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# Phytochemical screening and antioxidant activity of extracts of the leaf and stem of *Achillea millefolium*

**[1] Mohd Yaseen, [2] Mudasar Ahmad, [3] Tareq A. Wani, [4] Manzoor Ahmad, [5] B. A Gani, [6] Rashida Qureshi**
[1, 4] Department of Chemistry Saifia Science College Bhopal, Madhya Pradesh, India
[5, 6] Department of Clinical Biochemistry University of Kashmir, Srinagar, Jammu and Kashmir, India
[2, 5] Phytochemistry Laboratory, Centre of Department of Clinical Biochemistry University of Kashmir, Srinagar, India
[3] Department of Botany, University of Kashmir, Srinagar, Jammu and Kashmir, India

**Abstract**
*Achillea millefolium* L. sensu lato (yarrow) is the best-known species of the genus Achillea due to numerous medicinal applications both in folk and conventional medicine. It is also an important medicinal plant found in Kashmir. The present study was aimed to evaluate the phytochemical constituents and antioxidant activity of leaf and stem extracts of *Achillea millefolium.* Antioxidant activity was carried out by using 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) and Nitric oxide assay. The phytochemical screening of leaves and stems of *Achillea millefolium* revealed the presence of phenols, steroids, tannins, saponins and alkaloids in the hydroalcohol extracts. The percentage yield of hydroalcohol extract of the leaf of *Achillea millefolium* was higher (13.55) than that of the stem. Also quantitative analysis showed that percentage of phenols was higher (17.47) in the leaf extract. The results of DPPH scavenging activity for leaf hydroalcohol showed (14.87%) and stem hydroalcohol showed (12.95%) inhibition. When compared to standard the leaf hydroalcohol showed better DPPH scavenging activity (14.87%). The percentage antioxidant activity is high in leaf extract than stem extract and in Nitric oxide assay the percentage inhibition was also higher than the stem extract when compared to the Ascorbic acid (standard). It indicates the plant has the potency of scavenging free radicals and it may provide leads in the ongoing search for natural antioxidants from various medicinal plants to be used in treating diseases related to free radical reactions.

**Keywords:** phytochemicals, antioxidant, free radicals, *achillea millefolium*, DPPH, nitric oxide assay

## Introduction

Plants have been associated with the human health from time immemorial and they are the important sources of medicines since the down of human civilization. In spite of tremendous development in the field of allopathic medicines during 20$^{th}$ century, plants still remain one of the major sources of drugs in modern as well as in traditional systems of medicine. Herbal medicines are universally accepted because of the fact that Medicinal plants continue to play important role in healthcare system of a large number of world's population. In fact there are several medicinal plants all over the world which are being used traditionally in the prevention and treatment of several diseases. The medicinal plants are rich source of secondary metabolites like alkaloids, glycosides, steroids and flavonoids, which are potential source of drugs. Nearly one third of the pharmaceuticals are plant origin. As all the plants are able to synthesize a multitude of organic molecules/phytochemicals, they are referred to as "secondary metabolites" (Harborne, 1982) [1].

Plants derived compounds are playing an important role in the development of several clinically useful medicines (Madhuri and Pandey, 2009) [2]. Scientist first started extracting and isolating chemicals from plants in the 18th century and since the time have grown a custom of looking at herbs and their effects in terms of the active constituents they contain. It is generally accepted that plants based medicines are better than synthetic drugs as these are much safer for human beings. The ascendance of much touted synthetic drugs over phytomedicine thrived due to their fast action.

Phytochemicals are bioactive compounds found in plants that work with nutrients and dietary fiber to protect against diseases. They are non-nutritive compounds. These phytochemicals are the secondary metabolites present in smaller quantities in higher plants and they include the alkaloids, Steroids, flavonoids, terpenoids, tannins and many others (Peteros, 2010) [3]. Many phytochemicals have antioxidant activity and reduce the risk many diseases. It is crucial to know the type of phytochemical constituent, thus knowing the type of biological activity which might be exhibited by the plant (Agbafor and Nwachukwu, 2011) [4]. The importance of medicinal plants and the contribution of phytomedicine to the well-being of a significant member of the world's population have attracted interest from diverse disciplines.

