PHARMACODYNAMIC AND TOXICOLOGIC STUDIES OF INDIGENOUS PLANT - *Kalanchoe integra*

DISSERTATION

By

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M.V.Sc.

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Haryana Agricultural University

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submitted to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of :

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CERTIFICATE II

This is to certify that the dissertation entitled "Pharmacodynamic and Toxicologic Studies of Indigenous Plant - Kalanchoe integra" submitted by Dr. Ravindra Kishore Varma to the Haryana Agricultural University in partial fulfilment of the requirements for the degree of Ph.D., in the subject of Pharmacology, has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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External Examiner 7.10.77

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19.7.77.

(RAVINDRA KISHORE VARMA)

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CHAPTER I

INTRODUCTION

Plants constitute one of the major resources of the country. Proper exploitation of these resources on scientific lines is vital for the welfare and economic growth of the nation and can be tackled in different ways by the different scientific disciplines.

The plants are rich source of our indigenous medicines and they need extensive and systematic screening so as to explore cheaper and effective substitutes for the existing costly and often imported medicines. Besides, plants and weeds grown in pasture also pose a problem of plant poisoning in livestock and therefore, they also need to be investigated from the toxicity point of view so that economic losses are reduced.

Little data is available in India to show the total annual loss induced or influenced by poisonous plants to livestock industry. In the U.S.A., where sufficient knowledge of the poisonous plants and weeds are available and suitable preventive measures are undertaken, the statistics show that the annual losses caused by poisonous plants to livestock industry is of several million dollars. In India, where no systematic studies on plant toxicity to livestock in the different regions of the country have been conducted and consequently no specific preventive measures are undertaken, losses to livestock due to plant poisoning must be

considerable.

Some poisonous weeds and plants are very toxic and directly kill the animals when they eat these poisonous plants even in small quantity. The consumption of such weeds is increased under unfavourable weather conditions e.g. famine etc. Some other plants may not be too toxic to cause instantaneous death but may cause severe economic losses by causing reduction in milk yield or rendering milk unfit for human consumption, sterility, abortion and damage to skin and fleece etc. Veterinarians are often confronted with clinical cases of animals having eaten such plants and many a times fail to diagnose the trouble and give proper advice to the farmer. This is because, the syndrome of toxicity produced by these plants has not been investigated. Such handicaps to veterinarians can be overcome if the plants and weeds of the region are screened for their pharmacological and toxicological activities and the field staff properly educated.

Chopra and co-workers (1965) and several others have published books, monographs and glossaries on medicinal and toxic plants. These deal with the botanical and vernacular names, distribution and the informations about the possible therapeutic uses and toxicity. However, most of these informations are

incomplete, without any experimental data and scientific basis. Further the informations given do not indicate tha nature of the poisons and the possible way by which they produce toxic manifestations.

In view of the meagre informations available on the pharmacodynamic and toxicologic effects of a large number of plants available in our country particularly with respect to livestock industry, work on scientific lines to screen these plants is warranted. With this in view, the Department of Physiology and Pharmacology, Haryana Agricultural University, Hissar, in line with a few other institutions in the country has taken up a research project on indigenous plants. During the course of the investigation, cases of acute poisoning in sheep were reported from Himachal Pradesh with a plant locally known as 'Slundhru', which was later identified as Kalanchoe integra. Preliminary investigations regarding its toxicity and pharmacology was carried out by Singh et al. (1972, 1974) in this laboratory. The crude extract of the whole plant was found to be toxic to sheep and the symptoms resembled cyanide toxicity and that of cholinergics. The investigation being of preliminary nature, it was thought to take up detailed investigation of this plant with respect of phytochemical, pharmacodynamic and toxicological effects with an idea to suggest suitable remedial measures.

CHAPTER II

REVIEW OF LITERATURE

Some institutions in the country (Central Drug Research Institute, Regional Research Laboratories, Indian Veterinary Research Institute and a few veterinary and medical colleges) have taken up screening of indigenous medicinal plants to explore their possible therapeutic values both for human and animal use (Dhar et al., 1968; Bhakuni et al., 1969; Mukherjee and Pradhan, 1963 and Ahmad, 1976). So far, stress has been laid on screening of these plants for their medicinal values only and little attention has been paid to the toxicological aspect.

The Department of Physiology and Pharmacology, Haryana Agricultural University, Hissar has taken up a project in which the toxic and medicinal plants of this region are taken up for their systematic toxicological and pharmacological effects. So far, various plants like Lantana camara (Uppal, 1969), Anchusa strigosa (Garg et al., 1970), Eupatorium ayapana (Varma, 1973) and Albizzia lebbeck and Ipomoea carnea (Arora, 1973) have been taken up for the investigation. A plant Kalanchoe integra locally known as 'slundhru' in the Mandi District of Himachal Pradesh was reported to be toxic by the Disease Investigation Officer of Kamand Jersey Farm, Mandi, Himachal Pradesh and to cause acute death in sheep grazing on it. Preliminary work regarding its toxicity study in sheep and pharmacological activities

in laboratory animals was conducted by Singh *et al.* (1972, 1974). The review of literature show that no other work has been conducted with regard to the pharmacology and toxicology of *K.integra* and only the habitat, botanical characters, probable therapeutic uses and toxicity have been described (Chopra, 1955, 1956, 1958, 1965, 1969).

The genus *Kalanchoe* belongs to family Crassulaceae (synonym - Sedaceae) which is popularly known as Stone crop family.

Botanical Character of Family Crassulaceae :

Plants belonging to this family are mostly fleshy herbs and undershrubs. Leaves are sessile and exstipulate. The flowers are in the shades of yellow or red, more or less combined with green. Flower parts are typically free and distinct. Receptacle bears little scale at the base of each pistil.

According to Kirtikar and Basu (1933) there are 25 genera and 450 species while Mukherji and Ganguly (1964) reported that there are 30 genera and 1300 species. Benson (1957) has mentioned that *Bryophyllums* and *Kalanchoe* may be treated as one and the same genus.

Medicinal Value of Family Crassulaceae :

Kirtikar and Basu (1933) mentioned that the members of this family, in general, are vulnerary,

refrigerant, sedative, antiscorbutic and diuretic. A toxic acrid juice is contained in species of Sedum. Many crassulaceous plants yield malic acid. The following plants are used as official preparations in Portugal :

Cotyledon umbilicus Linn.

Umbilicus pendulinus De cand.

Sempervivum arboreum Linn.

(S. africanum Mill.)

S. tectorum Linn.

Botanical Character of Genus Kalanchoe :

Kalanchoe Adans are erect branched succulents, sometimes slightly woody at the base; leaves mostly opposite, fleshy; flowers medium to large, many in terminal panicled cymes, yellow, scarlet or purple; corolla urn-shaped or salverform, exceeding the calyx; stamens mostly 8, epipetalous, pistils 4, apocarpous; fruit a membranous follicle.

Species of Genus Kalanchoe :

Kalanchoe, the Chinese name of one of the genus contains about 70 species according to Kirtikar and Basu (1933) while Rendle (1959) and Cooke (1958) mentioned that it contains 100 and 25 species, respectively.

The important species of the genus Kalanchoe described by Kirtikar and Basu (1933) are as follows :

1. K.pinnata Pers.
2. K.spathulata
3. K.laciniata DC.
4. K.paniculata Harv.

The other species which are reported by different workers are listed below :

1. K.coccinia
2. K.blossfeldiana
3. K.daigremontiana
4. K.uniflora
5. K.glandulosa
6. K.floribunda
7. K.olivacea
8. K.bhidei
9. K.prolifera
10. K.integra (Medic.)Kuntze.
11. K.heterophylla Prain.
12. K.varians Haw.
13. K.verticillata
14. K.aegyptiaca

According to Hooker (1879) the following are the species found in India :

1. K.glandulosa
2. K.spathulata
3. K.floribunda
4. K.grandiflora
5. K.brasiliensis
6. K.laciniata

The species mentioned from 2 to 5 can be regarded as one species under the common name K.crenata.

Watt and Breyer - Brandwijk (1962) have reported the species found in Southern and Eastern Africa and are listed below :

1. K.glaberrima volkens.
2. K.hirta Harv.
3. K.laciniata DC.
4. K.oblongifolia Harv.
5. K.paniculata Harv.
6. K.prolifera R.Hamet.
7. K.rotundifolia Harv.
8. K.somalinesis Bak.
9. K.thyrsiflora Harv.

- According to Tutin and Heywood (1964) K.pinnata, Bryophyllum pinnatum Kurz. and B.calycinum salisb. are the same species. Dhar (1972, personal communication) reported that K.integra, K.spathulata and K.brasiliensis are one and the same species.

Distribution of Genus Kalanchoe :

The genus Kalanchoe is discontinuously distributed over a considerable part of tropical zone, South Africa and in the tropical regions of Asia. One species K.brasiliensis Camb. is found in Brazil, but also found in East Indies and tropical Africa. It is usually described as distinct and as a native, but it is possibly adventive. It is usually planted in gardens as ornamentals.

Medicinal Value of Genus Kalanchoe :

The following species are used medicinally according to Kirtikar and Basu (1933):
Indo-China and the Philippine islands - K.laciniata DC., K.pinnata Pers.-; in Brazil - K.brasiliensis Camb.-; in La Reunion - K.pinnata Pers. -; in the Gold Coast - K.crenata Haw. -; K.pinnata Pers.-; in South Africa - K.paniculata Harv., K.thyrsiflora Harv.

Kalanchoe integra

Botanical Character :

K.integra has stem 0.3 - 1.2 metre high, glabrous. Leaves spathulate - oblong, obtuse, crenate, cuneate at the base, glabrous; the lower petiolate, usually 7.5 - 12.5 by 3.8 - 5 cm. (sometimes reaching 25 by 10 cm.); the upper distant, becoming very narrow, 7.5 - 10 by 1.3 cm. (sometimes 3-foliolate), frequently sessile. Flowers in dense many - flowered cymose

panicles; lower bracts leaf-like. Calyx 6 - 10 mm. long, glabrous, divided almost to the very base; segments oblong - lanceolate or triangular from a broad base, acute or acuminate, 3 mm. broad at the base. Corolla clear yellow; tube glabrous, 1.3 cm. long; lobes broadly lanceolate, acute or shortly acuminate. Hypogymous scales narrow - linear, 33 mm. long, often bifid. Follicles glabrous (Fig. 1).

Vernacular Names :

The regional names of K.integra are as follows:

Hindi	-	Haize ka Patta, Haiza, Rungru, Tatara, Slundhru.
Kumaon	-	Bakal-patta, Pat-kuari.
Nepali	-	Hatho kane.
Punjabi	-	Haiza, Rungru, Tatra.

Distribution :

This plant is found in the tropical and sub-tropical Himalayas from Kashmir to Bhutan, generally between 1000 to 4000 feet, but near Simla and valleys below Simla it is found upto an altitude of 6000 feet. It is also found in Deccan. According to Duthie (1960), it is also found in Dehra Dun, Burma, the warmer parts of China and Java.

Pharmacology :

The expressed juice of the bitter variety is a drastic purgative (Bamber, 1916) and anti-periodic, toxic and purgative according to Chopra et al. (1969).

Fig. 1. Kalanchoe integra (Medic.) Kuntze.

It is poisonous to goats and is not eaten by cattle. Chopra and co-workers (1965) have prepared a list of important plants found wild or in a state of cultivation in India which are poisonous to man and livestock, which includes K.integra. Duthie (1960), Chopra and co-workers (1956, 1969) and Bamber (1916) also reported that it was poisonous to goat and cattle. The leaves are stated to be poisonous to insects. Chopra and co-workers (1941, 1969) reported that it had insecticidal and insect repellent properties while Chopra and Chopra (1955) claimed that it had piscicidal effect. The leaves are specific for cholera and prolonged fever. Chopra and co-workers (1955) and Bamber (1916) have confirmed it. In Kangra, they are burnt and applied to abscesses (Chopra, 1956).

Singh et al. (1972) studied the acute toxic effects of K.integra in sheep and its possible antidotal treatment was investigated. Crude powder and decoction of the whole plant was found to be lethal to sheep when given orally in dose of 3 - 5 g/kg. The symptoms were suggestive of cyanide poisoning and of cholinergic nature and the death was due to respiratory failure. Combination of sodium nitrite, sodium thiosulphate and atropine proved somewhat effective in combating the toxic effects of the plant.

The pharmacological studies of K.integra was done by Singh et al. (1974). The studies on isolated

guineapig ileum, frog heart, blood pressure of anaesthetised dog and the gross symptoms observed in the concious dogs, by them, indicated the presence of cholinergic activity in the plant.

Dhar (1972, personal communication) reported that the plant may have the possible anticancer activity.

K. pinnata

Vernacular Names :

Arabic	-	Kushnul hayat.
Bengali	-	Koppata.
Hindi	-	Zakhm haiyat.
Marathi and Gujarati	-	Ahiravana, Ghayamari, Mahiravana.
Persian	-	Chube hayat, Lakhm hayat.
Sanskrit	-	Asthi bhaksha
Tamil	-	Runakalli.
Telgu	-	Simajamuda.
Urdu	-	Chube hayat.

Distribution :

It is believed to be a native of tropical Africa but naturalised throughout the tropics of the world. In India, it is found in Sutlej valley on plains to an altitude of 3000 feet.

Pharmacology :

Kirtikar and Basu (1933) in their book "Indian Medicinal Plants" have described that the

leaves of K.pinnata are bitter and poisonous to insects. The bark is bitter and poisonous; tonic, alexipharmic, astringent to the bowels, analgesic, carminative; useful in diarrhoea, vomition and inflammations. Snakes and scorpions avoid this plant, hence its use in snake-bite and scorpion-sting is useful.

The leaves slightly toasted are used as an application to wounds, bruises, boils and bites of venomous insects. In the Konkan, the juice of the leaves is administered in 1/4 to 1/2 tola doses, with double the quantity of ghee in dysentery.

Decidedly beneficial effects follow their application to contused wounds and swellings; discolorations are prevented and union of the cut parts take place much more rapidly than it does with the ordinary treatment. Used in the form of poultice and powder for sloughing ulcers, it is a disinfectant.

Among the Mundas of Chota Nagpur, the leaves are gathered into a ball, baked, after which their juice is expressed. This is drunk against coughs, either alone or mixed with ghee and garlic.

In La Reunion, the plant is considered mucilaginous and emollient and is mostly used to prepare emollient baths.

The leaves are used in the Gold Coast to cure sore eyes. They are sometimes treated until soft and then applied to swellings which have first been treated with palm oil.

In Indo-China, the pounded leaves are applied to burns and scalds and also to corns.

The plant is not an antidote to either snake-venom or scorpion-venom. According to Chopra (1956), the leaves contain malic, isocitric and citric acids.

Bhakuni et al.(1969) reported the approximate LD_{50} (ALD_{50}) of the entire plant of K.pinnata to be more than 1000 mg/kg. They also subjected tha plant for the antibacterial, antifungal, antiprotozoal and anti-viral activities. The effects on the blood glucose, blood pressure, ganglia, respiration, guineapig ileum and central nervous system were also studied. The plant was screened for anticancer and diuretic activities.

K. laciniata

Vernacular Names :

Bengali and Hindi	-	Ham Sagar, Hem Sagar.
Marathi	-	Parnabij, Zakhm hyat.
Philippines	-	Siemprevica.
Sanskrit	-	Hemasagara.
Tamil	-	Malakalli.

Distribution

It is found in Bengal, Deccan, Burma, Ceylon, Java and tropical Africa.

Pharmacology :

According to Kirtikar and Basu (1933), the succulent leaves are valued as an application to wounds and sores; they allay irritation and promote cicatrization. In the Konkan, the juice of the leaves is given in bilious diarrhoea and lithiasis.

They are used for their good effects in cleaning ulcers and allaying inflammation. The juice is used externally in bruises and burns and to cure superficial ulcers. As a styptic, it is used on fresh cuts and abrasions. In Indo-China, the pounded leaves are applied in indolent ulcers.

Dey (1896) reported that the bruised leaves and juice in addition to have some reputation as an application to bruises and contusion had values in preventing discolouration.

K.paniculata

Vander (1944) reported that when tested for the presence of hydrocyanic acid, K.paniculata gave positive results.

K. prolifera

Clarke and Clarke (1975) have mentioned that K.prolifera was found to be toxic to cattle in Rhodesia

by Shone and Drummond (1965).

From all these informations, it appears that the pharmacological and toxicological studies of the plant K. integra have not been adequately conducted, except the preliminary work carried out in this Department, which is the objective of the present investigation.

CHAPTER III

MATERIALS AND METHODS

The plant Kalanchoe integra was collected during last week of September, 1975, from Kamand (District - Mandi of Himachal Pradesh) from where a few cases of poisoning in sheep with this plant had been reported earlier. The different parts of the plant (viz. leaf, stem, root and flower) were dried in the shade. After complete drying, these were powdered and stored in closed containers and used for subsequent phytochemical, toxicological and pharmacological studies.

A. EXTRACTION

The leaves of the plant were taken up for detailed extraction procedure (described later in this chapter) and depending upon the experience obtained with these procedures, the extraction of the other parts of the plant was undertaken. All the extracts or fractions obtained with the different extraction procedures were subjected to rapid selective testing in mice (Laurence and Bacharach, 1964). The criterion for rejection of any extract in the present investigation was the inability of the extract to produce toxicity in mice. The extract which was found to have maximum toxicity was taken up for the detailed toxicological and pharmacological studies.

