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EXTRACTION, ISOLATION, CHARACTERIZATION OF PHYTOCONSTITUENTS FROM *CURCUMA CAESIA* ROXB BY VARIOUS ANALYTICAL METHODS

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ABSTRACT

Curcuma caesia roxb a vegetatively propagated member of the Zingiberaceae family has been used extensively due to therapeutic properties. The phyto constituents are extracted and isolated by using soxhlet apparatus and preparative TLC respectively, and the isolated compound was characterized by FTIR. The new, simple and validated UV Spectroscopic method was developed for the isolated compound. Linearity of the isolated compound was found to be 10-50µg/ml, correlation co-efficient was 0.999, precision was found to be <2, LOD and LOQ were found to be 0.55µg/ml and 1.66µg/ml respectively. The anti-oxidant activity of *Curcuma caesia* roxb isolated compound was studied by free radical scavenging activity in comparison with ascorbic acid as standard.

KEYWORDS

Curcuma caesia roxb, Preparative TLC, FTIR and UV Spectroscopy.

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INTRODUCTION

Curcuma caesia roxb (Black turmeric) is a kind of turmeric with bluish-black rhizome, belonging to Zingiberaceae (Ginger) family. *Curcuma caesia* roxb is used for treating of piles, Leprosy, Bronchitis. *Curcuma caesia* roxb is also used for curing wounds, pimples, allergies, raw paste of rhizomes is applied externally. For migraine, 2-4 drops of fresh juice is poured in nose. For longevity, impotence, infertility, irregular menstrual flow, a spoonful powder from dried rhizomes is mixed with a spoonful of honey or

a cup of milk is taken twice a day. For gastric troubles, a fresh piece of rhizome is chewed.

EXPERIMENTAL WORK

Materials Required

Instruments

UV-Visible double beam spectrophotometer - 1800 (Shimadzu) Japan. Analytical balance (Denever), DS-852J, Mumbai. Soxhlet apparatus, Curcuma caesia Isolated compound, Methanol and Distilled water.

Apparatus

Beakers : 100ml
Standard flasks : 100ml and 10ml
Pipettes : 1ml, 5ml, 10ml

Extraction and Preliminary Phyto-Chemical Studies (Table No.1)

Selection and authentication of plant

The plant selected was C*urcuma caesia* roxb belonging to Zingiberaceae family. The plant was collected and powdered.

Reasons for selecting *curcuma caesia* roxb Plant

Plant with most important pharmacological actions. Easy availability of plant and plant parts. Very few literatures on the research work of the plant. Rhizome part of the plant is used for treating Piles, Leprosy, Bronchitis, Asthma, Cancer, Epilepsy, Fever, Wounds, Impotency, Fertility, Menstrual disorders, Tooth ache, Vomiting.

Selection of the part of the plant

The rhizome part of C*urcuma caesia* roxb had been selected for the analytical work because rhizomes of *Curcuma caesiaroxb* contain more amount of flavanoids and terpenoids which are responsible for the main pharmacological activities like anti-oxidant, anti-inflammatory, anti-fungal, etc. The other parts of the plant like leaves have anti-bacterial activity.

Preliminary extraction procedure

Preparation of powder for extraction

Rhizomes were collected. Then they are dried at room temperature to avoid the phytochemical degradation. The dried rhizomes were grinded well for getting fine powder.

Selection of solvents for extraction

The selection of the solvents for successive extraction was done by trial and error method. Small quantity of C*urcuma caesia* roxb rhizome powder is transferred to the series of test tubes. Various organic solvents are added to the each test tube containing the C*urcuma caesia* roxb rhizome powder. The solutions are mixed thoroughly and warmed for 5min. The solvents selected were n-hexane, methanol and water of increasing polarity.

Process of extraction

The *curcuma caesia* roxb rhizome powder was previously macerated overnight with Methanol in soxhlet apparatus. Then, the macerated drug was kept for extraction in soxhlet apparatus at 50^0C. The continuous hot percolation with Methanol was completed in 5 hours. Then, the extract was collected and concentrated by evaporating the solvent. The concentrated semi-solid extract yield was determined by weighing.

% yield of extract from crude drug = (weight of extract/weight of crude drug) X 100

Parameters of Extraction

Solvent	:	Methanol
Temperature	:	50^0C
Period of extraction	:	5 hours
Weight of crude drug	:	50 grams
Colour of extract	:	Yellowish brown
% yield	:	12.54% (w/w)

Thin-layer chromatographic analysis of extracts of *curcuma caesia* roxb

Procedure

Preparation of thin-layer plates

A thin-layer plate was prepared by spreading an aqueous slurry of the finely ground solid (silica gel G) on the clean surface of a glass. The plate was activated in an oven at 110^0C-120^0C for 30minutes prior to sample spotting.

