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Collection of the seeds:

Eucalyptus (Eucalyptus kirtoniana), Karanja (Pongamia glabra) and Akashmoni (Acacia auriculaeformis) seeds were collected from the local forests of Burdwan (W.B.), India and identified by the Botany Department of Burdwan University, Burdwan. The collected seeds were dried and powdered. Powdered seeds were completely defatted by solvent (n-hexane) extraction method in a soxhlet distillation apparatus for 72 hours. The oil was collected by distillation. Defatted seed powder was dried and stored in cold condition for experimentation.

Processing of the Eucalyptus seed meal:

Defatted Eucalyptus seed meal (CEM) contains approximately 6.4% tannins. Generally presence of tannins in seed meal reduced crede protein (CP) digestibility and caused various adverse nutritional or metabolic effects. Therefore, tannins must be removed or inactivated prior to the use of the seed meal in animal feed. For removal of tannin, seed meal was stirred with 15% acetone in water for 5 hours and discarded the filtrate. Approximately 500 g seed meal was taken in 1 litre of the acetone solution and acetone was recovered by distillation. The seed meal was then dried on hot plate and stored in cold condition. The acetone treated seed meal was almost devoid of tannins.
The drawbacks of this above mentioned process is the loss of approximately 15% solids including various nutrients, expensive and commercially impracticable.

A simple process was devised for the detoxification of Eucalyptus seed meal. In this process 500 g of the defatted seed meal was mixed with 45 g of calcium oxide and then subsequently autoclaved at 110°C for 5 minutes. The processed seed meal (PEM) was then cooled and stored.

Processing of Karanja seed meal:

The Karanja seed meal (RKM) contains approximately 0.35% karnjin and 4.1% saponins which showed some adverse nutritional and metabolic effects. These antinutritional factors must, therefore, be removed or inactivated by proper processing prior to the use of seed meal as animal feedstuff ingredient. The saponins and karanjin can be removed by soxhlet extraction with ethanol and acetone mixture (2 : 1 V/V) and it was found that the residue did not reveal any toxicity when given in the diet of rats for prolonged periods. The drawbacks of this process are, loss of about 30% solids including various nutrients, expensive, time consuming and commercial impracticability.

Therefore, an easy process was adopted, in which 500 g of the defatted seeds was refluxed for 5 hours with 1 litre of 4 per cent hydrochloric acid. The seed meal was then cooled
and 45 g of sodium hydroxide in 50 ml of water was added. The mixture was dried on hot plate and cooled again. This processed seed meal (PKM) was stored in cold condition until use.

Processing of Akashmoni seed meal:

Defatted Akashmoni seed meal also contains approximately 5% of tannins which was detoxified following the methods as described for Eucalyptus seed meal. In this process, 500 g of the Akashmoni seed meal was mixed with 30 g of calcium oxide and then subsequently autoclaved at 110°C for 8 minutes. The processed seed meal (ASM) was then dried, cooled and finally stored in cold for experimentation.

Preparation of the protein isolate:

From Eucalyptus seed meal: For the preparation of protein isolate, different standard methods were followed and a comparative study of the isolation of proteins by alcohol, salt solution and alkali solutions were carried out. The isolation of protein for the feeding experiments was done following the method which gave the maximum yield. The protein was isolated from the processed Eucalyptus seed meal following the method of Felker and Bandurski (1977) with slight modification. For the extraction of protein, a measured quantity (100 g) of defatted and processed seed meal was suspended in 1 litre cold dilute sodium hydroxide solution (pH 12) and stirred for 1 hour. The resulting suspension centrifuged at
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5,000 g for 15 minutes and supernatant was collected. The residue was washed once with 500 ml of water and the supernatant fraction collected after centrifugation. Protein in the combined supernatant fractions was precipitated by adding 10% solution of trichloro acetic acid (TCA). The process was repeated once and brown coloured protein was finally collected after washing three times with cold distilled water. All the above processings were done in ice-cold condition and freeze dried protein was stored in deep freeze (−4°C) until use.

From Karanja seed meal: Protein from Karanja seed meal was isolated following the method described earlier but with some modifications. For the extraction of protein, a measured quantity (100 g) of the processed Karanja seed meal was suspended in 1 litre of water containing 25 g sodium carbonate and 20 g sodium chloride. The pH of the resulting solution was adjusted to 10 by adding dilute solution of sodium hydroxide and the suspension was stirred for 1 hour. The suspension was centrifuged at 5,000 g for 15 minutes. The residue washed once with 500 ml of 1 M sodium chloride solution and supernatant was collected after centrifugation. Protein in the combined supernatant fractions was precipitated by adding 10% TCA solution. The process was repeated once and after washing three times with water, the white coloured protein was separated by centrifugation. The protein was freeze dried and stored in cold condition during the experimental period.
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From Akashmoni seed meal: Protein was isolated from the processed seed meal following the method of Felker and Bandurski (1977) and freeze dried protein was stored in cold until use.

