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BIOACTIVITY-GUIDED ISOLATION OF ACTIVE PRINCIPLES HAVING POTENT ANTIMICROBIAL ACTIVITY FROM THE RHIZOME OF *Curcuma caesia* Roxb. OF UPPER BRAHMAPUTRA VALLEY ZONE OF *ASSAM*

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ABSTRACT

The present study reported the bioactivity-guided isolation of biologically active curcumin from a widely used Zingiberaceae ethnomedicinal plant *Curcuma caesia* Roxb from the Upper Brahmaputra Valley Zone of Assam. A systematic approach that includes TLC – Bioautography (TLC-BA), column chromatography (CC), and preparative TLC (PTLC) techniques were employed in order to isolate and purify the potent biologically active compound from the rhizome extract of *C. caesia*. The crude ethyl acetate rhizome extract of *C. caesia* was partially purified through thin-layer chromatography (TLC) and bioautography. The positive band showing the highest antimicrobial activities from TLC was subjected to silica gel column chromatography for further purification. The isolated compound was analyzed for spectral studies like- GC-MS, LC-MS, FT-IR, 1H NMR, and 13C NMR for structural elucidation. The purified compound in *C. caesia* was chemically characterized as Curcumin, a curcuminoid having potent antimicrobial activity. Curcumin and its derivatives are considered potent therapeutic agents having antimicrobial, anticancer, anti-inflammatory, antitumor, anti-acidogenic, wound healing, and anti-oxidant activities.

Key words: *C. caesia*, TLC, Bioautography, Curcumin

INTRODUCTION

Medicinal plants have been used for thousands of years due to their healing strength and almost nil side effects. Medicinal plants contain some organic compounds which provide definite physiological action on the human body **[1]**, they include bioactive substances like

phenolics, alkaloids flavonoids, tannins, saponins, terpenoids, etc. Secondary metabolites in plants are the active compounds that are responsible for the biological properties of many plant species used throughout the globe for preventing and treating diseases. Medicinal plants are the source for the development of new medicine or they may be used as phytomedicine by different ethnic communities for the treatment of various common and frequently occurring ailments. Therefore, considerable attention has been given to the screening of medicinal plants all over the world as a means to identify cheap sources of new drugs. Phytochemical investigations are the most important chemical as well as biological investigations in modern-day plant research **[2]**. It helps us to know about the presence of various chemical constituents in a specific plant or plant parts. In the 21st century, the pharmacological effect of medicinal plants has been considered a promising future drug/medicine for the management of health care **[3]**. Isolation, purification, and characterization of bioactive compounds from medicinal plants is a major thrust area of research and development in the pharmaceutical sector worldwide. A number of bioactive compounds have been isolated, and purified and their chemical structure was elucidated **[4]**. The techniques generally employed for isolation or separation of bioactive compounds from crude extract include TLC (Thin layer chromatography, HPTLC (high-performance thin-layer chromate-graphy), Column chromate-graphy (CC), TLC – Bioautography, Bioactivity-guided TLC and column chromatography, etc.

Zingiberaceae, is a monocotyledonous family yielding spices, condiments, dyes, perfumes, and medicines besides having many ornamental species cultivated for their showy flowers **[5]**. The family is well known for its immense medicinal value and is widely distributed throughout the tropics, particularly in Southeast Asia. The genus Curcuma of the Zingiberaceae family is known for its high therapeutic potential. The genus in Assam is represented by *Curcuma longa* Linn., *C. aromatica* Salisb., *C. amada* Roxb., *C. angustifolia* Roxb., *C. caesia* Roxb. etc. *C. caesia* is used extensively by different ethnic communities of the study site to cure various ailments including dysentery, stomach ache, indigestion, constipation, worm infection, gastric, toothache, skin diseases, itching, sprains, leprosy, asthma, cancer, epilepsy, fever, wound, vomiting, menstrual disorder, etc.

The present work deals with isolation, purification, and characterization of bioactive principles having potent

antimicrobial activity from a widely used Zingiberaceae ethnomedicinal plant *C. caesia* of Upper Brahmaputra Valley, Assam India in order to ascertain the rationale of their use in traditional medicine.

MATERIALS AND METHODS

Plant material

Curcuma caesia Roxb, commonly known as black turmeric or black zedoary is a perennial herb with bluish-black rhizomes.

