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Research Article

# Immunopharmacological activity of flavonoids from *Lemna minor* (Duckweed) and determined its immunological activity

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## ABSTRACT

The main objective of our study is to conduct its immunopharmacological activity using flavonoids extracted from whole plant of *Lemna minor* (duckweed), medicinal plant in virally infected human whole blood against ovalbumin (OVA), specific protein antigen. For these studies, isolated secondary metabolite i.e. flavonoid from whole plant of duckweed and evaluated its immuno-pharmacological activity of flavonoid using variable concentration (i.e. 1-30 mg/ml; 50 µl) on infected human whole blood samples and determine its proliferation assay containing OVA and estimation of free haemoglobin content in blood plasma. In addition, antibody production was also estimated against OVA using Elisa method. The results of these studies showed that these flavonoids at higher doses showed immunosuppressive effect because of decline in proliferation, free haemoglobin content in the blood plasma and antibody production. Overall, this study claimed that these flavonoids from duckweed showed immunosuppressive activity against OVA.

**Keywords:** *Lemna minor*; Ovalbumin; Infected; Proliferation; Haemoglobin.

## 1. INTRODUCTION

Medicinal plants produce a variety of biologically active compounds that can be subdivided into two categories i.e. primary metabolites and secondary metabolites [1]. These metabolites especially primary that are essential for the survival of the medicinal plant which includes sugars, proteins and amino acids. On the other hand, secondary metabolites showed many uses or medicinal properties relevant to animal and human health, some of them are beneficial and few of them are toxic [1, 2]. The major difference between these two metabolites i.e. primary that directly involved in the growth and development of medicinal plant whereas secondary metabolites are present in the form of compounds that are produced in other metabolic pathways but these are not essential to the functioning of the medicinal plant. In other words, these secondary metabolites from medicinal plant products are also used in signaling and regulation of primary metabolic pathways. In general, these secondary metabolites are present in the form of flavonoids, terpenoids, alkaloids, saponin, glycosides etc. [3, 4].

One of the major components of secondary metabolites i.e. flavonoids and terpenoids that are present almost in every medicinal plant. Most familiar example of terpenoid i.e. artemisinin (malaria) and taxol (cancer) are widely used as medicine and manufactured by various pharmaceutical companies for these diseases [5-7]. All organisms naturally produce some terpenoids as a part of primary metabolism but many produce terpenoids via secondary metabolism [5-7]. Similarly, flavonoids (e.g. quercetin, kaempferol, catechins, and anthocyanidins) is well known for its antioxidant and anti-inflammatory properties [8-10]. In view of these secondary metabolites especially flavonoids that are present abundantly in most of these medicinal plant products and researchers start focusing on these immunopharmacological activities.

One of the aquatic plants i.e. duckweed that freely float on the surface of water. This plant is especially seen in ponds, waste water etc. but is present in the form of dense colonies. These colonies eliminated submerged plants and this could be due to the blockage of light penetration [11, 12]. Most of aquatic birds, snails and fishes consumed duckweed and it will transport to other bodies of water. In general, most of duckweed colonies that are present abundantly on the surface of water which is totally covered and eliminated oxygen level in the water and ultimately it leads to show some harmful or adverse effects related to aquatic animals. Duckweeds (monocotyledonous aquatic plants; family *Araceae*) are one of the world's smallest, fastest ever growing plant and can multiply in a very short period of time [13-15]. In this study, we discussed about its immunopharmacological activity of secondary metabolites especially flavonoids extracted from whole part of duckweed against OVA, specific protein antigen.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

Duckweed, whole plant material was collected from VSBT pond, Baramati, Maharashtra. First of all, whole part was washed thoroughly under tap water and then with distilled water. Thereafter, duckweeds were dried in a shady area and grounded into uniform powder using mortar and pestle. The powder was used for extracting secondary metabolites i.e. flavonoids and determined its immunopharmacological activity.

### 2.2. Extraction of flavonoids

Similarly, qualitative based studies of duckweed powder were done in order to estimate its flavonoid content. For confirmation of flavonoid using lead acetate test, take small amount of lead acetate solution is added into the duckweed powder and yellow colour precipitation will appear, it indicates the presence of flavonoid content.

In quantitative based studies, duckweed powder (1 g) was dissolved in methanol (80%, 10 ml) and then warm for 2 h at 100ºC. Afterwards, cool down the solution and then collect the filtrate using Whatman filter paper and add ethyl acetate (10 ml) along with distilled water (20 ml), shaking regularly for at least 5 minutes and then incubate the solution overnight at room temperature. After incubation, two different layers were observed i.e. upper layer (i.e. ethyl acetate) and lower layer (flavonoids). Finally, evaporate the upper phase i.e. ethyl acetate solution and then dried the plant extracts (flavonoids) settled at the bottom [16].

