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Isolation and Characterization of The Active Constituents Present In The *Chonemorpha macrophylla* Roots

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ABSTRACT

Ethanol extract of *Chonemorpha macrophylla* root was prepared using 50% ethanol. Preliminary phytochemical studies were performed and the presence of phenolics and flavonoids were detected. The extract was fractionated by column chromatography using petroleum ether, chloroform, ethyl acetate and methanol in the increasing order of polarity. Presence of gallic acid, quercetin and rutin were identified in methanol fraction using TLC. Presence of the above three components were confirmed and quantified by RPHPLC studies. Spectral studies were also performed on the above three isolated components to reaffirm their identity. LCMS studies were performed on the ethanol extract of the roots and m/z value was found out for five more components other than the above three identified components.

Key words: *Chonemorpha macrophylla*, isolation & characterization of the active constituents, RPHPLC, LCMS.

INTRODUCTION

In the first step, available literatures on *C. macrophylla* were reviewed to understand the traditional claims and the plant parts used in medicine[1]. Traditional claim is there in the antidiabetic property of the aqueous decoction prepared from the roots1. Anticancer activity[2], anti-amoebic activity[3] and skeletal muscle relaxant activity[4] of the plant were studied and reported earlier. According to the traditional claim, the plant also has antipyretic and anti-inflammatory effects. In addition to that, usefulness in the treatment of stomach disorders is also mentioned. Detailed studies on the active constituents present in the plant were not found. Hence, it was decided to perform this work.

Materials and Methods

Plant roots were collected from near the village areas of Aruvappara, Munippara and Kayattupa adjacent to the forests in Ernakulam district. The plant, *C. macrophylla* (Roxb.) G. Don, was identified by Dr. K V George, Professor of Botany, CMS College, Kottayam, and the voucher specimen number is assigned as KRK/UCP/CMS/505. Shade dried roots were powdered coarsely and two different extracts were prepared using 95% and 50% ethanol.

Procedure followed: Soxhlet extraction. Results are depicted in table 2.

Phytochemical screening

Chemical tests were performed in both the prepared extracts for the detection of various classes of phytoconstituents present in it.

Fractionation of the extract

Fractions of the *C. macrophylla* root ethanol (50%) extract were prepared by column chromatographic

technique. This method is adopted to overcome the problem of emulsion formation on following the fractionation method with two immiscible solvents.

Ethanol (50%) extract was loaded on a silica gel column and fractionated into petroleum ether (60-80 °C), chloroform, ethyl acetate and methanol eluates by using the above four solvents in the increasing order of polarity. The extract (540 g) was processed in batches of 30g each. The extract (30g) was dissolved in methanol (50 ml) in a beaker and treated with silica gel (50 g). It was mixed well and the solvent was allowed to evaporate. The dry powder thus obtained was loaded on a silica gel column prepared using silica gel (300 g) mixed with petroleum ether. Elution was carried out in a successive manner as explained above. Fractionation was performed with 500 ml each of the respective solvent and 20 ml of the eluate/fraction was collected every time. All the eluates found similar in composition on TLC (Silica gel G, solvent system (1) Ethyl acetate: Benzene (9:11)[5] and (2) Toluene: Ethyl acetate: Methanol (5:3:2)[6] and the spots were visualized in an iodine chamber) were pooled and concentrated by distillation to remove the solvent. The syrupy mass obtained was evaporated on a water bath and then kept in a desiccator.

The fractions collected using petroleum ether, chloroform, ethyl acetate and methanol were named as PE-CMRH, CH-CMRH, EA-CMRH and ME-CMRH respectively. Each of the fractions was subjected to TLC using the above mentioned methods.

Identification of the components present in the methanol eluate of ethanol (50%) extract of *C. macrophylla* **roots**

UV spectral studies

Separated components CM-1, CM-2 & CM-3 (1 mg each) were dissolved in methanol and recorded the spectrum in the range of 200 to 500 nm range using a UV double beam spectrophotometer (Shimadzu UV-1800). The solutions were diluted to get absorbance around 0.5 units.

Shift reagent study

In case of flavonoids in which the R_f values matched with those of known components, shift reagent study with 0.01 N NaOH was performed. After recording the spectrum in methanol one drop of 0.01N NaOH was added, mixed and the spectrum was recorded again immediately.

IR spectral studies

FTIR spectra were recorded in KBr pellet using Shimadzu-8400S spectrometer in the range 400-4000 cm-1.

NMR spectral studies

NMR spectrums of the isolated components were recorded using a BRUKER make 400 MHz instrument in the range of 0 to 15 ppm. TMS was used as the internal standard.

Estimation of total flavonoids in the methanol eluate obtained by column chromatography of ethanol (50%) extract of *C. macrophylla* **roots (ME-CMRH)**

Aluminium chloride colourimetric method was used for the determination of flavonoids. Aluminium chloride forms acid stable complexes with the C4 keto group of flavones and flavonols. In addition to that, aluminium chloride forms acid labile complexes with the ortho dihydroxyl groups in A and B ring of flavonoids. The mixture turns to pink colour and absorbance can be measured at 510 nm. Flavonoid content was determined from the calibration plot of rutin used as the standard.

Chemicals and Reagents.

Rutin (Sigma), aluminium chloride, sodium nitrite, sodium hydroxide and distilled water. Sodium nitrite (5% w/v solution), aluminium chloride (10% w/v solution) and sodium hydroxide (1 M) solution.

Preparation of the test solutions

Rutin (25 mg) was weighed, dissolved in methanol and made up to the volume in a 25 ml standard flask to get 1000 µg/ml stock solution. The above solution was diluted accordingly to get 10, 30, 50, 70 and 90 µg/ml of rutin and was used for the preparation of calibration graph.

In a similar way, the test solution (1000µg/ml) was prepared by dissolving the extract (10 mg ME-CMRH) in methanol.

Procedure[7]

In different 10 ml volumetric flasks, 1.0 ml of each dilution and 2.0 ml distilled water were taken. Sodium nitrite 0.3 ml (5% solution) was added to the above solution, mixed well and allowed to react. After five minutes, pipetted 0.3 ml of aluminium chloride (10% solution) and 2.0 ml of 1M sodium hydroxide solution to it. Then it was mixed well and the final volume was made up to the mark with distilled water.

In a similar way, the test solution (1.0 ml) of the extract was also reacted and measured the absorbance at 510 nm against reagent blank. The experiment was performed in triplicate.

Identification and estimation of the components present in methanol eluate of ethanol (50%) extract of *C. macrophylla* root (ME-CMRH) by HPLC

MATERIALS AND METHODS

All the chemicals and reagents used were of HPLC grade.

Gallic acid, Quercetin, Rutin, (Sigma chemicals USA), buffers and methanol (Merck) were used.

HPLC instrument make: Shimadzu LC-10 ATVP Software: Chromtech N 2000 data, Detector: UV Wavelength : 275 nm, Flow rate: 1 ml/minute Injection volume: 20 µ liter, Column dimension: RP C 18, 250 x 4.6 mm, 5 µs

Procedure

Mobile Phase: Methanol: Phosphate buffer (pH: 3) 70:30

Method followed: Isocratic elution

Gallic acid, quercetin and rutin standards 2 mg each were weighed and dissolved in 1 ml of methanol separately. From that 100 µl was taken, made up to 1 ml with methanol and 200 µl of this solution was injected.

The sample was weighed accurately (10 mg), transferred to a 10 ml standard flask and dissolved in methanol by warming, cooled and made up to the mark with methanol. Required dilutions were prepared from this and used. Results are depicted in table 6.

LC MS of *C. macrophylla* of ethanol (50%) extract

Instrument - Mass spectrometer: Thermo Fisher LCQ-Fleet – Ion Trap with Quadrupole Ion Trap Mass Analyser, HPLC: Accela High Speed Pump, Surveyor Plus Auto sampler. Experiment was performed at ambient temperature.

Table 1 LCMS conditions for CMRH

HPLC conditions			
Analytical column	C18 column, 3µm, 100 x 4.6 mm		
Injection volume	5 µl		
Flow rate	300 µl/min		
Mobile Phase:			
Solvent –A	0.1% Formic Acid		
Solvent –B	ACETONITRILE		
Gradient program	Time (min)	Solvent A	Solvent B
	00.01	75	25
	02.00	70	30
	08.00	40	60
	12.00	40	60
	15.00	75	25
	20.00	75	25
Mass spectrometry experiment conditions			
Ion probe	ESI		
Sheath gas	(22) Nitrogen		
Auxiliary gas	(8) Nitrogen		
Discharge current	5.0 K µA		
Capillary volt	7.00 V (positive), -16 V (negative)		
Tube Lens	55 V (positive & negative)		

RESULTS

Table 2 Extractive values of *C. macrophylla* root extract.

SI. No	Sample name	Weight of sample (g)	Weight of extract (g)	Yield % w/w
1.	*C. macrophylla* root ethanol (95%) extract	1000	120	12.0
2.	*C. macrophylla* root ethanol (50%) extract	4000	560	14.0

Phytochemical screening

Both the extracts were answering tests for carbohydrates, glycosides, saponin glycosides, flavonoids, phenolics and tannins.

Fractionation of the extract

Table 3 Fractions from *C. macrophylla* root ethanol (50%) extract.

SI. No	Sample Name	Weight of fraction (g)	Extractive value (%) w/w
1.	Petroleum ether soluble eluate (PE-CMRH)	11.88	2.2
2.	Chloroform soluble eluate (CH-CMRH)	37.80	7.0
3.	Ethyl acetate soluble eluate (EA-CMRH)	08.10	1.5
4.	Methanol soluble eluate (ME-CMRH)	49.14	9.1

TLC studies

Each of the eluate was subjected to TLC using the above mentioned methods. Methanol eluate (ME-CMRH) gave one spot on running with solvent system (1) and was designated as CM-1. Some matter was remaining at the point of sample application and a diffused faint spot was there with the solvent front also. In all the cases, heading, tailing of spots were observed. Spots matched with R_f values of standards are depicted in table 4.

