SGOT : Excel diagnostics Pvt Ltd. Mumbai

SGPT kit : Excel diagnostics Pvt Ltd. Mumbai

Estimation of Blood Glucose:

The determination of glucose is one of the most frequently performed tests in a clinical laboratory. The test based on the reducing property of glucose does not measure true glucose, as there is much interference. Subsequently other chemical and enzymatic methods were developed. Enzymatic methods are preferred because of their reliability and safety. This glucose kit is based on Tinder's method in which Glucose Oxidase and peroxidase enzymes are used along with the chromogenic 4-Aminoantipyrine and phenol. The method is one step, simple and rapid. It does not have any interference due to reducing substances or haemoglobin, etc.

Principle:

Glucose is oxidized by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of the enzyme peroxidase (POD) oxidized phenol which combines with 4-Aminoantipyrine to produce a red coloured quinoneimine dye. The intensity of the colour developed is proportional to glucose concentration in the sample.

D-glucose + H_2O + O_2GOD D-gluconic acid + H_2O_2

H_2O_2 + 4-AAP POD Quinoneimine dye + H_2O

Reagents	5×100ml	2×500ml	5ltr
Enzyme Reagent Buffer	5 vials	2 vials	1 vial
Solution	5×100ml	2×500ml	5000 ml
Standard (100mg%)	3ml	3ml	2 × 3 ml

The reagents are stable at 2-8 °C till the expire date is mentioned on the label.

Sample:

Serum/ Heparinized or EDTA plasma / CSF

Reagent Preparation:

Dissolve one vial of Enzyme Reagent (I) In one bottle of Buffer solution (2). Mix gently to dissolve. The prepared Working Enzyme Reagent is stable for at least 2 months at 2-8 °C.

Expected Range:

Random Glucose : 70-170 mg%
Fasting Glucose : 70-110 mg%
Post Lunch Glucose : up to 170 mg%
CSF Glucose : 50-80 mg%

Directions for Use on Analyzers:

Reaction type : End point with Std.
Wavelength : 505nm (Green Filter)
Incubation temp : 37°C
Incubation time : 10 min
Sample volume : 10µL
Reagent volume : 1ml
Light path : 1cm
Standard : 100mg%
Linearity : 500mg%
Unit : mg%

Procedure:

Pipette into clean, dry tubes labelled Blank (B) standard (S) and test (T) and add the reagents in the following order.

Reagents	B	S	T
Working Enzyme Reagent(ml)	1.0	1.0	1.0
Distilled water (ml)	0.01	-	-
Standard (ml)	-	0.01	-
Serum/Plasma/CSF (ml)	-	-	0.01

Mix well and incubate at 37 °C for 10 min or at R.T. for 20min. Measure the absorbance of Test (T) and Standard (S), against Blank (B) on a photo colorimeter with green filter or on a spectrophotometer at 505nm.

Calculations:

Glucose in mg% = A of (T) / A of (S) × 100 (std conc)

Estimation of Triglycerides:

Summary and Explanation of test:

Triglycerides circulate in blood as complexes with protein molecules called lipoproteins. Triglycerides reach maximum level in blood approximately 4 to 6 hours post prandial. Elevated levels of both cholesterol and triglycerides in blood have been identifies as risk factors related to atherosclerotic disease. The levels of cholesterol and triglycerides can vary independently; therefore, evaluation of hyper-lipidemia includes determination of both cholesterol and triglyceride.

Principle:

Triglycerides are usually assayed either by chemical methods or enzymatic methods. The chemical methods involve solvent extraction; chemical hydrolysis to release glycerol; oxidation of glycerol to form aldehyde which is then quantitated by coupling it with chromogens. These chemical methods are cumbersome, time consuming and are reliable only in expert hands. In contrast the enzymatic methods are simple, and easy to perform.

