Evaluation of oral Withaferin-A for prophylaxis against acute Graft versus Host Disease in murine model of allogenic hematopoietic stem cell transplantation

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Mr. Saurabh Kumar Gupta entitled "Evaluation of oral Withaferin-A for prophylaxis against acute Graft versus Host Disease in murine model of allogeneic hematopoietic stem cell transplantation" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Saurabh Kumar Gupta

List of Publications arising from the thesis

Journal

1. "Withaferin-A alleviates acute graft versus host disease without compromising graft versus leukemia effect", **Saurabh Kumar Gupta**, Dievya Gohil, Deepshikha Dutta, Girish Ch. Panigrahi, Puja Gupta, Kajal Dalvi, Twinkle Khanka, Subhash Yadav, Rajiv Kumar Kaushal, Akanksha Chichra, Sachin Punatar, Anant Gokarn, Sumeet Mirgh, Nishant Jindal, Lingaraj Nayak, Prashant R. Tembhare, Syed Khizer Hasan, Santosh Kumar Sandur, Lal Hingorani, Navin Khattry, Vikram Gota, *International Immunopharmacology*, **2023**, *121*, 110437.

2. "Safety, toxicity and pharmacokinetic assessment of oral Withaferin-A in mice", **Saurabh Kumar Gupta**, Shraddha Jadhav, Dievya Gohil, Girish Ch. Panigrahi, Rajiv Kumar Kaushal, Khushboo Gandhi, Anand Patil, Preeti Chavan, Vikram Gota, *Toxicology Reports*, **2022**, 9, 1204-1212.

3. "Withania Somnifera extract mitigates experimental acute Graft versus Host Disease without abrogating Graft versus Leukemia effect", **Saurabh Kumar Gupta,** Dievya Gohil, Mohd Bashar Momin, Subhash Yadav, Akanksha Chichra, Sachin Punatar, Anant Gokarn, Sumeet Mirgh, Nishant Jindal, Lingaraj Nayak, Lal Hingorani, Navin Khattry, Vikram Gota, [Manuscript under communication]

Conference abstracts (Published)

1. "Withaferin-a Alleviates Acute Graft Versus Host Disease By Regulating Immune Cell Proliferation, Differentiation and Cytokine Storm Via Inhibition of JAK2-STAT3 Signalling", Vikram Gota, **Saurabh Kumar Gupta**, Deepshikha Dutta, Kajal Dalvi, Twinkle Khanka, Akanksha Chichra, Sachin Punatar, Anant Gokarn, Sumeet Mirgh,

Nishant Jindal, Lingaraj Nayak, Prashant R. Tembhare, Syed Khizer Hasan, Lal Hingorani, Navin Khattry, Vikram Gota, Blood. **2022** Nov 15;140, 12669-70.

2. "Withaferin-A Prevents the Onset of Acute Graft versus Host Disease by Inhibiting JAK2-STAT3 Signaling Pathway. Indian Journal of Medical and Paediatric Oncology" **Saurabh Kumar Gupta,** Dievya Gohil, Navin Khattry, Vikram Gota, **2021** Dec 10;43:S1-9.

Chapters in books and lectures notes: None

Conferences

1. I participated in 46th Annual Conference of the Indian Immunological Society, Immunocon, at BARC, Mumbai, India in 2019

2. I presented a poster in 17th National Research Scholar Meet, at ACTREC, Navi-Mumbai, India in 2021

3. I participated in AAZPIRE workshop on Clinical Research Methods, at ACTREC, Navi-Mumbai, India in 2021

4. I won the **"Young Ethnopharmacologist Award"** for Best oral presentation in 9th International Congress of Society for Ethnopharmacology, at JSS college of Pharmacy, Mysore, India in 2022

5. I won the **"Best E-poster Presentation Award"** in 1st Global Cancer Consortium, held as a virtual event in 2022

6. I participated in 1st and 2nd Series of Advancement in Cell Therapy at ACTREC, Navi-Mumbai, India in 2022, 2023

7. I presented a poster in 27th congress of Asia Pacific Bone Marrow Transplantation at Kochi, India in 2022

8. I won the **"Best oral presentation award"** in 1st National Symposium on Integrating Traditional Knowledge in Evidence Based Medicine, 2023

Others:

1. **Gupta SK,** Gohil D, Panigrahi GC, Vaykar S, Rane P, Chavan P, Gota V. Comparison of different autoanalyzers for the determination of lymphocyte and neutrophil counts in mouse blood. Drug Metabolism and Personalized Therapy. 2021 Dec 1;37 (2):219-22.

2. Gohil D, Panigrahi GC*, **Gupta SK*,** Gandhi KA, Gera P, Chavan P, Sharma D, Sandur S, Gota V. Acute and sub-acute oral toxicity assessment of 5-hydroxy-1, 4-naphthoquinone in mice. Drug and Chemical Toxicology. 2022 Jul 27:1-4. (*equal contribution).

3. Gohil D, Gandhi K, **Gupta SK**…Gota V (2023). Immunomodulation by juglone alleviates acute graft versus-host disease without compromising the graft-versus-leukaemia activity in mice. [Manuscript under communication]

4. Sengupta S, Biswas M, Gandhi K, **Gupta SK**, Gera P, Gota V, Sonawane A (2023). Preclinical therapeutic evaluation of engineered L-asparaginase variants to improve primary and relapse ALL treatment. [Manuscript under communication]

5. Gaur T, Ali A, Sharma D, **Gupta SK**, Gota V….Hasan S (2023) Mitocurcumin Utilizes Oxidative Stress to Upregulate JNK/p38 Signaling and Overcomes Cytarabine Resistance in Acute Myeloid Leukemia. [Manuscript under communication]

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Saurabh Kumar Gupta

Dedicated to .....

Maa and Papa

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Saurabh Kumar Gupta

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Abbreviations

ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
aGvHD	Acute graft versus Host Disease
alloHSCT	Allogeneic hematopoietic stem cell transplantation
APCs	Antigen presenting cells
ATG	Anti-thymocyte globulin
BM	Bone marrow
BMT	Bone marrow transplantation
CAR	Chimeric antigen receptor
cGvHD	Chronic GvHD
CMC	Carboxymethylcellulose
CNIs	Calcineurin inhibitors
ConA	Concanavalin A
CS	Clinical score
CSA	Cyclosporine
DAMPs	Danger associated molecular patterns
DMSO	Dimethyl sulfoxide
GvL	Graft versus Leukemia
HLA	Human leukocyte antigen
HR	Hazard ratio
IS	Internal standard
IV	Intravenous
JAK	Janus kinase
LFS	Leukemia free survival

LFT	Liver function test
LI	Large intestine
LLOQ	Lower limit of quantification
LOQ	Limit of quantification
LPS	Lipopolysaccharides
MHC	Major histocompatibility antigen
MMP-9	Matrix metaloprotein-9
MTX	Methotrexate
NK	Natural Killer
OECD	Organisation for Economic Cooperation and Development
OS	Overall survival
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBSCs	Peripheral blood stem cells
PHA	Phytohemagglutinin
PK	Pharmacokinetics
PTCy	Post-transplant cyclophosphamide
QC	Quality control
RFT	Renal function test
RSD	Relative standard deviation
SD	Standard deviation
SEM	Standard error of mean
SI	Small intestine
STAT	Signal transducer and activator of transcription

TAC	Tacrolimus
US FDA	United States Food and Drug Administration
WA	Withaferin-A
WSE	Withania somnifera extract

Summary

Allogeneic hematopoietic stem cell transplantation (alloHSCT) is perhaps the only curative treatment option for several malignant and non-malignant haematological disorders. However, the success of alloHSCT is limited by acute graft versus host disease (aGvHD), a condition that typically manifests in the first 100 days of transplantation due to an exaggerated immune response of donor immune cells, mainly the activated T-cells, against the host tissue. Worldwide, every year approximately 30,000 patients undergo alloHSCT. Despite advances in treatment and supportive care, 30-70% of alloHSCT recipients develop aGvHD. Standard GvHD prophylactic and therapeutic regimens are not always effective and they are associated with significant clinical and/or financial toxicity. Therefore, development of safe and effective drugs against aGvHD remains an unmet medical need. Withaferin-A (WA), being an anti-inflammatory, anti-proliferative and immunomodulatory agent was investigated for this purpose.

We first investigated the safety, toxicity and pharmacokinetics of WA in mice. In the acute and sub-acute toxicity study, up to 2000 mg/kg and 500 mg/kg of WA was well tolerated without any signs of toxicity or death. Thereby confirming the LD_{50} >2000 mg/kg and No-Observed Adverse Effect Level (NOAEL) to be at least 500 mg/kg. Additionally, WA was found to be orally bioavailable.

Further, we investigated the efficacy of WA for the prevention and treatment of aGvHD using a murine model of alloHSCT. Prophylactic administration of WA to mice mitigated the clinical symptoms of aGvHD and improved survival significantly compared to the GvHD control [HR = 0.07 (0.01–0.35); $P < 0.001$]. Furthermore, WA group had better overall survival compared to standard prophylactic regimen of CSA + MTX [HR = 0.19 (0.03–1.1), $P < 0.05$]. At the same time, WA did not compromise

the beneficial Graft versus leukemia (GvL) effect. In addition, WA administered to animals after the onset of aGvHD could reverse the clinical severity and improved survival, thus establishing its therapeutic potential. Our findings suggest that WA reduced the systemic levels of Th1, Th2 and Th17 inflammatory cytokine and increased the anti-inflammatory cytokine IL-10 levels significantly ($P < 0.05$). WA also inhibited lymphocytes migration to gut, liver, skin and lung and protected these organs from damage. *Ex-vivo*, WA inhibited proliferation of human peripheral blood mononuclear cells (hPBMCs), modulated immune cell phenotype and decreased cytokine release. In addition, WA inhibited pJAK2 and pSTAT3 protein levels in mouse splenocytes and hPBMCs. These findings established that, WA is an attractive candidate to develop against GvHD. However, development of a pure compound as a drug is a long-drawn process associated with huge costs. On the other hand, extracts of *Withania somnifera* containing WA (WSE) are available as nutraceuticals around the world and therefore could be an alternative to pure WA should they have comparable activity. Therefore, we next evaluated the efficacy of WSE in the mouse model of aGvHD. In corroboration with above findings, WSE also showed protection of GvHD target organ and mitigated aGvHD severity without compromising beneficial GvL effect.

In conclusion, our study demonstrated the safety and utility of WA and WSE for the prevention and treatment of aGvHD, this prompted us to initiate two phase 2 clinical trials at our center for aGvHD prophylaxis and therapy.

Synopsis

Homi Bhabha National Institute

SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student:** Saurabh Kumar Gupta
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3. **Enrolment No. :** LIFE09201904009
4. **Title of the Thesis:** Evaluation of oral Withaferin-A for prophylaxis against acute Graft versus Host Disease in murine model of allogenic hematopoietic stem cell transplantation.
5. **Board of Studies:** Life Sciences

SYNOPSIS

Introduction: Bone marrow transplantation (BMT) is the only curative treatment option for patients with relapsed leukemias (1). However, the success of BMT is threatened by high treatment related mortality in the first 100 days of transplantation, with acute graft versus host disease (aGvHD) having a major stake in mortality (2). GvHD is an immunological condition caused by donor T-lymphocytes mounting an immune response against the host antigens leading to widespread tissue damage and functional impairment of internal organs (3,4). Standard GvHD prophylaxis such as calcineurin inhibitors, methotrexate, mycophenolate mofetil, cyclophosphamide, steroids are not always effective in preventing GvHD, as such 40-60% patients

undergoing alloBMT manifest GvHD symptoms (5,6). Therefore, there is an unmet need to develop novel drug candidate against GvHD.

Through our earlier work we demonstrated that *ex-vivo* treatment of donor graft with Withaferin-A (WA) reduces the incidence, severity and mortality associated with aGvHD in murine models of allogenic bone marrow transplantation (7).However, in order to be clinically acceptable, WA's efficacy following oral administration has to be established in mouse model of aGvHD. In addition, it is important to demonstrate that WA would not abrogate the GvL effect of the graft. Therefore, the present study has been proposed to investigate the oral efficacy of WA against aGvHD. As a prerequisite, oral pharmacokinetics, acute and repeat-dose toxicity of WA will also be investigated to determine the bioavailability and safe dose of WA.

Aim and Objectives:

Aim: Evaluation of oral WA for prophylaxis against aGvHD in murine model of allogenic hematopoietic stem cell transplant.

Objectives:

Objective 1. To determine the safety, toxicity and pharmacokinetics of oral WA in mice.

Objective 2. To evaluate the efficacy of Withaferin-A for the prophylaxis of acute aGvHD and compare with standard prophylactic regimens.

Objective 3. To investigate the impact of Withaferin-A on Graft versus Leukemia effect.

Objective 1. To determine the safety, toxicity and pharmacokinetics of oral WA in mice.

Methodology:

Acute toxicity: The acute oral toxicity study was conducted as per the Organisation for Economic Cooperation and Development (OECD) test guidelines 423 adopted on 17th December 2001. A single oral dose of 50 mg/kg, 300 mg/kg and 2000 mg/kg of WA suspended in 100µL of 0.5% carboxymethylcellulose (CMC) in 1X phosphate buffer saline was administered to mice using oral gavage. Mice were sacrificed after 14 days and blood samples was collected for clinical biochemistry and hematological investigation. Vital organs were harvested for histopathology examination.

Sub-acute toxicity: Sub-acute toxicity or 28 days repeat dose toxicity study were performed in accordance with OECD guideline 407 adopted on 3 October 2008. Thirty female mice were divided into six groups (five mice per group); 1. Vehicle control (CMC), 2. 10 mg/kg treatment (low dose), 3. 70 mg/kg treatment (medium dose) 4. 500 mg/kg treatment (high dose), 5. Vehicle control (satellite/recovery groups) and 6. 500 mg/kg treatment (satellite/recovery groups). At the end of the experiment, mice were sacrificed, blood samples were collected for biochemical, hematological investigation and vital organs were harvested for histopathology.

Pharmacokinetics:

Animals for PK study: Female BALB/c mice were used for the PK study. Mice were given single oral dose of 70 mg/kg of WA and their blood samples was collected in EDTA tubes at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 hours (5 mice per time points). To

another set of female BALB/c mice, intravenous injection (IV) of WA was given at dose of 10 mg/kg, blood samples were collected in EDTA tubes at 0.03, 0.17, 0.5, 1, 2, 4, 6, 8, 12, 24 hour (5 mice per time points). The level of plasma WA were quantified by using LC-MS/MS as per in house developed protocol.

Results:

Acute toxicity: WA treated group did not show any clinical symptoms of toxicity compared to the vehicle control group. There was no visible sign of toxicity in any animal in the WA group, and none of the animals died. Based on these findings, WA may be classified as GHS (Globally Harmonized System) category 5 (LD_{50} >2000 mg/kg body weight) compound as per OECD Guideline No. 423, December 2001.

Sub-acute toxicity (28 days repeat dose toxicity): Sub-acute toxicity were performed at dose of 10, 70and 500 mg/kg/day. Mice were dosed daily for 28 days. All animals were alive until necropsy in both the test group and the recovery (satellite) group. None of the animals in the test or recovery groups showed any visible signs of toxicity. These results suggest that oral administration of WA to mice every day for 28 days is safe and well tolerated. Therefore, 500 mg/kg oral WA shall be considered as No-Observed Adverse Effect Level (NOAEL) dose as per OECD Guideline No. 407, October 2008.

Pharmacokinetics of WA: Maximum plasma concentration (C_{max}) of WA were found to be 3996.9 ± 557.6 ng/mL and 141.7 ± 16.8 ng/mL for IV and oral doses respectively. The median T_{max} following oral administration was 0.5 h. Further, the percentage bioavailability of WA was found to be 1.8%.

Objectives 2: To evaluate the efficacy of Withaferin-A for the prophylaxis of aGvHD and compare with standard prophylactic regimens.

Methodology:

Induction of aGvHD and clinical scoring: The complete mismatched model of aGvHD were developed by caring out transplantation between donor C57BL/6 (H-$2K^b$) male and BALB/c (H-$2K^d$) female mice. Following myloablative 6.5 Gy of total body irradiation, BALB/c mice were injected with $15x10^6$ splenocytes and $5x10^6$ bone marrow cells of C57BL/6 mice. All mice were observed closely on a daily basis for GvHD severity. The clinical scoring system was based on the following six criteria; fur texture, skin integrity, posture, activity, weight loss and diarrhoea.

Study groups and intervention: After transplantation, mice were divided into GvHD control and treatment groups. WA and withania somnifera extract (WSE) was administrated orally to the treatment groups from day +1 of transplantation to day +21, at a dose of 1 mg/kg/day for aGvHD prophylaxis and +7 to +27 for treatment of aGvHD. The GvHD control mice were given vehicle (0.5% sodium corboxymethylcellulose). Cyclosporine (CSA) and Methotrexate (MTX) were injected according to the earlier published study (Mehta et al 2020).

Results:

Oral administration of WA and WSE mitigates aGvHD associated morbidity and mortality: aGvHD phenotype as well as clinical score (CS) in GvHD control and WA treated arm was monitored all along the study. WA treated mice showed significant decrease in clinical score (CS) compared to control mice ($P<0.001$). Furthermore, WA

treatment improved survival of mice significantly compared to the GvHD control [HR=0.07 (0.01-0.35); P<0.001]. We further analysed the engraftment of donor cells into WA treated mice and found complete presence of H-2K^b (FITC) positive cells in the host body, suggesting no compromise in donor cell engraftment by WA. Similar finding were observed with WSE as well.

WA and WSE protects GvHD target organ damage: The damage of aGvHD target organ such as liver, skin, SI, colon and lung were assessed. Upon histopathological examination, we observed protection of liver, skin, SI, colon and lung in WA and WSE treated animals compared to the control. WA treated group showed marked decrease in lymphocytic infiltration and had low infiltration score on all three-time points compared to GvHD control group. Similar observation were reported with WSE as well.

WA modulates cytokine secretion in-vivo and from hPBMCs: The cytokine storm is known to play a central role in GvHD and several other immune-pathological conditions. The Th1, Th2 and Th17 inflammatory cytokines were found to be significantly decrease at one or other time point. We next evaluated the effect of *ex-vivo* WA treatment on cytokine secretion from hPBMCs. Cytokines such as IFN-γ, IL-6, TNF-α, IP-10, IFN-L1, GM-CSF and IL-1β were found to be significantly decreased in WA treatment group compared to control group.

WA inhibits JAK2-STAT3 signaling and modulates immune cells phenotype. In order to gain aGvHD prophylactic mechanistic insight of WA, we *ex-vivo* treated the splenic lymphocytes of C57BL/6 mice. Upon western blot analysis the *ex-vivo* treated hPBMCs with WA also showed significant inhibition of pJAK2 and pSTAT3 protein

levels (P<0.01, 0.05 for pJAK2 and pSTAT3 respectively), but no significant difference were seen in tJAK2 and tSTAT3.

Furthermore, we evaluated the effect of *ex-vivo* WA treatment on hPBMCs immune cell modulation. Monocyte subsets, γδT-cells, PD1-CD4 cells and Tim3-CD8 cells were analysed. The absolute monocyte count and absolute classical monocyte count were found to be significantly decreased in WA treatment group compared to control group (P<0.01 and 0.01 respectively). The non-classical monocytes were significantly increased in WA treatment group compared to control group (P<0.05). Furthermore, absolute γδT-cells were found to be significantly increased in WA treatment group compared to control group (P<0.01), while in contrast, the absolute PD1-CD4 and Tim3-CD8 cells were found to be decreased significantly in WA treatment group compared to control group (P<0.01 and 0.05 respectively). Additionally, In presence of WA, the Ki67+ frequency within CD3+, CD4+ and CD8+ cells was found to be significantly decreased compared to control group (P<0.01, 0.01, 0.01 for CD3+, CD4+ and CD8+ cells respectively). Further, no significant difference in percent viability of hPBMCs were observed between control and WA group.

WA exhibit superior efficacy compare to standard prophylactic regimen: We further compared the efficacy of WA with standard prophylactic regimen of CSA+MTX. At day +14, median CS in control, WA, and CSA+MTX group was 7.5, 2 and 4 respectively. At the end of the experiment the percent survival in control, WA and CSA+MTX groups were 0, 83.3, 33.3 respectively. The median survival were 19 and 31 days in control and CSA+MTX group respectively. Interestingly, WA group does not reached median survival.

Oral administration of WA reversed aGvHD symptoms/signs and improve survival of mice. To test the anti-GvHD efficacy of WA, we allowed mice to develop GvHD and then WA was administered from day +7 of transplantation. WA significantly improved the overall survival [HR=0.2 (0.07-08)] and GvHD symptoms of the mice compared to GvHD control ($P<0.01$). Median survival time in GvHD control group was 19.5 days, however, WA group does not reached median survival. Percent survival in GvHD control and WA group were 0% and 58.3%. Similar findings were seen with WSE treatment as well

Objective 3: To investigate the impact of Withaferin-A on Graft versus Leukemia effect.

Methodology:

Development of GvL model: To assess the consequence of WA treatment on GvL effect, following total body radiation (6.5Gy) BALB/c recipient mice were transplanted with A20 ($3x10^6$ cells) alone or A20+ bone marrow transplantation (BMT) with $15x10^6$ splenocytes and $5x10^6$ bone marrow cells. Animals were divide in following three groups; [1] A20 [2] A20+BMT [3] A20+BMT+WA. The GvL effect were monitored as reported by Zhang et. al. and Snyder et. al. previously. Briefly, leukemic death was defined by the occurrence of hind-leg paralysis, however animal died due to clear sign of aGvHD but absence of leukemia (hind leg paralysis) were considered death due to aGvHD. All mice were monitored on daily basis for hind leg paralysis and aGvHD clinical symptoms. The day mice developed hind leg paralysis and become mortally ill, they were humanely sacrificed. At the end of the experiment, time to onset of leukemia (based on hind leg paralysis) and survival were evaluated.

Further, liver tissue were evaluated for any sign of tumor nodules or aGvHD associated findings to differentiate death either by leukemia or by GvHD.

Results: WA treatment preserves beneficial GvL effect of the graft: We observed that mice transplanted with A20 cells died due to leukemia as evidenced by development of hind leg paralysis. However, Mice received WA does not showed sign of leukemia. Similarly, mice transplanted with BMT+A20 does not showed sign of leukemia but all of them eventually died by GvHD confirmed with presence of aGvHD symptoms. As A20 cells are tend to home in liver and form tumor nodules, therefore, we evaluated histopathology of liver to differentiate death due to leukemia or GvHD. Histopathology of the liver tissue from A20 group showed multiple nodular deposits of a high grade malignant tumor composed of large cells with moderate to marked nuclear atypia, brisk mitosis including atypical forms and scant eosinophilic cytoplasm. However, liver tissue of A20+BMT group does not showed any sign of tumor but presence of mild to moderate chronic inflammation in the portal area, representing the GvHD sign. Interestingly, Liver tissue of A20+BMT+WA group neither showed any sign of tumor nor GvHD. This confirms WA does not hamper GvL response.

Conclusion:

1. Our results suggest that WA is safe at doses of 2000 mg/kg and 500 mg/kg in acute and repeat dose toxicity respectively and found to be orally bioavailable.

2. We further established that, WA and its formulation abrogates aGvHD manifestation and preserves GvL effect of the graft. Therefore, WA could be a potential treatment option for patients with aGvHD, and its efficacy either alone or in combination with standard regimens is currently under investigation in a prospective clinical trial.

References:

[1] J.L. Ferrara, J.E. Levine, P. Reddy, E. Holler, Graft-versus-host disease, Lancet. 373 (2009) 1550–1561.

[2] B.R. Blazar, W.J. Murphy, M. Abedi, Advances in graft-versus-host disease biology and therapy, Nat. Rev. Immunol. 12 (2012) 443–458. https://doi.org/10.1038/nri3212.

[3] L.A. Welniak, B.R. Blazar, W.J. Murphy, Immunobiology of Allogeneic Hematopoietic Stem Cell Transplantation, (2007).

[4] L. Qian, Z. Wu, J. Shen, Advances in the treatment of acute graft-versus-host disease, J. Cell. Mol. Med. 17 (2013) 966–975.

[5] L. Hill, A. Alousi, P. Kebriaei, R. Mehta, K. Rezvani, E. Shpall, New and emerging therapies for acute and chronic graft versus host disease , Ther. Adv. Hematol. 9 (2018) 21–46.

[6] S. Singhal, U. Vourka-Karussis, J. Mehta, S. Slavin, L. Weiss, Failure of cyclosporine to induce graft-vs-host disease or graft-vs-leukemia after syngeneic bone marrow transplantation in mice, Leuk. Res. 20(1996).

[7] M. Mehta, D. Gohil, N. Khattry, R. Kumar, S. Sandur, D. Sharma, R. Checker, B. Agarwal, D. Jha, A. Majumdar, V. Gota, Prevention of acute graft-versus-host-disease by Withaferin a via suppression of AKT/mTOR pathway, Int. Immunopharmacol. 84 (2020) 106575.

Publications in Refereed Journal:

a. Published

1. Gupta SK, Gohil D, Dutta D, Panigrahi GC, Gupta P, Dalvi K, Khanka T, Yadav S, Kaushal RK, Chichra A, Punatar S. Withaferin-A alleviates acute graft versus host disease without compromising graft versus leukemia effect. International Immunopharmacology. 2023 Aug 1;121:110437.

2. Gupta SK, Jadhav S, Gohil D, Panigrahi GC, Kaushal RK, Gandhi K, Patil A, Chavan P, Gota V. Safety, toxicity and pharmacokinetic assessment of oral Withaferin-A in mice. Toxicology Reports. 2022 Jan 1;9:1204-12.

b. Accepted: NA

c. Under preparation: **Gupta SK,** Gohil D, Momin MB, Yadav S, Chichra A, Hingorani L, Khattry N, Gota V. *Withania Somnifera* extract mitigates experimental acute Graft versus Host Disease without abrogating Graft versus Leukemia effect, (2023).

Other Publications:

1. Gupta SK, Gohil D, Panigrahi GC, Vaykar S, Rane P, Chavan P, Gota V. Comparison of different autoanalyzers for the determination of lymphocyte and neutrophil counts in mouse blood. Drug Metabolism and Personalized Therapy. 2021 Dec 1;37(2):219-22.

2. Gohil D, Panigrahi GC, Gupta SK, Gandhi KA, Gera P, Chavan P, Sharma D, Sandur S, Gota V. Acute and sub-acute oral toxicity assessment of 5-hydroxy-1, 4-naphthoquinone in mice. Drug and Chemical Toxicology. 2022 Jul 27:1-4.

a. Book/Book Chapter: NA

b. Conference/Symposium

1. I participated in 46th Annual Conference of the Indian Immunological Society, Immunocon, at BARC, Mumbai, India in 2019

2. I presented a poster in 17th National Research Scholar Meet, at ACTREC, Navi-Mumbai, India in 2021

3. I participated in AAZPIRE workshop on Clinical Research Methods, at ACTREC, Navi-Mumbai, India in 2021

4. I won the "Young Ethnopharmacologist Award" for Best oral presentation in 9th International Congress of Society for Ethnopharmacology, at JSS college of Pharmacy, Mysore, India in 2022

5. I won the "Best E-poster Presentation Award" in 1st Global Cancer Consortium, held as a virtual event in 2022

6. I participated in 1st Series of Advancement in Cell Therapy at ACTREC, Navi-Mumbai, India in 2022

7. I presented a poster in 27th congress of Asia Pacific Bone Marrow Transplantation at Kochi, India in 2022

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Chapter 1

Introduction and Review of Literature

1.1 Introduction of Graft versus Host Disease (GvHD): Allogeneic hematopoietic stem cell transplantation (alloHSCT) is been carried out with an intent to cure underlying haematological conditions such as leukemias (1,2). The ability of donor graft to identify and eradicate leukemic cells is known as graft versus leukemia (GvL) response, which is the beneficial outcome of alloHSCT (3). However, the way donor graft attacks leukemic cells, in similar manner it also attacks on host tissues as well. This phenomenon of host tissue damage by donor graft is known as graft versus host disease (GvHD) (3). In simple terms, GvHD is defined as reaction of donor immune cells over the host tissues (fig 1.1) (4). In 1956, Barnes and his colleagues identified that the alloHSCT recipient are less prone to develop relapse, but they died of secondary disease or wasting syndrome. Which is later known as GvHD (4,5). Further, in 1966, Billingham suggested three hallmarks of GvHD (7):

1. Presence of immunologically active cells in graft.
2. Recipient must be immunologically incompetent to reject the graft.
3. There should be disparity in histocompatibility between donor and the recipient.

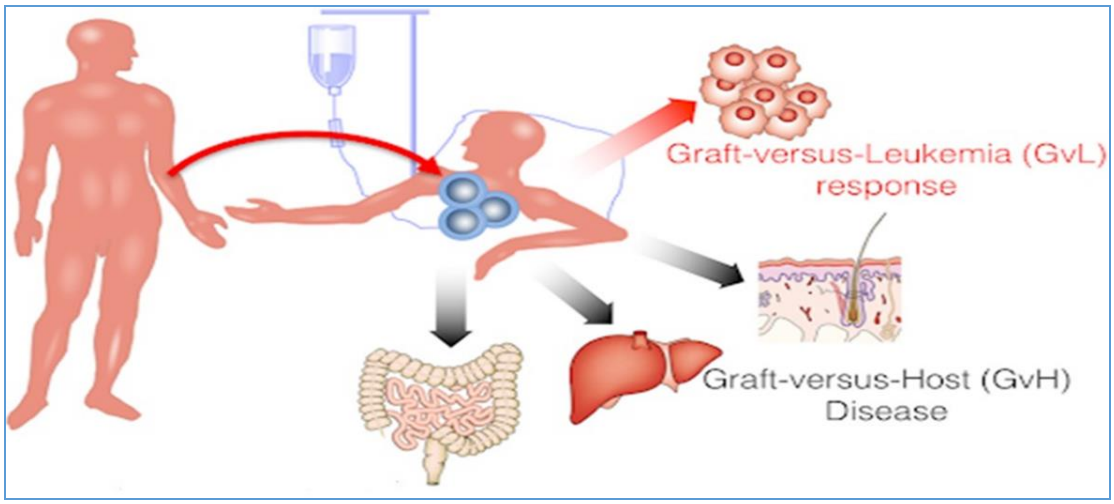


Figure 1.1: Concept of post-transplant GvHD vs GvL.

1.2 Classification of GvHD:

GvHD is mainly classified as acute and chronic. Earlier, classification of acute and chronic GvHD was based on the days when the symptoms appears. If the GvHD symptoms appears within 100 days of transplantation, it was considered as acute GvHD (aGvHD) (8). If it happens post 100 days of transplantation, it was said to be chronic GvHD (cGvHD) (8). This definition was recently revised by the National Institute of Health (NIH). The new classification is based on the organ involvements, this has also added a new class of GvHD called overlap syndrome, which is defined by the occurrence or persistence of acute GvHD post 100 days of transplantation (9). Old and new way of discriminating acute and chronic GvHD is mention in table 1.1.

Table 1.1: Classification of GvHD.

Category	Time of Symptoms	aGvHD Features	cGvHD Features
Classic acute	≤100 days	✓	✗
Persistent, recurrent or late-onset acute	>100 days	✓	✗
Classic chronic	—	✗	✓
Overlap syndrome	—	✓	✓

1.3 Acute GvHD: Acute GvHD (aGvHD) represents the most common cause of non-relapse mortality post-transplantation. It usually appears within the first 100 days of transplantation and mainly present with the involvement of skin (81%), gut (54%) and liver (50%) (10). A detailed clinical manifestation, stages/grading of aGvHD is mentioned in table 1.2 and figure 1.2.

Table 1.2: aGvHD grades and organ involvement.

Stage/Grade	Skin involvement	Liver involvement	Gut involvement
0 (none)	No rash or other skin involvement	No elevation of liver enzymes or other gut symptoms	No diarrhea
I (mild)	Rash covering < 25% of body surface area	Bilirubin < 2 mg/dL or 2-3 times upper limit of normal (ULN)	Diarrhea < 1 liter/day above baseline
II (moderate)	Rash covering 25-50% of body surface area	Bilirubin 2-3 mg/dL or 3-5 times ULN	Diarrhea 1-1.5 liters/day above baseline
III (severe)	Rash covering > 50% of body surface area with blistering/desquamation	Bilirubin 3-6 mg/dL or > 5 times ULN	Diarrhea > 1.5 liters/day above baseline
IV (Life Threatning)	Generalized erythroderma with bullous formation and/or involvement of face, palms, or soles.	Bilirubin > 6 mg/dL or refractory to treatment.	Profound diarrhea with ileus, GI bleeding, or perforation.

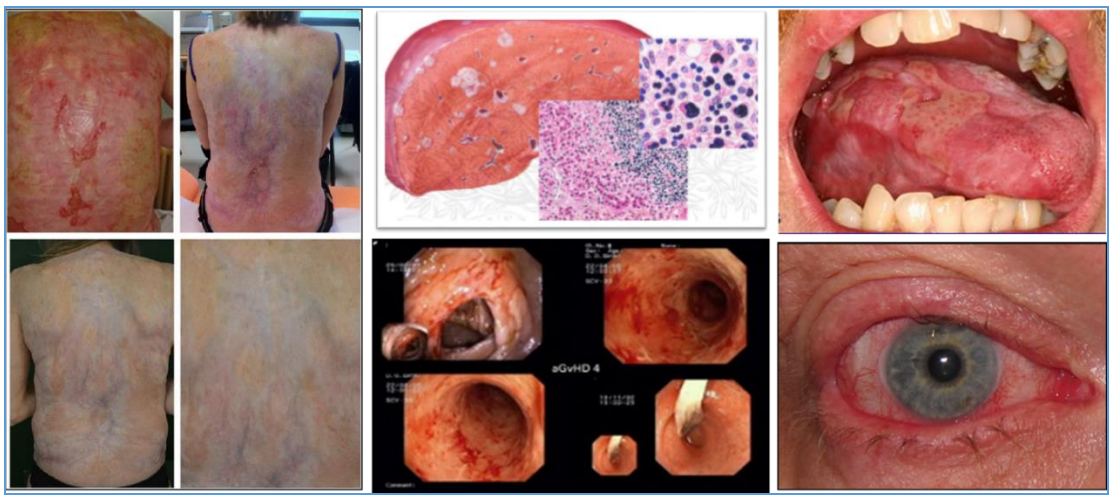


Figure 1.2: aGvHD symptoms and clinical manifestations in patients.

1.4 Epidemiology of aGvHD:

As per recent report by Centre for International Blood and Marrow Transplant Research (CIBMTR) and Hill et al., every year approximately 30,000 patients are undergoing alloHSCT (4). Out of which 40-50% patients are tend to develop aGvHD in case of matched donor. This incident rate extents up to 60% in unmatched transplantations (8). Steroid being the first choice of treatment for aGvHD, unfortunately close to 50% patients experience steroid refractory (2).

1.5 Risk Factor: The major risk factors associated with GvHD is as fallow:

A. Human leukocyte antigen (HLA) disparity

B. Patient age

C. Intensity of conditioning regimens

D. Female to male transplantation

E. Selection of GvHD prophylaxis regimens

F. Source of graft

1.5.1 Human leukocyte antigen (HLA) disparity: HLA are closely linked cluster of genes, which encodes the cell surface receptor proteins those are involves in self and non-self recognition of tissues. These genes are located on chromosome number 6 and 17 in human and mouse respectively. Mouse HLA are known as major histocompatibility antigen (MHC). MHCs are classified in three groups (11).

1. MHC class I: These molecules are present on all nucleated cells. The major MHC class I genes are HLA-A, HLA-B and HLA-C. These surface receptors recognise and binds to CD8+ receptors on T-cytotoxic cells and present endogenous antigens to these cells (11).

2. MHC class II: These class of genes are mainly present as surface receptor on antigen presenting cells (APCs). Major MHC II genes are HLA-DR, HLA-DQ and

HLA-DP. They recognize and binds to CD4+ T-helper cells and present exogenous antigens to them (11).

3. MHC class III: These class of genes do not involve in antigen recognition. There structure and functions are yet to be fully understood (11).

1.5.1.1 Role of MHC in GvHD: During the inception of GvHD, donor HLA recognises recipient antigens as foreign that ignite the activation of immune cells against host tissue and results in multi organ destruction. Therefore, HLA matched sibling remains the ideal donor for alloHSCT. However, for patients who unfortunately do not have matched sibling donors, matched unrelated donor is taken into the consideration for successful transplantation (12).

In a recent retrospective analysis of 10,035 patients having grade 2-4 aGvHD, Fuzi et al. demonstrated that HLA mismatched at more than 2 loci is associated with inferior survival outcomes (13). However, these risks can be avoided with the use of appropriate prophylaxis backbone and careful selection of graft source (12).

1.5.2 Patient age: Several findings suggested that older patients are at high risk of developing GvHD (14) (15). The exact phenomenon behind this is not so clear, but it is pertinent that older patients produce more danger signal including inflammatory cytokines due to exposure to conditioning regimens. Which can have direct hazardous effect on older patients. In addition, the old tissues are known to express more MHCs and are capable of elicit the excessive immune response (16). Other reason could be the failure of spleen to carry out the positive and negative selection of lymphocytes in older patients(15).

1.5.3 Intensity of conditioning regimens: The intensity of conditioning regimens used for myeloablation plays a crucial role in GvHD severity. Low dose or non-myeloablative chemo or radio-conditioning regimens are less likely to cause severe

GvHD, but limited by risk of graft rejection and high chances of relapse. However, high dose myeloablative chemo or radio-conditioning regimen ensures minimal chance of graft rejection and relapse, but this risk the patients for severe GvHD (16,17).

The association of GvHD severity with intensity of chemo/radio-conditioning regimens are well established (discussed later in this chapter; section 1.6). Briefly, a high intensity conditioning regimens can leads to the significant damage to organs, thereby release of more danger signals. These signals strongly activates the antigen presenting cells (APCs) for allo-reactivity which results in severe GvHD (4).

1.5.4 Female to male transplantation: A study by Randalph et al. on 3238 transplanted patients suggested that transplantation between female donor to male recipients are associated with higher risk of GvHD but lowest risk of relapse (19). Furthermore, in corroboration with this, Kim et al. reported the similar finding in a prospective study having a cohort of 11,797 patients (20). Both of these studies anticipated the involvement of minor MHC antigen behind this phenomenon (20,19). The donor T-cells are specific for recipient minor antigens encoded by Y-chromosomes, hence these T-cells exerts potent GvL activity with significant GvHD, which explains why female donor to male recipients are less prone to relapse but at risk of GvHD (19).

1.5.5 Selection of GvHD prophylaxis regimens: The GvHD prophylaxis backbone is mainly consist of calcineurin inhibitors (CNIs) such as cyclosporine (CSA) and/or tacrolimus (TAC) in combination methotrexate (MTX). Combination of CSA+MTX or TAC+MTX gives almost equal benefit but use of TAC is limited due to its toxicity. Therefore, CSA+MTX remains gold standard prophylaxis regimens for aGvHD (21). However, despite use of several other combination strategies the outcome of GvHD

remains poor (22). Prophylaxis backbone is not a direct risk factor for GvHD, but it is indeed important to choose right drug for better prophylaxis of GvHD (22). A details of drugs used for GvHD prophylaxis and its clinical outcomes are mentioned later in this chapter (section 1.7).