1. Aqueous Extraction :

(i) A 10 per cent solution of the water soluble portion of the plant material was prepared by macerating finely powdered material in glass distilled water for 24 hours. During maceration, vigorous shaking at different time intervals was applied and the extract filtered after 24 hours with a strainer (four folds of muslin cloth). The filtrate was left for an hour in order to allow insoluble portion to settle down. The supernatent fluid was used in this study. The volume of the filtrate was made up to original volume by adding distilled water.

(ii) Decoction was prepared by boiling 100 ml of a 10 per cent solution of the powdered plant material in glass distilled water for about 2 hours and then filtered through muslin cloth. Distilled water was added to bring the volume to original level.

2. Organic Extraction :

Powdered material was also subjected to organic extraction as described below :

(i) A continuous extraction procedure was followed using a reflux condenser. The powdered material and petroleum ether, b.p. 60 - 80°C (in the ratio 1 : 10), were kept in a round bottom flask attached to a vertical reflux condenser. The heat was

applied directly from a heating mantle for 4 hours continuously. The solvent was then decanted off from the flask in a pre-weighed beaker which was later kept on a water bath to evaporate the petroleum ether and the extract was obtained. The extractability which was expressed as the percentage of weight of the dry extract after evaporation to that of plant material was also noted. In the same manner, absolute alcohol and glass distilled water were sequentially added to the residue in order to prepare the alcohol and water soluble extracts. These extracts were prepared by evaporating the solvents and the percentage extractability noted. All the beakers containing the extracts were secured tightly and stored at $0^{o}C$.

(ii) A continuous extraction procedure was employed as described above with a difference that instead of using heating mantle, the round bottom flask was kept in boiling water. The solvents used were petroleum ether and glass distilled water sequentially.

The fraction extracted with distilled water (i.e. K.integra minus K.integra extracted with petroleum ether) was subsequently extracted with chloroform - ethyl acetate in one set and ethyl acetate - chloroform in another set using separating funnels.

(iii) Sequential soxhlet extraction was carried out using petroleum ether and absolute alcohol, respectively.

(iv) A cold organic extraction was prepared following the procedure adopted for the preparation of the aqueous extract. The solvents used sequentially were petroleum ether, absolute alcohol and glass distilled water.

The cold petroleum ether extract was subjected to column chromatography using alumina as the adsorbent and petroleum ether, petroleum ether + benzene (1 : 1), benzene, benzene + chloroform (1 : 1), chloroform, chloroform + methanol (1 : 1) and methanol as solvents. The different fractions were collected.

(v) The aqueous extract was further subjected to extraction with petroleum ether. The aqueous extract was mixed with an equal volume of petroleum ether in a separating funnel, vigorously shaken and left overnight after which the different layers were separated.

The stem was subjected to cold aqueous extraction and cold organic extraction. The root and flower were subjected to cold aqueous extraction only.

B. PHYTOCHEMICAL STUDIES

1. Quantitative Tests :

The leaf and stem were quantitatively analysed

for their ash, calcium, phosphorus and potassium contents. Solubility of the ash in water or acid was also determined.

(i) Ash value : A weighed amount of the powdered material in a pre-weighed china dish was kept in a muffle furnace. The furnace was maintained at a temperature of 650°C for 2.5 hours. The loss in the weight of the powdered material was noted and the ash value determined.

(ii) Solubility of ash in water : A weighed amount of ash was dissolved in a measured volume of hot distilled water, stirred well and then filtered through the pre-weighed whatman filter paper. The solubility of ash in water was noted by taking into account the insoluble portion remaining on the filter paper which was weighed after drying in the hot air oven at 60°C.

(iii) Solubility of ash in acid : The solubility of ash was seen in dilute hydrochloric acid (10 per cent solution) using the same method as described above.

(iv) Estimation of calcium and phosphorus : The estimation of calcium and phosphorus was done by the method described by A.O.A.C. (1960).

Ash of 2 gms of the powdered material was dissolved in 20 per cent hydrochloric acid (10 - 15 ml) and 5 ml of concentrated hydrochloric acid (36 per cent)

and evaporated on a hot plate (20°C). To this, was added 10 ml of 36 per cent (1+1) hydrochloric acid and the mixture kept on water bath for 30 minutes. This was filtered through whatman filter paper No. 44 and the filterate was made upto 100 ml by addition of 5 per cent hydrochloric acid. To 10 ml aliquot of the filterate was added 10 ml of saturated ammonium oxalate and a drop or two of methyl red. This was made neutral with ammonia, a faint yellow colour developed. It was again boiled with ammonia when the precipitate became granular. This was cooled and to it was added one or two drops of 20 per cent hydrochloric acid, a faint pink colour developed. This was left for 4 hours or preferably over-night after which it was filtered through whatman filter paper No. 40. Hot water washing was given to make it free from oxalate. The hot water washing was done till the white precipitate turned colourless. The precipitate alongwith the filter paper was kept in the same beaker. It was triturated with 20 ml of 10 per cent sulphuric acid. N/10 potassium permangnate was added to this. The temperature of the reaction mixture was maintained at 60 - 70°C. The volume of N/10 potassium permangnate required for giving the end point was noted and calculation done as under :

$$\% \text{ of Cal.} = \frac{.002 \times 100 \times 100 \times \text{ml of } \frac{N}{10} \text{ Pot.permangnate}}{\text{Weight of sample} \times \text{Volume of aliquot taken}}$$

To 10 ml of aliquot was added 10 ml of 20 per cent ammonium molybdate and 10 ml of concentrated nitric acid simultaneously. This was left over-night and then filtered through whatman filter paper No. 40. The residue in the filter paper was washed with 2 per cent potassium nitrate to make it free from acid for which a blue litmus paper was used.

The precipitate alongwith the filter paper was taken in the same beaker and was dissolved in a known volume of $\frac{N}{10}$ sodium hydroxide. The indicator phenolphthalein was added. This was titrated against $\frac{N}{10}$ sulphuric acid to know the exact volume of $\frac{N}{10}$ sodium hydroxide used to dissolve the precipitate and the calculation done as under :

$$\% \text{ of Phosph.} = \frac{.0001347 \times 100 \times 100 \times \text{ml of } \frac{N}{10} \text{ Sod.hydroxide}}{\text{Weight of sample} \times \text{volume of aliquot taken}}$$

(v) Estimation of potassium : A weighed quantity of ash was dissolved in a measured volume of hot distilled water and 10 per cent hydrochloric acid. The potassium content in these solutions was estimated with the help of flame photometer.

2. Qualitative Tests :

The leaves and stem were qualitatively tested for the presence of cyanide, alkaloids, glycosides, reducing sugars, fixed and essential oils and gases.

(i) Test for cyanide : The powdered plant material was dissolved in adequate volume of dilute sulfuric acid (12 per cent) in a round bottom flask and this was heated on a hot plate. The distillate was collected in a container using a Leibig condenser. The following reactions served to identify the presence of hydrocyanic acid in the distillate.

(a) The smell of the distillate gave the odour of bitter almond indicating that hydrocyanic acid was present in the plant material.

(b) Picric acid test paper* turned from yellow to brown within a period of 5 minutes, the intensity of the colour depending upon the concentration of hydrocyanic acid.

(ii) Tests for alkaloids : The presence of the alkaloids in the petroleum ether and the alcoholic extracts of the plant was tested with Mayer's** and Draggondorff's*** reagents. A little amount of the

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*Picric acid test paper were prepared by dipping strips of filter paper into a solution containing 1 gm picric acid in a 10 per cent sodium carbonate solution. The dipped paper was dried at room temperature.

**Mercuric chloride (1.36 g) was dissolved in 60 ml of distilled water. Potassium iodide (5 g) was dissolved in 10 ml of distilled water. These two solutions were mixed and diluted to 100 ml with distilled water.

***Bismuth nitrate ($Bi(NO_3)_2, 5H_2O$) (8 g) was dissolved in 20 ml of nitric acid (sp.gr. 1.18) and potassium iodide (27.2 g) in 50 ml of distilled water separately. These two solutions were mixed and allowed to stand. When potassium nitrate crystallized out, supernatant fluid was decanted off and was made upto 100 ml with distilled water.

petroleum ether or alcoholic extract was dissolved in dilute hydrochloric acid (10 per cent). To this was added a few drops of either of the reagents. The following reactions indicated the presence of alkaloids.

(a) Appearance of white precipitate on addition of the Mayer's reagent to the acidified solution of the extract indicated the presence of alkaloids.

(b) Appearance of orange red precipitate after the addition of Draggondorff's reagent indicated the presence of alkaloids.

(iii) Test for glycosides : To the aqueous solution of the petroleum ether or alcoholic extract was added two drops of an alcoholic solution of alpha-naphthol* and to the well cooled mixture, under a stream of cold water, 2 ml of sulphuric acid was carefully added. A violet ring at the junction of the acid and the water layer appeared, thus indicating the presence of glycosides or free sugars (Molisch's test).

(iv) Test for reducing sugars : The aqueous, petroleum ether and alcoholic extracts were tested for the presence of reducing sugars. To 2 ml of the aqueous solution of these substance were added 0.5 ml of Fehling's Solution (Fehling I and II mixed immediately before use) and 2 ml of 10 per cent sodium hydroxide

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*One gm alpha-naphthol in 100 ml of 95 per cent alcohol.

solution. These mixtures were heated on water bath for 10 minutes. Appearance of the red precipitate indicated the presence of reducing sugars.

(v) Test for fixed oils : Appearance of thick oily residue in the petroleum ether extract indicated the presence of fixed oils.

(vi) Test for essential oils : A little amount of the powdered material was dissolved in adequate volume of distilled water in a round bottom flask. A few glass beads were put in the flask to avoid bumping. The solution was heated and distilled. The distillate was then saturated with sodium chloride. Appearance of an oily layer over the aqueous layer indicated the presence of essential oils.

(vii) Test for gases : A 10 per cent solution of the aqueous extract was slightly warmed and the gas was collected in a graduated burette employing the water displacement method.

C. TOXICOLOGICAL STUDIES

1. Acute Toxicity and Gross Observable Effects :

The acute toxicity study was conducted in the laboratory animals viz. mice, rats, rabbits and dogs and the farm animals like sheep and buffalo calves. The mice, rats and rabbits were obtained from the Small Animal House of the Haryana Agricultural University; while dogs,

sheep and buffalo calves were purchased locally. The experimental animals were kept under standard management conditions.

(i) Mice and rats : Groups of albino mice (20 - 25 g) and rats (150 - 200 g) of both sexes were administered graded doses of the aqueous extracts of K.integra orally or intraperitoneally. After administration of the extracts, the animals were observed for gross effects for first 6 hours continuously and then at six hourly interval upto 72 hours. Gross effects including autonomic, neurologic and toxic effects were observed according to the method outlined by Irwin (1959). The number of animals dying during 24, 48 and 72 hours was noted. LD_{50} was calculated by the method of Litchfield and Wilcoxon (1949).

(ii) Dogs and rabbits : The aqueous extract of leaf was injected intravenously in the ear vein of rabbits (1 - 1.5 kg) and in the saphenous vein of dog (6 - 10 kg) and the animals were observed for gross effects and lethality.

(iii) Buffalo calves and sheep : Male buffalo calves (80 - 120 kg) and Nali sheep (10 - 15 kg) administered orally with different doses of aqueous extract of leaf, were observed for any gross effect continuously for 4 hours in the beginning and then at different time intervals for a week.

2. Sub-acute Toxicity Study in Sheep :

Thirty days sub-acute toxicity study in sheep was conducted with the aqueous extract of leaf administered daily (200 mg/kg, orally).

Seven healthy Nali sheep (10 - 15 kg) were kept under observation for a week. During the observation period, faecal and blood smear examination for the helminths and blood protozoan parasites, respectively was conducted to ensure that the sheep were healthy.

The weight of the animals were taken on alternate days, the rate of feed consumption observed and the symptoms noted. Before administration of the extract, the haematology and biochemical parameters (to be described later on) of the animals were studied. These haematological examinations and investigation of the biochemical parameters were further carried out on the 10th, 20th and 30th day or just before death in those animals which died earlier. The postmortem and histopathological examination of the dead animals were also carried out.

(i) Haematological parameters: The haematological examination included the estimation of haemoglobin, total erythrocytic count, packed cell volume, erythrocyte sedimentation rate (24 hours) and total and differential leucocytic counts.

All these examinations were conducted according to the standard methods while the packed cell volume and the erythrocyte sedimentation rate (24 hours) were seen according to the method described by Wintrobe and Landsberg (1935).

(ii) Biochemical parameters : The biochemical parameters included the estimation of the levels of glutamic pyruvate transaminase, glutamic oxaloacetate transaminase, alkaline phosphatase and lactic dehydrogenase in serum and the whole blood glucose.

(a) Serum glutamic pyruvate transaminase (SGPT) : The levels of enzyme SGPT was estimated by the method of Reitman and Frankel (1957). A standard curve was prepared as follows :

Five clean and dry test tubes were placed in a rack and numbered 1 to 5. Solutions were added in the following manner :

Tube Number	Alanine-ketoglutarate substrate	Distilled water	Pyruvate standard	SGPT units
1.	1.0 ml	0.2 ml	-	0
2.	0.9 ml	0.2 ml	0.1 ml	28
3.	0.8 ml	0.2 ml	0.2 ml	57
4.	0.7 ml	0.2 ml	0.3 ml	97
5.	0.6 ml	0.2 ml	0.4 ml	150

To all these five tubes were added 1 ml of colour reagent, 2,4-dinitrophenyl hydrazine and allowed to stand at room temperature for 20 minutes exactly, after which 10 ml of 0.4 N sodium hydroxide was added and allowed to stand for a further period of 10 minutes. The optical density was read at 505 mμ setting the photometer to read zero optical density with distilled water and a graph of optical density against SGPT units was plotted. This was used as the standard curve.

For the test samples, 1 ml of substrate solution was taken into a clean test tube and was placed in a water bath at 37°C for 5 minutes to equilibrate. Then 0.2 ml of unhaemolysed serum was added to the above tube and mixed. This was kept in the water bath at 37°C for exactly 30 minutes. At the end of the above incubation, the tube was taken out from the water bath and 1 ml of colour reagent was added. The rest of the procedure was same as that described for the standard curve. Interpolation of the reading in the calibration curve was done to obtain the level of SGPT enzyme in the sample.

(b) Serum glutamic oxaloacetate transaminase (SGOT): It was estimated by the method of Reitman and Frankel (1957). A standard curve was prepared as follows :

Five clean and dry test tubes were placed in a rack and numbered 1 to 5. The solutions were added

in the following manner :

Tube Number	Aspartate-ketoglutarate substrate	Distilled water	Pyruvate standard	SGOT units
1.	1.0 ml	0.2 ml	-	0
2.	0.9 ml	0.2 ml	0.1 ml	24
3.	0.8 ml	0.2 ml	0.2 ml	61
4.	0.7 ml	0.2 ml	0.3 ml	114
5.	0.6 ml	0.2 ml	0.4 ml	190

The rest of the procedures for the standard curve and unknown samples were the same as that of the SGPT enzyme estimation except that the incubation time for the unknown samples was 1 hour instead of 30 minutes.

(c) Alkaline phosphatase : The serum alkaline phosphatase activity was determined by the method of Kind and King (1954). In this method, the phenol released from the substrate disodium phenyl phosphate by the enzymatic hydrolysis at the pH 10 was estimated colorimetrically at 510 mμ setting to read zero optical density with blank. The following equation was used for the determination of KA units.

$$\text{KA units/ 100 ml} = \frac{\text{Optical density of test} - \text{Optical density of control}}{\text{Optical density of standard} - \text{Optical density of blank}} \times 10$$

The following schedule was followed :

Reagent sequence in ml	Test	Control	Blank	Standard
Buffer	1.0	1.0	1.1	1.1
Substrate	1.0	1.0	-	-
	incubated at 37°C for 3 minutes			
Water	-	-	1.0	-
Standard	-	-	-	1.0
Serum	0.1	-	-	-
	incubated at 37°C for 15 minutes			
Sodium hydroxide	0.8	0.8	0.8	0.8
Serum	-	0.1	-	-
Sod. bicarbonate	1.2	1.2	1.2	1.2
Antipyrine	1.0	1.0	1.0	1.0
Ferricyanide	1.0	1.0	1.0	1.0
	mixed thoroughly after addition of each reagent			

(d) Lactic dehydrogenase (LDH) : The serum lactic dehydrogenase enzyme level was determined by the colorimetric method of Cabaud et al. (1958). A standard curve was prepared. To six clean and dry test tubes, the pyruvate buffer and glass distilled water were added in the following manner :

Tube Number	Pyruvate buffer	Distilled water	LDH units
1.	0.5 ml	-	0
2.	0.4 ml	0.1 ml	300
3.	0.3 ml	0.2 ml	700
4.	0.2 ml	0.3 ml	1000
5.	0.1 ml	0.4 ml	1500
6.	0.05 ml	0.45 ml	2000

To each of the six tubes were added 0.5 ml of 2,4-dinitrophenyl hydrazine solution, mixed and left for exactly 20 minutes at room temperature. To this was added 5 ml of 0.4 N sodium hydroxide, mixed and allowed to stand for 30 minutes at room temperature. The optical density was noted at 505 mμ setting the photometer to read zero optical density with water. The optical density was plotted against the LDH units on a linear graph paper and the activity of the unknown was determined from this standard curve.

For the unknown samples, 0.01 ml of non-haemolyzed serum was added to 0.05 ml of pyruvate substrate (1 hour before use, 10 ml of pyruvate buffer was mixed with 0.010 g of $NADH_2$), mixed and incubated at 37°C for 45 minutes. The rest of the procedure was same as that described for the preparation of standard curve.

