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IN VITRO EVALUATION OF THE ANTI-INFLAMMATORY, ANTIOXIDANT, AND XANTHINE OXIDASE INHIBITORY POTENTIAL OF CALYCOSIN, A NATURALLY DERIVED ISOFLAVONE

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Abstract

Chronic inflammation and oxidative stress have significant contributions towards the course of various diseases including rheumatoid arthritis, cardiovascular disorders, diabetes, and some cancers. Plant-derived flavonoids have been of interest due to the capacity to affect inflammatory processes and minimize oxidative stress. Calycosin is a type of isoflavone that has been isolated in Astragalus membranaceus, and it has anti-inflammatory, antioxidant, and analgesic properties. This paper has studied the in vitro anti-inflammatory, xanthine oxidase (XO) and antioxidant properties of calycosin to determine its suitability as a natural therapy.

The protein denaturation assay was carried out to determine the anti-inflammatory activity. The DPPH free radical scavenging assay was used to determine the antioxidant potential. XO inhibitory activity was also determined spectrophotometrically; allopurinol was utilized as a positive control. Calycosin was subjected to the range of 10-300 ug/mL. We estimated the percentage of inhibition in all the assays and established the IC50 by the linear regression. The statistical analysis was conducted by one way ANOVA and post hoc test by Tukey.

These findings suggest that calycosin has notable anti-inflammatory and antioxidant activities, along with moderate XO inhibition. This supports its potential as a natural treatment for managing inflammation and oxidative stress-related disorders. Further research is needed to clarify its mechanisms and explore clinical applications.

Keywords: *Anti-inflammatory, Antioxidant, Calycosin, Flavonoids, Natural therapeutics, Xanthine oxidase inhibition*

INTRODUCTION

Inflammation is a vital and needed physiological phenomenon, wherein the immune system of a body reacts to a set of perceived attacks, such as pathogens, injured cells or irritants, like the chemicals. The acute inflammatory reaction is natural and localized and it is there to get rid of the stimulating agent and allow the wound to heal. Nevertheless, a long-term chronic inflammation is destructive to the body. Various autoimmune diseases that are linked to chronic inflammation include cardiovascular disease, diabetes and various types of cancers (1).

The fact that natural bioactive compounds of the plants are used in traditional medicine over centuries is the testimony to their various health-promoting effects. Flavonoids are one of such substances with a large number of pharmacological activities including anti-inflammatory, antioxidant and analgesic effects; this has led to an in-depth characterization of flavonoids. Calycosin is an isoflavone (compound) isolated in *Astragalus membranaceus*, which is one of the promising agents. It is a Chinese traditional medicine that is used to treat infections, inflammation and cardiovascular diseases (2). The scientific community has been interested in the bioactive properties of calycosin, which has a role in causing diseases that involve inflammation. It was also noted that calycosin might possess anti-inflammatory effects and the

action of this substance is aimed at the reduction of the synthesis of various pro-inflammatory cytokines like TNF-a and IL-6. These cytokines play a role in amplifying the processes of inflammatory responses and also in causing pain. Under the inhibition of these molecules, the sensitivity of nociceptors and the presence of immune cells in the inflamed tissues may be lowered, which subsequently decreases the pain caused by inflammation (3). It illustrates the potential of calycosin as a natural agent with the potential to regulate inflammatory pathways with little side effects linked to majority of the anti-inflammatory agents.

Calycosin had a great antioxidant activity, which might be explained by its antioxidant activity by means scavenging of free radicals and increasing the activity of the endogenous antioxidant enzymes. Calycosin exhibited an activity of an antioxidant according to the DPPH free radical scavenging in response to concentration and the greater was the concentration, the greater was the activity (4).

Calycosin is attracted as a possible treatment recently. The list of how it works in the body is ever-growing, with scientists making new findings and researchers having demonstrated anti-inflammatory effects, antioxidant effects, pain relief, to possibly block XO. All these qualities make it a good competitor to safer, plant-based medicines. In this experiment, we employed both the laboratory and animal experimentation that investigated the impact of calycosin on inflammation, oxidative stress, pain and XO activity.

