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Phytochemical and FT-IR spectral analysis of *In vivo* and *In vitro* root tuber extracts of *Holostemma annulare* (Roxb.) K. schum.

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Abstract
Medicinal plants have been used for centuries for the treatment of different diseases and disorders. The therapeutical application of these medicinal plants is attributed to the presence of different secondary metabolites or phyto-constituents in them. The present research was mainly concentrated on the preliminary phytochemical analysis and Fourier Transform Infra-Red (FTIR) spectral analysis of *in vivo* and *in vitro* root tuber extracts of *H. annulare*. The phytochemical screening of methanolic extracts of both *in vivo* and *in vitro* root tuber extracts revealed the presence of triterpenoids, flavonoids, alkaloids, tannins, proteins and carbohydrates. The FTIR analysis confirmed the presence of alkynes, alkanes, primary amines, aliphatic amines, alkenes and alkyl halides and the common compounds present on both the samples were primary amines, aliphatic amines alkanes and alkyl halides. The results confirm the fact that *H. annulare* possesses potential of bioactive compounds useful for our health and the *in vitro* root tubers can be used as alternatives for ayurvedic drug preparations.

Keywords: phytochemicals, FTIR spectroscopy, *Holostemmaannulare, in vivo* root tubers, *in vitro* root tubers

Introduction
Plants have been used as medicines for curing various illnesses in different cultures and religion for thousands of years [1]. The presence of phytochemical in medicinal plants make possible the cure and heal of different human diseases [2]. Based on their function in the plant metabolism these phytochemicals are basically categorized in to primary and secondary metabolites. Common sugars, amino acid, proteins and chlorophyll are constitute the primary metabolites while secondary metabolites includes alkaloids, terpenoids, saponins, phenolic compounds, flavonoids, tannins, etc.[3]. Phytochemical screening is the important step for the identification of phytochemical compounds present in the medicinal plants.
The medicinal properties of herbal plants are owing to the chemical nature of different phytochemical constituents. Identification of these chemical natures of compounds will provide some information about the functional groupspresent in it. To identify the active components in the sample powder based on the peak values in the region of IR radiation, FTIR spectrum is used.FTIR spectrum can be also used for determining the functional group and the chemical structure of the constituents [4]. By interpreting the infrared spectrum absorption the chemical bonds in a compound can be determined. The absorption radiation of most organic and inorganic compound is within 4000-400 cm^{-1}which lies in thecommonly used region for Infra-Red absorption spectroscopy [5].
The targeted plant of the study *Holostemmaannulare* (Roxb.) K. Schum. is used in the traditional system of medicine for maintaining youthful vigour and potentiality and is chiefly distributed in the tropical Western Ghats. It is a laticiferous climber belonging to the family Asclepiadaceae. The root tubers of this plant are medicinally important used for the preparation of theayurvedic drug *jivanthi* and are useful in ophthalmopathy, orichitis, cough, fever, burning sensation, stomachalgia and also used as expectorant, tonic, stimulant and galactagogue[6]. The terpenoid sugars present in the root tubers of the plant *Holostemma* are responsible for the medicinal properties. The tuberous roots of *Holostemma* have huge demand in South Indian pharmacies and the destructive collection for pharmaceutical preparations would lead to the decline in the population of the species. So it is crucial to develop technologies for producing root tubers under *in vitro* conditions and analyze the phyto-components in them and the present study attempted the phytochemical screening of *in vivo* and *in vitro* root tuber extracts and the powder samples by FTIR profiling.

Materials and methods
Collection of plant materials
Holostemmaannulare freshroots (*in vivo*) were gathered from the homestead cultivation at Vembayam, Thiruvananthapuram District, Kerala, India as well as *in vitro* samples collected from *in vitro* culture for phytochemical and FT-IR analysis.

Preparation of plant extracts for phytochemical analysis
Extraction of phytochemicals present in the root tuber of plant materials were done by distillation method by using Soxhlet apparatus. The collected root tubers (*in vivo* and *in vitro*) were washed well, dried and powdered and methanol were used as the solvents for the extraction of phytochemicals. The extract was concentrated to dryness and the residue was transferred to a sample bottle and was stored for further studies [6].

Preparation of plant materials for FTIR analysis
The root samples (*in vivo* and *in vitro*) were dried in an oven for 2 days at 60 ºC and powdered. These powdered samples were subjected to FTIR analysis. Tablets for FTIR spectroscopy were madeready in an agate mortars by mixing

the sample powder with (2 mg) with KBR (1:100 p/p). The absorbance spectra were calculated between 300 and 4500 cm^{-1}. For each sample, at least three spectra were achieved.

