MATERIALS AND METHODS
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I. Collection

Wild edible tubers, corms and rhizomes were collected from the Southern slope of Western Ghats, Kanyakumari district, Tamil Nadu (Vide area map). Field visits were made frequently. The data regarding the wild edibles mentioned above were collected by interviewing the Kanikkar tribals and their village leaders. Old experienced tribals were also interviewed. Specimens of tubers, corms and rhizomes were collected and brought to the laboratory for botanical identification and nutritive analysis. Voucher specimens were collected and deposited in the P.G. Department of Botany, V.O.Chidambaram College, Thoothukudi-8.

The specimens were identified using the following floras.


The wild edible tubers, corms and rhizomes were photographed and the photographs are affixed in appropriate places in the section “Wild edible plants and the Kanikkars—an investigation.”
II. Chemical Analyses

Chemicals

The chemicals used during the experiment were of BDH (AR) and Sigma Chemical Company, St. Louis, U.S.A. Casein and Poly Vinyl Pyrrolidone (PVP) were purchased from SISCO Research Laboratories Pvt. Ltd. Bombay. Porcine pancreatic α-amylase was purchased from E Merck, Germany.

Preparation of Samples

Wild edible samples washed free of dirt were chopped and about 50 g of each were weighed, dried and powdered in a Willey mill (Scientific Equipment Works, New Delhi, India) 60 mesh size. The powdered samples were stored in screw cap bottles at room temperature for further analysis.

Proximate analysis

Determination of Moisture content (AOAC, 1975)

The samples were weighed, chopped and incubated in a hot-air-oven at 80°C for 24 hours. Then the samples were cooled in a desiccator and weighed again. The loss in the weight of the sample was calculated as the moisture content and the average value of triplicate determinations are expressed on percentage basis.
**Determination of Crude protein content**

**Digestion**

The nitrogen content of the dried, powdered sample was determined by micro-kjeldahl method (Humphries, 1956). To 100 mg of the dried powdered sample taken in a microkjeldahl digestion flask, 2 ml of digestion mixture (5% (w/v) Salicylic acid in Con. H₂SO₄) was added and mixed well. After 20 min. 0.3 g of Sodium thio sulphate was added and heated gently until fumes disappeared. The contents of the flask were cooled and 60 mg of catalyst (a mixture of 1 g CuSO₄, 8 g K₂SO₄ and 1 g Selenium dioxide) followed by 1 ml of Con. H₂SO₄ were added. The contents were digested until they turned apple green in colour. The flask was cooled and the contents were made up to a known volume.

**Distillation**

10 ml of the digested solution from the volumetric flask was transferred to Paranas microkjeldahl distillation flask. To this, 10 ml of 40% (w/v) NaOH and 2 ml of distilled water were added and heated using Bunsen burner. The ammonia from the sample was steam-distilled for 5 min. into a receiver flask, which contained 5 ml of 2% (w/v) boric acid solution and indicator (83.3 mg of bromocresol green + 16.6 mg of methyl red dissolved in 10 ml of 95% ethanol).

The ammonia in the receiving flask produced by the breakdown of organic nitrogen-containing compounds in the sample was titrated against N/50 Sulphuric acid. A blank was run simultaneously using all the reagents and the value of the blank was deducted from the value of the
sample before calculation. One ml of N/50 H₂SO₄ equaled 0.00028 g of N, which forms the basis for calculation of N content in the sample.

The crude protein content, which is hereafter called simply the protein content was calculated by the equation.

\[
\text{Protein content (\%)} = \% \text{ nitrogen} \times 6.25
\]

**Crude Lipid (AOAC 1970)**

Dried powdered sample (2g) was extracted with ether in a Soxhlet apparatus for 16 hrs. The ether was evaporated and the residue was weighed. The average value of triplicate experiments was expressed as percentage of ether extract or total crude lipid content on dry weight basis.

