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Investigation of Immunomodulatory Potential of Methanolic and Hexane Extract of *Musa acuminate* Peel (Plantain) Extracts

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Abstract: The methanolic and hexane extracts of *Musa acuminate* peel (Plantain peel) were pharmacologically validated for immunomodulatory activity at dose (100, 300, 500mg/kg b.wt., *p.o.*) by carbon clearance method, neutrophil adhesion and footpad swelling method on Wistar albino rats. Results of present studies suggested that both hexane and methanolic extracts of *Musa acuminate* was found to be potent immuo-stimulant in a dose dependent manner when compared with control group. Though, the results were more pronounced in methanolic extract.

Key words: Carbon Clearance · Neutrophil Adhesion · Phagocytic Index · Immunomodulator

INTRODUCTION

Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles [1]. Natural products have been an important resource for the maintenance of life for ages. One of the best-known examples is Soma, a plant that was pressed to yield juice, which was used as a medicine [2, 3]. Immunomodulation is a therapeutic approach in which we try to intervene in auto regulating processes of the defense system; if it results in an enhancement of immune reactions it is named as an immunostimulative drug which primarily implies stimulation of non specific system, that is, granulocytes, macrophages, complement, certain T-Lymphocytes and different effector substances. Immunosuppression involves an act that reduces the activation or efficacy of the immune system [4]. Cragg *et al.* reviewed the role of natural products in drug discovery and concluded that for the disease indications anticancer and anti-infection; more than 60% of new approved drugs are derived from natural sources. Furthermore, there is a global concern about emerging infectious diseases and an urgent need to identify novel means for effective treatment thereof [5].

Medicinal plants are coming into prominence because of the conventional medicine such as antibiotics which have developed resistance to many of the infection organisms which are no longer responsive to conventional medicines. Herbal preparation can be more effective and safer than conventional medicines. Non-toxic could be administered for a long period [6]. Bananas are tree like perennial herbs 2-9 meters in height. They are vegetatively propagated from the rhizome. Peel contains potassium (K), calcium (Ca), sodium (Na), iron (Fe), manganese (Mn), Copper (Cu), bromine, rubidium, strontium, zirconium and niobium. Banana peel also demonstrated the present of various phenolic compounds such as gallocatechin and anthocyanins like peonidin and malvidine. Banana peels are potential sources of arabinoxylans with isolation yields of ≈40 and 10% when extracted with dilute alkali and inulin and oligofructose [7, 8]. Banana peels are used in bug bytes, topical infections, bruises, teeth whitening, warts removal, scrapes and scratches, as diuretic, psoriasis, headache, wrinkles, antideppresant, in total knee arthroplasty, wound healing, to filter pollutants out of water and garden compost (rich source of potassium) [9,10].

MATERIALS AND METHODS

Plant Materials: Musa acuminate peels were collected from Jaipur, Rajasthan, India, in the month of August, 2012. The plant was identified with the help of available literature and authenticated by Mr. Vinod Sharma, Taxonomist, Department of Botany, Rajasthan University, Jaipur, Rajasthan, India. A voucher specimen was deposited in the herbarium department, voucher specimen (RUBL21136) in University of Rajasthan.

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Preparation of Crude Extracts

Preparation of Methanolic Extract of Musa Acuminate Peel: Freshly collected *Musa acuminate* peels were dried in shade and coarse powder was prepared by maceration in methanol for one week. The macerated mixture was filtered through muslin cloth and evaporated at 40°C up to one third of initial volume, remaining solvent was completely evaporated at 40°C, using rotary vaccum evaporator (Superfit, India). The residue was designated as methanolic extract and used for further studies.

Preparation of Hexane Extract of Musa Acuminate Peel: Powdered peels were packed in Soxhlet apparatus. The drug was defatted with petroleum ether (60-80°C) for about 30-35 complete cycles. Defatted material was extracted with Hexane in Soxhlet apparatus. The extract was concentrated under vaccum to get solid crude mass. The dried crude extract was stored in a dessicator and used for further experiment after suspending in 2% sodium carboxy methyl cellulose (CMC).

