STUDY OF INTRASPECIES DIVERSITY

Introduction:

Intraspecific diversity is basically infraspecific in nature. It is a kind of difference which does not surpass the limit of the species circumscription, however, shows subtle variation amongst the individuals of the same species growing in different localities or conditions. In case such variation is found to be consistent with the variation in locality of occurrence, it may have implication suggesting them to be prospective morphotypes or ecotypes or chemotypes or cytotypes, if not a variety.

Intraspecific variation albeit having a cause stemming from a change at genetic level may have a reflection at the level of phenotype or chemotype etc.

Impacts of environmental factors to bring such changes may be multifarious and expressed at different levels of expression. To have a glimpse of it scrutiny of morphology, biomolecules, etc. can be useful. To determine the morphological variation many characters like, height of plant, length and width of leaf, length of fruit and weight of seed etc. can give some insight on it.

Quantity of DNA, RNA., and Protein amount of plant samples of different areas are taken into account to get an idea about biochemical diversity.

Alternation in environmental conditions and latitude play important roles for inducing variation in genetic makeup of a plant. Microelements and other soil nutrients are also having positive effect on growth of a plant.

In the present study an attempt has been made to work out morphological and biochemical diversity of different plants *e.g. Lantana camara* Linn., *Crotallaria pallida* Ait, *Ocimum* canum Sims and *Tephrosia purpurea* Linn. which are growing in natural contrasting habitats in district of Paschim Medinipur, West Bengal, India.

Material and Methods:

Fresh leaves and seeds of 40 plant samples of the selected every four-plant species were taken from each forest beat area. Leaves of these plant were used to determine the quantity of DNA, RNA and seeds for the determination of protein. Plant Protein extraction and quantity was studied according to Lowry's method (1951).

Protein Extraction:

Essential reagents for Protein extraction- 10% trichloroacetic acid (TCA), 20 mM DTT.

Plant tissue homogenate of 0.5 ml was taken with 1 ml of cold 10% trichloroacetic acid (TCA) and 20 mM DTT (Dithiothreitol) in acetone to precipitate to get it in pure form, prior to estimation of it. Mixture was centrifuged up to 10,000 x g for 10 minutes and then the supernatant was decanted. The precipitate, obtained thus, was dissolved in 5 ml of 0-1 N NaOH solution and 0.1 ml of it is used protein estimation.

Protein Estimation:

Essential Reagents for Protein Estimation –

I. 2.0 % Na_2CO_3 in 0.1N NaOH.

II. 0.3% $CuSO_4$. $5H_2O$ in 1% Sodium Potassium Tartarate.

III. Phenol reagent (Folin-Ciocalteau) 1.0 Normal.

IV. Standard Protein Solution: Bovine Serum albumin (BSA) at 0.2 mg/ml in 0.1N NaOH.

V. Mixture of reagents I and II (50:1) just before assay.

0.1 ml protein suspension (in NaOH) was mixed with reagent (V) and let stay it for 10 minutes. After that 0.5 ml of reagent (III) was added with the mixture and stirred it. Then the mixture was allowed to stay at room temperature (25^0C) for 30 minutes and read the absorbance at 660 nm. A standard curve was prepared by using 0.05 ml to 0.5

ml solution of BSA in test tubes, carry through whole procedure and determine the account of color development. Quantity of Protein was measured by absorbance at 660 nm. (Lowry, 1951).

DNA Extraction:

Essential reagents for DNA extraction -

CTAB buffer, 2 % cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone, 100 mM Tris-HCL, 1.4 M Nacl, 20 mM EDTA (CTAB Extraction Buffer), Isopropanol, 70% Ethanol, TE Buffer (10 mM Tris, pH 8, 1mM EDTA).

Plant samples are prepared by cryogenically grind in a mortar and pestle after freezing in liquid Nitrogen. Freeze dried plant samples kept at room temperature. 500 µl of CTAB buffer is mixed thoroughly vortex with 100 mg homogenized plant tissue and homogenate transferred to a 60^0 C bath for 30 minutes. Homogenate is centrifuged at 14000 x g for 5 minutes and supernatant is transferred in a new tube. 5 µl of RNase solution is added with it and allowed to incubate at 32^0 C for 20 minutes. Equal volume of Chloroform is added with it and vortex for 5 seconds. After that the sample is centrifuged at 14000 x g for 1 minutes and then aqueous upper portion placed to a new tube. This method repeated until the upper phase is clear. Then upper aqueous part is transferred in a new tube and 0.7 volume cold isopropanol is added with it to precipitate DNA allowed to incubate at 20 ^{0} C for 15 minutes. The sample is centrifuged at 14000 x g for 10 minutes. Supernatant is decanted without disturbing pellet and washed repeatedly with ice cold 70% ethanol. Ethanol is decanted and residual part of Ethanol is dried in a speedVac. Then DNA is dissolved in 20 µl TE buffer. (CTAB Protocol for the isolation of DNA from plant Tissues: (http://opsdiagnostics.com).

DNA Estimation:

Essential reagents for DNA estimation –

DNA Standard (0.5 mg / ml), Sample DNA, Saline Citrate (0.15 M NaCl, 0.015 M Na_3 Citrate) Solution, Diphenylamine reagent (Fresh or recrystallized Diphenylamine, Glacial Acetic Acid, Con. H_2SO_4).

Separate marked tubes having 1ml,2ml,3ml aliquots of the isolated DNA dissolved in Saline Citrate and similar aliquots of a 0.5 mg DNA /ml standard are prepared. All sample tubes and a separate blank made up to 3ml with H_2O. Then 6 ml of Diphenylamine reagent is added to every tube and after well mixing allowed them to heat in a Water bath for 10 minutes. After cooling of each tube, the absorbance measured at 600 nm against the blank. Then concentration of DNA dissolved in saline citrate solution is calculated with a standard graph A_{600}. (Ashwell, 1957).

RNA Extraction:

Essential reagents for RNA extraction –

Freshly redistilled Phenol, Extraction buffer pH 9.0: {Tris-HCL (0.1M) 1.21g, NaCl (0.075M) 0.44g, EDTA Na_2 (0.005M) 0.19g, H_2O}, Ethanol, SDS 10% (w/v in water), Ether.

5 g of sample is grinded in a mortar and pestle with liquid Nitrogen and extracted in 10 volumes of extraction buffer. Homogenate is centrifuged at 2000 x g for 3 minutes and supernatant is transferred to a volumetric flask. Then it stirred with 0.1 volume of 10% SDS for 3 minutes. After that an equal volume of buffered phenol (freshly redistilled phenol saturated overnight with 100 mM Tris – HCL pH 8.5) is added with it. Then the content centrifuged at 2000 x g for 5 minutes and upper aqueous phase is separated into a

flask. Then lower phase is centrifuged again with an equal volume of extraction buffer for 5 minutes at 2000 x g. Then aqueous phase is combined with previous upper aqueous phase and stirred with an equal volume of buffered -phenol for 5 minutes. These extraction and centrifugation steps are repeated at least 5 times. Finally, upper aqueous phase containing RNA is collected and dissolved in it about 250 mg NaCl by adding two volumes of cold ethanol (96%). This mixture is kept for overnight at -20^0 C for RNA precipitation. RNA is collected by centrifugation at 2000 x g for 10 minutes. The pellet (RNA) is washed with mixture of 70% ethanol and ether, ethanol: ether (1:1 v/v) and finally with ether. The pellets are gently dried 'in vacuo' for 3 minutes. (Brawerman, 1974).

RNA Estimation:

Essential reagents for RNA estimation –

Standard RNA and Sample RNA, Orcinol Acid reagent {2 ml of a 10% solution (w/v) of ferric chloride. 6 H_2 O to 400 ml of Conc. HCl., 6% acid Orcinol {6 g orcinol in 100 ml 95% ethanol}, 1o mM Tris-acetate, 1 mM EDTA buffer (pH 7.2).

A standard RNA solution (50µg RNA / ml) is prepared in ice chilled 10 mM Tris-acetate, 1 mM EDTA buffer. A series of test tubes containing 0.5 ml, 1 ml, 1.5 ml, and 3 ml of isolated RNA and similarly 0.5 ml, 1 ml, 1.5 ml, and 3 ml of 50 µg standard RNA / ml are prepared. All sample tubes and a separate blank made up to 3 ml with H_2O. 6 ml of orcinol acid reagent is added to each tube and after that similarly 0.4 ml of 6% alcoholic orcinol is added to every tube. All tubes are well shake to mix contents and allowed to heat all tubes in a water bath for 20 minutes. After cooling of all tubes, the absorbance measured at 660 nm against the blank. Then concentration of RNA is calculated with a standard graph A_{660}. (Ashwell, 1957).

Spectrophotometer Model: UV- 1800, made by SHIMADZU, Serial no: A11454703023, SHIMADZU CORP. is used to read absorbance for biomolecules (Protein, DNA and RNA) at respective web lengths.

Morphological Diversity Study:

External morphological studies of four plant species have been carried out. The habit of plant, height of plant, leave length and width (cm/mm), weight of fruit and seeds (gm) of selected plants were taken into account for this study.

Statistical Analysis:

The numerical data is statistically analyzed with help of MS Excel and SPSS version 16.0. LSD value of each parameter is calculated by 'One Way ANOVA' with the help of formula:

$LSD_{A,B} = t_{0.05/2} DFW \sqrt{MSW(1/n_A + 1/n_B)}$

t = Critical value from the t distribution table.

MSW = Mean square within, obtained from the results of ANOVA Test.

n = Number of scores used to calculate the means.

Results and Discussions:

Lantana camara plants of different sites showed considerable variation in height, leaf length and leaf width. Plants with least height were noted in Kalaikunda, while, significantly larger plants were witnessed at Bhadutala and still larger ones at Gurguripal. Whereas, in consideration of leaf length plant individuals of all sites were found to be different from each other. No significant difference was found in leaf width of plant individuals of Ramrama and Kalaikunda. But the fruit weight of the plants of Bhadutala and Gurguripal were noted to be devoid of even least difference and somewhat greater than that of Kalaikunda and Ramrama (Table 4.1).

Table No 4.1: Morphological diversity of *Lantana camara* Linn.

