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ASSESSMENT OF TOTAL PHENOLIC, FLAVONOID CONTENT AND *IN VITRO* ANTIOXIDANT PROPERTIES OF *ALCHEMILLIA VULGARIS* (LADY'S MANTLE)

Shilpee Jain[1], A. S. Yadav[2], Ragini Gothalwal[1]

[1]*Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India*

[2]*OSD, Higher Education, M.P. Govt., Bhopal, Madhya Pradesh, India*

**Corresponding author: jain.shilu620@gmail.com*

ABSTRACT

The current study was designed to evaluate the phytochemical profile and biological properties (antioxidant activity) of methanolic, ethyl-acetate and petroleum extracts of *Alchemilla vulgaris* which was traditionally used to alleviate and treat many diseases. Total phenolic content found in methanolic and ethyl acetate extract of *Alchemillia vulgaris* were 547.333 and 386.86 respectively. The phenolic content with respect to gallic acid was found to be 547.333 and 386.86 (µg gallic acid equivalent/ml of extract) for ethyl acetate and methanol extract. The flavonoid content was found to be as: 275.00 and 153.33 (µg rutin equivalents/ml of extract) in ethyl acetate and methanol extract. Free radicals are concerned in many disorders like neurodegenerative disease; cancer and AIDS. Antioxidants during their scavenging power are helpful for the management of those diseases. DPPH stable free radical method is an easy, rapid and receptive way tosurvey the antioxidant activity of a precise compound or plant extracts. $IC_{5\ 0}$of the standard compounds, ascorbic acid was 8.59µg/ml. The uppermost radical scavenging activity was showed by ethyl acetate and methanolic extracts of *Alchemillia vulgaris* IC_{50}= 66.71 and 23.47µg/ml respectively. The ethyl acetate extract has shown significantly low anti-radical activity compared to the methanolic extract. The results indicate that the extract reduces the radicals to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principle. The superior amount of phenolic compounds leads to further potent radical scavenging result as shown by *Alchemillia vulgaris* leaves extract.

Keywords: DPPH, Antioxidant**,** *Alchemillia vulgaris*, Ascorbic acid, Gallic acid.

1. INTRODUCTION

Free radicals donate to more than one hundred disorders in humans counting atherosclerosis, arthritis, ischemia and reperfusion damage of numerous tissues, central nervous system injury, gastritis, cancer and AIDS [1-2]. Free radicals are molecular species with unpaired electrons in their atomic orbital capable of independent existence [3]. Free radicals due to ecological pollutants, radiation, chemicals, toxins, profound fried and spicy foods as well as corporeal stress, cause exhaustion of immune system antioxidants, modify in gene expression and persuade abnormal proteins. Oxidation development is one of the most imperative routs for producing free radicals in food, drugs and still living systems. Catalase and hydroperoxidase enzymes change hydrogen peroxide and hydroperoxides to nonradical forms and purpose as natural antioxidants in human body. Owing to depletion of immune system natural antioxidants in dissimilar maladies, overwhelming antioxidants as free radical scavengers may be essential [4-9].

At present, available synthetic antioxidants similar to butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinon and gallic acid esters, have been supposed to cause or punctual negative health effects. Consequently, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Furthermore, these synthetic antioxidants also show low solubility and reasonable antioxidant activity [10-11]. Recently there has been an increase of interest in the therapeutic potentials of medicinal plants as antioxidants in dropping such free radical induced tissue injury. Polyphenolic compounds with known properties include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action [12]. A number of confirmations suggest that the biological actions of these compounds are related to their antioxidant activity [13]. An easy, rapid and sensitive method for the antioxidant screening

of plant extracts is free radical scavenging assay using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) stable radical spectrophotometrically. In the occurrence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases [14]. In particular, despite extensive use of wild plants as medicines in Iran, the prose contains few reports of antioxidant activity and chemical composition of these plants. In current study, we carried out a systematic record of the relative free radical scavenging activity in selected medicinal plant species, which are being used traditionally. *Alchemilla vulgaris* is herbaceous perennial found throughout Europe, especially on upland grassland and verges. Thin round green stems (up to 60 cm but usually less) bear bright green, palmately lobed leaves with toothed edges. There is a basal rosette and tufts of leaves encircle the stem at the apices. Tiny yellowy-green flowers occur in dense, terminal compound cymes with four sepals and stamens but no petals. The seed is an achene. The rhizome is woody and the plant spreads vegetatively and by seed. Characteristic water droplets are exuded by the leaves when air humidity is high.

