

IN VITRO ANTIOXIDANT AND IN VIVO ANTI-INFLAMMATORY ACTIVITY OF *Curcuma albiflora* THW.



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Abstract

Curcuma albiflora Thw. is a poorly explored plant which is termed as Harankaha locally. Among other Harankaha plants, *C. albiflora* is threaten and restricted to Sabaragamuwa province only. Therefore, current study was conducted to quantify phytochemicals in terms of total phenolic content (TPC), total flavonoid content (TFC) and prove or disprove its antioxidant and anti-inflammatory activity using ferric reducing antioxidant power (FRAP), DPPH, ABTS⁺, and oxygen radical absorbance capacity (ORAC) assays, and carrageenan-induced paw oedema, formaldehyde-induced paw oedema, and cotton pellet induced granuloma tests in Wistar rats respectively. Whole plant extract from composite samples was prepared using 50% ethanol (in water) and 50% dichloromethane (in methanol) as solvents mixture by continuous extraction (6 h). Antioxidant values of TPC, TFC, and FRAP were 31.25 ± 1.48 , 11.22 ± 0.13 , and 17.90 ± 0.38 mg/g respectively. IC₅₀ values (ppm) of DPPH and ABTS⁺ were 827.78 ± 6.06 , and 188.84 ± 2.99 respectively. ORAC value was 128.10 ± 3.29 mg/g. The correlation of TFC was shown that it may link to flavonoid content. Since, *C. albiflora* inhibited (61% at 1h) 1st phase in the acute model, and at the late phase on sub-chronic model considering insignificant on cotton pellet granuloma test was shown that its anti-inflammatory activity (as 19.5% on 400 mg/kg) may not relate on prostaglandin. Further experiments are warrant to identify a mechanism of anti-inflammatory activity of *C. albiflora*.

Key words: antioxidant, anti-inflammatory, albiflora, curcuma, paw oedema

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1. Introduction

Harankaha botanically known as *C. albiflora* Thw. is claimed to be used as an anti-inflammatory medicinal plant in traditional medicine (DOA, 2002). Under the same vernacular name, three plants (*Curcuma albiflora*, *C. zedoaria* Rosc., and *Zingiber zerumbet* Smith.) are being used (Dassanayaka, 1983). Amongst *C. albiflora* is an endemic, threaten and endangered plant (MOE, 2012). It is also an unexplored. The current study was conducted to study antioxidant activity of *C. albiflora* by using total phenolic content (TFC), total flavonoid content (TPC), ferric reducing antioxidant power (FRAP), DPPH, ABTS⁺, and oxygen radical absorbance capacity (ORAC), and also anti-inflammatory activity of *C. albiflora* by using formaldehyde-induced paw oedema, carrageenan-induced paw oedema, and cotton pellet induced granuloma tests in Wistar rats.

2. Material and Methods

2.1. Ethical approval

The study protocol and procedures were reviewed and approved by FGS, Colombo ethics committee (2015/MPhil-PhD/013). Since, *C. albiflora* is an endemic threaten specie, approval was taken from Forest department, Sampathpaya, Battaramulla (My ref. R&E/RES/NFSRC/12) to collect samples.

2.2. Collection of plant and preparation of extract

Plants were collected from 2016 to early 2017 in Sabaragamuwa province (Erathna: N 6° 50' 07", E 80° 24' 41", Kitulgala: N 6° 59' 41", E 80° 24' 20" and Bopathella: N 6° 48' 07", E 80° 22' 12"). Voucher specimens (No. 02, 03, 04) of the plants were authenticated from National Herbarium, Peradeniya, Sri Lanka. Whole plant extract from composite samples of *C. albiflora* was prepared using 50% ethanol (in water) and 50%

dichloromethane (in methanol) as solvents mixture by continuous extraction (6 h). 50g of powdered samples (40#) were extracted by Soxhlet apparatus (6 h) using 2 L dichloromethane/methanol (1:1) and ethanol/water (1:1). Dry samples were obtained by rot evaporator and stored at -20 °C for future experiments.

