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Research Article

EVALUATION OF HERBAL EXTRACT *EVOLVULUS ALSINOIDS ON* LYMPHOCYTE PROLIFERATION ASSAY, *IN VITRO*

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A B S T R A C T

Medicinal plants have been used for centuries and have become beneficial therapeutic medicines worldwide because of their potential health benefits. Some of their metabolites have been successfully used directly in the treatment and prevention of infectious diseases and cancer, or indirectly by stimulating the immune system. Most immunological studies necessitate evaluation of both humoral and CMI responses. Lymphocyte proliferation assays are widely used to assess the cell-mediated immunity.

Lymphocyte proliferation assay were carried out using the peripheral blood mononuclear cells (PBMC) with mitogen induction by phytohemagglutinin-L (PHA-L) and proliferation in cell cultures and whole blood were monitored using colorimetric (MTT) assay. Free radical scavenging capacity as a measure of antioxidant capacity was determined by DPPH inhibition method.

In vitro human peripheral blood mononuclear cells (PBMC) treated with methanol extract of *Evolvulus alsinoids* (62.5 to 500 µg/mL) showed lymphoproliferation at the concentration of 250 µg/ml, which were significantly higher proliferation when compared with media control ($p < 0.05$). The extract exhibited greater than 75% scavenging activity at 1000 µg/mL compared with the ethanol vehicle control.

The whole plant *Evolvulus alsinoids* extracts showed strongest effects on antioxidant activity compared to control.

INTRODUCTION

Innate and adoptive immune responses were the two major components of humoral and cell-mediated immunity (CMI). CMI is mediated by activated or sensitized reticuloendothelial (Phagocytic) and lymphoid (Lymphocytes) cells (Abbas, A. K. *et al* 2017).

Many herbal extracts established their anti-infective properties not just by affecting the pathogen directly, alternately, at least by stimulating innate and adaptive defensive mechanisms of the body (Shukla S *et al*. 2014). Humoral components can be easily monitored by use of precipitation, agglutination, neutralization and enzyme-linked immunosorbent assay (ELISA) methods

Efficacy of medicinal plants was most beneficial on improving innate immunity, enhancing antioxidant capacity, and inhibiting tumor cell growth (Lee SH, *et al* 2007, 2008 and Kim DK, *et al* 2013).

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The activation of the immune response in this model promotes cell proliferation with an increase in the number of cells present in the culture within a defined period (Rocha, K. C, *et al* 2007) and can be identified through MTT reduction by mitochondrial dehydrogenase of living cells (Mosmann T. 1983). Immunopotency determination of the proliferation and cytotoxic assays were widely used in drug discoveries, vaccines, diagnosis of infectious and immune deficiencies (Sitz, K. V. & Birx, D. L. 1999, Spohr, C. *et al*. 2015, Greene, N. *et al* 2010, El-Said, W. A., *et al* 2018) ,

Active compounds of *A. asphodeloides* as, timosaponin B and timosaponin B-II played an important role in the immune response by increasing proliferation through the extension of the S-phase of the cell cycle and that it has anti-inflammatory effects (Kon-Young Ji, *et al* 2019).

In practical terms, cells are stimulating by mitogen, antigen, toxin or drug, which may cause the cell activation or death. Then measurement of proliferating and/or surviving cells is conventionally determined by radiometric or colorimetric (Dye based) assays (Zhi-Jun, Y *et al* 1997).

In a living cell, to maintain energy and major natural mechanisms the redox reactions catalyzed by proteins or enzymes, during the activation and replication or apoptosis (cell death) the living cell will undergo alterations of metabolisms and membrane interactions with redox and ROS products

One of the studies indicated that *Evolvulus alsinoides [EA]* administration causes myocardial adaptation by augmenting endogenous antioxidants and protects rat hearts from oxidative stress associated with ISP induced myocardial injury, and justify its potential therapeutic value in the treatment of ischemic heart diseases in albino rats (Kumari. S, *et al* 2012).

In the present study, Evaluation of herbal extract, *Evolvulus alsinoids on* human lymphocyte proliferation, as to establish therapeutic potential dose which, could to an ideal candidate to treat inflammation and infectious diseases

METHODOLOGY

Plant extract preparation

Fresh mature whole plant *Evolvulus alsinoids* was collected from natural source in rural parts of South Karnataka regions of India. Routine pharmacognostic investigations were carried out to confirm authenticity of this material. The identification was carried out in sun biological lab.

