CHAPTER II
(Part - A)

NUTRIENT COMPOSITION, OXALATES AND NITRATE IN BRACHIARIA DECUMBENS AND BRACHIARIA DICTYONEURA
The genus *Brachiaria* belongs to tribe: Paniceae. Its species thrive well in tropical and subtropical environment. *B. decumbens* and *B. dictyoneura* are important species for introduction as improved pastures.

*B. decumbens* is stoloniferous. Its growth habit is erect or decumbent type (Sendulsky, 1978). *B. decumbens* is fairly drought resistant and grows well in India. When grazing in association with cattle and goat, sheep preferentially selected *B. decumbens* grass over legume in a mixed sward (Norton, 1984). However, bovine photosensitisation remains a problem in the widely grown *B. decumbens* grass (Garcia et al., 1982). *B. dictyoneura* is rhizomatous and stoloniferous with a rather tufted growth habit (Thomas and Groff, 1986). It is comparatively less popular in the country. Both the pastures grow better under moist conditions. These are grown either under irrigated conditions (in February-March) or under rainfed conditions (at the onset of monsoon) in heavy soil where the moisture holding capacity is high. Planting of rooted slips has been found to be more suitable as compared to seed sowing.

The two *Brachiaria* species were established in March at the Central Research Farm of the Institute. The crops were grown under irrigated conditions using standard agronomic practices. The samples for the study were collected from third regrowth pertaining to July and August. These were
material, dried at 60°C in forced-draft oven. The dried material was ground and stored in plastic bottles for exhaustive chemical analysis in respect of organic nutrients, inorganic constituents and toxic principles. Results on organic nutrients, ash and its fractions, major and trace minerals, and oxalates and nitrate-N of two *Brachiaria* species are shown in Tables 1 to 3.

(i) **Organic nutrients**

a) **Crude protein:**

The crude protein in air-dry grass samples was determined by the estimation of percent nitrogen by Kjeldahl method and multiplying the result by 6.25 (A.O.A.C., 1970). Its content in *B. decumbens* (15.2 percent) was appreciably higher than *B. dictyoneura* (9.1 percent) (Table 1). The recorded observations on crude protein in *B. dictyoneura* were 7.9 and 7.5 percent (Table 1). The former was reported to be the highest among 13 grass species (Arias *et al.*, 1985), while the latter was an average of twelve monthly cuttings (Abate *et al.*, 1984). Thus, *B. decumbens*, at sixty days' regrowth pertaining to monsoon months, excelled in crude protein than reported in literature.

b) **Ether extract:**

The ether extract in grasses was estimated by subje- cting the samples to continuous extraction with petroleum ether for 16 hours in soxhlet ether extraction apparatus (A.O.A.C., 1970). It was low (0.3 and 0.5 percent) in
### Table 1

<table>
<thead>
<tr>
<th>Nutritional parameter (%)</th>
<th>B. decumbens Present study</th>
<th>From literature (1)</th>
<th>(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>15.2</td>
<td>9.1</td>
<td>7.93</td>
</tr>
<tr>
<td>Ether extract</td>
<td>0.8</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>67.1</td>
<td>70.3</td>
<td>76.75</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
<td>43.3</td>
<td>40.0</td>
<td>-</td>
</tr>
<tr>
<td>Acid detergent lignin</td>
<td>4.8</td>
<td>5.9</td>
<td>-</td>
</tr>
<tr>
<td>Cellulose</td>
<td>33.8</td>
<td>31.3</td>
<td>-</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>23.8</td>
<td>30.3</td>
<td>-</td>
</tr>
<tr>
<td>Cellulose + hemicellulose</td>
<td>57.6</td>
<td>61.6</td>
<td>-</td>
</tr>
<tr>
<td>Soluble carbohydrate</td>
<td>14.4</td>
<td>17.5</td>
<td>-</td>
</tr>
</tbody>
</table>

(1) = Arias et al. (1985)
(2) = Abate et al. (1984)
Table 2

Chemical composition of two *Pachydia* species
(Inorganic constituents)

<table>
<thead>
<tr>
<th>Nutritional attribute (%)</th>
<th>B. decumbens</th>
<th>B. dictyoneura</th>
<th>Recommended level*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present study</td>
<td>From literature</td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>7.3</td>
<td>5.5</td>
<td>12.2</td>
</tr>
<tr>
<td>Acid insoluble ash</td>
<td>4.7</td>
<td>2.8</td>
<td>-</td>
</tr>
<tr>
<td>Acid soluble ash</td>
<td>2.6</td>
<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.4</td>
<td>0.6</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.23</td>
<td>0.22</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.18</td>
<td>0.2</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.11</td>
<td>0.31</td>
</tr>
<tr>
<td>Potassium</td>
<td>1.0</td>
<td>0.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Sodium</td>
<td>trace</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>Iron (ppm)</td>
<td>52</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Zinc (ppm)</td>
<td>32</td>
<td>26</td>
<td>20 to 40</td>
</tr>
<tr>
<td>Copper (ppm)</td>
<td>11</td>
<td>13</td>
<td>10</td>
</tr>
</tbody>
</table>

(1) = Arias *et al.* (1985)
(2) = Abate *et al.* (1984)

* = in the rations of finishing cattle weighing 300 to 500 kg (reproduced from Minson, 1981).
Table 3
Oxalates and nitrate - N content in two *Brachiaria* species

<table>
<thead>
<tr>
<th>Toxic principle (%)</th>
<th><em>B. decumbens</em></th>
<th><em>B. dictyoneura</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total oxalate</td>
<td>2.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Soluble oxalate</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Insoluble oxalate</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Nitrate - N (ppm)</td>
<td>3.3</td>
<td>4.0</td>
</tr>
</tbody>
</table>
c) **Cell wall and its fractions:**

These were determined as per the methods outlined by Goering and Van Soest (1970). The estimation of cell-wall or neutral detergent fibre consisted in boiling under reflux sample of plant material in neutral detergent solution for one hour and then weighing the insolubles after washing and drying. The cell-wall fractions, viz., acid detergent fibre, acid detergent lignin, cellulose and hemicellulose, were subsequently determined. The sample was boiled in acid detergent solution under reflux for one hour and filtered to recover the residue as acid detergent fibre. It was digested in 72 percent sulphuric acid at 15°C for 3 hr. The organic matter from the insoluble material is referred as acid detergent lignin. The difference between acid detergent fibre and acid detergent lignin + silica is the cellulose content of plant material. The difference between neutral detergent fibre and acid detergent fibre determines the hemicellulose content. The results on neutral detergent fibre showed that it was lower (67.1 and 70.3 percent) in the two grasses than recorded in *P. dictyoneura* (76.8 percent) by Arias et al. (1985) (Table 1). The acid detergent fibre (43.3 percent) and cellulose (33.8 percent) contents were higher and hemicellulose (23.8 percent; appreciably lower in *P. decumbens* than
B. dictyoneura. The acid detergent lignin (4.8 and 5.9 percent) was low and comprised of about 8 to 10 percent of cellulose + hemicellulose content in the grasses. Thus, the two pastures appeared to be nutritionally superior in respect of cell-wall and its fractions, as the nutritive availability of cellulose and hemicellulose is influenced to a considerable extent by the proportion of lignin present.

d) **Soluble carbohydrate:**

The residual forage dry matter after subtraction of neutral detergent fibre is termed as neutral detergent solubles. These mostly consist of cellular contents (starch, sugars, proteins, lipids, soluble ash, etc.) and are highly digestible. Soluble carbohydrate content in grass samples was like-wise computed by subtracting the sum of percentages of crude protein, ether extract and soluble ash from percent neutral detergent solubles (Harris, 1970). It was observed to be 14.4 and 17.5 percent in B. decumbens and B. dictyoneura respectively (Table 1).