Refining and hydrogenation of Karanja seed oil:

Slovent extractable oil content of the Karanja seed was 29 per cent. The oil was kept in freeze (4°C) for 3 days and during this time a brownish white semisolid mass was sedimented at the bottom part of the container. The brownish white coloured mass was removed carefully and examined chemically. It was karanjin, a toxic and major (1.25%) furano flavonoid of the seed oil. The remaining brown coloured oil was first steam distilled to remove the essential oil present therein. For the high acid value, one stage alkali refining was not possible for this oil. Hence the following three-stage alkali refining process was adopted. In the first stage, oil was treated with alcoholic caustic soda in concentration of 0.4 per cent. After settling overnight, the lower alcoholic layer containing the soap stock and the foots was removed. The clear oil was given a 2nd and 3rd stages of refining treatment with alcoholic caustic soda solution in concentrations of 0.2 and 0.1 per cent respectively. In every stage of alkali-refining, the alkali was added drop by drop during 15-20 minutes. During this period the oil was kept agitated with a stirrer run at a speed of C 400-500 rpm and this continued...
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for about half an hour afterward. The oil was heated to 70-75°C for soap to grain. Just after last stage operation, the oil was washed four times with hot water and then heated at 100°C to remove moisture. The final product was obtained in a yield of 91 per cent. The refined oil was further bleached with 3.5% fuller's earth according to the method of Sudbrough et al (1922). The refined Karanja oil was then hydrogenated. Hydrogenation was carried out in a three nacked round bottom glass flask at atmospheric pressure using rufort-nickel catalyst (0.75% catalyst on the weight of oil, in the preparation of catalyst 25% reduced nickel was employed). The oil was hydrogenated over the period of 5 hour at 190°C. Catalyst was carefully removed by filtration. The hydrogenated product (RHKO) was white in colour and semisolid in nature with mp 58.5°C. It was also found to be devoid of bitterness and unpleasant odour but contains 30% isomeric fatty acids. The hydrogenated product (RHKO) was examined for the presence of toxic karanjin and pongamol following their usual tests (Rangaswami and Seshadri, 1942; Jatkar and Mattoo, 1954) but it was found that the product was free from these two toxic constituents. The RHKO was stored in deep freeze (-4°C) during the time of experimentation.

Refining and hydrogenation of Eucalyptus seed oil:

Eucalyptus seed contains approximately 29 per cent oil which was red in colour. The oil was first steam distilled
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and then refined following the same method as employed for the refining of Karanja oil. Alkali refining was done in three stages with 0.5, 0.2 and 0.2 per cent alcoholic caustic soda solution respectively. Decolourization and deodourization was done by bleaching with 0.5% decolourizing carbon. The refined oil was light straw coloured and obtained in a yield of 85 per cent of the raw oil. The oil was hydrogenated using nickel catalyst at 110°C for a period of 10 hour. The oil was hydrogenated at the extent of 36 per cent only with 10 per cent isomerization. The hydrogenated product was yellowish white, odourless and semisolid in nature with mp 38°C and stored in cold condition until use.

Refining of Akashmoni seed oil:

Akashmoni seed oil contains 2.8% unsaponifiable matter and high amount of free fatty acids (acid value 13.4) and unusual colour. The solvent extracted oil was refined on a laboratory scale according to the method recommended by the AOAC (1973).

Test Animals:

Growing male albino rats were used throughout the studies. Rats were of local strain and inbred in the animal house of our own laboratory. Healthy rats were only used and all the animals were individually housed in wire net cages during experimental periods. Animals were maintained under
controlled temperature (21-24°C) and approximately 55% relative humidity conditions. At the start of the experiments, the animals were free from any disease or behavioral abnormality and weighing between 50-100 g. The animals were adopted to the laboratory conditions for a fortnight before commencement of experiments and during this period the animals were individually caged and their body weight gain and food intakes were recorded.

Feeding Experiment No. 1:

In this study, the nutritional status of Eucalyptus seed meal (CEM), processed Eucalyptus seed meal (PEM) and casein was evaluated in albino rats. The rats used for the bioassays were growing male and weighing between 50-60 g. The rats were divided into three groups of twelve animals in each. Animals of each of the three groups received the diet which was adequate with respect to protein (nitrogen of the test diet X 6.25), fat, carbohydrates, minerals and vitamins. Eucalyptus seed meal (CEM) and processed Eucalyptus seed meal (PEM) was incorporated at 300 g/kg diet along with 70 g casein/kg diet in the CEM and PEM groups diets respectively. Casein was given at 115 g/kg diet of the rats fed casein diet. A protein-free diet was also designed. The composition of group diets was given in table 7. Experimental food and water was offered ad libitum for 30 days. Food intakes and body weight gain were recorded daily and twice a week respectively. At the end of 30 days, experimental animals were sacrificed and
blood and liver was collected in chilled container for experimentation. Nutritional indices measured were protein efficiency ratio (PER), true digestibility (TD), biological value (BV) and net protein utilisation (NPU). For the determination of PER, TD, BV and NPU of processed Eucalyptus seed meal in rats to different levels of essential amino acid supplementation, three groups of rats containing six animals in each were individually caged and maintained on corresponding PEM diet (table 7) and water ad libitum for 30 days. Biological indices measured were blood haemoglobin, blood sugar, blood urea, total protein, total lipids, phospholipids, cholesterol of serum, liver and liver microsomes. Alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase activities of serum and liver were also measured. Organs such as liver, kidney, spleen, pancreas and intestines were excised and fixed in buffered 10 per cent neutral formalin and processed by conventional methods for histopathology. The paraffin sections were stained with haematoxylin-eosin and examined under Ortholux-Leitz microscope.

