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Pharmacognostic studies on *Holostemma ada-kodien* Schult. root tuber

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Abstract

The present work was carried out on the pharmacognostic characters, phytochemical localization and analysis of metabolites in *Holostemma ada-kodien* Schult. root tuber. Physicochemical parameters such as swelling and foaming indices were used for identifying the adulterants in the powdered samples. Microscopic observations of root tuber showed the presence of prismatic crystals, lignified wall, compound starch grains with centrally located hilum and the 'Maltese cross' as the characteristic features of the powdered sample. Metabolites such as starch, proteins, lipids, flavonoids, terpenoids, alkaloids, tannins and phenols in fresh root tuber gave clear demarcation of their localization in root tuber. The powdered root sample was extracted in soxhlet with various solvents such as petroleum ether, chloroform, acetone, methanol and water and was screened for various phytochemicals.

Keywords: Asclepiadaceae, *Holostemma ada-kodien*, Physicochemical, Organoleptic, Powder analysis

1. Introduction

Holostemma ada-kodien Schult. (Syn. *H. annulare* (Roxb.) K. Schum.) is a laticiferous perennial climber which belongs to the family Asclepiadaceae (Kirtikar and Basu, 1975; Sivarajan and Balachandran, 1994; Tuppad *et al*., 2017). The plant is known by different names such as *Jivanti, Arkapushpi, Kshira, Dodi, Suryavalli* and is widely distributed in the tropical rain forests in India (Sivarajan and Balachandran, 1994).

The plant is a twining shrub with opposite leaves and flowers are developed in axillary umbellate cyme. The plant is reported as rare (Matthew, 1983); as vulnerable (CAMP-1, 1995) in the first red list of medicinal plants of south India. The natural population is destroyed due to the collection of root tubers as a raw material for the Ayurvedic drug preparations and other anthropogenic reasons (Dan and Shanavaskhan, 1991). The tuberous root is used as rejuvanant, expectorant, stimulant, for stomach ache and ophthalmic disorders (Warrier *et al*., 1995). The plant is used for maintaining youthful vigour, strength and vitality (Gupta, 1997). The root tubers produce terpenoid sugars that are responsible for the medicinal properties (Ramiah *et al*., 1981). The Root tuber of the plant is reported to have antidiabetic and antipyretic (Janapati *et al.,* 2009), antibacterial (Irimpan, 2011), antihelminthic (Sadasivam *et al*., 2014) and hepatoprotective (Sunil *et al*., 2015) activities.

Plants have different chemical compounds like secondary metabolites with many biochemical and bioactive properties showing applications in various pharmaceuticals (Aktar and Foyzun, 2017). One of the impediments in the acceptance of herbal products worldwide is the lack of

standard quality control (Shinde *et al.,* 2009). Physicochemical characterization is important for maintaining the qualitative properties of the raw plant parts used in different formulations. Destruction of natural habitats and increasing demand of the herbal drugs may force the plant collectors to practice adulteration. Bioactivity of such adulterants not only reduce the effect of the particular action of the genuine drug but also adversely affect the health of the humans who depend on herbal medicines. So developing pharmacopoeial standards for medicinal plants helps in identification and authentication of the crude drugs obtained from plant sources (Surange and Deokule, 1987; Gupta *et al*., 2012; Saha and Rahaman 2013; Murti *et al*., 2010; Ray *et al*., 2018). Histochemistry helps us to identify the localization of metabolites when treated with respective reagents (Dhale, 2011) which may be useful for proper selection of the plant parts for the isolation of secondary metabolites.

2. Materials and Methods

Mature root tuber from the *H. ada-kodien* plant maintained at the Department of Botany, University of Kerala, Kariavattom was the source of material for the study (Fig.1 & 2). The accession were collected during spring season from localities of peechi, Thrissur District (76º 18′ East Longitude and 10º 28′ North Latitude. and Altitude 55.00 m), Kerala, India. The botanical identities were done by Curator, Department of Botany, University of Kerala, Thiruvananthapuram, Kerala, India and a voucher specimen was deposited at the Department Herbarium with an accession number KUBH 10456. Shade dried powdered root tuber was used for the pharmacognostic analyses and preliminary phytochemical screening (Fig.3-5). Histochemical localization was performed for starch, lipid, proteins, phenols and alkaloids and terpenoids, in fresh root tuber.

2.1. Pharmacognostic studies

2.1.1. Macromorphology

Morphological features of fresh and dried mature root tuber of *H. ada-kodien* were observed.

