METHODS
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The effects of khat on various biochemical parameters were studied under both *in vivo* and *in vitro* conditions. Male Albino Wistar rats were selected for the experiments conducted under *in vivo* conditions. The rats weighing 180-220g were housed in a room at 20-24°C and were maintained under standardized condition of light (12h L; 12h D) and on a Purina diet and water ad libitum. Rats were kept at laboratory conditions for 1 week prior to the experimentation.

Extract of khat at various doses/days were given to them orally through catheter. Both circulating and tissue levels of various biochemical parameters like superoxide dismutase, catalase, glutathione-s-transferase, glucose, uric acid, MDA, reduced glutathione, SGPT, SGOT, Creatine kinase and cortisol were determined.

For the phytochemical study the major constituents of khat were separated and similar studies were carried out as mentioned above.

Further the modulation of restraint stress induced oxidative damage was also evaluated by pre and post khat treatment.

**Preparation of *Catha edulis* extracts:**

500gm leaves and stem tips (dry) were soaked into 1 L distilled water. It was kept at room temperature for overnight, and then heated for 15 minutes on the water bath. Plant material was separated by filtration. Filtrate obtained was evaporated under reduced pressure till dryness. Dry aqueous extract obtained by this method was dark brown gummy in nature (10gm).

**Preparation of alkaloid and Flavonoid fractions:**

A sample of powdered dried leaves (500 gm) was soaked in methanol (1L) for one week. The methanol extract was evaporated under reduced pressure. The residue obtained was then dissolved in ethyl acetate (1L). This ethyl acetate solution was repeatedly subjected to extraction with 5 %
aq.HCl (3×100 ml) until the extract showed a weak reaction with
Dragendorff’s reagent, (Dragendorff’s reagent consisted of two stocks,
A&B. Stock A contained 0.6 gm Bismuth subnitrate dissolved in 2 ml
concentrated HCl and 10 ml distilled water was further added to it. 6 gm
Potassium Iodide was dissolved in 10 ml distilled water to yield stock B.
Now the two stock solutions were mixed and 7 ml of concentrated HCl and
15 ml of distilled water was added to the mixture of the stock solutions. The
volume was then made up to 400 ml with distilled water).

The acidic solution, after extraction with diethyl ether (500ml × 3), was
neutralized with 25 % NH₃ to PH 9-10 and exhaustively extracted with
chloroform. The combined chloroform extracts were washed twice with
water, dried over sodium sulfate, and evaporated to dryness to yield a crude
base alkaloid extract (800 mg). After getting the whole alkaloid fraction, the
remaining part of the extract was further neutralized by adding few drops of
concentrated HCl and then it was again exhaustively extracted with ethyl
acetate to remove all phenolic components. The whole process was repeated
four to five times until the ethyl acetate soluble extract showed no reaction
with ferric chloride. Alkaloid extract showed three spots on TLC in
benzene-acetone (1:1) when sprayed with Dragendorff’s reagent, while the
ethyl acetate extract showed at least 5 to 6 phenolic compounds (Flavonoids)
on TLC in toluene: ethyl formate: formic acid (5:4:1) when sprayed with
alcoholic solution of FeCl₃ and seen under UV light.

Effect of khat treatment:

The animals were classified into 5 groups of 15 rats each. The
controls group rats were given 2 ml of saline orally per kg body weight. The
experimental rats received a dose of 200, 400, 700 and 900 mg of khat per
kg body weight orally each day for 5 successive days. Another group
received a single dose of these quantities of khat and was sacrificed after 3
hrs.
Effect of stress on khat consumption:

Immobilization stress was accomplished by placing individual animals in wire mesh cages of their size attached to a wooden board. The rats were deprived of food and water during stress exposure (Hasan and Ali, 1980). The rats were exposed to 2, 4, 6 and 12 hours of restraint stress. For further studies a 4 hour stress period was selected. The animals were subjected to a 4 hr stress period beginning at the same time each day for 2 and 10 days, and then sacrificed 30 min after the last session. Controls were handled at the same time as the stressed animals and were placed in individual cages during the corresponding time.

For the repetitive stress plus drug challenge studies the animals were divided into two groups. One received the drug 30 min prior to 4 hrs stress session (post stress khat treated) while the other group received the drug 30 min after the session (pre stress khat treated). The animals were subjected to these sessions (at the usual time daily) as described above for 2 and 10 days.

