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Isolation and Pharmacological Activities of Curcumin from Curcuma longa L.

S. Mahalakshmi[1], V. S. Sruthi[2], N. G. Ramesh Babu[3], C. Sivaraj[4] and P. Arumugam[5]
[1]Department of Biotechnology, Adhiyamaan College of Engineering, Hosur-635109, INDIA
[2]Department of Biotechnology, Adhiyamaan College of Engineering, Hosur-635109, INDIA
[3]Department of Biotechnology, Adhiyamaan College of Engineering, Hosur-635109, INDIA
[4]Research Associate, Armats Biotek Training and Research Institute, Guindy, Chennai-600032, INDIA
[5]Research Associate, Armats Biotek Training and Research Institute, Guindy, Chennai-600032, INDIA

[4]Corresponding Author: shivaraj27@gmail.com

ABSTRACT

Curcuma longa commonly known as turmeric belongs to the family Zingiberaceae. It is mainly used for treating chronic diseases especially Diabetes mellitus. The present study aims at evaluating the anti-oxidant, anti-haemolytic, anti-angiogenesis and anti-bacterial activities of Curcumin fraction. Different antioxidant assays were carried out for evaluating the antioxidant capacity of aqueous extract of Curcuma longa. The maximum1, 1-diphenyl 2-picrylhydrazyl (DPPH) radical scavenging activity of curcumin fraction was 30.62±0.87 % at 60 µg/mL concentration. The inhibitory concentration (IC_{50}) values of $DPPH^{\cdot}$ radical scavenging activity was 97.97 µg/mL concentration. The maximum superoxide radical scavenging activity of Curcuma longa extract was 57.01±0.17 % at 60 µg/mL concentration and the inhibitory concentration (IC_{50}) was 46.91 µg/mL concentration. The maximum anti-haemolytic activity of curcumin fraction was 44.53±0.84 and the IC_{50} was 67.37 µg/mL. The maximum Fe^{3+} reduction and Mo^{6+} reduction were 54.85±0.35 % and 61.13±0.21 % at 60 µg/mL concentration and the reduction concentration (RC_{50}) were 51.59 µg/mL and 28.69 µg/mL concentration respectively.

Keywords-- Curcuma longa, Antibacterial activity, Antioxidant activity, Antihaemolytic activity, DPPH

I. INTRODUCTION

Curcuma longa commonly known as turmeric belongs to the family Zingiberaceae. Turmeric is native to the Indian subcontinent and tropical South Asia that requires temperature between 20° C and 30˚C. It is mostly used as a spice, a coloring agent and in traditional medicine such as Ayurveda, Unani[1]. Turmeric is a rhizomatous herbaceous perennial. The height of the plant reaches upto 1 m and has elongated leaves. Rhizomes of the plant are collected every year and are maintained to seed in the successive season. The turmeric derived from rhizome resembles a tuber with rutted and partitioned integument. The maturation of the rhizomes occur below the vegetation in the ground. It is captivated by its dusky and dull orange interior. The main rhizome is attenuated at the distal end which is metered 2.5-7.0 cm (1-3 inches) in length and 2.5 cm (1 inch) in diameter, with little roots spread out[2]. The dehydration and grounding of the turmeric rhizome yields yellow powder with a pungent and sweet taste.

India leads in the manufacture of turmeric crop consuming 80 % of it. A city in the Southern India of Tamil Nadu, Erode, is the largest producer in the world and the major market for turmeric. This city is also known by other names as “Yellow City”, “Turmeric City”, or “Textile City”. Sangli, a city in the state of Maharashtra which is only second in size to Erode, is an important production and trading site for turmeric. Turmeric is valued as a herbal medicine for chronic anterior uveitis, conjunctivitis, rheumatoid arthritis, small pox, skin cancer, urinary tract infections, chicken pox, liver ailments and wound healing. It is also used to treat digestive disorders to minimize menstrual difficulties, jaundice, colic and flatus, also reducing abdominal pain, dilatation as well as for bilious conditions such as anorexia, postpradial feelings of wholeness, and gall bladder and liver problems. The action of turmeric includes anti-inflammatory, choleric, antimicrobic and flatus-relieving problems. Another one of the main targets of turmeric are the intestinal digestive organs. Furthermore turmeric is also used for treating diseases such as familial adenomatous polyposis, inflammatory bowel disease and in treating colon cancer. In case of arthiritis, 8-60 g dosage of fresh turmeric tuber is recommended thrice daily. It is revealed that turmeric is a strong antioxidant, antimutagenic, antimicrobial, anticancer and anti-inflammatory agent in modern in vitro studies.

II. MATERIALS AND METHODS

Preparation of extract by Soxhlet method

The rhizomes of Curcuma longa was collected from the commercial market at Erode, Tamilnadu, India. About 50 g of rhizome was weighed, cut into small pieces and extraction was carried out with methanol by Soxhlet method. The small pieces of fresh rhizomes of

Curcuma longa was loaded and packed in a (12 x 3 cm) thimble made from a strong filter paper which was placed inside the thimble chamber of Soxhlet extractor. The solvent 250 mL of methanol was added to a round bottom flask, which was attached to a Soxhlet extractor and condenser. The whole set up was placed onto the heating mantle. The side arm of siphon was lagged with cotton. The solvent was heated using the heating mantle and will begin to evaporate, moving through the apparatus to the condenser. The condensate then drips back into the thimble container. Once the level of solvent reaches the siphon arm, the liquid contents emptied into the round bottom flask and the cycle begins again. The process should run till the thimble to be colourless. The supernatant in the round bottom flask was condensed in a rotary evaporator at 50° C to yield gummy extract.

