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Original Article

In vitro Evaluation of Curcumin and Quercetin in Combination on Oxidative Stress

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ABSTRACT

Background: Flavonoids are the natural product belongs to an important class of plant secondary metabolites. They are polyphenolic compounds and known for decades as a potent natural antioxidant. Curcumin (flavonoid polyphenol) and quercetin (flavonol) both are popular natural antioxidants and contributes a major role as an individual in the treatment of oxidative stress. This research study is more focused in their combination effect against oxidative stress. **Materials and Methods:** In this present research, we focused on hydro-alcoholic maceration of *Curcuma longa* rhizomes and *Morinda citrifolia* fruits to extract the curcumin and quercetin respectively. Also, their role in oxidative stress was studied using *in vitro* techniques. **Results:** The percentage (%) yield of hydro-alcoholic extract attained from *Curcuma longa* rhizomes and *Morinda citrifolia* fruits were found to be 23.06% and 21.08% respectively. **Conclusion:** Additionally, through DPPH (2,2-diphenyl-l-picrylhydrazyl) and Lipid peroxidation (LPO) assay, it was observed that curcumin and quercetin in combination gives the highest antioxidant activity when compared with their individual response at same concentration.

Keywords: Authentication, Hydro-alcoholic maceration, Curcumin, Quercetin, Flavonoids, Oxidative stress.

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INTRODUCTION

When the rate of oxidant generation outpaces the rate of oxidant scavenging, oxidative stress is the result. If this condition is not treated quickly it will ultimately leads to the irreversible cell damage and finally cell death.[1] In addition to its function in the emergence of problems, oxidative stress may be significant in diabetes because it can be triggered by prolonged hyperglycemia brought on by insulin resistance, which can then lead to the death of beta cells in type II diabetes.[2] The most recent data from the International Diabetes Federation (IDF) indicates that around 537 million adults, between the ages of 20 and 79, are currently living with diabetes. This number is expected to rise to 643 million by 2030 and reach 783 million by 2045.[3]

Flavonoids are a class of organic compounds that are categorized as secondary metabolites and are commonly present in various plants, fruits, vegetables, and beverages. They contain polyphenol in their structure and known for decades as a potent natural antioxidant, anti-carcinogenic, anti-inflammatory and anti-mutagenic.[4] The herbaceous plant *Curcuma longa*, also known as Turmeric or Haldi, is a member of the zingiberaceae family. There are four distinct curcuminoids found in commercial turmeric: Curcumin (77%) demethoxycurcumin (17%) bis-demethoxycurcumin (nearly 3%) and cyclocurcumin.[5] The rhizomes of *Curcuma longa* are used to extract the active ingredient curcumin, a flavonoid polyphenol whose first discovery was made by Vogel and Pelletier.[6] Curcumin (diferuloylmethane) is chemically described as [1,7-bis(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione] and it is represented as $C_{21}H_{20}O_6$.[7] The phenolic group in curcumin composition explains its ability to eliminate oxygen-derived free radicals and hence it is useful as a potent natural antioxidant.[8]

A few decades ago, *Morinda citrifolia*, a member of Rubiaceae family came into the public eye due to a wide range of medicinal actions that are particularly advantageous for the human race, including immunostimulatory, anti-diabetic, urinary tract ailments, gonorrhoea, tooth decay, antiseptic, antioxidant

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and neuroprotective effects.[9] Nearly 200 phytochemicals were discovered and isolated from *Morinda citrifolia* plant; quercetin is one of them.[10] Quercetin, chemically described as 3,3′,4′,5,7-Pentahydroxyflavone is a naturally derived flavonoid (flavonol) and known for its antioxidant, antiprotozoal, antimicrobial, anticancer, anti-inflammatory and hypoglycemic activity.[11] The antioxidant property of quercetin may be the cause of its hypoglycemic effects. By assisting in the neutralization of free radicals, this guards the pancreas against oxidative stress. The beta cells may proliferate and secrete more insulin as a result of the lessened oxidative stress on the pancreas.[12] Therefore, this present research study is focused on effect of flavonoids, curcumin (Figure 1A) and quercetin (Figure 1B) in combination on oxidative stress.

MATERIALS AND METHODS

Plant Material

The *Curcuma longa* rhizomes were purchased from Yucca Enterprises, Wadala, Mumbai, India and the *Morinda citrifolia* fruits were purchased from Green Earth Products Pvt Ltd., Greater Kailash Part- 1, New Delhi, India. Both the plant materials were authenticated by Dr Harshad M Pandit, PhD (Botany), Formerly Head and Associate Professor of Botany, Azadnagar Gem CHS Ltd., Mumbai, India (Rhizomes specimen: #:ak p 012290218; Fruits specimen: **#:**ak p 0922120720). The herbariums were submitted to the Department of Pharmacognosy, College of Pharmacy, JSS Academy of Technical Education, Noida, UP, India for further references.

