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Protective Effect of a Natural Coumarin (Aesculetin) on Adjuvant-induced Rats by Regulation of RA Factor and CRP Level

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Abstract

Background: Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disorder that can be associated with joint pain, swelling, stiffness, and progressive cartilage destruction. Coumarin derivatives are rich in antioxidant and anti-inflammatory properties and may be useful in the management of RA. **Aim:** The present study aimed to evaluate the character of antioxidant and anti-arthritic activity of aesculetin in Complete Freund's Adjuvant (CFA)-induced arthritic rats. **Methods:** Aesculetin was identified using chemical tests, melting point analysis, Thin-Layer Chromatography (TLC), and UV spectroscopy. Antioxidant activity was assessed by Nitric Oxide (NO) and DPPH free radical scavenging assays. Arthritis was induced in Swiss albino rats using CFA. Animals were treated orally with aesculetin (5 mg/kg and 10 mg/kg) and indomethacin (5 mg/kg) for 18 days. Anti-arthritic activity was evaluated through paw measurement, histopathological examination, and haematological biomarkers, including Rheumatoid Factor (RF) and C-Reactive Protein (CRP). **Results:** Aesculetin exhibited significant dose-dependent antioxidant and anti-arthritic activity. The compound showed approximately 50% inhibition of nitric oxide radicals at concentrations below 100 µg/ml and demonstrated concentration-dependent DPPH scavenging activity. Treatment with aesculetin significantly reduced paw, RF levels, and CRP levels compared with the arthritic control group. Histopathological studies showed reduced inflammatory changes and bone deformity in treated animals. **Conclusion:** The study confirms that aesculetin possesses significant antioxidant and anti-arthritic activity, which may be attributed to its polyphenolic coumarin structure and free radical scavenging ability. Aesculetin may serve as a promising lead compound for the management of rheumatoid arthritis, although further clinical studies are required.

Major Findings: Major findings include significant antioxidant activity in NO and DPPH assays, dose-dependent reduction in paw and inflammatory biomarkers, and histopathological improvement in arthritic rats, supporting the therapeutic potential of aesculetin in rheumatoid arthritis management.

Keywords: Aesculetin, Antioxidant Activity, Complete Freund's Adjuvant, Histopathological and Haematological Studies, Rheumatic Arthritis

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1. Introduction

Rheumatoid Arthritis (RA) is a progressive inflammatory autoimmune disease affecting one or more joints and is common in older people[1]. The types of arthritis are rheumatoid arthritis, osteoarthritis, juvenile arthritis, psoriatic arthritis, gout arthritis, and ankylosing spondylitis (axial arthritis). Irrespective of the cause, the pathophysiology involves the cartilage loss of smooth glide at the joint; an increase in friction

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during movement of the joint results in pain, swelling, and stiffness[2]. In a later stage, it leads to irreversible joint damage and disability[3]. Hydroxycoumarins are naturally occurring lactones, which are anhydrides of O-coumaric acid. Most of the coumarins are used as an anticoagulant (e.g., warfarin). Chemically, coumarin is a benzo-2-pyrone derived from cinnamic acid via the shikimic acid pathway in plants. The common coumarins are umbelliferone (7-hydroxycoumarin), aesculetin (6,7-dihydroxycoumarin), and scopoletin (6-methoxy-7-hydroxycoumarin)[4]. Coumarins and their related compounds have anti-inflammatory, antioxidant, antiviral, antimicrobial, antifungal, anticancer, and antitubercular activities. Coumarins act at the cellular level by inhibiting the Akt-mammalian target of rapamycin, NF-κB, MAPK, Nrf2, and nucleotide excision. Many coumarin derivatives can scavenge Reactive Oxygen Species (ROS). They also act on lipoxygenase (LOX) and cyclooxygenase (COX) pathways as anti-inflammatories[5,6]. Based on the above, the present work aims to study the protective effect of aesculetin in adjuvant-induced arthritic rats.

2. Materials and Methods

Aesculetin (Test drug) was procured from Sigma Aldrich Ltd., Bangalore. Standard Drug Indomethacin marketed formulation was purchased and used.