In Vitro Antioxidant Assays

DPPH˙ Radical Scavenging Activity

On the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical, the antioxidant activity of the Curcumin fraction of Curcuma longa (2-12 µg/mL) was taken with 1 mL of 0.1 mM DPPH solution in methanol and was incubated in the dark for 30 minutes[4]. 1 mL methanol and 1 mL DPPH solution was used as the control. The decreasing absorbance was measured using the UV-Vis Spectrophotometer at 517 nm. The percentage of DPPH radical inhibition was calculated using:

$$\%\ \text{of DPPH}^{\cdot}\ \text{radical inhibition} = \left(\frac{\text{Control} - \text{Sample}}{\text{Control}}\right) \times 100$$

Superoxide Radical Scavenging Activity

Superoxide radical scavenging activity was carried out by the method of Ravishankara et.al.,[5].The reaction mixture contains different concentrations of Curcumin fraction of Curcuma longa, 50 mM of phosphate buffer (pH 7.6), riboflavin-0.01 gm in 50 mL phosphate buffer, EDTA-1.6 gm in 40 mL phosphate buffer and NBT-10 mg in 10 mL of phosphate buffer accordingly. The reaction was initiated by treating the reaction mixture with UV illuminator for 15 minutes. Immediately, after illumination, the absorbance was measured at 590 nm and the IC_{50} was calculated. The positive control used was the ascorbic acid.

$$\%\ \text{of superoxide radical inhibition} = \left(\frac{\text{Control} - \text{Sample}}{\text{Control}}\right) \times 100$$

Phosphomolybdenum Reduction Assay

The Curcumin fraction of Curcuma longa was evaluated for its antioxidant capacity as explained by Prieto et al [6]. The varying concentrations of the plant extract(50-300 µg/mL) was blended with reagent solution consisting of ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). the mixture was incubated in water bath for 90 minutes at 90° C. the increasing absorbance of the colored complex was read at 695 nm using UV-Vis Spectrophotometer. The standard reference used was distilled water. The percentage of the phosphomolybdenum radical inhibition was estimated using the formula:

$$\%\ \text{of Phosphomolybdenum radical inhibition} = \left(\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right) \times 100$$

Ferric (Fe^{3+}) Reducing Power Assay

The Curcumin fraction of Curcuma longa was determined for its reducing power by the moderately modified method of Yen and Chen[7]. 1 mL of varying concentration of the plant extract (2-12 µg/mL) was combined with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1 % (w/v) potassium ferricyanide[$K_3Fe(CN)_6$]. The reaction mixture was incubated for 30 minutes in water bath.500 µL of 10% (w/v) trichloroacetic acid was added to each mixture. Then to the 1 mL mixture of 0.1% (w/v), $FeCl_3$ was added. The increasing absorbance was read at 700 nm using the UV-Vis Spectrophotometer. The control used was distilled water. The percentage of ferric radical inhibition was evaluated using the formula:

$$\%\ \text{of } Fe^{3+}\ \text{radical inhibition} = \left(\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right) \times 100$$

Antiheamolytic Activity

Heat Induced Haemolysis

The Curcumin fraction of Curcuma longa was evaluated for heat induced haemolysis in which varying concentrations (10 - 60 µg/mL) of the fraction was added, followed by 1% (w/v) saline addition upto 1 mL in all the test tubes. 200 µL of blood suspension was added and incubated at 50° C in water bath for 30 min. The decreasing absorbance was studied at 560 nm using the UV-Vis Spectrophotometer. The percentage of antihaemolytic inhibition was found by the formula:

$$\%\ \text{of antihaemolytic inhibition} = \left(\frac{\text{Control} - \text{Sample}}{\text{Control}}\right) \times 100$$

Antiangiogenesis Activity

Five day-old fertilized eggs were obtained from the local hatchery. Eggs were cleaned with ethanol and pre incubated at 37º C. The egg looks like a meta-ellipse with a relatively larger side and a smaller side. After disinfecting the shell with ethanol, a hole was gently drilled over the air sac with a nipper so as not to break the shell, and the vascular zones were easily identified on the Chorioallantoic membrane (CAM). 5 mL of albumin was aspirated and the eggs were incubated horizontally to allow the CAM to detach from the shell. Agarose containing discs were placed in sterile wash plates. Curcumin fraction was loaded in agarose discs at

concentration of 100 µg/disc. Discs containing the vehicle only (DMSO) were used as negative control. A small window opening was made in the shell, and the discs were directly applied onto the CAM. The square opening was covered with sterilized surgical tape and the embryos were incubated for 48 hours at 37º C. The CAMs were photographed under a dissecting microscope and blood vessels in each CAM were counted.

Antibacterial Activity

Microbial Strains

Bacillus subtilis, Micrococcus luteus, Escherichia coli and Proteus vulgaris were used for the evaluation of antibacterial activity.

Reference and Control

Tetracycline was chosen as the standard reference. The control consists of solidifying agar onto which solvent and the test compounds were soluble.