Hydro-alcoholic maceration of *Curcuma longa* rhizomes and *Morinda citrifolia* fruits

Prior to extraction, *Curcuma longa* rhizomes were shade dried for 1 week followed by oven dried at 70°C for 2 hr.[13] The dried and crushed rhizomes were pretreated with *n*-hexane for 2 hr and shade dried for 24 hr.[14] For hydro-alcoholic maceration pre-treated grinded rhizomes were soaked in the mixture of ethanol and water (70:30) for 7 days with occasional stirring and filtered. Filtrate was evaporated by placing the aliquots in petridish at 78.2°C by using water bath and residue was stored properly for further use (Figure 2A).[15] The reported procedure was followed for the hydro-alcoholic maceration of *Morinda citrifolia* fruits (Figure 2B) and the extract was stored properly for future use. The percentage (%) yield for both hydro-alcoholic extracts was then calculated.[16] Finally the phytochemical screening was done to establish the flavonoid content (Figure 3).[17,18]

Fractionation

The crude hydro-alcoholic extract of *Curcuma longa* and *Morinda citrifolia* were extracted successively by using hexane, chloroform, ethyl acetate and acetone as eluents using a separating funnel.[19] The maximum percentage yield was obtained in acetone fraction (14.5 g; 13.87 g) followed by ethyl acetate (6.1 g; 6.3 g), chloroform (4.8 g; 4.22 g) and hexane (4.3 g; 3.9 g).

Isolation of Curcumin from *Curcuma longa* rhizomes and Quercetin from *Morinda citrifolia* fruits

The acetone clear soluble fraction (14.5 g; 13.87 g) was utilized for column chromatography. The column was packed with 500 g of silica gel (60-120 mesh, Merck Pvt. Ltd., India), and a mixture of ethyl acetate and hexane (9:1 v/v) was used as the eluent. Through repeated chromatography, Fraction 1 with an R_f value of 0.71 and a yield of 0.168 g, and Fraction 2 with an R_f value of 0.81 and a yield of 0.198 g, were obtained.

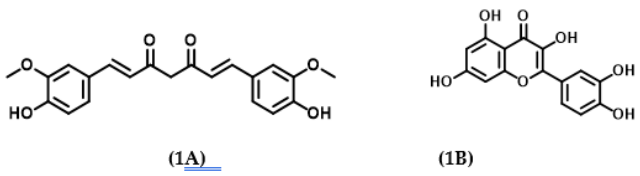
Purification

Fraction 1 was subjected to further purification by Thin Layer Chromatography (TLC) using a silica gel GF254 precoated plate (Merck Pvt. Ltd., India) and employing ethyl acetate and hexane (3:2 v/v) as the developing solvent (R_f value 0.723). As a result, curcumin was obtained with a yield of 320 mg. Similarly, Fraction 2 was purified using the same developing solvent (R_f value 0.804) with the same ratio on a TLC plate coated with silica gel GF254, resulting in the isolation of quercetin with a yield of 342 mg[20] (Figure 4).

Determination of antioxidant properties of isolated curcumin and quercetin

DPPH (2,2-diphenyl-l-picrylhydrazyl) and Lipid peroxidation (LPO) radical cation assay were adopted as an *in-vitro* models to study the antioxidant activity of isolated compounds.

The DPPH assay was carried out in a 96-well microtitre plate. To each well, 200 µL of DPPH solution was added followed by 10 µL of the test sample or the standard solution, separately. The final concentration of the test and standard solutions ranged from 1000 to 1.95 µg/mL. The microtitre plates were incubated at 37°C for 20 min, and subsequently, the absorbance of each well was measured at 490 nm using an ELISA reader, relative to the corresponding



(1A) (1B)

Figure 1: Chemical structure of curcumin (1a) and quercetin (1b).

test and standard blanks. The amount of remaining DPPH was then calculated. The IC_{50} (Inhibitory Concentration) refers to the concentration of the sample required to scavenge 50% of DPPH free radicals.[21]

$$\%\ antioxidant\ activity = \left(\frac{E_{DPPH} - E_{Sample}}{E_{DPPH}}\right) X\ 100$$