Triglycerides are hydrolysed by lipase to glycerol and free fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerol kinase (GK) to Glycerol-3-phosphate (G-3-P) which is oxidized by the enzyme Glycerol-3-phosphate oxidase (G-P-O) producing hydrogen peroxide. Hydrogen peroxide so formed reacts with 4-aminoantipyrine and ESPAS in the presence of the enzyme peroxidase (POD) to produce a Brown colour complex. The intensity of the colour developed is proportional to the tri-glycerides concentration.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + ATP \xrightarrow{GK} G\text{-}3\text{-}P + ADP$$

$$G\text{-}3\text{-}P + O_2 \xrightarrow{GPO} H_2O_2 + \text{dihydroxy acetone phosphate}$$

$$H_2O_2 + \text{ESPAS} + \text{4-AAP} \longrightarrow \text{Brown colour} + H_2O$$

Reagents:

1. Enzyme Reagent	5×10	2×50	2×100	5×100 ml
2. Standard (200 mg %)	1.0	1.0	1.0	2×1.0 ml

The reagents are ready to use and usable up to the expiration date when stored at 2-8 °C.
Sample: Serum / Plasma
Expected Range:
Serum triglycerides: Up to 150 mg%
Directions for use on Analysers:
Reaction type : end point with std
Wavelength : 546 nm/ green filter
Incubation temp : 37 °C
Incubation period : 10 min
Standard : 200 mg%
Linearity : 1000 mg%
Procedure:
Pipette into clean dry tubes labelled blank (B), standard (S), and test (T).

Reagents	B	S	T
Enzyme reagent	1.0 Ml	1.0 ml	1.0 ml
Standard	-	0.01ml	-
Serum/plasma	-	-	0.01ml

Mix well and incubate for 10 min at 37 °C. Read absorbance of Standard and Test against Blank on photo colorimeter at 546 nm/green filter.
Calculations:
Triglycerides conc. In mg %: A of (T) / A of (S) × 200 (std.conc)
SI conversion factor:mMol/L= mg % ×0.0113
Estimation of Cholesterol:
Summary and Explanation of test:
Cholesterol is both coming from food and synthesized by the human body, mainly in hepatic and intestinal cells. Cholesterol is a component of cells and organic membranes. It is a metabolic precursor of bile acids, vitamin D and steroid hormones. Cholesterol, insoluble molecule, circulates associated with lipoproteins (HDL, LDL, and VLDL).
Quantification of total cholesterol allows the detection of hyper-cholesterolemia, isolated of associated with hyper-triglyceridemia. High Cholesterol concentration are associated with a high risk for vascular accident and apparition of atherosclerosis. The LDL/HDL ratio should be taken into consideration for evaluating the risk of developing cardiovascular diseases.
Principle:
Enzymatic determination of total cholesterol according to the following reactions.

- Cholesterol ester + H_2O Cholesterol esterase cholesterol + fatty acids
- Cholesterol + O_2 Cholesterol oxidase 4-cholesterol-3-one + H_2O_2
- $2H_2O_2$+ phenol + 4-aminoantipyrine peroxidase Quinoneimine + 4 H_2O

Reagents:
1. Cholesterol reagent 5×10, 2× 50, 2× 50, 5×100ml
2. Cholesterol Standard 1 1 1 2×1ml
3. HDL PPT Reagent 5 10 2×10 50ml

The reagents are ready to use and usable to the expiration date when stored at 2-8 °C and protected from light, if contamination is avoided.
Sample:
Serum
Heparin or EDTA plasma from fasting patient
Expected Range:
Cholesterol Normal: up to 250 mg HDL cholesterol
Male: 30-63 mg% female: 35-75 mg%
Directions for use on Analysers:
Reaction type : end point with std
Wavelength : 500 nm (green filter)
Incubation temp: 37 °C
Incubation time: 5 min
Standard : 200 mg%
Linearity : 700 mg%
Unit : mg%

Total Cholesterol Procedure:

Pipette in a clean dry test tube labelled as blank (B), standard (S), and test (T)

B	Enzyme	S	T
1ml	Reagent	1ml	-
0.01 ml	De-ionized water	-	-
-	Standard	0.01 ml	0.01 ml
-	Serum/plasma	-	

Mix and read the optical density (OD) at 500 nm against blank after 5 min incubation (37 °C). The final colour is stable for at least 1 hr.

Calculation:

Cholesterol conc. In mg % = A of (T) / A of (S) × 200 (std.conc)

HDL cholesterol procedure:

Step 1: Precipitation

Serum	0.2 ml
HDL PPT reagent	0.3 ml

Mix well and stand at R.T. for 10 min. Centrifuge at 3000 RPM for 10 min.

