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GUIDELINE

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Guidelines for the in vitro determination of anti-inflammatory activity

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Abstract

Chronic inflammation contributes to various chronic diseases in humans. Currently used anti-inflammatory drugs have several limitations and are not suitable for long-term use. Anti-inflammatory foods or extracts can prevent inflammation-related diseases, offering a promising approach for the management of these pathologies. In vitro anti-inflammatory experiments have the advantages of short experimental period and low cost for screening anti-inflammatory foods or drugs. They measure pro-inflammatory enzyme activity such as lipoxygenase (LOX) and cyclooxygenase (COX), and the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukins (IL). This article reviews in vitro assays used to evaluate the anti-inflammatory effect, examining the effects of different inflammatory factors and the employed methods, and analyzing their principles, advantages, and disadvantages. Thus, it aims to provide guidelines for screening natural foods with anti-inflammatory potential.

KEYWORDS

anti-inflammatory, cyclooxygenase, hyaluronidase, in vitro, interleukins, lipoxygenase, nitric oxide, tumor necrosis factor

1 | INTRODUCTION

Chronic, low-grade inflammation can lead to serious diseases including cardiovascular disease, cancer, and type 2 diabetes. Approximately three out of every five people worldwide die from inflammation-related diseases, prompting global action. In 2011, the United Nations High-Level Meeting on Chronic Noncommunicable Diseases set a global goal of "reducing premature deaths from chronic diseases by 25% by 2025." Inflammation is the initial defensive response of the body consisting in cellular changes and immune responses that lead to elimination of pathogenic microbes, repair of damaged tissue, and the proliferation of cells in lesioned areas (Yi, 2016). However, if it persists or its regulatory mechanisms fail, inflammation can become chronic (Coussens & Werb, 2002).

The key intracellular mediators of inflammation are signal sensors and activators of transcription (Xiao et al., 2023). The most critical inflammatory transcription factors are nuclear factor (NF)-κB, nuclear factor of activated T cells, and activated protein (AP)−1. Measuring these intracellular factors is challenging (Lee et al., 2022). Easier-to-measure inflammatory biomarkers include anti-inflammatory enzymes and proteins such as cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), HAase, cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8), and nitric oxide (NO) synthesized by inducible NO synthases (iNOS) (Jeong et al., 2015).

Both COX-2 and 5-LOX use arachidonic acid (AA) as a substrate. COX-2 converts AA into prostaglandins (PG), most of which promote pain, fever, and other inflammatory symptoms (Nhiem et al., 2017). 5-LOX

metabolizes AA to leukotrienes (LT), significantly enhancing inflammatory and allergic reactions. INOS-derived NO provides protection against infectious pathogens and modulates immunity. However, NO produced in excess is generally oxidized to reactive nitrogen species, causing cell death in the surrounding tissues and disrupting tissular homeostasis. TNF-α and ILs are cytokines produced by inflammatory cells such as macrophages. They are involved in cell differentiation and apoptosis and are important pro-inflammatory mediators (Wang et al., 2021).

The prolonged presence of these pro-inflammatory enzymes and factors can induce inflammation-related diseases including Alzheimer's disease (AD) and atherosclerosis (AS). Nonsteroidal anti-inflammatory drugs (NSAIDs) used to treat acute inflammatory diseases, have limited efficacy in the treatment of chronic inflammatory diseases, including rheumatoid arthritis, while steroid anti-inflammatory drugs are more effective but also induce more severe adverse effects (Anyasor et al., 2019). Therefore, the development of safe and well-tolerated anti-inflammatory drugs has become imperative.

Diet is a major contributor to inflammation. Therefore, adopting an "anti-inflammatory diet" might be an effective approach to mitigating inflammation (Minihane et al., 2015). Identifying anti-inflammatory food ingredients could provide a superior option for preventing and treating chronic inflammation. Identifying such anti-inflammatory compounds, as well as the development of novel anti-inflammatory drugs, includes in vitro and in vivo assessment. In vivo experiments, mainly using mouse models, provide data more relevant to clinical applications but are demanding, expensive, and time-consuming (Han et al., 2022). For example, Lycium berries from *Lycium barbarum* significantly inhibited lipopolysaccharide (LPS)-induced production of NO, IL-6, and TNF-α in an AS mouse model, demonstrating therapeutic potential (Shen et al., 2020). Nootkatone, a sesquiterpene from Fructus Alpiniae Oxyphyllae, improved symptoms in mouse models of LPS-induced AD being associated with decreased brain levels of inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Wang et al., 2018).

In vitro experiments are easier to perform than in vivo experiments, offering simplicity, shorter timelines, and lower costs for rapid anti-inflammatory substance screening. For example, flavonoids in Fritillaria extract inhibited IL-6 and NO production in in vitro experiments. Further tests demonstrated that these flavonoids reduced lung inflammatory responses and attenuated LPS-induced lung injury in mice (Lee et al., 2015). A parthenolide-depleted extract of Feverfew directly inhibited the activity of 5-LOX and the release of NO, prostaglandin E_2 (PGE_2), and TNF-α in vitro (Sur et al., 2009).

This paper focuses on in vitro experiments used to assess the anti-inflammatory effects of various substances. We reviewed common and practical methods based on the determined key anti-inflammatory markers: lipoxygenase (LOX), cyclooxygenase (COX), hyaluronidase (HAase), NO, TNF-α, and interleukins (ILs). These insights could serve as valuable references for the development of anti-inflammatory drugs from natural sources.

2 | LOX INHIBITION TEST

LOXs are nonheme iron-containing enzymes widely distributed in plants and animals (Kuhn et al.,2015). In plants, they primarily metabolize linoleic acid (LA), whereas in mammals they biotransform AA (Landberg et al., 2020). In mammals, LOXs are found primarily in the lungs, platelets, and leukocytes. Based on their ability to insert oxygen atoms into AA, LOXs are divided into four main types: 5-LOX, 8-LOX, 12-LOX, and 15-LOX, with 5-LOX having direct links to human diseases (Haeggström & Funk, 2011).

In the presence of LOX, AA can be oxidized to 5-hydroperoxide 20-carbo4-enoic acid (5-HPete) at five sites. Subsequently, it is converted to a 5,6-epoxide (LTA_4) (Pergola & Werz, 2010). LTA_4 is hydrolyzed by hydrolase to 5,12-dihydroxy20-carbo4-enoic acid (LTB_4). Glutathione-S-transferase converts LTA_4 into leukotriene C_4 (LTC_4) which loses a glutamate to form leukotriene D_4 (LTD_4) via γ-glutamyltransferase. LTD4 loses glycine to form LTE_4 which can also bind to glucosyltransferase to form LTF_4 (Figure 1). LTC_4, LTD_4, LTE_4, and LTF_4 are leukotrienes combined with peptides, or peptide leukotrienes (pLTs) (Rådmark et al., 2015).