**Determination of Crude fibre content (AOAC, 1970)**

The left-out residue after extraction with ether was successively digested with 0.225N H₂SO₄ solution and 0.313N NaOH solution. After digestion, the sample was washed with boiling water followed by absolute ethanol in a Gooch crucible. The content of the crucible was dried to constant weight, cooled, weighed and ignited in an electric muffle furnace for 30 min. at 600°C. The contents were reweighed after cooling. The loss in weight was expressed as percentage of crude fibre on dry weight basis.

**Ash Analysis (AOAC, 1970)**

Two g of dried powdered sample was placed in a pre-weighed crucible and ignited at 600°C for 2 h. The contents of the crucible were cooled in a desiccator and weighed. The difference in weight of the crucible gives the
ash content. The ash content was expressed as percentage on dry weight basis.

**Determination of Nitrogen Free Extractives (NFE) Or Total Crude Carbohydrate content determination (Muller and Tobin, 1980)**

The carbohydrate content was obtained by the difference method: that is, by subtracting the sum of the protein, fat, ash and fibre from the total dry matter.

\[
\% \text{NFE} = 100 - (\text{CP}\% + \text{EE}\% + \text{CF}\% + \text{Ash}\%)
\]

where CP=Crude Protein; EE=Ether Extract; CF=Crude Fibre

**Calorific value determination**

The calorific values of the investigated samples were determined in KJ by multiplying the percentage of crude protein, crude lipid and NFE by the factors 16.7, 37.7 and 16.7 respectively.

**Soluble proteins**

**Extraction**

One g of the dried, powdered sample was extracted with 10 ml of 0.1N NaOH (The extract was slightly heated in a water bath by shaking the tubes) 2 replications of each sample were prepared and 2 aliquots from each replica were used for analysis.

**Estimation (Lowry et al., 1951)**

0.5 ml of extract was pipetted out into the test tubes and made upto 1 ml with distilled water. 5ml of alkaline CuSO₄ reagent was added mixed
well and kept for 10 minutes at room temperature. 0.5 ml of diluted Folin
ciocalteau reagent was added. The absorbance was measured after 30
minutes at 660 nm.

**Calculation**

The process was standardized using a bovine serum albumin
standard and the factor was calculated to be 1.0 mg. The soluble protein (g
100g⁻¹) was calculated according to the formula.

\[
\text{Soluble proteins} = 2 \times T \text{ for an aliquot of 0.5 ml.}
\]

Where T is the OD value for the corresponding sample.

**Starch and sugars (Moorthy and Padmaja, 2002)**

**Extraction and Estimation**

To 1 g of the dried powdered sample, 20 ml of 80% ethanol was
added and left overnight to extract the sugars.

The extracted sugars were separated from the residue by filtration
with Whatman No.1 filter paper. The filtrates were collected separately for
sugar estimation. The residue was transferred back into the conical flask
using 20 ml of 2N HCl.

The starch in the residue was then hydrolysed, cooled, and the
volume was increased to 100 ml using distilled water. This supernatant
was then directly used for titration.

The alcoholic sugar filtrate was treated with 1 ml Con.HCl and heated
(100°C). The volume of the sugar extract was raised to 50 ml with distilled
water and used for titration.
Four replicas of each sample were taken and 2 aliquots were taken from each replica for analysis.

**Titrimetric assay**

10 ml 1% potassium ferriyanide was pipetted and 5 ml NaOH (2.5 N w/v) was added and mixed. The contents were boiled and 3 drops of dilute methylene blue was added. The solution immediately turned blue green. The starch hydrolysate was taken in a 2 ml blow pipette and added drop by drop. The end point is the change of colour to golden yellow. The titre value is noted.

For sugar estimation, the sugar extract was taken in a 10 ml blow pipette and added drop by drop to boiling reagent. The end point is the rapid disappearance of violet colour. At this stage, the titre reading was noted.