Thus, based on organic nutrients, it can be stated that both the grasses were fairly nutritious and B. decumbens was additionally richer in crude protein. The crude protein and cellulose content in B. decumbens was comparable to B. ruziziensis (crude protein = 14.8 to 15.2 percent; cellulose 36.5 to 36.8 percent), when grown on a fertilised soil (Michel, 1960).
(ii) **Ash and its fractions:**

The ash content in grasses was determined by ignition of 3 g sample at 600°C until all carbon was removed. The residue is ash and is taken to represent the inorganic constituents of the sample and silica. The inorganic constituents and silica were fractionated by treatment of ash with dilute hydrochloric acid. The minerals form water-soluble chlorides and go in solution. Silica, either originating from the plant or due to soil contamination, is referable as acid insoluble ash (A.C.A.C., 1970). The ash content was considerably low (7.3 and 5.5 percent) in *B. decumbens* and *B. dictyoneura* in comparison to the value reported for the latter grass (12.2 percent) in literature (Table 2). The acid insoluble ash was higher (4.7 percent) in *B. decumbens* than *B. dictyoneura* (2.8 percent) and acid soluble ash (2.6 and 2.7 percent) was similar.

(iii) **Major and trace minerals:**

The samples were prepared for analysis of major minerals (percent calcium, potassium, sodium and phosphorus) by wet digestion method. Calcium, potassium and sodium were determined with the help of 'Elico' Digital Flamephotometer (Model CL 22 D). Phosphorus was estimated using ammonium metavanadate yellow colour method (Jackson, 1958). Sodium was present in traces and thus its concentration has not been reported. The calcium, phosphorus and potassium contents varied respectively from 0.4 to 0.6, 0.18 to 0.20 and 0.9 to 1.0 percent in the two grasses (Table 2). The trace minerals
(iron, zinc and copper) were estimated using Atomic Absorption Spectrophotometer model Varian Techtron, AA-120. The iron content in *B. decumbens* (52 ppm) was lower than *B. dictyoneura* (60 ppm). The concentration of zinc (26 to 32 ppm) and copper (11 to 13 ppm) varied within narrow limits in the two grasses (Table 2).

Recommended levels of major and trace minerals in the rations of finishing cattle weighing 300 to 500 kg are also shown in Table 2. A comparison of results of present study with those meant for finishing cattle revealed that these were higher in the case of calcium (0.4 to 0.6 versus 0.22 to 0.35 percent), potassium (0.9 to 1.0 versus 0.31 to 0.44 percent) and iron (52 to 60 versus 30 ppm); adequate in respect of phosphorus (0.18 to 0.20 versus 0.22 percent), zinc (26 to 32 versus 20-40 ppm) and copper (11 to 13 versus 10 ppm), and deficient in sodium (trace versus 0.05 percent) in the two grasses. Higher accumulation of calcium in *B. decumbens* and *B. dictyoneura* seemed possible as a concentration as high as 5.67 percent calcium was noted in *B. ruziziensis*, when grown on a fertilised soil (Michel, 1960). More (1982) stated that 3 percent potassium in dry forage had no adverse effect on grazing sheep. Thus, it can be stated that major and trace minerals in both the grasses were present in adequate amounts, no ill-effect was expected due to higher ingestion of potassium, and deficiency of sodium, which was present in traces, could be made good by feeding supplemental common salt to animals.
(iv) Oxalates and nitrate-N
   a) Oxalates:

   Water-soluble and total oxalates in samples were determined by the method described by Baker (1952). The oxalates in plant were converted into oxalic acid by hydrolysis with dilute hydrochloric acid. After de-proteination of the extract and removal of impurities, the oxalic acid was precipitated with calcium chloride buffer and its concentration was determined by titration with standard potassium permanganate solution. It will be seen from Table 3 that concentration of total and soluble oxalates (2.3 to 2.6 percent) was more or less similar in both the grasses. A level of two percent oxalate was expected to be well tolerated by animals. However, the total and soluble oxalates reported for Bracharia species ranged from 1.1 to 1.4 and 0.47 to 0.60 percent respectively (Mathews and Sutherland, 1952; Sood and Chopra, 1973; Ndyanabo, 1974). Oxalate is accumulated as secondary metabolite during metabolism and growth of the plant. It can be present in higher amounts in situations, where the uptake and synthesis of desirable nutrients is comparatively more. This seems to have occurred in the present study. Additional feed calcium counteracts the toxic effects of oxalate. In this connection, the calcium content in both the grasses was comparatively higher than what was reported for B. dictyoneura by Arias et al., (1985) or recommended for meeting the nutritional needs of actively fatten ing (finishing) cattle (Table 2). The insoluble oxalate was present in negligible amounts (0.2 to 0.3 percent).
Nitrate-N in samples was determined using the procedure of Peach and Tracey (1955). In it, nitrate, after removal of chlorides, was extracted in aqueous medium, which was further freed from impurities. The yellow colour due to nitrate was developed with 2,4 phenyl disulphonic acid in alkaline medium and compared with that of standard (prepared using KN03) at 420 μm.

It was present in traces. It was determined in terms of ppm. The concentration of nitrate-N in the two grasses ranged between 3.3 to 4.0 ppm (Table 3). The toxic level has been opined to be 0.2 percent in forage dry matter (Prasad, 1933). Nitrate toxicity on ingestion of these pastures was thus not anticipated.

The results of above investigation led to the conclusion that monsoon regrowths of *B. decumbens* and *B. dictyoneura* were fairly nutritious in respect of organic nutrients and major and trace minerals. *B. decumbens* was rich in crude protein. The toxicity, if any, due to higher ingestion of oxalates was likely to be mitigated by the presence of adequate amounts of calcium in both the pastures.


**EXPERIMENTAL**

*Brachiaria decumbens* and *Brachiaria dictyoneura* species are suitable for introduction as improved pasture grasses in tropical grasslands. *B. decumbens* belongs to stoloniferous species. Its growth habit is erect or decumbent type. *B. dictyoneura* is rhizomatous and stoloniferous with a rather tufted growth habit. The samples for the study were obtained from the Central Research Farm of the Indian Grassland and Fodder Research Institute, Jhansi.

**Dry matter and organic constituents**

(i) **Dry matter:**

Weighed 2.0 g ground sample and dried in electric oven at 102°C overnight. Dry matter in fresh forage was determined by quick drying of about 100 g sample at 60°C in forced-air-circulation type oven to constant weight.

(ii) **Crude protein:**

One g air-dry sample was digested with 25 ml concentrated sulphuric acid containing 10 g catalyst (7 percent copper sulphate - potassium sulphate). After addition of excess sodium hydroxide solution, distilled about two-thirds of contents of the flask in 25 ml four percent boric acid containing few drops of mixed indicator (0.1 percent methyl red and 0.2 percent bromocresol green in alcohol). The ammonia collected in distillate was titrated with 0.1N standard sulphuric acid to faint purple end point. Percent
crude protein was determined by multiplying the N content of the sample by 6.25.

(iii) **Ether extract:**

Introduced 1.5 g dried plant sample into clean alundum extraction thimble and extracted with petroleum ether in 'Goldfisch' soxhlet extraction apparatus for 16 hours. Dried the ether extract at 105°C for 30 min before weighing.