2.1 Identification of Aesculetin

2.1.2 Melting Point by Capillary Tube Method

The melting point of the test sample was determined. The temperature at which it starts melting has been noted as the reading for the determination of the melting point.

2.1.3 Thin Layer Chromatography (TLC)

Thin-layer chromatography is an analytical tool used for the separation and identification of phytocompounds. Several developing solvent systems were tried for the selected test sample, but the satisfactory resolution was found in the Mobile phase: Methanol: water (5:5) and the stationary phase: A precoated silica gel G plate was used as the stationary phase. The test sample was dissolved in methanol. After the development, the plate was dried and visualised using a UV chamber.

2.1.4 UV Absorption

The residue of the ethanolic solution of the test sample was dissolved in 0.5 ml of 10% ammonium hydroxide and spotted on filter paper and examined under UV light. Intense fluorescence indicates the presence of coumarins[7-9].

2.2 *In Vitro* Anti-oxidant Assay

2.2.1 Nitric Oxide Radical Scavenging Assay

Cells are prone to undergo damage due to oxidation, which produces free radicals, leading to chain reactions that may cause damage. Most of the free radicals come from the energy source by-products of normal and metabolic reactions. Nitric oxide radical inhibition was done by the standard method using Griess reagent[10]. The sample was used at various concentrations (100 – 500 µg/ml) in a final volume of 3 ml. 1 ml of Griess reagent was added after incubation at 25°C. The absorbance was measured spectrophotometrically at 546 nm. The percentage of inhibition was calculated by using a standard formula.

2.2.2 DPPH Assay

The sample was used at various concentrations (100, 200, 300, 400, and 500 µg/ml) in methanol. After 30 minutes of incubation in the dark at room temperature, the absorbance of the solution was read at 517 nm[11]. Percentage of inhibition = ([Absorbance of Control – Absorbance of Test] / Absorbance of Control × 100).

2.3 Anti Arthritic Activity

The study was conducted on both male and female Swiss albino rats weighing 150 to 250 g, which were obtained from the Veterinary and Animal Sciences University, Chennai. We were housed under the laboratory environmental conditions of 24°C, a relative humidity of 65 to 75%, and a 12-hour light/dark cycle. The experimental protocol adheres to the guidelines of the IAEC. IAEC Approval number: XXI/VELS/PCOL/04/2000/CPCSEA/IAEC/01.12.2017. CPCSEA Approval Number: 290/PO/RQ/S/2000/CPCSEA.

2.3.1 Acute Toxicity Study

Three Swiss albino rats weighing 150 to 250 g of both sexes were used. The acute toxicity study was performed based on earlier literature, and the sample

was administered with an initial single dose at a series of 5 mg/kg, 10 mg/kg, and 15 mg/kg to the rats once a day. The parameters such as colour of fur, eye dullness and colour, and consistency of faeces were observed along with its mortality for 3 days[12]. Arthritis was induced in rats by intraperitoneal injection of 0.1 ml of CFA (Complete Freund's Adjuvant) into the left hind paw. Inflammation is allowed to develop for three days. Rats were distributed into 5 groups with 3 rats in each group. 1% CMC (Carboxymethyl cellulose) was used as a vehicle for drug administration. Both the standard and test drugs were dissolved in Carboxymethyl Cellulose (CMC) and administered. Carboxymethyl Cellulose (CMC) solution was made as a fresh preparation daily before drug administration. Indomethacin at the dose level of 5 mg/kg body weight was used as the standard. All animals were administered it orally once daily for 18 days after the development of arthritis. A normal animal was injected with 0.1 ml of normal saline into the rat's left hind paw.

2.3.2 Histopathological Examination

After 18 days, the rats were anaesthetised and sacrificed, and the left hind paw was collected and rinsed with 0.9% saline sodium chloride and preserved in formalin solution. The histopathological parameter was performed.