Leukotrienes and their degradation products trigger leukocyte lysosomal enzyme release, amplifying inflammation. Thus, leukotriene concentration increases at inflammation sites (Pirault & Bäck, 2018). LTC_4, LTD_4, and LTE_4 are high-reactivity substances present during anaphylaxis (Nijkamp & Sitsen, 1982). They play an essential role in the occurrence of allergy-related inflammations. Consequently, inhibiting 5-LOX activity and regulating LT biosynthesis can provide an approach for treating diseases such as allergies and inflammation.

2.1 | Manometry (Warburg breathometer method)

LOX is an oxidoreductase that specifically catalyzes the formation of hydroperoxides of unsaturated fatty acids with a common double bond by molecular oxidation of polyunsaturated fatty acids with cis- and cis-pentadiene structures (Jacob P & Manju, 2020). The assay is based on maintaining the concentration of the substrate constant, while changes in dissolved oxygen levels are linearly related to the enzyme activity. Oxygen consumption occurs during the oxidation of polyunsaturated fatty acids by liquid oxygen, generating double-bonded

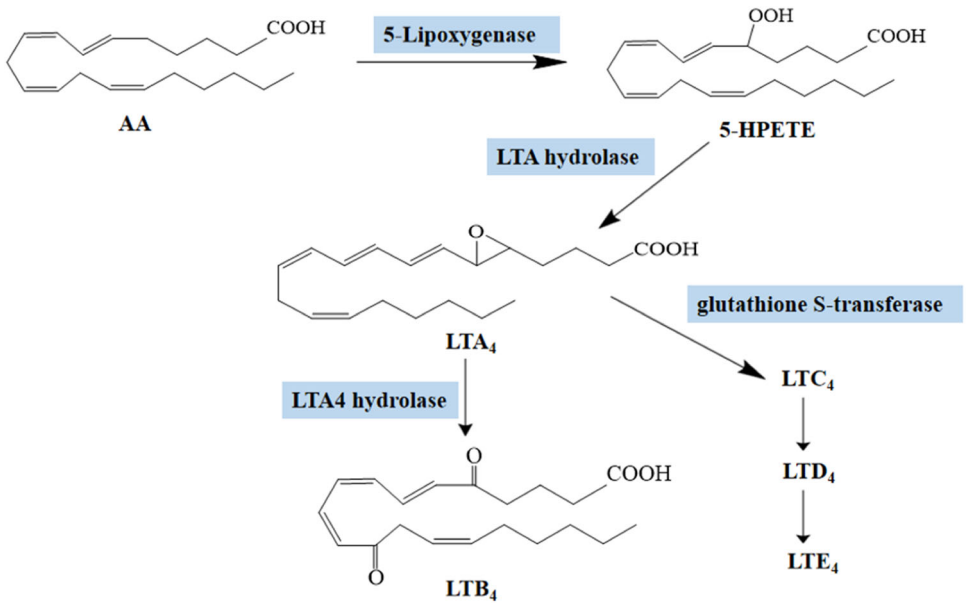

FIGURE 1 Leukotriene biosynthesis.

hydrogen peroxide. The rate of oxygen concentration decrease in the solution is proportional to the enzyme activity, making accurate measurement possible using an oxygen electrode. This method is simple and easy to operate (Table 1).

2.1.1 | Sample preparation

Fresh plant samples were collected, and any surface contaminants were carefully removed. The samples were then finely chopped, and precisely 0.5 g was weighed and placed in a mortar. A quantity of 5 mL Tris-hydrochloric acid buffer 25 mmol/L (pH 7.5) was added, and the mixture was ground in an ice bath, followed by homogenization. Subsequently, the mixture was centrifuged at 0–4°C (4000 r/min) for 5 min using a high-speed refrigerated centrifuge. The resulting supernatant was considered the crude enzyme extract.

2.1.2 | Experimental operation

In total, 0.4 mL enzyme solution (diluted 10 times with distilled water) was placed into the side arm of the reaction bottle. Within the main chamber, 1.0 mL of substrate emulsion (comprising 5 mL linoleic acid, 2 mL Tween-20, and 15 mL of distilled water thoroughly stirred) was added, along with 2.0 mL phosphoric acid buffer (0.1 mol/L, pH 6.5). Following this, the reaction bottle was vibrated and allowed to equilibrate at a constant temperature, using a water bath set at 30°C for 5 min.

Upon reaching equilibrium, the piston of the manometer was sealed, and the liquid level at the closed end was adjusted to match the reference point. Subsequently, the contents of the side arm were introduced into the main chamber, and the time was measured. After 3 min of reaction, the liquid column at the closed end of the barometer was adjusted to its original reference point. The height of the column at the open end was immediately measured and corrected using the temperature and barometric readings. The enzyme activity was expressed as the microliters of oxygen consumed in 1 min (Figure 2).

2.2 | Colourimetric method (Determination of product-hydrogen peroxide content)

LOX catalyzes the production of hydrogen peroxide which has a characteristic absorption peak at 234 nm. Linoleic, linolenic, and arachidonic acids are readily available substrates. This method is as simple and easy to operate as an oxygen content-based assay.

2.2.1 | Experimental operation

To initiate the experiment, 0.4 mL of linoleic acid was carefully dispensed, followed by the addition of 0.5 mL of 10% NaOH to facilitate complete dissolution. The total volume was adjusted to 100 mL, resulting in a clear and colorless solution. Twenty milliliters of this solution were mixed with 0.1 mL of Tween-60. Subsequently, 0.2 mol/L boric acid buffer (pH 9.0) was added, bringing the total volume to 100 mL. Before usage, the solution was diluted 40 times to achieve a linoleic acid concentration of 2.57 mmol/L.

TABLE 1 Principles, advantages and disadvantages of in vitro anti-inflammatory assay.