Standard Drug: Septilin (Himalaya Drug Co. Pvt Ltd.) was used as a standard drug at a dose of 500 mg/kg b.wt., *p.o.* Septilin, a proprietary herbal preparation has been reported to produce wound healing and immunomodulatory activities and it contanins *Balsamodendron mukul, Tinospora cordifolia, Emblica officinalis, Rubia cordifolia, Moringa plerygosperma, Glycyrrhiza glabra,* Shankh bhasma and maharasnadi quath [11].

Animal: Wistar albino rats (120-150 g) of either sex were used. The animals were housed under standard laboratory conditions maintained at 25±1°C and under 12/12 h light/ dark cycle and fed with standard pellet diet (Amrut feed, chakan) and water *ad libitum*. Animal experiments were approved by the Institutional Animal Ethical Committee.

Animals were divided into eight groups (I-VIII) viz. Group I: received 0.5 ml of 2% w/v sodium carboxymethyl cellulose suspension *p.o* daily for 14 days as a control group; Group II: received 500mg/kg *p.o* of Septilin daily for 14 days, Group III: received 100mg/kg *p.o* of methanolic *Musa acuminate* peel extract (MMAPE-1) daily for 14 days, Group IV: received 300mg/kg *p.o* of methanolic *Musa acuminate* peel extract (MMAPE-2) daily for 14 days, Group V: received 500mg/kg *p.o* of methanolic *Musa acuminate* peel extract (MMAPE-3) daily for 14 days, Group VI: received 100mg/kg *p.o* of hexane *Musa acuminate* peel extract (HMAPE-1) daily for 14 days, Group VII: received 300mg/kg *p.o* of hexane *Musa acuminate* peel extract (HMAPE-2) daily for 14 days, Group VIII: received 500mg/kg *p.o* of hexane *Musa acuminate* peel extract (HMAPE-3) daily for 14 days.

Determination of Neutrophil Adhesion [12]: After 14 days of drug treatment, blood samples were collected by puncturing retro-orbital plexus into heparinized vials and analysed for total leukocyte cell (TLC) and differential leukocyte cell (DLC) counts. After initial counts, blood samples were incubated with 8- mg/ml of nylon fibers for 15 minutes at 37°C. The incubated blood samples were again analyzed for TLC and DLC, respectively to give neutrophil index of blood samples. The present neutrophil adhesion was calculated by the following formula:

$$\text{Neutrophil adhesion (\%)} = \frac{\text{Difference of neutrophil count in untreated and fiber treated}}{\text{Blood / Neutrophil count of untreated blood}} \times 100$$

Determination of Cell Mediated Immune Response [13]: On day 0, all groups were immunized with SRBC's and all groups were fed with tap water and test samples respectively for 14 days. On day 15, all groups were challenged by injecting 0.5ml of cell suspension into left hind paw subcutaneously and the contra lateral paw received an equal volume of saline. The foot paw thickness (in mm) was measured at 24 hrs and 48 hrs after challenge using Mitutoyo Dail Caliper (Mitutoyo Manufacturing Company, Japan). The difference in the thickness of the right hind paw and left hind paw was used as a measure of DTH reaction.

Determination of Phagocytic Index [14]: On the day 14th, all groups were administered with 0.2 ml of carbon suspension (Pelikan Tuschea Ink, Germany) intravenously through tail vein individually on seventh day. Blood samples were collected from retro-orbital plexus immediately before and 5,10,15,20 minutes after the injection of carbon suspension. An aliquot of each and 25 µl of blood sample lysed with 2 ml of 0.1% acetic acid and absorbance was observed at 675 nm. The graph was plotted between absorbance against time for each animal and its respective test groups. The phagocytic index was calculated by the slope of time concentration curve.

Statistical Analysis: All data is expressed as Mean ± SD of each group, where n=6. $p<0.05$ was considered statistically significant. Data were analyzed using One Way ANOVA followed by tukey test.

