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Synthesis, Characterisation, Analgesic, Anti-inflammatory, Anti-ulcer, Wound healing and Antimicrobial effects of Curcuminoids

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Abstract: New Schiff bases of Curcumin have been synthesized by condensing it with *o*-phenyl enediamine/aniline. The structural features of the synthesized compounds have been determined from their elemental analysis, melting point, IR, UV-Vis, ^{1}H-NMR and mass spectral data. The investigated compounds were utilized to test the various biological activities like analgesic, anti-inflammatory, anti-ulcer and wound healing effects on albino rats. The biological effects of the compounds were compared with standard drugs like Pentazocin, Diclofenac sodium, Ranitidine and Curcumin. The *in-vitro* biological screening effects of the investigated compounds were tested against the bacteria, *Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli* and *Bacillus megatrium* and fungi *Candida albicans* by the well diffusion method and their zone of inhibitions at MIC values are reported.
Key words: curcumin, *o*-phenylenediamine, aniline, analgesic, anti-inflammatory, anti-ulcer, wound healing effect and antimicrobial activity.

Introduction

Curcumin (1,7-bis-(4-hydroxy-3-methoxy phenyl)-1,6-heptadiene- 3,5-dione) is a natural yellow pigment and extracted from the root of Curcuma longa which belongs to the family of Zingiberaceae. It is considered to be a stomachic, bitter aromatic cooling, astringent, and carminative[1]. Rhizomes are used externally in the form of paste over sprains and skin diseases[2]. Combined with other medicines, they are also used for improving the quality of blood[3]. Curcumin is the main active compound, possessing anti-inflammatory, hepatoprotective[4], nephroprotective[5], antimicrobial, wound healing, anticancer, anti-tumor and anti-viral properties[6&7]. Due to various important properties, Curcumin has been used for several clinical studies. Curcumin acts as a free radical scavenger and antioxidant, inhibiting lipid peroxidation and oxidative DNA damage[8]. Curcuminoids induce glutathione S-transferase and are potent inhibitors of cytochrome

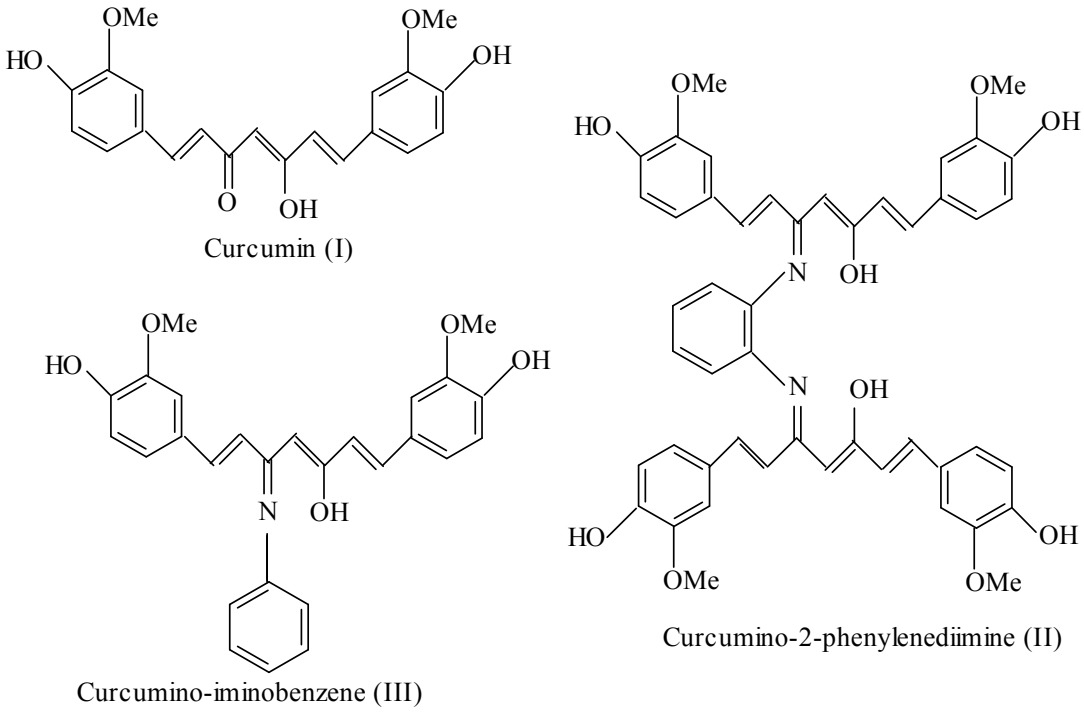
P450. It is a complexing agent which can give inter molecular and intra molecular bondings[9]. It is not stable to light, especially in solutions. It has poor bio availability, fast metabolism and requiring repetitive oral doses[10]. A search through the literature reveals that no work has been done on the condensation between Curcumin and o-phenylenediamine/aniline[11-13]. In order to analyse these problems, reduce the side effects[14] and improve the biological activities of Curcumin, this paper describes the synthesis, characterization, analgesic, anti-inflammatory, antimicrobial activities and wound healing effects of new Schiff bases derived by the condensation of Curcumin and o-phenylenediamine/aniline. The structure of the Curcumin and its Schiff bases is given in Scheme 1.