(iv) **Cell-wall and its fractions:**

a) **Cell-wall (Neutral detergent fibre):**

One g sample was refluxed with 100 ml neutral detergent solution (30 g sodium lauryl sulphate, USP; 18.61 g disodium dihydrogen ethylenediamine tetra-acetate dihydrate, L.R.; 4.56 g disodium hydrogen phosphate, L.R.; 6.31 g sodium borate decahydrate, L.R.; and 10 ml 2-ethoxy-ethanol, purified, in one lit distilled water), 2 ml decahydronapthalene and 0.5 g sodium sulphite for 60 min. The contents were then filtered through 2G1 sintered glass crucible applying mild suction as and when needed. The residue after washing is neutral detergent fibre. The crucible containing the fibre was dried at 105°C overnight and weighed.

b) **Acid detergent fibre:**

Refluxed 1 g sample with 100 ml acid detergent solution (20 g cetyl trimethyl ammonium bromide in one lit 1 N sulphuric acid) and 2 ml decahydronaphthalene for one hr.
Filtered on previously tared 2G1 sintered glass crucible using light suction. The acid detergent fibre was collected as residue in the crucible. Washed the residue and dried at 105°C for 8 hr and weighed.

c) Acid detergent lignin:
Covered the acid detergent fibre in crucible with cooled (5°C) 72 percent (12 moles) sulphuric acid and stirred with glass rod to smooth paste. The acid was allowed to slowly drain away. The crucible was refilled with 72 percent sulphuric acid and stirred at hourly intervals. The above treatment lasted for 3 hr. The residue was washed acid-free. Dried the crucible at 100°C overnight and weighed. Ignited the residue in muffle furnace at 500°C for 3 hr and weighed.

d) Cellulose:
It was estimated by subtracting the acid detergent lignin + silica content from percent acid detergent fibre in the sample.

e) Hemicellulose:
The concentration of hemicellulose was obtained by subtracting the acid detergent fibre content from percent neutral detergent fibre in the sample.

f) Cellulose + hemicellulose:
The value of cellulose + hemicellulose was derived by addition of cellulose and hemicellulose contents.
(v) **Soluble carbohydrate:**

The soluble carbohydrate in the sample was calculated by difference as follows:

\[
\text{Percent soluble} = 100 - (\text{percent cell-wall} + \text{percent crude protein} + \text{percent ether extract} + \text{percent soluble ash}).
\]

**Inorganic constituents**

(i) **Ash and its fractions:**

a) **Ash:**

Three g sample was ashed in muffle furnace at 600°C for four hours and weighed.

b) **Acid insoluble ash:**

After moistening the ash with distilled water and addition of 5 ml concentrated hydrochloric acid, the contents were boiled and transferred to 250 ml beaker. 5 ml concentrated hydrochloric acid was further added and solution evaporated to half its volume. After filtration and washing, the residue was acid insoluble ash. The filter paper along with residue was transferred to vitrēsil basin for drying and igniting at 550°C in muffle furnace for one hr. It was cooled in desiccator and weighed.

c) **Acid soluble ash:**

Acid soluble ash was computed by subtracting the acid insoluble ash content from percent ash in the sample.
(ii) **Major and trace minerals:**

**Wet digestion:**

To 1 g sample, added 15 ml tri-acid (concentrated nitric acid: sulphuric acid: 60 percent perchloric acid in the ratio of 9:2:2). Digested on hot plate. It took nearly four hours and 2-3 ml acid remained. It was diluted to 100 ml with 6 N hydrochloric acid.

a) **Calcium, potassium and sodium:**

Took 2.5 ml diluted acid extract in 25 ml volumetric flask, added 1 drop isopropyl alcohol and made up the volume. The 'Elco' Digital Flame Photometer (Model CL 22 D) was used for the estimation of calcium, potassium and sodium. The flame photometer was set at 100 using standard solution of the mineral of known strength (usually 25 ppm). The solutions of unknowns were then subjected to flame test and readings recorded using separate optical filters for calcium (623 nm wave length), potassium (766 nm wave length) and sodium (589 nm wave length). Standard solutions (1 ml = 1000 μg) of calcium, potassium and sodium were prepared by dissolving 2.5 g calcium carbonate (firstly in 5 ml concentrated nitric acid), 1.9069 g potassium chloride and 2.5419 g sodium chloride in one lit distilled water respectively.

b) **Phosphorus:**

One ml diluted acid extract was introduced in 25 ml volumetric flask and heated on hot plate to remove hydrochloric acid. The solution became colourless. It was cooled and diluted with 10 ml distilled water. Two ml Barton's
reagent was thereafter added and volume made up to mark with distilled water. The transmittance reading was recorded after 10 min in Spectronic 20 Bausch and Lomb colorimeter using blue filter (430 m/). The phosphorus content was calculated using standard curve, prepared from potassium dihydrogen phosphate solutions of varying strengths.

Barton's reagent:
Solution A : Dissolved 25 g ammonium molybdate in 400 ml distilled water

Solution B : Dissolved 1.25 g ammonium metavanadate in 300 ml boiling water. In it, 250 ml concentrated nitric acid added after cooling. Finally, solution A added in solution B and mixture diluted to one litre.

Wet digestion for analysis of trace minerals:

One g sample was digested with 20 ml digestion acid. The digestion acid comprised of concentrated nitric acid + sulphuric acid + 60 percent perchloric acid (5:2:1). Before digestion, the contents were left over-night. The digestion was continued till the digest became clear and further evaporation of acid resulted in formation dense white fumes within the flask. The contents were filtered in 50 ml volumetric flask through acid-washed filter paper using all-glass distilled water and volume made up to mark.

Iron, Zinc and Copper:

The diluted acid extract was used to determine the concentration (in ppm) of trace minerals (iron, zinc and copper)
in the sample using Atomic Absorption Spectrophotometer, model Varian Techtron, AA-120.

**Toxic principles (oxalates and nitrate-N):**

(i) **Oxalates:**

a) **Total oxalate:**

Boiled 2 g air-dry plant sample with ninety ml 1 N hydrochloric acid for 15 min under reflux. Transferred to 100 ml volumetric flask after cooling, and filtered on a dry filter paper after making up the volume with distilled water. To 12.5 ml aliquot in centrifuge tube, added 2.5 ml phosphoric acid-tungstate reagent (prepared by dissolving 24 g sodium tungstate in water, adding 40 ml syrupy phosphoric acid (Sp. Gr. 1.75) and diluting to one lit). The contents were mixed and kept for 5 hr. Centrifuged for 10 min at 3000 rpm (radius 15 cm). Transferred 10 ml supernatant in another centrifuge tube, added ammonia (Sp. Gr. 0.83) drop-wise till alkaline, followed by 3 ml calcium chloride buffer (containing 25 g anhydrous calcium chloride in 500 ml fifty percent (v/v) glacial acetic acid + 330 g sodium acetate in 500 ml water, pH: 4.5). Mixed the contents and kept over-night in refrigerator. Centrifuged for 15 min, carefully removed the supernatant and washed the loosened precipitate twice with 20 ml filtered wash solution (prepared by keeping dilute glacial acetic acid (5 percent, v/v) over calcium oxalate at room temperature and mixing periodically). After carefully removing the washings through centrifuging,
dissolved the precipitate in 2.5 ml ten percent sulphuric acid and titrated against standard (N/50) potassium permanganate solution to determine the oxalic acid content in the sample.

\[ 1 \text{ ml} \ 0.02 \text{ N KMnO}_4 = 0.0009 \text{ g oxalic acid (anhydrous)} \]

b) **Soluble oxalate:**

Two g air-dry plant sample was boiled with 90 ml distilled water for 15 min under reflux. The contents were cooled, diluted to 100 ml with water and mixed. Filtered using Whatman filter paper No. 40. Took 12.5 ml aliquot in a centrifuge tube, added 1.25 ml fifty percent concentrated hydrochloric acid and after mixing further added 1.25 ml phosphoric acid - tungstate reagent. The contents were thoroughly mixed and kept for 5 hr. The determination was completed as per the method described for total oxalate.

c) **Insoluble oxalate:**

The insoluble oxalate was determined by subtracting the soluble oxalate content from percent total oxalate in the sample.

(ii) **Nitrate-N:**

Reagents:

1) Copper sulphate solution-dissolved 5 g CuSO\(_4\cdot5\)H\(_2\)O in water and volume made upto 1 lit.

2) Silver sulphate solution - dissolved 3.5 g silver sulphate in 1 lit distilled water by heating.
3) Disodium hydrogen phosphate solution - dissolved 138 g Na₂HPO₄ in 500 ml water. Added strong sodium hydroxide solution to bring pH to 6.5 and volume made up to 1 lit.

4) Calcium hydroxide - magnesium carbonate mixture - mixed thoroughly 1 part Ca (OH)₂ with 2 parts MgCO₃.

5) 2:4 phenyl disulphonic acid reagent: DH

6) Standard potassium nitrate solution - dissolved 0.7221 g pure KNO₃ in 1 lit water (strength: 0.1 mg N/ml or 100 ppm N/ml) and stored as stock solution.

