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Immunomodulatory Potential Of Isolated Compound Of *Vigna Unguiculata* Leaves

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Abstract

Aim: The main aim of the present study is to investigate the immunomodulatory activity of phytochemical compound isolated and purified from methanolic extract of *Vigna unguiculata* leaves by different animal models.

Methods: The isolated compound of *Vigna unguiculata* leaves extract was compromised to acute toxicity according to OECD guidelines. Healthy albino Wistar rats of either sex was used for this study. The effect of the component on DTH response using SRBC was investigated by the footpad thickness, humoral antibody response to SRBC, carbon clearance assay or phagocytosis and cyclophosphamide induced neutropenia was investigated.

Result: The isolated compound of *Vigna unguiculata* leaves demonstrated the increase in paw thickness compared to control and standard group. The compound caffeic acid was having stimulatory effect on humoral immunity; this judgement was on the basis of effect on increment in HAT value. The increase in phagocytic index reflects the enhancement of the phagocytic function. The compound showed increase in total leukocyte count and differential leukocyte count.

Conclusion: This study revealed that the isolated compound, caffeic acid possessed immunomodulatory effect by stimulating cellular immunity, humoral immunity, phagocytosis and neutropenia.

Keyword: Immunomodulatory activity, *Vigna unguiculata*, Humoral antibody titer, Delayed type hypersensitivity reaction.

INTRODUCTION

Herbal medicine ("herbalism") is the study and use of medicinal properties of plants. Sometimes the use of herbal medicine is extended to include bee and fungal products, as well as shells, minerals and certain animal parts. The use of plants as medicines predates written human history. The study of traditional human uses of plants known as Ethno botany is recognized as an effective way to discover future medicines. In 2001, 122 compounds were identified by the researchers and used in modern medicine which were derived from "ethno medical" plant sources; 80% of the medicinal plants have had an ethno medical use identical or related to the active elements of the plant which is currently in use. Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including aspirin, digitalis, quinine, and opium. Among non-industrialized societies, the use of herbs to treat disease is almost universal, and is often more affordable and convenient than purchasing costly modern pharmaceuticals. The studies have shown that the use of clinical settings is less common in the United States and Europe, but as scientific evidence in recent years it has become increasingly more about the effectiveness of herbal medicine has become more widely available.[1]

Immunity is the ability of an organism to resist a particular infection or toxin by the action of specific antibodies or sensitized white blood cells or it is protection or exemption from something, especially an obligation or penalty. In biology immunity is the balanced state of having adequate biological defenses to fight infection, disease, or other unwanted biological invasion while having adequate tolerance to avoid allergy, and autoimmune disease. Humans have three types of immunity:

Innate Immunity-A type of general protection or the defense system with which everyone is born. There are many germs that affect other species but don't affect human. For example, the viruses that cause leukemia in cats or distemper in dogs don't affect humans. Innate or natural immunity working in both ways like there are some viruses that affect humans and make them ill — such as the virus that causes HIV/AIDS — does not affect to cats or dogs.

Adaptive Immunity- Another type of immunity to protect our body is adaptive (or active) immunity, which develops throughout our lives. Involvement of lymphocytes is there in adaptive immunity and by administering through vaccination, immunization develops as people are exposed to diseases or immunized against diseases.

Passive Immunity- Passive immunity is a type of immunity that occurs when "borrowed" from another source and it remains for a short time. For example, when a baby receives antibodies from mother through mother's breast milk and placenta with temporary immunity to diseases the mother has been exposed to. This can help protect the baby against infection during the early years of childhood.[2]