Feeding Experiment No. 2:

In this feeding experiment, rat bioassay procedure was used to assess the quality of the protein isolate obtained from the processed Eucalyptus seed meal and comparison were made with casein and soybean protein as protein sources. Thirty six male albino rats weighing between 50-60 g were divided into
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three groups. The animals were individually caged under controlled temperature and humidity conditions. Twelve animals of each of the three groups received the diets which were similar except the nature of protein. Casein, soybean protein and Eucalyptus seed protein isolate were used in separate diets as sole protein source and the protein content of each diet was approximately 10%. A protein-free diet was also designed which contains no protein. All the diets were adequate with respect to vitamins and minerals. Details of the diets are given in table 12. The animals were fed on experimental diets for 30 days with water ad libitum. Food intakes and body weight gain were recorded. At the end of experimental period, animals were sacrificed, blood and livers were collected for experimentation. PER, TD, BV and NPU were determined as nutritional indices. For the determination of PER, TD, BV and NPU of seed protein isolate in rats to different levels of essential amino acid supplementation, three groups of rats containing six animals in each were used and maintained under same conditions and offered Eucalyptus seed protein isolate diet (table 12) and water ad libitum for 30 days.

Blood haemoglobin, blood sugar, blood urea, serum total protein, serum albumin, serum total lipids, serum phospholipids and serum cholesterol contents were determined. Among the liver parameters, total protein, DNA, RNA, total
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Lipids, phospholipids and cholesterol contents of liver and liver microsomes were measured. Alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase activities of serum and liver were also assayed. Fresh tissues of liver, kidney, spleen, pancreas and intestine were taken for histopathological studies.

Feeding Experiment No. 3:

Nutritional evaluations of Karanja seed meal (RKM), processed Karanja seed meal (PKM) and protein isolate obtained from processed Karanja seed meal were carried out with albino rats in this feeding experiment. Comparison was made with casein as protein source. Growing rats weighing between 45-50 g were used in this study. The rats were assigned to four groups of six animals each, as RKM, PKM, protein isolate and casein diet. The animals of RKM group received the RKM at a level of 300 g/kg diet. PKM was also given at a level of 300 g/kg diet. Seed protein isolate and casein was given at 115 g/kg diet. All the experimental diets provided approximately 16 g N/kg diet i.e. 100 g protein/kg diet. The diets were also adequate with respect to vitamins and minerals. All the experimental animals were individually caged and maintained between 22 to 24°C and approximately 55% relative humidity. Body weight gain and food intakes were recorded. The animals received the corresponding group diet and water ad libitum over a 30 days period after which the animals were sacrificed, blood and livers were collected for haematological and biochemical estimations.
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Nutritional indices measured were PER, TD, BV and NPU. For the determination of PER, TD, BV and NPU of PKM and seed protein isolate in rats to different levels of essential amino acid supplementation, four groups of rats containing six animals in each, were also maintained on the corresponding group diets (table 18) and water ad libitum for 30 days. For NPU experiments, the rats were placed in individual cages equipped for separation of faeces and urine and measurement of food intake. A protein free diet for six animals were also used for determination of endogenous N.

Metabolic indices measured in the rat bioassay procedure were blood haemoglobin, blood sugar, blood urea, serum total protein, serum total lipids, serum phospholipids and serum cholesterol. To assess the effects of these proteins and seed meal on hepatic functions, total protein, total lipids, phospholipids, nucleic acids and cholesterol contents of liver were determined. Alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase activities were measured. Organs such as liver, kidney, spleen, pancreas, and intestines were excised for histopathological examination.

Feeding Experiment No. 4:

In this experiment, nutritional evaluation of processed Akashmoni seed meal (ASM) and its protein isolate was carried out with albino rats. Growing male rats weighing between 50-60 g were used throughout this study. The rats were divided into.
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three groups of twelve animals in each as ASM, ASM protein isolate and casein group. The animals were individually caged and maintained between 22 and 24°C and approximately 55% relative humidity. Dietary composition of three group diets are given in table 22. ASM was given at a level of 250 g/kg diet while ASM-protein isolate or casein was given at 115 g/kg diet. All the group diets provided 16 g N/kg or approximately 10% protein. The animals received the corresponding group diet (table 22) and water ad libitum for 30 days. Food intakes and body weight gain were recorded daily and twice a week respectively. At the end of 30 days, rats were sacrificed and blood and livers were collected for experimentation. Nutritional indices such as PER, TD, BV and NPU were determined to assess the nutritional status of the seed meal and seed protein isolate. For the determination of nutritional performance of ASM and ASM-protein isolate in rats to different levels of essential amino acid supplementation, six groups of rats containing 6 animals in each, were also individually placed in cages equipped for the separation of faeces and urine and maintained on corresponding group diet (table 22) and water ad libitum for 30 days.

Blood indices measured were: blood haemoglobin, blood urea, serum total protein, serum total lipids, serum phospholipids, serum cholesterol and serum FFA.

Liver biochemical estimations done were: total protein, total lipids, phospholipids, cholesterol and FFA.
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Enzymes assayed were alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase of serum and liver.

