CHEMISTRY AND PHARMACOLOGICAL STUDY OF *Cassia absus*

By

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A research report submitted in partial fulfilment of the requirement for the Degree of Bachelor of Science

In

Physical Science

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DECLARATION

The work described in this thesis was carried out by myself at The Research and Quality Assurances laboratory of Bandaranayaka memorial Ayurvadic Research Institute (B.M.A.R.I), under the supervision of Professor Ajith abeysekera and Dr.D.B.M.Wickramaratne. A report on this has not been submitted to another University for another degree.

Y. P. Peiris

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Signature of the student.

25/03/2002

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Date.

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The Director,
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ABSTRACT

The seed oil of *Cassia absus* (family fabaceae) is being used in Ayurvedic medical system to treat the decease Vitiligo. The plant is found in Sri Lanka at few locations and has no commercial value .The seeds of *Cassia absus* is being imported from India for the extraction of the oil for the remedies.

The decease Vitiligo is caused by the inhibition of melanin synthesis in melonocytes by an enzyme. Since the biological activity of the plant has not been yet properly investigated and having many important uses in the ayurvadic system, we thought of studying the chemical composition and biological activity of the seed oil. Later the plant *Psoralia corylifolia* was also found to contain psoralens, which are believed to be the active compounds against the decease Vitiligo. Therefore in this study a series of chemical investigations were conducted to detect the presence of several different classes of chemical compound such as Alkaloids, sterols, triterpens and polyphenols.

And the presence of psoralene was ruled out. As such the plant should contain other active ingredients, which is given time frame.

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1.0 INTRODUCTION

1.1 Introduction to the plant

Cassia absus belongs to the family Fabaceae (Leguminosae). The genus cassia is a large and predominantly tropical genus of about 580 species of herbs and trees, with about 20 representatives in India. Many of them are medicinal; a few provide tanning materials of economic value. The plant is distributed almost through out India ascending up to 1500m in the Himalayas. The plant *Cassia absus* is called "Bu thora" in sinhala and "Edikkol, Karunkanam" in Tamil and "Chaksu" in Hindi.

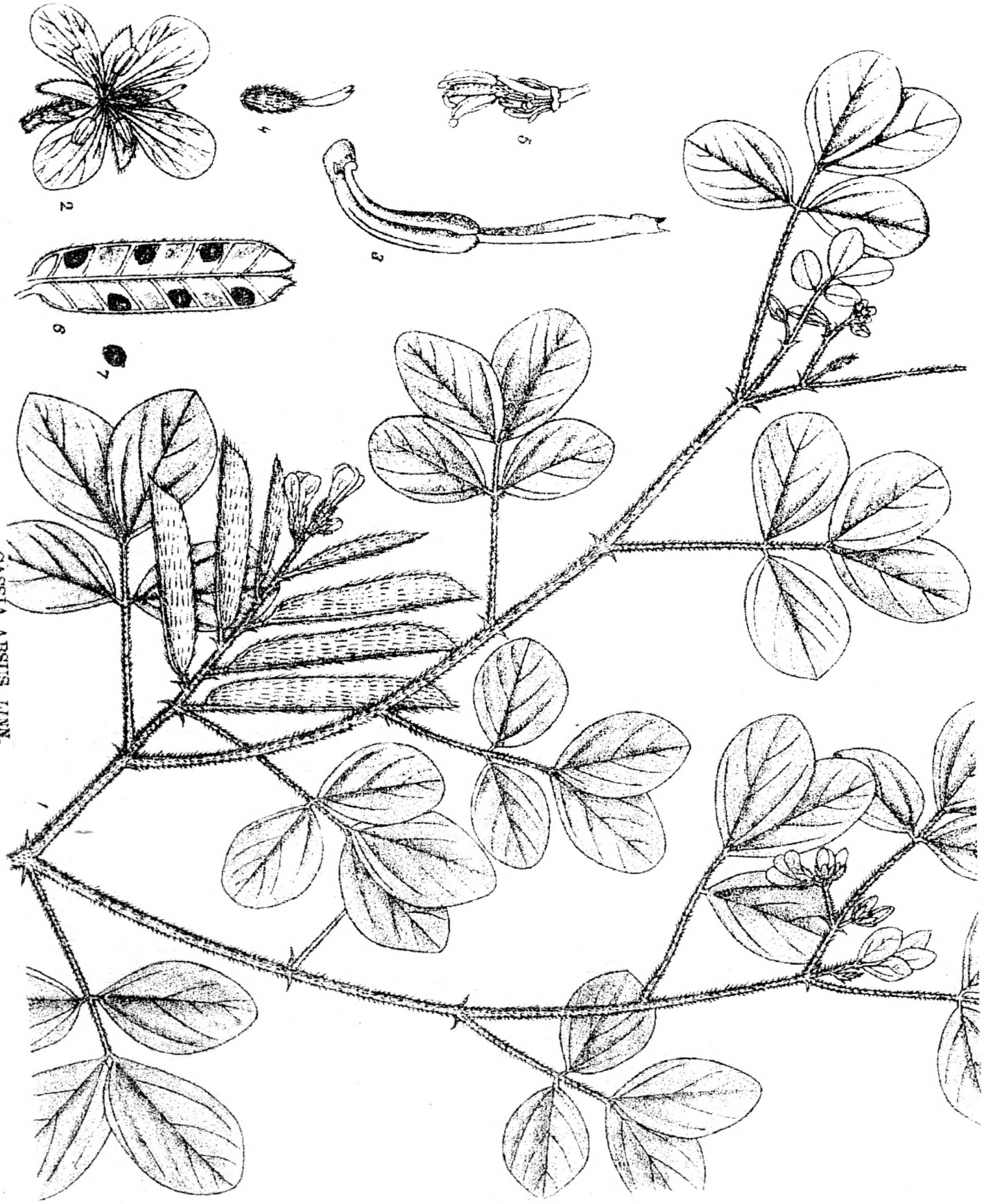
Plants annual: stems erect or sometimes decumbent, to about 1m long, viscidpilose with long and short hairs; stipules linear-attenuate, 2.5-4mm long, 1mm wide, persistent; leaves slender- petiolate with 2 pairs of leaflets, the rachis bearing a linear gland between the leaflets of 1 or both pairs; leaflets elliptic to ellipticobovate, 1-4 cm long, 1-3 cm wide, rounded at the apex, asymmetrical at the base, glabrous or subglabrous above, puberulent or submersed pubescent beneath; inflorescence viscid-pilose, racemose,leaf-opposed,2-13 cm long, few flowered; bracts ovate, about 2mm long; bracteoles 1mm long; flowers on peddles 3-5 mm long; sepals 3-4 mm long, viscid-pubescent; petals yellow, sometimes lined with red, appearing pink or reddish-yellow; stamens 2-7 , usually 5, subequal; fruit linear-oblong, elastically dehiscent, compressed, 2.5-4.5(-5.5)cm long,4-8 mm wide, sparsely setulose- pilose, 5-8-seeded; seed obviate or rhombic, black or dark brown, lustrous,4-5.5mm long,3.5-4.5mm wide, marked with several rows of minute puncta. (Dasanayaka, 1986)

These seed are called "Bodhi etta "in sinhala. The seed are black and polished, flat of an irregular oval or oblong shape. The end where the hilum is situated rather more pointed than the other length and breadth nearly alike, about 1/3-1/6 of an inch. [Welth Asia C-D]

1.2 medicinal uses

Seed oil of *Cassia absus* is being used in Ayurvadic medicine for the treatment of, Vitiligo. The oil "Bakuchi oil" which is extracted from the seed of *Psoralia corylifolia* is also being used to treat the same decease. Chemical and biological investigations have been conducted on the *Psoralia corylifolia* seed oil and the oil was reported to contain Coumarin compounds as Psoralens (1) (Chintolwar and Ramacrishan , 1992).