The whole plant was dried and cut into small pieces and crushed to a coarse powder using blender. Coarse powder was subjected to extraction in Sox let apparatus (in ratio of 1:5 to the quantity of raw material) for 6 h under reflux condenser at 45^{o} C using thermostat.

This extract was cooled to room temperature and evaporated using condenser then filtered through filter cloth (#100 mesh size), to get methanolic extract of *Evolvulus alsinoids* plant. The resulting extracts were stored in well-packed container at room temperature for future use. The yield of dried extract was approximately 40% and a homogenate was made in 50% ethanol. Three different concentrations of the extract were prepared, 100, 250 and 500 µg/ml

Estimation of Quercetine by Rp-HPLC

Mobile phase preparation

Mobile phase was prepared by mixing Methanol: 0.005mM phosphate buffer (70:30). pH of the solution was adjusted to 3 with dilute phosphoric acid. This solution was filtered using a 0.45-micron Millipore filter paper and was sonicated for 10mins. The total volume of the mobile phase prepared was 100ml.

Standard Preparation

10mg of Quercetine was taken in 10ml volumetric flask and make up the volume to 10 ml with methanol (the concentration of this solution is **1000** µg/ml).

From this above solution working solution 1ml was pipetted into 10ml volumetric flask and volume was made up to the mark with Methanol, this is a working solution (100 µg/ml). This was sonicated for 8 minutes then the solution was filtered using 0.45-micron Millipore filters.

Sample preparation

To 1mg of the given sample was taken in 1ml of respective solvents. The solution was vortex for 5mins. The sample was filtered using 0.45-micron Millipore filters. 20µl of this sample was injected in the HPLC system.

CALCULATION

The concentration of unknown sample was calculated as follows

= Sample area/ STD area x STD wt. / Sample wt. X Sample dilution /STD dilution X purity /100 x 100

Isolation of cells

The peripheral blood mononuclear cells (PBMC) from human blood was collected into 10 ml EDTA- through Ficoll- density gradient centrifugation method, within 8 h of collection of whole blood by centrifugation at 600 × g for 15 min. The white layer of cells at the plasma-Ficoll interface was harvested and washed three times with RPMI-1640 medium (Himedia, Mumbai) without fetal bovine serum (FBS), using trypan blue exclusion method Cell viability were determined with a hemocytometer.

Selection criteria for viable cells less than 95% and red blood cell and contamination more than 2% were excluded as per the ethical guidelines for research of the Indian Council of Medical Research (ICMR 2006)

Lymphocyte proliferation studies

Proliferation assay carried out by colorimetric MTT (tetrazolium) as 100µl of diluted cell suspension (2×10^6 cells/mL) in complete RPMI (RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin) then dispersed with incubation in 96-well micro titer plate.

Cells were seeded in 10 wells per sample in each test mitogen added (phytohemagglutinin-L, PHA-L; Sigma) and control negative experiment. The mitogen at the final working concentrations of 5, 10 and 20 µg/mL was added to test wells. In addition, cell-free media containing complete RPMI were maintained in parallel to test and control groups. The inoculated plates were incubated at 37 °C in a 5% CO2 incubator and checked for growth after 4, 24, 48 and 72 h using colorimetric assays.

Each assay is further evaluated for sensitivity to change in cell number and different concentrations of mitogen. Six different concentrations of cells in complete RPMI media ranging from 3×10^6 to 1.2×10^4 cells/mL were tested colorimetric MTT assays. All the experiments reported here were repeated four times.

Colorimetric MTT assay: Colorimetric lymphocyte proliferation was evaluated using MTT (3-(4, 5-dimethyl thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) method. After cell culture incubation (4, 24, 48 and 72 h), 10µl MTT (dissolved in RPMI-1640, 5 mg/mL) was added to well, and plates were further incubated at 37 °C for 4 h. Finally, the purple formazan crystals were dissolved by adding 100µl acid-isopropanol (0.04 N HCI in isopropanol) into each well. Absorbance was then measured at 550 nm against reference wavelength of 630 nm and stimulation index (SI) was determined. The SI is expressed with average OD value in the test group divided by average OD value in negative controls. control, the same serially diluted blood in RPMI media without PHA were run in parallel to test group.