ME-CMRH on running TLC with solvent system (2) gave two spots and were designated as CM-2 and CM-3 on the ascending order of R_f values.

CH-CMRH gave one spot on running TLC with solvent system (2). The R_f value of the spot was similar to that of the Rf of the spot CM-3 obtained in the TLC of ME-CMRH fraction. So this spot was also designated as CM-3.

EA-CMRH fraction gave two different spots on running TLC with solvent system (2). The R_f values were corresponding to the R_f values of the two spots CM-2 and CM-3 of ME-CMRH fraction. Thus the above two spots were designated as CM-2 and CM-3 respectively. Some matter was found unmoved at the point of sample application also. Results are depicted in table 4.

Table 4 R_f values of the spots obtained in Si-gel G TLC performed on ethanol (50%) extract of *C. macrophylla* root

Sample code	R_f value	Solvent system
ME-CMRH	Gallic acid - 0.40 Sample (CM-1) - 0.41	Ethyl acetate: Benzene (9:11) - solvent system (1)
ME-CMRH	Rutin - 0.29 Sample spot (CM-2) (1) - 0.28	Toluene: Ethyl acetate: Methanol(5:3:2) - solvent system (2)
ME-CMRH	Quercetin - 0.76 Sample spot (CM-3) (2) - 0.75	Toluene: Ethyl acetate: Methanol (5:3:2) - solvent system (2)
CH CMRH	Sample (CM-3) - 0.76	Toluene: Ethyl acetate: Methanol (5:3:2) - solvent system (2)
EA CMRH	Sample-0.76-spot (2) (CM-3) Sample - 0.28-spot (1) (CM-2)	Toluene: Ethyl acetate: Methanol(5:3:2) - solvent system (2)

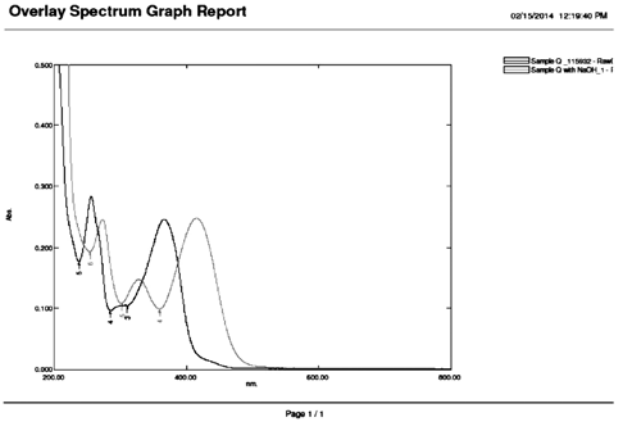

Fig.1 UV spectrum of CM-3 in methanol / + shift reagent (0.01N NaOH)

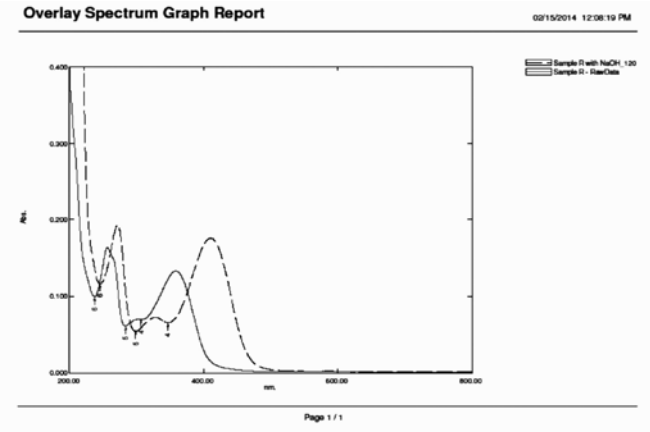

Fig.2 UV spectrum of CM-2 in methanol /+ shift reagent (0.01N NaOH)

DISCUSSION

The UV λ max of the white crystalline component (CM-1) separated from the methanol eluate recorded in ethanol (272 nm) matched well with the reported values (Harborne J B, third edition, 45) of gallic acid. The melting point recorded (250-253° C) was also in good agreement with the reported values. In the IR spectrum recorded in KBr disc method, bands at wave numbers in cm-1 at 3487, 2977, 2923, 2854, 2653,2533 (H-bonded –O-H stretch, 1712 (strong) (-C=O stretch), 1542, 1640 (-C=C- stretch 1203 (strong) (-C-O stretch or –O-H deformation) were observed. In the $_1$H NMR (400 MHz, DMSO), δ 12.25 (s, broad, -COOH proton), δ 9.2 (s, two protons, phenolic), δ 8.85 (s, one phenolic) and δ 6.92 (s, two Ar. ring protons) were present. The m/z value observed at 301 in the LCMS spectrum was confirmed as quercetin by using the above methods.

The UV spectrum recorded of the intense yellow microcrystalline powder (CM-3), separated from methanol eluate recorded in methanol (λ max 255 and 367 nm) and the spectrum recorded with the shift reagent 0.01 N NaOH (272, 324, and 411 nm) were matched well with the reported values of the flavonoid quercetin. Melting point of CM-3 was recorded as 317-319° C and was also matched well with the reported values. IR spectrum (KBr disc) show bands at wave numbers cm^{-1} 3425 (-O-H stretch), 1651 (-C=O stretch), 1604, 1512 (-C=C- stretch) were observed. In the $_1$H NMR (400 MHz, DMSO), δ 12.6, 10.8, 9.69 and 9.39 (multiplets, five different –O-H protons) and at δ 7.68, 7.55, 6.90, 6.41 and 6.2 (doublets, five different Ar. ring protons) were observed.

The UV spectrum recorded in methanol of the pale yellow crystals (CM-2) separated from the methanol eluate showed UV λ max at 255 and 358 nm and the spectrum recorded with the shift reagent 0.01 N NaOH showed a shift of UV max to 272, 328 and 410 nm were also in agreement with the reported values of rutin. The melting point recorded (190-193°C) was also in good agreement with the reported values. IR spectrum (KBr disc) show bands at wave numbers in cm^{-1} 3450 (broad, -O-H stretch, hydrogen bonded), 2908 (-C-H stretch), 1651 (-C=O stretch), 1604, 1504 (-C=C- stretch) were observed. In the ^{1}H NMR (400 MHz, DMSO), δ 12.6, 10.86, 9.70 and 9.20 (singlets, four different phenol –O-H protons), δ 7.54, 6.86, 6.84, 6.39 and 6.2 (doublets, five different Ar. ring protons), δ 5.36, 5.34, 5.31, 4.41and 3.72 (two multiplets and two singlets, six different –O-H protons of sugars), δ 4.46 (doublet, C-1''' proton of 3-O-rutinoside, δ 4.39, 3.70, 3.08 (one multiplet and one singlet, eleven different rutinose protons) and δ 1.007 (doublet, three protons of rhamnosyl –CH_3) were observed[8].

Table 5 Estimation of total flavonoids in the methanol eluate obtained by column chromatography of ethanol (50%) extract of *C. macrophylla* **roots (ME-CMRH)**

Sl.No.	Concentration of rutin in ± g/ml	Absorbance ± SD
1	10	0.082 ± 0.007
2	30	0.251 ± 0.006
3	50	0.432 ± 0.009
4	70	0.629 ± 0.007
5	90	0.792 ± 0.006
Unknown	24.45	0.211 ± 0.004

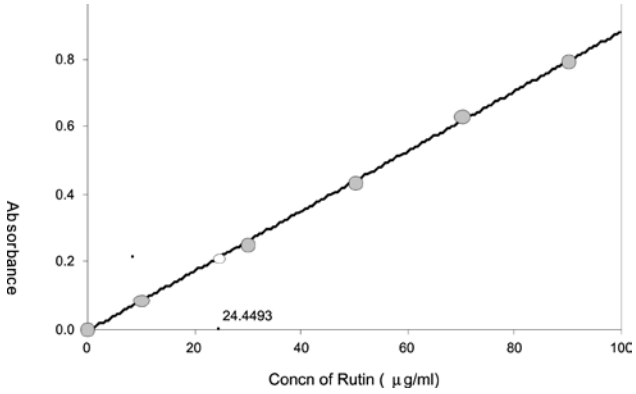

Fig. 3 Calibration plot of rutin for estimation of flavonoids

In the total flavonoid content estimation by aluminium chloride colorimetric method, a content of 24.45 µg/ml of total flavonoid equivalent to rutin was found to be present. In the HPLC method for the estimation of flavonoids 0.143%w/w & 0.236%w/w of quercetin & rutin were estimated in ME-CMRH fraction.

Table 6 Identification and estimation of the components present in methanol eluate of ethanol (50%) extract of *C. macrophylla* root (ME-CMRH) by HPLC

Type Marker	Details	
Gallic acid	sample area	9932756
	standard area	1032040.125
	sample dilution	10000
	standard dilution	100
	% of Gallic acid in active fraction	9.624
Quercetin	sample area	98080.25
	standard area	685859.375
	sample dilution	10000
	standard dilution	100
	% of Quercetin in active fraction	0.143
Rutin	sample area	471806.375
	standard area	1997017.875
	sample dilution	10000
	standard dilution	100
	% of Rutin in active fraction	0.236

Table 7 LC MS of *C. macrophylla*, methanol eluate of ethanol (50%) extract

Sl No	Retention time in minutes	m/z
1	5.56	276.97
2	8.05	518.99
3	8.73	260.94
4	6.54	510.49
5	8.08	538.14
6	9.57	301.15
7	10.56	359.10

Note: As the concentrations of the compounds are less, the individual m/z has been extracted and separate chromatograms were generated

CONCLUSION

The current study resulted in the isolation, identification and quantification of, gallic acid, quercetin and rutin from the ethanol (50%) extract of *C. macrophylla* roots. It is already reported the antioxidant and other related medicinal uses of the above constituents. In addition to the above three constituents, m/z values of six other molecules are also reported. They are not identified and quantified in this study. Studies can be continued further for the isolation and identification of the above unidentified components.