The seed extract of *Cassia absus* used as remedy for cough, asthma, bronchitis and yaw. The powdered leaves are reported to heal venereal ulcers and haemorrhoids.



CASSIA ABSUS, LINN.

Seed are employed as a cathartic in habitual constipation. They are also used for ringworm, venereal ulcers and other disease of skin, for pains, headache, bronchitis, leucoderma, asthma, hemorrhoids and anthelmintic. The seed extract is used to purify blood and in mucous disorders. A decoction of seed is used in eye diseases, a plaster made from the seeds is recommended as an application to wounds and sores, especially of the penis. The kernels show a general depressant action on heart, respiration and nerves in experimental animals and the action on the nerves resemble that of the belladonna groups of drugs. The baked kernels are used in the treatment of purulent opthalmaia and conjunctivitis. Chaksine, which is a compound that is extracted from the seeds of Cassia absus, is found to be antibacterial against micrococcus pyogenes. It stimulates contraction of different tissues of plain muscles, like uterus, intestine, bladder, and muscles, in blood vessels. It depresses the parasympathetic nerve –ending of certain organs like intestine and bladder. Chaksine has ganglion blocking property and showed a marked anti-nicotinic and anti-5-hydroxytryptamine effect. Chackine and isochackine possess a local aesthetic effect intradermally. (Welth Asia, C-D)They also produce histamine like anesthetic effect intradermaly. Chackine chloride produces a sustained fall in blood pressure of the anaesthetized animals and produces a weak anti-acetylcholine effect.

1.3 The decease vitiligo

The decease vitiligo is caused by the inhibition of melanin synthesis by the melanocytes.

The pigment melanin is responsible for the colour of the skin. Due to the lack of melanin synthesis non-palpable localized area of change in skin colour can be visible. These maculae's vary in shape and can be localized on any part of the skin especially on mucous membranes of the lips & genitals.

Some of the lesions or some part of the lesions may be hypo pigmented and not completely depigmented and in some lesions the hairs may also be white (leucotrichia). The margins of the lesions are generally well defined and may be hyper pigmented. In some cases some patients have only a few small lesions which are static or progressing very slowly, while others have multiple lesions located all over the body. In some patients the disease progresses very fast to cover the entire skin surface within few months. Thus, the course of the disease is very variable and in some patients even spontaneous repigmentation of the lesions may also be observed. The lesions are not usually present at birth, but may appear at any time thereafter.

Vitiligo lesions can be caused by a verity of factors. Evidence for a direct genetic transmission of Vitiligo from parent to the children is observed in less than 10% of the patients. Vitiligo can occur due to pressure of the cloths or shoes, rubber/plastic gloves etc. Action of monobenzyl ether of hydroquinone (MBH) and several other similar chemicals used as accelerators and anti-oxidants the manufacture of rubber and plastics which may still be present in the products made from materials could also act as a factor that lead to vitiligo lesions. (Pasricha, 1992)