Sample application and chromatogram development

The collected extract of *Curcuma caesia* rhizome powder was applied as a spot 1cm from the edge of the plate by using capillary tube. The plate was then

allowed for evaporating sample solvent. The plate was placed in a closed container saturated with vapours of developing solvent Toluene: Ethylacetate [97:3%v/v] with care being taken to avoid direct contact between the sample and the developer but it showed poor resolution. The other mobile phase composition prepared was chloroform: ethanol: glacial acetic acid [95:4:1%v/v/v] which showed good separation of components. After developing the plate up to two-third of the length of the plate, it was removed from the container and dried. After development, the plate was examined in ultra-violet chamber.

Observation

The TLC of ME is shown in the Figure No.1. The chromatogram of extract was scanned under UV light and spots were observed at various R_F values which are tabulated in Table No.2. The methanolic extract was selected as it contains Curcumin as a major phyto-constituent and exhibits more fluorescence spots.

Observation

The mobile phase composition with chloroform: Ethanol: glacial acetic acid [95:4:1%v/v/v] showed good separation. So the same mobile phase composition was selected for the isolation by TLC.

Isolation by TLC

TLC was performed for the ME in order to get the isolated compounds which can be used as the standard marker for analytical works like method development, comparative studies, *invitro* studies, etc. The process of isolation was done using preparative TLC plates and the parameters are as below.

Parameters of Preparative TLC

Solvent system	:	Chloroform: Ethanol:Glacial acetic acid [95:4:1%v/v/v]
Chamber saturation	:	30 minutes
Temperature	:	29°-33°C
Solvent front	:	8.1cm
Detection	:	UV at 256nm

Procedure

20cm glass plates were coated with silica gel G and activated at 110°C-120°C for 30minutes in hot air oven. ME was spotted (1 spot per plate) over the pre-activated plates. The plates were developed in the pre-saturated mobile phase Chloroform: Ethanol: Glacial acetic acid [95:4:1%v/v/v]. Greenish-yellow fluorescence spots with R_F value of 0.60 was good and prominent for isolation (Table No.3). Those spots were isolated by scrapping the silica gel exhibiting greenish-yellow fluorescence in UV chamber. The isolated compound was separated from silica gel by dissolving the scrapped silica gel in Methanol and filtered. The filtrate was then evaporated to dryness and weighed. The amount of isolated compound obtained from ME was 40mg. The component isolated was checked for the spot and these can be used as unknown standard marker for analysis of formulations.

Characterization of isolated compound by FTIR

KBr pellet was prepared for blank by pressing in hydraulic press at 3000atm pressure. Test was prepared by mixing isolated powder of Methanolic extract and KBr. KBr was used for holding the test sample. By keeping these pellets in sample holder, IR was performed. The graphs obtained were FTIR spectrum, Finger print region: (500-1500cm^{-1}) (Figure No.2), Functional Group region: (1500-4000 cm^{-1}) (Figure No.3). Then the graphs obtained were interpreted.

Observation

From the above obtained functional and finger printing spectra, interpretation was done (Table No.4 and 5). The results suggest that the isolated compound may be a Flavanoid, Terpenoid or Tannin derivative. Further studies should be made for the structural confirmation of the isolated compound.

Estimation of isolated compound by UV-visible spectrophotometric method

Estimation of isolated compound by UV-visible spectrophotometer was done by determining the maximum wavelength (λ_{max}) of that compound. Maximum wavelength of a compound is unique and

so it can be used for qualitative determination of a compound.

Maximum wavelength of a compound is defined as the wavelength at which the compound shows maximum absorbance.

For the methanol the maximum wavelength (λ_{max}) was determined by preparing series of concentrations in the following manner.

Stock solution 1

40mg of dried extract was weighed accurately. Then it was transferred to 100ml standard flask. It was dissolved well with the small amount of methanol. The final volume was made with methanol to get a concentration of 400µg/ml.

Stock solution 2

From stock solution 1, 25ml was taken and transferred to 100ml standard flask. The volume was made up to 100ml by using methanol to get a concentration of 100µg/ml.

Preparation of standard solutions

From the stock solution 2 serial concentrations of 10, 20, 30, 40 and 50µg/ml were prepared with the solvent methanol.