Method:

To 100 mg ground sample, added 8 ml silver sulphate solution and swirled around in 100 ml beaker. Immediately added exactly 1 ml sodium phosphate solution and allowed to stand for 2 hr. Filtered through Whatman Filter paper No.42. Took 2 ml extract in 15 ml centrifuge tube, added 2 ml copper sulphate solution and 2 ml water. Introduced 0.5 g calcium hydroxide-magnesium carbonate mixture. Allowed to stand for 1 hr, centrifuged the contents. Measured 2 ml supernatant into 25 ml beaker and evaporated to dryness on water bath. Cooled and quickly added 1 ml 2:4 phenyl disulphonic acid, making certain that all the residue comes into immediate contact with the acid. Allowed to stand for 10 min. Added 10 ml water and then excess 50 per cent ammonium hydroxide solution. Made up the volume to 25 ml. Compared colour with that of standard at 420 m/μ (standards were prepared in
centrifuge tubes with 0, 1, 2 and 3 ml stock solution, making up its volume up to 4 ml, adding 2 ml copper sulphate solution and calcium hydroxide - magnesium carbonate mixture and proceeding as with unknown solution described above).
CHAPTER II

(Part - B)

CHEMICAL CONSTITUENTS IN
BRACHIARIA DECUMBENS AND
BRACHIARIA DICTYONEURA
(a) **Chemical constituents in *B. decumbens* Stapf.**

Shade-dried 1.5 kg ground leaves of *B. decumbens* grass were extracted with ethanol (95 percent). The combined extract was separated into hexane, benzene and ethyl acetate soluble fractions. Each fraction was investigated separately.

1. **Hexane SULuble Fraction:**

   The hexane soluble fraction was dark green and syrupy in nature. It was column chromatographed over Brockmann alumina, neutral, and eluted with solvents of increasing polarity and their mixtures, resulting into two crystalline compounds (A and B).

**Compound A:**

Eluants (hexane-benzene, 50:50 and 20:30) of similar Rf value on TLC were mixed and after evaporation of solvent the residue crystallised from acetone as white shining flakes (104 mg), TLC homogeneous (Rf 0.53, solvent system, benzene-ethyl acetate, 5:95). It melted at 80° and analysed for $C_{26}H_{38}O$, M+ 382 (EIMS).

\[
\text{IR} \quad \text{KBr} \quad \max \quad 3345, 2855, 2805, 1050, 740, 715 \quad \text{and} \quad 705 \text{ cm}^{-1}.
\]

**Acetylation of compound A:**

On acetylation (50 ml compound A + pyridine and acetic anhydride 1.5 ml each; steam bath, 3 hr; usual work up; crystallisation from methanol), it yielded a
a crystalline derivative, m.p. 65°, analysed for C_{28}H_{56}O_{2}, M^+424 (EIMS).

\[ \text{IR} \quad \text{KBr} \quad \text{max} \quad 2925, 2835, 1735, 1450, 1365, 1255 \]

and 725 cm\(^{-1}\).

The above physico-chemical data of compound A and its acetate showed it to be hexacosanol (Sharma and Gupta, 1983). It was further confirmed through co-tlc, mixed m.p. and super-imposable IR with authentic sample.

\[
\text{CH}_3 \cdot \left[\text{CH}_2\right]_{24} \text{CH}_2 \text{OH}
\]

**COMPOUND A**

**Compound B:**

The eluants obtained from solvent systems benzene, benzene-ethyl acetate, 80:20 and 50:50 and ethyl acetate (fractions No. 19-24, 25-30, 31-34 and 45-50 respectively) were of similar Rf value on TLC and therefore combined and solvent evaporated. The residue after usual work up gave crystalline compound (193 ml), TLC homogeneous (Rf 0.61, solvent system, benzene-ethyl acetate, 70:30). It melted
at 136-37°, analysed for C_{29}H_{50}O, M^{+}414 (SIMS). It gave bluish green colour in Liebermann-Birchard reaction (Steiner and Holtzen, 1955) for steroidal moiety and was positive for tetranitromethane test (Fieser and Fieser, 1966).

\[ \text{IR (KBr)} \]
\[ \text{max} \quad 3390, 2915, 1635, 1455, 1360, 1045, 955 \text{ and } 810 \text{ cm}^{-1}. \]

**Acetylation of compound B:**

100 mg compound on treatment with 1 ml pyridine + 3 ml acetic anhydride, keeping overnight, isolation by normal procedure and crystallisation from methanol, yielded acetate, m.p. 132°, molecular formula, C_{31}H_{52}O_{2}, M^{+}456 (SIMS).

\[ \text{H NMR (CDCl}_{3}) \]
\[ \delta 5.20 \text{ (m, 1H, } -\text{C=OH}), \delta 4.40 \text{ (m, 1H, CHOOAc)}, \delta 1.95 \text{ (s, 3H, } -\text{OOCH}_{3}), \]
\[ \delta 0.65-1.20 \text{ (ov.ripping s, 15H, 6 x CH}_{3}. \]

The chemical shifts obtained from \(^1\text{H NMR spectrum of acetyl derivative of compound B were identical with } \beta\text{-sitosterol acetate. The compound B was thus identified as } \beta\text{-sitosterol (Jain and Gupta, 1981) on the basis of mixed m.p., co-tlc and superimposable spectral data with authentic sample.}
2. **Benzene Soluble Fraction:**

The benzene soluble fraction was a brown sticky mass and showed three spots on TLC examination, using \( C_{6}H_{2}MeOH, 80:20 \), as the solvent system and \( I_{2} \) vapours as visualising agent. It was chromatographed over silica gel (60-120 mesh), column, and eluted with mixtures of benzene and ethyl acetate. It yielded two compounds (C and D).

**Compound C:**

(Fractions 4-11). The residue gave colourless crystalline compound (230 mg) on repeated crystallisations from methanol, m.p. 300°. It analysed for \( C_{35}H_{60}O_{6}, M^{+} 576 \) (EIMS). The compound gave positive \( \text{Liebermann-Burchard} \), \( \text{Feigl (1954)} \) and \( \text{Molisch's tests} \).

\[
\text{IR}^{\text{max}} \text{ KBr} \quad 3415, 2960, 2840, 1460, 1380, 1350, 1265, 1100, 1075, 1020, 965, 870 \text{ and } 785 \text{ cm}^{-1}.
\]
Acetylation of compound C:

(50 mg compound C + pyridine and acetic anhydride
1.5 ml each, oil bath, 120°, 4 hr, crystallised from
methanol after normal work up), m.p. 168-70°, analysed for
C_{43}H_{68}O_{10}, M^{+}744 (EIMS). The peaks at m/z 169 and 331 in
the EIMS were typical of acetylated glucose (Budzikiewicz
et al., 1967).

\[ \text{IR} \]

\[ \text{max} \] 2965, 2845, 1735, 1245, 1215, 1165,
1100, 1075 and 870 cm\(^{-1}\).

\[ ^1H \text{NMR} \]

\( \text{CDCl}_3 \)

\[ S \ 4.5-1.5 \ (s, 18H, 6 \times \text{CH}_3), S 1.94, 1.95, 
1.97, 1.99 \ (s, 3H, 4 \times\text{OCH}_3), 
S 3.58 \ (m, 1H, -\text{CDOH}), S 4.1 \ (m, 1H, 
C-1'H), S 4.5 \ (m, 1H, C-2'H), 
S 4.9-5.1 \ (m, 4H, C-3'\text{H}, C-4'\text{H}, 
C-5'\text{H} \text{and} C-6'\text{H}) \text{and} S 5.25 \ (m, 1H, 
-C=\text{CH}-\text{CH}_2^-). \]

Acid hydrolysis of compound C:

Refluxing 50 mg compound C in 150 ml rh flask with
5 percent ethanolic H\(_2\)SO\(_4\), steam bath, 6 hr; distilling off
ethanol; diluting the contents with water; extraction with
ethyl acetate; crystallisation from benzene-methanol (1:1)
gave an aglycone, m.p. 136°, analysed for C\(_{29}\)H\(_{50}\)O, M\(^+\)414
(EIMS).

