III RESULTS AND DISCUSSION

1. Meiotic Study :

Genus Tribulus belonging to the family zygophyllaceae has 25 species distributed in the warmer dry regions of old world. The genus was first described by Linnaeus is characterised by pinnately compound leaves, leaflets having markedly unequal bases, presence of intrastaminal glands and the fruit being a capsule at maturity, separates into 5 bony, spiny, tubercled or winged mericarps (Nayar & Giri, 1982). The fact that it is confine to old arid region itself is indicative of its restricted distribution, possibly delimited by ecogeographical requirement. That means the genetic variation in genus does not appear to be wide. The chromosome number in the genus Tribulus especially Tribulus terrestris was reported by Nigodi (1939), describing from old world tropics as X = 12. However, till 1971 Shukla studied Tribulus terrestris complex noting its distribution in different habitats and considered two distinct forms in it. Neither genetical nor cytological work is hitherto known. However, he used the term complex on the basis of s[illegible]ght flora variation, which he attributed to the genetic variation. According to him one form has a long style while the other has short style. The flowers

of short styled plants are smaller with small pedicels and the long styled form are larger with long pedicels. He recorded these observation after field study. Further Shukla (1971) collected seeds of these two forms and sown them separately and examined upto third generation. When no variation even in F_3 generation was noted, he concluded that variations are genetically maintained. However, Nair and Giri (1982) examined good number of Indian specimens of different habitats and concluded that long styled form is Tribulus lanuginosus while the short styled form is Tribulus terrestris. This agrees with the diagnosis of Boissier (1867), one of the earliests to describe genus Tribulus. What concerns is not the distinction between Tribulus lanuginosus and T.terrestris, but the term complex which is cytotaxonomical word and mostly attributed, not to genetic variation, but, variation induced by the chromosome number. Now it is very clear that Shukla was missled in identifying the two distinct species as two genetic forms of same species; nevertheless the two species grow side by side. What is noteworthy here is, in this region of Maharashtra only T.terrestris L. has been growing. Since seeds have very high dormancy of germination is a great problem. And therefore, in order to know the chromosomal variation the meiotic study in this species has been carried out.

What is striking here is the plants raised in the garden from seeds collected from various regions, started flowering

Plate 6.1 : Meiocyte of pollen mother cell exhibiting 12 II's (6.3 x 100 x).

6.2 : Photograph of meiocyte of PMC of Tribulus terrestris showing nucleolar organiser (3.2 x 100 x).

6.3 : Photograph showing meiotic metaphase-I showing in pollen mother cell showing precocious movement of chromosomes, (6.3 x 100 x).

6.4 : Photograph showing anaphase I of pollen mother cell of Tribulus terrestris (6.2 x 100 x).

Plate 7.1 : Photograph showing Metaphase-I in pollen mother cell of Tribulus terrestris L. (6.2 X 100 x)

7.2 : Photograph showing Metaphase-I in pollen mother cell of Tribulus terrestris (6.2 X 100 x).

7.3 : Meiocyte showing Anaphase II of meiosis note, no cytokinesis is seen after meiosis-I. (6.2 x 100 x).

7.4 : Metaphase-II of pollen mother cell (6.2 x 100 x). Note - orientation of metaphase plate in plain of division perpendicular to the first meiosis (6.2 X 100 x).

Plate 8.1 : Photograph exhibiting telophase II of meiosis note that one of the diad exhibiting disjunction of chromosome after meiotic metaphase II. (6.2 x 100 x).

Plate 9.1 : Plate showing low power magnification acetolysed pollen grains of Tribulus terrestris L. (100 x.)

9.2 : High magnified picture of pollens taken under scanning electron microscope (200 x).

9.3 : More magnified picture of pollens showing under scanning electron microscope (100 x).

9.4 : Photograph of pollen grain under SEM dark field illumination (600 x).

Plate 10.1 : Moderately magnified picture of pollen grain of Tribulus terrestris seen under SEM (200 X).

10.2 : Pollen grain of Tribulus terrestris exhibiting colpi (6.3 x 100).

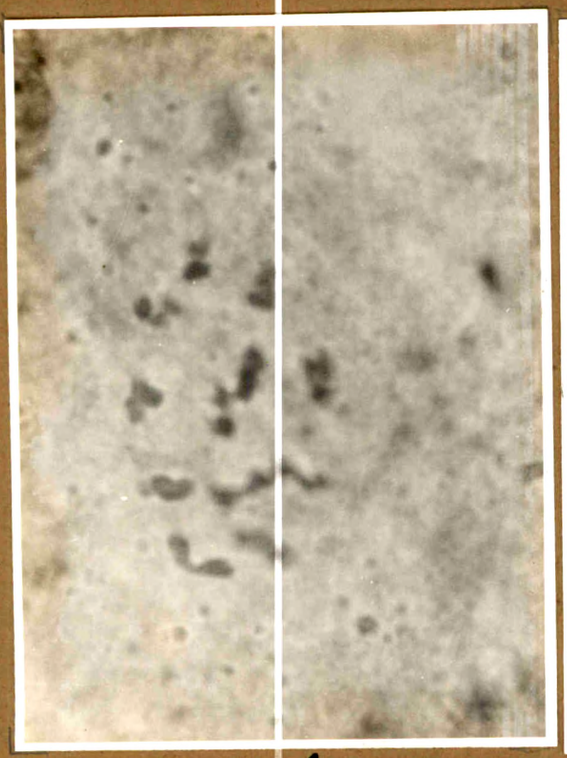
10.3 : Photograph under SEM exhibiting sulcus. (100 X).

10.4 : Reticulate exine ornamentation as seen in the SEM (4000 X).

Plate 11.1 to 11.4 :

Magnified picture of exine ornamentation as seen under SEM (4000X, 2000X, 4000X, 4000X, respectively).

PLATE - 6



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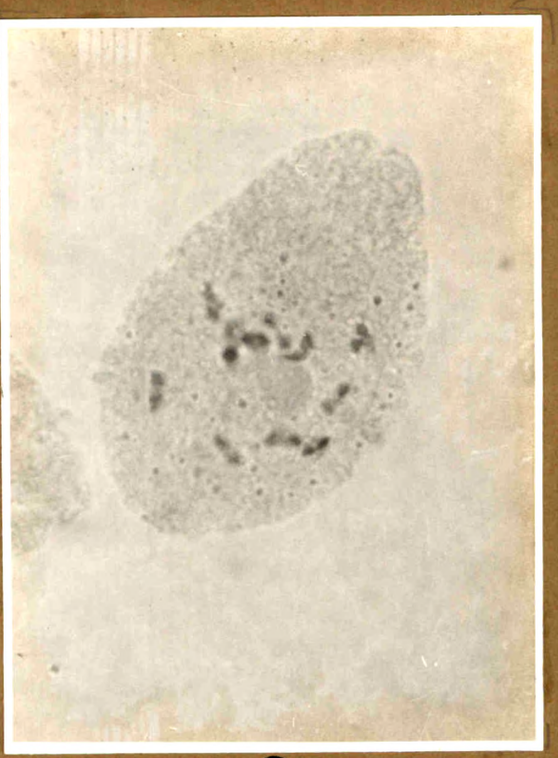

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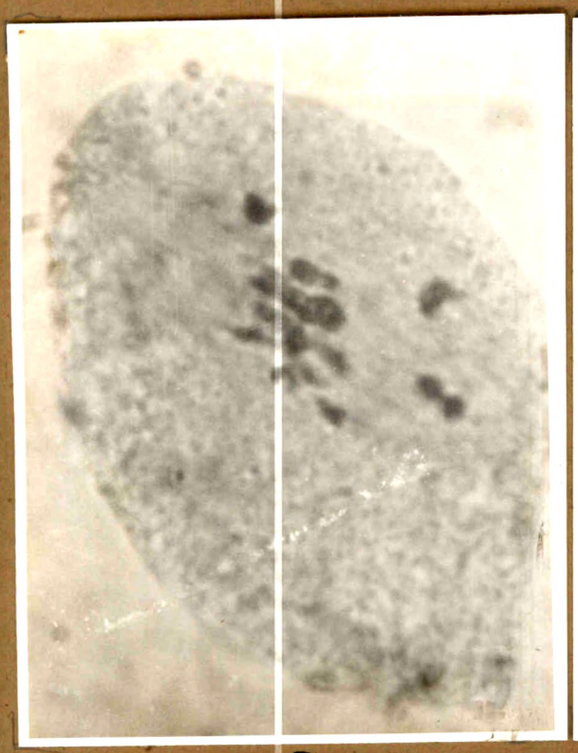

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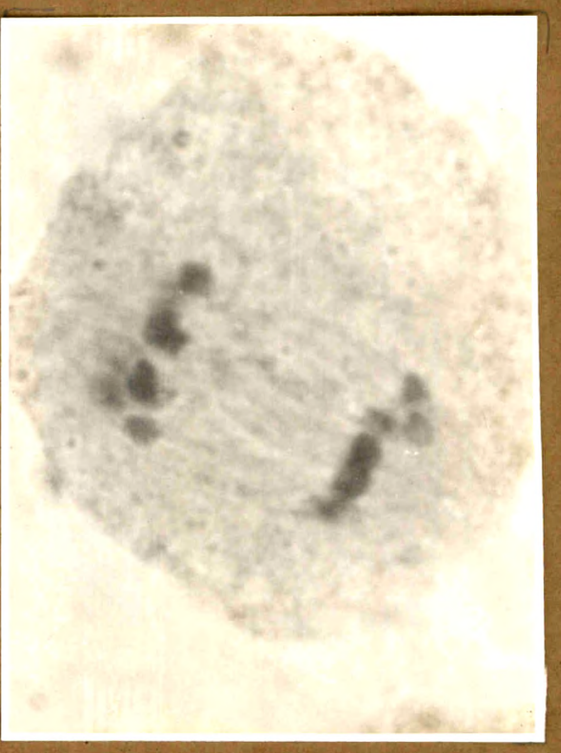

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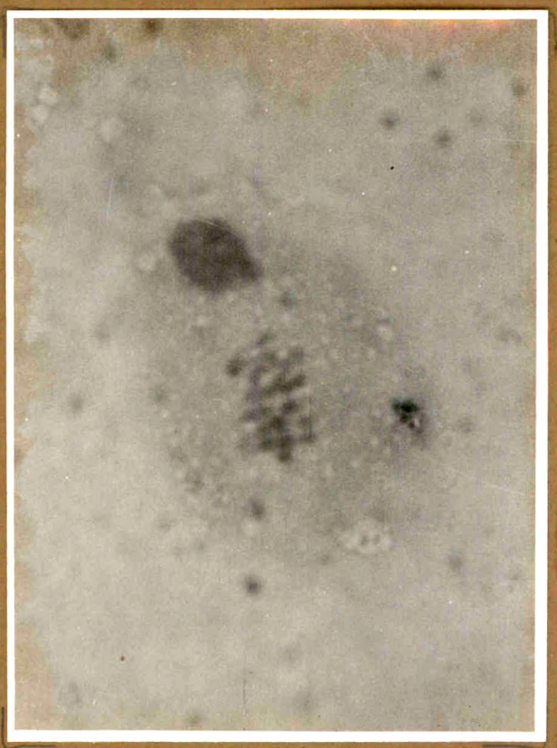
PLATE - 7



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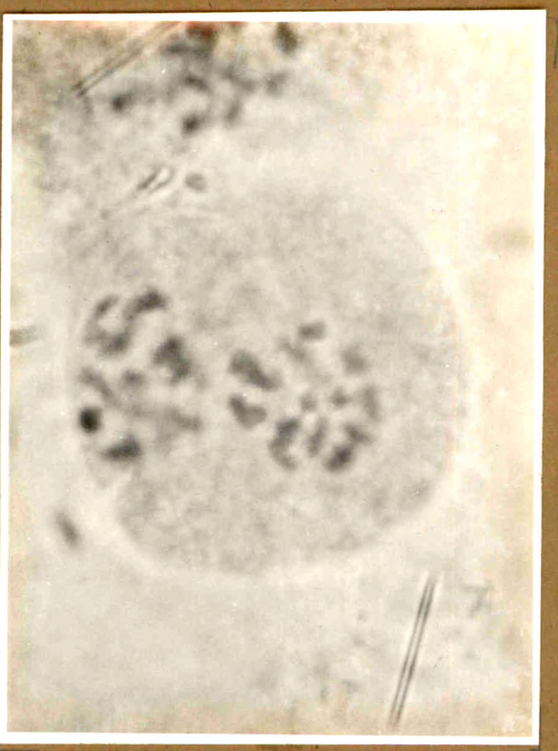

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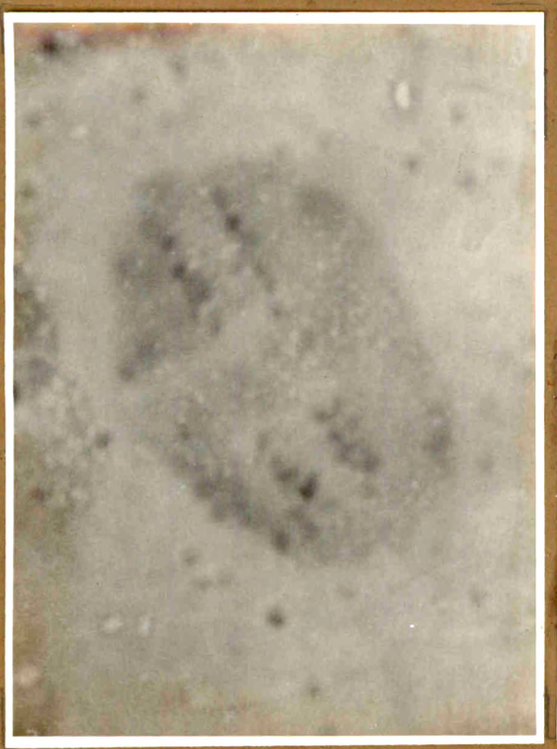

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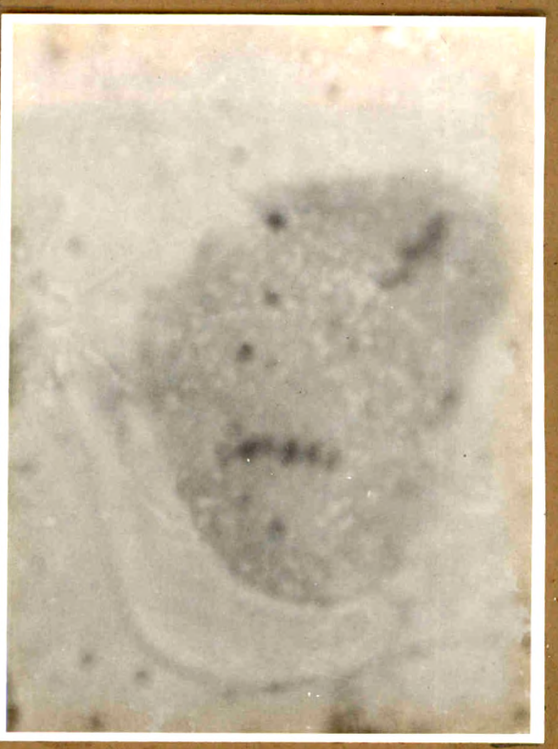

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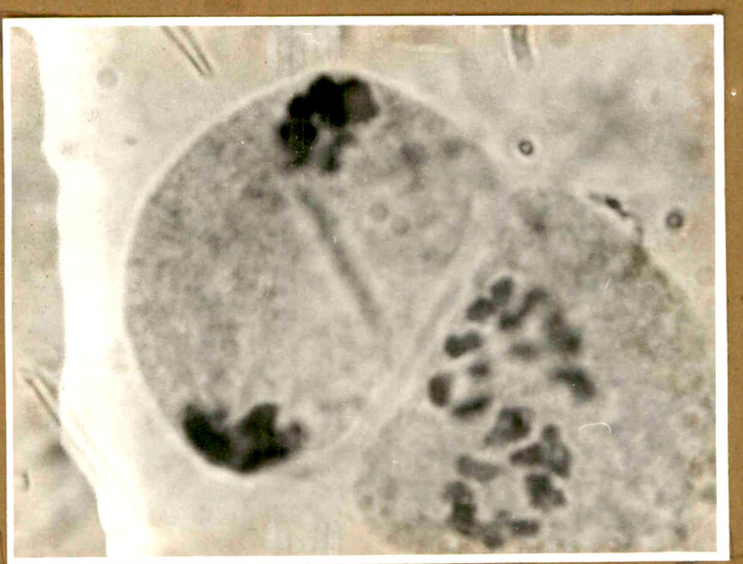
PLATE -8



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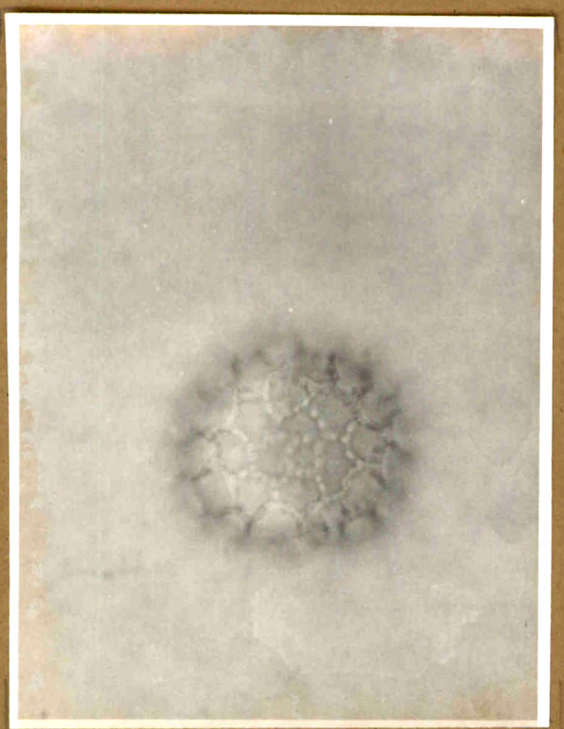

