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Developing a Herbal Elixir Comprising Methanol Extracts of Sage and Rosemary and Evaluation for its Therapeutic Activity in Oxidative Stress and Inflammation

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1. Introduction

The body's complicated and necessary physiological response to adverse stimuli like viruses, tissue damage, or irritants is inflammation. It is an essential component of the body's defensive system, working to get rid of the injury's cause and start the healing process (Schmid-Schönbein, 2006). Inflammation is a closely controlled sequence of molecular and cellular events that is organised by the immune system and is stereotypically hallmarked by swelling, redness, discomfort, and heat in the pretentious area. Immune cells release signalling molecules, such as cytokines and chemokines, in response to tissue damage or infection. These molecules cause a chain of events that draw white blood cells to the site of damage or infection. These immune cells strive to eliminate cellular waste and kill invasive germs. While short-lived and protective, acute inflammation can be a problem and a factor in a number of chronic conditions, such as autoimmune illnesses, malignancies, and cardiovascular conditions (Lisa M. Coussens & Zena Werb, 2002; Ocampo-Gallego et al., 2023). When the immune reaction lasts for a long time, it can possibly harm healthy tissues and increase the likelihood that a disease will emerge. This is known as chronic inflammation. Genetics, lifestyle choices, nutrition, and environmental exposures can all have an impact on the ratio of pro-inflammatory to anti-inflammatory signals. For optimal health, inflammation must be controlled and its delicate balance upheld (Ahmed, 2011). A balanced diet high in antioxidants, frequent exercise, stress management, getting enough sleep, and minimising exposure to dangerous pollutants are all ways to reduce chronic inflammation. For the purpose of creating preventative strategies and focused treatments to treat diseases caused by inflammation and enhance general health, understanding the complex relationship between inflammation and health is crucial (Ahmed, 2011).

Within the body's physiological reactions, the interaction between oxidative stress and inflammation creates a complicated and important dynamic. Reactive oxygen species (ROS) generation and the body's antioxidant defence mechanisms are out of balance, which causes oxidative stress (Federico, Morgillo, Tuccillo, Ciardiello, & Loguercio, 2007). These ROS, which also include the free radicals superoxide and hydrogen peroxide, can

harm DNA, lipids, and proteins in addition to other biological components. When cells are damaged, the immune system recognises them and launches an attack to try and fix and get rid of them. Ironically, inflammation itself can make oxidative stress worse by drawing in immune cells that produce more ROS while battling infections or mending injured tissues. The relationship between oxidative stress and inflammation has important effects on a number of medical diseases. Chronic inflammation, as observed in illnesses like obesity, autoimmune disorders, and cardiovascular diseases, causes ongoing immunological responses that lead to continued ROS generation, which perpetuates oxidative stress. In contrast, oxidative stress can cause inflammatory pathways to be active (Chaudhari, Talwar, Parimisetty, Lefebvre d'Hellencourt, & Ravanan, 2014) and encouraging the release of cytokines and chemokines that cause inflammation. This reciprocal link creates the conditions for a vicious cycle in which oxidative stress and inflammation feed off of one another, advancing a variety of chronic diseases (Chaudhari et al., 2014; Federico et al., 2007).

It is essential to comprehend this complex interaction in order to create potent therapeutic approaches. Antioxidants have been investigated for their ability to control inflammation because they counteract oxidative stress by neutralising ROS. Similar to this, anti-inflammatory treatments that focus on particular pathways may subtly lower oxidative stress by calming the immune system. Oxidative stress and inflammation can both be impacted by lifestyle changes such eating a balanced diet high in antioxidants, exercising frequently, and managing stress (Chaudhari et al., 2014; Steven et al., 2019). In essence, oxidative stress and inflammation are inversely correlated and are essential for the initiation and progression of many diseases. It is clear that controlling oxidative stress and inflammation is essential for preserving general health and preventing chronic diseases, even though the exact processes underlying this interaction are still being investigated.