Liver, kidney, spleen, pancreas and intestines were examined for histopathology under Ortholux-Leitz microscope.

Feeding experiment No. 5:

Refined and hydrogenated Karanja seed oil (RHKO) and refined and hydrogenated Eucalyptus seed oil (RHEO) were nutritionally evaluated following well-established rat bioassay procedure in this feeding experiment. Young male albino rats weighing between 80-100 g were used in this study. Animals were maintained under controlled temperature and humidity conditions. Sixty rats were divided into five groups, 12 animals each. Five diets were used; one normal control (N) diet and four fat-supplemented diets. In the fat-supplemented diets, different kind of fats were used and they were coconut oil, corn oil, RHKO and RHEO. The fat was incorporated at a level of 150 g/kg diet. In the normal (N) diet, a mixture of fatty acids (g/kg diet) was given: lauric acid 2.4, myristic acid 3.1, palmitic acid 15.5, stearic acid 2.4, oleic acid 13.9, linoleic acid 20.2 and arachidonic acid 2.5. This mixture of fatty acids considered adequate for normal growth of experimental animals and used according to Tsang et al (1980). The animals were maintained on their corresponding group diet (table 30) and water ad libitum for a period of 30 days. Food intakes were recorded daily and
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the animals weighed twice weekly. At the end of 30 days, the animals were sacrificed under similar conditions. Blood and livers were collected for haematological and biochemical estimations. Digestibility of fats were measured by determining fecal fat excretion.

Blood parameters measured were: blood haemoglobin, blood sugar, blood urea, serum total protein, serum total lipids, serum phospholipids, serum cholesterol and serum FFA.

Biochemical estimations of liver carried out were: total lipids, phospholipids, cholesterol and FFA.

Fatty acid analysis of serum and depot fat of rats fed on different fat-supplemented diets or a normal diet were also done.

Feeding Experiment No. 6:

The present study was undertaken to evaluate the nutritional quality of the refined Akashmoni seed oil in albino rats. Growing rats, weighing between 50-60 g were used throughout the study. A group of six animals were used as experimental and fed on diets containing 10 per cent refined Akashmoni seed oil. A similar group of 6 animals receiving 10 per cent of groundnut oil in the diet served as control. Other ingredients of the diets of both experimental and control were (g/100 g): casein
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20, starch 40, sucrose 20, cellulose powder 5, salt mixture 4 and vitamin mixture 1. The animals were fed on their respective diets and water ad libitum for 30 days. Food intakes were recorded daily and the animals weighed twice weekly. At the end of 30 days, the animals were sacrificed. Blood and livers were collected for biochemical estimations.

Blood and liver lipid parameters such as total lipids, phospholipids, cholesterol and FFA were measured. The coefficient of digestibility was measured by determining fecal fat excretion. Liver, kidney, spleen, pancreas and intestines were examined for histopathology. Isolation of Eucalyptus seed protein for gel and paper electrophoresis or sephadex G-150 filtration:

Dried seeds were powdered and completely defatted by repeated solvent (n-hexane) extration in cold condition and air dried. 10 g of defatted seed meal was stirred with 100 ml of 1 M sodium chloride solution for 1 hour. The insoluble residue was separated by centrifugation at 3,500 g for 30 minutes and the clear supernatant dialysed against distilled water for 6 hours. The water insoluble proteins in the dialysis tubes were separated by centrifugation at 20,300 g for 60 minutes and supernatant discarded. Protein was washed 2-3 times with small portions of glass distilled water (GDW), dissolved in 5-10 ml of phosphate buffer (pH 8.3) and dialysed against the same buffer. The clear protein solution was used for sephadex filtration or electrophoresis.
Separation by gel electrophoresis:

Polyacrylamide gel electrophoresis (PAGE) was carried out by the procedure of Davis (1964) using 7.5% gels. The experiments were done in 0.025 M tris-glycine buffer at pH 8.3. Three milliamperes (mA) constant current per gel was used for 5-6 hours. After electrophoresis, the gels were removed from the tubes by squirting water from a syringe between gel and glass wall and by using a pipette bulb to exert pressure. The length of the gel and the distance moved by the dye, bromophenol blue was measured. The gels were stained with coomassie brilliant blue for 2-5 hours. Staining solution contained 1.25 g coomassie brilliant blue in 454 ml of 50% aqueous methanol and 46 ml of glacial acetic acid. Gels were removed from the staining solution and rinsed with water and placed in destaining solution (mixture of 75 ml of acetic acid, 50 ml of methanol and 875 ml of water) for 30 minutes and stored in 7.5% acetic acid solution. 10-15 μl of sample solution in 20% sucrose was applied at the top of the gels (0.5 X 6 cm). Protein isolate obtained from Eucalyptus seed meal was examined by gel electrophoresis to determine the number of protein fractions and their molecular weights. For the determination of molecular weight different standard and purified protein samples were also run side by side. The standard samples run were: serum albumin (mol wt 69,000), aldolase (mol wt 160,000), pyruvate kinase (230,000) and 11S protein fraction (mol wt 300,000) of sunflower seed protein. Protein fraction of Eucalyptus seed protein obtained by sephadex G-150 filtration was also individually
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examined by PAGE. Number of protein fraction was determined from the number of bands and molecular weight was determined from the graph of mobility vs molecular weights of the standards.