Assay	Method	Principle	Advantage	Shortcomings	References
LOX	Manometry	Determine the oxygen consumption of the reactant	Responsive and easy to operate	The result will be affected by air leakage and improper operation of the instrument	(Zhong, Wang, & Xu [2001]) (Galliard & Phillips [1971])
	Colorim-etric method	The hydrogen peroxide produced by enzyme catalysis has a characteristic absorption peak at 234 nm	The operation is simple, the substrate is easy to obtain, and the margin of error is minimal	/	(Zhong, Wang, & Xu [2001])
COX	^{14}C-labeling	^{14}C is used to identify the presence and distribution of radioactive materials	Simple operation, accurate positioning	It can only be used for relative quantification and requires a long exposure time under low-content conditions	(Lapetina et al. [1978])
	Kit method	Detection of COX enzymes using specific chemiluminescent substrates (substrate chemiluminescence method)	Convenient and versatile method	/	(Hitchcock et al. [2021])
		The reaction between AA-derived PGG_2 and ADHP catalyzed by COX produces fluorescent substances (fluorescence quantification)	Higher sensitivity	/	(Destefanis et al. [2019])
HAase	HAase activity inhibition method	PDMAB and HAase products can produce color reaction	Convenient operation, strong versatility	/	(Aoshima et al. [2012]) (González-Peña et al. [2013])
NO	Griess colorimetry	NO_2^{2-} reacts with sulfonamide to form a diazo compound under alkaline conditions, and then reacts with n-1-naphthalene ethylenediamine dihydrochloride to form a colored compound	Simple and fast reactions, simple instrumentation and high operability	Lack of sensitivity	(González-Peña et al. [2013]) (Qiao et al. [2005])
TNF	Crystal violet method	The concentration of TNF-induced cell death is determined by crystal violet staining	Easy to operate, strong versatility, simple instrument	/	(Shen et al. [2020])
IL	ELISA Kit method	The antibody is fixed onto a plate, the antigen is captured in solution, and then a distinct epitope of the same antigen is detected using another enzyme-coupled antibody	Strong sensitivity and convenient operation	/	(Shen et al. [2020]) (Xiang et al. [2021])

Abbreviations: AA, arachidonic acid; ADHP, 10-acetyl-3, 7-dihydroxyphenoxazine; COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LOX, lipoxygenase; PDMAB, p-dimethylaminobenzaldehyde; PGG_2, prostaglandin G_2; TNF, tumor necrosis factor.

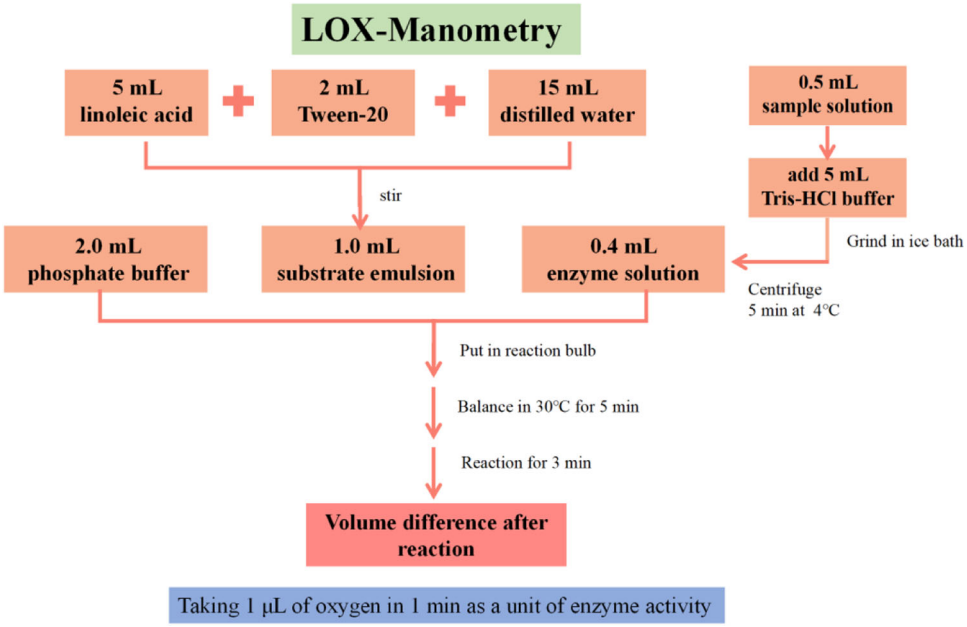

FIGURE 2 Flow diagram of lipoxygenase (LOX) activity tested by pressure measurements.

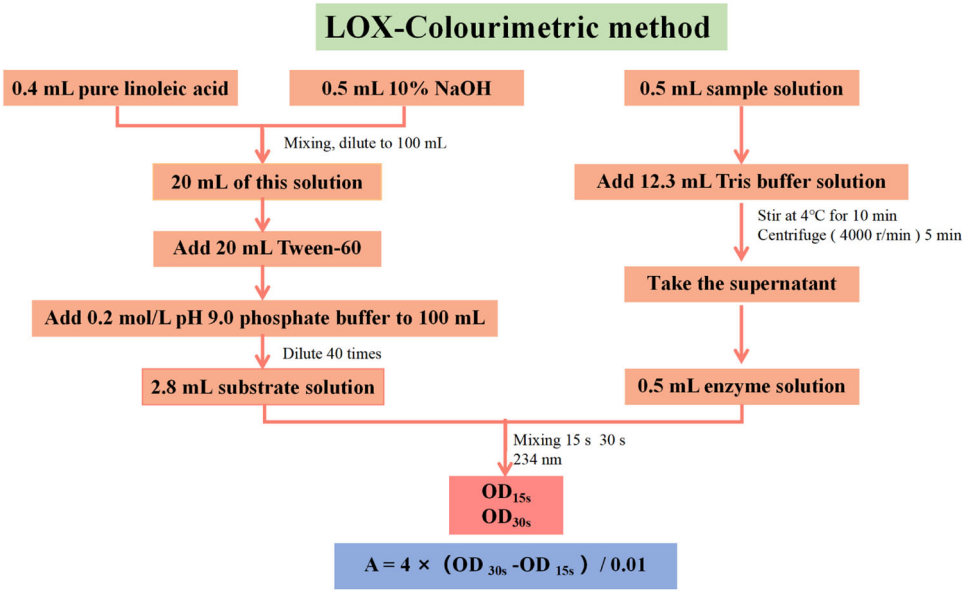

FIGURE 3 Experimental flow chart of determination of lipid oxidase activity by colorimetry. LOX, lipoxygenase.

For the sample solution, 0.5 mL was combined with 12.3 mL of Tris buffer (consisting of 0.015 mol/L $CaCl_2$ and 13% sucrose at pH 8.2), stirred at 4°C for 10 min, centrifuged at 4000 r/min for 5 min, yielding the crude enzyme extract. This extract was refrigerated for subsequent utilization.

In the determination step, 2.8 mL of the previously prepared substrate and 0.2 mL of the enzyme extract were thoroughly mixed. Changes in optical density (OD) at 234 nm were monitored, and data was recorded at 15-second intervals (Figure 3).

2.2.2 | Calculation

The calculation of enzyme activity (A) was based on the formula:

$$A = 4 \times (OD_{30s} - OD_{15s})/0.01,$$

A represents the enzyme activity in units; OD_{30s} and OD_{15s} are the OD values at 30 s and 15 s, respectively. The constant 0.01 represents the activity required to increase the absorbance by 0.01 per min, serving as a unit of LOX activity.