In the field of medicine, medicinal plants have become important resources, especially for treating inflammation and oxidative stress. These plants include bioactive substances with powerful antioxidant and anti-inflammatory activities, including as polyphenols, flavonoids, and terpenoids (Hassan et al., 2017). These constituents neutralise including scavenging of ROS (reactive oxygen species) and aid in reestablishing the proper ratio of antioxidant defences to oxidative stress condition. By halting the production of cytokines and enzymes that trigger inflammation, they can also regulate inflammatory pathways. The value of medicinal plants resides not only in their holistic and natural approach to health, but also in their capacity to offer complementary or alternative therapies to traditional medical treatments (Fard et al., 2022). Numerous conventional treatments, some of which have their origins in ancient customs, have been proven beneficial in treating inflammatory and oxidative stress-related diseases by scientific research. Additionally, using medicinal plants promotes biodiversity and lessens the environmental impact of synthetic medications, offering a sustainable approach to healthcare. By utilising the potential of medicinal plants, researchers and medical experts are exploring cutting-edge methods to alleviate oxidative stress and inflammation. With a route to holistic wellbeing and a seamless synthesis of nature's healing powers and contemporary medicine, these natural therapies continue to show their therapeutic potential. By utilising the potential of medicinal plants, researchers and medical experts are exploring cutting-edge methods to alleviate oxidative stress and inflammation. With a route to holistic wellbeing and a seamless synthesis of nature's healing powers and contemporary medicine, these natural therapies continue to show their therapeutic potential.(Fard et al., 2022; Palipoch, 2013).

Salvia officinalis and *Rosmarinus officinalis*, two herbs that have gained attention, may both be helpful in reducing oxidative stress and inflammation (Palipoch, 2013). Polyphenols, flavonoids, and essential oils are just a few of the bioactive elements which are plentiful in both the plants and support their protective, antioxidant and anti-inflammatory actions. It is believed that the strong antioxidant content of rosemary and sage aids the body's defence against dangerous free radicals. These antioxidants, such as rosmarinic acid in sage and rosmarinol in rosemary, minimise oxidative stress and shield cells from ROS-related cell damage (Amaral et al., 2019; Bozin & Mimica-Dukić, 2007; El-Hadary, Elsanhoty, & Ramadan, 2019; Khare, 2007; Kontogianni et al., 2013). By preventing the production of cytokines and enzymes that trigger inflammation, the bioactive substances in sage and rosemary had been established to possess anti-inflammatory efficacy. By modifying inflammatory pathways, which are frequently linked to chronic diseases, inflammation is reduced. It has been shown that sage and rosemary strengthen the body's defence mechanisms by enhancing the action of antioxidant enzyme complex including superoxide dismutase (SOD) and catalase. These enzymes are essential for

preserving cellular health and fending off oxidative damage (Adzet, Caiñigueral, & Iglesias, 1988; Bouaziz, Yangui, Sayadi, & Dhouib, 2009; Khare, 2007).

Sage and rosemary may have digestive-system-specific anti-inflammatory effects that could provide relief from gastrointestinal inflammation and pain. Sage and rosemary have a number of positive effects, but it's crucial to remember that these effects might change depending on things including dosage, preparation, and personal health circumstances. These herbs can be used as seasonings, teas, supplements, or as part of a balanced diet to promote general health and reduce inflammation and oxidative stress. In light of these findings, the current study's objectives were to create a herbal blend using 1:1 methanol extracts of the herbs sage and rosemary and to test it for mechanistic antioxidant and anti-inflammatory properties.