Experimental

All the chemicals and solvents were Merck products and were used without purification. Spectroscopic grade solvents were used for spectral measurements. Elemental analyses and mass spectral data of the samples were measured at Sophisticated Analytical Instrumentation Facilities, IIT, Mumbai. The ^{1}H- NMR spectra of the samples were recorded on a Brucker 300 spectrometer using $CDCl_3$ as solvent. Chemical shifts (δ) are reported in ppm relative to tetramethyl silane at Madurai Kamaraj University, Madurai. The IR spectra of the samples were recorded in KBr pellets using a Shimazdu 8400 Spectrophotometer. The UV-Vis. spectra of the investigated compounds were recorded on a Shimadzu UV-1604 spectrophotometer. The animal study was approved by the animal ethical committee (509/02/C/CPCSEA/2002).

Synthesis of curcumino-2-phenylenediimine

Curcumin (0.1 mol) and *o*-phenylenediamine (0.05 mol) was dissolved in 50 ml of ethanol and refluxed for 6 h at 70^{o}C. The resulting solution was concentrated and collected in a beaker. To this mixture, 20 ml of petroleum ether (40-60^{o}C) was added and kept at 0^{o}C for 48 h. A light brown solid mass obtained was collected and recrystallised in hot ethanol. Curcumin was named as compound I and the synthesized compound was named as compound II.



Scheme 1: The structure of curcumin, curcumino-2-phenylenedimine and curcumino-iminobenzene.

Table 1. Physical characterization, P^H, elemental analysis, λ_{max}, IR and ^1H NMR spectra data of the synthesized compound

S. No	Description		Compound code	
			II	III
1.	Yield (%)		85	72
2.	Empirical formula		$C_{48}H_{44}O_{10}N_2$	$C_{27}H_{25}O_5N$
3.	M. Wt.		808	443
4.	Melting point (^oC)		155	190
5.	Colour		Dark Brown	Brown
6.	pH		9.47	8.6
7.	Found (Calc)	C	71.23 (71.29)	64.14 (64.54)
		H	5.37 (5.45)	4.75 (4.95)
		N	3.49 (3.47)	3.19 (3.16)
8.	λ_{max} (nm)		360, 440	435, 465
9.	IR data (cm^{-1})		-OH : 3600 – 2800, -OMe : 1373, -C=N : 1583, -C=C- : 1510, aromatic ring –C=C- : 1460, - C - O- C- for OMe : 1217, enolic –C-O- : 975, Phenolic stretch : 1350-1275. Aliphatic –C=CH stretch: 750, 815 and 850.	-OH : 3500 – 2700, -OMe : 1375, -C=N : 1593, -C=C- : 1512, aromatic ring –C=C- : 1465, - C - O- C- for OMe : 1225, enolic –C-O- : 968, Phenolic stretch : 1340-1280. Aliphatic –C=CH stretch: 696, 752 and 820.
10.	^1H NMR data (PPM)		-OMe (s) : 3.9, -OMe azomethine side (s) ; 4.0, phenolic – OH (s) : 7.4, enolic – OH (s) : 11.1, active –CH (s) : 6.05, -C=CH on enolic side (d,d) 5.7 & 6.2, -C=CH on azomethine side (d,d) : 6.7, 7.0, Benzene ring in curcumin moiety (s,d,d) : 7.3, 7.5, 7.6, Benzene ring in o-phenylenediamine moiety (d,d) : 7.9 and 8.1.	-OMe merged (s) : 4.1 phenolic – OH (s) : 7.8, enolic – OH (s) : 10.1, active –CH (s) : 7.05, -C=CH on enolic side (d,d) 5.9 & 6.5, -C=CH on azomethine side (d,d) : 6.9, 7.2, Benzene ring in curcumin moiety (merged s,d,d) : 7.54, 7.4, 7.5, Benzene ring in aniline moiety (merged d,q,q) : 7.9, 8.1 and 8.2.