2.3. Experimental animals

Wistar female healthy rats (180-240g) about 1-2 months aged were obtained from Medical Research Institute, Borella, Sri Lanka. Anti-inflammatory experiments were performed at the research room (temperature: 26 °C, relative humidity: 50%) in animal house, the department of zoology, university of Colombo. Rats were fed commercial pelleted diet and water ad libitum. Thirty rats were assigned into six groups. Groups were treated in the following manner; control (distilled water), three *C. albiflora* (CA) drug groups; 200 mg/kg, 400 mg/kg, and 600 mg/kg, standard group; indomethacin 4 mg/kg (Dharmasiri et al., 2003; Ratnasooriya et al., 2015).

2.4. Total phenolic content (TPC)

DCM/methanol extract of *C. albiflora* was determined by Folin-Ciocalteu colorimetric method according to the Singleton et al. (1999) with some modifications. A reaction volume of 200 µL, containing Folin-Ciocalteu (110 µL), extract (20 µL), and 10% Na₂CO₃ solution (70 µL) were incubated at room temperature for 30 min. Absorbance was measured at 765 nm using micro-plate reader (Spectra Max +384). Gallic was used as standard, and water was used as blank. Replicated (5) results expressed as gallic acid equivalents (mg/g).

2.5. Total flavonoid content (TFC)

DCM/methanol extract of *C. albiflora* was determined according to Siddhuraju et al. (2003) with slight modification. Briefly, 100 µL of extract, 70 µL of AlCl₃ (2%)

were mixed and incubated at room temperature for 10 min. Absorbance was measured at 415 nm using micro-plate reader (Spectra Max +384). Quercetin was used as standard, and water was used as blank. Replicated (5) results expressed as quercetin acid equivalents (mg/g).

2.6. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power of *C. albiflora* DCM/methanol extract was determined according to Benzine *et al.* (1999) with slight modification. Briefly, 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine), and Trolox (1 mg/mL) were prepared. FRAP reagent was prepared by mixing acetate buffer, ferric chloride, TPTZ (10:1:1) and incubating at 37 °C for 10 min. A reaction volume of 200 µL, containing FRAP reagent (150 µL), acetate buffer (30 µL), and extract (20 µL) were incubated at room temperature for 8 min and absorbance was measured at 600 nm using micro-plate reader (SpectraMax+384). Trolox was used as standard and buffer was used as blank. Replicated (5) results were expressed as Trolox equivalents (mg/g).

2.7. DPPH radical scavenging assay

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging in plant material was determined according to Blois *et al.* (1958) with slight modification. DPPH (0.5 mM) was prepared using methanol. A reaction volume of 200 µL, containing DPPH radical (65 µL), Methanol (85 µL) and extract (50 µL) were incubated at room temperature for 10 min and absorbance was measured at 517 nm using micro-plate reader (Spectra Max +384). Trolox was used as standard and methanol was used as control. DPPH radical scavenging activity percentage was measured using the formula;

$$\text{Percentage radical scavenging activity} = (1 - A/B) \times 100$$

where A: absorbance of extract at concentration and B: absorbance of control.

IC₅₀ at each concentration of the extract/standard were calculated.

2.8. ABTS⁺ radical scavenging assay

The ABTS⁺ radical scavenging in plant material was determined according to Re *et al.* (1999) with slight modification. Briefly, 50 mM PBS (phosphate buffer saline, pH 7.4) was prepared. Fresh ABTS⁺ (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical solution was prepared. A reaction volume of 200 µL, containing PBS (110 µL), extract (50 µL), and ABTS⁺ solution (40 µL) were incubated at room temperature for 10 min. Absorbance was measured at 734 nm using micro-plate reader (SpectraMax+384). Trolox was used as standard, and water was used as control. PBS and water were used as the blank. ABTS⁺ radical scavenging activity percentage and IC₅₀ was calculated as per section 2.4.4.