2.1.2. Anatomy

Freehand sections of secondary root were stained

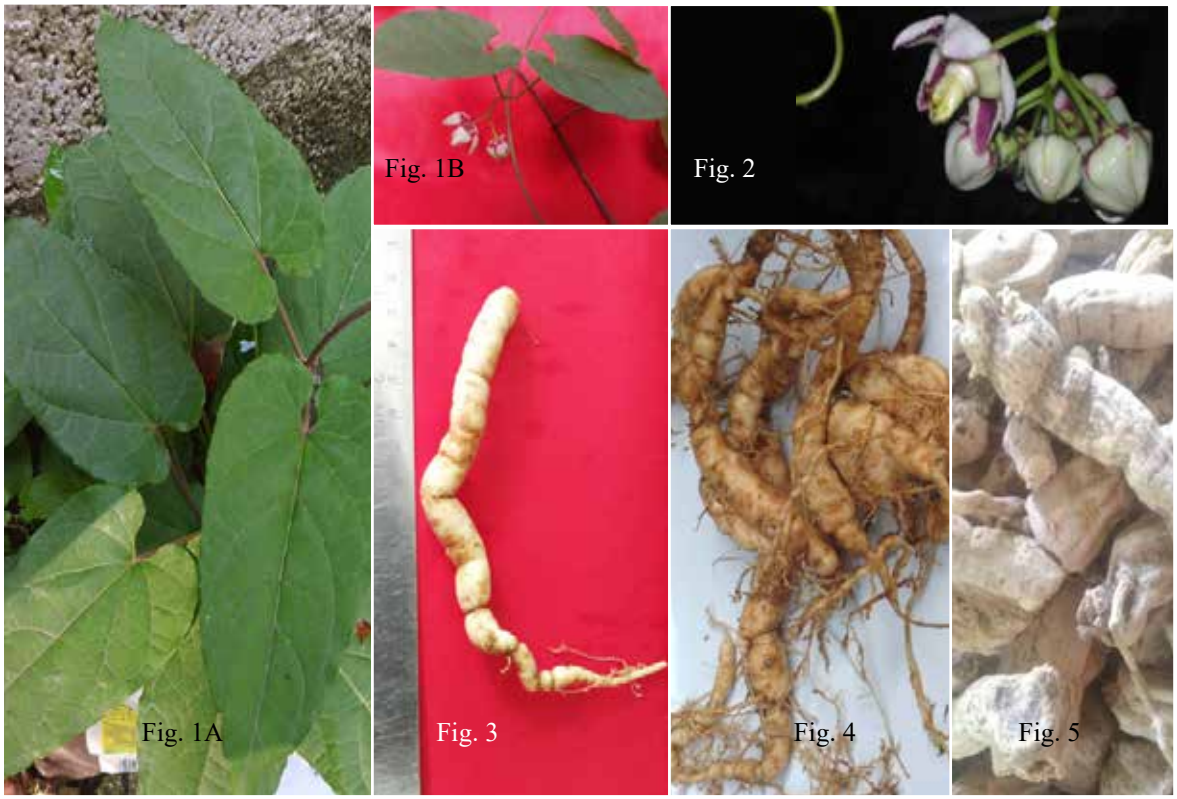


Fig. 1A & **1B.** Habit of *H. ada-kodien*; **Fig. 2.** Cymose inflorescence; **Fig. 3**. Root tuber; **Fig. 4.** Root tuber after one year growth; **Fig. 5.** Dried root tuber

with safranine, observed and photomicrographs were taken using an image analyzer (Leica DM 2000).

2.1.3. Tissue Maceration
Equal portions of freshly prepared 8 to 10% nitric acid and chromic acid were used for maceration (Jeffrey, 1917).

2.1.4. Organoleptic study of powdered sample
Colour, texture, odours and taste of powdered sample were identified with sense organs (WHO, 1998).

2.1.5. Drug Powder microscopy
Microphotographs of powder analysis were made by adding 1-2 drops of phloroglucinol and a drop of concentrated hydrochloric acid to the powdered drug, covered with a coverslip and observed.

2.1.6. Determination of physical constants
2.1.6.1. Determination of moisture content, foaming and swelling index
Physicochemical parameters such as loss on drying at 105ºC, foaming index, swelling index were calculated based on Indian Pharmacopoeia (1996).

2.1.6.2. Determination of ash values
Total ash, water-soluble ash, acid insoluble ash, sulphate ash, etc., were studied in accordance with procedures in Indian Pharmacopoeia (1996).