Fig 1.2

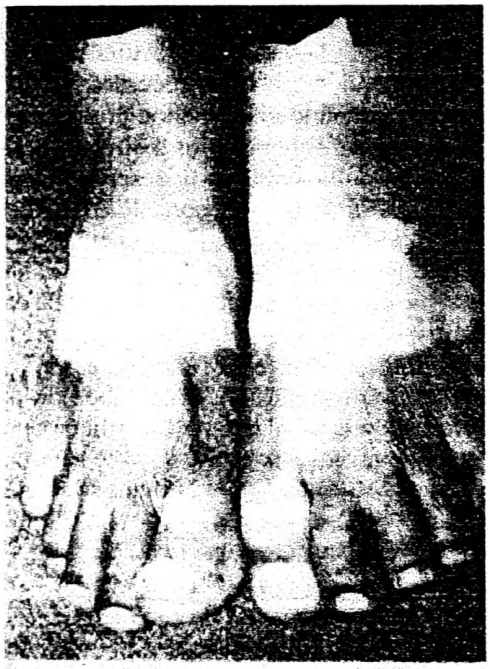


Fig. 58 Vitiligo due to chemicals in the slippers

fig 1.3

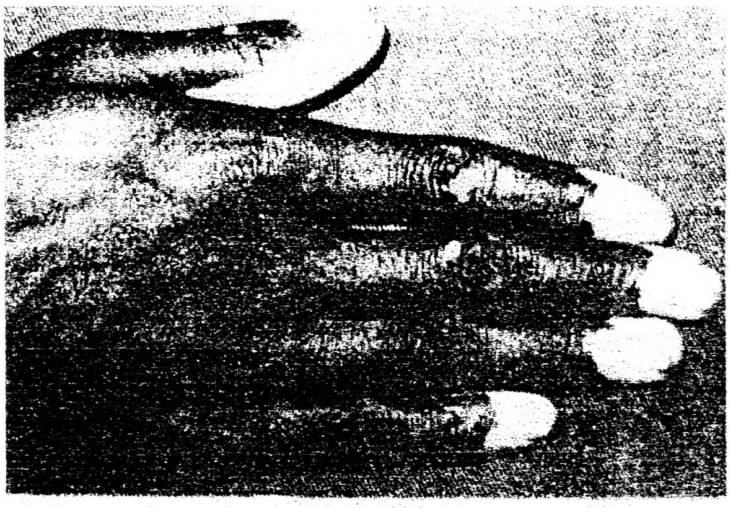


Fig. 63 Vitiligo on finger tips

Fig 1.4

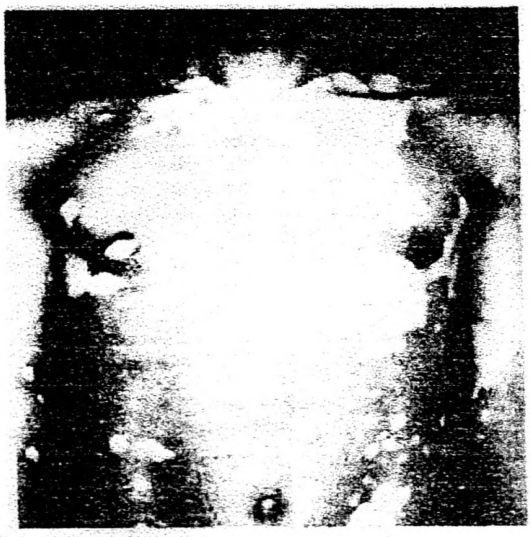


Fig. 60 Generalized vitiligo

1.1 Treatment for the decease vitiligo

psoralens have been the mainstay of treatment of Vitiligo for the last four to five decades. Psoralens are groups of photosensitizing compounds, which make the skin, sensitize to ultraviolet rays. (Pasricha, 1992) These agents were originally obtained from the plant *Psoralea corylifolia* (Babchi) in India and *Ammi majus* in Egypt. These compounds are now available as synthetic products, in the form of tablets for oral administration, and an ointment or solution for topical application. The three main compounds, psoralen, 8-methoxy psoralen (8-MOP) and trimethyl psoralen (TMP) available for the above purpose do not differ much in their therapeutic effect or toxicity. Therefore these products can be used interchangeably. If the lesions are few and small, local drugs may be used. The patient should apply the solution or the ointment on the depigmented area and after ½ to 2 hours expose this area to sunlight (Pasricha, 1992). Oral psoralens are used if lesions are multiple. Ten to thirty milligrames of any of the psoralens can be taken as a single dose followed two to four hours later, by the exposure of the vitiligo lesions to sunlight for15-30 minutes after 2-4 hr in the same way as explained for topical therapy (Pasricha, 1992). With out exposure to sunlight, the topical application of psoralens or their oral ingestion is useless, because psoralens alone cannot initiate repigmentation. . (Pasricha, 1992)

Corticosteroids used topically or systematically are another method for treating vitiligo and the results are at least as good if not better. For localized vitiligo, corticosteroids should be used topically. The ointment should be massaged in to the lesion and one application a day is adequate (Pasricha, 1992).

The third approach to vitiligo patients is the immuno-modulator levamisole. Levamisole can be used as a dose of 150 mg orally on two consecutive days per week. This treatment is effective in nearly half the patients' .It is not to be used in-patients where the disease is static or not spreading very fast.

Another reagent that was recently introduced for the treatment of Vitiligo is an alcoholic extract of placenta. The active component of this extract has not been yet purified, Local applications of this solution on the vitiliginous patches thrice a day followed by infrared ray exposures have been reported to lead to repigmentation.

Good results have been obtained by excising or demabrading the depigmented area and replacing it with split thickness graft taken from another skin area. Alternatively, 2 mm circular areas of skin can be excised from different areas of the vitiliginous skin with the help of a skin punch as for taking a skin biopsy, and replacing with a slightly larger (3 mm) circular pieces of normal skin taken from some other site. These pieces are held in place under a porous tape, which is removed only after 7 days. With time the pigment from the

grafted skin starts extending into the adjoining vitiligineous skin. Further punch graft can be used in certain portions of the skin still remain unpigmented (Pasricha, 1992).

For camouflage, the most ideal method is to have a cream incorporating yellow, red, black and white pigments in a proportion to match the skin colour of the individual. Such creams have to be applied every time the skin is washed or wiped (Pasricha, 1992). The skin area can be painted with 1-% aqueous solution of potassium permanganate, or silver nitrate. Local application of henna paste can also stain the skin in reddish brown. The paste has to be applied for 4-6 hours. Some people also undertake tattooing of the skin with a black pigment to match the skin colour (Pasricha, 1992).

Chapter - 02

2.0 Litrerature Review.

Cassia absus leaves contain quercetin(2), rutin(3), chaksine(fig 2.2) & Isochaksine. The seed contain 2 isomeric quaternary bases chaksine & isochaksine along with palmatic(21),gentisic(4) 5-O-D-glucopyranosyl gentisic acids, ethyl-D-galactopyranoside, apigenin(6), luteolin(5), hydnocarpin(7), isohydnocarpin(8),β-Sitosterol(9), β-D-glycoside(17) and two galactomannans. The mucilage of the seeds contains mannose, galactose, galacturonic acid and xylose. The kernel contains raffinose. (Welth Asia, C-D)

Cassia absus seed is a good source of polysides. A purified polyose fraction from West African C*assia absus* seeds was prepared by water extraction of the peripheral mucilaginous tissues. Investigation by chromatographic methods showed 42% mannose (10); 21% galactose(17); 10% galacturonic acid and 1% xylose. Pilot scale extraction of the seed yielded 19% of extracts similar substances that used in the pharmaceutical and other industries (Kappor, Mukherjee, 1971).

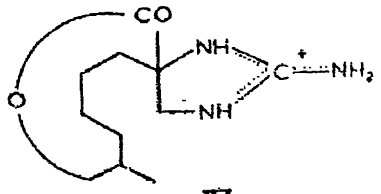
Solvent extraction of *Cassia absus* seeds gave yellow oil (yield 6.0%). The seed oil is a rich source of hitherto unknown keto acid (52%) and characterized as 9-ketoacetadec-cis-15-enoicacid. It also contains palmatic acid (12.3%) stearic acid (2.5%), arachidic acid (1.4-%) oleic acid (7%) and linoleic acid (24.8%). (Med & Arom. Plant 1994, 16) the acid soluble fraction contains acetic acid-soluble and insoluble galactomannans in the ratio 3:1. (Kapoor and Mukeherjee 1972) With the use of various techniques the ratio of D-galactose to D-mannose in the acid-insoluble portion was found to be 1:3. Xylose was present in trace levels. (Kappor, Mukherjee, 1971) A degraded galactomannan composed of D-galactose (1 mole) and D-mannose (3 moles) has been isolated from *cassia absus* seed. Methylation and hydrolysis of the degraded galactomannan yielded 2,3,4,6-tetra-O-methyl-D-mannopyranose (0.97 moles), 2,3,4,6-tetra-O-methyl-D-galactopyranose (4.07moles), 2,3,6-tri-O-methyl-D-mannose, (7 moles), 2,3-di-O-methyl-D-mannose (2.10 moles) and 4,6-di-O-methyl-D-mannopyranose (1.95 moles). Periodate consumption is 1.29moles for each hexose unit with concomitant liberation of 0.41 moles of HCOOH .The degraded galactomannan is a branched polysaccharide composed of 16 hexose units in which 12 are mannose units from the main chain and 4 are galactose units which are present as single branch. Most of the D-mannose units are attached through (1→4) linkages (~80%) and the rest through 1→3, whereas the branches of single D-galactose units are attached through (1→6) (~50%) and (1→2) linkages (Kappor, Mukherjee, 1972).