(e) Whole blood glucose level : The whole blood glucose level in the protein free blood filterate was determined colorimetrically at 420 mμ using Spectronic 20 by the method of Folin and Wu (1920).

For the haematological examination and the whole blood glucose, the blood samples were collected in clean and sterilized rubber cork stoppered vials of 10 ml capacity. About 5 ml of blood was collected using sodium fluoride (10 mg/ml of blood) as a preservative and anticoagulant as described by Oser (1965). All haematological examinations were conducted on the same day. For biochemical examination, about 20 ml of blood was collected in a 50 ml clean and sterilized test tube. The tube was immediately kept in a slanting position and care was taken to avoid minimum possible jerks to it. In this position the tubes were kept for a minimum of an hour at the place of collection after which they were transferred to laboratory where these were kept for another 3 to 4 hours. The tubes were then kept in an upright position in a test tube stand and were chilled in a refrigerator over-night. The next morning, serum was collected in well stoppered test tubes with the help of a pasteur pipette and stored in freezing chamber of the refrigerator. All the estimations were carried out as early as possible but not later than 5 days of collection.

D. PHARMACOLOGICAL STUDIES

1. Effect on Forced Co-ordinated Motor Activity :

The effect of the aqueous extract of leaf on the forced coordinated motor activity in mice was tested by the rota-rod method as described by Kinnard and Carr (1957). The mice were trained to stay on a horizontal rotating rod (2 cms in diameter and rotated at 12 RPM) for at least 3 minutes. The extract, in different doses, was administered intraperitoneally to groups of trained mice (six in each), while saline was injected in control group. The treated mice were subjected to rota-rod test at every 30 minutes interval upto a period of 6 hours and then at six hourly interval upto a period of 48 hours. Treated mouse falling off within 120 seconds was considered to have been affected.

2. Effect on Spontaneous Motor Activity :

The spontaneous motor activity of mice before and after administration of different doses of the aqueous extract of leaf was measured with photocell activity cage (Actophotometer)*. Groups (six in each) of albino mice of either sex were administered intraperitoneally different doses of the extract. The control animals were administered saline only. The activity of each group was measured by placing them in the actophotometer cage for 5 minutes, before administration and at the

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*Adair Dutt and Co. (India) P.Ltd.

interval of 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 8, 12 and 24 hours, after treatment.

3. Analgesic Effect :

The hot plate method of Eddy and Leimbach (1953) was employed to test the analgesic activity. An individual mouse was placed on the thermostatically controlled hot plate*, maintained at a temperature of 55 ± 0.5°C and the reaction time to the heat stimulus through the sensory nerve endings of its feet was determined. The reaction time was considered to be the time elapsing between placing of the mouse on to the hot plate and the time when the signs of acute discomfort appeared. The acute discomfort was characterised either by kicking with hind legs, licking the fore paws or frisking about the restraining enclosure.

The reaction time of each mouse was noted. If the reaction time was more than 6 seconds, the animal was discarded. After screening, the animals were divided in groups (six in each) and were administered different doses of leaf extract. The saline treated animals served as control. The reaction time of each mouse for a maximum period of 10 seconds was recorded before and 1, 2, 3, 4, 6, 8, 12 and 24 hours after the administration of the extract. The results were analysed on a quantal basis.

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*The Techno Electronic Ltd., Lucknow.

4. Effect on Supramaximal Electroshock Induced Seizures (SMES) :

SMES was induced in mice by electro-convulsiometer* through the electrodes attached to pinnae according to the method outlined by Swinyard et al.(1952). Only those mice which showed extensor tonic spasm to electroshock (48 mA for 0.2 seconds), 24 hours before the test, were used in the experiment. Different doses of leaf extract were administered to groups of mice (six in each). Abolition of the extensor tonic spasm of the hind leg was kept as the criterion for anticonvulsant effect. Treated mice were subjected to electroshock at an interval of 1, 2, 3, 4, 6, 8, 12 and 24 hours.

5. Effect on Chemically Induced Seizures :

The chemically induced seizures in mice was induced with pentylene tetrazol (metrazol) given subcutaneously at the rate of 85 mg/kg according to the method described by Swinyard et al.(1952). Groups of mice (five in each) were injected the different doses of leaf extract intraperitoneally. At the time of peak effect, the animals were challenged with metrazol and the pattern of convulsions was noted.

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*The Techno Electronic Ltd., Lucknow.

6. Effect on Pentobarbitone Induced Sleeping Time :

The aqueous extract of leaf was tested for its effect on the duration of hypnosis produced by pentobarbitone sodium (50 mg/kg, i.p.). The experiment was conducted at room temperature of 21 ± 1°C. The criterion for hypnosis was the loss of righting reflex. The mice were gently placed on their back as soon as they lost the righting reflex. Criterion for awakening from sleep was the correction of the posture at least 3 times within a period of 30 seconds when they were placed on their back . The time elapsing between the loss of righting reflex and awakening of animals was taken as sleeping time. The aqueous extract of leaf was administered intraperitoneally to groups of mice (six in each), 1 hour before the administration of pentobarbitone sodium. Duration of the sleeping time was recorded in minutes.

7. Effect on Conditioned Avoidance Response (CAR):

To study the effect of aqueous extract of leaf on the conditioned avoidance response, method of Cook and Weidly (1957) was employed. Only those rats which climbed the pole within 5 seconds of sound stimulus were considered trained and taken up for this study. In one group of trained rats, the aqueous extract of leaf was injected intraperitoneally while in other,

normal saline was injected. The animals were tested for CAR after 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 hours of treatment. CAR was considered to be blocked in rat which did not respond to sound stimulus within 30 seconds. Affected rats were subjected to unconditioned stimulus i.e. shock.

8. Cardiovascular and Respiratory Effects :

Healthy mongrel dogs (6 - 10 kg) of either sex, anaesthetised with pentobarbitone sodium (30 mg/kg, i.v.), were employed to study the effects of the extracts on blood pressure, respiration, myocardium and electrocardiogram.

(i) Effect on blood pressure and respiration : The trachea of the anaesthetised dog was cannulated and connected to a pressure manometer for recording the rate and depth of respiration. The right carotid artery was cannulated and connected to a mercury manometer through a rubber tube filled with 12 per cent sodium citrate solution which acted as an anticoagulant. The venous cannula was inserted in one of the femoral veins, the other end of which was attached to a burette, containing normal saline solution, from which 2 ml saline was released in order to push the drugs or extracts into the general circulation every time after the injection of drugs.

Initially, normal responses of the carotid occlusion, acetylcholine, adrenaline and histamine to the blood pressure and respiration were recorded kymographically. The effect of the extracts were then observed on the blood pressure and respiration.

Any alteration in the responses to the carotid occlusion, acetylcholine, adrenaline and histamine was also seen. Atropine and promethazine were used as blockers for investigating hypotensive effect of the extracts.

(ii) Effect on myocardium : Anaesthetised dogs were maintained on artificial respiration and the thoracic cavity was opened giving a longitudinal incision over the sternum. The walls of the thoracic cavity were retracted to expose the heart completely. Hooks tied with long threads were inserted into the apex of the left ventricle and right auricle separately and the threads were passed over the pulleys to connect the Starling levers. Normal auricular and ventricular movements were recorded kymographically after which the extracts were injected intravenously.

(iii) Effect on electrocardiogram : Electrocardiogram was recorded using standard limb lead II with dog in the lateral recumbency at different time intervals.

(iv) Effect on blood vessels (Rat hind leg perfusion): A rat was stunned with hammer blow and the abdominal cavity was opened. The posterior aorta, just before the bifurcation to iliac artery, was exposed and cannulated with a cannula, the other end of which was connected with a tube leading to the reservoir containing Ringer Solution*. The upper portion of the body above the cannulation was separated. The pressure of the fluid was controlled with the help of Murffe's drip and pinch-cock between reservoir and cannula. The perfusion rate (120 drops/minute) was adjusted. The venous outflow was collected in a graduated measuring cylinder every minute. Both the number of drops and the volume of the fluid were measured. The extracts were injected in different concentrations only after the rate of the returning venous fluid became constant for three consecutive minutes. The effect was noted for 5 minutes. The next injection was given only when the rate of drops/minute returned either to original or to a constant level. The experiments were conducted at room temperature of 35 ± 2°C.

*Sodium chloride: 9.00 g; Potassium chloride: 0.42 g; Calcium chloride: 0.24 g; Sodium bicarbonate: 0.50 g; Glucose: 1.00 g and Distilled water: 1000 ml.

9. Effect on Isolated Frog Heart :

A frog was stunned with a hammer blow on the head and abdominal cavity opened by a median ventral incision. The heart was exposed by cutting the sternum and clavicles and retracting these to the sides. The sinus-venosus was exposed by turning the heart upside down. The other end of the sinus-venosus was ligated. A venous cannula connected with a rubber tube to the reservoir, containing frog Ringer Solution modified by Starling* at room temperature (30 ± 2°C), was inserted and fixed in the sinus-venosus. The heart was then freed from all its attachments and removed from the thoracic cavity. The pressure of fluid was controlled with the help of Murffe's drip and pinch-cock in between the reservoir and the venous cannula. The flow of the perfusion fluid was regulated to 120 drops per minute and was kept constant throughout the experiment. A hook was inserted into the apex of the ventricle and the thread tied to other end of the hook was connected to a Starling heart lever, fixed below the level of the heart. Rate and amplitude of the heart were recorded on smoked

*Sodium chloride: 6.5 g; Potassium chloride: 0.14 g; Calcium chloride: 0.12 g; Sodium bicarbonate: 0.2 g; Sodium dihydrogen phosphate: 0.01 g; Glucose: 2.0 g; and Distilled water: 1000 ml.

drum. The extracts/drugs were instilled into the perfusion fluid in different concentrations.

10. Effects on Smooth Muscle (Guineapig ileum) : Guineapigs (300 - 400 g), fasted over-night, were used for this experiment. A sharp blow with a hammer was applied to stun the animal. The abdominal cavity was opened by a mid-line incision. The caecum was lifted and the ileo-caecal junction was identified. The ileum was cut at this point. A length of ileum was removed and transfered to a petri-dish containing Tyrode Solution*, tying a thread at the duodenal or jejunal end, to serve as a marker. The mesentery was trimmed away and pieces (about 3 cm each) were cut from the length of the ileum, starting above the Peyer's patch. Washing of the lumen of its contents, if necessary, was done with the help of a syringe applying minimum hydrostatic pressure and using Tyrode solution. A thread was tied at each end of ileum taking care that the threads did not close its lumen and mounted in an organ bath of 30 ml capacity maintained at 35 ± 0.5°C. The tissue was continuously

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*Sodium chloride: 8.0 g; Potassium chloride: 0.2 g; Calcium chloride: 0.2 g; Sodium bicarbonate: 1.0 g; Sodium dihydrogen phosphate: 0.05 g; Glucose: 1.0 g; Magnesium chloride: 0.1 g and Distilled water: 1000 ml.

aerated and allowed to equilibrate for about 30 minutes, after which a few responses of acetylcholine were obtained till three consecutive responses, for the same concentration of acetylcholine, were constant. Acetylcholine was allowed to act for 15 seconds and then washed. Three minutes rest to the tissue was given before adding another dose of acetylcholine. The extracts were added to the organ bath in different concentrations and allowed to remain in contact with the tissue for 5 minutes, after which acetylcholine, in the same concentration used previously, was added and allowed to act for exactly 15 seconds, after which it was washed twice. Any change in the response to acetylcholine was noted. Similarly, the effect of extracts on histamine and barium chloride induced contractions was also studied.

11. Effect on Skeletal Muscle (Frog rectus abdominis muscle) : A frog was stunned by sharp blow on the head, pithed and mounted on wax board. The rectus abdominis muscle was carefully dissected out and transferred to a petri-dish containing Frog Ringer solution modified by Starling. Rectus abdominis muscle was tied at both the ends and mounted in organ bath of 20 ml capacity, maintained at room temperature ($28 \pm 1^{\circ}C$). The tissue was continously aerated. Eserine salicylate (10 µg/ml of perfusion fluid)

was added in the reservoir, containing Frog Ringer solution, to inhibit the acetylcholine esterase enzyme, in order to get a greater response of acetylcholine. The tissue was considered to be stabilized when three consecutive responses to a perticular concentration of acetylcholine were constant. The extracts were tested for its own effect, as well as their effects on acetylcholine response.

12. Effects on Blood Coagulation

(i) Bleeding time : Bleeding time in rabbits was determined by the method of Duke (1915) as described by Kolmer et al.(1969). The hair at the margin of the ear was clipped and cleaned with xylene. A standard puncture was made, using a spring lancet, so that, blood flowed freely without squeezing. A whatman filter paper was used to soak/mop the oozing blood at an interval of 15 seconds. The lapse of time when the blood first appeared and time when the blood ceased to ooze was considered to be bleeding time.

(ii) Coagulation time : Coagulation time in rabbits was determined by the capillary tube method of Wright and Colebrook (1921) with slight modification. The hair at the tip of the ear was clipped and cleaned with xylene. A fairly deep puncture was made quickly, using a spring lancet, so that, blood flowed freely without

squeezing. The first two drops of the blood were discarded and then a capillary tube (0.5 mm x 5 cm) was filled upto three fourth of its total length with blood by capillary action. The capillary tube was moved upside down and when a diminished movement of the blood inside the capillary was noticed, a portion of the capillary tube was then gently broken off from one end, at frequent intervals of time, until a thin line of unbroken coagulum was seen stretched between the broken ends. The time elapsing between the appearance of stretched unbroken coagulum and the time when blood first entered the capillary tube was taken as the coagulation time. The coagulation time was determined before and after administration of extracts at different time intervals.

The tubes were not sealed and kept in warm distilled water at 37°C because the coagulation time in rabbit is low. However, all the experiments were conducted at a room temperature of 35 ± 2°C.

13. Effect on Blood Glucose Level :

The whole blood glucose level in rabbits was determined according to the method of Folin and Wu (1920) as described by Oser (1965). The blood was collected from the marginal ear vein, of an over-night fasted rabbit, and used for the determination of the blood glucose, before and after the administration of the extracts, at 2 hours interval upto a period of 6 hours.

14. Antibacterial Effects :

The antibacterial activity of the extracts was tested by means of the agar cup plate method as described by Merchant and Packer (1967). A flask of plain nutrient agar was seeded with 1 ml of 24 hours broth culture of Bacillus anthracis, Staphylococcus aureus, Pseudomonas aeroginosa, Salmonella hvittingfoss and Escherichia coli respectively, obtained from the Department of Veterinary Bacteriology and Hygiene, Haryana Agricultural University, Hissar. This inoculated agar was poured into sterile petri-dishes using approximately 50 ml per plate and was allowed to harden. With a sterile tube of 0.5 cm diameter, provided with a rubber bulb, four wells were made on each nutrient agar plate aseptically. The bottom of the cut area was sealed with melted agar.

The aqueous extracts prepared in sterile glass distilled water and further filtered through Seitz filter, were used in different concentrations. In control experiments, for the gram positive organisms, penicillin (5 I.U.) was used while for the gram negative organisms, streptomycin (S_{25}) antibiotic discs were used. The agar plates were then incubated at 37°C for 24 hours and appearance of the zone of inhibition around the margin of the cup, if any, was noted.

E. ANTIDOTAL TREATMENT

Attempts were made to investigate into antidotal treatment for the acutely poisoned animals (mice and sheep). For this, a 100 per cent lethal dose of the aqueous extract of leaf was administered to mice (500 mg/kg, i.p.) and sheep (5 g/kg, orally). In case of mice the treatment was given at the peak hour (2.5 hours post administration of the extract) and 5 and 10 hours after administration while in case of sheep, the treatment was resorted when the sheep showed the toxic symptoms (usually 20 to 30 minutes post administration). The details of the treatment have been given in the result section.

CHAPTER IV

RESULTS

A. EXTRACTION

The results showed that the cold aqueous extraction of leaf resulted in maximum yield (40.95 per cent). The hot petroleum ether gave 6.64 per cent extractability, while on successive extraction of the residue left after petroleum ether extraction, with absolute alcohol and distilled water, the percentage extractability was 3.61 and 35.45, respectively. The percentage extractability on Soxhlet extraction with petroleum ether and absolute alcohol sequentially were 4.85 and 3.72, respectively. Cold petroleum ether extraction gave the extractability of 2.51 per cent. The yield from the residue after cold petroleum ether extraction, when treated subsequently with absolute alcohol was 2.01 per cent. The petroleum ether extraction of aqueous extract had much smaller extractability i.e. 0.23 per cent.

The results of the rapid selective test in mice with the different extracts of leaf showed that the ALD_{50} with cold petroleum ether extract was 50 mg/kg, i.p. and with aqueous extract, LD_{50} was 230 mg/kg, i.p. (Table 3). However, keeping in view the extremely low extractability of petroleum ether as compared to distilled water, difficulty in making the petroleum

ether extract in injectable form and its toxicity in proportion to the extractability, the aqueous extract of leaf was taken up for the further toxicological and pharmacological studies.

Similarly, rapid selective test in mice with the organic as well as the aqueous extracts of stem showed that aqueous extract of stem was more toxic as compared to organic extracts. The LD_{50} of aqueous extract was found to be 60 mg/kg, i.p. (Table 5).

The root and flower had the same order of toxicity as that of leaf and ALD_{50} of aqueous extracts of root and flower was found to be 230 mg/kg, i.p. in mice.