3 | COX INHIBITION TEST

COX, an important rate-limiting enzyme in the synthesis of PG from AA, possesses both catalase and oxygenase activities (Yasir Khan et al., 2022). Two COX isoenzymes, structural COX-1 and inducible COX-2, have been identified, each encoded by distinct genes and possessing different biological effects (Yasir Khan et al., 2022). COX-1 exists in various human tissues and cells, primarily regulating physiological PG synthesis and cell functions (Daniel et al., 2016). Conversely, COX-2 is under-expressed in normal tissues but becomes overexpressed in most inflamed tissues and nearly all tumor tissues (Luo et al., 2017). Given the distinct functionalities of these enzymes, in vitro investigations to discern the selectivity of a potential anti-inflammatory compound accurately are needed.

Currently, the evaluation of anti-inflammatory activities of foods and natural drugs commonly relies on COX-2 measurement. Therefore, the following experimental description predominantly focuses on COX-2.

The production of AA under the influence of various physiological and pathological stimuli is catalyzed by phospholipase A_2 (PLA_2). Subsequently, AA undergoes a sequential transformation into the prostaglandin intermediate metabolite PGG_2 through the epoxidizing and peroxidizing activities of COX, ultimately being reduced to PGH_2 (Niu et al., 2015). Following metabolic processing by different downstream prostaglandin synthetases, PGH_2 gives rise to various bioactive prostaglandins, including PGI_2, PGE_2, $PGF_{2\alpha}$, PGD_2, and thromboxane A_2 (TxA_2). The synthesis of these prostaglandins is facilitated by distinct prostaglandin synthetases, including PGI_2 synthetase (PGIS), PGE_2 synthetase (PGES), $PGF_{2\alpha}$ synthetase (PGFS), PGD_2 synthetase (PGDS), and TxA_2 synthetase (TxS), respectively (Ambati et al., 2021)(Figure 4).

COX inhibition in a sample is commonly measured using radioautography (utilizing ^{14}C labeling) measure the conversion rate of radioactive AA, while LOX activity is generally assessed using kits.

3.1 | ^{14}C labeling method (Measurement of the conversion of radioactive AA)

The assessment of COX inhibition typically involves quantifying reactants or products to evaluate a sample's capacity to inhibit COX, thereby gauging its anti-inflammatory potential. A method employed for this purpose is the measurement of radioactive AA conversion through radioautography using ^{14}C labeling. Radioautography, initially introduced in biology by E.S. London in 1940 (Lapetina et al., 1978), allows for precise identification and distribution mapping of radioactive materials, offering a straightforward operational procedure. However, it can only be used for relative quantitative measurements, requiring lengthy exposure times in low-content conditions. Hence, this method is seldom used.

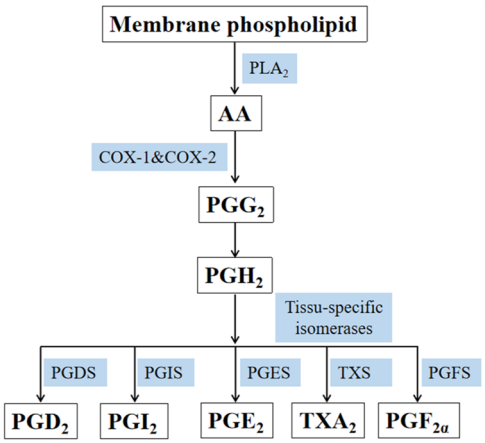

FIGURE 4 Cyclooxygenase (COX) involvement in the arachidonic acid pathway. AA, arachidonic acid; PGDS, PGD_2 synthetase; PGES, PGE_2 synthetase; PGFS, $PGF_{2\alpha}$ synthetase; PGG_2-prostaglandin; PGIS, PGI_2 synthetase; TXS, TxA_2 synthetase.

3.1.1 | Experimental operation

A mixture containing 5 μL epinephrine (1.3 mg/mL), 10 μL glutathione (3 mg/mL), and 127.5 μL tris buffer (0.1 mol/L) was mixed in the test tube and placed on ice for 5 min. After adding 10 μL of either COX-1 or COX-2 enzyme and 10 μL of the test sample, the mixture was preincubated on ice for 10 min.

After the preincubation step, 10 μL [1-^{14}C] AA was added to the reaction mixture which was further incubated at 37°C for 20 min. To halt the reaction and facilitate the extraction process, 10 μL HCl 2 mol/L and 800 μL ether were added and mixed for 30 s. Subsequently, the mixture was centrifuged at 2500 r/min for 1 min. In total, 800 μL of the supernatant was collected and kept at 37°C. A quantity of 30 μL was dissolved in acetone. The resulting solution contained ^{14}C-labeled prostaglandins, which were quantified using electron autoradiography (Figure 5) after being developed by thin layer chromatography (expander-Chloroform: methanol: acetic acid =18:1:1).

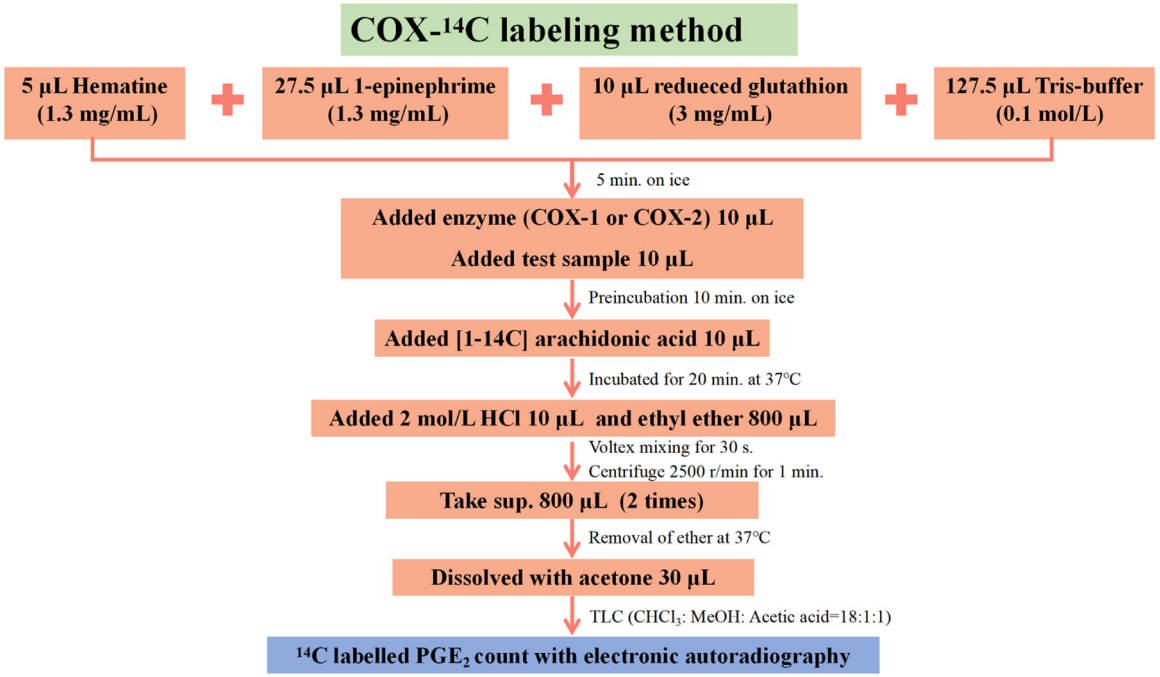

FIGURE 5 Flow chart of measurement of radioactive arachidonic acid conversion using ^{14}C labeling method. COX, cyclooxygenase.