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Validated HPTLC Method for the Simultaneous determination of Phenylephrine and Levocetirizine in Drug Formulation

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ABSTRACT

A simple, precise, specific and accurate high-performance thin-layer chromatographic method has been developed for the simultaneous determination of Phenylephrine (PHE) and Levocetirizine (LEV) in pharmaceutical dosage form. The separation was carried out on Merck HPTLC aluminum plates of silica gel 60 F254, (20×10 cm) with 250 µm thickness using Methanol: Ethyl acetate: Ammonia in the ratio of 3:6.5:0.5 (v/v/v) as mobile phase. HPTLC separation of the two drugs followed by densitometric measurement was carried out in the absorbance mode at 230 nm. The drugs were resolved satisfactorily with Rf values of 0.19 and 0.34 for PHE and LEV, respectively. The linear regression analysis data for the calibration plots showed good linear relationship with r2 = 0.9987 and 0.9989 for PHE and LEV, respectively, in the concentration range of 50–400ng spot−1 for PHE and 100–600ng spot−1 for LEV. The method was validated for precision, robustness, specificity and accuracy. The limits of detection and quantitation were 0.24 and 0.70ng spot−1, respectively, for PHE and 0.13 and 0.37ng spot−1, respectively, for LEV. The proposed HPTLC method can be applied for identification and quantitative determination of PHE and LEV in bulk drug and drug formulation.

Key words: Phenylephrine, Levocetirizine, HPTLC, Formulation, Validation

INTRODUCTION

Phenylephrine (PHE) is m-Hydroxyphenyl-2-methylamino-ethanol (Figure 1). It is a α-adrenergic agonist most commonly used either topically or orally for symptomatic relief of nasal congestion. It is used as decongestant in treatment of fecal incontinence. In ophthalmology it is used as mydriatic[1,2]. Levocetirizine (LEV) (Figure 2) is chemically [2-[4-[(4-Chlorophenyl)-phenylmethyl]-1-piperazinyl] ethoxy] acetic acid. It is a non-sedating type histamine H1-receptor antagonist used for symptomatic relief of allergic conditions including rhinitis and chronic urticaria.[3,4] Literature survey reveals that only spectrophotometric methods[5-7] and RP-HPLC methods[8-10] have been reported for the simultaneous estimation of PHE and LEV. According to literature review no method has been reported for simultaneous determination of PHE and LEV by HPTLC. HPTLC method is cost effective, rapid, and less time consuming. In HPTLC many samples are simultaneously used and solvent requirement is low. The present work reports the development and validation of simple, precise and accurate HPTLC method for the simultaneous determination of PHE and LEV in tablet formulation. The proposed method is validated as per ICH guidelines[11].

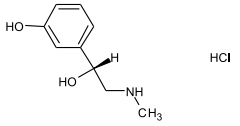

Figure 1. Structure of PHE

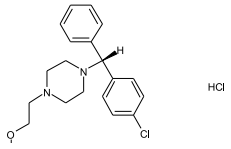

Figure 2. Structure of LEV

EXPERIMENTAL

Materials:

Working standards of pharmaceutical grade PHE (99.60%, w/w) and LEV (100.0%, w/w) were obtained as gift samples from Centaur Pharmaceuticals Ltd., Pune and Emcure Pharmaceutical Ltd.,Pune. Fixed dose combination tablets (Sinarest Levo, B.No. BD034, Centaur Ltd., MFG. 05/2013, EXP. 04/2015) containing 10 mg PHE and 2.5 mg LEV were purchased from local pharmacy, Pune, India. All chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India.

Instrumentation and Chromatographic Conditions:

The HPTLC plates were prewashed with methanol and activated at 110◦C for 5min prior to chromatography. The samples were spotted in the form of bands 6mm width with a Camag 100 microlitre sample syringe (Hamilton, Bonaduz, Switzerland) on silica-gel-precoated HPTLC aluminum plate 60 F254, ((20 × 10 cm) with 250 μm thickness(E. Merck, Darmstadt, Germany, supplied by Anchrom Technologies, Mumbai) using a Camag Linomat V applicator (Switzerland). A constant application rate of 0.1 μLs−1 was used and the space between two bands was 6 mm. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The mobile phase consisted of Methanol: Ethyl acetate: Ammonia in the ratio of 3:6.5:0.5 (v/v/v). The optimized chamber saturation time with mobile phase was 30 min using saturation pads at room temperature (25◦C). Densitometric scanning was performed using a Camag TLC scanner III in the reflectance-absorbance mode and operated by winCATS software (V1.1.4, Camag). The slit dimension was kept at 5mm × 0.45mm and the scanning speed was 10mm s−1. The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 200 and 400 nm. All determinations were performed at ambient temperature with a detection wavelength of 230 nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was by peak areas with linear regression.

Calibration procedure:

For calibration, mixed stock standard solution containing 12 mg mL−1 of PHE and 1mg mL−1 of LEV was prepared in methanol by dissolving 300 mg of PHE and 25 mg of LEV in 25mL methanol. Mixed stock standard solution was further diluted with methanol to obtain working standard solutions in a concentration range of 50-400 ng spot−1 for PHE and 160-400ng spot−1 for LEV. Each concentration was applied six times on the HPTLC plate. The plate was then developed using the previously described mobile phase. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs. Linear calibration curves were generated using least-squares linear-regression analysis.

Analysis of Commercial Formulation:

To determine the content of PHE and LEV simultaneously in pharmaceutical dosage form SINAREST LEVO (label claim: 10 mg PHE and 2.5 mg LEV per tablet, B.No. BD034, Centaur Ltd.), twenty tablets were weighed and finely powdered. Quantity equivalent to 13.5 mg of phenylephrine was taken in 50 mL of volumetric flask and dissolved in 40 mL methanol and the final volume was made with the same solvent. The solution was filtered through a 0.45 μm nylon syringe filter. From the resultant solution 1 mL was diluted to 10 mL which contains 300 and 75 ppm of PHE and LEV respectively. 0.2 μL volume was spotted for six times to achieve a final concentration of 360 ng spot−1 for PHE and 240 ng spot−1 for LEV. The plate was developed in the previously described chromatographic conditions. The peak area of the spots was measured at 230nm for PHE and LEV, respectively and the concentrations in the samples were determined using multilevel calibration developed on the same plate under the same conditions using linear regression equation.

Method Validation:

The optimized HPTLC method was validated for precision, robustness, accuracy and specificity as per the ICH guidelines. The precision of the method was verified by repeatability (intraday) and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations of working

standard of 40, 50, and 60 ng spot−1 for PHE and 160, 200 and 240 ng spot−1 for LEV. Method repeatability was achieved by repeating the same procedure six times on the same day for intraday precision. The intermediate (interday) precision of the method was checked by performing same procedure on different days under the same experimental conditions. The repeatability of sample application and measurement of peak area were expressed in terms of relative standard deviation (% RSD).The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. Following the introduction of small changes in the mobile phase composition (±0.1mL for each component), the effect on the results was examined. Mobile phases having different compositions, for example, Methanol: Ethyl acetate: Ammonia (3:6.8:0.5 v/v/v), (3.3:6.5:0.5 v/v/v), and (3:6.5:0.3 v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied over the range of ±5%. The time from spotting to chromatography and from chromatography to scanning was varied by +10 min. In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), the signal-to-noise ratios of 3 : 1 and 10 : 1 were regarded as LOD and LOQ. To assess specificity, the peak purity of PHE and LEV was determined by comparing their respective spectra at peak start, peak apex and peak end position of the spots. Accuracy of the proposed method was carried out by recovery studies by standard addition. To the solution of the formulation (PHE and LEV combination tablets) known amounts of PHE and LEV standard powder corresponding to 80, 100, and 120% of label claim were added and the absolute recovery was calculated by comparing the peak areas obtained from standard solution of PHE and LEV with the peak areas of samples of different concentration.

RESULTS AND DISCUSSION

Selection of Analytical Wavelength:

UV spectrum of PHE and LEV showed maximum absorbance at 275nm and 230 nm, respectively. Further, in situ HPTLC spectral overlain of PHE and LEV was taken and 230nm was selected as scanning wavelength **(Figure 3)**.

Optimization of Mobile Phase:

Optimization of mobile phase was done with a view to separate PHE and LEV drugs. The composition of solvent constituting the mobile phase in this work was varied in order to study its effect on resolution of drugs. The analyte should have distinct Rf values for good resolution. Initially, different mobile phases such as dichloromethane: ethyl acetate: methanol: ammonia (1.0:3.5:2:3.5v/v/v/v), (1.5:3.5:1.5:3.5v/v/v/v), (2.0:3.5:1:5:3.5v/v/v/v) and (3:2.5:1:3.5v/v/v/v) were tried. But in this system both drugs did not move. Then toluene: methanol: ammonia (6.5:3:0.5v/v/v/), (4.5:5:0.5v/v/v/), (3.5:6.5:0.5v/v/v) were tried, in this mobile phase LEV moved but PHE was retained at the base. For effective separation of both drugs various ratios of the mobile phase were tried and the optimal mobile phase ratio was Methanol: Ethyl acetate: Ammonia (3:6.5:0.5 v/v/v) which gave compact, symmetrical, well-resolved spots with Rf values of 0.19±0.4 and 0.34±0.6 for PHE and LEV, respectively **(Figure 4).** The development chamber was saturated for 30min. The development was done for 80mm on the plate and the development time was 20 min. After development, drying of the plates was done using air. Simultaneous detection of PHE and LEV was performed at 230nm since both compounds are well known to exhibit sufficient ultraviolet absorption at this wavelength.