Step II: Colour Development

Take 3 clean glass tubes labelled as blank (B), standard (S), and test (T)

Sample	B	S	T
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Standard	-	0.01 ml	-
Serum/ plasma	-	-	0.01 ml

Incubate for 5 min at 37 °C.

Read the optical density at 500 nm against blank.

Calculation:

HDL conc. = abs of test / abs of standard × 5.

LDL cholesterol procedure:

Below is general example of the direct LDL test procedure for an automated analyzer. All analyzer applications should be validated in accordance with NCEP and CLIA recommendations.

Use 3ul sample with 300 ul of direct LDL cholesterol reagent 1.

1. Equilibrate to 37 °C for 5 min
2. Add 100 ul of direct LDL cholesterol reagent 2
3. Equilibrate to 37 °C for 5 min
4. Measurement (absorb. Difference between 660nm and 546nm)

Take 3 clean glass tubes labelled as blank (B), Standard (S), and test (T)

Reagent	B	S	T
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Standard	-	0.01 ml	-
Serum/ plasma	-	-	0.01 ml

Mix well and incubate for 10 min at 37 °C. Read absorbance of standard and test against blank on automated analyzer between 660 nm and 546 nm.

Calculation:

LDL conc. = Abs of test / Abs of standard × 5

SGOT (ASAT) Estimation:

Summary:

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of enzyme in blood stream. Elevated levels are found in myocardial infarction, cardiac operations, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases, primary muscle disease. Decreased levels may be found in Pregnancy, Beri-beri an Diabetic ketoacidosis.

Principle:

SGOT converts L-Aspartate and a Ketoglutarate to Oxaloacetate and glutamate. The oxaloacetate formed reacts with 2,4-dinitropheny hydrazine to produce a hydrazine derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured. The reaction does not obey Beer's law and hence a calib0ration curve is plotted using a pyruvate standard. The activity of SGOT (ASAT) is read off this calibration curve.

L-Aspartate + keto glutarate $\xrightarrow[\text{pH 7.4}]{\text{SGOT}}$ oxaloacetate + L-Glutamate

oxalo acetate + 2, 4, DNPH —(alkaline medium)→ 2, 4, Dinitro phenyl hydrazone (brown coloured complex)

Normal Reference Values

Serum: 8-40 units/ml

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	**40 assays**
L1: Substrate reagent	25 ml
L2: DNPH reagent	2×12.5 ml
L3: NaOH reagent (4N)	25 ml
S: Pyruvate standard (2mM)	5 ml

Storage/ stability

Contents are stable at 2-8 °C till the expiry mentioned on the labels. NaOH can be stored at R.T. till the expiry mentioned.

Reagent Preparation

All the reagents are ready to use except NaOH reagent (4N) which has to be diluted 1:10 with distilled/ deionized water. Working NaOH reagent: Dilute the NaOH to 250 ml or for every 1.0 ml of NaOH reagent (4N) add 9.0 ml of dist. Water.

The working NaOH reagent is stable at R.T. till the expiry mentioned, in a plastic bottle.

Sample Material

Serum free form hemolysis SGOT (ASAT) is reported to be stable in serum for 3 days at 2-8 °C.

Procedure

Wavelength / filter : 505nm (Hg 546 nm) / green

Temperature : 37 °C and R.T.

Light path : 1cm

Assay:

Pipette into clean dry test tubes labelled as blank (B) and test (T):

Addition sequence	**B (ml)**	**T (ml)**
Substrate reagent (L1)	0.50	0.50
Incubate for 37 °C for 3 min		
Sample	-	0.10
Mix well and incubate at 37 °C for 60 min		
DNPH reagent (L2)	0.50	0.50
Mix well and allow to stand at R.T. for 20 min		
Distilled water	0.10	-
Working NaOH reagent (L3)	5.00	5.00

Mix well and allow to stand at R.T. for 10min. Measure the absorbances of the test (T) against blank (B) and read the activity of the test form the calibration curve plotted earlier.

SGPT (ALAT) Estimation:

Summary:

SGPT is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction.