Common name: Black Turmeric

Assamese: Kala haladhi

Hindi: Kali Haldi, Nar Kachura

Manipuri:Yaingang Amuba

Marathi: Kala-haldi

Telugu: Nalla Pasupu

Kannada: Kariarishina, naru kachora

Bengali: Kala haldi

Mizo: Aihang, Ailaihang

Nepali: Kaalo haledo

Ayurvedic name: Narkachur

Unani name: Siyah haldi,Kali haldi

Trade name: Black zedoary,Kali haldi

C. caesia is a perennial rhizomatous herb. The rhizome is externally brownish with bluish-black colour inside. The plant height is about 1.0–1.5 m in height. Matured leaves are up to 17 × 45 cm in size, leaves are lanceolate or oblong oblong-elliptic or oblong-lanceolate. The inflorescence appears from the base of the rhizome; the flowers are pale yellow in colour with reddish at the outer border. Leaves generally appear after the flowers. Green foliage dies in late autumn and the rhizomes remain dormant in winter.

Botanical Classification:

Kingdom: Plantae

Division: Angiospermae

Class: Monocotyledone

Order: Zingiberales

Family: Zingiberaceae

Genus: Curcuma

Scientific name: *Curcuma caesia*

Rank: Species

Techniques employed for Isolation, Purification, and characterization of bioactive principles:

A systematic approach that includes TLC – Bioautography (TLC-BA), column chromatography (CC), and preparative TLC (PTLC) techniques was employed in order to isolate and purify the potent biologically active compound from the rhizome extract of *C. caesia* from UBV zone of Assam. The crude ethyl acetate rhizome extract of *C. caesia* was partially purified through thin layer chromatography (TLC) and bioautography. The positive band showing the highest antimicrobial activities from of TLC was subjected to silica gel column chromatography for further purification. The isolated compound was analyzed for spectral studies like- GC-MS, LC-MS, FT-

IR, 1H NMR, and 13C NMR for structural elucidation.

Thin Layer Chromatography (TLC):

TLC was performed with crude ethyl acetate extract, with gradient polarity of solvents using silica gel. 50 µl extract from each sample was spot on the TLC plate 2 cm above its bottom with the help of capillary tubes and dried. The TLC chamber was filled with 75-80 ml of the solvent system and the TLC plate was allowed to run for about 30 min. The plate was removed from the chamber and the solvent front was marked immediately with a pencil for calculating Rf. The solvent was dried up from the plate and visualized under a hand-held UV lamp at 254 nm and 365 nm and active spots were marked. Bands were scraped off from the TLC plate and collected in a fresh tube. After that these bands (silica gel contains band) were dissolved in methanol and centrifuged at 3000 rpm for 10 minutes. After, those supernatants were transferred to a fresh tube; this process was repeated two times. Finally, the supernatant was allowed to dry and store at 4°C for further analysis.

The extraction procedure:

TLC was performed with Ethyl acetate crude extracts (EA). The fractions or bands on the TLC plate were scrapped. Antimicrobial activity assay was carried out with the materials of these scrapped bands and the band showing the highest antimicrobial activity was selected for re-chromatography. Different solvent systems with different ratios were applied as mobile phases in order to determine the functional eluent for chromatographic separation. The solvents were hexane: ethyl acetate (1:1), water: methanol: ethyl acetate (0.5:1.5:8), ethyl acetate: methanol: acetic acid (7:1:2), toluene: ethyl acetate (4:1), benzene: ethyl acetate (9:1), Chloroform: Ethanol (1:1), Chloroform-methanol (7: 3), Chloroform: Methanol (9.5: 0.5) etc.

Bioautography of TLC scrapped samples:

Contact bio autography was carried out following, Kagan and Flythe, (2014), Jesionek *et al*., (2017) Sakunpak and Sueree, (2018), Jayalakshmi *et al*., (2021) **[6-9]** with slight modification. The antimicrobial activities of TLC scrapped samples were checked by following the zone inhibition method. For which bacterial *strains Bacillus subtilis, Enterococcus. faecalis, Escherichia coli,* and fungal strains *Candida albicans* were selected. For bacteria MHA (Muller Hilton's Agar) plates were spread and inoculated with 100 µl of log cultures of bacteria followed by placing the discs containing a concentration of 1000µg of samples, one disc was loaded with solvent (ethyl acetate) alone which served as

vehicle control and ciprofloxacin solution (10µg/disc) was taken as a positive control for bacteria.