The aqueous portion of the hydrolysate was neutralised
with BaSO\(_3\) and BaSO\(_4\) was filtered off. Upon concen-
tration, the filtrate was positive for sugar by chemical
tests and showed single spot identical with D-glucose by co-paper chromatography (Rf 0.19, n-butanol-acetic acid-water, 4:1:5, v/v, upper).

**Quantitative estimation of sugar in compound C:**

Quantitative estimation of sugar in compound C was carried out according to Mishra and Hao (1960). It showed that the compound in its molecular form contained one unit of D-glucose.

**Enzymatic hydrolysis of compound C:**

The enzymatic hydrolysis of compound C in alcoholic medium with emulsin liberated the aglycone and D-glucose. It indicated β-linkage between the non-sugar residue and D-glucose.

The aglycone was identified as β-sitosterol by comparison (mixed m.p., TLC and spectral data) with an authentic sample. Thus, on the basis of above facts, the compound C was characterised as β-sitosterol - β-D-glucoside (Swift, 1952; Kind and Celentano, 1953).
Compound C:

The fractions (16-24) obtained from eluant benzene-ethyl acetate, 30:70, were of same Rf value on TLC. These were mixed, crystallised from chloroform-methanol as yellow crystalline solid (134 mg), m.p. 145°, analysed for C_{11}H_{12}O_{4}, M^+ 208 (EI MS). The colour reactions with NaOH (yellow) and FeCl₃ (green) indicated its phenolic nature.

UV \( \lambda_{\text{max}} \) MeOH: 244, 292 and 346 nm.
\textbf{Acetylation of compound D:}

The acetylation of compound D (by refluxing 50 mg compound + 1 ml pyridine + 2 ml acetic anhydride; 100°; 5 hr; crystallised from hexane after usual work up) yielded a derivative, m.p., 72°, analysed for C_{15}H_{16}O_{6}, M^{+} 292 (EIMS).

\begin{align*}
\text{IR} & \quad \text{KBr} \\
\text{max} & \quad 3400, 3100, 1675, 1605, 1555, 1435, \\
& \quad 1410, 1320, 1280, 1175, 1165, 1125, \\
& \quad 1055, 985, 870, 775, 735 and 685 \text{ cm}^{-1}.
\end{align*}

\textbf{Acid hydrolysis of compound D:}

The compound on acid hydrolysis (50 mg compound + 5 ml seven percent H_{2}SO_{4}, refluxed, boiling water bath, 30 min; extracted with ether; crystallised from aqueous methanol) gave yellow crystalline compound, m.p. 201°, analysed for C_{9}H_{8}O_{4}, M^{+} 180 (EIMS). It gave effervescence with NaHCO_{3} and green colour with FeCl_{3}.

\begin{align*}
\text{IR} & \quad \text{KBr} \\
\text{max} & \quad 3435, 1630, 1440, 1200, 1170, 1110, \\
& \quad 975, 905, 855 and 810 \text{ cm}^{-1}.
\end{align*}

The hydrolysed compound formed an acetate (crystallised from acetone), m.p. 198°, analysed for C_{13}H_{12}O_{6}, M^{+} 264 (EIMS). Its identity as caffeic acid was established by mixed m.p., co-tlc and superimposable spectral data with authentic sample.

On the basis of above physico-chemical data, the compound D was identified as ethyl caffeate (Heilbron et al., 1965). The leaves on extraction with methanol and similar
processing also yielded ethyl caffeate indicating it to be originally present as ethyl ester.

\[
\begin{align*}
\text{COMPOUND D}
\end{align*}
\]

3. **ETHYL ACETATE SOLUBLE FRACTION:**

The ethyl acetate soluble fraction was freed from organic solvent under reduced pressure. It showed yellow colour in dilute NaOH, red colour with Mg+HCl in alcoholic medium and was positive for Feigel test. These tests indicated the presence of flavonol glycoside. It was isolated by first precipitating as lead salt and then decomposing it by H₂S. It was crystallised as yellow compound (Compound D) from methanol (277 mg), m.p. 222-225°C, analysed for C₂₁H₂₀O₁₁, M⁺ 448 (EI-MS).
UV  
\[ \text{H}_2\text{O} \]
252, 270 and 345 nm.
\[ \text{NaOMe} \]
275, 339 (sh) and 408 nm.
\[ \text{AlCl}_3 \]
270, 300 (sh), 334 and 422 nm.
\[ \text{AlCl}_3/\text{HCl} \]
258 (sh), 274, 300 (sh), 350 and 395 nm.
\[ \text{NaOAc} \]
270, 280, 364 and 424 nm.
\[ \text{NaOAc/H}_3\text{BO}_3 \]
262, 293 (sh) and 370 nm.

IR  
\[ \text{KBr} \]
\[ \text{max} \]
3300, 2910, 1640, 1600, 1500, 1280, 1215, 1150 and 800 cm\(^{-1}\).

The bathochromic shift of 25 nm in band I with addition of sodium acetate in presence of boric acid (relative to methanol spectrum) suggested the presence of ortho-dihydroxyl group in the flavone nucleus. Its confirmatory evidence was provided by the bathochromic shift of 34 nm in band I with AlCl\(_3\) (relative to AlCl\(_3/\)HCl spectrum). The presence of free C-4' hydroxyl group in the molecule was indicated by the bathochromic shift of 63 nm in band Ia with addition of sodium methoxide (relative to band I of methanol spectrum). Further, a 50 nm bathochromic shift observed in band Ia with AlCl\(_3/\)HCl indicated the presence of free hydroxyl groups at C-3 and C-5 positions. The intense peak at 3300 cm\(^{-1}\) in the IR spectrum also suggested the presence of -OH groups. Thus the spectral data showed that free hydroxyls were present at C-3, C-5, C-3' and C-4' positions in the molecule.
Acid hydrolysis of compound \( B \):

On acid hydrolysis (75 mg compound + 20 ml six percent methanolic HCl, refluxed, steam bath, 4 hr; reaction mixture freed from methanol; diluted; extracted with ethyl acetate; aqueous layer preserved for separate analysis), the compound \( B \) furnished an aglycone, crystallised from acetone-methanol (1:1) as light yellow crystals, m.p. 314-15\(^\circ\), analysed for \( \text{C}_{15}\text{H}_{10}\text{O}_{7}, M^+302 \, (\text{WIMS}) \) and was positive for Shinoda test.

\[
\begin{align*}
\text{UV} & \quad \lambda_{\text{KOH}} \quad 254, 266, 300 \, (\text{sh}) \, \text{and} \, 368 \, \text{nm}.
\lambda_{\text{NaOAc}} & \quad 245 \, (\text{sh}) \, \text{and} \, 320 \, \text{dec. nm}.
\lambda_{\text{AlCl}_3} & \quad 274, 306 \, (\text{sh}), \, 335 \, \text{and} \, 453 \, \text{nm}.
\lambda_{\text{AlCl}_3/\text{HCl}} & \quad 258, 304 \, (\text{sh}), \, 359 \, \text{and} \, 427 \, \text{nm}.
\lambda_{\text{NaOAc}} & \quad 259, 272, 327 \, \text{and} \, 338 \, \text{dec. nm}.
\lambda_{\text{NaOAc/H}_3\text{PO}_4} & \quad 264, 305 \, (\text{sh}) \, \text{and} \, 390 \, \text{nm}.
\end{align*}
\]

\[
\begin{align*}
\text{IR} & \quad \tilde{\lambda}_{\text{B R max}} \quad 3335, 1650, 1610, 1495, 1445, 1350,
1260, 1190, 1120, 1035, 1015, 905, 845 \, \text{and} \, 750 \, \text{cm}^{-1}.
\end{align*}
\]

\[
\begin{align*}
\text{H NMR} & \quad (\text{Acetone-d}_6) \quad \delta 7.65 \,(J = 2.5 \, \text{Hz}, \, \delta, \, 1 \text{H}, \, \text{H-2'},
\delta 7.54 \,(J = 9.0 \, \text{Hz} \, \text{and} \, 2.5 \, \text{Hz}, \, \text{dd}, \, 1 \text{H}, \, \text{H-6'}),
\delta 6.83 \,(J = 9.0 \, \text{Hz}, \, \delta, \, 1 \text{H}, \, \text{H-5'}),
\delta 6.42 \,(J = 2.5 \, \text{Hz}, \, \delta, \, 1 \text{H}, \, \text{H-8}) \, \text{and}
\delta 6.19 \,(J = 2.5 \, \text{Hz}, \, \delta, \, 1 \text{H}, \, \text{H-6}).
\end{align*}
\]

The UV spectrum showed absorption maxima at 368, 300 (sh), 266 and 254 nm similar to flavonols (Mabry et al., 1970). A bathochromic shift of 85 nm in band I a with
AlCl₃ and its reduction in AlCl₃/HCl to 59 nm (both relative to band I in MeOH) were indicative of C-3 and C-5 hydroxyls along with an ortho-dihydroxyl function in the molecule. In presence of sodium acetate, a 6 nm bathochromic shift of band II was caused by C-7 hydroxy function. Thus, it appears that the -OH group at C-7 position was rendered free by acid hydrolysis.