Effect of major constituents of khat and stress on various biochemical parameters:

The major constituents of khat were separated as mentioned elsewhere. The rats were treated with alkaloid (200mg/kg body weight) and flavonoid (200mg/kg body weight) similarly as the khat treated groups mentioned above. The effect of stress was also evaluated on these treatments.

Quantitative Estimation of Protein (Lowry et al., 1951)

A suitable aliquot of the protein sample was diluted to 1ml with distilled water. To this was added 5.0 ml freshly prepared alkaline copper reagent. The alkaline copper reagent was prepared by mixing 0.5% copper sulphate 1% (w/v), sodium potassium tartarte 2% (w/v), and sodium carbonate in 0.1 N NaOH in the ratio of 1:1:100. After incubation for 10
33

minutes at room temperature, 0.5 ml of 1 N Folin's reagent was added. The contents were rapidly mixed and color intensity was read after 30 minutes against reagent blank at 660 nm. The concentration of protein in the samples was determined using standard curve with BSA.
Methods for estimation of various biochemical parameters: catalase, SOD, GST, glucose, uric acid, MDA, SGPT, SGOT, CK, GSH and cortisol were estimated by known standard methods as mentioned below:

Catalase (EC 1.11.1.6)
(Beers and Sizer, 1952)

Principle:

The enzyme catalase catalyzes the following reaction

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2
\]

In the UV range \(\text{H}_2\text{O}_2\) shows a continual increase in absorption with decreasing wavelength with a maximum at 240 nm. The decomposition of Hydrogen peroxide was followed by the loss of its light absorbance at 240 nm.

Procedure:

30 mM of Hydrogen peroxide was prepared in 50 mM Potassium phosphate buffer (pH 7.0), 3.0 ml of \(\text{H}_2\text{O}_2\) phosphate buffer was pipetted into cuvette, 0.025 ml of samples were added as enzyme source, and the contents were mixed thoroughly. The decrease in absorbance at 240 nm was recorded after every 30 seconds for 3 minutes.

Calculation:

The specific activity of catalase is defined in terms of micromoles of hydrogen peroxide consumed per minute per milligram of protein sample. The conversion of initial velocity (change in absorbance at 240 nm/min) to catalase specific activity is done as follows:
Specific Activity (units/mg) = \frac{\Delta A_{\text{min}}^{-1} \times 1000}{43.6 \times \text{mg protein} / \text{ml reaction mix}}

where 43.6 M^{-1} cm^{-1} represents the molar extinction coefficient of hydrogen peroxide.

**Superoxide dismutase (EC 1.15.1.1)**
*(Marklund and Marklund, 1974)*

**Principle:**
Procedure depends upon autooxidation of pyrogallol:

\[
\text{Pyrogallol} + \text{O}_2 \xrightarrow{\text{autooxidation}} \text{oxidation products} + \text{O}_2^- \\
2\text{O}_2^- + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2
\]

The increase in absorbance was recorded at 420 nm.

**Procedure:**
Tissues were homogenized (10% w/v) in chilled 0.15M KCl and centrifuged in cold at 10,000 rpm for 15 min. To 0.05 ml of supernatant 2.85 ml 0.05 M tris-succinate buffer (pH 8.2) was added, mixed well and incubated at 25°C for 20 minute. The reaction was started by adding 0.1 ml of 8 nM pyrogallol solution. Change in absorbance per minute was immediately recorded for initially 3 minutes at 420 nm on Beckman DU-6 UV/VIS spectrophotometer. A reference set consisting of 0.05 ml distilled water instead of the sample solution was also run simultaneously.

**Calculation:**

\[
\text{SOD Activity} = \frac{(\Delta A/\text{min ref} - \Delta A/\text{min sample}) \times 30}{(\Delta A/\text{min ref}/2 \times 0.05 \times \text{units}/10 \text{ mg tissues})}
\]
Where $\Delta A/\text{min ref} =$ Change of absorbance per min. in reference set.
$\Delta A/\text{min sample} =$ Change of absorbance per min in sample.
Activity Unit: One unit of the enzyme is defined as the amount of enzyme which causes 50% inhibition of pyrogallol autoxidation under assay conditions.

