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Anti-Inflammatory Activity of Flavonoids Isolated from the Seed Extract of Glycine Max (Black) by In-Vitro and In-Vivo methods.

Pramod Kumbhar[1,2]*, Chandraprabhu Jangme[3], Abhinandan Patil[3], Kajal Shinde[1], Manjusha Karepaa[1]

[1]Department of Pharmaceutical Science, Centre for Interdisciplinary Research, D Y Patil Education Society (Deemed to be University), Kolhapur, Maharashtra, India.
[2]Vilasrao Deshmukh Foundation, School of Pharmacy, Latur, Maharashtra, India.
[3]D Y Patil College of Pharmacy, Kolhapur, Maharashtra, India.
*Correspondence: Pramod Kumbhar
Department of Pharmaceutical Science, Centre for Interdisciplinary Research, D Y Patil Education Society (Deemed to be University), Kolhapur, Maharashtra, India.
Email: pramodkumbhar2001@gmail.com

ABSTRACT:

Background: The Glycine max (L.) Merrill (black soybean) belongs to the family Fabaceae, from a high altitude geographic region is an abundant source of flavonoids. The flavonoids are well known for its diverse activities like anticancer, antidiabetic, anti-inflammatory, and hepatoprotective. The present study is carried out to investigate the anti-inflammatory efficacy of isolated flavonoids from seed extract of Glycine Max from Himalayan region (Uttarakhand, India) by in-vitro as well as in-vivo methods.

Methods: The anti-inflammatory activity of flavonoids isolated from 70 % hydroalcoholic seed extract was evaluated using a carrageenan (0.1 ml of 1% w/v carrageenan suspended in 1% carboxymethylcellulose) induced paw edema model in Wistar rats N=6 (Group I, II and III) having either sex with 170-200 gm body weight by in-vivo method. Group I served as control group and treated with distilled water orally, group II as test group treated with test extract dissolved in distilled water orally (250 mg/kg) and group III as standard group treated with Diclofenac sodium (10 mg/kg). Protein denaturation assay was used to screen the in-vitro anti-inflammatory activity of test extract.

Results: The paw edema was measured in volume (ml) at 0, 1st, 2nd, 3rd, 4th, and 5th hours after carrageenan injection by digital plethysmometer by in-vivo method. The obtained results were expressed in mean + SEM and statistical analysis was done by applying a one way ANOVA test followed by a post hoc Tukey test. The test group demonstrated significant anti-inflammatory efficacy as compared to the control and standard group by ($p < 0.001$) and ($p < 0.05$) respectively from in-vivo study. The protein denaturation assay showed highest inhibitory activity of Diclofenac sodium (80.37%) and the test extract (73.43%) at 100 µg/mL. The IC_{50} values, calculated by linear interpolation, were 66.52 µg/mL for Diclofenac sodium and 69.92 µg/mL for the test extract.

Conclusions: The present study scientifically justifies flavonoids isolated from seed extract of Glycine Max have anti-inflammatory activity.

INTRODUCTION:

Inflammation is a complex protective response of the body against infection, tissue injury, and cellular stress. In its acute form, inflammation facilitates the removal of harmful stimuli and supports tissue repair. The persistent or dysregulated inflammation may contribute to tissue damage and the progression of chronic diseases. Activation of inflammatory cells stimulates the release of mediators like tumor necrosis factor-α (TNF-α), interleukins, prostaglandins, nitric oxide (NO), and reactive oxygen species (ROS). Several intracellular pathways, particularly nuclear factor-ϰB (NF-ϰB), mitogen-activated protein kinase (MAPK), cyclooxygenase-2 (COX-2), and inflammasome signaling, are responsible for regulating these inflammatory events [1, 2].

Pharmacological management of inflammation includes non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease-modifying antirheumatic drugs. NSAIDs act through inhibition of cyclooxygenase enzymes and reduction of prostaglandin synthesis, which provides analgesic and anti-inflammatory effects. Prolonged NSAID administration may cause gastrointestinal injury, renal dysfunction, fluid retention, and cardiovascular complications [3]. Corticosteroids provide potent suppression of inflammatory responses and are useful in several inflammatory diseases. But long-term corticosteroid therapy is associated with metabolic disturbances, osteoporosis, hypertension, impaired immune function, and increased susceptibility to infection [4, 5]. These limitations stimulate the researchers for safer and naturally occurring compounds, which are capable of modulating inflammation through multiple biological mechanisms.

