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Original Article

Effect of IMT-03, an Herbal Formulation in Cyclophosphamide-induced Immunosuppression in Mice

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Abstract: Immumomodulatory actions of natural ingredients have been recognized from the time of early civilization. Though herbal medicines are equally used to treat deadly diseases, but till date there are lacks of evidences. In the present work, an herbal preparation IMT-03 was used to find out its immunostimulatory efficacy against chemotherapeutics. Although, most of the individual ingredients present in IMT-03 have been reported for their protective roles against chemotherapeutics, but there is no report in combination. Cyclophosphamide (300 mg/kg, intraperitoneally) induces immunosuppression through myelosuppression. Pretreatment with of IMT-03 at dose of 100 mg/kg and 200 mg/kg in Cyclophosphamide induced immunosuppressive mice showed dose dependently and significantly ($p<0.001$) improvement in total WBC and absolute neutrophil counts. It also protects the general health of animals from chemotherapeutic induced serious adverse events like signs of sickness, lethargy, immobility, reducing food habit, infections in nostrils and pinna etc. Moreover, IMT-03 has the abilities to counter the macrophages surge during the LPS challenge. The test drug also showed safe up to the oral dose of 2 g/kg. *In vitro* studies revealed IMT-03 has polyphenols and also radical scavenging actions. There are several evidences that polyphenols can able to modulate cytokines and chemokines signaling pathways in immune cells. Therefore, it is assumed that polyphenols present in IMT-03 either modulate the inflammatory signaling pathways or protect from oxidative stress related DNA damage in myeloid tissues.

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INTRODUCTION

Immune system is an extremely complicated defense system to protect human body from invading agents like, viruses, bacteria, fungi, protozoa etc. It is able to produce varieties of cells and molecules capable of recognizing and eliminating limitless varieties of foreign and undesirable agents (Majali *et al*., 2015 ; Marshall *et al.*, 2018). On the other hand, immunomodulator is such a substance that can influence any constituent or function of the immune system in a specific or nonspecific manner (Wu *et al.*, 2019). Immunomodulators are two types, namely immunostimulant and immunosuppressant. Immunostimulant is required during conventional chemotherapy when the host defense mechanisms are to be activated under conditions of impaired immune responsiveness. In addition, it may help in prophylaxis of opportunistic infections in risk-prone, sensitive patients (Khan *et al*, 2020). On the other hand, immunosuppressant may be of choice in treatment of autoimmune disorders such as rheumatoid arthritis, multiple sclerosis etc (Notarangelo, 2010).

Medicinal herbs and their bioactive metabolites have shown to be an important source of natural immune functions. A range of immunomodulatory agents from herbs has been reviewed. Most of these herbs possessed a range of pharmacological properties such as antibacterial, antiviral, antimicrobial, anti-inflammatory, anti-arthritic, anti-allergic, antitumor, anticancer, immunostimulant and so on (Gulati *et al.*, 2002; Alamgir and Uddin, 2010; Archana *et al.*, 2011; Jantan *et al.*, 2015). In Indian System of Medicine, Ayurveda recommended polyherbal formulation to boost immune functions, rather than a single herb. Chyavanprash has been practiced to vitalizing the body function from ancient time (2000 BC) and still relevant (Sur *et al.*, 2004).

Based on the traditional knowledge and scientific validation, a new herbal formulation IMT-03 was prepared (M/s Isha Natural & Herbal Products Pvt. Limited, Hyderabad, India) with the purified and standardized extracts of *Achyranthes aspera, Andrographis paniculata, Asparagus adscendens, Asparagus racemosus, Centella asiatica, Emblica officinalis, Ocimum sanctum, Piper longum, Terminalia bellerica, Tinospora cordifolia* and *Withania somnifera* for clinical use during impaired immune function. Bioactive constituents, such as arabinogalactan polysaccharide of *T. cordifolia* stem and glycowithanolides and sitoindosides of *W. somnifera* root helpful in the treatment of myelosuppression (Srikumar *et al.*, 2005; Davis and Kuttan, 1998); *C. asiatica stem* stimulated phagocytic-index and antibody formation (Mediratta *et al.*, 2002); piperine of *Piper longum* fruits effectively kills Dalton's lymphoma ascites and Ehrlich's ascites (Devan *et al.*, 2007). The components of IMT-03 with supportive evidence based research information on immune function are given in Table 1. Therefore, the aim of the present study was to find out the immunomodulatory activity of IMT-03 in immune suppressed animals and establish potential mode of actions.