For fungi PDA plates were spread inoculated with 100 µl of log cultures of fungi followed by placing the discs containing a concentration of 1000µg of samples, one disc was loaded with solvent (ethyl acetate) alone which served as vehicle control and amphotericin solution (10µg/disc) was taken as a positive control for bacteria. The plates were incubated at 30 °C for 16-24 h and zones created around the discs were measured and recorded.

Column Chromatography:

The semi-purified compound obtained through TLC- Bioautography of ethyl acetate rhizome extract of *C. caesia* was further purified with silica gel column chromatography **[10 - 12]**.

Sample selection:

The positive and bioactive fraction obtained through preparative TLC – Bioautography was bulked and this bulk quantity of fraction was further purified with silica gel column chromatography.

Preparation of sample for column chromatography:

A small amount of silica gel mesh was taken in a petridish. The partially purified sample (w/v) was added gently to the petridish having 60-120 mesh silica gel. The solvent is allowed to evaporate to get the dry sample. This sample was loaded on the top of the packed column.

Preparation and Processing of the column:

The partially purified compound was further purified with silica gel column chromatography. For this purpose, a dry and clean column was placed on a stand in a vertical position. A loose cotton plug was pushed down to the bottom of the column with the help of a wooden stick. A layer of silica gel mesh was placed over the cotton. The column is pre-eluted with petroleum ether. The partially purified sample was loaded on top of the packed column. A conical flask was placed under the column outlet for the collection of fractions. Chloroform: Methanol in the ratio of 9.5:.5 was used as the eluting solvent.

Fractions were collected separately in a conical flask and numbered consecutively for further analysis. The samples are concentrated and loaded into thin layer chromatography to check the homogeneity of various fractions and to study the biological activity.

Antimicrobial study of collected fractions:

The antimicrobial activities of collected fractions were checked by following the zone inhibition method by using bacterial strains *B. subtilis*, *E. coli*. MHA (Muller Hilton's Agar) plates were spread

inoculated with 100 µl of log cultures of bacteria followed by placing the discs containing concentration 1000µg of samples, one disc was loaded with solvent alone which served as vehicle control and ciprofloxacin solution (10µg/disc) was taken as a positive control for bacteria.

Thin Layer Chromatography (TLC) of active fraction:

Preparative TLC of the bioactive fractions obtained by column chromatography followed by bioassay was carried out for further separation by adopting the methods mentioned earlier. After TLC analysis, the solvent was dried up from the plate and visualized under a hand-held UV lamp at 254 nm and 365 nm, and active spots were marked. The retention factor (Rf) was calculated.

Bands were scraped off from the desired location of the TLC plate and collected in a fresh tube. After that these bands (silica gel contains band) were dissolved in methanol and centrifuged at 3000 rpm for 10 minutes. After, those supernatants were transferred to a fresh tube; this process was repeated two times. Finally, the supernatant was allowed to dry.

The dried fraction was analyzed for spectral analysis like- FT-IR, 1H and 13 C NMR, LCMS, GCMS, etc for structural elucidation analysis for identifying the active compound isolated **[13 - 14]**.

RESULT AND DISCUSSION

Thin Layer Chromatography (TLC) of Ethyl acetate extract:

The ethyl acetate extract of *C. caesia* exhibited the highest antimicrobial activity in comparison to the acetone, methanol, and aqueous extracts. Therefore, ethyl acetate extract was selected for further study. TLC was performed with Ethyl acetate crude extracts by using different solvent systems with different ratios as mobile phases in order to determine the functional eluent for chromatographic separation. Five bands (B1 – B5) were obtained in the Benzene: Ethyl acetate (9:1) solvent system **(Table 1)**.

Contact bioautography of TLC Fractions of Ethyl acetate extract:

After TLC separation of ethyl acetate extract, the antimicrobial activity of the TLC scrapped bands was detected with the direct bioautography method. Out of the five bands scrapped from the TLC plate the band showing the highest antimicrobial activity against the tested microorganisms was ‘B4’ (Rf= 0.74).

Re - Thin Layer Chromatography (TLC) of fraction ‘B4’:

Fraction B4 was selected for re- thin layer chromatography. Different solvent systems were applied with different ratios as mobile phase. The bands were obtained

in Chloroform: Methanol (9.5:5). Two bands 'B4a' (Rf= 0.47) and 'B4b' (Rf= 0.77) were scrapped and collected.

