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SCREENING OF IMMUNOMODULATORY ACTIVITY HYDROALCOHOLIC LEAVES EXTRACT OF *LAGERSTROEMIA PARVIFLORA*

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Abstract

Lagerstroemia parviflora Roxb leaf extracts showed the presence of phytochemical constituents such as terpenoids, steroids, phytosterols, flavonoids, carbohydrates, proteins and saponin. There are no previous preliminary phytochemical and other reports in this plant. Also, previous studies on the immunomodulatory activity of *Lagerstroemia parviflora* were done in different geographical regions of the world and this has been reported to affect the nutritional and the phytochemical composition of the plant and hence its medicinal value effectiveness may differ geographically. The study therefore, determined the immunomodulatory activity of methanolic leaf extracts of the *Lagerstroemia parviflora* in nonimmunosuppressed Wistar albino rats.

Total phenols and flavonoids contents, as well as scavenging activity of DPPH in Hydroalcoholic extract obtained from *Lagerstroemia parviflora* leaves, was evaluated. The total phenol and flavonoids content of the Hydroalcoholic extract *Lagerstroemia parviflora* leaves shows the excellent amount. Hydroalcoholic extract *Lagerstroemia parviflora* leaves showed the high radical scavenging activity of DPPH. The in-vivo Immunomodulatory activity was performed on Hydroalcoholic extract *Lagerstroemia parviflora* (leaves).Various parameters have been evaluated such as Neutrophils adhesion test, Carbon clearance test, Indirect heamagglutination test, Mice lethality test**.** The study was carried out using four different methods, each of which provides information about effect on different components of the immune system. The variety of plant products can modulate immune reaction either by stimulation or suppression and may assist as a supportive therapy along with conventional drugs in immune compromised patients.

Key words: Immunomodulatory Activity, *Lagerstroemia parviflora*, Hydroalcoholic Extract

Introduction

Various disease conditions like cancer, atherosclerosis, diabetes, neurological disorders, liver disorder, nephrotoxicity, inflammation, arthritis, ageing etc., occurs as a result of oxidative stress caused by free radicals. These free radicals are unstable molecules and in the phase of becoming stable, they abstract the hydrogen form biomolecules and cause their damage resulting in diseases. These radicals include various ROS and RNS. Superoxide radical is an initial free radical (ROS) formed and is involved in the synthesis of other ROS, such as hydrogen peroxide, singlet oxygen etc., (Powers & Jackson, 2008).

The process of degradation of polyunsaturated fatty acids of the membranes involving the formation of lipid radicals and resulting in lipid membrane damage is called lipid peroxidation (Baskar *et al.,* 2007). It also induces the damage to genomic and mt DNA as a result of elevated oxidative stress in cell and finally leads to unstable cytological conditions. The damage of lipid membrane, affects the signal transduction cascade by altering the stability of ligand binding domains, distrupting membrane transport proteins and associated enzymes. Thus, lipid peroxidation associated damage results in various diseases. Superoxide radical also reacts with nitric oxide and produces peroxynitrite. These radicals, non-radicals and the aldehydes become critical to the bio molecules. Thus inhibition of these becomes very much important and are usually deactivated by endogenous antioxidant system. But, certain conditions results in overproduction of these radical resulting in oxidative stress and the body demands for external supplementation.

The immune system is a complex network of specialized cells, molecules and organs that are involved in protecting the body against foreign antigens such as bacteria, viruses, fungi, parasites, cancer cells and toxins. The most important physiological function of the immune system is to protect the body against infection and to remove infections which are already developed A healthy immune system has the ability to distingnish between the body's own cells (self) and foreign cells (non- self).

However, the immune system is considered as a double-edged sword in that the highly active immune system can not only destroy foreign invaders, but also destroy body tissues. When the immune system functioning properly,it fights against infections and when it malfunctions leading to the development of a variety of diseases such as allergy,

arthritis,multiple sclerosis, cancer and so on (Devereux, 2002).

Currently, worldwide, there is an increase in diseases especially infectious diseases that requires efficient body defense mechanisms to control them through the process of immunomodulation. Malnutrition and infectious diseases have remained a challenge especially in developing nations as they greatly compromise the body's immune system responses in the affected individuals.