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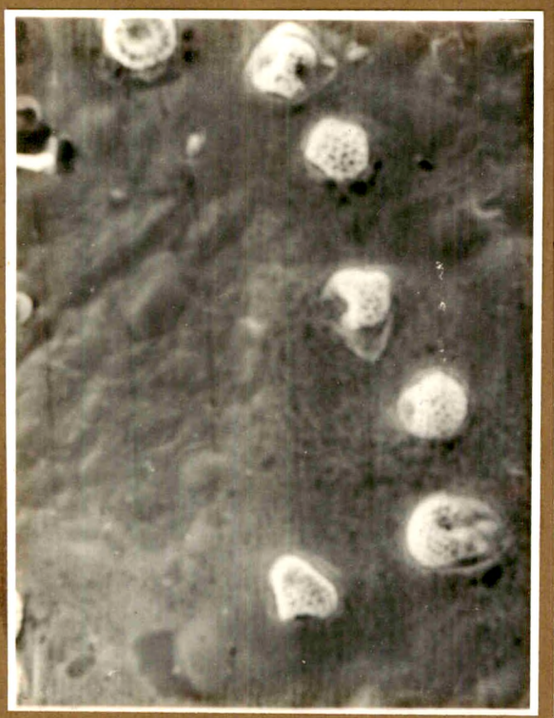

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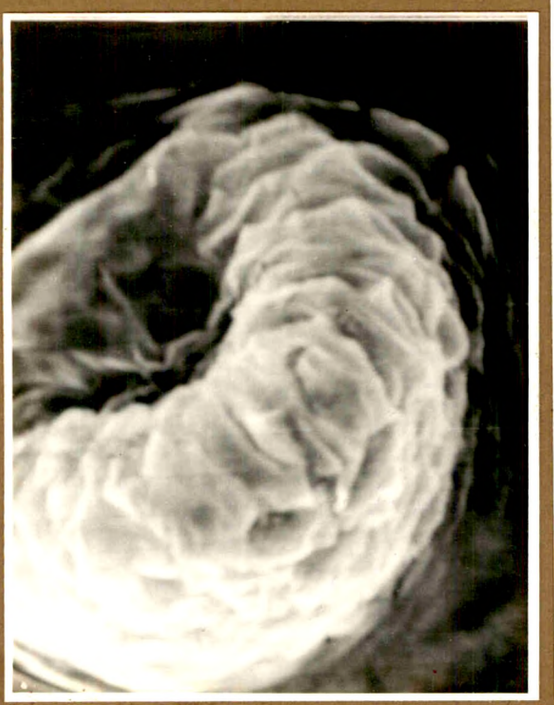

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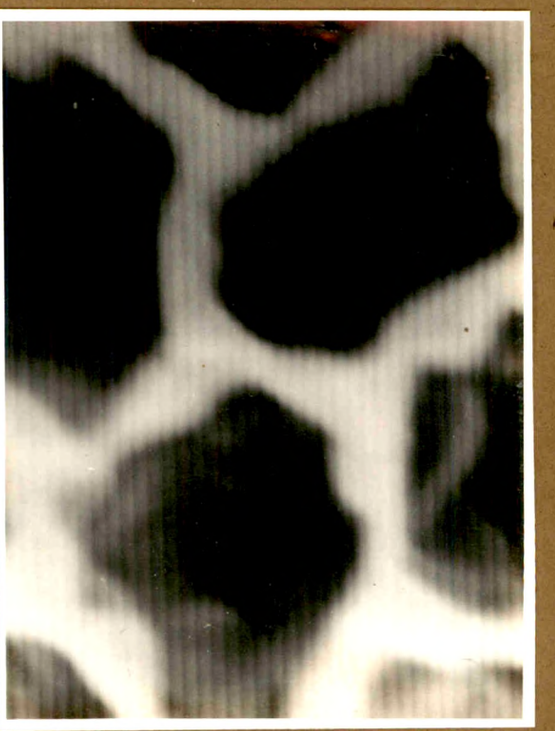

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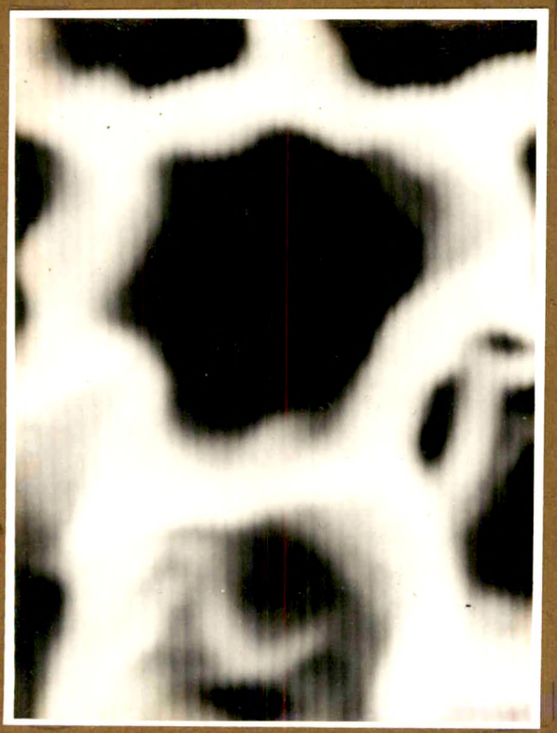
PLATE-11



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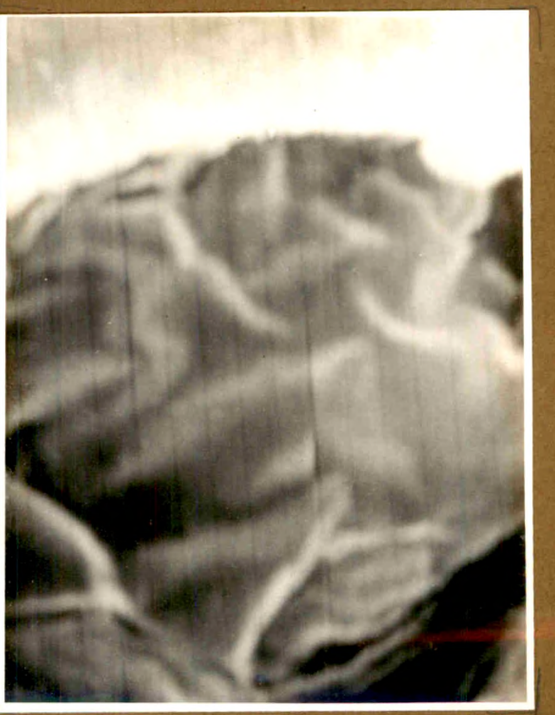

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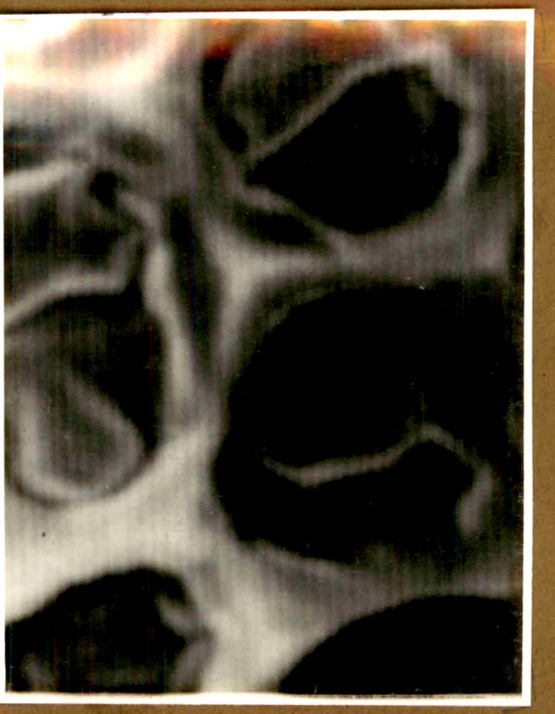

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uniformely and all of them exhibit distinctly '12' bivalents (Plate 6.1 & 6.2). However, some of the bivalents are relatively large though no greater variation in chromosome morphology can be envisaged; with result the organisation, orientation and distribution of chromosome on the equator of meiotic metaphase isnormal (Plate 6.3). The typical of dicot the PMC are relatively small with comparatively small number of chromosomes. The PMC exhibit rapid advancement of prophase-I, the pairing of homologous chromosomes in meiosis appear to be normal. The fact that all the bivalents exhibiting distinct chiasma formation irrespective of their size (Plate 6.4), and, rarely if any, exhibit asynaptic condition the resultant genetic segregation of characters is ensured. With absence of karyomorphological study of somatic chromosomes, though it is difficult to identify chromosome pairs, having the nucleolar organiser, it distinctly exhibits at least two bivalents and their possible association with organization of nucleolus (Plate 7.1).

The characteristic feature of meiosis in the species is the chromosomal orientation in meiotic metaphase-I. The bivalents orient on the equator in meiotic metaphase-I, which is prompt and normal (Plate -7.2). However, when the homologues start disjoining during anaphase, some exhibit more faster movement towards pole than the others. Nonetheless this rate of movement does not bring about failure of disjunction

and eventually no legards or false univalents could be seen on equator (Plate 7.3).

After the first telophase the diad formation is not marked by cytokinensis. Even at the first reduction division the sister chromosomes that have reached the pole slowly revolved in the plane 90°C to the first plane of division (Plate 7.4). It is the first perfect form of metaphase-II. In other word the prophase I is not discernable at all.

Occasionally not the first, but the second division fails with the result the sister chromatids of one of the diads, instead of being distributed into two products they remain on the equator, reflecting on distinct failure in meiosis.II. In one of the two products of meiosis I (Plate 8.1), this disjunction is likely to induce ploidy at gametic level itself. However, the stainability test to determine fertility of pollens studied, clearly indicate that there are no abortive pollens. This reflects on the fact that such gametes with anomalous number of chromosomes are possibly eliminated during the early differentiation, from tetrad to two celled pollen which would be certainly interesting to examine.

In conclusion, it can be said that, though not much cytological variation can be seen to identify certain marker characters it can be very well induced by means of irradiation. In a way normal meiotic pattern reflects on the genetic

stability of the plant. For the purpose of identifying the isogenic line, it is quite a steady character. Another, important noteworthy point is the distribution of Tribulus terrèstris and T.lanuginosus indicate that their ecogeographical requirement is same (Nayar and Giri, 1982). The two species are known to grow side by side and have subtle differences in morphology of flower. The chromosome number is reported as n = 12. Therefore, it is quite predictable that they may have free hybridization and intermediate stage might be evolving, such transgressive hybridization is not unknown phenomenon in the species population (Stebbins, 1950).

2. Pollen Study :

i) Pollen morphology : In order to know whether there is greater genetic variation, this study has been carried out. It is needless to say that examination of pollen morphology and fertility establishes a primafecia about the genetic variability or cytological variability of species. If the pollen grains have uniform side, it indicates that there is no greater cytological variation. It also indicates that possibility of interspecific hybridization is rare. Such variation is sharply distinguish when the pollen grain were examined under scanning electron microscope (SEM). It has been established phenomenon that the ornamentation of sporoderm or sporoderm stratification or sporoderm arm is a

characteristic feature of species and is consistents with it. With this view the study has been under taken in Tribulus terrestris L. The samples examined for pollen grain size, class, shape, ornamentation etc. in Tribulus terrestris L. as given in the table and the photographs showing the nature of pollen can be seen from the plates (9,10,11). As given in the table the size of pollen grain has been worked out after examining '25' different samples. The average diameter of the pollens is 82.6 µm ± 1.33. This reflects that there is no much variation in pollen size dispite the fact that certain abnormal division in Meiosis II does occur (discussed elsewere).

The average total area (l x b) is 6639.73 µm ± 130.64. The pollen grains are prolate spheroidal with P/E ratio 1.1 The size 82.6 µm lead to categorise these pollens are fairly large 'MA' is magnae as revealed by the scanning electron microscope (SEM) the exine sporoderm stratification is distinctly straiate, reticulate, polyforate. Aperture is circular, sulcus, ragged

The earlier description of pollen grain of Tribulus terrestris is not possibly based on SEM picture
The high resolution of pollen grain under SEM has revealed the clear-cut picture of its ornamentation as well as nature of pore as given in the Plate .

Table No.1

Summary of pollen morphological features as revealed under SEM in Tribulus terrestris L.

Genus species	*Pollen grain size µm	*l x b average µm	*Ratio of $\frac{P}{E}$	Pollen class	Pollen shape	Ornamentation
Tribulus terrestris L.	82.6 (1.33)	6639.73 (130.64)	1.1 (0.0009)	MA	Prolate spheroidal	Polyforate reticulate

* Means of 25 readings.

Values in paranthesis are S.E.

3. Nitrogen Content :

The plant is herbaceous and prostrate in habit. Being confine to tropical region is exposed to vagaries of nature. It grows naturally in the dry regions especially on road side, pavements and on the lateritic soil which is naturally poor in nitrogen content. Because of this the plants are also poor in nitrogen. In other words, though it is a herbarious plants since it grows in low rain fall area in it's habit, it is similar to that of grass, which posses greater ability to retain moisture. In other words it is not a nitrophilous plant. However, nitrogen is very important nutrient which profoundly influences any sink product. In this case it is flavonoid, glycosides which is a sink product. Therefore, the plant has been examined for nitrogen content both in leaves as well as in seeds. The results are given in Table 2.

What we see from the table is that the leaves have 33.8 mg/100 g nitrogen of dry tissue while seed 13.02 mg/100 g nitrogen dry tissue. These values as worked out by Kjeldahl distillation method reflects on poor content of nitrogen in both leaves as well as seeds. As discuss earlier whatever may be the sink content nitrogen plays profound role in determining it in the present plant. The sink is mainly flavonoid, glycosides, and then some sugars. The former is mainly derived from aromatic amino acid pathway such as phenylalanin through shikimic acid where the carbon skeleton comes from the photo-

Table No.2

Nitrogen content of leaf and seed of
Tribulus terrestris L.

Genus species	Nature of material	N_2 content mg/100 g
Tribulus terrestris L.	Leaf	33.8
	Seed	13.02

synthesis and the amino group by transamination. Depending upon the nature nature of plant whether is leguminous or non-leguminous the nitrogen made available in the system. In conclusion it may be said that the poor nitrogenous soil which sustains the plants limit even the sink. It is quite that if the nitrogen content of soil is increased or the system is manipulated to take up more nitrogen mediated through mineral it may be able to increase its sink product. As such the plant is C_4 plant which has inherently more efficient photosynthetic system.

4. Flavonoid glycosides content of the plant :

Tribulus terrestris is ~~of~~ well known plant for its medicinal value, especially in India. The old Ayurvedic literature emphasizes its diuratic effect. In fact the Himalayan drug company has certain standard medicines for kidney stone in which the seeds of T.terrestris L. is a major ingredient. This has attracted many biochemist and chemist to study and analyse the ingredients and characterised its chemical nature. One of the pioneers in this direction is Seshadri and his associates who first published comprenssive treatise on flavonoids of fruit and leaves of T.terrestris (Bhutani et al., 1969; N.A.M.Saleh, 1982). The latter authors have identified flavonoid glycosides both in Tribulus terrestris and T.pentandrus. However, Tribulus pentandrus may have a

medicinal value, but it is not widely used for the purpose. However, the Indian flora does not report Tribulus pentandrus to be growing.

Since T.terrestris is adapted to the tropical warm climate it even grows in Rajasthan desert. Autecological study have also been carried out by number of workers (Joshi et al., 1967). However, autecological study of T.terrestris carried out by Joshi et al.,' (1969) did not lead to identify variation found in flavonoid glycosides content of those plants growing at different regions. Bhutani et al. (1969) have identified 4-flavonoid glycosides in the fruits and leaves of this plant where as N.A.M. Saleh have identified as 25 flavonoid glycosides. As it is known that these compounds are secondary metabolites the syntheses take place slowly. Many of them are intermediates substitutes, analogues etc. and their synthesis is profoundly influenced by the external environmental factor such as nutrients, nature of soil etc. As such Gasic et al. (1978) have shown that the micronutrient contents of soil profoundly influence synthesis of the secondary metabolites. Therefore, it was thought worthwhile to investigate the plants growing in different ecological conditions especially the influence of soil factors on flavonoid glycosides synthesis. It was experimentaly examined the variation induced by external application of trace element on glycosides. This aspect has been investigated

Table No.3

Paper cromatographic separation of flavonoid glycosides from leaves and seeds of Tribulus terrestris L. naturally growing in Kolhapur region.

Nature of material	Solvent system used	Rf.value x 100	Identification	*Intensity
Fresh leaves	B:A:W	48	(K)-3-rutinoside	+++
		88	Unknown	++
	CCl_4:C_6H_6:MeOH	27	(Q)-3-rhamnogentiobioside	++
		72	Unknown	++
		91	Unknown	+++
Fresh seed	B:A:W	79	(K)-3-rutinoside	++
		84	(K)-3-D-coumaroglycoside	+++
	CCl_4:C_6H_6:MeOH	60	(I)-glucoside	++
		70	Unknown	+++
Dry seed	B:A:W	87	Unknown	+++
	CCl_4:C_6H_6:MeOH	84	(K)-coumaroglucoside	+++

* \+ = Weak
++ = Strong
+++ = major

Fig.No.1 :

Paper chromatogram showing flavonoid glycosides separation in B:A:W solvent system of Tribulus terrestris (fresh leaves) naturally growing in Kolhapur region.

A -- (K)-3-rutinoside

B -- Unknown.