1.5.6 Source of graft: Several studies have compared the severity of GvHD following transplantation using various graft source including peripheral blood stem cells (PBSCs), cord blood stem cells and bone marrow (BM) (18). In a multicentre, phase III randomised trial Anasetti et al. compared the overall survival and GvHD incident between PBSCs and BM transplanted group. The result of this study suggested an increase incident of cGvHD in PBSCs transplanted group compared to BM (53% vs 41%). Interestingly, incident of aGvHD does not differ between both the groups. But, the survival were better in PBSCs group compared to BM (51% vs 46%) (23). In consistence with this study, Flowers et al. also reported the greater incidence of cGvHD in patients transplanted with PBSCs compared to BM, but no difference in aGvHD incident between two groups were seen (15). Gupta et al. demonstrated that infusion of high total nucleated cells are associated with severe grade of GvHD (24). Chen et al. reported that patients received cord blood transplantation had higher risk of aGvHD (39%) than the cGvHD (27%) (25). Hence, choice of graft shall be considered before transplantation to decrease post-transplant complications mainly GvHD.

1.6 Pathophysiology of aGvHD: aGvHD pathophysiology is broadly defined in three phases as illustrated in figure 1.3 (25,26,13).

A. Phase 1. Conditioning regimens mediated damage of tissues and activation of antigen presenting cells

B. Phase 2. Donor T-cells activation, differentiation and migration to target organ (Afferent phase)

C. Phase 3. Cellular and inflammatory effector phase (Efferent phase)

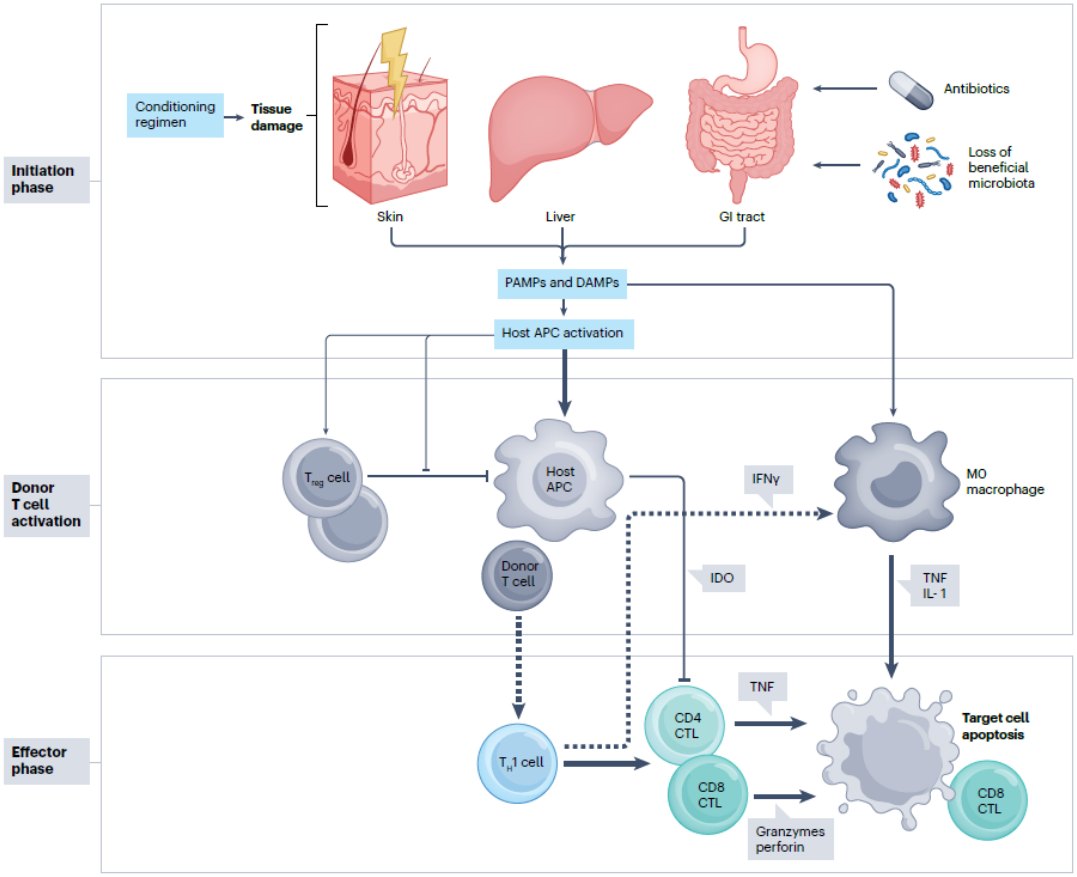


Figure 1.3: Pathophysiology of acute graft versus host disease.

1.6.1 Phase 1: Conditioning mediated damage of tissues and activation of antigen presenting cells: Due to high dose chemo or radio-conditioning regimens, host tissues undergoes damage. These damage tissues release danger signals in form of cytokines storm, pathogen associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides (LPS) from gut and danger associated molecular patterns (DAMPs) such as uric acid and ATP (10). These signals activates the host antigen presenting cells (APCs) (figure 1.3). This also explains why intensity of conditioning regimes is associated with severity of GvHD (28). The extent of tissue damage from conditioning regimes activates downstream cascade of events and controls the

magnitude of GvHD. This has led to the beginning of reduced intensity conditioning era and use of low dose cyclophosphamide (29).

1.6.2 Phase 2: Donor T-cells activation, differentiation and migration to target organ (Afferent phase): The activated APCs in phase 1 reaction interact with donor T-cells and triggers their proliferation, differentiation and migration of these cells to GvHD target organs mainly; liver, gut, skin and lung (7) (figure 1.3). This also hints T-cells as major culprit behind GvHD. A study by Ordemann et al. showed higher magnitude of GvHD in older mice due to enhanced allo-reactivity by APCs (30). This explains why older recipient are at higher risk of developing severe GvHD. The donor and recipient HLA dependent event and activation of type of T-cells is elaborated in section 1.5.1.

The activated and differentiated Th1, Th2 and Th17 cells secretes excessive cytokines, which acerbate the GvHD progression. Principle T-cell cytokines are IL-2, IFN-γ, TNF-α (Th1), IL-4, IL-6 (Th2) and IL17A (Th17) (31).

1.6.3 Phase 3: Cellular and inflammatory effector phase (Efferent phase): This phase is also defined as cell death/target organ damage phase, wherein cytotoxic T-lymphocytes and other killer cells such as NK cell mediates target cell lysis via perforin/granzyme and Fas/FasL pathway (6,31). The concept of perforin mediated cell death in GvHD were demonstrated by Baker et al., wherein, transplantation of perforin deficient T-cell to host does not induced target cell damage (33). Similar, findings were reported when granzyme-B deficient T cells was transplanted in experimental aGvHD model (34). Further, few studies have design handicapped immune effector cells with single deficient T-cells (only FasL) or double deficient (FasL +perforn/granzyme) T-cells. These cells could not elicit the immune response against host organs in preclinical studies (34,35). Therefore highlighting the

importance of immune effector cells mainly T-cells in onset and progression of aGvHD.

NK cells, having licence to kill, play a crucial role in aGvHD and GvL as well. NK cells harbour potent GvL response and insures less aGvHD by targeting the host APCs. It also decreases the chance of graft rejection through killing of host T-cells. Currently NK cells expressing chimeric antigen receptors (CAR) are under investigation to mitigates GvHD and improve GvL (37).

The opportunity to combat aGvHD lies in each of above steps wherein blockade of APCs, T-cell proliferation, differentiation and cytokine storm can be useful approach to mitigate GvHD. In this regards several groups have tried to control GvHD by blocking one or many above events using drugs which is listed in table 1.3 and 1.4.

1.6.4 Molecular events in manifestation of aGvHD: Several signaling cascade and cross talk between them orchestrates the pathogenesis of aGvHD. One of the key pathway that play a vital role in the onset and progression of aGvHD is JAK/PI3K/NF-kB/Akt/mTOR axis (fig 1.4A) (38). JAK2-STAT3 signaling are central to all three phases of aGvHD mentioned above (fig 1.4B). Activation of JAK2 in immune cells leads to the activation of genes responsible for proliferation, differentiation and cytokine secretion mediated through pSTAT3 or the PI3K/Akt/mTOR/NF-Kb axis (fig 1.4A-B) (38,39). Thus, any intervention which could inhibit this cascade can be potentially useful in the prevention and treatment of GvHD. In this regards, Betts et al. demonstrated that either pharmacological or genetic disruption of JAK2 results in decreased morbidity and mortality of bone marrow transplanted mice without compromising GvL effect (38). JAK-STAT inhibitors such as ruxolitinib, nifuroxazide,

SHR0302, tofacitinib have shown therapeutic effect in mouse models of acute and chronic GvHD (40,41,42,43). Ruxolitinib, the first FDA approved drug against acute GvHD is JAK2-STAT3 inhibitor (45). FDA approved this in 2019 for steroid refractory GvHD (46). However, considering the role of JAK2-STAT3 involvement in every step of GvHD, several trial are currently investigating the use of ruxolitinib for prophylaxis of aGvHD as well (47).

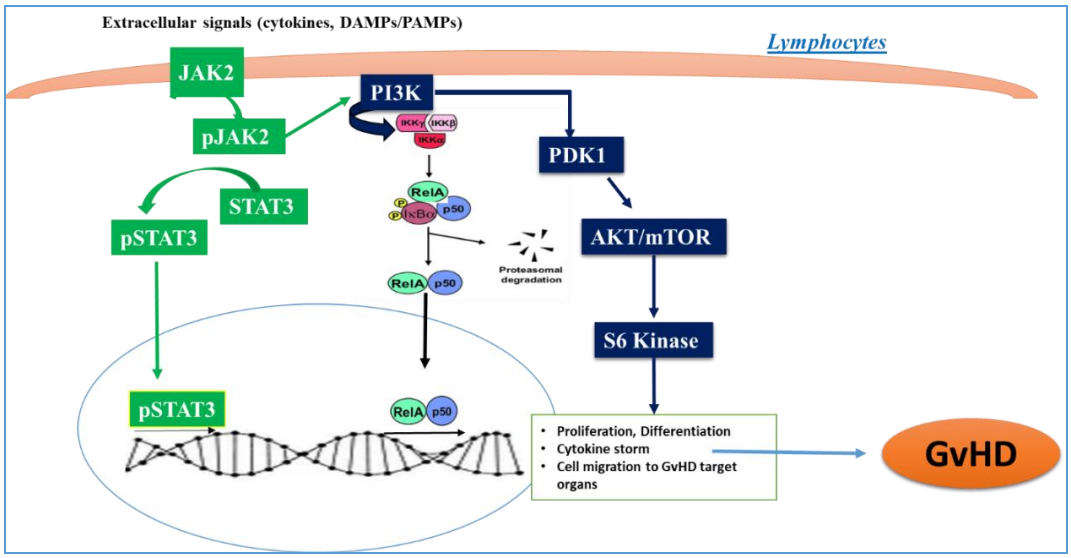


Figure 1.4A: Role of JAK2-STAT3 signaling in pathogenesis of aGvHD.

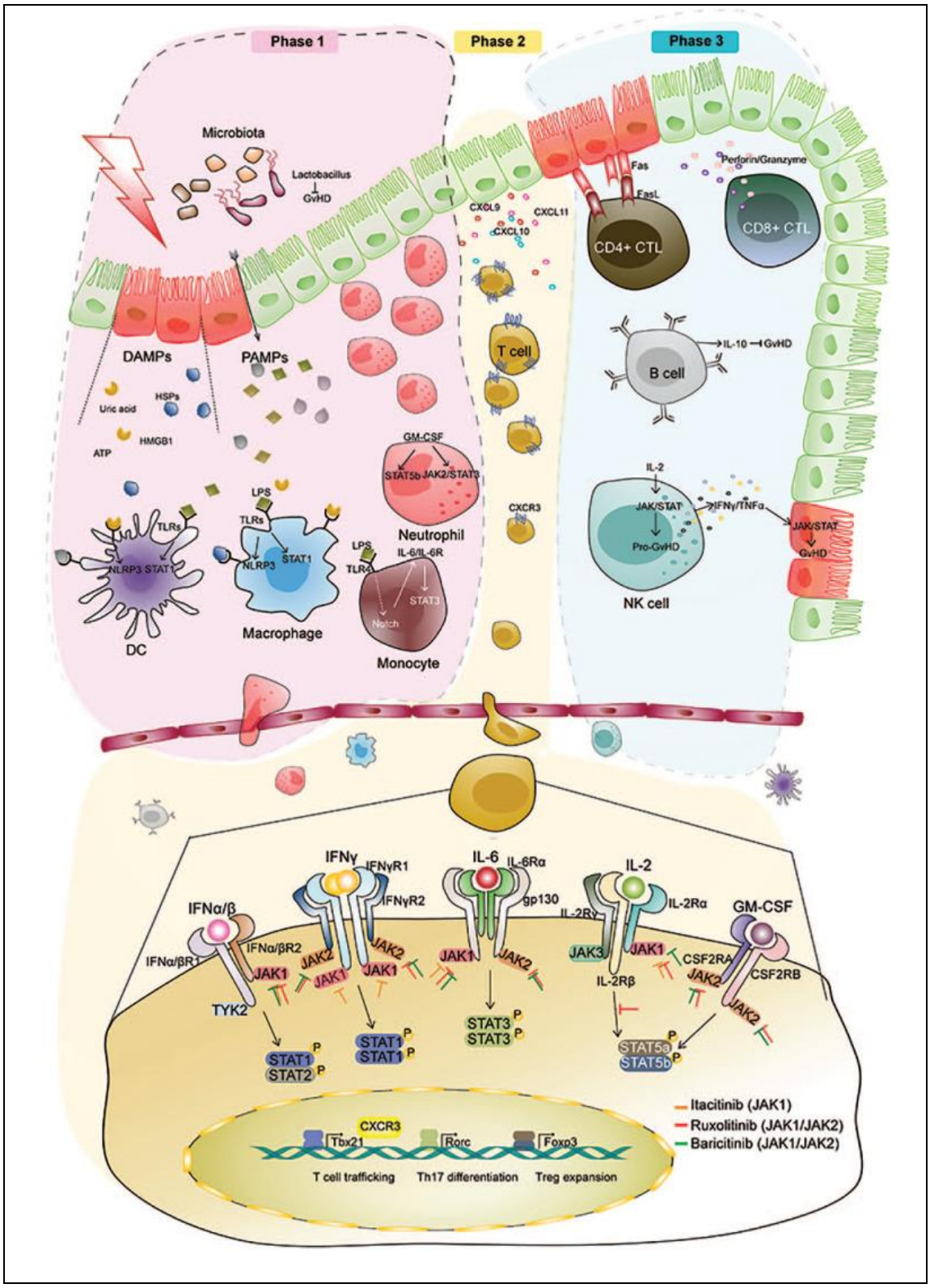


Figure 1.4B: Role of JAK2-STAT3 signaling in all three phases of aGvHD pathogenesis.

1.7 Current standard of care for aGvHD: The key to prevent or treat GvHD is suppression of overzealous allo-reactive immune cells. The most commonly used drug for prevention and treatment of GvHD is CNI+MTX and steroid respectively (21,8). The detailed list of drugs used against aGvHD including its mechanism of action is mentioned in figure 1.5, table 1.3 and 1.4.

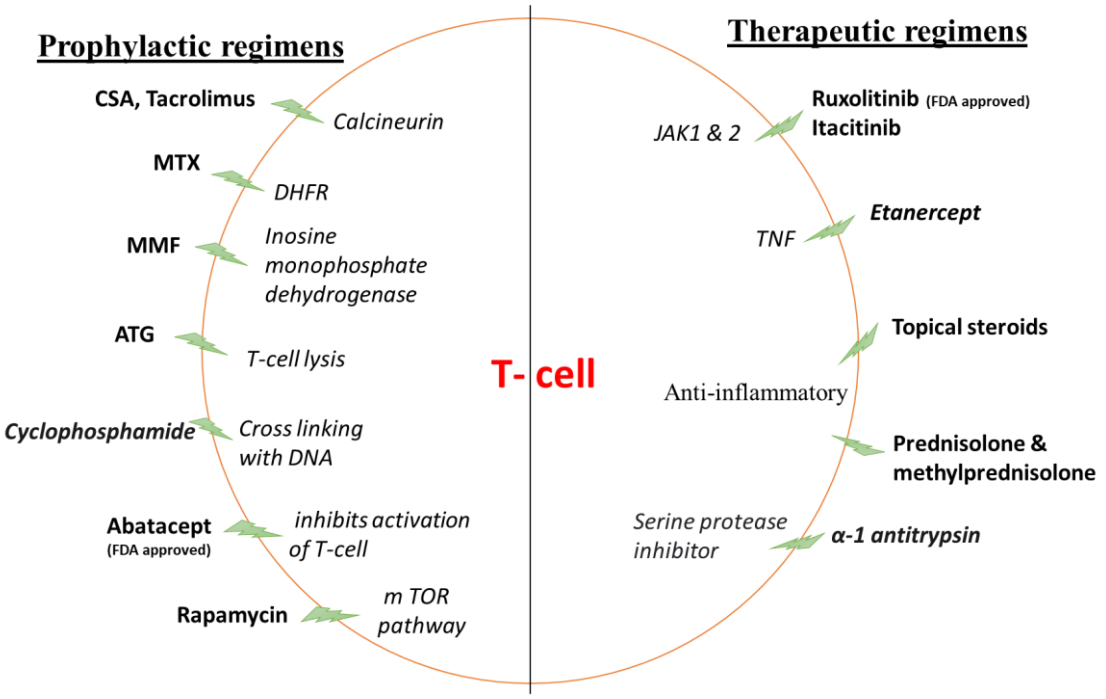


Figure 1.5: aGvHD prophylaxis and therapeutic agents and their mechanism of action.

In 1988 storb et al. demonstrated the clinical superiority of CSA+MTX compared to CSA alone for the prevention of aGvHD and survival benefit to the patients (17). A decade later Ratanatharathorn et al. compared tacrolimus (TAC)+MTX vs CSA+MTX in a phase III study. They found the superiority of TAC+MTX compared to CSA+MTX (31.9% vs 44.4%) for the prevention of aGvHD. In contrast to aGvHD, overall survival (50.4% vs 40.5%) and disease free survival rate (57.2% vs 46.9%) were higher in CSA group compared to that of TAC. TAC related toxicity were also found to be higher in the TAC+MTX cohort (17). This study led to the belief

CSA+MTX as gold standard prophylaxis regimens. Further, several other immunosuppressant have been tested in combination with known prophylactic regimens or with newer pharmacological agents, some of these interventions are mentioned in the table 1.3 (25,47,48,20). Recently, FDA approved first aGvHD prophylaxis drug, abatacept (co-stimulatory inhibitor of T-cells) based on a phase II results from Watkins et al. (22). This study was a randomized, double blind placebo-control trial with CNI+MTX+abatacept in one arm and CNI+MTX+placebo in other arm and concluded a significant decrease in grade 3-4 GvHD in abatacept arm compared to placebo (22).

Steroid remains the gold standard for the treatment of aGvHD. Recently, FDA approved ruxolitinib (JAK1-JAK2 inhibitor) for the treatment of steroid refractory aGvHD. Considering the role of JAK2-STAT3 signaling in onset and progression of aGvHD, several clinical and non-clinical studies are ongoing to establishing the role of ruxolitinib for prophylaxis of aGvHD as well. Other novel strategies for treatment of aGvHD is mentioned in table 1.4 (49,25,47,48,20).

Table 1.3: Novel pharmacological agents for aGvHD Prophylaxis.

Prophylactic interventions	**Molecular mechanism**
PTCy	DMA alkylating agent
Vedolixumab	Target α4β7 integrin on lymphocytes
Alpha-1 antitrypsin	Serine protease inhibitor
Statins	Hamper the development of Th-1 pro-inflammatory lymphocytes, stimulate Treg proliferation, and suppress APC function.

Vorinostat	HDAC inhibitor, suppress histone deacetylation, leading to diminished inflammatory cytokines, enhanced Treg efficiency and a decrease in GVHD cases with intact GVL activity
Tregs	Limiting the response of allo-reactive T cells
Selective deletion of T-cells	Allo-reactive T-cell eradication and keeping NK cells and γδT cells intact.
Tocilizumab	Specifically targets the receptor of IL-6 and suppresses downstream inflammatory responses.
Abatacept	CD28: CD80/86 co-stimulation blockade suppresses T cell-mediated immune responses.
JAK inhibitors (itacitinib, ruxolitinib)	Diminishing cytokine levels, T-cell activity, and preserves Tregs and GVL potency.
TAC	Inhibits calcineurin
ATG-F	Selective depletion of T-cells in the donor graft
MTX	Inhibition of dihydrofolate Reductase
Sirolimus	Inhibition mTOR signaling
MMF	Inhibition of lymphocyte proliferation through depletion of guanosine nucleotides
CSA	Inhibits calcineurin activity and T-cell activation by binding to cyclophilin.

Table 1.4: Novel pharmacological agents for the treatment of aGvHD.

Therapeutic interventions	**Molecular mechanism**
JAK inhibitors (ruxolitinib, itacitinib)	Suppression of pro-inflammatory cytokines, T-cell activation, preservation of Tregs, and maintenance of GvL
a-1 antitrypsin	Serine protease inhibitor
Sirolimus	mTOR inhibition
Natalizumab and Vedolizumab	Inhibtion α4-integrin inhibition
Mesenchymal stromal cells	Target activated lymphocytes mainly B and T
IL-22	Strengthening of epithelial barrier function and tissue repair
Prednisolone	Suppression of pro-inflammatory cytokine production and inhibition of T-cell activation and migration
Methylprednisolone	Immunosuppressive Effects on B-cells and other immune cells
Itolizumab	CD6-ALCAM pathway
Natalizumab	Target α4β7 integrin on lymphocytes
RIP1	By inhibiting RIP1 mediated apoptosis
JAK inhibitors (ruxolitinib, itacitinib)	Suppression of pro-inflammatory cytokines, T-cell activation, preservation of Tregs, and maintenance of GvL

a-1 antitrypsin	Serine protease inhibitor
Sirolimus	mTOR inhibition
IL-22	Strengthening of epithelial barrier function and tissue repair
Prednisolone	Suppression of pro-inflammatory cytokine production and inhibition of T-cell activation and migration
Methylprednisolone	Immunosuppressive Effects on B-cells and other immune cells
Itolizumab	CD6-ALCAM pathway
Natalizumab	Target α4β7 integrin on lymphocytes
RIP1	By inhibiting RIP1 mediated apoptosis
Treatment of steroid refractory GvHD	
Ruxolitinib	JAK2-STAT3 inhibitor
Apraglutide	GLP-2 analogue
BET inhibitor	Inhibition of inflammatory genes
Neiulizumab	PSGL-1 (target T-cell migration)

1.8 Limitations of current standard of care for acute GvHD prevention and treatment: Acute GvHD prophylactic or/and treatment agents are not always effective due to various reasons as listed below (49,50,20,51,1):

1. These drugs (current standard of care) have a response rate of approximately 50% patients. This poor outcome is due to associate high-grade clinical severity. Detailed of toxicity related to standard drugs are mentioned in table 1.5.

2. These drugs also increases the chance of opportunistic infections.

3. They sometime compromise the GvL effect of graft.

4. Steroids only response in approximately 35-50% aGvHD patients, rest develops steroid refractory. Ruxolitinib approved for steroid refractory, but this have various toxicities and is also an expensive drug making it unaffordable to a majority of patients in resource limited countries.

5. Despite all available options, 35-50% patients are still at stake of developing aGvHD.

Table 1.5: Communally used drugs in GvHD and their toxicity.

Drugs	Toxicity
Methotrexate	Nausea, stomach pain, diarrhea, hair loss, chills, headache
Cyclosporin, Tacrolimus	Tremor, kidney damage, hypertension, infection, headache, nausea
Ruxolitinib	Liver damage, dizziness, breath shortening
Abatacept	Diarrhea, stomach pain, dizziness
Anti-thymocyte globulin	Headache, fever, anaphylaxis, infection
Rapamycin	Peripheral edema, hypertension, thrombocytopenia
Steroids	Glaucoma, high BP, edema, delirium

1.9 Need of the hour: Graft versus Host Disease (GvHD) is the most lethal complication of alloHSCT. Current standard of care are associated with significant toxicities, and response only in 35-50% cases. Hence, there is an unmet medical need to develop a novel drug with limited toxicity and a complementary mechanism of action with existing drugs, which shall also preserve the GvL efficacy of the graft.

1.10 Withaferin-A

Withaferin-A (WA) is the most biologically active constituent of *withania somnifera,* also known as Ashwagandha or Indian winter cherry. Structurally, WA is a steroidal lactone. Lactone is the major pharmacophore of WA, which is responsible for most of its biological activity. WA was first isolated in 1962 by Lavie and Yarden. Chemical structure of WA is given in figure 1.6 (53).

Almost all part of Ashwagandha contains WA, but root is thought to be richer source of WA. WA is well documented for its activity in cancer, inflammation, immune-modulation and organ protection (53). A detailed biological role of WA is depicted in figure 1.7.

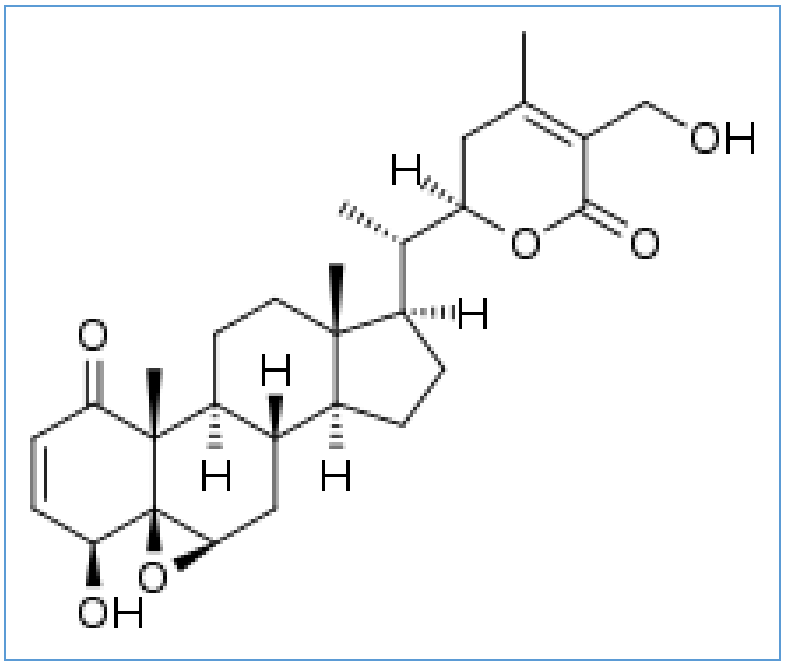


Figure 1.6: Chemical structure of withaferin-A.

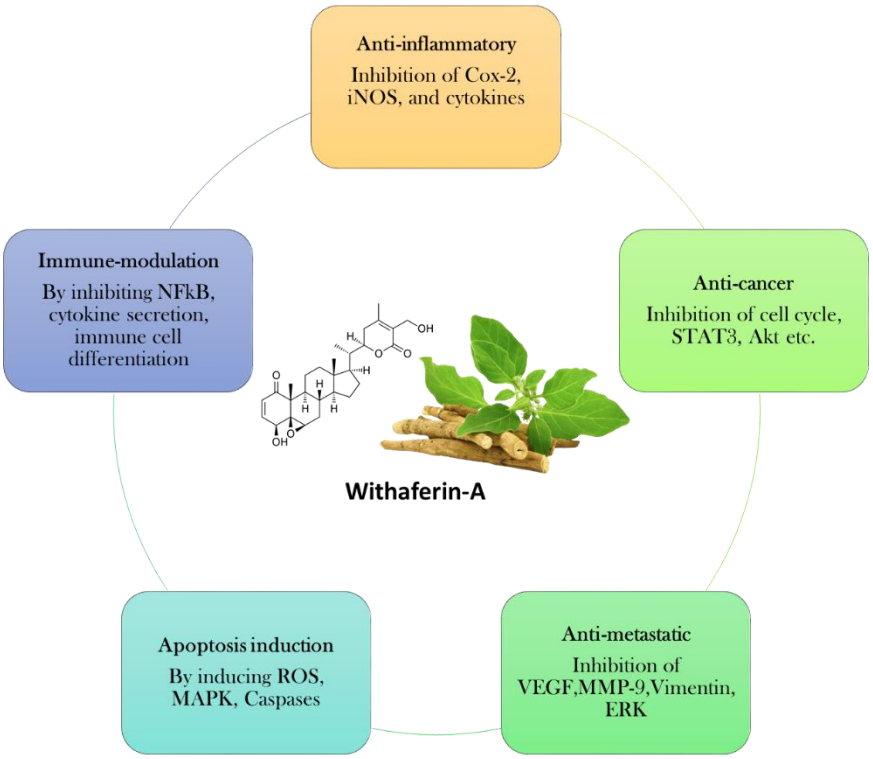


Figure 1.7: Various biological activity of withaferin-A.

1.10.1 Anti-proliferative and anti-cancer activity of WA:

WA has proven to be efficacious in several cancers including prostate, leukemias, gynaecological, gastrointestinal and other malignancies (fig 1.8) (54). WA induced cancer cell apoptosis by arresting cell cycle progression at G2/M phase. Several molecular incidences have been proposed behind its anti-proliferative mechanism, which involves inhibition of NFkB, Akt, mTOR and other key molecular signatures. Modulation of reactive oxygen species is also one of the key events that happens as a results of altered cellular signaling by WA (54). A detailed anti-cancer mechanism by WA is represented in figure 1.9 (54).

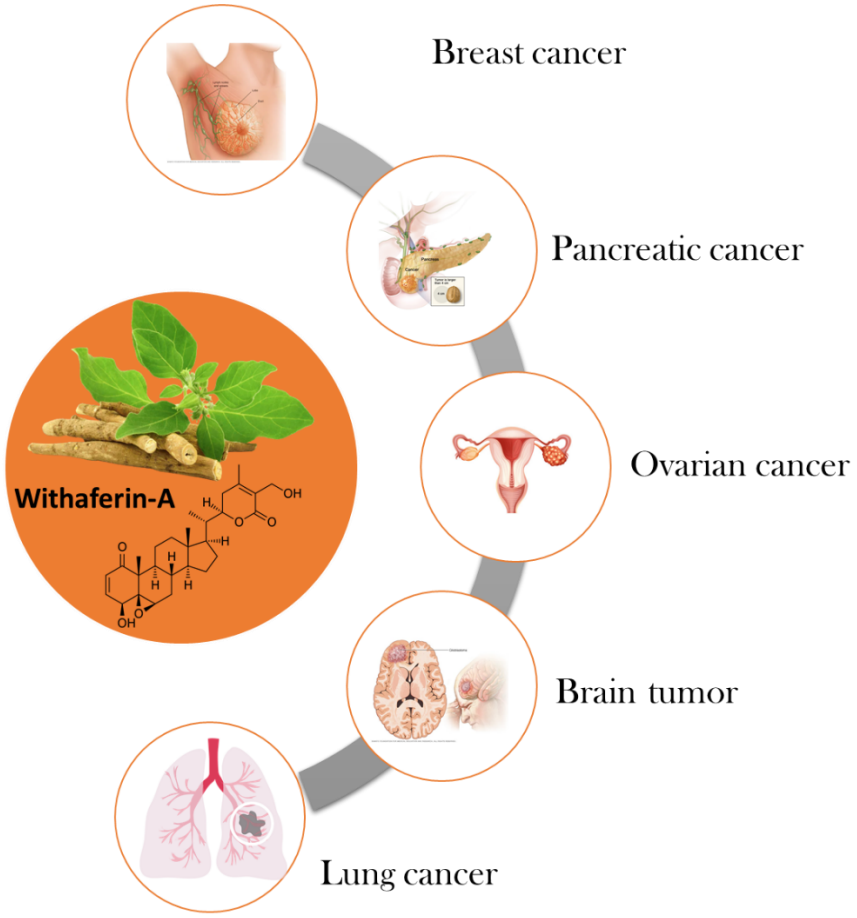


Figure 1.8: Anti-cancer efficacy of withaferin-A against various cancer.

WA also exhibits anti-metastatic properties through inhibition of epithelial to mesenchymal transition. A study by Lee et al. reported that WA inhibits TNF-α and TGF-β induced EMT phenotypes of MCF-10A cell lines. WA also decreases the protein levels of vimentin in mice model of MDA-MB-231 and MMTV-*neu* tumor (54). Further, WA showed inhibition of cervical cancer cell migration and invasion by suppression of matrix metaloprotein-9 (MMP-9) (55). Several other studies have highlighted the synergistic role of WA with approved anti-cancer drugs. For instance, Kakar et al. reported the inhibition of ovarian cancer growth and metastasis by WA in

combination with cisplatin in the orthotropic animal model (56). In another study, WA showed reversal of doxorubicin resistant leukemic cells and induces cell death (57). These findings collectively suggest that WA is a potential anti-cancer and anti-metastatic candidate that also exhibits synergistic effect with known anti-cancer drugs and could reverse the resistance cancer to sensitive one.

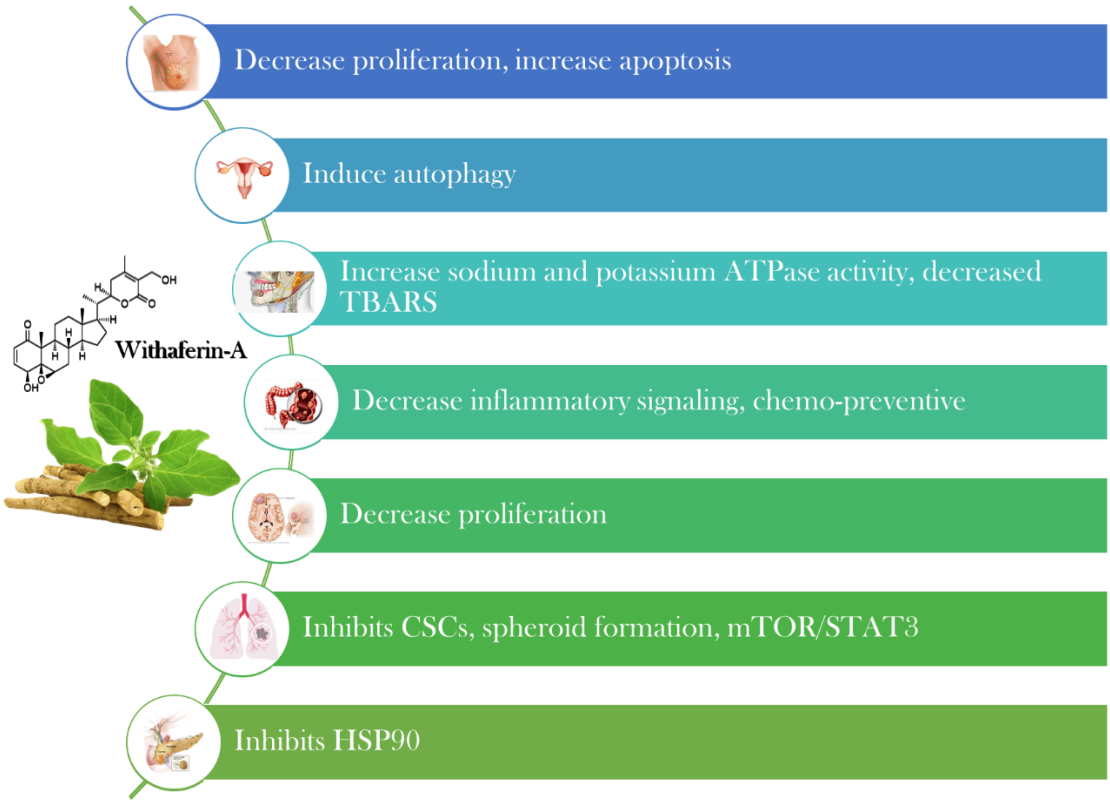


Figure 1.9: Anti-cancer mechanism of withaferin-A.

1.10.2 Organ protective effect of WA: Several studies have demonstrated the protective role of WA towards liver, skin, gut, brain, kidney and other vital organs. Neuroprotective ability of WA is demonstrated in several studies wherein it preserves the motor neurons integrity through increasing the levels of dopamine and other neuronal messengers (58,59). Vedi et al. demonstrated the hepato-protective and nephro-protective role of WA using bromobenzene induced liver and kidney damage model. They also demonstrated that WA keeps mitochondrial function intact, balanced ROS, decreases inflammatory cytokines and keeps hepatic and kidney function enzymes well within the normal range (57). Furthermore, WA showed protection of liver from acetaminophen-induced toxicity. Wherein, induction of cytoprotective enzymes through NRF2 were reported upon WA administration (60). Additionally, WA was found to protect liver against alcoholic and non-alcoholic injury in experimental mouse model (61).

In an animal model, WA attenuated scleroderma through FoxO3a-Akt-NF-κβ/IKK axis, suggesting its protective role on skin (61). Apart from this, protection of gut has also been studied by WA. Wherein, WA protects gastric epithelium by inhibiting NF-κβ mediated H.pylori inflammation (62). Further, WA showed prevention of inflammation and carcinogenesis of colon and intestine in an experimental mice model. This was found to be mediated through inhibition of IL-6, TNF-α, Cox-2 via pAKT, Notch1 and NF-kβ cascade (62). Role of WA against other diseases are mentioned in figure 1.10.

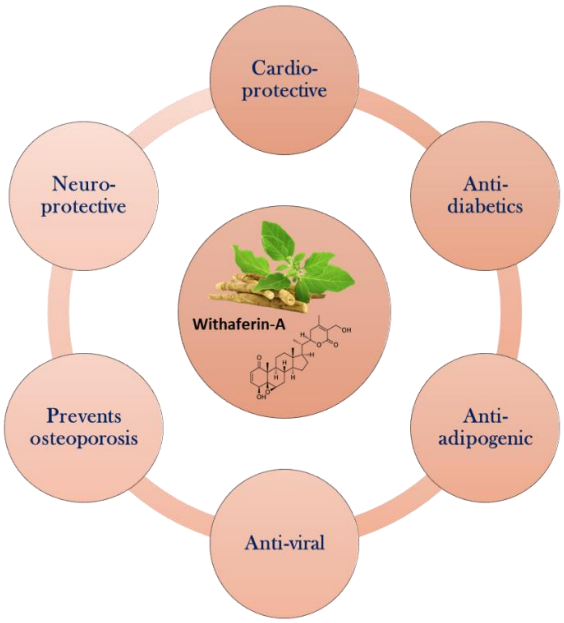


Figure 1.10: Effect of WA against various disease conditions.