Principle:

SGPT (ALAT) catalyzes the transfer of amino group between L-Alanine and a ketoglutarate to form Pyruvate and Glutamate. The pyruvate formed reacts with NADH in the presence of Lactate Dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGPT (ALAT) activity in the sample.

Normal reference value:

Serum: Male: up to 40 U/L at 37 °C

Female: up to 31 U/L at 37 °C

It recommended that each laboratory established its own normal range representing its patient population.

Contents	**25 ml**	**75 ml**
L1: enzyme reagent	20ml	60 ml
L2: starter reagent	5 ml	15 ml

Storage / stability

Contents are stable at 2-8C till the expiry mentioned on the label.

Reagent preparation: Reagents are ready to use.

Working Reagent:

For sample start assays a single reagent is required, pour the contents of 1 bottle of L2 (starter reagent) into 1 bottle of L1 (enzyme reagent). This working

reagent is stable for at least 3 weeks when stored at 2-8˚C. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (enzyme reagent) and 1 part of L2 (starter reagent). Alternatively, 0.8 ml of L1 and 0.2 ml of L2 may also be used instead of 1 ml of the working reagent directly during the assay.

Substrate start assay:

Pipette into a clean dry test tube labelled as test (T):

Addition sequence	**(T) 25 °C / 30˚C**	**(T) 37 °C**
Enzyme reagent (L1)	0.8 ml	0.8 ml
Sample	0.2 ml	0.1 ml
Incubate at the assay temperature for 1 min and add		
Starter reagent (L2)	0.2ml	0.2 ml

Mix well and read the initial absorbance A and repeat the absorbance reading after every 1, 2 and 3 min. Calculate the 0 mean absorbance change per min (D A/min).

Sample start Assay:

Pipette into a clean dry test tube labelled as test (T).

Addition sequence	**(T) 25˚C / 30˚°C**	**(T) 37˚°C**
Working reagent (L1)	1.0 ml	1.0ml
Incubate at the assay temperature for 1min and add		
Sample	0.2 ml	0.1 ml

Mix well and read the initial absorbance A after 1min and repeat the absorbance reading after every 1, 2 and 3min. Calculate the mean absorbance change per min (ΔA/min).

Calculations:

Substrate / Sample start:

SGPT (ALAT) activity in U/L 25 °C / 30°C = A/min × 952

SGPT (ALAT) activity in U/L 37 °C = A/min × 1746.

Experimental study design:

The rats were randomly divided into 5 groups (n=6) as follows:

Group I : control animals (sod. Carboxymethyl cellulose – 1%, orally).

Group II : diabetic animals

Group III: ethanolic extract of *Tecomariacapensis* 200 mg/kg

Group IV: ethanolic extract of *Tecomariacapensis* 400 mg/kg

Group V : metformin 100 mg/kg

Biochemical evaluation:

Blood samples were collected from the retro orbital puncture of rats on 0 and 8th days of control, diabetic and ethanolic extract of *Tecomariacapensis* treated diabetic rats and determined blood glucose levels, lipid profiles and SGPT were estimated after 8 days treatment.

Statistical analysis:

The data are presented as mean ± S.D Statistical comparisons were made by one-way analysis of variance (ANOVA) and followed by Student-Neuman-Keuls test. Data was considered significant when *p* values were lower than 0.05.

Sample Material:

Serum free from haemolysis. SGPT (ALAT) is reported to be stable in serum for 3 days at 2-8˚C.

Procedure:

Wavelength / filter	: 340nm
Temperature	:37˚C / 30 °C / 25 °C
Light path	:1cm

RESULTS AND DISCUSSION

The herbal medicines are effective in the treatment of various life-threatening diseases. Very often these drugs are unscientifically exploited and / or improperly used. Therefore, these plant drugs deserve detailed studies in the light of modern science. The detailed investigation and documentation of plants used in total health traditions and pharmacological evaluation can lead to the development of invaluable plant drugs for many treated drugs.