### 2.3. Lymphocyte proliferation assay

Anti-coagulant, EDTA human blood samples (virally infected) were collected from Mangal Pathology Laboratory, Baramati, Maharashtra, India. In this study, lysed human whole blood (100 µl) were cultured with variable doses of flavonoids (1-30 mg/ml, 50 µl) extracted from duckweed powder along with or without ovalbumin (1 mg/ml, 50 µl). Incubate 96-well plates for 48 h at 37°C. OVA used as standard for these immunological studies. Centrifuging (2500 rpm for 10 minutes at 4°C) the plates and then add fresh complete medium was added into the 96-well plates. Again, incubating the plates for another 4 h along with MTT (5 mg/ml, 10 µl) continued. After incubation, the plates were suddenly centrifuged with discarding the supernatant, collecting the pellet and finally dispersing in dimethyl sulphoxide (DMSO) solution. The optical density was measured at 570 nm [17, 18].

### 2.4. Estimation of free haemoglobin

Lysed virally infected human whole blood (n = 6; $10^5$ cells/well; 100 µl) were collected and cultured in 96 well flat bottom tissue culture plate for 48 h incubation along with variable doses of flavonoids (1-30 mg/ml; 50 µl). Collect and transfer the samples from culture plate into 3 ml falcon tube. Centrifuge the samples at 6000 rpm at 4ºC and then washed with PBS pertaining to observe the free haemoglobin in the supernatant. Finally samples were analysed through UV visible spectrophotometer at 570 nm [19].

### 2.5. ELISA

Indirect Elisa was performed for estimating antibody production against ovalbumin (OVA, 100 µg/well) using variable doses of flavonoids and terpenoids (1-30 mg/ml; 50 µl). OVA used as coating antigen and incubate the plate for overnight at 4ºC. After incubation, first of all block this plate with 1% bovine serum albumin (BSA). Incubate the plate for one hour at room temperature and then wash the plate with PBS (2-3 times). Thereafter, add variable concentration of flavonoids and terpenoids (1-30 mg/ml; in 96 well plate. Incubate the plate for another 4 h incubation at carbon dioxide incubator. Afterwards, again wash the plate with PBS (2-3 times) and then add secondary antibody (horse antiserum; 1:10000 dilution). Incubate the plate for another 1h at carbon dioxide incubator. After incubation, wash the plate with OBS and then add substrate, TMB. Incubate the plate for another 10-15 minutes in dark at room temperature. Afterwards, stop solution was added and optical density was measured at 450 nm [20].

### 2.6. Statistical analysis

The difference between control and treated group of flavonoids extracted from duckweed is determined by one way ANOVA test (Bonferroni multiple comparison test). *P <0.05; *P<0.01; ***P<0.001.

## 3. RESULTS

### 3.1. Lymphocyte proliferation assay

The effect of variable doses of flavonoids from duckweed on antigen (OVA) specific immune response in virally infected lysed human whole blood as shown in Fig. 1. The results showed its decline in proliferation rate at higher doses of flavonoids and terpenoids. Overall, this study showed its immunosuppressive effect.

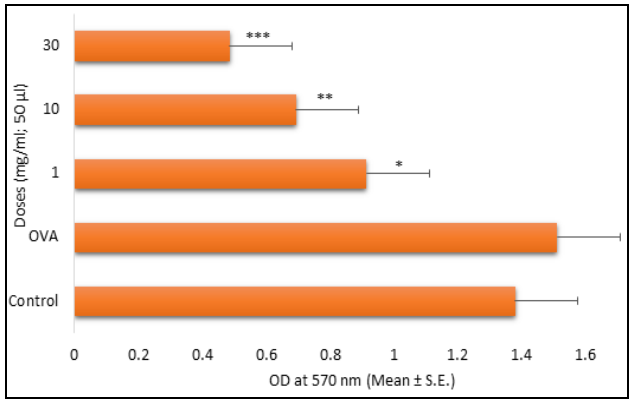


**Figure 1.** Proliferation assay. To determine the effect of variable doses of flavonoids (1-30 mg/ml, 50 µl) extracted from whole plant of duckweed on virally infected lysed human whole blood. Values are expressed as Mean ± S.E. The difference between the controls versus variable doses of flavonoid is determined by one way ANOVA test (Bonferroni multiple comparison test).).*P<0.05; **P<0.01, ***P<0.001.

### 3.2. Estimation of free haemoglobin

At higher doses of flavonoids showed decline in free haemoglobin content as shown in Fig. 2 in virally infected lysed human whole blood as compared to control.

### 3.3. ELISA

The results of these studies related to flavonoids on antibody production against OVA as shown in Fig. 3. At lower doses, there is slightly enhancement in antibody production but at higher doses, there is decline in antibody production as compared to control.

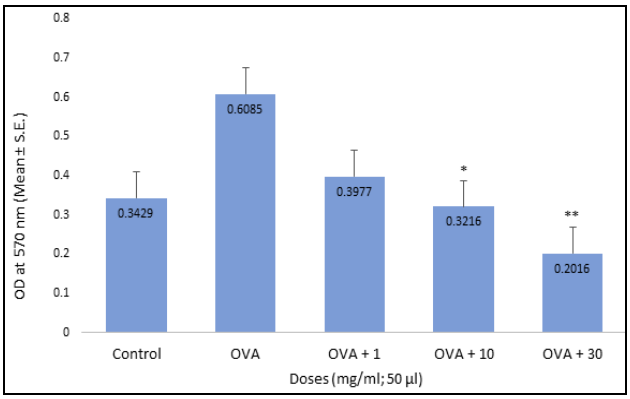


**Figure 2.** Estimation of free haemoglobin content. To determine the effect of variable doses of flavonoids (1-30 mg/ml, 50 µl) extracted from whole plant of duckweed on total haemoglobin content in virally infected lysed human whole blood. Values are expressed as Mean ± S.E. The difference between the controls versus variable doses of flavonoid is determined by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01, ***P<0.001.