1.10.3 Anti-inflammatory and immuno-modulatory activity of WA: Inflammatory cascade is key phenomenon of several immunological and cellular dysfunctions. WA shown to suppress inflammation through inhibition of NF-kβ, AP-1, Cox-2 and pro-inflammatory cytokines (63). Nearly a decade ago, SoRelle et al. reported the beneficial effect of WA on islet transplantation model. Their data suggest that WA improves the survival of syngeneic islet graft through inhibition of NF-kβ, pro-inflammatory cytokines and chemokines (63). Further, our group reported that WA binds with thiol group of NF-kβ and inhibits its migration to the nucleus, thereby, prevents cytokine secretion and inflammatory events (64). Wherein, WA inhibited T-cells and B-cells proliferation without affecting the cell viability. We also reported its immunosuppressive effect through suppression of activation markers of these cells and inhibition of Th1, Th2 cytokine secretion (64). Taking clue from these findings we next *ex-vivo* treated the donor mice graft with WA and transplanted into the immunosuppressed recipient mice (65). This resulted in prevention of aGvHD with significant improved in morbidity and survival of the mice. Wherein, we also demonstrated the probable involvement of Akt-mTOR signaling and suppression of

inflammatory cytokine storm by WA (65). In this line, Kumano et al. reported that WA improved the survival of allo-islet graft through inhibition of immune cell proliferation, dendritic cell maturation and cytokine suppression (66). Similar to our earlier findings, recently it is been reported that WA modulates the immune system via NF-kβ and AkT signaling and inhibits inflammatory cascades (66). Additionally, in this study WA showed inhibition of LPS induced inflammation by suppressing the macrophage ability to induce cytokine and nitric oxide secretion (66). These data strongly suggests potential role of WA in immune-modulation and hints towards its use in transplantation setting.

As indicated above in sections, aGvHD is mainly associated with immune cell mediated inflammation, damage of target tissue namely liver, skin, and gut. Several studies have emphasized upon anti-inflammatory, immune-modulatory and organ protective role of WA. These findings open the avenue to test the efficacy of WA against aGvHD.

Aim and objectives of the study

Aim: To evaluate the aGvHD prophylactic efficacy and GvL effect of Withaferin-A after oral administration in murine model of allogenic hematopoietic stem cell transplantation.

Objectives:

Objective 1. To determine the safety, toxicity and pharmacokinetics of oral Withaferin-A in mice.

Objective 2. To evaluate the efficacy of Withaferin-A for the prophylaxis of aGvHD and compare with standard prophylactic regimen.

Objective 3. To investigate the impact of Withaferin-A on Graft versus Leukemia effect.

Objective 4. To investigate the efficacy of ready-to-use formulation of Withaferin-A for prophylaxis and treatment of the aGvHD

Chapter 2

Objective 1. To determine the safety, toxicity and pharmacokinetics of oral Withaferin-A in mice

2.1 Introduction: Plants have served as a source of medicine for over 4000 years (67). Phytochemicals from plants represents the excellent source of biologically active compounds and stand significant for pharmaceutical industry (68). They exhibit various therapeutic benefits against diseases such as infection (viral & bacterial), cancer and diabetes (55,70). The systematic collection of scientific data on phyto-constituents began in the 1950s as documented by Kurup and his colleagues (71). According to the estimates from the World Health Organization (WHO), approximately 80% of the global population relies on herbal medicine for some aspect of their health care needs. Among all ancient cultures, India represent richest repository of medicinally valued plants (72). One such plant of great medicinal importance is *Withania somnifera* also known as Ashwagandha/Indian Ginseng/Indian Winter cherry. Ashwagandha has been used since ancient times in Ayurveda and considered as most important and prominent traditional medicinal plant of alternative medicine in India (73). It exhibits numerous biological activities including neuroprotection, anti-inflammation, immune-modulation, antitumor etc. (73,74). Ayurvedic remedy of Ahswagandha can be prepared from any part of this plant (75). Several biologically active components are present in the root extract of Ashwagandha including alkaloids (isopelletierine, anahygrine etc.), saponins and steroidal lactones (withanolides and withaferins) (76).

Withaferin-A (WA), a steroidal lactone, is the most biologically active component of *Withania somnifera* (64). WA was first isolated by two Israeli chemist, Lavie and Yarden. Other members of Solanaceae family also serve as source of WA (64). WA has been shown to exhibit diverse pharmacologic activities, including anti-cancer, anti-diabetic, anti-stress, anti-oxidant, neuroprotective, cardio-protective and immuno-

modulatory properties (53,55). It is also used as a potential candidate for flavour and aroma (75). This beneficial activity of WA is greatly attributed to its double bond and epoxide ring (structure of WA given chapter 1, section 1.10, figure 1.6) (79). Our recent study showed that *ex-vivo* treatment of donor cells with WA could ameliorate onset of Graft versus Host Disease (GVHD) by modulating the Akt-mTOR signaling pathway (65). Straughn et al., reported the efficiency of WA against SARS-CoV-2 infection, and suggested that WA could be used for cancer patient with SARS-CoV-2 infection as WA harbour anticancer as well as anti-viral activity (80). The anti-cancer activity of WA and its molecular mechanism have been extensively evaluated in several studies, wherein, WA showed a direct binding with numerous intracellular signaling molecules such as NFkB, Par-4, STAT3 etc. and regulates the cell fate, such as proliferation, migration, invasion and metastasis (53). WA treatment to the breast cancer cell lines (MDA-MB- 231 and MCF-7) increases the expression of autophagy markers and initiates the autophagy induced cell death in *in-vitro* and in tumor xenograft model (81). Currently, in era of cancer immunotherapy a wide variety of treatment modalities have emerged, but this excellent mode of treatment is limited by the numerous side effects and toxicity due to hyperactive immune cells mainly T-cells. A recent study by Gambhir et al. demonstrated that WA reduced the secretion of Th1 and Th2 cytokines and inhibited the mitogen-induced T-cell and B-cell proliferation. This immunosuppressive effect of WA was mediated through its binding with cysteine-62 residue of p50 and subsequent inactivation of the NF-κB pathway in T lymphocytes (64). This immunosuppressive property of WA suggest that it could be a potential candidate to aid with cancer immunotherapy compounds in overcoming the limitations of current immunotherapy drugs. In animal model system, WA exhibit

protection against liver, kidney and skin inflammation related disorders (82) (detailed in chapter 1, section 1.10.2).

Despite its pharmacological effects, the translation of WA to the bedside has not been accomplished. Establishing the non-clinical toxicity and pharmacokinetics (PK) of a molecule is an important aspect of drug development, and provides vital insights about its safety and posology in humans. Several groups have worked extensively on understanding the toxicity of *Withania somnifera* extract (83,84). However, there is no data available on oral safety and toxicity of WA, which is the most biologically active component of *Withania somnifera*. With the objective of advancing WA towards clinical use, the present study aimed to evaluated the oral safety, toxicity and pharmacokinetics of WA in mice.

2.2 Aim and Objectives:

2.2.1 Aim:

To established the safety, toxicity and pharmacokinetics of oral Withaferin-A in mice

2.2.2 Objectives:

Objective 1a. To evaluate the acute toxicity of oral Withaferin-A in mice.

Objective 1b. To evaluate the sub-acute or repeat dose toxicity of Withaferin-A in mice.

Objective 1c. To determine the pharmacokinetics of Withaferin-A in mice

2.3 Material and methods:

2.3.1 Chemicals and reagent: Withaferin-A and LC-MS/MS internal standard (fluoximesterone) was obtained from Pharmanza herbal Pvt. Ltd, Gujrat, India. LC-MS grade ammonium acetate, ethyl acetate, acetonitrile, and sulfosalicylic acid was used in the study. Filtered milli-Q water was filtered in-house and used for LC-MS/MS.

2.3.2 Experimental Animals: The study was commenced following the approval from Institutional Animal Ethics Committee of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial centre (TMC), Mumbai, India (project no. 17/2020). In consideration of the heightened sensitivity of females in toxicology studies, BALB/c female mice of 8-10 weeks old, weighing 20±2 grams were selected in this study. Mice were housed in the small animal facility of the ACTREC and provided with standard chow and water *ad libitum*, with a light/dark cycle of 12 hours at a temperature of 22-25^{o} C and humidity of approximately 50%. All experiment were initiated only after a week of acclimatization period for the mice. At the end of experiments, mice were humanely sacrificed, and blood was collected for biochemistry and haematology analysis in toxicity experiments and bioavailability evaluation for pharmacokinetics (PK) study. All procedures were carried out in accordance with CPCSEA guidelines and the study was performed in compliance with the ARRIVE guidelines.

2.2.3 *In silico* Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) analysis: ADMET properties of WA was evaluated using online tool ADMETlab2.0 (https://admetmesh.scbdd.com/). In this regard, first we retrieved the 2D structure of WA from PubChem database. Further, we generated the SMILES

format of WA structure using SwissADME (http://www.swissadme.ch/index.php). This SMILES was then acquiesced to ADMETlab2.0 for ADMET analysis.

2.3.4 Prediction of toxic hazards: The investigation of toxic hazards in WA was conducted using Toxtree (v3.1.0). Toxtree decision tree was employed for Verhaar scheme, skin irritation and corrosion prediction, eye irritation and corrosion prediction, Benigni/Bossa rule base (for mutagenicity and carcinogenicity), skin sensitization alerts, START biodegradability, cytochrome P450-mediated drug metabolism, DNA binding alerts, protein binding alerts, etc. It can estimate the threshold of toxicological concern (TTC) of the compound or their possible toxicity. The molecule site(s) labile to metabolism by cytochrome P450 isoform 3A4 was predicted by SMARTCyp reactivity model.

2.3.5 Acute toxicity: In order to assess acute oral toxicity, we followed Organisation for Economic Cooperation and Development (OECD) test guidelines - 423 Adopted on 17th December 2001. Animals were kept on fasting before and after 4 hour of dosing. Three female mice were allocated in each group, which were administered with 50 mg/kg, 300 mg/kg and 2000 mg/kg of WA via oral gavage. Carboxymethylcellulose (CMC) were used as vehicle. Following dosing each group was observed at four intervals of half-hour each and daily once untill 14^{th} day for any signs of clinical toxicity. Mice weight were recorded on day 0, 7 and 14. After the 14^{th} day mice were sacrificed and blood was collected for biochemistry and hematology evaluation, while the organs were harvested for histopathology. The acute toxicity scheme is illustrated in figure 2.1.

Figure 2.1: Schematic representation of method to assess the acute toxicity of withaferin-A in mice.

2.3.6 Sub-acute toxicity: Sub-acute toxicity or 28 days repeat dose toxicity study was conducted in accordance with the OECD guideline 407 adopted on 3 October 2008. Thirty female mice were divided in six groups (five mice per group); 1. Vehicle control (CMC) 2. 10 mg/kg treatment (low dose) 3. 70 mg/kg treatment (medium dose) 4. 500 mg/kg treatment (high dose) 5. Vehicle control (satellite/recovery groups) 6. 500 mg/kg treatment (satellite/recovery groups). Mice were administered WA for 30 days via oral gavage. Two satellite groups after 30 days of dosing were kept under observation for 14 more days to check the reversibility or delayed toxic effect. The mice were observed daily for any clinical sign of toxicity, and their weight were recorded on day 0,7,14 and 28. For satellite group, weight were also recorded on day 42. At the end of the experiment, mice were terminally sacrificed, blood was collected for biochemical and haematological analysis. Organ were harvested for histopathology. The sub-acute toxicity scheme is depicted in figure 2.2.

Figure 2.2: Schematic representation of method to assess the sub-acute toxicity of withaferin-A in mice.

2.3.7 Biochemical evaluation: Following 400-500µL of blood collection in clot activator tube, serum was harvested by centrifugation at 3000 rpm for 10 minutes. Liver function test (LFT) and renal function test (RFT) were analyzed using Dimension EXL 200-Siemens (Germany) autoanalyzer.

2.3.8 Hematological analysis: For CBC (complete blood count), a sample of blood measuring 100 µL was collected in an EDTA tube. Following the blood collection the CBC parameters were analysed using ADVIA 2120i (USA) autoanalyzer.

2.3.9 Percentage lymphocytes and neutrophil count: Manually slides were prepared for the purpose of counting lymphocytes and neutrophils in according to the protocols described by Hoppe et al. (85). Briefly, blood smears were made on a glass slide utilising 10 µl of blood. Subsequently, by using the wright stain, slides were stained and allowed to air-dry prior to counting. The cells were counted under microscope by two independent experts from clinical hematology laboratory of ACTREC.

2.3.10 Histopathology: Immediately after sacrificing the mice, tissues for histopathology were collected in 10% formalin and sent to the histopathology facility

of ACTREC for the preparation and staining of sides. Further the slides were evaluated by two independent pathologists from our centre to investigate any possible signs of tissue toxicity.

2.3.11 Pharmacokinetics:

2.3.11.1 Animals for PK study: In this study, female BALB/c mice were utilized for the purpose of pharmacokinetic analysis. A single oral dose of 70 mg/kg of WA was administered to these mice, and their blood was collected in EDTA tubes at various time intervals, including 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, and 24 hours, with 5 mice per time point. Additionally, a separate group of female BALB/c mice was given an intravenous (IV) injection of WA at a dose of 10 mg/kg, and their blood was collected in EDTA tubes at the same time intervals as the oral group (having one additional time point of 5 min), with 5 mice per time point. The collected blood samples from both groups were centrifuged at 3000 rpm for 10 minutes at 4°C, and the resulting plasma was stored at -80°C until further analysis. The plasma concentration of WA was determined using LC-MS/MS, as described in the method below. The schematic representation of the pharmacokinetic study is depicted in figure 2.3. The pharmacokinetic parameters were evaluated using non-compartmental analysis with Phoenix WinNonlin (CERTARA) software (version 8.3., NJ, USA). The absolute oral bioavailability (F) of WA was calculated using the following equation…

$$F\ (\%) = AUC_{0\text{-}inf}(Oral)*Dose\ IV\ /\ AUC_{0\text{-}inf}(IV)*Dose\ Oral\ *100$$

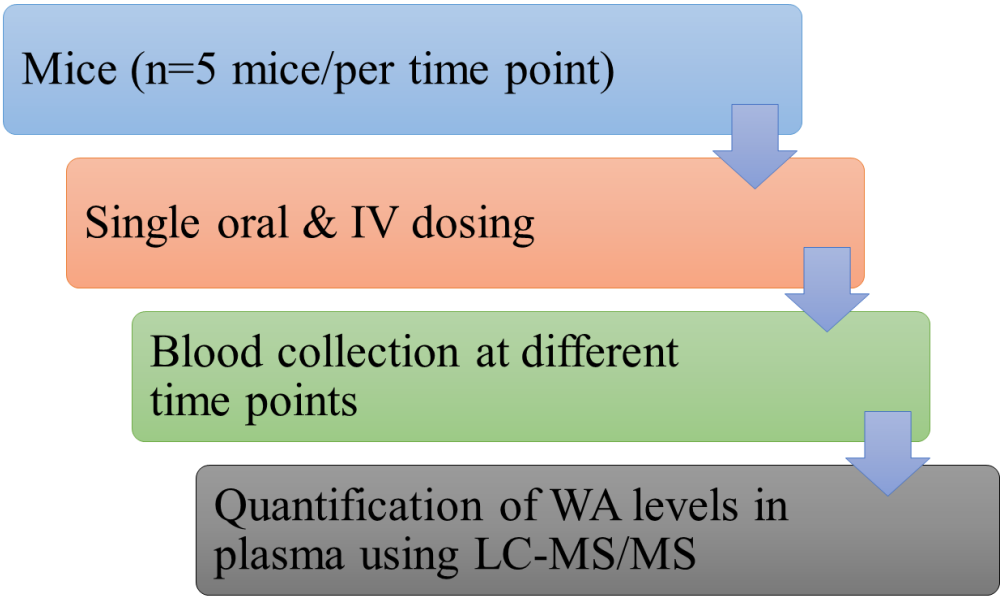


Figure 2.3: Schematic representation of WA pharmacokinetics study.

2.3.11.2 Mass spectrometry and chromatographic conditions: LC-MS/MS method was performed using the AB SCIEX QTRAP-4500 LC-MS/MS instrument, LC SHIMADZU Nexera X2 Micro LC. A Kinetex® 1.7µm C18 100Å (100*3 mm, S/No. H20-111310, Batch No. XD-4475-YO) column was used for chromatographic separation. The mobile phase consisted of acetonitrile and 10mM ammonium acetate in milli-Q water (60:40 v/v). Mass spectrometer was operated in the positive ion mode. The aqueous phase was eluted at a flow rate of 0.2 mL/min and the analytes were quantified in MRM mode using the following mass transitions i.e., WA - Q1/Q3 471.400/ 281.200 and fluoxymesterone, the internal standard (IS), -Q1/Q3 337.200/91.100. Quantitation was achieved by measurement of the peak area ratios of the drug to the internal standard. Data acquisition was performed with Analyst version 1.6.1 software.

2.4 Bioanalytical method development and validation

2.4.1 Standard solutions for Pharmacokinetics study: Using separate weighing, Stock solution of Withaferin-A (WA) and fluoxymesterone were prepared for standard calibration curve and quality control (QC) samples. Dimethyl sulfoxide (DMSO) was used to prepare stock solution of WA and fluoxymesterone at concentration of 1 mg/mL, and stored at -20º C until further analysis.

2.4.2 Sample preparation for Pharmacokinetics: In a 1.5mL Eppendorf tube 20µL of internal standard (IS) were added to 0.2mL plasma making a final solution of (20 µg/mL). Next these were vortexed for 30 seconds. Followed by addition of 20µL of 4% sulfosalicylic acid, vortexed for 1min. 1ml of absolute ethyl acetate was added for extraction, followed by 60 second of vertex and centrifugation for 15 minutes at 13000 rpm. The resultant supernatant were taken in fresh tube and subjected to evaporation using nitrogen gas. The residue was reconstituted in 100µL of 60% acetonitrile in milli-Q water, vortexed and centrifuged at 13000 rpm for10 minutes and 5 µL of the final clear solution was injected into the LC-MS/MS.

2.4.3 Calibration curves and quantitation for Pharmacokinetics: Calibration samples of Withaferin-A were prepared in blank plasma. A serial dilution of IS (5, 10, 25, 50, 100, 500, 1000, 2500 and 5000 ng/mL) was prepared and stored at -20°C. Preparation procedure described above was followed for each sample and 5 µL of the final solution was injected into the column. Plasma drug concentrations of WA was determined based on the ratio of peak area of WA (x) to the internal standard (y) using linear regression to calculate the unknown concentration levels from the calibration curve.

2.4.4 Mass Spectrometry conditions: Optimized LC-MS/MS parameters used to detect the analyte and IS is tabulated below (table 2.1).

Table 2.1: The optimized MS conditions for Multiple Reaction Monitoring (MRM) mode.

Ionization mode	Positive ESI
Ion source	Turbo spray
Curtain gas	40
Collision gas	Medium
Ion spray voltage	5500.00
Temperature (^{0}C)	500.00
GS1	50
GS2	50

ESI; electrospray ionization, GS1; Ion source gas1, GS2; Ion source gas2.

2.4.5 Pharmacokinetic method validation: A 5-day validation analysis of WA was performed. Microsoft excel were used to calculate %RSD and %DEV. The acceptance criteria for method validation were followed as per the bioanalytical method validation guidelines, May 2018.

2.4.5.1 Selectivity: Blank plasma were obtained to examine the presence of any possible interfering endogenous peak. Sample preparation were carried out as per the method mentioned in section 2.4.2. As depicted in table 2.2 area of the interference peak was found to be ≤ 20% of peak area of the lower limit of quantification (LLOQ) for analyte and ≤ 5% for IS (table 2.2).

Table 2.2: The average percentage Interference for analyte and IS.

Levels	**Analyte area**	**IS area**	**% Interference for analyte**	**% Interference of IS**
Plasma blank	39	45	0.21	0.06
LLOQ	18732	78313		

2.4.5.2 Carryover: Carry over in the blank sample was well within the range of ≤20% of the LLOQ for analyte and ≤ 5% for the internal standard.

2.4.5.3 Linearity: Calibration curves were constructed from calibration standard and concentration of analyte in unknown and QC samples were analysed. The calibration model was accepted if percent accuracy was within ± 20% for limit of quantification (LOQ) and within ± 15% for all other standard concentrations (table 2.3).

Table 2.3: The mean correlation coefficients of the linear regression analysis of calibration curve.

Levels (ng/mL)	Mean	SD	%RSD (± 20%)	% Accuracy (85-115%)
1	1.2	0.1	16.4	116.36
5	5.7	0.8	13.1	113.05
50	56.8	9.0	13.6	113.61
500	571.8	32.4	14.4	114.36
1000	1000.2	74.3	0.0	100.02
2500	2195.2	355.6	-12.2	87.81
5000	5242.5	104.4	4.8	104.85

The calibration curves were constructed by plotting the ratio of WA peak areas to that of IS versus standard WA concentrations. The method demonstrated linearity ranging from 1-5000 ng/mL in plasma. Regression equations for the calibration curve of WA in plasma was given by y=mx+c; (y= ratio of peak area of WA to IS) and (x= WA concentration). Mean correlation coefficient (R^2) of the calibration curve were 0.9970. **Acceptance criteria:** Non-zero calibrator should be within ± 15% RSD, except for LOQ for which it should be within ± 20% RSD. RSD: Relative standard deviation.

2.4.5.4 Sensitivity [limit of quantitation or Limit of detection (LOD)]: LOD was determined using the signal-to-noise ratio by comparing known concentrations of analyte to blank samples. Signal-to noise ratio of 3:1 produced by analyte concentration was accepted as the LOD. The LOQ is define as the lowest plasma concentration of the standard curve that could be quantified with acceptable accuracy, precision, and variability with acceptable accuracy and precision of ± 20% (table 2.4).

Table 2.4: The LOQ for withaferin-A in extracted plasma

Levels (ng/mL)	Average mean area	SD	%RSD (± 20%)
1	0.4129	0.004	0.97

The LOQ for WA in extracted plasma was 1 ng/mL with precision expressed as a %RSD of 0.97%. **Acceptance criteria:** The accuracy and precision should be within ± 20%. SD: Standard deviation, RSD: Relative standard deviation.

2.4.5.5 Precision and Accuracy: Using the same stock solutions and plasma batches, inter and intra-day precision and accuracy of analytical method were determined for WA (n=5 replicate for all quality control samples). The percent RSD of the assay were calculated. **Acceptance criteria**: Intra and inter-day precision & accuracy should be within ± 15% of nominal concentrations, except within ± 20% at LLOQ (table 2.5).

Table 2.5: The intra and inter-day precision and accuracy for plasma samples.

	Levels	Concentration (ng/mL)	Mean	SD	%RSD (± 15%)	% Accuracy (85-115%)
Intra-day	LLOQQC	1	1.1	0.11	13	113
	LQC	5	5.6	0.60	11.1	111.1
	MQC	500	497.7	132.07	-0.457	99.543
	HQC	2500	2640.6	113.18	5.622	105.622
Inter-day	LLOQQC	1	1.104	0.061	10.37	110.37
	LQC	5	5.263	0.155	5.27	105.27
	MQC	500	513.737	187.259	2.75	102.75
	HQC	2500	2357.283	239.702	-5.71	94.29

The intra and inter-day precision and accuracy for plasma samples are presented in Table 2.5. In plasma, quality control samples the mean precision were 5.36% and -3.64%, and accuracy were 105.36% and 96.36%. LLOQQC: Lower limit of quantification quality control, LQC: Lower quality control, MQC: Middle quality control, HQC: High quality control, SD: Standard deviation, RSD: Relative standard deviation.

2.4.5.6 Recovery: WA's recovery was calculated by comparing the peak area obtained following extraction of known concentration of WA from plasma with that of the area obtained from the same concentration of WA in aqueous solution (table 2.6).

Table 2.6: Recovery of WA.

Levels	Un-extracted area of analyte	Levels	Extracted area of analyte	% Recovery
AQ-LLOQQC	18411	PLS-LLOQQC	12050	65.45
AQ-LQC	65438	PLS-LQC	49210	75.20
AQ-MQC	301169	PLS-MQC	213488	70.89
AQ-HQC	1245622	PLS-HQC	777689	62.43

The overall mean recovery of Withaferin-A was 69.76 % after plasma extraction. AQ: Aqueous, PLS: Plasma, LLOQQC: Lower limit of quantification quality control, LQC: Lower quality control, MQC: Middle quality control, HQC: High quality control.

2.4.5.7 Stability: The stability of WA was assessed by analyzing samples under various conditions like auto-sampler, wet extract, dry extract, benchtop, freeze thaw, short term (6hr) and long term (7^{th}, 15^{th} day) stability and was checked the variation in the quality control samples respectively (table 2.7). **Acceptance criteria:** Percent RSD should be within ± 15% for quality control samples. RSD: Relative standard deviation.

Table 2.7: Stability of WA.

Stability	Levels	Concentration (ng/mL)	Mean	%RSD (± 15%)
Auto-sampler	LQC	5	4.50	0.25
	HQC	2500	2450	0.51
Wet extract	LQC	5	4.95	1.05
	HQC	2500	2438	1.25
Dry extract	LQC	5	5.05	1.05
	HQC	2500	2499	0.85
Benchtop	LQC	5	4.95	1.24
	HQC	2500	2581	0.85
Freeze thaw	LQC	5	5.05	1.25
	HQC	2500	2492	2.5
Short and long term	LQC	5	5.12	2.51
	HQC	2500	2483	2.51

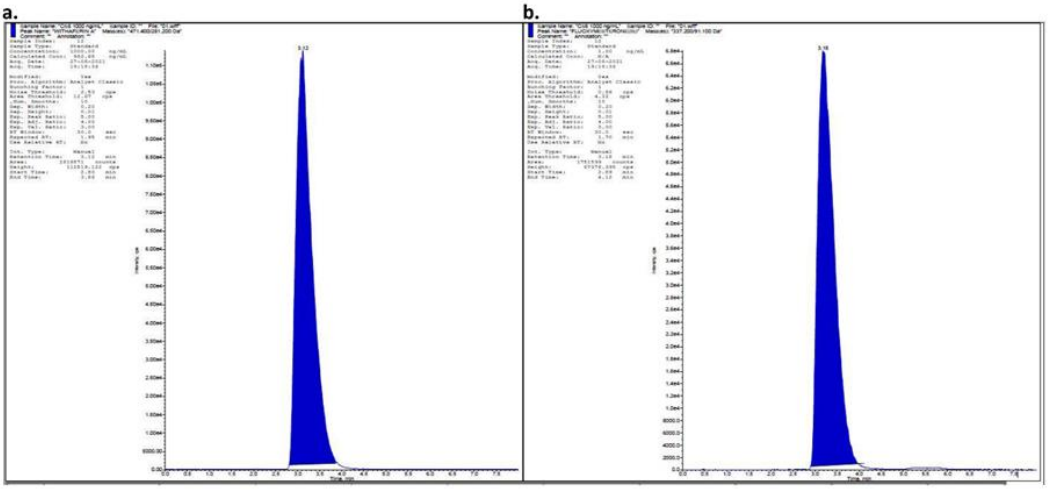


Figure 2.4: Chromatograms of plasma spiked with WA and the IS Fluoxymesterone. a) Withaferin-A and **b)** Fluoxymesterone eluted with high resolution within the run time of 8 minutes at 3.12 min and 3.18 min, respectively.

2.5 Statistical analysis: GraphPad Prism version 8.0 were used to analyse all the data. All data is expressed in mean ± SEM. Comparison between multiple groups were done using one-way ANOVA and Tukey's tests. A *p* value of <0.05 considered as statistically significant. Other than T_{max} alll PK parameters were expressed as Mean ± SEM. T_{max} was represented in median (range).

2.6 Results:

2.6.1 *In silico* ADMET analysis: ADMET properties describes the therapeutic action of a molecule that depends on its reach to the target site in the body at an adequate amount. ADMETlab2.0 is a freely accessible software that provides fast and robust predictive data about the physicochemical properties, pharmacokinetics and drug-likeness of a compound. We utilized this to compute the physicochemical properties and bioavailability of WA. Detailed physicochemical and ADMET parameters are mentioned in table 2.8-2.16. Concisely, topological polar surface area (TPSA) of WA falls in the acceptable zone of 0-40 whereas logS (aqueous solubility) was found to be slightly lower and logP (partition coefficient) was moderately higher than the optimum range. WA obeys Lipinski, Pfizer and Golden triangle rule that suggests the drug-likeness of a molecule. Absorption profile showed that WA is highly gastro-intestinal (Caco2 and MDCK) permeable and also acts as a strong P glycoprotein -inhibitor with a probability of 0.994. Distribution data outlined the plasma protein binding (PPB) and fraction unbound (Fu) of WA were 84.60% and 4.713% respectively. However, the probability of blood brain barrier (BBB) permeability is 0.725. Metabolism profile explained WA to be a CYP3A4 substrate with a probability of 0.895. WA showed high clearance of 16.18 ml/min/kg that was computed from the excretion profile of the ADMET analysis. The ADMET prediction suggest very low probability of being hERG (human Ether-a-go-go-Related Gene) blocker. The human hepatotoxicity of WA were estimated to be very low, similarly oral toxicity in rat were also predicted to be scarce. WA also determined to be noncorrosive to the eye.

2.6.2 Computation of toxic hazards: Cytotoxicity by CYP450-mediated drug metabolism for WA predicted 4 sites of metabolism viz. epoxidation and aliphatic hydroxylation. START biodegradability and Verhaar scheme explained WA to be a

persistent chemical and possess unspecific reactivity based on its structural annotation. The presence of α,β-unsaturated carbonyls in WA explained the structural alerts for genotoxic carcinogenicity and S. typhimurium mutagenicity computed by Benigni/Bossa rule base (for mutagenicity and carcinogenicity). The protein binding and DNA binding alerts for Michael acceptor and SN2-nucleoplilic aliphatic substitution were also identified.

Table 2.8: Physicochemical properties of WA.

Property	**Value**	**Comment**
Molecular Weight	470.27	Contain hydrogen atoms. Optimal:100~600
Volume	483.701	Van der Waals volume
Density	0.972	Density = MW / Volume
nHA	6	Number of hydrogen bond acceptors. Optimal:0~12
nHD	1	Number of hydrogen bond donors. Optimal:0~7
nRot	3	Number of rotatable bonds. Optimal:0~11
nRing	6	Number of rings. Optimal:0~6
MaxRing	18	Number of atoms in the biggest ring. Optimal:0~18
nHet	6	Number of heteroatoms. Optimal:1~15
fChar	0	Formal charge. Optimal:-4 ~4
nRig	31	Number of rigid bonds. Optimal:0~30
Flexibility	0.097	Flexibility = nRot /nRig
Stereo Centers	10	Optimal: □ 2
TPSA	93.2	Topological Polar Surface Area. Optimal:0~140
logS	-4.621	Log of the aqueous solubility. Optimal: -4~0.5 log mol/L
logP	3.787	Log of the octanol/water partition coefficient. Optimal: 0~3
logD	3.709	logP at physiological pH 7.4. Optimal: 1~3

Table 2.9: Medicinal chemistry of WA.

Property	Value	Decision	Comment
QED	0.498	●	▪ A measure of drug-likeness based on the concept of desirability; ▪ Attractive: > 0.67; unattractive: 0.49~0.67; too complex: < 0.34
SAscore	5.698	●	▪ Synthetic accessibility score is designed to estimate ease of synthesis of drug-like molecules. ▪ SAscore □ 6, difficult to synthesize; SAscore <6, easy to synthesize
Fsp3	0.821	●	▪ The number of sp3 hybridized carbons / total carbon count, correlating with melting point and solubility. ▪ Fsp3 □0.42 is considered a suitable value.
MCE-18	143.0	●	▪ MCE-18 stands for medicinal chemistry evolution. ▪ MCE-18□45 is considered a suitable value.
NPscore	3.196	-	▪ Natural product-likeness score. ▪ This score is typically in the range from □5 to 5. The higher the score is, the higher the probability is that the molecule is a NP.
Lipinski Rule	Accepted	●	▪ MW □ 500; logP □ 5; Hacc □ 10; Hdon □ 5 ▪ If two properties are out of range, a poor absorption or permeability is possible, one is acceptable.
Pfizer Rule	Accepted	●	▪ logP > 3; TPSA < 75 ▪ Compounds with a high log P (>3) and low TPSA (<75) are likely to be toxic.
GSK Rule	Rejected	●	▪ MW □ 400; logP □ 4 ▪ Compounds satisfying the GSK rule may have a more favorable ADMET profile
Golden Triangle	Accepted	●	▪ 200 □ MW □ 50; -2 □ logD □ 5 ▪ Compounds satisfying the Golden Triangle rule may have a more favorable ADMET profile.
PAINS	0 alerts	-	▪ Pan Assay Interference Compounds, frequent hitters, Alpha-screen artifacts and reactive compound.
ALARM NMR	2 alerts	-	▪ Thiol reactive compounds.
BMS	0 alerts	-	▪ Undesirable, reactive compounds.
Chelator Rule	0 alerts	-	▪ Chelating compounds.

Table 2.10: Absorption of WA.

Property	Value	Decision	Comment
Caco-2 Permeability	-5.067	●	▪ Optimal: higher than -5.15 Log unit
MDCK Permeability	1.5e-05	●	▪ low permeability: < 2 × 10□6 cm/s ▪ medium permeability: 2–20 × 10□6 cm/s ▪ high passive permeability: > 20 × 10□6 cm/s
Pgp-inhibitor	0.994	●	▪ Category 1: Inhibitor; Category 0: Non-inhibitor; ▪ The output value is the probability of being Pgp-inhibitor
Pgp-substrate	0.709	●	▪ Category 1: substrate; Category 0: Non-substrate; ▪ The output value is the probability of being Pgp-substrate
HIA	0.004	●	▪ Human Intestinal Absorption ▪ Category 1: HIA+(HIA < 30%); Category 0: HIA-(HIA < 30%); The output value is the probability of being HIA+
F20%	0.133	●	▪ 20% Bioavailability ▪ Category 1: F20%+ (bioavailability < 20%); Category 0: F20%- (bioavailability □ 20%); The output ▪ value is the probability of being F20%+
F30%	0.975	●	▪ 30% Bioavailability ▪ Category 1: F30%+ (bioavailability < 30%); Category 0: F30%- (bioavailability □ 30%); The output ▪ value is the probability of being F30%+

Table 2.11: Distribution of WA.

Property	Value	Decision	Comment
PPB	84.60%	●	▪ Plasma Protein Binding ▪ Optimal: < 90%. Drugs with high protein-bound may have a low therapeutic index.
VD	0.679	●	▪ Volume Distribution ▪ Optimal: 0.04-20L/kg
BBB Penetration	0.725	●	▪ Blood-Brain Barrier Penetration ▪ Category 1: BBB+; Category 0: BBB-; The output value is the probability of being BBB+
Fu	4.713%	●	▪ The fraction unbound in plasms ▪ Low: <5%; Middle: 5~20%; High: > 20%

Table 2.12: Excretion of WA.

Property	Value	Decision	Comment
CL	16.186	●	▪ Clearance ▪ High: >15 mL/min/kg; moderate: 5-15 mL/min/kg; low: <5 mL/min/kg
T1/2	0.82	-	▪ Category 1: long half-life ; Category 0: short half-life; ▪ long half-life: >3h; short half-life: <3h ▪ The output value is the probability of having long half-life.

Table 2.13: Metabolism of WA.

Property	Value	Comment
CYP1A2 inhibitor	0.033	▪ Category 1: Inhibitor; Category 0: Non-inhibitor; ▪ The output value is the probability of being inhibitor.
CYP1A2 substrate	0.509	▪ Category 1: Substrate; Category 0: Non-substrate; ▪ The output value is the probability of being substrate.
CYP2C19 inhibitor	0.188	▪ Category 1: Inhibitor; Category 0: Non-inhibitor; ▪ The output value is the probability of being inhibitor.
CYP2C19 substrate	0.894	▪ Category 1: Substrate; Category 0: Non-substrate; ▪ The output value is the probability of being substrate.
CYP2C9 inhibitor	0.249	▪ Category 1: Inhibitor; Category 0: Non-inhibitor; ▪ The output value is the probability of being inhibitor.
CYP2C9 substrate	0.045	▪ Category 1: Substrate; Category 0: Non-substrate; ▪ The output value is the probability of being substrate.
CYP2D6 inhibitor	0.008	▪ Category 1: Inhibitor; Category 0: Non-inhibitor; ▪ The output value is the probability of being inhibitor.
CYP2D6 substrate	0.131	▪ Category 1: Substrate; Category 0: Non-substrate; ▪ The output value is the probability of being substrate.
CYP3A4 inhibitor	0.866	▪ Category 1: Inhibitor; Category 0: Non-inhibitor; ▪ The output value is the probability of being inhibitor.
CYP3A4 substrate	0.895	▪ Category 1: Substrate; Category 0: Non-substrate; ▪ The output value is the probability of being substrate.

Table 2.14 Toxicity of WA.

Property	Value	Decision	Comment
hERG Blockers	0.233	●	▪ Category 1: active; Category 0: inactive; ▪ The output value is the probability of being active.
H-HT	0.263	●	▪ Human Hepatotoxicity ▪ Category 1: H-HT positive(+); Category 0: H-HT negative(-); ▪ The output value is the probability of being toxic.
DILI	0.562	●	▪ Drug Induced Liver Injury. ▪ Category 1: drugs with a high risk of DILI; Category 0: drugs with no risk of DILI. The output value is the probability of being toxic.
AMES Toxicity	0.115	●	▪ Category 1: Ames positive(+); Category 0: Ames negative(-); ▪ The output value is the probability of being toxic.
Rat Oral Acute Toxicity	0.35	●	▪ Category 0: low-toxicity; Category 1: high-toxicity; ▪ The output value is the probability of being highly toxic.
FDAMDD	0.429	●	▪ Maximum Recommended Daily Dose ▪ Category 1: FDAMDD (+); Category 0: FDAMDD (-) ▪ The output value is the probability of being positive.
Skin Sensitization	0.804	●	▪ Category 1: Sensitizer; Category 0: Non-sensitizer; ▪ The output value is the probability of being sensitizer.
Carcinogenicity	0.764	●	▪ Category 1: carcinogens; Category 0: non-carcinogens; ▪ The output value is the probability of being toxic.
Eye corriosion	0.028	●	▪ Category 1: corrosives ; Category 0: noncorrosives ▪ The output value is the probability of being corrosives.
Eye irritation	0.138	●	▪ Category 1: irritants ; Category 0: nonirritants ▪ The output value is the probability of being irritants.
Respiratory Toxicity	0.964	●	▪ Category 1: respiratory toxicants; Category 0: respiratory nontoxicants ▪ The output value is the probability of being toxic.

Table 2.15: Environmental toxicity of WA.

Property	Value	Comment
Bioconcentration Factors	0.372	▪ Bioconcentration factors are used for considering secondary poisoning potential and assessing risks to human health via the food chain. ▪ The unit is □log10[(mg/L)/(1000*MW)]
IGC50	3.366	▪ Tetrahymena pyriformis 50 percent growth inhibition concentration ▪ The unit is □log10[(mg/L)/(1000*MW)]
LC50FM	4.204	▪ 96-hour fathead minnow 50 percent lethal concentration ▪ The unit is □log10[(mg/L)/(1000*MW)]
LC50DM	3.965	▪ 48-hour daphnia magna 50 percent lethal concentration ▪ The unit is □log10[(mg/L)/(1000*MW)]

Table 2.16: Toxicophore Rules for WA.

