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Research Paper

In-Vitro Antioxidant Activity and Erythrocyte Membrane Stabilization Activity of Baicalein

N. Thenmozhi*, G. Ramyashree, C. Subbulakshmi, M. Lokesh, K. Vishnuprasath, Dr. N. Gnanasekar, Dr. D. Rajalingam

Department of Pharmacology, Kamalakshi Pandurangan College of Pharmacy, yyampalayam, Tiruvannamalai, Tamil Nadu – 606 603, Affiliated to The TN Dr. M.G.R. Medical University, Chennai - 32.

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ABSTRACT

Baicalein is a naturally occurring flavonoid isolated from the roots of Scutellaria baicalensis. It exhibits various pharmacological activities including antioxidant, anti-inflammatory, and anticancer properties. The present study aims to evaluate the in-vitro antioxidant activity and erythrocyte membrane stabilization activity of baicalein using DPPH radical scavenging assay, hydrogen peroxide scavenging assay, and hypotonicity-induced hemolysis method.MethodTo evaluate the in-vitro antioxidant activity and erythrocyte membrane stabilization activity of baicalein using DPPH radical scavenging assay, hydrogen peroxide scavenging assay, and hypotonicity-induced hemolysis method.ResultThe sample identified as Baicalein demonstrates significant, dose-dependent anti-inflammatory activity. By stabilizing the HRBC membrane, the sample effectively inhibits lysis, which serves as an in vitro model for the stabilization of lysosomal membranes during inflammation.Also, Baicalein demonstrates exceptionally high antioxidant activity, characterized by its potent ability to scavenge DPPH radicals. The test results indicate that the compound is an effective free-radical scavenger even at very low concentrations.

INTRODUCTION

Antioxidants are natural or synthetic compounds that neutralize free radicals—unstable molecules that damage cells and contribute to chronic diseases like cancer and heart disease. Found in fruits, vegetables, and nuts, they include vitamins A, C, E, minerals like zinc and selenium, and phytochemicals. A diet high in these, rather than supplements, is recommended for health.The body's trillion or so cells face formidable threats, from lack of food to infection with a virus. Another

***Corresponding Author:** N. Thenmozhi
Address: *Assistant Professor, Department of Pharmacology, Kamalakshi Pandurangan College of Pharmacy, Ayyamapalyam, Tiruvannamalai – 606 603*
Email ✉: shine.then@gmail.com

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constant threat comes from chemicals called free radicals. In very high levels, they are capable of damaging cells and genetic material. The body generates free radicals as the inevitable byproducts of turning food into energy. Free radicals are also formed after exercising or exposure to cigarette smoke, air pollution, and sunlight. [1]Free radicals come in many shapes, sizes, and chemical configurations. What they all share is a voracious appetite for electrons, stealing them from any nearby substances that will yield them. This electron theft can radically alter the "loser's" structure or function. Free radical damage can change the instructions coded in a strand of DNA. It can make a circulating low-density lipoprotein (LDL, sometimes called bad cholesterol) molecule more likely to get trapped in an artery wall. Or it can alter a cell's membrane, changing the flow of what enters the cell and what leaves it. An excessive chronic amount of free radicals in the body causes a condition called oxidative stress, which may damage cells and lead to chronic diseases. [2]We aren't defenseless against free radicals. The body, long used to this relentless attack, makes many molecules that quench free radicals as surely as water douses fire. We also extract free-radical fighters from food. These defenders are labeled "antioxidants." They work by generously giving electrons to free radicals without turning into electron-scavenging substances themselves. They are also involved in mechanisms that repair DNA and maintain the health of cells.There are hundreds, probably thousands, of different substances that can act as antioxidants. The most familiar ones are vitamin C, vitamin E, beta-carotene, and other related carotenoids, along with the minerals selenium and manganese. They're joined by glutathione, coenzyme Q10, lipoic acid, flavonoids, phenols, polyphenols, phytoestrogens, and many more. Most are naturally occurring, and their presence in food is likely to prevent oxidation or to serve as a natural defense against the local environment.But using the term "antioxidant" to refer to substances is misleading. It is really a chemical property, namely, the ability to act as an electron donor. Some substances that act as antioxidants in one situation may be pro-oxidants—electron grabbers—in a different situation. Another big misconception is that antioxidants are interchangeable. They aren't. Each one has unique chemical behaviors and biological properties. They almost certainly evolved as parts of elaborate networks, with each different substance (or family of substances) playing slightly different roles. This means that no single substance can do the work of the whole crowd.