2. Material and Methods

2.1 Assortment and authentication of the Plants: Preparation of the extracts

Salvia officinalis and *Rosmarinus officinalis* leaves were gathered in the Dehradun area in the late spring. A botanist recognized, identified and confirmed the plant material, and the voucher specimens (SR/SO/2021/13 and SR/RO/2021/14) were kept for later use. The leaves were mechanically ground, then dried in shade, cut, and pulverized into powder form. Petroleum ether was used to remove the fat from the 2.5 kg of powdered leaves (at room temperature; for 48 hour). This technique was carried out three times in order to completely remove the fatty components. The same methanol extraction procedure was then used after allowing it to air dry. The methanol extract was fully extracted, then collected and concentrated at 45–50 °C under decreased pressure. Two concentrated methanol extracts of *Salvia officinalis* and *Rosmarinus officinalis* leaves, yielding 0.72 percent and 0.81 percent, respectively, of the dried starting material, were produced. Prior to usage, the finished product was then kept at 4°C. The herbal mixture known as HB-SR was created by combining the methanol extracts of *Salvia officinalis* and *Rosmarinus officinalis* in a 1:1 ratio.

2.2 Drugs and chemicals

ABTS (2, 2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (1, 1-diphenyl-2-picryl hydrazyl hydrate) were bought from Himedia, India. The Zodley Pharmaceuticals in Haryana set up free samples of quercetin and vitamin C. All other unlabeled chemicals and reagents were of analytical quality and were readily available in the market (SRL Mumbai, E. Merck India).

2.3 Determination of total phenolic compounds

The Folin-Ciocalteau method was used to quantify the total amount of soluble phenolic in the herbal blend containing the methanol extracts, with quercetin serving as a reference phenolic component (Slinkard & Singleton, 1977). In a volumetric flask, 1.2 mL of blend solution (10 mg herbal blend) was combined with 47 mL of water (grade distilled). A total of 1.2 mL of the Ciocalteau-Folin reagent was further added and properly mixed. Next, 180 seconds later, 3.2 mL of 2.2 percent sodium carbonate was added. The mix was then permitted to stand for three hours while being periodically shaken. Using a spectrophotometer, the mixture's absorbance was observed at 762 nm (1601 Shimadzu, Japan). The unit of measurement for total phenolic compounds per gram of extract was milligrams (mg/g). The total amount of phenolic compounds estimated in the mix (HB-SR) as grammes of quercetin equivalent was calculated using a formula derived from the equation of the regression line of the standard quercetin graph (QE):

$Y = 0.0024x + 0.0638$, $r^2 = 0.9662$

Where, x was the concentration, and y was the absorbance.

2.4 Anti-inflammatory activity

2.4.1 Cycloxygenase–1 (COX–1) and cycloxygenase–2 (COX–2) assays

The COX-1 assay was carried out in accordance with the procedure detailed elsewhere (Aguilar et al., 2002; Redl, Breu, Davis, & Bauer, 1994). The sample solution (12 µL) was added to 192 µL of 0.2 M Tris-HCL, 19 µL of L-adrenaline-D-hydrogentartrate, and 12 µL of hematine. After COX-1 (0.3 U) was added and incubated

for 6 minutes, arachidonic acid (6 µL) was added to the mixture. After 30 minutes of incubation at 37 °C, 12 µL of 12 percent formic acid was added to stop the process. Then, a PGE_2 enzyme immunoassay (Caymen sys) was used to measure PGE_2 levels. The previously reported approach was used to conduct the COX-2 assay (Aguilar et al., 2002; Redl et al., 1994). A 0.2 M Tris-HCL buffer (192 µL), 19 µL of L-adrenaline-D-hydrogentartrate, 12 µL of sodium edetate (Na_2-EDTA), and 12 µL of hematine were added to the sample solution (12 µL). The combination was then supplemented with 0.3 U of COX-2 and pre-incubated for 6 minutes. Six µL of arachidonic acid were again added to this. The incubation at 37 °C was terminated after 30 minutes by adding 12 µL of formic acid (12 percent). In the end, the concentration of PGE_2 was determined using the PGE_2-enzyme-immunoassay (Caymen sys).

