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EVALUATION OF IMMUNOMODULATORY ACTIVITY OF *CRATAEVA NURVALA*

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Abstract

The body's immune system is one of its most intricate biological systems. An alteration in the strength of the immune response is referred to as immunomodulation. A wide range of allopathic medications are used to alter the immune system. But these medications are prohibitively expensive for the underprivileged, difficult to obtain, and frequently linked to negative drug reactions. Therefore, using herbal formulation as a positive immunomodulator may be advised. Thus this study aims at evaluation of immunomodulatory activity of *crataeva nurvala.* The plant material was gathered and proccesed properly. Further the extraction ohytochemical and quantitative study along with assessment of parameters associated with immunomodulatory activity**.** The % loss after drying was estimated to be 30%. After performing defatting and extraction % yield was also calculated. The percentage yield for pet ether and hydroalcoholic extract was found to be 4.4% and 7.2% respectively. The phytochemical screening suggested the presence of alkaloid, flavonoid, saponin, phenolics, protein and carbohydrates. Total phenol & flavonoid content was estimated to be 0.647 and 0.801 respectively. It was observed that, in case of animals treated with HACN 50 and 100mg/kg the % neutrophil adhesion was 46.7 ± 1.2 % and 32.7± 2.0 %respectively which is comparable to that of standard drug Levamisole(0.68 mg/kg) with 38.55 ± 0.6 neutrophil adhesion. The phagocytic index for HACN 50 mg/kg and 100 mg/kg was observed to be 0.0468 ± 0.0027 and 0.0415 ± 0.0016 respectively. The Heamagglutination titer was observed to be 0.0017 ± 0.0003 and 0.0043 ± 0.0008 in HACN 50 mg/kg and 100 mg/kg treated rats. The mice lethality test showed that mortality % 66.66 in plant extract treat group which is equal to the animals treated with drug. Thus it can be inferred that *Crataeva nurvala* possesses potent immunomodulatory activity.

Keywords: Immunomodulatory, Medicinal plants, *Crataeva nurvala***,** Heamagglutination, mice lethality test, HACN (Hydroalcoholic extract of , *Crataeva nurvala),* Phytochemicals

Introduction

The body's immune system is one of its most intricate biological systems. The immune system is attacked by numerous viruses, bacteria, and fungi during an infection. The body's immune system uses a particular receptor to identify pathogens and triggers an instantaneous response by activating immune cells, cytokines, chemokines, and the release of inflammatory mediators.

An alteration in the strength of the immune response is referred to as immunomodulation. Compounds that enhance immune responses and stimulate the immune system non-specifically are known as immunostimulants. Conversely, substances known as immunosuppressive agents have the ability to reduce an individual's resistance to infections and stress brought on by external circumstances or chemotherapy. Immunomodulators are substances or compounds that have the ability to modify the immune system's activity and functionality in a specific or nonspecific way. Furthermore, because of their increased propensity to multiply in culture and high sensitivity, lymphocytes—which are frequently employed in studies examining immune function—can also be made to proliferate more readily (Van der Meide PH and Schellekens, 1996; Gerard *et al*., 1993).

Immunomodulators function on the immune system at various levels. As a result, several medication types have been created that specifically suppress or boost particular immune response cell populations and subsets, such as neutrophils, natural killer (NK) cells, lymphocytes, macrophages, and cytotoxic T lymphocytes (CTL). Immunomodulators have an impact on the cells that generate cytokines and other soluble mediators. Hence, immunotherapy targets the immune system to aid in the recovery of a specific illness (Reuman, 2001).

A wide range of allopathic medications are used to alter the immune system. But these medications are prohibitively expensive for the underprivileged, difficult to obtain, and frequently linked to negative drug reactions. The use of these medications is linked to a number of adverse effects (Brindha, 2016).

In order to get around some of these risks—such as their unavailability in some developing nations, the possibility of misuse leading to drug resistance, environmental pollution, and food residues—naturally produced medicinal plant products offer as an

alternative immunomodulatory and therapeutic agent. As a result, they may be environmentally acceptable and sustainable. Both the immunosuppressive and the immunostimulatory aspects of immunomodulation are equally significant in clinical medicine. Immunopotentiation is the best option in conventional chemotherapy when it comes to activating the host defense mechanisms in situations where the immune response is compromised (Azad *et al*., 2018; Sharma *et al*., 2017).