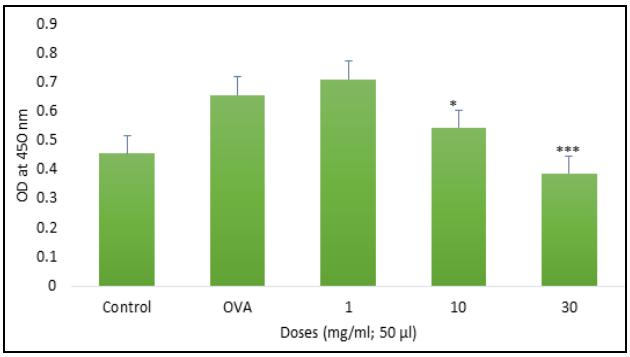


**Figure 3.** ELISA. Indirect ELISA was assayed using OVA as coating antigen using variable doses of flavonoid from whole plant of duckweed for determining antibody titre. Horse anti-serum used as secondary antibody. The difference between control and variable doses of flavonoid is determined through one way ANOVA test (Bonferroni multiple comparison test). *P < 0.05; **P < 0.01 and ***P < 0.001.

## 4. DISCUSSION

The use of various medicinal plant products is believed to be an age-old tradition. Numerous studies were conducted by various researchers and proved that these natural plant based products are useful for diverse ailments and diseases. Most of the health care professionals including researchers all over the world have shown some interests in the medicinal uses of these plant products but lot of confusion related to its identification, effectiveness, efficacy, dosage, toxicity, standardization, and regulation. As per WHO, traditional medicine is more popular in all regions of the world and its use is rapidly expanding even in developed countries as well. In view of this, variety of medicinal plant products that are reported in Baramati region, Maharashtra, India and its knowledge about its medicinal properties has been accumulated regarding various diseases e.g. rheumatoid arthritis, cancer, autoimmune diseases etc. Recently, more than two thousand medicinal plants are mentioned in Ayurvedic systems of medicine [1]. Out of these, number of medicinal plants that are reported and claimed its immunosuppressive properties. In view of this, we worked on various medicinal plants especially *Lemna minor* (duckweed) [11, 12] and extracted secondary metabolites i.e. flavonoids and determined its immunological activity against specific protein antigen. In this study, our results showed that these flavonoids showed immuno-suppressive effect in case of virally infected human whole blood samples at higher doses. This activity of this fraction especially flavonoids and terpenoids may be attributed due to the presence of active molecules in the extract [9, 10]. The capacity of these active molecules may have some useful applications in various disease disorders e.g. autoimmune disease, organ transplant rejection etc.

In this study, exposure of variable doses of flavonoids isolated from duckweed, medicinal plant products caused a reduction in free haemoglobin content at higher doses in case of lysed virally infected human whole blood samples. Due to sudden decline in free haemoglobin content in virally infected blood clearly showed its immunosuppressive effect. Further immunological studies are needed in order to confirm its immunosuppressive activity of this medicinal plant and evaluate its activity or potential in the treatment of various disorders. According to the literature, major factors that are associated with virally infected blood profile i.e. high haemoglobin concentration in blood plasma. Regulation of these components using flavonoids, secondary metabolite from duckweed, medicinal plant is the major goal of this study.

Immunological validation of these flavonoids isolated from duckweed and proved its efficacy in

order to reducing the free haemoglobin content in virally infected blood and also reduction in antibody production against specific protein antigen. From these results related to its potential effectiveness against virally infected blood samples, it is assumed that these flavonoids isolated from medicinal plant products that played in the management of infectious diseases, which needs further exploration for necessary development of drugs and nutra-ceuticals from natural resource.

## 5. CONCLUSION

This study showed its immunosuppressive effect of flavonoid from duckweed against specific protein antigen. Further immunological investi-gation is also required to extract the active compound which can be observed as a potent immunosuppressive drug.

## AUTHOR'S CONTRIBUTION

This work was carried out in collaboration between three authors. SS, AG and VM designed the study, wrote the protocol and interpreted the data where SS and AG anchored the field study, gathered the initial data related to his M.Sc. Microbiology dissertation work under AG guidance and performed preliminary data analysis. AG, SS, BS and VM managed the literature searches whereas AG and SS produced the initial draft. The final manuscript has been read and approved by all authors.

## TRANSPARENCY DECLARATION

Authors have declared that no conflict of interests exists.

## ETHICAL APPROVAL

These studies were conducted under IBSC guidelines and approved by Savitribai Phule Pune University.

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