Antioxidants are substances that may protect our body cells against the effects of free radicals. Free radicals are molecules produced when our body breaks down food. They can also be produced by environmental exposures like tobacco smoke and radiation. Free radicals can damage cells, and may play a role in heart disease, cancer and other diseases. Oxidative damage can lead to a break down or even hardening of lipids, which is the major composition of all cell walls. This breakdown or hardening is due to lipid peroxidation and leads to death of cell or loss of normal cell function. In addition, other

biological molecules including RNA, DNA and protein enzymes are also susceptible to oxidative damage. Environmental agents initiate free radical generation, which leads to different complications in body. The toxicity of lead, pesticides, cadmium, ionizing radiation, alcohol, cigarette smoke, UV light and pollution may all be due to their free radical initiating capability (Aruoma, 1998; Bandoniene *et al*., 2000; Kuçukboyacı *et al*., 2012) [7, 5, 6].

Antioxidants cause protective effect by neutralizing free radicals which are toxic byproducts of natural cell metabolism. The human body naturally produces antioxidants but the process is not 100% effective in case of over whelming production of free radicals and that effectiveness also declines with age (Sies, 1999) [8]. Increasing the anti-oxidant intake can prevent diseases and lower health problems. Research is increasingly showing that antioxidant rich foods and herbs have health benefits. Medicinal herbs are the richest sources of antioxidants compounds (Sies *et al*., 1992) [9].

Despite some folklore use of this plant, there is no scientific report documented in the information on its phytochemical and antioxidant properties of this plant to the best of our knowledge. In this study, we sought to interrogate the antioxidant and screening of phytoconstituents of *Achillea millefolium.*

## Materials and Methods

### Collection of plant material

The leaves and stem of *Achillea millefolium* were collected from the wild Pahroo Budgam and the specimen deposited in the department of Botany university of Kashmir. The fresh leaves and stems of *Achillea millefolium* were authenticated by department of Botany University Kashmir

### Extraction of the plant material

The fresh plant materials were washed with running tap water and shade dried. The leaves and stems were crushed to coarse powder by a grinder. The coarse powder (25g) was then subjected to successive extraction in 250 ml of each solvent hexane, ethyl acetate and hydroalcohol by using Soxhlet apparatus. The collected extracts were stored and then taken up for further investigations. Dimethyl sufloxide (DMSO) is used as solvent for dissolving the extracts.

### Quantitative phytochemical analysis

### Estimation of flavonoids

The total flavonoid content in the sample was estimated by the method of Chang *et al*. (2002) [10]. The extract prepared for the estimation of total phenolics was used as sample for this assay. A volume of 0.25 ml of the sample was diluted to 1.15 ml with distilled water. A volume of 75 µl of 5% sodium nitrite was added and after six minutes 0.12 ml of aluminium chloride solution was added. A volume of 0.3 ml of 0.1M NaOH was added after 5 min and made up to 2.2 ml with distilled water. The solution was mixed well and the absorbance was read at 510 nm in comparison with standard quercetin at 4-25 µg concentration. The results are expressed as mg of flavonoids as quercetin equivalent/ gm of dried sample.

### Estimation of alkaloids

Alkaloid was determined using Harborne (1998) [11] method. Five grams of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

### Determination of saponin

Twenty grams of plant sample was dispersed in 150 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. A volume of 60 ml of normal butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the sample were dried in the oven into a constant weight. The saponin content was calculated in percentage [12]**.**

### Estimation of saponins

Plant extract was dissolved in 80% methanol, 2 ml of Vanillin in ethanol was added, mixed well and the 2 ml of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 60°C for 10 min, absorbance was measured at 544 nm against reagent blank. Diosgeninis was used as a standard material and the result was compared with Diosgenin equivalents.

### Determination of total phenols

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. A volume of 5 ml of the extract was pipetted into a 50 ml flask, then 10ml of distilled water was added. A volume of 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark and left to react for 30 min for color development. This was measured at 505 nm.

### Estimation of steroids

A volume of 1ml of the plant extract of steroid solution was transferred into 10 ml volumetric flasks. Sulphuric acid (4 N, 2 ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water-bath maintained at 70±2°C for 30 min with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank.