Determination of the maximum wavelength (λ_{max})

The standard solutions prepared were measured for absorbance in UV-Visible Spectrophotometer in the UV region (200nm - 400nm). Then, the graph was plotted between absorbance and wavelength. The peak obtained from the graph was taken as the maximum wavelength of that compound. Then the same was repeated for precision studies (Figure No.4 and 5).

Linearity and Calibration

From trial and error method, the concentration range from 10-50µg/ml was found to give better linearity. So aliquots of 1 to 5 ml standard stock 2 solution were transferred to a series of 10ml volumetric flasks and volume in each flask were make up to 10ml with distilled water obtain the concentration range from 10-50µg/ml. Calibration curve for *Curcuma caesia* roxb., isolated compound-1 was obtained by measuring the absorbance at 278nm. Statistical parameters like the slope, intercept and co-efficient of correlation (Table No.6) (Figure No.6).

Validation of developed method

Validation is a method of documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. Method was validated for different parameters like linearity, precision, LOD, LOQ (Table No.7).

The method developed was more specific, precised and reproducible which can be used for the routine analysis of *Curcuma caesia* roxb., isolated compound.

***Invitro* anti-oxidant studies of isolated compound of *curcuma caesia* roxb**

DPPH free radical scavenging activity

Free radical scavenging potentials of isolated compound of methanolic extract of *Curcuma caesia* roxb., were tested against the methanolic solution of DPPH. 0.002% of solution of DPPH in methanol was prepared and 1ml of this solution was added to the test concentrations (10-50mcg/ml) of isolated compound prepared using distilled water. It was incubated at room temperature for 30 minutes and the absorbance was measured at 517nm against the corresponding blank solution. Ascorbic acid was taken as the reference (Table No.8). Percentage inhibition of DPPH free radical was calculated based on the control reading, which contained DPPH and distilled water without any sample using the following equation:

% Scavenging activity = [(Ac – As)/ Ac] × 100

Where,

Ac = is the absorbance of the control reaction and

As = is the absorbance in presence of the sample.

The antioxidant activity was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in mcg/ml) of sample that inhibits the formation of DPPH radicals by 50%.

Observation

DPPH scavenging activity has been used by various researchers as a quick and reliable parameter to

access the *in vitro* antioxidant activity of the isolated compound of plant extracts. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in methanol was at 517nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidants molecules and radical, progresses, which results in the scavenging of the radical by hydrogen donation. IC_{50} for standard ascorbic acid was found to be 29 and sample was found to be 35. Thus the anti-oxidant activity of sample was less than the standard (Table No.9).

Table No.1: Preliminary phyto-chemical tests for C*urcuma caesia* roxb extracts

S.No	Phyto-chemical tests	Observation	Inference
1 i. ii.	**Tests for Tannins** Extract + $KMnO_4$. Extract + $FeCl_3$.	i. Decolourisation was seen. ii. Greenish or Bluish precipitate not formed.	**Tannins were present**
2 i.	**Tests for Flavanoids** Drug (Section, powder or extract) +NaOH+ H_2SO_4 (80%V/V).	Yellow was formed	**Flavanoids were present**
3 i. ii.	**Tests for Alkaloids** Extract + Wagner's reagent. Extract + Hager's reagent	i. Reddish-brown precipitate was formed. ii. Yellow precipitate was formed.	**Alkaloids were present**
4 i.	**Tests for Triterpenoids** **Libermann Storch Morasky Test** Extract + 0.5ml acetic acid + a drop of H_2SO_4 and Heat it.	Red to blue colour was seen.	**Triterpenoids were present**
5 i.	**Tests for Aminoacids** Drug / Extract + Ninhydrin reagent.	Purple colour was not formed.	**Aminoacids were absent**
6 i.	**Tests for Saponins** Alcoholic drug + 20ml distilled water and shake for 15-20 minutes.	Foam was formed	**Saponins were present**

Table No.2: Detection and R_f Values of Extracts

S.No	Extract	Mobile phase composition	R_f values	Detection under UV light (256nm)
1	ME	Toluene: Ethyl acetate [97:3%v/v]	Tailing was observed	Yellowish-blue fluorescence
2	ME	chloroform: Ethanol : glacial acetic acid [95:4:1%v/v/v]	0.60	

Table No.3: Percentage yield of isolated compounds and their R_f values

S.No	Sample	Amount of Cc rhizome powder(g)	Amount of isolated compounds(mg)	% of isolated compounds	R_f value
1	Isolated compound	50	116	8.41	0.60