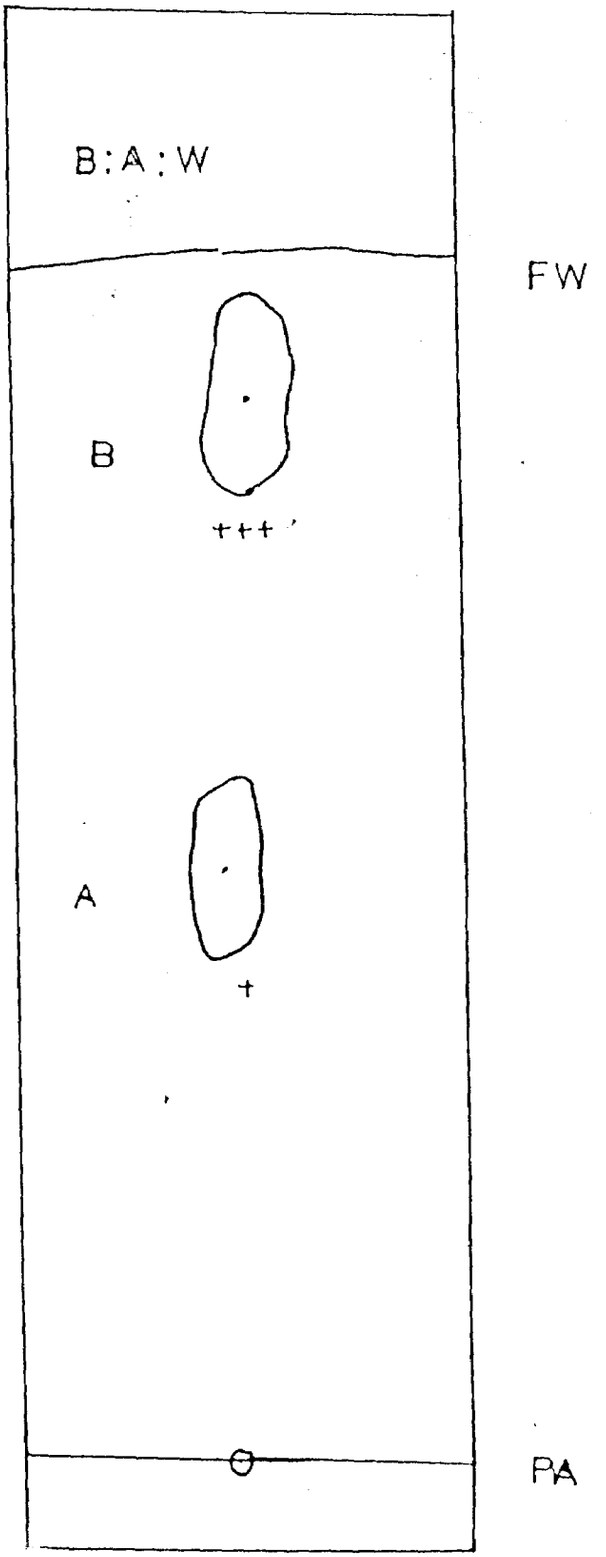

Fig-1

PC

FRESH LEAVES. (gK)

Fig.No.2 :

Paper chromatogram showing flavonoid glycosides separation in CCl_4 : C_6H_6 : MeOH solvent system of Tribulus terrestris L. (fresh leaves) naturally growing in Kolhapur region.

A : (Q)-3-rhamnogentiobioside.
B : Unknown
C : Unknown.

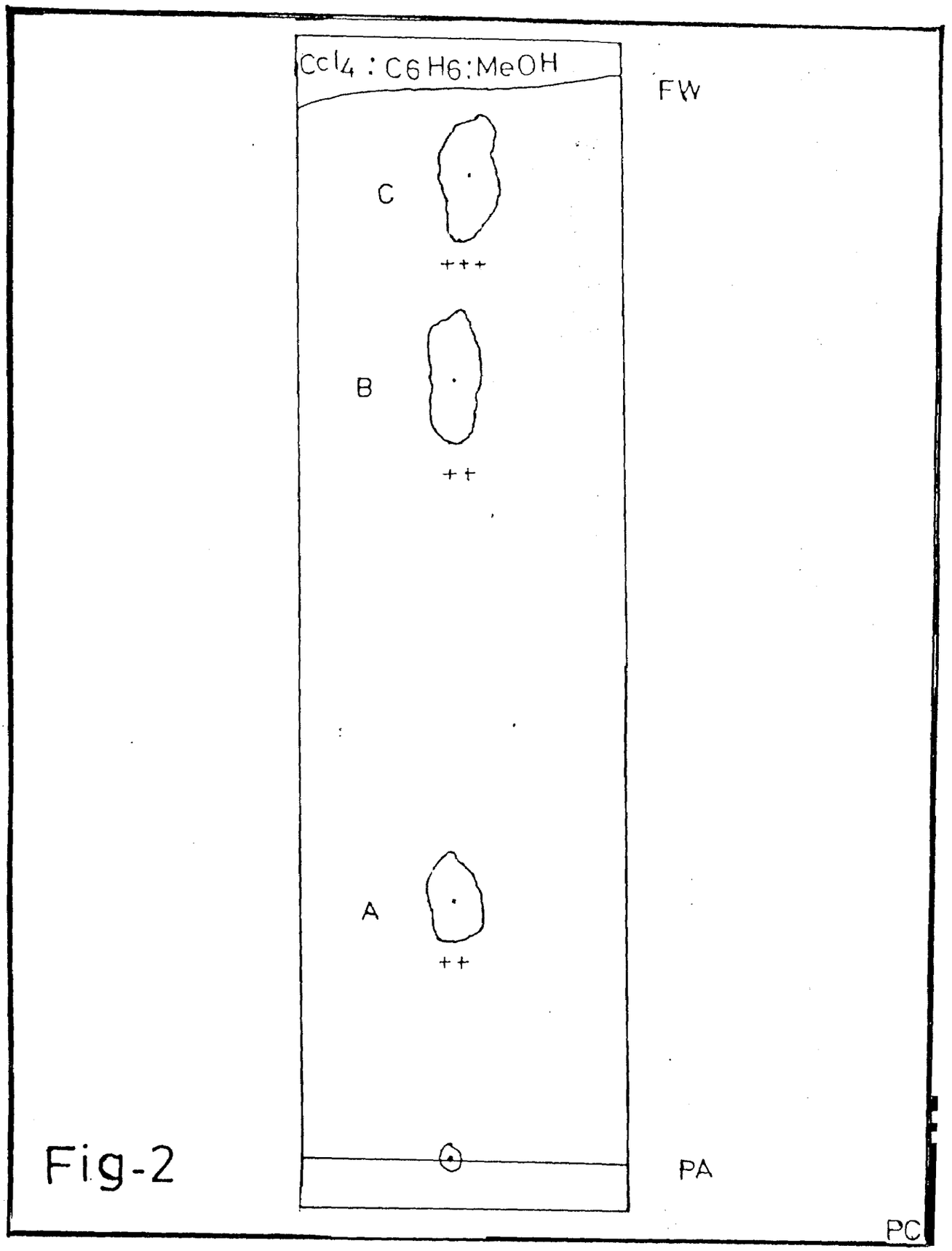

Fig-2

FRESH LEAVES (K)

with two different approaches. (i) The seed and leaves of plant were collected from different localities viz. Kolhapur, Solapur, Satara and they were analysed for flavonoid glycosides both paper chromatographically as well as by TLC method.

ii) The plants collected from different region were raised in garden of the department and they were given a foliar spray of mixture of Zn and Mn. After 3 weeks the plant both leaves and seeds were analysed for flavonoid glycosides by the same method maintained above.

A) Flavonoid glycosides from seed collected from Kolhapur Region :

These studies give fairly good idea of the fluctuation in level of flavonoid glycosides as the soil and other factors change. The result of these studies are given in Table 3. It is clear from the table that when the naturally growing plant of Kolhapur region was examined for the flavonoid glycosides of the fresh leaves (K)-3-rutinoside develops clear spot on the chromatogram, when the B:A:W. Solvent system is used. Besides one more spot develops, whose Rf value does not tally with any of the known compound (Fig.1) of T.terrestris. Where as when the same extract was run on chromatogram with solvent system CCl_4:C_6H_6: MeOH a spot, (Q) - rhamnogentiobioside readily develops. Besides two spots higher up in the cromatogram with Rf value 72, 91, which are not talling with other known (Fig.2) also develop.

The fresh seed extract when was chromatogramed under

Fig.No.3 :

Paper chromatogram showing flavonoid glycosides separation in B : A : W solvent system of Tribulus terrestris L. (fresh seed) naturally growing in Kolhapur region.

A : (K)-3-rutinoside

B : (K)-3-D-coumaroglycosides.

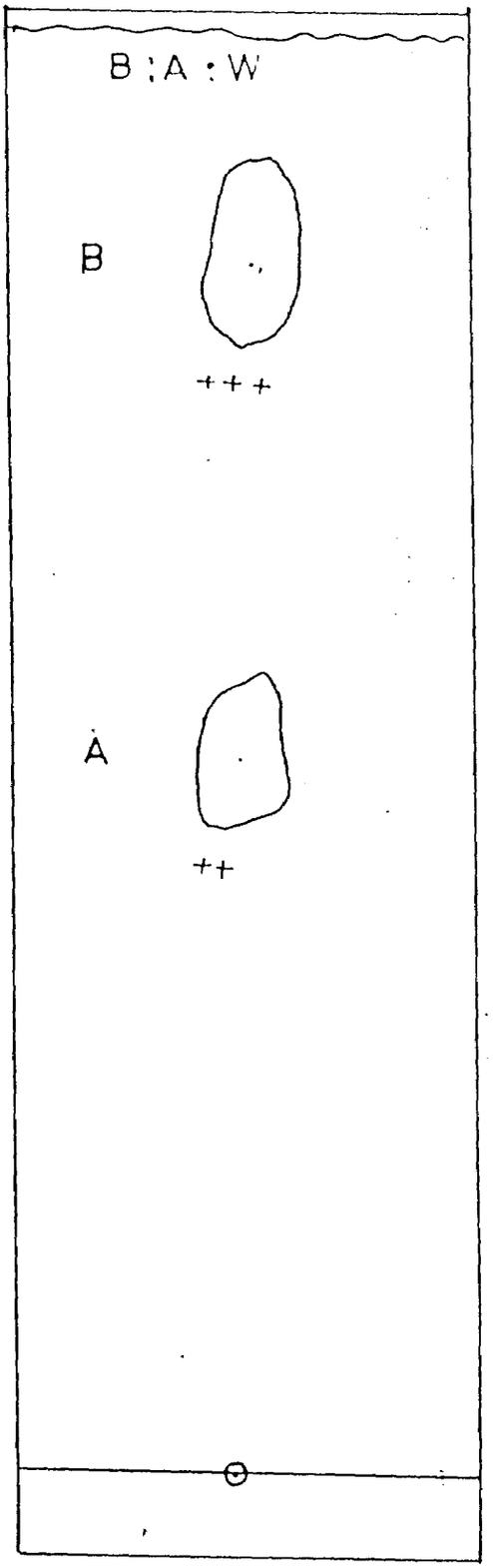

Fig. 3

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FRESH SEED (K)

Fig.No.4 :

Paper chromatogram showing flavonoid glycosides separation in CCl_4 : C_6H_6 : MeOH solvent system of Tribulus terrestris (fresh seed) naturally growing in Kolhapur region.

A - (I)-3-glucoside,
B - Unknown.

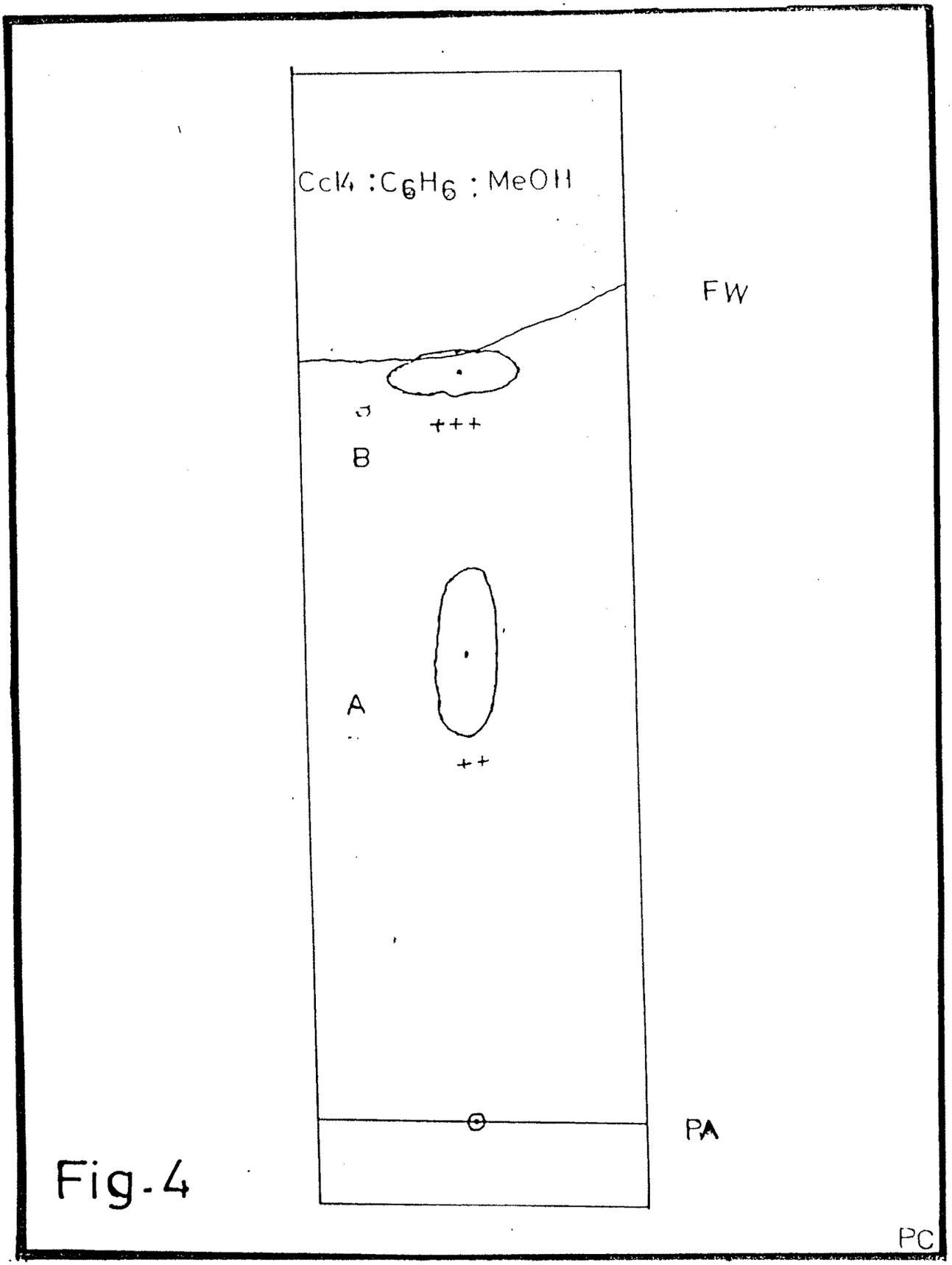


Fig. 4

FRESH SEED (K)

Fig.No.5 :

Paper chromatogram showing flavonoid glycosides separation in B:A:W solvent system of Tribulus terrestris (dry seed) naturally growing in Kolhapur region.

A - Unknown.

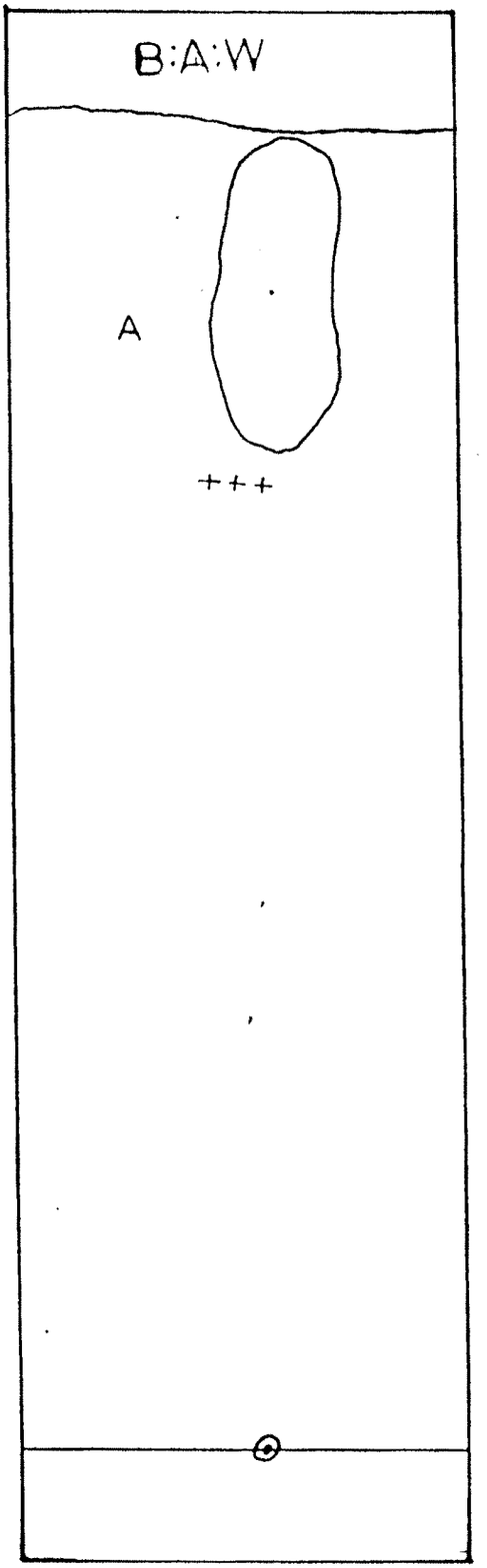


Fig. 5

DRY SEED (K)

Fig.No.6 :

Paper chromatogram showing flavonoid glycosides in CCl_4 : C_6H_6 : MeOH solvent system of Tribulus terrestris (dry seed) naturally growing in Kolhapur region.

A - (K)-coumaroglucoside.