2.4.2 Effect on 5–lipoxygenase (LOX) enzyme

The 5–LOX assay was performed according to the method described elsewhere (Kulkarni, Mitra, Chaudhuri, Byczkowski, & Richards, 1990). 5 nM of the 5-LOX enzyme was added to 3 mL of HCL-Tris buffer (pH = 8.5) to create the assay mixture (Soybean; Sigma-type V, 110 KD). At room temperature, the test (HB-SR) and standard substance were then incubated with this enzyme mixture for an additional 15 minutes. After the incubation period was over, the enzyme mixture received an addition of freshly synthesized arachidonic acid as 90 µM in 50 mM (pH = 9) Tris-HCL buffer. For three minutes, the assay mixture's absorbance was observed at 234 nm using a spectrophotometer (1601 Shimadzu, Japan), in order to determine the enzyme inhibition.

2.5 Antioxidant activity

2.5.1 Determination of DPPH (1, 1–diphenyl–2–picryl hydrazyl) radical scavenging activity

DPPH was used to assess the free radical scavenging capacity of HB-SR using the previously reported methodology (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Three millilitres of test (HB-SR) solution in water were mixed with varying concentrations (50–250 µg/mL) of a 0.2 mM DPPH•. ethanol solution. Before allowing the mixture to stand at room temperature for 25 minutes, it was thoroughly dazed. The absorbance at 517 nm was then measured using a spectrophotometer (1601 Shimadzu, Japan). An improved ability to scavenge free radicals was suggested by the reaction mixture's decreased absorbance. In order to calculate the percent DPPH scavenging effect, the following equation was utilized**:**

Percentage scavenging of $DPPH^{\bullet}$ radical = $[(A_c - A_t / A_c) \times 100]$

Where A_t = absorbance when the extract or reference is present and A_c = absorbance of the control reaction system.

2.5.2 Reducing power

The reducing power of HB-SR was determined according to the method described previously (Oyaizu, 1986). The various HB-SR concentrations (50-250 µg/mL) were combined with potassium ferricyanide $[K_3Fe(CN)_6]$ and phosphate buffer in 1.5 mL of distilled water (2.6 mL, 0.3 M, pH 6.7). (1.1% in 2.6 mL). For 25 minutes, the mixture was incubated at 50 °C. The mixture was centrifuged at 4000 rpm for 12 minutes after being added 2.6 mL of 12 percent trichloroacetic acid. The top layer of the solution was prepared by mixing 0.6 mL of $FeCl_3$ (0.2%) with 2.6 mL of distilled water. Then, a spectrophotometer was used to detect the absorbance at 700 nm (1601 Shimadzu, Japan). Greater reducing power was shown by the reaction mixture's higher absorbance.

2.5.3 Evaluating superoxide radical ($O_2^{\bullet -}$) scavenging activity

Based on the ability of HB-SR to prevent the synthesis of blue formazon, the assay was conducted. Superoxide radical was produced in the riboflavin-light-NBT (Nitroblue tetrazolium) system (Beauchamp & Fridovich, 1971). The reactant combination had a 4 mL of entire volume. This reaction concoction was volume up of 4 mL of 0.2 mg NBT, 25 µg of riboflavin, 13 mM EDTA, buffer of sodium phosphate (60 mM, pH 7.7), and sample solution at 1.2 mL. Various concentrations of HB-SR (50-250 µg/mL) were shone onto the reaction liquid for 90 seconds to begin the reaction. At 590 nm, the absorbance was measured immediately after illumination. A box with an aluminium foil interior contained the reaction assembly. As a blank, reaction mixture-filled identical

tubes with no illumination were used. The suppression and inhibition of superoxide anion production was calculated using the following formula.

Percentage inhibition of superoxide anion = $(A_c - A_t / A_c) \times 100$

Where A_t = the absorbance in presence of standard or extract, and A_c represents the control's absorption (without extract).

2.6 Statistical analysis

The data and results had been shown as mean ± SD (n = 6). The software program GraphPad Prism was used to conduct the statistical analyses, which included one-way analysis of variance (ANOVA) and *post hoc* "Dunnett's Multiple Comparison Test." *P* values of 0.05 or less denoted as $p<0.05$ were employed to estimate the statistical significance.

3. Results and findings

3.1 Total phenolic compounds determination

The HB-SR contained 246.22 µg/mL of total phenolic compounds, according to the report (articulated as equivalents of quercetin per gram of herbal blend) (Figure 1).