Extraction of seed by neutral salt solution or by 40% NaOH and charactarization by Paper chromatography showed arginine, glutamic acid, tyrosine, and valine as free amino acids. Alanine, histidine, leucine, phenylalanine, and proline were present in the proteins. Aspartic acid, glycine, and isoleucine were also found. (Chemical Abst. 1968,69,57454e)

β-D-glycoside m.p (293-4°), $[\alpha]^{31}{}_{D}$ was isolated from the pet- ether extract of the seed. (Quresh et al, 1964) Eight components have been isolated and characterized on the basis of chemical and spectral evidences. They are palmatic acid, gentisic acid, 5-O-D-glucopyranosyl gentisic acid, ethyl α-D-galactopyranoside, apigenin(5), luteolin(6), hydnocarpin (7) and isohydnocarpin (8). (Kostova, Rangaswami 1977)

The pharmacological investigation of) isochaksine (0.5-2.0 mg/kg) produced hypotension in anaesthetized dogs and cats (Faure,Demande 1973). Chaksine iodide produces a fall of blood pressure chiefly by causing dilation of capillaries. The fall can be completely prevented by suitable doses of anti-histaminics, viz.phenergan and anthisan. The pharmacological properties of chaksine iodide differ in many respects from those of the sulfate (Pradhan *et al*, 1953), has shown to stimulate respiration. This is primarily due to compensatory reflexes brought by sharp fall of blood pressure. The drug stimulates contraction of different plain muscle tissues like uterus, intestine, bladder, and muscle in blood vessel; etc. the degree of stimulation varies, but is prominently exhibited in case of uterus. Has shown to stimulate respiration depresses parasympathetic nerve endings of certain organs like intestine and bladder. The action here is similar to that of atropine, which removes the heightened tone of these organs without significantly hampering peristalsis or normal contraction. (J.Sci.Ind.Res 1953,12B)

The formula C_{11} H_{21} O_3 N_3, was confirmed for chaksine, When chaksine was heated with potassium hydroxide at 200 °C it yielded ammonia, α-methylpimelic acid, and atribasic chaksic acid, $C_{10}H_{16}O_6$, M.P 145-147° C (Singh *et al.*, 1956). These findings are at variance with those reported, by Siddiqui *et al* 1956, who have shown that the water –heating chacksine iodide with cupper generates chloroform soluble acid or silver filing is p-isopropylbenzonic acid. The strongly basic nature of alkaloid is not due to its quaternary nitrogen but due to the fact that it is a guanidine derivative. Hydrolysis of chackin with alkali gave a positive ninhydrin test for amino acid and yielded ureido acid, $C_{14}H_{26}N_2$ (M.P.164°C). The data that have been interpreted



IV

Fig 2.1

in terms of the monoterpenoid structure (fig 2.1) in which the site of the potential hydroxylis tentative. (Wiesner *et. al*, 1957).The structure is suggested as an alternative for this alkaloid. Many important reactions could be explained by this reaction (sing et al, 1958). Structure of chaksinic acid was confirmed by a synthesis to be Heptane-1, 2, 6 -tricar-boxylic acid (sing *et. al*, 1958). Later structure was confirmed by NMR-spectroscopy.

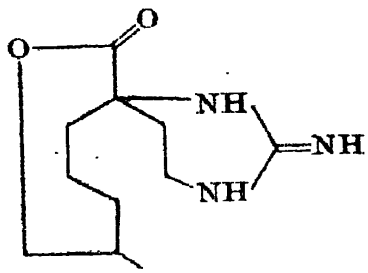


Fig-2.2

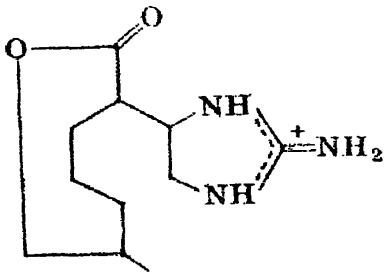
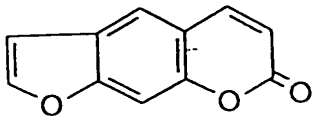


fig-2.3

Figure 2 4-chemical stracture



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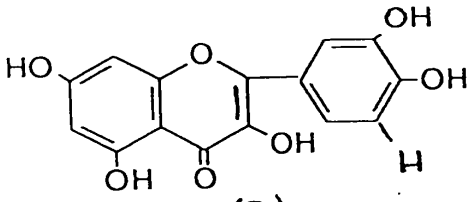

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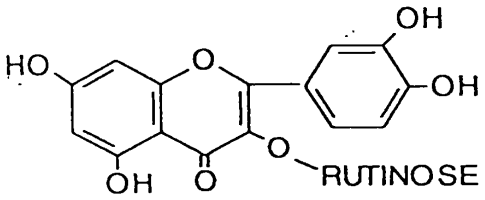

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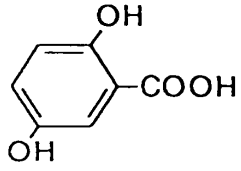

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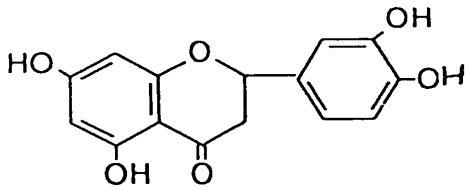

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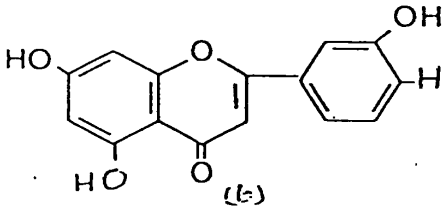

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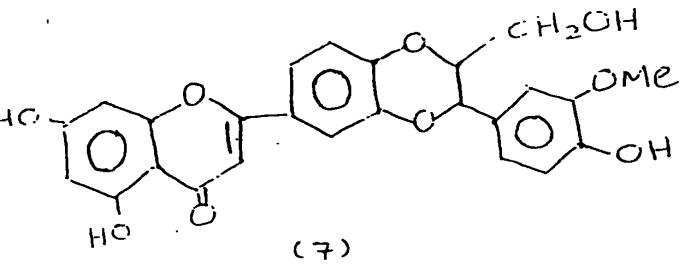

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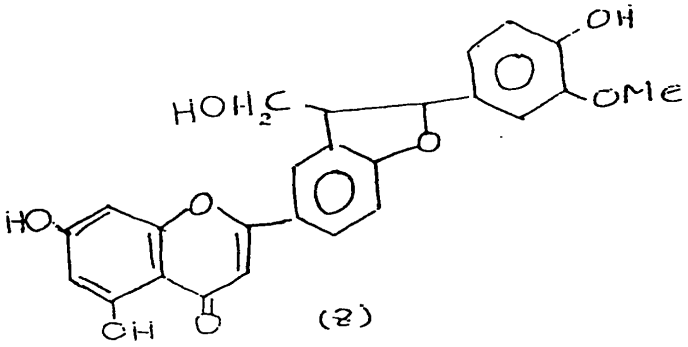