3.1.2 | Calculation

The inhibition rate is calculated by the following formula:

$$\text{Inhibition (\%)} = (C_B - C_A)/C_B \times 100\%$$

C_B represents the concentration of [1-^{14}C] AA, and C_A, the concentration of ^{14}C-labeled prostaglandin.

3.2 | Kit method

3.2.1 | Substrate chemiluminescence method

The chemiluminescence method is based on the detection of COX enzyme peroxidation activity using a specific chemiluminescent substrate. Residual COX activity was measured following NSAID inhibition by introducing a proprietary luminescent substrate and AA. The light emission, directly proportional to COX activity in the sample, begins immediately. This method is convenient and versatile compared to fluorescence quantification-based methods, with fluorescence assays potentially providing higher sensitivity.

Due to the specialized chemiluminescent substrate, this method usually employs kits. For instance, Hitchcock et al. used the COX activity assay kit (Abcam ab139432), to assess the anti-inflammatory activity of Z-ajoene obtained through the thermal rearrangement of allicin. They reported a dose-dependent inhibitory effect of Z-ajoene on COX-2, with a half-maximal inhibitory concentration (IC_{50}) of 32.6 ± 6.1 μmol/L (Hitchcock et al., 2021).

The procedure involved preincubating the buffer, hematin solution, COX-2 preparations, and the sample at 37°C for 5 min. Subsequently, the mixture was incubated at 25°C for 5–120 min in a 96-well plate. Cold COX chemiluminescence substrate was injected, followed by an immediate injection of diluted cold AA solution. Determine integrated light output for the 5 s read time in Relative Light Units (RLU). The experimental setup included blank, control, and inactive groups, with each sample undergoing three replicates. The inhibition rate was calculated by the following formula:

$$\text{Inhibition (\%)} = [1 - (RLU_A - RLU_B)/RLU_C] \times 100\%$$

RLU_A indicates the average of RLU in the control group, RLU_B represents the average of RLU in the inactive group, and RLU_C represents the average of RLU in blank group.

3.2.2 | Fluorescence quantification

The most commonly employed method for quantifying prostaglandin levels is based on fluorescence measurement. This technique capitalizes on the conversion of AA into PGG_2 by COX. Subsequently, PGG_2 reacts with 10-acetyl-3,7-dihydroxyphenoxazine, yielding the fluorescent substance trihalin. Fluorescence can be generated at specific wavelengths. COX enzyme activity can be

reflected by detecting the fluorescence intensity of haloperidol. Subsequently, this can be used to assess the COX inhibitory activity of the tested drug. This reliable and sensitive approach allows efficient quantification.

In the process of experimenting with the Abcam, Cambridge, UK (ab204699) kit, a research found out that downregulation of heme export protein in colorectal cancer cells did not affect the expression and activity of COX-2. Conversely, administration of 5-aminolevulinic acid decreased the expression of COX-2 (Destefanis et al., 2019). For this procedure, a mixture comprising 150 μL reaction buffer, 10 μL heme, 10 μL COX-2, and 10 μL sample solution was incubated at 25°C for 10 min. Subsequently, 10 μL ADPH was added to each well. To start the reaction, 10 μL AA was added, and the reaction mixture was incubated at room temperature for 5 min. The fluorescence intensity was measured at excitation wavelengths of 530–540 nm and emission wavelengths of 585–595 nm. A positive control group was set up, and all samples were generated in triplicate.

4 | HAASE INHIBITION TEST

HAase comprises a group of enzymes that enable the production of low molecular weight hyaluronic acid which regulates various physiological processes and pathological conditions, including inflammation. HAase induce the release of inflammatory cytokines when activated by Ca^{2+} (Kiss et al., 2011). Therefore, inhibiting HAase activity is a promising strategy for alleviating anti-inflammatory and other related diseases. Furthermore, inhibiting HAase activity may serve as an indicator for assessing the anti-inflammatory activity of different plant-derived compounds (Aoshima et al., 2012). P-dimethylaminobenzaldehyde (PDMAB) can produce a color reaction with hyaluronase products. The enzyme activity is proportional to the intensity of the color.

4.1 | Sample and reagent preparation

Samples were dissolved in dimethyl sulfoxide (DMSO) and diluted in acetate buffer solution (100 mmol/L, pH 3.6, 50 μL). Sodium hyaluronate was prepared using acetic acid buffer as a solvent at 1.2 mg/mL. PDMAB solution was prepared by dissolving 4 g PDMAB in a mixture containing 50 mL of HCl 10 mol/L and 350 mL of glacial acetic acid.

4.2 | Experimental operation

Diluted samples were mixed with bovine HAase (50 μL) in the experimental group, while the control group was incubated at 37°C for 20 min with 50 μL DMSO 5% instead of the extract. Calcium chloride (12.5 mmol/L, 50 μL) was added to the reaction mixture which was incubated at 37°C for 20 min. Ca^{2+}-activated hyaluronase was added to 250 μL sodium hyaluronate and incubated at 37°C for 40 min. After incubation, sodium hydroxide (0.4 mol/L, 50 μL) and sodium borate (0.2 mmol/L, 100 μL) were added. The mixture was inactivated in a boiling water bath for 3 min and then cooled to 25°C. Subsequently, 1.5 mL PDMAB solution was added to the reaction mixture and incubated at 37°C for 20 min. The absorbance was measured at 585 nm using an ultraviolet spectrophotometer when color developed. The experiment was performed in triplicate (Figure 6).

4.3 | Calculation

The inhibition rate was calculated by the following formula:

$$\text{Inhibition (\%)} = (OD_B - OD_A)/OD_A \times 100\%$$

OD_A was the absorbance of sample group and OD_B was the absorbance of control group.