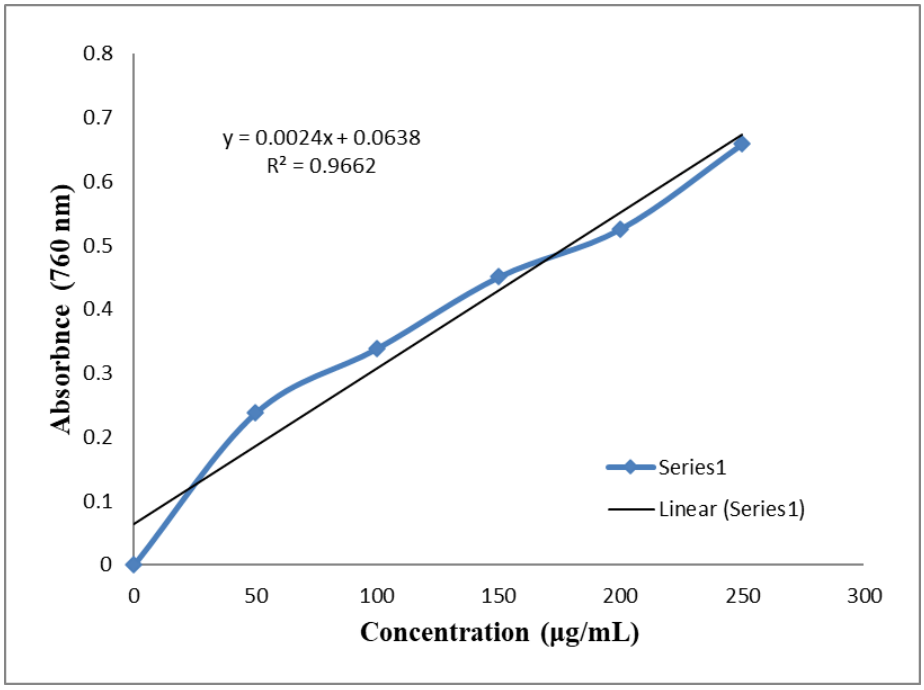

Figure 1. Estimation of total phenolic compounds in HB-SR

3.2 Evaluation of Anti-inflammatory activity

3.2.1 Measuring the COX-1 (Cyclooxygenase-1) and COX-2 (cyclooxygenase-2) enzymes

Additionally, it was shown that the anti-inflammatory action in the pathway for arachidonic acid system is linked to cessation of the COX (both COX-1 & -2) enzyme systems. As a result, the potential COX inhibitory capacity of the herbal blend was also assessed in the current investigation at four concentration levels (50, 100, 150, 200, an d 250 µg/mL). Interestingly, at a concentration of 250 µgmL-1, HB-SR showed a considerable suppression of cyclooxygenase -1 and -2 of (97.801±0.064) percent and (89.386±0.156) percent, respectively. Calculated values for the IC 50 were 179.38 µgmL-1 (for COX-1) and 187.27 µgmL-1 (COX-2) (Table 1).

3.2.2 5-lipoxygenase (LOX) enzyme assay

The activity of the 5-LOX enzyme was shown to be inhibited by HB-SR (250 µg/mL) to a degree of 68.99±0.456 %. IC_{50} value for HB-SR was calculated and computed to be 212.82 µg/mL (Table 1).

Table 1. Percentage enzyme inhibition of the COX system by the herbal blend (HB-SR).

Concentration (µg/mL)	% Enzyme Inhibition		
	COX-1	COX-2	LOX
50	3.941±0.024	5.894±0.111	3.297±0.001
100	8.082±0.167	13.336±0.122	7.91±0.046
150	25.026±0.048	30.750±0.177	19.796±0.084
200	48.010±0.060	47.980±0.181	43.874±0.161
250	97.801±0.064	89.386±0.156	68.99±0.456
IC_{50}	179.38 µg/mL	187.27 µg/mL	212.82 µg/mL

3.3 Estimation of Antioxidant activity

3.3.1 Results from Reducing power measurement

Figure 2 compares the power of reducing of HB-SR to that of Quercetin and Vitamin C (Figure 2). Using a previously published technique, Fe^{3+}-Fe^{2+} transformation in the presence of herbal blend samples (HB-SR) were studied in the reductive ability assessment (Oyaizu, 1986). With increasing HB-SR concentration, the blend's reducing power rose and was discovered to be concentration dependent.