2.1.6.3. Determination of extractive value
About 100g dried and powdered root tuber was used for soxhlet extraction with various solvents such as petroleum ether, chloroform, acetone, methanol and water based on the polarity of the solvents. The extracts were filtered and dried separately at room temperature. Extractive value of each extract was determined according to the following formula (Evans, 2008; Ghosh and Rahaman, 2016).

$$\text{Extractive value (\%)} = \frac{\text{Weight of the residue obtained}}{\text{Weight of the plant material taken}} \times 100$$

2.1.6.4. Fluorescence study
Fluorescence study was carried out on powdered root tuber with various chemicals under long UV (365nm), short UV (254nm) and under visible light. For this powdered sample was placed in a glass slide and mixed with the chemicals such as petroleum ether, chloroform, acetone, methanol and water. The slide with samples were placed in a UV chamber for the characteristic colour (Ansari, 2006).

2.1.7. Histochemical localization
The collected root tuber of *H. ada-kodien* was sectioned and observed without any stain, as the control. It was stained with safranine to identify various tissues.

2.1.7.1. Test for starch: Sections were placed in 5% potassium iodide solution containing 0.5% Iodine for 2 minute and rinsed in distilled water. They were mounted in glycerine, observed and photographed.

2.1.7.2. Test for protein: Fresh sections were stained in 1% mercuric iodide and 0.5g bromophenol blue dissolved in 100ml double distilled water for 10 minutes. They were washed, differentiated in1% acetic acid, mounted in glycerine and observed.

2.1.7.3. Test for lipid: Sections were placed in Sudan IV solution, mounted in glycerine, observed under the microscope and photographed.

2.1.7.4. Test for alkaloids: The fresh hand sections of root were placed in few drops of Dragendorff's reagent on a clean slide and kept for 2–4 min. The sections were rinsed with distilled water and mounted on glycerine and water in the ratio 9:1, covered with coverslip and observed under the microscope.

2.1.7.5. Test for phenol: Sections of root were stained with a few drops of Toluidine Blue for 2–4 minutes, rinsed with distilled water, covered with a coverslip, were observed under the microscope.

2.1.7.6. Test for terpenoids: Fresh hand sections of root were placed on a glass slide and stained with a few drops of DPH (Diphenyl Hexatriene) for 2 minutes. Stained sections were rinsed with distilled water, mounted in glycerine and observed under microscope.

2.1.8. Phytochemical extraction and qualitative screening of secondary metabolites
Powdered root tuber was used for soxhlet extraction with various solvents such as petroleum ether, chloroform, acetone, methanol and water. The extract was filtered and dried using a rotary

evaporator under vacuum at 45° C. Qualitative chemical test for screening phytochemicals in various extracts were carried out according to Harborne (2007) are shown in Table 1.

3. Results and Discussion

3.1. Pharmacognostic studies

3.1.1. Macromorphology of root

The mature root of *H. ada-kodien* are tuberous creamish colour and nodulated with tapering ends. Odour soil type with a neutral taste. Morphological characters play significant roles for correct identification, characterization and delimitation of taxa on the basis of available markers (Fazal *et al*., 2013). Morphological characters are useful in rapid identification of plant material for pharmaceutical industries.

3.1.2. Anatomy

Transverse section of root tuber shows outer cork, middle cortex and inner vascular region. The cork is with 10-12 layers, outer 4-6 thick walled reddish brown phellem, a distinct phellogen is not evident and a narrow zone of phelloderm is present which is composed of oblong or rectangular, tangentially elongated thin walled cells with starch grains and some of the cells of the innermost rows contain rosette crystals of calcium oxalate (Fig. 6). Cortex was differentiated into two distinct zones, the outer and inner cortex. The outer cortex is with calcium oxalate crystals and inner cortex having 3-4 stone cells (Fig. 7). Sclereids in the cortex are characteristic feature of Apocynaceae and Asclepiadaceae and are present throughout all life forms (Schweingruber *et al*., 2011). Stone cells were rectangular to pentagonal in shape, aligned more or less continuous as a broken ring in the cortex. Each group consisted of 2-3 stone cells, with a broad band of parenchymatous tissues intervening the group of sclereids. The cortical cells of root tuber were filled with plenty of simple and compound starch grains. In cross section of the root tuber the secondary xylem of root is with narrow lumen with intraxylery parenchyma cells as reported by Kolammal (1979). Presence of stone cells with wide lumen,