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\text{mobility} = \frac{\text{Distance of protein migration}}{\text{Length of the gel after destaining}} \times \frac{\text{Length of gel before destaining}}{\text{Distance of dye migration}}
\]

Separation by Paper electrophoresis:

Paper electrophoresis of Eucalyptus seed protein was carried out on Whatman No. 3 MM paper (27 X 60 cm). Seed protein was found to be homogeneous in 0.025 M tris-glycine buffer at pH 8.3. Electrophoresis was usually carried out for a period of 5-6 hours at 300 V, 90-100 mA in a Savant high-voltage electrophoresis apparatus. Paper strips were stained with 0.5% amido black in 7.5% acetic acid solution for 1 hour and then destained.

Separation by Sephadex G-150 column chromatography:

Protein fractionation and molecular weight of protein fractions of Eucalyptus seed protein was determined by Sephadex G-150 filtration as described by Andrews (1964). Sephadex G-150 was suspended in phosphate buffer (pH 8.3), allowed to swell for 48 hours and then smallest particles were removed by decantation. Column was prepared by pouring a thin slurry of gel particles in buffer solution in a vertical column (40 X 3 cm), already partly filled with buffer and at the same time allowing excess
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of liquid to percolate through the growing gel bed. The column was washed with the same phosphate buffer for 2-3 days and equilibrated. The flow of buffer maintained at a rate of 10 ml/hour.

The column was calibrated using 11 S protein fraction (300,000) of sunflower protein, pyruvate kinase (230,000), aldolase (160,000) and bovine serum albumin (69,000). Two ml of each protein solution (5 mg protein in 2 ml of phosphate buffer, pH 8.3) was passed through the column using phosphate buffer. Fraction volume collected was 2.5 ml. A solution of 0.25% blue dextran (2 ml) was used to measure the void (V₀) volume. Absorbance was determined at 280 nm. For Eucalyptus seed protein, 2 ml of protein solution (5 mg of the protein was homogeneous in 2 ml of phosphate buffer, pH 8.3) was passed through the pre-equilibrated column and upto 160 fraction of elute (160 x 2.5 ml) was collected. Vₑ - V₀ was plotted against molecular weight of the standards. Molecular weight of the protein fractions of Eucalyptus seed protein was calculated by referring the standard graph. Vₑ is the elution volume and V₀, void volume is the elution volume of a solute completely excluded from the internal cavities of gel.

Heat denaturation of Eucalyptus seed protein and their fractions:

Eucalyptus seed protein isolate and the protein fractions recovered from sephadex G-150 column chromatography was examined
for heat denaturation. Five ml of 0.5% protein isolate or protein fraction solution in 0.025 M tris-glycine buffer of pH 8.3 were heated for 10 minutes at various temperatures, in the range of 30-90°C, in a thermostatic waterbath. For each temperature a separate aliquot was used. The protein solution was then cooled immediately in an ice-bath and rewarmed to room temperature. The turbidity of the solution was measured by determining the transmittance at 540 nm in a spectronic 20 spectrophotometer. From the transmittance value, a quantity \((T_0 - T)/T \times 100\), was calculated where \(T_0\) is the transmittance of the unheated sample solution. The effect of heating interval on turbidity was also determined i.e. protein solution was heated for 5 minutes and more than 10 minutes and the change of turbidity was also determined on the basis of transmittance.

Analysis of carbohydrate moieties of the glycoprotein fraction (EglyP) of Eucalyptus seed protein isolate:

Glycoprotein fraction obtained from sephadex G-150 column chromatography was evaporated to dryness in reduced pressure and freeze dried. Glycoprotein (EglyP) was treated with alkaline borohydride according to Carlson (1966) with the following modification. Freeze dried glycoprotein (10-25 mg) was treated with 10 ml of 0.1 M sodium hydroxide solution containing 1 M NaBH₄ at 37°C. The mixture was left for a period of 50 hours and then the excess of borohydride was destroyed with dilute acetic acid (to pH 5) and concentrated under reduced
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pressure. Borate was removed by repeated evaporation with methanol. The borate free material was applied on a column of Dowex 50 X 2 (H\(^+\)) (10 X 0.5 cm) and carbohydrate moieties were obtained by passage through the column after washing with GDW. The washings were concentrated and then subjected to preparative paper chromatography (n-butanol-ethanol-water = 10:1:2) and the various mono-, di- and trisaccharides were isolated. Monosaccharides were qualitatively determined by reffering with authentic samples in paper chromatography. Di- and trisaccharide was individually hydrolysed in a sealed tube mixing with 2 N sulphuric acid and kept at 110°C for a period of 10 hours. Then the solution was neutralised with sodium carbonate solution (to pH 7) and filtered. The filtrate was concentrated and examined by paper chromatography (n-butanol : water : acetic acid 4:5:1) for corresponding monosaccharides. Staning reagent used was saturated aquous solution of aniline hydrogen oxalate.

The carbohydrate free protein portion was eluted from the Dowex column with repeated washing with 1 M pyridine acetate (pH 5.0), evaporated to dryness and subjected to amino acid analysis.