**Glutathione-S-Transferase (EC 2.5.1.18)**

*(Habig et al., 1974)*

**Principle:**

The enzyme activity is measured by following the increase in absorbance at 340 nm of CDNB-GSH conjugate generated as a result of GST catalysis between glutathione and 1-chloro-2,4-dinitrobenzene (CDNB).

$$\text{CDNB} + \text{GSH} \xrightarrow{\text{GST}} \text{CDNB} - \text{GSH conjugate}$$

**Procedure:**

Different tissues of rats were homogenized in chilled 0.1 M phosphate buffer pH 6.5 (10% w/v) and centrifuged in cold for 15 min at 15000 rpm. To 0.1 ml of supernatant, 2.7 ml glutathione solution (0.1 M in phosphate buffer, pH 6.5) and 0.2 ml CDNB (1.0 mM in acetone) were added and mixed thoroughly. The change in absorbance at 340 nm was recorded at room temperature against blank containing all the reagents except the enzyme. Protein content in enzyme source was also determined by Lowry’s method.

**Calculation:**

The values were calculated on the basis of molar extinction coefficient of CDNB (9.6 mM$^{-1}$ cm$^{-1}$) and specific activity of enzyme was expressed in nmoles of GSH-CDNB conjugate formed per minute per mg protein.
Creatine Kinase (EC 2.7.3.2)  
(Olivers, 1985)

This test was done by using reagent kit from RECKON DIAGNOSTICS Pvt. Ltd. (India).

Principle:

The principle is based on coupled enzyme assay utilizing the enzymes, Hexokinase and glucose-6-phosphate dehydrogenase. Creatine Kinase, which catalyzes the formation of ATP from Creatine Phosphate and ADP:

\[
\text{Creatine phosphate} \quad \text{Creatine} \\
+ \quad \text{CK} \quad + \\
\text{ADP} \quad \text{ATP}
\]

Glucose is converted to Glucose-6-Phosphate by Hexokinase using ATP as a source for phosphate moiety. Glucose-6-phosphate is oxidized by G-6PDH to 6-phospho- gluconate reducing NADP to NADPH. The reaction after the lag phase was monitored by the increase in absorbance at 340 nm and is directly proportional to the creatine kinase activity. (i.e. the formation of NADPH is in equimolar amount as that of formation of creatine).

\[
\text{ATP} \quad \text{HK} \quad \text{G-6-P} \\
+ \quad \text{Glucose} \quad \text{ADP} \\
\text{G-6-P} \quad 6\text{-Phosphogluconate} \\
+ \quad \text{G6PDH} \quad + \\
\text{NADP} \quad \text{NADPH}+\text{H}
\]

CK = Creatine Kinase  
HK = Hexokinase  
G6P = Glucose-6-phosphate  
G6PDH = Glucose-6-phosphate-dehydrogenase
N-acetylcysteine acts as a thiol activator and Diadenosine 5' Penta Phopsphate (DAPP) & Adenosine Monophosphate (AMP) inhibit the interfering myokinase activity.

**Procedure:**

1 ml of working reagent was prepared by mixing enzyme, activator and buffer (100 mM imidazole acetate buffer, pH 6.5, 30 mM phosphocreatine, 2 mM ADP, 20 mM D-glucose, 2 mM NADP⁺, 3500 U/L Hexokinase, 2000 U/L D-glucose-6-phosphate dehydrogenase, 5 mM AMP and 20 mM N-acetylcysteine). To this 0.05 ml of serum was added and mixed immediately. The reaction was followed by measuring absorbance at an interval of 30 seconds for 2 minutes at 340nm.

**Calculation:**

\[
\text{CK Activity (U/L)} = F \times \Delta A/\text{minute}
\]

Where \( F = 3376 \) (based on the millimolar absorption of NADPH at 340 nm).

**Aspartate transaminase (AST or SGOT) (EC 2.6.1.1)**

(*Ladue et al., 1954*)

The activity of aspartate transaminase was measured using kit from RECKON DIAGNOSTIC PVT. LTD. (India).