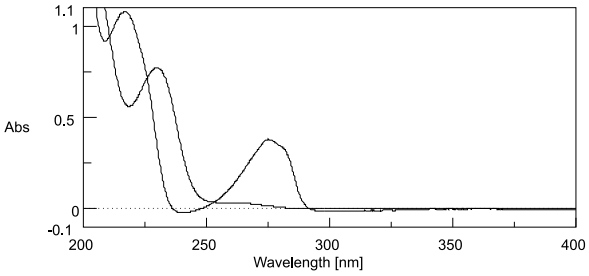

Figure 3. UV spectrum overlay of PHE and LEVO

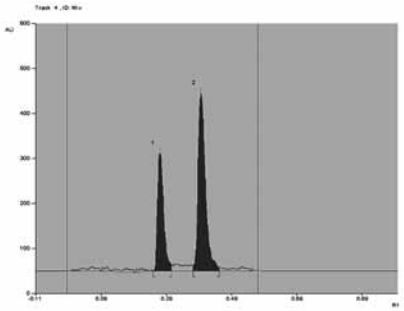

Figure 4. Densitogram of PHE and LEV

Validation of the Method:

Linear relationships were observed by plotting drug concentration against peak areas for each compound. PHE and LEV showed linear response in the concentration range of 50-400 ng spot-1 and 100-600 ng spot-1, respectively with square of correlation coefficient (r2) of 0.9987 and 0.9989 for PHE and LEV, respectively. (Table - 1)

Table 1. Linear regression data for calibration curves (n = 6)

Parameter	PHE	LEV
Linearity range ng/spot	50-400	100-600
r2	0.9987	0.9989
Slope	33.68	37.64
Intercept	561.4	972.4

The % RSD values depicted in Table - 2 show that the proposed method provides acceptable intraday and interday variation of PHE and LEV with respect to working standard. The repeatability of real sample application and measurement of peak areas were expressed in terms of % RSD and were found to be 0.61 and 0.58 for PHE and LEV, respectively.

The standard deviation of the peak areas was calculated for each parameter and the % RSD was found to be less than 2%. The low values of the % RSD, as shown in Table - 3, indicate the robustness of the method. The signal/noise ratios 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 0.24, 0.70 ng spot−1 and 0.13, 0.37 ng spot−1 for PHE and LEV, respectively (Table - 4)

Table 2. Intraday and interday precision of PHE and LEV (n = 6)

Drug	Concentration ng/spot	Intraday precision % RSD	Interday precision % RSD
PHE	40	0.81	0.61
	50	0.73	0.68
	60	0.68	0.58
LEV	160	0.72	0.68
	200	0.61	0.63
	240	0.53	0.58

Table 3. Robustness testing of method (n = 3)

Parameters	SD of peak area for PHE	% RSD	SD of peak area for LEV	% RSD
Mobile phase composition (±0.1ml)	13.76	0.4	8.33	0.5
Amount of mobile phase (± 5 %)	16.80	0.5	9.5	0.6
Time from application to development (+10 min)	17.9	0.6	8.4	0.5
Time from development to scanning (+ 10 min)	16.2	0.5	10.2	0.7

Table 4. Limit of Detection(LOD) and Limit of Quantitation(LOQ)

Parameter	PHE	LEV
LOD (ng/spot)	0.24	0.13
LOQ (ng/spot)	0.70	0.37

The specificity was noticed by the complete separation of PHE and LEV peaks. The peak purity of was assessed by comparing their respective spectra at the peak start, apex, and peak-end positions of the spot, that is, r(S, M) = 0.9999 and r(M, E) = 0.9999 for PHE and r(S, M) = 0.9997 and r(M, E) = 0.9998 for LEV.As shown from the data in Table - 5, satisfactory recovery percentage in the limit of 98–99% with small relative standard deviations (% RSD) is obtained at various added concentrations. The results indicate that the method is highly accurate for simultaneous determination of PHE and LEV.

Analysis of Marketed Formulation:

Using the proposed chromatographic method, assay of PHE and LEV in their tablets (SINAREST LEVO, label claim: 10 mg PHE and 2.5 mg LEVO per tablet, B.No BD034, Centaur Ltd.) was carried out. The peaks at Rf 0.19 for PHE and 0.34 for LEV were observed in the densitogram of the drug samples extracted from tablets.

There was no interference from the excipients commonly present in the tablets. Satisfactory results were obtained for both drugs which are in good agreement with the label claim. The drug content was found to be 99.40% (%RSD of 0.24) and 99.25% (% RSD of 0.26) for PHE and LEV, respectively (Table - 6)

Table 5. Accuracy studies for the determination of PHE and LEV

Drug ng/ spot	Amount added ng	Total amount ng	Amount ng	% Recovery	% RSD
PHE 50	40	90	88.6	98.44	0.36
	50	100	99.4	99.40	0.24
	60	110	108.7	98.81	0.34
LEV 200	160	360	357.3	99.25	0.26
	200	400	395.8	98.85	0.33
	240	440	434.2	98.6	0.38

Table 6. Analysis of marketed formulation

Drug	Label claim (mg)	Drug content (%) ±S.D	% RSD
PHE	10	98.78 ± 1.33	0.85
LEV	2.5	99.36 ± 0.674	0.62

CONCLUSION

The developed HPTLC technique is precise, specific, robust and accurate and can be conveniently used for the routine analysis of PHE and LEV in pharmaceutical preparations. The procedure can be readily used for selective analysis of drugs and repeatable results are obtained without interference from auxiliary substances. The method is rapid and cost-effective and the results also meet the ICH guidelines for validation of pharmaceutical TLC methods.

ACKNOWLEDGEMENTS

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Isolation, Characterization and Enzymes Inhibitory Activity of Flavonoid Present in *Clerodendrum Viscosum Vent.*

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ABSTRACT

The plant Clerodendrum viscosum (CV) of Family Verbenaceae a medicinal plant which contains flavonoids is used in the treatment of diabetes in the Indian system of medicine. The main objective of the study is to isolate the flavonoid responsible for the antidiabetic activity in the plant and evaluate the role of the ethyl acetate extract of Clerodendrum viscosum (EAECV) in controlling blood glucose level. In the study, the root EAECV was prepared by successive cold maceration process by petroleum ether, ethyl acetate, chloroform and ethanol, followed by isolation of flavonoids by column chromatography and HPLC .The isolated compound was subjected to FTIR, GCMS and NMR analysis. The EAECV was subjected to alpha amylase and alpha glucosicdase enzymes inhibition study and carbohydrate tolerance test. The studies reveal that the ethyl acetate extract contains flavonoid 5, 7-dihydroxy-2-(hydroxyphenyl)-4H-chromen-4-one. In vitro studies showed that crude ethyl acetate extract of CV has good inhibitory activity on alpha amylase and alpha glucosicdase enzymes where IC50 values are 7.91µg/ml and 10.54 µg/ml respectively when compared to the reference drug acarbose where IC 50 ¬ value is 20.42µg/ml. The carbohydrate tolerance test also showed good result in inhibiting the absorption of starch and sucrose in normal and diabetic rats. Data from our study suggest that the EAECV containing flavonoid can inhibit the enzymes responsible for the absorption of carbohydrate and thereby controlling the blood glucose level.

Key words: flavonoids, ethyl acetate extract, column chromatography, alpha glucosicdase, alpha amylase.

INTRODUCTION

In India knowledge of medicinal plants is very rich and it has been described in Rigveda and Atharvanaveda (3500-1500 BC) from which Ayurveda has developed.[1] WHO has listed 21,000, which was used for medicinal purposes around the world Out of these 2500 species was found from India and 150 species are used commercially.[2]

There has been an exponential growth in the field of herbal medicine and is gaining popularity among the developing and developed countries due to its natural origin and less side effect. Most of the traditional medicines that are in use from time immemorial are found to be derived from various natural sources.[3]

Medicinal plants play an important role in the management of diabetes mellitus. Ethano botanical studies on traditional herbal medicine identified nearly 1200 species of medical plants that have been reported for its antidiabetic activity out of which some of them are also used in commercial preparations. Eighty five percent of the antidiabetic plants used most widely around the world are prescribed in India.[4] For the present study we have selected the important plant

Clerodendrum viscosum-vent (CV), from family: Verbenaceae. Synonym Clerodendrum infortunatum.L is known as Bhandirah in Sanskrit and Perugilai in Tamil. It has been used traditionally for the treatment of type 2 diabetes. It is an important plant in the Indian system of medicine. It is traditionally used in ethno medicine for its various medicinal properties which include the treatment of scorpion sting,[5] certain tumors, leprosy and skin diseases[6] Previous phytochemical investigation of the plant revealed the presence of alkyl sterols and 2,-(3,4-dehydroxyphenyl) ethanol 1-O-α-2 rhamnopyranosyl-(1→3)-β-D-(4-O-caffeoyl) glycopyranoside (acteoside).[7] Clerodendrum viscosum leaves yielded flavone glucuronides (about 0.1 %) scutellarin and hispidulin-7-0-glucuronide with practically no free aglycones. The flavonoid pattern of C. indicum and C. infortunatum is similar to C.phlomides and C. nerifolium 4 in having the 6-oxygenated flavones occurring mainly as their glucuronides.

Moreover in the literature review, there is no report on isolation of 5, 7-dihydroxy-2-(hydroxyphenyl)-4H-chromen-4-one (apigenin) from Clerodendrum viscosum vents roots and to our knowledge there was no report on the alpha amylase and alpha glucosidase inhibitory activity. Therefore, the present study was designed to isolate the active compound and investigate the anti diabetic activity through alpha amylase and alpha glucosidase.