2.9. Oxygen Radical Absorbance Capacity (ORAC) assay

The oxygen absorbance capacity in plant material was determined according to Ou *et al.* (2001) with slight modification. Briefly, 75 mM PB (phosphate buffer, pH 7.4), fluorescein solution (16 mg in 100 mL PB and 1 mL diluted to 100 mL with PB), and AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) solution (40 mg/mL in PB) were prepared. A reaction volume of 200 µL, containing PB (40 µL), extract (10 µL), fluorescein (100 µL), and AAPH solution (50 µL) were incubated at 37 °C for 5 min. Trolox (0.75 µL/ mL) was used as standard and PB was used as the blank.

Decay of florescence was scanned for 35 min at 1 min interval at 37 °C (Ex 494 nm, Em 535 nm) using micro-plate reader (Spectra Max Gemini EM). The area was recorded under the curve for extracts and Trolox. ORAC value was calculated using the formula;

$$\text{ORAC} = \frac{\text{Net AUC extract}}{\text{net AUC Trolox}} \times \left(\frac{\text{Trolox concentration}}{\text{extract concentration}} \right)$$

where net AUC extract = AUC extract - AUC blank, net AUC Trolox = AUC Trolox - AUC blank, and AUC = area under the curve.

2.10 Anti-inflammatory experiments

Required concentrations of *C. albiflora* (200 mg/kg, 400 mg/kg, and 600 mg/kg) drug groups were prepared using water as solvent. The animal dose was calculated based on human equivalent dose (HED) using the formula $\text{HED (mg/kg)} = \text{animal dose (mg/kg)} \times \text{animal } K_m \text{ factor (6)} / \text{Human } K_m \text{ factor (37)}$ (Reagan-Shaw *et al.*, 2007). Anti-inflammatory experiments were performed according to published procedures (Dharmasiri *et al.*, 2003; Ratnasooriya *et al.*, 2015).

2.11 Carrageenan induced paw oedema in Wistar rats

Inflammations were induced by 0.05 ml of 1% carrageenan powder was dissolved in 1% methyl cellulose (analytical grade, from Sigma-Aldrich) into the plantar surface of the left hind paw under mild anesthetic ether anesthesia. Treatments were performed prior 1h of carrageenan injection. The paw volumes of pretreatment of the paws and 1h, 2h, 3h, and 4h after the treatment were measured. The percentage inhibition was determined using the formula;

$$\text{Inhibition percentage} = \frac{(\text{control-treated})}{\text{control}} \times 100$$

2.12 Formaldehyde induced paw oedema in Wistar rats

Inflammations were induced by 0.1 ml of 2% formaldehyde in distilled water into the plantar surface of the left hind paw under mild anesthetic ether anesthesia on days 1 and 3. Treatments were performed for 7 consecutive days. The paw volumes of these rats were measured prior to the injection of formaldehyde, at 4 h after the injection on day 1 and at 1 h of oral treatment from days 2-7.

2.13 Cotton pellet induced granuloma in Wistar rats

Granulomatous lesions were induced by surgically implanting two cotton pellets subcutaneously in the dorsal region of the rats near the axilla. *C. albiflora* extract was administered orally before 1 h of the surgery. Rats were anaesthetized using ketamine (0.6 ml kg⁻¹) and autoclaved sterile pellets of cotton (8 ± 0.5 mg each) were implanted. The rats of the control group were administered with water and standard group by Indomethacin (5 mg/kg). Drugs and water were administered for consecutive 7 days. Rats were anaesthetized on the eighth day using ketamine and the pellets were dissected out carefully and dried at 60 °C (3 d). Mean weight of the granuloma tissue formed around each dried pellets were recorded.

