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Evaluation of an Antioxidant Activity of *Achillea millefolium* Linn. Extract via DPPH Assay

Zahoor Ahmad Malik [1], Muskan Samaiya [2], Dr. Jitendra Bajaj [3]

[1, 2, 3] Sagar Institute of Pharmaceutical Technology and Research, Gandhi Nagar, Bhopal, Madhya Pradesh, India

Corresponding Author; **Zahoor Ahmad Malik**

Abstract

Historically, herbs have been valued for their aromatic properties in culinary applications, with little consideration for their nutritional benefits. However, recent research highlights their richness in phytochemicals, many of which possess strong antioxidant properties. As a result, these natural ingredients may play a significant role in supporting antioxidant defense mechanisms and redox signaling in the body. *Achillea millefolium* L. is known for its potential in treating inflammatory conditions and other health issues. This study focuses on isolating antioxidants using the DPPH radical scavenging assay to highlight its significance as a bioactive medicinal plant. Each part of *Achillea millefolium* possesses therapeutic properties, including antibacterial activity, making it a valuable candidate for further research. The DPPH assay is a simple, fast, and cost-effective method for evaluating antioxidant capacity by assessing a compound's ability to donate hydrogen or act as a free-radical scavenger, providing insights into its potential health benefits. In this study, the antioxidant activity of *Achillea millefolium* L. were analyzed using the DPPH radical scavenging assay compared to the phenolic compound that is gallic acid (GA). Research shows GA to be more effective than ascorbic acid, Trolox, caffeic acid, sinapic acid, and vitamin E in the DPPH radical neutralization assay.

Keyword: *Achillea millefolium* L, DPPH, Antioxidants, Methanol, Free Radicals, Gallic

1. Introduction

Our bodies naturally produce reactive oxygen species (ROS) and free radicals during various biochemical processes, with environmental factors also playing a role. While enzymatic and non-enzymatic antioxidants help neutralize them, excessive free radical production can lead to oxidative stress, damaging vital biomolecules like lipids, proteins, and DNA. If the body's defense mechanisms fail, oxidative stress may contribute to various health issues. Researchers are exploring natural antioxidants from edible and medicinal plants as safer alternatives to synthetic options. These natural compounds help reduce harmful free radicals, supporting overall health and protecting against oxidative damage [3]. Phenolic compounds are very powerful antioxidants with redox properties that enable them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers.

Antioxidants are essential compounds that help protect biological systems from oxidative damage by donating electrons to free radicals, rendering them harmless. Free radicals, primarily linked to oxidative stress, are generated when oxygen interacts with specific chemicals. Once formed, these unstable molecules can cause significant harm by interacting with critical cellular components such as DNA, proteins, and cell membranes. However antioxidants counteract this threat by neutralizing free radicals before they inflict damage. Plants naturally produce a diverse range of secondary metabolites, many of which function as potent antioxidants [12]. Antioxidants are thus effective in protecting organisms from oxidative stress due to free radicals. Natural

antioxidants are therefore very well-known and widely used these days in tea, fruits, vegetables, and spices, with more commercial additions or nutraceutical uses as well. Despite extensive research on antioxidant properties of various kinds of plant species, it remains worth investigating more plant species to understand further the antioxidant potential [1].

The Achillea genus, belonging to the Asteraceae family, includes around 85 species, mainly found in India, Europe and Asia, with some also present in North America. In 22 species exist, with *Achillea millefolium* being the most widespread. Traditionally, yarrow has been used in folk medicine for treating various ailments, including fever, asthma, bronchitis, wound healing, skin inflammation, digestive issues, and menstrual disorders [2]. Extracts from this plant are widely utilized in skincare due to their anti-inflammatory properties, which aid in skin lightening, wound healing, and rejuvenation while also offering antimicrobial benefits. The *Achillea millefolium* are recommended as key ingredients in cosmetic formulations designed for cleansing, moisturizing, soothing, conditioning, masking, and refreshing the skin. Additionally, A. millefolium extracts are rich in natural antioxidants and bioactive compounds, making them valuable for anti-aging and skin-protective cosmetic applications 8. This study aims to evaluate the antioxidant capacity of hydroalcoholic extract of *Achillea millefolium* L [2].