RESULTS AND DISCUSSION

Neutrophil Adhesion Test: Neutrophil adhesion test is an indicative of the marginalization of phagocytic cells in the blood vessels, *i.e.* an indication of immunostimulation [15]. The % neutrophil adhesion in control group animals was 18.99, in *Musa acuminate* Peel Extracts treated group with the doses of HMAPE-1, HMAPE-2 and HMAPE-3; it was 32.52, 36.56, 41.57 respectively and with the doses of MMAPE-1, MMAPE-2, MMAPE-3; it was 47.85, 52.39 and 55.57 respectively. As was evident from the results of neurophil adhesion test, significant increase in neutrophil adhesion was observed after administration of methanolic *Musa acuminate* peel extracts as compared to hexane *Musa acuminate* peel extracts in dose dependent manner, when compared with control and septilin treated animals as shown in Table 1 and Figure 1.

When blood samples were incubated with nylon fibres, a reduction in neutrophil percentage due to the adhesion of neutrophils to the nylon fibres was observed. The percentage reduction in neutrophil count in nylon fibre treated blood samples from the treatment with methanolic *Musa acuminate* Peel extracts was significantly more than the hexane *Musa acuminate* Peel extract. The adhesion of neutrophil to nylon fibers indicates the migration of cells in the blood vessels and the number of neutrophils reaching the site of inflammation. Methanolic and hexane *Musa acuminate* peel extracts at selected doses in the albino rats have showed a significant increase in the neutrophil adhesion to the nylon fibres. This may be due to upregulation of the â2 integrins that are present on the surface of the neutrophils through which; they adhere firmly to the nylon fibres [16, 17]. Hence it can be inferred that methanolic and hexane *Musa acuminate* peel extracts causes the stimulation of neutrophils towards the site of inflammation.

Delayed Type Hypersensitivity: The influence of immunomodulators on T-cell function can be assessed by measuring the delayed type hypersensitivity reaction. SRBC's were used for inducing the hypersensitivity reaction. The delayed type hypersensitivity was the first experimental evidence of transferable immunity carried only immune cells. Robert Koch first discovered the reaction in 1882, but it was not until 1940's that Landsteiner and chase proved that the reaction was mediated by the cellular and not the humoral arm of immune system [18].

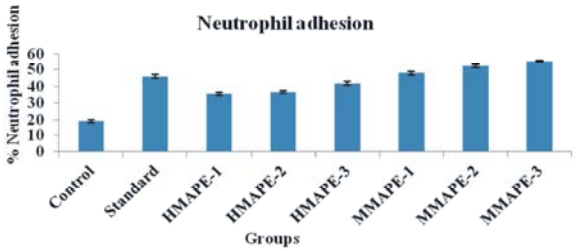

Fig. 1: Graphical representation of effect of *Musa acuminate* peel extracts on neutrophil adhesion in rats

The molecular mechanisms involved in DTH are upon injection of the antigen, Langerhan's cells process the antigen and present it to local memory T-cells, whether they are CD4+ and CD8+. These T-cells in concert with activated Langerhan's cells secrete numerous cytokines that cause the early hallmarks of inflammation [19]. Within 2 hours neutrophils begin to infiltrate the infection site. During the early stages of inflammation, leukocytes migrate exclusively through the post capillary venules and do not follow an obvious gradient of cytokine. Although cytokines such as members of cytokine family that are secreted by T-cells and macrophages are chemo-attractant for numerous immune cells, it is unlikely these cytokines are directly regulating the influx of cells from the vasculature. Instead, it is probable that the venular endothelium is recruiting the cells to the local site. The endothelial cells secrete vasodilators such as prostacyclin. The vasodialation caused by the prostacyclin optimizes delivery of immune cells to the site of challenge. The endothelial cells undergo changes as a result of TNF and IFN acting in concert. The endothelial cells remodel the basement membrane and allow the extravagation of plasma macromolecules, especially fibrinogen. The increase in fluid volume slows the blood flow and allows the Lymphocytes to attach more readily to the endothelium [18, 20]. In the present investigation, SRBC induced delayed type hypersensitivity was used to assess the effect of the fraction on cell mediated immunity. In rats treated with hexane *Musa acuminate* extract for 14 days; the footpad thickness due to SRBC induced DTH by HMAPE-1, HMAPE-2 and HMAPE-3 were found to be 26.13, 24.4 and 21.39 respectively at 24 hrs after challenge when compared with that of control (23.31) and standard drug (20.65). It was reduced to 11.2, 8.3 and 5.6, respectively after 48 hrs as depicted in Table 2.