Globally, it was estimated that about 870 million people were undernourished in the period from 2010 to 2012 and this represented 12.5% of the global population of which about 852 million people live in developing countries, where malnutrition is estimated at 14.9%. However, malnutrition greatly affects the individual's immune system physiology and in most cases, there is need to stimulate it in circumstances of immunosuppression or suppress it in case of overexaggerated stimulation as in case of autoimmune disease conditions.

Lagerstroemia parviflora Roxb leaf extracts showed the presence of phytochemical constituents such as terpenoids, steroids, phytosterols, flavonoids, carbohydrates, proteins and saponin. There are no previous preliminary phytochemical and other reports in this plant. Also, previous studies on the immunomodulatory activity of *Lagerstroemia parviflora* were done in different geographical regions of the world and this has been reported to affect the nutritional and the phytochemical composition of the plant and hence its medicinal value effectiveness may differ geographically. The study therefore, determined the immunomodulatory activity of methanolic leaf extracts of the *Lagerstroemia parviflora* in nonimmunosuppressed Wistar albino rats.

Material and methods

Chemicals and their sources

Leishmann's stain and gluteraldehyde were purchased from Merck (NS Scientific, Bhopal). Indian ink from HIMEDIA (NS Scientific, Bhopal). WBC diluting fluid and EDTA from (Gupta Pathology, Bhopal). Pasteurella multocida of bovine origin and its vaccine and Nylon fibers From (Bhopal Memorial Hospital and research Centre, Bhopal).

Collection and Identification of Plant Material

To identify crude drugs, organoleptic characteristics, morphological characteristics,

and microscopic investigation were used. Herbariums and renowned botanical gardens are very useful for identifying unknown medications. The leaves of chosen plant were collected in the month of January 2022, From Bhojpur (M.P.) The leaves of the, *Lagerstroemia parviflora* were identified by Dr. Saba Naaz, HOD Department of Botany, Safiya Science College Bhopal.

Extraction by Soxhletion Method (Mukharjee, 2007)

100 gram of powdered stems of *Lagerstroemia parviflora* was exhaustively extracted with different solvent petroleum ether and Hydroalcohol (Ethanol: water;70:30) by Soxhletion method. The extract was evaporated above their boiling points. Finally, the percentage yields were calculated of the dried extracts.

Phytochemical Analysis

Qualitative photochemical analysis

Photochemical examinations were carried out extracts as per the following standard methods.

1. Detection of alkaloids: Extracts dissolved individually in dilute Hydrochloric acid and filtered.

a) Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Alkaloids confirmed by the formation of yellow coloured precipitate.

2. Detection of carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a) Fehling's Test: Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

a) Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Finding of pink to blood red colour indicates the presence of cardiac glycosides.

4. Detection of saponins

a) Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the incidence of saponins.

5. Detection of phenols

a) Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

6. Detection of flavonoids

a) Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the occurrence of flavonoids.

7. Detection of proteins

a) Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

8. Detection of diterpenes

a) Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes (Roopashree *et al.,* 2008; Obasi *et al.,* 2010; Audu *et al.,* 2007).

Quantitative studies of phytoconstituents

Total phenol content estimation

Principle: The total phenol content of the extract was determined by the modified folin-ciocalteu method.

Preparation of Standard: 10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 10- 50µg/ml was prepared in methanol

Preparation of Extract: 10 mg of dried extract was dissolved in 10 ml methanol and filter. Two ml (1mg/ml) of this extract was for the estimation of phenol.

Procedure: 2 ml of extract and each standard was mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexes for 15s and allowed to stand for 10min for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Total flavonoids content estimation

Principle: Determination of total flavonoids content was based on aluminum chloride method

Preparation of standard: 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol.

Preparation of extract: 10 mg of dried extract was dissolved in 10 ml methanol and filter. Three ml (1mg/ml) of this extract was for the estimation of flavonoids.

Procedure: 1 ml of 2% $AlCl_3$ solution was added to 3 ml of extract or each standard and allowed to stand for 15min at room temperature; absorbance was measured at 420 nm.