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Standard: - DPPH (2,2-Diphenyl-1-picrylhydrazyl), Methanol, Gallic acid

2.1.2 Instrument: UV-VIS Spectrophotometer, Shimadzu analytical India Pvt. Ltd.

2.1.3. DPPH free radical scavenging activity: - The 1, 1-diphenyl-2- picrylhydrazil (DPPH) radical has played a crucial role in antioxidant research since its discovery by Goldschmidt and Renn in 1922. In 1958, Blois developed a method using this stable free radical to evaluate antioxidant activity through spectrophotometric measurements. This assay, later refined by Brand-Williams and colleagues in 1995, became widely adopted for assessing the ability of antioxidants to neutralize DPPH radicals.

Gulcin's research group further modified the method to enhance its effectiveness. The DPPH radical is unique due to its single unpaired electron on the nitrogen atom, which makes it remarkably stable and gives its solution a deep violet color. This color results from π–π electronic transitions*, with the unpaired electron contributing significantly to the visible spectrum. The DPPH assay works on a simple principle: when an antioxidant donates a hydrogen atom, DPPH• is reduced to its hydrazine form (DPPH-H), causing the violet color to fade to pale yellow. This color change, which indicates radical scavenging activity, can be precisely measured using UV-Vis spectroscopy.

The stability of the DPPH radical is due to steric hindrance around the divalent nitrogen atom and the push-pull effect between its electron-donating diphenylamino group and electron- accepting picryl group, which stabilizes its canonical structure. Electron Paramagnetic Resonance (EPR) spectroscopy confirms this stability by measuring spin densities at the hydrazyl nitrogen atoms, which are both large and nearly equal. The radical's UV-Vis spectrum features two distinct absorption bands, mainly due to π–π* electronic transitions, with one band in the visible region responsible for its intense violet hue.

Widely recognized for its reliability and simplicity, the DPPH assay is one of the most frequently used methods for evaluating antioxidant capacity, especially in plant extracts, phenolic compounds, and pure antioxidant molecules. Beyond antioxidant studies, DPPH has also found applications in polymer chemistry and EPR spectroscopy. Its ability to produce observable and measurable color changes makes it a valuable tool for understanding free radical scavenging mechanisms and assessing the antioxidant potential of natural and synthetic compounds [10].

2.2. Methods

2.2.1 DPPH Solution: To prepare the solution, 3.81 mg of DPPH was added to a 100-mL amber volumetric flask and made up to 100 mL with MeOH, and then kept in a dark place to obtain the solution [7].

2.2.2 Preparation of Standard Solution: Standard stock: To obtain a 100 µg/ml solution, 1 milligram of gallic acid was dissolved in 5 ml methanol, and its final volume made up to 10 ml with methanol. Substock 1: A 10 µg/ml solution was made by taking 1 ml of gallic acid stock and diluting it to 10 ml in MeOH.

Substock 2: To make a 1 µg/ml solution, 1 ml of Substock-1 was diluted to 10 ml in MeOH.

Dilutions for gallic acid: From Substock-2, various dilutions of concentrations 0.1-1 µg/ml were prepared, similar to stock and substock. In addition, gallic acid concentrations of 2, 3, 4, 5, 6, 7, and 8 µg/ml from Substock-1 were also prepared [11].

2.2.3 Preparation of Plant Extract Solution

Standard stock: 100 mg/ml in a final volume of 10 ml was prepared by diluting 5 ml with methanol and making up to 10 ml with methanol.

Substock 3: A 1 ml aliquot of plant extract stock solution was diluted with 10 ml of methanol to yield a concentration of 10 µg/ml.

Substock 4: From Substock 3, 1 ml was diluted with 10 ml of methanol, yielding a concentration of 1 µg/ml.

From Substock 4, similarly as before, dilutions of 0.1 µg/ml, 0.5 µg/ml, and 1 µg/ml were prepared from stock and substock. From Substock 3, dilutions of 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL, 6 µg/mL, 7 µg/mL, and 8 µg/mL were made [12, 13].

2.2.4 Procedure for DPPH Assay

- The absorbance of the DPPH solution was first measured by UV-Vis spectroscopy for the negative control (A control).
- After 15 minutes, the absorbance value of a solution containing 2 mL of DPPH and 0.1 µg/mL gallic acid was observed through UV-Vis spectroscopy at 517nm.
- After 15 minutes, the absorbance of a solution containing 2 mL of DPPH and 0.5 µg/mL gallic acid was measured with the previous DPPH solution containing 0.1 µg/mL gallic acid.
- Following similar procedures, the 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL, 6 µg/mL, 7 µg/mL, and 8 µg/mL gallic acid solutions were mixed with 2 mL of DPPH at 5-minute intervals, and the absorbance for each solution was measured every 15 minutes.
- In a volumetric flask, 2 mL of DPPH solution was added in a 2-mL aliquot of the plant extract solution of concentration 0.1 µg/mL, and this was left for 15

minutes. After this time, the absorbance of the solution was measured.