**Calculation**

Each lot of Potassium ferriyanide was calibrated using Std. Glc. solution and the relation.

10 mg of Glc. = 10 ml of Potassium ferriyanide.

The starch content was calculated by the formula.

\[
\text{Starch content} = \frac{10^a \times 100^b \times 0.9^c \times 100}{T \times 2^d \times 1000}
\]

- \(a\) - Titre obtained for ferriyanide reagent against Glc. Std.
- \(b\) - Total volume of starch hydrolysate
c-Morris factor for converting sugar to starch.
d-Weight of sample (g)
T-Titre value for starch hydrolysate.

The sugar content was calculated as

\[
\text{Sugar (g100g}^{-1}) = \frac{10^a \times 50^b \times 100}{T \times 2^c \times 1000}
\]

a—Titre obtained for ferricyanide reagent against Glc. solution.
b—Total volume of hydrolysate.
c—Weight of the sample (g).
T—Titre value for sugar hydrolysate.

Mineral Analysis

Sample Digestion

Five-hundred mg of dried powdered sample was mixed with 10ml of Con. HNO₃, 4 ml of 60% perchloric acid and 1 ml of Con. H₂SO₄ and the contents were kept undisturbed overnight.

After that it was heated on a hot plate containing Con. H₂SO₄ in a beaker until the brown fumes ceased coming out and then allowed to cool. After cooling it was filtered through Whatman No.42 filter paper. After filtration the filtrate was made upto 100 ml with distilled water.

Estimation of Sodium and Potassium

Sodium and Potassium were estimated by using Flame Photometer (Model Elico). The sodium and potassium contents were calculated by
referring to the calibration curves of sodium and potassium respectively and expressed as mg 100 g\(^{-1}\) of powdered samples.

**Estimation of Calcium and Magnesium (Jackson, 1973)**

**Calcium**

Five ml of triple acid digested extract was taken in a china dish. To this 10 ml of 10% (w/v) NaOH and 0.1 g of murexide indicator powder [40 g of potassium sulphate or potassium chloride was added and ground with 10 g ammonium chloride and 0.2 g of murexide (ammonium purpurate)]. The solution was then titrated against 0.02 N versenate (19 g of EDTA was dissolved in 5 litres of distilled water) and standardized against 0.2 N Na\(_2\)CO\(_3\) solutions and adjusted until the colour changed from red to violet.

**Calcium and Magnesium**

Five ml of triple acid digested extract was taken in a china dish. To this 10 ml of ammonium chloride—ammonium hydroxide buffer (pH 10) and a few drops of Erichrome black T indicator (0.1 g of Erichrome black T dissolved in 25 ml of methanol containing 1 g of hydroxylamine hydrochloride) were added and titrated against 0.02 N versenate solution until the colour changed from red to blue.

**Calculation**

Percentage of calcium in the sample = Titre value of calcium \(\times\) 100/5 \(\times\) 100/0.5 \(\times\) 0.0004

Percentage of magnesium = Titre value of calcium + magnesium - Titre value of calcium

or

\[
\text{Titre value of calcium + magnesium} \times 0.96
\]
Calcium and Magnesium contents were expressed as mg 100g⁻¹ of sample.

**Estimation of Phosphorus (Dickman and Bray, 1940)**

One ml of triple acid digested extract was pipetted into 100ml volumetric flask. To this 50 ml of distilled water was added, followed by 5 ml ammonium molybdate-sulphuric acid reagent (Solution A: 25g of ammonium molybdate dissolved in 100ml of distilled water. Solution B: 280 ml of Con. H₂SO₄ diluted to 800 ml. Solution A was added slowly with constant stirring to solution B and the volume was made upto 1000 ml with distilled water). Blue colour developed after adding 6 drops of 2.5% (w/v) stannous chloride solution. The total volume was made upto 100 ml. The intensity of the blue colour was measured at 650 nm in a Spectrophotometer. The phosphorus content present in the sample was calculated by referring to a standard graph of phosphorus using potassium dihydrogen phosphate (KH₂PO₄) as standard and expressed as mg 100g⁻¹ of powdered samples.