Materials and Methods

Clerodendrum viscosum vent roots were collected from Palakkad district, Kerala, India in the months of September and October 2010. The roots were inspected to be healthy and botanically identified and authenticated by Dr. G.V.S. Moorthy, Plant Biotechnologist, Botanical Survey of India, Coimbatore. The herbarium Clerodendrum viscosum Vent roots were deposited in Botanical Survey of India (BSI) against voucher no. BSI/SRC/5/23/10-11/Tech1152. After collection ,the roots of Clerodendrum viscosum Vent were dried at room temperature (27-30º C) for 40-50 days. After complete drying, the dried root materials were grounded into coarse powder using domestic electric grinder and used for extraction

Preparation of plant extract

Coarse powders of Clerodendrum vicosum root (30g) was subjected to successive maceration process with 300 ml of pet.ether, chloroform, ethyl acetate and ethanol in a shaker system at room temperature. Then each extracts was filtered using and Whatman No.1 filter paper. The filtrate was subjected to evaporation under reduced pressure to obtain dried extracts.

Phytochemical studies and isolation of flavonoids

In our earlier studies[8] flavonoids were isolated from the ethyl acetate extract of CV by column chromatography studies and determined by HPTLC methods .The fractions F2, F3 and F4 showed single peak were subjected to HPLC studies.

Separation of Flavonoids by preparative HPLC

HPLC separation of single band fraction (F2, F3 and F4) of ethyl acetate extract of Clerodendrum viscosum vent was performed in Waters 515 pump, with manual rheodyne injector, (volume of injection is 10µl run time 10 min at a flow rate of 1ml/mn) using acetonitrile and water as the mobile phase in the ratio 70:30. The column used was C18 waters sun fire column, (15X5µmX4.6mm) and the detector was UV (2489) with the detection wavelength of 214 nm. The software used was waters Empower 2. The peak was eluted at the retention time of 5.2 min. The eluted fractions were collected in an eppendrof tube, directly from the column outlet and the organic acetonitrile was allowed to evaporate and the aqueous phases were dried by freeze drying.

FTIR studies

The freeze dried powder sample was mixed with potassium bromide, which was kept in hot air oven for 1 hour at 90 degree Celsius, in the ratio of about 100 times its weight of powdered potassium bromide. The finely ground mixture was then passed through KBR pellet press under a pressure of about 25,000 Psi and maintained for about 10 seconds. The resulting transparent pellet was subjected to FTIR analysis. The instrument used was FTIR 8400s (CE) Shimadzu and it was calibrated by measuring power spectrum, polystyrene film and blank KBR pellet. This takes about

2 minutes and the obtained spectrum along with the instrument generated report was monitored for pass result. The made sample pellets were subjected to FTIR analysis, resulting in IR spectra of the compound.

GCMS analysis

The GCMS used was a Thermo Scientific DSQ II series single quadrupole GC-MS. The GC was operated in the splitless injection mode with a flow of 1 ml/min through a DB-35ms capillary standard non-polar column (30 m length x 0.25 mm i.d. x 0.25µm film thickness). Mass spectrometry was used in selected ion monitoring (SIM) scan mode and operated in the electron impact (EI) mode at 40eV. Oven temperature was programmed to 50°C (1 min. isothermal) to 260°C (at 10°C / min.) and carrier gas: helium at 1ml/min. Xcalibur version 2, software was used to analysis the data.

NMR analysis

NMR spectra was obtained using Bruker Avance 400 equipped with BBI Inverse Probe, equipped with an automatic tuning and matching (ATM) as well as Z-gradients. 2 Channel amplifier system (20-420) MHz each) communication control unit with high speed RISC processor, 16 MB memory, dedicated enternet connection ports for communication link to workstation. The spectra were acquired and processed using PC/NT workstation with NMR suite, NT software. The spectra were recorded in DMSOd6 solvent systems at 300 K and the chemical shift calibration was carried out either on the TSP signal or residual solvent peak. The size of all 1D spectra was 65 K and the number of transients varied for different types of spectra. The standard 1D H NMR spectra were acquired with 30 pulse length and a relaxation delay of 2s, while the 1 H NMR45 spectra were acquired with 45 pulse length and a relaxation delay of 60 s to enable an accurate quantification of peaks .

In vitro α Amylases inhibitory study

In vitro alpha amylase assay was performed by taking a tube containing 0.2 ml of 0.5MTris-HCl buffer (pH-6.9) containing 0.001 M calcium chloride (substrate) and dissolve 2 mg of starch azure in the above test tube. Then the test tube was boiled for 5 min and then pre-incubated at 37˚C for 5 min. The ethyl acetate extract of CV was dissolved in 1 ml of solution containing 0.1% of dimethyl sulfoxide and make a serial dilution of 10, 20, 40 and 60µg/ml. Then 0.2 ml of a particular concentration was put in the test tube containing the substrate solution. 0.1 ml of procaine pancreatic amylase in TrisHCl buffer (2units/ml) was added to the tube containing the CV and substrate solution. The process was done at 37˚c for 10 min. The reaction was ended by adding 0.5 ml of 50% acetic acid in each tube. The reaction mixture was then centrifuged at 3000 rpm for 10 min at 4˚C and supernatant liquid was separated and absorbance was measured at 595nm using spectrophotometer[9]

The alpha amylase inhibitory activity was measured by the formula [(Ac+)- (Ac-)-(As-Ab) / (Ac+)-(Ac-) ×100] where in Ac+,Ac – As-Ab are defined as the absorbance of 100% enzyme activity(only solvent with enzyme), 0% enzyme activity (only solvent without enzyme), a test sample (with enzyme) and a blank (a test sample without enzyme) respectively.

In vitro β glucosidase inhibitory activity

The in vitro alpha glucosidase inhibitory assay was performed by the following protocol. Alpha glucosidase (2U/ ml) was premixed with 20 ul of ethyl acetate extract of CV to prepare serial dilution of various concentrations of 10,20,40 and 60 µg/ml and incubated for 5 min at 37˚C. A combination of 1m M para-nitrophenylgluco-pyanoside (pNPG) (20µl) and 50mM of phosphate buffer (ph. 6.8) was added to the above test tubes. The mixture was further incubated at 37˚C for 20 min. The reaction was ended by adding 50µl of 1M sodium carbonate and the final volume was made up to 150µl. Alpha glucosidase activity was determined spectrophotometrically at 405nm on micro plate reader.[10]

Animal study

In this study, male adult Wistar (200-250g) was used. The animal experiment was performed in PSGIMS&R animal house, Coimbatore. They were housed 6 per cage under (25±2°C, 50% humidity) laboratory conditions, maintained on a 12 hour day-night cycle with free access to standard food and water. The animals were adapted to laboratory conditions before the test. The

experiment protocol was approved by the Institutional Animal Ethical Committee (158/99/CPCSEA-141) and conducted according to the CPCSEA guidelines on the use and care of experimental animals.

Oral carbohydrate tolerance test

Oral carbohydrate tolerance test was performed separately for starch, sucrose and glucose to respective groups of normal and diabetic animals.[11] Male Wistar rats weighing 200-250 gm were selected and divided into four groups of six each. Group-1 was treated with 4ml /kg of CMC. Group-2 was treated orally with standard acarbose 10mg/kg (positive control) Group-3 was treated orally with 200mg/kg of test drug and Group-4 was treated orally with 400mg/kg of test drug. After 10 min, all the groups were treated with 3g/kg starch and the tail was snipped and blood glucose levels were measured at 0, 30, 60 and 120 min. Peak blood glucose was determined and area under curve (AUC) was determined. The maximum blood glucose determined was taken as PBG. The AUC was determined by the following formula

$$\mathrm{AUC}\left(\mathrm{m}\cdot\frac{\mathrm{mol}}{\mathrm{l}\cdot\mathrm{h}}\right)=\frac{\mathrm{BGO}+\mathrm{BG30}}{2}X\,0.5+\frac{\mathrm{BG30}+\mathrm{BG60}}{2}X\,0.5+\frac{\mathrm{BG60}+\mathrm{BG120}}{2}X1$$

Oral sucrose tolerance tests:

Male Wistar rats weighing 200-250gm were selected and divided into four groups of six in each. Group-1 was treated with 4ml /kg of CMC. Group-2 was treated orally with standard acarbose 10mg/kg (positive control), Group-3 was treated orally with 200mg/kg of test drug and Group-4 was treated orally with 400mg/kg of test drug (table 3). After 10 min, all the groups were treated with 4g/kg sucrose and the tail was snipped and blood glucose levels was measured at 0, 30, 60 and 120 min. Peak blood glucose was determined and area under curve (AUC) was determined. The maximum blood glucose determined was taken as PBG. The AUC was determined by the above said formula.

Oral starch tolerance test:

Male Wistar rats weighing 250-300 gm were selected and divided into four groups of six each. Group-1 was treated with 4ml /kg of CMC. Group-2was treated orally with standard acarbose 10mg/kg (positive control), Group-3 was treated orally with 200mg/kg of test drug and Group-4 was treated orally with 400mg/kg of test drug. After 10 min, all the groups were treated with 2g/kg starch and the tail was snipped and blood glucose levels was measured at 0, 30, 60 and 120 min. Peak blood glucose was determined and area under curve (AUC) was determined. The maximum blood glucose determined was taken as PBG. The AUC was determined by the following said above. The results were tabulated in Table No3.

Induction of diabetes in rats

Type 2 diabetes was induced in overnight fasted rats by single intra-peritoneal injection (IP) of Streptozotocin (STZ) 65mg/kg in ice cold citrate buffer(pH- 4.5) in dark room(STZ is light sensitive) 15 min after administration of nicotinamide 110mg/kg. Fasting blood glucose levels of animals were checked 7days after induction of diabetes[12]. Animals having fasting blood glucose level more than 250mg/dl were selected and used for experiments

Result

HPLC separation of ethyl acetate fraction of Clerodendrum viscosum vent root

The single peak obtained fractions from HPTLC study (F2, F3 and F4) were separated by using HPLC, a single band compound obtained at 5,2 min ,the peak was collected at the column out let, (Figure 1) acetonitrate was evaporated in room temperature and the compound was precipitated by freeze drying technique.