Aseptic Chamber

Using 70 % ethanol, the aseptic chamber which is composed of a wooden box (1.3m x 1.6m x 0.6m) with a door and was illuminated with short wave UV light (from light)

Nutrient Broth Agar Medium

The standard method was used to formulate the nutrient agar broth medium.

Agar well Diffusion Method

The agar well diffusion method was implemented for the antimicrobial activity of Curcumin fraction of Curcuma longa[8]. The solidified nutrient agar in the petriplate was inoculated by using sterilized cotton swab, which was immersed in the inoculum containing test tubes and spread evenly onto the solidified agar medium. The help of sterile template of 8 mm diameter was used to create 5 wells in each plate. The plant extract of concentration (100, 150 and 200 µg/mL) was poured into each well. The positive control used was tetracycline (25 µg/mL). The extract loaded wells in each plate were incubated at 37° C for 24 hours. The diameter of the inhibition zone formed around the well was measured for its antimicrobial activity.

Thin Layer Chromatography

Thin Layer Chromatography (TLC) was performed for the Curcumin fraction of Curcuma longa in Merck TLC aluminium sheets, silica gel 60 F254 (20 x 20 cm), preloaded plates. The plant extract was dotted at 0.3 mm form above the bottom of the TLC plate. A mixture of suitable solvent system was used to generate the chromatogram. The spots were viewed under the UV light at 356 nm. The retention factor (R_f) values of the coloured spots were recorded. The ratio in which distinct bands appeared was optimized and the retention factor (R_f) values were calculated[9].

Calculation of R_f value:

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Gas Chromatography–Mass Spectrometry (GC–MS)

For Gas Chromatography- Mass Spectrometry analysis, the samples were were introduced into a HP-5 column (30 m X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. The chromatographic conditions were used consequently: Helium as carrier gas, flow rate of 1 mL/min; and the injector was handled at 200° C and 50° C- 250° C column oven temperature was scheduled at a rate 10° C per minute injection mode. The given MS conditions were followed.: Ionization voltage of 70 eV; ion source temperature of 250° C; interface temperature of 250° C; mass range of 50-600 mass units[10].

Identification of Components

The connotation on mass spectrum of GC-MS was estimated from the database of the Ntional Institute Standard and Technology (NIST) which has more than 62,000 patterns. The mass spectrum of the unknown components was differentiated from that of the known components reserved in the NIST library.

III. RESULTS AND DISCUSSION

DPPH˙ Radical Scavenging Assay

Scavenging of DPPH free radical is one of the popular antioxidant assays. DPPH radical scavenging assay is a decolourization assay that will measure the capacity of antioxidants to directly scavenge DPPH˙ radicals by monitoring its absorbance using UV-Vis Spectrophotometer at a wavelength of 517 nm[11]. The ability of curcumin fraction to scavenge free radicals formed was assessed using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). At 60 µg/mL concentration, 69.045±0.95 % was the maximum DPPH radical scavenging activity (Table 1, Fig 2). The stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was reduced to the yellow colored 1,1-diphenyl-2-picrylhydrazyl (DPPH) by the Curcumin fraction which exhibited high capacity for scavenging free radicals. With the increase in the concentration of the extract the reducing capacity also increases. The inhibitory concentration (IC_{50}) was found to be 97.97 µg/mL concentration which was correlated with the standard ascorbic acid (IC_{50} = 11.98µxg/mL concentration).

Table 1: DPPH˙ radical scavenging activity of Curcumin fraction

Sl. No.	Concentration (µg/mL)	Curcumin fraction
1	10	1.77±0.33
2	20	9.67±0.62
3	30	14.3±0.43
4	40	16.67±0.34
5	50	19.1±0.18
6	60	30.62±0.87

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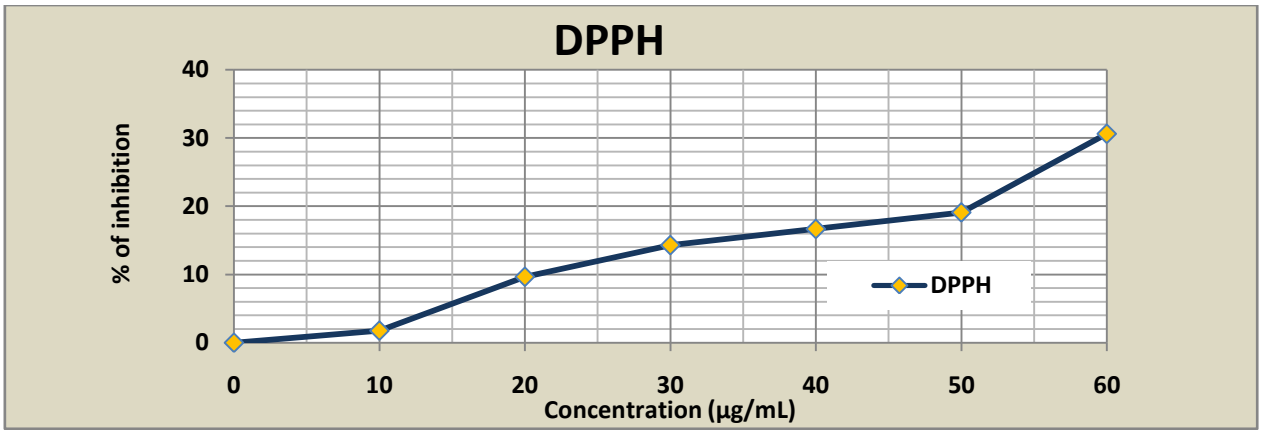