Beat / Parameter	RAMRAMA	KALAIKUNDA	GURGURIPAL	BHADUTALA	LSD VALUE
HEIGHT (ft.)	4.5 ± 0.4	3.6 ± 0.9	6.3 ± 0.4	5.9 ± 0.7	0.3
LEAF LENGTH (cm)	3.4 ± 0.5	4.6 ± 1.2	5.5 ± 0.6	6.4 ± 0.4	0.4
LEAF WIDTH (cm)	2.5 ± 0.4	2.7 ± 0.6	3.9 ± 0.5	4.2 ± 0.8	0.3
FRUIT WEIGHT (g)	0.0170 ± 0.005	0.015±0.007	0.020±0.007	0.020±0.006	0.003
FRUIT SIZE (mm)	4.72 ± 0.61	4.52 ± 0.51	5.57 ± 0.49	5.15 ± 0.54	0.29

Variability in biochemical content in reference to *Lantana camara* Linn was discussed by Soliman. M.S.A. *et al* (2018). Even though in respect of morphological traits the plants of different localities showed subtle variation, the biochemical contents showed remarkable variation as depicted in Table No. 4.2.

Table No 4.2: Biochemical diversity of *Lantana camara* Linn.

Beat / Parameter	RAMRAMA	KALAIKUNDA	GURGURIPAL	BHADUTALA	LSD VALUE
DNA (µg/g fr.wt.)	18.04 ± 0.41	16.01 ± 0.08	21.07 ± 0.23	19.07 ± 0. 34	0.631
RNA (µg/g fr.wt.)	39.02 ± 0.51	35.03 ± 0.72	41.02 ± 0.37	37.06 ± 0.57	0.762
PROTEIN (mg/g fr.wt.)	190.22 ± 0.73	186.43 ± 0.69	202.05 ± 0.76	198.03 ± 0.57	1.977

In *Crotalaria pallida* population of different locations also exhibited difference in height; though the plants of Bhadutala and Gurguripal forest beats showed almost no difference. Plants of this species in Ramrama beat were of shortest height out of four locations. In respect of leaf width of *Crotalaria pallida* showed no remarkable difference in the plants of Kalaikunda and Bhadutala forest areas. Whereas, in consideration of leaf length plant individuals of all sites were found to differ from each other except plants of Gurguripal and Bhadutala. Fruit weight of *Crotalaria pallida* of all sites showed significant difference, but in fruit size, no significant difference was found to exist between the plants of Kalaikunda and Gurguripal. Extract of all morphological data have been displayed in Table 4.3

Table No 4.3: Morphological diversity of *Crotalaria pallida* Ait.

Beats / Parameters	RAMRAMA	KALAIKUNDA	GURGURIPAL	BHADUTALA	LSD VALUE
HEIGHT (cm)	124.7 ± 5.2	136.1 ± 4.9	132 ± 6	130. ± 5.4	2.9
LEAF LENGTH (cm)	10.8 ± 1.5	7.7 ± 1.3	9.2 ± 1.6	8.6 ± 1.6	1. 4
LEAF WIDTH (cm)	5.8 ± 0.6	4.0 ± 0.8	3.0 ± 0.2	4.5 ± 0.5	0.6
FRUIT WEIGHT (g)	0.0178± 0.0061	0.0544 ± 0.0281	0.0485 ± 0.0221	0.0476 ± 0.0182	0.001
FRUIT LENGTH (cm)	3.6 ± 1.2	4.4 ± 1.1	4.2 ± 1.0	3.2 ± 1.2	0.4

Regarding morphological traits the plants of Ramrama and Bhadutala showed least difference, though the biochemical contents of plants in all sites exhibited considerable variation.

Table No 4.4: Biochemical diversity of *Crotalaria pallida* Ait

Beats / Parameters	RAMRAMA	KALAIKUNDA	GURGURIPAL	BHADUTALA	LSD VALUE
DNA (**µg/g fr.wt.)**	12.04 ± 0.89	13.86 ± 0.69	14.36 ± 0.72	16.12 ± 0.34	0.598
RNA (**µg/g fr.wt.)**	25.37 ± 0.54	27.44 ± 0.29	29.19 ± 0.16	30.48 ± 0.67	0.796
PROTEIN (**mg/g fr.wt.)**	120.11 ± 0.65	128.24 ± 0.72	132.01 ± 0.86	138.44 ± 0.62	1.322

Ocimum canum plants of different sites showed considerable variation in height; and excepting the absence of variation in regard of height of the plants of Kalaikunda and Bhadutala. Leaf length, fruit weight and biochemical content (DNA, RNA and Protein) of *Ocimum canum* populations of most of the four sites, under study showed significant difference. Still then, leaf width of the plants of Gurguripal and Bhadutala showed no remarkable difference. (Table 4.5 & 4.6)

Table No 4.5: Morphological diversity of *Ocimum canum* Sims.

Beats / Parameters	RAMRAMA	KALAIKUNDA	GURGURIPAL	BHADUTALA	LSD VALUE
HEIGHT (cm)	54 ± 11	63 ± 10	39 ± 16	69 ± 13	7
LEAF LENGTH (cm)	2.6 ± 0.7	3.1 ± 0.5	3.4 ± 0.3	2.8 ± 0.4	0.3
LEAF WIDTH (cm)	1.1 ± 0.2	1.3 ± 0.2	1.5 ± 0.1	1.4 ± 0.3	0.2
FRUIT WEIGHT (g)	0.0050 ± 0.002	0.0064 ± 0.003	0.0118 ± 0.004	0.0074 ± 0.002	0.012

Table No 4.6: Biochemical diversity of *Ocimum canum* Sims.

Beats / Parameters	RAMRAMA	KALAIKUNDA	GURGURIPAL	BHADUTALA	LSD VALUE
DNA (**µg/g fr.wt.)**	8.43 ± 0.39	9.56 ± 0.48	12.02 ± 0.92	11.08 ± 0.47	0.511
RNA (**µg/g fr.wt.)**	13.76 ± 0.43	14.91 ± 0.88	18.09 ± 0.58	16.02 ± 0.24	0.581
PROTEIN (**mg/g fr.wt.)**	104.02 ± 0.76	108.33 ± 0.21	120.06 ± 0.98	110.01 ± 0.62	1.672

Tephrosia purpurea plants of different sites show considerable variation in height. Leaf length of plant individuals of Kalaikunda varies with other three sites and leaf width of plants of Ramrama and Bhadutala showed no remarkable difference. Regarding other morphological traits the plants of four localities showed considerable difference, though DNA, RNA and protein contents explore absolute consistency. (Table 4.7 & 4.8).

Table No 4.7: Morphological diversity of *Tephrosia purpurea* Linn.

Beat / Parameter	RAMRAMA	KALAIKUNDA	GURGURIPAL	BHADUTALA	LSD VALUE
HEIGHT (cm)	35 ± 12	40 ± 6	60.8 ± 5.2	47 ± 4	4.1
LEAF LENGTH (cm)	6.2 ± 2.1	8.8 ± 2.4	7.4 ± 1.5	6.4 ± 2.1	1.2
LEAF WIDTH (cm)	3.6 ± 1	4.2 ±1.2	5.2 ± 0.6	3.8 ± 1.2	0.5
FRUIT WEIGHT (g)	0.0116 ± 0.0038	0.0127 ± 0.0031	0.0096 ± 0.0036	0.0072 ± 0.0038	0.013
FRUIT SIZE (cm)	2.6 ± 0.4	3.2 ± 0.2	3.6± 0.6	4.4 ± 0.4	0.3

Table No 4.8: Biochemical diversity of *Tephrosia purpurea* Linn.

Beat / Parameter	RAMRAMA	KALAIKUNDA	GURGURIPAL	BHADUTALA	LSD VALUE
DNA (**µg/g fr.wt.)**	10.07 ± 0.18	12.92 ± 0.76	11.05 ± 0.82	15.02 ± 0.43	0.761
RNA (**µg/g fr.wt.)**	22.34 ± 0.53	26.64 ± 0.65	25.09 ± 0.83	28.01 ± 0.74	0.895
PROTEIN (**mg/g fr.wt.)**	138.07 ± 0.77	144.68 ± 0.38	142.73 ± 0.57	148.94 ± 0.61	1.965

Summary:

In the present study four weedy herbs growing wild in forest namely, *Lantana camara* Linn, *Crotalaria pallida* Ait. *Ocimum canum* Sims and *Tephrosia purpurea* Linn showed both morphological and biochemical diversities amongst the individuals of same species of four different locations in Paschim Medinipur. Intraspecific diversity of these species indicates the potential of selection to be practiced with them in search of the best form. Best form may be settled based on the trait of the plant species mostly sought after, however, the spectrum of diversity may facilitate this search as an associated one.

Fig 4.1a: Graphical representation of average height of *Lantana camara* Linn in four Forest Beat.

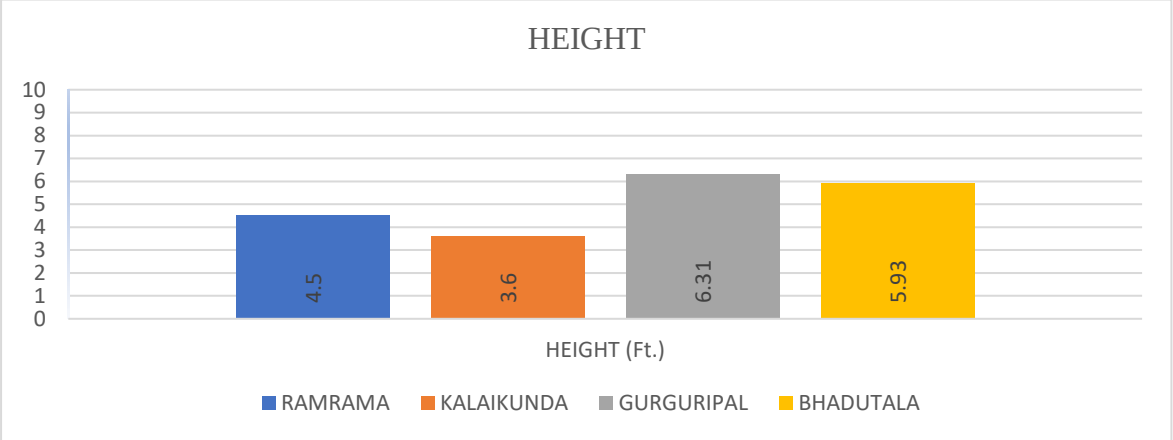

Fig 4.1b: Graphical representation of average Leaf length of *Lantana camara* Linn in four Forest Beat.