DPPH radical scavenging assay

Antioxidant activity of plant extract was analyzed using DPPH free radical scavenging assay (Brand-Williamis W, *et al* 1995), 167 µL of 4mM ethanol solution of DPPH was mixed with 33 µL analyzed samples in different concentrations (125 µg/ml to 1000 µg/ml), the absorbance was measured at λ = 516 nm in every 5min for 30 min using UV-Vis spectrophotometer Filter Max 5 (Thermo Scientific). DPPH solution mixed with equal volume of distilled water was served as a control (Zofia Nizioł-Łukaszewska, *et al* 2018). The percentages of the DPPH radical scavenging were calculated using the equation:

%DPPH scavenging = [Abs control - Abs sample] / Abs control X 100%

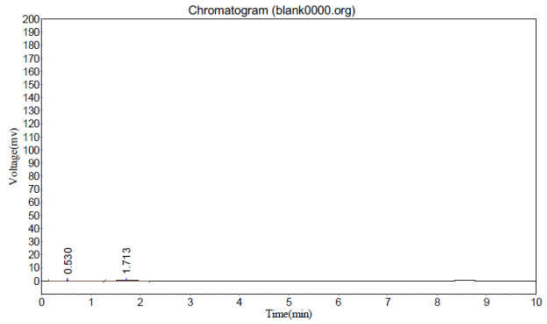
Statistical Analysis

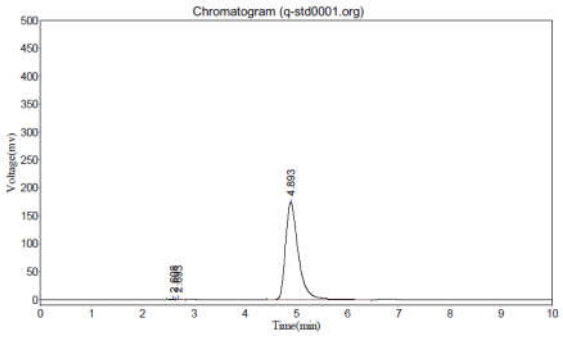
All experiments were carried out in triplicate and repeated three times. Statistical analysis was performed using SPSS software (SPSS 22.0 for Windows, Chicago, IL, USA), and all data were expressed as the mean ± SD of triplicate cultures.

Analysis of variance (ANOVA) and t-tests were used to evaluate differences between the mean value of negative control-treated and extracted samples. Differences with p values of less than 0.05 were considered statistically significant.

RESULTS

Estimation of Quercetine by RPHPLC





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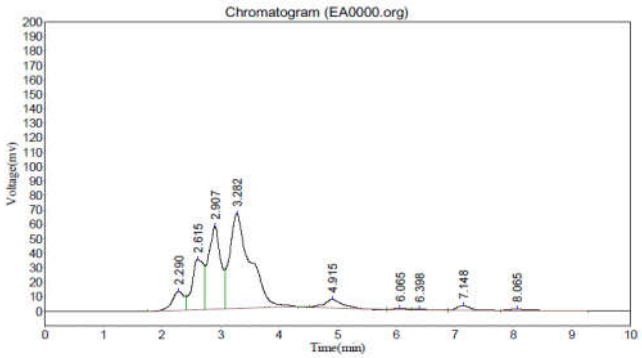

Test – Run *Evolvulus alsinoids*

Table 1 Content of Quercetine

Assessment	*Evolvulus alsinoids*
purity	98
sample area	122133.102
standard area	2988727.5
sample weight in mcg	1000
standard weight in mcg	100
sample dilution (ml)	1
standard dilution ml	1
Content of Quercetine µg/ml	**0.400**

Table 2 Lymphocyte proliferation studies

Sl. No	Groups	MTT assay
1	Control	0.71 ± 0.08
2	Mitogen induced Treated	0.86 ± 0.07
3	Evolvulus *alsinoids* (250 µg/ml) + Mitogen induced	0.74 ± 0.05

Table 3 DPPH radical scavenging assay

Concentrations (µg/ml)	% DPPH Inhibition
125	16
500	35
750	55
1000	75

DPPH radical scavenging assay

The free radical scavenging capacity of *Evolvulus alsinoids* were determined by DPPH assay is shown in table.3. The extract exhibited greater than 75% scavenging activity at 1000 µg/mL compared with the ethanol vehicle control.