Figure 1: DPPH˙ radical scavenging activity of curcumin fraction

Superoxide ($O_2^{\cdot-}$) Radical Scavenging Activity

The superoxide anions have the capability to produce various kinds of free radicals and oxidizing agents which are toxic to the biological material and their effects can be intensified[12]. The scavenging of superoxide anion is done strongly by the flavonoids. The reduction of NBT in a system is done by the superoxide anion, attained from dissolved oxygen by the riboflavin-light-NBT system. In this technique, the yellow dye (NBT^{2+}) is reduced to blue formazon via superoxide anion that is measured by the UV-Vis Spectrophotometer at 590 nm. The decrease in absorbance with antioxidant specifies the inhibition of blue NB formation which depletes the superoxide anion in the mixture. At 60 µg/mL concentration, 57.01±0.17 % was the maximum superoxide radical scavenging activity of Curcumin fraction (Table 2, Fig 3). The inhibitory concentration (IC_{50}) was found to be 46.91 µg/mL concentration with the standard ascorbic acid (IC_{50} = 9.65 µg/mL concentration).

Table 2: Superoxide ($O_2^{\cdot-}$) radical scavenging assay of Curcuma longa fraction of Curcuma longa

Sl. No.	Concentration (µg/mL)	Curcumin fraction
1	10	3.82±0.35
2	20	7.46±0.45
3	30	21.2±0.31
4	40	46.20±0.30
5	50	53.29±0.39
6	60	57.01±0.17

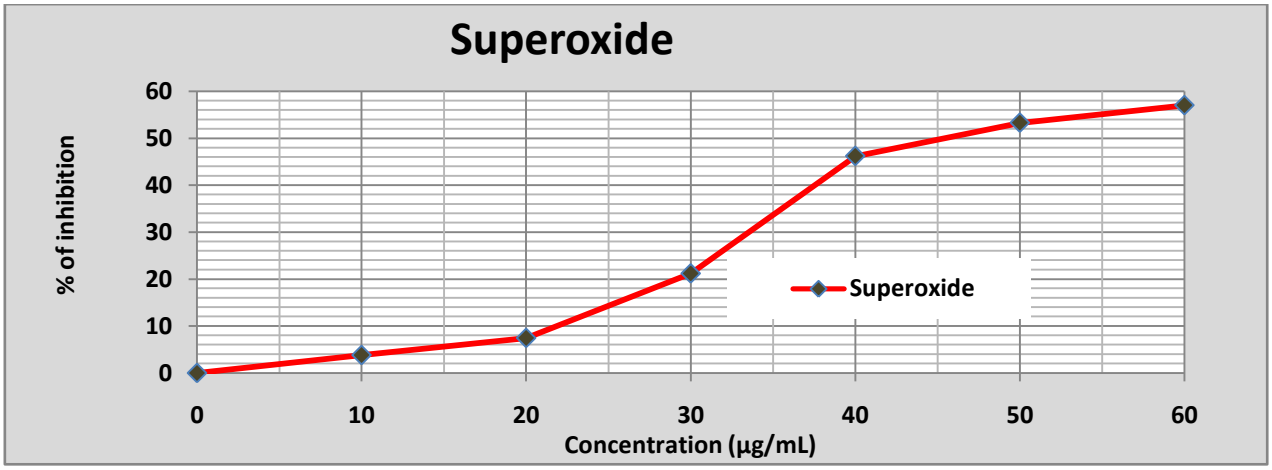

Figure 2: Superoxide radical scavenging activity of Curcumin fraction of Curcuma longa

Phosphomolybdenum Reduction Activity

The phosphomolybdenum reduction assay was followed to measure the total antioxidant capacity of Curcumin fraction of Curcuma longa which depends on the Mo (VI) to Mo (V) reduction characterized by the appearance of green phosphate/Mo (V) complex at acidic pH with maximum absorbance found at 695 nm[13]. At 60 µg/mL concentration 61.13±0.21 % was the maximum phosphomolybdenum reduction of curcumin fraction (Table 3, Fig 4). The reduction concentration RC_{50} was found to be 28.69 µg/mL concentration which was compared with that of the standard ascorbic acid (RC_{50} = 6.34µg/mL concentration). This assay is a significant method to examine the reduction rate among oxidant, antioxidant and molybdenum ligands. During prolonged incubation period at a high temperature, it leads to thermally generating auto-oxidation.

Table 3: Phosphomolybdenum reduction activity of Curcumin fraction of Curcuma longa

Sl. No.	Concentration (µg/mL)	Curcumin fraction
1	10	5.45±0.61
2	20	14.91±0.36
3	30	18.65±0.46
4	40	36.87±0.34
5	50	48.45±0.60
6	60	54.85±0.35

Table 4: Ferric (Fe^{3+}) reducing power activity of Curcumin fraction of Curcuma longa

Sl. No.	Concentration (µg/mL)	Curcumin fraction
1	10	14.7±0.59
2	20	25.24±0.34
3	30	52.28±0.39
4	40	55.28±0.38
5	50	59.83±0.23
6	60	61.13±0.21