Table 1: Effect of *Musa acuminate* peel extracts on neutrophil adhesion in rats

S.No.	Groups	Total Leukocyte Count		Total Neutrophil Count		%
		UB	NFTB	UB	NFTB	Neutrophil adhesion
1	Control	6.1±2.49	5.6±2.09	26.43±1.45	21.41±1.28	18.99±0.98
2	Standard Drug	8.0±2.98	6.1±0.98	34.98±1.54	18.92± 1.45	45.9±1.02*
3	HMAPE-1	6.9±0.98	6.2±1.93	30.96±1.97	20.89±0.97	32.52±0.87
4	HMAPE-2	7.3 ±1.98	6.54±0.78	31.01±2.1	19.67± 1.09	36.56±0.76
5	HMAPE-3	8.01±2.02	6.24± 0.97	32.67±0.98	19.04±1.42	41.7 ±1.22
6	MMAPE-1	7.5±2.49	6.02± 0.12	34.98±1.39	18.24±1.2	47.85±1.09*
7	MMAPE-2	8.09±1.78	5.98± 0.73	36.28±1.45	17.27±0.93	52.39±0.89**
8	MMAPE-3	8.14±0.29	5.93± 0.09	38.29±0.93	17.01± 1.4	55.57±0.37***

All data is expressed as Mean ± SD for each group, where n=6, *p<0.05, **p<0.01, ***p<0.001 as compared to control; Data were analysed using One Way ANOVA followed by tukey test

Table 2: Effect of *Musa acuminate* peel extracts on cell mediated immunity in rats.

S.No.	Groups	Delayed Type Hypersensitivity (Footpad thickness)	
		24 hrs	48 hrs
1	Control	22.31±0.74	15.7±0.26
2	Standard Drug	20.65±0.80***	4.5±0.64***
3	MMAPE-1	25.12±0.34***a2	10.97± 0.22***a2
4	MMAPE-2	23.74±0.28***a2	7.82±0.16***a2
5	MMAPE-3	20.14±0.19***	4.4± 0.32***
3	HMAPE-1	26.13±0.21***a2	11.2 ±0.28***a2
4	HMAPE-2	24.21±0.46***a2	8.3±0.45***a2
5	HMAPE-3	21.39±0.28*	5.6±0.26***a2

All data is expressed as Mean ± SD for each group, where n=6, *p<0.05, ***p<0.001 as compared to control; ^{a2}p<0.001 as compared to standard; Data were analysed using One Way ANOVA followed by tukey test

Table 3: Effect of *Musa acuminate* peel extracts on phagocytic index in rats

S.No.	Groups	Phagocytic Index
1	Control	0.00
2	Standard Drug	0.671
3	HMAPE-1	0.783
4	HMAPE-2	0.735
5	HMAPE-3	0.710
6	MMAPE-1	0.792
7	MMAPE-2	0.768
8	MMAPE-3	0.706

In rats treated with methanolic *Musa acuminate* peel extract for 14 days; the footpad thickness due to SRBC induced by MMAPE-1, MMAPE-2 and MMAPE-3 were found to be 25.12, 23.74 and 20.14, respectively at 24 hrs after challenge when compared with that of control and standard drug. It was reduced to 10.97, 7.82 and 4.4, respectively after 48 hrs as shown in Table 2.

Cell mediated immunity (CMI) involves effector mechanisms carried out by T-lymphocytes and their products (lymphokines). CMI responses are critical to defence against infectious organisms, infection of foreign grafts, tumor immunity and DTH. Therefore, increase in DTH reaction in rats response to T-cell dependent antigen revealed the stimulatory effect of methanolic and hexane *Musa acuminate* peel extracts on T-cells [21]. Methanolic *Musa acuminate* peel extracts exhibited more immunoprotective activity than hexane *Musa acuminate* peel extracts against impaired DTH conditions. This study clearly indicates that methanolic *Musa acuminate* peel extracts is more immunoprotective than hexane *Musa acuminate* peel extracts in a dose dependent manner when compared with control group.