Health benefits of antioxidants:

Antioxidants came to public attention in the 1990s, when scientists began to understand that free radical damage was involved in the early stages of artery-clogging atherosclerosis. It was also linked to cancer, vision loss, and a host of other chronic conditions. Some studies showed that people with low intakes of antioxidant-rich fruits and vegetables were at greater risk for developing these chronic conditions than were people who ate plenty of those foods. Clinical trials began testing the impact of single substances in supplement form, especially beta-carotene and vitamin E, as weapons against chronic diseases.Even before the results of these trials were in, the media and the supplement and food industries began to hype the benefits of "antioxidants." Frozen berries, green tea, and other foods labeled as being rich in antioxidants began popping up in stores. Supplement makers touted the disease-fighting properties of all sorts of antioxidants.The research results were mixed, but most did not find the hoped-for benefits. Most research teams reported that vitamin E and other antioxidant supplements

didn't protect against heart disease or cancer. [3] One study even showed that taking beta-carotene supplements actually increased the chances of developing lung cancer in smokers. On the other hand, some trials reported benefits; for example, after 18 years of follow-up, the Physicians' Health Study found that taking beta-carotene supplements was associated with a modest reduction in the rate of cognitive decline. [4]These mostly disappointing results haven't stopped food companies and supplement sellers from banking on antioxidants. Antioxidants are still added to breakfast cereals, sports bars, energy drinks, and other processed foods, and they are promoted as additives that can prevent heart disease, cancer, cataracts, memory loss, and other conditions.Often the claims have stretched and distorted the data: While it's true that the package of antioxidants, minerals, fiber, and other substances found naturally in fruits, vegetables, and whole grains helps prevent a variety of chronic diseases, it is unlikely that high doses of antioxidant supplements can accomplish the same feat.Antioxidant supplements and disease prevention: little supportive evidenceRandomized placebo-controlled trials, which can provide the strongest evidence, offer little support that taking vitamin C, vitamin E, beta-carotene, or other single antioxidants provides substantial protection against heart disease, cancer, or other chronic conditions. The results of the largest trials have been mostly negative.

Antioxidants in food

One possible reason why many studies on antioxidant supplements do not show a health benefit is because antioxidants tend to work best in combination with other nutrients, plant chemicals, and even other antioxidants.For example, a cup of fresh strawberries contains about 80 mg of vitamin C, a nutrient classified as having high antioxidant activity. But a supplement containing 500 mg of vitamin C (667% of the RDA) does not contain the plant chemicals (polyphenols) naturally found in strawberries like proanthocyanins and flavonoids, which also possess antioxidant activity and may team up with vitamin C to fight disease. Polyphenols also have many other chemical properties besides their ability to serve as antioxidants. There is a question if a nutrient with antioxidant activity can cause the *opposite* effect with pro-oxidant activity if too much is taken. This is why using an antioxidant supplement with a single isolated substance may not be an effective strategy for everyone Differences in the amount and type of antioxidants in foods versus those in supplements might also influence their effects. For example, there are eight chemical forms of vitamin E present in foods. However, vitamin E supplements typically only include one form, alpha-tocopherol. [1]Epidemiological prospective studies show that higher intakes of antioxidant-rich fruits, vegetables, and legumes are associated with a lower risk of chronic oxidative stress-related diseases like cardiovascular diseases, cancer, and deaths from all causes. [30-33] A plant-based diet is believed to protect against chronic oxidative stress-related diseases. [2] It is not clear if this protective effect is due to the antioxidants, other substances in the foods, or a combination of both. The following are nutrients with antioxidant activity and the foods in which they are found:

- **Vitamin C:** Broccoli, Brussels sprouts, cantaloupe, cauliflower, grapefruit, leafy greens (turnip, mustard, beet, collards), honeydew, kale, kiwi, lemon, orange, papaya, snow peas, strawberries, sweet potato, tomatoes, and bell peppers (all colors)