Preliminary phytochemical analysis
The phytochemical screening of the different extracts (*in vivo* and *in vitro* root tuber) were done for the identification of the following phytochemical constituent as per the standard methods [7].

Test for Alkaloids
Approximately 50 mg of root extract was dissolved in 5 ml of distilled water. Further hydrochloric acid was added until an acid reaction occurred and it was filtered. The filtrate was tested for the presence of alkaloids as described below.

a. Dragendroff's test: 1 ml of Dragendroff's reagent was added to 2 ml of the filtrate along the side of the test tube. Reddish brown precipitate was formed which indicated the test as positive.

b. Wagner's test: To 1 ml of the test solution added 2 drops of Wagner's reagent along the side of the test tube. Yellow or brown precipitate was formed and it confirmed the test as positive for alkaloids.

Test for flavanoids
A small amount of root extract was heated with 10 ml of ethyl acetate in boiling water bath for 3 minutes. The mixture was filtered differently and the filtrates were used for the following tests.

a. Ammonium test: The filtrate was shaken with 1 ml of 1% (v/v) dilute ammonia solution (1%, v/v). The layers were allowed to separate and yellow colour detected at ammonia layer indicates the presence of flavanoids.

b. Alkaline reagent test: 2 ml of root extract was treated with few drops of 20% (w/v) NaOH solution. Intense yellow color was formed which becomes colourless on adding dilute HCl which indicates the occurrence of flavanoids.

c. Shinoda test: A few magnesium turnings and 5 drops of concentrated HCl was added drop wise to 1 ml of test solution. After few minutes a pink, scarlet, crimson red or occasionally green to blue colour appeared which confirmed the test.

Test for Phytosterols/ terpenoids
a. Liebermann-Burchard's test: 2 mg of the rootextract was dissolved in 2 ml of acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated H_2SO_4 was added along the test tube side. A brown ring was formed at the junction confirmed the test for the presence of phytosterols.

Test for Tannin
Ferric chloride test
A few drops of 5% (w/v) ferric chloride solution was added to 2 ml of the root extract. Bluish black colour formed indicated the presence of hydrolysable tannin.

Test for Phenol
Extract of roots were treated with 3-4 drops of 10 % (w/v) ferric chloride solution and greenish black colour was formed which indicated the presence of phenol.

Test for Triterpenoids
Salkowski test: Approximately 2 mg of dry extracts was shaken with 1 ml of chloroform and a few drops of concentrated sulfuric acid was added along the side of test tube. Formation of a red brown colour at the interface indicated the test as positive for triterpenoids.

Test for Anthraquinone glycosides
To 1 ml of the extract, a few drops of 10 % (w/v) potassium hydroxide solution was added and the formation of red colour confirmed the test.

Test for carbohydrates
a. Molisch's test: To 1 ml of the test solution a few drops of 1% α-naphthol and 2-3 ml concentrated sulphuric acid was added along the side of test tube. A reddish violet or purple ring at the junction of two liquids confirmed the presence of carbohydrates.

b. Fehling's test: 2 mg dry extract was dissolved in 1 ml of distilled water and 1 ml of Fehling's (A and B) solution was added, shaken and heated on a water bath for 10 minutes. The brick red precipitate formed confirmed the test.

Test for proteins
Biuret test
To 2 ml of the test solution added 5 drops of 1% (w/v) copper sulphate solution and 2 ml of 10% NaOH and mixed thoroughly. Purple or violet color was formed which confirmed the presence of protein.

Test for saponin
Foam test
5 ml of the test solution was taken in a test tube. A drop of sodium bicarbonate solution was added to the test tube containing about 5 ml of root extract and then test tube was shaken vigorously and left for 3 minutes. Formation of honey comb like froth indicates the presence of saponins.

Results and Discussion
Preliminary phytochemical screening
Qualitative analysis
Phytochemical screening of *H.*annulare*in vivo* and *in vitro* root tubers indicated the existence of flavanoids, tannins, saponins, anthocyanins, steroids, alkaloids and phenols [8,9]. Medicinal properties of *H. ada-kodien*was as a result of the occurrence of various complex chemical substances of different composition which occur as secondary metabolites and the preliminary phytochemical screening of the leaf aqueous extract of the plant exhibited positive test for alkaloids, tannins, phloba tannins, flavanoids, terpenoids, glycosides and phenol [10]. The presence of phytochemicals such asflavanoids and tannins may be responsible for the antidiabetic activity. In agreement with the findings of the present study, preliminary phytochemical screening of stem and leaf samples of the herb showed positive test for flavanoids, hydrolysable tannins, phenols, saponins, sterols and terpenoids[11]. The leaves exhibited the presence of flavanoids, saponins,tannins, anthocyanins and phenols in the methanolic extract and hydro-alcoholic extracts [12, 13]. Also, preliminary phytochemical screening of *H. ada-kodien* leaves conclude that *H. ada-kodien* is a promising source of potential antioxidants which can be used for various diseases [14].