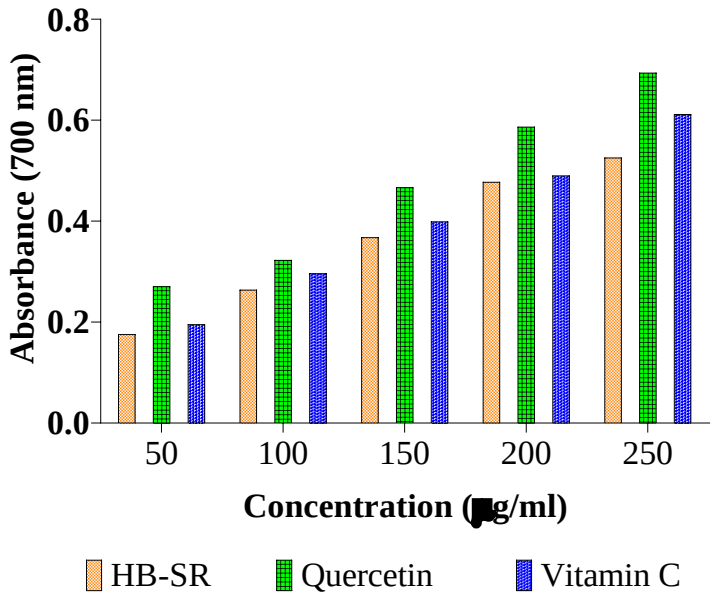

Figure 2. Reducing power assay results of HB-SR compared to Quercetin and Vitamin C

3.3.2 Evaluating the scavenging of DPPH radical

As traditional and standard radical scavengers, quercetin and vitamin C were utilized in the current study. It was shown that HB-SR's capacity to neutralize DPPH radicals was only slightly inferior to quercetin and vitamin C. Quercetin had a 97.11±0.59 percent DPPH scavenging effect, HB-SR had a 95.87±0.38 percent scavenging effect, and 96.88±0.68 percent scavenging effect at a concentration of 250 µg/m (vitamin C). The results demonstrated the blend's capability as a strong DPPH radical scavenger on par with industry norms. The IC_{50} values for the herbal mixture, standard compounds (quercetin, and vitamin C) were computed to be 98.57, 70.99, and 80.52 µg/mL, correspondingly, utilizing the equation produced from a linear regression study (Table 2 and Figure 3).