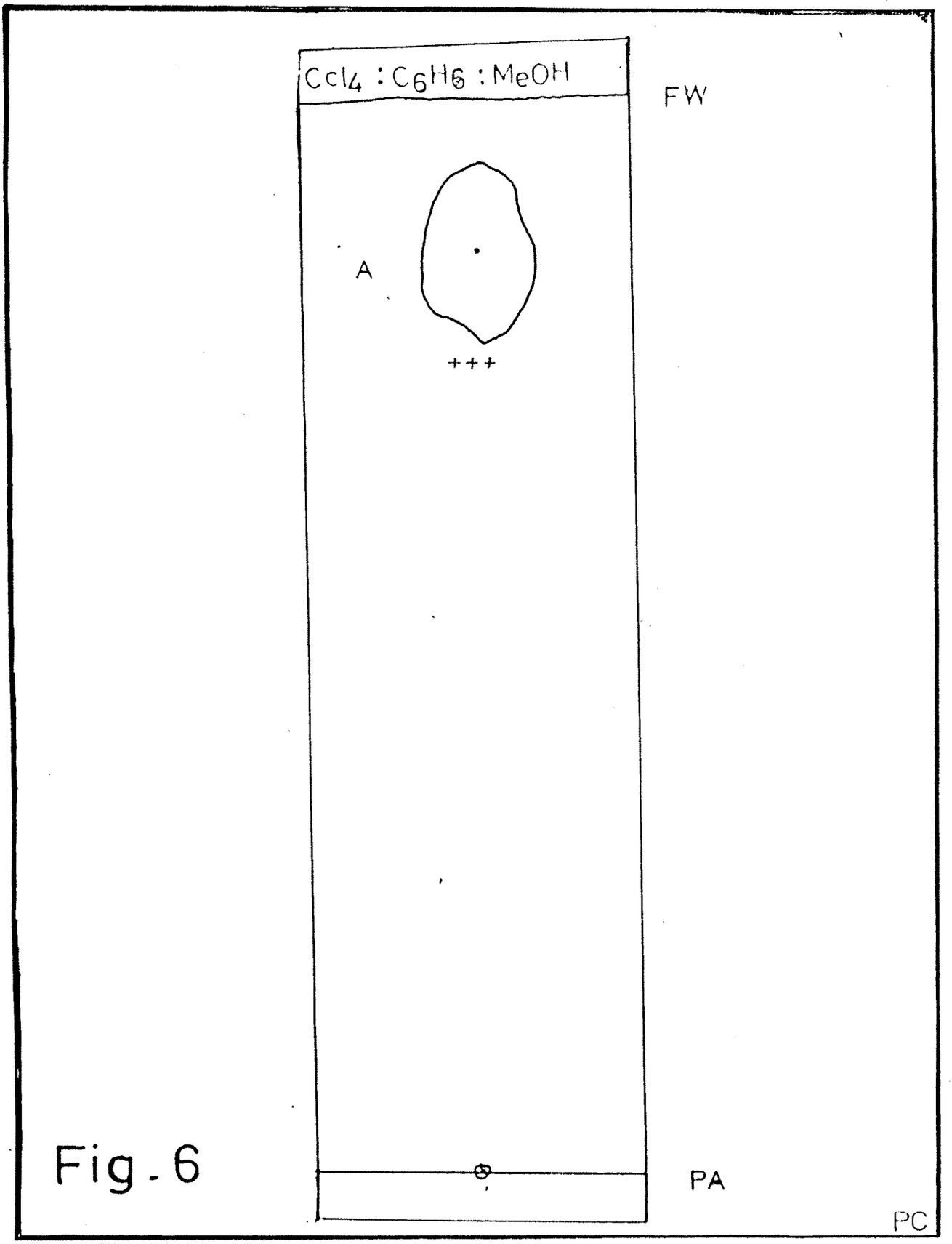


Fig. 6

DRY SEED (K)

two different solvent system, B:A:W and CCl_4:C_6H_6:MeOH in the former solvent system (K)-3-rutinoside and (K)-3-coumaro-glucoside both distinctly separated with Rf value 49 and 84 respectively (Fig.3). Where as in the latter system two spots were distinct,one with Rf value 60 which is identified as (I)-3-glucoside and the other with Rf value 70 which was not talling with any of the known flavonoid glycosides (Fig.4) hitherto describe by N.A.M.Saleh et al (1982) and Bhutani (1969). We hold the conviction that with the compound Rf value 70 is the distinct one and the derivative of known series developing in response to the ecological condition. But when seeds were dried and the flavonoid glycoside are extracted from dry seed in the same way as that of fresh seed, and chromatogramed in two different solvent systems described above, in B:A:W a bright large spot develops with Rf.value 87 (Fig.5); where as in other solvent system (K)-coumaroglucoside develops with Rf value 84 (Fig.6) which has been identified by N.A.M.Saleh (1969). However, the spot that develops in BAW at Rf value 87 is not tallying with any of the known flavonoid glycosides described by those authors. In other word even after using the same solvent system as used by them, it would be identified with any of the known flavonoid glycoside. This led to conclude that it is a derivative of one of the known compounds appearing in the Kolhapur region or may be a very close allie of (K)-coumaroglucoside.

Table No. 4

TLC separation of flavonoid glycosides from the leaves and Seed of *Tribulus terrestris* L. in Kolhapur region

Nature of material	Solvent system used	Rf x 100 values	Identification	*Intensity
Fresh leaves	$MeOH:NH_4OH$	66	Unknown	+
		66	Unknown	++
		66	Unknown	++
		69	Unknown	+++
		66	Unknown	+
	B : A : W	81	Unknown	++
		84	(K)-3-P-coumaroglycoside	++
		87	Unknown	++
		80	Unknown	++
Fresh seed	$MeOH:NH_4OH$	56	Unknown	+
		57	Unknown	++
		61	Unknown	++
		52	Unknown	+++
	B : A : W	71	Unknown	++
		72	Unknown	++
		70	Unknown	++
		71	Unknown	++
Dry seed	$MeOH:NH_4OH$	47	(K)-7-glucoside	+
		52	Unknown	+
		61	(I)-3-glucoside	++
		61	Unknown	+++
	B : A : W	77	Unknown	++
		79	(I)-C-3-P-coumaroglycoside	+++
		82	Unknown	++
		76	Unknown	++

* + = Weak, ++ = Strong, +++ = major.

Fig.No.7 :

TLC - Plate showing the separation of flavonoid glycosides in MeOH:NH4OH solvent system of Tribulus terrestris (Fresh leaves) naturally growing in Kolhapur region.

1. Unknown,
2. (K)-3-P-coumaroglycosides,
3. Unknown,
4. Unknown.

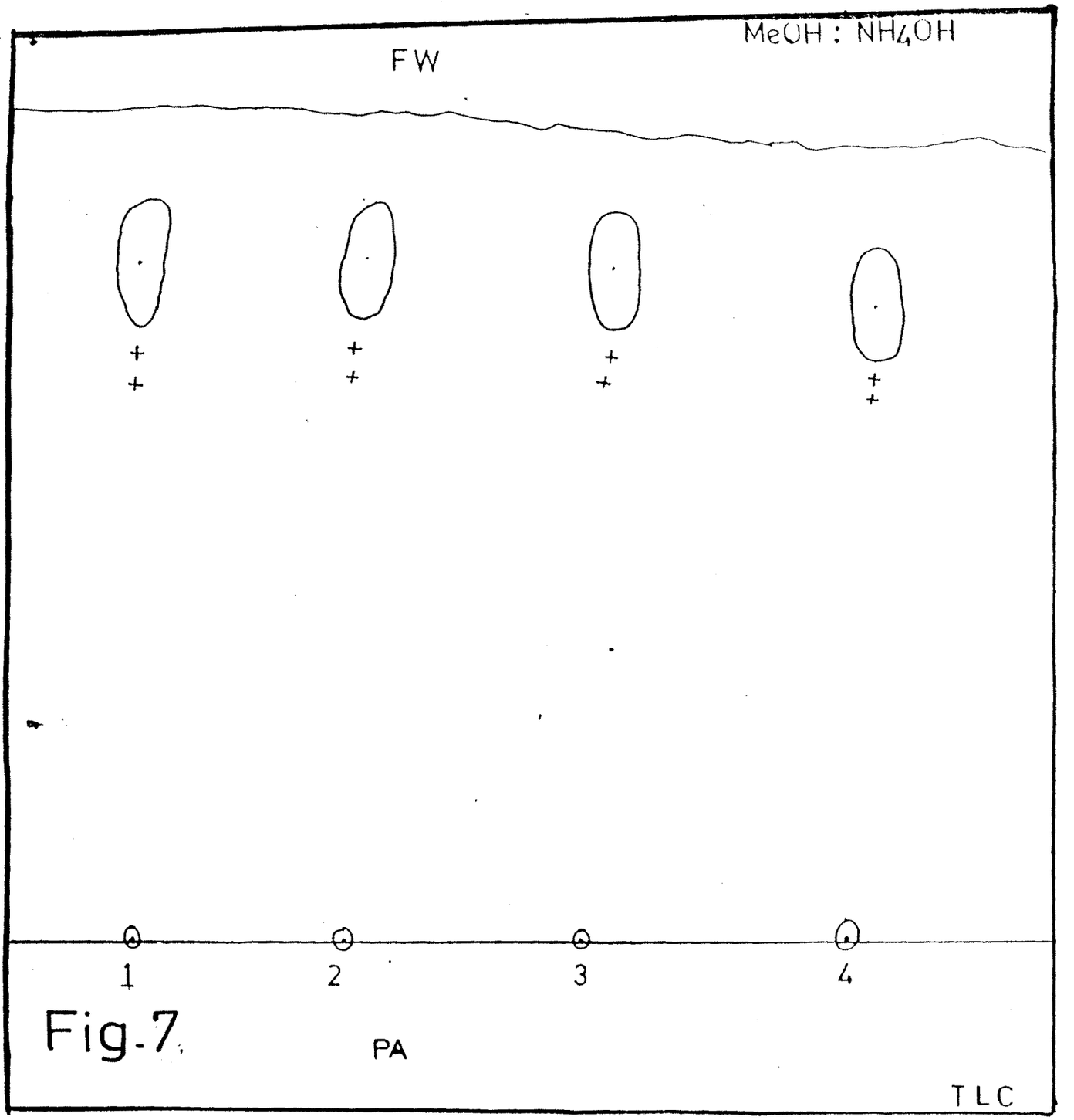


Fig.7

• FRESH LEAVES (K)

Fig.No.8 :

TLC Plate showing the separation of flavonoid glycosides in MeOH : NH_4OH solvent system of Tribulus terrestris (Fresh leaves) naturally growing in Kolhapur region.

1. Unknown
2. Unknown
3. Unknown
4. Unknown

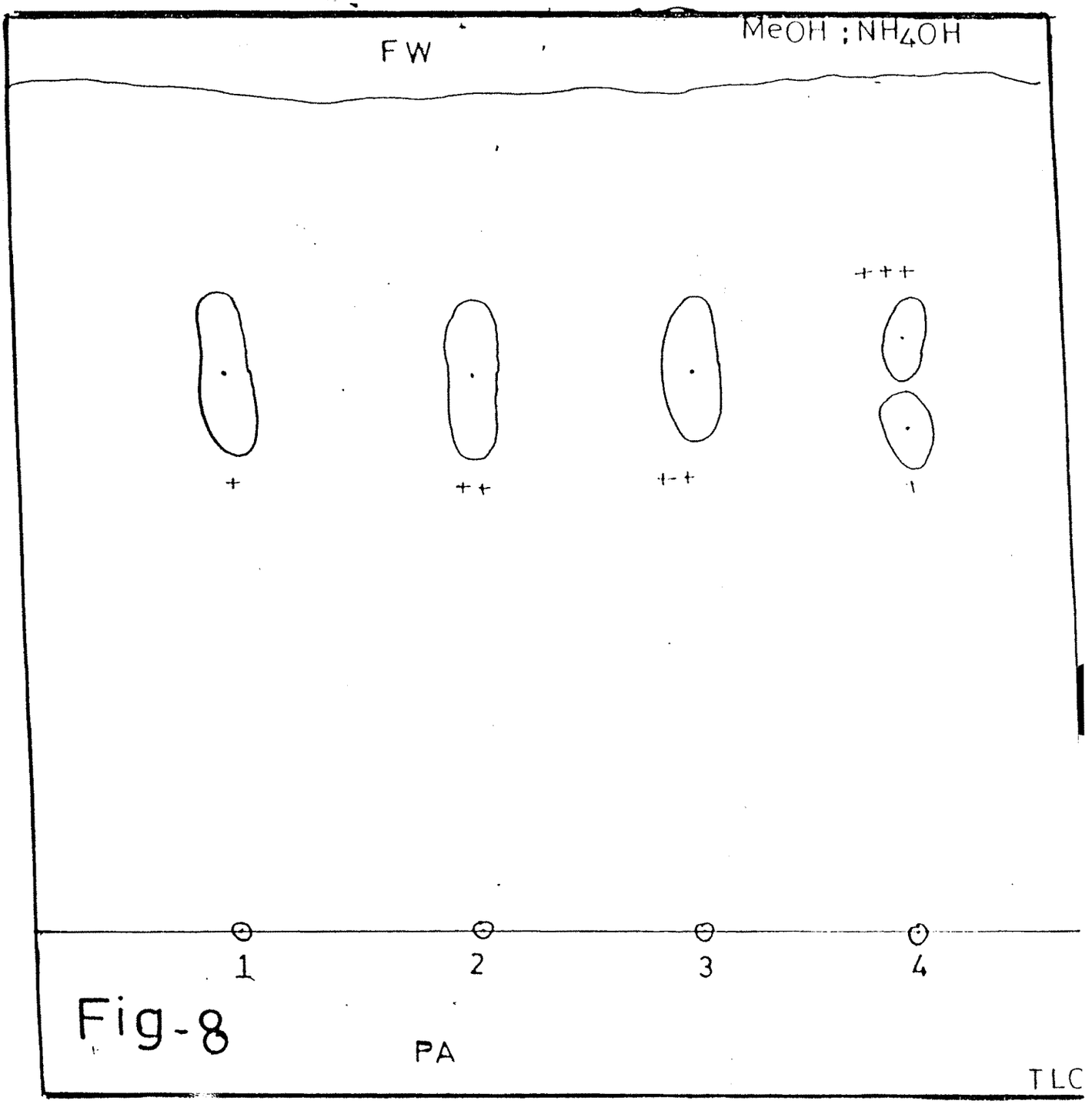


Fig-8

FRESH LEAVES (K)

Fig.No.9 :

TLC Plate showing the separation of flavonoid glycoside in B:A:W solvent system of Tribulus terrestris (fresh seed) naturally growing in Kolhapur region -

1. Unknown,
2. Unknown,
3. Unknown,
4. Unknown.

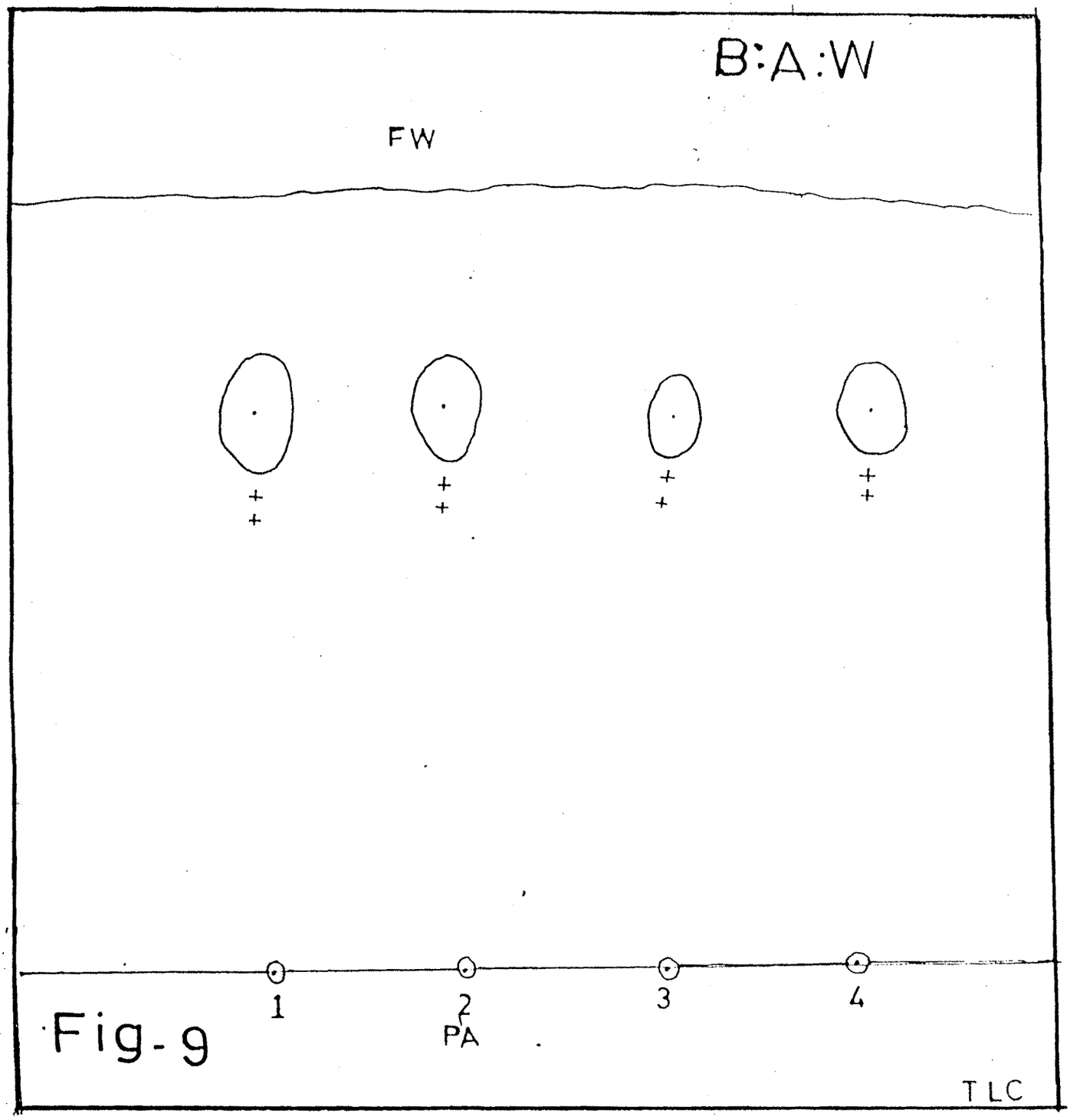


Fig-9

• FRESH SEED (K)

Fig.No.10 :