- **Vitamin E:** Almonds, avocado, Swiss chard, leafy greens (beet, mustard, turnip), peanuts, red peppers, spinach (boiled), and sunflower seeds

- **Carotenoids** including beta-carotene and lycopene: Apricots, asparagus, beets, broccoli, cantaloupe, carrots, bell peppers, kale, mangos, turnip and collard greens, oranges, peaches, pink grapefruit, pumpkin, winter squash, spinach, sweet potato, tangerines, tomatoes, and watermelon

- **Selenium:** Brazil nuts, fish, shellfish, beef, poultry, barley, brown rice

- **Zinc:** Beef, poultry, oysters, shrimp, sesame seeds, pumpkin seeds, chickpeas, lentils, cashews, fortified cereals

- **Phenolic compounds:** Quercetin (apples, red wine, onions), catechins (tea, cocoa, berries), resveratrol (red and white wine, grapes, peanuts, berries), coumaric acid (spices, berries), anthocyanins (blueberries, strawberries)

- **Coenzyme Q10**

Coenzyme Q10 (CoQ10), or ubiquinone, is actually a vitamin or substance similar to vitamin. It is found in small amounts of a variety of foods and is absorbed into all tissues. The biosynthesis of CoQ10 from the amino acid tyrosine is a multi-phase process that requires at least eight vitamins and a few trace elements.CoQ10 decreases in the body as people age or develop certain diseases (such as other heart conditions, Parkinson's disease, and asthma). But that does not mean that low levels of CoQ10 cause disease or that extra CoQ10 will fight the disease or slow down the effects of aging. Some medications, including certain cholesterol-lowering statins, beta-blockers, and antidepressants, can lower CoQ10 levels in the body, but there is no evidence that this causes any side effects.

- **Alpha-lipoic acid**

Alpha-lipoic acid can effectively combine vitamins C and E as part of the first line of defense against free radicals. The body often converts alpha-lipoic acid into di hydro lipoic acid, which appears to be a more potent antioxidant. The therapeutic dose of lipoic acid is 600 mg/day in Europe. In the United States, it is marketed as a dietary supplement, usually as a 50- mg pill. A rich source of alpha-lipoic acid is red meat.

- **Ellagic acid**

Ellagic acid is a plant polyphenol and a highly effective antioxidant that inhibits hydroxyl radicals. It is usually found in pomegranates. Pomegranates have grown in Asia and the Middle East for thousand years for spiritual and health reasons. Western medicine has recently come to realize the importance of this powerful drug, which is gaining popularity in preventing and treating cancer and heart disease. Recent scientific studies suggest that pomegranate may be helpful in preventing and treating various cancers, such as prostate cancer.

- **Green tea**

Green tea has been used for centuries in India, China, Japan and Iran, and in traditional Chinese and Indian medicines, it has been used as a stimulant (somnolence), diuretic (to promote urination), astringent (to control bleeding and help heal wounds), and to improve heart health. Other traditional uses of green tea include treating constipation, regulating body temperature and blood glucose levels, promoting digestion, and improving mental processes.

- **Vitamin C**

Vitamin C reaches all parts of the body, and the level of vitamin C in both blood serum and tissue is very high. In fact, the nutrient plays a vital role in shaping and protecting our connective tissue, the upper matrix that holds the body together. Studies show that antioxidant supplements for vitamin C can help prevent cancer in many ways. For example, vitamin C fights the peroxidation of lipids, which have been linked to degeneration and aging. Vitamin C can also reduce the development of nitrosamines from nitrates, chemicals that are widely used in digested foods.

- **Selenium**

Selenium is a mineral trace that supports the healthy functioning of the immune system, acts as a powerful antioxidant glutathione, and is essential for good thyroid health.[34]

INFLAMMATION

Inflammation acts as an initial safeguard of inherent immunological system of anatomy counter to numerous detriment, septicity, and anxiety. Persistent inflammation is responsible for myriad degenerative sickness like gout, pyrexia, neurological problems, asthma, hardening of arteries, and even cancer. Currently, steroid along with non-steroidal anti-inflammatory drugs employed to manage for reducing pain. The drawback of these drugs is that they possess many serious side effects on hepatic system, orthopedic system, immunosuppression, hypertension, and so forth. Natural products possess pharmacological activities like antiviral, antitussive, immunostimulatory, immunomodulatory, anticancer, antibacterial, antioxidant, antidepression, and anti-inflammatory activities.