The signals in ¹H NMR spectrum suggested that the aromatic protons of ring A appeared at 8 6.19 and 6.42 (2H, AB system, J = 2.5 Hz, H-6 and H-8) and of ring B at 8 6.83 (1H, J = 9.0 Hz, H-5'), 8 7.54 (1H, J = 9.0 Hz and 2.5 Hz, H-6') and 8 7.65 (1H, J = 2.5 Hz, H-2').

On the basis of above physico-chemical data, the identity of aglycone was established as quercetin (Clark and Simon, 1953; Bhardaria and Gupta, 1981 b). It was further confirmed by co- TLC and mixed m.p. with an authentic sample.

Identification of sugar moiety:

The aqueous portion from acid hydrolysis of compound E was neutralised with barium carbonate and barium sulphate was filtered off. Upon concentration, the filtrate was positive for sugar by chemical tests and showed a single spot identical with L-rhamnose by co-paper chromatography (RF 0.33, n-butanol-acetic acid-water, 4:1:5, v/v, upper).
Quantitative estimation of sugar in compound E:

The quantitative estimation of sugar in compound E was conducted by following the procedure of Mishra and Rao (1960). It indicated that the compound in its molecular form contained one unit of L-rhamnose.

Enzymatic hydrolysis of compound E:

It was carried out in alcoholic medium with tokadiastase, which liberated the aglycone and L-rhamnose. It indicated α-linkage between quercetin and L-rhamnose.

Thus, the compound E was characterised as quercetin-7-O-α-L-rhamnoside (Clark and Simon, 1953).
In conclusion, it can be stated that detailed investigations on the isolation and identification of naturally-occurring chemical constituents in *Brachiaria decumbens* grass revealed the presence of known compounds of widely varying nature. The hexane fraction of leaves contained a long chain aliphatic alcohol (hexacosanol) and a plant sterol (\(\beta\)-sitosterol). A sterol glucoside (\(\beta\)-sitosterol-\(\beta\)-D-glucoside) and phenolic acid ester (ethyl caffeate) were present in benzene fraction, while a flavonol glycoside (quercetin-7-O-\(\alpha\)-L-rhamnoglucoside) was isolated from the ethyl acetate fraction. The compounds were identified on the basis of spectral analysis, preparation of derivatives, and degradation studies. They were present as minor plant constituents and thus were not expected cause any health problem when the animals were allowed to graze the pasture.
**EXPERIMENTAL**

Air-dried powdered leaves (1.5 kg) of *B. decumbens* grass were exhaustively extracted with ethanol (95 percent, 4x2 lit). The combined alcoholic extract was concentrated under reduced pressure below 50°. The combined residue (23 g) was successively fractionated into hexane (3x1 lit), benzene (2x1 lit) and ethyl acetate (2x500 ml) soluble components. Those on concentration afforded the following fractions:

1. Hexane soluble (7.3 g)
2. Benzene soluble (4.5 g)
3. Ethyl acetate soluble (4.2 g)

**Column chromatography of hexane soluble fraction:**

- **Length of column** - 160 cm
- **Dia. of column** - 5.0 cm
- **Weight of crude extract** - 7.0 g
- **Volume of each fraction collected** - 150 ml
- **Weight of neutral alumina** - 200 g.

**Table 1. Column chromatography of hexane soluble fraction**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction No.</th>
<th>Eluant</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1-4</td>
<td>Hexane</td>
<td>Mixture</td>
</tr>
<tr>
<td>2.</td>
<td>5-10</td>
<td>Hexane:benzene (80:20)</td>
<td>Greenish material</td>
</tr>
<tr>
<td>3.</td>
<td>11-14</td>
<td>Hexane:benzene (50:50)</td>
<td>Contained compound A</td>
</tr>
<tr>
<td>4.</td>
<td>15-18</td>
<td>Hexane:benzene (20:30)</td>
<td>Contained compound A</td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction No.</th>
<th>Eluant</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>19-24</td>
<td>Benzene</td>
<td>Contained compound B</td>
</tr>
<tr>
<td>6.</td>
<td>25-30</td>
<td>Benzene:ethyl acetate</td>
<td>Contained compound B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(80:20)</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>31-34</td>
<td>Benzene:ethyl acetate</td>
<td>Contained compound B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50:50)</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>35-44</td>
<td>Benzene:ethyl acetate</td>
<td>Complex mixture</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20:80)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45-50</td>
<td>Ethyl acetate</td>
<td>Contained compound B</td>
</tr>
</tbody>
</table>

Column chromatography of benzene soluble fraction:

Length of column - 160 cm

Diameter of column - 5.0 cm

Weight of crude extract - 4.0 g

Volume of each fraction collected - 100 ml

Weight of silica gel - 150 g.

Table 2. Column chromatography of benzene soluble fraction

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction No.</th>
<th>Eluant</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1-3</td>
<td>Benzene:ethyl acetate</td>
<td>Mixture</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(70:30)</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>4-7</td>
<td>Benzene:ethyl acetate</td>
<td>Contained compound C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(60:40)</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>8-11</td>
<td>Benzene:ethyl acetate</td>
<td>Contained compound C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50:50)</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>12-15</td>
<td>Benzene:ethyl acetate</td>
<td>Brown residue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(40:60)</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>16-24</td>
<td>Benzene:ethyl acetate</td>
<td>Contained compound D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(30:70)</td>
<td></td>
</tr>
</tbody>
</table>
Ethyl acetate soluble fraction:

The concentrated residue (4.2 g) was taken up with 100 ml hot water and filtered. The filtrate was cooled and mixed with basic lead acetate solution. It resulted in a precipitate, which was removed and washed with water. The precipitate was suspended in 100 ml ethanol and $\text{H}_2\text{S}$ gas was bubbled for one hr. The precipitated lead sulphide was filtered off. The filtrate, after concentration under reduced pressure, was thoroughly extracted with ethyl acetate (3x200 ml). The combined ethyl acetate extracts on concentration yielded a mixture of pigments. These were applied along the breadth of Whatman 3 mm (46x97 cm) filter paper in the form of about 3 cm layer and allowed to dry. The pigments were then subjected to ascending chromatography at room temperature with n-butanol-acetate-acid-water (4:1:5, v/v, upper) as the developing reagent. It took 18 hr to complete the experiment. The different zones on the chromatogram were detected by UV light after drying them in air. The one showing the maximum area (Rf, 0.73) was cut and extracted with methanol (2x50 ml, 7 hr, each time). The combined methanol extracts were concentrated to 4-5 ml, when the compound $\xi$ appeared. It was crystallised from methanol (277 mg), m.p. 222-25°.
(b) Chemical constituents in *P. dictyoneura* Stapf.

The leaves of *P. dictyoneura* grass were dried in shade and ground to pass through 1 mm screen for the study. 1.5 kg dried leaves were exhaustively extracted with ethanol (95 percent). The combined extract was concentrated under reduced pressure below 50°. It was fractionated into hexane, benzene and ethyl acetate soluble fractions. Each fraction was studied separately for the occurrence of chemical compounds.