METHODOLOGY

PROTEIN DENATURATION ASSAY

To test the anti-inflammatory ability of calycosi, the classic test of protein denaturation test was applied to identify the compounds that inhibit the loss of structure of proteins which is an early sign of inflammation. Calycosin was added in 10, 50, 100, and 300 ug/mL concentrations in order to see the denatured protein in heating condition. Our positive control was diclofenac sodium, which is a famous anti-inflammatory medication.

The assay was done by the interaction of egg albumin (the protein), phosphate buffer, and different concentrations of calycosin. The mixtures were incubated after which they were heated to 70°C in order to cause protein denaturation to be done followed by cooling. The absorbance of each mixture was determined spectrophotometrically after the mixture had cooled. A common anti-inflammatory agent was diclofenac sodium, which was used as our positive control.

The proportion of protein denaturation inhibition was computed by using the following equation:

% Inhibition = (Absorbance of control - Absorbance of sample) / Absorbance of control × 100

DPPH FREE RADICAL SCAVENGING ASSAY

The DPPH assay was used to determine the antioxidant capacity of calycosin, it is an examination of how effective a compound is in neutralizing the free radicals. Various solutions of calycosin i.e: 10, 50, 100 and 300 ug/mL were taken. To carry out the assay, calycosin and mixtures were incubated in the dark at room temperature with freshly prepared DPPH solution in methanol before 30 minutes. The absorbance at 517 nm was then taken after incubation using a spectrophotometer. Ascorbic acid (vitamin C) was stored as the positive control. Radical scavenging activity was determined in the following way.

% Inhibition = (Absorbance of control - Absorbance of sample) / Absorbance of control × 100%

XANTHINE OXIDASE (XO) INHIBITION ASSAY

To explore more on how calycosin can be used to prevent xanthine oxidase (XO), an enzyme that produces reactive oxygen species in the course of purine metabolism. The XO inhibition is an excellent antioxidant ability marker. In order to test the calycosin the following mixtures were prepared i.e xanthine (substrate), XO enzyme, calycosin at various concentrations, and the reaction was allowed to take place in a period of 30 minutes. The allopurinol was served as the XO inhibitor and standard. The formation of uric acid was measured at 290 nm. To determine the extent of XO inhibition, the results were compared from calycosin-treated samples to the control (without calycosin). Data are presented as mean ± SD.

STATISTICAL ANALYSIS

All experimental results were reported as mean ± SEM or mean ± SD, according to the specific assay requirements. Statistical comparisons among the groups were conducted using one-way ANOVA, followed by Tukey's post hoc test to assess the significance between treatments. A p-value of < 0.05 was regarded as statistically significant. IC_{50} values for antioxidant activity were determined using linear regression analysis.

RESULTS

INHIBITION OF PROTEIN DENATURATION

To assess the anti-inflammatory nature of calycosin, an *in-vitro* protein denaturation assay was utilized and the% age of inhibition was determining at various concentrations shown in Table I. Calycosin showed a high inhibitory effect with 79.44% and 80.68% inhibition being observed at the concentration of 50 ug/mL and 100 ug/mL respectively ($p = 0.001$). As expected, the standard Diclofenac exhibited greater inhibition with 89.88 at 100 ug/mL and 88.91% at 300 ug/mL being statistically significant ($p< 0.001$). These findings indicated that calycosin is a good anti-inflammatory agent, which is effective in inhibiting the process of protein denaturation.

Table I. Effect of calycosin on inhibition of protein denaturation

Compounds	Concentration (µg/ml)	Protein denaturation	Protein denaturation (Inhibition %)
Control	-	0.616 ± 0.010	-
Diclofenac	10	0.124 ± 0.001*	79.87
	50	0.152 ± 0.0008*	75.27
	100	0.062 ± 0.001*	89.88
	300	0.068 ± 0.001*	88.91
Calycosin	10	0.213 ± 0.001*	65.40
	50	0.126 ± 0.002*	79.44
	100	0.119 ± 0.007*	80.68

Results are expressed as Mean ± SEM where n=5 for each group. Significant value: *p<0:001 compared to the control group done by ANOVA followed by Tukey's post hoc test