Since Neolithic times, one of the most ancient food sources and has probably been used as a crop plant is Vigna unguiculata (Cowpea).[3] Like other legumes, the seeds of cowpea are the most economically valuable plant part and due to their ascribed nutritional and medicinal properties are well-known. Cowpea is also rich in important vitamins, minerals, and soluble and insoluble dietary fiber and also known to be an excellent source of protein. For the purpose of food or fodder all parts of cowpea plants are used. During the fruiting stage, the consumption of immature pods and seeds is there while as the tender shoot tips and leaves reach the seeding stage they are consumed. Cowpea cake has been made by slurry of grounded harvested dry seeds, or deep fried into bean balls, or the seeds could be boiled, mixed with sauce or stew and consumed directly. Plant residues are used as fodder for farm animals.[4] In the Indo-Pakistan sub-continent cowpea is used in culinary dishes. The cowpea seeds consumption is common among the rural people in Pakistan, after processing such as soaking, dry heating, followed by cooking along with cooked rice, is a common cuisine. Fresh young leaves, immature pods, and seeds are used as vegetables, while dry grain is used to prepare main meal dishes and snacks.[5] For common cold, the cooking liquor of the seeds with spices is considered to be a potential remedy. For later use, leaves are boiled, drained, sun-dried and then stored. Seed oil exhibit antidiabetic properties.[6] Seeds possess nematocidal and antifungal properties.[7] Sometimes green cowpea seeds are roasted like peanuts. In Sudan and Ethiopia, the roots are eaten. Occasionally as a coffee substitute Scorched seeds are used. The seed is diuretic and after eating after boiling is considered to destroy worms in the stomach.[8]

The objective of the present study is to investigate the immunomodulatory activity of *Vigna unguiculata* leaves extract and the possible underlying mechanisms using in vivo models.

MATERIAL AND METHODS

Fresh leaves of *Vigna unguiculata* were collected locally from near region Jaipur, Rajasthan, India. Plant was identified and authenticated by **Botanical Survey of India**, Jodhpur, India (12012/Tech./2024-25(Pl. Id.)/509).

Extraction and Isolation

The coarsely powdered leaves material was extracted with Methanol by cold maceration technique. Their phytochemical component was isolated by Thin Layer Chromatography and purified using UV-vis spectroscopy, FTIR, Mass and NMR.[9-12]

For pharmacological screening, the isolated compound was dissolved in methanol.

Experimental animals

Wistar albino rats weighing between 130-250 gm and mice weighing between 25-30 gm of either sex were used for immunomodulatory activity. They were obtained from **Central Animal House of KLR Pharmacy College, Paloncha, Bhadradri Kothagudam (Dt), Telangana**. The experimental animals were housed in standard polypropylene cages with sterile husk as bedding. Animals were housed in relative humidity of 30.7 % at 22 ± 2^0 C with 12 hrs light and 12 hrs dark cycle. All the animals were fed with standard rat feed (Golden feeds, New Delhi, India) and water was available *ad libitum*. The litter in the cages was renewed thrice a week to ensure hygenicity and maximum comfort for animals. Ethical clearance for handling the animals was obtained from Institutional Animals Ethics Committee prior to the beginning of the project work bearing, studies were performed in according to guidance of CPCSEA registration number is **CPCSEA/IAEC/KLRCP/07**. Protocol approval reference number is **KLRCP/IAEC/006/2022-2023**.

Acute oral toxicity

Acute oral toxicity was performed as per OECD 423 guidelines. For acute oral toxicity study, guidelines are regulated by Organization for Economic Co-operation and Development (OECD). With the aim of reducing both the number of animals and the level of pain associated with acute toxicity testing, this international organization works. In an acute toxicity study of isolated compound caffeic acid, animals were given single doses of drug. The albino Wistar rats were divided into groups. All animals fed with standard rat pelleted diet had free access to tap water *ad libitum*. The doses selected for the study were 2000 mg/Kg, 300 mg/Kg, 50 mg/Kg, 5 mg/Kg. animals were observed for mortality in next 72 hours after sample administration.[13]

Immunomodulatory activity

The animals were divided into four groups consisting of six animals each. A group of six untreated rats were taken as control (Group I). A group of six rats treated with standard drug levamisole at a dose of 2.5 mg/kg b.w. were considered as standard (Group II). The isolated compound was fed orally for six days at a dose of 50 mg/kg (Group III), 100 mg/kg (Group IV) for the assessment of immunomodulatory activity.