**Principle:**

The principle was based on the reaction of L-Aspartate and alpha-ketoglutarate in the presence of GOT present in the sample to yield oxaloacetate and L-glutamate. The oxaloacetate thus produced was reduced by malate dehydrogenase (MDH) to yield L-malate with the oxidation of NADH to NAD⁺. The rate of the reaction was monitored by measurement of the decrease in absorbance of NADH at 340nm.
L-Aspartate + Alpha-ketoglutarate \[\rightarrow\] Oxaloacetate + L-Glutamate

Oxaloacetate + MDH \[\rightarrow\] Malate + NADH \[\rightarrow\] NAD^+

The rate of decrease in absorbance of NADH is proportional to GOT activity in the sample.

**Procedure:**

1 ml of working reagent was prepared by mixing enzyme/coenzyme and buffered substrate (90 mM Tris-buffer, pH 7.8, 200 mM L-aspartate, 2 mM alpha-ketoglutarate, 600 U/L MDH and 0.15 mM NADH). To this 0.05 ml of serum was added and mixed. After incubation at 37°C for 60 seconds, the absorbance at an interval of 30 seconds was recorded for 2 minutes at 340 nm against distilled water taken as blank.

**Calculation:**

Serum GOT activity (IU/L) = \(\Delta A\)/min \(\times F\)

where \(F = 3376\) (based on the millimolar extinction coefficient of NADH at 340 nm).

**Pyruvate transaminase (ALT or SGPT) (EC 2.6.1.2)**

(Henry et al., 1960)

This test is done using kit from RECKON DIAGNOSTICS PVT. LTD. (India).
**Principle:**

The GPT present in the sample reacted with L-alanine and alpha-ketoglutarate to yield pyruvate and L-glutamate. Pyruvate thus produced was reduced by lactate dehydrogenase to yield lactate with the oxidation of NADH to NAD⁺. The reaction was monitored by measurement of the decrease in absorbance of NADH at 340 nm.

\[
\begin{align*}
\text{L-Alanine} & \quad + \quad \text{GPT} & \quad \to & \quad \text{Pyruvate} & \quad + \\
\text{Alpha-Ketoglutаратate} & \quad \to & \quad \text{L-Glutamate} \\
\text{Pyruvate} & \quad + \quad \text{LDH} & \quad \to & \quad \text{Lactate} & \quad + \\
& \quad + \quad \text{NADH} & \quad \to & \quad \text{NAD⁺} & \quad + 
\end{align*}
\]

The rate of decrease in absorbance was proportional to GPT activity in sample.

**Procedure:**

The procedure was similar to SGOT except the difference was in enzyme/coenzyme and buffered substrates used (90 mM Tris-buffer, pH 7.3, 200 mM L-alanine, 2 mM alpha-ketoglutarate, 2400 U/L LDH and 0.15 mM NADH).

**Calculation:**

Serum GPT activity (IU/L) = \( \Delta A/\text{min} \times F \)

Where \( F = 3376 \) (based on the millimolar extinction coefficient of NADH at 340 nm.)
Serum Cortisol (Breuer et al., 1976)

The test is performed using cortisol microtitre plate enzyme immunoassay kit.

**Principle:**

The cortisol microtitre plate enzyme immunoassay (MP EIA) is a direct assay of limited reagent (competitive) design. A specific agent is used to displace cortisol from serum binding proteins, thus making it available for binding by antibody.

**Procedure:**

50 μl of sample or standard were added to the wells of ELISA plate. 0.2 ml of cortisol EIA enzyme label was also added: plates were covered then kept on a shaker for 2 hours. The plates were removed from shaker after the stipulated time and washed four times with 0.3 ml diluted cortisol wash buffer, then 0.1 ml of cortisol EIA substrate was added then wells were covered and placed on shaker for 20 minutes: the plates were then removed from shaker and 0.1 ml of cortisol EIA stop solution was added. The plates were then placed on microtitre plate reader and the absorbance was recorded at 450 nm.

**Calculation:**

The concentration of cortisol was expressed in terms of ng/ml.