One of the plant, that have medicinal quality to provide the rational means for the treatment of many diseases, is *Alchemilla vulgaris* L. syn. *Alchemilla xanthochlora* Rothm., commonly known as lady's mantle and bear's foot, well-known species from the genus Alchemilla (Rosaceae) [15]. *Alchemilla vulgaris* is an aggregate species divided into 12 sections of apomictic microspecies that are clones arising from seed produced by asexual reproduction [16]. The microspecies are often not distinct morphologically and vary genetically where the microspecies are widespread [17]. As microspecies hybridize, there are different opinions on the extent to which this has resulted in new species and thus as to the number of species in the genus. Stace (1991) distinguishes 15 species that are native or have been introduced to the UK. A study of 23 widespread microspecies in Estonia found that the best characteristic for distinguishing species is the degree and type of hairiness [18]. The aim of this work was to determine the quality (types), quantity (amount) of bioactive compounds and in vitro antioxidant activity of leaf of *Alchemilla vulgaris*.

2. MATERIAL AND METHODS

2.1. Collection and authentication of Plant

The plant *Alchemilla vulgaris* was procured from Bristol Botanicals Ltd. The herbarium of plant *Alchemilla vulgaris* was prepared. The plant was authenticated from the department of Botany, Saifia Science College Bhopal with serial no. 159/saif/sc./college/Bpl (59).

2.2. Extraction of plant *Alchemilla vulgaris*

Extraction of *Alchemilla vulgaris* was carried out by the maceration process. The crushed plant of *Alchemilla vulgaris* (500.28 gm.) was used for the extraction process. The powdered *Alchemilla vulgaris* was successively extracted with soxhlet extraction using solvents of increasing polarity; petroleum ether, ethyl acetate and methanol. The solvents were removed under reduced pressure on rotary evaporator until it became complete dry. The extract was transferred to beaker and evaporated and excessive moisture was removed. Then, the dried extract were placed and labelled in air tight container for further studies [19].

2.3. Qualitative phytochemical analysis of plant extract

Petroleum ether, ethyl acetate and Methanolic extracts of leaves of *Alchemilla vulgaris* were subjected to the preliminary phytochemical analysis [20-21]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

2.4. Total Phenolic Content Estimation

The total phenols content of the extract was determined by the Folin-Ciocalteu's method. The concentration gradient of gallic acid was prepared as standard solution (20–100 µg/ml), and calibration curve was established using gallic acid. The AV extract was diluted with deionized water as sample solutions. The 400µl of deionized water and 100µl of sample or standard solution was added to 5-ml centrifuge tube and mixed well. The diluted extract or gallic acid was added to 0.2 ml Folin-Ciocalteu reagent and mixed for 6 min, followed by the addition of 4 ml of 20% sodium carbonate and 800 µl deionized water. The mixture was placed for 90 min at room temperature. Every experiment was performed in triplicate. The absorbance of the mixture was measured at 760 nm using a UV spectrophotometer. The concentration of free phenols and bound phenols in the sample was calculated, according to the regression equation of standard curve. The sum concentration of free phenols and bound phenols was total phenols content, and the results were

expressed as mg of gallic acid equivalent per 100 g dry weight [22].

2.5. Total Flavonoid Content Estimation

The total flavonoid content of crude extract was determined by the aluminium chloride colorimetric method. In brief, 50 µL of crude extract (1 mg/mL methanol) were made up to 1 mL with methanol, mixed with 2 mL of distilled water and then 0.15 mL of 5% $NaNO_2$ solution; 0.3 mL of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution were added, and the final volume of the mixture was brought to 5 mL with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was measured at 510 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per g dry weight [23].

2.6. Antioxidant Assay

2.6.1. DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The scavenging reaction between (DPPH•) and antioxidant (H-A) can be written as:

(DPPH•) + (H-A) ⟶ DPPH-H + (A)
(Purple (Yellow)

Antioxidants reacts with DPPH, which is stable free radical and is reduced to DPPHH and as consequence, the absorbance's decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in term of hydrogen donating ability. To assess the scavenging ability on DPPH, each extract (20-100µg/ml) in water and ethanol was mixed with 1 ml of methanolic solution containing DPPH radicals (0.2mM). The mixture was shaken vigorously and left to stand for 30 mins in the dark before measuring the absorbance at 517nm against a blank. Then the scavenging ability was calculated using the following equation:

$$I\ \% = 100\times (A\ blank - A\ sample/\ A\ blank)$$

Where, I (%) is the inhibition percent, a blank is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound [23].

3. RESULTS AND DISCUSSION

3.1. Calculation of extraction yield

Alchemilla vulgaris plant extracts were obtained from different solvents including petroleum ether, ethyl acetate and methanol. The maximum yield was found from the methanolic solvent.