5 | METHOD FOR DETERMINATION OF NO

NO is associated with various physiological and pathophysiological conditions. Its role in inflammation includes vasodilation, formation of edema and local erythema, increased exudation, development of sepsis, and activation of prostaglandin synthase. Furthermore, NO is involved in the development of rheumatoid disease (González-Peña et al., 2013), promoting inflammatory toxicity. Three forms of NO synthase (NOS) have been identified. The constitutively expressed endothelial NOS (eNOS) and neuronal NOS (nNOS) yield low concentrations of NO that mediate tissue homeostasis. iNOS, on the other hand, produces high levels of NO, being involved in various pathological states (Ryu et al., 2003), including chronic inflammatory diseases such as rheumatoid arthritis and asthma. Therefore, the regulation of iNOS is a potential target for inflammatory disease management (Min et al., 2009).

In the human body, NOS catalyzes the transformation of L-arginine to NO, as follows: L-arginine+ Reductive coenzyme II $(NADPH) + O_2 \rightarrow$ Citrulline $NO + NADP^+$. However, in vivo or in aqueous solution, NO is easily oxidized into $NO_2^{2-}$and NO_3^{3-}. The latter can be reduced to NO_2^{2-} by cadmium (Figure 7). Nitrite and nitrate are end products of the NO oxidation pathway, and their concentrations are often evaluated as indicators of NO production in biological liquids and cell cultures.

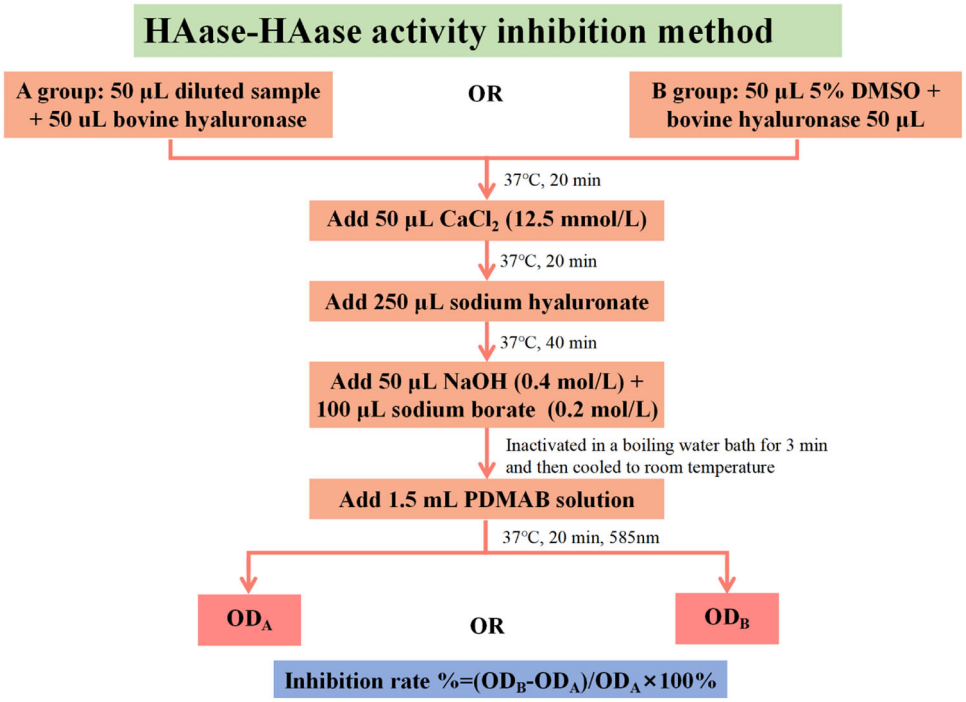

FIGURE 6 Experimental flow chart of measuring HAase activity through the chromogenic method.

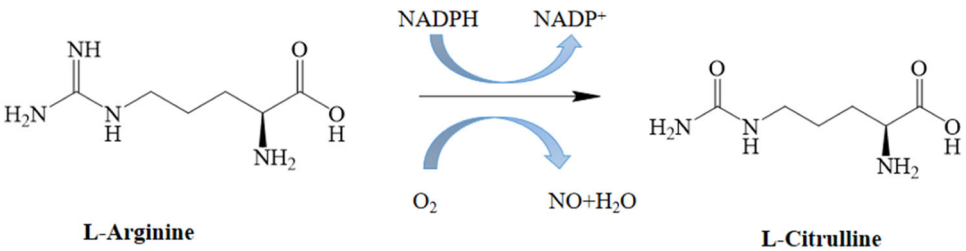

FIGURE 7 NO production catalyzed by iNOS in the human body.

Several methods have been developed for the measurement of NO_2^{2-}, including Griess colorimetry, chemiluminescence, gas chromatography, hemoglobin capture, and fluorescence (Ambati et al., 2021). The most commonly used and convenient method for analyzing NO_2^{2-} in aqueous solutions is the Griess colorimetric method.

5.1 | Griess colorimetry

The Griess method is a well-known two-step diazotization reaction. NO_2^{2-} reacts with sulfonamide under alkaline conditions to produce a diazo compound, which undergoes a coupling reaction with N-1-Naphtholethylenediamine dihydrochloride (nitrogen retention reaction) to produce a colored compound. Absorption is measured at 546 nm with a spectrophotometer, and a standard curve is generated to determine the NO content in the sample (Miles et al., 1996) (Figure 8). Cell viability experiments confirmed that inhibition experiments did not hinder NO production by affecting cell activity.

While the Griess method is simple and rapid, its detection limit for NO_2^{2-} is only 1 μM (Nagano, 1999). This limited sensitivity restricts its use for quantifying micromolar levels of $NO_2^{2-}$and NO_3^{3-} in biological samples (Li et al., 2000). However, it remains the most commonly used method for NO_2^{2-}detection due to its simple instrumentation, ease of use, and other practical advantages.

5.1.1 | Configuration of reagents

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% antibiotics and 10% fetal bovine serum (FBS).

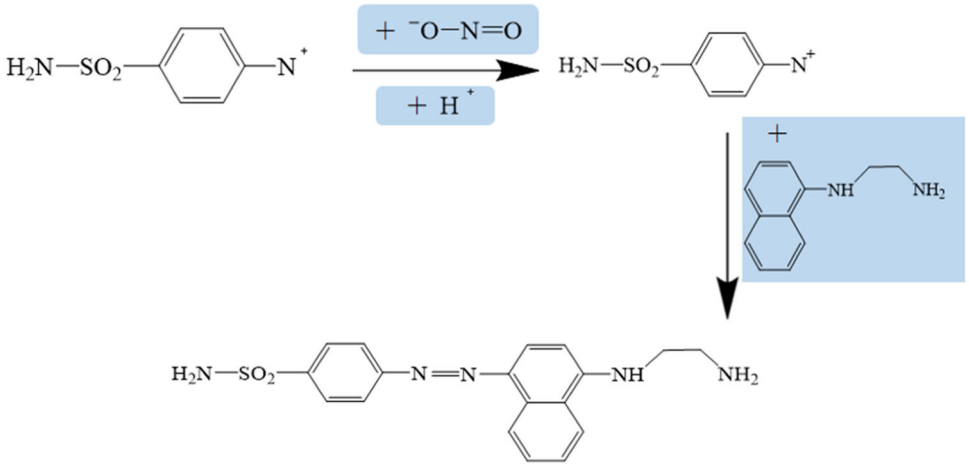

FIGURE 8 Griess colorimetric reaction principle.