B. PHYTOCHEMICAL STUDIES

The results of phytochemical studies are presented in table 1 and 2.

1. Quantitative Tests:

The leaf on quantitative analysis showed that it had a higher percentage of ash (27.65 per cent) and its solubility in water and dilute hydrochloric acid was 14 and 60 per cent, respectively. It was observed to have a higher percentage of calcium (14.05 per cent) and the level of phosphorus was found to be 0.498 per cent. The potassium levels in ash, soluble in water and acid were estimated to be 26.0 and 11.2 per cent, respectively.

Table 1

Phytochemical tests of Kalanchoe integra leaf.

Sr. No.	Quantitative Tests	Content Percent	Sr. No.	Qualitative Tests	Present/ Absent
1.	Ash	27.65	1.	Cyanide	Traces
a)	Water Soluble	14.00	2.	Alkaloids	Present
b)	Acid* Soluble	60.00	3.	Glycosides	Present
2.	Calcium	14.05	4.	Reducing Sugars	Absent
3.	Phosphorus	0.498	5.	Fixed Oils	Present
4.	Potassium in		6.	Essential Oils	Present
a)	Water Soluble Ash	26.00	7.	Gases	Present
b)	Acid* Soluble Ash	11.20			

*Dilute hydrochloric acid - 10 per cent solution.

Table 2

Phytochemical tests of Kalanchoe integra stem.

Sr. No.	Quantitative Tests	Content Percent	Sr. No.	Qualitative Tests	Present/ Absent
1.	Ash	13.35	1.	Cyanide	Present
a)	Water Soluble	23.00	2.	Alkaloids	Present
b)	Acid* Soluble	60.00	3.	Glycosides	Present
2.	Calcium	7.00	4.	Reducing Sugars	Present
3.	Phosphorus	0.468	5.	Fixed Oils	Present
4.	Potassium in		6.	Essential Oils	Present
a)	Water Soluble Ash	17.60	7.	Gases	Present
b)	Acid* Soluble Ash	17.20			

*Dilute hydrochloric acid - 10 per cent solution.

The stem had fairly good percentage of ash (13.35 per cent) and its solubility in water and acid was found to be 23 and 60 per cent, respectively. The calcium content of ash was 7.0 per cent while that of the phosphorus was 0.468 per cent. The potassium levels in the water and acid soluble ash was 17.6 and 17.2 per cent, respectively.

2. Qualitative Tests :

The leaf was found to contain traces of cyanide and gave positive colour reactions for alkaloids and glycosides. The test for reducing sugars was negative. Presence of fixed oils, essential oils and gases was also noted.

The stem gave positive reactions for cyanide, alkaloids, glycosides, reducing sugars, fixed and essential oils and this emitted gas.

C. TOXICOLOGICAL STUDIES

1. Acute Toxicity and Gross Observable Effects :

(i) Mice and rats : The 24 hours LD_{50} of the aqueous extract of leaf in mice and rats by intraperitoneal route was found to be 230 (172.90 - 305.90) and 560(482.75 - 649.60) mg/kg, respectively. The 48 and 72 hours LD_{50} in mice were 118 (70.23 - 198.24) and 50 (35.21 - 71.00) mg/kg intraperitoneally, respectively.

The results pertaining to the acute toxicity and LD_{50} of the aqueous extract of leaf are summarised in table 3 and 4 and illustrated in figures 2 and 3.

Although, the 24 hours LD_{50} in mice was less than that in rats but the mice died in 10 - 12 hours while rats died in about 5 to 6 hours.

The extract was found to be ineffective orally in mice and rats as it did not produce any gross observable effect in doses upto 2000 mg/kg.

The mice were observed for the gross effects and the main symptoms included increased respiratory rate, writhing, decreased alertness, mild passivity, decreased spontaneous motor activity, crouching, staggering gait and decreased palpebral opening. These symptoms varied from mild to moderate depending upon the dose. The death was preceded by anoxic convulsions and gasping and was due to respiratory failure.

The toxic signs observed in rats were not markedly different from those in mice.

No gross postmortem finding was observed except congestion of liver in mice and rats.

The 24 hours LD_{50} of the aqueous extract of stem in mice by intraperitoneal route was found to be 60 (37.03 - 97.20) mg/kg. The results are presented in table 5 and figure 4. The gross symptoms in mice

Table 3

Lethal effects of the aqueous extract of *Kalanchoe integra* leaf in mice

Dose (mg/kg,i.p.)	24 hours toxicity			48 hours toxicity			72 hours toxicity		
	No. of mice		Percent	No. of mice		Percent	No. of mice		Percent
	Dead	Taken	effect	Dead	Taken	effect	Dead	Taken	effect
25	-	-	-	3	22	13.6	5	22	22.7
50	-	-	-	5	22	22.7	11	22	50.0
100	1	10	10.0	5	10	50.0	16	22	72.7
200	6	16	37.5	10	16	62.5	-	-	-
300	7	10	70.0	-	-	-	-	-	-
LD_{50} (mg/kg) with 95% confidence limit	230.0 (172.90 - 305.93)			118.0 (70.23 - 198.24)			50.0 (35.21 - 71.00)		

Table 4

Lethal effects (24 hours) of the aqueous extract of *Kalanchoe integra* leaf in rats.

Dose (mg/kg,i.p.)	No. of Rat Dead	Taken	Percent effect	LD_{50} (mg/kg) with 95% confidence limit
500	3	7	42.9	
600	3	6	50.0	
650	4	6	66.7	560.0 (482.75 - 649.60)
700	5	6	83.3	

Table 5

Lethal effects (24 hours) of the aqueous extract of *Kalanchoe integra* stem in mice.

Dose (mg/kg,i.p.)	No. of mice Dead	Taken	Percent effect	LD_{50} (mg/kg) with 95% confidence limit
25	1	6	16.7	
50	2	6	33.3	
75	4	6	66.7	60.0 (37.03 - 97.20)
100	5	6	83.3	

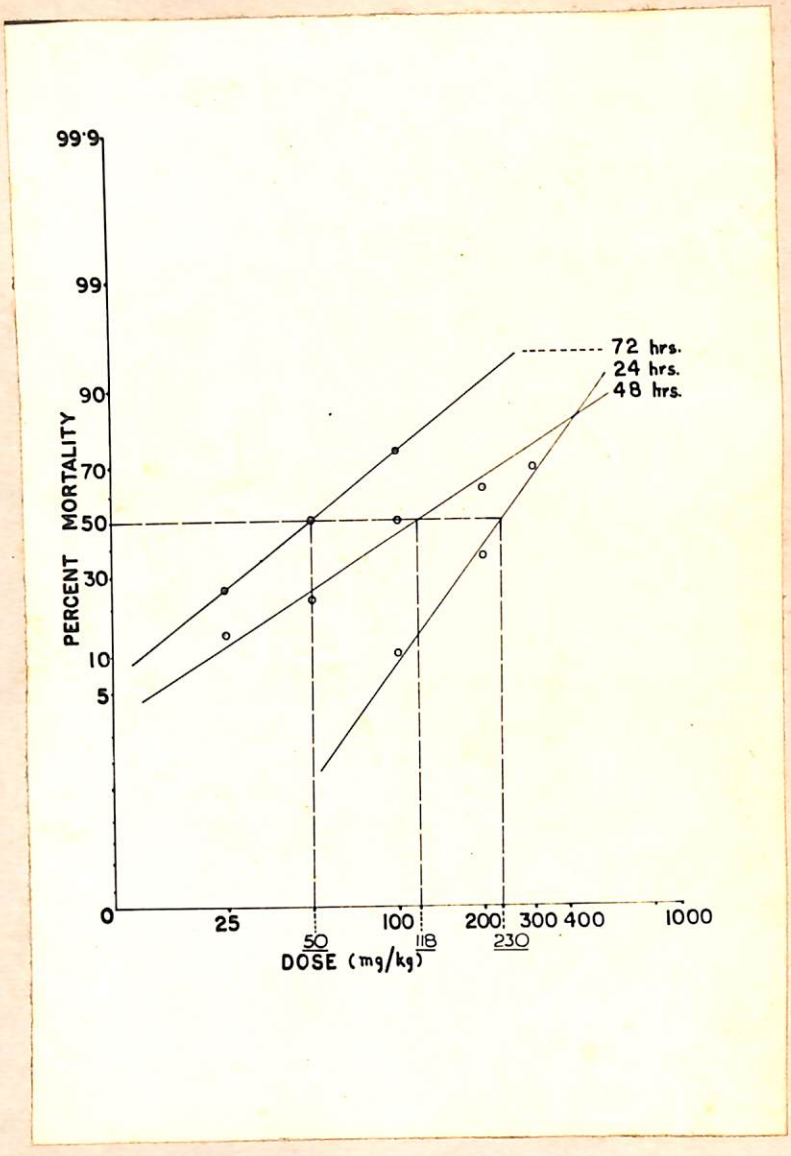

Fig. 2. Dose-mortality curves of the aqueous extract of Kalanchoe integra leaf in mice by intraperitoneal route. Separate curves for 24, 48 and 72 hours mortality have been shown.

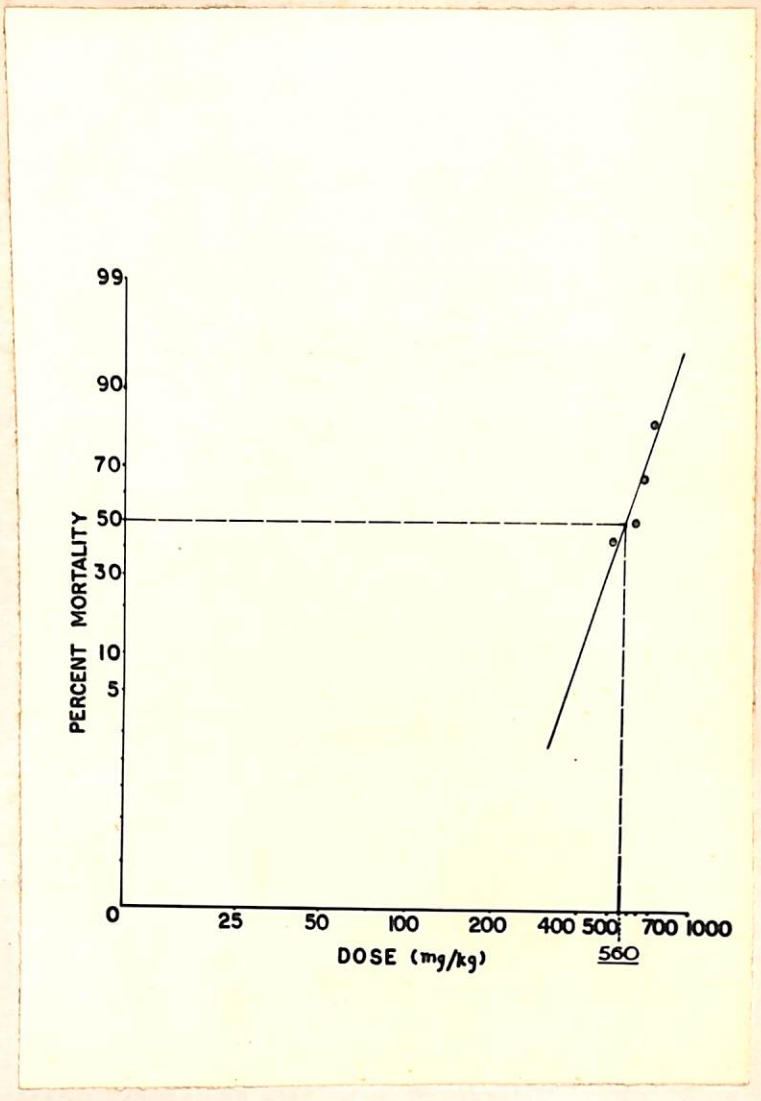

Fig. 3. Dose-mortality curve (based on 24 hours mortality data) of the aqueous extract of Kalanchoe integra leaf in rats by intraperitoneal route.

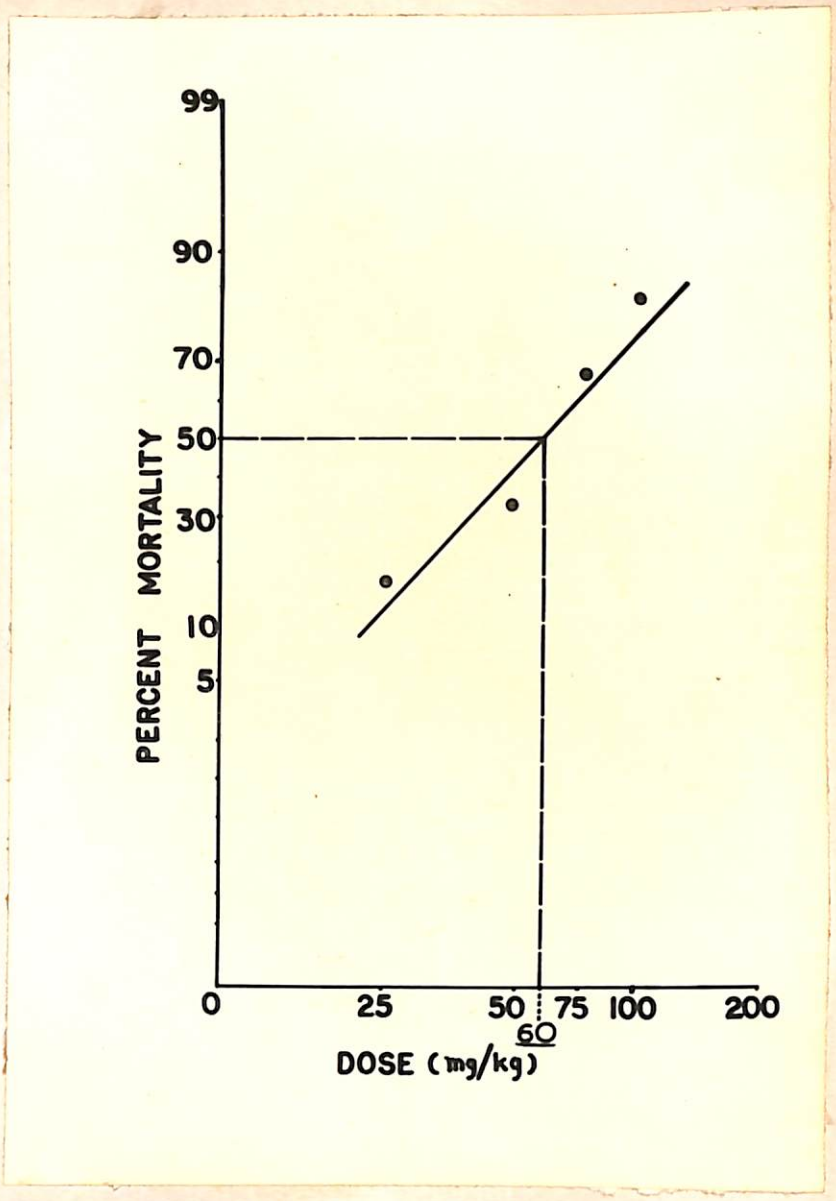

Fig. 4. Dose-mortality curve (based on 24 hours mortality data) of the aqueous extract of Kalanchoe integra stem in mice by intraperitoneal route.

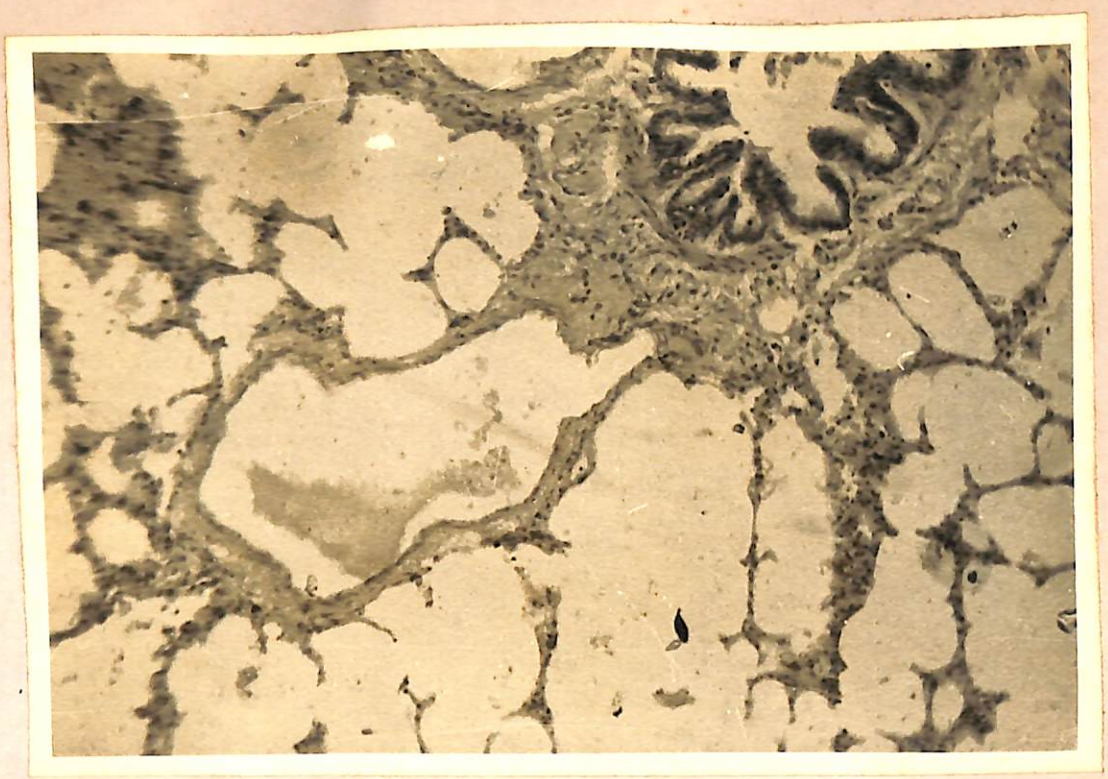

Fig. 5. Effect of the aqueous extract of Kalanchoe integra leaf (5 g/kg, orally) on the lung of sheep showing emphysema of alveoli and bronchiolar constriction (H. & E. 100X).