Analytical Procedure:

protein estimation: The seed protein was extracted from the seed meal either by salt solution or alkali solution, precipitated with 10% TCA solution, freeze dried and weighed. Total protein
content of serum and liver was estimated either following the Biuret method (Gornall et al., 1949) or Lowry et al. (1951). Crude protein (CP) content of the seed meal or experimental diets were determined on the basis of their nitrogen content (analyses by micro-kjeldahl method) and multiplying it with a constant factor of 6.25 e.g. CP = N X 6.25.

Fat: Fat was extracted by solvent extraction method in soxhlet distillation apparatus using petroleum ether (bp 40-60°C) or n-hexane and estimated by evaporating the measured amount of extract.

Carbohydrate: Protein free seed meal was used for the determination of carbohydrates. Protein was removed from the sample following the above mentioned method. Total carbohydrate content was determined following the method of Carrol et al. (1956) using anthrone reagent.

Nitrogen: Nitrogen was determined by micro-kjeldahl method. In this method, 100-120 mg sample was digested with 1-2 ml of hot concentrated H₂SO₄ and a pinch of catalyst mixture for 2-4 hours at 380°C in a kjeldahl flask. After digestion, cooled the mixture and 5 ml of GDW was added. The mixture was then transfered quantitatively to steam distillation unit with 5 ml of sodium hydroxide-thiosulphate mixture solution (50 g of sodium hydroxide and 5 g of sodium thiosulphate in 100 ml of GDW). Liberated ammonia was collected in 5 ml of 4% boric acid containing one
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drop of indicator dye (100 mg of methyl red and 25 mg of methylene blue in 100 ml of 95% ethanol). Collection of ammonia was done till the colour of boric acid changes to green. Back titration of the green coloured boric acid was done with 0.01 N HCl till it revives the original colour.

Nitrogen % = titre value X 0.14008 X 100/Amount of sample in mg

Catalyst mixture : 2 g of SeO₂, 2 g of CuSO₄•5H₂O and 8 g K₂SO₄ was mixed and powdered.

Total moisture : Moisture content of the seed meal or seed protein isolates was determined following the method of AACC (1969). In this method test sample was allowed to dry by keeping it in a vacuum desiccator maintaining 0-5% relative humidity till it reaches a constant weight.

Total ash : Total ash was determined according to the AACC (1969) method. Dried test sample was taken in a porcelain crucible and heated at about 500-600°C in an incinerator for 4 hours. Then it was cooled in a vacuum desiccator and weighed.

Crude fibre : Crude fibre content was determined following the AACC (1969) method.

Saponin : 75 ml of distilled water was added to 15 g of dried, finely powdered seed meal (defatted). The mixture was allowed to stand for 5 hours. 210 ml of 95% ethanol was then added to it and the mixture was kept on a shaker for 20 hours. This was followed by the addition of 53 ml of 95% ethanol and 162 ml of
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water so that the total volume of the liquid was 500 ml and the final strength of alcohol was 50%. The liquid was filtered after standing for 1 hour. One gram of activated charcoal was added to 50 ml of this alcoholic extract (equivalent to 1.5 g of the seed meal sample) which was warmed over steam for 15 minutes with occasional stirring. This was filtered in vacuo and the residue washed with 100 ml of 50% ethanol. To the filtrate (including washing) 1.5 g of activated charcoal was added and the mixture stirred and warmed over steam for 5 minutes. This was filtered and the charcoal washed successively with 20 ml of 10 and 20% ethanol. The filtrate and washings were discarded and the adsorbed saponins were eluted from the charcoal with a mixture of pyridine and absolute ethanol (3:7 V/V). The charcoal in the column was not allowed to dry between two successive additions of solvent. The last portion of the elute was tested with Libermann-Burchard reagent, a negative reaction confirming complete removal of saponin from charcoal. About 200 ml of solvent mixture was found to be adequate. The pyridine-alcohol eluate was then evaporated under vacuum to give the crude saponins. Purification of saponin was done by paper (Whatman 3 M M) chromatography using n-butyl alcohol : 1 M ammonium hydroxide : 90% ethanol (60:30.5:13) as developing solution and a mixture of sulphuric acid and acetic anhydride (Libermann-Burchard reagent) as staining reagent. Rf values of seed saponins were ranged from 0.25 to 0.62. The saponin area on the paper was excised and cut into small pieces. Saponins were recovered from these
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pieces by warming them gently for 5-7 minutes with 60% ethanol repeatedly. The combined portions and washings (60% ethanol washing) were evaporated in vacuo and obtained the saponins in a purified form.

Tannin: Qualitative testing for tannins: Plant extract (extraction was done with 50% ethanol), was added 1% ferric alum solution (3 to 5 drops) and the occurrence of a blue or green colouration indicated the presence of tannins. This was confirmed by the formation of precipitate on adding a few drops of gelatin-salt solution to the extract.

Quantitative estimation of tannins: The official gravimetric method based on the absorption of tannins by hide power, has been adopted. It depends on the determination of the solubles in a filtered plant infusion before and after the removal of tannins by hide power; the difference of two values giving the tannin content of the plant extract.