Among naturally occurring bioactive compounds, flavonoids have attracted considerable attention because of their antioxidant, anti-inflammatory, and cytoprotective properties. Flavonoids can influence several inflammatory processes, including oxidative stress, cytokine production, NF-ϰB activation, MAPK signaling, and expression of inflammatory enzymes. Their multi-target activity is particularly relevant because chronic inflammation is not controlled by a single mediator or pathway. The plant-based flavonoids are increasingly being investigated as potential complementary sources of anti-inflammatory agents [6].

Black soybean Glycine max (L.) Merr. is a promising source of such compounds. Its dark seed coat contains considerably higher concentrations of phenolic compounds and anthocyanins. Anthocyanins and isoflavones constitute important flavonoid groups in black soybean [7]. Recent studies indicate that the bioactive constituents of black soybean are associated with antioxidants and other potential health-promoting effects [7, 8]. High content of isoflavones in black soybean are reported at higher altitudes than in low altitude genotypes [9]. Hence this topic is taken into consideration.

2. MATERIALS AND METHOD:

Chemicals and standard drugs:

Chemicals and standard drugs used in this study were of analytical grade and were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Deionized water was used in all experiments.

Preparation of plant extract:

The seeds of black soybean were collected from the Himalayan region of Uttarakhand, India, in January 2024 and authenticated by the Department of Botany, Agriculture College, Latur, Maharashtra. The seeds were shade-dried at room temperature to reduce their moisture content and washed thoroughly to remove dirt and other extraneous matter. The dried seeds were pulverized into a coarse powder and passed through sieve No. 60 to obtain a relatively uniform particle size. The powdered material was defatted with n-hexane for 24 h to remove the lipid fraction [10]. The defatted powder was then extracted separately with 70% ethanol, distilled water, and acetone using a Soxhlet apparatus maintained at 60 °C for 5 h, with a sample-to-solvent ratio of 1:10 (w/v). The selection of aqueous ethanol and acetone as extraction solvents was based on their established ability to recover phenolic and flavonoid constituents from soybean and other plant matrices [11–12]. After extraction, the individual solvent extracts were concentrated under reduced pressure by using a rotary vacuum evaporator to obtain the corresponding ethanolic, aqueous, and acetone crude extracts. Similar vacuum-evaporation procedures have been employed for concentrating soybean phenolic extracts while minimizing prolonged exposure to elevated temperatures [11]. The flavonoid-rich constituents were subsequently separated from the crude extracts by column chromatography [13].

Animals:

This study involved healthy Wistar rats of either sex, each weighing between 170 and 200 g. The animals were maintained in hygienic polypropylene cages under controlled environmental conditions, including a room temperature of 25 ± 2°C, a relative humidity of 45–55%, and a regulated 12-hour light/dark cycle. Except during specific experimental phases, animals received

the standard laboratory diet and water (*ad libitum*). All animal care practices are strictly followed by the rules established by the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). The experimental work was done at the animal facility of D Y Patil Medical College, Kolhapur, following official ethical approval from the Institutional Animal Ethics Committee (IAEC) of D Y Patil Medical College, Kolhapur, under registration number 1800/PO/ReBi/15/CPCSEA.

Acute oral toxicity study:

An acute oral toxicity study was done by following the procedures given in OECD Guideline 423 to determine the safety profile of the test extract. Healthy female Wistar rats which were non-pregnant, aged in between 8 to 10 weeks and weighing approximately 170 to 200 g, were selected for the study. The animals were randomly divided into groups, (n = 3). Before administration of the test extract, the animals were fasted overnight and provided unrestricted (ad libitum) access to drinking water.

***In-vivo* Evaluation of Anti-inflammatory activity:**

The anti-inflammatory activity of test extract was investigated by using carrageenan-induced hind-paw edema model in Wistar rats [14–17]. The animals were selected randomly and allocated into three groups (n = 6). Group I served as the carrageenan control and received the vehicle only. Group II was administered the test extract at a dose one-tenth of its LD_{50}. Group III received the standard Diclofenac sodium (10 mg/kg) body weight [15, 18, 19].