2.14 Statistical analysis

Calculation and statistical analysis was performed using Minitab 17 (version 17.1.0.0). Results were expressed as mean ± standard deviation. DPPH, TPC, and TFC values were further analyzed by Pearson correlation coefficient. Anti-inflammatory activity results were analyzed by ANOVA comparing control group values using Turkey test.

3. Results

3.1 Antioxidant activity

TPC, TFC, FRAP, and ORAC values were reported in Table 1. IC₅₀ values of DPPH, and ABTS⁺ were reported in Table 2. The standard curves were included in Plate 1.

Table 1: Measured TPC, TFC, FRAP, and ORAC values of *C. albiflora* DCM/methanol (1:1) extract

<i>In-vitro</i> antioxidant assay	Measured value
TPC	31.25 ± 1.48 mg GAE/g of extract
TFC	11.22 ± 0.13 mg QE/g of extract
FRAP	17.90 ± 0.38 mg TE/g of extract
ORAC	128.10 ± 3.29 mg TE/ g of extract

Values: Mean ± SD, n=5

Table 2: Measured DPPH, ABTS⁺, and Trolox values of *C. albiflora* DCM/methanol (1:1) extract

	Trolox (Standard)	DPPH	ABTS ⁺
IC ₅₀ (ppm)	8.68 ± 1.54	850.41 ± 20.72	188.84 ± 2.99

Values: Mean ± SD, n=5

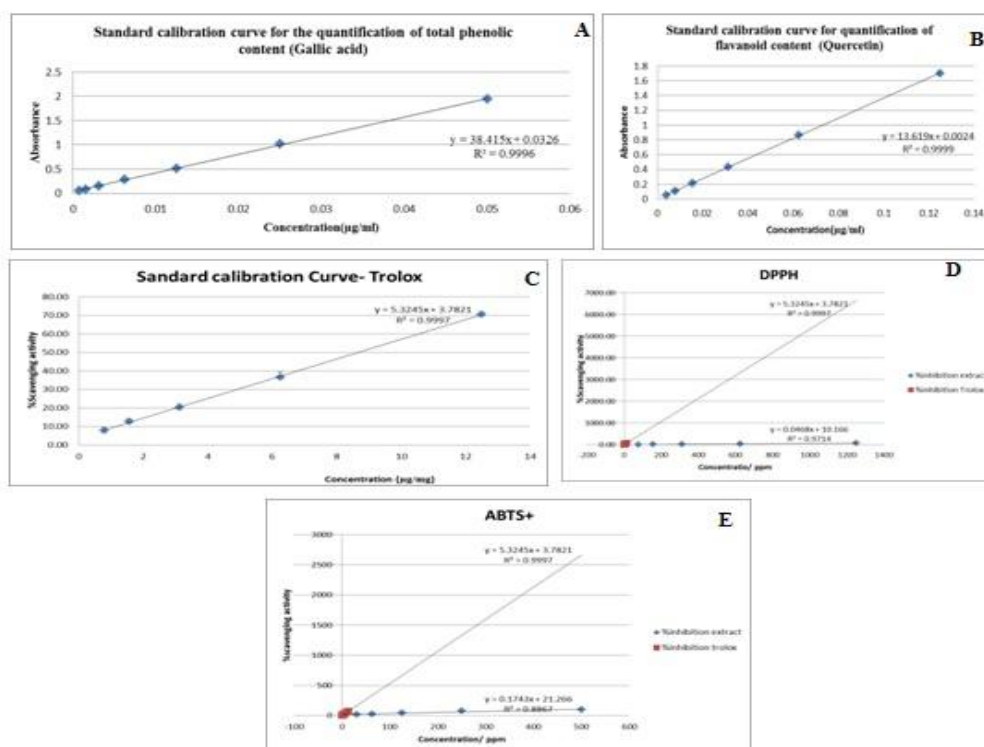


Plate 1: A; Standard calibration curve of Gallic acid for the quantification of total phenolic content, B; Standard calibration curve of Quercetin for quantification of total flavonoid content, C; Standard calibration curve for Trolox, D; Inhibition percentage of *C. albiflora* (DCM/ methanol (1:1) extract and Trolox on DPPH assay, E; Inhibition percentage of *C. albiflora* (DCM/ methanol (1:1) extract and Trolox on ABTS⁺ assay.