A weighed quantity of powdered dry seeds was taken which would give 3.7 to 4.2 g of tanning matter per litre of infusion. It was extracted in Proctor Extractor first at room temperature, then at 50°C and finally the temperature was raised to boiling. The quantity of solution collected was made up to volume of 2000 ml. The extract was filtered, first 150 ml of filtrate was rejected and the subsequent portion was used for analysis.
Materials and Methods

Chromed hide powder was prepared by digesting dry hide powder (6.25 g) with 10 times its weight of distilled water for 1 hour. A 3% chrome alum $\text{Cr}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ solution (6.25 ml) was added, the contents were frequently stirred for several hours and then allowed to stand overnight. Thereafter the material was transferred to a linen cloth, drained and squeezed. The cloth was opened and water equal to 15 times the weight of air dry powder was added to chromed material and digested for 15 minutes after which the cloth and powder were lifted out, drained and squeezed. This process of digestion and squeezing was repeated 3 more times. Finally the weight of squeezed material was adjusted to 26.25 g by adding distilled water. 50 ml of the filtered infusion was evaporated in tared flat china dish and dried at 100°C in an oven to a constant weight (total solubles). Another 100 ml of infusion was added to the prepared chromed hide powder and shaken for 10 minutes. The contents were immediately filtered through a linen cloth. Kaolin (2 g) was thoroughly mixed in the filtrate and repeatedly filtered through Whatman filter paper, until it is clear. This filtrate (50 ml) was pipetted into a tared dish, evaporated and dried to constant weight (non-tannins).

The tannins absorbed by hide powder was obtained by the difference between percentages of total solubles and non-tannins.

Amino acid analysis: Amino acids were determined by both paper chromatography and column chromatography using a Beckmann 120-B
amino acid analyser (Beckman Instruments, Palo Alto, Calif., USA). Protein fractions or seed meal was first acid hydrolysed. The samples were weighed and 6 M HCl added in glass-tubes, frozen in cold condition and vacuum sealed. The ratio of sample to HCl was 1 mg sample: 1 ml acid and the hydrolysis continued for 24 hour at 110°C. The hydrolysate was then filtered and the acid removed by evaporation under reduced pressure.

For paper chromatography (Whatman No 3 M M) the residue was dissolved in 10% aqueous isopropanol and used it for spotting (Smith and Seakins, 1976). Two dimensional ascending paper chromatography was done and solvent systems used were (i) butanol: acetic acid: water 12:3:5 and (ii) phenol (160 g in 40 ml of GDW): ammonia 200:1. After completion of the second run the paper was dried in air and then the chromatogram was stained by spraying of 0.2% ninhydrin reagent (0.2 g ninhydrin in 100 ml of acetone). For the detection of proline or hydroxy proline 0.5% isatin reagent was used as staining reagent (Ravindranath, 1981). Qualitative detection of different amino acids were only done in paper chromatography and those were quantitatively determined by column chromatography.

For the analysis of amino acids by amino acid analyser, the acid hydrolysed residue was taken up in 6 ml of 0.2 M sodium citrate buffer, pH 2.2 and applied. For the determination of sulphur containing amino acids the samples were first oxidised with performic acid. Performic acid was prepared according to
Schram et al (1954) by adding 1 vol 30% aqueous H₂O₂ (V/V) to 9 vol of 88% aqueous formic acid (W/W) for 18 hours according to the method of Lewis (1966) before acid hydrolysis.

Fatty acid analysis: Fatty acid analysis of RHKO, RHEO, refined Akashmoni seed oil and serum and depot fat of rats fed fat-supplemented diets were done. For fatty acid analysis, 100 mg of fats or oils and 0.5 ml of plasma were used. Lipid materials were extracted according to the method of Folch et al (1956) using chloroform-methanol (2:1 V/V). The lipid extract was washed with 0.1 M potassium chloride solution. It was evaporated to dryness under reduced pressure and saponified at 100°C for a period of 12 hours in 5 ml ethanolic potassium hydroxide solution. After saponification, unsaponifiable matter was extracted with ether repeatedly. The saponified portion was acidified with dilute hydrochloric acid and the liberated fatty acids were extracted with redistilled ether. The fatty acids were methylated with diazomethane. The mixture of methyl esters were analysed by Gas Liquid Chromatography (GLC) (Phillips, 1956). A 20% diethylene glycol succinate (DEGS) chromosorb packed column (6 ft) was employed; column temperature 200°C, injection temperature 300°C, attenuation 32 X 100, carrier gas nitrogen, speed of carrier gas 2.6 kg/hour and chart speed 60 cm/hour. The peaks were identified by comparing their retention times with that of a reference sample. The peak areas were calculated by triangulation method.
Nutritional Indices:

Protein efficiency ratio (PER): PER was calculated according to the AOAC (1960) method, is based on growth rate and crude protein (CP) intake. CP was calculated as the nitrogen content of the test diet X 6.25.

\[
\text{PER} = \frac{\text{Gain in body weight (g)}}{\text{Protein intake (g)}}
\]

True digestibility (TD) of protein and fat:

The term digestibility of protein and fat refers to the percentage of the ingested protein or fat absorbed into the bloodstream after the process of digestion is complete.

\[
\text{TD} = \frac{\text{N intake} - (\text{N in faeces} - \text{endogenous faecal N})}{(\text{Protein}) \times \text{N intake}}
\]

N intake: Food nitrogen intake (based on actual N analysis of test diet)

N in faeces: Total faecal N excreted (based on the nitrogen analysis of faeces excreted) by the animals fed on diets containing 16 g N/kg diet (food was offered ad libitum).