- To another flask of 0.5 µg/mL plant extract, 2 mL of DPPH was added five minutes after the first addition of DPPH to the volumetric flask of 0.1 µg/mL plant extract. After 15 minutes, the absorbance of this solution was measured using.
- In the same manner, 2 mL of DPPH was added at 5-minute intervals, respectively, to plant extract solutions of 1, 2, 3, 4, 5, 6, 7, and 8 µg/mL. The absorbance was measured later at 15 minutes.
- All solutions were made in amber volumetric flasks of 10 mL 12.

2.2.5 Antioxidant Activity

% Antioxidant Activity was calculated using the following formula:

$$\text{Scavenging Activity \%} = \frac{AC - AS}{AC} \text{ X } 100$$

Where

- To another flask of 0.5 µg/mL plant extract, 2 mL of DPPH was added five minutes after the first addition of DPPH to the volumetric flask of 0.1 µg/mL plant extract. After 15 minutes, the absorbance of this solution was measured using.

AC = absorbance of the control (without extract)
AS = absorbance with the plant extract The IC50 value indicates the concentration of the extract required to achieve 50% inhibitory action [7].

3. Results and Discussion-

3.1. To determine the antioxidant capacity of a sample, the concentration of the extract required to scavenge 50% of the DPPH radical (IC50 value) is calculated. The IC50 value is a crucial indicator of the effectiveness of an antioxidant, with lower IC50 values suggesting stronger antioxidant activity. In the experiment, various concentrations of the extract are prepared and mixed with a fixed concentration of DPPH solution. After an incubation period, the decrease in absorbance is measured, and the IC50 value is determined by plotting the percentage of DPPH scavenging against the concentration of the extract. The concentration at which 50% of the DPPH radical is scavenged corresponds to the IC50 value. Gallic acid, a well-known phenolic compound with strong antioxidant properties, is used as a reference standard in this assay. Its known IC50 value serves as a benchmark for comparing the antioxidant potential of the test extracts. The IC50 value of the test extract can be compared to that of gallic acid to assess the relative antioxidant activity. A lower IC50 indicates that the extract requires a lower concentration to achieve the same level of DPPH radical scavenging, thus demonstrating higher antioxidant activity compared to gallic acid or other standards.