The extent of DPPH and ABTS⁺ radical scavenging at various concentrations of *C. albiflora* extracts were reported in Table 3. DPPH, TPC, and TFC values were further analyzed by Pearson correlation coefficient and reported in Table 4.

3.2 Anti-inflammatory activity

3.2.1 Carrageenan induced paw-oedema in Wistar rats

The effect of oral treatment of *C. albiflora* whole plant extract of rats was reported in Table 5.

Table 3: DPPH and ABTS⁺ free radical scavenging activity of the extract *C. albiflora*

Trolox Standard/ (ppm)	DPPH		ABTS ⁺		
	Inhibition (%)	Concentration extract/ (ppm)	Inhibition (%)	Concentration extract/ (ppm)	Inhibition (%)
12.50	70.56 ± 0.30	1250	65.76 ± 0.72	500	99.19 ± 0.72
6.25	36.58 ± 1.73	625	44.95 ± 1.39	225	82.56 ± 1.39
3.125	20.26 ± 0.26	312.5	27.11 ± 1.42	125	48.87 ± 1.42
1.563	12.69 ± 0.38	156.25	16.15 ± 0.76	62.5	28.72 ± 0.76
0.781	7.78 ± 0.96	78.13	10.12 ± 0.32	31.25	15.81 ± 0.32

Values: Mean ± SD, n=5

Table 4: Correlations between the IC₅₀ values of DPPH assay, ABTS⁺ assay, FRAP, and ORAC assays with phenolic and flavonoids content of *C. albiflora*

Pearson correlation coefficient	TPC	TFC
DPPH	-0.794	0.293
ABTS ⁺	-0.686	0.479
ORAC	-0.934	0.867
FRAP	-0.150	0.822

Table 5: Effect of oral treatment of *C. albiflora* whole plant extract on the carrageenan induced paw oedema

Dose (mg/kg)	Paw oedema (ml)			
	1h	2h	3h	4h
Control	0.33 ± 0.02	0.37 ± 0.03	0.48 ± 0.05	0.55 ± 0.06
200 CA	0.19 ± 0.06	0.31 ± 0.04*	0.40 ± 0.05	0.40 ± 0.06
400 CA	0.14 ± 0.05	0.28 ± 0.06	0.38 ± 0.05	0.52 ± 0.06*
600 CA	0.18 ± 0.07	0.20 ± 0.12	0.35 ± 0.08	0.48 ± 0.02
4 Indomethacin	0.12 ± 0.02	0.04 ± 0.01*	0.11 ± 0.04*	0.23 ± 0.03*

Values are means ± SEM (n=6), *p values < 0.05 as compared with control (Turkey's test)

3.2.2 Formaldehyde induced paw-oedema in Wistar rats

The effect of *C. albiflora* extract on the formaldehyde-induced paw oedema was reported in Table 6.

3.3.3 Cotton pellet induced granuloma in Wistar rats

Wet cotton pellets which were dissected out on the 8th day and mean dry weight of cotton pellets were shown in the Plate 2. The effect of *C. albiflora* on cotton pellet granuloma test in Wistar rats was reported in Table 7.