**Estimation of Micronutrients by Atomic Absorption Spectrophotometer (Issac and Johnson, 1975)**

By feeding the sample on an Atomic Absorption Spectro-photometer ECIL (Electronic Corporation of India Ltd.) the following elements were estimated with appropriate wavelengths.

<table>
<thead>
<tr>
<th>Name of the Mineral</th>
<th>Wavelength used for estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>248.3 nm</td>
</tr>
<tr>
<td>Copper</td>
<td>324.5 nm</td>
</tr>
<tr>
<td>Zinc</td>
<td>213.9 nm</td>
</tr>
<tr>
<td>Manganese</td>
<td>279.4 nm</td>
</tr>
</tbody>
</table>
The mineral contents were expressed as mg 100g\(^{-1}\) powdered samples.

**In vitro protein digestibility determination (Padmaja, 2001)**

100g of the dried powdered sample was weighed and 10 ml distilled water was added. The sample was allowed to hydrate for 1 hour and equilibrated to pH 8.0 at 37\(^{\circ}\)C. Panzymon-N (Enzyme tablet) was dissolved in 5 ml of 0.02 M sodium phosphate buffer solution (pH 6.9) 1ml of enzyme solution was added and incubated at 37\(^{\circ}\)C for 1 hr. The reaction was stopped by heating to 100\(^{\circ}\)C. Control was run without the sample. The aminoacids released were estimated by Lowry's method (1951).

**Calculation**

The process was standardized using a bovine serum albumin standard and the factor was calculated to be 1mg.

**In vitro** protein digestibility was calculated as

Protein liberated in test sample as a result of enzyme digestion = T x F mg

\[ T = \text{Test OD} - \text{Control OD} \]

\[ F = \text{Factor (1 mg)} \]

**In vitro starch digestibility determination (Padmaja, 2001)**

100 mg of powdered sample was weighed and 10 ml of the buffer (0.02 M Sodium phosphate buffer) was added. The volume was then made upto 20 ml using the buffer after the samples were homogenized over a boiling water bath.

**Colorimetric assay**

To nullify the effect of free reducing sugars, controls were set up 0.2ml of the samples were pipetted out and the volume was made to 1.0 ml
using distilled water. To the remaining sample 0.5 ml of pancreatic amylase was added and incubated at room temperature for 30 seconds. The flasks were placed in a boiling water bath immediately to inactivate the enzyme. On cooling 0.2 ml aliquots from each sample were pipetted out to obtain the test values. The reducing sugars formed by the action of α-amylase on the starch were estimated by Nelson-Somogyi’s method and the absorbance was read at 520 nm.

The *in vitro* starch digestibility was expressed as mg reducing group formed/hr/g starch taken.

**Calculation**

\[
\text{In vitro digestibility units} = T \times 407.33 \text{ units}
\]

where 100 mg of the sample was used

\[
T = \text{Test OD value} - \text{Control OD value}
\]

**Anti-nutritional components**

**Extraction and Estimation of Total free phenolics**

**Extraction (Maxon and Rooney, 1972)**

Five hundred milligram of air-dried powdered sample was taken in a 100 ml flask, to which 50 ml of 1% (v/v) HCl in methanol was added. The samples were shaken on a reciprocating shaker for 24 hrs. at room temperature. The contents were centrifuged at 10,000 x g for 5 minutes. The supernatant was collected separately and used for further analysis.
**Estimation of Total free phenolics (Sadasivam and Manickam, 1996)**

One ml of aliquot of the above extract was pipetted into different test tubes to which 1ml of Folin-ciocalteu’s reagent followed by 2 ml of 20% (w/v) Na$_2$CO$_3$ solution were added and the tubes were shaken and placed in a boiling water bath for exactly 1 min. The test tubes were cooled under running tap water. The resulting blue solution was diluted to 25 ml with distilled water and the absorbance was measured at 650 nm with the help of a Spectrophotometer. If precipitation has occurred, it was removed by centrifugation at 5000 x g for 10 min. before measuring the absorbance. The amount of phenolics present in the sample was determined from a standard curve prepared with catechol. A blank containing all the reagents minus plant extract was used to adjust the absorbance to zero. Average value of triplicate estimation was expressed as g 100g$^{-1}$ of powdered sample on dry weight basis.