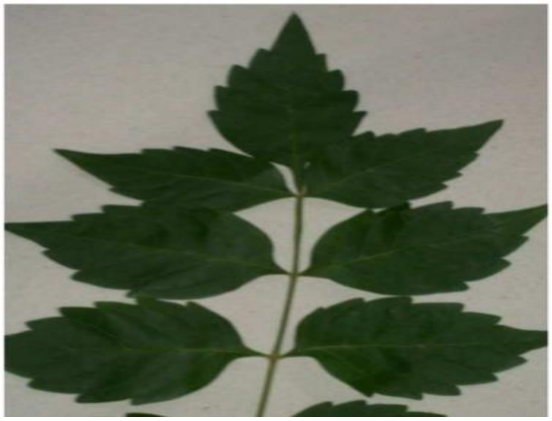
Macroscopic Characters

Leaf	**:** Isobilateral
Shape	**:** Ovate
Size- length	**:** 6 - 7.5 cm
Width	**:** 2.5 – 3.5 cm
Texture	**:** Glabrous
Apex	**:** Acute
Margin	**:** Serrated
Base	**:** Decurrent
Petiole	**:** Expetiolate
Surface	**:** Glossy
Colour- outer	**:** Dark green
Inner	**:** Light green
Venation	**:** Pinnate
Odour	**:** Characteristic

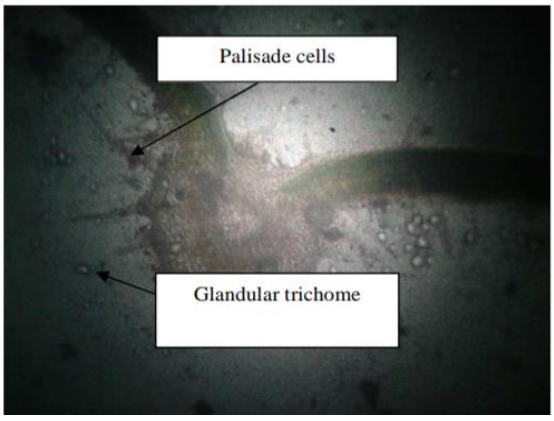
Microscopic Characters

Transverse section of leaves of *Tecomariacapensis*

Type: Isobilateral, convex type of leaf.
Upper epidermis: It is of single layer non-lignified rectangular shape of cells arranged transversally.
Lower epidermis: It is of single non-lignified rectangular shape of cells arranged transversally.
Trichomes: Both covering as well as glandular trichomes are present. Covering trichomes are present just above the epidermal layer. Glandular trichomes are present in more number in the midrib portion.
Stomata: Anamocytic stomata.
Palisade cells: Below the upper epidermis and above the lower epidermis palisade cells are longitudinally compactly arranged and they are terminated up to the lamina portion or mesophyll portion.
Calcium Oxalate Crystals:
Mesophyll region contains calcium oxalate crystals. Isolate or cluster of prism of calcium oxalate crystals.

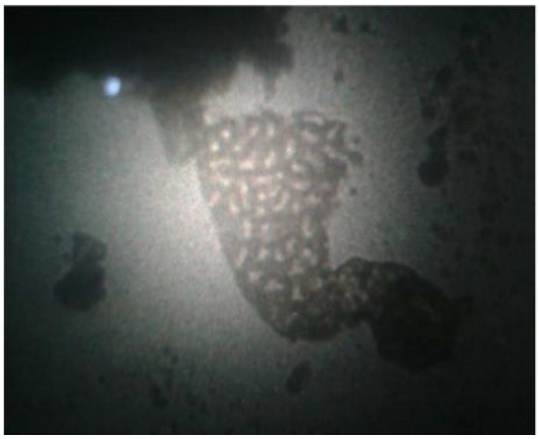


Leaves of *Tecomaria capensis*



T.S of *Tecomaria capensis*

Anamocytic stomata



Spongy parenchymatous cells

Physiochemical constituents:

Determination of physiochemical constituents are performed as per the standard protocol followed in the Ayurvedic pharmacopoeia. The values are tabulated in **Table 1 and 2**.

Table 1: Different Extractive Values

Extractive Value	value
Alcohol soluble extraction	0.25 gm
Water soluble extraction	0.36 gm
Ether soluble extraction	0.04 gm

Table 2: Different Ash Values of *TecomariaCapensis* leaves

Ash values	In gms
Total ash value	0.93
Acid insoluble ash value (dil. HCl)	0.01
Sulphate ash value (H_2SO_4)	0.06
Water soluble ash value (H_2O)	0.05

Percentage yield of *Tecomariacapensis* extracts

Table 3: % yield of different leaves extracts of *Tecomariacapensis*.