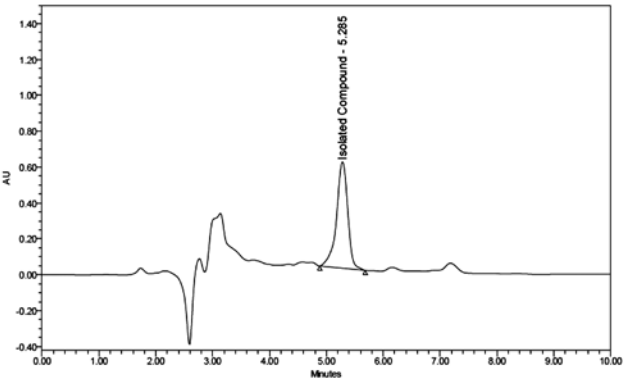


Figure:1 HPLC study of isolated fraction of ethyl acetate extract of *Clerodendrum viscosum* vent root

FTIR analysis of the isolated compound

A white crystalline compound was obtained after freeze drying was subjected to FTIR studies to find out the functional groups present in the compound. IR spectra shows following vibrations at 1654 and 1610 for the presence of carbonyl groups(C=O), vibration frequency at 1584 cm-1, 1555cm-1, 1450cm-1 and 1396cm-1 for the presence of aromatic ring and the wave numbers at 1356 cm-1-1298cm-1and 1112cm-1-1172 cm-1conforms the presence of phenolic (C-OH) hydroxyl group. This conforms the presence of various functional groups present in the isolated compound (Figure 2).

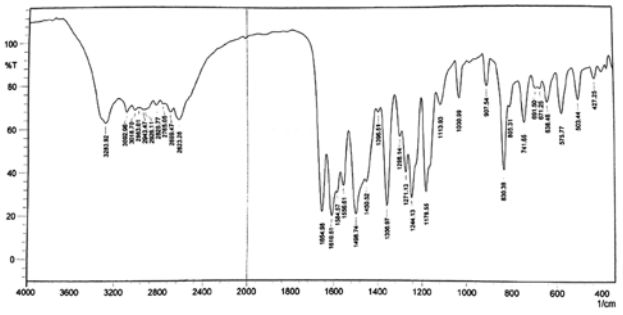


Figure: 2 FTIR spectrum of isolated compound from ethyl acetate extract of *Clerodendrum viscosum* vent root

GCMS Analysis of isolated compound

Selected ion monitoring scan spectra of the isolated compound showed ions with m/z a 270.0 → 242.0 → 153.0. In comparison with the NIST library which corresponded with a molecular ion composition of $C_{15}H_{10}O_5$ to ion with m/z at 270.0 for the assigned structure of the isolated compound .(Figure 3)

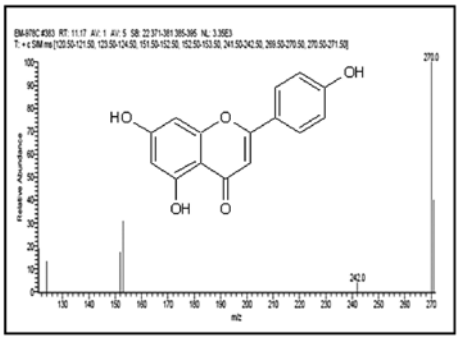


Figure: 3 GCMS analysis and Chemicla structure of the isolated compound from ethyl acetate extract of Clerodendrum viscosum

NMR Analysis of isolated compound

The NMR analysis of the isolated compound was down .The H NMR was based on the proton correlation. H coupling data and selective decopling of H6 and H2',6'. there is an nuclear overhauser from H2',6' to H3 and H8 but not in H6 provied good evidance for $_1$H assignment the datas were at tabel 1.

Tabel 1 : H NMR data of the isolated compound from ethyl acetate extract of Clerodendrum viscosum root.

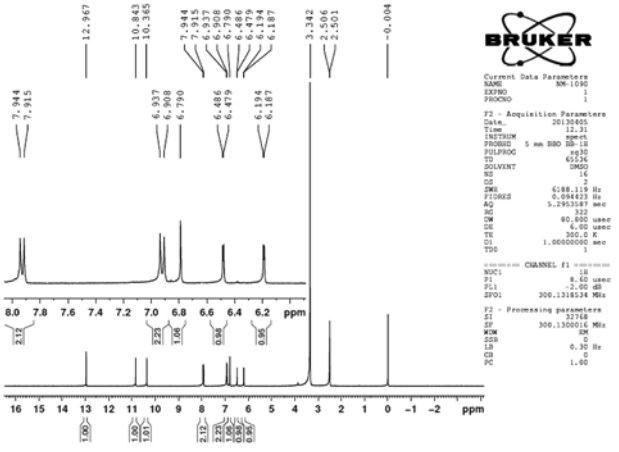
S.No	δ (ppm)	Splitting pattern	Area under integration	No of protons
1	6.187, 6.194	Doublet (d)	0.95	1H
2	6.479, 6.486	Doublet (d)	0.98	1H
3	6.790	Singlet (s)	1.06	1H
4	6.908, 6.937	Doublet (d)	2.23	2H
5	7.915, 7.944	Doublet (d)	2.12	2H
6	10.365	Singlet (s)	1.01	1H
7	10.843	Singlet (s)	1.00	1H
8	12.967	Singlet (s)	1.00	1H

$_1$H NMR (300 MHz, DMSO) - δ 6.187, 6.194 (1H-d), δ 6.479, 6.486 (1H-d), δ 6.790 (1H-s), δ 6.908, 6.937 (2H-d), δ 7.915, 7.944 (2H-d), δ 10.365 (1H-s), δ 10.843 (1H-s), δ 12.967 (1H-s).

Note:-

1. Peak at δ -0.004 is due Tetra methyl Silane
2. Peak at δ 2.501 is due to DMSO
3. Peak at δ 3.342 is due to Moisture

The NMR data firmly established the structure of the isolated compound. (Figure 4)



Instrument Make: BRUKER NMR (Nuclear Magnetic Resonance Spectrometer)

MHz: 300

Nuclei: Proton NMR ($_1$H NMR)

Solvent: DMSO

Figure: 4 NMR data of the isolated compound from ethyl acetate extract of *Clerodendrum viscosum*

In vitro alpha amylase and alpha glucosidase assay

The in vitro alpha amylase inhibitory studies demonstrate the extract having good alpha amylase inhibitory activity. The percentage of inhibition at 10, 20, 40 and 60µg/ml concentrations of Clerodendrum viscosum showed a concentration dependent reduction in the activity. The highest concentration of 60µg/ml showed maximum inhibition of nearly 90%. The percentage inhibition varied from 50-90% from the lowest concentration to highest concentration.(10-60 µg/ml) the result was demonstrated in the Figure 5 .The IC50 value was calculated (7.91µg/ml) and the effect was compared with the standard drug acrabose (16.42µg/ml).The result was tabulated in Table 2 .

Table 2: The IC50 values of the ethyl acetate extract of Clerodendrum Viscosum root.

Sl No	Enzymes	Ethyl acetate extract	Acarbose
1	Alpha Amylase	7.91 µg/ml	20.42µg/ml
2	Alpha Glucosidase	10.54 µg/ml	20.42µg/ml

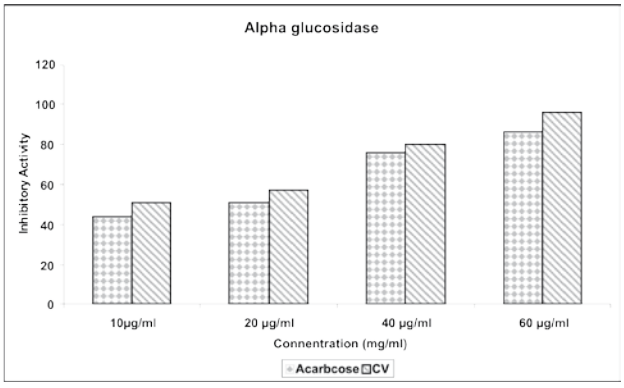


Figure: 5 Effect of ethyl acetate of Clerodendrum viscosum vent on alpha amylase inhibitory assay

The alpha glucosidase inhibitory assay of ethyl acetate extract of Clerodendrum viscosum root exhibits a good inhibitory property. The percentage of inhibition at 10, 20, 40 and 60µg/ml concentrations of Clerodendrum viscosum showed a concentration dependent reduction in the activity. The highest concentration of 60µg/ml showed maximum inhibition of nearly 85%. The percentage inhibition varied from 45-90% from the lowest concentration to highest concentration.(10-60 µg/ml). The result was demonstrated in (Figure 6)

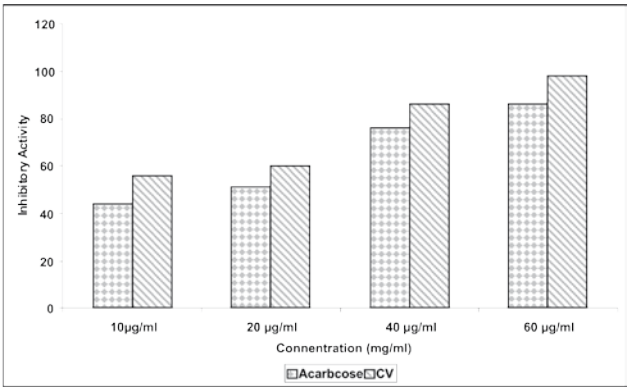


Figure: 6 Effect of ethyl acetate extract of *Clerodendrum viscosum* vent on alpha glucosidase inhibitory assay.

The IC50 value of the ethyl acetate extract was calculated (10.54µg/ml) and the effect was compared with the standard drug acarbose (20.00µg/ml). The result was tabulated in Table 2.

Oral carbohydrate tolerance test

In the carbohydrate tolerance test group of animals feed with starch showed no significant effect on the AUC in the 200 mg/kg extract treated animals. But there was a significant decrease in the 400mg/kg treated animals ($P<0.05$) when compare with the control group. But in the sucrose feed group, the extract treated animals did not show any significant decrease in the AUC when compare with the normal animals. Standard drug agarbose reduced the AUC significantly ($P<0.001$) when compare with the control groups. The results were at Table 3. In diabetic animal the extract treated groups (200 and 400mg/kg) significantly lower ($P<0.05$) the AUC (Table 4) in both sucrose and starch feed animals when compare with the control group of animals.