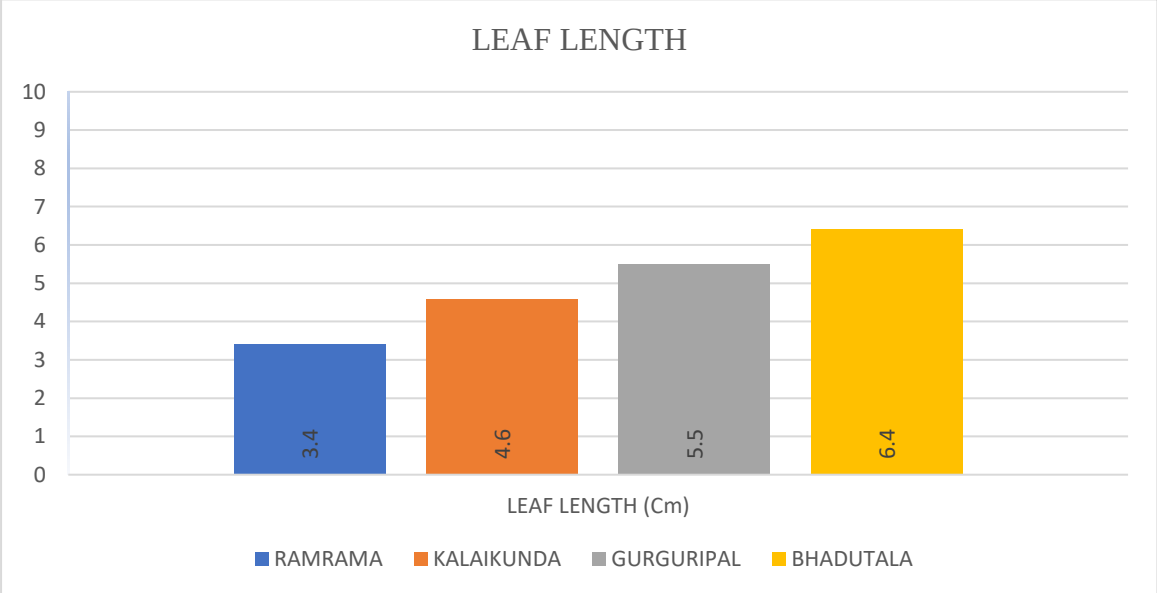

Fig 4.1c: Graphical representation of average Leaf width of *Lantana camara* Linn in four Forest Beat.

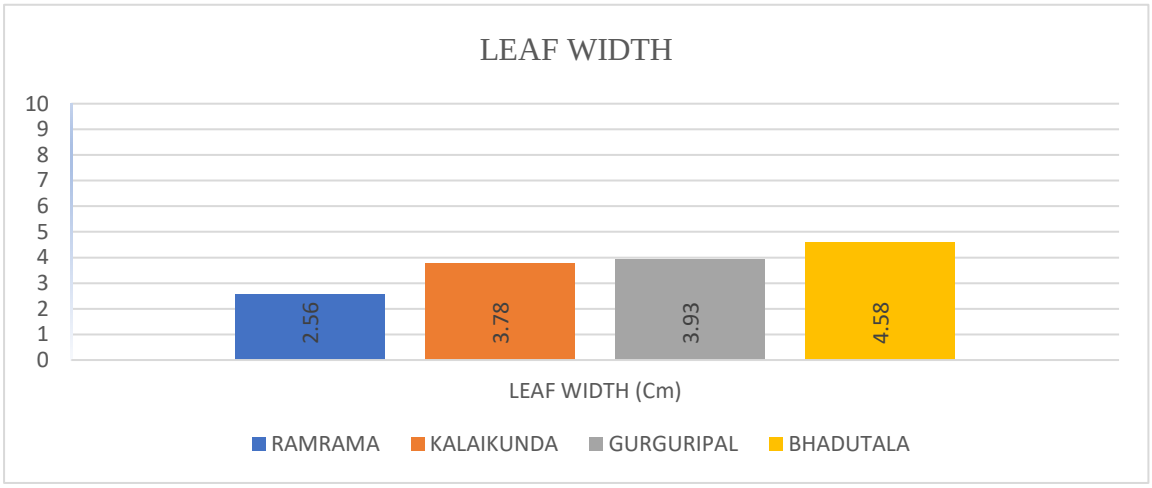

Fig 4.1d: Graphical representation of average Fruit weight of *Lantana camara* Linn in four Forest Beat.

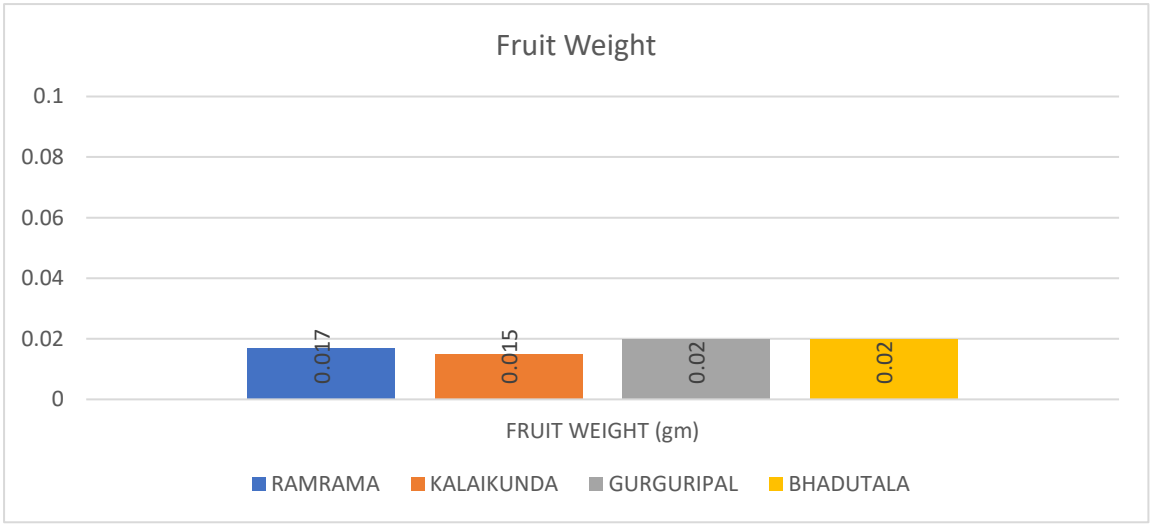

Fig 4.1e: Graphical representation of average Fruit size of *Lantana camara* Linn in four Forest Beat.

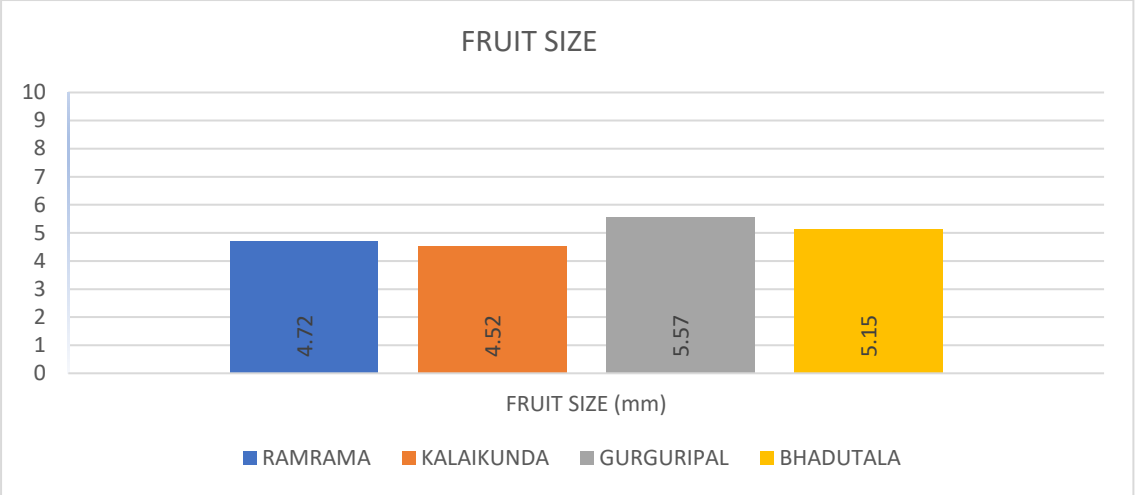

Fig 4.2a: Graphical representation of Average DNA amount of *Lantana camara* Linn.

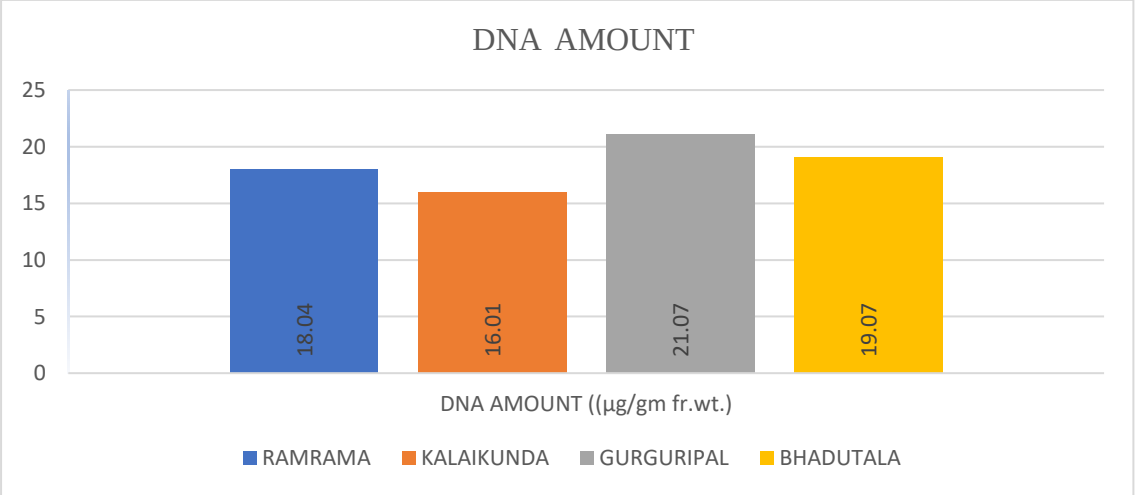

Fig 4.2b: Graphical representation of Average RNA amount of *Lantana camara* Linn.