In the Lipid Peroxidation (LPO) assay, various concentrations of test samples (100 µL) were added to a mixture of egg lectin (1 mL), while a control sample was prepared without the test sample. The induction of lipid peroxidation was achieved by adding $FeCl_3$ (400 mM) and L-ascorbic acid (200 mM) in the quantity of 10 µL each. Following an hour-long incubation at 37°C, the reaction was terminated by adding 2 mL of 0.25 N HCl, containing 15% TCA and 0.375% TBA. Subsequently, the reaction mixture was boiled for 15 min, allowed to cool, and centrifuged, and the supernatant's absorbance was measured at 532 nm.[22]

In-vitro cytotoxicity assay

Determination of mitochondrial synthesis by MTT assay

The cell culture was first subjected to centrifugation, and its cell count was subsequently adjusted to $1.0x10^5$ cells/mL using DMEM medium, containing 10% FBS. A volume of 100 µL of the diluted cell suspension (approximately 10,000 cells/well) was then added to each well of a 96-well flat-bottomed micro titre plate. After allowing 24 hr for the cell population to attain sufficient density, the cells were again subjected to centrifugation, and the resulting pellets were then suspended in 100 µL of various test sample concentrations, prepared in maintenance media. The plates were then placed in a 5% CO_2 atmosphere at 37°C and incubated for 48 hr, during which microscopic examinations were performed, and observations were recorded every 24 hr. Following the completion of the incubation period, 20 µL of MTT (2 mg/mL) in MEM-PR (MEM without phenol red) was added to each well. The plates

Figure 2: Hydro-alcoholic maceration of *Curcuma longa* rhizomes (2A) and *Morinda citrifolia* fruits (2B).

were then gently shaken and incubated for 2 hr, again in a 5% CO_2 atmosphere at 37°C. Next, 100 µL of DMSO was added to each well, and the plates were again gently shaken to solubilize the formed formazan. The absorbance of each well was measured using a microplate reader, with the wavelength set to 540 nm.[23]

RESULTS AND DISCUSSION

Percentage yield of hydro-alcoholic extracts

The percentage yield of hydro-alcoholic extract obtained from *Curcuma longa* rhizomes was found to be 23.06% and the percentage yield of hydro-alcoholic extract obtained from *Morinda citrifolia* fruits was found to be 21.08%.

Phytochemical Screening

The phytochemical screening was performed for the hydro-alcoholic extract of *Curcuma longa* rhizomes and *Morinda citrifolia* fruits and results were tabulated in Table 1.

Characterization of Curcumin and Quercetin

Isolated compounds curcumin and quercetin were characterized by using ^{1}H NMR, ^{13}C NMR and HRMS techniques. The results obtained were as shown in figures provided in supplementary material (Figure S1- S6).

Curcumin: MP:183°C; ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.64 (s, 2H), 7.55 (t,*J* =8.0 Hz, 3H), 7.32 (s, 2H), 7.15 (d,*J* =8.1 Hz, 2H), 6.83 (d,*J* =8.1 Hz, 2H), 6.75 (d,*J* =15.8 Hz, 2H), 6.05 (d,*J* =6.1 Hz, 1H), 3.84 (s, 6H).; ^{13}C NMR (101 MHz, DMSO-d_6) δ 183.67, 149.82, 148.46, 141.17, 130.79, 126.81, 123.57, 121.57, 116.38, 116.18, 111.84, 101.29, 56.16.; HRMS: *m/z*calcd for $[M+H]^+$ $C_{21}H_{21}O_6$ 369.1333 found 369.1338. It was characterized as curcumin by comparison of physical and spectroscopic properties with previous published data.

Quercetin: MP: 316-318°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.76 (s, 1H), 9.57 (s, 1H), 9.34 (s, 1H), 9.29 (s, 1H), 7.68 (d,*J* =2.2 Hz, 1H), 7.54 (dd,*J* =2.2, 8.5 Hz, 1H), 6.89 (d,*J* =8.5 Hz, 1H), 6.41 (d,*J* =2.0 Hz, 1H), 6.19 (d,*J* =2.0 Hz, 1H).; ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.87, 163.91, 160.75, 156.17, 147.72, 146.83, 145.08, 135.76, 121.99, 120.00, 115.63, 115.10, 103.05, 98.21, 93.38.; HRMS: *m/z* calcd for $[M+H]^+$ $C_{15}H_{11}O_7$ 303.0449 found 303.0506. The compound was characterized as quercetin by comparison of physical and spectroscopic properties with previous published data.