Endogenous faecal N: Total faecal N excreted (based on nitrogen analysis of faeces) by the animals fed on protein-free diet.
Materials and Methods

\[ \text{DC} = \frac{\text{Fat intake} - (\text{Fat in faeces-endogenous faecal fat})}{\text{Fat intake}} \]

\[ \text{Biological Value (BV)} : \]

\[ \text{BV} = \frac{\text{N digested} - \text{N lost in metabolism}}{\text{N digested}} \]

\[ \text{N digested} : \text{N intake} - \left[ \text{N in faeces (on the protein diet)} - \text{endogenous faecal N (on protein-free diet)} \right] \]

\[ \text{N lost in metabolism} : \text{N in urine (on protein diet)} - \text{N in urine (on protein-free diet)} \]

\[ \text{Net Protein Utilisation (NPU)} : \]

\[ \text{NPU} = \text{TD} \times \text{BV} \]

\[ \text{Biological Indices :} \]

Blood haemoglobin was estimated either by acid hematin method or by the help of haemoglobinometer (Coulter Electronics, Hialeah, Florida, USA). Blood sugar was measured by the method of Somogyi (1945). Estimation of blood urea was done on the basis of the method of Netelson (1957). Lipid materials were extracted from the serum or liver tissues following the method of Folch et al (1957). Total lipid content was estimated by evaporating the measured amount of extract. Phospholipid was assayed by the estimation of inorganic phosphorus according to the method of Fiske and Subbarow (1925). The cholesterol was estimated by the method of Sperry and Webb (1950). Free fatty
acid (FFA) was estimated according to the method of Chakrabarty et al (1969). Liver microsomes were prepared from 0.25 M sucrose liver homogenates (1:10 W/V) by differential centrifugation at 0-4°C as described by Schenkman et al (1967). A 10% homogenate of liver in 0.25 M sucrose was centrifuged at 10,000 g for 30 minutes at 0-4°C and the supernatant fraction again centrifuged at 16,000 g for 60 minutes. The resulting supernatant fraction of two above mentioned operations served as microsomal source of biochemical estimations. Liver homogenate and microsomal suspensions of liver were processed by the method of Schmider for the estimation of nucleic acids (Schmider, 1957). Estimation of RNA was carried out by the orcinol method of Mejbaum (1939). DNA was estimated by Burton's (1956) modification of Disches' diphenylamine method (Dische, 1930). Alanine aminotransferase and aspartate aminotransferase activities of liver and serum were estimated according to the method of Reitman and Frankel (1957). Serum alkaline phosphatase activity was measured according to the method of King and Angstron (1925). Alkaline phosphatase activity of liver and serum was estimated by determining the rate of hydrolysis of p-nitrophenol phosphate as advocated by Bessey et al (1946).

Studies in in vitro digestion of seed protein isolate:

Substrate: Seed proteins (Eucalyptus, Karanja and Akashmoni) and casein.

Enzymes: Pepsin (Costantino, Italy) and trypsin (Merck).
Materials and Methods

In vitro digestibility of the seed proteins by pepsin, trypsin and pepsin followed by trypsin was determined. The extent of hydrolysis of proteins was measured by the spectrophotometric method of Kunitz (1947; 1948).

Peptic digestion: The reaction mixture of 1 ml of 1 per cent protein in M/20 hydrochloric acid-potassium chloride buffer (Clark and Lubs, 1917) (pH 1.8) and 1 ml of 1 per cent pepsin (in the same buffer) was incubated at 37°C, and the extent of digestion estimated at different intervals. Digestion was stopped at selected intervals by the addition of 3 ml of 5 per cent TCA solution. A control was run in which TCA was added before the addition of pepsin to the protein solution. The tubes were allowed to stand for 1 hour after the addition of TCA and the suspensions were then filtered. The concentration of split products in the clear filtrate was determined by measuring the optical density of the solution after diluting it five-fold at 280 nm in a Beckmann spectrophotometer (DU model).

Tryptic digestion: The procedure followed for the tryptic hydrolysis was the same as that for peptic hydrolysis, the reaction mixture being 1 ml of 1 per cent protein in M/20 Sorensen's phosphate buffer (Sorensen, 1909) pH 7.6 and 1 ml of 1 per cent trypsin (in the same buffer).
Materials and Methods

Peptic digestion followed by tryptic digestion: For evaluating the true digestibility of proteins, the in vivo sequence of enzymic hydrolysis of proteins, i.e., peptic digestion followed by tryptic digestion, was carried out in vitro. The protein was digested first by pepsin for 2 hours and then by trypsin for 22 hours. The concentration of the materials in the reaction mixtures were the same as those used in the previous two experiments. The reaction mixture consisted of equal amounts of protein and pepsin solutions (13 ml). Aliquots (2 ml X 2) were withdrawn to determine the extent of peptic hydrolysis, the pH of the digestion mixture was adjusted with sodium hydroxide solution to 7.6 followed by the addition of 9 ml of trypsin solution. Aliquots were withdrawn at selected intervals and enzymic action arrested by the addition of TCA. TCA filtrates, diluted five-fold, were used for the spectrophotometric estimation at 280 nm.