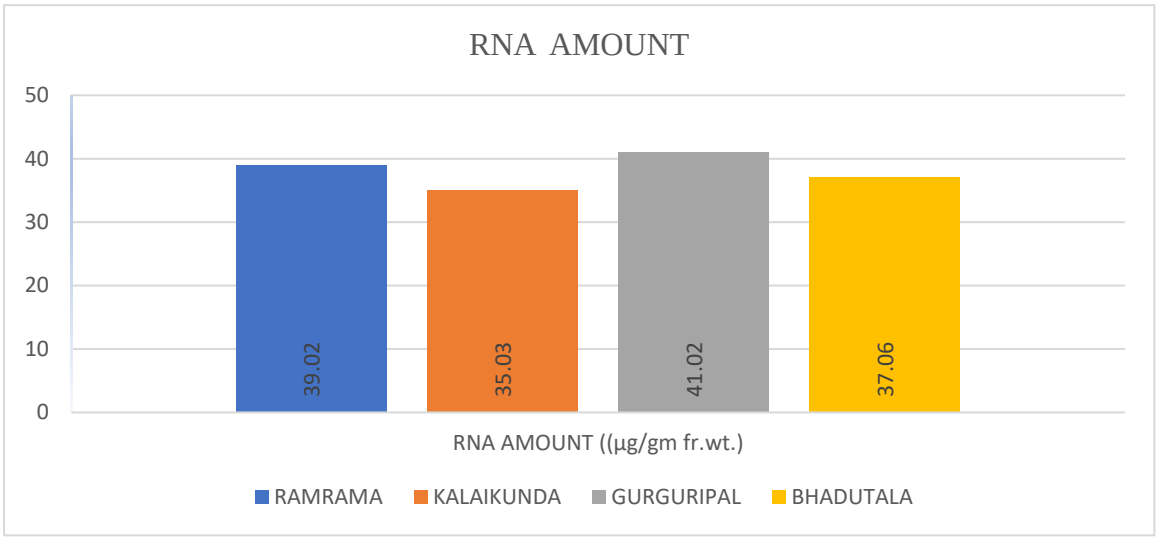

Fig 4.2c: Graphical representation of Average Protein amount of *Lantana camara* Linn

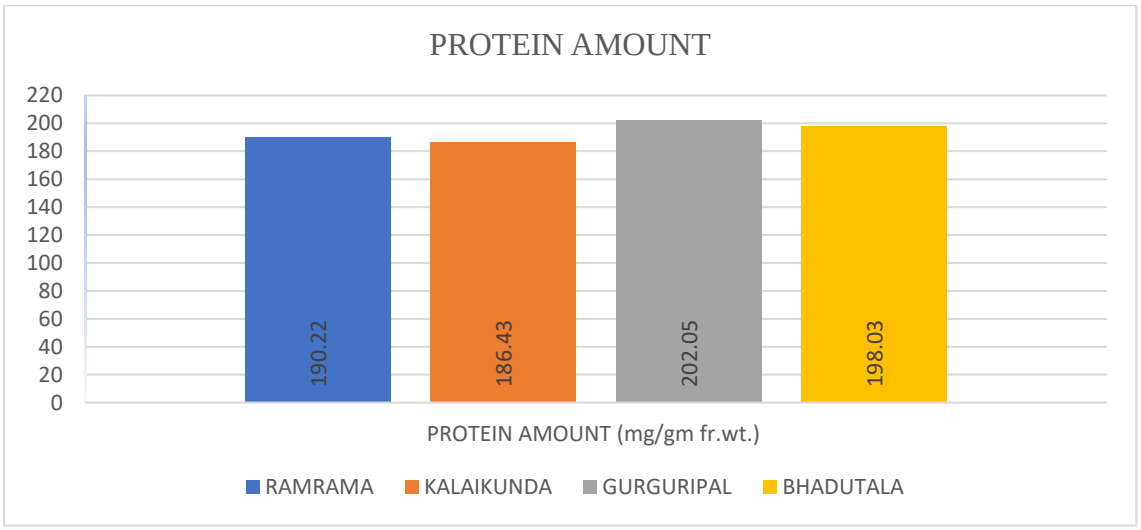

Fig 4.3a: Graphical representation of average height of *Crotalaria pallida* Ait. in four Forest Beat.

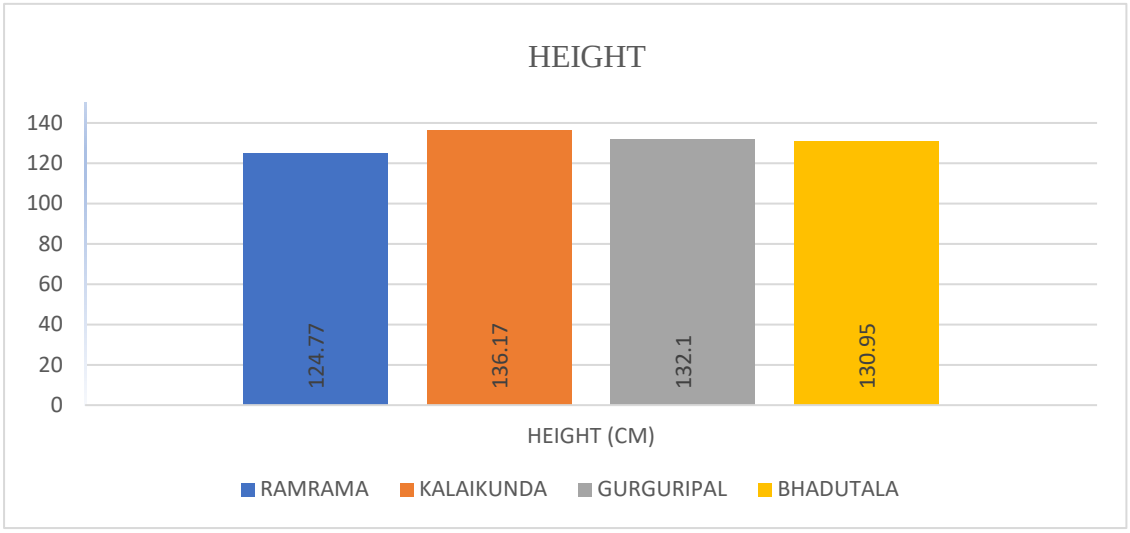

Fig 4.3b: Graphical representation of average leaf length of *Crotalaria pallida* Ait. in four Forest Beat

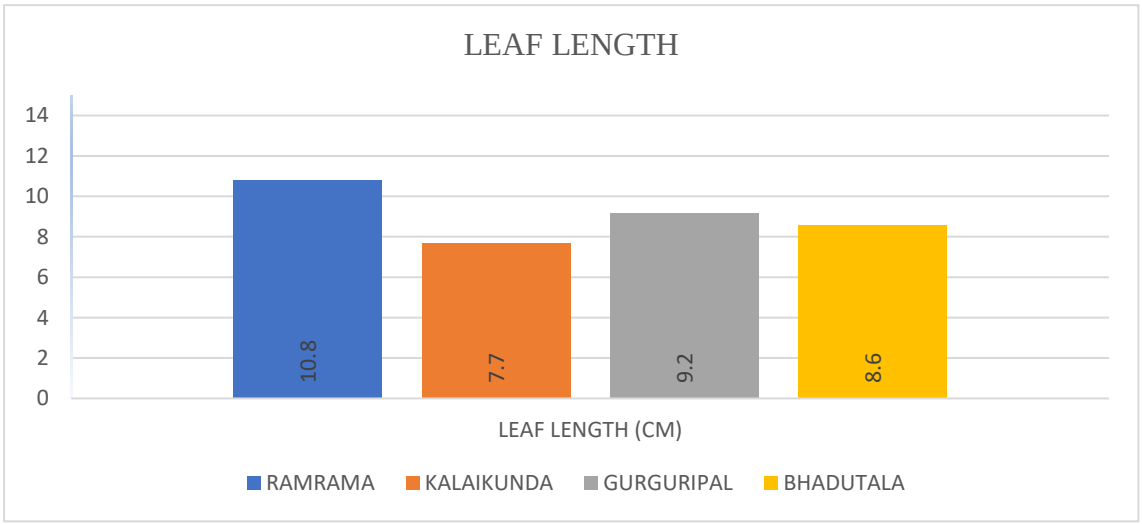

Fig 4.3c: Graphical representation of average leaf width of *Crotalaria pallida* Ait. in four Forest Beat.

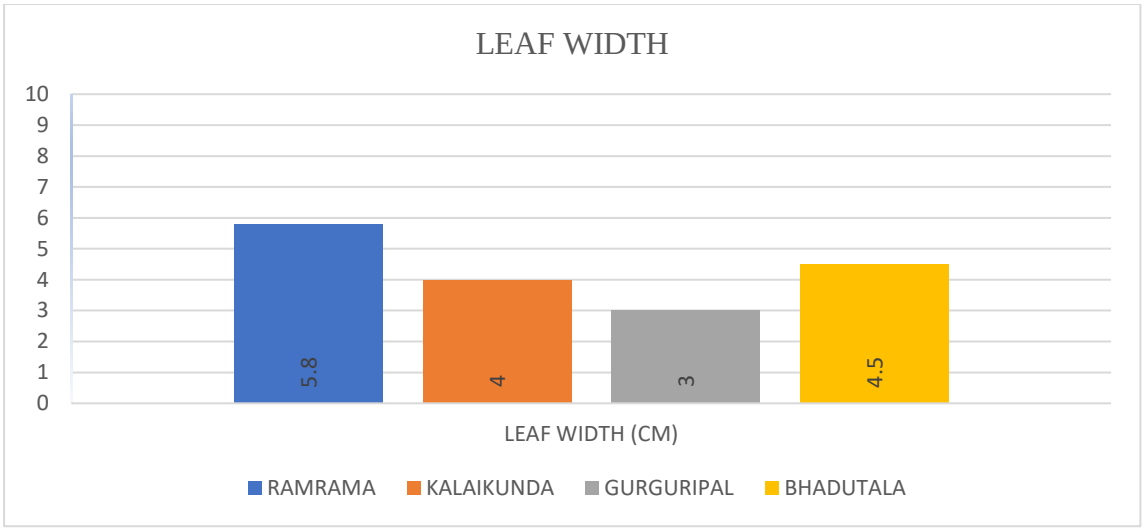

Fig 4.3d: Graphical representation of average fruit weight of *Crotalaria pallida* Ait. in four Forest Beat.