In-vivo Immunomodulatory activity

Animals

Laboratory bred Wistar albino rats (180–200 g) and albino mice (20–25 g) of either sex were housed at 25° ± 5 °C in a well-ventilated animal house under 12/12 h light/dark cycle. The mice were procured from College of Veterinary Science and Animal Husbandry Mhow, Indore M.P, (India). The animals had free access to standard food pellets (Bird House Bhopal, India) containing (% w/w) protein 22.10, oil 4.13, fiber 3.15, ash 5.15, sand (silica) 1.12, and water ad libitum. Bedding material was removed and replaced with fresh paddy husk as often as necessary to keep the animals clean and dry. The animals were maintained under standard conditions in an animal house approved by Committee for the purpose of control and supervision on experiments on animals (CPCSEA). The experimental protocol was approved by Institutional ethical committee. The animals were subjected for quarantine (10 days) prior to experimentation

Antigen preparation

Fresh sheep blood was collected from the local slaughterhouse. Sheep red blood cells (SRBCs) were washed three times in large volumes of pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5 x 10^9cells/ml for immunization and challenge (Thomas et al., 2004).

Acute toxicity studies (Ghosh. 1984)

The acute toxicity study was carried out to select the dose, by using up and down or stair case method. Two mice were selected with a dose of 50 mg/kg orally and examined for a period of 24 h for mortality. The subsequent doses are then increased by 1.5 factors to attain maximum non-lethal and minimum lethal dose. The Isolated compounds was found to be safe at the dose of 5 g/kg p.o According to office of pollution prevention and toxics (OPPT) guidelines, 1/10th of the maximum safe dose (5 g/kg).

Experimental protocol

The drug solutions were prepared in distilled water for oral administration. Immunomodulatory activity was checked both at cellular and humoral levels. Cellular immunity was evaluated by Neutrophils adhesion test and carbon clearance assay, whereas, humoral immunity was analyzed by mice lethality test and indirect heamagglutination assay. All the experimental models had four common groups consisting of six animals each.

Group I, was served as control and received (vehicle 1 ml/100 g, p.o), group II, received the Levamisole (0.68 mg/kg, p.o), whereas groups III and IV were administered Hydroalcoholic extract of leaves of *Lagerstroemia parviflora* (HALP) 50 and 100 mg/kg, oral, respectively. However, in mice lethality test, an additional negative control group was also present.

Neutrophils adhesion test (Fulzele et al., 2003 and Shinde et al., 1999)

The rats were pre-treated orally with vehicle or isolated compounds for 14 days. At the end of treatment day 14, blood samples were collected from the retro-orbital plexus into heparinized vials and analyzed for differential leukocyte count (DLC). After the initial counts, blood samples were incubated with 80 mg nylon fibers/ml for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC, respectively to give Neutrophils index of blood samples. The percent Neutrophils adhesion was calculated as follows:

$$\textbf{Neutrophil adhesion } \% = \frac{\textbf{Nlu} - \textbf{Nlt}}{\textbf{NIu}} x100$$

Where NIu is the Neutrophils index of untreated blood samples and NIt is the neutrophil index of treated blood samples.

Carbon clearance test (Jayathirtha et al., 2004 and Gokhale et al., 2003)

Swiss albino mice were administered Hydroalcoholic extract of leaves of *Lagerstroemia parviflora* (HALP) 50 and 100 mg/kg, and Levamisole treatment orally for 10 days in their respective groups. Forty- eight hours after the last dose of the drug, animals of all the groups received intravenous injection of (0.3 ml per 30 g) Indian ink (colloidal carbon) via the tail vein. Blood samples were withdrawn from each animal by retro-orbital plexus at an interval of 0 and 15 min after the ink injection. Blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm. The phagocytic index K was calculated using the following formula:

$$K = \frac{(\text{Loge OD1} - \text{Loge OD2})}{15}$$

Where OD1 and OD2 are the optical densities at 0 and 15 min, respectively

Mice lethality test (Ramanatha et al.,1995)

Swiss albino mice were pretreated with Hydroalcoholic extract of leaves of *Lagerstroemia parviflora* (HALP) 50 and 100 mg/kg and Levamisole orally for 21 days in their respective groups. On the 7th and 17th day of the treatment, the animals were immunized with haemorrhagic septicaemic vaccine (HS vaccine) through subcutaneous route. On the 21st day, the animals were challenged subcutaneously with 0.2 ml of lethal dose (25x LD_{50}) of Pasteurella multocida (bovine origin) containing 10^7 cells per ml. The animals were observed for a period of 72 h and the mortality percentage was determined.