Sl.no	Solvents	Nature of extracts	Colour	% yield
1	Pet-ether	Semisolid	Dark yellow	0.35
2	N-Hexane	Semisolid	Dark yellow	0.13
3	Chloroform	Semisolid	Dark green	0.20
4	Ethyl acetate	Semisolid	Dark green	0.70
5	Ethanol	Semisolid	Dark green	0.52
6	Aqueous	Semisolid	Dark brown	0.25

Table 4: Preliminary Phyto Chemical Screening

Phyto constituents	Pet.ether	N-hexane	Chloroform	Ethyl acetate	Ethanol	Water
alkaloids	--	--	--	--	--	--
Flavonoids	--	--	++	++	++	++
Cardiac glycosides	++	++	++	++	++	--
Saponin glycosides	++	++	++	++	--	--
Coumarin glycosides	--	--	--	++	++	--
Tannins	--	--	--	--	++	--
Steroids & terpenoids	--	++	++	++	++	--
Carbohydrates	--	--	--	++	--	--
Protein	--	--	--	++	++	--
Inulin	--	--	--	--	++	++
Volatile oil	++	++	++	++	++	--
Waxes	--	--	--	--	--	--
Mucilage	--	--	--	--	++	++

++ present, -- absent

Phytochemical studies of *Tecomariacapensis* leaves revealed the presence of cardiac glycosides, saponin glycosides and volatile oils in pet-ether extract. In n-hexane extract cardiac glycosides, steroids, tri-terpenoids and volatile oils are present. In chloroform extract, flavonoids, cardiac glycosides, saponin glycosides, steroid, tri-terpenoids, volatile oils are present. In ethyl acetate extract, flavonoids, cardiac glycosides, coumarin glycosides, saponin glycosides, steroids, terpenoids, carbohydrates, proteins and volatile oils are present. In ethanolic extract terpenoids, flavonoids, cardiac glycosides, coumarin glycosides, tannins, steroids, terpenoids, protein, inulin, volatile oils and mucilage are present. In water extract, flavonoids, inulin and mucilage are present. In ethanol and ethyl acetate extracts maximum phytochemical constituents have been observed.

Pharmacological Screening

In-vitro alpha-amylase inhibition assay:

The below table shows the inhibition of α-amylase activity for the methanol and pet. ether extracts and are compared with standard Acarbose. All the tested extracts showed dose dependent enzyme inhibition. The extracts exhibited IC $_{50}$µg/mL will be considered active in comparison with other tested extracts showed enzyme inhibition activity with IC $_{50}$ 131.33±1.8, 123.59±1.2, 133.81±0.7, 127.62±53, 128.00±0.6, 127.22±1.4, 128.43±0.8, 117.52±1.2, 119.64±1.5, 110.45±1.2, 121.93±0.8, 114.26±1.2 and standard shows 0.32 µg/mL respectively.

Table 5: Comparison of compounds with Acarbose IC $_{50}$ in µg/mL against α-amylase activity.

Sample	Concentration	IC $_{50}$ (µg/mL)
Petroleum ether extract	100 (mg/ml)	131.33±1.8
Petroleum ether extract	200(mg/ml)	123.59±1.2
n-hexane extract	100(mg/ml)	133.81±0.7
n-hexane extract	200(mg/ml)	127.62±53
Ethyl acetate extract	100(mg/ml)	128.00±0.6
Ethyl acetate extract	200(mg/ml)	127.22±1.4
Chloroform extract	100(mg/ml)	128.43±0.8
Chloroform extract	200(mg/ml)	117.52±1.2
Ethanol extract	100(mg/ml)	119.64±1.5
Ethanol extract	200(mg/ml)	110.45±1.2
Aqueous extract	100(mg/ml)	121.93±0.8
Aqueous extract	200(mg/ml)	114.26±1.2
Acarbose	2.5 (µg/ml)	0.32±0.12

All values reported as Mean ± S.E.M (n=3)

A-amylase activity was measured in-vitro by hydrolysis of starch in presence of α-amylase enzyme. This process was quantified by using iodine, which gives blue colour with starch. The reduced intensity of blue colour indicates the enzyme-induced hydrolysis of starch into monosaccharides. If the substance/extract process α-amylase inhibitory activity, the intensity of blue colour will be more. In other words, the intensity of blue colour in test sample is directly proportional to α-amylase inhibitory activity.