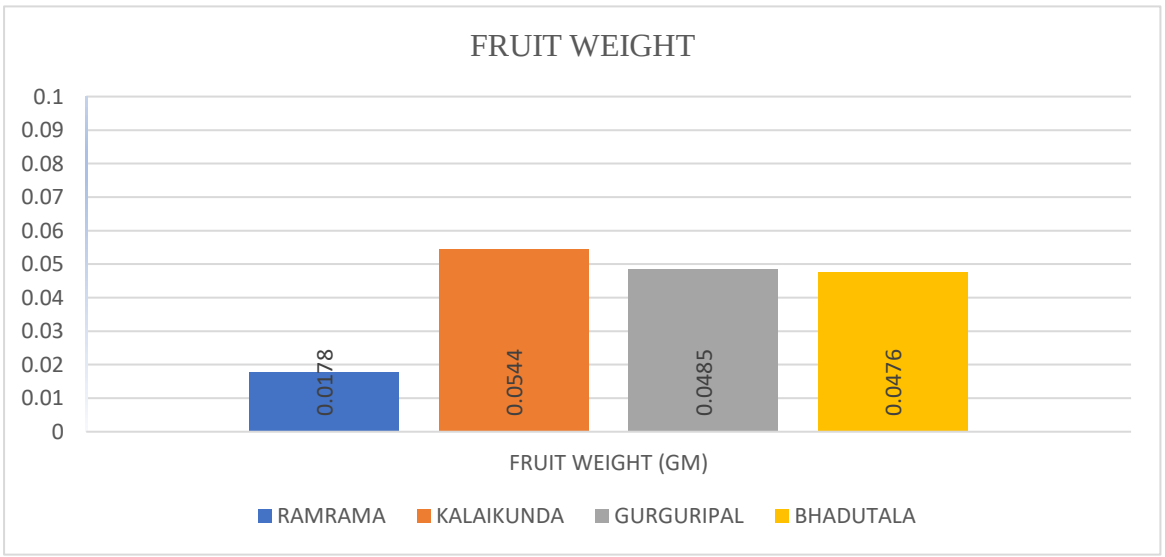

Fig 4.3e: Graphical representation of average fruit size of *Crotalaria pallida* Ait. in four Forest Beat.

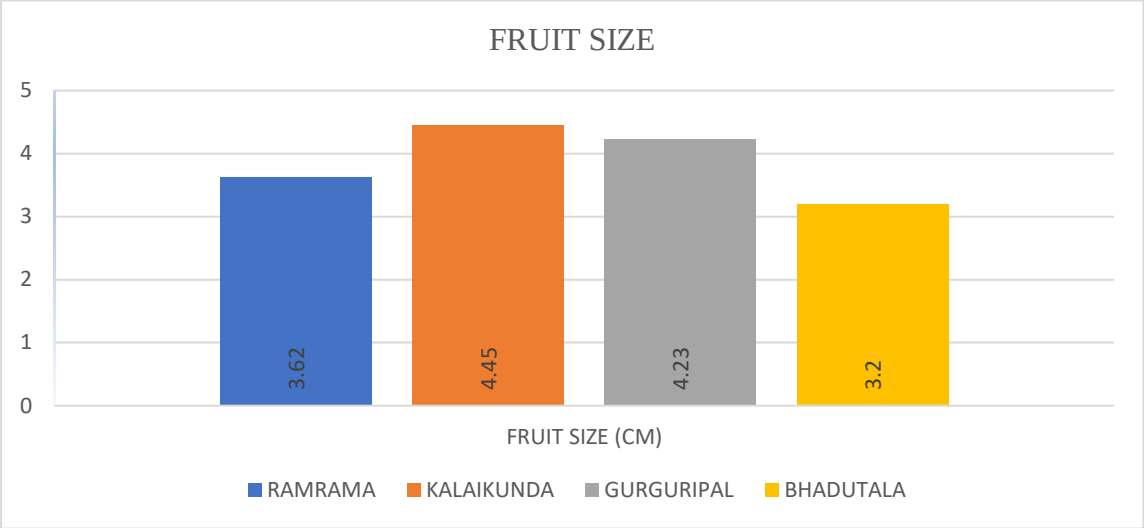

Fig 4.4a: Graphical representation of Average DNA amount of *Crotalaria pallida* Ait.

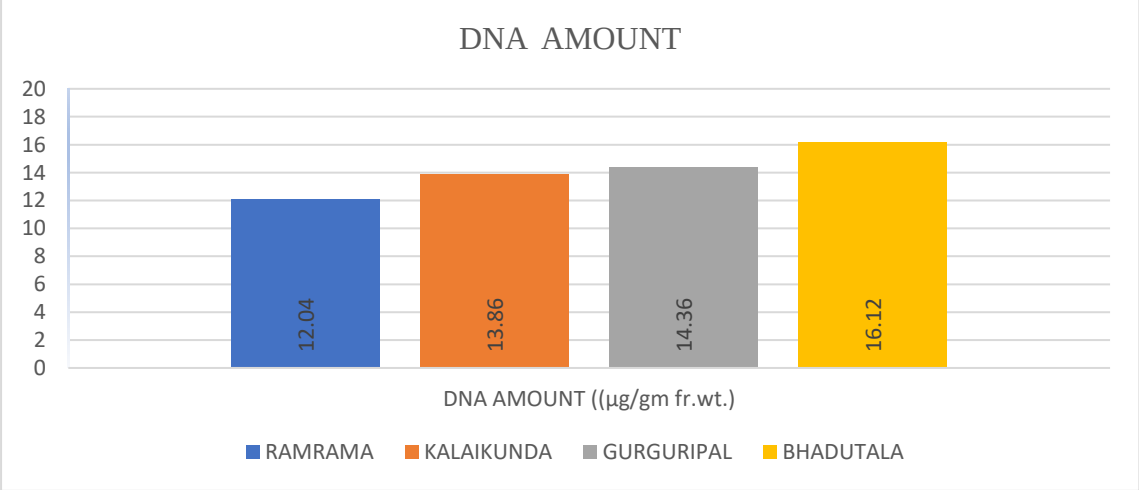

Fig 4.4b: Graphical representation of Average RNA amount of *Crotalaria pallida* Ait.

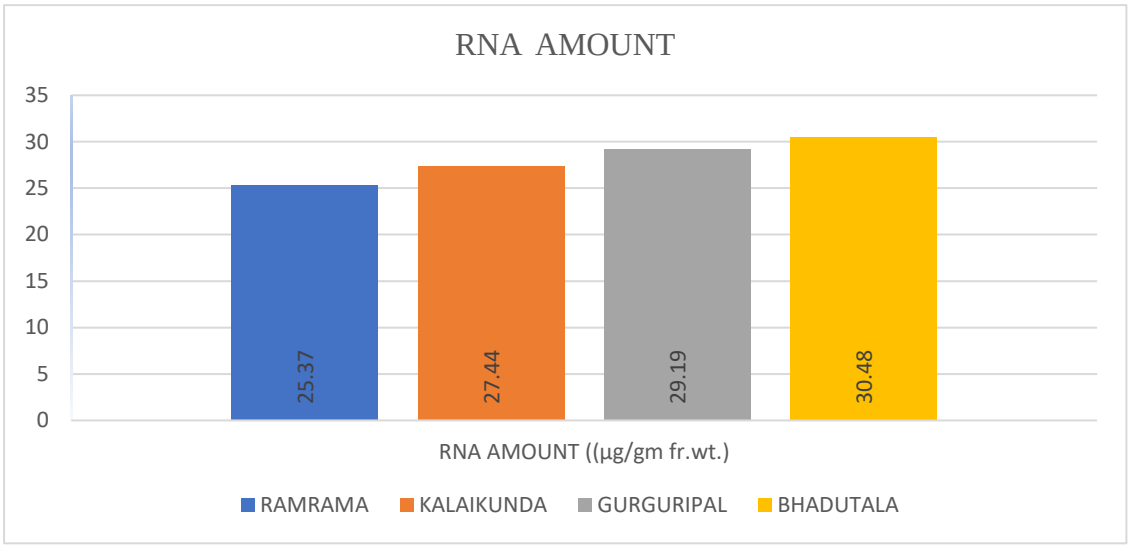

Fig 4.4c: Graphical representation of Average Protein amount of *Crotalaria pallida* Ait.

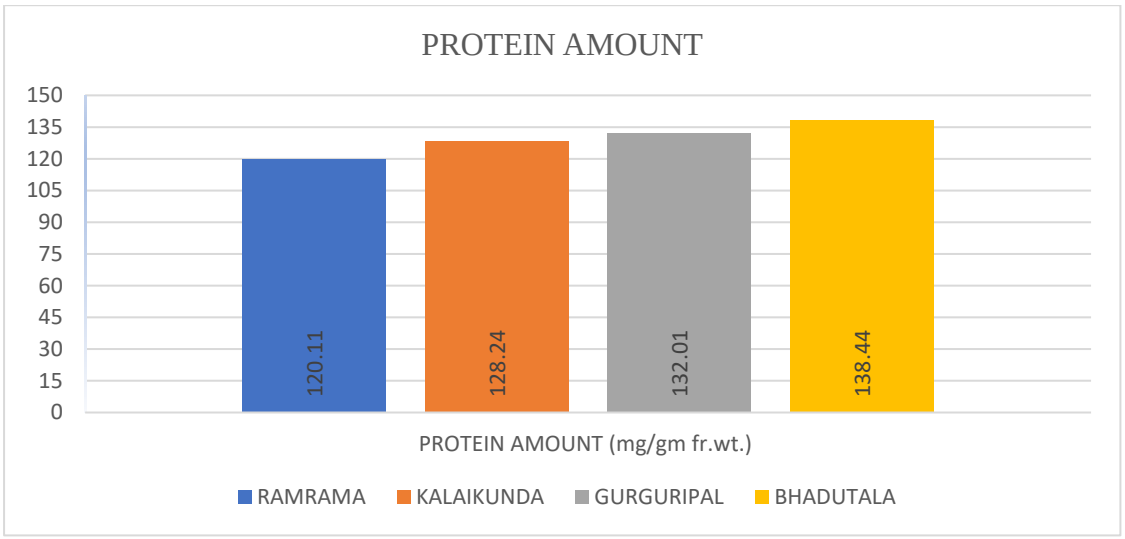

Fig 4.5a: Graphical representation of average height of *Ocimum canum* Sims in four Forest Beat.