Indirect heamagglutination test (Fulzele et al., 2003)

Rats of various groups were pretreated with the drugs for 14 days and all rats of entire groups were immunized with $0.5x10^9$ sheep red blood cells (SRBCs) intraperitoneal. The day of immunization was referred to as day 0. The drug treatment was continued for 14 more days and blood samples were collected from each rat at the end of the drug treatment and the titre value was determined by titrating serum dilutions (50–100 µl)

with SRBC (0.025 x10^9 cells) in microtitre plates. The plates were incubated at room temperature for 2 h and examined visually for agglutination. The minimum volume of serum showing heamagglutination was expressed as heamagglutination (HA) titer.

Statistical analysis

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's comparison test. The values were expressed as mean ± SEM and $P < 0.05$ was considered significant.

Results and Discussion

Since the dawn of time, medicinal plants have been praised for their wide range of pharmacological effects. This praise is due to the existence of secondary plant metabolites such alkaloids, flavonoids, glycosides, tannins, steroids, etc. By scavenging free radicals, which are implicated in the aetiology of many diseases, several of these plants are significant sources of natural antioxidants that have been demonstrated to lower the risk and progression of some acute and chronic diseases, such as cancer, heart disorders, and stroke. The yields were found to be (11.91 % w/w of crude drug) of petroleum ether extract with semisolid mass of brown colour, (13.58 % w/w of crude drug) of methanolic extract with orange black colour semisolid mass for *Lagerstroemia parviflora* leaves.

Total phenols and flavonoids contents, as well as scavenging activity of DPPH in Hydroalcoholic extract obtained from *Lagerstroemia parviflora* leaves, was evaluated. The total phenol and flavonoids content of the Hydroalcoholic extract *Lagerstroemia parviflora* leaves shows the excellent amount. Hydroalcoholic extract *Lagerstroemia parviflora* leaves showed the high radical scavenging activity of DPPH.

The in-vivo Immunomodulatory activity was performed on Hydroalcoholic extract *Lagerstroemia parviflora* (leaves).Various parameters have been evaluated such as Neutrophils adhesion test, Carbon clearance test, Indirect heamagglutination test, Mice lethality test **.**

In Neutrophils adhesion test, incubation of blood with nylon fibers (NF) produced a decrease in the Neutrophils counts due to adhesion of Neutrophils to the fibers. Both doses Hydroalcoholic extract of leaves of *Lagerstroemia parviflora* (HALP) 50 and 100

mg/kg and Levamisole showed significant increase in the Neutrophils adhesion when compared to control. The 100 mg/kg dose of (HALP) was found to be more effective than 50 mg/kg dose of (HALP)

In Carbon clearance test both doses of Hydroalcoholic extract of leaves of *Lagerstroemia parviflora* (HALP) and Levamisole showed significant increase in the phagocytic index when compared to control indicating that there was increase in the clearance of colloidal carbon from the blood after administration of these drugs. However, the clearance was best with 100 mg/kg dose of (HALP) and Levamisole

In the indirect heamagglutination test, the heamagglutination antibody (HA) titer value was significantly increased in animals that received vaccination along with 50 and 100 mg/kg dose of (HALP) or Levamisole compared to animals that received vaccination alone. Mortality was found to be 100% within 72 h in control group upon administration of Pasteurella multocida. There was 83.33% mortality in vaccinated group without any prior treatment of drug. The 50 and 100 mg/kg doses of (HALP) as well as Levamisole reduced the mortality percentage to 66.66%

Table 1: Extractive values obtained from *Lagerstroemia parviflora* leaves using different solvents

S.N.	Solvent	Time of extraction (Hours)	Color of extract	% Yield
1	Petroleum ether	12	Brown	11.91%
2	Methanol: Water	28	Orange-Black	13.58 %

Table 2: Preliminary phytochemical screening of *Lagerstroemia parviflora* leaves

S.N.	Phytoconstituents	Test Name	Hydroalcoholic Extract
1	Alkaloids	Mayer's Test	Absent
		Dragendorff's Test	Present
2	Glycosides	Raymond's Test	Present
		Killer Killani Test	Present