ANTIOXIDANT ACTIVITY

To evaluation of the antioxidant capacity of calycosin was observed through the DPPH free radical scavenging assay, ascorbic acid used as the reference standard. The percentage inhibition of compounds is compared in Fig. 1a. Calycosin revealed a significant concentration-dependent increase in radical scavenging property, attained maximum inhibition of 74.37% and 76.12% at concentrations of 100 and 300 µg/mL respectively. An analogous finding was observed in ascorbic acid which had a better scavenging capacity and had the ability of 81.84% and 88.15% inhibition in the same concentrations. Using the linear regression analysis, the half-maximal concentration of Calycosin and ascorbic acid were established. The IC_{50} of ascorbic acid was determined as 2.08 ug/mL (Fig. 1b) and calycosin was found to have a moderate but significant antioxidant activity at a relatively high IC50 of 2.41 ug/mL.

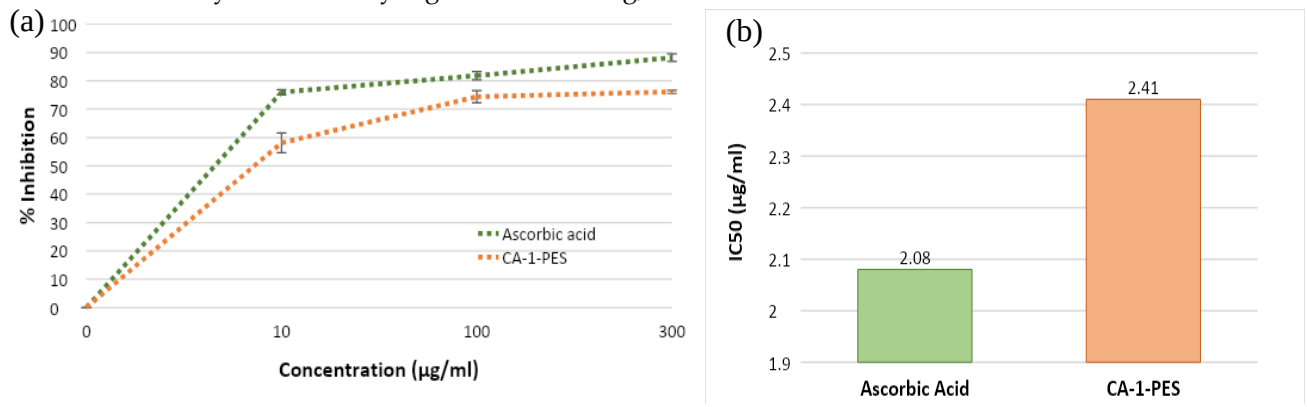

Fig. 1(a). Anti-oxidant activity of calycosin, values are expressed as Mean±SD **(b).** IC50 of calycosin and ascorbic acid

XANTHINE OXIDASE POTENTIAL

Calycosin was tested on xanthine oxidase (XO) as an inhibitor with the help of different concentrations and the results were recorded in Table II. The positive control was allopurinol, which is a famous XO inhibitor. There was moderate inhibitory power of calycosin with 39.75 and 39.04 percent inhibition 300 ug/mL and 100 ug/mL respectively. Comparatively, allopurinol showed much greater potency with a 97.6% inhibition level at 50 and 100 ug/mL, which justifies its usefulness as a standard xanthine oxidase inhibitor.

Table II. Xanthine oxidase activity of Calycosin

Compound	Concentration (µg/ml)	Inhibition % (Mean±SD)
Allopurinol	10	18.27 ± 0.27
	25	29.69 ± 0.77
	50	97.62 ± 1.61
	100	97.63 ± 0.83
Calycosin	10	15.43 ± 0.89
	30	15.20 ± 0.55
	100	39.04 ± 0.45
	300	39.75 ± 0.81