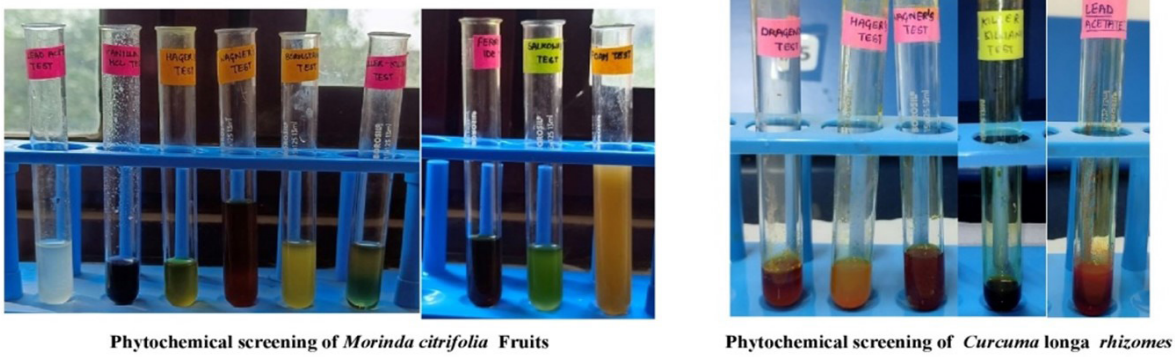


Figure 3: Phytochemical screening of hydro-alcoholic extracts.

Table 1: Phytochemical Screening of Hydro-alcoholic Extracts.

Sl. No.	Constituents	Test Applied	Hydro-alcoholic Extracts and isolated compound	
			Curcuma longa	*Morinda citrifolia*
1	Alkaloids	Dragendroff's Test	+	+
		Hagers Test	+	+
		Wagners Test	+	+
2	Glycosides	Borntragers Test	+	+
		Keller Killiani Test	+	+
3	Steroids	Salkowaski Test	-	-
		Ferric Chloride Test	-	-
4	Flavonoids	Lead acetate test	+	+
		Vanillin Test	+	+
5	Saponins	Foam Test	+	+

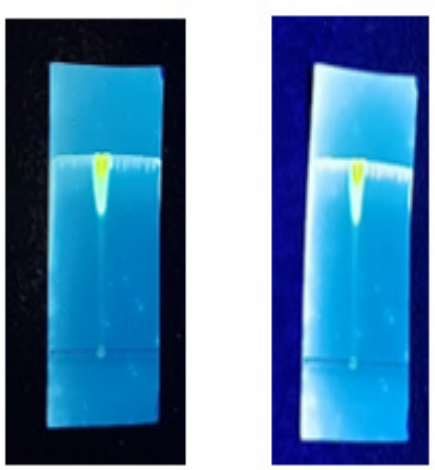


Figure 4: Thin Layer Chromatography.

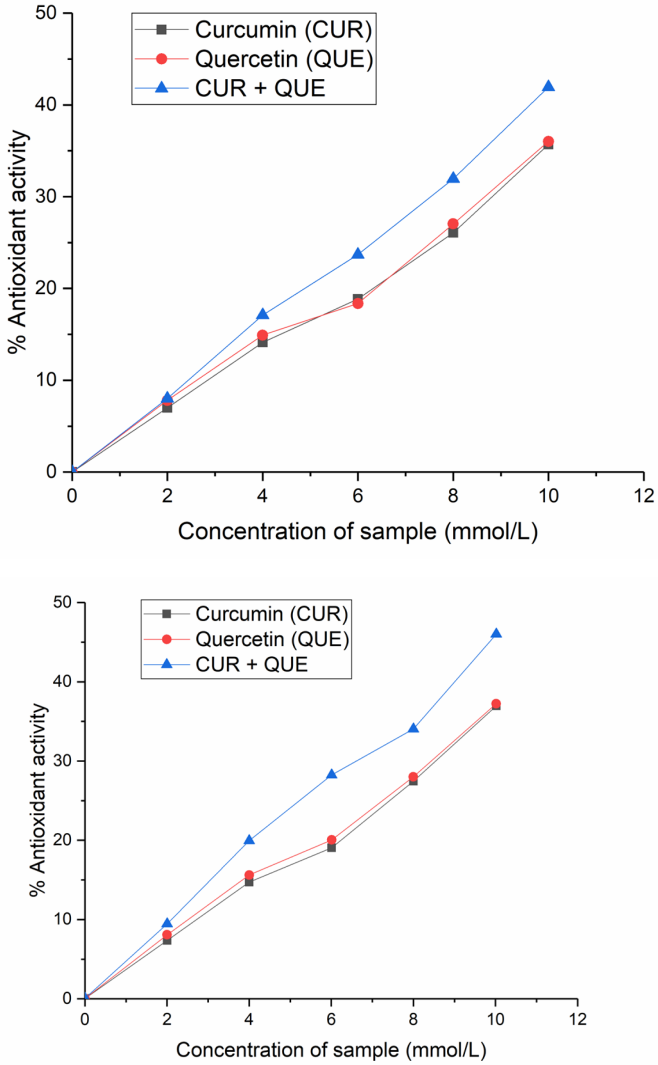


Figure 5: (A) *In vitro* antioxidant activity of isolated compounds using DPPH assay model, (b) *In vitro* antioxidant activity of isolated compounds using LPO assay model.