DISCUSSION

Restoration of redox balance is critical for removing cellular oxidants, Reactive oxygen species (ROS) are to be generated by exogenous stimuli such as drugs, pollutants, UV radiation and smoke, endogenously produced by intracellular metabolic processes, which triggers the cell defense systems when the intracellular oxidative status increases, and undergoes apoptosis (R. K. Gupta, *et al* 2014, T. Finkel and N. J. Holbrook, 2000).

Numerous cellular processes including core signaling pathways were influenced by oxidative stress responses which are leading to a systematic or chronic disorder i.e., aging and cancer (T. Finkel and N. J. Holbrook, 2000, B. Halliwell, *et al* 1995).

Metabolism of the cells can be affected by culture conditions such as longer incubation time can increase sensitivity, variation in pH, deprivation of essential nutrients which may lead to the accumulation of toxic by-products (Wright, M. M., *et al* 2018).

Anemarrhena asphodeloides extract exhibited anti-inflammatory effects, including the inhibition of the production of NO, ROS, and pro-inflammatory cytokines through the suppression of mitogen-activated protein kinase and nuclear factor kappa B phosphorylation downstream of the toll-like receptor 4 signaling pathway (Kon-Young Ji, *et al* 2019).

An association between green tea (*C. sinensis*) consumption showed on several epidemiological studies which reduce the risk of different kinds of human cancer (Katiyar SK, *et al* 1996, Yang CS, *et al* 1993, Chan H, *et al* 2003).

Human epidermal keratinocytes tested exponentially growing and aged primary in response to EGCG or a mixture of the four major green tea-polyphenols (Hsu S, *et al* 2003).

The highest lymph proliferative response exhibited in *O. basilicum* extracts up to 80 to 83% for the methanol and aqueous extracts, respectively (R. Gomez-Flores, *et al* 2008). Moreover *O. basilicum´*s main reported activity is anti-inflammatory (Singh S. 1999).

The methanol extract of *C. brasiliense* induced an increase in cell proliferation in a dose-dependent response. The average percentage of cell growth ranged from 5.8 to 88.2% and from 15.3 to 96.3%, for the extract alone and extract together with PHA in the cell culture, respectively (R. H. Zandonai, *et al* 2010).

In the present study in vitro human peripheral blood mononuclear cells (PBMC) treated with methanol extract of *Evolvulus alsinoids* (62.5 to 500 µg/mL) showed lymph proliferation at the concentration of 250 µg/ml, which were significantly higher proliferation when compared with media control ($p < 0.05$) (Table: 2).

Proliferation of lymphocytes were promoted by 5 herbal extracts tested on normal healthy lymphocytes, the plant (green tea) is the only one which was not cytotoxic to lymphocytes (KN Varalakshmi, *et al* 2011).

The preliminary phytochemical screening of *E.alsinoides* contains some secondary metabolites such as glycosides, alkaloids, poly phenols, carbohydrates, amino acids and proteins, saponins, volatile oil, flavonoids and tannins (Omogbai B. A. and Ze. F. A. 2011).

These plant metabolites have shown significant in vitro antioxidant activity. Therefore, *Evolvulus alsinoids* may hold great potential in preventing clinical deterioration in stress induced oxidative load and related disorders in our study since rich flavonoids component is Quercetine in plant extract showed 0.400 µg/ml (table-1)

Plant extracts is mainly associated with phenolic, flavonoids, isoflavonoids and anthocyanins contents possess strong antioxidant capacity. Many studies had indicated a correlation between the content of flavonoids and phenolic compounds of plants were proven antioxidant activity (Orzechowski A, *et al* 2002, Zhao Y, *et al* 2015), in the present study plant extract Evolvulus Alsinoides exhibited rich Anti -oxidant potential in DPDH inhibition 1000µg/ml with 75% (Table:3).

CONCLUSION

The high antioxidant capacity of *Evolvulus Alsinoides* extracts may indicate its potential use in the treatment of diseases, which were reducing the excessive production of reactive oxygen species (ROS). The most promising activities observed on the proliferation of human peripheral blood mononuclear cells (PBMC) by methanol extracts of *Evolvulus Alsinoides*.

The regulation of immune parameters induced by plant extracts may be clinically relevant in numerous disease processes including chronic viral infections, tuberculosis, AIDS, and cancer.

Conflict of interest

The authors declare that they have no conflict of interest related to the publication of the manuscript.

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