Property	Value	Comment
Acute Toxicity Rule	0 alerts	▪ 20 substructures ▪ acute toxicity during oral administration
Genotoxic Carcinogenicity Rule	7 alerts	▪ 117 substructures ▪ carcinogenicity or mutagenicity
NonGenotoxic Carcinogenicity Rule	1 alerts	▪ 23 substructures ▪ carcinogenicity through nongenotoxic mechanisms
Skin Sensitization Rule	7 alerts	▪ 155 substructures ▪ skin irritation
Aquatic Toxicity Rule	7 alerts	▪ 99 substructures ▪ toxicity to liquid(water)
NonBiodegradable Rule	3 alerts	▪ 19 substructures ▪ non-biodegradable
SureChEMBL Rule	2 alerts	▪ 164 substructures ▪ MedChem unfriendly status

2.6.3 Acute toxicity: WA treated group did not show any clinical symptoms of toxicity compared to the vehicle control group. There was no visible sign of toxicity in any animal in the WA group, and none of the animals died. Based on these findings, WA may be classified as GHS (Globally Harmonized System) category 5 (LD_{50} >2000 mg/kg body weight) compound as per OECD Guideline No. 423, December 2001.

2.6.4 Sub-acute toxicity (28 days repeat dose toxicity): Sub-acute toxicity were performed at dose of 10, 70 and 500 mg/kg/day. Mice were dosed daily for 28 days. All animals were alive until necropsy in both the test group and the recovery (satellite) group. None of the animals in the test or recovery groups showed any visible signs of toxicity. These results suggest that oral administration of WA to mice every day for 28 days is safe and well tolerated. Therefore, 500 mg/kg oral WA shall be considered as No-Observed Adverse Effect Level (NOAEL) dose as per OECD Guideline No. 407, October 2008.

2.6.5 Body weight: Upon oral WA administration, mice in both acute toxicity as well as in sub-acute toxicity studies did not show any changes in body weight compared to their respective controls (fig 2.5 a-b).

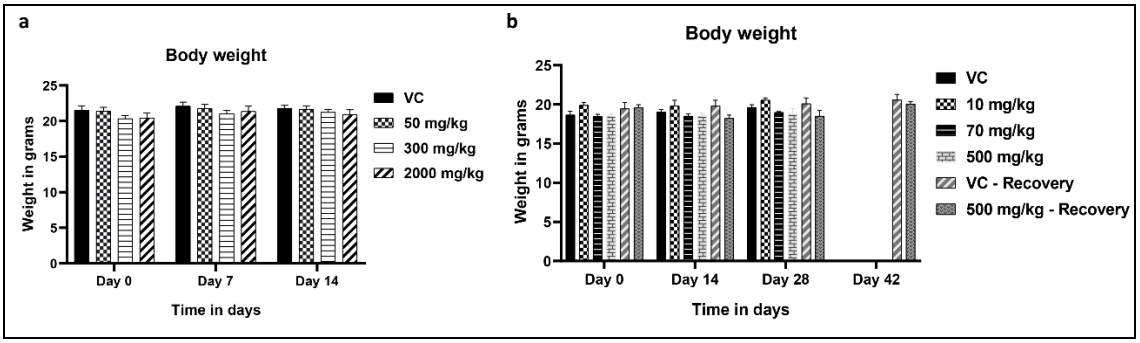


Figure 2.5: Body weight of mice in **(a)** acute and **(b)** sub-acute toxicity. All values are represented as mean ± SEM.

2.6.6 Serum Biochemistry: Serum RFT and LFT parameters were investigated. RFT parameters included blood urea nitrogen (BUN), uric acid (URCA) and creatinine (CREA). LFT parameters included total protein (TP), albumin (ALB), alkaline phosphatase (ALP), total bilirubin (TB), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In acute and sub-acute toxicity, no changes were observed in LFT and RFT parameters in WA treated groups compared to their respective control groups (table 2.17 and 2.18).

Table 2.17: Effect of orally administered withaferin-A on RFT and LFT parameters in acute toxicity.

Test	Unit	VC	50 mg/kg	300 mg/kg	2000 mg/kg
TP	g/dl	5.9 ± 0.5	4.9 ± 0.4	5.4 ± 0.1	5.4 ± 0.1
ALB	g/dl	1.0 ± 0	1.1 ± 0	1.1 ± 0	1.1 ± 0
ALP	U/L	105.0 ± 8.5	103.3 ± 6.3	115.6 ± 6.3	130.6 ± 9.1
TB	mg/dl	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
AST	U/L	118.0 ± 18.2	107.3 ± 17.8	134.3 ± 22.8	121.3 ± 38.4
ALT	U/L	40.3 ± 2.7	47.7 ± 7.8	49.7 ± 4.4	45.7 ± 4.9
BUN	mg/dl	46.0 ± 1.5	45.0 ± 3.6	51.0 ± 2.1	56.3 ± 5.5
URCA	mg/dl	2.8 ± 0.6	2.8 ± 0.4	2.6 ± 0.1	2.8 ± 0.6
CREA	mg/dl	0.3 ± 0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.04

All values are represented as Mean ± SEM. TP: total protein, ALB: albumin, ALP: alkaline phosphatase, TB: total bilirubin, AST: aspartate aminotransferase, ALT:

alanine aminotransferase, BUN: blood urea nitrogen, URCA: uric acid, CREA: creatinine.

Table 2.18: Effect of orally administered withaferin-A on RFT and LFT parameters in sub-acute toxicity.

Test	Unit	VC	10 mg/kg	70 mg/kg	500 mg/kg	VC Recovery	500 mg/kg Recovery
TP	g/dl	4.9 ± 0.3	5.1 ± 0.0	4.9 ± 0.5	4.8 ± 0.4	5.3 ± 0.1	5.5 ± 0.1
ALB	g/dl	1.6 ± 0.1	1.4 ± 0.0	1.5 ± 0.4	1.7 ± 0.1	1.2 ± 0.2	1.3 ± 0.3
ALP	U/L	145.3 ± 8.0	123.8 ± 3.7	138.2 ± 6.2	138.6 ± 11.5	121.6 ± 3.9	133.4 ± 4.5
TB	mg/dl	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
AST	U/L	185.5 ± 13.8	181.4 ± 5.2	169.8 ± 31.9	162.4 ± 19.0	158.0 ± 9.4	173.4 ± 5.1
ALT	U/L	48.8 ± 5.9	42.4 ± 2.0	43.8 ± 4.7	46.4 ± 1.4	41.0 ± 1.7	51.0 ± 6.6
BUN	mg/dl	46.5 ± 5.9	50.4 ± 2	55.2 ± 2.3	50.6 ± 5.7	49 ± 2.0	45.6 ± 5.2
URCA	mg/dl	2.0 ± 0.3	2.4 ± 0.2	1.8 ± 0.1	1.2 ± 0.2	1.9 ± 0.3	2.1 ± 0.4
CREA	mg/dl	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.1	0.4 ± 0.1

"TP: total protein, ALB: albumin, ALP: alkaline phosphatase, TB: total bilirubin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, BUN: blood urea nitrogen, URCA: uric acid, CREA: creatinine. All values are represented as Mean ± SEM."

2.6.7 Hematological Parameters: We analyzed the CBC and differential counts in acute and sub-acute toxicity animals following oral administration of WA. At any given acute toxicity dose none of the CBC parameters were altered, barring a non-significant dose dependent decrease in total WBC count (table 2.19). Hematological parameters in sub-acute toxicity also did not show any changes at any dose level tested compared to control groups (table 2.20).

Table 2.19: Effect of orally administered withaferin-A on hematological parameters in acute toxicity.

Test	Unit	VC	50 mg/kg	300 mg/kg	2000 mg/kg
WBC	$10^3/\mu l$	5.1 ± 0.9	4.8 ± 0.5	3.5 ± 1.2	2.3 ± 0.5
RBC	$10^6/\mu l$	9.9 ± 0.1	9.3 ± 0.1	9.22 ± 0	9.3 ± 0.2
Hb	g/dl	14.6 ± 0.1	13.7 ± 0.3	13.6 ± 0.1	13.6 ± 0.2
HCT	%	49.2 ± 0.3	46.1 ± 0.2	47.5 ± 0.3	46.3 ± 1.1
MCV	fl	49.4 ± 0.4	49.5 ± 0.7	50.9 ± 0.4	50.0 ± 0.4
MCH	pg	14.6 ± 0.1	14.7 ± 0.3	14.8 ± 0.1	14.7 ± 0.1
MCHC	g/dl	29.6 ± 0.1	29.9 ± 0.4	29.0 ± 0.1	29.4 ± 0.2
PLT	$10^3/\mu l$	839.3 ± 28.2	759.3 ± 35.4	782.0 ± 49.1	710.3 ± 51.5
NEUT	%	14 ± 2	15 ± 5	12 ± 2	18 ± 7
LYMPH	%	81 ± 2	81 ± 5	83 ± 2	77 ± 9

All values are represented as Mean ± SEM. WBC: white blood cell count, RBC: red blood cell count, Hb: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, PLT: platelet, NEUT: neutrophils, LYMPH: lymphocytes.

Table 2.20: Effect of orally administered withaferin-A on hematological parameters in sub-acute toxicity.

Test	Unit	VC	10 mg/kg	70 mg/kg	500 mg/kg	VC Recovery	500 mg/kg Recovery
WBC	$10^3/\mu l$	3.7 ± 0.3	4.1 ± 0.4	3.4 ± 0.5	3.9 ± 0.4	3.8 ± 1.0	3.9 ± 0.4
RBC	$10^6/\mu l$	8.5 ± 0.2	8.1 ± 0.2	8.9 ± 0.2	9.2 ± 0.3	8.2 ± 0.2	7.5 ± 0.2
HGB	g/dl	13.4 ± 0.3	11.9 ± 0.1	13.3 ±0.3	13.7 ± 0.5	12.4 ± 0.2	11.2 ± 0.2
HCT	%	44 ± 0.5	40 ± 0.7	44.6 ± 1	45 ± 1	42 ± 0.8	39 ± 0.7
MCV	fl	50.8 ± 0.4	49.7 ± 0.4	50.1 ± 0.4	49.3 ± 0.5	51 ± 0.4	50.7 ± 0.7
MCH	pg	13.6 ± 0.8	11.8 ± 2.7	15 ± 0.2	14.9 ± 0.1	15.1 ± 0.2	14.9 ± 0.2
MCHC	g/dl	30.1 ± 0.1	29.3 ±0.2	29.9 ± 0.3	30.3 ± 0.5	29.7 ± 0.3	29.3 ± 0.1
RDW	%	11.7 ± 0.4	11.7 ± 0.3	11.6 ± 0.2	11.6 ± 0.3	12.2 ± 0.1	12.4 ± 0.3
PLT	$10^3/\mu l$	649.0 ± 17.2	639.6 ± 33.4	666.5 ± 18.6	684.7 ± 52.9	678.8 ± 22.2	652.0 ± 23.3
NEUT	%	13 ± 3	16 ± 5	14 ± 1	16 ± 3	15 ± 4	14 ± 2
LYMPH	%	82 ± 2	79 ± 5	81 ± 2	80 ± 4	80 ± 3	82 ± 3

All values are represented as Mean ± SEM. WBC: white blood cell count, RBC: red blood cell count, Hb: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, LT: platelet, NEUT: neutrophils, LYMPH: lymphocytes.

2.6.8 Histopathology: Upon histopathological observation of vital organs such as brain, heart, lungs, liver, kidney, spleen, bone, small and large intestine, none of them showed any sign of drug induced toxicity in acute as well as in sub-acute toxicity studies (fig 2.6 and 2.7).

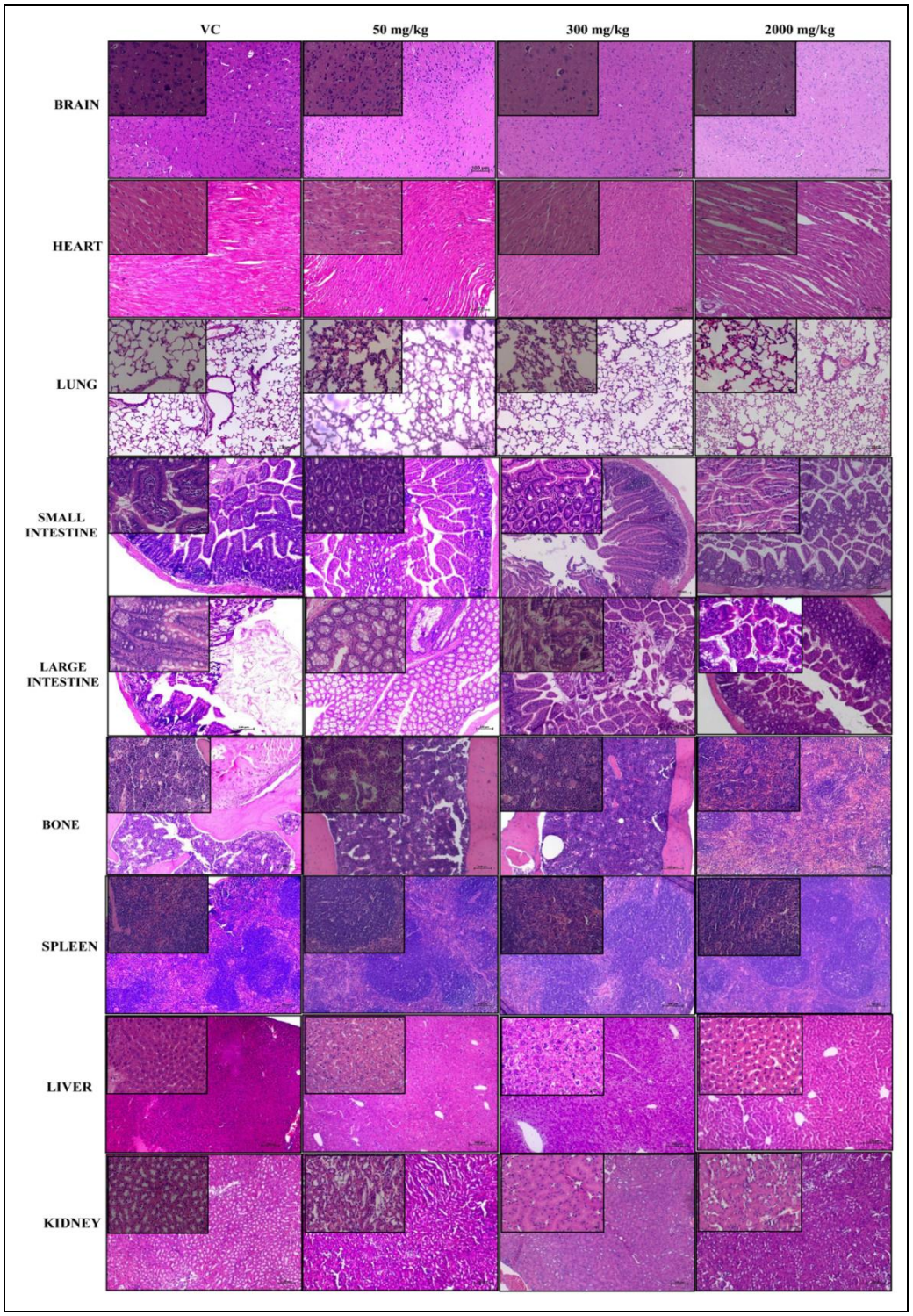


Figure 2.6: Histopathology of vital organs in acute toxicity study. H and E magnification × 100 and × 400 (Inset). VC: vehicle control.

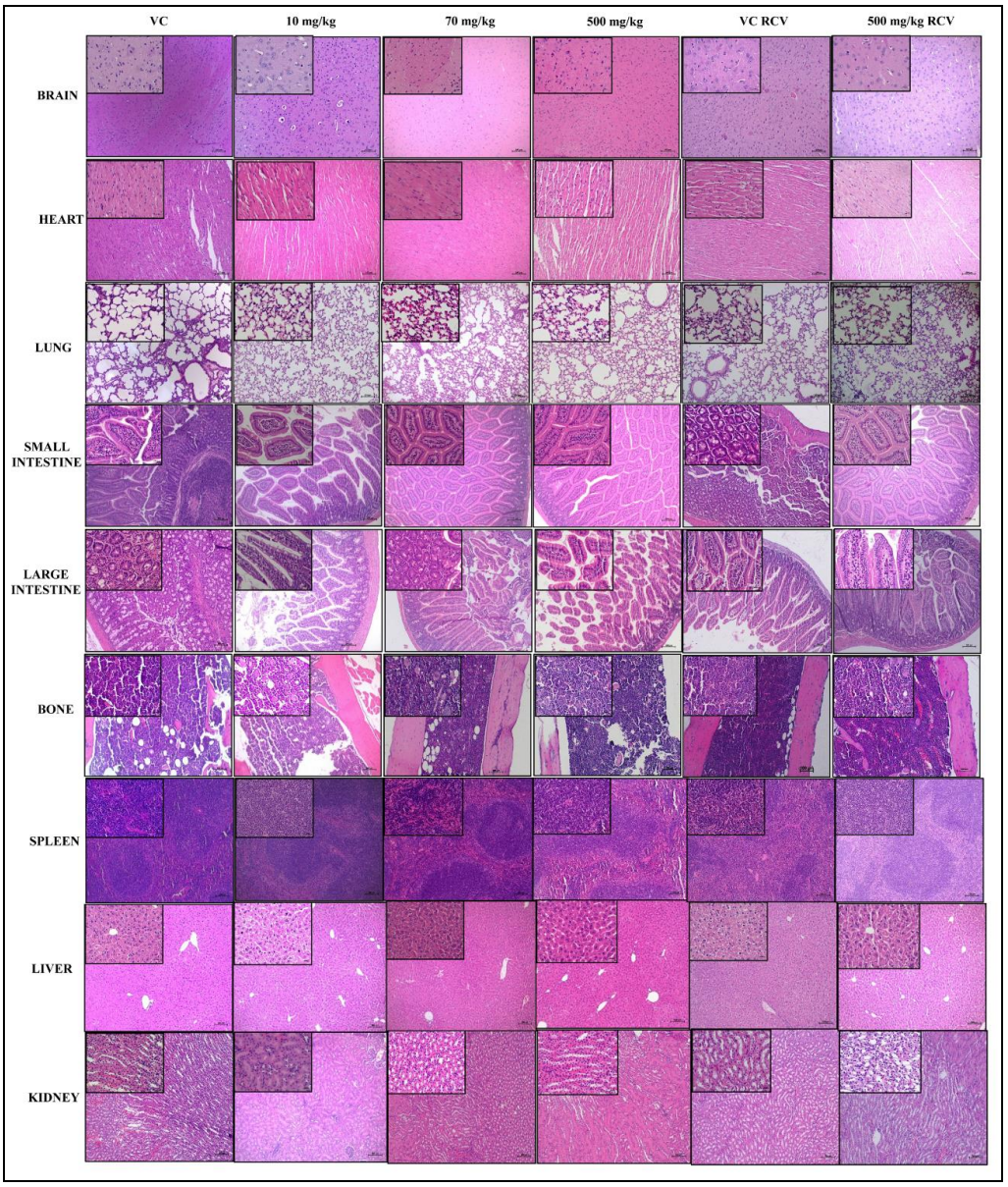


Figure 2.7: Histopathology of vital organs in sub-acute toxicity study. H and E magnification × 100 and × 400 (Inset). VC: vehicle control, RCV: recovery.

2.6.9 Pharmacokinetics of WA: PK samples were analyzed for WA levels using a validated LC-MS/MS technique. Maximum plasma concentration (C_{max}) were found to be 3996.9 ± 557.6 ng/mL and 141.7 ± 16.8 ng/mL for IV and oral doses respectively. The median T_{max} following oral administration was 0.5 h. The mean plasma concentration-time curves of WA are illustrated in figure 2.8a-b and the PK parameters are shown in table 2.21. Further, the percentage bioavailability of WA was found to be 1.8%.

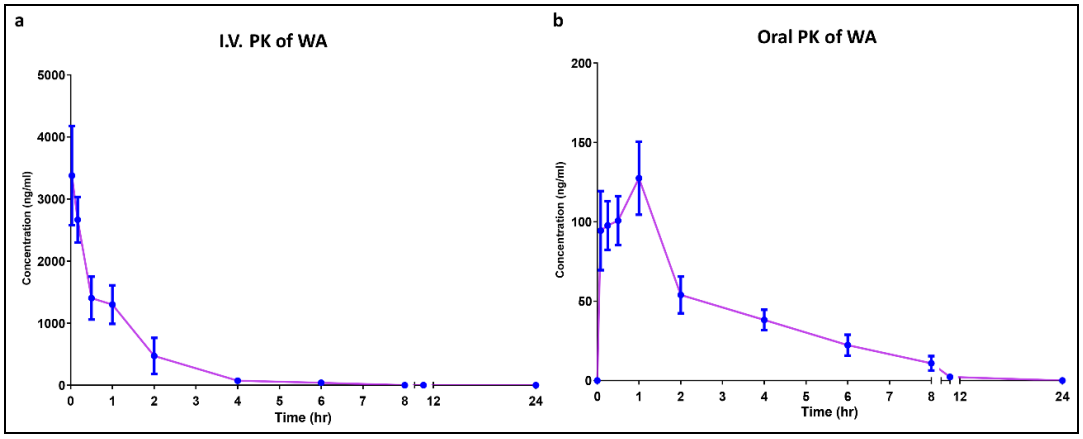


Figure 2.8: Mean plasma concentration versus time curve for WA after **(a)** intravenous and **(b)** oral administration. Values are expressed as means ± SEM (n = 5 mice/time points).

Table 2.21: Pharmacokinetic parameters in mice after IV and oral administration of withaferin-A.

Parameters	**I.V. WA (10 mg/kg)**	**Oral WA (70 mg/kg)**
C_{max} (ng/mL)	3996.9 ± 557.6	141.7 ± 16.8
T_{max} (h)	-	0.5 (0.25-1.0)
$t_{1/2}$ (h)	0.6 ± 0.4	2.7 ± 0.4
AUC 0-∞ (ng/mL*h)	3509.8 ± 302.4	436.1 ± 60.9
CL(L/hr/kg)	2.9 ± 0.2	171.1 ± 19.5
V (L/kg)	5.2 ± 1.2	656.6 ± 107.7
% F	-	1.8

All data presented in Mean ± SEM, T_{max} presented as median ± range. AUC: Area under the concentration–time curve; $t_{1/2}$: half-life; T_{max}: time to peak concentration; CL: clearance; V: volume of distribution; C_{max}: peak concentration; F: bioavailability.

2.7 Discussion:

Phytochemicals are proven to have significant beneficial role in prevention of disease and its progression (86). Most of the time application of phytochemicals become limited due to underline toxicity and poor bioavailability (87). Therefore, it is desirable to have toxicity and pharmacokinetics profiling to most widely applicable plant derived compound used in medicine. *Withania somnifera* (Ashwagandha) is one of the well-known medicinally valued plant used in Ayurvedic practice. WA, being a steroidal lactone is the active component of Ashwagandha which are responsible for its therapeutic properties (83,84). WA has shown several beneficial role against numerous fatal conditions such as cancer, hepatitis, Alzheimer's disease etc. (87,88,89).

This is the first study wherein a comprehensive toxicity evaluation of pure WA is been carried out. Our results suggest that WA did not produce any mortality up to 2000 mg/kg and 500 mg/kg in acute and sub-acute toxicity respectively. Body weight, physical and behavioural appearance of mice did not indicated any sign of toxicity in acute as well as in sub-acute toxicity study. Biochemistry and hematology of blood, and histopathology of vital organs provide information about overall health status of the animal (93). None of these parameters were altered in acute and sub-acute toxicity animals, suggesting WA is safe up to 2000 mg/kg in acute and 500 mg/kg in sub-acute dose. Based on these finding LD_{50} of WA was found to be more than 2000mg/kg and NOAEL was found to be 500mg/kg. As per the Globally Harmonized classification System (GHS) of drugs, WA falls in category 5 which is a relatively safe category of the drugs. Further, we evaluated the PK of WA and observed that WA had a C_{max} of 3996.9 ± 557.6 Considering the

favorable toxicity profile, the current study will help the future development of pure WA for clinical use, highlighting the significance of the study.

While to the best of our knowledge this is the first study to conclusively demonstrate the toxicity profile of WA in mice, Prabhu et al., reported the safety of a hydro alcoholic extract of Withania somnifera root (WSR) up to 2000mg/kg in acute and sub-acute toxicity studies in rats (83). No significant hematological, biochemical and histological changes were observed in Wister rats at these doses (83). Similar results were reported by Shruti et al., using methanolic extract of WSR containing 4.5% of WA (84). Despite the high doses of WSR used in these studies, the absolute dose of WA present in these extracts was significantly less than that used in our study, although WSR will have other withanoloids whose toxicity profile vis-à-vis WA is yet to be studies. Nevertheless, all these studies taken together clearly demonstrate the safety of WA in rodents at high doses. Of course, oral administration may not be a true reflection of a drug's safety, particularly if it has low oral bioavailability. Therefore, the reports from Sharada et. al., and Shohat et. al., assumes significance in this context as both groups reported LD_{50} of WA at sub-100 mg/kg dose in acute toxicity studies following intraperitoneal (IP) administration (80 mg/kg and 54 mg/kg respectively) (94,95). The difference in LD_{50} between the two studies is perhaps attributable to differences in environment, method of isolation and species variation between herbs grown in India and Israel (95).

Pharmacokinetic study showed low oral bioavailability of WA which also corroborated the outcome of *in silico* analysis. *In silico* analysis predicted a high first-pass effect due to extensive metabolism by CYP3A4 in the liver, while absorption across the GI tract itself was not expected to be low given a high Caco-2 and MDCK-permeability. Dai et al., demonstrated rapid metabolism of WA in liver microsomes which explains the first-pass effect (96). True to this phenomena, in our study the peak concentration following oral administration was achieved rapidly but was less than 1% of the maximum

concentration achieved through intravenous route (normalized for dose). Similar findings were also reported by Patil et al., who observed a rapid (T_{max}=20 min) but low C_{max} of 16.69 ng/mL following oral administration of 1000 mg/kg of an aqueous root extract of *Withania somnifera* (97). Surprisingly, Dai et al., reported an exceptionally high average C_{max} of 619 ng/mL with a 10 mg/kg oral dose of WA. Further, they reported oral bioavailability of approximately 32% (96). The difference in PK parameters could be possibly explained by the vehicles used in the two studies. We administered oral WA in CMC which forms drug suspension while Dai et al formulated WA in ethanol–solutol HS 15–distilled water (10:5:85, v:v:v). They observed an extremely rapid oral absorption (T_{max}= 6 min) which could have possibly saturated the CYP enzymes, leading to high C_{max}, and consequently a higher AUC. We have earlier established the safety of WA up to 216 mg/day in a phase I human trial (74).

2.8 Conclusion: our results suggest that WA is safe at doses of 2000 mg/kg and 500 mg/kg in acute and repeat dose toxicity respectively, albeit with oral bioavailability. Considering a wide safety window of WA, the current study encourage it development for clinical use via systemic route.

Chapter 3

Objective 2.
To evaluate the efficacy of Withaferin-A for the prophylaxis of acute Graft versus Host Disease and compare with standard prophylactic regimen

3.1 Introduction: Allogeneic hematopoietic stem cell transplantation (alloHSCT) is perhaps the only curative treatment option for several malignant and non-malignant haematological disorders (1,2). However, the success of alloHSCT is limited by acute graft versus host disease (aGvHD), a condition that typically manifests in the first 100 days of transplantation due to an exaggerated immune response of donor immune cells, mainly the activated T-cells, against the host tissue (3,2). Despite use of rigorous immunosuppressive therapy, approximately 30-70% of alloHSCT recipients experience aGvHD (4,5).

Among others, steroids remain the first line treatment of choice for aGvHD. Unfortunately, approximately 35-50% aGvHD patients become steroid refractory (2). More recently ruxolitinib was approved by the United States Food and Drug Administration (US FDA) for the treatment of steroid refractory aGvHD (103). Ruxolitinib, is known to have various toxicities and is also an expensive drug making it unaffordable to a majority of patients in resource limited countries. Other pharmacological agents used for treatment of aGvHD such as etanercept, anti-thymocyte globulin (ATG), pentostatin, sirolimus, mycophenolate mofetil and other immunosuppressive drugs have shown promise but are limited by their toxicities including increased susceptibility to various life threatening infections (104). Some of the above drugs may even compromise the beneficial Graft versus Leukemia (GvL) effect (9,7). Therefore, this opens avenues to develop novel pharmacological interventions for the prevention and treatment of aGvHD.

Withaferin-A (WA), principle active component of *Withania somnifera* (ashwagandha or Indian ginseng), has shown anti-proliferative, anti-inflammatory, anti-viral, hepatoprotective, nephroprotective, neuroprotective and several other biological activities in non-clinical studies (detailed in chapter 1, section 1.10). WA is known to

engage a number of molecular targets, which explains its pleiotropic effects (53). Among others, the anti-inflammatory activity of WA is well documented through inhibition of NF-kB, NLRP3 inflammasome complex, pro-inflammatory cytokines, and immune cells modulation (64,65,105). Recently, the beneficial effect of WA in a mouse model of islet cell transplantation has been established, where WA treatment increased the islet allograft survival by inhibiting the maturation of dendritic cells (66). These observations provided a strong basis to explore the role of WA for the prevention and treatment of GvHD.

Through our earlier work, we established proof of WA's utility for aGvHD prophylaxis. We demonstrated that WA inhibits proliferation and decreases cytokine secretion from mouse splenic cells without affecting the viability of lymphocytes (64). Further, we reported that, *ex-vivo* treatment of donor graft with WA reduces the incidence, severity and mortality associated with aGvHD in murine models of alloHSCT (65). However, in order to be clinically acceptable, the present study was conducted with an aim to establish the efficacy of systemically (oral) administered WA for the prophylaxis and therapy of aGvHD.

3.2 Aim and Objectives:

3.2.1 Aim

To evaluate the efficacy of Withaferin-A for the prophylaxis of aGvHD and compare with standard prophylactic regimen.

3.2.2 Objectives

Objective 2a: To evaluate the efficacy orally administered Withaferin-A for the prophylaxis of aGvHD in murine model

Objective 2b: To evaluate the aGvHD prophylactic effect of Withaferin-A in comparison with standard prophylactic regimen

Objective 2c: To evaluate the aGvHD prophylactic mechanism of Withaferin-A

Objective 2d: To evaluate the utility of Withaferin-A for the treatment of aGvHD in murine model

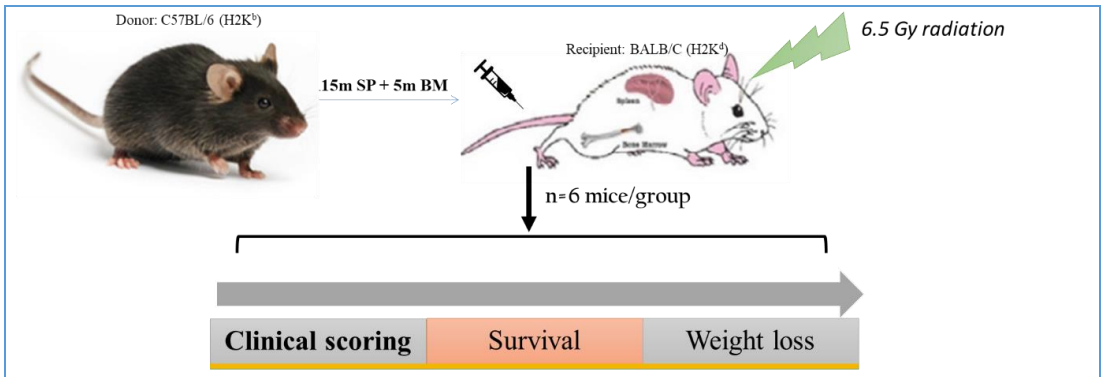
3.3 Materials and methods:

3.3.1 Reagents and antibodies: PhosphoJAK2 (pJAK2; Y1007/1008, cat: 3771), total JAK2 (tJAK2) (cat: 3220), phosphoSTAT3 (pSTAT3; Y705, cat: 9145) and total STAT3 (tSTAT3) (cat: 3230) was purchased from Cell Signaling Technology (CST). H-2K^b (cat: 562002), H-2K^d (cat: 553566) and 7AAD (cat: 559925) were procured from BD Biosciences. Pharmanza Herbal Pvt. Ltd. provided Withaferin-A (WA). Concanavalin-A (conA; cat: C5275) was procured from Sigma-Aldrich. Cytokine measurement were done using BD CBA mouse Th1/Th2/Th17 cytokine kit (cat: 560485). Phytohaemagglutinin (PHA, cat: 10576015) was procured from Gibco.

3.3.2 Experimental Animals: The study was approved by the institutional animal ethics committee of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) (project no: 04/2021, 21/2021). All animals were acclimatized for at least one week prior to start of the experiments. BALB/c female and C57BL/6 male mice of 8-10 weeks old having weight of 20±2 grams were used in the study. All animals were housed in the laboratory animal facility of the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC). Standard chow and water were given *ad libitum*. 55±15% humidity, 22-25^o C temperature with a 12 h light/dark cycle were maintained in the facility. All animal procedures were carried out in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and ARRIVE guidelines.

3.3.3 Induction of aGvHD and clinical scoring: The complete mismatched model of aGvHD were developed by carrying out transplantation between donor C57BL/6 (H-2K^b) and recipient BALB/c (H-2K^d) mice. Following 6.5 Gy of myloablative total body irradiation, BALB/c mice were injected with 15x10^6 splenocytes and 5x10^6 bone marrow cells of C57BL/6 mice. All mice were observed closely on a daily basis for

onset and severity of GvHD. aGvHD severity were assessed by clinical scoring system on a total score of 11. The clinical scoring system was based on the following six criteria: fur texture, skin integrity, posture, activity, weight loss and diarrhoea as defined by Lai et al. (1). Development of GvHD model is depicted in figure 3.1.



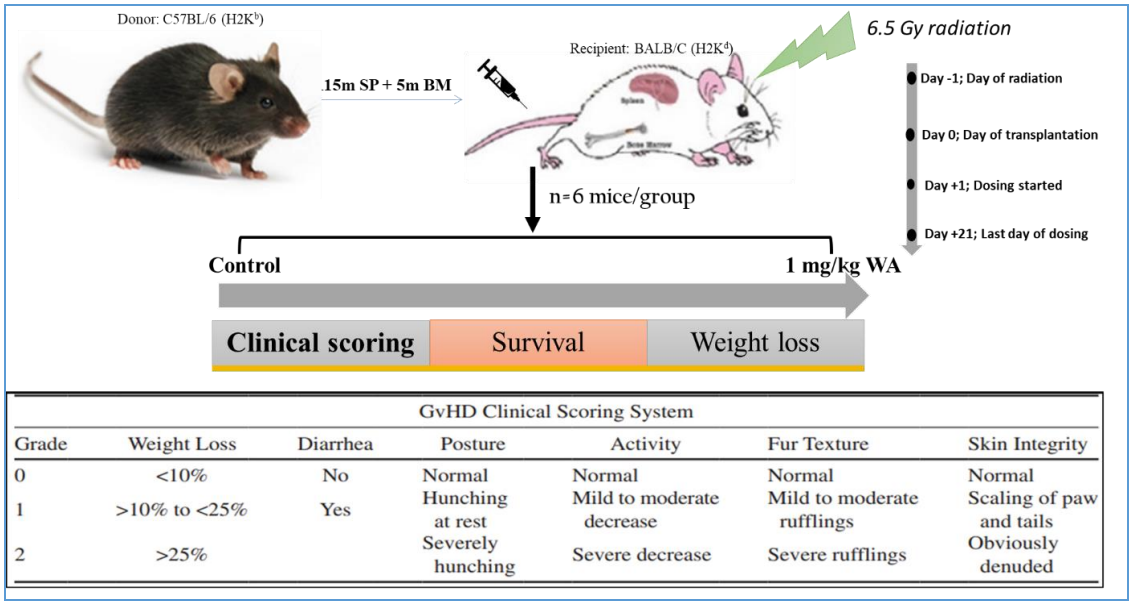
GvHD Clinical Scoring System

Grade	Weight Loss	Diarrhea	Posture	Activity	Fur Texture	Skin Integrity
0	<10%	No	Normal	Normal	Normal	Normal
1	>10% to <25%	Yes	Hunching at rest	Mild to moderate decrease	Mild to moderate rufflings	Scaling of paw and tails
2	>25%		Severely hunching	Severe decrease	Severe rufflings	Obviously denuded

Figure 3.1: Development of aGvHD murine model.

3.3.4 Study groups and intervention:

3.3.4.1 ***GvHD prophylaxis:*** After transplantation, mice were divided into GvHD control and WA (treatment) group. WA was administrated orally to the treatment group from day +1 of transplantation to day +21, at a dose of 1 mg/kg/day suspended in 0.5% sodium carboxymethylcellulose (CMC). The GvHD control mice were administered vehicle alone (fig 3.2).



GvHD Clinical Scoring System

Grade	Weight Loss	Diarrhea	Posture	Activity	Fur Texture	Skin Integrity
0	<10%	No	Normal	Normal	Normal	Normal
1	>10% to <25%	Yes	Hunching at rest	Mild to moderate decrease	Mild to moderate rufflings	Scaling of paw and tails
2	>25%		Severely hunching	Severe decrease	Severe rufflings	Obviously denuded

Figure 3.2: Experimental approach to test WA for prophylaxis of aGvHD.

We also compared the efficacy of WA with standard prophylactic regimen of Cyclosporine A (CSA) in combination with Methotrexate (MTX). CSA+MTX were injected to mice according to the earlier published study (65) (fig. 3.3).