Table 1: Theoretical weight of Plant

Solvent used	Actual weight of plant (gm.)	Theoretical weight of plant (gm.)
Petroleum ether	3.513	500.26
Ethyl acetate	4.503	495.18
Methanol	45.425	480.55

Table 2: Extraction yields of different extract

Solvent used for extraction	Quantity of *Alchemillavulgaris* (gm.)	Extraction Yield (%)
Petroleum Ether	500.26	0.7022
Ethyl Acetate	495.18	0.9093
Methanol	480.55	9.4527

Table 3: Phytochemical test result of *Alchemilla vulgaris*

Test for Carbohydrates			
Chemical test/Reagent	**Methanolic extract**	**Ethyl acetate**	**Petroleu mether**
Molish	**+ve**	**-ve**	**-ve**
Fehling's	**+ ve**	**-ve**	**-ve**
Benedict's	**+ve**	**-ve**	**-ve**
Test for protein and amino acid			
Biuret	**-ve**	**-ve**	**-ve**
Million's test	**-ve**	**-ve**	**-ve**
Ninhydrin	**-ve**	**-ve**	**-ve**
Test for glycosides			
Legal's test	**+ve**	**-ve**	**-ve**
Keller-killani	**+ve**	**-ve**	**-ve**
Test for alkaloids			
Mayer's	**+ve**	**+ve**	**-ve**
Hager's	**+ve**	**+ve**	**-ve**
Wagner's	**+ve**	**+ve**	**-ve**
Test for saponins			
Froth test	**+ve**	**-ve**	**-ve**
Test for flavonoids			
Lead acetate	**+ve**	**+ve**	**-ve**
Alkaline reagent	**+ve**	**+ve**	**-ve**
Test for triterpenoids and steroids			
Salkowski's	**+ve**	**-ve**	**-ve**
Libermann-burchard's	**+ve**	**-ve**	**-ve**
Test for Tanin and phenolic compounds			
Ferric chloride	**+ve**	**+ve**	**-ve**
Lead acetate	**+ve**	**+ve**	**-ve**
Gelatin	**+ve**	**+ve**	**-ve**
Dilute iodine solution test	**+ve**	**+ve**	**-ve**

Table 4: Total phenolic content in ethyl acetate and methanolic extractof *Alchemilla vulgaris*

S. No.	Ethyl acetate	MeOH
Mean	0.839	1.160
SD	0.013503	0.000577
TPC Value	386.833	547.333

Table 5: Total flavonoid content in ethyl acetate and methanolic extract

S. No.	Ethyl acetate	MeOH
Mean	0.245	0.367
SD	0.002	0.001
TFC Value	153.33	275.00

Table 6: DPPH assay for antioxidant activity at different concentrationof *Alchemilla vulgaris*

Ascorbic acid (std.)		
S. No.	**Concentration**	**% Inhibition**
1.	10 µg/ml	51.644
2.	15 µg/ml	56.359
3.	20 µg/ml	61.513
4.	25 µg/ml	68.969
5.	30 µg/ml	71.710
	IC_{50}	8.59

Table 7: DPPH radical scavenging activity of *Alchemillia vulgaris* ethyl acetate and methanolic extracts

% Inhibition by *Alchemilia vulgaris* extract			
S. No.	**Concentration**	**Ethyl acetate**	**Methanolic**
1.	20 µg/ml	39.47368	48.135
2.	40 µg/ml	40.02193	57.565
3.	60µg/ml	47.25877	60.416
4.	80µg/ml	51.20614	68.750
5.	100µg/ml	62.5	75.657
	IC_{50}	66.71	23.47

The phenolic content with respect to gallic acid was found to be 547.333 and 386.86 (µg gallic acid equivalent/ml of extract) for ethyl acetate and methanol extract. The flavonoid content was found to be as: 275.00 and 153.33 (µg rutin equivalents/ml of extract) in ethyl acetate and methanol extract. Free radicals are concerned in many disorders like neurodegenerative disease; cancer and AIDS. Antioxidants during their scavenging power are helpful for the management of those diseases. DPPH stable freeradical method is an easy, rapid and receptive way to survey the antioxidant activity of a precise compound or plant extracts [24-27]. IC_{50} of the standard compounds, ascorbic acid was 8.59 ml^{-1}. The uppermost radical scavenging activity was shown by ethyl acetate and methanolic extracts of *Alchemillia vulgaris* IC_{50}= 66.71 23.47 $mgml^{-1}$ respectively which is higher than that of ascorbic acid ($P<0.05$). The radical scavenging activity in the plant extracts decreased in the subsequent order ethyl acetate extract < methanolic extract of *Alchemillia vulgaris*. Most of the plants extracts at dissimilar concentrations exhibited more than 70 % scavenging activity (Table 7). Therefore, the antioxidant effect of ethyl acetate extracts *Alchemillia vulgaris* was 3 times greater than that of methanolic extract *Alchemillia vulgaris*.

4. CONCLUSION

The result of the current study showed that the extract of *Alchemilla vulgaris*, which hold highest amount of flavonoid and phenolic compounds, exhibited the maximum antioxidant activity in ethyl acetate extract. The high scavenging property of *Alchemilla vulgaris* may be due to hydroxyl groups accessible in the phenolic compounds chemical structure that can offer the necessary component as a radical scavenger. Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented. A potent scavenger of free radicals may serve as a probable anticipatory intervention for the diseases [28]. Ethyl acetate extract of *Alchemilla vulgaris* showed a higher potency than ascorbic acid in scavenging of DPPH free radical. This may be related to the high amount of flavonoid and phenolic compounds in this plant extract.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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