Table 2: Antioxidant activity using DPPH method.

Sl. No.	Plant extracts	IC_{50} (µg/mL)
1.	Quercetin	5.38±0.02
2.	Curcumin	9.00±0.74
3.	Quercetin+Curcumin	5.29±0.43
4.	Ascorbic acid	2.69±0.05

Table 3: Antioxidant activity using LPO method.

Sl. No.	Plant extracts	IC_{50} (µg/mL)
1.	Quercetin	6.64±0.04
2.	Curcumin	5.93±0.59
3.	Quercetin+Curcumin	5.25±0.37
4.	α- tocopherol	9.66 ± 1.12

Table 4: *In vitro* Cytotoxicity Study.

Sl. No.	Sample Description	IC_{50} (µg/mL)	
		SHSY5Y	HeLa
1.	Quercetin	27.34	24.47
2.	Curcumin	16.65	17.45
3.	Quercetin + Curcumin	15.11	16.74

In-vitro antioxidant activity of isolated Compounds using DPPH and Lipid peroxidation (LPO) assay

The DPPH and LPO models were used to examine the individual and combined *In-vitro* antioxidant activities of curcumin and quercetin. It was concluded from the results depicted in (Tables 2 and 3) that, when curcumin and quercetin were used in combination, their combined percentage antioxidant activity was significant than their individual percentage antioxidant effects at the same concentration. Thus, it was determined that using curcumin and quercetin as combination had a synergistic antioxidant effect (Figure 5).

In-vitro cytotoxicity assay

Cytotoxicity assays are commonly employed to assess the capacity of cells to withstand exposure to toxins. These assays rely on the assumption that dead cells or their metabolites will not reduce tetrazolium. The accuracy of the assay is influenced by both the quantity of cells present and the mitochondrial function of each individual cell. The process of the assay involves the quantification of the conversion of MTT, a yellow tetrazolium salt, to a blue formazan derivative by viable cells. From this assay, it was concluded that, curcumin and quercetin in combination are more potent as shown in (Table 4) (Figure 6).