AUC (m.mol/lit)		
Group	Sucrose	Starch
Control	12.50±0.02	11.91±0.49
Std(Acarbose 10mg/kg)	10.40±0.06*	7.3±0.21***
200mg/kg CV	11.4±0.39	11.20±0.40
400mg/kg CV	12.15±0.53	10.20±0.40*

All the values were expressed in Mean±SEM . All the values were compared with the control and * denotes statistical significance at P<0.05 and *** denotes statistical significance at P<0.001

Table: 3 Effect of ethyl acetate extract of Clerodendrum viscosum on starch and sucrose loading test in normal animals

AUC(m.mol/lit)		
Group	Sucrose	Starch
Diabetic control	22.7± 0.91	23.40± 0.53
Std(Acarbose10mg/kg)	18.02± 0.31**	18.5 ±0.45***
200mg/kg CV	19.3 ±0.87*	22.1 ±0.5*
400mg/kg CV	18.32 ±0.31**	21.1 ±0.45*

All the values were expressed in Mean±SEM. All the values were compared with the control and * denotes statistical significance at P<0.05, **denotes statistical significant at P<0.01, *** denotes statistical significance at P<0.001.

Table: 4 Effect of ethyl acetate extract of *Clerodendrum viscosum* on starch and sucrose loading test in diabetic animals

Discussion

The 2nd, 3rd and 4th fractions showed single band green fluorescent peak. The fractions obtained from the column chromatography (2, 3 and 4th) were taken for HPLC study and found that the extract showed single peak in the HPLC at 5.2min. The single peak was collected from the column outlet.

The IR spectra of the isolated compound from the ethyl acetate extract of Clerodendrum viscosum shows the following vibration which confirmed certain functional groups for flavonoids[13] Carbonyl group has vibration from 1659cm-1 to 1649cm-1 and some characteristic vibrations of the compound which fall in these range confirmed the presence of carbonyl groups. The aromatic ring vibrations were confirmed by the vibrations appearing at 1584cm-1,1555cm-1,1450cm,-1 and 1396cm-1. Other characteristic bands come from the phenol group vibration at 1356cm-1, 1298 cm-1, 1112cm-1 and 1172cm-1.

Gas Chromatography /Mass Spectral (GCMS) selected ion monitoring scan spectra showed ion with m/z 270, 242 and 153.0 In comparison with the NIST library which corresponded with a molecular ion composition of C15H10O5 to ions with m/z at 270.0 for the assigned structure of the isolated compound with IUPAC name 5 ,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one.

NMR studies have been done in DMSO-d6 solvent which gives an optimum viscosity and high resolution. The peaks obtained by the H NMR studies integrate with 7 protons. The doublet peaks at 7.275, 6.964, 6.946, and 7.875 conform the following positions for aromatic protons (2', 3', 5', 6') and the peak at 6.642(S) confirm the 3 position -H in the structure and the 6.214 and 6.476 confirm the 6th and 8th protons in the structure. The spectral analysis data confirmed the structure of the compound.

Alpha amylase found in small intestine digests starch to oligosaccharides and further digested to glucose by alpha glucosidase resulting in hyperglycemic condition. In vitro alpha amylase and alpha glucosidase inhibitory effect of ethyl acetate extract of Clerodendrum viscoscum was evaluated by using standard protocol. The extract showed excellent inhibitory effects on alpha amylase at IC50 (7.91µg/mg) and alpha glucosidase at IC50 (10.54 µg/mg) When compared to standard drug acarbose of IC50 (20.22µg/mg) value which inhibits the activity of enzymes. This effect of the extract was confirmed by carbohydrate tolerance test in normal and diabetic rats. In this study the normal animal showed no significant reduction in the sucrose level but there was a significant reduction in the starch level at 400mg dose. The carbohydrate tolerance test in diabetic rats shows significance in both sucrose and starch in 200 and 400mg/kg dose when compared with the normal animals. In the present study ethyl acetate extract of

CV showed a good effect on alpha amylase and alpha glycosidase activity when compared with the standard drug acarbose. Postprandial hypoglycemia is one of the characters of type 2 diabetes. Uncontrolled post postprandial hypoglycemia can contribute to factors like atherosclerosis and cardiovascular disease.[14] It is well known that alpha amylase and alpha glucosidase inhibitors, reduced the absorption of carbohydrate and thereby decrease the postprandial blood glucose level. Postprandial hyperglycemia increases the glucose toxicity and thereby increases the production of superoxide by mitochondrial electron transport chain. The over production of superoxide accompanied by the production of nitric oxide and other free radicals damage different cells.[15] Clerodendrum viscosum can be used to treat the postprandial blood glucose level and also the plant poses a good free radical scavenging activity which can contribute to the oxidative stress produced during elevated glucose level.

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Stability of Quercetin and P-Coumaric Acid Content of *Aegle marmelos* Extracts

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ABSTRACT

Aegle marmelos belongs to the species of rutaceae, used in Ayurvedic formulations to treat various ailments. There is a serious concern about the determination of stability of the constituents present in the crude drugs as per WHO guidelines. The successive extracts obtained from the roots of *Aegle marmelos* were subjected to various stress conditions. The plain extracts and the stressed extracts were spotted on Silicagel 60F 254 pre coated plates along with chemical markers (quercetin and p-coumaric acid) using HPTLC. The spots were developed using the mobile phase Toluene: Ethyl acetate: Methanol: Formic acid in the ratio of [12:6:0.4:0.8 (v/v)] and the resultant spots were detected in UV and scanned through WINCATS 3 software. Through the analysis of the chromatograms it is found that the constituents present in the ethyl acetate extracts were more stable than other extracts.

INTRODUCTION

Natural products have, until recently, been the primary source of commercial medicines and drug leads. Quality and efficacy of herbal medicines are directly linked to the quality of the medicinal herb raw materials.

The stability is aimed at assuring that the drug/drug extract remains within the specifications established to ensure its identity, strength, quality and purity. Each ingredient, whether therapeutically active or inactive in a dosage form can affect stability. Environmental factors such as temperature, light, air (specifically oxygen, carbon di oxide and water vapors) and humidity can affect stability[1].

The objective of the project work is to analyze the stability of successive extracts of *Aegle marmelos*.

The bael tree is one of the sacred trees of Hindus. Leaves are offered in prayers to Shiva and Parvathi since ancient times. It has its own place in indigenous systems of medicine. Roots and bark parts are used in ayurvedic system of medicine for the treatment of various ailments[2]. The phytochemicals reported from the roots of *Aegle marmelos* were skimmiamine,

The plan made to carry out the stability studies are as follows

- Successive solvent extraction of dried roots of *Aegle marmelos* using solvents with increasing polarity such as n-hexane, chloroform, ethyl acetate and methanol.
- Then induce stress to the each extract by using various agents like Hydrochloric acid, Ammonia, Hydrogen peroxide, Tri fluoro acetic acid, light and water content.
- And analyze the extracts using HPTLC by comparing with the control drug[3].

2. Materials and Methods

Phytochemical markers used are quercetin and para-coumaric acid, the solvents used is n-hexane, chloroform, ethyl acetate and methanol.

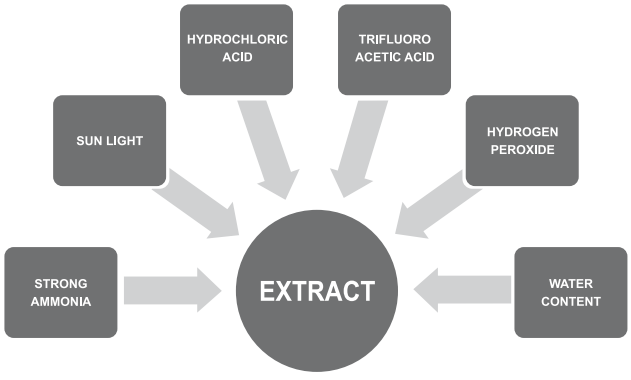
2.1 Preparation of extracts

The roots of *Aegle marmelos* tree were collected and authentified from Tamil Nadu Agricultural University, Coimbatore. The dried roots were coarsely powdered. The powdered roots were defatted with n-hexane and the successive extracts were prepared using chloroform, ethyl acetate and methanol by cold maceration process[4].

The duration of maceration was 48 hours for each solvent. The extracts were filtered and the solvents were distilled off, using rotary evaporator under vacuum. The resultant products were stored in a vacuum desiccator.

2.2 Preparation of Sample

The extracts were dissolved in methanol and individually treated with hydrochloric acid, Strong. ammonia, hydrogen peroxide, trifluro acetic acid, further exposed to light.



2.3 Preparation of Standard Solution

The selected phytochemical markers of quercetin and para-coumaric acid were dissolved in methanol to obtain the concentration of 2mg per 10 ml.

2.4 Thin - Layer Chromatography[5]

TLC was performed on Silica gel 60F 254 TLC plates (E Merck, Germany) with Toluene: Ethyl acetate: Methanol: Formic acid in the ratio of 12:6:0.4:0.8 (v/v) as mobile phase. The standard and the prepared samples were applied to the plates as 5 mm wide from the bottom, by means of pressurized nitrogen gas (150 kg/cm2) through CAMAG Linomat V fitted with a 100 µl syringe.

Ascending development was performed in a twin –trough glass chamber (10x10cm) obtained from CAMAG, which is previously saturated with the mobile phase for 30 minutes at room temperature (25 ± 2o C) and relatively humidity (60 ± 5%) for a distance of 8 cm. The bands were visualized in CAMAG UV cabinet at 254 nm and 366 nm and photo documented.

3. RESULTS AND DISCUSSION

Successive extracts of *Aegle marmelos*, prepared by cold maceration, were evaluated for the stability of their chemical markers: quercetin, para-coumaric acid.