TLC Plate showing the separation of flavonoid glycosides in MeOH : NH_4OH solvent system of Tribulus terrestris (fresh seed) naturally growing in Kolhapur region -

1. Unknown,
2. Unknown,
3. Unknown,
4. Unknown.

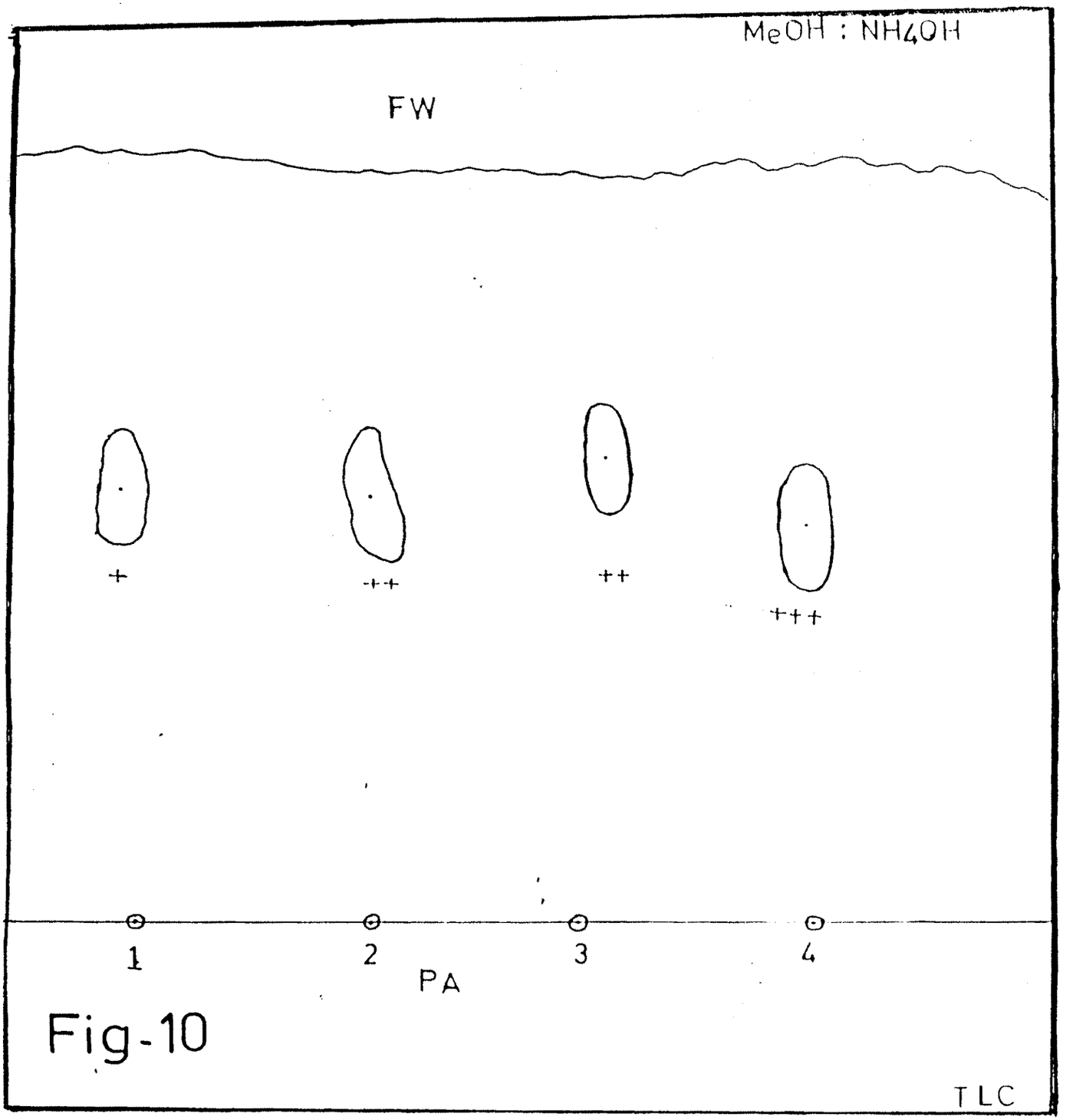


Fig-10

FRESH SEED (K)

Fig.No.11 :

TLC Plate showing the separation of flavonoid glycosides in B:A:W solvent system of Tribulus terrestris (Dry seed) naturally growing in Kolhapur region.

1. Unknown,
2. (I)-C-3-P-coumaroglycoside,
3. Unknown,
4. Unknown.

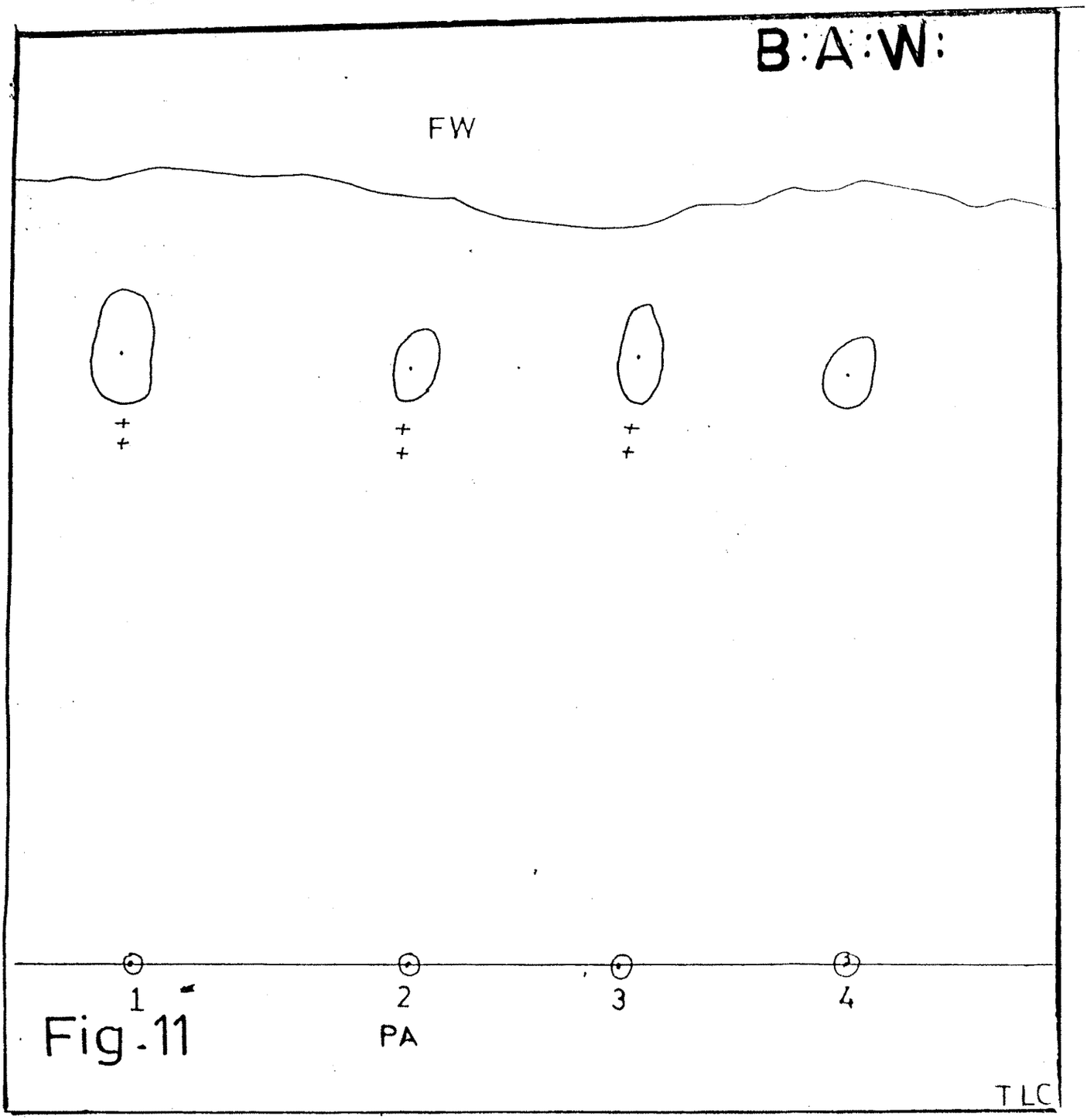


Fig.11

• DRY SEED (K)

a) TLC separation of flavonoid glycosides :

The fresh leaves extract when loaded on the TLC plate and run in to two different solvent system viz. B:A:W and MeOH : NH_4OH in the former system four spots develop while in the latter system two spots develop. In BAW solvent system only one spot with Rf value 84 was identified as (K)-3-P-coumaroglycoside and rest of three do not tally with any known ones. Whereas in the latter solvent system both spots one with Rf value 66 and other 69 remain unknown, they could not be identified with any of the described glycosides (Fig.7 & 8), (Table 4).

When the extract of fresh seed was run by the same system on the TLC plate in B:A:W three spots with Rf value 70,71,72 developed while in MeOH:NH_4OH solvent system four spots developed but none of the spots could be identified with any known flavonoid glycosides. This reflects on the fact that large number of derivatives are resolved on the same solvent system, while on the paper they do not (Fig.9 and 10).

When dry seed extract was run in B:A:W solvent system 4 spots with Rf value 76,77,79,82 developed out of which only Rf value of 79 is identified as (I)-3-P coumaroglycoside and rest of three were not comparable with spots of fresh seed extract (Fig.11). Similarly in MeOH : NH_4OH solvent system four spot developed one with Rf value 47 identified as

Fig.No.12 :

TLC Plate showing the separation of flavonoid glycosides in MeOH : NH_4OH solvent system of Tribulus terrestris (Dry seed) naturally growing in Kolhapur region.

1. (K)-7-glucoside,
2. Unknown,
3. (I)-3-glucoside,
4. Unknown.

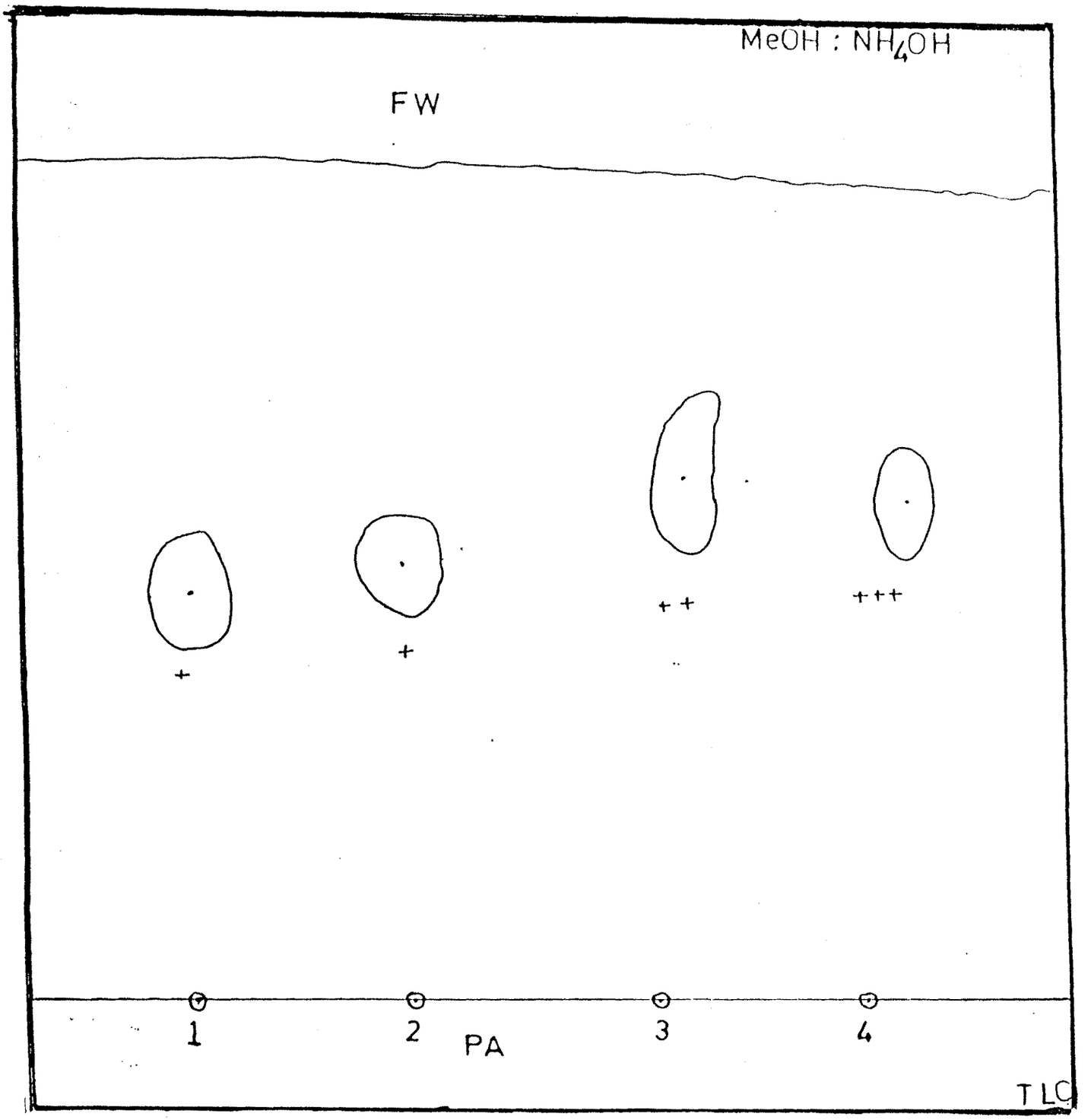


Fig.12

DRY SEED (K)

Fig.No.13 :

Paper chromatogram showing flavonoid glycosides separation in B:A:W solvent system of Tribulus terrestris (Dry seed) naturally growing in Solapur region.

A - Unknown,
B - Unknown.

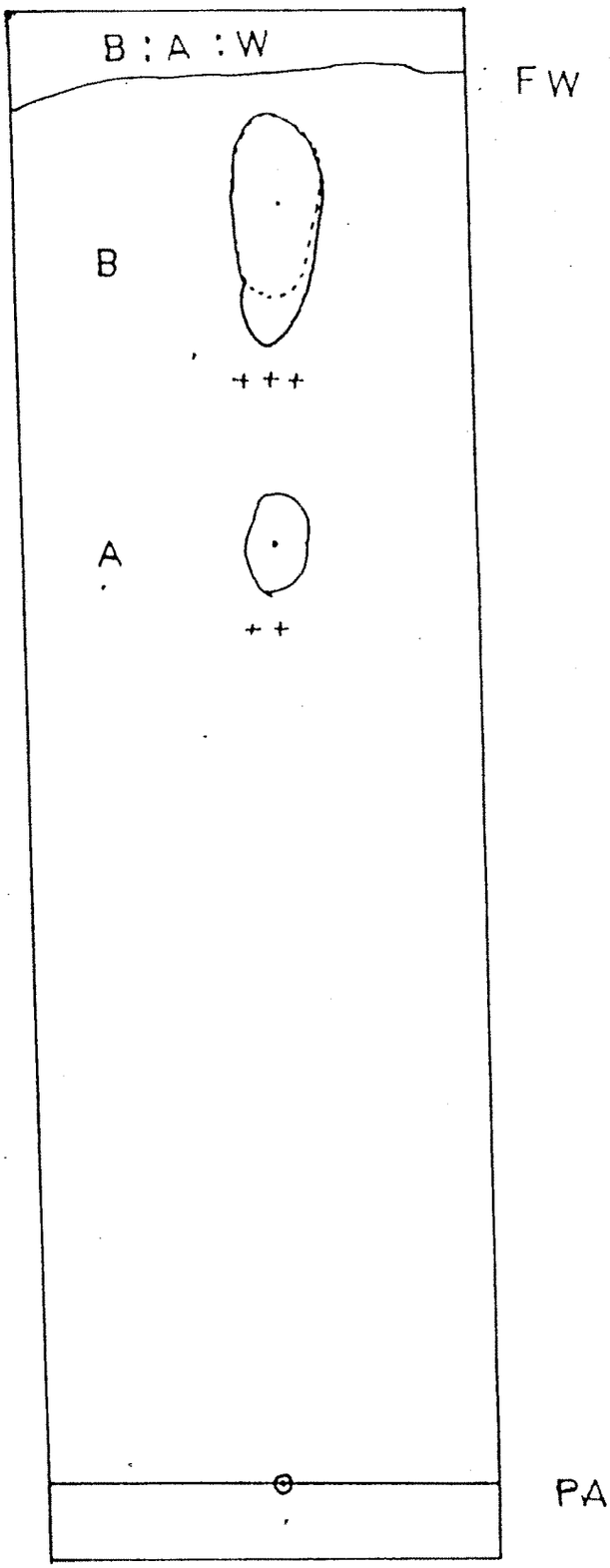


Fig- 13

PC

DRY SEED (S)

(K)-glucoside, another value with 61-identified as (I)-3-glucoside and rest two unknown (Fig.12). This again indicates that except one with Rf value 52 rest of them are not seen, in fresh seed extract though run in the same solvent system. This leads us to conclude that (i) by the TLC method greater resolution of compound can be obtained than that of paper chromatography. (ii) a sort of transformation, perhaps, in the conformation of compound occurs when physical changes in substance is induced i.e. in the fresh material, in both the solvent system, though, several spots could be seen number of them were identifiable in as much as the dry seed. (iii) Important point to be emphasized here is the environmental condition is likely to be induce the change and hence many spots are not talliable with known compounds.

B) Flavonoid glycosides from seeds collected from Solapur Region :

Paper chromatographic as well as TLC separation of flavonoid glycosides made from the seeds of Tribulus terrestris collected from Solapur region have been given in Table 4 and 6 respectively. When the sample is loaded on chromatogram and run in B:A:W solvent system two spot developed. One with Rf value 66 and the other is at Rf value 90. These spots do not tally with any of the hitherto known compound (Fig.13). On the other hand the dry seeds when collected from Kolhapur region and its extract was chromatogramed in B:A:W solvent

Table No.5

Paper chromatographic separation of flavonoid glycosides from leaves and seed of Tribulus terrestris L. naturally growing in Solapur region.