Acute inflammation was induced in all rats by sub-plantar administration of 0.1 mL of 1% (w/v) carrageenan into the left hind paw [14, 16, 17]. The Carrageenan was prepared in a 1% (w/v) carboxymethyl cellulose (CMC) suspension. Paw thickness was recorded by using a digital plethysmometer immediately before carrageenan administration (0 min) to keep the baseline value and subsequently at 60, 120, 180, and 240 min after inflammation induction [15, 17, 20]. The changes in thickness of paw relative to the baseline measurement were used to determine the inhibitory effect of the test extract on carrageenan-induced edema in comparison with the carrageenan control and diclofenac-treated standard group.

Statistical Analysis:

Experimental data are presented as mean ± standard error of the mean (SEM). To determine statistical variance one-way ANOVA followed by the Tukey's post hoc test was performed. Statistical significance was defined by 'p' value.

***In vitro* Evaluation of Anti-inflammatory activity by protein denaturation assay:**

The in vitro anti-inflammatory potential of the test extract was done by heat-induced denaturation of bovine serum albumin (BSA). Protein denaturation is considered as one of the processes linked with inflammatory responses [21, 22]. In the present assay, different concentrations of the test extract are incubated with 1% BSA. The controlled heating is provided to induce protein denaturation. After cooling the reaction mixture to room temperature, the turbidity is measured by the spectrophotometric method at 660 nm, with diclofenac sodium used as the reference standard [21, 23]. The percentage inhibition of protein denaturation was calculated by comparing the absorbance of the treated samples with that of the control, and a higher percentage inhibition indicates greater protection against protein denaturation. The IC_{50} value, representing the concentration required to inhibit 50% of protein denaturation, may also be determined to compare the relative anti-inflammatory potency of the extract with the standard drug [22, 23]. The percentage inhibition of protein denaturation was calculated using the following formula:

$$\text{Percentage Inhibition} = \frac{\text{A control - A sample}}{\text{A control}} \times 100$$

3. RESULTS:

Acute oral toxicity study:

The outcomes of acute oral toxicity for the test extract demonstrated high safety margins. During the first phase of evaluation of three female rats treated with 2000 mg/kg, the extract did not induce any clinical symptoms of toxicity or mortality. In the subsequent confirmatory phase at the same dose level resulted in one mortality. All animals were observed during the first 30 minutes continuously for critical clinical signs. Periodically the first 4 hours post-administration, and daily thereafter for 14 days.

The histopathology of vital organs, including the liver, kidneys, heart, lungs, and spleen, showed no structural lesions, tissue congestion, or treatment-related abnormalities. As per the OECD Guideline 423 a 1/6 mortality rate at 2000 mg/kg excludes the extract from high-toxicity categories (Categories 1–4). Hense, the median lethal dose (LD_{50}) was determined to be 2500 mg/kg.

***In-vivo* Evaluation of Anti-inflammatory activity:**

Table 1: *In vivo* anti-inflammatory effect of the test extract and standard Diclofenac.

Groups	Rat paw edema volume in ml at different time interval (hrs)					
	0 hr	**1 hr**	**2 hr**	**3 hr**	**4 hr**	**5 hr**
I. Control	0.94 ± 0.00j	1.83 ± 0.01ad	1.89 ± 0.00a	1.85 ± 0.01ab	1.77 ± 0.01bd	1.68 ± 0.01ce
ll. Test	0.93 ± 0.00j	1.71 ± 0.06cgh	1.76 ± 0.03abc	1.65 ± 0.05cfh	1.48 ± 0.05egh	1.41 ± 0.02gh
III. Standard	0.93 ± 0.00j	1.38 ± 0.04gi	1.45 ± 0.02fg	1.40 ± 0.02fg	1.35 ± 0.03gh	1.33 ± 0.02hi

All values are given as Mean ± SEM N=6. Means with different letters differ significantly at $P < 0.05$ while means with the same letter do not differ significantly at $P < 0.05$ by using one-way ANOVA with Tukey's post hoc test.