The development of analytical conditions for the analyses must necessarily go through a specific validation. Considering that the stability assay was developed with aim to get an initial response about the chemical stability of the extracts for research and development purposes, the used protocol allowed the extracts to be evaluated under stress conditions.

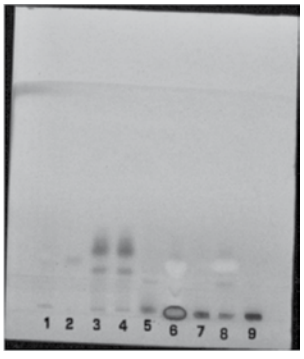


FIG: 1

1.QUERCETIN
2.ρ-COUMARIC ACID
3.CHLOROFORM EXTRACT
4.LIGHT
5.HYDROGEN PEROXIDE
6-HYDRO CHLORIC ACID
7-AMMONIA
8-TRI FLUORO ACETIC ACID(TFA)
9-WATER CONTENT

In chloroform extract, there are no degradation and changes due to light. But the constituents were lightly varying under hydrogen peroxide, hydrochloric acid and trifluoro acetic acid stress conditions. Stress due to water content and ammonia were indicating highly degraded constituents as shown in the figure 1.The chromatogram had been documented in fig: 2

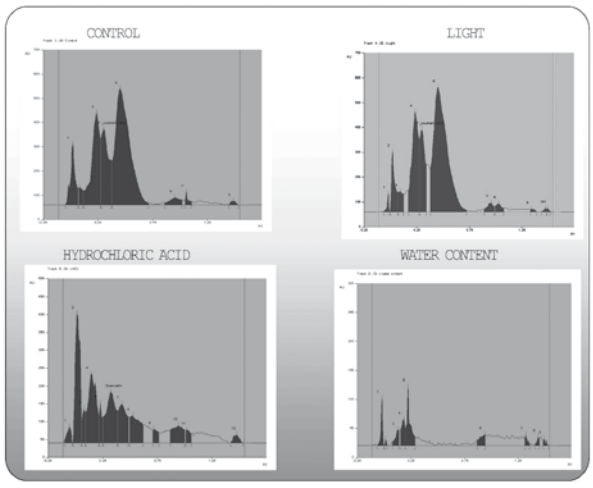
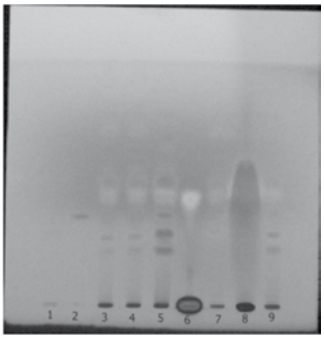


FIG: 2



1.QUERCETIN
2.ρ-COUMARIC ACID
3.CHLOROFORM EXTRACT
4.LIGHT
5.HYDROGEN PEROXIDE
6-HYDRO CHLORIC ACID
7-AMMONIA
8-TRI FLUORO ACETIC ACID(TFA)
9-WATER CONTENT

In ethyl acetate extract there are no degradation and changes due to light and Hydrogen peroxide. But the constituents were slightly varying under ammonia and water stress. Stress due to trifluoroacetic acid was indicating highly degraded constituents as shown in the figure 3.the chromatogram is shown in Fig: 4

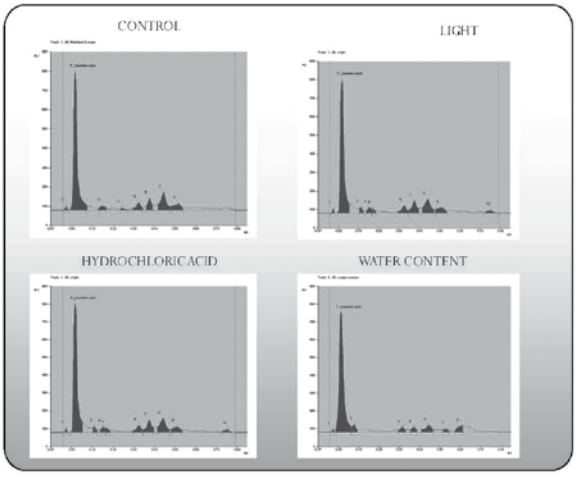
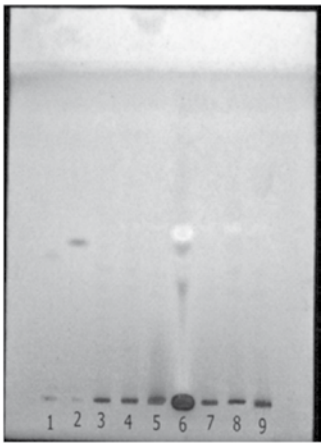


FIG: 4



1.QUERCETIN
2.ρ-COUMARIC ACID
3.METHANOLIC EXTRACT
4.LIGHT
5.HYDROGEN PEROXIDE
6-HYDRO CHLORIC ACID
7-AMMONIA
8-TRI FLUORO ACETIC ACID(TFA)
9-WATER CONTENT

FIG: 5

In Methanolic extract there is no degradation and contents were uniformly observed in all other stress conditions but degradation is high when treated with hydrochloric acid as shown in the figure 5.The chromatogram were denoted in fig 6.

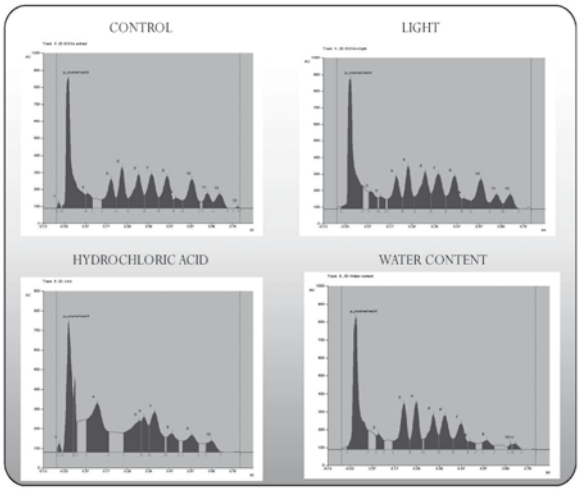


FIG: 6

The results suggest that the interaction occurred in all the extracts produce less alteration with para-coumaric acid. However they show that the quality control of extracts containing desired chemical marker which are more physically stable.

4. CONCLUSION

As a conclusion, para-coumaric acid is an appropriate compound to use as a chemical marker in quality control of the dried extract of *Aegle marmelos*. From the above screening study ethyl acetate extract of

Aegle marmelos showed good compatibility with various stress conditions. This enhances in processing of *Aegle marmelos* formulation using ethyl acetate extracts.

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INSTRUCTIONS TO AUTHORS
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All the references, figures (Fig.) and tables (Table) in the test shall be numbered consecutively as they first appear.

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Abstract: the abstract is limited to 250 words, and should describe the essential aspects of the investigation. In the first sentence the background for the work should be stated; in the second sentence the specific purpose or hypothesis shall be provided; followed sequentially by summary of methods, results and conclusions. No references should be cited.

Introduction: A brief background information on what has been done in the past in this area and the importance of the proposed investigation shall be given. Introduction shall end with a statement of the purpose or hypothesis of the study.

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Results: this section maybe divided into subsections if it facilitates better reading of the paper. All results based on methods must be included. Tables, graphic material and figures shall be included as they facilitate understanding of the results.

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Tables: Each table should be given on a separate page. Each table should have a short, descriptive title and numbered in the order cited in the text. Abbreviation should be defined as foot notes in italics at the bottom of each table. Tables should not duplicate data given in the text or figures. Tables should show lines separating columns with those separating rows. Units of measurement should be abbreviated and placed below the column headings. Column headings or captions should not be in bold face. It is essential that all tables have legends, which explain the contents of the table. Tables should not be very large that they run more than one A4 size page. If the tables are wide which may not fit in portrait form of A4 size paper, then it can be prepared in the landscape form, tables should be numbered as Table No. 1- Title, Table No. 2- title, etc. Tables inserted in word document should be in tight wrapping style with alignment as centre.

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Biological nomenclature:- Names of plants, animals and bacteria should be in italics.

Enzyme nomenclature:- The trival names recommended by the IUPAC-IUB Commission should be used.

Physical Quantity	Base Unit	SI Symbol
Length	meter	m
Mass	gram	g
	kilogram	kg
	microgram	pg
Time	second	s
	minute	min
	hour	h
	day	d
	week	w
	month	mo
	year	y
Amount of substance	mole	mol
Area	square meter	m^2
Volume	cubic meter	m^3
	liter	l
	milliliter	ml
	microliter	µl

Specification	Example	Correct Style
Use lowercase for Symbols or abbrevations,	kilogram	kg
Symbols are not followed by a period, exception end of sentence	meter	m
Do not pluralize symbols	kilograms	kg
When numbers are printed symbols are preferred	100 meters	100 m
Space between number and symbol	2mol	2 mol
Place a zero before a decimal	10mg	10 mg
Decimal numbers are preferable to fractions	0.75	
Space used to separate long number exception four digit numbers		15000001000

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Editor(s), Compiler(s) as author:- Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996.

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Conference proceedings:- K. Velayutham, V. Harikrishnan, R. Prasath. Drug Delivery and Drug Targeting Research. Proceedings of the 1st Pharm Tech IAPST International Conference; 2008 Jan 19-20; Kolkota, India.

Dissertation:- KaplanSJ. Post-hospital home health care; the elderly's access and utilization [dissertation]. St. Louis (MO): Washington Univ.; 1995.

Patent:- Nilani. P, Sankar. V, Chandrasekharan. A. K. Herbal Ant Repellant. INDIA patent: 2007-09-14, filed 2004-09-29.

Electronic journal articles:- Morse SS. Factos in the mergence of infectious diseases. Emerg Infec Dis [serial online] 1995Jan-Mar [cited 1996 Jun5]; L(1):[24 screens].

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