Nature of material	Solvent system used	Rf x 100 value	Identification	*Intensity
Dry seed	B : A : W	66	Unknown	++
		90	Unknown	+++
	CCl_4:C_6H_6:MeOH	42	(Q)-3-rutinoside	+
		65	Unknown	+++
		88	Unknown	+++

* \+ = weak
++ = strong
+++ = major

Table No.6

TLC separation of flavonoid glycosides from seed of Tribulus terrestris L. naturally growing in Solapur region.

Nature of material	Solvent system used	Rf x 100 values	Identification	*Intensity
Dry seed	MeOH:NH_4OH	36	(K)-3-gentiobioside	+
		37	Unknown	++
		36	(K)-3-gentiobioside	+++
		35	(K)-3-7-diglucoside	+++
		23	Unknown	+
		21	Unknown	++
		17	(I)-3-gentiobioside-7-glycoside	++
		18	Unknown	+++

* + = weak
++ = strong
+++ = major

Fig.No.14 :

Paper chromatogram showing flavonoid glycosides separation in CCl_4:C_6H_6: MeOH solvent system of Tribulus terrestris (dry seed) naturally growing in Solapur region.

A - (Q)-3-rutinoside
B - Unknown
C - Unknown.

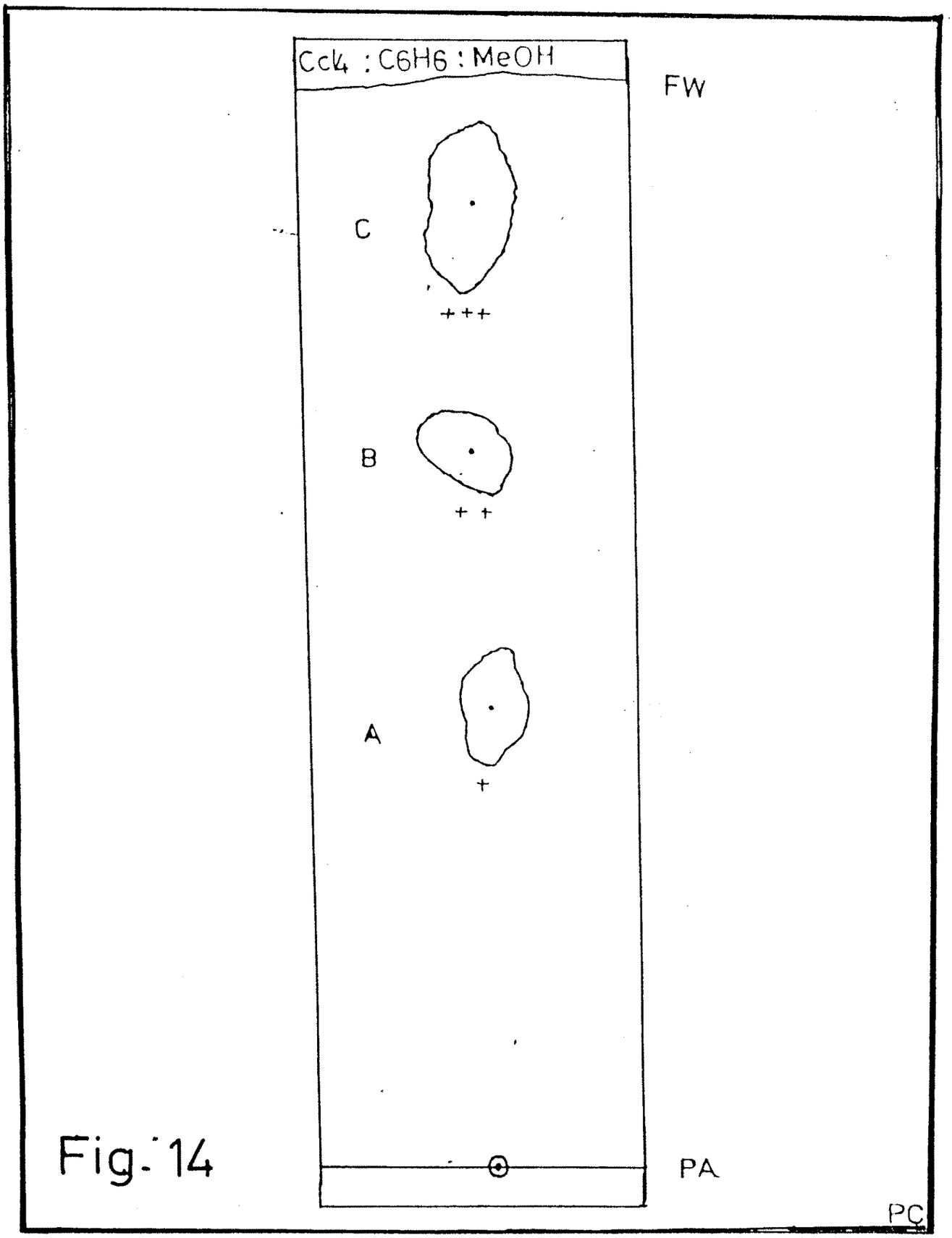


Fig. 14

DRY SEED (S)

Fig.No.15 :

TLC Plate showing the separation of flavonoid glycosides in MeOH : NH_4OH solvent system of Tribulus terrestris (Dry seed) naturally growing in Solapur region.

1. (K)-3-gentiobioside
2. Unknown
3. (K)-3-gentiobioside
4. (K)-3-7-diglucoside

1' Unknown
2' Unknown
3' (I)-3-gentiobioside-7-glucoside
4' Unknown.

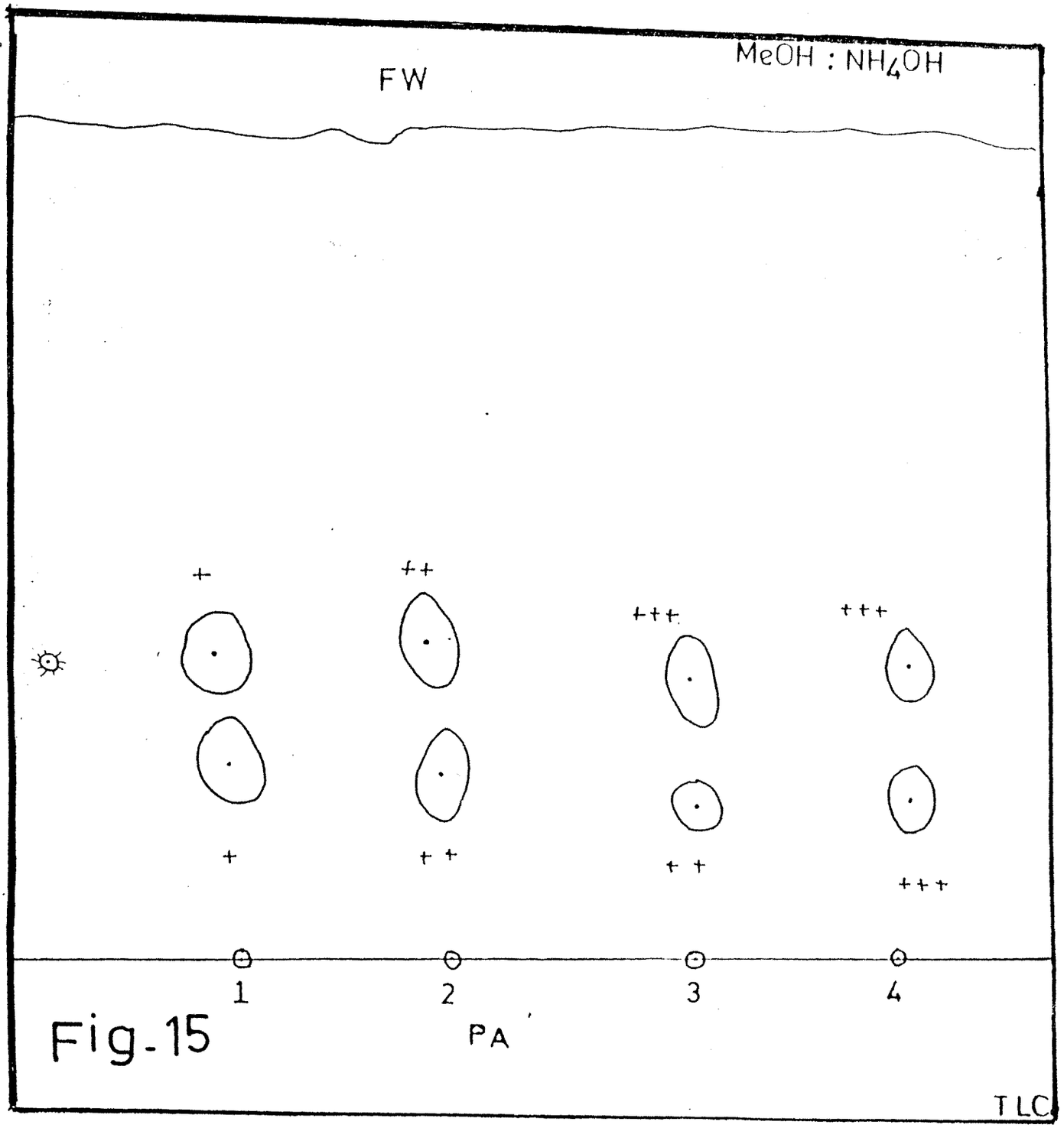


Fig-15

DRY SEED (S)

system only one spot developed which is also unknown. That means the seeds of T.terrestris grown in Solapur region has two additional compound appeared to be quite distinct from that of plants growing in Kolhapur region. In the former with(BAW solvent system) two spots one with Rf value 66 and the other with Rf value 90 while in the latter, it is only one spot with Rf value 87; but the same extract when was run in CCl_4:C_6H_6: MeOH solvent system the sample of Kolhapur showed one compound namely (K)-coumaroglycoside, while that Solapur has 3-compounds out of which one separated at Rf value 42 which is identified as (Q)-3-rutinoside, and other two with Rf values 65 and 85 are unknown (Table 5, Fig.14). In brief, the plants growing in drier region such as Solapur have more number of compounds.

When the same sample was run on TLC plate 7 spots separated. In the sample of Solapur region, there are several spots : one with Rf value 36, identified as (K)-3 gentiobioside, other two with Rf value 35 and 17, identified as (K)-3-7-diglucoside and (I)-3-gentiobioside-7-glycoside respectively, and rest with Rf value 37,23,21,18 (Table 6), which are unknown. This clearly indicated that the sample exhibits different compound varying with ecological condition (Fig.15).

Table No.7

TLC separation of flavonoid glycosides from dry seeds of Tribulus terrestris L. naturally growing in Satara region.

Nature of material	Solvent system used	Rf x 100 values	Identification	*Intensity
Dry seed	MeOH:NH_4OH	61	(I)-3-glucoside	++
		65	Unknown	++
		64	Unknown	++
		63	Unknown	+++

* + = weak
++ = strong
+++ = major

Fig.No.16 :

TLC Plant showing flavonoid glycosides separation in MeOH : NH_4OH solvent system Tribulus terrestris (Dry Seed) naturally growing in Satara region.

1. (I)-3-glucoside
2. Unknown
3. Unknown
4. Unknown

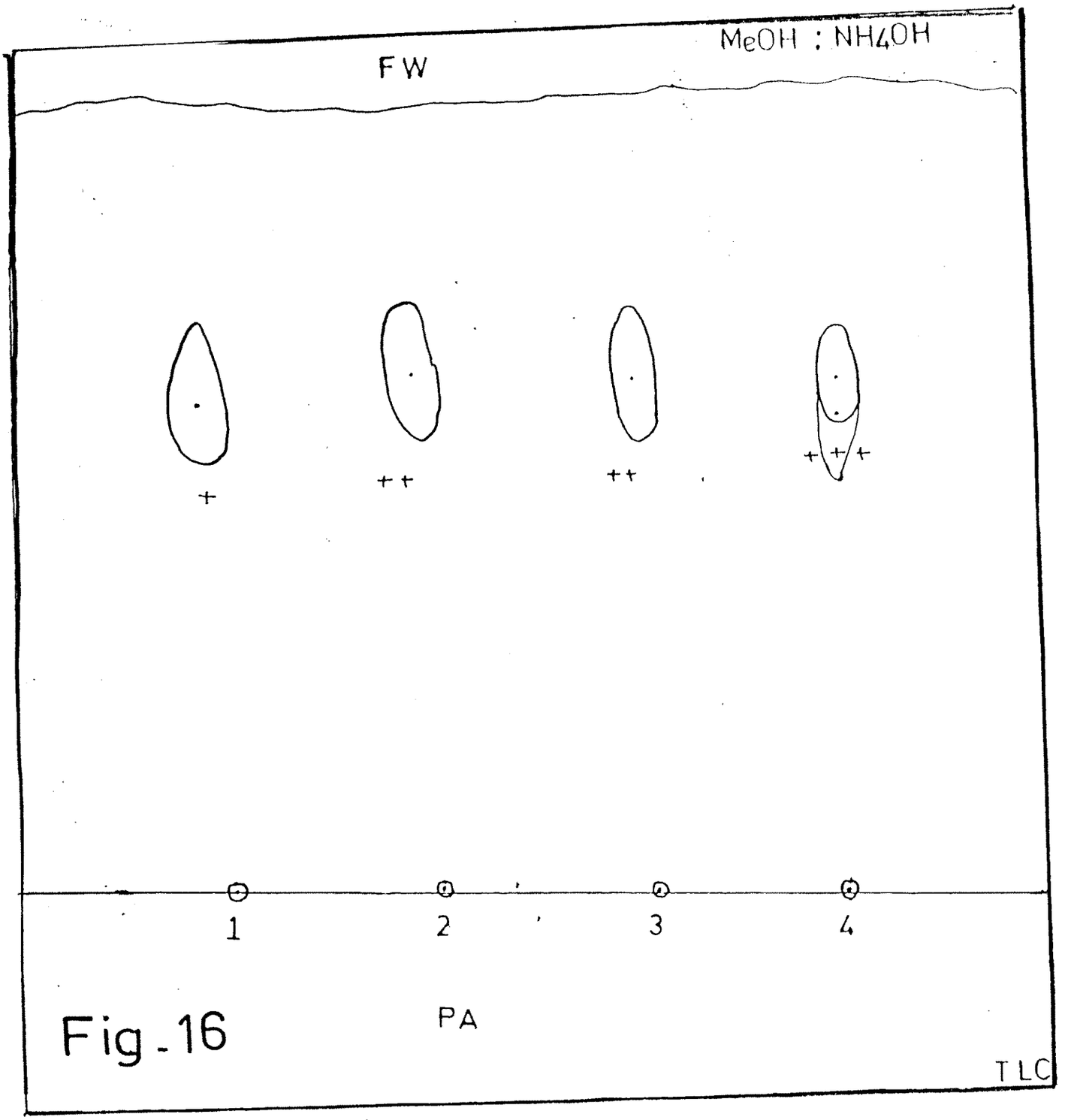


Fig.16

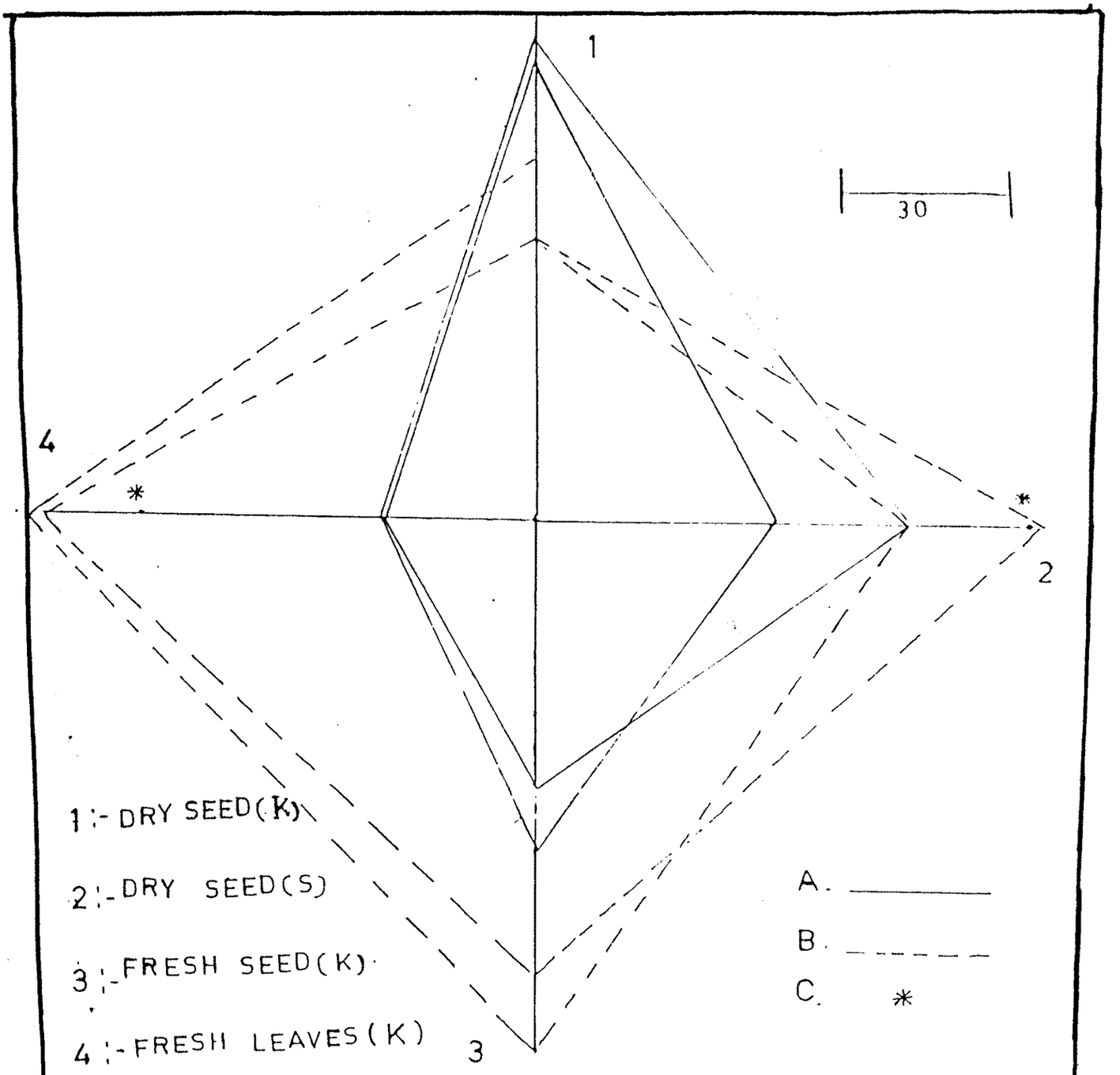
DRY SEED (SAT)

C) Flavonoid glycosides of Dry seeds collected from naturally growing population in Satara Region :

The dry seeds of Tribulus terrestris L. collected from naturally growing population of Satara region was extracted and the flavonoid glycosides separated by TLC with solvent system MeOH:NH_4OH. The values are given in the table (Table 7). Four spots each one separated at Rf value 61,63,64,65 were identified out of which Rf value 61 is identified as (I)-3-glucoside while rest were not comparable. This clearly indicates that it is a same compound which appears in the dry seed of the Kolhapur region which also appeared in the dry seeds collected from Satara region. In other words one compound namely (K)-7-glucoside, which separated at Rf value 47 is missing in the seeds collected from Satara region (Fig.16). It is essential to point out here that (I)-3-glucoside is predominantly synthesized while (K)-7-glucoside is not. It is possible that (I)-3-glucoside readly appear in plant of Kolhapur as well as Satara while does not occur in dry seeds of Solapur, because, the ecogeographical condition of Kolhapur and Satara is more or less identical. In conclusion it can be said that the ecological condition with predominance of the nature of soil rain fall and humidity profoundly influences the flavonoid glycoside metabolism. Polygraph of paper chromatogram showing Rf x 100 values in B:A:W and CCl_4: C_6H_6 : CH_3OH solvent system at various localities of Tribulus terristris

Fig.No.17 :

Polygraph of paper chromatogram showing Rf x 100 values in B:A:W and CCl_4:C_6H_6:CH_3OH solvent system at various localities of Tribulus terrestris seed and leaves.