### Antioxidant activity

***DPPH radical scavenging activity***

The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple color). When Antioxidants react with DPPH, which is a stable free radical, it becomes paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging antioxidant) and is reduced to the DPPH and as a consequence, the absorbance's decreased from the DPPH.

**The scavenging reaction between DPPH and antioxidant**
A mass of 4.3 mg of DPPH (1, 1-Diphenyl –2-picrylhydrazyl) was dissolved in 3.3 ml methanol; it was protected from light by covering the test tubes with aluminum foil. A volume of 150 µl DPPH solutions was added to 3 ml methanol and absorbance was taken immediately at 517 nm for control reading. A volume of 50 µl of various concentrations of coumarin compounds as well as standard compounds (Ascorbic acid) were taken and the volume was made uniformly to 150 µl using methanol. Each of the samples was then further diluted with methanol up to 3 ml and to each 150 µl DPPH was added. Absorbance was taken after 15 min. at 517nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. The IC50 values for each compounds as well as standard preparation were calculated. The DPPH free radical scavenging activity was calculated using the following formula:
% scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] × 100

**Nitric oxide free radical scavenging activity**
The procedure is based on the principle that, sodium nitroprusside in hydroalcohol solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Large amounts of NO may lead to tissue damage. A volume of 50 µl of each of the concentrations of coumarin compounds previously dissolved in DMSO, as well as Ascorbic acid (standard compound) were taken in separate tubes and the volume was uniformly made up to 150 µl with methanol. To each tube, 2.0 ml of sodium nitroprusside (10 mM) in phosphate buffer saline was added. The solutions were incubated at room temperature for 150 min. The similar procedure was repeated with methanol as blank which served as control. After the incubation, 5 ml of griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV-visible spectrometer Shimadzu, UV-1601, Japan. Ascorbic acid was used as positive control.

**Results**
The results of the phytochemical screening revealed the presence of phenols, steroids, tannins, saponins and flavonoids in the hydroalcohol leave and stem extracts of *Achillea millefolium.* The percentage yields of the constituents of leaf hydroalcohol are alkaloids with 0.10%, flavonoids with 10.42%, steroids with 31.15%, saponin with 25.90%, phenols with 38.78%, tannin with 28.58%. The percentage yield of the constituents of stem hydroalcohol are alkaloids with 0.16%, flavonoids with 5.70%, steroids with 26.33%, saponin with 19.50%, phenols with 30.31% and tannin with 26.15%. Results of quantitative analysis showed that percentage with reference to phenol in leaf (38.78%) was more than stem (30.31%). (Table 1 and Figure 1).

**Table 1:** Quantitative Phytochemical analysis of *Achillea millefolium*

| Name of the phytochemical constituents (%) | Extracts of *Achillea millefolium* | |
|---|---|---|
| | Leaf hydro alcohol | Stem hydro alcohol |
| Alkaloids | 0.10 | 0.16 |
| Flavonoids | 10.42 | 5.70 |
| Steroids | 31.15 | 26.33 |
| Saponin | 25.90 | 19.50 |
| Phenol | 38.78 | 30.31 |
| Tannin | 28.58 | 26.15 |