Table 1. Test for screening phytochemicals in *H. ada-kodien* root tuber

Sl. No.	Secondary metabolites	Test	Colouration
1	Terpenoids	Extract with 5 ml conc. Sulphuric acid	Reddish brown
2	Phenols	Alcoholic Ferric Chloride solution	Bluish green or red
3	Flavones	Shinoda test	Magenta
4	Steroids	Libermann Burchard	Blue green
5	Quinones	Sodium Hydroxide	Red
6	Anthraquinones	Borntrager's test	Pink
7	Glycosides	Anthrone and a drop of conc. Sulphuric acid and warm over water bath	Dark green
8	Alkaloids	Dragendroff's reagent and Sulphuric acid	Orange red precipitate
9	Tannins	Extract with water kept in water bath and add Ferric Chloride	Dark green
10	Lignans	Phloroglucinol and conc. Sulphuric acid	Red to pink colour
11	Saponin	Foam test	Presence of foam

lignified and pitted walls, presence of compound starch grains with 'maltese cross', calcium oxalate crystals were strongly agree with the work of Sudhakaran 2017. While in mature root the secondary xylem was fissured by radial and tangential strips, like the spokes of a wheel so that xylem core appeared irregular and wedge-shaped in the mature root (Fig. 8&9).

3.1.3. Tissue Maceration

Anatomical studies of stems or other parts, rarely convey an accurate picture of the real nature of the cells of which they are composed (Mahesh *et al*., 2015). In *H. ada-kodien* root tuber maceration gave a clear picture about stone cells (Fig. 10), which is noticed in the outer cortex. Parenchyma cells filled with starch grains and long xylem groups (Fig. 11) with interconnected pitted vessels were noted (Fig. 12).

3.1.4. Organoleptic study on powdered drug

Organoleptic properties dealt with the quality aspects of food or other substances which are experienced by the senses, including taste, sight, smell, and touch. Organoleptic analysis of the powdered sample is one of the methods to identify adulterants in the pharmaceutical industry (Sreelekshmi *et al*., 2017). The result of the organoleptic analysis of root tuber of *H. ada-kodien* is given in Table 3. Powdered sample of root tuber of *H. ada-kodien* is cream coloured with a neutral taste and coarse in texture. Deviation in these properties gives a primary indication of quality variation (Mukhi *et al*., 2016).

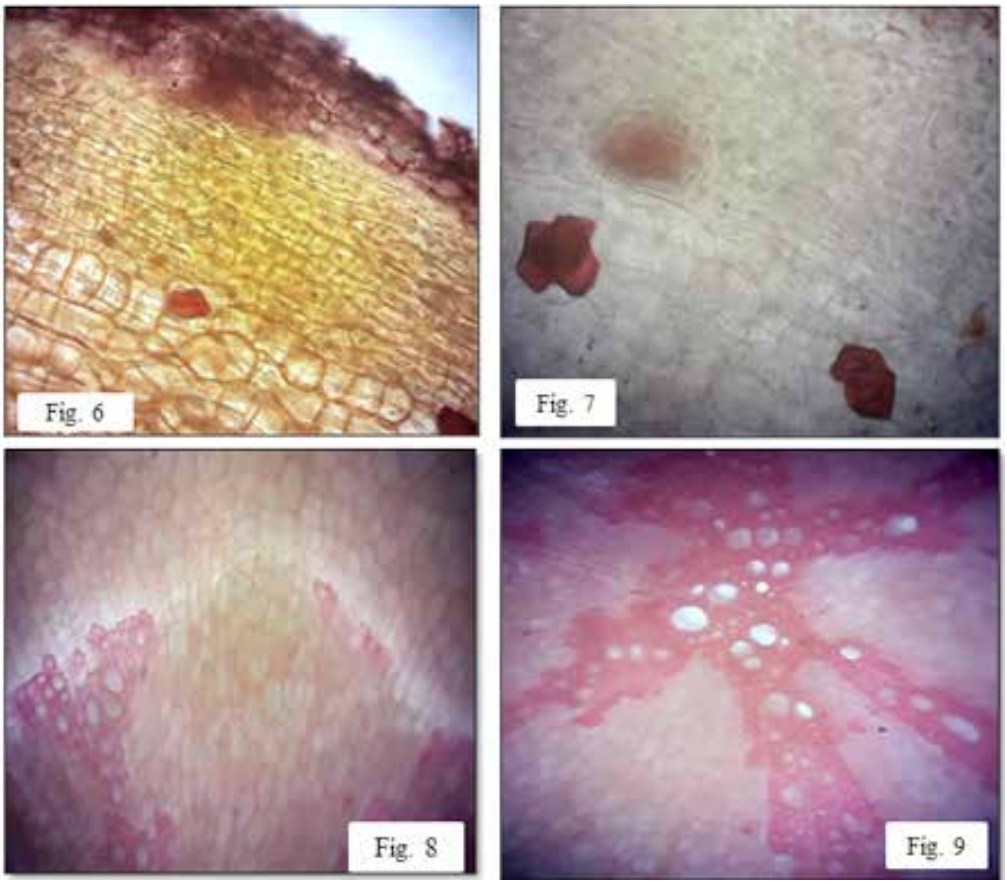


Fig. 6. Periderm of root tuber of *H. ada-kodien;* **Fig. 7.** Stone cells in the outer cortex; **Fig. 8.** Formation of xylem vessels from cambium; **Fig. 9.** Xylem forms irregular wedge shape without pith in the root tuber after one year