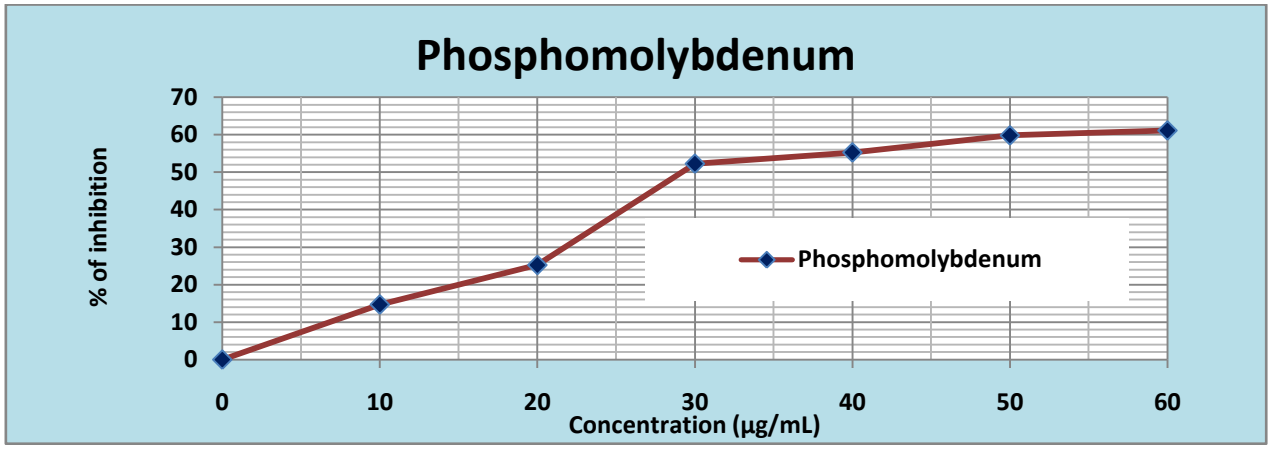

Figure 3: Phosphomolybdenum reduction activity of Curcumin fraction of Curcuma longa

Ferric (Fe^{3+}) Reducing Power Activity

The Curcumin fraction of Curcuma longa helps in the reduction of the Fe^{3+} to Fe^{2+} by the consequent production of ferro-ferric complex. The increase in concentration of the extract also increases the reduction ability[14]. At 60 µg/mL concentration, 54.85±0.35 % was the maximum Fe^{3+} reduction power of Curcumin fraction (Table 4, Fig 5). The reduction concentration (RC_{50}) was found to be 51.59 µg/mL concentration which was compared with that of standard ascorbic acid (RC_{50}= 7.72 µg/mL concentration). The higher reduction potential in this activity is revealed by the higher absorbance of the reaction mixture. The reducing capacity of aqueous extract holds as an important indicator of its potential antioxidant activity. In this assay, the colour change from yellow to green or blue colour is brought by the reduction of Fe^{3+} to Fe^{2+} which is dependent on the concentration of antioxidant. This trait of the antioxidant is revealed by the components such as phenolic acids, sand flavonoids present in the extract of Curcuma longa in small amounts which indicated the reducing activity that depends on the concentration.

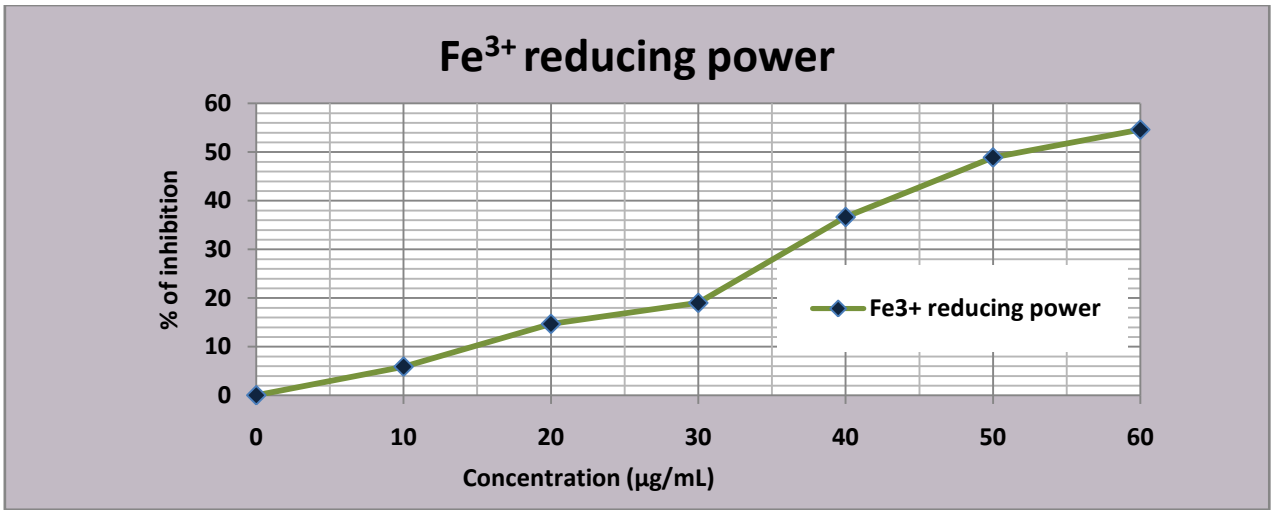

Figure 4: Ferric (Fe^{3+}) reducing power activity of Curcumin fraction of Curcuma longa

Antiangiogenic Activity

Antiangiogenesis is the quantification involved measuring of the length and number of branching microblood vessels. Curcumin fraction seems to specifically inhibit the microvascular formation that occurs normally during embryogenesis. The CAMs were photographed under a dissecting microscope and blood vessels were counted. Angiogenesis is an energetic propagation and differentiation procedure, which have need of endothelial propagation, passage and tube development. Tumors with physically powerful angiogenic action are associated with a lesser cancer patient survival speed. The combination of this technique and new strategies in medication discovery has large prospective in medication of the future. Anti-angiogenic medicines have fewer side effects than other procedures for curing cancer. The CAM regions were reduced in treated eggs by using Curcumin fraction. The diameter of treated eggs blood vessels and the length of Abbott curve and standard surface roughness were also reduced in the egg, which was treated.