Table 6: Effect of *C. albiflora* extract (water) on the formaldehyde-induced paw oedema in Wistar rats

Dose (mg/kg)	Paw oedema (ml)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	0.39±0.05	0.60±0.03	0.65±0.05	0.77±0.07	0.94±0.08	0.54±0.06	0.47±0.05
200 CA	0.29±0.03	0.75±0.07	0.64±0.03	0.86±0.05	1.02±0.12	0.61±0.06	0.51±0.05
400 CA	0.22±0.05*	0.13±0.05**	0.32±0.04**	0.55±0.04*	0.09±0.01**	0.07±0.02**	0.04±0.01**
600 CA	0.20±0.04*	0.15±0.04**	0.36±0.03**	0.57±0.06*	0.16±0.08	0.13±0.03**	0.12±0.04**
4 Indomethacin	0.19±0.05*	0.23±0.05**	0.37±0.09*	0.52±0.06*	0.07±0.07**	0.04±0.02**	0.02±0.01**

Values are means ±SEM (n=6), *p values < 0.05 and **p values < 0.01 as compared with control (Turkey's test)

Table 7: Effect of *C. albiflora* on cotton pellet granuloma test in Wistar rats

Dose mg/kg	Mean of wet cotton pellets(mg)	Percentage inhibition	Mean of dry cotton pellets(mg)	Percentage inhibition
Control Group	37.57 ±0.93		27.60±1.02	
200 CA	34.48±0.26	8.2%	24.88±0.69*	8.1%
400 CA	30.75±0.12	18.2%	21.78±0.37**	19.5%
600 CA	33.56±0.65	10.7%	23.44±0.74**	13.4%
4 indomethacin	22.03±0.35	41.4%	13.18±0.25	51.3%

Values are means ± SEM (n = 6). *p < 0.05 and **p<0.01 as compared with the control (Turkey's test)

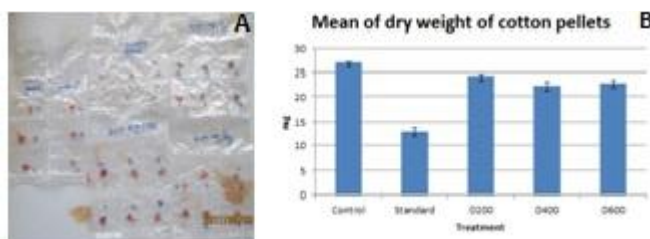


Plate 2: Cotton pellet experiment: A: Picture of wet cotton pellets after surgical removal according to group, B: mean of dry weight of cotton pellets of each treatment groups

4. Discussion

4.1 Antioxidant activity

All *in vitro* antioxidant experiments were performed on dichloromethane/methanol (1:1) extract of *C. albiflora* in the concentration of the 200 mg/kg. Polar and non-polar dichloromethane/methanol solvent system was used to extract polar and non-polar compounds present in *C. albiflora*. The standard and the plant extracts were shown their maximum %inhibitory activity against concentration: standard was shown 70.56 ± 0.30 at 12.50 ppm, and *C. albiflora* DCM/methanol (1:1) extract was shown 65.76 ± 0.72 % at 1250 ppm assay concentration on DPPH bioassay (Table 3). Moreover, *C. albiflora* DCM/methanol (1:1) extract was shown 99.19 ± 0.42 % at 500 ppm assay concentration on ABTS⁺ bio assay. *In vitro* antioxidant studies were shown that the radical scavenging effect (DPPH, ABTS⁺) was found to increase with increasing concentrations (Table 3). The correlations of TFC against the antioxidant activity based on the DPPH, ABTS⁺, FRAP, and ORAC assay were significant (Table 4). Further, the negative correlation between TPC and antioxidant activity were suggested that it could be related to other antioxidant compounds contained in the plants (Kolar *et al.*, 2011). Although TPC, and TFC values of *C. albiflora* were reported as 31.25 ± 1.48 (mg GAE/g of extract), and 11.22 ± 0.13 (mg QE/g of extract), in terms of *C. longa* were reported as 260 ± 0.25 (mg GAE/ g of extract) and 79.36 ± 0.01 (mg QE/g of extract) respectively (Rajeswari *et al.*, 2013). Whereas the concentration of 1250 µg/mL of *C. albiflora*. DCM/methanol extract was shown that 65.76 ± 0.72 %, the concentration of 100 µg/mL of water extract of *C. zedoaria* reported to have 98.95% inhibition (Himaja *et al.*, 2010). Although, IC₅₀ values of *C. albiflora* DCM/methanol extract on DPPH and ABTS⁺ assays were 827.78 ± 6.06 ppm, and 188.84 ± 2.99 ppm respectively, Water extract of *C. aromatica* was