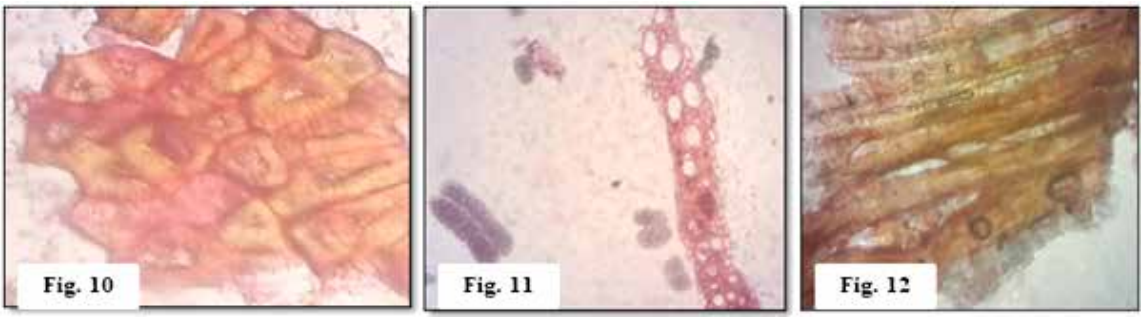


Fig. 10. Stone cells in root tuber of *H. ada-kodien*; **Fig. 11.** Parenchyma cells and xylem vessels; **Fig. 12.** Xylem vessels showing inter connected pits

Table 2. Observations on fluorescent characters of powdered root tuber of *H. ada-kodien*

Sl. No.	Chemicals	Long UV (365nm)	Short UV(254nm)	Visible
1	Petroleum ether	Light Brown	Yellow	Light Brown
2	Chloroform	Light Brown	Yellow	Light Brown
3	Acetone	Light Brown	Yellow	Light Brown
4	Methanol	Brown	Cream	Brown
5	Aqueous	Light Brown	Yellow	Light Brown
6	Acetic Acid	White	Yellow	Light Brown
7	Benzene	White	Yellow	Light Brown
8	Hexane	Light Brown	Cream	Light Brown
9	100% H_2SO_4	Black	Olive Green	Black pasty
10	50% H_2SO_4	Light Brown	Cream	Light Brown
11	100% HCl	Black	Dark Green	Brown
12	50% HCl	Cream	Yellow	Light Brown

3.1.5. Powder microscopic study

Root powder showed prismatic crystals (Fig. 13), compound starch grains (Fig. 14), spiral thickening of tracheids (Fig.15) and secondary cells with lignin (Fig.16). Starch grains were simple and compound types with central hilum.

Table 3. Organoleptic features of the powdered drug

Sl. No.	Organoleptic features	Observations
1	Colour	Cream
2	Odour	Soil type
3	Taste	Neutral
4	Texture	Coarse

The 'Maltese cross' is a characteristic feature for the identification of the powdered sample in *H. ada-kodien* root tuber. Similar observations were made by Sudhakaran (2017) in *H. ada-kodien*. Powder microscopic study helps to identify the genuine samples and adulterated samples. Presence or absence of crystals, types of crystals, the arrangement of phloem, nature of tracheids and medullary rays, etc., are used for detecting the adulteration in samples (Ravindran and Jayasree, 2016).

3.1.6. Determination of Physical constant

Data on the Swelling and Foaming Index can be used as a reference value for identifying adulteration. In *H. ada-kodien*, dried root shows no foaming index and 4 ml swelling index. The result of the physicochemical analysis was given in Table 4. The test for the percentage of moisture content (loss on drying) determines both water and volatile matter. Extractive values are useful for evaluation consistency of nature and amount of chemical constituents present in the drug.