Table No.4: The functional group region of the isolated compound was found to be consisting the following groups

S.No	Functional Group	Frequency(Cm^{-1})
1	C=O (keto)	1736
2	O-H	2920
3	C-H	3680

Table No.5: The finger printing region of the isolated compound was found to be consisting the following groups

S.No	Finger Print Region	Frequency(Cm^{-1})
1	Aromatic	802
2	Carboxylic acids	1099
3	C=C	1458

Table No.6: Determination of calibration curve for *Curcuma caesia* roxb isolated compound Correlation co-efficient: 0.998

S.No	Concentration (µg/ml)	Absorbance
1	10	0.064
2	20	0.124
3	30	0.186
4	40	0.252
5	50	0.319

Table No.7: Optimized validation parameters of developed method *Curcuma caesia* roxb

S.No	Validation Parameters	Results
		***Curcuma caesia* roxb., isolated compound**
1	Linearity	10-50 µg/ml
2	Correlation coefficient	0.999
3	Precision	< 2
	Intra-day precision (n = 6)	1.860 (10 µg/ml) 0.461 (30 µg/ml) 0.499 (50 µg/ml)
	Inter-day precision (n = 6)	0.926 [1st day] 0.876[2nd day] 0.685 [3rd day]
4	LOD	0.55 µg/ml
5	LOQ	1.66 µg/ml

Table No.8: DPPH free radical scavenging activity standard Ascorbic acid
Control absorbance (Ac): 0.445

S.No	Sample	Concentration (µg/ml)	Absorbance at 517nm (As)	% Anti-Oxidant Activity	IC_{50}
1	Ascorbic acid (Standard)	10	0.353	20.5	29
		20	0.255	42.5	
		30	0.161	63.7	
		40	0.088	80.2	
		50	0.048	98.9	

Table No.9: DPPH free radical scavenging activity of isolated compound-2 of Curcuma *caesia* roxb
Control Absorbance (Ac): 0.445

S.No	Sample	Concentration (µ g/ml)	Absorbance at 517nm (As)	% Anti-Oxidant Activity	IC_{50}
1	*Curcuma caesia* roxb., isolated compound-2	10	0.365	17.8	35
		20	0.300	32.5	
		30	0.223	49.8	
		40	0.157	64.5	
		50	0.097	78.2	