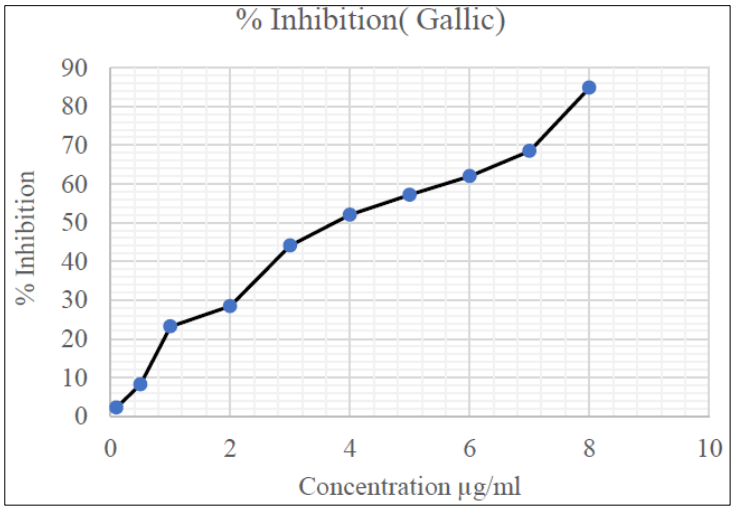


Fig 1: Standard curve of Gallic acid

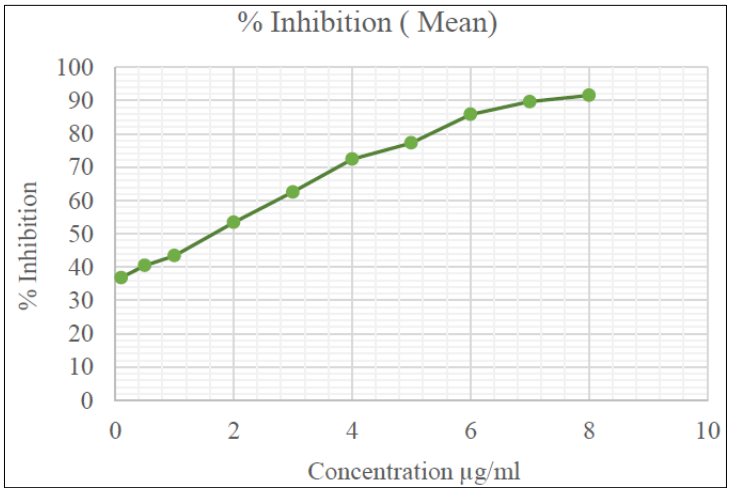


Fig 2: Graphical representation of DPPH Scavenging activity of the extract

3.2. The antioxidant activity of the extract was assessed over three consecutive days using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay, a widely recognized method for measuring the ability of compounds to neutralize free radicals. The experiment aimed to determine the extract's efficacy in scavenging free radicals and compare its activity with that of a standard antioxidant, gallic acid. The extract was obtained through maceration using 80% methanol as the solvent. The results demonstrated a strong antioxidant potential, as indicated by high inhibition percentages: 95.12% on day 1, 91.25% on day 2, and 85.12% on day [3]. The decreasing trend over time suggests a slight reduction in the extract's scavenging activity, which may be attributed to potential degradation of active compounds or changes in the extract's chemical stability over the three-day period.

Interestingly, the antioxidant activity of the extract was comparable to that of gallic acid, which exhibited a free radical inhibition of 84.78%. This similarity suggests that the bioactive compounds in the extract possess significant antioxidant properties, making it a potential candidate for further studies on natural antioxidants.

The IC50 value, which represents the concentration required to scavenge 50% of the DPPH radicals, is a key parameter for evaluating antioxidant strength. A lower IC50 value indicates a higher free radical scavenging ability. In this study, the IC50 value of gallic acid was determined to be 84.78, serving as a reference standard. The extract exhibited a mean IC50 value of

91.54±1.46, which, while slightly higher than that of gallic acid, still suggests notable antioxidant activity. This result implies that although the extract is slightly less potent than gallic acid, it retains a strong ability to neutralize free radicals.

Table 1: % Inhibition of Gallic Acid and Extract (*Achillea millefolium* L.)

Concentration µg/ml	% Inhibition of Gallic acid	% Inhibition of Extract (Mean±S.D)
0.1	2.3	36.80±0.33
0.5	8.22	40.47±0.46
1	23.19	43.40±1.09
2	28.46	53.38±0.66
3	44.04	62.51±0.79
4	52.02	72.38±1.30
5	57.17	77.266±1.84
6	61.96	85.80±1.07
7	68.46	89.64±0.71
8	84.78	91.54±1.46

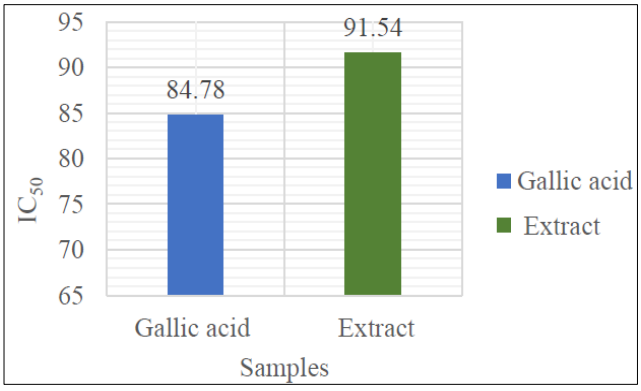


Fig 3: IC50 values of Standard and Extract

4. Conclusion

The results of the experimentation emphasized that the hydroalcoholic extract of *Achillea millefolium* L. was composed of phenols and flavonoids, manifesting a strong free radical scavenging ability, comparable with that of gallic acid. According to this, herbal plants are of utmost significance for all who advocate their use as a natural and easy source of antioxidants and are thus contributing in the prevention of diseases such as cancer, diabetes, dementia, and myocardial infarction, in which free radicals have been shown to play a pivotal role. The results demonstrated that *Achillea millefolium* exhibited the highest antioxidant activity, with an IC50 value of 91.54±1.46 µg/mL, outperforming Gallic acid, which had an IC50 value of 84.78 µg/mL.This suggested that *Achillea millefolium* L. could be a promising natural source of antioxidants, potentially beneficial for combating oxidative stress and related health conditions.

5. Conflicts of Interest

The authors declare no conflict of interest.

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