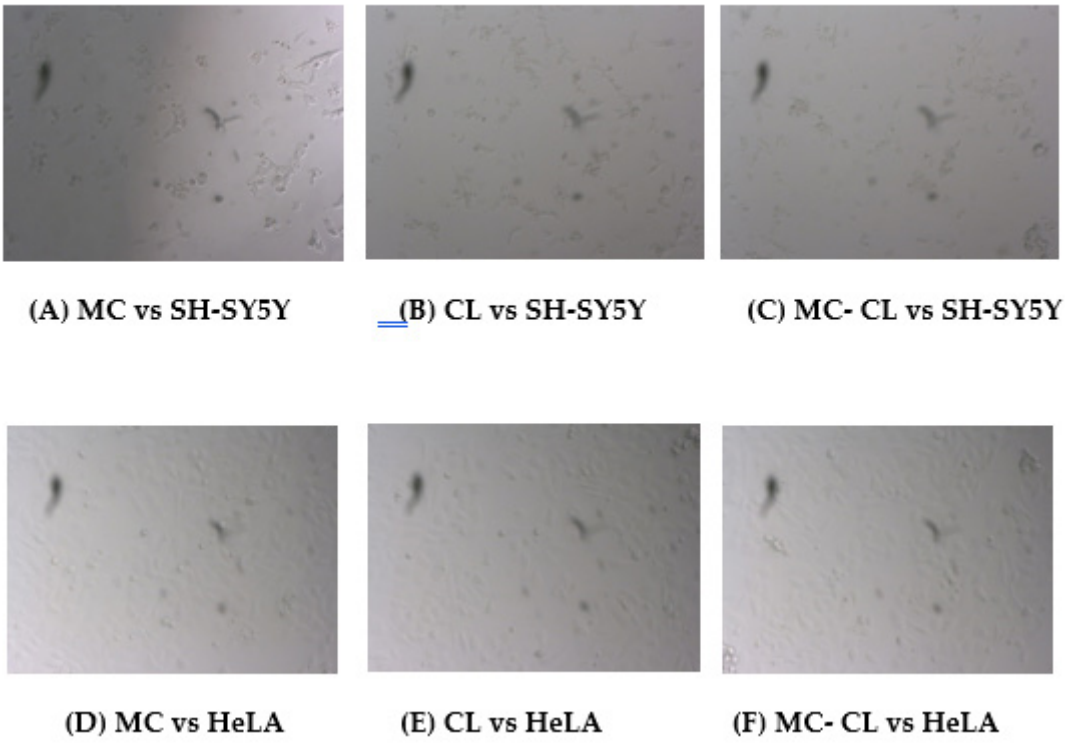


Figure 6: *In vitro* cytotoxicity assay using SH-SY5Y and HeLA cell lines. It comprises of six subfigures (A-F) showing cytotoxicity studies on SH-SY5Y and HeLA cell lines.

CONCLUSION

In the present research work, we focused on the isolation of two flavonoids through hydro-alcoholic maceration technique that is curcumin (flavonoid polyphenol) and quercetin (flavonol) from *Curcuma longa* rhizomes and *Morinda citrifolia* fruits respectively. Additionally, the antioxidant activity of isolated compounds were performed via DPPH (2,2-diphenyl-l-picrylhydrazyl) and Lipid Peroxidation (LPO) radical assay *in-vitro* antioxidant models. According to the results, the % yield of hydro-alcoholic extract obtained from *Curcuma longa* rhizomes and *Morinda citrifolia* fruits were found to be 23.06% and 21.08% respectively. The antioxidant studies showed significant activity of curcumin and quercetin in combination as compared to when used individually at same concentration. Hence, the combination of both will be more effective in the treatment of oxidative stress associated with hyperglycemia.

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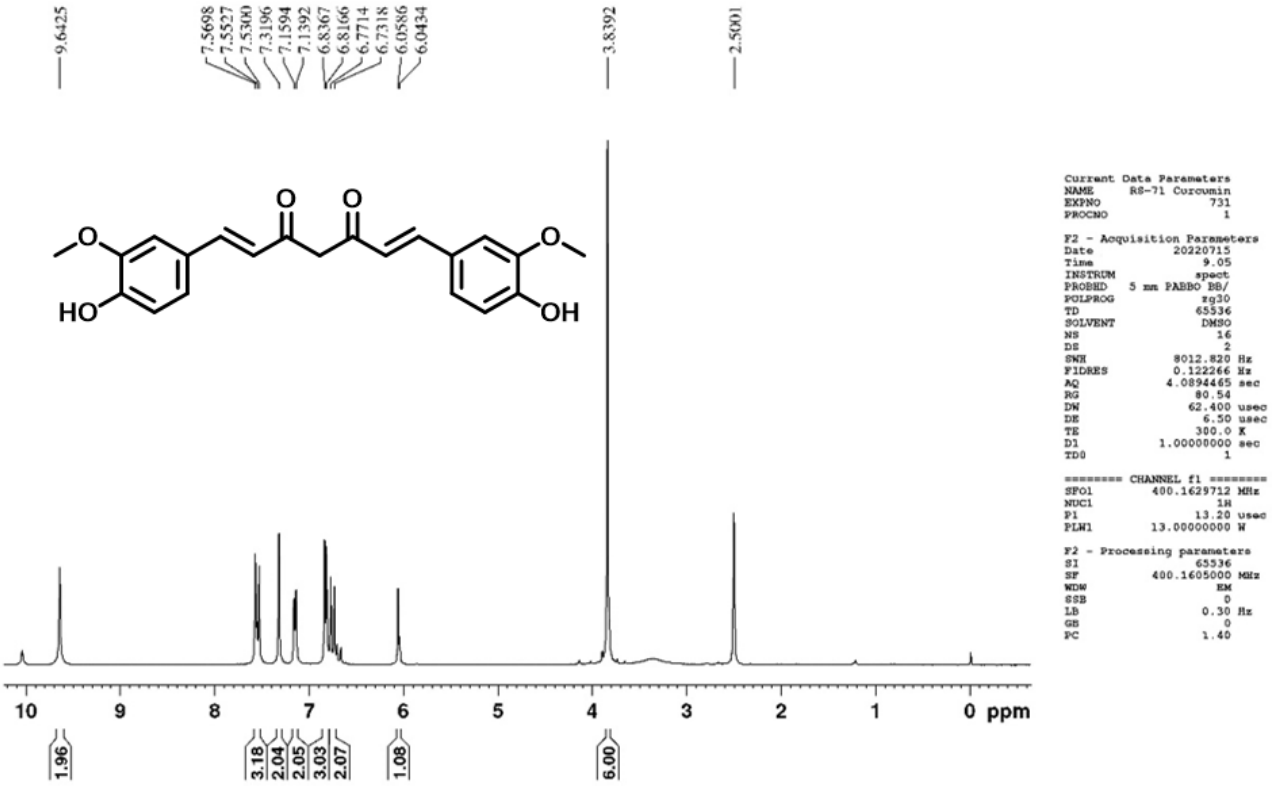

Figure S1: [1]H-NMR spectrum of 1 (400 MHz, DMSO).