Polygraph of paper chromatogram showing Rf X 100 values in B:A:W and $CCl_4 : C_6H_6 : CH_3OH$ Solvent system at various localities of Tribulus terrestris · L · Seed and leaves.

Fig - 17

Fig.No.18 :

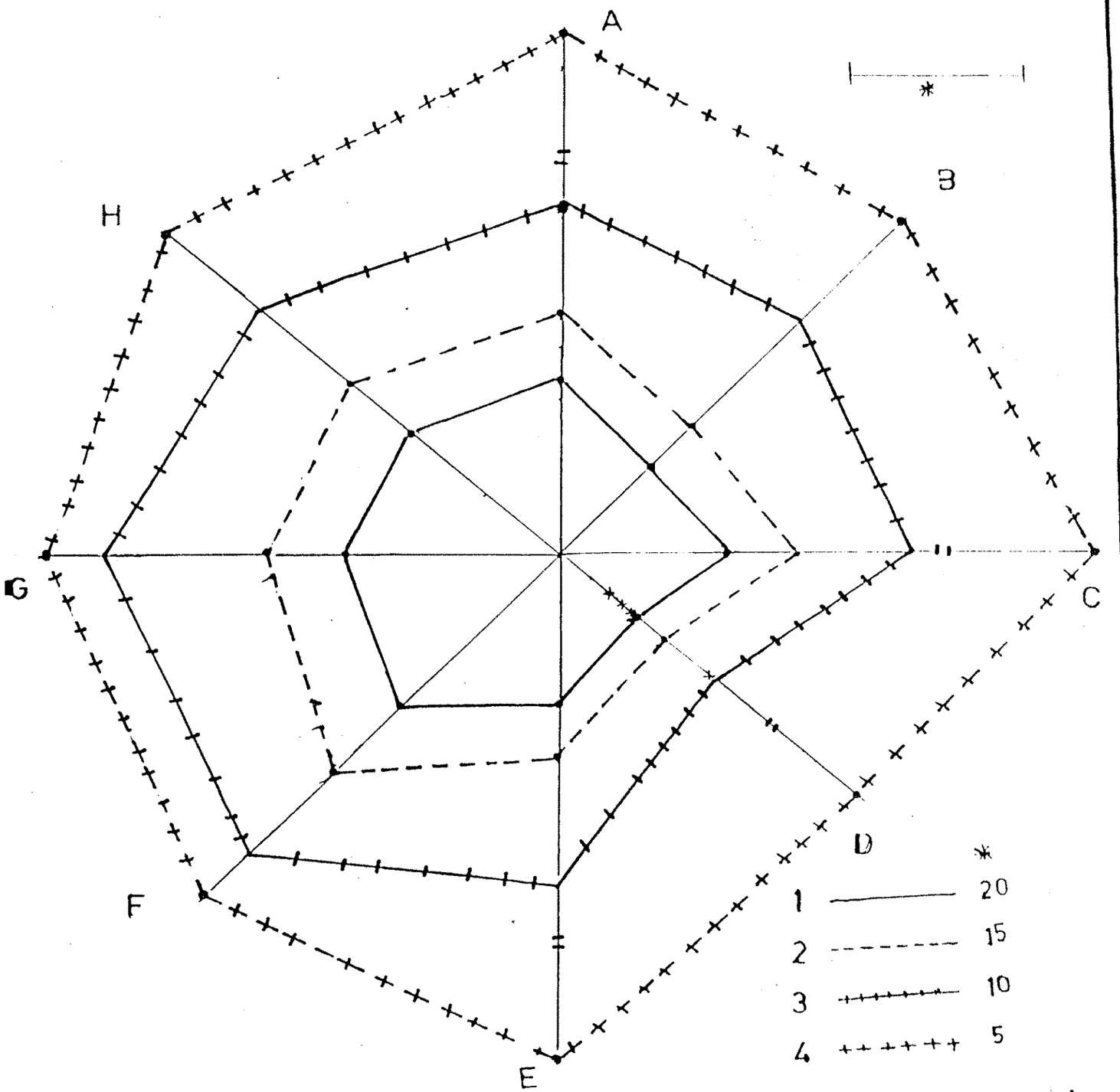
Polygraph of TLC showing Rf x 100 values of flavonoid glycoside in MeOH : NH_4OH and B:A:W solvent system at various localities of Tribulus terrestris.

A. Fresh leaves of Kolhapur
B. Dry seed of Kolhapur
C. Dry seed of Satara
D. Dry seed of Solapur

} MeOH:NH_4OH solvent sy

E. Fresh seed of Kolhapur
F. Fresh leaves of Kolhapur
G. Dry seed of Kolhapur
H. Fresh seed of Kolhapur

} B:A:W solvent ssystem



Polygraph of TLC showing Rf × 100 values in MeOH : NH4OH and B: A: W solvent system at various localities of Tribulus terrestris L. seed and leaves.

D - ✲ - ×

Fig - 18

Fig.No.19 :

The biosynthatic pathway of the flavonoid (shikimic acid pathway).

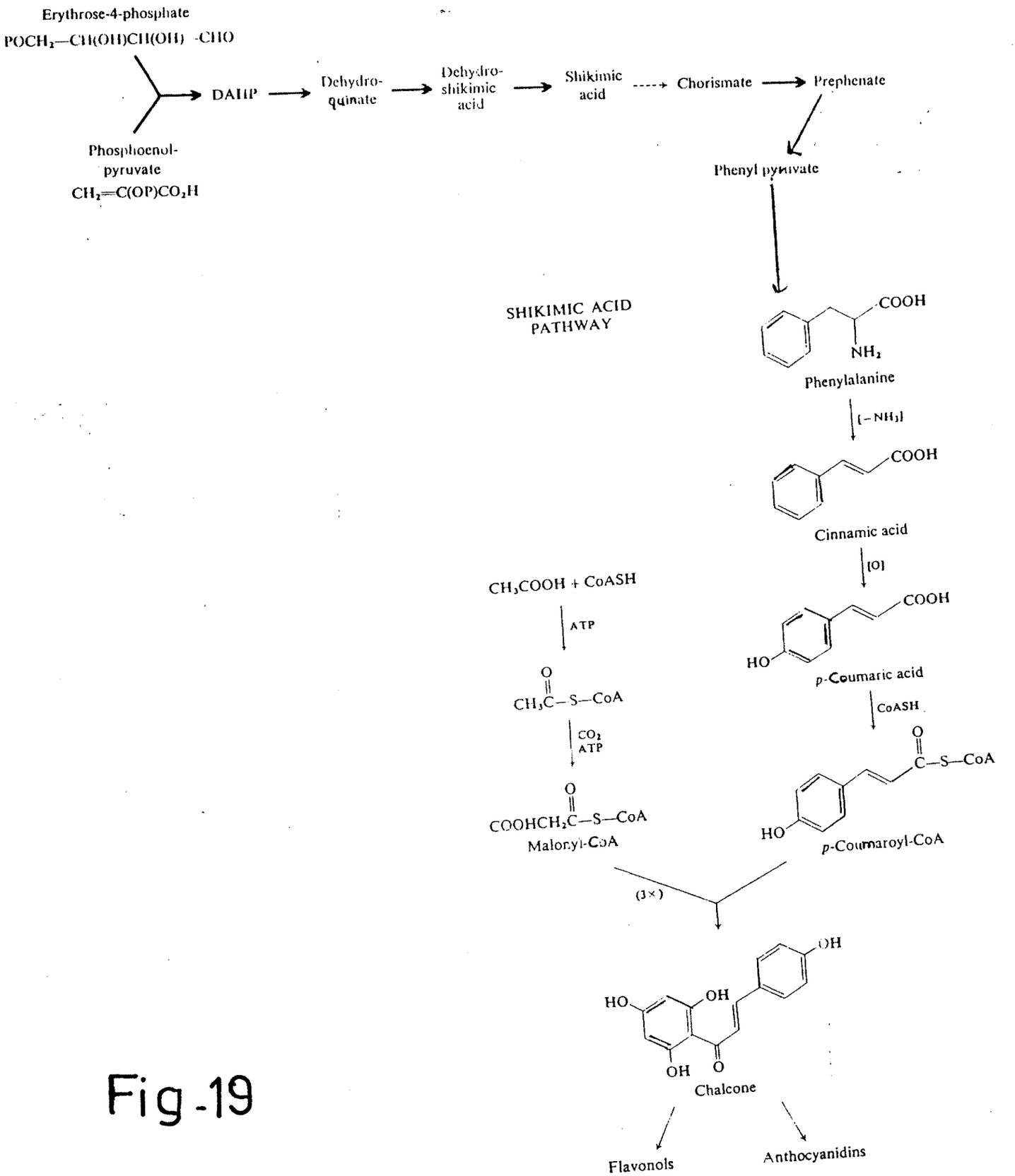


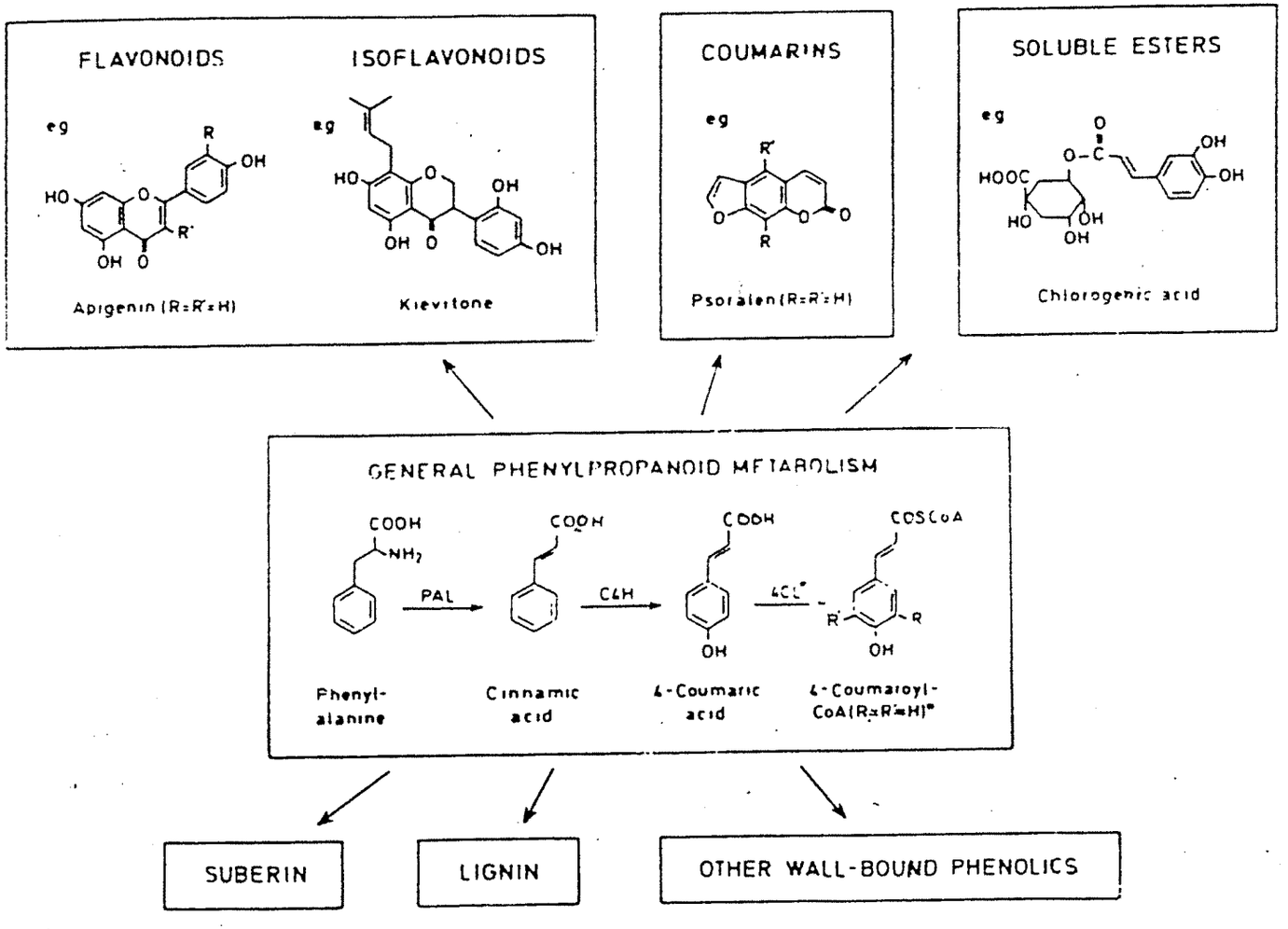
Fig-19

THE BIOSYNTHETIC PATHWAY OF THE FLAVONOID

Fig.No.20 :

Scheme illustrating the Flux of Phenylalanine derived intermediates from the core reaction of general phenylpnopanoid metabolism to some of the major branch pathway.

(Adapted from Hahlbrock & Schell 1989).



Scheme illustrating the flux of phenylalanine-derived intermediates from the core reactions of general phenylpropanoid metabolism to some of the major branch pathway

(Adopted from Hahlbrock and Schell, 1989)

Fig 20

seed and leaves in Fig.17 and polygraph of TLC showing Rf x 100 values in B:A:W and MeOH:NH_4OH solvent system at various localities of Tribulus terrestris seed and leaves (Fig.18).

5. Effect of foliar application of trace element, Zn and Mn on flavonoid glycosides content of Tribulus terrestris L.

It is well established fact that a complex cyclic compounds in plants such as Alkaloid, Flavonoids, anthocyanins, terpenes, steroids, which are called secondary metabolites, trace their origin to aromatic amino acids, since these compounds are cyclic in nature it requires large number of carbon molecules and hence the building blocks are derived from glucose or hexose derivative products of respiration. Mainly Erythrose-4. Phosphate and phosphoenol pyruvic acid contribute carbon skeleton for cyclization and this pathway way is called shickimic acid pathway which is very lenthy one (Fig.19). All the three aromatic aminoacid, are mainly formed through shikimic acid pathway. Among the amino acids belonging to this phenylalanine plays pivotal role and as a precursor for the synthesis of flavnoids, isoflavonoids, coumarins on one hand, and suberin, lignin and other wall bound phenolix on other hand (Fig.20), Hahlbrock and Scheel (1989). In brief it is very important biochemical pathway. However, the plant takes lot of time in synthesizing these compounds because of their very lenthy nature. Nonetheless these compounds are readly synthesized even in the early

period of growth during seedling stage itself. This is because the differentiation requires some of these compounds and flavonoids play, again, a very important role in protection or in preventing the diseases that is likely to occur during the juvenile stage of plant.

Gasic et al. (1968) working on colchicine yielding plant Colchicum autmnale established a positive correlation between the colchicine content of the plant and trace elements available in the soil. He collected Colchicum autmnale from natural population growing at different regions and analyse the soil sample for Cu, Zn and observed that these trace elements of soil profoundly influence the colchicine content. It is well established fact that some of the trace elements such as Zn^{++} and Mn^{++} are functioning as cofactors in the enzymes of aromatic amino acid metabolism and therefore, they can be used as a mediators in the synthesis of compound where these amino acid serve as precorsors. In present investigation therefore, an effort has been made to supply these trace elements externally as a foliar spray and their effect on synthesis of flavonoid glycosides has been examined. Since, it is complex biosynthetic pathway a few enzymes which are directly involved in synthesis are known. However, enzymes involved in various pathways and their metal dependance both specific as well as multiple have been listed long ago by McElroy and Nason (1950). Smith (1973) has listed large number

Table No.8

Paper chromatographic separation of flavonoid glycoside exhibiting the foliar spray of Mn^{++} and Zn^{++} combination in Tribulus terrestris L. leaf and seed growing in Kolhapur region.