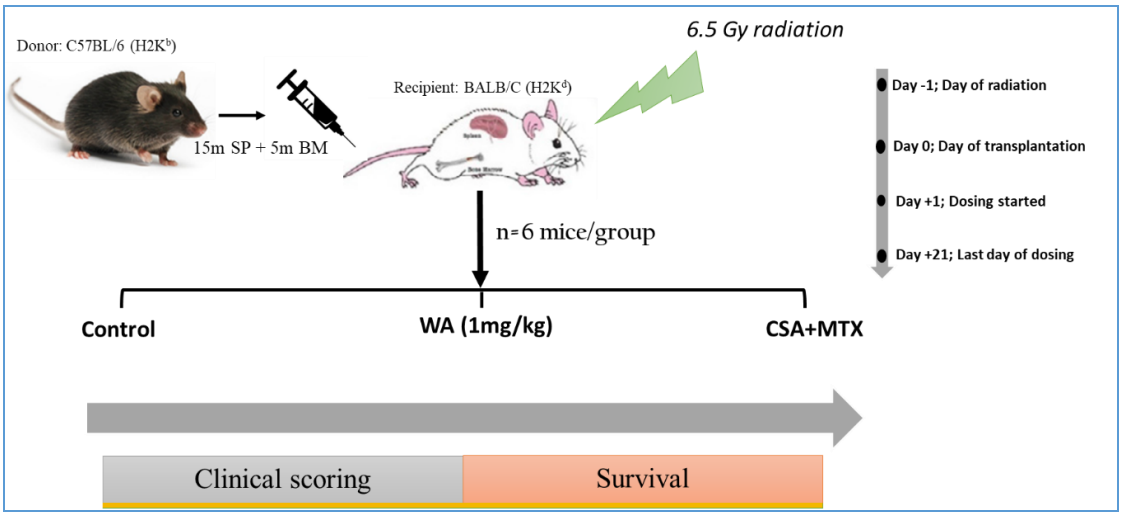
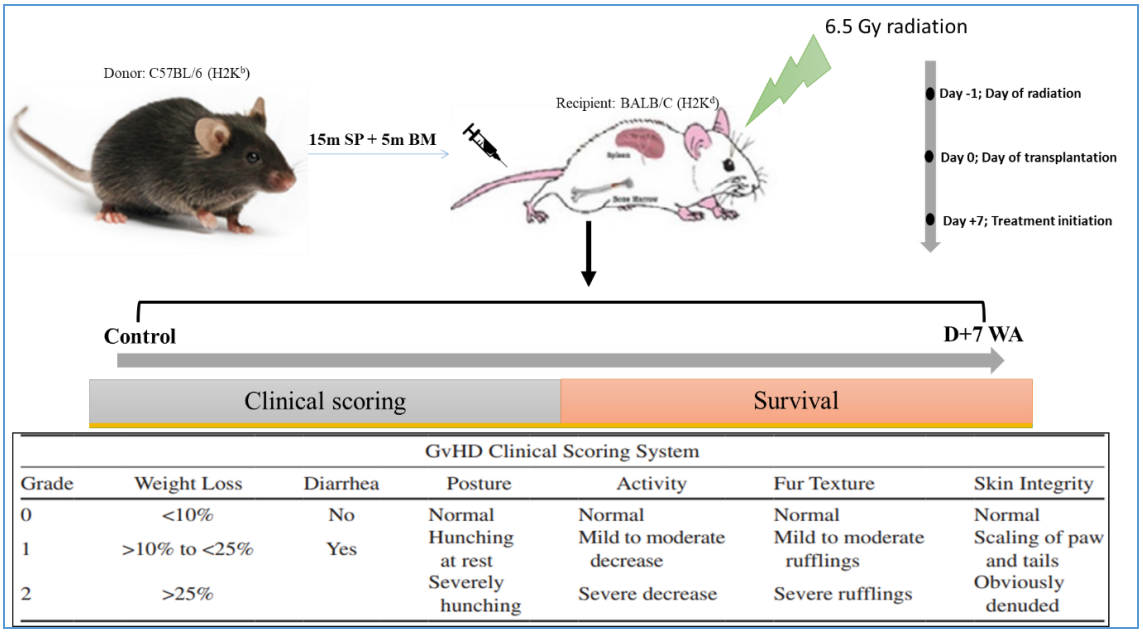


Figure 3.3: Experimental approach to compare efficacy of WA with standard prophylactic regimens.

3.3.4.2 ***GvHD treatment:*** Following transplantation, mice were allowed to develop GvHD. At day +7 mice were randomized in GvHD control and the WA (+D7), WA was administered from day +7 to +27 for treatment of aGvHD at a dose of 1 mg/kg/day. The GvHD control mice were given CMC as vehicle (fig 3.4).



GvHD Clinical Scoring System

Grade	Weight Loss	Diarrhea	Posture	Activity	Fur Texture	Skin Integrity
0	<10%	No	Normal	Normal	Normal	Normal
1	>10% to <25%	Yes	Hunching at rest	Mild to moderate decrease	Mild to moderate rufflings	Scaling of paw and tails
2	>25%		Severely hunching	Severe decrease	Severe rufflings	Obviously denuded

Figure 3.4: Experimental approach to test WA for treatment of aGvHD.

3.3.5 Histopathology: In a separate cohort of mice, to investigate the effect of WA treatment on target organ protection from GvHD. On day +7, +14 and +21, aGvHD target organs namely liver, skin, small intestine (SI), colon and lung were harvested from GvHD control and WA treated group and fixed in 10% neutral buffer formalin. The slides were stained with haematoxylin and eosin (H and E) in the animal histopathology laboratory at ACTREC. The tissue pathology evaluation and scoring were done as per method reported earlier (107,108,109). Specifically liver, SI and colon's scoring was performed as per Cooke et al.(107), Skin and lung was scored as per Sina et al.(108) and Cook et al. respectively (109). The same H and E slides were used to score the lymphocytic infiltration in the GvHD target organs as per method

mentioned in table 3.1. The scoring was done by trained pathologists who were blinded to the group assignment.

Table 3.1: Scoring method for tissue lymphocytic infiltration.

Absence	0-<5 %	Score 0
Mild	>5-<20 %	Score 1
Moderate	>20-<50 %	Score 2
Severe	>50 %	Score 3

3.3.6 Human peripheral blood mononuclear cells (hPBMCs) collection and isolation: Buffy coat from whole blood was collected from healthy donors after written informed consent. hPBMCs were isolated from whole blood sample using ficoll gradient method. Next, hPBMCs were counted (8-10 $x10^6$) and plated in following groups; (1) VC [dimethyl sulfoxide] (2) WA (3) PHA (4) WA+PHA [2h treatment with WA (1µM) followed by PHA stimulation]. PHA were used at a concentration of 0.25% for 72h (unless specified). At the end of the experiment, cells were collected and processed for evaluation of immune cell phenotyping. The supernatant media was taken for cytokine analysis. This study was approved by the institutional ethics committee of ACTREC, Tata Memorial Centre.

3.3.7 Flow cytometry:

3.3.7.1 ***In-vivo serum cytokine quantification:*** Serum Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-6, IL-10) and Th17 (IL17A) cytokines from GvHD control and WA treated groups on day +7, +14, +21 was measured using cytometric beads array as per manufacture's instruction. Briefly, 50 µl of mixed capture beads and 50 µl of detection reagent were added to 50 µl of serum sample. Following 2h incubation and washing of

excess antibody, cells were acquired on flow cytometry (FACSAria I, BD biosciences USA). The data were analysed using FCAPArray software version 1.0.0.

3.3.7.2 ***Evaluation of donor cells engraftment:*** The engraftment of donor positive cells (H-2K^{b}) was evaluated using flow cytometry. Briefly, peripheral blood was collected from recipient BALB/c mice through retro-orbital plexus. Following blood collection, RBC were lysed and washed thrice and cells were stained with fluorochrome labelled antibody. Donor positive cells expressing H-2K^{b} MHC class I were stained with FITC fluorochrome labelled antibody against it. Host cells expressing H-2K^{d} MHC class I on their cell surface were stained with PE fluorochrome antibody against it. These cells were acquired on attune NxT (Thermofisher, USA), and data were analysed using FlowJo software version 10.0.

3.3.7.3 ***Immune cell phenotyping of hPBMCs following WA treatment:*** The immune cell subsets such as monocytes, classical monocytes, non-classical monocytes, γδT-cells, PD1-CD4 and Tim3-CD8 cells were acquired on DxFlex (Beckman Coulter, Inc.). hPBMCs viability (using cell viability dye 7AAD) and frequency of Ki67+ CD3, CD4 and CD8 in all experimental arm were also evaluated. The assay and gating strategy were performed as per previously published method (110) (detailed list of marker is provided in table 3.2), and the data was analysed on a predesigned template using Kaluza software (version 2.1; Beckman Coulter, Inc.).

Immune Phenotype	Markers
T-cell	CD3
Th	CD4
Tc	CD8
hPBMC viability	7AAD dye
Proliferation	Ki67
GD-Tcell	CD3, CD45RA
TIM3	CD366
PD1	CD269
Monocytes	Absolute: CD11b Classical: CD11b, CD14++, CD16++ Non-classical: CD11b, CD14 (dim), CD16+

3.3.7.4 ***Cytokine measurements of hPBMCs:*** Cytokine analysis (IFN-γ, IL-6, TNF-α, IP-10, IFN-L1, GM-CSF and IL-1β) from hPBMCs following treatment with WA was performed on supernatant media using BioLegend® human anti-virus response panel 13-plex (BioLegend®, USA) cytometric bead array according to the manufacturer's instructions. Briefly, 25 µl of sample was incubated with 25 µl of capture bead mixture and 25 µl of assay buffer at room temperature (RT) for 2h. Samples were washed with wash buffer and centrifuged at 250g for 5 mins at RT. Further, detection reagent (25 µl) was added to this mixture and incubated at RT for 1h. Following this, 25 µl of SA-PE (secondary antibody) were added for 30 mins and sample were centrifuged at 250 g for 5 mins. Incubation of the samples with capture beads and then the detection reagent leads to the formation of a "sandwich complex" of the three. These complexes were acquired on LSR-Fortessa (BD Biosciences), and the data were analyzed using LEGENDplex8.0 software (Bio Legend, USA).

3.3.8 Western Blotting: For assessment of tJAK2, pJAK2, tSTAT3 and pSTAT3 levels in mouse splenic lymphocytes: following single cell suspension preparation and RBC lysis, cells were divided into following groups. (1) VC [dimethyl sulfoxide] (2) WA (3) ConA (4) ConA+WA. Cells were first exposed to WA for 2h at a concentration of 1µM. following which ConA was added for 3h at a concentration of 5 µg/mL.

In order to assess the effect of WA on protein levels of tJAK2, pJAK2, tSTAT3, and pSTAT3 on hPBMCs. hPBMCs were counted (8-10 $x10^6$) and plated in following groups; (1) VC [dimethyl sulfoxide] (2) WA (3) PHA [18h PHA stimulation] (4) WA+PHA [2h treatment with WA (1µM) followed by 18h PHA stimulation]. PHA was used at a concentration of 0.25%.

At the end of the experiment, cells were collected and lysed using RIPA buffer in the presence of protease inhibitors. The total protein concentration was measured using Bradford assay. Following SDS-PAGE electrophoresis, the proteins were transferred onto nitrocellulose membrane. Non-specific binding site was blocked with 5% BSA in TBST. Primary antibody for pJAK2, tJAK2, pSTAT3, tSTAT3 were added to the membrane and incubated for 48h at 4ºC, following which peroxidase conjugated secondary antibody was added. Immuno-reactive bands were developed by using ECL kit and visualized by Chemi Doc MP imaging system. Blot densitometric analysis was done using image J software version 4.0.1.

3.4 Statistics: Data was analysed using GraphPad Prism 8.0 software and represented as mean ± standard error of mean (SEM). Comparison between two groups was done using unpaired t-test. Comparison across multiple groups was done using one way ANOVA, followed by post hoc test. Kaplan–Meier plots and Mantel-Cox log-rank test were used for analysis of time to event data. $P < 0.05$ was considered statistically significant.

3.5 Results:

3.5.1 Prophylactic administration of WA mitigates aGvHD associated morbidity and mortality: Following transplantation, aGvHD phenotype as well as clinical score (CS) was monitored throughout the study in WA and GvHD control groups (fig 3.5A-B). A significant difference in CS was observed between the two groups of mice ($P<0.001$). Median CS on day +14 in the GvHD control and WA group was 9.5 and 2.0 respectively (fig 3.5B). Upon analysis of body weight alteration, GvHD control group showed significant weight loss compared to WA treated group ($P<0.05$ at day +14) (fig 3.5C). Furthermore, WA treatment improved survival of mice significantly compared to the GvHD control [HR=0.07 (0.01-0.35); $P<0.001$] (fig 3.5D). All mice in GvHD control group were dead by day +24 with a median survival time of 17.5 days. Median survival in WA treated arm remains undefined as 83.3% animals were alive till the end of experiment (fig 3.5D).

We further analyzed the engraftment of donor cells in WA treated mice and found complete presence of H-2K^b (FITC) positive cells in the host body, indicating that WA did not affect donor cell engraftment (fig 3.6A-B).

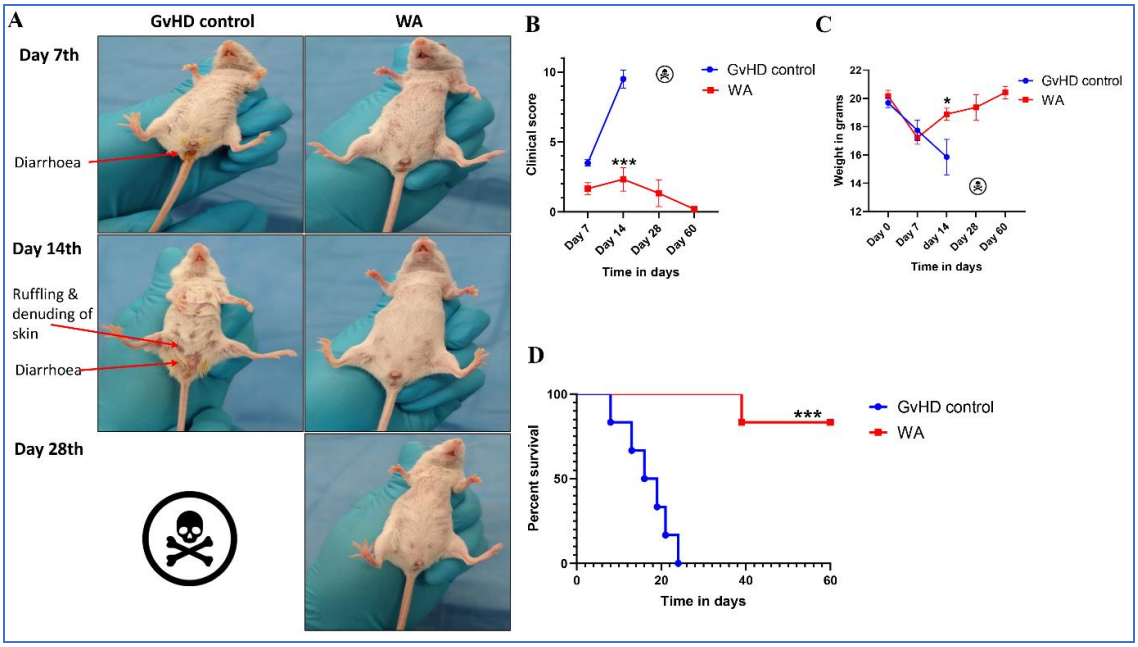


Figure 3.5: WA administration mitigates clinical aGvHD and improves survival of mice. Following transplantation of $15x10^6$ splenocytes and $5x10^6$ bone marrow cells from C57BL/6 to myeloablated BALB/c, mice were divided into GvHD control and WA treatment group. Vehicle or WA was given from +1 to +21 days through oral gavage. **(A)** aGvHD phenotype **(B)** clinical score and **(C)** weight of mice in GvHD control and WA treatment group were recorded periodically as shown. **(D)** Kaplan–Meier plot showing the overall survival in GvHD control and WA group. Data is represented as mean ± SEM. n=6 mice/group. *p= < 0.05, ***p= < 0.001.

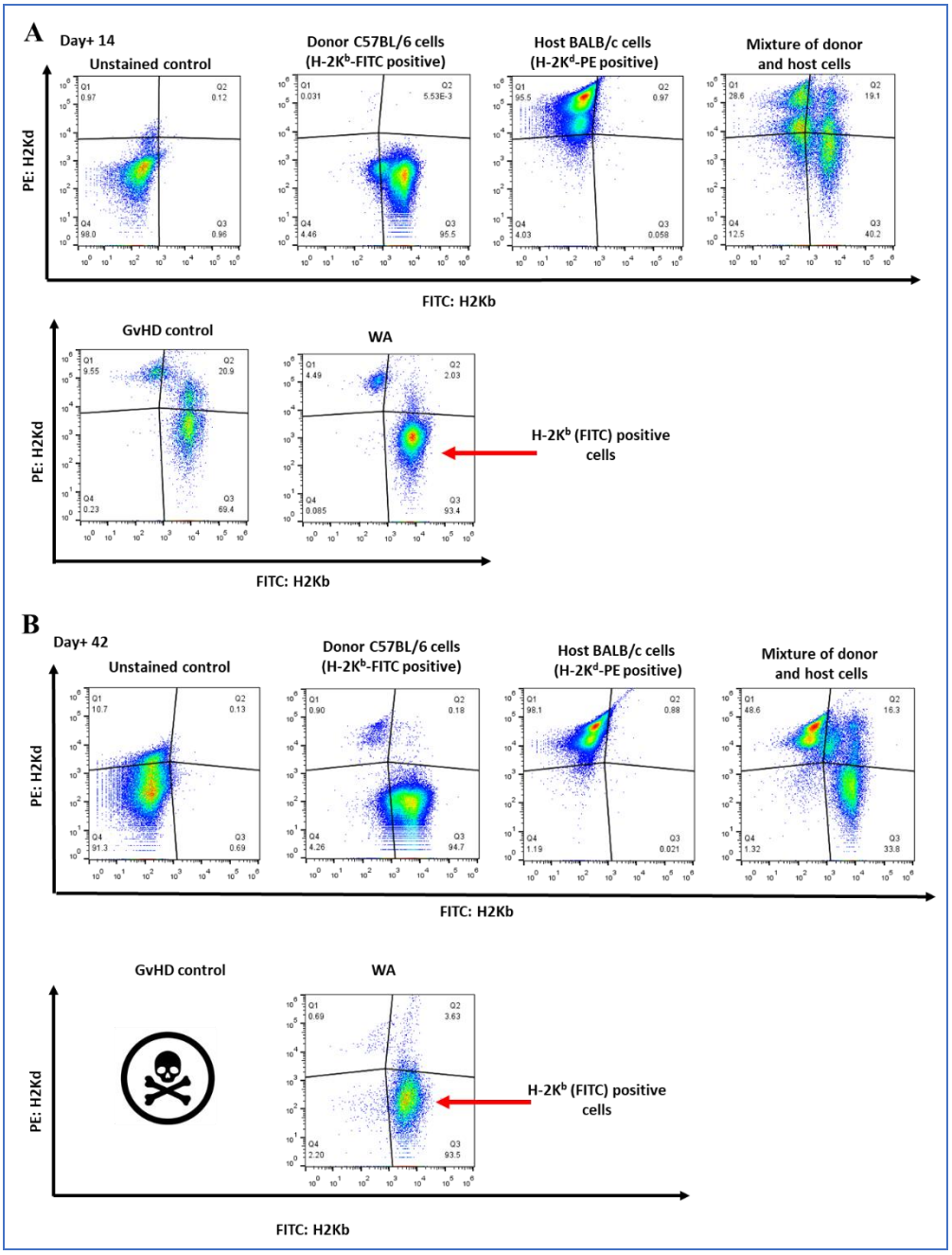


Figure 3.6: Effect of WA treatment on donor cell engraftment in the host. Engraftment was assessed on **(A)** day +14 and **(B)** +42. Briefly, following transplantation peripheral blood cells from mice were collected and subjected to H-2K^b (FITC) and H-2K^d (PE) staining as per protocol mentioned above. Donor mice cells express H-2K^b class of MHC antigen on their cell surface and recipient mice cells express H-2K^d class of MHC antigen on their cell surface. By day +14 mice in both the

groups showed engraftment of donor positive cells, which remains consistence even later course of time (+42) in WA group, wherein, mice in GvHD control group were dead by day +42.

3.5.2 WA protects GvHD target organ damage: The damage to target organs such as liver, skin, SI, colon and lung was assessed on day +7, +14 and +21 of transplantation. Upon histopathological examination in GvHD control group, the liver was characterized by chronic inflammation in portal area and lobular inflammation with neutrophilic infiltrates. Skin showed moderate-severe hyperkeratosis, severe chronic inflammation, focal basal vacuolar degeneration, thinning and ulceration of epidermis, mild-moderate chronic inflammation, diffuse desquamation and marked fibrosis of the dermis. The SI showed moderate chronic inflammation, marked blunting of villi and crypt loss. Colon showed severe chronic inflammation, lymphoid aggregates, fibrosis, diffuse ulceration, necrosis and marked crypt loss. Lung showed evidence of focal mild chronic inflammation composed of lymphocytes. Mice treated with WA showed marked protection of these organs compared to GvHD control (fig 3.7). Pathology score of these organs on +7, +14 and +21 is represented in figure 3.8. WA treated group also showed minimal lymphocytic infiltration in target organs and had low infiltration score compared to GvHD control group (fig 3.9).

Spleen weight is inversely proportional to the severity of aGvHD (111), therefore we also examined the weight of spleen on day +7, +14 and +21 in both the groups. As depicted in figure 3.10A-C, GvHD control animals had significant decrease in spleen weight and size compared to WA group ($P<0.05$, 0.01, 0.001 respectively at day +7, +14 and +21).

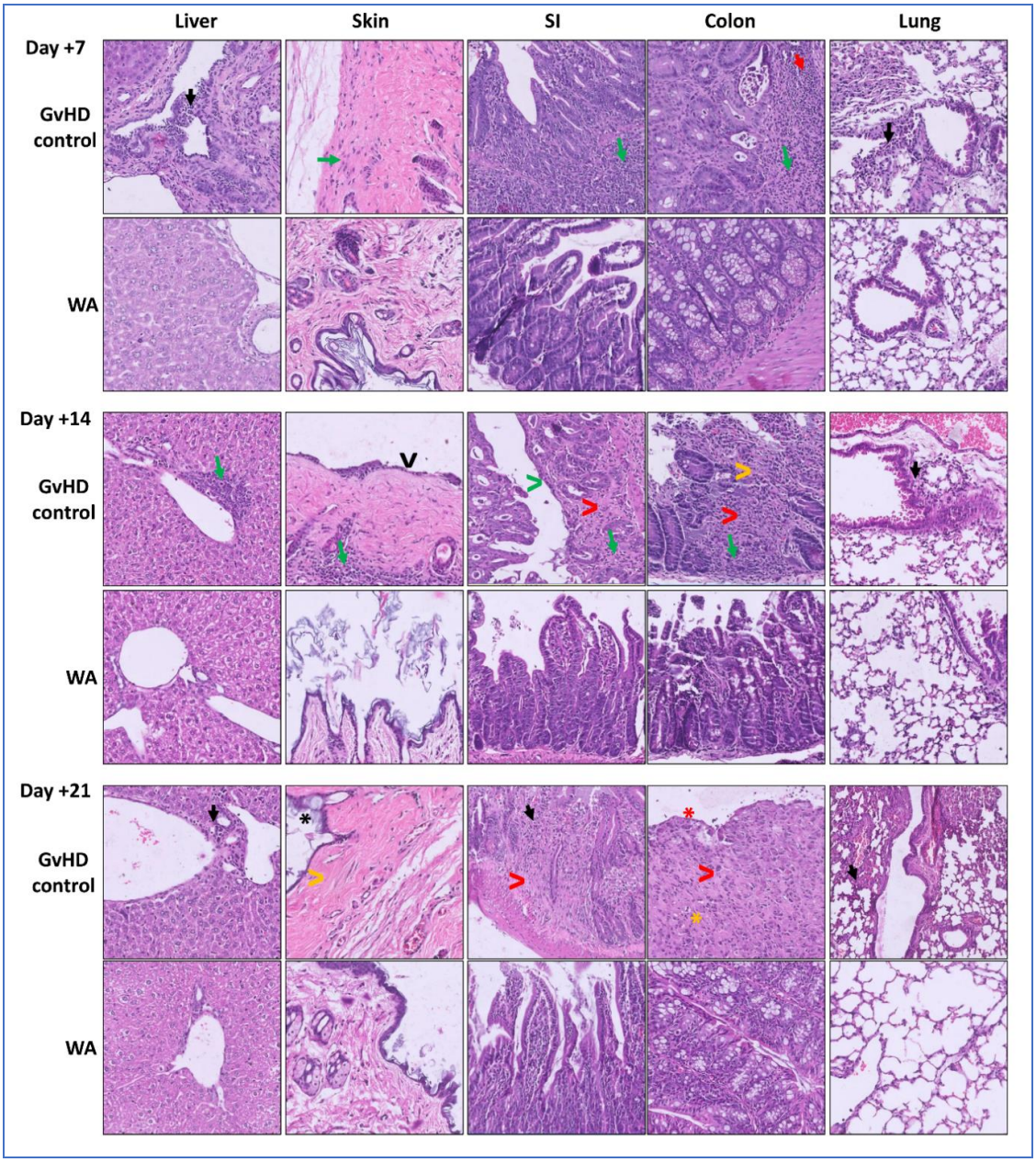


Figure 3.7: WA protects target organ damage from aGvHD. Following transplantation, on day +7, +14 and +21 mice from GvHD control and WA treatment groups were sacrificed and GvHD target organs were harvested. Tissues were fixed in 10% formalin buffer until preparation of H and E slide. The pathologist who were blinded to the group assignment evaluated tissue sections. Control group showed moderate to severe damage in live, skin, SI, colon and lung. Mice treated with WA showed intact architecture of GvHD target tissues suggesting marked protection. SI -

small intestine; Black arrow - mild chronic inflammation; Green arrow - moderate chronic inflammation; red arrow - lymphoid aggregate; black arrowhead - thinning and ulceration of epidermis; green arrowhead - blunting of villi; red arrowhead - crypt loss; yellow arrowhead - fibrosis; black star - desquamation; red star - ulceration; yellow star - severe chronic inflammation. H and E magnification 20x.

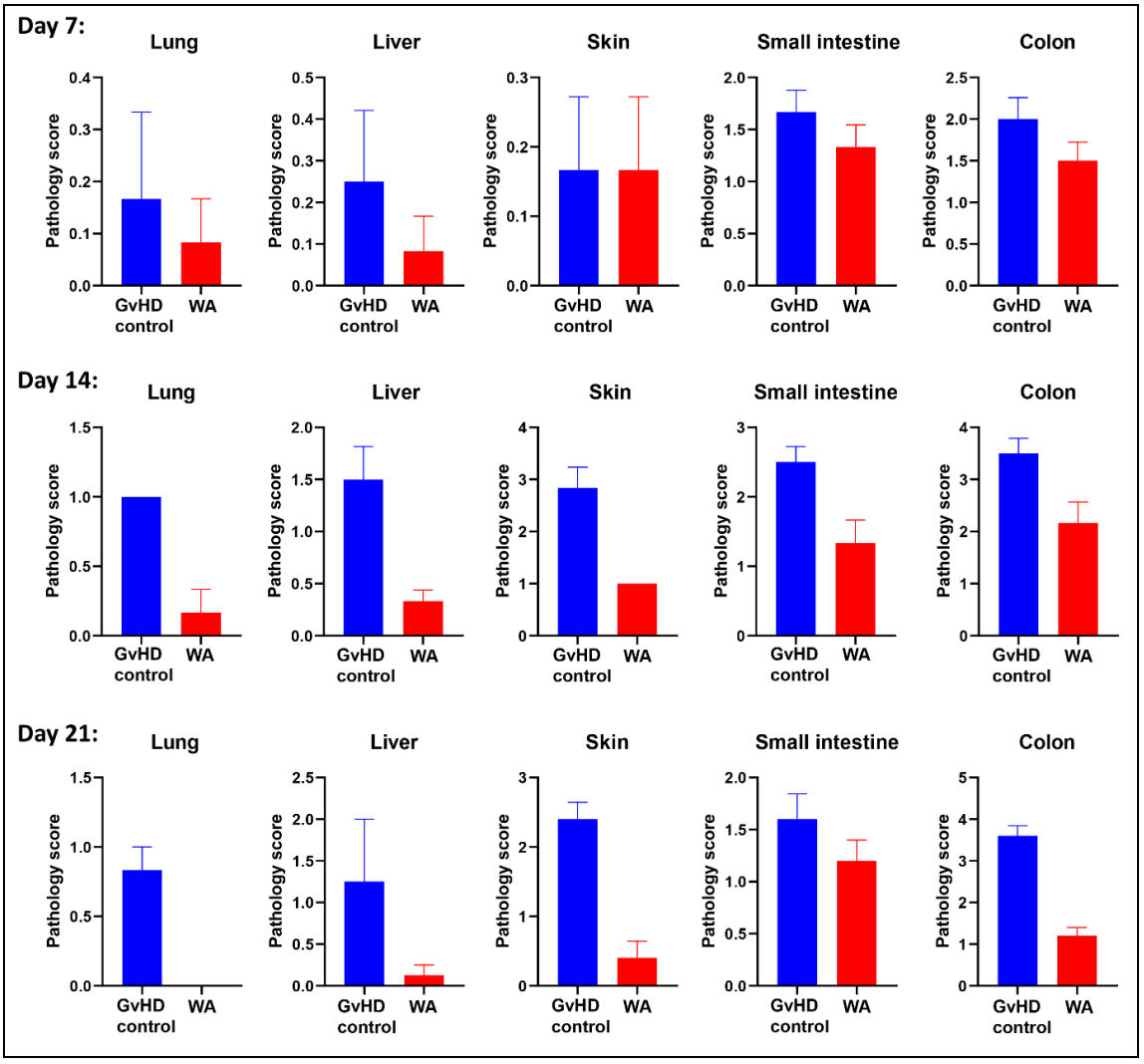


Figure 3.8: Pathology score of target tissue in WA group compared to GvHD control. The H and E slides were scored by pathologists for the extent of damage of organs as mentioned in Supplementary method 2. GvHD control group showed moderate to severe damage of liver, skin, small intestine, colon and lung. In contrast,

mice treated with WA showed marked protection of GvHD organs and had lower pathology scores. Data is represented as mean ± SEM. n=6 mice/group on day +7, +14 and 4 mice/group on day +21. Statistical test was not applied because of the high likelihood of survivorship bias in the control arm since only those animals that survived till the assessment day were included for scoring.

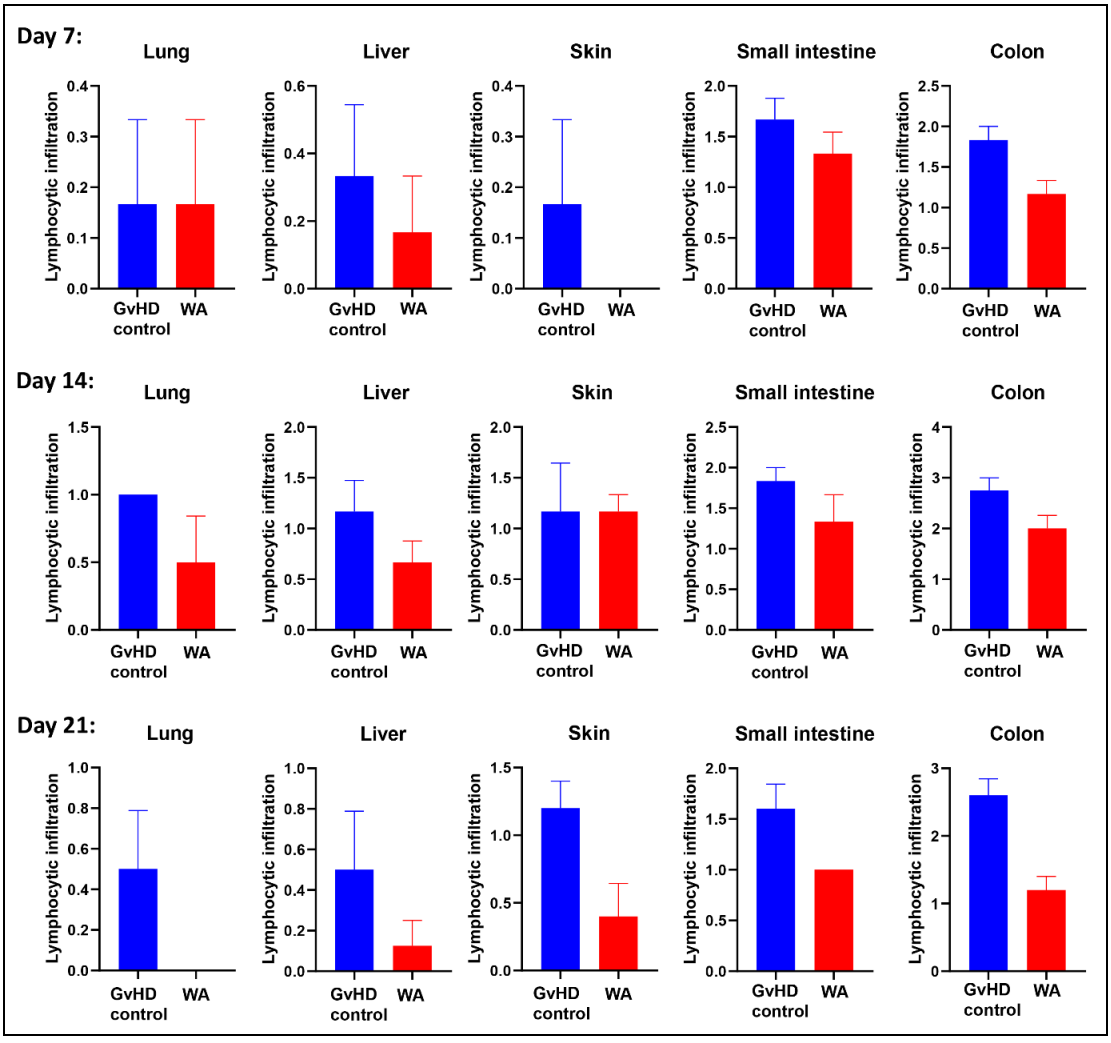


Figure 3.9: Effect of WA treatment on lymphocytic infiltration in aGvHD target organ. Following transplantation, at day +7, +14 and +21 mice from GvHD control and WA treated groups were sacrificed and GvHD target organs was harvested. Organs were fixed in 10% formalin buffer and H and E slides were prepared. Pathologists evaluated the tissue sections for lymphocytic infiltration based on method mentioned in

supplementary table 1. Markedly high lymphocytic invasion in liver, skin, small intestine, colon and lung tissues of GvHD control group was seen, and less so in the WA treated group. Data is represented as mean ± SEM. n=6 mice/group at day +7, +14 and 4 mice/group at day +21. Statistical test was not applied because of the high likelihood of survivorship bias in the control arm since only those animals that survived till the assessment day were included for scoring.

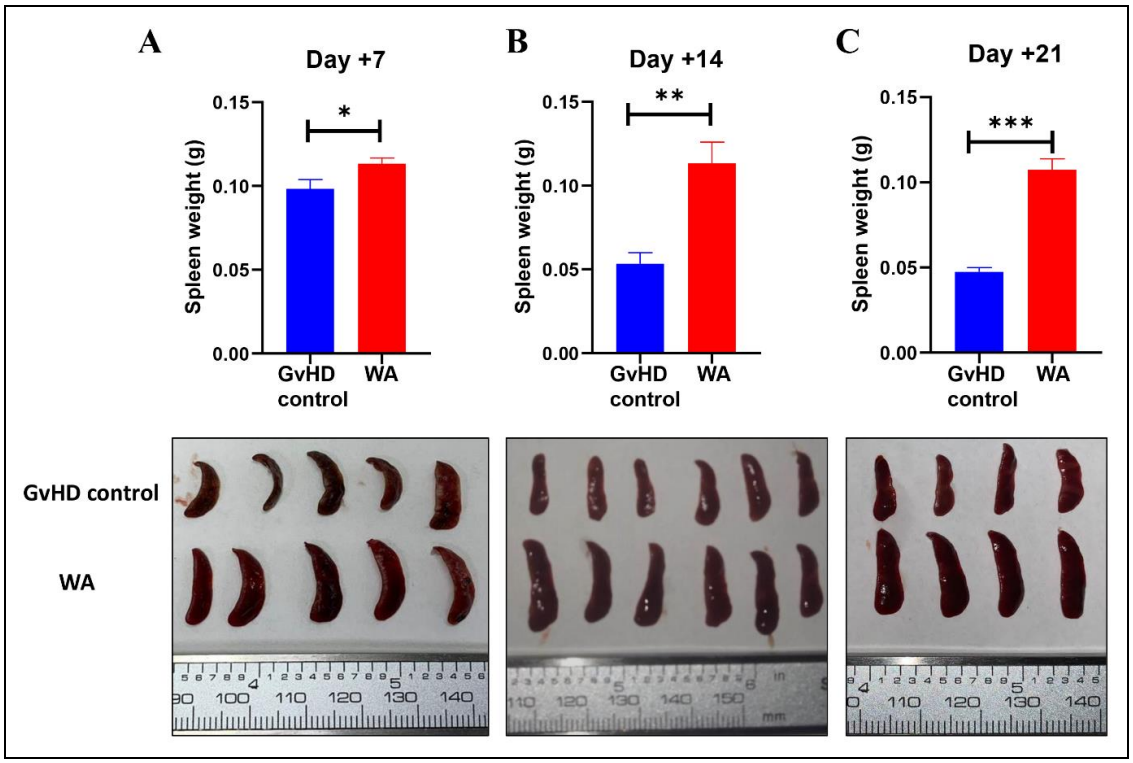


Figure 3.10: Effect of WA treatment on Spleen weight. Following transplantation at day **(A)** +7, **(B)** +14 and **(C)** +21, mice from GvHD control and WA treated group were sacrificed, and the spleen was collected for weight and size. As illustrated above, GvHD control animals had significant decrease in spleen weight and size compared to WA group. Data is represented as mean ± SEM. n=6 mice/group at day +7, +14 and 4 mice/group at day +21. *p= < 0.05, **p= < 0.01 ***p= < 0.001.

3.5.3 WA modulates cytokine secretion: Cytokine storm is known to play a central role in GvHD and several other immune-pathological conditions (112). Cytokine levels in GvHD control and WA treated groups are shown in figure 3.11 (day +7) and figure 3.12A-B (Day +14 and +21). Of note, pro-inflammatory cytokines such as IL-2, IFN-γ, TNF- α, IL-4, IL-6, IL-17A were found to be significantly decreased in WA treated mice compared to GvHD control on one of more occasions ($P<0.05$). In contrast, IL-10, an anti-inflammatory cytokine was found to be significantly increased on day +14 in WA group compared to GvHD control group ($P<0.05$) (fig 3.12A-B).

We next evaluated the effect of *ex-vivo* WA treatment on cytokine secretion from hPBMCs. Cytokines such as IFN-γ, IL-6, TNF-α, IP-10, IFN-L1, GM-CSF and IL-1β were found to be significantly decreased ($P< 0.001$, 0.001, 0.001, 0.05, 0.01, 0.01, and 0.05 respectively) in WA+PHA treatment group compared to PHA alone group (fig 3.13A-G).