Uric Acid (Caraway, 1963)

**Principle:**

Uric acid in the protein free filtrate reacted with phosphotungstic acid reagent in the presence of sodium carbonate (alkaline solution) to form a blue colored complex. The intensity of the color was measured at 710 nm.
Procedure:

For the uric acid assay, the deproteinisation of serum was done by taking 1.0 ml of serum, 8.0 ml distilled water, 0.5 ml sulphuric acid (2/3 N) and 0.5 ml sodium tungstate (10% w/v). After mixing and standing for 10 minutes, the mixture was centrifuged at 3000 rpm for 10 minutes. 3.0 ml of the supernatant was used for further assay. To this, 1.0 ml sodium carbonate (14% w/v) and 1.0 ml phosphotungstate were added. Standard tube was treated similar to sample containing 3.0 ml of working standard of uric acid (100 mg%), after mixing and standing in dark for 15 minutes. Absorbance was measured against the blank treated similar to samples containing 3.0 ml of distilled water that replaces the sample at 710 nm.

Calculation:

\[
\text{Serum Uric Acid in mg/100 ml} = \frac{\text{O.D. test}}{\text{O.D. std}} \times 10
\]

Reduced glutathione (Nagi et al., 1992) (GSH)

Principle:

The assay is based on the reduction of 5,5'-dithiobis-(2- nitrobenzoic acid) (DTNB) by SH groups of glutathione to form 2-nitro-S-mercaptobenzoic acid per mole of glutathione.

Procedure:

0.5 ml of homogenate (1g tissue/10 ml 0.9% NaCl) was deproteinised by the addition of 0.5 ml of 5% perchloric acid. The mixture was then centrifuged at 3000 rpm for 5 min. To 0.1 ml of supernatant, 0.02 ml of DTNB (4 mg/ml in 0.1 M potassium phosphate, pH 8.0) and 1.88 ml of 0.1 M potassium phosphate buffer (pH 8.0) was added. After mixing, the absorbance was read at 412 nm against the blank treated similar to samples containing 0.1 ml of distilled water that replaces the sample.
Calculation:

The result was calculated using the product extinction coefficient of 13.7 mM\(^{-1}\) cm\(^{-1}\) glutathione contents were expressed as (μmole/g tissue).

Measurement of lipid peroxidation (Ohkawa et al., 1979)

Principle:

One molecule of malondialdehyde (MDA) reacted stoichiometrically with two molecules of 2-thiobarbituric acid (TBA) at pH 3.5 according to the following mechanism:

\[
\begin{align*}
\text{HS} &+ \text{CHO} \\
2 \text{OH} &+ \text{CH}_2 \text{CHO} \\
\text{TBA} &\rightarrow \text{Product}
\end{align*}
\]

The pink chromogen can be detected spectrophotometrically with extinction coefficient of 156 mM\(^{-1}\) cm\(^{-1}\) at 532 nm.

Procedure:

The final volume of the reaction mixture was 4 ml, which consisted of 1.5 ml acetic acid (20%), 0.2 ml SDS (8.1%), 1.5 ml TBA (0.8%), 0.7 ml distilled water and 0.1 ml of tissue homogenate (10% w/v). It was incubated at 95°C for 60 min, then cooled and centrifuged at 4000 rpm for 10 min. The optical density of MDA formed was read in the supernatant at 532 nm against the blank, treated similarly containing 0.1 ml of distilled water instead of the sample. The protein concentration in each sample was determined by the method of Lowry et al. (1951).
Calculation:

The values were calculated on the basis of molar extinction coefficient of MDA 156 mM$^{-1}$ cm$^{-1}$ and the results were expressed in nmoles of MDA formed per mg protein.

**Determination of glucose (Trinder, 1969)**

**Principle:**

For estimation of glucose, coupled enzymatic method was employed. The aldehyde group of glucose was oxidized by glucose oxidase to give gluconic acid and hydrogen peroxide, which was broken to water and oxygen by peroxidase. The oxygen, thus produced reacted with 4-aminophenazone in the presence of phenol to form a pink colored compound, the intensity of which was determined at 530 nm.

**Procedure:**

Four sets of tubes were taken as unknown (test sample), standard (in duplicate) and blank, 3 ml of glucose reagent (glucose oxidase, peroxidase, 4-aminophenazone, sodium azide and phenol reagent) was added to each test tube, 0.02 ml of serum, 0.02 ml of glucose standard (100 mg/dl) and 0.02 ml of distilled water were added to the tubes respectively. The reagents were mixed and tubes were kept at 37°C for 15 minutes. The intensities of the color produced were recorded at 530 nm.