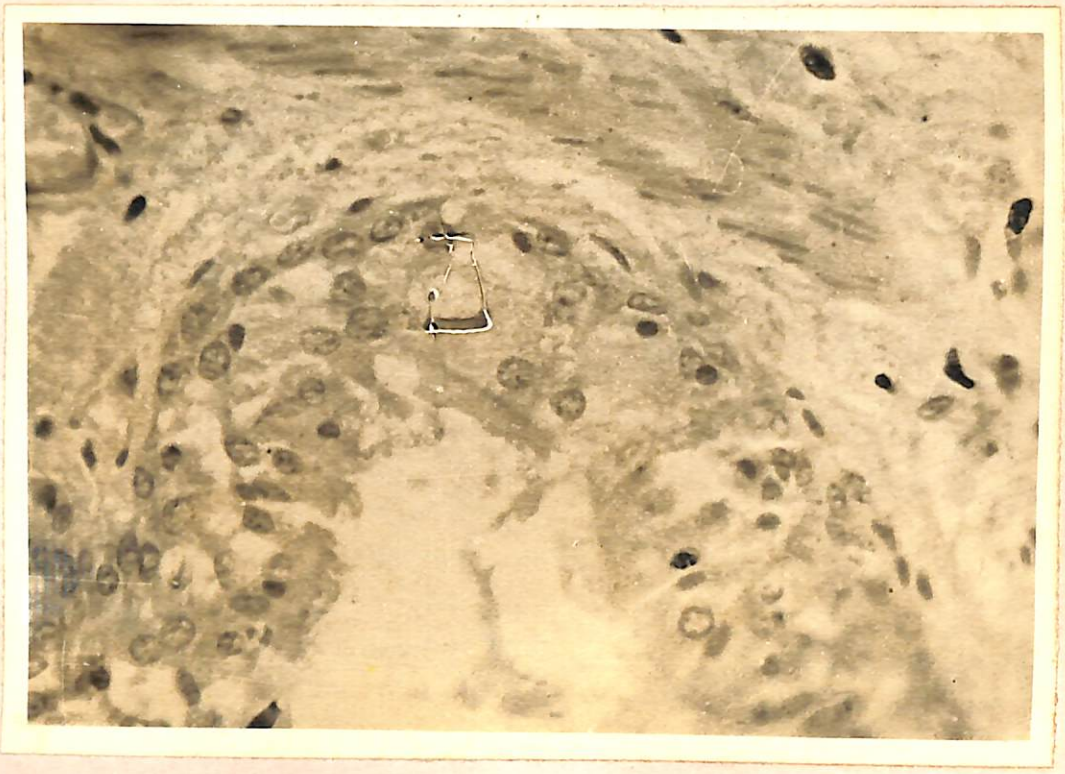

Fig. 6. Effect of the aqueous extract of Kalanchoe integra leaf (5 g/kg, orally) on the bronchus of sheep showing hyperplasia and degenerative changes in goblet cells (H. & E. 400X).

was also observed. Extensive dilatation of perivascular space and compression of capillaries of cerebrum was noticeable.

2. Sub-Acute Toxicity Study :

The results of the 30 days sub-acute toxicity study in sheep have been summarised in Tables 6 to 8 and figures 7 and 8.

(i) Gross symptoms : Daily administration of the aqueous extract of leaf (200 mg/kg, orally) for 30 days reduced the body weight of sheep. The reduction in body weight was 32.15 per cent on the 25th day in comparison to its pretreatment value. The feed consumption of the animals declined after 10th day and was severely reduced by the 20th day. The animals showed general debility, dehydration, loss of fat around the eye ball (shunken eye ball), tough inelastic skin, 'break' i.e. wool fibres pulled out on gentle manipulations, inability to support their body, muscular incoordination, bronchitis, muconasal discharge, diarrhoea, dysentry, urination, coma, gasping, anoxic convulsions and death due to respiratory failure. Four out of 5 animals died between 21st to 28th day. Two of the animals died on the 21st day while two died on the 27th and 28th day, respectively.

(ii) Effect on haematological parameters : The haemoglobin, total erythrocytic count and packed cell volume all increased in comparison to their pre-treatment values on the 10th and 20th day while these showed a slight decrease in the value just before death. The erythrocyte sedimentation rate showed a trend of decrease which was maintained throughout the experiment. The total and differential leucocytic counts did not show any appreciable change.

(iii) Effect on bio-chemical parameters :

(a) SGPT : The serum glutamic pyruvate transaminase enzyme level increased from the pretreatment level of 27.6 ± 10.88 RF units/ml to 30.1 ± 11.50 units on the 10th day of the experiment while its levels on the 20th day and just before death were 39.8 ± 11.01 and 40.7 ± 25.25 units, respectively.

(b) SGOT : The serum glutamic oxaloacetate transaminase enzyme level increased from 102.6 ± 28.14 RF units/ml on zero day to 115.8 ± 5.64 units on the 10th day. The levels on the 20th day and just before death were 171.2 ± 5.53 and 191.0 ± 47.00 units, respectively.

(c) Alkaline phosphatase : The level of the enzyme alkaline phosphatase increased from the pre-treatment value of 3.66 ± 1.07 KA units/100 ml to

Table 6

Effect of the aqueous extract of Kalanchoe integra leaf (200 mg/kg, orally for 30 days) on the body weight of sheep.

Parameter	Mean* body weight in kg on day 0	5	10	15	20	25**	30
Body Weight	10.76 (0.35)	10.62 (0.41)	9.78 (0.35)	8.46 (0.32)	7.74 (0.27)	7.30 (0.11)	-

*Values are mean of five animals
**Values are mean of three animals
Figure in parentheses denote the standard error

Table 7

Effect of the aqueous extract of Kalanchoe integra leaf (200 mg/kg, orally for 30 days) on some haematological parameters in sheep.

Parameters	Mean* values on day 0	10	20	Before Death**
Haemoglobin (g/100 ml)	10.5 (0.22)	10.7 (0.41)	11.7 (0.30)	9.8 (0.20)
Total erythrocytic count (millions/c mm)	10.16 (0.11)	10.32 (0.15)	10.79 (0.11)	9.88 (0.07)
Packed Cell volume (percent)	35.0 (1.58)	37.8 (1.42)	43.0 (4.01)	35.0 (3.00)
Erythrocyte sedimentation rate/24 hours in mm.	5.0 (0.55)	3.8 (0.37)	3.2 (0.37)	4.75 (1.25)

*Values are mean of five animals
**Values are mean of two animals
Figure in parentheses denote the standard error.

7.98 ± 2.88 units on the 10th day while on the 20th day and just before death the levels were 5.31 ± 1.95 and 8.02 ± 1.08 units, respectively.

(d) LDH: The enzyme lactic dehydrogenase showed a decline in its level on the 10th day, a rise on the 20th day and just before death. Its level on the day 0, 10, 20 and just before death were 706 ± 82.56, 584 ± 80.66, 990 ± 112.52 and 915 ± 195.00 units/ml, respectively.

(e) Blood glucose : The blood glucose level showed a continuous rise upto the 20th day while just before death, its level was lowered. The blood glucose levels on day 0, 10, 20 and just before death were 114.2 ± 6.66, 118.0 ± 7.21, 142.2 ± 14.41 and 81.5 ± 29.50 mg/100 ml, respectively.

(iv) Post-mortem findings : The gross post-mortem findings were loss of subcutaneous fat, slight hydroperitoneum, lungs mildly congested with emphysema, trachea showing slight congestion and frothy exudate and pale liver with peticheal haemorrhages on surface. The cortex and medulla of kidney were pale. There was mild hydropericardium, heart was flabby and at the apex ecchymotic haemorrhages were present. Excessive serous fluid in the perimeningeal area was observed. On histopathological examination of lung sections, congestion,

Table 8

Effect of the aqueous extract of *Kalanchoe integra* leaf (200 mg/kg, orally for 30 days) on some biochemical parameters in sheep.

Parameters	Mean* values on day 0	10	20	Before death**
SGPT (RF units/ml)	27.6 (10.88)	30.1 (11.50)	39.8 (11.01)	40.7 (25.25)
SGOT (RF units/ml)	102.6 (28.14)	115.8 (5.64)	171.2 (5.53)	191.0 (47.00)
Alkaline phosphatase (KA units/100 ml)	3.66 (1.07)	7.98 (2.88)	5.31 (1.95)	8.02 (1.08)
Lactic Dehydrogenase (units/ml)	706 (82.56)	584 (80.66)	990 (112.52)	915 (195.00)
Glucose (mg/100 ml)	114.2 (6.66)	118.0 (7.21)	142.2 (14.41)	81.5 (29.50)

*Values are mean of five animals
**Values are mean of two animals
Figure in parentheses denote the standard error

haemorrhage, emphysema of alveoli and stenosis of bronchioles was revealed (Fig.7). The liver sections revealed that there were focal areas of haemorrhages and cloudy swelling was present particularly around the portal tract. Tubular epithelium of kidney showed cloudy swelling. Sections of the heart showed congestion of epicardial blood vessels, haemorrhages in epicardial fat and cloudy swelling of myofibrils. Spleen showed moderate haemosiderosis. There was distinct oedema of Purkinje cell layer of cerebellum and the Purkinje cells were undergoing necrosis. Vacuoles of varying size were present separating the granular layer (Fig.8). The perivascular spaces were extensively dialated.

D. PHARMACOLOGICAL STUDIES

1. Effect on Forced Coordinated Motor Activity :

The aqueous extract of leaf in the doses of 25, 50, 100 and 200 mg/kg, i.p. in mice affected the motor coordinated activity except at the lowest dose. At 50 mg/kg, the impairment of motor activity was observed in one out of six animals after 60 minutes of the administration of the leaf extract. At 100 and 200 mg/kg dose levels, the effect appeared after 30 minutes in one and two animals, respectively (Table 9).

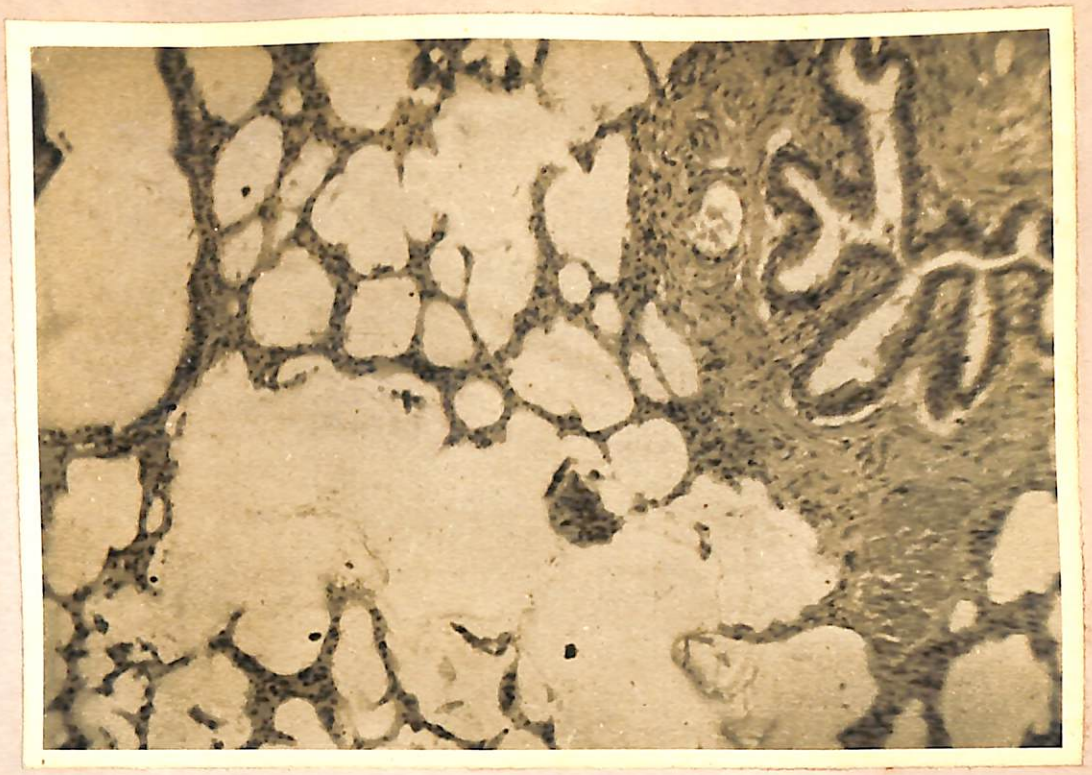

Fig. 7. Effect of the aqueous extract of Kalanchoe integra leaf (200 mg/kg, orally for 30 days) on the lung of sheep showing emphysema of alveoli and stenosis of bronchioles (H. & E. 100X).

Fig. 8. Effect of the aqueous extract of Kalanchoe integra leaf (200 mg/kg, orally for 30 days) on the cerebellum of sheep showing oedema of Purkinje cell layer and necrosis of Purkinje cells (H. & E. 100X).

Table 9

Effect of the aqueous extract of *Kalanchoe integra* leaf on the forced coordinated motor activity in mice.

Dose (mg/kg,i.p.)	Percent* of animals affected at minute						
	0	30	60	90	120	180	240
Normal Saline	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0
50	0	0	16.66	0	0	0	0
100	0	16.66	0	0	0	0	0
200	0	33.33	0	0	0	0	0

*Each dose was tested on a group of six mice.

Note: Out of six mice, one died between 24 to 48 hours at 100 mg/kg dose level, while at 200 mg/kg, two died within 24 hours and the rest died between 24 to 48 hours. All the animals showed motor incoordination before death.

Out of six mice, one mouse died between 24 to 48 hours at 100 mg/kg, while at 200 mg/kg, two died within 24 hours and the rest four died between 24 to 48 hours. All the animals exhibited motor incoordination before death.

2. Effect on Spontaneous Motor Activity :

The spontaneous motor activity was decreased by the aqueous extract of leaf at 10 and 20 mg/kg dose levels. At the lower dose, the effect started after 30 minutes while at the higher dose, after 15 minutes only. The peak effect was observed at 150th minute in both the cases after which there was a trend to recover, however, even at the 24th hour the spontaneous motor activity did not recover to the control level.(Fig.9).

3. Analgesic Effect :

The aqueous extract of leaf at 10 mg/kg dose level had no analgesic effect, however, at the higher dose level (20 mg/kg) slight analgesic activity was observed. The reaction time was prolonged upto a period of 3 hours. The results are presented in table 10.

4. Effect on Supramaximal Electroshock Induced Seizures (SMES) :

No protection against the SMES was observed with the aqueous extract of leaf (10 and 20 mg/kg, i.p.) upto a period of 24 hours.

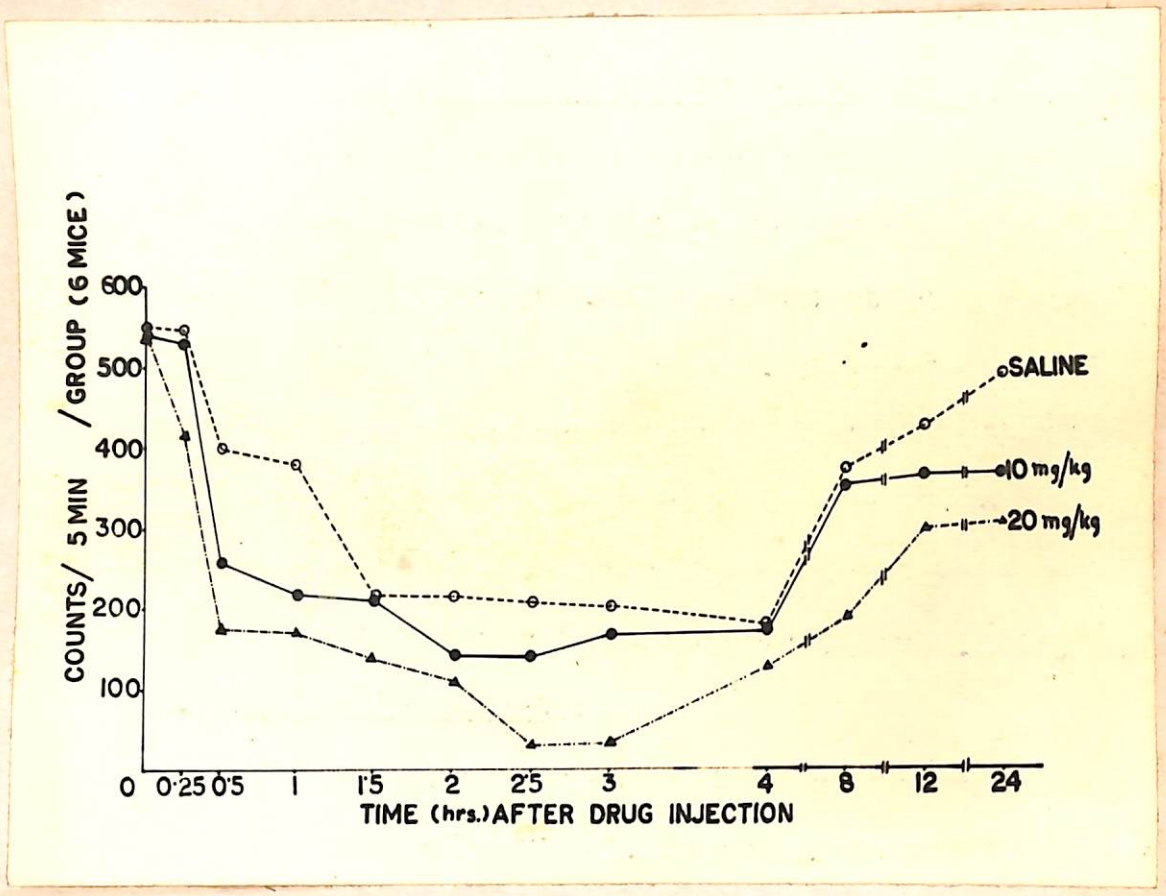

Fig. 9. Effect of the aqueous extract of Kalanchoe integra leaf (10 and 20 mg/kg, i.p) on the spontaneous motor activity in mice.