Figure S2: [13]C-NMR spectrum of 1 (101 MHz, DMSO).

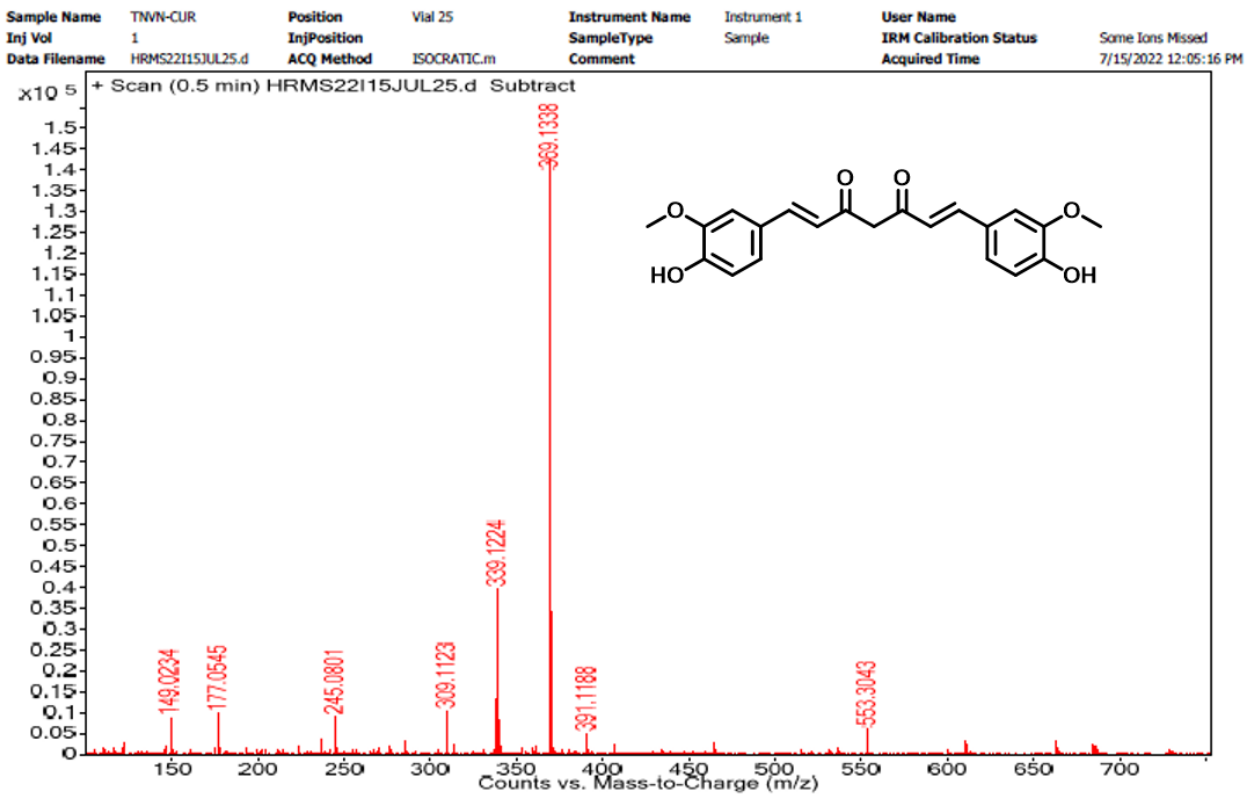

Figure S3: HRMS Spectrum of compound 1.

Figure S4: ^{1}H-NMR spectrum of compound 2 (400 MHz, $CDCl_3$).