Contact bioautography of TLC fractions 'B4a' and 'B4b':

Antimicrobial activity of the fractions 'B4a' and 'B4b' of *C. caesia* scrapped from the TLC plate with fraction 'B4' was carried out with contact bioautography and fraction 'B4b' was found most active against the tested microorganisms.

Column chromatography of partially purified compound 'B4b':

The partially purified compound obtained through TLC- Bioautography of ethyl acetate rhizome extract of *C. caesia* was further purified with silica gel column chromatography. Chloroform: Methanol in the ratio of 9.5:.5 was used as the eluting solvent. Five no of fractions (1-5) were separated and collected.

Antimicrobial activity analysis of different fractions obtained by Column chromatography:

Antimicrobial analysis of five different fractions obtained by silica gel column chromatography of *C. caesia* was carried out and fraction no. 2 was found positive against the tested microorganisms.

Thin layer chromatography of fraction no. 2 obtained by silica gel column chromatography:

The fraction no.2 obtained by silica gel column chromatography of *C. caesia*, which was found positive during antimicrobial analysis was subjected to TLC. The plates were visualized under a hand-held UV lamp at 254 nm and 365 nm and active spots were marked. A single spot appears with Rf 0.79. As a single compound was developed in the TLC plates it is considered a pure compound. The purified compound from the TLC plates was bulked and used for character elucidation.

On the interpretation of GC-MS **(Figure 1)** purified antimicrobial fraction, the M+1 peak was observed at 369.0491 m/z ratios with 10% intensity.

The FT-IR spectrum of the fraction **(Figure 2)** showed the IR band at 1625.1 cm-1 was found for >C=O stretching. A conjugated dienes C=C can be assigned with 1599 -1. C-C in ring can be assigned to 1423.8 1 cm-1 for out of the plane. An Ar-O-R C-O stretching was found at 1230 cm-1. Ar-OH, C-O stretching out of the plane was observed at 1203.9 cm-1 whereas 1151.7 and 1025 cm-1 was observed for C-O stretching. R-O-R was found at 1114.5 cm-1. Out of the plane bending for HC=CH was observed at 715.6 cm-1.

The 1H NMR (DMSO, 400 MHz, δ in ppm) spectra of fraction **(Figure 3)** recorded the following chemical shifts- δ 3.812, 3.830(s, 3+3 H, -OCH3); δ 6.650, 6.690(d, 2H, J = 16 Hz, 2,6-H); δ 6.811,

6.832(d, 2H, J = 8.4 Hz, Ar-H); δ 7.130, 7.149(d, 2H, J = 7.6, Hz, Ar-H); δ 7.311 (s, 2H, Ar-H); δ 7.538, 7.566(d, 2H, J = 11.2 Hz, 1,7-H); δ 9.645(s, 2H, Phenolic -OH). On interpretation of recoded 1H NMR, 6 Hydrogen of –OCH3 were found at 3.812, 3.830 ppm with singlet. 2 hydrogen of alkyl were found at 6.650 and 6.690 ppm with coupling constant of 16 Hz whereas other two were observed at 7.538 and 7.566 ppm with coupling constant of 11.2 Hz. Ar-H with doublet were observed with 7.130 and 7.149 ppm with coupling constant of 7.6 Hz whereas singlet is found with 7.311 ppm. 2 hydrogens of phenolic –OH were found at 9.645 ppm with singlet.

13C NMR (DMSO, 400 MHz, δ in ppm) spectra of fraction **(Figure 4)** recorded δ 56.280, 101.433, 111.980, 116.319, 121.690, 123.684, 126.943, 141.294, 148.591, 149.953, 183.791. On interpretation of 13C NMR, carbon was recorded at 56.280, 101.433, 111.980, 116.319, 121.690, 123.684, 126.943, 141.294, 148.591, 149.953 and 183.791 ppm.

LC-MS study (m/z, %) **(Figure 5)** recorded 369.0491 (M+1, 10), 368.003 (M, 25); RT 3.74 with 95.91% area. As far as LC-MS is concerned, 95.91% of the area was observed with a retention time of 3.74 sec.