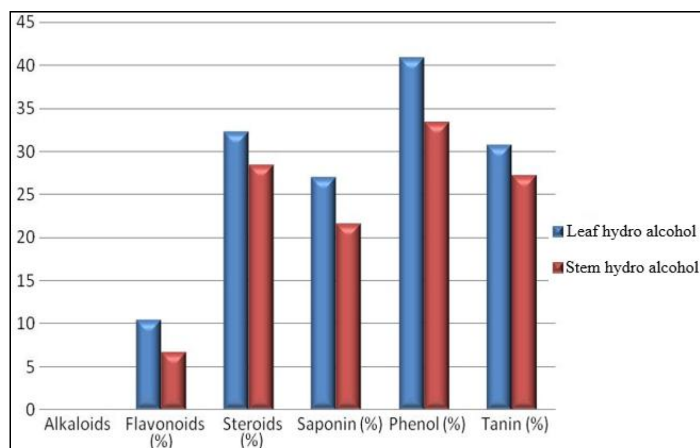


**Fig 1:** Quantitative Phytochemical analysis of *Achillea millefolium*

The results of the free radical scavenging activity of the 1,1-diphenyl - 2 picryl - hydrazyl (DPPH) assay showed percentage antioxidant activity (% AA) is 14.80% in leaf extract and 12.30% in stem extract and in Nitric oxide assay the percentage inhibition was 12.88% and 10.90% in leaf and stem extract. The Nitric oxide assay of the leaf and stem extract was found to be higher than the Ascorbic acid (standard) value of 11.05% (Table 2).

**Table 2:** Antioxidant activity of *Achillea millefolium*

| Samples | Percentage of inhibition (%) | |
|---|---|---|
| | DPPH | Nitric oxide |
| Leaf Hydro alcohol | 14.80 | 12.88 |
| Stem hydro alcohol | 12.30 | 10.90 |
| Standard | | |
| Ascorbic acid | 11.05 | 9.89 |

**Discussion**
The Medicinal plants are rich in secondary metabolites which include alkaloids, flavonoids, steroids and related active metabolites which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry. Recently number of studies had been reported on the phytochemistry of medicinal plants, particularly on the vegetative parts like leaves and stems etc (Balakumar *et al*., 2011 and Rajan *et al*., 2011) [13, 14]. Phytochemicals especially

plant phenolics constitute a major group of compounds that act as primary antioxidants (Hatano *et al*., 1988) [15]. The phenolic compounds have high redox potential which allow them act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kahkonen *et al*., 1999) [16]. The antioxidant effects of leaf and stem extracts may be due to its phenolic content. The delocalization of electrons over the phenolics and stabilization by the resonance effect of the aromatic nucleus prevents the continuation of the free radical chain reaction (Tsao and Akhtar, 2005) [17]. The DPPH assay has been largely used as a quick, reliable and reproducible parameter to search for the *in vitro* general antioxidant activity of pure compounds as well as plant extracts (Koleva *et al*., 2002; Goncalves *et al*., 2005) [18, 19]. The decrease in absorbance by the DPPH radical with increase in concentration of the extract which manifested in the rapid discolouration of the purple DPPH, suggest that the hydro alcoholic extracts of *Achillea millefolium* has antioxidant activity due to its proton donating ability. The extract was found to highly scavenge free radicals when compared to standard antioxidants. The reducing capacity of compounds could serve as indicator of potential antioxidant properties [20]. and increasing absorbance could indicate an increase in reducing power. Nitric oxide is a free radicals product and involved in the regulation of various physiological processes, however excess production of nitric oxide is associated with several diseases (Lalenti *et al*., 1994) [21].

Considering the phytochemical screening, total phenolics, reducing capacity and the DPPH radical scavenging activity as indicates the antioxidant activity of the extract, these findings revealed the potential of *Achillea millefolium* as a source for natural antioxidants. The phytochemical like flavonols or tannins are known to occur in plant species belonging to the Leguminosae - Mimiosoideae and Asteraceae family (Mensor *et al*., 2001) [30] to which *Achillea millefolium* belongs to it. It indicates that the plant could be a promising agent in scavenging free radicals and treating diseases related to free radicals reactions.

## Conclusions

Despite ongoing scientific research on this species, this study constitutes the first attempt to compile the phytochemical compositions as well as the antioxidant, activities of *Achillea millefolium* leaf and stem hydro alcoholic extracts that could be found despite the throughout literature survey so far as we know. The knowledge of phytochemical constituents of the plant is the basic approach to identify novel secondary metabolites as unmodified form, semi-synthetic or drug templates. This study delineates that hydro alcohol extracts could be a potentials in free-radical scavenging activity. So, it can be assumed that different active secondary metabolites were present in these extracts. Furthermore, the activity of this plant constituent can help to elucidate the justification for the ethnomedicinal use of this plant species scientifically. Based on our findings, further studies are necessary to elucidate the mechanism lying with these effects of the plant extracts and could be open a new window in the search for new bioactive drug lead components of this plant extracts.

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