Acarbose shows the best when compared to extracts, among all concentrations of different extracts, the ethanol extract of 200 mg/ml showed the superior anti-diabetic activity when compared to rest of the concentrations of different extracts.

***In-vitro* evaluation of antidiabetic[92] activity:**

The effects of Ethanolic extract of *Tecomariacapensis*100mg/kg on blood glucose, SGOT, SGPT levels (0 & 8 days) of control, diabetic and ethanolic extract of treated diabetic rats were summarized in Table 1. Lipid profiles were significantly ($p<0.001$) reduced the blood glucose and SGOT and SGPT concentration diabetic groups ($p< 0.001$). The ethanolic extract of *Tecomariacapensis* was significantly reduced, the triglycerides, LDL-cholesterol, and total cholesterol but increased HDL cholesterol levels after treatment. *Tecomariacapensis* occurs throughout the tropic and sub-tropic regions. It contains chemical constituents such as triterpenes, tannins, anthroquinones, flavonoids, saponins, steroids, resins, lectins, glycosides, sugars, phenolic compounds and alkaloids and these are more beneficial in diabetes and its associated complications, holding hope of the new generation anti-hyperglycaemic drug.

Table 6: Blood glucose levels were estimated in Normal, Diabetic control & Treatment groups.

Groups/ Parameters (mg/dl)	Normal	Metformin (25 mg/kg)	CG-I (100 mg/kg)	CG-II (200 mg/kg)
Blood glucose	83.1±11.2	111.3±19.4	199.6±38.6	169±47.1
TC	46±2.7	61±5.3	72.3±5.2	63±6.9
TG	54.7±4.3	60.9±4.2	73.8±3.8	66.7±4.7
HDL	23.6±1.6	17.7±0.3	13±0.4	15.1±0.5
LDL	11.1±0.5	11.8±0.8	21.0±0.6	11.5±0.6
VLDL	11.64±1.1	11.27±0.9	15.36±0.51	12.78±5.4
SGOT	25±3	31.5±4.1	52.6±6.7	41.6±3.7
SGPT	41.9±9	54.9±3.1	75.9±4.7	60.9±4.7

SUMMARY AND CONCLUSION

India is one of the largest producers of medicinal herbs in the world. The Indian traditional healthcare system, Ayurveda provides relatively organized database and more exhaustive description of botanical materials, many of which have been used as templates for novel drug development. Nature has provided a complete storehouse of remedies to cure all ailments of mankind by providing us drugs in the form of herbs, plants and algae to cure the incurable diseases without any toxic effect. Research on medicinal plants is an important fact of biochemical research in India because of several reasons.

Review of literature was done on herbal plant *Tecomariacapensis* species and its literature was collected and reviewed. Form the review of literature we identified that less biological activity was estimated. So, we have made an attempt for *in-vitro* evaluation of anti-diabetic activity.

The plant was selected from in and around of Guntur district and it was authenticated by professor Dr.M. Raghu Ram, Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur. The leaves were dried and powered. The powdered material was extracted by using non-polar (petroleum ether) to polar (water) solvents. Phytochemical screening revealed that in pet ether extracts glycosides and in ethanol extract, tannins and steroids were presented.

At present *in-vitro* evaluation of anti-diabetic activity on extracts of leaves of *Tecomariacapensis* was performed.

We concluded that the ethanol extracts of leaves of *Tecomariacapensis* have revealed that a significant activity against diabetes. Further-work has to be done for the phyto constituent's responsible for the activities.

Our results shown that oral administration of *Tecomariacapensis* extract has a beneficial effect on the reducing hyperglycaemia, SGOT & SGPT levels, total cholesterol, triglycerides levels, LDL-cholesterol, VLDL-cholesterol and improving the HDL status. This study suggests that the induction of diabetes mellitus may be prevented by flavonoids of plant constituent's administration. There is a need to continue to explore the mechanisms for anti-diabetes.

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