*Results are expressed as Mean ± SD where n=5 in each group

DISCUSSION

The present study provides the insights of calycosin as a therapeutic agent. It demonstrates numerous effects- anti-inflammatory, antioxidant and analgesic. These properties particularly the anti-inflammatory and antioxidant effects enable calycosin to become a novel solution to the treatment of chronic inflammatory diseases, as inflammation and oxidative stress are indeed the sources of the disease in these cases. In this case, there is an emphasis on the pure bioactivity of calycosin, therapeutic worth, and its relative efficacy compared to traditional therapies. Calycosin in this research produced good anti-inflammatory effects. Its primary mechanism of action included inhibitions of protein denaturation, a reduction in pro-inflammatory cytokines, and the attack on COX-2 enzymes. In case the proteins lose structure, the immune system intervenes and kick-starts inflammation (5). By preventing that procedure, calycosin manages the inflammation, which may prove to be a substantial benefit to such disorders as arthritis. This is supported by the numbers: the inhibition rate of calycosin was 79.44% when the concentration was 50 ug/mL and 80.68% when the concentration was 100 ug/mL, which is almost identical to diclofenac, a common NSAID. Calycosin should therefore be seen as a natural substitute to anti-inflammatories, synthetic and better with fewer side effects, which NSAIDs are infamous with (6). The research also had a closer examination of the anti-inflammatory effect of calycosin in living model, using the formalin induced paw edema. This test has an immediate and prolonged inflammation. Calycosin was effective, reducing swelling as effectively as indomethacin. It also reduced the major inflammatory cytokines, TNF-a, and IL-6 which are the usual suspects in stimulating inflammation and the immune cells (7). Reducing the production of these cytokines, yet not reducing the normal functioning of the body, calycosin may provide some defense to tissues in chronic diseases such as rheumatoid arthritis and inflammatory bowel disease. Its mechanism of action with regard to cytokines is a pointer to true potential in illnesses which are not always sensitive to conventional anti-inflammatory medication. The most interesting fact about calycosin is that it is selective on COX-2. The majority of NSAIDs inhibit both CoX-1 and CoX-2 which mostly results in stomach and heart problems. Calycosin, however, is specific to COX-2, which is the one that is increased when there is inflammation (8, 9). Such a selective fold may render it safer to use over the long-term and place it in a good position among the natural COX-2 modulators. Another giant in the disease process such as heart problems, cancer, and neurodegenerative disorders is oxidative stress (10). In this case, calycosin was a potent antioxidant and it exhibited 76.12% DPPH radical scavenging at 300 ug/mL. That is, it is rather effective in neutralizing free radicals, or, in other words, in protecting cells and reducing oxidative damage. This is in line with our knowledge of flavonoids. Plant-based polyphenols possess a reputation on reducing oxidative stress and

preventing lipid peroxidation (11). The antioxidant advantage of calycosin is also due to the fact that it activates the Nrf2 signaling pathway, which is akin to switching on the antioxidant protective mechanisms of the body itself (12). Once Nrf2 is activated, it increases the concentration of such essential enzymes as SOD, catalase, and GPx, which causes cells to be more resistant to oxidative stress. With this, calycosin may be used to provide defense in diseases in which oxidative stress never relents, such as diabetes and heart disease. Top of that, calycosin was found to be capable of inhibiting xanthine oxidase (XO), an enzyme that takes part in purine metabolism also producing reactive oxygen species on uric acid generation. Although it is not as potent in this application compared to allopurinol, the combination of antioxidant and anti-inflammatory effects of calycosin would also allow it to be used as an add-on to various conditions such as hyperuricemia and gout (13). Calycosin could help prevent issues related to oxidative stress by reducing the ROS level and maintaining the uric acid concentration.

CONCLUSION

Current findings indicate that calycosin demonstrates strong in vitro anti-inflammatory, antioxidant, and xanthine oxidase (XO) inhibitory effects. These results suggest that calycosin may have potential as an anti-inflammatory, pain-relieving, and anti-gout agent with fewer side effects. However, additional in vivo studies, clinical trials, and mechanistic research are necessary to confirm its effectiveness and establish its potential as a therapeutic candidate for diseases related to inflammation and xanthine oxidase activity

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Author s' contributions:

HKM Conducted the experimental work and data collection; SM Conceptualized and Supervised the study; FT done the analysis of results; NHS and SW worked for write up; AS provided the experimental support and supervision.

Declaration of generative AI-Assisted Tools:

No AI-assisted tools were used.

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