When used in conjunction with a scheduled vaccination regimen, specific plant extracts and herbal fed additives may help produce and develop more effective cell-mediated immune responses that provide protection against a range of bacterial, viral, and other diseases, as well as higher protective antibodies against various infections. Therefore, using herbal formulation as a positive immunomodulator may be advised. Numerous botanical products possess the potential for therapeutic use due to their high efficacy, affordability, and low toxicity (Bopana and Saxena, 2008).

Traditionally, *C. nurvala* has been used to treat a variety of conditions, including poor blood flow, respiratory issues, metabolic disorders, fever, joint lubrication, skin moisture, wound healing, memory loss, heart and lung weakness, and weakened immune system. The bark is used in the Unani medical system to increase appetite and reduce phlegm and bile secretion. Its potential applications as an oxytosic, diuretic, laxative, anti-periodic, and bitter tonic are suggested by folklore (Tolsarwad *et al*., 2020).

In light of the plant's advantages, the assessment of *Crataeva nurvala*'s immunomodulatory activity is the focus of this study.

Materials and Methods

Selection and collection of plant material

Stem bark of *Crataeva nurvala* were collected from ruler area of Bhopal (M.P.) in the month of February, 2023. The stem bark of plant sample were separated and washed with sterile distilled water to remove the adhering dust particles and other unwanted materials. Drying of fresh plant parts were carried out in sun but under the shade.

Percentage loss

The weight of fresh sample and dried powder was determined and percentage loss due to drying and loss of water was calculated. The percentage loss was calculated by dividing loss in weight of sample by actual weight of sample.

Defatting of plant material

All materials were shade dried at room temperature. 45 gram of dried stem bark of *Crataeva nurvala* was coarsely powdered and subjected to extraction with petroleum ether in a maceration method.

Extraction by maceration process

Defatted dried stem bark of *Crataeva nurvala* were extracted with hydroalcoholic solvent (methanol: water; 70:30v/v) by maceration method. The extract was evaporated above their boiling points. Finally, the percentage yields were calculated of the dried extract (Casassa and Harbertson, 2014).

Total phenolic content estimation

The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method. 10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 10- 50µg/ml was prepared in methanol. 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. 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 vortexed for 15s and allowed to stand for 10min for colour development. The absorbance was measured at 765 nm using a spectrophotometer (Slinkard and Singleton, 1977).

Total flavonoids content estimation

Determination of total flavonoids content was based on aluminium chloride method. 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 10-50µg/ml were prepared in methanol. 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. 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 (Saeed *et al*., 2012)

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 animals had free access to standard food pellets 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.,* 2009).

Acute toxicity studies

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) (Ghosh *et al*., 2019).

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 *Crataeva nurvala* (HACN) 50 and 100 mg/kg, oral, respectively. However, in mice lethality test, an additional negative control group was also present.

Neutrophils adhesion test

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 (Forrester and Lackie , 1981).

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

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

Carbon clearance test

Swiss albino mice were administered Hydroalcoholic extract of *Crataeva nurvala* (HACN) 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 (Jayathirtha *et al.,* 2004 and Gokhale *et al.,* 2003). 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

Swiss albino mice were pretreated with hydroalcoholic extract of *Crataeva nurvala* (HACN) 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 (Finco-Kent *et al*., 2001).

Indirect heamagglutination test

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 (Levett and Whittington, 1998).

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

The % loss after drying was estimated to be 30%. After performing defatting and extraction % yield was also calculated. The percentage yield for pet ether and hydroalcoholic extract was found to be 4.4% and 7.2% respectively. The phytochemical screening suggested the presence of alkaloid, flavonoid, saponin, phenolics, protein and carbohydrates. Total phenol & flavonoid content was estimated to be 0.647 and 0.801

respectively. Further the immunomodulatory activity of plant extracts in experimental animal was checked by assessing various parameters, one of them is neutrophil adhesion step. It was observed that, in case of animals treated with HACN 50 and 100mg/kg the % neutrophil adhesion was 46.7 ± 1.2 % and 32.7± 2.0 %respectively which is comparable to that of standard drug Levamisole(0.68 mg/kg) with 38.55 ± 0.6 neutrophil adhesion.