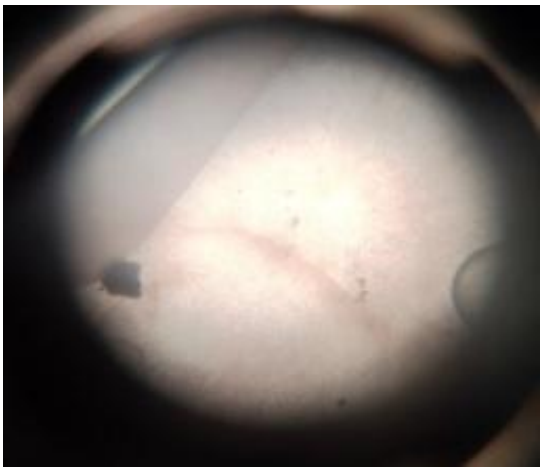

Figure 5: Antihaemolytic activity of Curcumin fraction of Curcuma longa

Antihaemolytic Activity

Most of the cells in the human body are found to be the erythrocytes and they have been widely exploited in drug transport. The polyunsaturated fatty acids (PUFA) and hemoglobin molecules which are redox active oxygen transport molecules and potent promoters of activated oxygen species mainly target the erythrocytes. Oxidative mutilation to the erythrocyte membrane lipids and proteins may be responsible for hemolysis accompanying with several factors viz. hemoglobinopathies, oxidative drugs, excess of transition metals, various radiation, and deficiencies in erythrocyte antioxidant coordination. The maximum antihaemolytic activity of curcumin fraction of Curcuma longa was 57.01±0.17% at 60 µg/mL concentration and the IC_{50} was 46.91 µg/mL concentration.

Table 5: Antihaemolytic activity of Curcumin fraction of Curcuma longa

Sl. No.	Concentration (µg/mL)	Curcumin fraction
1	10	3.82±0.35
2	20	7.46±0.45
3	30	21.2±0.31
4	40	46.20±0.30
5	50	53.29±0.39
6	60	57.01±0.17

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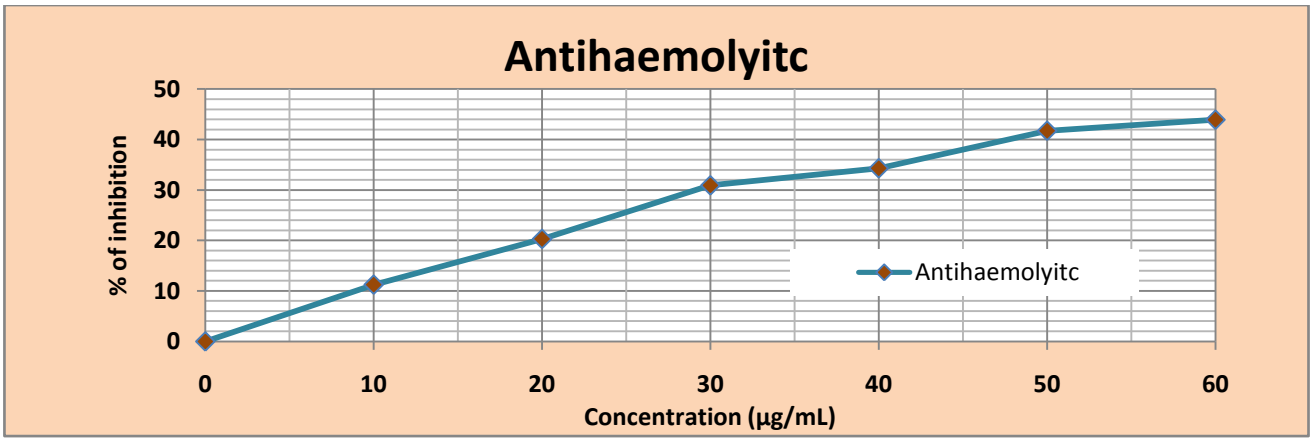
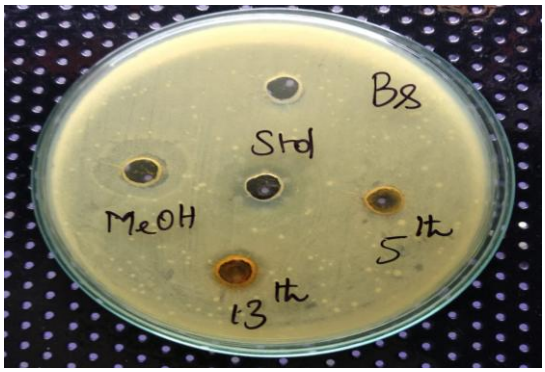