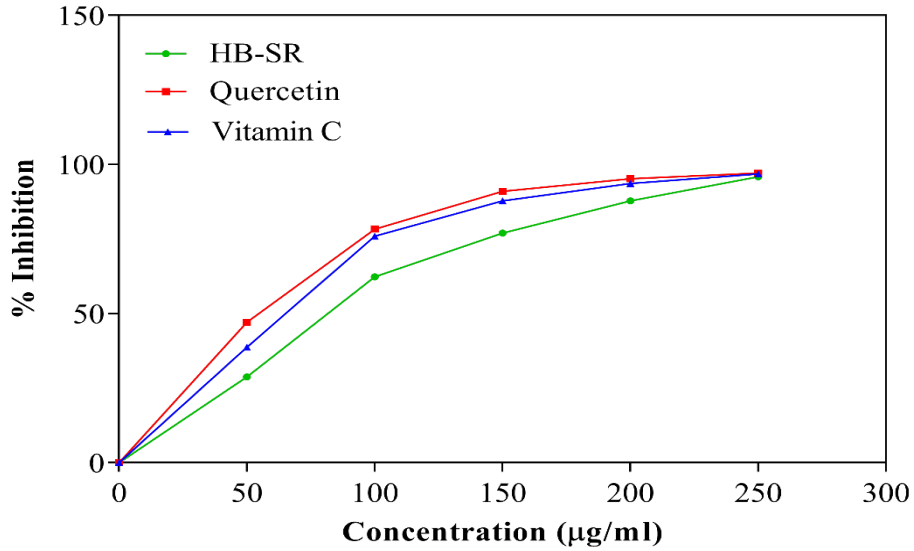

Figure 3. Scavenging of DPPH radical of HB-SR compared to Quercetin and Vitamin C

3.3.4 Evaluating the scavenging of superoxide radical ($O_2^{\bullet -}$)

It has been discovered that phenolic components, in particular catechins and flavonoids, are significant scavengers for superoxide and antioxidants. The concentration of phenol and the quantity and distribution of the hydroxyl groups play major roles in the scavenging effectiveness of these compounds (Ashokkumar, Thamilselvan, GP, Mazumder, & Gupta, 2008; Erasto, Grierson, & Afolayan, 2007). The physiological system produces the very harmful superoxide anion as a result of several biological processes. The current investigation shows that the feeding of superoxide radical anion in the reaction concoction is indicated by a drop in absorbance at 590 nm with antioxidants. The outcomes demonstrated that HB-SR and conventional chemicals inhibited the production of superoxide radicals in a concentration-dependent manner. Although HB-SR had good scavenging activity for superoxide radical, it was discovered that HB-SR's scavenging activity was inferior to that of conventional standard entities (vitamin C and quercetin). The calculated IC_{50} values of HB-SR, vitamin C and quercetin were estimated and computed to be 134.34, 100.84 and 80.25 $\mu g \cdot mL^{-1}$, respectively (Figure 4 and Table 2).

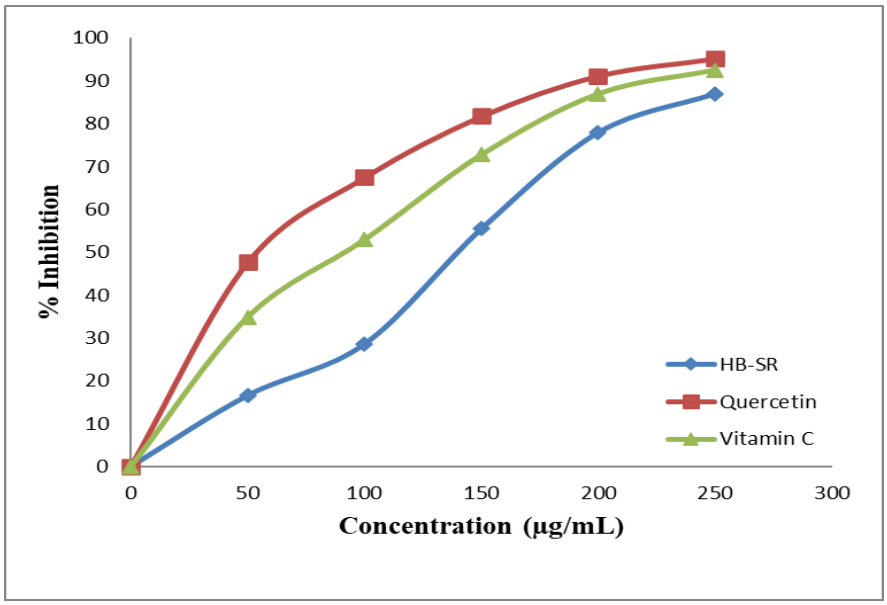

Figure 4. Superoxide radical (O2• –) scavenging activity of HB-SR compared to Quercetin and Vitamin C

Table 2. Calculated IC_{50} values for scavenging activity of DPPH and superoxide radicals by HB-SR (mean ± SD, n = 3)