Table 1: Composition of IMT-03

Botanical name	Indian name	Parts used	Wight (mg)*	Reference
Achyranthes aspera	Apamarg	Whole plant	10	Chakraborty *et al.*,2002
Asparagus adscendens	Safed mushli	Root	10	Tandon and Shukla, 1995
Andrographis paniculata	Kalmegh	Leaf	6	Rao *et al.*, 2004
Asparagus racemosus	Shatavari	Root	10	Gautam *et al.*, 2004
Centella asiatica	Mandookparni	Whole plant	10	Punturee *et al.*, 2005
Emblica officinalis	Amla	Fruit	10	Haque *et al.*, 2001
Ocimum sanctum	Tulsi	Leaf	10	Mediratta *et al.*, 2002
Piper longum	Pippali	Fruit	10	Devan *et al.*, 2007
Terminalia bellerica	Bibhitaki	Fruit	4	Srikumar *et al.*, 2005
Tinospora cordifolia	Gudichi	Stem	10	Sainis *et al.*, 1997
Withania somnifera	Ashwagandha	root	10	Davis and Kuttan, 1998

*100 mg IMT-03 powder

MATERIALS & METHODS

Animals: Male Swiss albino mice were used. The recommended guidelines for the care and use of the animals were strictly followed (CPCSEA 2003). Briefly, room temperature was maintained at 22±2°C, humidity between 50 and 65% and 12 h light-dark cycle was maintained. The animals were fed supplementary feed for animal and water *ad libitum*. The plan of animal experiments was approved by the Institutional Animal Ethic Committee (IAEC/AH-2/2011/UCM-72).

Preparation of Test Drug: IMT-03 and *W. somnifera* was supplied in powder form and was dissolved in required volume of deionized water and kept in 4ºC.

***In vitro* Pharmacological Study:**

Total Phenolic compound: The total phenolic compound in IMT-03 was determined by Folin-Ciocalteu method (Sur *et al.,* 2016). 0.5 ml of Folin-Ciocalteu reagent in 2.4 ml deionized water was mixed in 0.1 ml IMT-03 (1mg/ml) solution in methanol and incubated in the dark for 3 minutes. Thereafter, 2 ml of 20% sodium carbonate solution was added, mixed and further incubated in the dark for 5 min at 50ºC and absorbance was read at 650 nm. The total phenolic compound in the extract was expressed as gallic acid equivalent (GAE).

DPPH radical inhibition: The scavenging activity of IMT-03 was determined using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl). 0.1 ml IMT-03 at different known concentrations was mixed with 3.9 ml of 0.1 mM DPPH solution and was allowed to stand in dark for 30 min. The absorbance was read at 517 nm and expressed as IC_{50} (Banerjee *et al.,* 2013).

Nitric oxide radical inhibition: NO radical was generated from sodium nitroprusside and measured by Griess' reaction (Sur *et al.,* 2015). 2 ml sodium nitroprusside (10 mM) and 0.5 ml phosphate buffer (0.025M, pH 7.4) was mixed in 0.5 ml IMT-03 at different known concentrations and incubated at 25ºC for 150 min. From the mixture 0.5 ml solution was taken and diluted with 1 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min. Then, 1 ml of napthylethylene diamine dihydrochloride (0.1% w/v) was added, mixed and allowed to stand for 30 min at 25ºC. The absorbance was read at 546 nm.

***In vivo* pharmacological study:**

Acute toxicity study: IMT-03 was given to 16 h fasted mice in the graded doses (0.5 g, 1 g, 1.5 g and 2 g per kg body weight) by oral route and observed for 3 days for any significant signs of acute toxicity and mortality (OECD No: 423, 2001).

Cyclophosphamide-induced immunosuppression:

Immunomodulatory properties of IMT-03 and WS were studied in CYP-induced immunosuppressive mice. Animals were divided in to five groups of 6 mice each and treated for 5 consecutive days as follows: (i) normal control-treated with 0.2 ml deionized water; (ii) CYP control-treated with 0.2 ml deionized water; (iii) treated with WS at 200 mg/kg; (iv) treated with IMT-03 at 100 mg/kg and (v) treated with IMT-03 200 mg/kg. On day 5, all mice (except normal control) were injected CYP (Sigma, St Louis, MO) intraperitoneally at the dose of 300 mg/kg of body weight (Santosuosso *et al* 2002; Khan et al., 2020). Total count of WBC and neutrophil were examined in blood on the day of 0, 5 and 10 after CYP injection. Endotoxin (LPS) derived from *E. coli* at the dose of 25 µg/kg was challenged to mice intraperitoneally at day 10 and after 24 h, leucocytes in blood were determined (Rutten and Thiemermann, 1997).

Statistical Analysis: The data were presented as mean ± standard deviation. P-value less than 0.05 were considered statistically significant. All values were compared using one-way analysis of variance (ANOVA) followed by Tukey's HSD as a post-hoc test.