Table 1: Qualitative phytochemical analysis of *in vivo* and *in vitro* root tuber extracts of *H. annulare*

Phytochemicals	Test	Inference of phyto-constituents	*In vivo* root tuber	*In vitro* root tuber
Alkaloids	Wagner's test	Yellow or brown precipitate formed	+++	++
	Dragendroff's test	Reddish brown precipitate formed	++	++
Flavanoids	Alkaline test	A yellow colour observed at ammonia layer	+	++
	Shinoda test	After few minutes a pink, scarlet, crimson red or occasionally green to blue colour appeared	+++	+
Triterpenoids	Salkowski test	A red brown color formed at the interface	++	+
Tannins	$FeCl_3$ test	Formation of bluish black colour	+	+
Phenols	-	Formation of greenish black colour	+	+
Antraquinones	-	Appearance of Red colour	-	-
Carbohydrates	Molisch's test	At the junction of two liquids a reddish violet or purple ring formed	++	+
Saponins	Foam test	Stable foam	-	-
Protein	Biurette test	Purple or violet color formation	+	+
Phytosterol	Libermann-burchards	formation of a brown ring at the junction	++	-

(+++ High; ++ Moderate; + Low; - Nil)

Quantitative analysis

Quantitative analysis of different phyto-constituents showed comparatively highest quantities of soluble sugar (12.68 mgg^{-1}) compared to the protein and the amount of reducing sugar were found to be 0.986 mgg^{-1} and 0.080 mgg^{-1} respectively) in the methanol extract of *in vivo* root tubers of *H. annulare*. The results suggested that methanol extracts of *in vivo* and *in vitro* root tuber contained 1.88 and 1.65 mgg^{-1} of flavanoids, 7.62 and 7.01 mgg^{1} of alkaloids respectively. Quantity of tannin estimated as tannic acid equivalent (TE mgg^{-1}) in the *in vivo* and *in vitro* root tubers were found ranging from 0.42mgg^{-1} and 0.74 mgg^{-1}. In the present study, the phenolic content in methanol extract of *in vivo* sample was slightly high (10.47 mgg^{-1}) than *in vitro* sample (10.29 mgg^{-1}). In comparison to alkaloids, phenolics, flavonoids and tannin the total triterpenoid content detected was significantly low in methanol extract of *in vitro* sample (0.29 mgg^{-1}) than the *in vivo* sample and was found to be in the order *in vivo* triterpenoid (0.312 mgg^{-1}) > *in vitro* triterpenoid (0.285 mgg^{-1}) (Table 2). The results indicated that the *in vitro* root tuber samples can also be used as a better source of phytocomponents similar to the *in vivo* plant.