Anti-inflammatory activity refers to the ability of a substance, compound, or treatment to reduce inflammation, which is the body's protective response to injury, often characterized by redness, swelling, pain, and heat. These agents work by inhibiting inflammatory markers, such as cytokines and histamine, or by modulating signaling pathways to alleviate chronic or acute inflammation.

ERYTHROCYTE MEMBRANE STABILIZATION

Erythrocyte membrane stabilization is a key anti-inflammatory assay measuring a substance's ability to prevent red blood cell membrane rupture (hemolysis) under heat or hypotonic stress. Because erythrocyte membranes resemble lysosomal membranes, stabilization prevents the release of inflammatory mediators. This method is frequently used for preliminary screening of plant extracts, often showing promising, concentration-dependent activity comparable to standard drugs like diclofenac sodium.

Mechanism and Utility

- **Anti-inflammatory Screening:** Membrane stabilization protects against hemolysis, indicating a potential mechanism for reducing inflammation.
- **Lysosomal Analogy:** Because erythrocyte membranes resemble lysosomal membranes, stabilization prevents the release of substances that cause inflammation.
- **Methodology:** The most common techniques involve testing against hypotonicity-induced or heat-induced lysis.
- **Typical Results:** Effective compounds or extracts show increased inhibition of hemolysis as concentration increases.

METHODS

1. HRB membrane stabilization test

HRB membrane stabilization test was performed by the following described method proposed by Siddique et al. (1989).Fresh whole human blood (10ml) was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with an equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline. The reaction mixture 2ml consists of 1 ml of test sample solution and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56ºC for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. Percent membrane stabilization activity was calculated by the formula

Percentage Inhibition = (A of Control – A of Sample)/A of Control x100.

2. Free radical scavenging ability by the use of a stable DPPH radical (1,1-diphenyl-2-picrilhydrazyl)

The effect of given samples on DPPH radical was estimated according to the procedure described by Von Gadow *et al.* (1997). Two mL of 6 $\times 10^{-5}$ M methanolic solution of DPPH were added to 50 µl of the sample solution with different concentrations (0.2 mg, 0.4 mg, 0.6 mg, 0.8 mg and 1 mg). The decrease of absorbance at 515 nm was recorded in a spectrophotometer for 16 min at room temperature. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time and the percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 16 m in duration as follows:All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula of Yen and Duh (1994).

$$IP = [(A_{C(0)} - A_{A(t)} / A_{C(0)})] \times 100$$

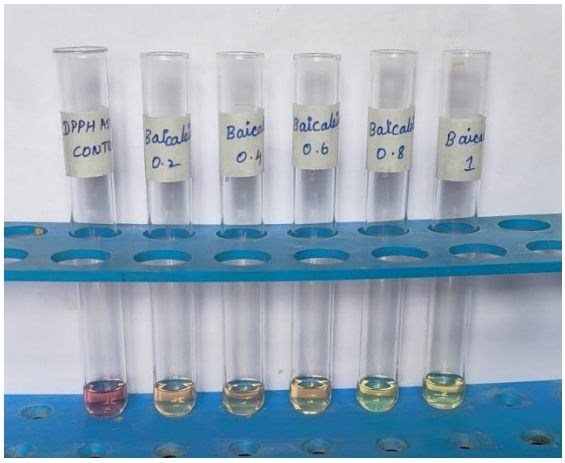
Where $A_{C(0)}$ is the absorbance of the control at t = 0 min; and $A_{A(t)}$ is the absorbance of the antioxidants at t = 16 min.