Phagocytic Index: Virtually every cell except the mature erythrocyte ingests particulate materials from its surrounding by receptor-mediated pinocytosis. Phagocytic ability is an important element of cellular immunity and it differs from pinocytosis by bigger size of ingested particles and stronger dependence on the inhibitory effects of cytochalasins and low temperature.

Phagocytosis provides the first line of defence of the host against infectious microorganisms. In the body of higher species, there are two mostly recognized 'professional phagocytosis: polymorphonuclear (PMN) leukocytes (neutrophils and eosinophils) and mononuclear phagocytes (monocytes and macrophages). Macrophages have a major role in immunomodulation. The primary target of most immunomodulators is believed to be macrophages which play a major role by engulfing pathogens (or) foreign particles and initiating innate immune response which in turn orchestrate the adaptive response. The PMN cells emerge from the marrow as mature cells, which circulate in the blood for about 10 hrs before migrating to the tissues where they perform their effector functions for 1 or 2 days. In contrast, mononuclear phagocytes emerge from the marrow as immature cells monocytes, circulate in the blood and then enter tissues.

In the experimental animals, carbon clearance test can determine the influence of immunomodulators on change in macrophage phagocytic activity through reticuloendothelial system. The absorbance of lysed blood samples at 675 nm was proportional to the amount of residual carbon suspension in the blood. The phagocytic index was determined by carbon clearance method. When the carbon suspension was injected intravenously, the rate of clearance of carbon from blood by macrophage was governed by an exponential equation. This seems to be the general way in which inert particulate matter was cleared from the blood. The results of carbon clearance clearly indicated that the rate of elimination of carbon suspension by methanolic *Musa acuminate* peel extracts, MMAPE-1, MMAPE-2, MMAPE-3 was found to be 0.792, 0.768, 0.706 respectively. In case of hexane *Musa acuminate* peel extracts, HMAPE-1, HMAPE-2, HMAPE-3, the phagocytic index was observed to be 0.783, 0.735, 0.710 respectively (Table 3).

The study demonstrated that 7 days treatment of methanolic *Musa acuminate* peel extracts potentiated more elimination of foreign particles from its surrounding by enhancing the phagocytic activity of the macrophages (the phagocytosis of reticulo endothelial system) than hexane *Musa acuminate* peel extracts in a dose dependent manner. The present study established the immunostimulatory activity of the methanolic *Musa acuminate* peel extracts more than the hexane *Musa acuminate* peel extracts in a dose dependent manner. Prophylactic treatment of methanolic and hexane *Musa acuminate* peel extracts enhanced the rate of carbon clearance from the blood when compared to septillin treated animals. The result is owing to a mechanism related to phagocytosis by macrophages. The process of phagocytosis of macrophages includes opsonisation of the foreign particulate matter with antibodies ad compliment C3b, leading to a more rapid clearance of foreign particulate matter from the blood [22-25].

SUMMARY AND CONCLUSION

Neutrophil adhesion test is an indicative of marginalization of phagocytic cells in the blood vessels, i.e. an indication of immunostimulation. As was evident from the results of neutrophil adhesion test, more profound increase in neutrophil adhesion was observed after administration of methanolic extract than the hexane extract in a dose dependent manner.

The phagocytic index was determined by carbon clearance method. The study demonstrated that 7 days treatment of methanolic extract potentiated the phagocytic activity of the macrophages more than the hexane extract. The influency of immunomodulators on T-cell function can be assessed by measuring the delayed type hypersensitivity reaction. The increase of DTH reaction in response to T-cell dependent antigen revealed the stimulatory effect of the both extracts. Hexane extract showed the less protective activity as compared to the methanolic extract against the impaired DTH conditions. This study clearly indicated that the methanolic extract is more immunoprotective than the hexane extract.

Thus the immunostimulatory effect produced by both the extracts may be due to cell mediated and humoral antibody mediated activation of T and B cells. It is therefore concluded that the methanolic extract is a potent immunostimulator than the hexane extract of *Musa acuminate* peel and can be used as a complimentary therapeutic agent.

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