1. **Hexane Soluble Fraction**:

The hexane soluble fraction was brownish, semi-solid mass. It was column chromatographed over neutral alumina (Brockmann) and eluted with hexane, benzene and ethyl acetate and in-between with their mixtures, resulting in compounds A and B.

**Compound A:**

Fractions (6 - 10 and 14 - 19) obtained from elution with hexane-benzene (80:20 and 20:80) were of same Rf value on TLC. So, these were mixed. Evaporation of solvent yielded white shining flasks from acetone (230 mg), m.p. 68°, analysed for C\text{31}H\text{64}, M^+436 (λIMS).

IR \( \text{max} \) 2925, 2845, 1455, 1370, 1035, 745 and 715 cm\(^{-1}\).

\( ^1H \text{ NMR} \) (CDCl\text{3}) 8 0.9 and 8 1.25 (s, CH\text{3} protons).
$^1$H NMR spectrum manifested singlet at $\delta$ 0.9 and
$\delta$ 1.25 indicating the compound to be a straight chain

The compound A was thus identified as hentriacontane
(Heilbronne et al., 1965) and its physico-chemical data
corresponded to reported values in literature.

$$
\text{CH}_3 \cdot \text{CH}_2 \cdot [\text{CH}_2]_{27} \cdot \text{CH}_2 \cdot \text{CH}_3
$$

**COMPOUND A**

**Compound B:**

(Fractions 25-43). The residue yielded colourless
waxy solid on crystallisation from hexane after removal of
impurities (193 mg). It melted at 33-34°, analysed for
$C_{31}H_{64}O$, M$^+$452 (EI-MS).

IR $\nu_{\text{max}}$ KBr

$$
\begin{align*}
3310, & 2390, 2845, 1475, 1065 \text{ and } 725 \text{ cm}^{-1}.
\end{align*}
$$

**Acetylation of compound B:**

The compound B on acetylation (50 mg compound B +
1 ml pyridine + 1.5 ml acetic anhydride, refluxed, oil bath,
120-30°, 3 hr; usual work up; crystallised from ethanol) yielded a derivative, m.p. 72°. It analysed for C_{33}H_{66}O_{2}, M^{+} 494 (EIMS).

IR (KBr) max 2930, 2825, 1745, 1460, 1375, 1230, 910 and 735 cm^{-1}.

The above physico-chemical data of compound B and its acetate conformed to the reported values for hexatriacontanol (Heibronnan et al., 1965) and was further confirmed by comparison (co-tlc, mixed m.p. and superimposable IR) with authentic sample.

\[ \text{CH}_3 \cdot [\text{CH}_2]_{29} \text{CH}_2 \text{OH} \]

**COMPOUND B**

2. **Benzene Soluble Fraction**:

The benzene soluble fraction was brown, syrupy material and found to be a mixture of two compounds on TLC examination. It was subjected to column chromatography over silica gel (60-120 mesh) and eluted with benzene and ethyl acetate in different proportions, resulting into two crystalline compounds C and D.
Compound C:

Fractions 7-13 eluted from benzene-ethyl acetate (60:40). The residue crystallised from benzene (110 mg), m.p. 35-36°, analysed for \( \text{C}_{30}\text{H}_{62}\text{O} \), \( M^+433 \) (EIMS).

\[
\text{IR (KBr)} \quad \max \quad 3348, 2900, 2820, 1460, 1050, 380, 730 \text{ and } 715 \text{ cm}^{-1}.
\]

\[
\begin{align*}
1^1\text{H NMR} \\
(\text{CDCl}_3)
\end{align*}
\]

\[
\delta 0.38 \text{ (t, 3H, }-\text{CH}_3), \delta 1.18 \text{ (s, 56H, }-(\text{CH}_2)_2\text{)-}, \text{ and } \delta 3.63 \text{ (t, 2H, }-\text{CH}_2\text{OH}).
\]

The \( 1^1\text{H NMR} \) spectrum showed a triplet at \( \delta 0.38 \) for one terminal methyl group and a broad singlet at \( \delta 1.18 \), integrating for 56 protons, due to \( -(\text{CH}_2)_2\text{-} \) chain. The two methylene protons attached to oxygen atom \( -(\text{CH}_2\text{-OH}) \) appeared as triplet at \( \delta 3.63 \).

Acetylation of compound C:

(50 mg compound C + 1.5 ml pyridine + 1.5 ml acetic anhydride, refluxed, oil bath, 120-130°, 3 hr; isolated by normal procedure; crystallised from ethanol), m.p. 72°, analysed for \( \text{C}_{32}\text{H}_{64}\text{O}_2 \), \( M^+430 \) (EIMS).

\[
\text{IR (KBr)} \quad \max \quad 2910, 2335, 1735, 1460, 1365, 1235 \text{ and } 725 \text{ cm}^{-1}.
\]

\[
\begin{align*}
1^1\text{H NMR} \\
(\text{CDCl}_3)
\end{align*}
\]

\[
\delta 0.89 \text{ (t, 3H, }-\text{CH}_3), \delta 1.20 \text{ (s, 56H, }-(\text{CH}_2)_2\text{)-}, \text{ and } \delta 2.95 \text{ (s, 3H, }-\text{COCH}_3) \text{ and } \delta 4.13 \text{ (t, 2H, }-\text{CH}_2\text{OAc}).
\]
The $^1$H NMR spectral data of compound C and its acetate were in complete conformity with that of myricyl alcohol (Rhadoria and Gupta, 1977; Jain and Gupta, 1991). Identity of compound as myricyl alcohol was further established by co-tlc, mixed m.p. and super-imposable IR with authentic sample.

\[ \text{CH}_3\cdot[\text{CH}_2]_{28}\text{CH}_2\text{OH} \]

**COMPOUND C**

**Compound D:**

Fractions 18-21 and 22-25 (eluted with benzene-ethyl acetate, 40:60 and 20:80). Furnished colourless solid with acetone, rich in compound D. Its PLC on silica gel using benzene-methanol (1:1) as the developing system afforded compound D, crystallised from hexane-benzene (1:1) as colourless needles (103 mg). It melted at 30-33°, analysed for C$_{45}$H$_{80}$O$_2$, M$^+$ 652 (EI-MS).

IR (KBr)  
$\text{max}$ 2920, 2865, 1755, 1470, 1400, 1105, 910 and 715 cm$^{-1}$. 
EIMS

m/z 652, 638, 396, 255, 229, 215, 146, 111, 97, 85, 83, 71 and 57.

It showed prominent even mass ion peaks at m/z 396, 638 and 652. The peaks at m/z 57, 71, 83, 85, 97, 111, etc. suggested the presence of an aliphatic chain in the molecule and the peaks at m/z 255 and 396 were indicative of a steroid nucleus.

**Alkaline hydrolysis of compound D:**

(50 mg compound D + 3 ml 25 percent methanolic sodium hydroxide, refluxed, steam bath, 1 hr; reaction mixture extracted with chloroform after distilling methanol; crystallized from 1:1 chloroform - methanol), m.p. 135°, analysed for C_{29}H_{50}O, M^+14 (EIMS). It was characterised as β-sitosterol on the basis of mixed m.p. and co-tlc with authentic sample.

The saponified fraction on usual work up afforded an acid, m.p. 62-63°, analysed for C_{16}H_{32}O_{2}, M^+256 (EIMS). It was identified as palmitic acid by its superimposable IR spectrum, co-tlc and mixed m.p. with authentic sample.

On the basis of above facts, compound D was characterized as β-sitosterol palmitate (Tiwari and Richard, 1979).
3. **ETHYL ACETATE SOLUBLE FRAGMENT**:

Removal of solvent from the ethyl acetate fraction yielded a residue which answered Shinoda test for flavonoids and Molisch's test for sugar. It was isolated as Compound D through formation of Pb salt (by basic lead acetate) and then recovering the compound by precipitating Pb as PbS by H₂S.
Compound E:

Crystallised as yellow granules from methanol (183 mg), m.p. 189-90°, analysed for C_{27}H_{30}O_{16}, M^{+}610 (EIMS). It gave cherry red colour with Mg/HCl in alcoholic medium and was positive for Molisch's and Feigel tests for sugar.