**Estimation of Tannins (Burns, 1971)**

From suitable aliquots of the above extract tannin content was quantified by the Vanillin-HCl method of Burns (1971) using phloroglucinol as a standard at 500nm with a Spectrophotometer. The average values of triplicate estimates of all samples were expressed as g 100g$^{-1}$ powdered samples on dry weight basis.
Extraction and Estimation of Hydrogen cyanide (Jackson, 1967)

Extraction

Three gms of air-dried powdered sample was mixed thoroughly with 62.5 ml of distilled water and 3 to 4 drops of chloroform in a distillation flask. The above suspension was steam distilled. The delivery end of the condenser was kept below the surface of 5 ml of 2% KOH solution in a beaker. Approximately 30 ml of distillate was collected and it was used as the source material.

Estimation

Five ml of aliquots of the above extract was pipetted into different test tubes and 5 ml of alkaline picrate solution was added to each test tube. The contents of the test tubes were mixed and digested in boiling water bath for 5 min. and the absorbance was measured at 520 nm against a reagent blank. The Hydrogen cyanide content present in the sample was calculated by referring to a standard graph of potassium cyanide as standard and expressed as mg 100g⁻¹ of powdered samples.

Total oxalate estimation (AOAC, 1984)

One gm dried powdered sample was extracted twice with 0.25 N HCl in a water bath (60°C) for 1 hour each. The centrifugate was collected in a conical flask. This extract was precipitated by adding tungstophosphoric acid kept overnight and centrifuged. Then it was neutralized with 1:1 dil. ammonium solution. It was precipitated overnight by adding 5 ml of acetate buffer with CaCl₂ (pH 4.5). The precipitate was centrifuged and washed
twice with wash liquid (Dilute acetic acid + Calcium oxalate, decanted and filtered).

The precipitate was then dissolved in 15 ml 2 N H₂SO₄ and titrated against 0.01 N KMnO₄ solution at 60°C.

**Calculation**

\[
\text{Total oxalate (\% (dry weight))} = \frac{0.063 \times \text{vol. of 0.01 N KMnO}_4}{\text{Weight of sample (gm)}}
\]

**Trypsin inhibitor assay (Sasikiran and Padmaja, 2003)**

Caseinolytic assay was used for the study of trypsin inhibitor. Three types of assay systems were used. The control system consisted of casein (2%, 2.0ml), trypsin inhibitor extract (0.3 ml) and 1.7ml sodium phosphate buffer (0.01 M, pH 8.0), trypsin (0.2 ml from a 1mg/10ml stock). In the inhibited system, trypsin (0.2 ml) and the inhibitor extract (0.3 ml) were pre-incubated for 30 min. for maximum inhibitor response. To this, casein (2.0 ml) was added followed by 1.5ml buffer. The system was incubated for 15 min. at 30°C and the reaction was stopped by adding 4.0 ml of 10% TCA. The TCA system was kept for one hour to complete protein precipitation and the precipitate was centrifuged off at 1000 x g for 10 min. One ml of the supernatant was used for the quantification of TCA soluble peptide fragments by the method of Lowry et al., (1951).
**Calculation of trypsin inhibitor activity**

A calibration curve was prepared using bovine serum albumin (BSA) as standard. One unit of trypsin activity is defined as one mg peptide fragments released per minute at 30°C under the assay conditions.