Synthesis of curcumino-iminobenzene

50 ml of ethanolic solution, curcumin (0.1mol) and aniline (0.05mol) were taken in a round bottom flask and refluxed for 6 h at 70^{o}C and the resulting solution was concentrated. To this mixture, 20 ml petroleum ether (40-60^{o}C) was added and kept at 0^{o}C for 48 h days. A light brown solid mass was collected and recrystallised in hot ethanol and named as compound III. The physical characterization and various spectral data of these synthesized compounds were tabulated in Table 1.

Analgesic activity

The analgesic activity of the synthesized compounds was carried out in albino rats by Tail flick method[15&16] using 0.5 % solution of CMC as vehicle and Pentazocin was used as a standard. Healthy albino rats of either sex (150-200 g) were taken and divided into 5 groups (5 animals in each group). They were housed in polypropylene cages and maintained under standard conditions (12 h light/dark cycles at (25±3^{o}C). The animals were fasted for 24 h before the experiment. The basal reaction time of each rat was noted by placing the tip of the tail of rat on the hot water at 55^{o}C. Group 1 animals were received 1 ml of 0.5 % solution of CMC orally and group 2 animals were received Pentazocin (30mg/kgi.p). The Curcumin and synthesized drugs (30 mg/kg) were suspended in the vehicle and administered orally into groups 3, 4 and 5. The basal reaction times of albino rats were noted at different interval of time (15, 30, 45 and 60 min.) and the percentage of increase in basal reaction time was calculated and summarized in Table 2.

Table 2. Analgesic activity of synthesized compounds by Tail flick method

Compound code		Basal reaction time (Mean ± SEM, sec.)				% Index of Analgesia $= \frac{V_t - V_c}{V_t}$
	Dose/kg	15 min.	30 min.	45 min.	60 min.	
Vehicle	1ml	3.20± 0.4	2.92 ±0.14	2.54 ±1.16	2.22± 1.2	-
Standard	30mg	6.93± 0.48*	6.85± 0.23*	7.02 ±0.42*	8.24 ±1.04*	73.06
I	30mg	5.84± 0.39*	7.73± 0.47*	8.36± 0.28*	8.9 ±0.19*	76.26
II	30mg	5.20± 0.24*	5.60 ±0.97*	5.88 ±1.64*	6.22± 1.06*	66.45
III	30mg	6.93 ±0.90*	6.52± 1.02*	6.80±0.72*	7.02± 0.40*	68.38

*P<0.05 is considered as significant, V_t = value of test substance and V_c= value of control

Table 3. Anti-inflammatory studies of synthesized compounds

		Paw volume (Mean ± SEM, ml)					
S.No.	Groups	Dose/kg	0 h	1 h	2 h	3 h	% protection $= \frac{V_c - V_t}{V_c}$
1	Vehicle	1 mL	0.9±0.04	1.2±0.02	1.5±0.01	1.7±0.02	-
2	Standard	100 mg/kg	1.1±0.03	1.8±0.01**	1.5±0.16**	1.2±0.01**	55.56
3	I	100 mg/kg	1.8±0.03	2±0.01**	1.2±0.2**	1.0±0.03**	62.96
4	II	100 mg/kg	1.6±0.03	3.2±0.01*	2.8±0.20*	1.4±0.03*	48.15
5	III	100 mg/kg	1.2±0.01	2.6±0.03*	2.4±0.12*	1.5±0.12*	44.44

Significant **P < 0.01 and *P < 0.02, V_t = value of test substance and V_c= value of vehicle