Further, carbon clearance test was performed. The carbon clearance test was used to evaluate the effects of drugs on the reticuloendothelial system. The diffuse reticuloendothelial system (RES) is composed of phagocytic cells. RES cells have a vital role in the elimination of particles from the bloodstream. The rate at which macrophages remove carbon from blood when ink containing colloidal carbon particles is injected directly into the bloodstream is determined by an exponential equation. The phagocytic index for HACN 50 mg/kg and 100 mg/kg was observed to be 0.0468 ± 0.0027 and 0.0415 ± 0.0016 respectively.

The Heamagglutination titer was observed to be 0.0017 ± 0.0003 and 0.0043 ± 0.0008 in HACN 50 mg/kg and 100 mg/kg treated rats. One important metric for evaluating the animal's humoral immune response is the hemagglutination antibody titer assay. In the current study, sheep red blood cells were used to clarify the production of antibody against RBC because it is anticipated that the antigen will cause the production of serum against it. When an individual's immune system is primed, a higher titer of antibody against a specific antigen is anticipated.

The mice lethality test showed that mortality % 66.66 in plant extract treat group which is equal to the animals treated with drug.

Table 1: Showing the results of percentage loss of *Crataeva nurvala*

S. No.	Description	Weight in (gms.)	% Loss
1.	Weight of plant material in wet, fresh condition	70	30%
2.	Weight of plant material after drying at room temperature	49	
3.	Loss in weight on drying	70-49=21	

Table 2: Results of percentage yield of *Crataeva nurvala*

Extracts	Percentage yield (%)
Pet. ether	4.4%
hydroalcoholic	7.2%

Table 3: Result of phytochemical screening of hydroalcoholic extract of *Crataeva nurvala*

S. No.	Constituents	Hydroalcoholic extract
1.	Alkaloids A) Wagner's Test: B) Hager's Test:	 -Ve +Ve
2.	Glycosides A) Legal's Test:	 -Ve
3.	Flavonoids A) Lead acetate Test: B) Alkaline Reagent Test:	 +Ve +Ve
4.	Saponins A) Froth Test:	 +Ve
5.	Phenolics A) Ferric Chloride Test:	 +Ve
6.	Proteins A) Xanthoproteic Test:	 +Ve
7.	Carbohydrate A) Fehling's Test:	 +Ve
8.	Diterpenes A) Copper acetate Test:	 -Ve

Table 4: Estimation of total phenol and flavonoids content of hydroalcoholic extract of *Crataeva nurvala*

S. No.	Total phenol content (mg/100mg of dried extract)	Total flavonoids content (mg/ 100 mg of dried extract)
1.	0.647	0.801

Immunomodulatory activity

Table 5: Effect of Hydroalcoholic extract of *Crataeva nurvala* (HACN) 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***
(HACN) 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***
(HACN) 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 *Crataeva nurvala* (HACN) 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***
(HACN) 50 mg/kg,po	0.0468 ± 0.0027***	0.0017 ± 0.0003***
(HACN) 100 mg/kg,po	0.0415 ± 0.0016***	0.0043 ± 0.0008***

Table 7: Effect of Hydroalcoholic extract of *Crataeva nurvala* (HACN) 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
(HACN)50mg/kg,po + vaccination	-	2	2	66.66
(HACN)100mg/kg, po+vaccination	-	3	1	66.66

Conclusion

According to the current study, extracts from the stem of *C. nurvala* may boost humoral as well as cellular immune responses. The extracts successfully enhance humoral and cell-mediated immunity in addition to amplifying nonspecific immune response. Therefore, based on the data acquired, it can be said that *C. nurvala* has medicinal potential and may be a useful candidate for immunomodulation.

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