Table 10

Analgesic activity of the aqueous extract of *Kalanchoe integra* leaf in mice

Dose (mg/kg, i.p.)	Reaction time (seconds) at hour					
	0	1	2	3	4	6
Normal Saline	6.0	3.8	4.2	4.5	4.0	5.5
	3.6	2.5	2.5	3.5	2.0	4.0
	4.0	3.5	4.5	2.0	3.0	4.3
	3.5	3.5	4.5	4.0	3.0	3.2
	3.8	3.0	2.0	1.5	2.5	3.9
	5.0	2.5	2.5	3.5	3.5	4.0
Mean	$\overline{4.32}$	$\overline{3.13}$	$\overline{3.36}$	$\overline{3.16}$	$\overline{3.00}$	$\overline{4.15}$
	(0.40)	(0.22)	(0.47)	(0.48)	(0.28)	(0.31)
10	3.0	2.5	2.5	2.0	2.5	2.7
	6.0	5.4	5.3	5.5	5.0	5.8
	5.5	3.5	3.6	3.5	4.0	5.0
	3.5	3.2	3.0	4.0	3.5	4.0
	6.0	7.8	7.0	7.3	7.5	8.0
	5.0	4.0	4.5	4.0	4.5	5.2
Mean	$\overline{4.83}$	$\overline{4.40}$	$\overline{4.32}$	$\overline{4.38}$	$\overline{4.50}$	$\overline{5.12}$
	(0.52)	(0.78)	(0.67)	(0.74)	(0.70)	(0.73)
20	5.0	8.5	8.3	8.0	7.5	8.0
	2.5	9.0	9.5	9.0	4.2	3.0
	5.5	13.5	14.5	8.5	4.5	4.3
	6.5	12.5	14.0	13.0	9.0	8.2
	7.0	9.0	15.5	12.0	8.0	7.0
	3.5	7.0	8.0	7.6	4.0	3.0
Mean	$\overline{5.00}$	$\overline{9.91}$	$\overline{11.63}$	$\overline{9.68}$	$\overline{6.20}$	$\overline{5.58}$
	(0.71)	(1.03)	(1.40)	(0.92)	(0.90)	(0.99)

Figure in parentheses denote the standard error.

5. Effect on Chemically Induced Seizures :

All the mice pretreated with the aqueous extract of leaf (10 and 20 mg/kg, i.p.) and subsequently challanged with metrazol exhibited clonic convulsions. This indicated the inability of the extract to protect the mice from chemoshock. However, it was observed that there was a little delay in the onset of convulsions and increase in survival time (Table 11).

6. Effect on Pentobarbitone Induced Sleeping Time :

The aqueous extract of leaf at 10 mg/kg, i.p. dose produced slight increase in the pentobarbitone induced sleeping time (9.79 per cent) in mice, while at 20 mg/kg dose level marked increase in the sleeping time (83.83 per cent) was observed (Table 12).

7. Effect on Conditioned Avoidance Response (CAR) :

The CAR was impaired in two out of six (33.33 per cent) trained rats treated with the aqueous extract of leaf (100 mg/kg, i.p.). Such an effect was observed one hour after the administration of the extract. The unconditioned avoidance response (UAR) was not affected. One out of six rats died within 24 to 48 hours.

8. Cardiovascular and Respiratory Effects :

(i) Effect on blood pressure and respiration: The aqueous extracts of both leaf and stem showed marked hypotensive effect in anaesthetised dog at a dose rate

Table 11

Effect of the aqueous extract of Kalanchoe integra leaf on the metrazol (85 mg/kg, s.c.) induced seizures in mice.

Dose (mg/kg,i.p.)	Time (minutes) taken for the occurance of* Preconvul-siveness	Clonic convulsion	Tonic convulsion	Death
Normal Saline	5.6 (0.81)	10.4 (0.98)	15.0*** (1.53)	23.0*** (4.72)
10	5.6 (0.81)	11.2 (1.56)	16.0*** (2.00)	25.0*** (6.80)
20	9.8 (1.65)	22.4 (4.92)	38.5** (6.81)	40.0 (5.68)

*Values are mean of five mice
**Values are mean of four mice
***Values are mean of three mice
Figure in parentheses denote the standard error.

Table 12

Effect of the aqueous extract of Kalanchoe integra leaf on the pentobarbitone sodium (50 mg/kg, i.p.) induced sleeping time (minutes) in mice.

Mouse Number	Normal saline	Aqueous extract of leaf (10 mg/kg,i.p.)	(20 mg/kg,i.p.)
1	60.0	68.0	83.0
2	43.0	33.0	100.0
3	38.0	74.0	105.0
4	73.0	43.0	66.0
5	21.0	40.0	78.0
Mean	47.0	51.6	86.4
S.E.	(8.99)	(8.14)	(7.17)

of 25 mg/kg, i.v. The range of the fall of the blood pressure was 53.71 to 66.66 per cent and 33.33 to 83.08 per cent with the aqueous extract of leaf and stem, respectively. In the pilot experiments, it was found that a dose lesser than 25 mg/kg did not lower the blood pressure appreciably. It was also observed that the hypotensive effect produced by the extracts was not repeatable upto a period of 6 hours. Both the extracts did not change the blood pressure response to carotid occlusion, acetylcholine or histamine. However, there was a little exaggerated response of adrenaline (Fig.10,11).

Atropine (1.5 mg/kg, i.v.) and promethazine (5 mg/kg, i.v.) were used in separate experiments to find whether they blocked the hypotensive action and it was found that both the blockers failed to alter the blood pressure response of the leaf and stem extracts (Fig.12,13). An increase in the rate as well as depth of the respiration was noted with the aqueous extracts of leaf and stem. The magnitude was greater with stem.

(ii) Effect on myocardium : The extracts produced negative inotropic effect on the intact dog heart (Fig.14). This effect was also not reproducible as on blood pressure.

(iii) Effect on electrocardiogram : No appreciable effect was observed on the electrocardiogram of dog at a dose rate of 25 mg/kg, i.v.

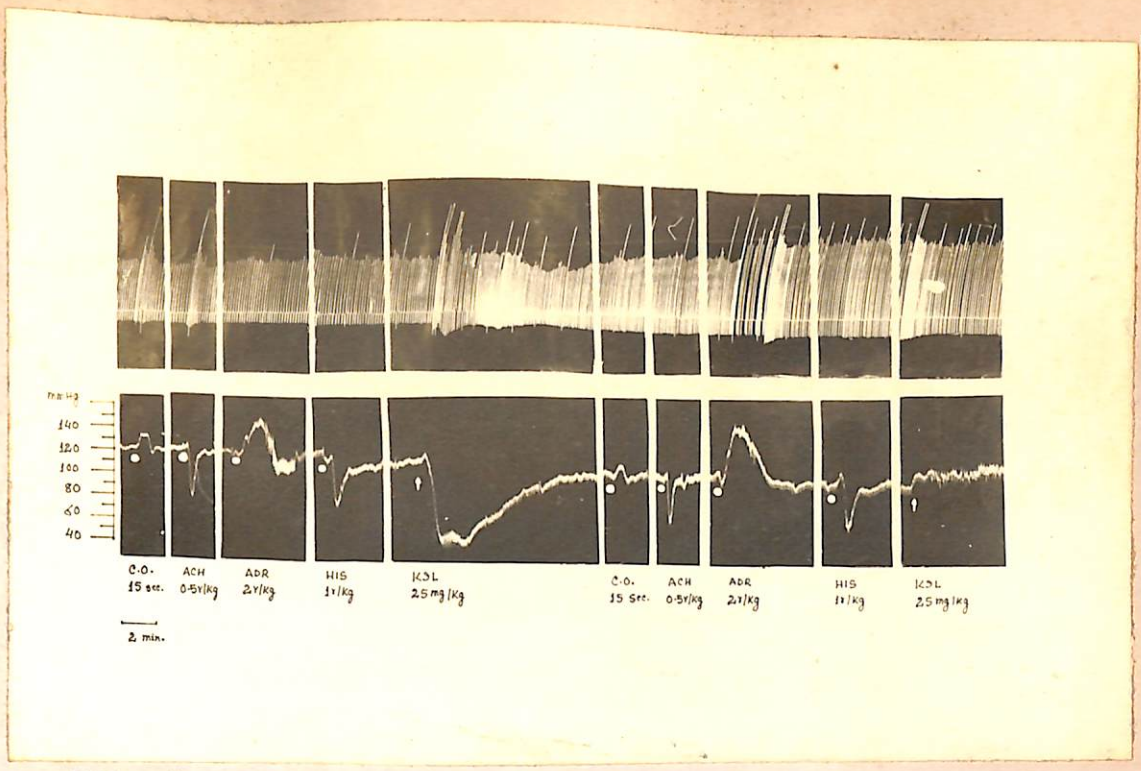

Fig. 10. Effect of the aqueous extract of Kalanchoe integra leaf on the carotid blood pressure (lower tracing) and respiration (upper tracing) in pentobarbitone sod. (30 mg/kg, i.v.) anaesthetised dog (8 kg). C.O. - adrenaline; HIS - histamine; KIL - Kalanchoe integra leaf. Drugs and extract were administered through cannulated femoral vein.

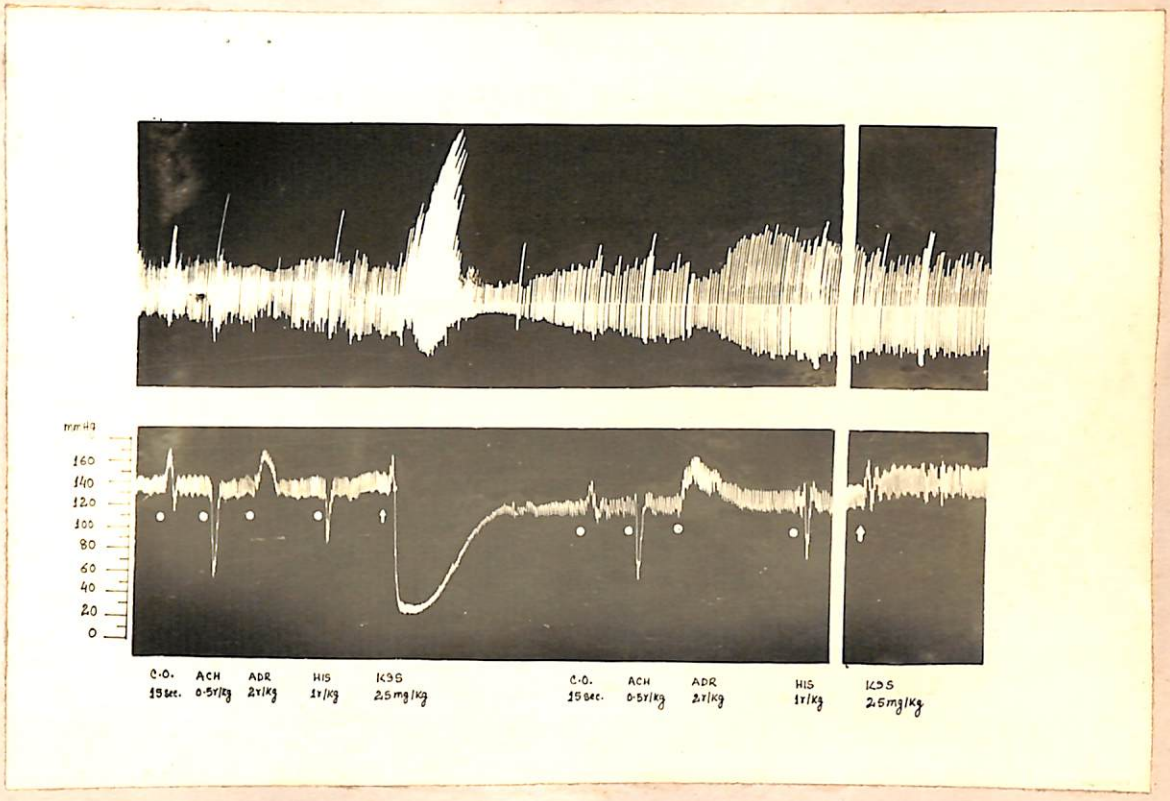

Fig. 11. Effect on the aqueous extract of Kalanchoe integra stem on the carotid blood pressure (lower tracing) and respiration (upper tracing) in pentobarbitone sod. (30 mg/kg, i.v.) anaesthetised dog (7 kg). KIS - Kalanchoe integra stem.

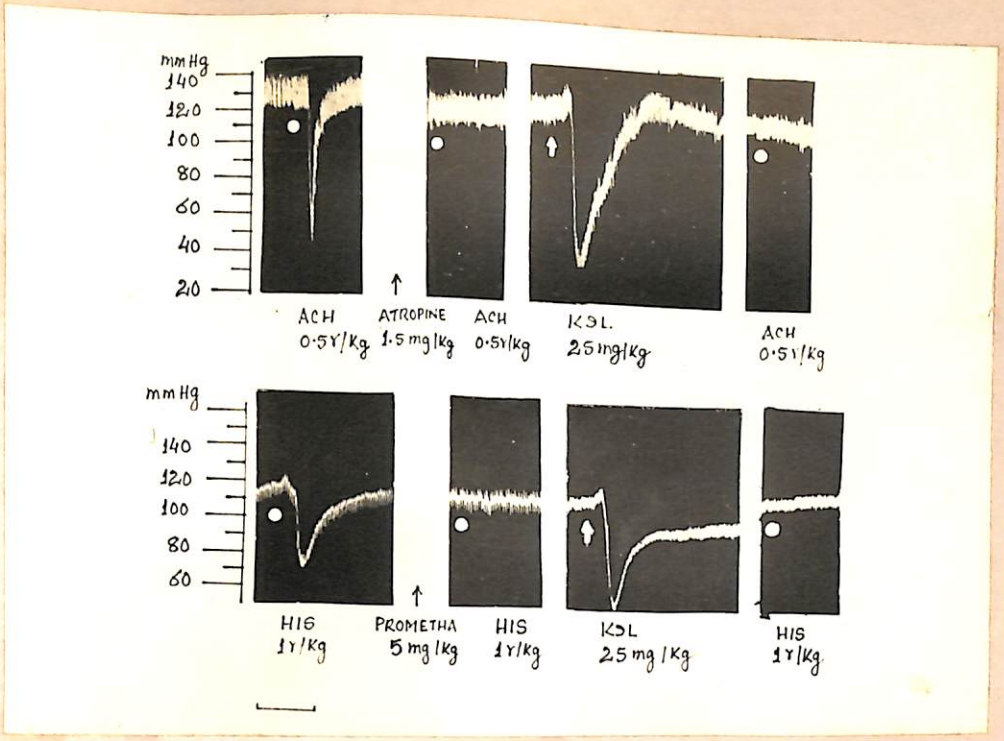

Fig. 12. Effect of atropine (upper tracing) and promethazine (lower tracing) on the blood pressure response to the aqueous extract of Kalanchoe integra leaf in anaesthetised dog.