Anti-inflammatory activity

The investigated compounds were utilized to test the anti-inflammatory activity by Carrageenan induced rat hind paw edema method[17] using 0.5% solution of CMC as vehicle and Diclofenac sodium (100mg/kgi.p) as standard. 25 Healthy albino rats (150–200 g) were taken and divided into 5 groups (5 animals in each group). The animals were fasted for 24 h before the experiment. The initial paw volume of each rat was found by mercury displacement method. Group 1 received 1 ml of the vehicle orally and group 2 animals were received Diclofenac sodium (100mg/kgi.p). The curcumin and synthesized drugs (100mg/kg) were suspended in the vehicle and administered orally into groups 3, 4 and 5. After 30 min., acute inflammation was induced in rats by injecting 0.1 ml of 1%(w/v) solution of Carrageen an into the sub plantar region of the left paw. The paw volumes were measured at 1, 2 and 3 h interval and the percentage protection of paw edema was calculated and mentioned in Table 3**.**

Anti-ulcer effects

The anti-ulcer effects of the investigated compounds were studied [18-20] in aspirin induced ulcers in albino rats by using 2% gum acacia solution as vehicle and Ranitidine as standard. Albino rats (150-200 g) were housed 5 in each group in a standard laboratory conditions. They were fasted for 24 h before doing the experiment. They were deprived of food and water during experiment. Group 1 animals received 1mL of gum acacia solution and group 2 animals received Ranitidine (20mg/kgi.p) orally. Group 3, group 4 and group 5 animals received I,II and III compounds which were suspended in gum acacia solution (20 mg/kg). After 4 h the stomach of albino rats was opened and the gastric content was collected followed by counting the number of ulcers. The volume and P^H of the collected gastric juice were measured. Free and total acidity were estimated by titrating it with 0.01 N standard NaOH solutions using methyl orange followed by phenolphthalein indicators. The number of ulcers was counted and ulcer index (UI) was calculated using the formula:

$$\text{Ulcer index} = \frac{10}{X}$$

$$\text{Where, } X = \frac{\text{Total mucosal area}}{\text{Total ulcerated area}}$$

The P^H, free acids, total acidity and volume of acid secretion of gastric juice and ulcer index were analyzed by using ANOVA followed by multiple range test and were reported in Table 4.

Table 4. Anti-ulcer effect of synthesized compounds in aspirin induced ulcers in albino rats

S. No.	Treatment	Dose	P^H	Free acids mEq./L	Total acidity mEq./L	Ulcer index (UI)
1	Vehicle	1ml	3.40±0.05	28.23±0.26	45.67±1.21	4.35±0.30
2	Standard	20mg	3.4*±0.12	13.2*±0.21	31.46*±0.85	0.008*±0.02
3	I	20mg	3.6*±0.2	22.9*±0.19	43.26*±0.62	2.72*±0.12
4	II	20mg	2.87*±0.025	19.37*±0.58	40.47*±0.40	2.35*±0.17
5	III	20mg	3.10*±0.18	15.18*±0.42	33.52*±0.37	2.28*±0.15

All values represent Mean ± SEM, n = 5 in each group, *p= <0.001

Table 5. The wound healing effect of the Curcumin and synthesized compounds by excision wound model method

Days	Control	I	II	III
1	2.5 ± 0.1	2.6 ± 0.2	2.5 ± 0.2	2.5 ± 0.1
5	2.3 ± 0.1	2.2 ± 0.1	2.0 ± 0.2	2.2 ± 0.1
10	2.1 ± 0.1	1.9 ± 0.2*	1.5 ± 0.2*	1.7 ± 0.2
15	2.0 ± 0.1	1.7 ± 0.1*	0.9 ± 0.01*	1.1 ± 0.1*
20	1.9 ± 0.1	1.2 ± 0.2*	0.7 ± 0.01*	1.0 ± 0.1*

Values are expressed as Mean ± SEM, n = 5, Values are significant at *P<0.05.

Wound healing effect

The wound healing effect of the Curcumin and synthesized compounds were tested with cream base[21] by excision wound model method.[22,23] The Curcumin (I) and synthesized compounds (II) and (III) were thoroughly mixed separately with the cream base (10% w/w) by the trituration method for topical application.