Nature of Material	Treatment	Rf x 100 values	Identification	*Intensity
Fresh leaves	Control	45	(I)-3-rutinoside	++
	15 ppm Zn + Mn	45	(I)-3-rutinoside	+++
	20 ppm Zn + Mn	i) 38	Unknown	++
		ii) 48	(K)-3-rutinoside	++
	25 ppm Zn + Mn	48	(K)-3-rutinoside	++
Fresh seed	Control	41	(K)-3-rutinoside	++
	15 ppm Zn + Mn	41	(Q)-3-rutinoside	+++
	20 ppm Zn + Mn	55	(K)-coumaroglycoside	++
	25 ppm Zn + Mn	36	(K)-gentiobioside	++

* \+ = weak
++ = strong
+++ = major

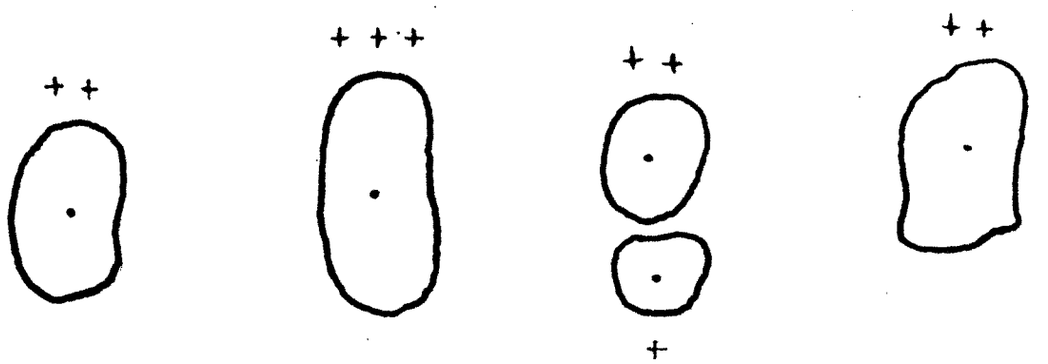
Fig.No.21 :

Paper chromatogram showing separation of flavonoid glycoside exhibiting the foliar spray of Mn^{++} and Zn^{++} combination in Tribulus terrestris L. (fresh leaves) naturally growing Kolhapur region.

1. Control : (I)-3-rutinoside
2. 15 ppm Zn + Mn : (I)-3-rutinoside
3. 20 ppm Zn + Mn : i) Unknown,
   ii) (K)-3-rutinoside
4. 25 ppm Zn + Mn : (K)-3-rutinoside.

B : A : W

FW



1 2 3 4

Fig.21

PA

PC

FRESH LEAVES

of enzymes of flavonoid biosynthesis while discussing their light dependance, however, he did not refer to their metal dependence.

It is already been maintained that Tribulus terrestris seedlings were given foliar spray of a mixture of Zn and Mn in concentration 15, 20, 25 ppm in the form of $ZnCl_2$ and $MnCl_2$. The fresh leaf and fresh seeds after protracted treatment were extracted separately and extract was run on chromatogram using solvent system B:A:W. The result is given in Table 8

What we see from the result is mainly irrespective of the constitution of trace element in all the two known and one unknown compound are seen, in control at 15 ppm is (I)-3-rutinoside which distinctly developed. Where as when concentration increased to 20 ppm two spots developed one with Rf value 38 and other with 48 and the former is not talliable with any of the known flavonoid glycoside while latter is identified as (K)-3-rutinoside. When the concentration increased to 25 ppm the unknown spot disappeared but large (K)-3-rutinoside develops on chromatogram (Fig.21). What is achieve through this experiment is, there may not be consistancy in the compounds being synthesized when the concentration of trace element is changed but the quantum of the compound increased to almost double. For instance in the control (I)-3-rutinoside is seen which has doubled in quantity when 15 ppm of Zn^{++}, Mn^{++} was sprayed. However, this has reduced when the concentration is increase.

Fig.No.22 :

Paper chromatogram showing separation of flavonoid glycoside exhibiting the foliar spray of Mn^{++} and Zn^{++} combination in Tribulus terrestris (fresh seed) naturally growing in Kolhapur region.

1. Control : (Q)-3-rutinoside
2. 15 ppm Zn + Mn : (Q)-3-rutinoside
3. 20 ppm Zn + Mn : (K)-coumaroglycoside
4. 25 ppm Zn + Mn : (K)-gentiobioside.

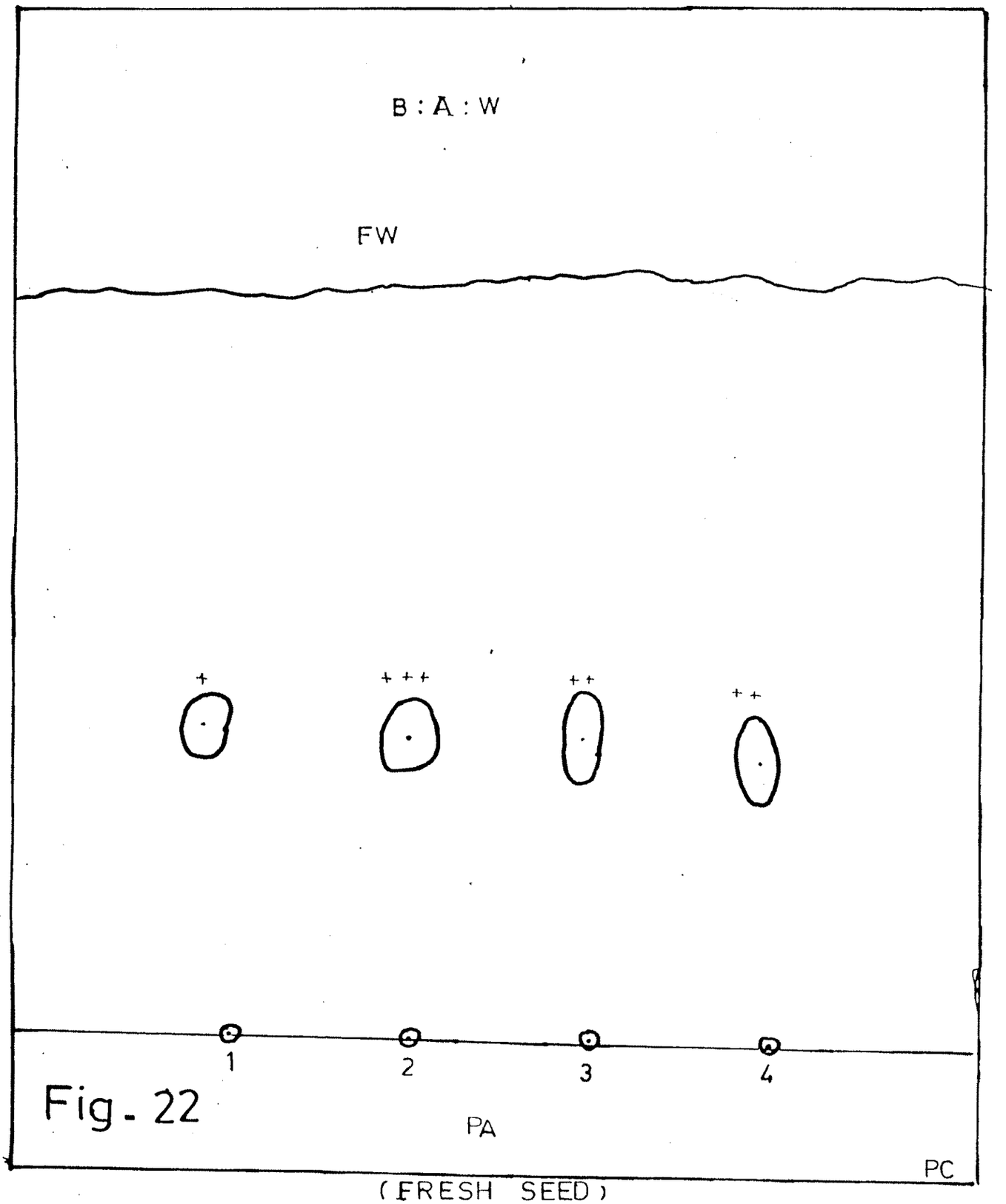
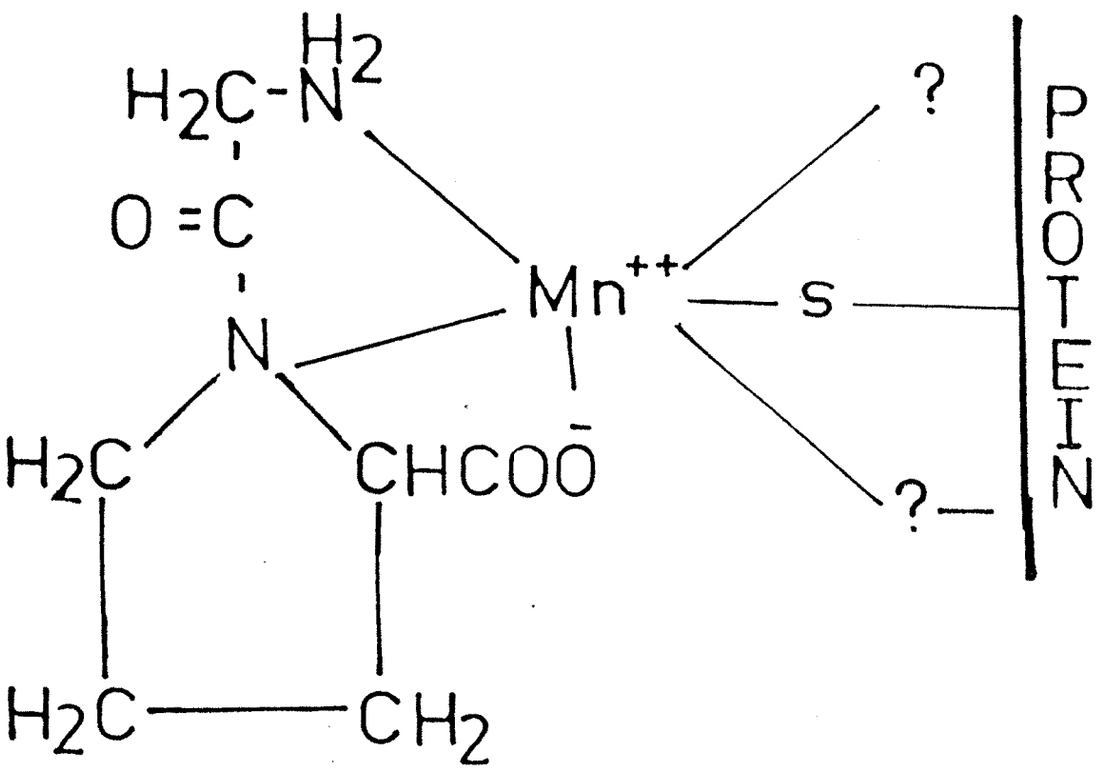


Fig. 22

(FRESH SEED)

Fig.No.24 :

Polygraph of paper chromatogram in B:A:W solvent system showing Rf x 100 values of flavonoid glycoside in seed leaves of Tribulus terrestris L. sprayed with micronutrient.



Postulated Coordination of glycyl-L- Proline with Prolidase

Fig-23

When the fresh seeds were extracted and chromatogramed, in the control (Q)-3-turinoside developed. Its quantity has increased almost one and half time when plant is sprayed with 15 ppm trace element mixture; but, when concentration increase to 20 ppm (K)-coumaroglucoside developed and with concentration of 25 ppm (K)-gentiobioside develop (Fig.22). These results clearly indicate that at low concentration of 15 ppm whether it is leaf or seed the synthesis of compound increases but the some other compounds of the same groups appears. That means although apparently we identify these compound as on the basis of Rf value, they are very close derivatives possibly of the same generic compound. It is essential to point out here that availability and non-availability of trace elements possibly play a very important role.

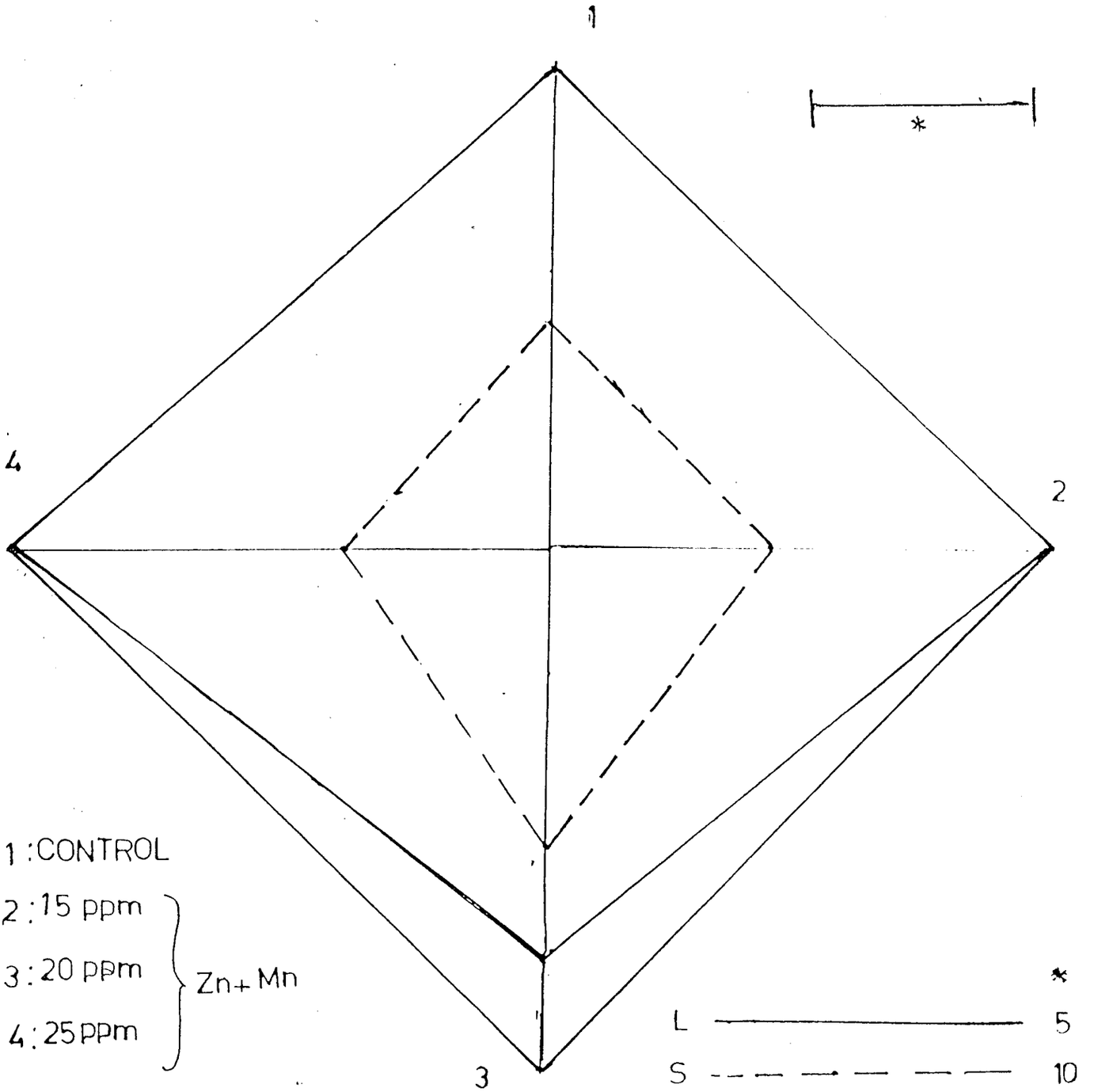
Mcelroy and Nason (1950) reviewal the mechanism of action of micronutrient in enzyme system. According to the trace element metals have very important role in enzyme catalysed reaction. Their mechanism action in catalysis is after combining with proteins. In many cases Zn^{++}, Mn^{++}, Cu^{++}, Ni^{++}, Co^{++} etc. form a chealates with dye in the absences of protein. They have discuss how Mn^{++} plays role in some of the enzymes which catalyse hydrolysis of glycil-l-proline and leucine aminopeptides which will hydrolyze many amino acid amides. The usual and most active substrate being L-leucinamide (Fig.23). In many catalytic activity Mg^{++} is often replaced

by Mn^{++}. For instance Mg^{++} or Mn^{++} condenser for Acetyl-CoA and oxalic acid to form citrate in citric acid cycle. Leucine amino peptidase can be activated either by Mn^{++} or Mg^{++} but for its full activity Mn^{++} is required rather than Mg^{++}. The predominent metal involved in the general enzymatic decarboxilation and hydrolysis reaction is Mn^{++}. Its mode of action is mainly by forming the metal chelate. It is established fact that Mn^{++} and Zn^{++} are required for the full activity of enzymes concerned with aromatic amino acids. For instance Zn^{++} is not constituent of pyruvic carboxylase but is necessary for synthesis of enzyme itself. It has also been shown that the activity of the enzyme concenned in the synthesis of tryptophan is completely suppressed in safely extracted Zn^{++} deficient Nerospora. This reflects on the essentiality of trace element. In conclusion the Mn^{++} is required primarily for those reactions involving group transfer particularly those in which phosphates participate. It is established however, that enzymes participates intimately in group transfer by serving as intermediate carriers. And in hydrolytic reaction Mn^{++} and certain extent Zn^{++} and Mg^{++} play an important role. Based on this we may say that the trace elements which penetrate through foliage stimulate certain catalytic enzymes or function as chelate.

For secondary metabolites such as flavonoid glycosides, genetic manipulation is difficult task because of the fact that

Fig.No.23 :

Postulated coordination of glycyl-L. proline with prolidase.



Polygraph of paper chromatogram in B : A : W solvent system showing Rf x 100 values of glycosides in seed and leaves of Tribulus terrestris L sprayed with micronutrient.

Fig - 24

there are inmumerable number of compounds. Besides the biochemical pathway leading to synthesis is extremely complex. It is spaculated that spontaneous isomerisation or conversion of one form to an other among the closely related derivatives or intermediates might be taking place in response to the natural environment. However, the broad genetic type may not alter. The foregoing discussion state that manipulation with the help of micromutrient can easily be achieved besides stimulating certain synthetic enzymes and thereby increase the turn over.

Polygraph of paper chromatogram in B:A:W solvent system showing Rf x 100 value of flavonoid glycosides in seed, and leaves of Tribulus terrestris sprayed with micronutrient (Fig. 24).