2.3.3 Haematology Study

The blood was collected from the heart of the rat after sacrifice. Commercially available RF and CRP turbinate kits were used for the estimation of biomarkers due to their high sensitivity, specificity, and reproducibility in quantitative analysis. The blood was stored in a heparinised tube and stored at 2-8°C. The haematological parameters for RF and CRP were measured.

2.3.3.1 Turbilatex RF

It is a turbidimetric test for the measurement of RF in human serum or plasma by comparison to the RF content of a sample that can be quantified from a calibrator of a known RF concentration (range from 20 to 160 IU/ml). The below-mentioned RF calibrator dilutions in normal saline were obtained by multiplying the concentration of the RF calibrator by the corresponding factor to obtain the RF concentration of each dilution. One-point calibration: (linear range up to 100 IU/ml). RF calibrator dilution was prepared with 30 µl RF calibrator + 70 µl normal saline. The RF concentration of the diluted calibrator was obtained by multiplying the RF calibrator concentration by 0.30. The calibration curve was plotted based on the absorbance difference (A2 - A blank reagent). Rheumatoid factor concentration in the sample is calculated by the interpolation method. Absorbance was measured after 2 min of the sample addition.

2.3.3 2 CRP Turbilatex

The CRP level in a rheumatoid arthritic condition was measured by CRP-turbidimetry in human serum or plasma. Specific anti-human CRP were coated over latex particles and were agglutinated when mixed with samples containing CRP. The agglutination causes an absorbance change dependent upon the CRP content that can be quantified by comparison with a calibrator of known CRP concentration.

3. Results and Discussion

3.1 Identification of Aesculetin

The chemical test results showed the presence of a phenolic group and coumarin, and the absence of a flavonoid linkage in the test drug. The drug aesculetin melted at a temperature of 207°C, which was compared with the standard value, which is 209°C[13]. This confirms the test sample is qualitatively acceptable for its purity and values. The R_f value for aesculetin is 0.67, showing a brown-colored spot before exposure to UV and a blue-colored spot after exposure to a UV light source[14].

3.2 Anti-oxidant Activity

3.2.1 Nitric Oxide Scavenging Assay

The effective concentration for 50% inhibition was found to be less than 100 µg/ml of the test sample. The test drug showed a significant percentage of inhibition in nitric oxide (NO). The results of the nitric oxide scavenging activity of aesculetin are shown in Table 1.

3.2.2 DPPH Method

A concentration-dependent increase in percentage inhibition was also observed in the DPPH assay. Scavenging activity IC50 is less than 100 compared to the DPPH method, which was more than 100 µg/ml[14]. The

results of the free radical scavenging assay of aesculetin by the DPPH method are exhibited in Table 1.

3.3 Acute Toxicity Study

No abnormal behaviour or mortality was observed; it does not induce mortality in all tested animals. So, the tested dose was found to be safe for the animal model. The left hind paw of the rat was observed and measured using a Vernier calliper every day from the initial administration of the drug and for about 18 days during the dosage period. The results of thickness were compared among groups; it indicates that the drug at the dose level inhibited the from day 1 till the end of instillation. The test drug showed a significant reduction with a P value of 0.0028 compared to the control value of 0.001. The results were also compared on day 1 to day 12. The test drug significantly reduced the amount from the 1st day to the 18th day, with a P value of 0.001 compared to the control group's 0.154. The effect of aesculetin on the animal model rat by histopathology examination showed normal architecture with moderate bone deformity at the low dose level, as compared to the normal group. The collected blood was studied for haematological parameters like RF and CRP[15-17]. The amount of rheumatic factor (RF) IU/ml of the control and tests was compared. The control *vs*. Test-I is 22.5 *vs*. 18.24 IU/ml, and the control *vs*. Test-II is 22.5 *vs*. 14.02 IU/ml. This shows dose-dependent activity of aesculetin in treating arthritis[18,19] (Table 2). The amounts of C-reactive protein (CRP) mg/L in the control and test groups were compared. The control *vs*. Test-I is 7.03 *vs*. 5.02 mg/l, and the control *vs*. Test-II is 7.03 *vs*. 4.10 mg/l. This shows dose-dependent activity of aesculetin in treating arthritis (Table 2).