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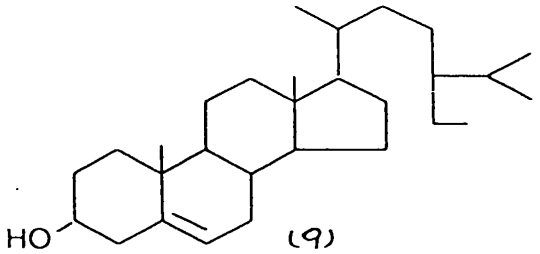

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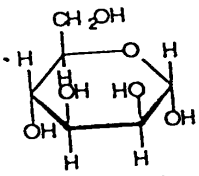

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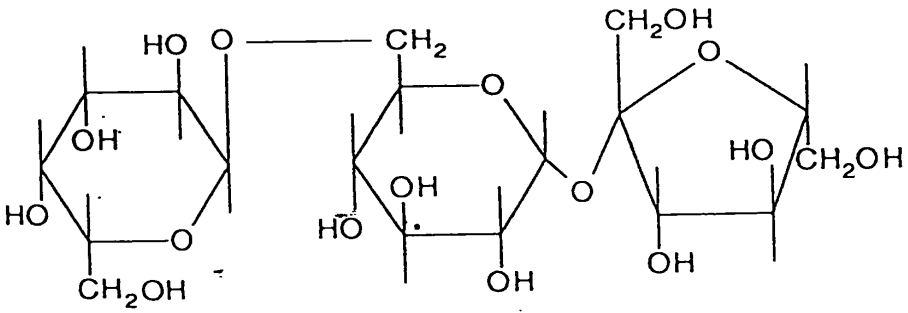

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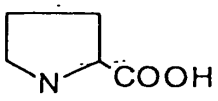

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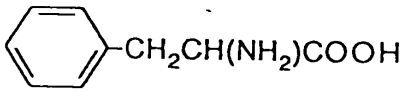

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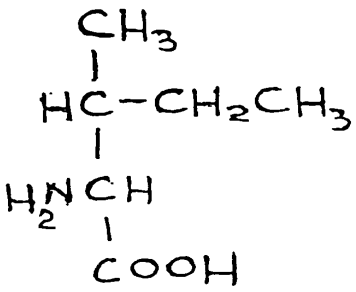

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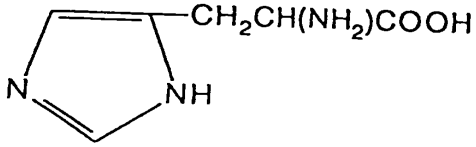

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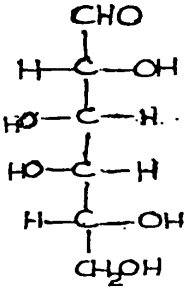

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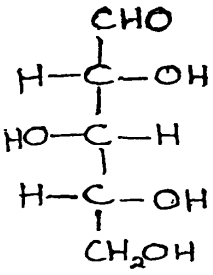

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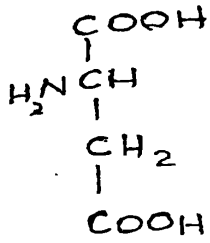

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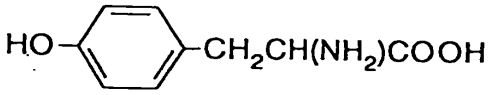


(20)

$CH_3(CH_2)_{14}COOH$ (21)

$CH_3(CH_2)_{12}COOH$ (22)

$CH_3(CH_2)_{16}COOH$ (23)

$CH_3(CH_2)_{18}COOH$ (24)

$CH_3(CH_2)_{20}COOH$ (25)

$CH_3(CH_2)_{22}COOH$ (27)

$CH_3(CH_2)_7CH{=}CH(CH_2)_7\ COOH$ (28)

$CH_3(CH_2)_4CH{=}CHCH_2\ CH{=}\ CH(CH_2)_7\ COOH$ (29)

$CH_3(CH_2)_5CHCH_2\ CH{=}\ CH(CH_2)_7\ COOH$ (30)

$(CH_3)_2CHCH(NH_2)\ COOH$ (31)

$(CH_3)_2CH\ CH_2CH(NH_2)\ COOH$ (32)

$CH_3\ CH(OH)\ CH(NH_2)\ COOH$ (33)

$CH_3\ S(CH_2)_2CH\ (NH_2)\ COOH$ (34)

$NH_2\ (CH_2)_4\ CH(NH_2)\ COOH$ (35)

Chapter- 03

3.0 Materials and methods.

Dried seeds were purchased from ayurvadic medicinal stores in Colombo. Prior to the extraction the seeds were ground using a grinder.

3.1 Preparation of extract of *Cassia absus* for photochemical screening.

The powdered material (100 g) was extracted with 80% hot aqueous methanol (300 ml) under reflux condition for a period of one hour. One hundred and fifty milliliter of extract was evaporated using rotary evaporator until a syrupy extract was obtained.

3.2 Screening for Alkaloids.

3.21 Preliminary alkaloid test.

Ten milliliters of HCl (2 N) was added to the extract while heating on a steam bath. Extract was cooled to room temperature, powdered sodium chloride (0.5g) was added stirred until dissolved and the solution was filtered. Few drops of Mayer's reagent **were added to the solution.

3.22 Confirmed Alkaloid tests

The sample test positive to the above alkaloid test was used in confirmed alkaloid test. Ammonium hydroxide 28% was added to filtrate until alkaline. Solution was transferred in-to separatory funnel and extracted using chloroform. Chloroform extract was then evaporated and 5 ml HCl (2N) was added, stirred for 2 minutes on a steam bath, filtered and was tested for alkaloid using Mayer's reagent.

**Mayer's reagent

To prepare Mayer's reagent Mercuric chloride (1.358g) and Potassium iodide (5.0 g) were disslved in Distilled water 100.0ml

3.23 Test for quaternary and/or amine oxide bases.

Part of the aqueous alkaline extract remaining in the separating funnel was taken and make acidic by adding HCl (2N) was added drop wise. Solution was then tested using Mayer's reagent.

3.3 TLC for Alkaloid

3.31 Preparation of alkaloid extract for thin layer chromatography (TLC).

Method 1

Plant extract (300ml) was evaporated and of HCl (2N) 10 ml was added, the solution was partitioned with $CHCl_3$ (10 mi 3). Aqueous layer was decanted and NH_4 OH (25%) was added to the solution until alkaline. The solution was once again partitioned with $CHCl_3$ (10ml x 3). The organic layer was dried using $MgSO_4$ and evaporated, using rotary evaporator, analyzed on TLC (Kieselgel 60G plates were used.) The TLC was visualized using Dragendroff reagent and L.B. reagent. The solvent systems and reagents used are summarized on table 3.1

Table-3.1 Used solvent systems and spray reagents

Solvent systems.	Spray reagents.
1) Ethyl acetate: Methanol 100: 13.5	Dragendroff reagent
2) Ethyl acetate: Methanol: Diethyl amine 100: 13.5: 0.1	Dragendroff reagent
3) Ethyl acetate: Methanol: water 100: 13.5: 10	Dragendroff reagent

Dragendroff reagent

Basic bismuth nitrate (0.85g) was dissolved in water (40 ml) and glacial acetic acid (10 ml) and 8g of KI dissolved in water (20 ml) were added.