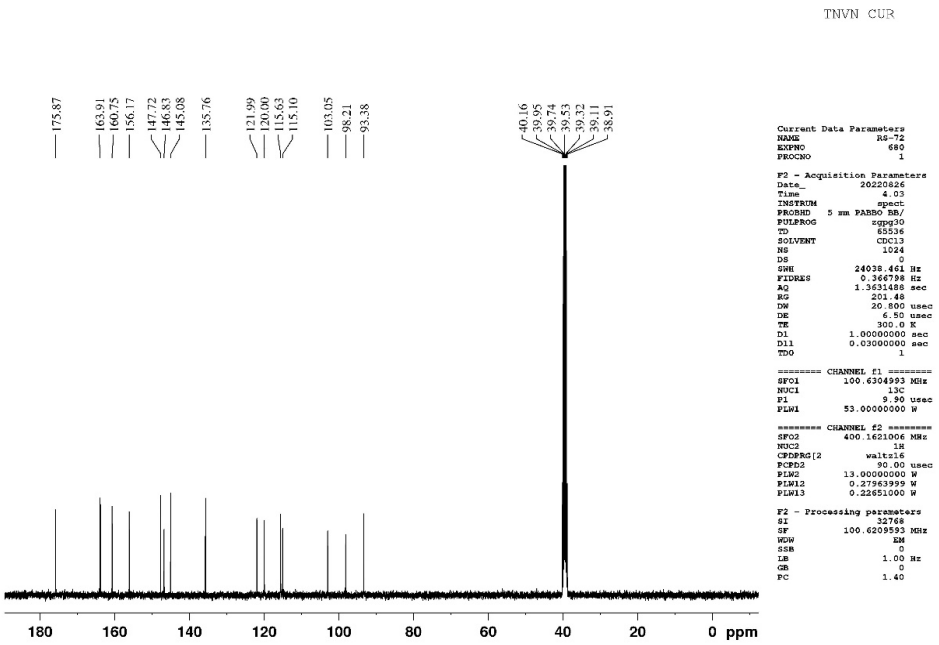

Figure S5: ^{13}C-NMR spectrum of compound 2 (100 MHz, $CDCl_3$).

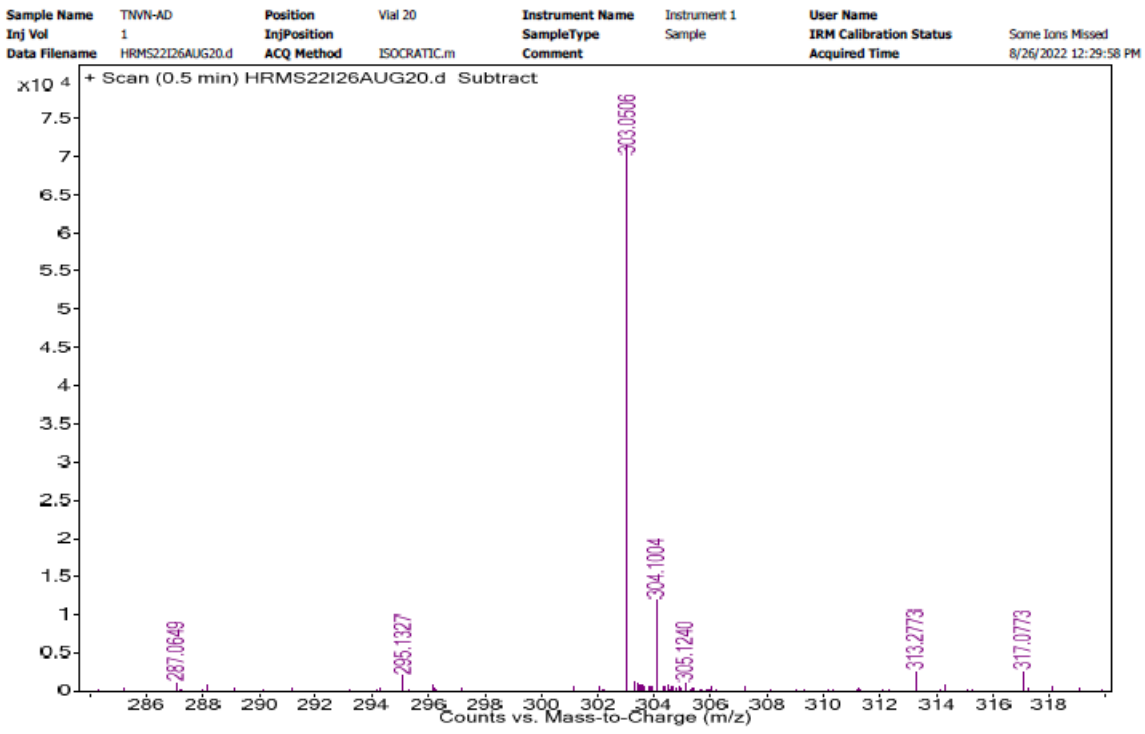

Figure S6: HRMS Spectrum of compound 2.