reported to have 427.75 ± 1.43 ppm and 11.674 ± 1.98 ppm respectively (Ammayappan *et al.*, 2012). Low IC₅₀ values were shown higher antioxidant activity. Although, ORAC value of *C. albiflora* was reported as 128.10 ± 3.29 mg TE/g of extract in *C. longa*. It was reported 1592.77 µM TE/g of extract (Reşat *et al.*, 2013). According to the results obtained from the current study, *C. albiflora* was shown a low antioxidant active species, comparatively to *C. longa*, *C. aromatica* and *C. zedoaria*.

4.2 Anti-inflammatory activity

4.2.1 Carrageenan induced paw-oedema in Wistar rats

The 200 mg/kg was significantly impaired the paw oedema, at 1h (by 61%). In contrast, the 400 and 600 mg/kg tested were significantly inhibited the paw oedema measured; 1h (by 45-58%), 2h (by 24-46%), 3h (by 21-27%). Therefore it was shown that the anti-inflammatory effect of *C. albiflora* was inversely dose dependent (See Table 1). Indomethacin induced significantly impairment of oedema at all-time points measured (58-89%). Initial phase lasting primarily mediated via production of cox-1, histamine, serotonin, bradykinins etc (Ratnasooriya *et al.*, 2015). Since, *C. albiflora* inhibited 1st phase, the anti-inflammatory effect may relate with above mentioned pathways. *C. zedoaria* shows 54- 56% inhibition in the initial phase and 56-59% in late phase by the concentration 200 mg/kg of petroleum ether on carrageenan induced paw oedema test. However it was shown that the 58% inhibition at 2h by the concentration of 200 mg/kg of chloroform (Kaushik *et al.*2011), Therefore, it was shown that the anti-inflammatory activity of *C. zedoaria* on both the initial and late phases. In contrast, *C. albiflora* was shown its anti-inflammatory activity in initial phase.

4.2.2 Formaldehyde induced paw-oedema in Wistar rats

The drug group (400 mg/kg and 600 mg/kg) significantly ($P < 0.05$) reduced the paw oedema from the day 5 to 7 by 400 mg/kg when compared with the control (see Table 5). This effect related prostaglandin synthesis can be proved by cotton pellet induced granuloma test. From the present study, it can be concluded that 400 mg/kg treated group was shown higher inhibition percentage than 200 and 600 mg/kg treated groups. Since, *C. albiflora* was shown low (as 19.5% on 400 mg/kg) anti-inflammatory activity on Cotton pellet granuloma test, it can be concluded that anti-inflammatory activity of *C. albiflora* is not linked with prostaglandin synthesis.

4.2.3 Cotton pellet induced granuloma in Wistar rats

Inhibition percentages were found as 51.3 % (Standard), 8.1 % (200 mg/kg treated group), 19.5 % (400 mg/kg treated group), and 13.4 % (600 mg/kg treated group). From the present study, it can be concluded that 400 mg/kg treated group was shown higher inhibition percentage than 200 and 600 mg kg⁻¹ treated groups.

5. Conclusions

It can be concluded from the current study that *C. albiflora* was shown correlation with flavonoid content which is responsible for its antioxidant property. *C. albiflora* inhibited 1st phase in the carrageenan-induced paw oedema test, but with low concentrations. By formaldehyde induced paw oedema and cotton pellet granuloma, tests were shown anti-inflammatory activity at the late phase (from 5 to 7 day) and its activity does not relate to prostaglandin synthesis, on other autacoids. Further experiments have to be conducted to identify a mechanism of anti-inflammatory activity of *C. albiflora*.

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