For obtaining the Griess reagent, 0.5 g of p-aminobenzenesulfonic acid and dissolved in 150 mL of 10% hydrochloric acid to formulate reagent A. 0.2 g of 1-Naphthylamine were mixed with 150 mL of 10% hydrochloric acid and 20 mL of DH_2O to formulate reagent B. Equal proportions of reagents A and B were mixed and filtered to obtain Griess reagent, which is colourless when fresh; if it turns red, it is an invalid reagent. The reagent should be prepared and used immediately.

5.1.2 | Cell culture

Mouse macrophages (RAW 264.7) were cultured in DMEM at 37°C with 5% CO_2 (v/v) in a humidified environment. After 1–3 days of cell culture, the cell density was around 80% to passaging at a ratio of 1:3 using a cell scraper. The experiments were carried out using cells in logarithmic growth phase with good morphological growth under an inverted phase contrast microscope. They were inoculated into 96-well plates at 6×10^4 cells/well and cultured for 24 h.

5.1.3 | Cell viability assay

The 3-(4,5-dimethyl-2-thiazolyl)−2,5-diphenyl-2H-tetrazolium bromide (MTT) assay is commonly used to assess cell viability. After cell culturing as previously described, the supernatants were removed and a gradient concentration of sample was added (control and blank group without samples and blank group without cells) for 24 h. Then the supernatant of each well was removed and the attached cells were rinsed with PBS- and FBS-free medium. A mixture containing 20 μL MTT solution (5 mg/mL MTT dissolved in PBS) and 180 μL FBS-free DMEM was added to each well. After 4 h of incubation in a humid environment with 5% CO_2 at 37°C, the medium was carefully removed, and the formation crystals were dissolved by adding 100 μL DMSO. The absorbance was measured at 570 nm.

5.1.4 | Determination of nitrite

The nitrite concentration in the cell culture supernatant was used to evaluate the concentration of NO produced by the macrophage. Generally the sample concentration was chosen as the concentration with cell viability greater than 80% for subsequent experiments. After culturing the cell as previously described, the supernatant was discarded before being treated with samples (initially dissolved in DMSO and diluted with medium) for 2 h, and then cultured in a medium containing LPS (1 μg/mL) for 24 h. In the control group, cells were cultured only in LPS- and DMEM-containing medium under the same conditions. Then 100 μL of cell culture supernatant from each well of the cell culture plate was mixed with 100 μL Griess reagent in a 96-well microtitration plate. After 10 min, absorbance was measured at 540 nm. A standard curve, using the concentration of diluted sodium nitrite solution as the x-axis and the corresponding absorbance as the y-axis, was used to quantify the concentration of nitrite. The inhibitory rate of plant extracts on NO production was calculated relative to the control group (Figure 9).

5.1.5 | Calculation

Cell survival was calculated relative by the following equation.

$$\text{Cell survival\%} = (OD_A - OD_B)/(OD_C - OD_B) \times 100\%$$

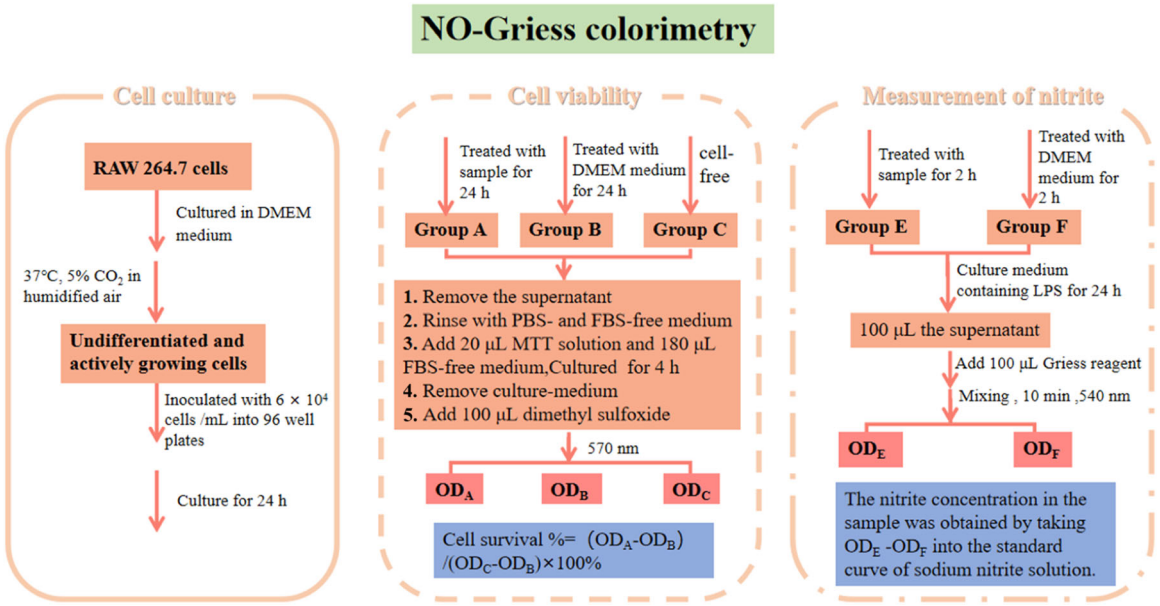

FIGURE 9 Griess colorimetric method experimental procedure.

Group A is the experimental group, including cells, medium, MTT and sample. Group B is the control group, including cells, medium and MTT. Group C is the blank group, including medium and MTT.

The nitrite concentration in the sample was obtained by taking OD_E -OD_F into the standard curve of sodium nitrite solution.

6 | ASSAY OF TNF

TNF is a cytokine associated with various infections and diseases, including inflammation, and includes two types, TNF-α and TNF-ß, which have extremely similar biological activities (Huyghe et al. (2023)). TNF is biologically active in vitro and in vivo by killing some tumour cells, but has no cytotoxic effect on the normal cells. Based on the cytotoxic effect of TNF on sensitive target cells, a classical and commonly used crystal violet staining method for the determination of TNF activity in L-929 cells was compiled in this paper. The experimental principle is that TNF binds to its specific receptor, enters the cell, destabilises the lysosome and allows the extravasation of various enzymes, leading to cell lysis.