Table 1. Free radical scavenging activity of aesculetin

Concentration (µg/mL)	Nitric Oxide Scavenging Activity (% Inhibition)	DPPH method (% Inhibition)
100	69.15	12.22
200	75.53	24.44
300	80.85	43.33
400	93.62	52.22
500	98.94	75.56
EC_{50}:	< 100 µg/mL	354.59 µg/ml

Aesculetin (6,7-dihydroxycoumarin) is a genin of the coumarin glycoside aesculin. It is a naturally occurring coumarin that possesses anti-inflammatory, antioxidant, and antinociceptive activity. It was identified by a chemical test; it showed a positive test for a phenolic group and a coumarin nucleus. It gave a negative response for flavonoids and glycosidic linkage. So, the results of the chemical test confirmed aesculetin. The purity of the sample was determined by TLC[20], melting point, and UV spectroscopy. The observed melting point was 2070°C; it was compared with the standard. TLC was performed by standard protocol. It showed a very clear brown spot before exposure to UV radiation and a blue fluorescence spot after UV exposure. The TLC data confirmed its purity. Further, it was confirmed by UV spectroscopy; it showed a λ max at 400 nm, indicating the presence of a coumarin nucleus[21,22]. The chemical structure of aesculetin is shown in Figure 1.

3.4 Antioxidant Activity

3.4.1 Nitric Oxide Scavenging Assay

Inflammatory diseases are mostly associated with free radical nitric oxide production. It is very unstable under aerobic conditions. It reacts with oxygen to produce a stable product; it was estimated by using a Gives reagent in the presence of the test compound. In the present study, nitrite produced by sodium nitroprusside was reduced by the test compound. This may be due to the antioxidant principle of the test sample, which competes with oxygen to react with NO; these inactivated the NO synthase enzyme (NOS), thereby the test compound inhibited the generation of nitrite for inflammation. Excess nitric oxide production is very common in most of the inflammatory diseases, so in the present study, nitrite

Table 2. Effect of aesculetin on rheumatoid factor and C-reactive protein in an arthritic animal (rat)

S. No.	Sample	RF IU/ml	CRP mg/l
1	Normal	5.01	2.02
2	Control	22.5	7.03
3	Standard	11.58	3.80
4	Test I	18.24	5.02
5	Test II	14.02	4.10

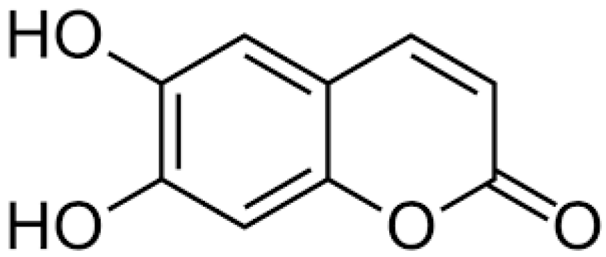


Figure 1. Chemical structure of aesculetin.

produced by sodium nitroprusside was reduced by the test compound due to scavenging of the nitric oxide free radical of the test sample, which competes with oxygen to react with NO. This inactivated the NO synthase enzyme (NOS). The percentage inhibition of nitric oxide radicals increased with increasing concentration of aesculetin, indicating dose-dependent antioxidant activity[23].

3.4.2 DPPH Method

This assay provides information on the reactivity of the test compound with the stable free radical. Such reactivity has been widely used to test the ability of the compound to act as a free radical scavenger. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm. A decrease in absorption of the reaction mixture indicates higher free radical scavenging activity of the two tested free radical inhibition assay methods.

3.5 Acute Toxicity Study

The test compound does not show any undue effect or mortality in all tested animals. So, the tested dose was found to be safe for the animal model.