Delayed type hypersensitivity reaction (Effect on immunity)

Procedure: Animals will be sensitized with 0.1 ml of 10% SRBC (1×10^8 cells) at day 0 by i.p. route. Test sample will be administered -4 days to + 4 days of SRBC immunization. On day 9, animals will be challenged with 1×10^8 SRBC cells, intradermally into the left footpad of each animal, while PBS (pH 7.4) will be injected into right hind paw. DTH response was measured by the increase in footpad thickness (FPT) 24 h after SRBC challenge by digital vernier caliper.[14]

The degree of DTH reaction will expressed as the percentage increase in FPT over the control values.[15]

Hemagglutination Antibody Titer (Effect on Immunity)

Procedure: Animals of group II, III & IV were pretreated with test sample for six days and each animal was immunized with 0.2 ml of 5×10^9 SRBC by i.p. route, including control rats. Day 0 was considered as the day of immunization. The animals were treated with test sample for six more days. Control group received equal volume of vehicle. On day 7, from retro-orbital plexus blood samples were collected. Titrating serum dilutions with SRBC and the titre was determined. To two-fold dilutions of serum samples made in 25 µl volumes of normal saline containing 0.1% BSA (BSA saline) in V bottom hemagglutination plates were added 25 µl of 0.1% suspension of SRBC in BSA saline. Mix it well and after thorough mixing, at room temperature SRBC were allowed to settle for 90 min until small button of cells (negative pattern) were showed in control wells.[14]

The values of the highest serum dilution, causing visible hemagglutination was considered as the antibody titer.[15]

Carbon clearance assay

Preparation of carbon colloidal suspension

Carbon suspension was made by suspending 1.6 ml of Indian ink in 8.4 ml of 1% gelatin (gelatin was dissolved in 0.9% pyrogen-free NaCl solution).[16]

Procedure

Test samples will be administered for five days by IP route. On day six, through the tail vein 0.1 ml of carbon ink suspension were given to all the groups. At 0 and 15 min immediately after the injection of carbon suspension, from individual animal, blood was collected from the retro-orbital plexuses. Blood (25 μl) was lysed with 2 ml of 0.1% sodium carbonate and for determination of optical densities the absorbance was measured spectrophotometrically at 675 nm.[17]

Calculated the rate of carbon clearance known as phagocytic index (K) by using following equation.

$$K = (\text{In OD1} - \text{In OD2}) / t2 - t1$$

Where OD_1 Optical density after blood collection from mice tail vein at a time (t_1) 0 min
(Optical density is the absorbance of an optical element for a given wavelength).
OD_2 Optical density after blood collection from mice tail vein at a time (t_2) 15 min

Cyclophosphamide induced neutropenia
Cyclophosphamide was dissolved in normal saline (0.9% NaCl) and administered at the dose of 30 mg/kg body weight.

Procedure
Test samples will be administered orally for four days. On day five, all the groups were given cyclophosphamide drug solution according to their body weight through the IP route. After 1hr from individual animal, blood was collected from the retro-orbital plexuses. Immediately after the cyclophosphamide injection. Blood (25 μl) was lysed with 100 mcl. of 1% EDTA and the hematology was measured by autoanalyser. After 72 hrs. of blood collection the procedure was repeated same.[18]
Difference in the count of lymphocyte and the WBC (white blood cells) between 1 hr. and 72 hrs. Observation was recorded.[15]

Statistical Analysis
The data were expressed as mean ± S.E.M. The differences were compared using one-way ANOVA followed by Bonferroni test using Sigma Stat software. The results were considered significant when $P < 0.05$.

RESULT AND DISCUSSION

Acute toxicity
Maximum tolerated dose of 2000 mg/Kg b.w. were observed by acute toxicity studies in animals with no noticeable lethality changes in all groups. Therefore, 1/40th and 1/20th of the maximum tolerated dose 50 mg/Kg b.w. and 100 mg/Kg b.w. respectively were chosen for further studies.

Immunomodulatory effect
Basic aim of present study was to evaluate immunomodulatory effect of caffeic acid, isolated from methanolic extract of *V. unguiculata* leaves. This effect was ascertain on the basis of effect on cellular immunity, humoral immunity, neutropenia and phagocytosis.