Four groups of (5 rats in each group) of healthy albino rats (150–200 g) were used in this experiment. The rats were anaesthetized with ether and the excision wound was made by sterilized surgical blade in dorsal thoracic part of the rats (circular area of 2.5 cm length and 0.2 cm depth). The wound was left undressed to open environment. The prepared creams were applied daily till the day of epithelium formation was completed. The wound area were measured on different interval of days (1, 5, 10, 15 and 20 days) followed by the initial wound. The observed period of wound closure and the epithelium formation for all the animals were **reported in Table 5** and the results were analyzed by using ANOVA followed by multiple range tests.

Antimicrobial activity

In vitro biological screening effects of the investigated compounds were tested against the bacteria, *Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli* and *Bacillus megatrium* by the well diffusion method [24-26] using agar nutrient as the medium and ciprofloxin as control. The antifungal activity of the investigated compounds were evaluated by the well diffusion method against the fungi *Candida albicans* cultured on Sabouraud agar medium and ketaconazole is used as control. The stock solutions were prepared by dissolving the compounds in Dimethyl sulfoxide. It is serially diluted and used to found the minimum inhibitory concentration values. In a typical procedure, 6 mm diameter well was made on the agar medium inoculated with the microorganism. The well was filled with test solution using a micropipette and the plates were incubated at 35°C for 24 h for the bacteria and 72 h for the fungi. During this period, the test solution was diffused through the medium and the growth of the inoculated **microorganisms was affected.** The inhibition zone developed on the plate was measured and reported in the Table 5. All the experiments were done in triplicate.

Result and Discussion

Physical characterization, pH, elemental analysis and spectral data[27] of the synthesized compound were summarized in Table 1. The FAB mass spectra of the compound II showed a molecular ion peak M^+ at m/z = 808 (43 %). The molecular ion peak for the compound III was observed at m/z = 443 (38 %). From the elemental analysis, Mass, IR, UV – Visible and ^1H – NMR spectral data structure of the compounds were confirmed as II and III.

Table 6. Antimicrobial activity of the compounds by well diffusion method (Zone of inhibition at MIC in mm)

S. No.	Organisms	Zone of inhibition in mm			
		Control	I	II	III
1	*Staphylococcus aureus*	30	18	14	16
2	*Proteus vulgaris*	29	12	10	11
3	*Pseudomonas aeruginosa*	24	14	12	14
4	*Klebsiella pneumonia*	22	12	R	R
5	*Escherichia coli*	24	18	16	17
6	*Bacillus megastrum*	24	17	9	15
7	*Candida albicans*	18	14	11	13

R=Resistant

Analgesic effect

The synthesized curcuminod III has shown higher significant analgesic effect than II. It is mainly due to presence of imino groups, more number of polar groups, less steric effect and extensive conjugation. Compound II also has more conjugation than III but its larger size decreases its absorption.

Anti-inflammatory property

Compound II has maximum anti-inflammatory activity than the III compound which is compared with standard and curcumin. Compound III also has better effect in reducing the inflammation. It is mainly due to the presence of imino and phenyl groups[28]. Comparatively, curcumin has maximum anti-inflammatory effects than the synthesized compounds and standard.

Anti-ulcer effects

The anti-ulcer effects of the investigated compounds were studied in aspirin induced ulcers in albino rats by using 2% gum acacia solution as vehicle and Ranitidine as standard. The result shows that new curcuminiods has better effects than the parent curcumin. In addition to this, compound III has greater anti-ulcer effect than the compound II. The observed data of II suggested that it increases the acidity in stomach due to the presence of more number of hydroxyl groups.

Wound healing effect

The wound healing effect of the curcumin and synthesized compounds were tested with cream base by excision wound model method and the data are reported in Table 5. From the observed data, compound II shows significant wound healing effect than all other compounds which is due to the presence of imino and polar phenolic hydroxyl groups. These groups form a hydrogen bonding between the skin systems and induce its growth.

Antimicrobial activity

On treatment with various microorganisms like *Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli* and *Bacillus megastrum* against the synthesized curcuminoids (II, III) and curcumin (I) have shown significant effect of inhibition on the growth of organisms. curcumin has maximum inhibitory effect than compound II and III. In comparison of compound II and III, former has higher sensitivity than the later one because of its lipid solubility. Decrease in activity of compound III is due to the increase in water solubility and polarization[29].

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