UV
- MeOH: 253, 264 (sh), 300 (sh) and 357 nm.
- NaOMe: 274, 320 and 408 nm.
- AlCl_{3}: 276, 304 (sh) and 435 nm.
- AlCl_{3}/HCl: 273, 302, 364 (sh) and 400 nm.
- NaOAc: 270, 327 and 390 nm.
- NaOAc/H_{3}BO_{3}: 264, 300 and 382 nm.

IR
- KBr: 3405, 1650, 1605, 1560, 1355, 1295, 1200, 1095, 1050, 1010 and 795 cm^{-1}.

The UV spectrum of compound E showed absorption maxima at 253, 264 (sh), 300 (sh) and 357 nm characteristic of flavonoids (Nabry et al., 1970). The peak at 1650 cm^{-1} in the IR spectrum also indicated the flavone nucleus.

The appearance of bathochromic shift of 12 nm in band II with addition of sodium acetate indicated the presence of -OH group at C-7. The bathochromic shift of +3 nm in band I a (with AlCl_{3}/HCl) relative to band I (in MeOH) was caused by C-5 hydroxyl group. The presence of bathochromic shift of 25 nm of band I with sodium acetate in presence of boric acid suggested ortho-dihydroxyl group in B ring at C-3' and C-4' positions. It is also supported
by bathochromic shift of 35 nm of band I a with AlCl₃ (relative to AlCl₃/HCl). The IR spectrum of compound E also showed an intense peak at 3405 cm⁻¹ for the -OH groups. The above spectral data further suggest that hydroxyl group at C-3 position is substituted.

**Acid hydrolysis of compound E:**

The acid hydrolysis of compound E (50 mg compound E + 15 ml three percent methanolic H₂SO₄, refluxed, boiling water bath, 3 hr; extracted with ethyl acetate after distilling off methanol and dilution with water; aqueous layer preserved for separate analysis) yielded an aglycone, crystallised from acetone-methanol (1:1) as yellow crystals, m.p. 314-15⁰, analysed for C₁₅H₁₀O₇, M⁺ 302 (ESMS). The UV, IR and ¹H NMR spectra of the aglycone showed identical results to the observations recorded earlier for quercetin.

**Study of sugar moiety (ies):**

The aqueous portion from acid hydrolysis of compound E was neutralised and the sugar moiety (ies) was (were) identified as D-glucose and L-rhamnose by co-paper chromatography as per the method already described.

**Periodate oxidation of glycoside (compound E):**

The sodium metaperiodate oxidation of the glycoside (compound E) consumed 3.04 moles of periodate and liberated 1.09 moles of formic acid. It indicated that one molecule of L-rhamnose and one molecule of D-glucose were attached.
to one molecule of aglycone, both the sugars were in pyranose form and attached to the same C-atom as a disaccharide.

The compound E was identified as rutin (Hattori, 1962) and confirmed by direct comparison with authentic sample.
Thus, it will be seen that detailed studies on isolation and identification of naturally-occurring chemical constituents in *B. dictyoneura* revealed the presence of known compounds. These varied widely in nature. The hexane, benzene and ethyl acetate soluble fractions of leaves of *B. dictyoneura* contained a long chain hydrocarbon (hentriacontane) and related alcohol (hentriacontanol); another long chain alcohol (myricyl alcohol), plant sterol ester (β-sitosterol palmitate), and a flavonol glycoside (rutin) respectively. The compounds were present as minor plant constituents and were not likely to cause any toxic effect when the pasture was grazed by animals. The pattern of isolated compounds in *B. dictyoneura* and *B. decumbens* (studied earlier) was, however, similar and thus may be of chemotaxonomic interest.
EXPERIMENTAL

Air dried powdered leaves (1.5 kg) of P. dictyoneura pasture were thoroughly extracted with 95 percent ethyl alcohol (5x2.5 lit) at room temperature. Concentration of total alcoholic extract at a temperature below 50° and under vacuum furnished 32.5 g residue. It was exhaustively shaken successively with hexane, benzene and ethyl acetate (4x1 lit, 2x500 ml and 3x500 ml respectively) for separation of different organic solvent-soluble fractions. Upon concentration, following amounts of various soluble components were recorded:

1. Hexane soluble (9.1 g),
2. Benzene soluble (5.9 g)
3. Ethyl acetate soluble (4.7 g).

Column chromatography of hexane soluble fraction:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of column</td>
<td>160 cm</td>
</tr>
<tr>
<td>Dia. of column</td>
<td>5 cm</td>
</tr>
<tr>
<td>Weight of crude extract</td>
<td>9 g</td>
</tr>
<tr>
<td>Volume of each fraction collected</td>
<td>150 ml</td>
</tr>
<tr>
<td>Weight of Al₂O₃</td>
<td>225 g</td>
</tr>
</tbody>
</table>
Table 3. Column chromatography of hexane soluble fraction

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction No.</th>
<th>Eluant</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1-5</td>
<td>Hexane</td>
<td>Mixture</td>
</tr>
<tr>
<td>2.</td>
<td>6-10</td>
<td>Hexane:benzene (80:20)</td>
<td>Contained compound A</td>
</tr>
<tr>
<td>3.</td>
<td>11-13</td>
<td>Hexane:benzene (50:50)</td>
<td>Oily residue</td>
</tr>
<tr>
<td>4.</td>
<td>14-19</td>
<td>Hexane:benzene (20:80)</td>
<td>Contained compound A</td>
</tr>
<tr>
<td>5.</td>
<td>20-24</td>
<td>Benzene</td>
<td>Complex mixture</td>
</tr>
<tr>
<td>6.</td>
<td>25-30</td>
<td>Benzene:ethyl acetate (80:20)</td>
<td>Contained compound B</td>
</tr>
<tr>
<td>7.</td>
<td>31-34</td>
<td>Benzene:ethyl acetate (50:50)</td>
<td>Contained compound B</td>
</tr>
<tr>
<td>8.</td>
<td>35-43</td>
<td>Benzene:ethyl acetate (20:80)</td>
<td>Contained compound B</td>
</tr>
<tr>
<td>9.</td>
<td>44-50</td>
<td>Ethyl acetate</td>
<td>Mixture</td>
</tr>
</tbody>
</table>

Column chromatography of benzene soluble fraction:

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of column</td>
<td>160 cm</td>
</tr>
<tr>
<td>Dia. of column</td>
<td>5 cm</td>
</tr>
<tr>
<td>Weight of crude extract</td>
<td>5.5 g</td>
</tr>
<tr>
<td>Volume of each fraction collected</td>
<td>100 ml</td>
</tr>
<tr>
<td>Weight of silica gel</td>
<td>150 g</td>
</tr>
</tbody>
</table>
Table 4. Column chromatography of benzene soluble fraction

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction No.</th>
<th>Eluant</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1-6</td>
<td>Benzene:ethyl acetate</td>
<td>Mixture</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(80:20)</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>7-13</td>
<td>Benzene:ethyl acetate</td>
<td>Contained</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(60:40)</td>
<td>compound C</td>
</tr>
<tr>
<td>3.</td>
<td>14-17</td>
<td>Benzene:ethyl acetate</td>
<td>Complex oily</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50:50)</td>
<td>mixture</td>
</tr>
<tr>
<td>4.</td>
<td>18-21</td>
<td>Benzene:ethyl acetate</td>
<td>Contained</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(40:60)</td>
<td>compound D</td>
</tr>
<tr>
<td>5.</td>
<td>22-25</td>
<td>Benzene:ethyl acetate</td>
<td>Contained</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20:30)</td>
<td>compound D</td>
</tr>
</tbody>
</table>

Ethyl acetate soluble fraction:

It afforded compound \( \beta \) (rutin). It was processed in a manner described earlier for the isolation of flavonoid compound from \( P. \ decumbens \) using the ethyl acetate soluble fraction.