METHOD 2: Direct 80% methanolic extract. The sample was analyzed using TLC (Kieselgel 60G plates were used.) The TLC was visualized using Dragendroff reagent and L.B. reagent. The solvent systems and reagents used are summarized on table 3.2

Table-3.2 Used solvent systems and spray reagents.

Solvent system	Spray reagent
1) Ethyl acetate: methanol: water 100: 13.5: 10	Dragendroff reagent
2) Ethyl acetate: methanol: water 100: 13.5: 2	Dragendroff reagent Libermann burchard reagent
3) Chloroform: methanol 10: 1	Dragendroff reagent

Method –3:

Powdered seed (10g) was moistened with 10% ammonia solution (10 ml) and methanol (50 ml) was added. The solution was refluxed for 15 minutes on a heating mantle. The solution was concentrated and used for TLC analysis (Kieselgel 60G plates were used.) The TLC was visualized using Dragendroff reagent and L.B. reagent. The solvent systems and reagents used are summarized on table 3.3

3.4 Screening for unsaturated sterol & triterpenes.

Forty milliliter of 80% methanolic plant extract was evaporated until syrupy. Petroleum ether (10 ml) was added to the sample and, stirred for few minutes and allowed to settle. The supernent was decanted and discarded. The above procedure was repeated for 3 times. Ten milliliters of chloroform was added to the residue and stirred thoroughly for about five minutes and decanted into a test tube. $CaCl_2$ (100mg) were added, swirled gently and filtered in to test tube. The filtrate was divided in to 3 test tubes and tested for sterol and terpens. The samples were also analyzed using TLC

Table -3.3 used solvent systems and spray reagents

Solvent system	Spray reagent
Ethyl acetate: methanol: water 100: 13.5 : 10	Dragendroff reagent Liebermann- burchard reagent
Ethyl acetate: methanol: water 100: 13.5 : 5	Dragendroff reagent
Ethyl acetate: methanol: water 100: 13.5 : 2	Dragendroff reagent
Ethyl acetate: methanol 10: 1 Chloroform: methanol 10: 1	Dragendroff reagent Dragendroff reagent
Chloroform: methanol 10: 2	Dragendroff reagent
Chloroform: methanol 10: 5	Dragendroff reagent
Chloroform: methanol 10: 8	Dragendroff reagent
Chloroform: methanol 1: 1	Dragendroff reagent

3.41 The Llibermann-Burchard (L-B) test for unsaturated sterols and triterpenes:

Three drops of acetic anhydride were added to the test sample containing the above extract, mixed gently and one drop of concentrated sulfuric acid was added.

3.42 The salkowski test for unsaturated sterols:

Test tube containing the extract was held at 45^0 angles. 1-2 ml of concentrated H_2SO_4 was allowed to run down the side of the test tube. Immediate colour change was noted, at the junction of the sulphuric and extract. The sample was gently mixed and observed for immediate and/or gradual colour changes over a period of one-hour.

3.5 TLC for sterols and triterpenes

The sample was analyzed using TLC (Kieselgel 60G plates were used.) The TLC was visualized using Dragendroff reagent and L.B. reagent. The solvent systems and reagents used are summarized on table 3.5

After sprayed with Libermann-burchard reagent plates were heated 15 minutes in 85^0 C.

3.6 Screening for Tannins and Polyphenols.

Methanolic directs extract (30 ml) was evaporated to dryness and distilled water (25 ml) was added to the residue. The sample and I, 10% NaCl (3-4 drops) was added. Resulting solution was filtered. Filtrate was tested using the 1-% gelatin solution, gelatin salt solution, Ferric chloride test solution and Folin-Denis reagent

Table -3.4 used solvent systems and spray reagents

Extract	Solvent system	Spray reagent
3.4 Extract	Chloroform: methanol 10 : 1	Libermann-burchard reagent Dragendroff reagent
3.4 extract	Hexane : ethyl acetate 1 : 1	Libermann-burchard reagent Dragendroff reagent
3.11 extract	Ethyl acetate: methanol: water 100 :13.5 : 2	Libermann-burchard reagent Dragendroff reagent
3.11 extract	Chloroform: methanol 10 : 1	Libermann-burchard reagent Dragendroff reagent Carr price reagent

3.7 _Preparation of extract for flavanoids

Method 1

Ground seed (10g) was reacted with of 2M HCl (50 ml) for 30 minutes. Extract was filtered and partition with ethyl acetate in a separating funnel. Organic layer was decanted dried with ($MgSO_4$) and evaporated under vacuum. The concentrated sample was analyzed using TLC. (Kieselgel 60G plates were used). The TLC was visualized using Folin-Denis reagent and 10%methanolic KOH reagent. The solvent systems and reagents used are summarized on table 3.5.

Folin-Denis reagent

Into a 1-liter round bottom flask, add 750 ml deionized water, sodium tungstate (100g), phosphomolybic acid (20g) and or thoposphoric acid (50 ml) was added. Refluxed for 2 hours, cooled and diluted to 1L with deionized water.

Table -3.5 Used solvent systems and spray reagents

Solvent system	Spray reagents
Ethyl acetate: HCl: water 10: 3: 30	Folin- Denis reagent
n-Butanol : acetic acid :water 4: 1 : 5	Folin Denis reagent
10% acetic acid in chloroform	Folin Denis reagent

Method 2

Ethyl acetate (40ml) was added to the ground seed (10g) and placed on the heating mental to reflux for 30 minutes. The resulting solution was filtered and evaporated to a concentrate sample and was analyzed using TLC. (Kieselgel 60G plates were used.) The TLC was visualized using Folin-Denis reagent and 10%methanolic KOH reagent. The solvent systems and reagents used are summarized on table 3.6

3.8 TLC for Anthocyanins

Methanolic extract was analyzed using TLC for anthocyanins. The TLC was eluted with Ethyl acetate: formic acid: HCl (2M) (85: 6: 9) and visualized using naked eye. Kieselgel $60HF_{254}$ plates were used

Table –3.6 used solvent systems and spray reagents.