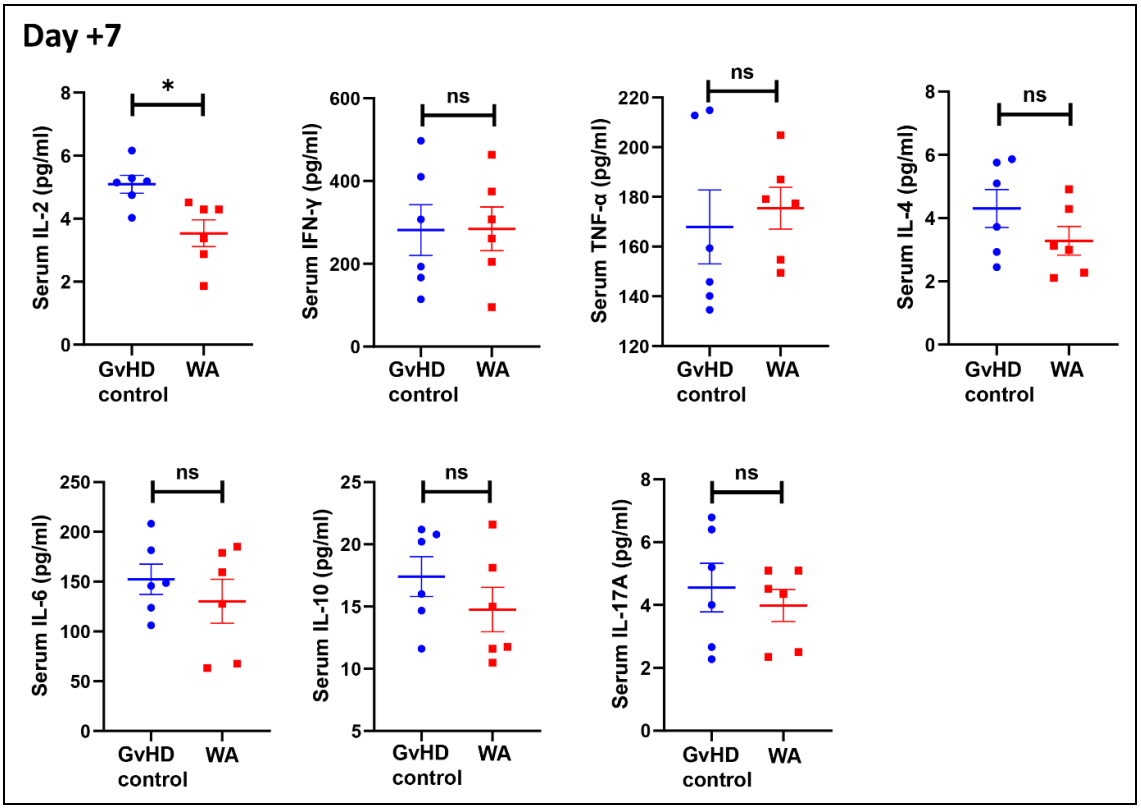


Figure 3.11: Effect of WA treatment on cytokine secretion *in-vivo*. At day +7 of transplantation, serum Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-6, IL-10) and Th17 (IL17A) cytokines levels were measured between GvHD control and WA treated groups using cytometric beads array. Data is represented as mean ± SEM. n=6 mice/group, ns= not significant. *p= < 0.05.

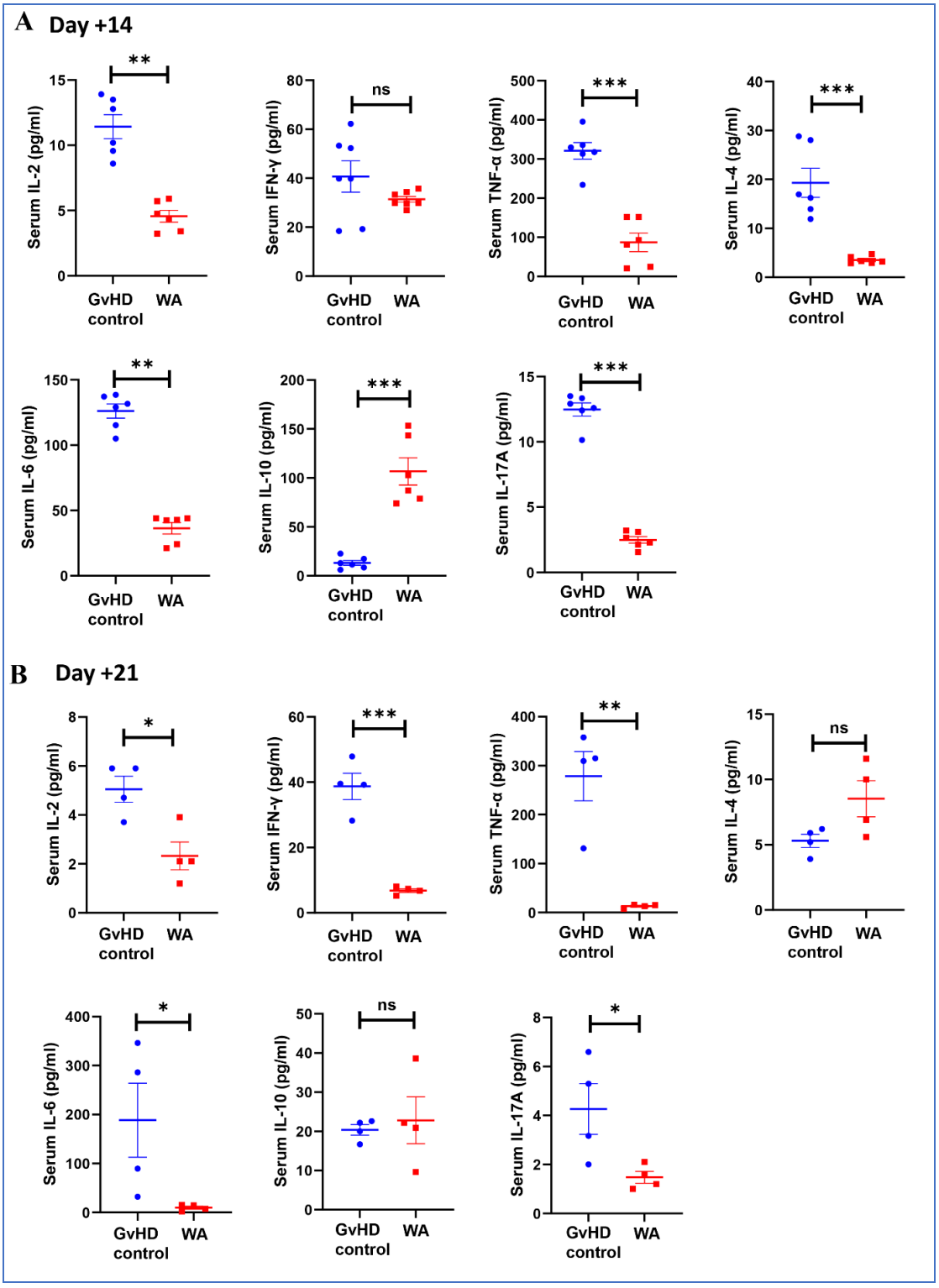


Figure 3.12: WA administration to mice modulates systemic cytokine secretion. After **(A)** day +14, and **(B)** +21 of transplantation, serum Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-6, IL-10) and Th17 (IL17A) cytokine levels were measured between

GvHD control and WA treated groups using cytometric beads array. Data is represented as mean ± SEM. n=6 mice/group at day +14 and n=4 mice/group at day +21. ns= not significant, *p= < 0.05, **p= < 0.01 ***p= < 0.001.

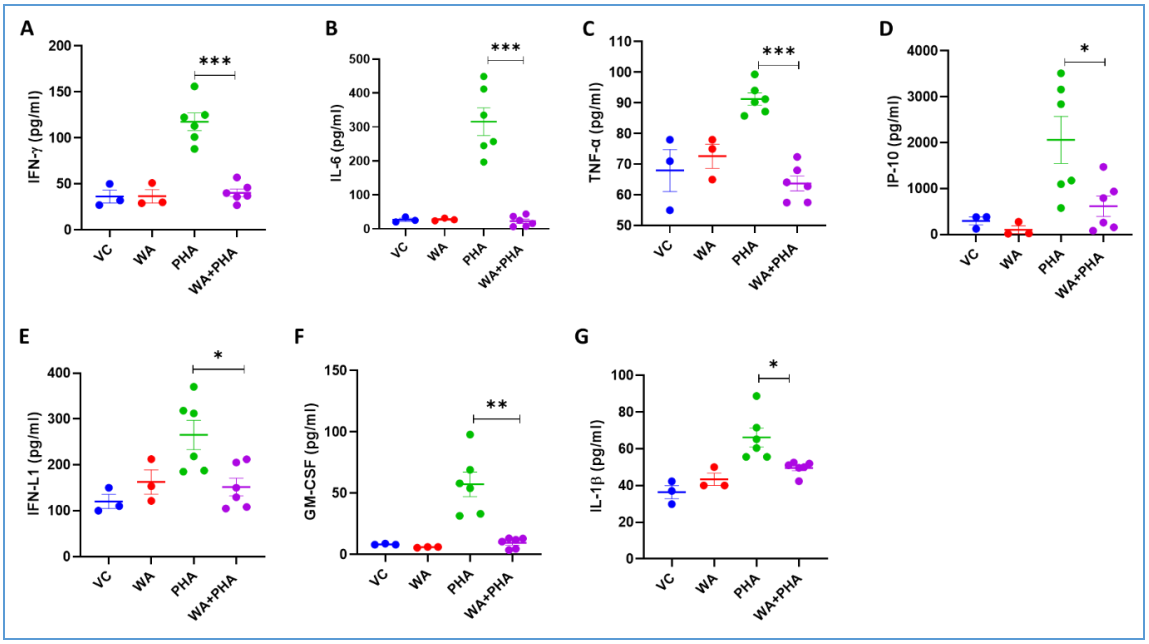


Figure 3.13: Effect of *ex-vivo* treatment of WA on cytokine secretion from human peripheral blood mononuclear cells (hPBMCs). Following treatment of hPBMCs for 2h with vehicle or 1µM WA and subsequent stimulation with PHA for 72 h, **(A-G)** cytokine levels were measured using cytometric bead assay in the media supernatant. Data is represented as mean ± SEM. N=6 independent experiments; PHA and WA+PHA, N=3 independent experiment; VC and WA. *p= < 0.05, **p= < 0.01, ***p= < 0.001.

3.5.4 WA inhibits JAK2-STAT3 signaling and modulates immune cell phenotype. In order to gain mechanistic insight, splenic lymphocytes of C57BL/6 mice were treated with WA *ex-vivo*. Upon western blot analysis, significant decrease in the protein levels of pJAK2 and pSTAT3 ($P<0.05$) was observed in ConA+WA group compared to ConA alone (fig 3.14A-B). The total protein levels of JAK2 and STAT3 remained unchanged (fig 3.14A-B).Consistent with this observation, hPBMCs treated with WA (WA+PHA group) *ex-vivo* also showed significant inhibition of pJAK2 and pSTAT3 protein levels ($P<0.01$), but no significant difference were seen in tJAK2 and tSTAT3 (fig 3.15A-B).

Furthermore, we evaluated the effect of *ex-vivo* WA treatment of hPBMCs on immune cell landscape. Monocyte subsets, γδT-cells, PD1-CD4 cells and Tim3-CD8 cells were analysed. The absolute monocyte count and absolute classical monocyte count were found to be significantly decreased in WA+PHA treated group compared to PHA alone group ($P<0.01$ and 0.01 respectively) (fig 3.16A-B). The non-classical monocytes were significantly increased in WA+PHA group compared to PHA group ($P<0.05$) (fig 3.16C). Furthermore, absolute γδT-cells were found to be significantly increased in WA+PHA treated group compared to PHA group ($P<0.01$) (fig 3.16D), while in contrast, the absolute PD1-CD4 and Tim3-CD8 cells were found to be decreased significantly in WA+PHA group compared to PHA group ($P<0.01$ and 0.05 respectively) (fig 3.16E-F). Additionally, in presence of WA (WA+PHA group), the frequency of Ki67+ in CD3+, CD4+ and CD8+ T-cells was found to be significantly decreased compared to PHA group ($P<0.01$, 0.01, 0.01 for CD3+, CD4+ and CD8+ cells respectively) (fig 3.16G-I). Further, no significant difference in percent viability of hPBMCs were observed between control and WA group (fig 3.16J).

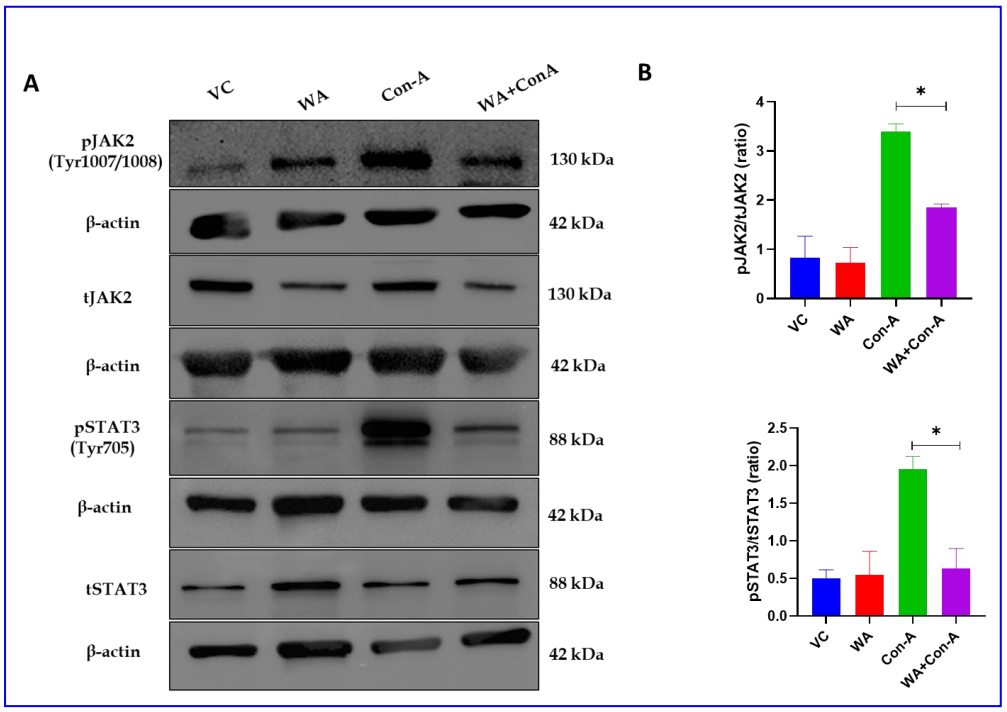


Figure 3.14: Effect of WA treatment on JAK2-STAT3 protein levels in mouse splenic cells. Following single cell suspension preparation, mouse splenic cells were divided into; (1) VC (2) WA (3) conA (4) conA+WA. Cells from all groups were harvested and subjected for western blotting. **(A)** Representative western blots showing the expression of pJAK2, tJAK2, pSTAT3 and tSTAT3 protein levels in mouse splenic cells. **(B)** Bar diagram showing ratio of relative intensity of pJAK2/tJAK2 and pSTAT3/tSTAT3 protein levels. Data is represented as mean ± SEM. n=3 biological replicates. ns= not significant, *p= < 0.05.

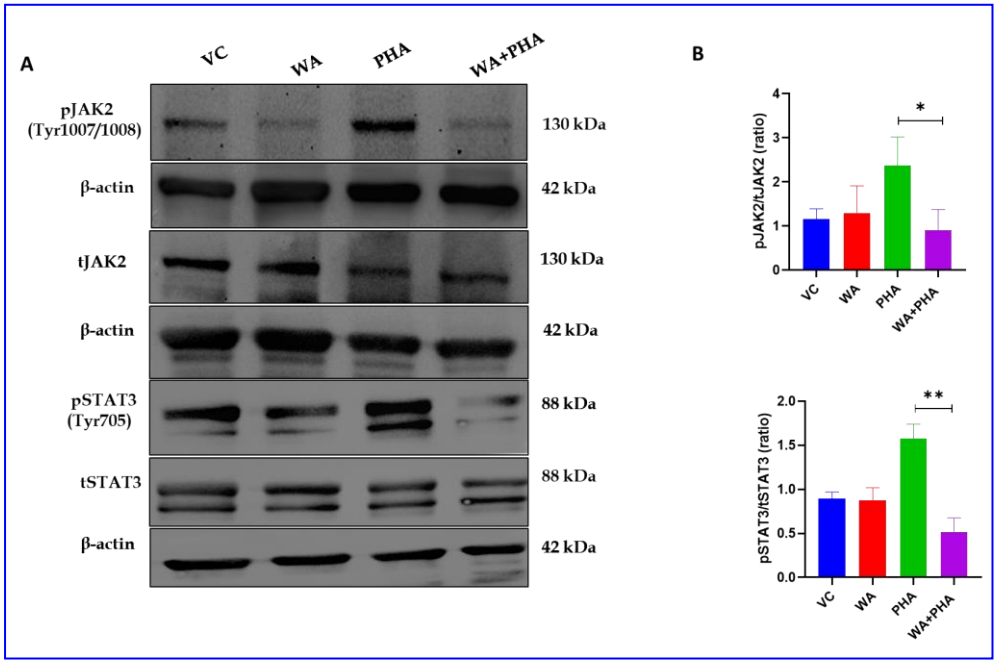


Figure 3.15: Effect of WA treatment on JAK2-STAT3 protein levels in hPBMCs. Following treatment of hPBMCs with (1) VC (2) WA (3) PHA (4) WA+PHA, proteins levels of pJAK2, tJAK2, pSTAT3 and tSTAT3 were measured using western blot. **(A)** Representative western blots showing the expression of pJAK2, tJAK2, pSTAT3 and tSTAT3 protein levels. **(B)** Bar diagram showing ration of relative intensity of pJAK2/tJAK2 and pSTAT3/tSTAT3 protein levels. Data is represented as mean ± SEM. N=3 independent experiments. **p= < 0.01.

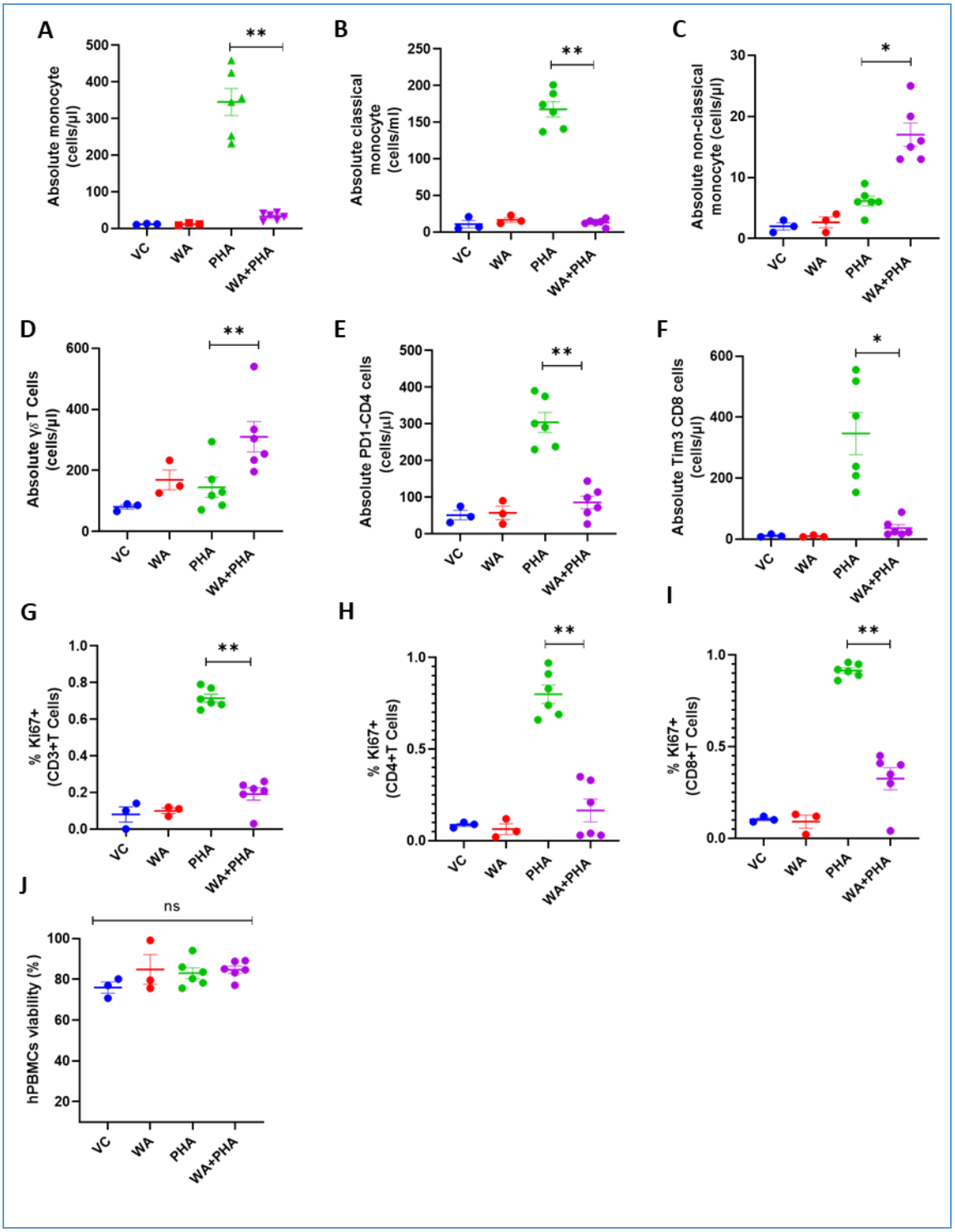


Figure 3.16: Effect of WA treatment on immune cell modulation. Human peripheral blood mononuclear cells (hPBMCs) were treated with (1) VC (2) WA (3) PHA (4) WA+PHA. After 72 hrs of incubation hPBMCs were collected. **(A-F)** Absolute counts of immune cells such as monocyte subset, γδT-cells, PD1+CD4 cells and TIM3+CD8

cells were analysed between control and treatment groups using flow cytometry. **(G-I)** Frequency of Ki67+ T-cell subsets were analysed using flow cytometry. **(J)** Percent viability of hPBMCs in control and WA treated group. Data is represented as mean ± SEM. N=6 independent experiments; PHA and WA+PHA, N=3 independent experiment; VC & WA. *p= < 0.05, **p= < 0.01.

3.5.5 WA exhibits superior efficacy compared to standard prophylactic regimen: We compared the efficacy of WA with standard prophylactic regimen of CSA+MTX. At day +14, median CS in control, WA, and CSA+MTX group was 7.5, 2 and 4 respectively. WA alone was superior to CSA+MTX in lowering the CS of aGvHD (P<0.05) (fig 3.17A). Nevertheless, CSA+MTX could also ameliorate aGvHD symptoms significantly compared to control (P<0.001) (fig 3.17A). We also observed initial weight loss in all the study groups, but animals in the treatment groups could regain their weight after day +14 (fig 3.17B). Further, survival analysis suggested better overall survival in WA arm compared to standard prophylactic regimen [HR=0.19 (0.03-1.1)] (fig 3.17C). At the end of the experiment, the percent survival in control, WA and CSA+MTX groups were 0, 83.3 and 33.3 respectively (fig 3.17C). The median survival was 19 and 31 days in control and CSA+MTX group respectively, while median was undefined in the WA group (fig 3.17C). Comparative CS and survival among the study groups is shown in figure 3.17D.

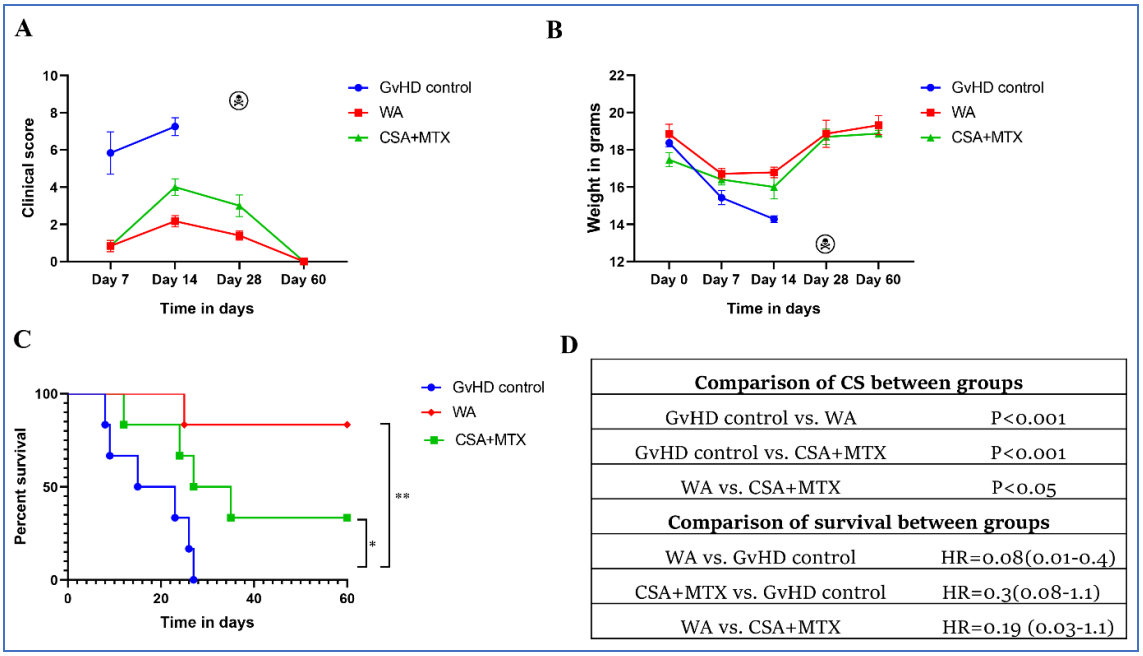


Figure 3.17: Comparison of WA's efficacy with standard prophylactic regimen. Following total body radiation and bone marrow transplantation, recipient mice were divided into (1) GvHD control (2) WA and (3) CSA+MTX. **(A)** GvHD clinical score (CS) in all groups were monitored periodically. **(B)** Comparison of weight changes among study groups. **(C)** Kaplan–Meier plot showing the overall survival of mice in all experimental groups. **(D)** Comparison of CS and survival among the study groups. Data is represented as mean ± SEM. n=6 mice/group. *p= < 0.05, **p= < 0.01.

3.5.6 WA can reverse aGvHD and improve survival of mice. To test the anti-GvHD efficacy of WA, we allowed mice to develop aGvHD and then WA was administered from day +7 of transplantation. The aGvHD phenotype was monitored throughout the experiment, representative animals of each group at various point of time are shown in figure 3.18A. Median CS in the GvHD control and WA group was 7 and 3.5 respectively ($P<0.01$ at day +14) (fig 3.18B). Next, we analysed the weight changes in the two experimental groups. As shown in figure 3.18C, continuous weight loss was observed in GvHD control mice, which was reversed in WA treated group (fig 3.18C). Median overall survival was significantly higher in the WA treated group compared to GvHD control (undefined vs. 19.5 days; HR=0.2 (0.07-0.8); $P<0.01$) (fig 3.18D).

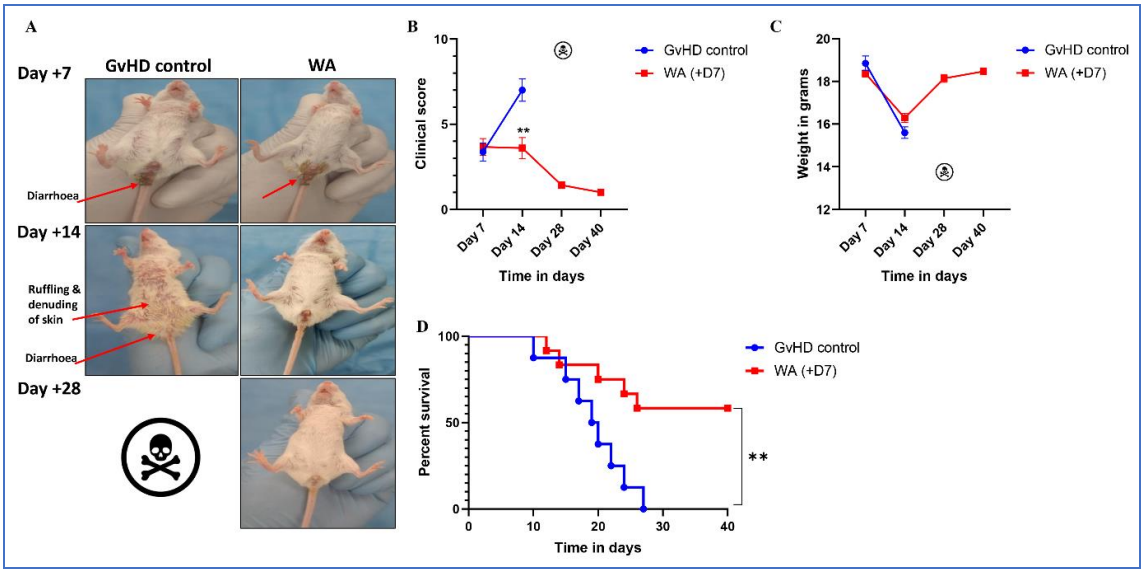


Figure 3.18: **WA exhibits anti-GvHD potential and reverses established aGvHD.** GvHD was induced as described in the methods section (n=20) and WA treatment was initiated in n=12 animals on +7 day of transplantation. The remaining 8 animals acted as GvHD control **(A)** GvHD phenotype in both the study groups were recorded periodically. **(B)** Clinical score and **(C)** weight of mice in both the groups at various time points post-transplant are shown. **(D)** Survival analysis was carried out by Kaplan-Meier plots and compared between WA and GvHD control groups using the log-rank test. Clinical score and weight of the mice is represented as mean ± SEM. **p= < 0.01.

3.6 Discussion:

Withaferin-A is a phytochemical having well documented anti-inflammatory, immune-modulatory and anti-proliferative properties (64,65,105,113). In the current study for the first time, we established the aGvHD prophylactic and therapeutic potential of WA administered systemically. WA significantly mitigated aGvHD associated morbidity and mortality without compromising donor cell engraftment into the host body. WA could also ameliorate the aGvHD target organ damage by preventing the lymphocyte migration to these organs and limiting the serum cytokine storm probably through inhibition of JAK2-STAT3 signaling cascade - the principal signaling pathway implicated in the onset of aGvHD. We also demonstrated that WA modulates immune cell landscape along with suppression of pro-inflammatory cytokines from hPBMCs without affecting their viability. Furthermore, we could demonstrate the superiority of WA over CSA+MTX, which is the most widely used standard prophylactic regimen in clinics.

Several signaling cascades and crosstalk between them are involved in the pathogenesis and progression of aGvHD (114). One such signaling pathway is JAK2-STAT3 (114). Activation of JAK2-STAT3 leads to the migration of pSTAT3 into the nucleus, while at the same time activated JAK (pJAK2) triggers JAK/PI3K/NF-kB/Akt/mTOR axis (114,45). These series of events lead to immune cell proliferation, differentiation, cytokine storm and lymphocytic migration to GvHD target organ. Herein, we demonstrated the ability of WA to inhibit JAK2-STAT3 along with inhibition of cytokine release and lymphocytic migration to target organs. While JAK2-STAT3 inhibition by WA is already known (115,116), our findings hints at possible mechanistic association between WA, JAK2-STAT3 and immune modulation required for anti-GvHD activity. Earlier studies from our group have shown that WA also inhibits NF-kB

and Akt-mTOR signaling (64,65). Importantly, WA is known to bind and inhibit JAK2, STAT3 and NF-kB separately and independent of each other (64,116). Therefore, we postulate that WA exerts pleotropic action along the JAK/PI3K/NF-Kb/Akt/mTOR axis. The proposed immunomodulatory and anti-GvHD mechanism of WA is illustrated in figure 3.19.

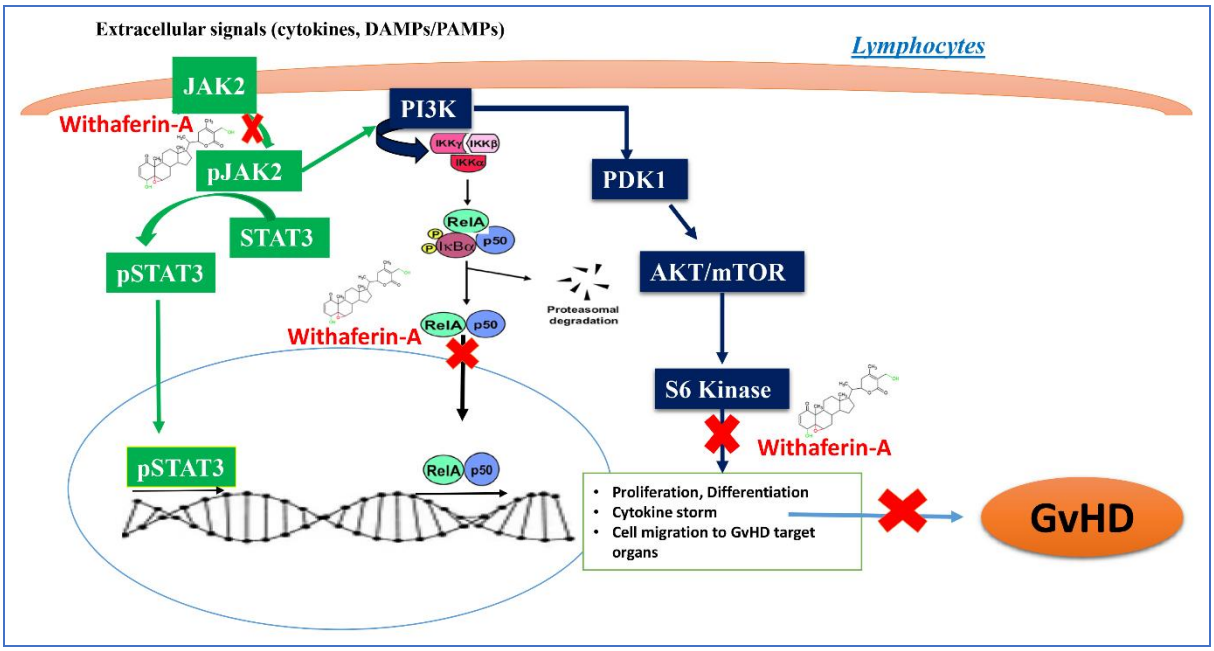


Figure 3.19: Proposed immunomodulatory and anti-GvHD mechanism of WA.

The exaggerated immune response in aGvHD is manly driven by antigen presenting cells (APCs), predominantly the monocyte subsets, and T-cells (100). Therefore, pharmacological interventions to inhibit APCs and T-cells are useful in the prevention and treatment of aGvHD (100). Corroborating the earlier report by Kumano et al. (66), we also found that WA inhibited the proliferation of human T-cell subsets. There was also a significant decrease in classical monocyte counts following treatment with WA. At the same time, significant increase in non-classical monocytes was observed which might again be important in the context of GvHD for their anti-inflammatory properties (117).

While WA modulates the immune system, it should not kill the lymphocytes functional graft. Therefore, we evaluated its effect on hPBMCs survival and found that it does not effects the viability of the hPBMCs. Also, the monocytes were not isolated and cultured separately in these experiments, it was the hPBMC (having monocytes along with other cells) which was cultured using standard protocol (10% FBS, IMDM media, incubation: 5% CO_2 at 37°C).

aGvHD prophylactic agents such as CSA, MTX, tacrolimus, mycophenolate mofetil, and post-transplant cyclophosphamide are not always effective and they are associated with severe adverse events such as drug induced toxicity of vital organs, opportunistic infection and loss of beneficial GvL effect (103,104,118,119). WA can be a safe alternative as seen from its tolerability reported by us in rodents and humans (120,74). It is pertinent to note that the superior immunomodulatory efficacy of WA vis-à-vis standard immunosuppressant such as tacrolimus is already established in murine model of allogenic islet transplantation (66). Our findings reiterates this observation, albeit in a model of aGvHD, thus firmly asserting its potential as a candidate for pharmacotherapy in these indications. The suppression of inflammatory cytokine secretion reported by Sorelle et al. and Kumano et al. earlier in line with our findings reaffirms the mechanistic basis of immunomodulation by WA (106,66). As discussed above, non-classical monocytes are widely accepted as anti-inflammatory cells (117), which are known to secrete IL-10 (121), an anti-inflammatory cytokine (122). In the current study, we observed increase in the levels of IL-10, which correlated with the increase in non-classical monocytes count, suggesting WA not only suppresses inflammatory milieu but also augments the anti-inflammatory response.

One of the major T-cell population i.e. Treg cells plays a crucial role in mitigation of GvHD. Earlier work from our group has assessed the impact of WA on Tregs and showed showed that WA increases the Treg population independent of NRF2 (DOI:10.1182/blood.V130.Suppl_1.3174.3174).

In a phase I clinical trial in patients with high grade osteosarcoma, the clinical safety of WA has already been demonstrated up to 216 mg/day (74). Besides, there are several disorders having similar immunological basis as GvHD, wherein, JAK2-STAT3 signaling is predominantly implicated such as cancer, COVID-19 and cytokine release syndrome (80,114,123). This lends support to a systematic development plan of this important phytochemical against these indications as a low-cost alternative to the currently approved standards of care.

3.7 Conclusion: Our findings established that WA abrogates aGvHD manifestation and could be a potential treatment option for patients with aGvHD. WA's efficacy either alone or in combination with standard regimens should be tested in a prospective clinical trial.

Chapter 4

Objective 3.
To investigate the impact of Withaferin-A on Graft versus Leukemia effect

4.1 Introduction

AlloHSCT is being performed with an intent to cure underlying haematological disorders such as leukemias (124). The ability of donor graft to recognise residual leukemic cells and to eradicate them is known as graft versus leukemia response (GvL). However, GvHD occurs at cost of purging underlying disease (3). In 1956 Barnes and his colleagues defined the GvL for the first time in murine experiment, wherein transplantation of leukemic mouse with bone marrow cured the leukemia but mice died because of diarrhoea (5). This led to the beginning of treating leukemias using bone marrow transplantation. After a decayed, Mathe and his group tested this phenomenon in human, where they found that the patients are dying because of secondary disease, later this known as GvHD (125). This challenged the scientist to study GvHD and GvL closely to find out what causes these outcomes. A series of experiments over a decayed emphasised that donor T-cells are responsible for GvL effect but remains the same for GvHD as well (Fig 4.1). These T-cells recognise leukemic cells through binding with their receptor and MHC present on leukemic cell surface. This also explains why earlier strategy of depleting T-cells for preventing GvHD was failed due to increase in relapse rate of leukemia (49).

Despite rigorous research, it is still unclear what separates GvHD from GvL. However, recent technology advances have made scientist to understand these phenomenon to some extent. With the help of immune cell phenotyping scientist have proposed certain immune cells which seems to be prominent for GvL effect but not for GvHD. These cells are mainly natural killers (NK) and γδT-cells. Research exploring the anti-leukemic activity of NK and γδT-cells have paved the way to utilise these cells for immunotherapy in haematological and solid tumors (126).