Calculation:

\[
\text{Serum glucose (mg/dl)} = \frac{A_{\text{unknown}}}{A_{\text{standard}}} \times 100
\]

where \( A \) = Absorbance at 530 nm.
(a) Effect of khat on BSA:

The reaction mixture consisted of protein (3 mg/ml) and khat extract (15, 30, 100 and 120 mg) prepared as described earlier, in a total volume of 1 ml. After incubation at 37 °C for 3 hours, the reaction was terminated by the addition of 0.25 ml of SDS-sample dye that contained 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue. A protein sample without khat as control was also run for the same period of time. In some experiments, 100 mM of free radical scavengers like sodium azide, potassium iodide, thiourea and sodium formate were included in the reaction mixture of protein with khat extract as indicated. The samples thus prepared were incubated at 37°C for 45 minutes, and boiled for five minutes before loading to SDS PAGE.

(b) Separation of degraded protein by SDS-PAGE:

The samples were separated on 10% (w/v) SDS-polyacrylamide gels essentially performed by the method of Laemmli (1970). The khat treated samples and untreated BSA were loaded on the gel. Electrophoresis was performed at 100 V till the tracking dye reached the bottom of the gel. Running buffer used during electrophoresis contained 1% SDS in addition to 192 mM glycine and 25 mM Tris-HCl pH 8.8. After the electrophoresis was complete, protein bands were detected by staining the gels with 0.1% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid. Destaining was carried out with 10% glacial acetic acid.

Treatment of supercoiled plasmid pBR322 DNA with khat extracts:

Reaction mixture (30 µl) containing 10 mM Tris-HCl (pH 7.5), 0.5 µg of pBR322 plasmid DNA and khat extract (1.5 and 3 mg) were incubated for three hours. The reaction was terminated by adding 10 µl of tracking dye, containing 40 mM EDTA, 0.05% bromophenol blue and 50% (v/v) glycerol.
The mixture was then subjected to electrophoresis on 1% agarose gel for 2 hours. After this, the gel was stained with ethidium bromide dye (0.5μg/ml), and viewed on UV transilluminator (Photodyne, USA) and photographed.

**Preparation of Pea Seeds Nuclease:**

For pea seed nuclease preparation, the method of Wani and Hadi (1979) was followed with slight modification.

Pea seeds were allowed to germinate after surface sterilization by 0.01% HgCl₂. Germination was carried out for 7 days at 25±5°C. The seedlings were removed after 7 days and the crude homogenate was prepared by suspending 88 grams excised embryo axes in three volumes of Tris-HCl buffer (0.02 M, pH 8.0) containing 0.001M β-mercaptoethanol. Then they were grounded in a chilled warring blender under cold conditions. The slurry was passed through cheesecloth and the filtrate was then centrifuged at 10,000 g for 10 minutes. The supernatant was subjected to 40% and 80% ammonium sulfate fractionations. The precipitate was removed after centrifugation at 15,000 g for 20 minutes and dissolved in 60 ml of the buffer A. Sixty ml of dissolved precipitate was extensively dialyzed against 3 changes of buffer A, and finally against buffer B (0.01 M Tris-HCl, pH 7.5) for 12 hours each.

**Digestion of Calf Thymus DNA by khat extract and Pea Seeds Nuclease (Schneider, 1957)**

The experiment was performed to determine the acid soluble nucleotides released from DNA if any as a result of damage caused to DNA by khat using pea seed nuclease digestion. Native calf thymus DNA prepared in 10 mM Tris-HCl was incubated with varying concentration of khat (50, 100, 150 and 200mg) at 37°C for overnight. The reaction mixture for pea seed nuclease digestion contained in a total volume of 1.0 ml, 0.1 M
Tris-HCl, pH 7.4, 0.001 M MgCl₂, distilled water and enzyme. The reaction mixture was incubated for 2 hours at 37°C. At the end of the incubation the reaction was stopped by adding 0.2ml of 10mg/ml BSA, mixed thoroughly by shaking, and then added 1 ml of chilled 14% perchloric acid. The tubes were kept in ice for half an hour, and centrifuged to remove undigested DNA and proteins.

To 1.0 ml aliquot, 2.0 ml diphenylamine reagent (freshly prepared by dissolving 1 gm of recrystallised diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of concentrated sulfuric acid) was added. The tubes were heated in a boiling water bath for 20 minutes. The intensity of blue color was read at 600 nm.