Solvent systems	Spray reagent
Ethyl acetate: methanol: water 100: 16.5 : 13.5	Folin-Denis reagent 10% methanolic KOH
Ethyl acetate: methanol: water 100: 10 : 10	Folin-Denis reagent 10% methanolic KOH
Ethyl acetate: methanol 19: 1	Folin-Denis reagent 10% methanolic KOH
Ethyl acetate: methanol 10: 2	Folin-Denis reagent 10% methanolic KOH
Ethyl acetate: toluene 3: 1	Folin-Denis reagent
Ethyl acetate: toluene 1: 1	Folin-Denis reagent
Ethyl acetate	Folin-Denis reagent
Benzene : ethyl acetate 3 : 1	Folin-Denis reagent 10% methanolic KOH
10% acetic acid in chloroform	Folin-Denis reagent 10% methanolic KOH

. 3.9 TLC for anthraquinones

Methanolic direct extract was analyzed using TLC for anthraquinones. The TLC was eluted with Ethyl acetate: formic acid: HCl (2N) (100:16.5: 13.5) visualized using 10% methanolic KOH. Kieselgel 60HF_{254} plates were used.

Chapter - 4

4.0 RESULTS AND DISCUSSION

The methanolic extract of seed (3.11) was brown-orange in colour. The purpose of preparing this extract was for the convenient of handling and this concentrating the substances to a desired level for subsequent phytochemical screening exercises

Table –4.1

4.1 Results of screening for alkaloid

Test	When Mayer's reagent added;
Preliminary test	Slight turbidity while adding the reagent colour reduced and black precipitate was formed.
Confirmed test	
1^0,2^0,3^0/amine oxide	Slight turbidity
4^0/ amine oxide	Definite turbidity

When Mayer's reagent is added, the following changes can be observed. Indicating the presence of alkaloids

According to the result, the screening test for alkaloids showed the presence of alkaloids. Presence of alkaloids will give orange spots in yellow background with visualized using dragendroff reagent. Result of TLC, alkaloids is present in the seed extract. Therefore the TLC results confirmed the present of at least 2 alkaloids in the seed extract (with MeOH)

4.2 Results of TLC for alkaloids

Table -4.21

Method 1

Solvent system	Spray reagent	Observations
Ethyl acetate: methanol 100:13.5	Dragendroff	Bright yellow spots of Rf =0.8 & 0.1
Ethylcetate: methanol: diethyl amine 100: 13.5: 0. 1	Dragendroff	Bright yellow spots of Rf=0.8 & 0.1 and below the spotted place of the plate was become red orange color
Ethyl acetate: methanol: water 100: 13.5: 10	Dragendroff	Orange spot of Rf=0.5 was appeared in yellow background

Table –4.22

Method 2

Solvent system	Spray reagent	Observation
Ethyl acetate: methanol: water 100: 13.5: 10	Dragendroff	Orange red spot of Rf=0.7
Ethyl acetate: methanol: water 100: 13.5: 2	Dragendroff Libermann-buchared	Orange spot of Rf=0.6 Brownish yellow spot of Rf=0.2 Maroon spot of Rf=0.6 Brown spot of Rf=0.8
Chloroform: methanol 10: 1	Dragendroff	Orange spots were formed Rf=0.1 & 0.7

Table –4.23 Method 3

Solvent system	Spray reagent	Observations
Ethyl acetate: methanol: water 100: 13.5: 10	Dragendroff	Orange spot of Rf=0.8
Ethyl acetate: methanol: water 100: 13.5: 5	Dragendroff Libermann bucharred	Orange spot of Rf=0.4 and the spotted place was maroon color . Yellow spot of Rf=0.4 and brown spot of Rf=0.9
Ethyl acetate: methanol: water 100: 13.5: 2	Dragendroff	Orange spot of Rf=0.4
Ethyl acetate: methanol 10: 1	Dragendroff	Orange spot of Rf=0.3
Chloroform: methanol 10: 1	Dragendroff	Two Orange spots were formed Rf=0.1 and Rf =0.7
Chloroform: methanol 10: 2	Dragendroff	Orange spot was formed Rf=0.2 the place spotted was brown red color.
Chloroform: methanol 10: 5	Dragendroff	Orange spot of Rf=0.5 and brown red spot of Rf=0.1
Chloroform: methanol 10: 8	Dragendroff	Orange spot of Rf=0.6 and brown red spot of Rf=0.2
Chloroform: methanol 1: 1	Dragendroff	Orange spot of Rf=0.6 and brown red spot of Rf=0.1

4.3 Results of screening for sterols & triterpenes

Libermann Burchard test (L-B)

If sterols and triterpenes are present L.B. test give greenish blue colour, but the immediate change was olive green after One hour it become greenish black

Salkowski test

If sterols are present salkowski test give cherry red colour, after one hour it becomes cherry red color. Cherry red color is usually indicative of the presence of unsaturated sterols.

Table –4.3

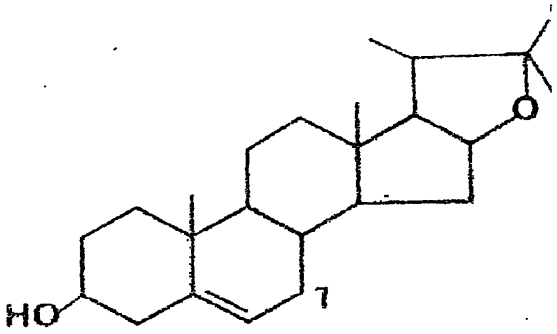
4.4 Results of TLC for sterols & triterpenes

Extract	Solvent system	Spray reagents	Observations
3.4 extract	Chloroform: methanol 10: 1	Libermann-burchard dragendroff	Black spot of Rf=0.8 Orange spot of Rf = 0.1
3.4 extract	Hexane: ethyl acetate 1: 1	Libermann-burchard Dragendroff	Black brown spot of Rf=0.2 Spotted place was orange
3.11 extract	Ethylacetate:methanol:water 100: 13.5: 2	Libermann-burchard Dragendroff	Brownish yellow spot of Rf=0.2 Maroon spot of Rf=0.6 Brown spot of Rf=0.8 Orange spot of Rf=0.6
3.11 extract	Chloroform:methanol 10: 1	Libermann-burchard Dragendroff reagent Carr price reagent	Yellow brown spot of Rf=0.8 & maoon spot of Rf=0.3 Orange spot of Rf=0.1 and Rf=0.7 No color change

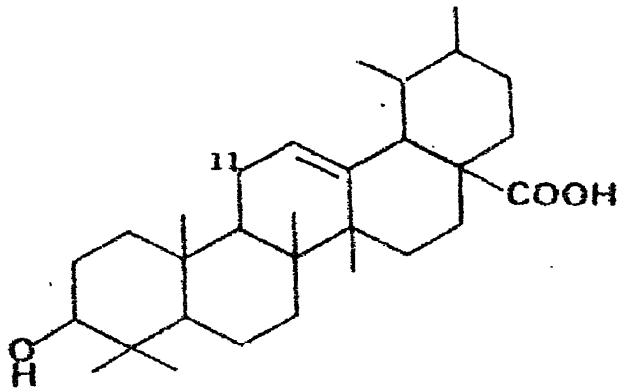
The TLC result does not confirm the existence of triterpenes and unsaturated sterols in the seed extract.