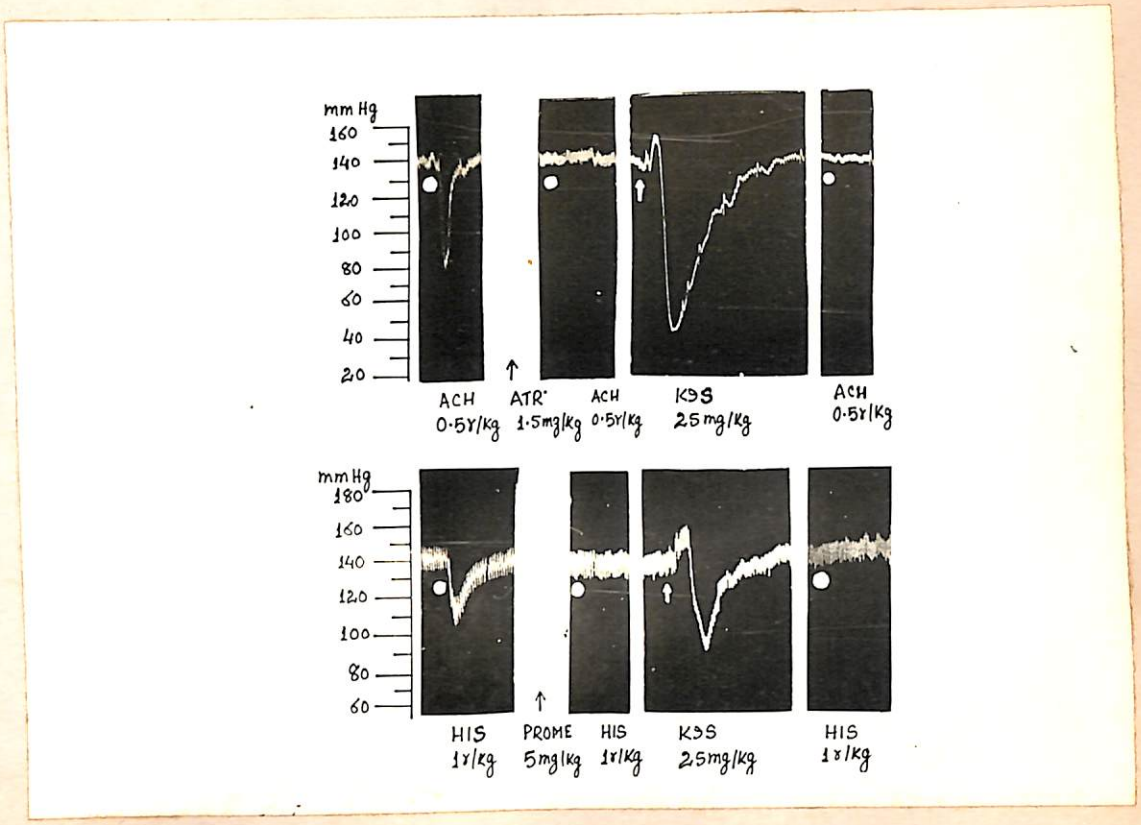

Fig. 13. Effect of atropine (upper tracing) and promethazine (lower tracing) on the blood pressure response to the aqueous extract of Kalanchoe integra stem in anaesthetised dog.

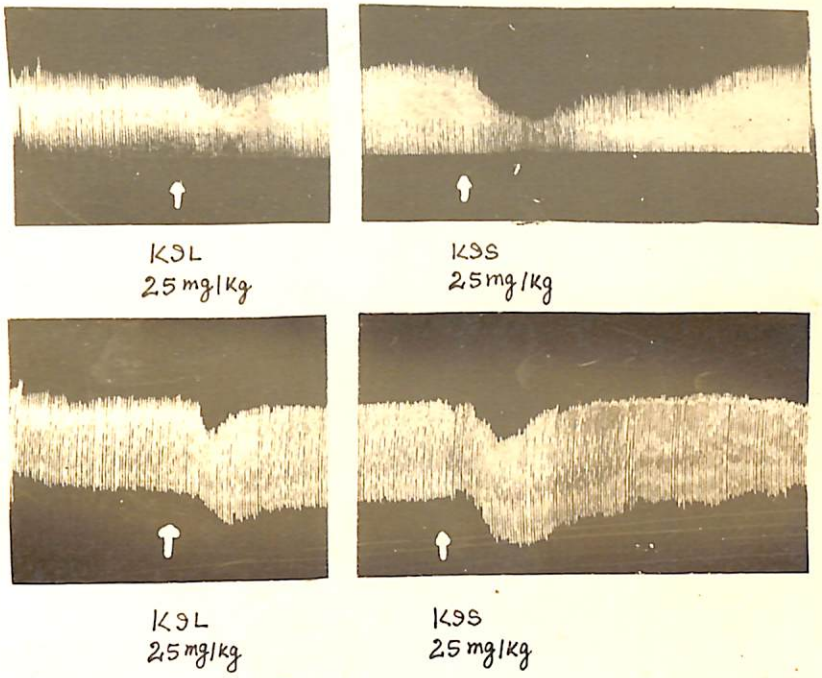

Fig. 14. Effect of the aqueous extracts of Kalanchoe integra leaf (left side) and stem (right side) on the myocardium of anaesthetised dog.

Upper tracing - auricular contraction.
Lower tracing - ventricular contraction.

(iv) Effect on blood vessels : The aqueous extract of leaf as well as stem used in the concentrations of 1, 3, 10 and 30 mg produced no significant effect on the blood vessels, however, some degree of collapse of the blood vessels was noticed particularly with leaf.

9. Effects on Isolated Frog Heart :

The aqueous extracts of leaf and stem in the concentrations of 100, 300 and 1000 µg produced positive inotropic effect. This effect was not blocked by simultaneous perfusion of propranolol (0.3 µg/ml in the perfusion fluid) and promethazine (1 µg/ml in the perfusion fluid) and prior administration of atropine (300 µg) in separate experiments (Fig.15, 16).

10. Effect on Smooth Muscle (Guineapig ileum) :

The aqueous extracts of leaf and stem in the concentrations of 100, 300 and 1000 µg/30 ml bath had no effect of its own but produced a dose dependant inhibition of contractions produced by acetylcholine (0.1 µg/30 ml bath) on the isolated guineapig ileum (Fig.17).

The extracts also produced inhibition of contractions produced by histamine (1 µg/30 ml) and barium chloride (1 mg/30 ml) (Fig. 18, 19). The mean percentage inhibition at different dose levels for different agonists are presented in tables 13 and 14.

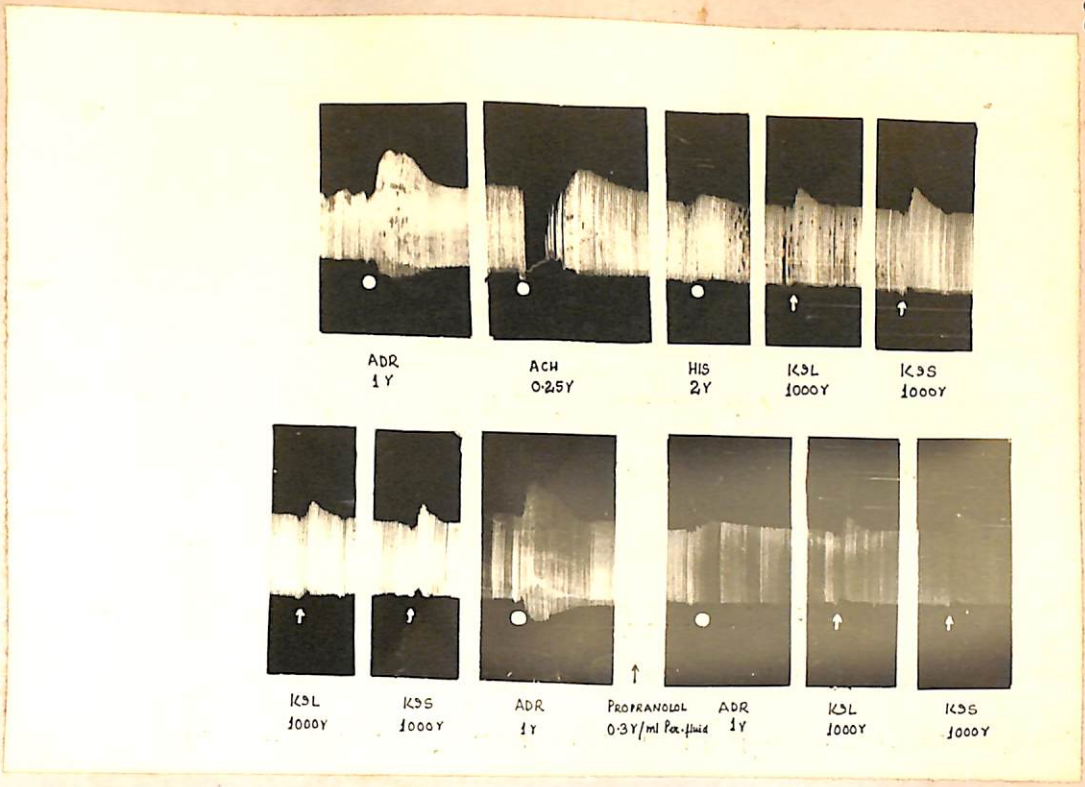

Fig. 15. Effect on the isolated frog heart.
Upper tracing - effect of adrenaline, acetylcholine, histamine and the aqueous extracts of Kalanchoe integra leaf and stem.
Lower tracing - effect of the aqueous extracts of Kalanchoe integra leaf and stem before and after propranolol.

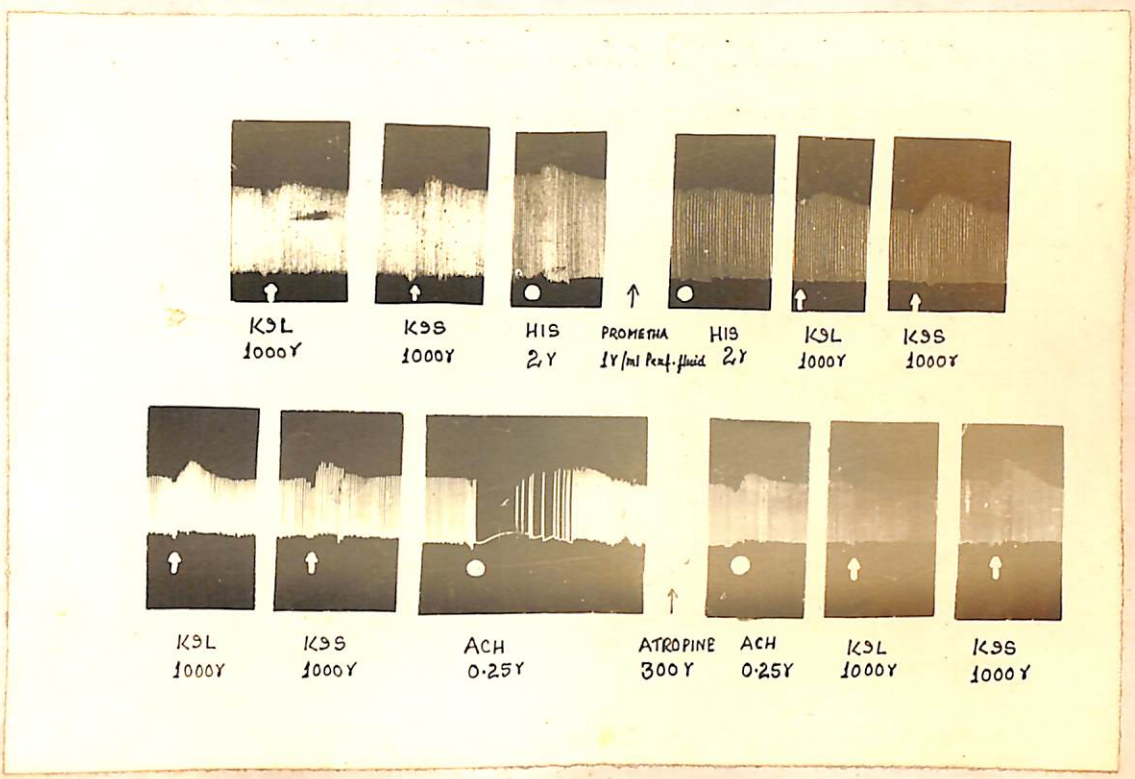

Fig. 16. Effect of the aqueous extracts of Kalanchoe integra leaf and stem on the isolated frog heart before and after promethazine (upper tracing) and atropine (lower tracing).

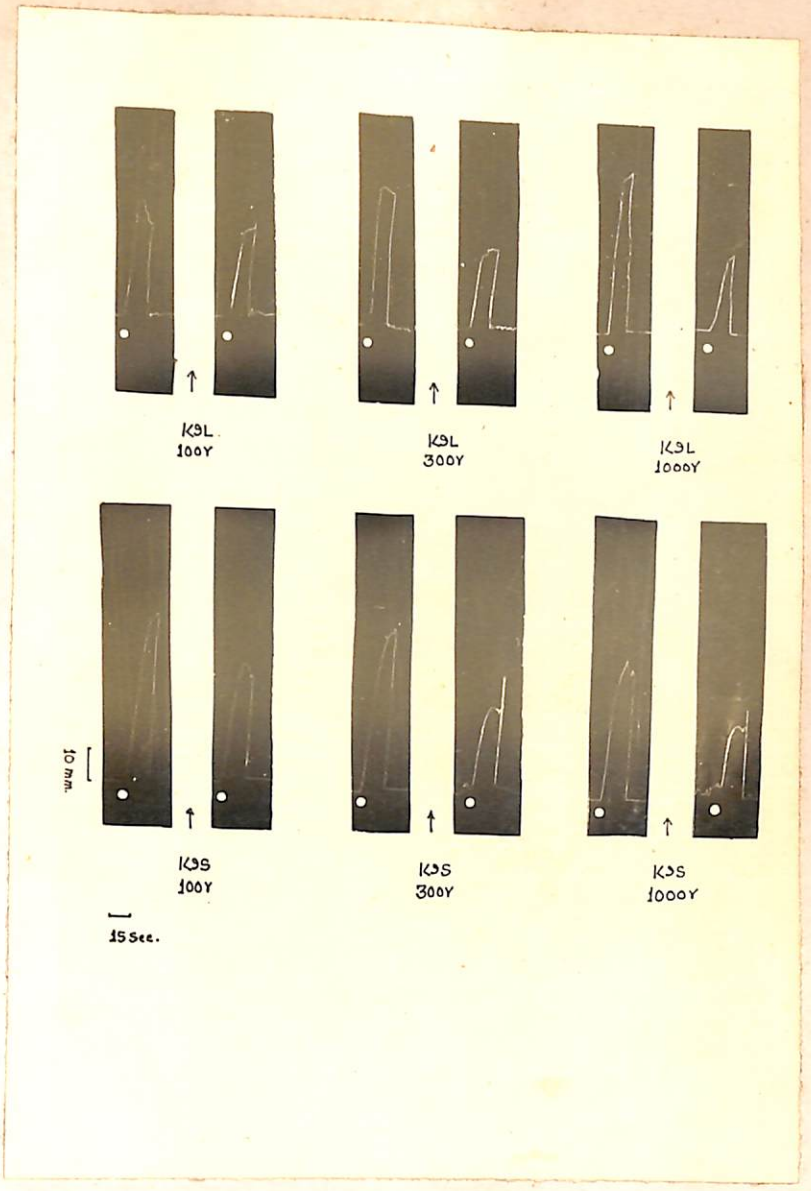

Fig. 17. Effect of the aqueous extracts of Kalanchoe integra leaf (upper tracing) and stem (lower tracing) on the acetylcholine (0.1 µg/30 ml bath) induced contractions of guineapig ileum. At white dots acetylcholine was added.

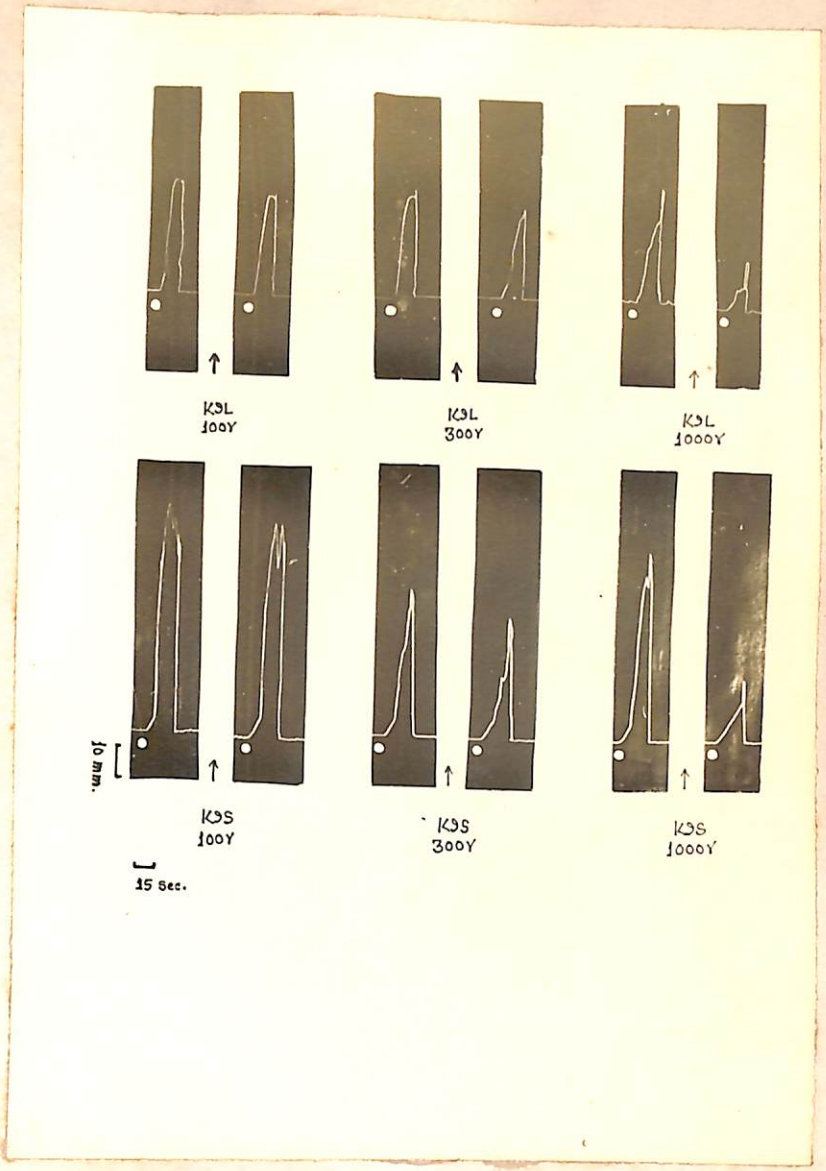

Fig. 18. Effect of the aqueous extracts of Kalanchoe integra leaf (upper tracing) and stem (lower tracing) on the histamine (1 µg/30 ml bath) induced contractions of guineapig ileum. At white dots histamine was added.