In the present study, quantitative analysis of the different phytochemicals in the *in vivo* and *in vitro* samples were analyzed and revealed that the amount of different phytochemicals varied in little extent in both the samples (Table 2) and these phyto constituents are responsible for the medicinal activities of *H. annulare*. In the present study the amount of carbohydrates in *in vivo* and *in vitro* samples were 12.68 mgg^{-1} and 11.23 mgg^{-1} respectively. Nutritional evaluation was conducted in *H. ada-kodien* and it was found that the plant possess highest amount of carbohydrates [15]. There are previous reports indicating that the root tubers of the *H. ada-kodien* contain 32.54% of starch [16]. The quantitative estimation in methanolic extract of *in vivo* and *in vitro* root tuber samples contains 0.80 and 0.76 mgg^{-1} of reducing sugar. Earlier studies reported that root tubers of *H. adakodien* contain 24% of sugar [16]. The result of the present study indicated that the amount of proteins were 0.98 mgg^{-1} in in *in vivo* samples and 0.94 mgg^{-1} in *in vitro* samples comparatively high than 4.07% of protein as per the previous report [16]. Substantiating the results in *H. annulare*, *Leptadenia reticulata* contains 35.80% of proteins [17]. Alkaloid contents in methanolic*in vivo* and *in vitro* root tuber extracts of *H. annulare* were 7.62 mgg^{-1} and 7.01 mgg^{-1} respectively. Agreeing this, methanolic extracts of leaves of *Ageratinaa denophora* contain the 6.07 mgg^{-1} alkaloid [18]. The methanolic extracts of *in vivo* samples showed slightly high phenolic content (10.47mgg^{-1}) compared to *in vitro* samples (10.29 mgg^{-1}). Contrastingly the total phenol content in stem and leaf extracts of *in vivo* plant *Ceropegia thwaitesii* (Asclepiadaceae) were 10.27 mgg^{-1} and 11.21 mgg^{-1} and that of *in vitro* derived plants were 14.94 and 13.82 mgg^{-1} respectively [19]. The amounts of flavonoids in *in vivo* samples of *H. annulare* was 1.88mgg^{-1} while 1.65 mgg^{-1} in *in vitro* samples. However, the total flavonoid contents in ethanolic root extracts of *Calotropis gigantea* and *Calotropis procera* were comparatively low, i.e., 1.516 and 1.271 mg per 100 mg [20]. The total terpenoid content detected in *H. annulare* was substantially low in methanolic extracts of *in vitro* roots (0.31mgg^{-1}) than the *in vivo* samples (0.29mgg^{-1}). Terpenoid sugars are reported for the anti-inflammatory activity of *Holostemma* species and their analgesic and anti-inflammatory activity is already established [21].

Table 2: Quantitative analysis of phytochemicals in methanol root tuber extracts of *H. annulare*

Phytochemicals	Quantity (mgg^{-1})	
	In vivo root tuber	*In vitro* root tuber
Carbohydrate	12.68 ± 0.010^{a}	11.23 ± 0.005^{a}
Reducing sugar	0.80 ± 0.010^{f}	0.763 ± 0.005^{f}
Proteins	0.98 ± 0.002^{e}	0.94 ± 0.001^{e}
Alkaloids	7.62 ± 0.010^{c}	7.01 ± 0.015^{c}
Phenols	10.47 ± 0.005^{b}	10.29 ± 0.020^{b}
Flavanoids	1.88 ± 0.010^{d}	1.65 ± 0.030^{d}
Tannin	0.42 ± 0.050^{g}	0.74 ± 0.015^{g}
Triterpenoid	0.31 ± 0.009^{h}	0.29 ± 0.020^{h}

Data represents mean values of six replicates repeated thrice. Mean values followed by the same letter in the superscript do not differ significantly based on ANOVA and t- test at $p\leq 0.01$.

Fourier Transform Infra-Red (FTIR) analysis

Identification of functional groups present in the sample molecule is done by FTIR spectroscopy which is also known as vibrational spectroscopy. FTIR is an analytical technique for identifying functional groups of the active components, based on the peak value in the region of infrared radiation. In IR spectral analysis, functional groups like –OH, C=O, N-H, CH_3 etc are present in the absorption bands in the range of 4000-2000 cm^{-1}. The region between 2000-500 cm^{-1} in the

IRspectrum are considered as the fingerprint region. The result of FTIR analysis confirmed the presence of characteristic band at 3276.92 cm^{-1} shows =C (triple bond) C-H:C-H stretch and 2927.10cm^{-1} shows C-H stretching. The peak at 1636.92 cm^{-1} shows the N-H bending, while those at 1331.29 cm^{-1}, 1149.61 cm^{-1}, 1076.36 cm^{-1} shows C-N stretch in the *in vivo* root extract of *H. annulare* (Table 3, Fig. 1). The *in vitro* root sample exhibited characteristic bands at 2934.84 cm^{-1} that shows C-H stretch, and the bands at 1737.47 cm^{-1}, 1637.18 cm^{-1}, 1366.20 cm^{-1} and 1217.15 cm^{-1} denoted C=O stretch, N-H bend, C-H rock and C-N stretching respectively (Table 4, Fig. 2). In *in vivo* and *in vitro* samples, alkyl halides were seen at 522.45, 572.56 cm^{-1} and at 529.09 cm^{-1} and 567.13 cm^{-1}. Alkynes, alkanes, primary amines, aliphatic amines, alkenes and alkyl halides are the compounds present on the FTIR spectrum of *in vivo and in vitro* root tuber samples and the common compounds seen on both the samples are primary amines, aliphatic amines, alkanes and alkyl halides.