The L-B test has been used to detect unsaturated sterols and also used to distinguish between triterpenoid and steroidal spaonins. The blue or blue- green colour is formed in the L-B test with steroidal saponins; and red, pink, or purple colours result if triterpinoids are present. The L-B test was performed directly to the powdered of the plant material or to a solution of extracted material, colour change was observed. Confirm the presence of steroids using chloroform extracts of plant material; show the presence of phytosterols in the presence of interfering substances such as carotin and xanthophyll. Showing immediate colour change in the L-B test, as also do saturated sterols. However the interfering

steroids using chloroform extracts of plant material; show the presence of phytosterols in the presence of interfering substances such as carotin and xanthophyll. Showing immediate colour change in the L-B test, as also do saturated sterols. However the interfering substances are absent, unsaturated sterols give a minimal colour density at the start of the test, and slowly reach a maximum after about 15 minutes. An investigation of mechanism of the L-B reaction, found the following features essential for colour formation in the steroids, two conjugated double bonds in ring B, or one double bond and an unhindered methylene group at C7 which can undergo oxidation and dehydration, are essential. In penta cyclic triterpenes, however, it is methylene group at C11 in ring C that is involved. Steroid esters were shown to give amore intense color reaction in the L-B test then the corresponding alcohols.



Steroid Sapogenin



Pentacyclic Triterpenoid Sapogenin

Steroid sapogenin

Pentacyclic triterpenoid sapogenin

4.5 Result of screening for tanning and polyphenols

The seed extract did not show precipitation with gelatin solution and it also did not show Precipitation with gelatin-salt solution. Confirmed the absence of tannins in the extract. Treatment of extract with ferric chloride showed appearance of deep brown red colour and also the Folin test on the extract showed a colour change to bluish green. Confirmed the presence of the polyphenolic compounds. The results are tabulated on table 4.41

Tannins are detected most simply in plant extract by the use of the so called gelatin-salt block test both the gelatin and gelatin salt reagent is indicative of the presence of tannins. The basis for the reaction is that tannins precipitate protein (gelatin); the reaction being made move sensitive by the addition of sodium chloride to enhance "salting out" of the protein-tannin complex. If precipitation is observed only with the salt solution, a false positive test is indicated. Test is confirmed by the addition of ferric chloride solution to the extract and show result in a blue, blue black, green, or blue green colour and precipitate.

The blue, blue black, green, or blue green coloured tannin precipitate produced by the addition of frric chloride solution is due to the chemical reaction between ferric chloride and the phenolic functions of the tannin. Phenolic or polyphenolic compounds other than the tannins are present, one would observe negative gelatin-salt test. But coloured solution would be produced on the addition of the ferric chloride solution.

4.6 Result of TLC for phenolic compounds

Table –4.41 **Method 1**

Solvent system	Spray reagents	observations
Ethyl acetate : HCl : water 10 : 3 :30	Folin denis reagent	Blue spot of Rf=0.8
n-Butanol : acetic acid :water 4 : 1 : 5	Folin denis reagent	Blue spot of Rf=0.8
10%acetic acid in chloroform	Folin denis reagent	Two blue spots were formed One is with high intensity and Rf=0.6 and the other is Rf=0.1

Table –4.42 -

Method 2

Solvent systems	Spray reagent	observations
Ethyl acetate :methanol : water 100 :16.5 :13.5	Folin-denis reagent 10% methanolic KOH	Half of the greenish spot of Rf=0.59 was established on the half of the blue spot of Rf=0.47 Visible colour becomes bright.
Ethyl acetate :methanol : water 100 :10 :10	Folin-denis reagent 10% methanolic KOH	Half of the greenish spot of Rf=0.65 was established on the half of the blue spot of Rf=0.47 Visible colour becomes bright.
Ethyl acetate :methanol 19 : 1	Folin-denis reagent 10% methanolic KOH	Half of the greenish spot of Rf=0.47 was established on the half of the blue spot of Rf=0.35 Visible colour becomes bright.
Ethyl acetate :methanol 10 : 2	Folin-denis reagent 10% methanolic KOH	Half of the greenish spot of Rf=0.56 was established on the half of the blue spot of Rf=0.48 Visible colour becomes bright.
Ethyl acetate : toluene 3 : 1	Folin-denis reagent	Tow separate spots were formed. one was greenish ,Rf=0.73 and the other was blue ,Rf=0.25

Ethyl acetate : toluene 1 : 1	Folin-denis reagent	Tow separate spots were formed. one was greenish ,Rf=0.77 and the other was blue ,Rf=0.20
Ethylacetate	Folin-denis reagent	Tow separate spots were formed. one was greenish ,Rf=0.44 and the other was blue ,Rf=0.81
Benzene : ethyl acetate 3 : 1	Folin-denis reagent 10% methanolic KOH	Tow separate spots were formed. one was greenish ,Rf=0.76 and the other was blue ,Rf=0.13
10% acetic acid in chloroform	Folin-denis reagent 10% methanolic KOH	Half of the greenish spot of Rf=0.48 was established on the half of the blue spot of Rf=0.35 Visible colour becomes bright.

Under UV_{365} we observed that the blue spot appeared blue in colour and greenish spot in dark pink colour.

There was no visible colour on TLC for anthocyanins. When the TLC was sprayed with 10% methanolic KOH to visualize anthraquinones, it showed bright yellow spot with a Rf=0.2.

Chapter - 5

5.0 Conclusion

Presence of alkaloids

According to the screening test performed on methanol extract of seed of *Cassia absus* confirmed the present of alkaloids by TLC analysis using dragendroff reagent. According to the results of the test performed two alkaloids are present in the extract

Presence of flavonoids

The TLC performed on the acid hydrolyzed fraction of the seed extract showed the presence of flavonoids. This was confirmed by the TLC visualization using Folin-Denis reagent and 10% methanolic KOH that changed the colour intensity to a bright yellow colour. According to the results of the test performed two flavonoids were present in the extract

Presence of Terpenes and Tannin

I was unable to confirm the presence of terpenes and tannins using TLC method. The conformation test performed for tannin using a gelatin solution also did not show the existence of tannin in the extract. There for I cannot confirm the existence of terpetens or tannin in the extract. But both these compounds may exist in trace levels. The analyzing procedures used were not capable of identifying them.

Presence of sterols

Both Llibermann Buchard test and Salkowski test confirmed the presence of steroidle compounds in the extract

From the data obtained on the methanolic extract of the seeds of *Cassia absus* shows the presence of compounds alkaloids, steroids, flavonoids and polyphenols. Therefore it is important to further investigate the extract to isolate and identify each of these compounds and test their biological especially as a cure for vitiligo.

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