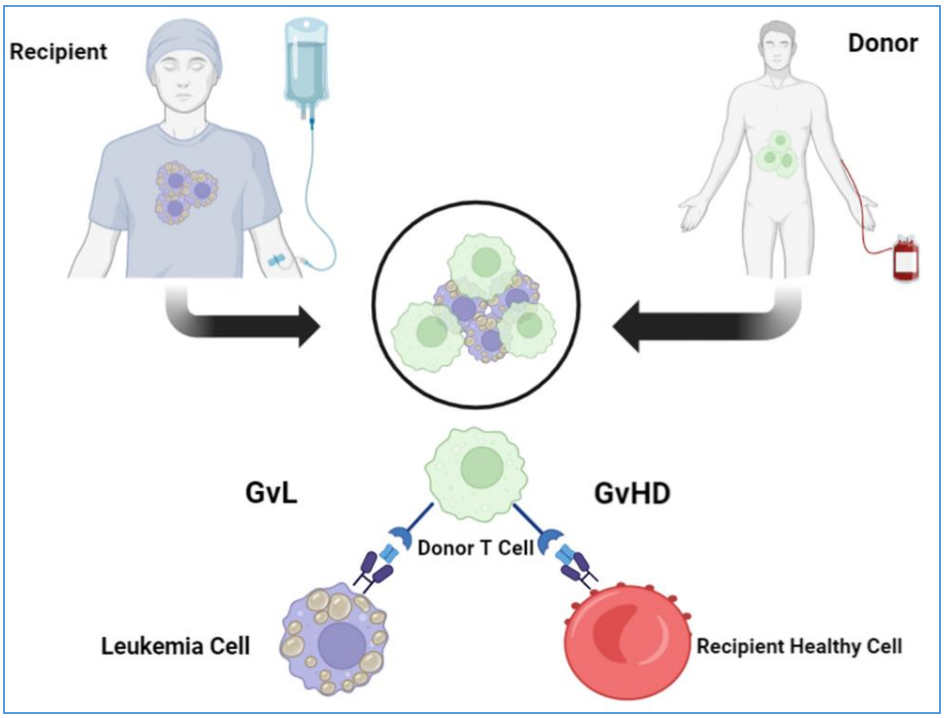


Figure 4.1: Role of T-cell in GvHD and GvL.

In a recent report, increase γδT-cells showed positive correlation with increased leukemia free survival in patients undergone bone marrow transplantation (127). Therefore, several study have proposed to use in-vivo or ex-vivo expanded γδT-cells for enhancing the GvL without GvHD. This strategy has also proven successful for developing γδ T-cells as an immunotherapy candidate (127). In a recent preclinical study, Song et al. showed that donor γδT-cells promotes GvL without causing GvHD. This study also evaluated that Vγ4 subset of γδT-cells was major phenotype responsible for GvL maintenance through partial dependency on IL17-A (128). Despite this, further investigation on other immune cells are required to establish the role of immune landscapes in maintenance of GvL.

A recent review by Harries et al. emphasised on role of transcription factors in regulation of GvHD and GvL (129). They also focused on how targeting these transcription factors can be helpful in decreasing GvHD and keeping the GvL intact. A

list of transcription factors its effect on outcome of GvHD/GvL is tabulated below (table 4.1)

Table 4.1: List of transcription factor, its role and implementation in GvHD and GvL response.

Factor	Function Impacted	Role	Impact of Loss
JAKs	Differentiation, Proliferation, Cytokine Production	Controls Th1/Th2 polarization, Treg cell fate, proinflammatory cytokines, and T cell proliferation	Reduced GVHD, maintained GVT
STATs	Differentiation, Proliferation, Cytokine Production	Controls Treg/Th subset fate, cytokine production, and proliferation	Reduced (STAT1/3) or enhanced (STAT4/5/6) GVHD, maintained GVT (STAT5/6)
Nf-kb	Differentiation, Cytokine Production, Proliferation	Regulates Treg development, cytokines, and T cell expansion	Reduced GVHD, maintained GVT
Nfat	Proliferation, Migration, Cytokine Production, Differentiation	Regulates migration, Treg development, cytokine production, and proliferation of T cells	Reduced GVHD, maintained GVT
Nrf2	Differentiation, Migration	Inhibits Helios+ Tregs, controls migration to periphery	Reduced/enhanced GVHD ()?, maintained GVT
Bcl6	Differentiation, Proliferation	Regulates GC formation by Tfh cells and effector CD4+ expansion	Reduced cGVHD
Foxp3 (and Tregs)	Differentiation, Proliferation	Lineage factor for Tregs, stability regulates Treg function	Increased GVHD, maintained GVT
c-Rel	Differentiation, Proliferation, Apoptosis, Migration, Cytokine Production	Regulates migration to target organs, formation of Tregs/Th1/Th17, cytokine production, and apoptosis	Reduced GVHD, maintained GVT
c-Fos/c-Jun (Ap-1) and c-Myc	Differentiation, Migration, Cytokine Production	May regulate migration, Treg fate, and cytokine production	Reduced GVHD (human studies showpossible contradiction)
Batf	Differentiation, Proliferation	Controls IL-7/GM-CSF-producing T cells	Reduced GVHD
Ezh2	Proliferation, Differentiation	Regulates long-term proliferation/expansion, promotes differentiation to IFN-g+ effector fate	Reduced GVHD, maintained GVT
Smad3	Migration, Differentiation, Cytokine Production	Controls Th fate decisions, T cell migration, and proinflammatory cytokines	Reduced allograft rejection, enhanced GVHD
Hif1a	Differentiation, Survival	Inhibits Tregs and enhances Th17/Th1 cells	Reduced GVHD, maintained GVT
Plzf	Apoptosis, Differentiation	Regulates apoptosis, Th2 fate, and Treg cells	Reduced GVHD, maintained GVT
Fli-1	Differentiation, Activation	Regulates CD4+ T cell differentiation to Treg/Tfh fates, regulates effector CD4+ T cells	Reduced GVHD, maintained GVT
Bhlhe40	Cytokine Production	Regulates GM-CSF production in CD4+ T cells	Reduced GVHD

The beneficial GvL effect by donor graft and a toxic outcome in form of GvHD, indicates that an ideal drug which is to be developed against GvHD shall keep GvL intact. In earlier chapter we discussed that WA prevented aGvHD but does it preserves GvL is remains a question. Therefore, we next aim to evaluate effect of WA treatment on GvL response of the graft.

4.2 Aim and Objectives

4.2.1 Aim

To evaluate the outcome of Withaferin-A treatment on beneficial GvL effect

4.2.2 Objective

Objective 3. To investigate the impact of Withaferin-A on Graft versus Leukemia effect.

4.3 Materials and methods:

4.3.1 Animal maintenance: Animals were maintained as mentioned in chapter 2 and 3. Briefly, institutional animal ethics committee of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) approved this study (project no. 19/2022). All experimental animals were acclimatized for at least one week before the initiation of experiments. In this study, BALB/c (H-2K^{d}) female mice and C57BL/6 (H-2K^{b}) male mice of 8-10 week old having a weight of 20±2 grams were used. All animals were housed in the laboratory animal facility of ACTREC. Standard chow and water was given *ad libitum*. 55±15 percentage humidity, 22-25°C temperature with a 12h light/dark cycle were maintained in the facility. All animal procedures were carried out in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and ARRIVE guidelines.

4.3.2 Cell line and maintenance: A20 cell line was procured from ATCC (cat: ATCC-TIB-208). These cells are derived from BALB/c and were injected to BALB/c mice in this study. Hence, this represents a syngeneic model to study GvL. Cells were maintained in complete RPMI media containing 10% fetal bovine serum (FBS). A final concentration of 0.05 mM 2-mercaptoethanol were added in media while passaging. After passaging cells were incubated at 37°C with maintenance of 5% CO2.

4.3.3 Development of GvL model: To assess the consequence of WA treatment on GvL effect, myeloablated recipient BALB/c mice were either injected with A20 ($3x10^{6}$) cells alone or A20 + allogenic bone marrow transplantation (BMT) as described earlier in chapter (section 3.3.3). Further, animals in A20+BMT group were divided into WA treated and untreated groups (fig 4.2). The final experimental groups were [1] A20 [2] A20+BMT and [3] A20+BMT+WA. The GvL effect was monitored as per arlier

published method (131). Briefly, all mice were monitored on daily basis for sign of leukemia (based on hind-leg paralysis) and clinical signs of aGvHD. The day mice developed leukemia and became mortally ill, they were humanely sacrificed. Time to event outcomes comprised of (a) overall survival defined as time to death due to leukemia or GvHD, and (b) time to onset of leukemia defined by the occurrence of hind-leg paralysis. Further, liver tissue was evaluated for any sign of tumor nodules or aGvHD associated findings to differentiate death either by leukemia or by GvHD (fig 4.2).

4.3.4 Drug administration: Withaferin-A (WA) was administered prophylactically from day +1 of transplantation to day +21 at a dose of 1 mg/kg.

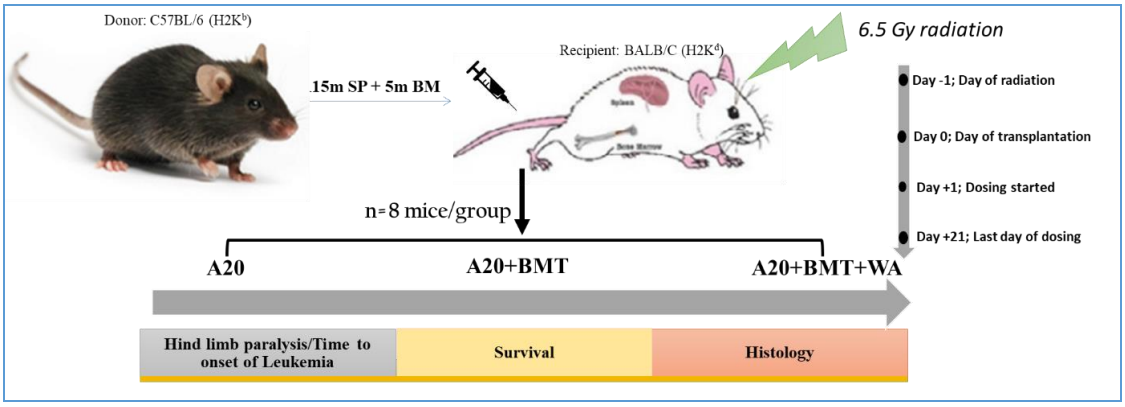


Figure 4.2: Experiment design for evaluation of WA treatment on beneficial GvL response.

4.4 Statistics: Time to event data such as survival and onset of leukemia were analysed using Kaplan–Meier plots and Mantel-Cox log-rank test. P=<0.05 was considered statistically significant. Data analysis was done using GraphPad Prism version 8.0.

4.5 Results:

4.5.1 WA treatment preserves beneficial GvL effect of the graft: We assessed the effect of WA treatment on GvL response. Leukemic and aGvHD manifestations were monitored throughout the study and representative phenotypes of each group are shown in figure 4.3A. Death due to leukemia was characterized by hind-leg paralysis and presence of tumor nodules in liver. Death due to GvHD was typically characterized by features suggestive of aGvHD and absence of hind-leg paralysis or tumor nodules in the liver. We observed that mice transplanted with A20 cells alone died due to leukemia by day +13 (median overall survival = 12 days). Mice transplanted with A20+BMT did not show signs of leukemia but all of them eventually died of aGvHD by day +24 (median overall survival = 17 days). Similarly, mice in the A20+BMT+WA group did not show signs of leukemia, however, 2 out of 8 animals in this group died of GvHD. The leukemia free survival (LFS) and overall survival (OS) of the 3 study groups are shown in figure 4.3B-C.

Histopathology of the liver tissue from A20 group showed multiple nodular deposits of a high-grade malignant tumor composed of large cells with moderate to marked nuclear atypia, brisk mitosis including atypical forms and scant eosinophilic cytoplasm. However, liver tissue of A20+BMT group showed mild to moderate chronic inflammation in the portal area suggestive of GvHD, but no tumor nodules. Notably, the surviving animals in the A20+BMT+WA group neither showed tumor nodules nor features of GvHD (fig 4.3D), thereby confirming that WA does not interfere with the GvL response.

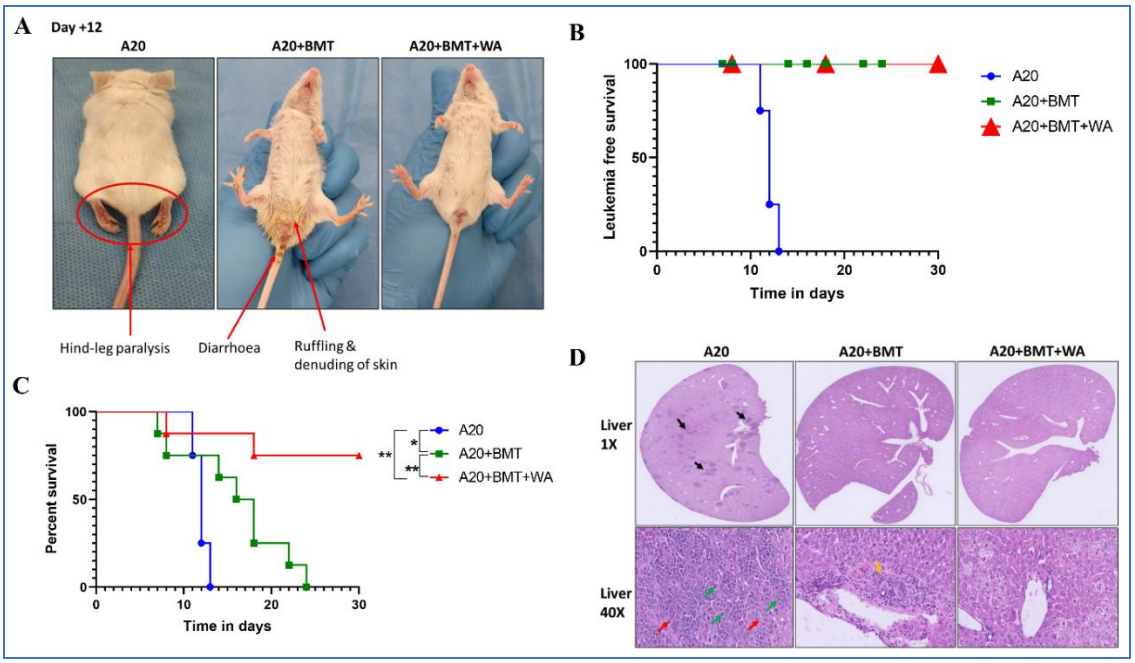


Figure 4.3: WA treatment maintains GvL effect. Following radiation, mice were transplanted either with A20 or A20+BMT. A20+BMT mice were further divided into WA treated (A20+BMT+WA) or untreated (A20+BMT) group. **(A)** At day +12 leukemic and aGvHD phenotypes were captured in all groups. A representative animal from each group is shown. **(B)** At the end of the experiment, leukemia free survival (based on onset of hind-leg paralysis) and **(C)** overall survival was analysed in all the study groups using Kaplan-Meier plots. Survival between groups was compared using the log-rank test **(D)** Histopathology of liver tissue showing presence of tumor nodules in A20 group but not in A20+BMT or A20+BMT+WA group. n=8 mice/group. *p= < 0.05, **p= < 0.01. Black arrow - tumor nodules; red arrow - nuclear atypia; green arrow – mitosis; yellow arrow - moderate chronic inflammation. H and E magnification 1x and 40x.

4.6 Discussion

Prevention of GvHD without compromising the beneficial GvL effect remains the "holy grail" of anti-GvHD measures (52). The γδT-cells are known for their anti-leukemic activity and for maintenance of GvL response (127). High counts of γδT-cells in patients undergoing alloHSCT correlated with longer survival (127). Incidentally, patients with high γδT-cell count also had lower rate of aGvHD (132). Therefore, several pharmacological agents such as aminobisphosphonates are being exploited for stimulating the *ex-vivo* proliferation of γδT-cells from hPBMCs in allo-transplantation (127). In the current study, we observed increase in γδT-cells following WA treatment with a corresponding decrease in PD1-CD4+ and Tim3-CD8+ cells (chapter 3, section 3.5.4, fig 3.16D-F). PD1 and Tim3 both are attractive target for immunotherapy as their co-blockade exerts potent anti-tumor response (133). Therefore, we anticipate that, decrease in PD1 and Tim3 along with suppression of T-cells and monocytes by WA treatment probably restores the balance between the G vL and anti-GvHD responses. Thus, WA has the hallmarks of an ideal drug for GvHD.

Oikawa et al. demonstrated the importance of Tim3 in aGvHD experimental mice. They reported the significant upregulation of hepatic Tim3-CD8+ cells in GvHD mice. Tim3 were also found to be up regulated in CD4 cells and dendritic cells. Furthermore, they used anti-Tim3 monoclonal antibody to target Tim3 and reported acceleration of GvHD (134). This could be because, Tim3 interaction with its partner galectin causes T-cell apoptosis (135), therefore, inhibition of this interaction keeps the T-cell hyperactive could explain why mice treated with anti-Tim3 monoclonal antibody had severe GvHD. On the other hand, inhibition of Tim3 may fall in favor of GvL due to active T-cells, which might be happening in our case where WA decreases Tim3 levels with intact GvL response.

Pan et al., recently reported the rationale of using PD1 antibody with potential anti-GvHD drug ruxolitinb to decrease GvHD and improve GvL. Enhancing the T-cell activity post-transplantation may help to diminish the relapse and infections, but it comes with a cost of increase GvHD (130). Therefore, a strategy that can decrease GvHD with improved GvL is required. Considering this, Pan et al., combined the ruxolitinib with PD1 antibody and reported a significant improved GvL and decrease GvHD response in mice (130). Our results suggested that WA can decrease the PD1 and reduces the GvHD severity. Therefore, a single molecule is ample for PD1 inhibition with protection of GvHD.

Another important strategy to improve GvL with reduced GvHD is use of γδT cells. Song et al. demonstrated that infusion of donor γδT cells can improve the GvL and mitigates the GvHD in a non-clinical study. The major subpopulation of γδT cells responsible for this effect was Vγ4 γδT cells (128). In a study by Lamb et al. in early 19s reported that the leukemic patients who underwent bone marrow transplantation and had more than 10% γδT cells showed advantage in disease free survival in first 6 month of transplantation (136). In addition, no differences in acute or chronic GvHD were seen in this patients. Subsequently, they reported that, patients with high γδT cells had better 5-year leukemia free overall survival (136). In our study, we also found an increase levels of γδT cells (chapter 3, section 3.5.4, fig 3.16D) suggesting a strong GvL effect by WA. However, these findings will be evaluated further during the clinical trial of WA.

4.7 Conclusion: The current study suggest that WA do not compromised with the beneficial GvL effect of the graft and keeps a balance between anti-GvHD and GvL measures (fig 4.4).

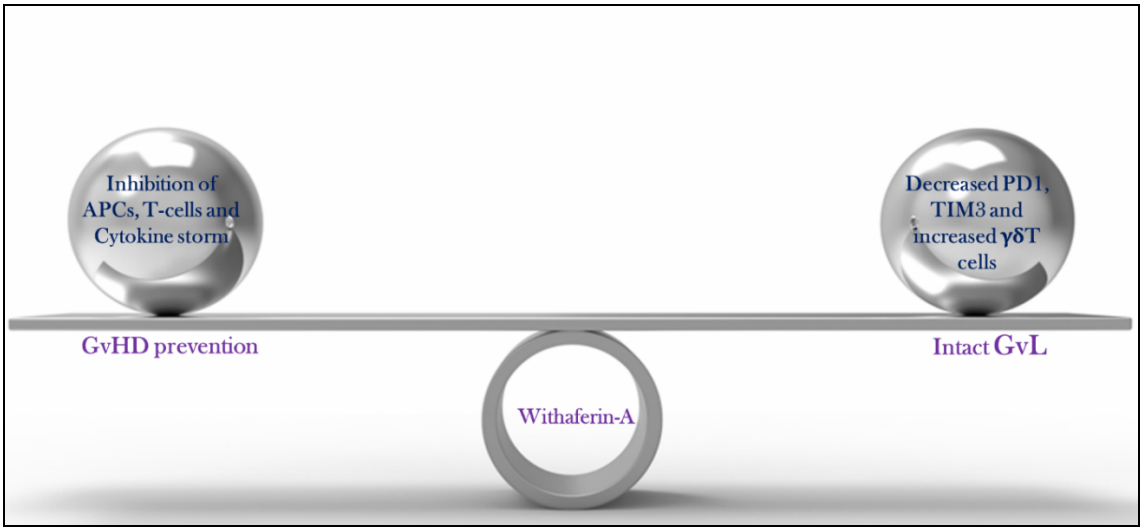


Figure 4.4: Effect of WA on GvHD and GvL. WA prevents GvHD through inhibition of APCs, T-cells and cytokine storm and it keeps GvL intact by decreasing PD1, TIM3 and by increasing γδT cells.

Chapter 5

Objective 4.
To investigate the efficacy of ready-to-use formulation of Withaferin-A for prophylaxis and treatment of acute Graft versus Host Disease

5.1 Introduction: Allogeneic hematopoietic stem cell transplantation (AlloHSCT) is often the only curative treatment option for several hematological disorders including leukemias (101). Worldwide, every year approximately 30,000 patients undergo alloHSCT. Despite advances in treatment and supportive care, 30-50% of alloHSCT recipients develop acute graft versus host disease (aGvHD), the most common cause of death following transplantation (101). aGvHD mainly causes the destruction of skin, gut, liver, and lung due to an overarching immune response of donor cells against these host tissues (99). Standard aGvHD prophylactic and therapeutic regimens are not always effective (137), thereby highlighting the need to develop novel pharmacological agents against aGvHD.

Medicinal plants have often attracted the attention of medical scientists due to their safety profile and short development time (138,139,140). In recent years, many plant products such as curcumin (141), extract of *Tripterygium hypoglaucum* (142), green tea catechin epigallocatechin gallate (143), and polyphenolic extract from olive oil (144), have been tested against aGvHD in preclinical models due to their anti-inflammatory and immunomodulatory efficiency. *Withania somnifera* (Indian ginseng or Ashwagandha) also has anti-inflammatory, immune-modulatory, and anti-proliferative properties (145,146,147). The use of *Withania somnifera* for aGvHD, a condition orchestrated by an inflammatory milieu and a hyperactive immune response (101), is therefore appealing. In the current study, a root extract of *Withania somnifera* (WSE) was investigated for its utility against aGvHD, both for prophylaxis and treatment. In addition, we also elucidated the role of WSE in GvHD target organ protection, cytokine storm, and graft versus leukemia (GvL) effects.

5.2 Aim and Objectives:

5.2.1 Aim

To investigate the efficacy of ready-to-use formulation of Withaferin-A *(Withania somnifera extract)* for prophylaxis and treatment of the acute Graft versus Host Disease

5.2.2 Objectives

Objective 4a. To evaluate the effect of *Withania somnifera* extract (ready-to-use formulation of Withaferin-A) for prevention and treatment of aGvHD.

Objective 4b. To evaluate effect of *Withania somnifera* extract on cytokine storm and GvHD target organ protection in-vivo.

Objective 4c. To investigate the GvL effect in the mice treated with *Withania somnifera* extract.

5.3 Rational of the study: In the previous experiments (chapter 3 and 4), we established that, WA is an attractive candidate for development as an anti-GvHD agent. However, development of a pure compound as a drug is a long-drawn process associated with huge costs. On the other hand, extracts of *Withania somnifera* containing WA are available as nutraceuticals around the world and therefore could be an alternative to pure WA should they have comparable activity. In fact, WSE has been tested in several human trials, and its safety is well established up to doses equivalent to 216 mg of WA per day. For these reasons, we aim to investigate efficacy of WSE in the mouse model of aGvHD.

5.4 Materials and methods:

5.4.1 Reagents and antibodies: H-2K^b (cat: 562002) and H-2K^d (cat: 553566) antibodies were procured from BD Biosciences. Pharmanza Herbal Pvt. Ltd. provided WSE. Cytokine measurements was performed using the BD CBA mouse Th1/Th2/Th17 cytokine kit (cat: 560485). A20 cell lines were obtained from ATCC (cat: ATCC-TIB-208).

5.4.2 Experimental animals: Institutional animal ethics committee of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) approved this study (project no. 19/2022). All experimental animals were acclimatized for at least seven days before the initiation of experiments. In the study, BALB/c (H-2K^d) female mice and C57BL/6 (H-2K^b) male mice of 8-10 week old having a weight of 20±2 grams were used. All animals were housed in the laboratory animal facility of ACTREC. Standard chow and water was given *ad libitum*. 55±15% humidity, 22-25°C temperature with a 12h light/dark cycle were maintained in the facility. All animal procedures were carried out in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and ARRIVE guidelines.

5.4.3 Induction of aGvHD: Development of aGvHD models was carried out as described earlier in chapter 3 (section 3.3.3). Briefly, aGvHD was induced by allogeneic transplantation between donor C57BL/6 (H-2K^b) and recipient BALB/c (H-2K^d) mice. One day before transplantation, BALB/c mice were exposed to total body myeloablative 6.5Gy of irradiation. Transplantation was carried by intravenous injection of $15x10^6$ splenocytes and $5x10^6$ bone marrow cells obtained from C57BL/6 mice. After transplantation, all mice were closely monitored for aGvHD symptoms and the clinical score (CS) of aGvHD was recorded based on the following six criteria: fur texture, skin

integrity, posture, activity, weight loss and diarrhoea (fig 5.1). Clinical scoring method was adopted from Lai et al. (1).

5.4.4 Drug administration for prevention and treatment of GvHD: Two separate experiments were carried out to investigate the utility of WSE in the prophylaxis and treatment of aGvHD. WSE was suspended in 0.5% sodium carboxymethylcellulose (CMC). In the prophylaxis study, WSE was administered by oral gavage from day +1 to day +21 of transplantation. The following four groups were employed: **(1)** GvHD control **(2)** 25 mg/kg of WSE **(3)** 75 mg/kg of WSE and **(4)** 250 mg/kg of WSE (fig 5.1).

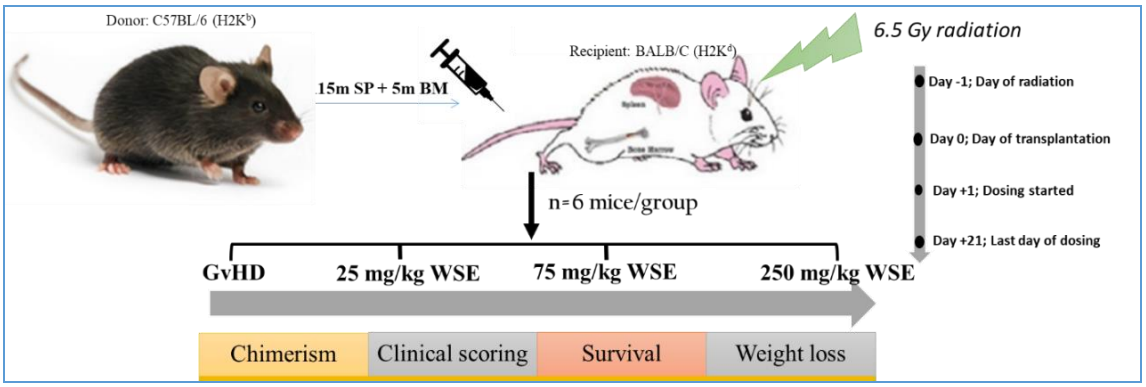


Figure 5.1: Development of aGvHD model and testing of WSE for prevention of aGvHD.

For therapeutic purpose, WSE was administered from day +7 to day +27 of transplantation and the final study groups were **(1)** GvHD control **(2)** 75 mg/kg WSE **(3)** 250 mg/kg WSE and **(4)** 500 mg/kg WSE. GvHD control group in both experiments received vehicle (CMC) only (fig 5.2). Total withanoloid content in WSE was 15%, standardized for 4.5% withaferin-A w/w. The remaining 10.5% consisted of other withanoloids such as withanolide A and 12-Deoxy-withastramonolide.

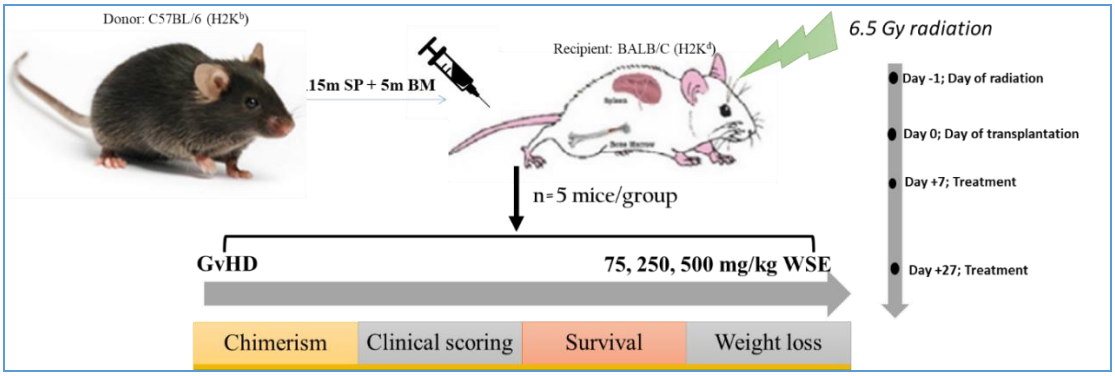


Figure 5.2: Testing of WSE for treatment of aGvHD.

5.4.5 Effect of WSE on graft versus leukemia (GvL) response: Development of GvL models was carried out as described earlier chapter 4 (section 4.3.3). Briefly, to investigate the effect of WSE on anti-leukemic response of the graft, 28 mice were divided equally into the following 4 groups: (1) A20 (2) A20+BMT (bone marrow transplantation) (3) A20+WSE and (4) A20+BMT+WSE. After exposing animals to 6.5Gy total body radiation, $3x10^6$ A20 cells were injected with or without BMT according to group allocation. WSE was administered prophylactically to groups 3 and 4 at a dose of 75 mg/kg. The GvL effect was monitored according to earlier published method (131,141). Leukemic death was defined by the presence of hind leg paralysis and the presence of tumor nodules in the liver. On the other hand, GvHD deaths were characterized by presence of GvHD symptoms but absence of hind-leg paralysis and tumor nodules in liver.

5.4.6 Analysis of donor cell engraftment using flow cytometry: Presence of donor positive cell (H-2K^b) in recipient mice were evaluated as per protocol mentioned in chapter 3 (section 3.3.7.2.). Briefly, peripheral blood was collected from recipient mice through retro-orbital puncture. Following RBC lysis and washing, cells were incubated with fluorochrome labelled antibodies against donor H-2K^b (FITC) and recipient H-2K^d (PE) MHC class I antigen and acquired using flow cytometry [attune NxT (Thermofisher, USA)].

5.4.7 Histopathology of aGvHD target organs: In a separate set of experiment, on day +7 and +14 of transplantation, aGvHD target organs (liver, gut, skin and lung) were harvested and processed for H&E. Trained pathologist evaluated and scored the aGvHD severity and lymphocytic infiltration to the target organs as per method mentioned in chapter 3 (section 3.3.5). The pathological evaluation were carried out in a blinded fashion.

5.4.8 *In vivo* cytokine measurement: In the same cohort of animals used for histopathology, blood from recipient mice was collected on day +7 and +14 of transplantation. Serum was separated and Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-6, IL-10) and Th17 (IL17A) cytokines were measured using cytometric beads array as per manufacture's instruction and method mentioned in chapter 3 (section 3.3.7.1).

5.5 Statistical analysis: Mean ± standard error of the mean (SEM) was used to represent the data. Comparison between two groups was done using unpaired t-test. Time to event data such as survival and onset of leukemia were analysed using Kaplan–Meier plots and Mantel-Cox log-rank test. P=<0.05 was considered statistically significant. Data analysis was done using GraphPad Prism version 8.0.

5.6 Results:

5.6.1 Prophylatic administration of WSE alleviates aGvHD associated morbidity and mortality. All mice were monitored periodically for aGvHD sign and survival. The median CS on day 14 in all experimental groups was 9, 3.5, 2, and 2.5 respectively in the GvHD control, 25, 75 and 250 mg/kg WSE (fig. 5.3A). Furthermore, continuous weight loss was observed in the GvHD control and the 25 mg/kg WSE group, the other groups had initial weight loss but was paused over a period of treatment (fig. 5.3B). Survival analysis suggested a significant increase in survival in all the three treatment arms compared to GvHD control (fig. 5.3C). Significant survival was higher in 75 mg/kg WSE [HR=0.15 (0.03-0.68), P=<0.01] and 250 mg/kg WSE [HR=0.16 (0.03-0.73), P=<0.01] group compared to the GvHD control than that of 25 mg/kg WSE arm [HR=0.26 (0.06-1.03), P= <0.05]. Median survival in GvHD control and 25 mg/kg WSE was 14 and 28.5 days respectively, however, it was undefined in 75 and 250 mg/kg WSE group (fig 5.3C). Furthermore, we analysed donor cell engraftment in WSE treated animals and found the complete presence of donor positive cells in the host body, suggesting that WSE does not interfere with the engraftment (fig 5.4). Based on these observation 75 and 250 mg/kg WSE was found to be equally efficacious, therefore, further prophylactic experiments was conducted at 75 mg/kg WSE dose.

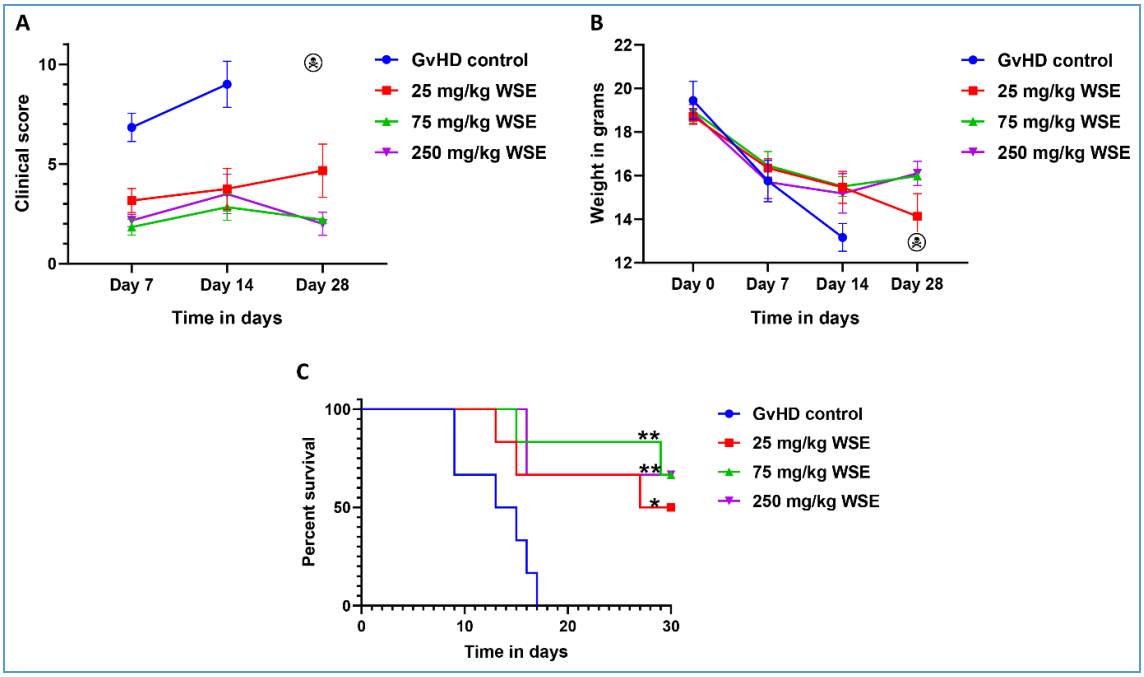


Figure 5.3: Effect of WSE on aGvHD prophylaxis. Following radiation and transplantation, mice were divided into GvHD control and WSE treatment (25, 75, and 250 mg/kg doses) groups. **(A)** The severity of aGvHD was monitored all along the study and CS was documented. **(B)** Body weight alteration in all experimental arms were also recorded throughout the study. **(C)** At the end of the study, overall survival was analysed in all experimental arms using Kaplan–Meier plot and Mantel-Cox log rank test. Data is represented as mean ± SEM. n=6 mice/group. *P= < 0.05, **P= < 0.01.

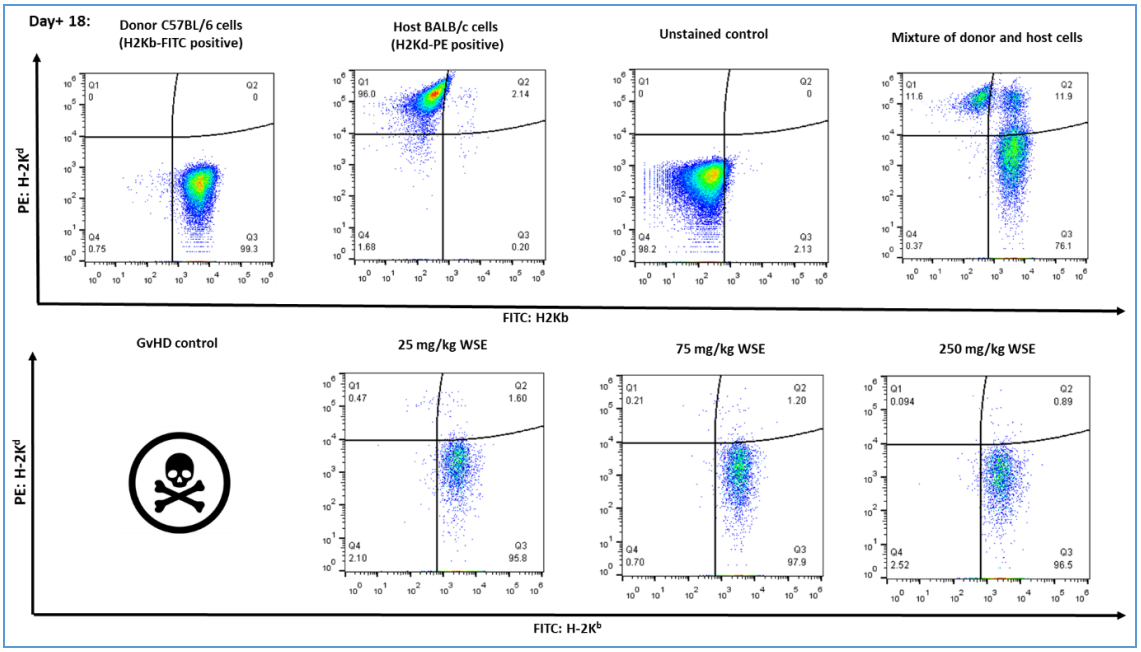


Figure 5.4: Effect of WSE on donor cells engraftment in recipient body. We investigated the presence of donor positive antigen post +18 days of transplantation. By this time mice in GvHD control arm were dead but animals received WSE were alive and subjected for analysis of engraftment and they showed complete presence of H-2K^b positive cells.

5.6.2 WSE averts aGvHD target organ damage. In a separate cohort of mice at day +7 and day +14 of transplantation, aGvHD target organ from GvHD control and WSE treated arms were harvested and evaluated histologically. Mice in GvHD control arm showed mild chronic inflammation in the liver and lung, vacuolar degeneration and desquamation of skin, mild-moderate chronic inflammation, ulceration, crypt loss, fibrosis, and sloughing of the small intestine and colon (Fig 5.5). In contrast, WSE treated group showed marked protection of these organs (Fig 5.5). Histopathological scoring based on the extent of damage to organs in GvHD control versus treatment arm is shown in 5.6. Additionally, the same tissue was evaluated for lymphocyte infiltration into these organs. Lymphocyte infiltration in GvHD control group were higher

compared to WSE arm, suggesting protection of these organs possibly via inhibition of lymphocyte infiltration (fig 5.7).