Figure No.1: ME in normal light

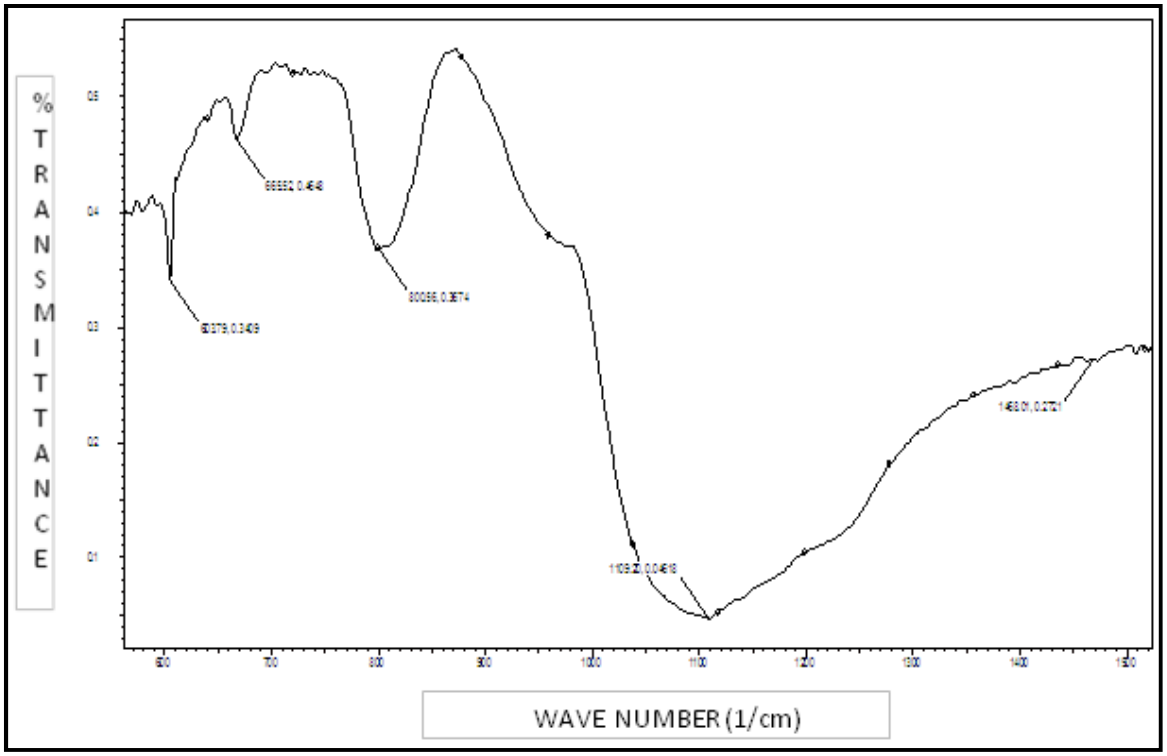

Figure No.2: Finger Print Region: (500-1500cm^{-1})

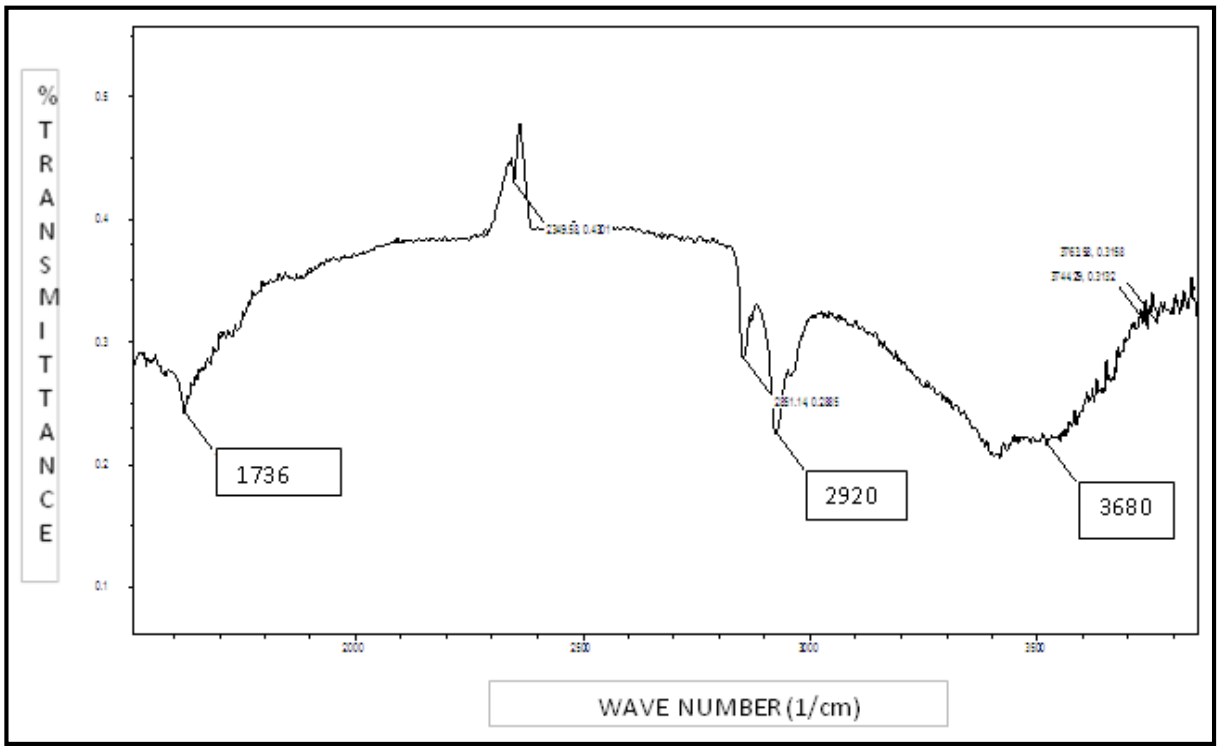

Figure No.3: Functional group region: (1500-4000 cm^{-1})