3	Carbohydrates	Molisch's Test	Absent
		Fehling's Test	Absent
4	Tannins	Vanillin- HCl Test	Present
		Gelatin Test	Absent
5	Flavonoids	Lead acetate	Present
		Shinoda Test	Present
6	Resins	Color detection with ferric chloride	Absent
		Turbidity Test	Absent
7	Steroids	Libermann- Bur chard Test	Present
		Salkowski Reaction	Present
8	Proteins & Amino acids	Biuret Test	Present
		Precipitation test	Absent
		Ninhydrin Test	Present
9.	Phenols	Ellagic Acid Test	Present

Table 3: Total Phenolic Content of Hydroalcoholic extract of leaves of *Lagerstroemia parviflora*

Sample	Total phenolic content GAE mcg/ml
Hydroalcoholic extract 100µg/ml	18.24± 0.154

n=3, values are given in SEM

Table 4: Total Flavonoid content of Hydroalcoholic extract of leaves of *Lagerstroemia parviflora*

S. No.	Extracts 100µg/ml	Flavonoid content Quercetin equivalent mcg/ml
1	Hydroalcoholic extract (100µg/ml)	28.45 ± 0.121

n=3, values are given in SEM

Table 5: Effect of Hydroalcoholic extract of *Lagerstroemia parviflora* (HALP) and Levamisole on Neutrophils adhesion test

Treatment	**TLC (103/mm³) (A)**		**Neutrophils% (B)**		**Neutrophils index (A x B)**		**Neutrophils adhesion (%)**
	UB	**NFTB**	**UB**	**NFTB**	**UB**	**NFTB**	
Control	5.6 ± 0.16	5.5 ± 0.16	23.3 ± 0.80	22.5 ± 0.8	130.48± 5.4	123.75± 4.5	5.15 ± 0.6
Levamisole(0.68 mg/kg)	6.6 ± 0.18	5.8 ± 0.15	26.6 ± 1.08	18.6 ± 0.4	175.56 ± 6.4	107.88± 4.2	38.55 ± 0.6***
(HALP) 50 mg/kg	6.7 ± 0.12	5.8 ± 0.13	27.0 ± 1.33	16.6 ± 1.0	180.9± 4.7	96.28± 8.5	46.7 ± 1.2***
(HALP) 100 mg/kg	6.3 ± 0.86	5.9 ± 0.49	24.5 ± 1.23	17.6 ± 1.2	154.35± 8.8	103.84± 9.1	32.7± 2.0***

[[[[

Table 6: Effect of Hydroalcoholic extract of *Lagerstroemia parviflora* (HALP) and Levamisole on phagocytic index and HA titer

Treatment	Phagocytic index in carbon clearance assay	Heamagglutination (HA) titer (µl)
CONTROL	0.0163 ± 0.0036	0.0874 ± 0.2562
Levamisole(0.68 mg/kg,po)	0.0483 ± 0.002***	0.0018 ± 0.0003***
(HALP) 50 mg/kg,po	0.0468 ± 0.0027***	0.0017 ± 0.0003***
(HALP) 100 mg/kg,po	0.0415 ± 0.0016***	0.0043 ± 0.0008***

All values are expressed as mean ± SEM of six observations. *** P < 0.001 when compared to control.

Table 7: Effect of Hydroalcoholic extract of *Lagerstroemia parviflora* (HALP) and Levamisole on mice lethality test

Treatment dose	Mortality first day	Second day	Third day	Mortality percentage
No drug, no vaccination	2	4	-	100
No drug, vaccination	1	3	1	83.33
Levamisole(0.68mg/kg,po)+ vaccination	-	1	3	66.66
(HALP)50mg/kg,po + vaccination	-	2	2	66.66
(HALP)100mg/kg, po+vaccination	-	3	1	66.66

Conclusion

In this study we found that (HALP) possesses Immunomodulatory activity in experimental models of cellular and humoral immunity. The study was carried out using four different methods, each of which provides information about effect on different components of the immune system. The variety of plant products can modulate immune reaction either by stimulation or suppression and may assist as a supportive therapy along with conventional drugs in immune compromised patients.

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