3.6 Antiarthritic Activity

The effect of aesculetin on an adjuvant-induced arthritic rat model was studied. After the full development of joint inflammation, the test sample was administered. The daily oral administration of aesculetin significantly inhibited the increase in hind paw thickness[24]. It was associated with the reduction of CRP and rheumatoid factor. So far, several studies have been carried out and shown that the reduction of LTB4 level and IL-1β, TNF-α, by Aesculetin. It was shown that this compound potently reduces RA factor and CRP level in resorbing cartilage. The

Table 3. Effect of aesculetin in thickness of paw odema lin CFA induced arthritic rat

Group	Odema on Day (Cm)			
	1	6	12	18
Standard (Indomethacin 5 mg/kg)	1.10	1.02	0.84	0.74
	1.01	0.93	0.83	0.74
	1.02	0.93	0.94	0.64
Test-I (5 mg/kg)	1.01	0.91	1.02	0.61
	1.02	0.93	0.92	0.61
	1.10	1.01	0.91	0.72
Test-II (10 mg/kg)	1.01	0.92	0.82	0.63
	1.01	0.90	0.72	0.51
	1.01	0.91	0.71	0.52
Control (CFA Induced)	1.02	1.03	0.92	0.93
	1.11	1.12	1.01	1.01
	1.12	1.12	1.01	1.02

results of thickness were compared among groups; it indicates that the drug at the dose level inhibited the growth from day 1 till the end of instillation. The test drug showed a significant reduction with a P value of 0.0028 compared to the control value of 0.001. The results also compared day 1 to day 12 (Table 3). The test drug significantly reduced the amount from the 1st day to the 18th day, with a P value of 0.001 compared to the control group's 0.154. So, the present study concluded that the test sample significantly reduces the formation of free radicals, and the process is a key factor for inflammation[25-29]. Our results also reveal that the compound may be effective for the treatment of inflammation-associated diseases, like arthritis. In the chronic inflammation of 21 days, induced by CFA. Aesculetin promoted inhibition in the paw, accompanied by a reduction in the incidence of RA factor and CRP compared to the control animal group (Table 4). The effect of aesculetin on arthritis may be related to the antioxidant and reduction in NO production. Thereby, it reduces the activity of the MAPK pathway, which is a prime factor for arthritis. However, the observed results provide preliminary evidence supporting the anti-arthritic potential of aesculetin. Further studies with larger sample sizes are warranted.

Table 4. Comparison between the groups-unpaired comparison between the groups-unpaired

Group	Odema On Day (Cm)			
	1	6	12	18
Normal	0.413 ± 0.006	0.413 ± 0.006	0.413 ± 0.006	0.427 ± 0.012
Control	1.08 ± 0.055	1.09 ± 0.052	0.98 ± 0.052	0.99 ± 0.049$$$
Standard	1.04 ± 0.049	0.96 ± 0.052**	0.87 ± 0.061	0.71 ± 0.058$$$***
Test-I	1.04 ± 0.049	0.95 ± 0.053**	0.95 ± 0.061	0.65 ± 0.064$$$***
Test-Ii	1.01 0.006	0.91 ± 0.01**	0.75 ± 0.061**Andand	0.55 ± 0.067***#
One-Way ANOVA	0.0369	0.001	0.0028	0.0029

One-way ANOVA statistical analysis was used, n = 6. The values are compared between the groups, unpaired comparison between the groups-unpaired and comparison with test 1.
*Comparison with control
$comparison with normal #comparison with standard

4. Conclusion

Our observation confirms that aesculetin may effectively reduce hind paw thickness in adjuvant-induced arthritis in rats and modulate RA factor level and CRP level in plasma. All may be attributed to the antioxidant property of NO due to the polyphenolic nature of coumarin. So, in the present study, the tested compound is a lead molecule candidate for rheumatoid arthritis after the clinical trial study.

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Author Contribution

Conceptualisation: S. Jayakumari and Dr M. Mahesh; methodology: S. Jayakumari and Sreenath V.; validation: S. Jayakumari and MD Alimoddin; investigation: Sreenath V., S. Jayakumari, Resources Dr M. Mahesh, MD Alimoddin; writing—original draft preparation Jayakumari, writing—review and editing, Sreenath V and S. Jayakumari; visualisation Dr M. Mahesh 1; MD Alimoddin supervision. All authors have read and agreed to the published version of the manuscript.