A tentative structure for the fraction was elucidated from the foregoing spectral data i.e., $[HOC_6H_3(OCH_3)CH{=}CHCO]_2CH_2$

Table 1: Rf value of TLC bands scrapped for bio autography from TLC plate of Ethyl acetate extract

TLC Plate	Solvent System	Bands	Rf value
TLC of Ethyl acetate extract of *C. caesia.*	Water: methanol: Ethyl acetate (0.5:1.5:8),	Smear formation	--
	Hexane: ethyl acetate (1:1)	No separation	--
	Chloroform: methanol: water (6:3.5 :0.5)	Smear formation	--
	Toluene: ethyl acetate (4:1)	No separation	
	Benzene: Ethyl acetate (9:1)	B1	3.6/9.3 = 0.41
		B2	5.4/9.3 = 0.58
		B3	6.5/9.3 = 0.69
		B4	6.9/9.3 = 0.74
		B5	8.2/9.3 = 0.88

Table 2: Antimicrobial activity of the materials scrapped from TLC plate

Test Organism	ZOI in mm						
	B1	B2	B3	B4	B5	"+" Control	" – " Control
B. subtilis	2	4	-	9	7	22	-
B. faecalis	3	4	-	6	4	21	-
E. coli	1	-	3	7	5	19	-
C. albicans	-	3	-	4	3	21	-

Table 3: Rf value of TLC bands scrapped from TLC plate with band 'B4'

TLC Plate	Solvent System	Bands	Rf value
TLC Plate with band 'B4'	Chloroform: Ethanol	No separation	--
	Chloroform-methanol (7: 3)	Smear formation	--
	Chloroform-methanol (9: 1)	B4a	4.7/8.9 = 0.47
		B4b	6.9/8.9 = 0.77

Table 4: Antimicrobial activity of the materials scrapped from TLC plate with 'B4' band.

Test Organism	ZOI in mm			
	B4a	B4b	"+" Control	"-" Control
B. subtilis	2	5	22	-
B. faecalis	3	5	24	-
E. coli	3	7	23	-
C. albicans	3	8	25	-

Spectral Characterization of the purified fraction:

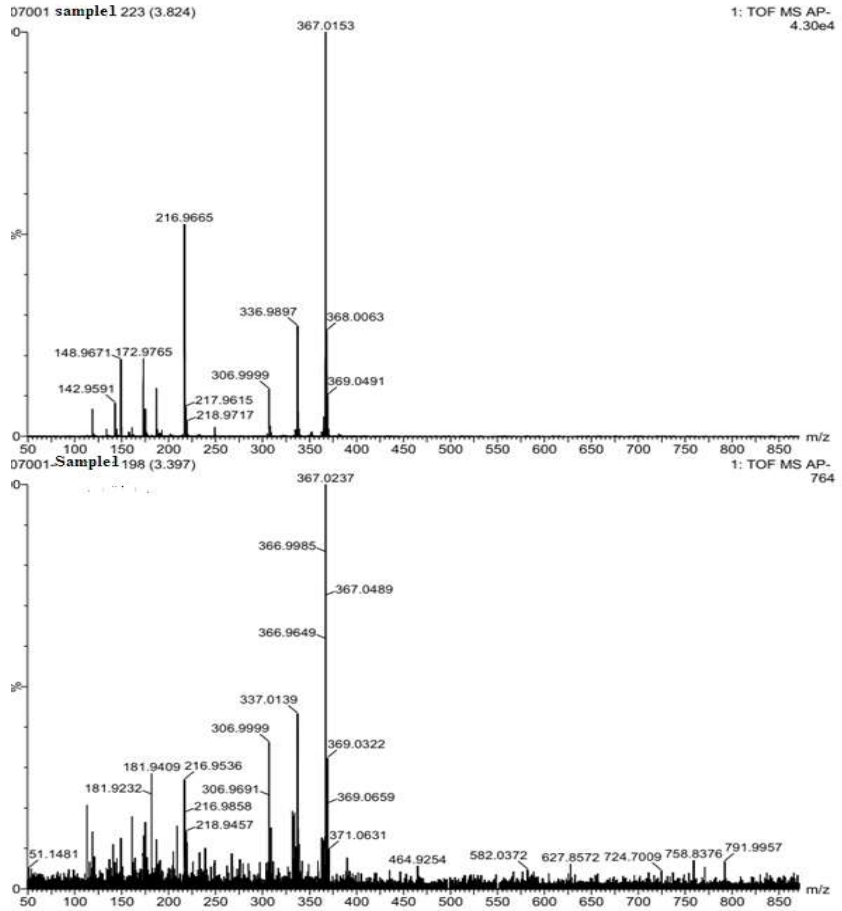

Figure 1: GC-MS spectrum of the purified fraction of *C. caesia*

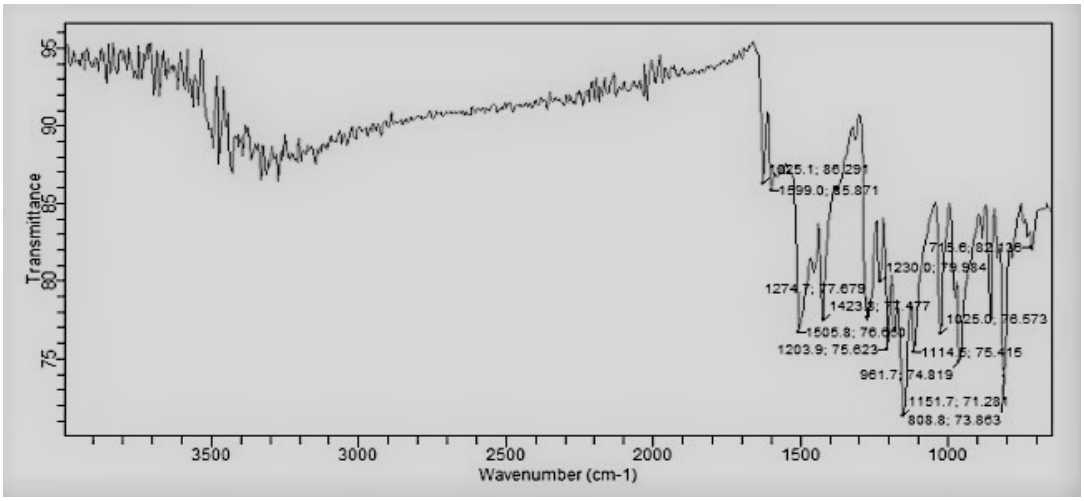

Figure 2: FT-IR spectrum of of the purified fraction of *C. caesia*

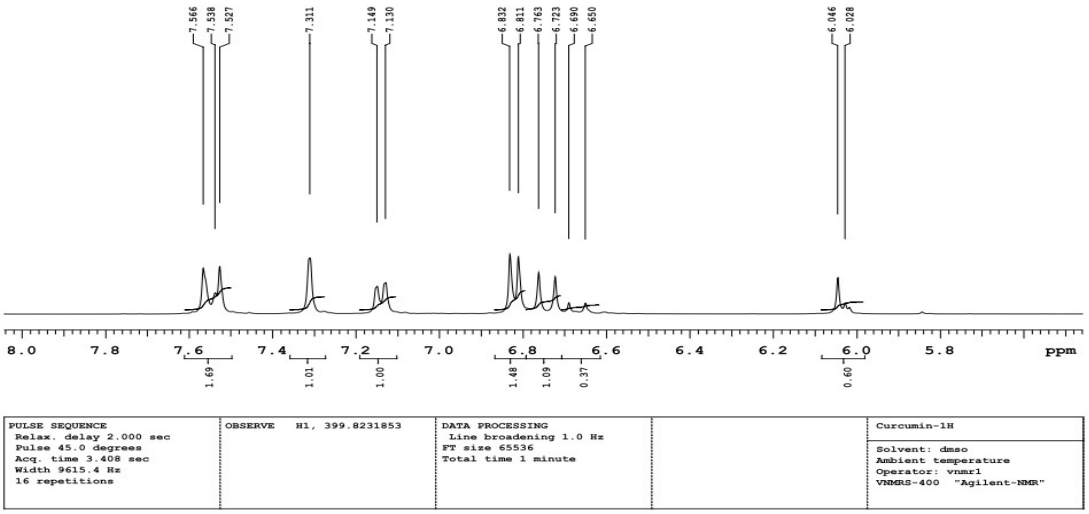

Figure 3: ^{1}H NMR of the purified fraction of *C. caesia*

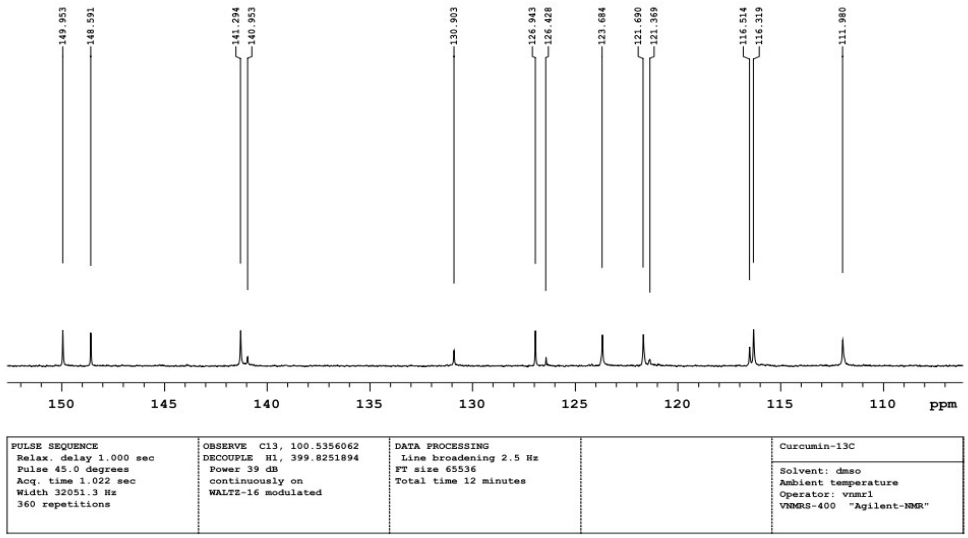

Figure 4: ^{13}C NMR spectrum of the purified fraction of *C. caesia*

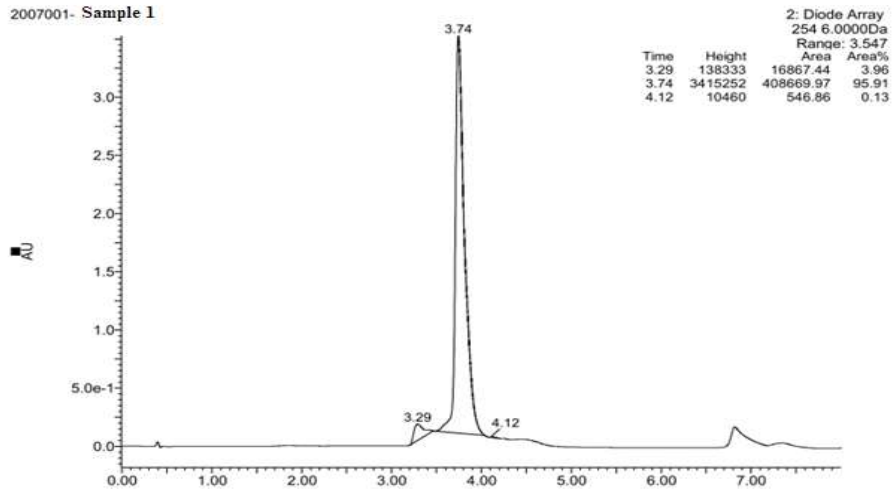

Figure 5: LC-MS spectrum of the purified fraction of *C. caesia*

CONCLUSION

C. caesia is a wild rhizomatous ethnomedicinal plant of the Zingiberaceae family. Rhizomes of these plants in various forms of preparation are traditionally used against different ailments. The present study focused on the isolation and identification of the active compound (s) in rhizome extract. Thin layer chromatography coupled with bio autography was employed for the identification of active principles from the plant. TLC was performed with Ethyl acetate crude extracts (EA). The fractions or bands on the TLC plate were scrapped. Antimicrobial activity assay was carried out by direct bioautography with the materials of these scrapped bands and the band showing the highest antimicrobial activity was selected for re-chromatography. The fractions showing the highest bioactivity were bulked and subjected to silica gel column chromatography. The fractions of silica gel column chromatography were subjected to antimicrobial analysis and the fraction showing the highest bioactivity was further purified in TLC. As the TLC showed a single band it was considered as a pure compound. Which were scrapped and bulked for spectral analysis. The purified compound in *C. caesia* was chemically characterized as Curcumin a curcuminoid having potent antimicrobial activity. Curcuminoids including curcumin are phenolic compounds, Curcumin and its derivatives are considered potential therapeutic agents having antimicrobial, anticancer, anti-inflammatory, antitumor, anti-acidogenic, wound healing, and anti-oxidant activities. The presence of bioactive components in the rhizome of *C. caesia* from the Upper Brahmaputra Valley Zone of Assam strongly validates

the traditional knowledge of the different ethnic communities of the Upper Brahmaputra Valley Zone of Assam regarding the application of this plant as traditional medicine.

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