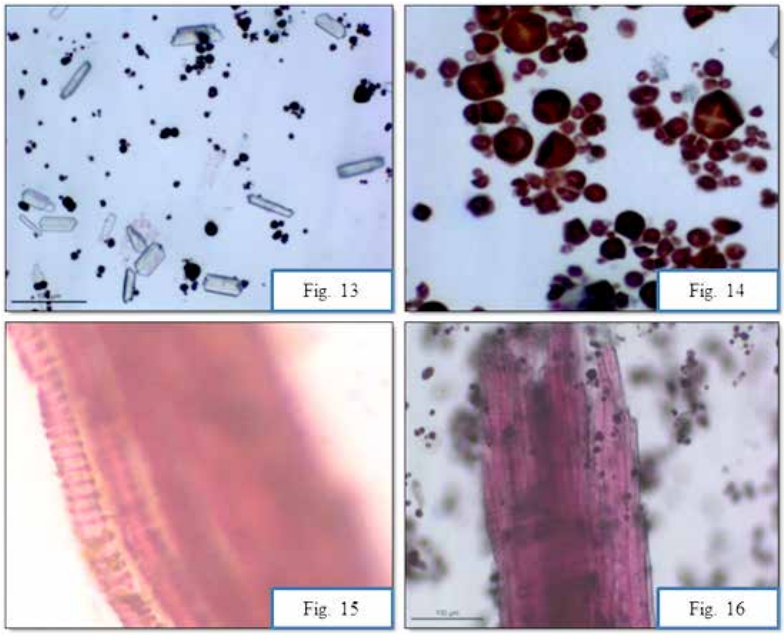


Fig. 13. Prismatic crystals; **Fig. 14.** Compound starch grains; **Fig. 15.** Tracheids showing spiral thickening; **Fig. 16.** Lignified secondary elements

The data collected based on physicochemical parameters on the root tuber of *H. ada-kodien* are useful in identifying the adulterants. According to Husain *et al.,* (2019) foaming and swelling indices are very important in identifying the adulteration while in the formulation of the drug.

Table 4. Physicochemical parameters of root tuber of *H.ada-kodien*

Sl. No.	Test	Value
1	Loss on drying at 105^{0}C (%)	15.34
2	Alcohol soluble extractive (%)	2.25
3	Water soluble extractive (%)	5.89
4	Total ash (%)	4.78
5	Acid insoluble ash (%)	0.12
6	Water soluble ash (%)	2.82
7	Sulphate ash (%)	8.86
8	Foaming index	Nil
9	Swelling index (ml)	4
10	pH at 4% aqueous	5.8

3.1.7. Fluorescence study

Qualitative assessment of crude drug is an important parameter in pharmacognostic evaluation. Fluorescence is an important phenomenon exhibited by various chemical constituents present in many plant materials (Chase and Pratt, 1949). For the herbal drug preparation, purity of drug without adulteration is important for the quality of the drug. Fluorescence characteristics of any powder drug is very distinctive and its distinguishing features will help in the determination of adulterants in drug powder (Jahan, *et al*., 2008; Jalaj and Radhamany, 2014). The result of fluorescence analysis of powdered root tuber is recorded in Table 2. Here, the powder sample in acetic acid and also in benzene, under visible light gives light brown, in short UV it is yellow and in long UV, it is white. In 100% HCl, in visible light, the powder appears brown and in long and short UV, it appears as black and dark green respectively. While in 50% HCl, the powder is light brown under visible light and cream under UV and yellow under short UV. The acetic acid, benzene, 100% HCl and 50% HCl is used for authenticating the root powder. Hashmi and Singh (2003) reported that 'Ceylon cinnamon' was easily distinguished from Chinese and Saigon variety by their characteristic fluorescence. In the present study, data on the fluorescence analysis will be helpful for the determination of adulterant.

3.2. Histological localization test

Many plants contain medicinally important secondary metabolites (Dhar *et al*., 1968). Among the different group of phytochemicals, phenols, alkaloids, flavonoids, glucosides and terpenoids are therapeutically important for various biological activities. Considering the deleterious effects of synthetic antibiotics, the isolation, purification and characterization of novel types of plant secondary metabolites could be a safer alternative to synthetic compounds (Bajpai and Kang, 2011). In *H. ada-kodien*, the localization of secondary metabolites in different tissues of root tuber are given in Table 5.