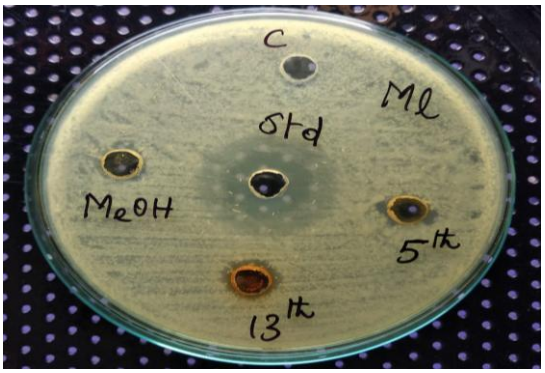
Figure 6: Antihaemolytic activity of Curcumin fraction of Curcuma longa

Antibacterial Activity

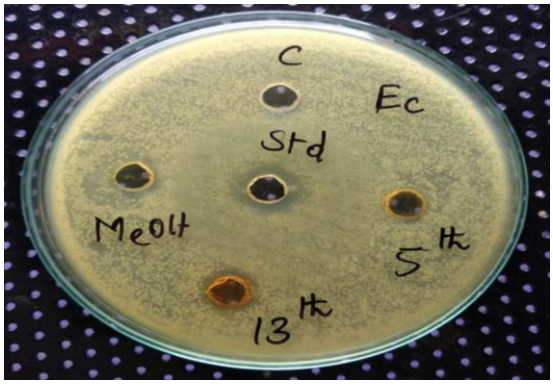
The Curcumin fraction of Curcuma longa was investigated for in vitro antibacterial activity against microorganisms such as Bacillus subtilis and Micrococcus luteus which are Gram-positive bacteria, and Escherichia coli and Proteus vulgaris which are Gram-negative bacteria. The effectiveness and the antimicrobial susceptibility of the crude extract was analysed quantitavely by measuring the diameter of the clear zone in cultures contained in petriplates. At 500 µg/mL concentration, the maximum zone of inhibition was 20 mm for Escherchia coli. Due to the presence of phenolic compounds, tannin, alkaloids and terpenoids which are secondary metabolites, the antibacterial activity may show the inhibition of bacterial growth[15].



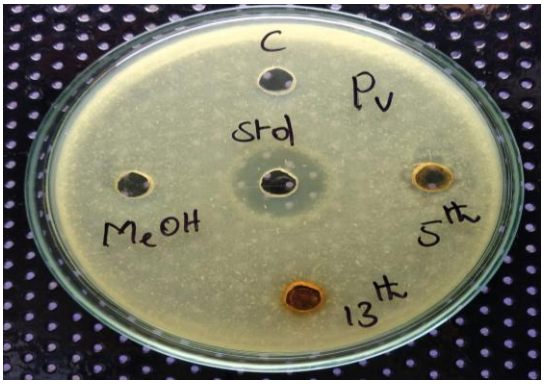
Bacillus subtilis



Micrococcus luteus



Escherichia coli



Proteus vulgaris

Figure 7: Antibacterial activity of Curcumin fraction of Curcuma longa

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Table 7: Antibacterial activity of Curcumin fraction of Curcuma longa

Sl. No	Bacteria	Zone of inhibition mm			Standard (Tetracycline) 25 µg/mL
		250 µg/mL			
		5^{th} fraction	13^{th} fraction	Methanol fraction	
1	Bacillus subtilis	10	10	13	13
2	Micrococcus luteus	10	10	-	15
3	Escherichia coli	10	12	13	13
4	Proteus vulgaris	10	10	-	14

Thin Layer Chromatography

The analysis of the extract in solvent system, Methanol:Chloroform in the ratio of 1.5:0.5 was performed using thin layer chromatography (TLC) and the separated compounds are shown in the Figure below and the values are expressed in terms of R_f.

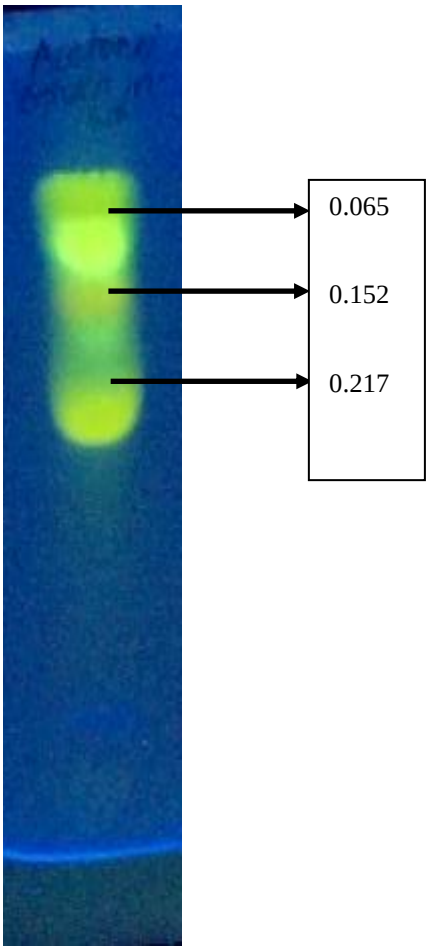


Figure 8: Compounds separated by Thin Layer Chromatography

Gas Chromatography-Mass Spectrometry Analysis

Gas Chromatography-Mass Spectrometry analysis of Curcumin fraction of Curcuma longa is shown in the following Table 8.