RESULTS

The phenolic content was 43.63 µg GAE/mg in IMT-03. The IC_{50} of IMT-03 on DPPH radical formation was 116.8 µg/ml. Moreover, NO radical scavenging action, IC_{50} of IMT-03 was 42.47 µg/ml (Table 2).

Table 2: Antioxidant actions of IMT-03

	Total phenolics (µg GAE/mg extract)	IC_{50}	
		DPPH inhibition (µg/ml)	Nitric oxide scavenging (µg/ml)
IMT-03	43.63±0.004	116.8±0.009	42.47±0.002

Results are mean ± standard deviation; N=6;

Oral acute toxicity studies of IMT-03 in mice exhibited any signs of toxicity (activity, tremors, writhing, sedation, ptosis, lacrimation, diarrhoea etc.) or mortality up to 2.0 g/kg oral dose (limit dose).

CYP injection significantly depleted peripheral blood WBC to 51.73% within 5 days and 49.81% within 10 days; but it was drastically enhanced to 82.12% within 24 h after LPS injection. Moreover, neutrophil number was also reduced to 52.72% at 10th day of CYP injection compared to normal mice. Indeed, IMT-03 dose dependently (100 mg/kg and 200 mg/kg) and significantly (P<0.001) recovered the peripheral WBC to 37.77% and 56.11% within 5 days and to 49.74% and 65.80% within 10 day after CYP injection, while reference drug, WS (200 mg/kg) recuperated only 33.57% and 48.65% at the same interval (Table 3).

Table 3: Effect of IMT-03 on WBC in cyclophosphamide induced mice

Groups	Total WBC Count (per mm^3)		
	Day 0	Day 5	Day 10
Normal	6598 ± 38.51	6610 ± 22.21	6601 ± 20.56
CYP	6531 ± 49.89(a)	3190 ± 43.81(a)* [-51.73]	3313 ± 34.60(a) * [-49.81]
CYP+WS 200 mg/kg	6538 ± 61.06(b)	4261 ± 74.09(b)* [33.57]	4925 ± 79.61(b)* [48.65]
CYP+IMT-03 100 mg/kg	6540 ± 55.91(b)	4395 ± 84.76(b)* [37.77]	4961 ± 34.60(b)* [49.74]
CYP+IMT-03 200 mg/kg	6528 ± 50.16(b)	4980 ± 67.57(b)* [56.11]	5493 ± 92.40(b)* [65.80]

Results are mean ± standard deviation; N=6; (a) compared to normal control and (b) to CYP; NS= Not significant; * indicates p<0.05; data in parenthesis indicates percent change

Neutrophil number was reduced to 53.25% after 5 days of CYP treatment, but it was recovered to 44.67% and 62.60% in IMT-03 treated mice, while, reference drug WS enhanced only 26.52% (table 4). The significant increment (59.72% and 76.81%) of neutrophil was observed after 10 days treatment with IMT-03. Further surge of 67.62% and 72.58% in peripheral WBC count was noted after endotoxin injection in IMT-03 treated mice (table 5).

Table 4: Effect of IMT-03 on CYP induced peripheral neutrophils

	Total Neutrophils Count (per mm^3)		
	Day 0	Day 5	Day 10
Normal	1985 ± 24.32	1968 ± 8.33	1980 ± 6.83
CYP	1964 ± 19.45(a)	920 ± 7.30(a)* [-53.25]	936 ± 6.14(a)* [-52.72]
CYP+WS 200 mg/kg	1981 ± 25.22(b)	1256 ± 8.81(b)* [26.52]	1466 ± 17.63(b)* [56.62]
CYP+IMT-03 100 mg/kg	1986 ± 44.47(b)	1331 ± 23.01(b)* [44.67]	1495 ± 19.79(b)* [59.72]
CYP+IMT-03 200 mg/kg	1990 ± 44.06(b)	1496 ± 22.45(b)* [62.60]	1655 ± 32.53(b)* [76.81]

Results are mean ± standard deviation; N=6; (a) compared to normal control and (b) to CYP; NS= Not significant; * indicates p<0.05; data in parenthesis indicates percent change

DISCUSSION

The development of new immunomodulator from natural resources has now a striking endeavor. There is an immense possibility to innovate more effective immunomodulator from herbal resources to mimic or antagonize the cytokines and interleukins pathways (Khan *et al.,* 2020). All the individual herbal components used in IMT-03 are well-known and well documented for immune function (API, 2004). But, their role in combination is still not known.