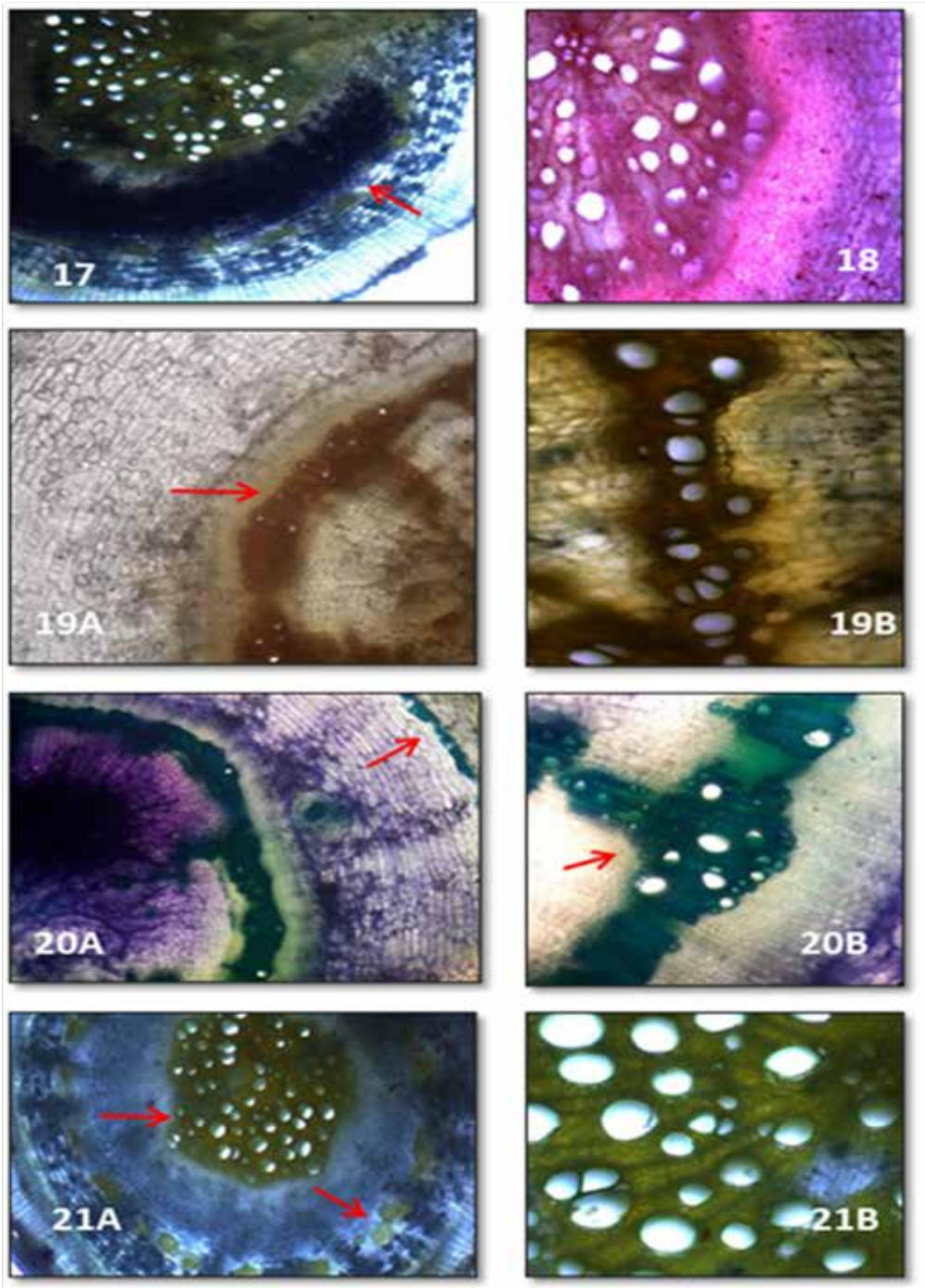
In root tuber, starch was localized in the region as reddish-brown colour, outer to secondary tissues and also in few cells of periderm (Fig. 17). Proteins were detected as blue colour in almost all cells but meagre in secondary vessels and periderm of the root. The lipid was localized as red colour in almost all cell types of the root (Fig. 18). Histological location of alkaloids in the root of *H. ada-kodien* shows clear demarcation not only at stelar region (Fig. 19A & B), but also in certain cortical cells when treated with Dragendorff's reagent. Alkaloids are chemically heterogenous group of natural substances and pharmacologically active compounds, compose more than six thousand basic nitrogen containing organic compounds, which occur in more than 150 different plant families (Visweswari *et al*., 2013). It has been reported that total alkaloids extracted from *Withania somnifera* roots, caused relaxant and antispasmodic effects against various agents that produce smooth muscle contractions in intestinal, uterine, tracheal, and vascular muscles (Mishra *et al*., 2000). In root tuber secondary xylem vessels and few cells of the inner cortex *ie*; outer to phloem shows greenish-blue colouration indicating the presence of phenol (Fig. 20A & B). The terpenoids were noted as orange-yellow colour in the walls of xylem vessels and outer cortex of root (Fig. 21A & B). Terpenoids are small molecular products synthesized by plants and are probably the most widespread group of natural products. Terpenoids show significant pharmacological activities, such as antiviral, antibacterial, antimalarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities (Mahato and Sen, 1997).