RESULTS

1. DPPH radical Scavenging Assay

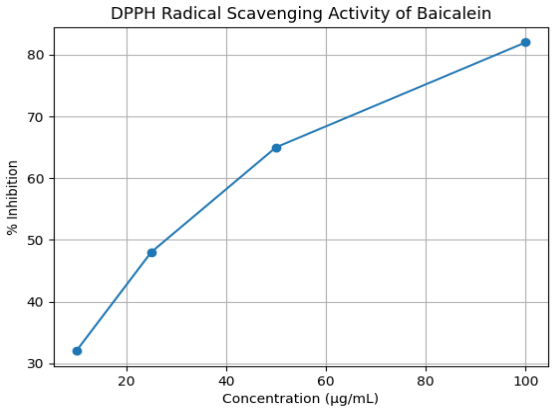
Sample ID: Baicalien

Concentration (mg/mL)	Method	Optical Density	Results (Inhibition %)
Control	DPPH radical Scavenging Assay (Von Gadow et al., 1997)	0.612	0.00
0.2		0.102	83.33
0.4		0.089	85.46
0.6		0.076	87.58
0.8		0.072	88.24
1		0.054	91.18



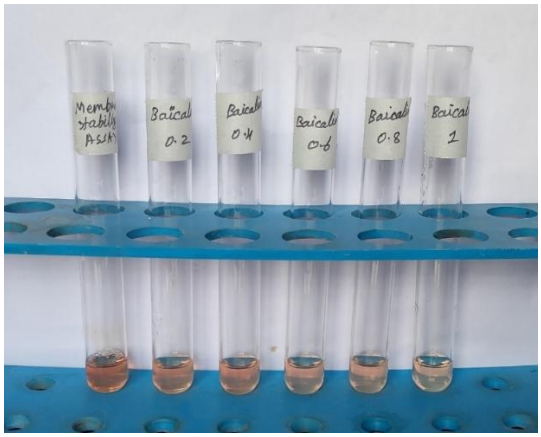
IC50 VALUES

					% Inhibition
	0	0.612	0.612	0	0.00
	0.2	0.102	0.612	0.51	83.33
DPPH	0.4	0.089	0.612	0.523	85.46
	0.6	0.076	0.612	0.536	87.58
	0.8	0.072	0.612	0.54	88.24
	1	0.054	0.612	0.558	91.18



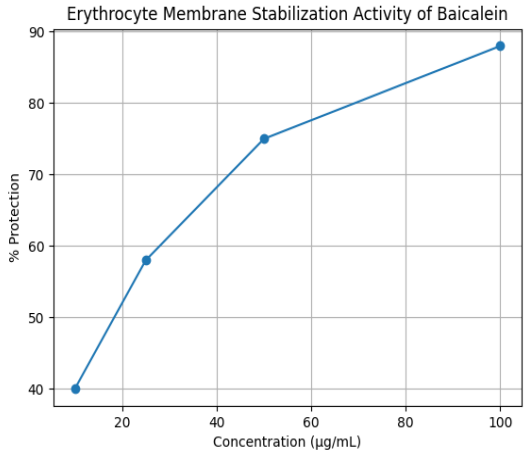
2. HRB Membrane stabilization Assay SAMPLE: G

Concentration (mg/mL)	Method	Optical Density	Results (Inhibition %)
Control	HRB Membrane stabilization Assay (Sadique *et al.,* 1989)	0.546	0.00
0.2		0.324	40.66
0.4		0.301	44.87
0.6		0.257	52.93
0.8		0.211	61.36
1		0.158	71.06



IC50 VALUES

					% Inhibition
	0	0.546	0.546	0	0.00
	0.2	0.324	0.546	0.222	40.66
Membrane Stabl.	0.4	0.301	0.546	0.245	44.87
	0.6	0.257	0.546	0.289	52.93
	0.8	0.211	0.546	0.335	61.36
	1	0.158	0.546	0.388	71.06



CONCLUSION

The sample identified as Baicalein demonstrates significant, dose-dependent anti-inflammatory activity. By stabilizing the HRBC membrane, the sample effectively inhibits lysis, which serves as an *in vitro* model for the stabilization of lysosomal membranes during inflammation.

Membrane Protection: The sample successfully prevented the release of lysosomal enzymes and inflammatory mediators by stabilizing the red blood cell membrane.

Concentration Efficiency: The highest tested concentration of **1 mg/ml** yielded a robust inhibition of **71.06%**

Statistical Trend: There is a clear linear correlation between the concentration of Baicalein and the percentage of inhibition

The sample Baicalein demonstrates exceptionally high antioxidant activity, characterized by its potent ability to scavenge DPPH radicals. The test results indicate that the compound is an effective free-radical scavenger even at very low concentrations.

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