Table 5: Effect IMT-03 after LPS challenge on CYP Leucopenia

	Total WBC Count (per mm^3)		
	Before LPS	24 h after LPS	% increase
Normal	6601 ± 20.56	12418 ± 118.69(c)*	88.12
CYP+LPS	3313 ± 34.60(a)*	5005 ± 57.83(c)*	51.07
CYP+WS+LPS 200 mg/kg	4925 ± 79.61(b)*	8091 ± 97.65(c)*	64.42
CYP+IMT-03+LPS 100 mg/kg	4961 ± 34.60(b)*	8316 ± 95.97(c)*	67.62
CYP+IMT-03+LPS 200 mg/kg	5493 ± 92.40(b)*	9480 ± 165.36(c)*	72.58

Results are mean ± standard deviation; N=6; (a) compared to normal control, (b) to CYP+LPS and (c) before and after LPS injection; NS= Not significant; * indicates $p<0.05$; data in parenthesis indicates percent change

The protective function against external pathogens carried out by the immune system is by itself a source of reactive oxygen species (ROS), since activated neutrophils, produce free radicals to a significant extent. Moreover, neutrophil-derived oxidants may promote tissue injury indirectly by altering the protease/antiprotease equilibrium that normally exists within the intestinal interstitium (Mileo *et al.,* 2019). An adequate intake of vitamins and antioxidant elements seems to be essential for an efficient function of the immune system. Polyphenolic compounds, widely present in fruits, vegetables, cereals, beverages and medicinal plants have potential benefits for human health and are protective agents against infections (Brambilla *et al.,* 2008). The immunomodulatory properties of polyphenols are able to modulate cytokines and chemokines production and activation of immune cells (Hazra *et al.,* 2018). In the present study, IMT-03 exhibited free radical scavenging antioxidant properties. Earlier studies supported that *A. paniculata, O. sanctum, E. officinalis, P. longum, A. racemosus, C. asiatica* and *T. cordifolia* have rich source of phenolic compounds and all of them have strong antioxidant activities and that may be one of the reasons of their immunomodulatory actions (Khan *et al.,* 2019). Moreover, *P. emblica, O. sanctum, A. adscendens, W. somnifera* and *T. cordifolia* reported for nitric oxide radical (NO) scavenging activities. NO is a proinflammatory agent which produced within tissues, giving rise to the highly toxic radical peroxynitrite ($ONOO^-$) and other reactive nitrogen species (RNS). Immune function may be reduced via the stimulation of apoptosis of neutrophils accelerated by the generation of NO (Pacher *et al.,* 2007).

CYP is alkylating agent, which react with grouping like amino, sulfhydryl, hydroxyl or phosphate in the physiologically important molecules in the cells and renders them unavailable for the normal metabolic reactions (Chabner, 2001). CYP can react with the nucleic acid bases and inhibit DNA synthesis and also bring about cross-linkage of DNA strands in resulting as well as in dividing cells and thus interfere with cell replication. At over dosage and use, CYP produces an acute myelosuppression. Both cellular and humoral immunity are suppressed by CYP, which has been used to treat various autoimmune diseases (Buckner *et al.,* 1972; Wang *et al.,* 2002). It was found that CYP rapidly reduced the number of total leukocytes and neutrophils in peripheral blood within 10 days. The CYP-treated mice showed less profound effect compared to IMT-03 treated mice in terms of increasing WBC and neutrophils count. Moreover, CYP infected mice revealed signs of sickness and lethargy, which was diminished on IMT-03 treated animals. CYP regimen had a chronic and profound suppressive effect on macrophage populations. Previous studies demonstrated that *T. cordifolia* has the ability to decrease NO and TNF-α production in macrophages and responsible for averting the DNA damage inducing induced by LPS treatment (Kapil and Sharma, 1997). Furthermore, major ingredients of IMT-03 like, *A. paniculata, C. asiatica, E. officinalis, O. sanctum, T. cordifolia* and *W. somnifera* have been reported for their role in CYP induced immunosuppression (Thatte *et al.,* 1987).

The macrophage is usually is in a quiescent state in a healthy individual, but in the presence of a pathogen or when treated with drug macrophage becomes activated. LPS is an integral part of the bacterial cell wall and known to cause severe respiratory bursts in phagocytes (Berenbaum *et al.,* 1973). LPS also induces NO production in macrophages, both directly and by inducing other cytokines like TNF-α and IFN-β and causing DNA strand breaks while inhibiting DNA repair enzymes (Huang *et al.,* 2017). Present findings suggest that IMT-03 has good effect on the residential leucocytes and macrophage population and their growth. IMT-03 modulates macrophage responses and protects mice against LPS induced endotoxic shock.

CONCLUSION

The present study demonstrated that IMT-03 have the potential chemo-preventive and immunomodulatory actions that either be mediated through inactivation of ROS generation by inhibiting the induction of iNOS or enhancing the non-specific host innate immunity.

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Conflict of Interests

The authors declare that they have no competing interests.

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