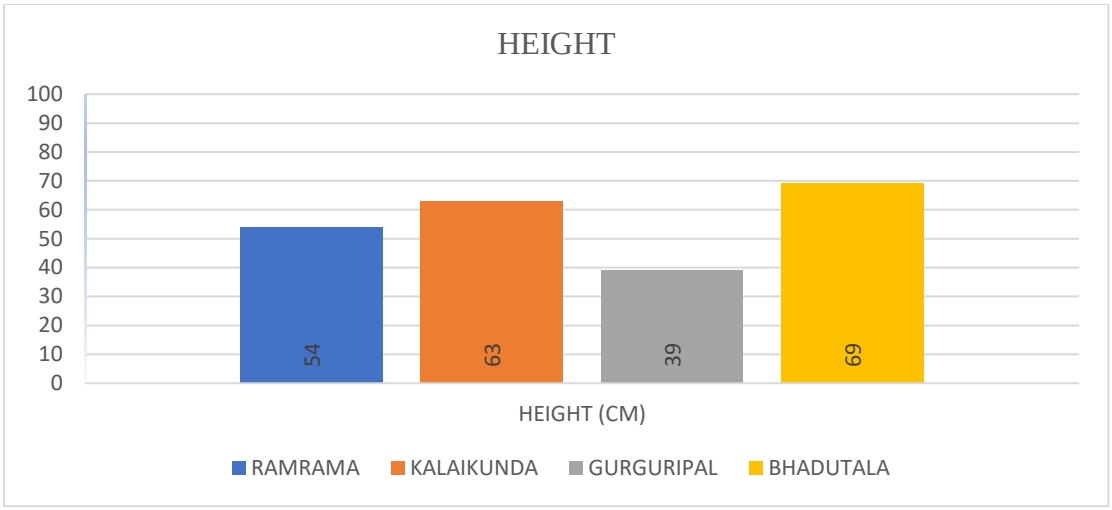

Fig 4.5b: Graphical representation of average leaf length of *Ocimum canum* Sims in four Forest Beat.

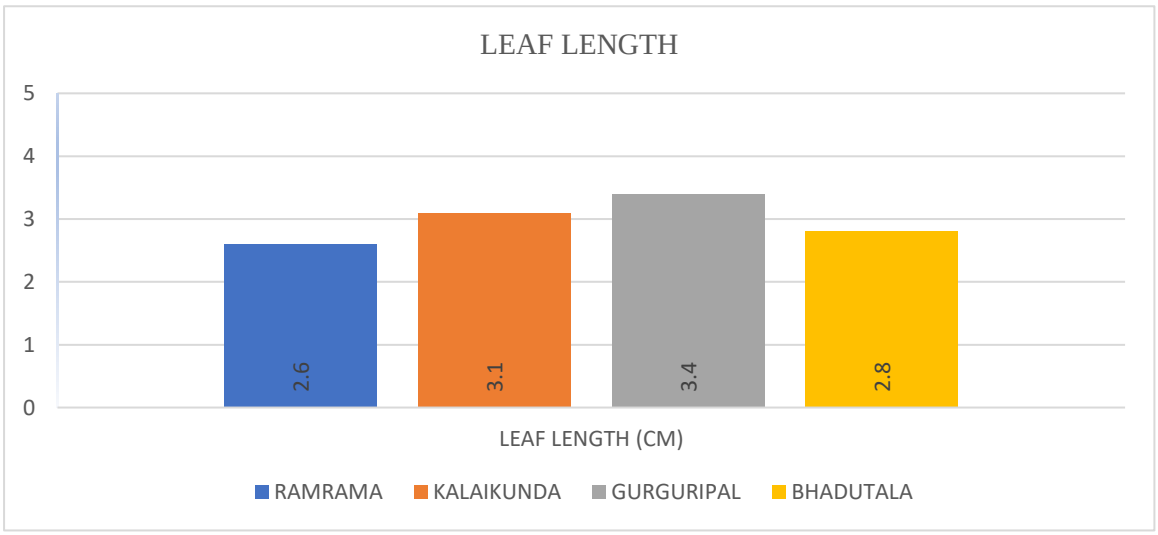

Fig 4.5c: Graphical representation of average leaf width of *Ocimum canum* Sims in four Forest Beat.

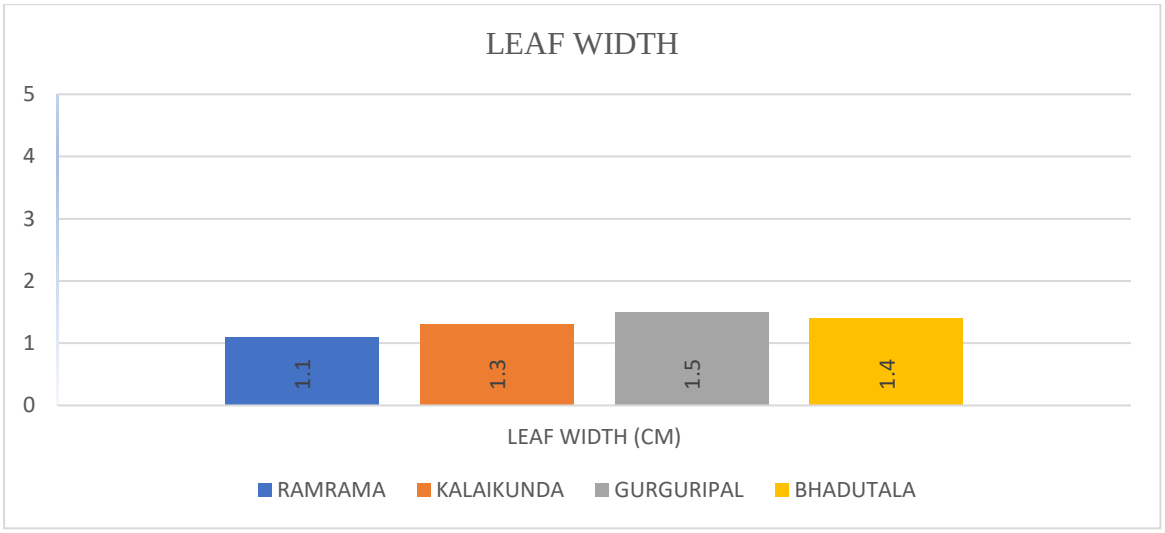

Fig 4.5d: Graphical representation of average fruit weight of *Ocimum canum* Sims in four Forest Beat.

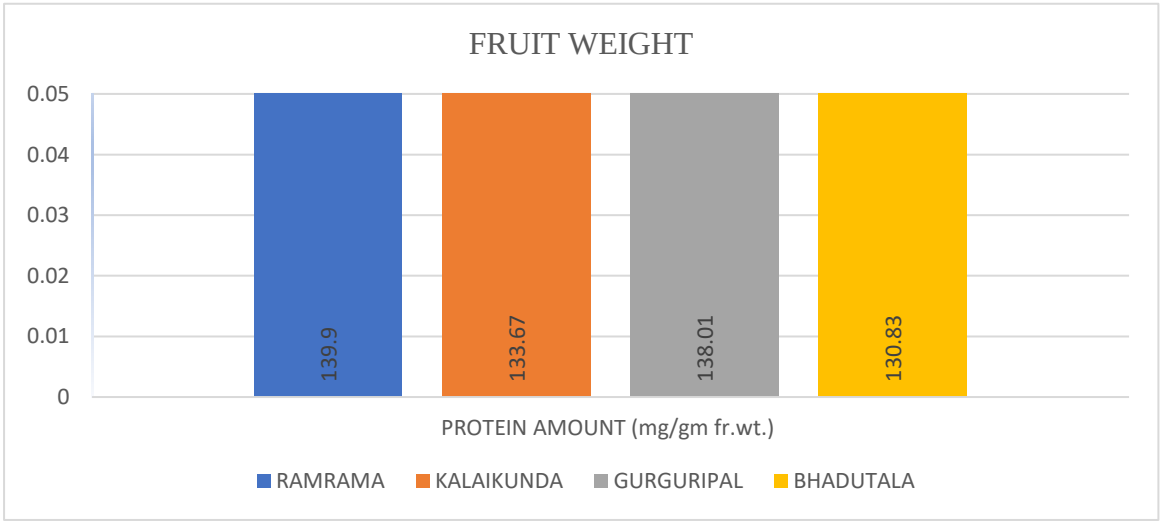

Fig 4.6a: Graphical representation of Average DNA amount of *Ocimum canum* Sims.

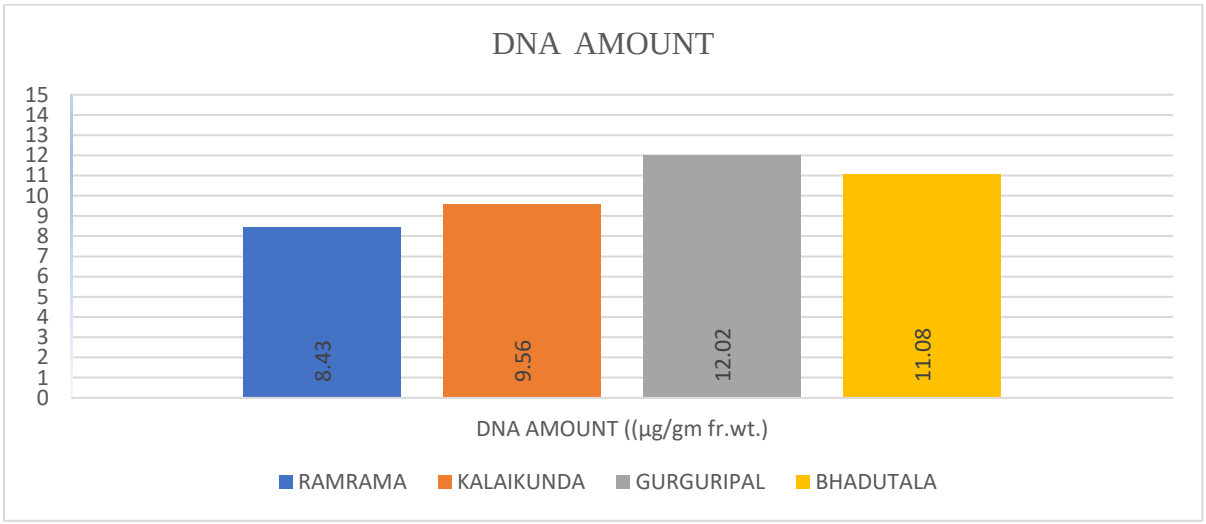

Fig 4.6b: Graphical representation of Average RNA amount of *Ocimum canum* Sims.