6.1 | Preliminary preparation

L-929 cells were digested with 0.25% trypsin and adjusted to a concentration of 2×10^5 cells/mL in 10% calf serum and 1 μg/mL Penicillin-Streptomycin RPMI1640 medium. 0.1 mL of cell solution was added into each well of a 96-well culture dish and cultured for 24 h at 37°C with 5% CO_2.

6.2 | Experimental operation

Experimental, control and blank groups were established respectively. Add 0.1 mL actinomycin D (1 μg/mL) solution, and incubation for 14 h. After remove the medium, 1 drop of 0.5% crystal violet was added in each hole, and the mixture was incubated for 10 min. Subsequently, it was rinsed slowly under running water. Cells were then dissolved in 0.2 μL of 1% sodium dodecyl sulfonate (SDS), and absorbance was measured at 570 nm. (Figure 10). Standard curve Preparation of 8 series diluents with standard TNF (100 U/mL) is completed in the above steps.

6.3 | Calculation

Cell survival was calculated relative by the following equation.

$$\text{Cell survival\%} = (OD_A - OD_B)/(OD_C - OD_B) \times 100\%$$

Group A is the experimental group, including cells, medium, actinomycin D and sample. Group B is the control group, including cells, medium and actinomycin D. Group C is the blank group, including medium and actinomycin D.

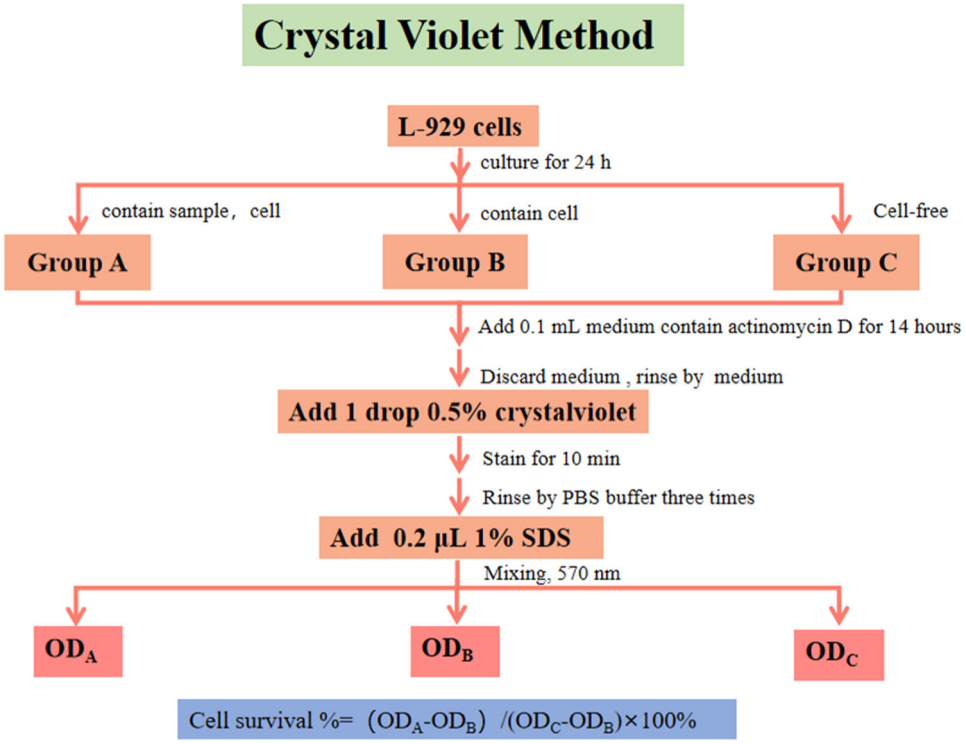

FIGURE 10 Experimental procedure of TNF assessment using crystal violet method. SDS, sodium dodecyl sulfonate.

By calculating the cell survival rate and comparing it with the TNF standard, the TNF activity unit of the sample to be tested can be detected.

7 | IL ASSAY (ELISA)

ILs are lymphoid factors that mediate the interaction between various immune cells. They are crucial in conveying information, activating, and regulating immune cells such as T and B cells. Thus, they play a key role in the development of the inflammatory response (Ouyang et al., 2011). ILs comprise a high number of molecules, but IL-1β, IL-6, and IL-10 are widely assessed in in vitro anti-inflammatory studies.

7.1 | ELISA kit method

ELISA is a common method for antibody or antigen detection. The technique relies on detecting enzyme-labeled antibodies that bind to antigens immobilized on solid surfaces while washing away unbound material between steps (Tabatabaei & Ahmed, 2022). The most commonly used enzymes are alkaline phosphatase and horseradish peroxidase (HRP), with HRP often preferred due to its low detection limit (Lin, 2015). There are three ELISA methods, with the sandwich ELISA being a classic in vitro method for assessment of the anti-inflammatory effect.

This method involves immobilizing an antibody on a plate, capturing the antigen in solution, and then recognizing different epitopes of the same antigen with another enzyme-coupled antibody, offering maximum sensitivity for specific antigen detection in complex samples.

Currently, ELISA reached maturity, its applications typically being available in kit form. For example, using IL-6 (EK0411) and IL-10 (EK0417) ELISA kits from Broadtech Bioengineering Co, LTD, a research studied the anti-inflammatory effects of Mytilus coruscus polysaccharide (MP) on a mouse model of ulcerative colitis and lipopolysaccharide-stimulated RAW264.7 cells. The results demonstrated that MP effectively promoted the proliferation of RAW264.7 cells and mitigated the overproduction of inflammatory cytokines IL-6 and IL-10 (Xiang et al., 2021). Selection of kits should be based on the studied organism/cell type and on kit parameters, such as sensitivity, detection range, and suitability for serum or plasma.

8 | CONCLUSIONS

This review aims to function as a guideline, describing several in vitro assays used in research laboratories to assess the anti-inflammatory effects of various compounds. These assays include LOX, COX, and HAase inhibition tests, and NO, TNF, and IL measurements. This manuscript provides a thorough explanation of the

purpose of each assay and includes the assessment of cellular viability and the determination of substance concentrations.

Table 1 summarizes the principles, advantages, and disadvantages of these assays. Ideally, such an assay should be rapid, reliable, efficient, time-saving, cost-effective, sensitive, and based on relatively simple experimental conditions. When selecting an assay, available laboratory equipment should be considered, as well as the type of method employed. Due to the complexity and diversity of anti-inflammatory pathways, multiple indicators of inflammatory enzyme activity and inflammatory factors should be used to assess the anti-inflammatory activity of foods.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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