The anti-inflammatory activity of the test extract was evaluated using the carrageenan-induced rat paw edema model. The paw edema volume was measured over a period of 5 hours. The results are presented as mean ± SEM (n = 6) and analyzed using one-way ANOVA followed by Tukey's post hoc test as given in Table 01. At baseline (0 h), there was no significant difference in paw edema volume of the control, test, and standard groups, indicating comparable initial conditions before carrageenan administration. After carrageenan injection, the control group exhibited an increase in paw edema progressively. This pattern is characteristic of acute inflammation induced by carrageenan in the control group.

The standard group treated with Diclofenac sodium showed a marked reduction in paw edema throughout the experimental period compared with the control group. The edema volume was significantly lower at all post-treatment time points. These reductions were highly significant ($p < 0.05$) compared with the control group, exploring the potent anti-inflammatory effect of Diclofenac Sodium as a standard drug.

The test extract treated group also demonstrated a reduced paw edema volume compared to the control group ($p < 0.05$). Comparison with the standard group revealed no significant difference at time period 1, 3, 4, and 5 hrs ($p > 0.05$). It indicates the anti-inflammatory effect of the test extract was comparable to that of the standard drug during 3,4 and5 h time points. However, at 2 h, the test group showed a significant difference from the standard group ($p > 0.05$), suggesting that the comparatively lower inhibitory effect during the peak phase of inflammation.

***In vitro* Evaluation of Anti-inflammatory activity by protein denaturation assay:**

The in vitro anti-inflammatory activity of the test extract was evaluated using the protein denaturation assay and compared with the standard anti-inflammatory drug, Diclofenac sodium in Figure 01. The results revealed that both the standard drug and the test extract inhibited protein denaturation in a concentration-dependent manner, indicating their ability to stabilize proteins against heat-induced denaturation.

Diclofenac sodium exhibited percentage inhibition values of 25.07%, 33.57%, 40.87%, 68.87%, and 80.37% at concentrations of 20, 40, 60, 80, and 100 µg/mL, respectively. Similarly, the test extract showed increasing inhibitory activity with increasing concentration, producing 11.57%, 34.17%, 43.53%, 56.57%, and 73.43% inhibition at the corresponding concentrations. As shown in Figure 01 the highest inhibitory activity of Diclofenac sodium (80.37%) and the test extract (73.43%) was observed at 100 µg/mL. The IC_{50} values are calculated by linear interpolation, as shown in Figure 2, were 66.52 µg/mL for Diclofenac sodium and 69.92 µg/mL for the test extract.

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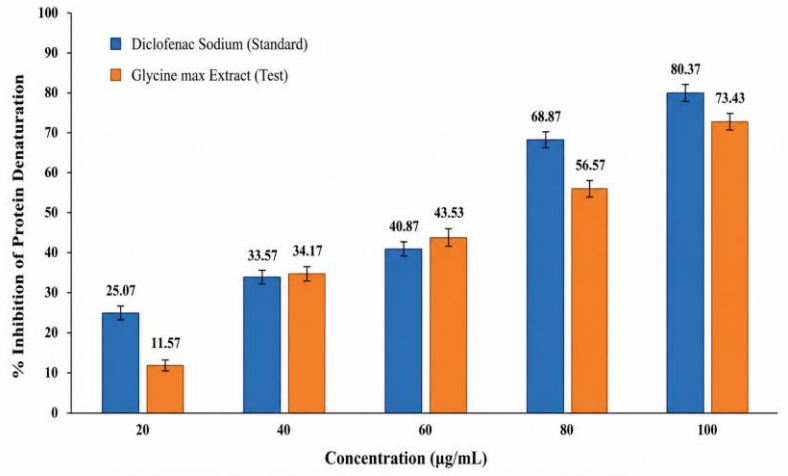

Figure 1: *In vitro* anti-inflammatory effect of the test sample and standard Diclofenac.