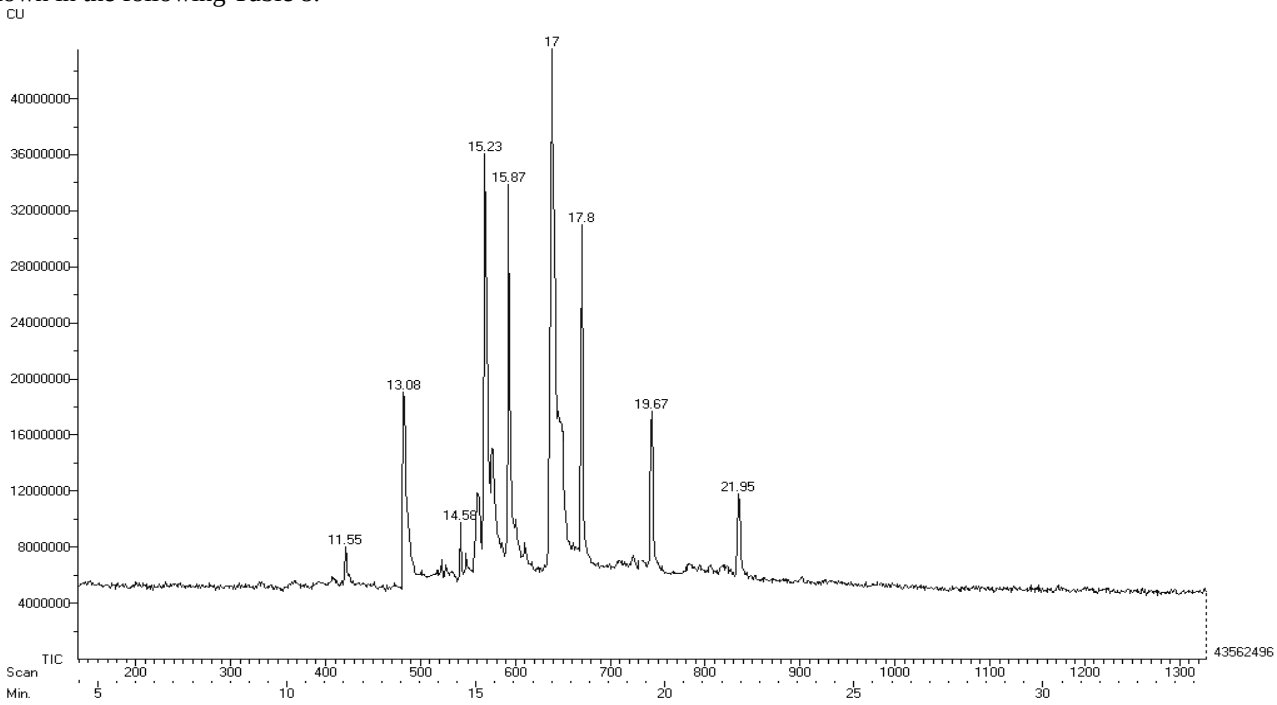
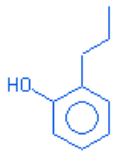
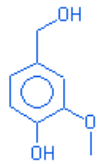
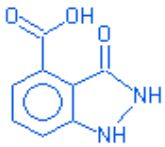
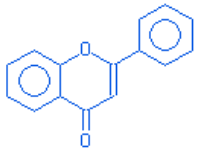
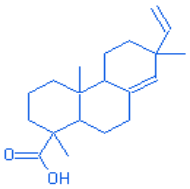
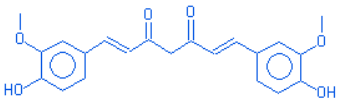


Fig.9 GC- MS Chromatogram of Curcumin fraction of Curcuma longa

Table 8: GC- MS analysis of Curcumin fraction of Curcuma longa

S. No	Compound name	RT	Compound structure	Mol. weight g/mol	Mol. formula
1.	Phenol, 2-propyl-	11.55		136.19	$C_9H_{12}O$
2.	4-Hydroxy-3-methoxybenzyl alcohol	13.08		154.16	$C_8H_{10}O_3$
3.	3-Oxo-2,3-dihydro-1H-indazole-4-carboxylic acid	14.58		178.14	$C_8H_6N_2O_3$

4.	Pentadecane	15.23		212.42	$C_{15}H_{32}$
5.	Flavone	15.87		222.24	$C_{15}H_{10}O_2$
6.	E-6-Tetradecen-1-ol acetate	17		272.42	$C_{16}H_{32}O_3$
7.	1-Eicosene	17.8		280.53	$C_{20}H_{40}$
8.	Dextropimaric acid	19.67		302.45	$C_{19}H_{29}COOH$
9.	1,6-Heptadiene-3,5-dione, 1,7-bis(4-hydroxy-3-methoxyphenyl)-	21.95		368.38	$C_{21}H_{20}O_6$

IV. CONCLUSION

Antioxidants are substances that significantly delay or prevent the oxidation of an oxidisable substrate when present in low concentrations. Plants are potential sources of invaluable antioxidants. The results of the present study indicate that Curcumin fraction of Curcuma longa has significant antioxidant activities in reducing harmful effect of radicals. The results of the present study provide promising hope to use Curcuma longa as an antioxidant agent. The curcumin fraction of Curcuma longa also revealed the properties of antihaemolysis, antimicrobial activity and antiangiogenesis, which is a major futuristic novelty in the therapeutic industry as well.

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