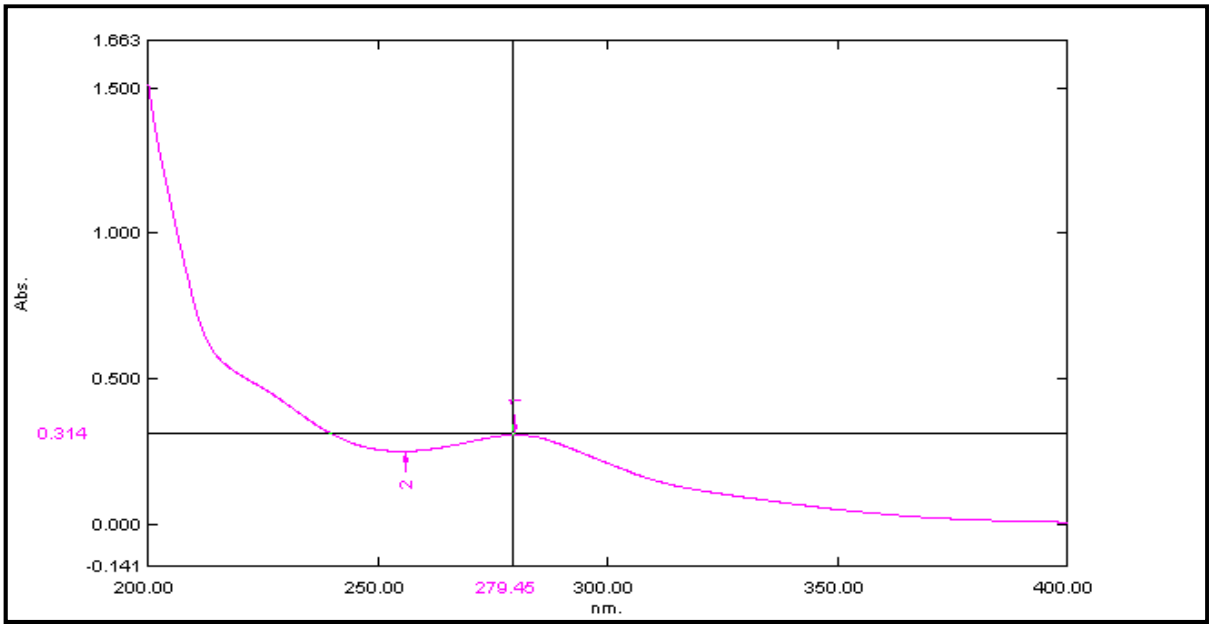

Figure No.4: Determination of λ_{max} for the isolated compound 2

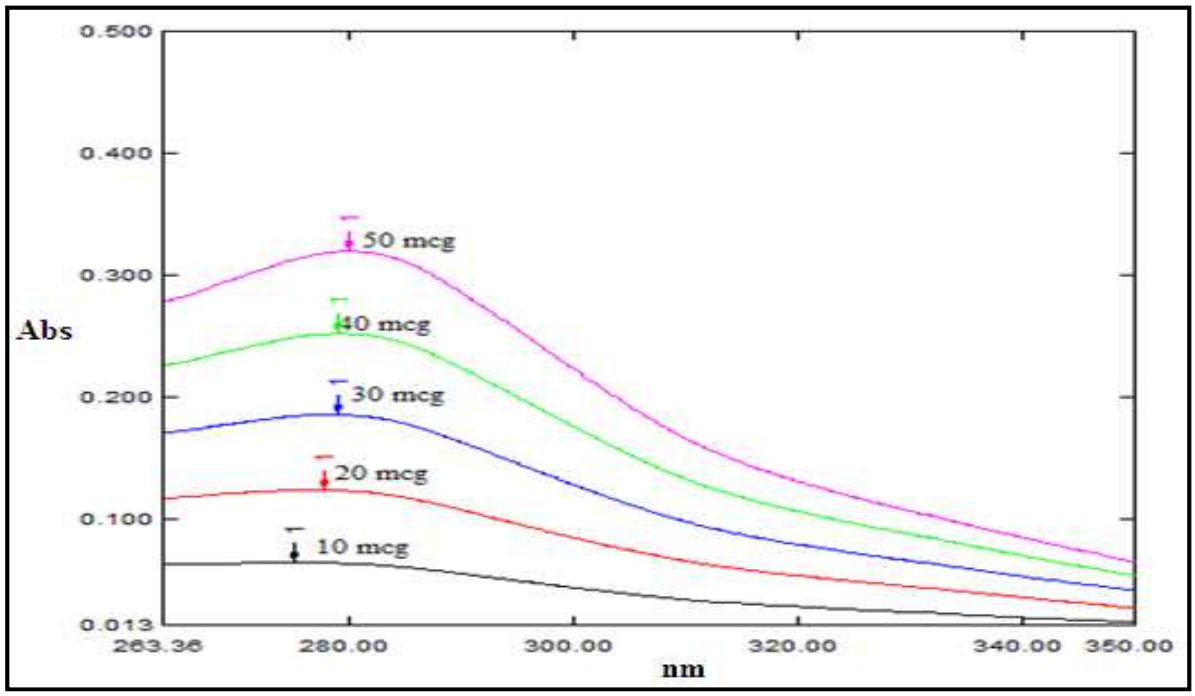

Figure No.5: Overlay spectrum of isolated compound

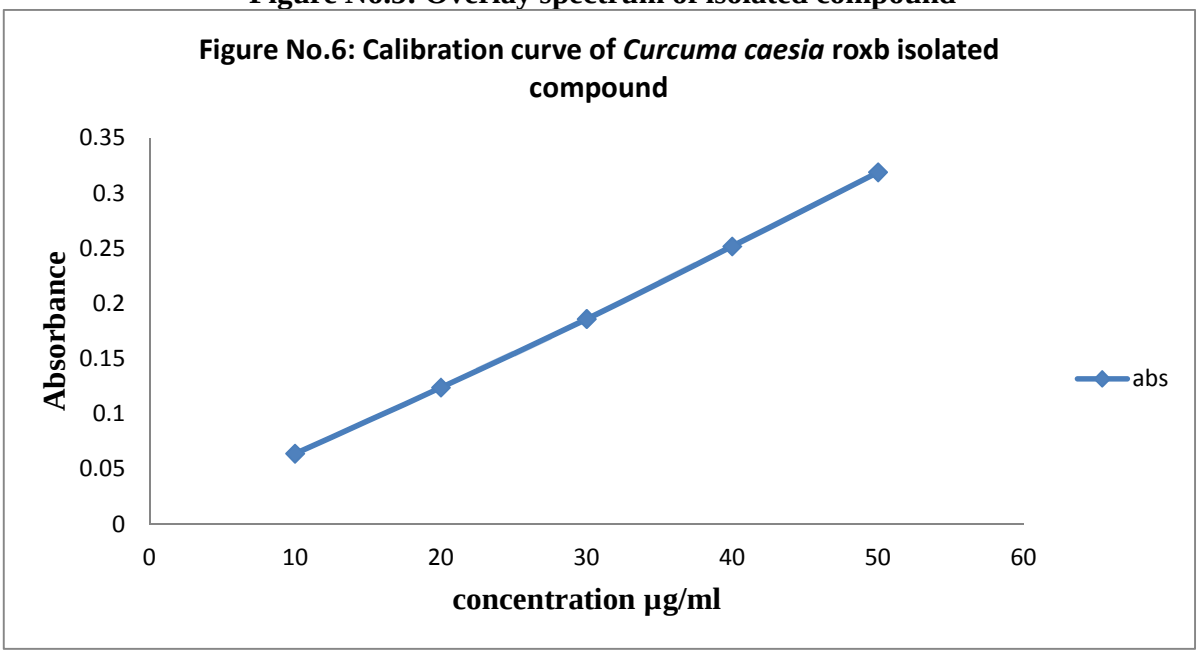

Figure No.6: Calibration curve of *Curcuma caesia* roxb isolated compound

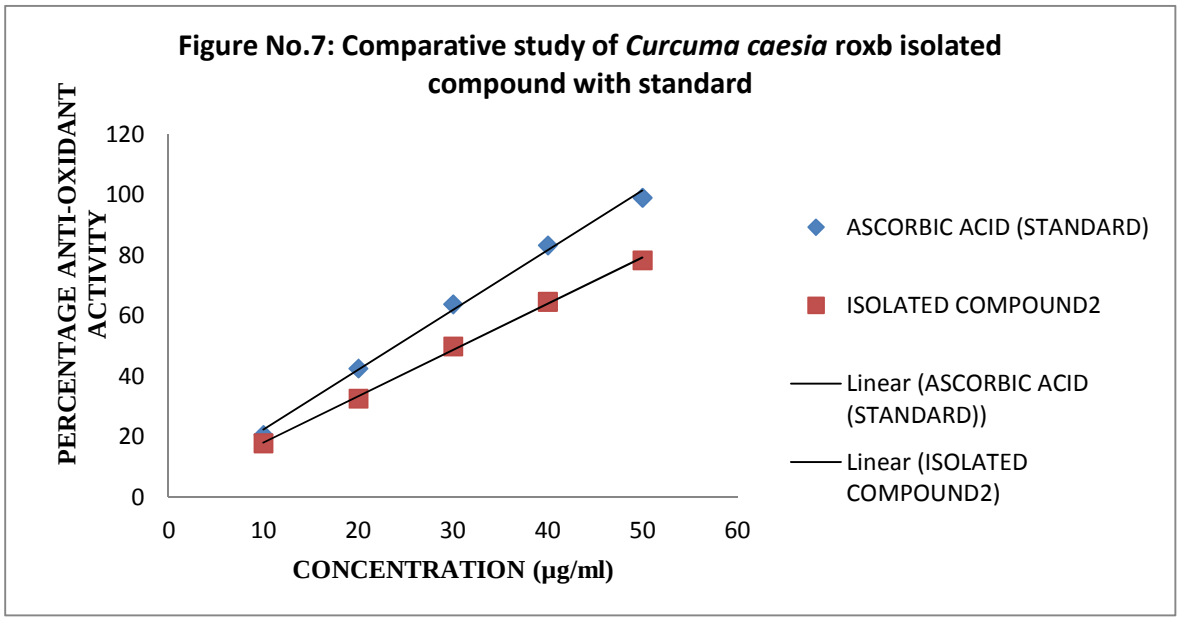

Figure No.7: Comparative study of *Curcuma caesia* roxb isolated compound with standard

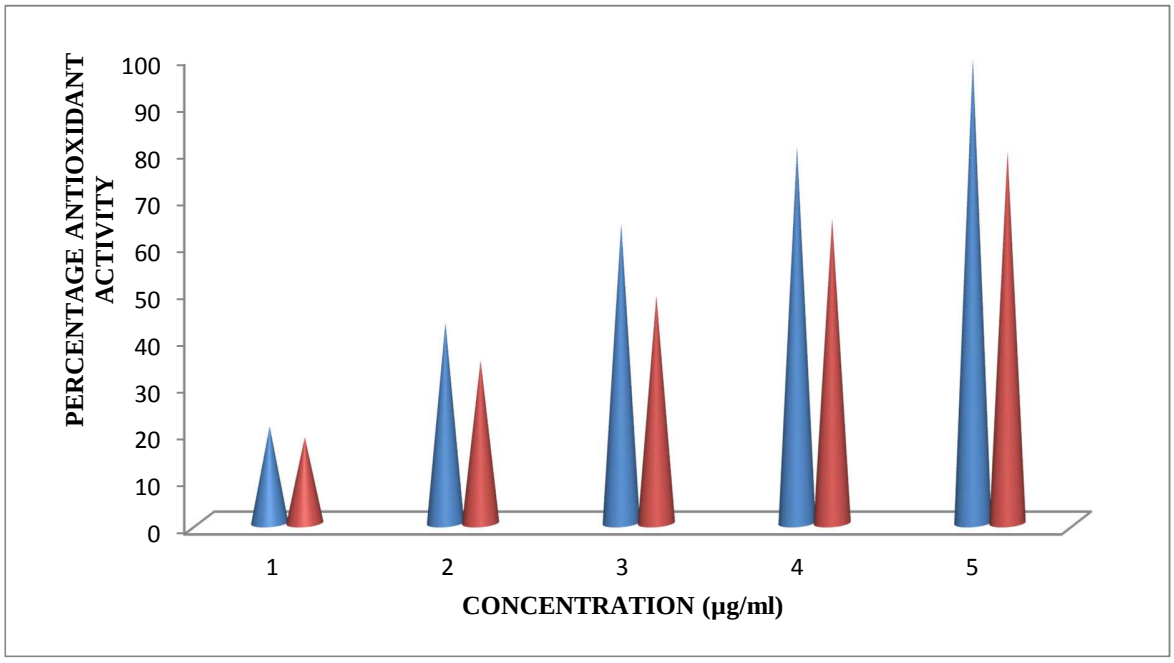

Figure No.8: comparative study of *curcuma caesia* roxb isolated compound with standard

CONCLUSION

The percentage yield of extract was found to 12.5%. By using extract compound was isolated by preparative TLC with R_f value 0.60. FTIR was performed for the isolated compound and interpreted. The results suggest that the isolated compound may be a Flavanoid, Terpenoid or Tannin derivative.

UV- Spectrophotometric method was performed for isolated compound, the method developed was more specific, précised and reproducible. Linearity of the isolated compound was found to be 10-50 µg /ml, correlation co-efficient was 0.999, precision was found to be <2, LOD and LOQ were found to be 0.55µg/ml and 1.66 µg /ml respectively.

The anti-oxidant activities of *Curcuma caesia* roxb., isolated compound were studied by free radical scavenging activity in comparison with ascorbic acid as standard. Since most of the chronic diseases are caused by free radicals this study would be of very useful in research field. So, the natural anti-oxidants plays major role in research side when compared with synthetic anti-oxidants because of their carcinogenicity.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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