Trypsin inhibitor unit (TIU) is defined as the number of trypsin units inhibited and is expressed on a dry weight basis. The effect of free amino acids in the inhibitor was nullified by subtracting the readings of control system from the inhibited system readings.

**Assay of amylase inhibitor (Rekha and Padmaja, 2002)**

The amylase inhibitor activity was studied using 0.5 % soluble starch as substrate. Porcine pancreatic α-amylase (Emerck, Germany) was used as the enzymatic source uniformly throughout the study. Three assay systems were used to quantify the amylase inhibitor activity. The control system consisted of starch (0.5 ml) and 0.02 M sodium phosphate buffer pH 6.9 (2.25 ml). In the uninhibited system, 0.5 ml starch and 2.0 ml buffer were allowed to react with α-amylase (0.25 ml from a solution containing 100 μg porcine pancreatic α-amylase/ ml). After incubation for 10 min. the reaction was stopped by adding 1.0ml of 0.1N HCl. In order to elicit maximum inhibitor response, the inhibitor extract (0.5 ml) was pre-incubated with the buffer (1.5 ml) and α-amylase (0.25 ml) for 30 min. at room temperature (30±2°C). To this inhibited system, 0.5 ml starch was added to initiate enzyme action. After incubation for 10 min, the reaction was terminated by adding 1.0 ml 1 N HCl.

The residual starch in the uninhibited and inhibited system as well as the starch in the control system was quantified by the method of
Mohammed and Sharma (1985) using iodine reagent (0.5 ml of an iodine solution containing 0.2 g iodine crystals and 2g potassium iodide in 100 ml distilled water). The volume was increased to 20ml with distilled water and the absorbance of the blue colour was measured at 620 nm in an ATI-Unicam UV—Vis Spectrophotometer.

**Calculation of amylase inhibitor activity**

A calibration curve was prepared using soluble starch (500-3000 µg), phosphate buffer and iodine reagent.

One α-amylase unit is defined as one µg starch hydrolysed per minute at 30°C under the assay conditions.

One α-amylase inhibitor unit (AIU) is defined as the amount of inhibitor that reduces the α-amylase activity by one unit.

The amount of starch hydrolyzed in the uninhibited system was computed by subtracting the respective readings from the control system readings.

Three replications were maintained for each sample and duplicate analysis was performed for each replicate.

**Treatments/ Processing**

**Soaking**

The tubers were peeled and cut into small pieces and were soaked in distilled water and 0.02% (w/v) sodium bicarbonate (NaHCO₃) solution (pH 8.6) for 3, 6 and 9 hrs. in the ratio of 1:10 (w/v). The water was drained off, and the samples were dried at 55°C.
**Cooking**

Separate batches of the sample were cooked in distilled water (100°C) in the ratio of 1:10 (w/v) for 30, 60 and 90 min. The cooked samples were rinsed and dried at 55°C.

**Autoclaving**

The samples were autoclaved at 15 lb pressure (121°C) in distilled water (1:10 w/v) for 15, 30 and 45 min. The samples were rinsed with distilled water and dried at 55°C.

All the treated as well as raw samples were powdered in a Willey mill to 60 mesh size.

**Statistical analysis**

Proximate analysis, mineral analysis, soluble protein, starch, sugars, and anti-nutritional factors like total free phenolics, tannins, hydrogen cyanide and total oxalate were estimated on triplicate determinations. Estimates of mean and standard error for the above stated parameters were calculated.

Mean was calculated by using the formula

\[
\bar{X} = \frac{\Sigma x}{N}
\]

Where

- \(\bar{X}\) = Mean
- \(\Sigma\) = Summation
- \(x\) = Observations
- \(N\) = Number of observations
Standard error was calculated by using the formula

\[ S.E. = \frac{SD}{\sqrt{N}} \]

Where

\[ S.D. = \text{Estimates of Standard Deviation} \]

\[ N = \text{Number of observations.} \]