3.3. Characterization and preliminary phytochemical screening of extract

The consistency and yield of the various extract are characterized and the colour of the extract is authenticated by referring colour chart (Wilson, 1938). The result is given in Table 6. Extractive values are also useful to evaluate the chemical constituents present in the crude drug and also help in the estimation of specific constituents soluble in particular solvents (Kumar *et al*., 2017). Previous studies confirmed that the solvent plays a vital role in the extraction of the plant constituents (Fayaz *et al*., 2017). The colour of the extract and yield varies for five solvents but consistency in methanol is semi-solid and in water solid while in the other three solvents it is sticky. Maximum yield was noted in water and less in chloroform. Phytochemical screening of petroleum ether, chloroform, methanol, acetone and water extract confirmed the presence of terpenoid, alkaloids, phenols, flavones, steroids, glycosides, lignans and saponins in the extracts but in varying concentration which was analysed qualitatively. The present study indicates that

Table 5. Result of histochemical localization of secondary metabolites

Ergastic content	Reagent	Colour changes	Tissue zones with localized metabolites
Alkaloids	Dragendorff's reagent	Brown colour	Stelar region and certain cortical cells
Phenols	Toludene blue	Bluish green	Secondary xylem vessels and few cells of the inner cortex, outer to secondary phloem
Terpenoids	Diphenyl Hexatriene	Orange yellow	Secondary xylem vessels and outer cortex



Figures showing histochemical localization: Fig. 17. Showing localization of starch (outer to secondary tissues and in periderm); **Fig. 18.** Localization of lipid as reddish colouration; **Fig. 19A.** Alkaloid localization at stelar and cortical region; **Fig. 19B.** Enlarged view of localization of alkaloid at stelar region; **Fig. 20A.** Localization of phenol at inner cortex and vascular region; **Fig. 20B.** Enlarged view of xylem vessels showing localization of phenol; **Fig. 21A.** Localization of terpenoids at stelar and outer cortex; **Fig. 21B.** Enlarged view of xylem showing localization of terpenoid.

maximum phytochemicals were present in methanol extract. Terpenoids and alkaloids were present in all the five solvents but terpenoid shows better result in chloroform extract, while alkaloids show best in methanol. Result of qualitative phytochemical screening among various solvents is given in Table 7.

4. Conclusion

The results obtained from the pharmacognostic studies on root tuber of *H. ada-kodien* will be a tool for the authentification and quality control assessment of root tuber collected for pharmaceutical industries. The presence of prismatic crystals and the 'Maltese cross' are characteristic features for the identification of the powdered root tuber of *H. ada-kodien*. Methanol was the best solvent for the isolation of phytoconstituents and the presence of terpenoids and alkaloids in all solvents drives the scope of isolating the active principle from the *H. ada-kodien* root tuber. Thus pharmacognostic studies increase the quality and reliality in phytodrugs which could extend nationally and internationally leading to the research for discovering more bioactive compounds.

Acknowledgements

The authors acknowledge the Head of the Department of Botany, University of Kerala, Karyavattom, for the facilities provided to carry out the work.

Table 6. Characteristics of root tuber extract of *H. ada-kodien* in successive solvents

Solvent	Consistency	Yield(%)	Colour
Petroleum Ether	Sticky	0.234	Scheels green 860/2
Chloroform	Sticky	0.144	Fern green 862/1
Acetone	Sticky	0. 273	Oxblood red 00823/3
Methanol	Semi solid	0.680	Garnet brown 00918/2
Water	Solid	0.907	Leek green 000858

Table 7. Qualitative phytochemical analysis of root tuber extract of *H. ada-kodien*

Phytochemicals	Petroleum Ether	Chloroform	Acetone	Methanol	Aqueous
Terpenoids	++	+++	++	++	+
Phenols	-	+	+	+	+
Flavones	-	-	-	++	+
Steroids	-	-	++	++	+
Quinones	-	-	-	-	-
Anthraquinones	-	-	-	-	-
Glycosides	-	-	-	+++	++
Alkaloids	++	++	++	+++	+
Tannins	-	-	-	-	-
Lignans	-	+	++	++	+
Saponins	+++	++	-	+	-

(+) slightly present, (++) Moderately present, (+++) Highly present, (-) absent. All the test were carried out thrice.

Conflict of interest

We declare that there is no conflict of interest.

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