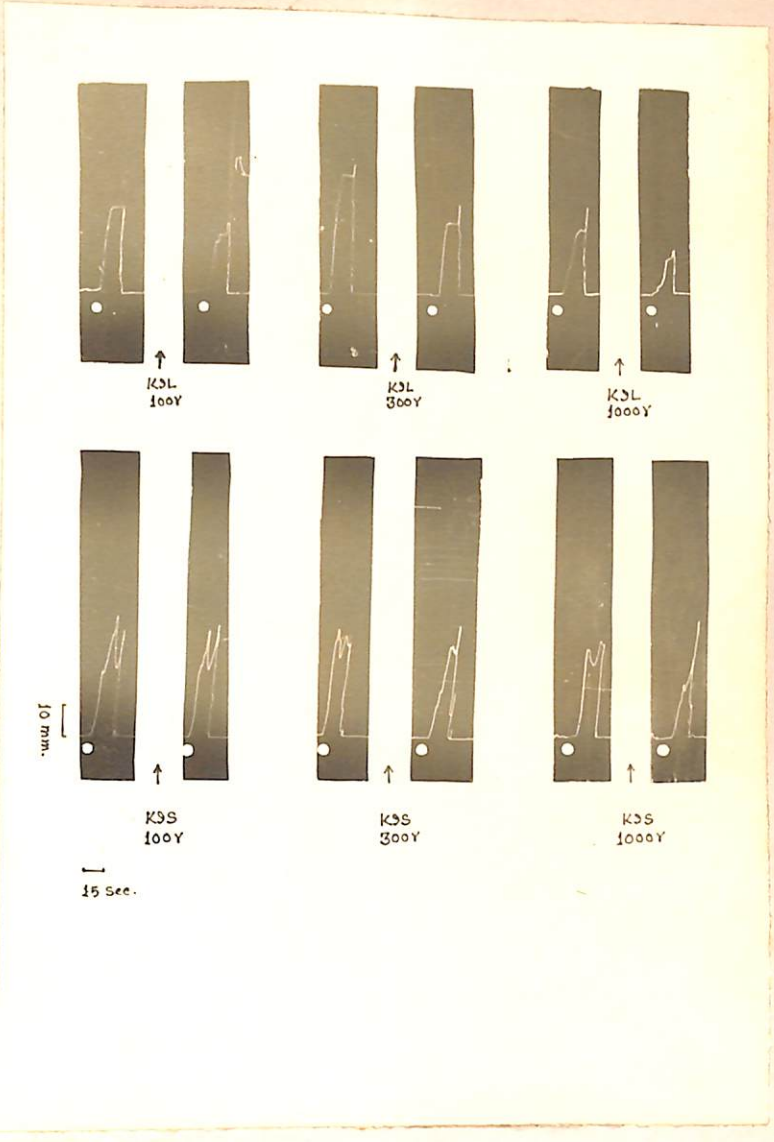

Fig. 19. Effect of the aqueous extracts of Kalanchoe integra leaf (upper tracing) and stem (lower tracing) on the barium chloride (1 mg/30 ml bath) induced contractions of guineapig ileum. At white dots barium chloride was added.

Table 13

Effect of the aqueous extract of Kalanchoe integra leaf on acetylcholine, histamine and barium chloride induced contractions of isolated guineapig ileum.

Agonists	Concentrations of K.integra leaf used (µg/30 ml)	Mean* percentage inhibition in agonists effect
Acetylcholine (0.1 µg/30 ml)	100	19.13
	300	34.81
	1000	57.51
Histamine (1 µg/30 ml)	100	10.49
	300	14.47
	1000	26.88
Barium chloride (1 mg/30 ml)	100	17.84
	300	34.70
	1000	57.93

*Values are mean of three experiments.

Table 14

Effect of the aqueous extract of Kalanchoe integra stem on acetylcholine, histamine and barium chloride induced contractions of isolated guineapig ileum.

Agonists	Concentrations of K.integra stem used (µg/30 ml)	Mean* percentage inhibition in agonists effect
Acetylcholine (0.1 µg/30 ml)	100	22.18
	300	33.22
	1000	41.69
Histamine (1 µg/30 ml)	100	8.67
	300	14.90
	1000	37.66
Barium chloride (1 mg/30 ml)	100	8.15
	300	12.39
	1000	25.86

*Values are mean of three experiments.

11. Effect on Skeletal Muscle (Frog rectus abdominis muscle) :

The aqueous extracts of leaf and stem in the concentrations upto 3 mg/20 ml bath had no effect of their own on the frog rectus abdominis muscle preparation nor could they alter the response produced by acetylcholine (4 µg/20 ml bath).

12. Effects on Blood Coagulation :

(i) Bleeding time : The aqueous extract of leaf in dose of 0.5 mg/kg, i.v. produced considerable decrease of the bleeding time in the first hour and then marked increase in the second hour. At the fourth hour there was a trend towards recovery. The extract in dose of 0.25 mg/kg, i.v. produced initial decrease in bleeding time without any significant rise. The effect at the higher dose (0.5 mg/kg) persisted beyond 24 hours while at the lower dose (0.25 mg/kg), the bleeding time was nearly comparable to its pretreatment value at the eighth hour. The results are presented in figure 20.

The aqueous extract of stem in the doses of 0.125 and 0.25 mg/kg, i.v. increased the bleeding time without initial decrease as noticed with the leaf. At the lower dose, the bleeding time increased in the first hour and showed a decline in the second hour and the pretreatment bleeding time was obtained at the 12th hour.

At the higher dose level, bleeding time showed a tendency of increase in the second hour also and a phase of decline was seen in the fourth hour. The effect persisted upto 24 hours (Fig.21).

(ii) Coagulation time : The aqueous extract of leaf at the dose levels of 0.25 and 0.5 mg/kg, i.v. decreased the coagulation time in the first two hours and it was slightly above the control value in the fourth hour. From the fourth hour onward, a gradual decrease was noted and the coagulation time was nearly normal at the eighth hour and 24th hour in cases of lower and higher dose levels, respectively. The results are presented in figure 20.

The aqueous extract of stem in the doses of 0.125 and 0.25 mg/kg, i.v. produced slight decrease in the coagulation time in the first and second hour. A trend towards recovery was observed in the fourth and eighth hour with the lower and higher doses, respectively. At lower dose level, the coagulation time was comparable to the pretreatment value in the 12 hour while at the higher dose level, the effect persisted upto 24 hours (Fig. 21).

13. Effect on Blood Glucose Level :

The aqueous extract of leaf had no effect on the blood glucose level of rabbits at a dose rate of 0.5 mg/kg, i.v. The blood glucose level at 0, 2, 4 and

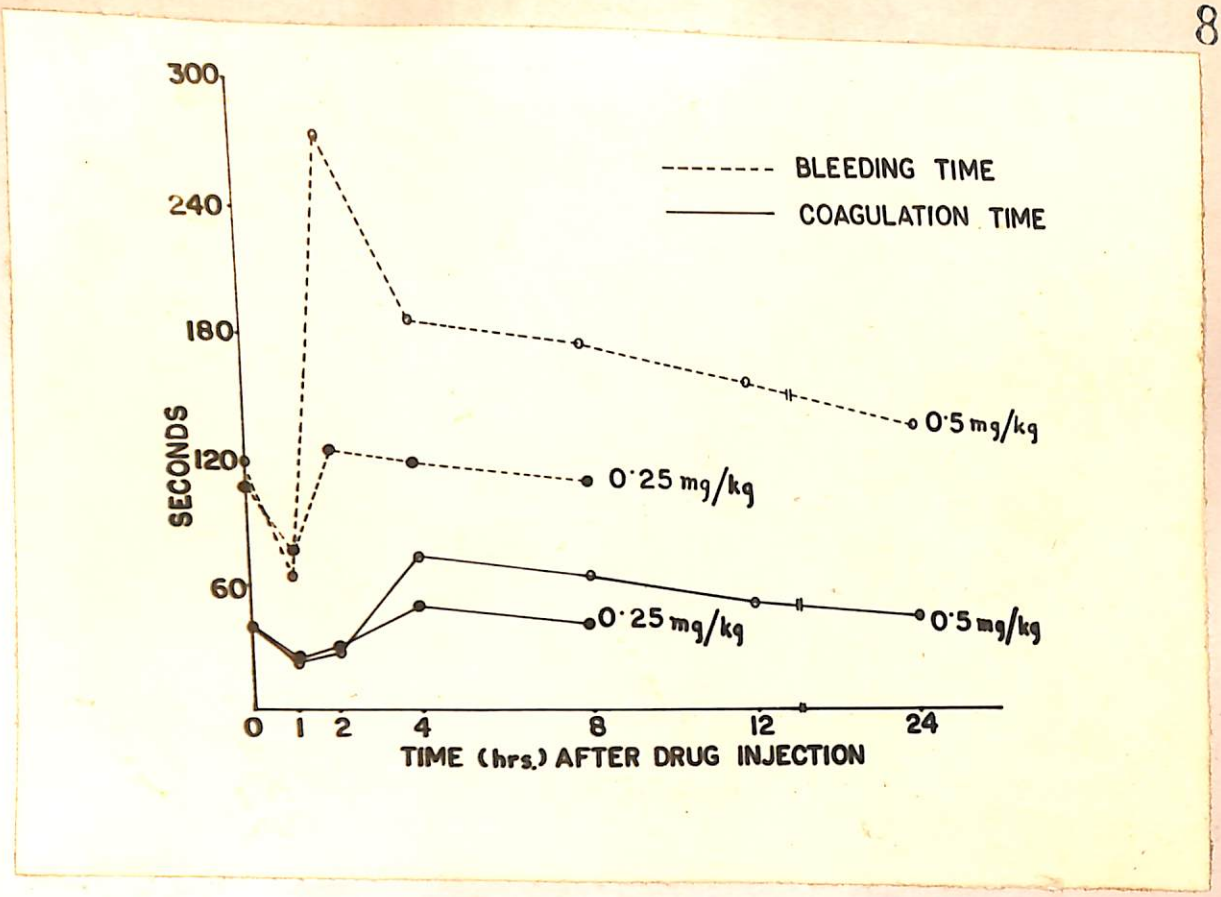

Fig. 20. Effect of the aqueous extract of Kalanchoe integra leaf (0.25 and 0.5 mg/kg, i.v.) on the bleeding and coagulation time of rabbit.

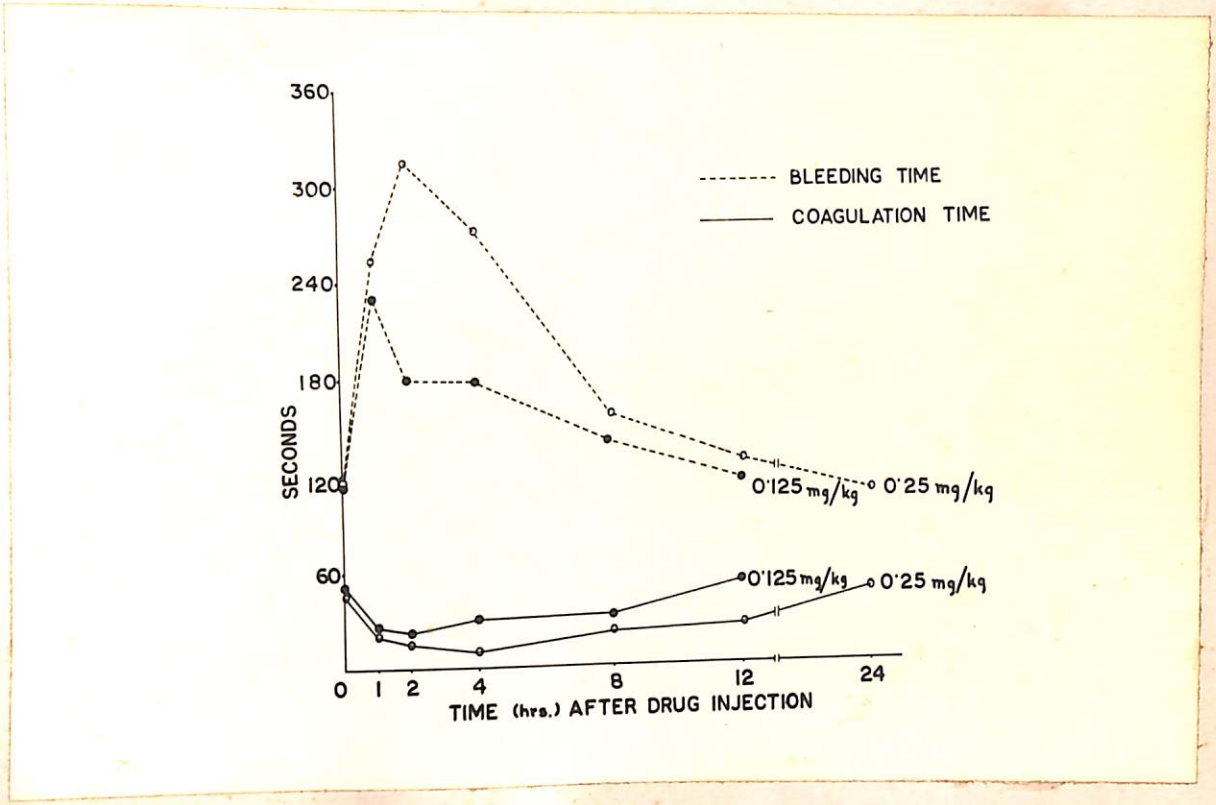

Fig. 21. Effect of the aqueous extract of Kalanchoe integra stem (0.125 and 0.25 mg/kg, i.v.) on the bleeding and coagulation time of rabbit.

6 hours were 109.5, 118.5, 118.5 and 114.0 mg/100 ml of blood, respectively.

The aqueous extract of stem also did not produce any effect. The glucose levels at 0, 2, 4 and 6 hours after administration of extract were 106.5, 118.5, 104.0 and 108.0 mg/100 ml of blood, respectively.

14. Antibacterial Effects :

The aqueous extracts of leaf and stem in the concentrations of 0.3 to 20 mg were unable to produce any antibacterial effect against Bacillus anthracis, Staphylococcus aureus, Pseudomonas aeroginosa, Salmonella hvittingffoss and Escherichia coli. No zone of inhibition was visible upto 24 hours, the period of observation.

E. ANTIDOTAL TREATMENT

The results of the antidotal treatment carried out in acutely poisoned mice and sheep are summarised in table 15. The results of the antidotal treatment in mice indicated that the treatment for cyanide toxicity and against cholinergic stimulation were ineffective. Central nervous system stimulant and reducing agent also could not give any protection. However, in sheep ephedrine exerted some effect by prolonging the survival time. The non-specific measure protected the acutely poisoned sheep.

Table 15

Effect of various treatments against acute toxicity of *Kalanchoe integra* leaf aqueous extract in mice and sheep.

Species	Drugs used	Dose (mg/kg)	Results
Mice			
	i) Sodium nitrite (1% solution)	108	Death occured earlier in comparison to control animals.
	Sodium thiosulphate (25% solution)	6 g/kg	
	ii) Sodium nitrite Sodium thiosulphate Atropine sulphate	2	
	iii) Atropine sulphate		Death occured as in control animals.
	iv) Amphetamine	5	
	v) Amphetamine Atropine sulphate		
	vi) Methylene blue	20	
Sheep	i) Ephedrine	30 (Total dose)	Survival time was slightly prolonged.
	ii) Universal antidote containing		The animals survived.
	Activated charcoal	10 g	
	Light magnesium oxide	5 g	
	Kaolin	5 g	
	Tannic acid	5 g	
	Water	250 ml	

A. EXTRACTION

The results of the rapid selective test in mice with the different extracts and fractions of leaf indicated that both the hot aqueous as well as hot organic extracts were non-toxic while cold extracts were toxic. This showed that the leaf contained some heat labile toxic principle. It was observed previously in our laboratory that the decoction of the whole plant in the doses upto 10 g/kg, i.p. in rats was non-toxic and produced no observable symptoms.

B. PHYTOCHEMICAL STUDIES

The quantitative tests of leaf and stem for ash, calcium and phosphorus revealed that the ash and calcium content of leaf was double than that of the stem while the phosphorus content was roughly equal in both the leaf and stem.

On qualitative examination of stem, the presence of cyanide, alkaloids, glycosides, reducing sugars, fixed and essential oils and gases were evident. However, in leaf, only traces of cyanide was present while reducing sugars were absent. The presence of cyanide in appreciable quantities in stem as compared to leaf may account for the more toxicity of K. integra stem in comparison to leaf which contained only traces

of cyanide. The presence of cyanogenetic glycosides in the whole plant K.integra was demonstrated by Talwar (1970, personal communication) and was determined to be 8 mg of potassium cyanide per 100 gms of the material. Singh et al. (1972) attributed the death in sheep by whole plant K.integra to be due to the presence of this cyanogenetic glycoside. However, Clarke and Clarke (1975) have stated that material containing less than 20 mg of hydrocyanic acid per 100 gms was not dangerous to livestock. It will be worth mentioning here that while hydrocyanic acid is the most toxic and rapidly acting, its sodium and potassium salts are slightly less toxic. The absence of typical syndrome of cyanosis after administration of leaf aqueous extract in the different species of animals, therefore, may be attributed to its comparatively lower content of cyanide. It may be mentioned here that the gases which emitted, when the powdered leaf was dissolved in water, may account for mild tympany observed in the acutely poisoned sheep.

It was found that while the cold petroleum ether extract of leaf was the most toxic of the extracts prepared, the cold aqueous extract of stem was more toxic than the cold petroleum ether extract of stem.