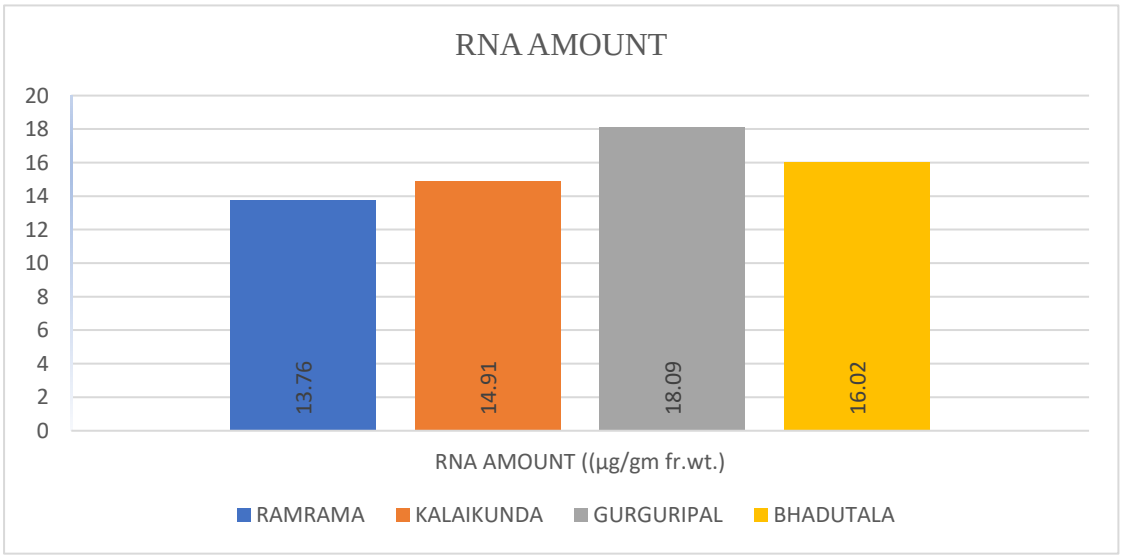

Fig 4.6c: Graphical representation of Average Protein amount of *Ocimum canum* Sims.

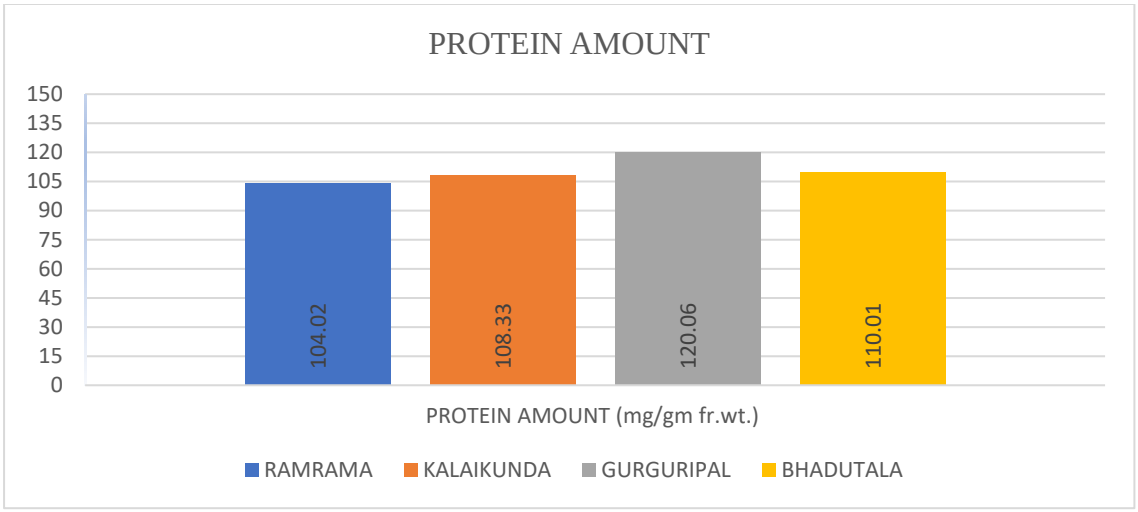

Fig 4.7a: Graphical representation of average height of *Tephrosia purpurea* Linn in four Forest Beat.

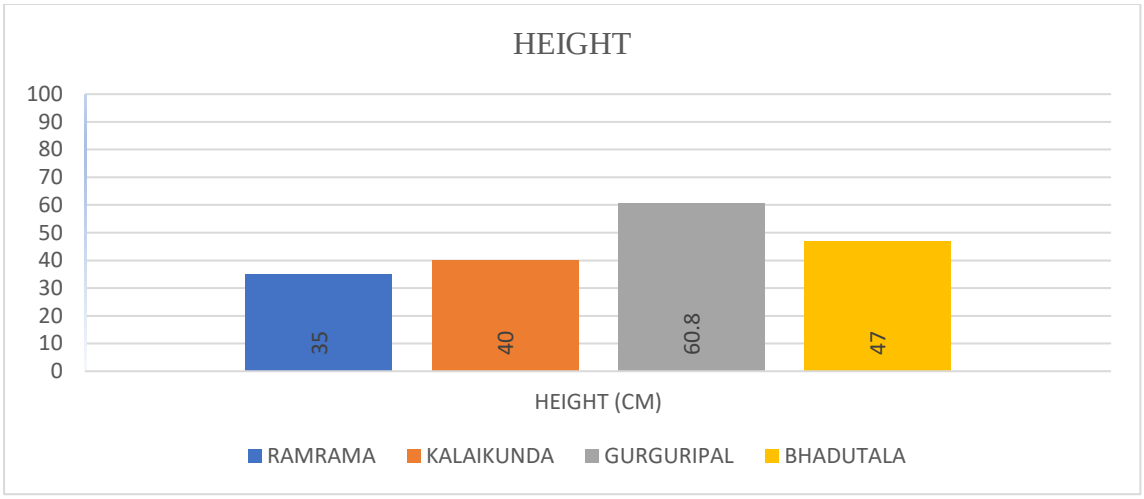

Fig 4.7b: Graphical representation of average leaf length of *Tephrosia purpurea* Linn in four Forest Beat.

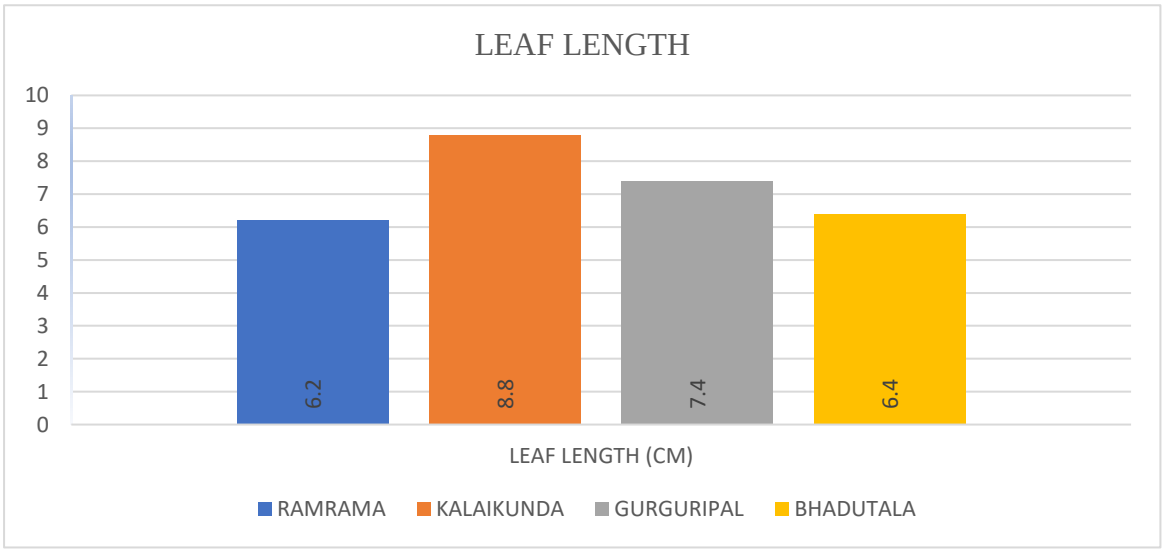

Fig 4.7c: Graphical representation of average leaf width of *Tephrosia purpurea* Linn in four Forest Beat.

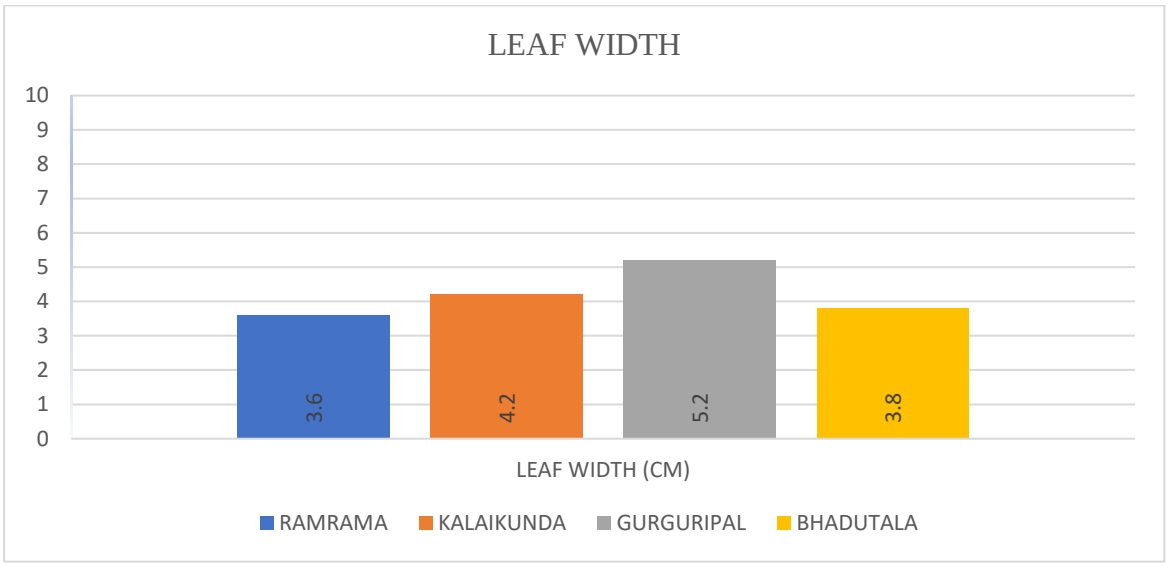

Fig 4.7d: Graphical representation of average fruit weight of *Tephrosia purpurea* Linn in four Forest Beat.