Drugs	IC_{50} (μg·mL^{-1})	
	DPPH radical	Superoxide radical
HB-SR	98.57 ± 0.67	134.34 ± 0.93
Quercetin	70.99 ± 0.94	80.25 ± 0.26
Vitamin C	80.52 ± 0.39	100.84± 0.54

4. Discussions

The drugs that are most frequently recommended for treatment of inflammation and pain include traditional NSAIDs. A number of negative symptoms are connected to the use of these substances. NSAIDs are the main pharmacological option for managing pain and inflammation despite their potentially harmful side effects. Numerous innovative strategies for treating inflammation and pain that reduce the likelihood of side effects and other harmful manifestations are suggested by growing bodies of literature (Hotter et al., 1997; Khodr & Khalil, 2001; Martínez-Cayuela, 1995). It is therefore becoming clear that better anti-inflammatory compounds with fewer adverse effects need to be designed and developed. COX and LOX inhibition is a very popular mechanistic strategy for assessing anti-inflammatory and analgesic effectiveness since it is a well acknowledged, well-researched, and effective notion (Brito & Antonio, 1998). It is well known that the production of several inflammatory mediators, including histamine, prostaglandins, leukotrienes, PAF (Platelet Activating Factor), and other cyclooxygenase and lipooxygenase products, is associated with the development of inflammation brought on by toxins and infections (Brito & Antonio, 1998). Migration of neutrophils and leukocytes is also related to the inflammatory process. Free radicals may play a significant part in the process of inflammation, according to pathophysiology. As evidenced by the enzyme inhibition assays involving COX and LOX system, the current data demonstrated a strong inhibitory impact and recommended that the key mode of act of the HB-SR may include arachidonic acid pathway products inhibition and cessation. HB-SR appears to be just as effective as NSAIDs at reducing inflammation. According to the results of the COX inhibitory capacity, HB-SR significantly inhibited COX (both COX-1 and -2) whereas also suggestively obstructing 5-LOX enzyme. These outcomes amply illustrated the HB-SR's anti-inflammatory mechanism.

Superoxide (O_2^-) radical production during leukocyte and macrophage migration to the site of injury was another hypothesis for the pathophysiology of inflammation (L M Coussens & Z Werb, 2002; Hussain, Hofseth, & Harris, 2003; Khodr & Khalil, 2001). The physiological system has been proven to be extremely toxic to superoxide radical. In the presence of sufficient transitional elements, this superoxide radical generated hydrogen peroxide (H_2O_2), which in turn produced a hydroxyl radical (Martínez-Cayuela, 1995). Other inflammatory mediators can be produced as a result of the second messenger function that these radicals can perform (Hotter et al., 1997; Hussain et al., 2003; Khodr & Khalil, 2001). There are many *in vitro* evaluation techniques or models available, such as superoxide anion radical and DPPH free radical scavenging. The HB-SR displayed considerable superoxide anion radical and DPPH free radical scavenging capabilities in the current data, which were concentration dependent. Additionally, it was discovered that the HB-SR has strong reductive and superoxide radical scavenging abilities (F. F. Benzie & Strain, 1996; I. F. F. Benzie, Chung, & Strain, 1999). By reducing oxidative stress, neutralizing, and scavenging free radicals, and inhibiting the enzyme systems of the arachidonic acid pathway viz., COX and LOX systems, the herbal mixture (HB-SR) including methanol extracts of *Salvia officinalis* and *Rosmarinus officinalis* leaves is said to have an anti-inflammatory effect.

5. Conclusions

The herbal mixture's anti-inflammatory effects, which include methanol extracts of *Salvia officinalis* and *Rosmarinus officinalis* leaves (HB-SR) may therefore be ascribed to its repressive action on the enzymes systems of COX and LOX pathways' arachidonic acid byproducts. The herbal blend's (HB-SR) significant

ability to scavenge free radicals had a positive and significant impact on how well it reduced inflammation. To identify and characterize the chemical elements in charge of the reported inhibition of COX and LOX as well as the free radical scavenging capabilities, more study is already being conducted.

Acknowledgements

Conflict of interest

In this work, the authors say they have no competing interests.

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