Table 3:FTIR peak values and functional groups of *in vivo* root tuberextracts of *H. annulare*

Sl. No.	Frequency	Bond	Functional group
1	3276.92	=C(triple bond) C-H:C-H stretch, narrow, strong	Alkynes (Terminal)
2	2927.10	C-H stretch, medium	Alkanes
3	1636.92	N-H bend, medium	Primary amines
4	1331.29	C-N stretch, strong	Aromatic amines
5	1149.61	C-N stretch, medium	Aliphatic amines
6	1076.36	C-N stretch, medium	Aliphatic amines
7	999.54	=C-H bend, strong	Alkenes
8	522.45	C-Br stretch, medium	Alkyl halides
9	572.56	C-Br stretch, medium	Alkyl halides

Table 4: FTIR peak values and functional groups of *in vitro* root tuber extracts of *H. annulare*

Sl. No	Frequency	Bond	Functional group
1	3282.39	=C(triple bond) C-H:C-H stretch, narrow, strong	Alkynes (Terminal)
2	2934.84	C-H stretch, medium	Alkanes
3	1737.47	C=O stretch, strong,	Esters, saturated aliphatic
4	1637.18	N-H bend, medium	Primary amines
5	1366.20	C-H rock, medium	Alkanes
6	1217.15	C-N stretch, medium	Aliphatic amines
7	1018.98	C-O stretch, strong	Alcohols, carboyli aids, esters, ethers
8	529.09	C-Br stretch, medium	Alkyl halides
9	567.13	C-Br stretch, medium	Alkyl halides

In the present study, the FTIR spectrum of *in vivo* and *in vitro* samples confirmed the presence of alkynes, alkanes, primary amines, aliphatic amines, alkenes and alkyl halides and the common compounds present on both the samples were primary amines, aliphatic amines alkanes and alkyl halides. The FTIR spectra of both samples of *H. annulare* revealed almost similar absorption of IR radiation with little variation only regarding the area of absorption. FTIR has a great value because of its simplicity, rapidity, sensitivity and low expense and in the FTIR studies, the methanolic extract of *Ceropegiapussila*contain 28 compounds with different chemical structures [22]. FTIR is undertaken to identify the functional groups present in the root samples of*H. ada-kodien*whichhas a major role in the field of medicinal plant analysis [23]. There are previous reports regarding the FTIR analysis in the family Asclepiadaceae like *Calotropisgigantea*, *Carallumageniculata* and *Carallumanilagiriana*[24,25,26] and *Gmnelinaasiatica*[27].

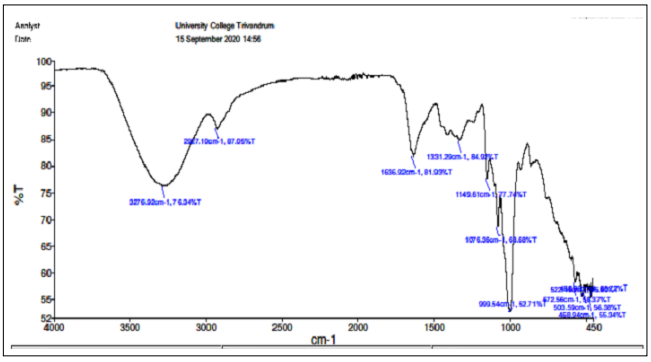

Fig1: FTIR spectrum of *H. annulare in vivo* root tuber extract

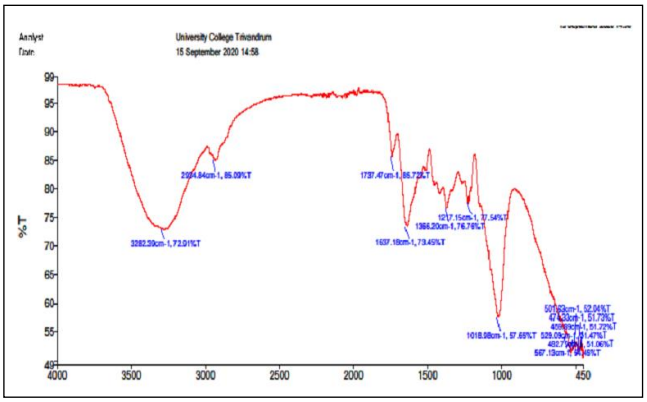

Fig2: FTIR spectrum of *H. annulare in vitro* root tuber extract

Conclusion

The phytochemical investigation studies of roots of *H. annulare* revealed the presence of medicinally important metabolites that are of great value in the pharmaceutical industries**.** One of the most important analytical device used for the qualitative and quantitative analysis of biological materials were spectroscopic techniqueand in the present study the FTIR profiling and the spectrum provide a tool for identification of the bioactive compounds present in *H. annulare* root tuber.

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