Delayed type hypersensitivity

Table 1: DTH readings of all four groups

S. No.	Group	Paw thickness (24 hrs.)	Paw thickness (48 hrs.)
1.	Control	0.52±0.172	0.43±0.156
2.	Levamisole (2.5 mg/Kg)	0.99±0.146*	0.79±0.152*
3.	Compound (50mg/Kg)	0.80±0.136*	0.68±0.080*
4.	Compound (100mg/Kg)	0.94±0.096*	0.71±0.057*

Value expressed as mean±standard deviation (SD) at no=6, one way ANNOVA, followed by Bonferroni test * $P< 0.05$ significant compared to the control group.

In isolated compound (Caffeic acid) treated animals paw thickness was also found to be more ($P<0.05$) as compared to vehicle treated animals and even in standard drug treated animals paw thickness was significantly more ($P<0.05$). This confirmed that the extract was modulating cellular immunity and this effect was definitely stimulating. The DTH that was measured in this experiment has only some major components- sensitization, release of cytokines and inflammation. DTH reaction is characterized by hefty invasion of non-specific inflammatory cells, in which the macrophage is a major participant. It is a type 4 hypersensitivity reaction that develops when antigen make active sensitized T- cells. Activation of t cells by antigen results in the discharge of a variety of cytokines including interlukin-2, interferon-α, macrophage migration inhibition factor and tumor necrosis factor-ß. The taken as whole effect of these cytokines are to recruits macrophages into the vicinity and turn on them, encouraging increased phagocytic activity and increased concentration of lytic enzymes for more effective killing. Several lines of evidence suggest that in host defense against bacteria and parasites that can live and proliferate intracellularly, DTH reaction is important.

Hemagglutination antibody titer assay

Table 2: HAT readings of all four groups

S. No.	Group	Dilution no.
1.	Control	2.67±0.816
2.	Levamisole (2.5 mg/Kg)	6.83±1.941*
3.	Compound (50mg/Kg)	5.83±0.753*
4.	Compound (100mg/Kg)	6.17±1.169*

Value expressed as mean±standard deviation (SD) at no=6, one way ANNOVA, followed by Bonferroni test * P< 0.05 significant compared to the control group.

Effect of humoral immunity was confirmed on the basis of effect of hemagglutination antibody titer. In extract treated and levamisole treated animals. Dilution factor increased significantly (P<0.05). This showed that extract was having stimulatory effect on humoral immunity; this judgement was on the basis of effect on increment in HAT value. According to principle of precipitation reactions agglutination reactions are similar; with the exception that; precipitation reactions involve soluble antigens, while agglutination involves particulate antigens, they depend on the cross linking of polyvalent antigens. A phase change was represented in precipitation reaction, while clumping of antigen/antibody complexes was manifest in the agglutination reactions.

Hemagglutination antibody titer assay is an important tool for investigation of components acting on humoral immunity. Antibody molecules, a product of B-lymphocytes and plasma cells, are central to humoral immune response; IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins.[19] At neutral pH, negative ions clouds possess in red blood cells that makes the cells repel from one another, this repulsive force is known as zeta potential. Because of its size and pentameric nature, IgM can overcome the electric barrier and crosslink red blood cells, leading to subsequent agglutination.[20]

Carbon clearance assay

Table 3: Carbon clearance readings of all four groups

S. No.	Groups	Phagocytic index
1.	Control	0.0034±0.0040
2.	Levamisole (2.5 mg/Kg)	0.0106±0.0036*
3.	Compound (50mg/Kg)	0.0086±0.0023*
4.	Compound (100mg/Kg)	0.0099±0.0025*

Value expressed as mean±standard deviation (SD) at no=6, one way ANNOVA, followed by Bonferroni test * P< 0.05 significant compared to the control group.

Animal treated with ethanolic extract of *V. unguiculata* leaves showed significant increase in phagocytic index (P<0.05) as compared to vehicle treated animals. In levamisole treated animals level of phagocytic index was found to be also elevated significantly (P<0.05). This confirmed that extract was stimulating immunity mediated through phagocytosis inside the body.

The increase in carbon clearance index reflects the enhancement of the phagocytic function of mononuclear macrophage and non-specific immunity. Innate immune cells like Macrophages with well-established role in tissue homeostasis, the primary response to pathogens, coordinate of the adaptive immune response, inflammation, esolution and repair. These cells are capable of recognizing danger signals through receptors capable of inducing specialized activation programs. IFN-α induced the classically known macrophage activation, to kill intracellular pathogens it triggers a harsh proinflammatory response that is required. IL-4 and IL-13 show alternative activation to, a different phenotype was triggered that is important from the immune response to parasites.

Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by the oponisation of parasites with antibodies complementing C3b, leading to a more rapid clearance of parasites from the blood. From the blood stream the rate of removal of carbon particles were measured for the phagocytic activity of the reticuloendothelial system. Macrophages are integral part of innate immunity.

Macrophages respond to activation by microbial products that are required for innate immunity and priming of the acquired immune response.[21]

Cyclophosphamide induced neutropenia

Table 4: CYP assay readings of all four groups

S. No.	Group	WBC (Before)	WBC (After)	LYM (Before)	LYM (After)
1.	Control	3.68±0.436	1.58±0.488	1.93±0.363	1.13±0.333
2.	Levamisole (2.5 mg/Kg)	4.85±0.442*	3.97±0.579*	3.05±0.418*	2.48±0.655*
3.	Compound (50mg/Kg)	4.33±0.753*	2.87±0.695*	2.50±0.352^{NS}	1.35±0.217^{NS}
4.	Compound (100mg/Kg)	4.58±0.884*	3.02±0.799*	2.55±0.295*	1.65±0.274*

Value expressed as mean ± standard deviation (SD) at no=6, one way ANNOVA, followed by bonferrony test
* P< 0.05 significant compared to the control group.

A decreased WBC reveals an infectious process that has overwhelmed the immune system or a chronically depressed immune system that is not functioning properly. WBCs helps the body to fight illness or infections. As part of the immune system, they recognize and fight things that are foreign to the body. The hematopoietic system suppresses by Cytotoxic chemotherapy, impairing mechanisms for host protection and limiting the doses of chemotherapy that can be tolerated.

CYP is a prodrug requiring metabolic transformation to generate activity alkylating species. Hepatic cytochrome P-450 enzymes mediate the initial activation and for parent drug it is the major pathway of elimination. Hydroxylation at the carbon-4 position of the oxazaphosphorine ring produces 4-hydroxycyclophosphamide in equilibrium with the tautomer aldophosphamide, which spontaneously degrades to phosphoramide mustard and acrolein.[22] These alkylating species bind to DNA and induce strand breakage and cross-linking, killing actively replicating cells.[23] Cyclophosphamide is an anticancer drug and it causes neutropenia. Leukocyte possess very important role in immunity. If pathogens get through all the barriers to infection a second line defense is activated. To give you active or acquired immunity the immune system responds to a particular pathogen. Increase in total leukocyte count and differential leukocyte count demonstrated that caffeic acid plays an important role in modulation of immunity mediated through immunity this confirmed that extract possess significant stimulatory effect on immunity.

CONCLUSION

In present investigation immunomodulatory potential of caffeic acid, isolated from methanolic extract of *Vigna unguiculata* leaves was confirmed on various animal models. Extract was prepared using maceration technique. Initially extract was defatted with pet ether and defatted marc was further extracted with methanol as solvent. Safety of extract was confirmed as OECD 423 guidelines which revealed that extract was safe up to 2000 mg/kg. Hence its 1/40th i.e. 50 mg/kg and 1/20th i.e. 100 mg/kg was selected as dose for further in vivo investigation.

Effect on immunity was ascertain non cellular immunity, humoral immunity, WBC count and phagocytosis. Animal models used were hemagglutination antibody titer, delayed type hypersensitivity, cyclophosphamide induced neutropenia and carbon clearance assay. It was observed that caffeic acid at 50mg/kg and 100mg/kg significantly (P<0.05) increase dilution factor value in HAT assay, paw thickness in DTH assay, WBC and lymphocyte count in cyclophosphamide induced neutropenia and phagocytic index in carbon clearance assay as compare to vehicle treated group.

Thus from present investigation it can be concluded that caffeic acid, isolated from methanolic extract of *Vigna unguiculata* leaves possess significant immunostimulant activity and this effect may be mediated through cellular immunity, humoral immunity and phagocytosis stimulation. In future study is required ascertain mechanism of action for said activity.

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