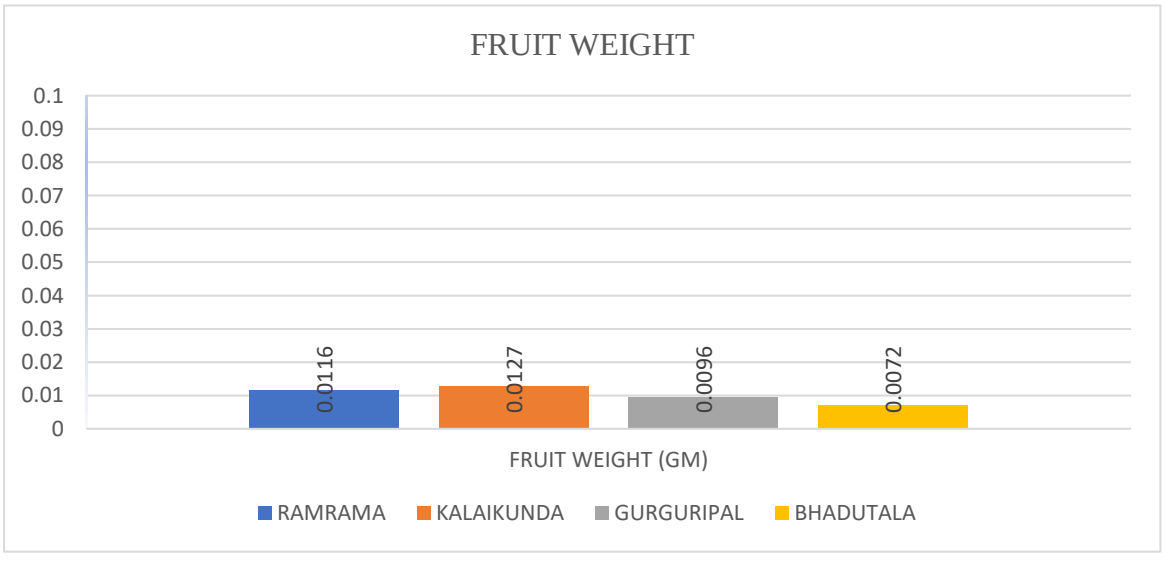

Fig 4.7e: Graphical representation of average fruit size of *Tephrosia purpurea* Linn in four Forest Beat.

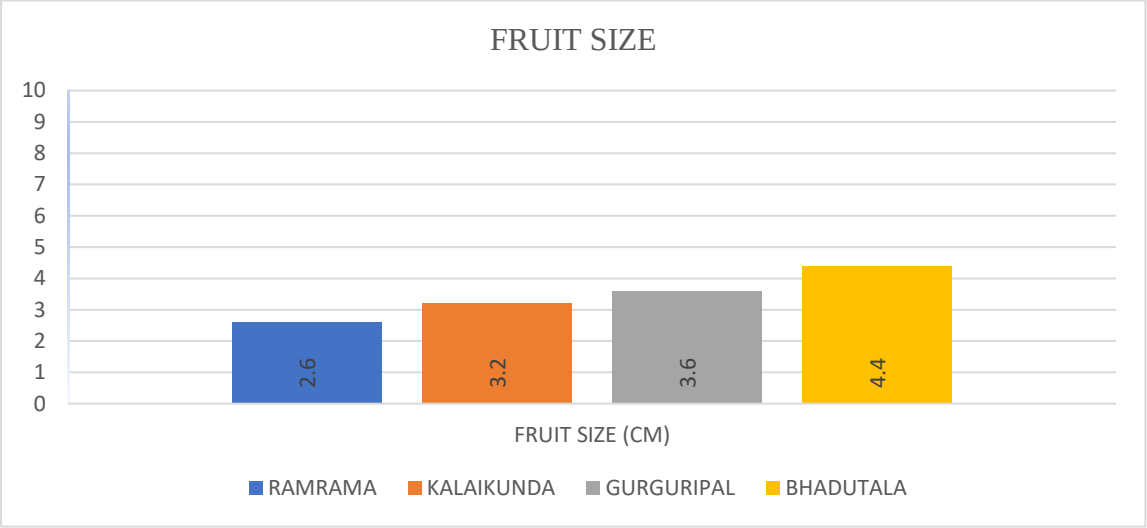

Fig 4.8a: Graphical representation of Average DNA amount of *Tephrosia purpurea* Linn.

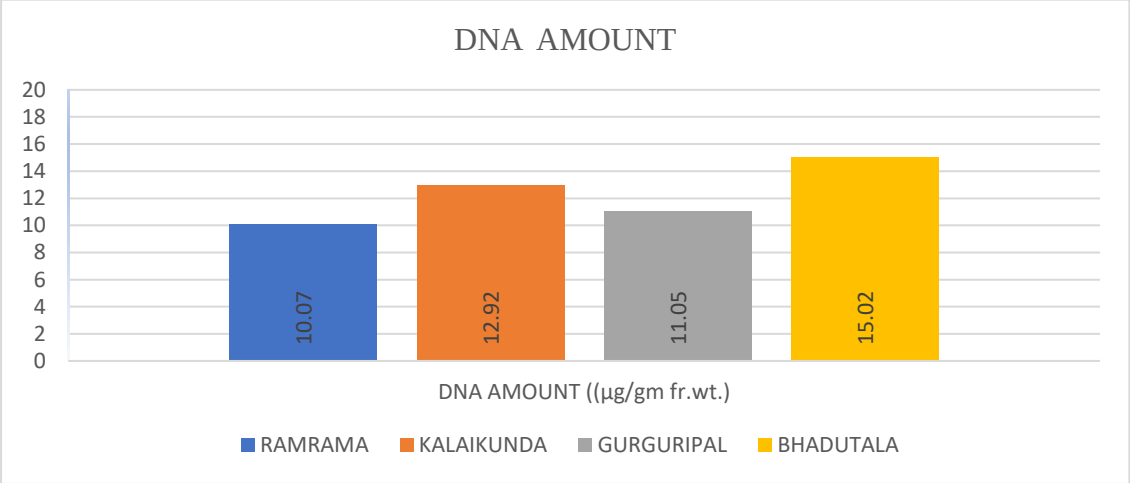

Fig 4.8b: Graphical representation of Average RNA amount of *Tephrosia purpurea* Linn.

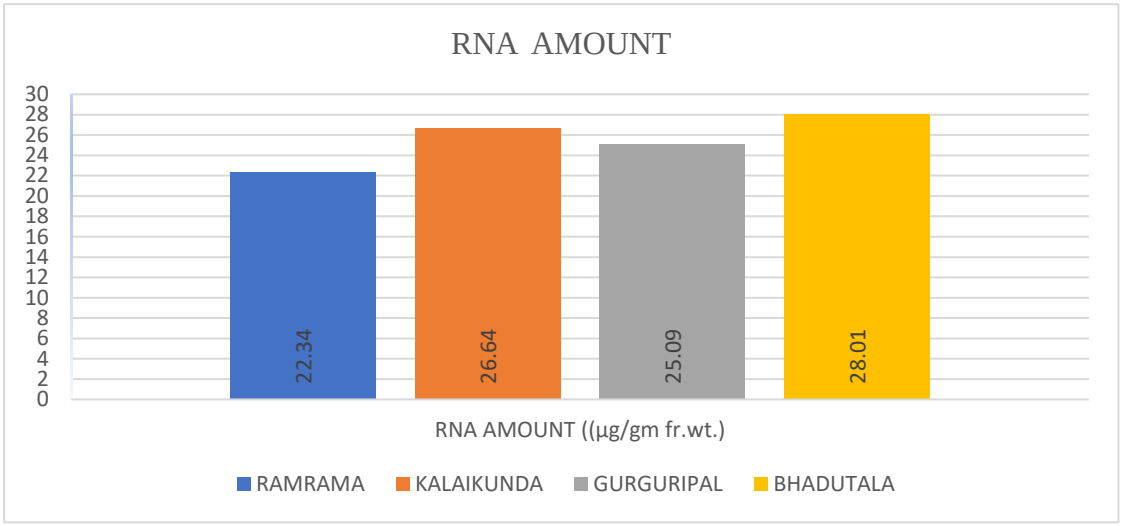

Fig 4.8c: Graphical representation of Average RNA amount of *Tephrosia purpurea* Linn.

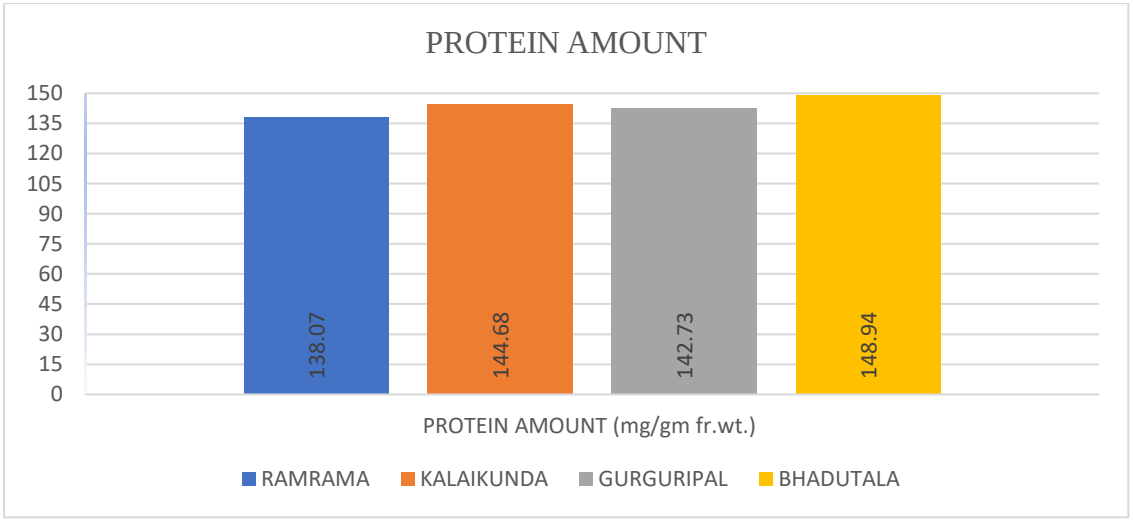

Fig 4.9: Mature fruit of *Lantana camara* Linn.

Fig 4.10: Mature fruit of *Crotalaria pallida* Ait.

Fig 4.11: Mature fruit of *Ocimum canum* Sims.

Fig 4.12: Mature fruit of *Tephrosia purpurea* Linn.

Fig 4.13: Leaves of *Lantana camara* Linn.

Fig 4.14a: Leaves of *Crotalaria pallida* Ait.

Fig 4.14b: Leaves of *Crotalaria pallida* Ait.

Fig 4.15: Leaves of *Ocimum canum* Sims

Fig 4.16: Leaves of *Tephrosia purpurea* Linn