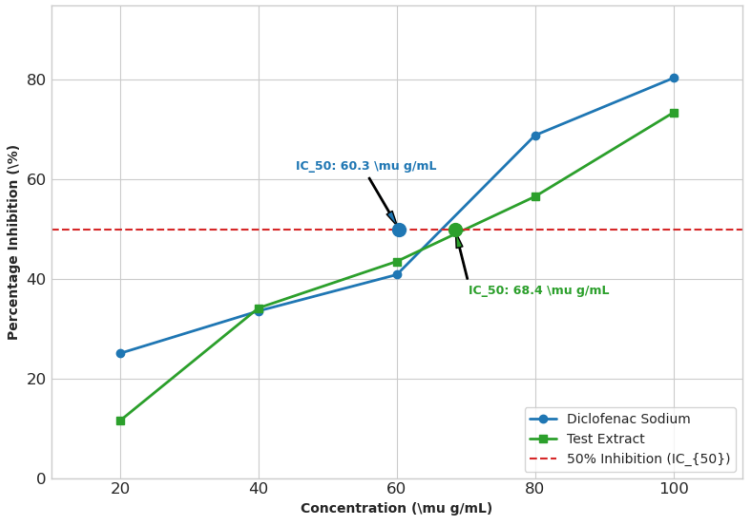

Figure 2: IC50 value of the test sample and standard Diclofenac.

4. DISCUSSION:

The present study demonstrated that flavonoids which are isolated from the seed extract of *Glycine max* (black soybean) possess potential anti-inflammatory activity in both *in-vitro* and *in-vivo* experiments with high safety profile. The acute oral toxicity findings suggest that the test extract has a relatively more favorable safety profile at a dose of 2000 mg/kg. One mortality was observed among the six treated animals. The surviving animals did not show any noticeable signs of toxicity or abnormal behavior during the observation period. The animals were closely monitored during the initial 30 minutes, at regular intervals for the first 4 hours, and daily for 14 days, according to OECD Guideline 423. The absence of treatment-related histopathological changes in the liver, kidneys, heart, lungs, and spleen further supports the lack of significant organ toxicity. These findings indicate that the extract can be considered reasonably safe for further pharmacological investigations.

In the *in-vivo* study, the test group exhibited a highly significant reduction in inflammatory response compared with the control and standard groups with ($p < 0.001$) and ($p < 0.05$) respectively. It indicates substantial anti-inflammatory efficacy of the isolated flavonoids in *Glycine max*, which are known for the several molecular pathways involved in the inflammation process [24, 25].

Flavonoids are reported to modulate important inflammatory mediators and signaling pathways, including nuclear factor-kappa B (NF-$\varkappa$B), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and pro-inflammatory cytokines like tumor

necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [24, 26]. Inhibition of these pathways can reduce the production of inflammatory mediators and decrease tissue edema.

The *in-vitro* findings supported the biological activity of the test extract. At 100 µg/mL, the highest inhibition was observed with standard drug diclofenac sodium 80.37%, where the test extract demonstrated 73.43% inhibition. The IC_{50} values calculated using linear interpolation were found to be 66.52 µg/mL for diclofenac sodium and 69.92 µg/mL for the test extract. The relatively close IC_{50} values indicate that the test extract possessed considerable activity under the experimental conditions. The difference in IC_{50} between the standard and test extract was approximately 3.4 µg/mL, suggesting that the isolated flavonoid fraction had appreciable pharmacological potential.

The findings in present study are consistent with previous investigations on soybean-derived phytochemicals. Soybean extracts have been reported to suppress inflammatory responses by reducing nitric oxide production and the expression of inflammatory mediators such as IL-1β, IL-6, TNF-α and COX-2 [27]. Similarly, soybean isoflavones, particularly daidzein and genistein, have demonstrated inhibitory effects on inflammatory responses in macrophages through modulation of NF-κB and MAPK signaling pathways [28]. These observations provide a possible mechanism for the anti-inflammatory activity observed in the present study.

The outcome of the literature survey says that modification of soybean processing conditions can increase the concentration of biologically active aglycone isoflavones and ultimately anti-inflammatory activity [29]. Flavonoids may additionally contribute through their antioxidant properties, since oxidative stress and inflammation are closely interconnected and reactive oxygen species can stimulate inflammatory signaling [24, 25].

5. CONCLUSION:

This study demonstrates that flavonoids isolated from *Glycine max* seeds exhibit potent anti-inflammatory activity by effectively reducing carrageenan-induced rat paw edema by *in vivo* as well as *in vitro* method. These findings suggest that *Glycine max* seed extract could serve as a valuable foundation for discovering new treatments to prevent and manage inflammatory disorders.

6. CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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