An inversely proportional relationship between spleen size and GvHD severity has been established earlier (111). Therefore, we also measured the spleen size and weight, which was found to be significantly decreased in GvHD control group compared to WSE group (5.8A-B).

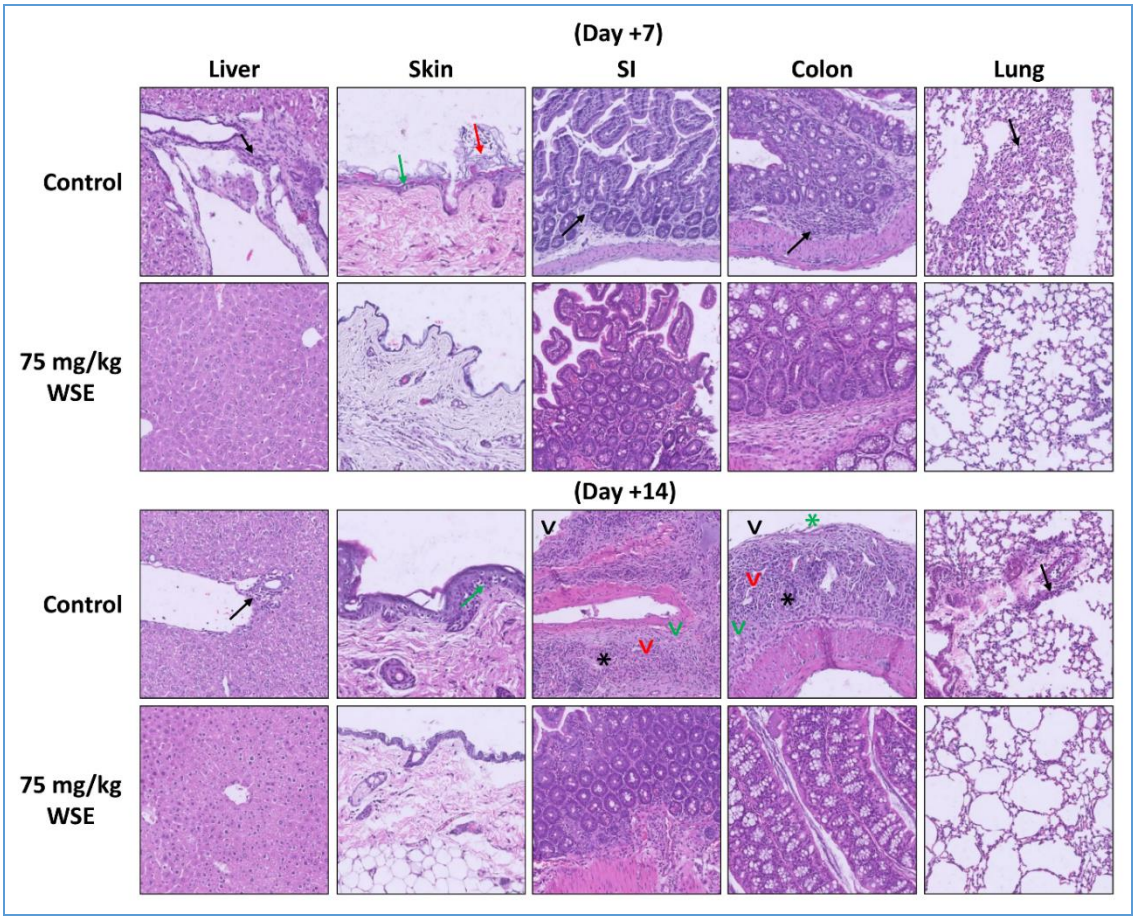


Figure 5.5: Protection of aGvHD target organs by WSE. In a separate cohort of mice, on days +7 and +14 of transplantation, aGvHD target organs were collected from GvHD control and WSE treated mice. aGvHD-associated damage were assessed by a pathologist in a blinded manner. Mice that received WSE prophylaxis showed intact tissue histology; however, mice in GvHD control arm had marked damage of target organs. n=6 mice/group/time point. Black arrow – Mild-moderate chronic inflammation;

green arrow - vacuolar degeneration; red arrow - desquamation; black arrowhead - ulceration; green arrowhead - fibrosis; black star - crypt loss; green star - sloughing.

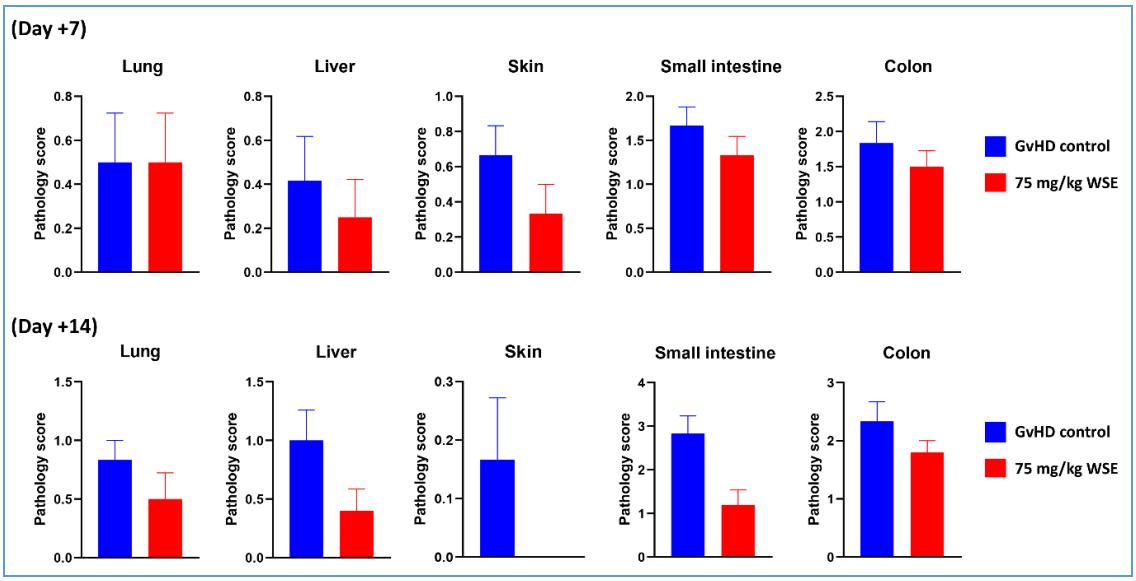


Figure 5.6: Pathology score of target tissue in WSE treated group compared to GvHD control. Following transplantation, on day +7 and +14 target organ namely liver, gut and skin were harvested. Upon histological analysis, moderate to severe damage of liver, skin, small intestine, colon and lung were observed in GvHD control mice. However, mice received WSE treatment showed marked protection of GvHD organs and had lower pathology scores. Data is represented as mean ± SEM. n=6 mice/group/time point. Animal those survived until the assessment of study were included for scoring which may have resulted in survivorship bias; hence statistical test was not applied.

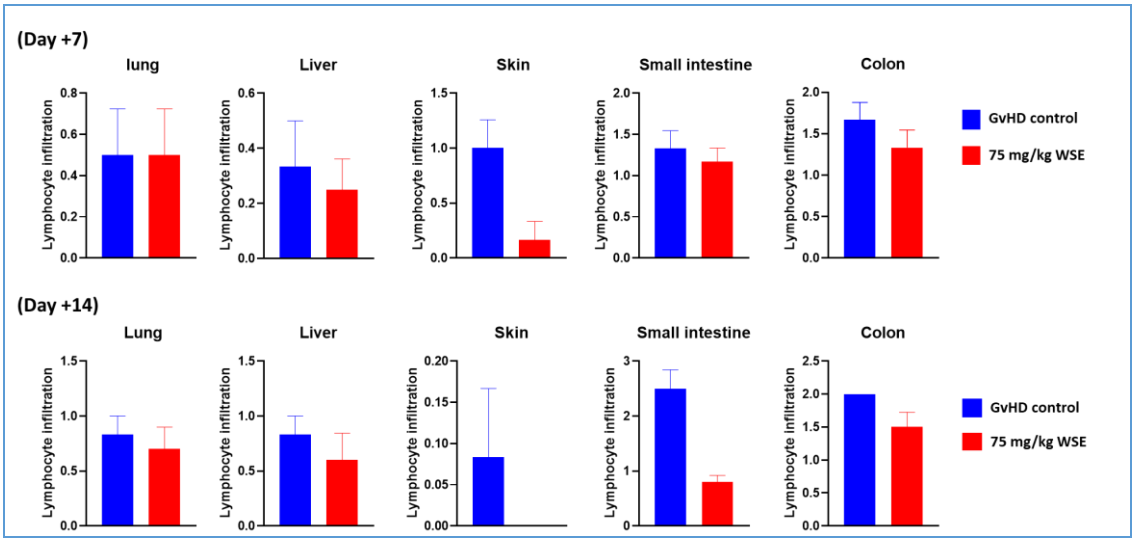


Figure 5.7: Effect of WSE treatment on lymphocytic infiltration on aGvHD target organ. The same histology slide that was used for analysis of organ damage was used to assess the lymphocytic infiltration. Evaluation of infiltration was done as per protocol mentioned in materials and methods. High lymphocytic infiltration was observed in liver, skin, small intestine, colon and lung tissues of GvHD control mice, and less so in the WSE treated group. Data is represented as mean ± SEM. n=6 mice/group/time point. Animal those survived until the assessment of study were included for scoring which may have resulted in survivorship bias; hence statistical test was not applied.

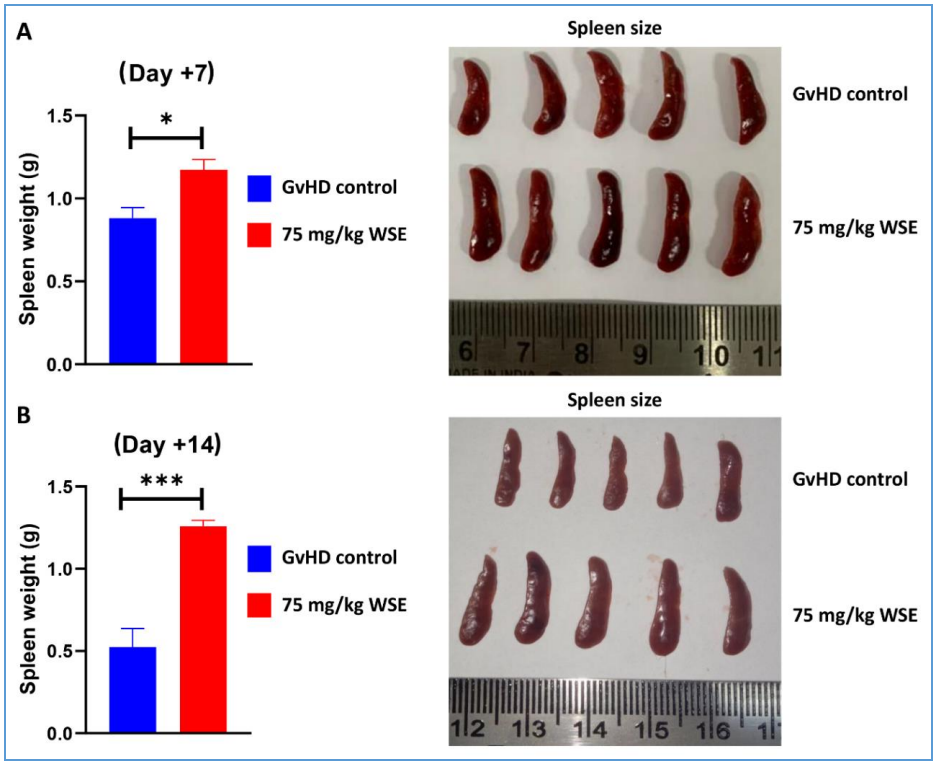


Figure 5.8: Effect of WSE treatment on Spleen weight and size. At day **(A)** +7 and **(B)** +14 post-transplantation, spleen was collected for weight and size from GvHD control and WSE treated mice. As depicted above, GvHD control animals had significant decrease in spleen weight and size compared to WSE group. Data is represented as mean ± SEM. n=5 spleen/group/time point. *p= < 0.05, ***p= < 0.001.

5.6.3 WSE modulates *in-vivo* serum cytokine storm. Excess cytokine secretion is one of the hallmarks of GvHD, and inhibiting their release is critical for the prevention of GvHD (148,149). Herein, we evaluated the effect of WSE treatment on cytokine levels at day +7 and +14 of transplantation and found a significant decrease in inflammatory cytokines such as IL-2, IFN-γ, TNF-α, IL-6 and IL-17A in WSE treated group compared to GvHD control (P=<0.05) (fig 5.9A-B). IL-10, an anti-inflammatory cytokine, was found to increase in mice treated with WSE compared to the GvHD control (P=<0.05) (fig 5.9A).

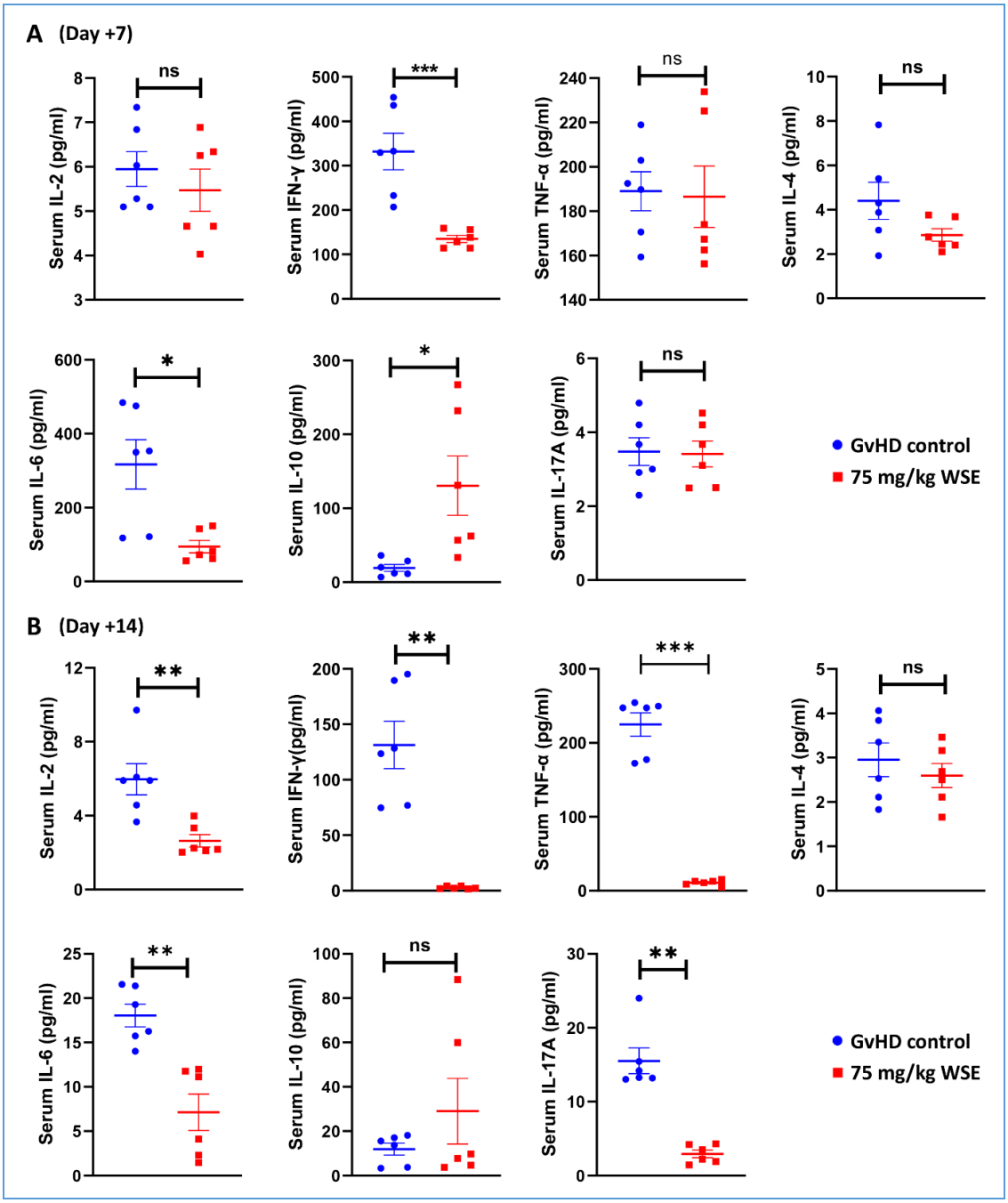


Figure 5.9: Effect of WSE treatment on *in-vivo* cytokine secretion. Serum Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-6, IL-10) and Th17 (IL17A) cytokine levels from GvHD control and WSE treated arms were measured at **(A)** day +7 and **(B)** day +14 of transplantation using cytometric beads array. Data is represented as mean ± SEM. n=6 mice/group/time point. *P= < 0.05, **P= < 0.01, ***P= < 0.001.

5.6.4 WSE does not hamper GvL effect. A prerequisite for any pharmacotherapy to be developed against GvHD is that the drug should not compromise the beneficial GvL effect of the graft (52). Therefore, we evaluated the effect of WSE treatment on GvL. Mice in all experimental arms were monitored daily for any sign of leukemia (based on hind-leg paralysis) or aGvHD. Phenotype of leukemia or aGvHD in each group is represented in figure 5.10A. Mice in A20 alone and A20+WSE group showed signs of leukemia (fig 5.10A-B). Mice in A20+BMT and A20+BMT+WSE group did not showed any sign of leukemia, suggesting no disruption of GvL effect by WSE (fig 5.10A-B). Mice in A20+WSE group showed delayed onset of leukemia (fig 4B), and better overall survival (fig 5.10C) compared to A20 alone group [HR=0.55 (0.18-1.65), P=0.06]. Although this difference is not statistically significant, the trend possibly hints at anti-leukemic effect of WSE. The median survival in the experimental groups was 16, 17 and 14 days respectively in the A20, A20+WSE, A20+BMT groups. However, the median survival remained undefined in the A20+BMT+WSE group (fig 5.10C).

A20 cells tend to form tumor nodules in the liver. We further confirmed the death due to leukemia or GvHD by histological analysis of liver (fig 5.10D). Liver of mice in A20 and A20+WSE showed multiple nodular deposits of a high-grade malignant tumor composed of large cells with moderate to marked nuclear atypia, brisk mitosis including atypical forms and scant eosinophilic cytoplasm. Consistent with leukemia free survival, A20+WSE group had less tumor burden compared to A20 alone group. A20+BMT group showed signs of aGvHD in the form of moderate chronic inflammation and portal vein congestion. However, A20+BMT+WSE group neither showed leukemic nor signs of aGvHD (fig 5.10D). These results suggest that WSE do not hamper GvL effect while mitigating aGvHD.

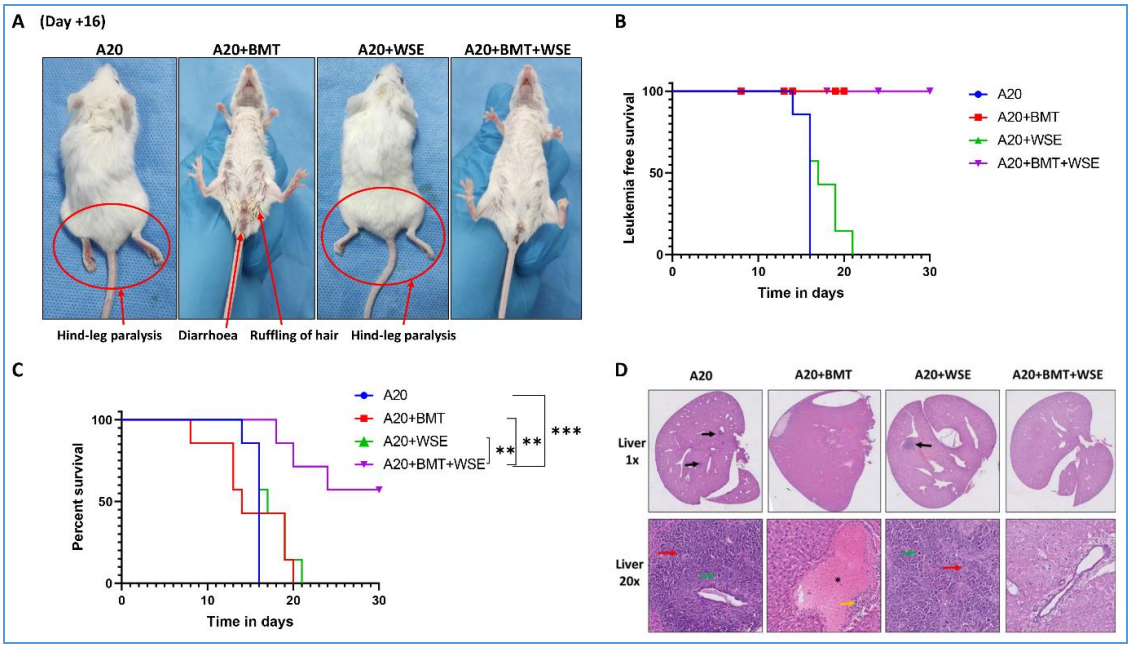


Figure 5.10: Effect of WSE treatment on GvL. Following radiation, mice were divide into (1) A20 (2) A20+BMT (3) A20+WSE (4) A20+BMT+WSE groups. **(A)** All mice were closely monitored for any signs of leukemia (based on hind leg paralysis) or aGvHD throughout the study. **(B)** Time-to-event data such as leukemia free survival and **(C)** overall survival were analysed in all experimental arms using Kaplan–Meier plot and Mantel-Cox log-rank test. **(D)** As and when mice became morbid either due to leukemia or GvHD, they were humanely sacrificed. Liver was collected for microscopic examination for the presence of tumor nodules or features of aGvHD. In figure 2B, death due to non-leukemic causes were censored. Censored points are represented with blue circle, red square, green triangle and inverted purple triangle in A20, A20+BMT, A20+WSE and A20+BMT+WSE groups respectively. Data is represented as mean ± SEM. n=7 mice/group. *P= < 0.05, **P= < 0.01, ***P= < 0.001. Black arrow - tumor nodules; red arrow - nuclear atypia; green arrow – mitosis; yellow arrow - moderate chronic inflammation; black star – portal vein congestion. H and E magnification 1x and 20x.

5.6.5 WSE treats established GvHD. We next evaluated the anti-GvHD efficacy of WSE, wherein we first allowed mice to develop GvHD and then WSE treatment was initiated from day +7 of transplantation. The median CS after two weeks of treatment with WSE was 6.5, 3, 1, and 3 respectively in the GvHD control, 75 mg/kg of WSE, 250 mg/kg of WSE and 500 mg/kg of WSE (fig. 5.11A). Continuous loss of weight was observed in the GvHD control group, however, treatment with WSE at all doses halted weight loss (fig 5.11B). Furthermore, overall survival was found to increase significantly in treatment groups compared to the GvHD control arm (P=<0.05). Hazard ratio (HR) in comparison with GvHD control was 0.32 (0.10-0.95) for 75 mg/kg of WSE, 0.16 (0.05-0.5) for 250 mg/kg of WSE, and 0.25 (0.08-0.75) for 500 mg/kg of WSE. The median survival was 21.5, 30, 30 days respectively in the GvHD control, 75 mg/kg and 500 mg/kg WSE groups (Fig. 5.11C), whereas it was undefined in 250 mg/kg WSE group (fig. 5.11C).

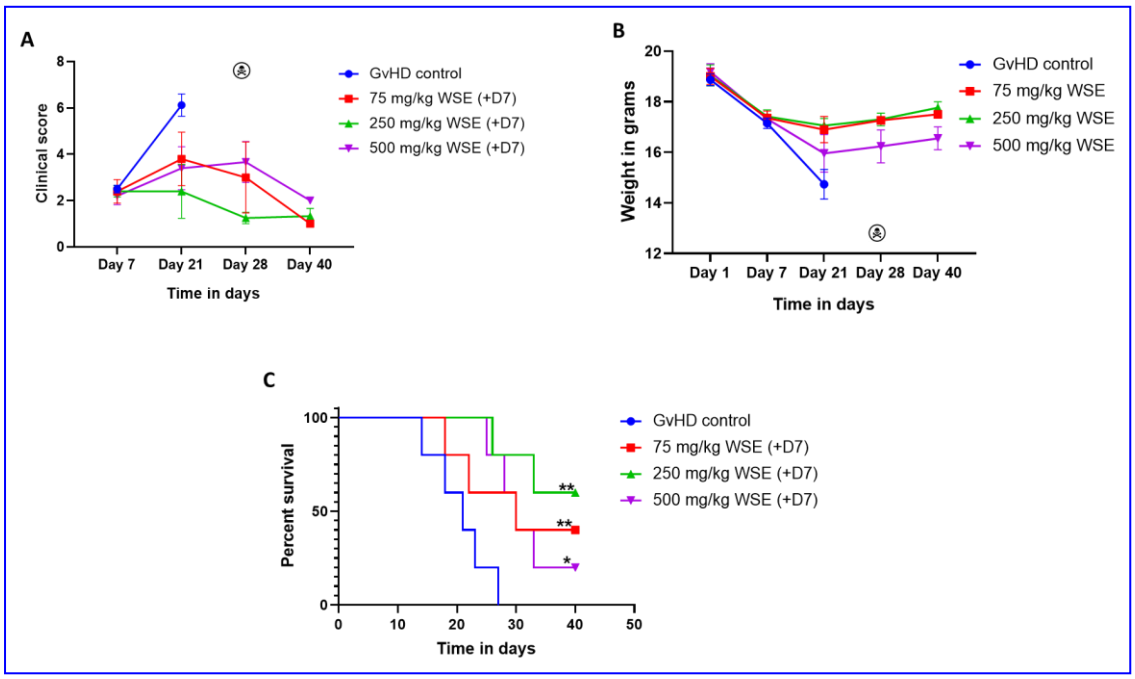


Figure 5.11: Anti-GvHD efficacy of WSE. To evaluate the anti-GvHD efficacy of WSE, post- transplantation mice were allowed to develop GvHD and WSE treatment at three different dose levels (75, 250, and 500 mg/kg) was started from day +7. **(A)** aGvHD clinical score and, **(B)** body weight were recorded at different time points as shown. **(C)** Overall survival among study groups was analysed using Kaplan–Meier plot and Mantel-Cox log rank test. Data is represented as mean ± SEM. n=5 mice/group. *P= < 0.05, **P= < 0.01.

5.7 Discussion: The development of novel interventions to prevent and treat aGvHD remains a challenge (150). Herein, we demonstrated the utility of WSE against aGvHD. Our finding suggests that early (prophylactic) or late (therapeutic) administration of WSE to mice can mitigate the severity of aGvHD and improve survival significantly. In addition, WSE could also inhibit inflammatory cytokine secretion and protect aGvHD target organs. We also demonstrated that WSE does not obstruct the beneficial GvL effect.

Earlier, we established the inhibition of lymphocyte proliferation and cytokine secretion from mouse lymphocytes treated *ex-vivo* with withaferin-A (WA), a principle component of WSE (64). Next, we established that *ex-vivo* graft manipulation with WA could prevent aGvHD (65). Further, to bring WA one-step closure to clinic, we established safety, pharmacokinetics and anti-GvHD efficacy of oral WA (chapter 2 and 3). Pharmacodynamically, WA was shown to regulate JAK/PI3K/NF-kB/Akt/mTOR signaling and exert potent immune-modulatory response (detailed chapter 3). Thus, WA is an attractive candidate for development as an anti GvHD agent. However, development of a pure compound as a drug is a long-drawn process associated with huge costs. On the other hand, extracts of *Withania somnifera* containing WA are available as nutraceuticals around the world and therefore could be an alternative to pure WA should they have comparable activity. In fact, WSE has been tested in several human trials (146), and its safety is well established up to doses equivalent to 216 mg of WA per day (74). For these reasons, we went on to test the efficacy of WSE in the mouse model of aGvHD.

WSE is the main component of tradition Rasayana and serves as a remedy against a plethora of human diseases (152). It is well known for its ability to enhance age, vitality,

and protection against numerous diseases (153). In addition to this, WSE also has anticancer, anti-inflammatory and immune modulatory properties (152,154). Turrini et al. reported the anti-leukemic activity of WSE (155). Our observations that WSE resulted in a slight increase in survival and reduced tumor burden in mice transplanted with leukemic cells (A20) corroborates these findings. This anti-tumor efficiency of WSE may synergise with anti-leukemic activity of the graft in patients with leukemia who undergo transplantation, and could decrease the chance of disease relapse as well. The immune-modulatory ability of WSE was reported by Singh et al. through inhibition of NFkB and AP-1 proteins (156). Furthermore, Singh et al. and Naidoo et al. reported inhibition of inflammatory cytokine secretion from human peripheral blood mononuclear cells following *ex-vivo* treatment with WSE (156,157). A recent study by Kaur et al. reported the inhibition of inflammatory molecules including PPARγ, MCP-1, iNOS, IL-1β, TNFα and IL-6 by WSE via inhibition of JAK2-STAT3 and NFkB signaling (158). Consistent with these studies, we also found a significant decrease in pro-inflammatory cytokine secretion in mice treated with WSE. Additionally, we also reported increased IL-10 levels, an anti-inflammatory cytokine. We further, reported that mice treated with WSE showed protection of gut, liver, and skin from GvHD induced destruction of these tissues. In line with our results, several preclinical studies have demonstrated the protection of the gut, liver, and skin by WSE in different experimental settings other than GvHD (159,160).

In the current study, we tested WSE at three dose levels (25, 75 and 250 mg/kg) for the prophylaxis experiment, wherein we found that 75 and 250 mg/kg of WSE give equal protection. Therefore, for further prophylactic experiments 75 mg/kg WSE dose was chosen. On the other hand, for anti-GvHD experiment 75, 250 and 500 mg/kg WSE doses were selected and we found highest efficacy at 250 mg/kg of WSE. A three-fold

higher effective dose of WSE for GvHD therapy compared to prophylaxis could be explained by lower absorption associated with GvHD of the gut.

The current standard of care for GvHD is associated with significant clinical and/or financial toxicities (161,162). Therefore, a safe and cost effective intervention for the management of GvHD is appealing. WSE has gained popularity in the nutra-health sector, it being the 12th bestselling nutraceutical of 2020 (163), which is also a reflection of its affordability. Safety of the product used in our study is already established up to 2000 mg/kg/day in non-clinical acute and sub-acute toxicity studies (84), and up to 4800 mg/day in a phase 1 clinical trial (74). Recently, pharmacokinetics of WSE in rats demonstrated oral bioavailability of withanolides such as withanoside IV, WA, 12-deoxy-withastramonolide, and withanolide A (77). Based on our extensive preclinical safety, efficacy, pharmacokinetics, pharmacodynamics and phase I trial data on WSE a phase II trial (CTRI/2023/07/055361) for the treatment of aGvHD is currently under investigation at our centre.

5.8 Conclusion: In the current study, we explored the prophylactic and therapeutic efficacy of WSE against aGvHD. WSE mitigated the severity of aGvHD and improved survival of mice by protecting target organ damage and inhibiting the inflammatory milieu without compromising the beneficial GvL effect. Based on these findings and its compelling safety profile, WSE is currently under investigation in a phase 2 clinical trial for the treatment of aGvHD at our centre.

Chapter 6

Overall summary, conclusion and Way forward

6.1 Overall summary:

- The success of alloHSCT is limited by the complication of aGvHD.
- Worldwide, 35-50% patients tend to develop aGvHD post transplantation.
- Standard drugs are not always effective and they are associated with significant clinical and/or financial toxicity.
- Therefore, development of safe and effective drugs against aGvHD remains an unmet medical need.
- WA, being an anti-inflammatory, anti-proliferative and immunomodulatory agent was investigated for this purpose.
- We first established the acute, sub-acute toxicity and pharmacokinetics of WA (fig 6.1).
- In acute toxicity, WA was found to be safe up-till 2000 mg/kg dose.
- Based on acute toxicity assay, LD_{50} of WA was determined to be >2000 mg/kg.
- In subacute-toxicity, WA was found to be safe up to 500 mg/kg.
- Based on sub-acute toxicity, NOAEL of WA was established >500 mg/kg.
- In pharmacokinetics study, WA was found to be orally bioavailable.

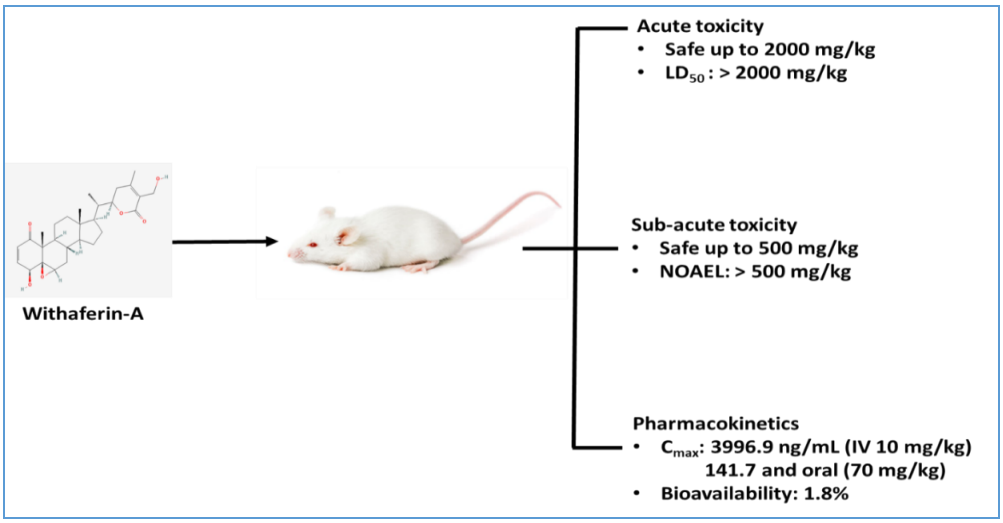


Figure 6.1: Graphical illustration of safety, toxicity and pharmacokinetic findings of WA.

- We next went on establishing aGvHD prophylactic and therapeutic efficacy of WA and its effect on GvL response (fig 6.2).
- Prophylactic administration of WA was found to prevent onset of aGvHD without compromising the engraftment of donor cells.
- Further, WA prevents cytokine storm and migration of lymphocytes to GvHD target organs and protested organs from damage.
- In addition to this, WA could also revert the established GvHD, which provides basis for its utility for the therapy of GvHD.
- Furthermore, WA administration to mice do not compromised with beneficial GvL effect of the graft.

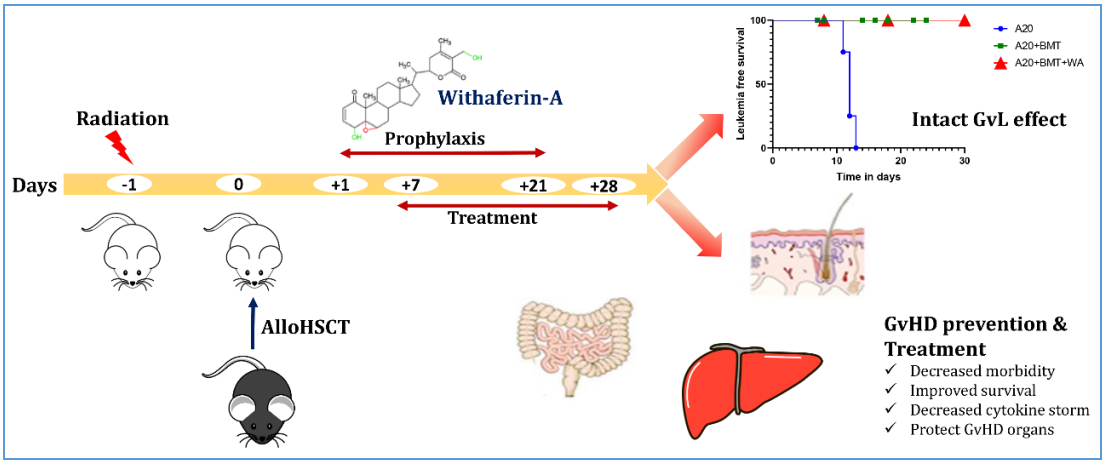


Figure 6.2: Graphical representation of WA's effect on aGvHD and GvL.

❖ Mechanistically, WA inhibits JAK2-STAT3 signaling and regulates immune cells proliferation, differentiation and cytokine secretion (fig 6.3).

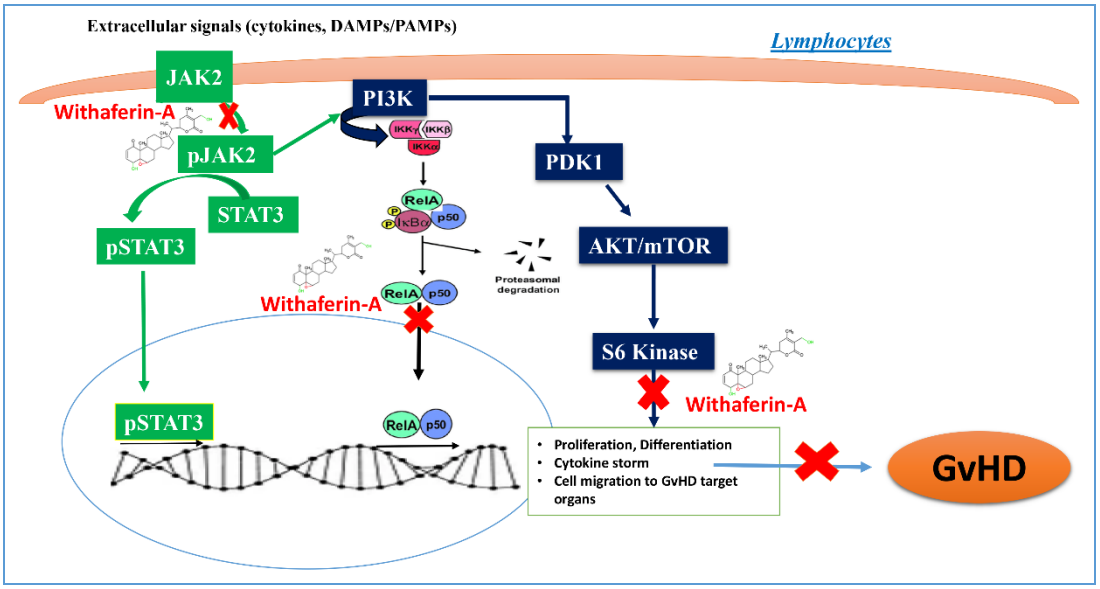


Figure 6.3: Immunomodulatory and anti-GvHD mechanism of WA.

❖ We next investigated the GvHD prophylactic, therapeutic and GvL effects using ready-to-use formulation of WA i.e. WSE (fig 6.4).

❖ WSE also showed prophylactic and therapeutic benefits against aGvHD and keeps GvL intact.

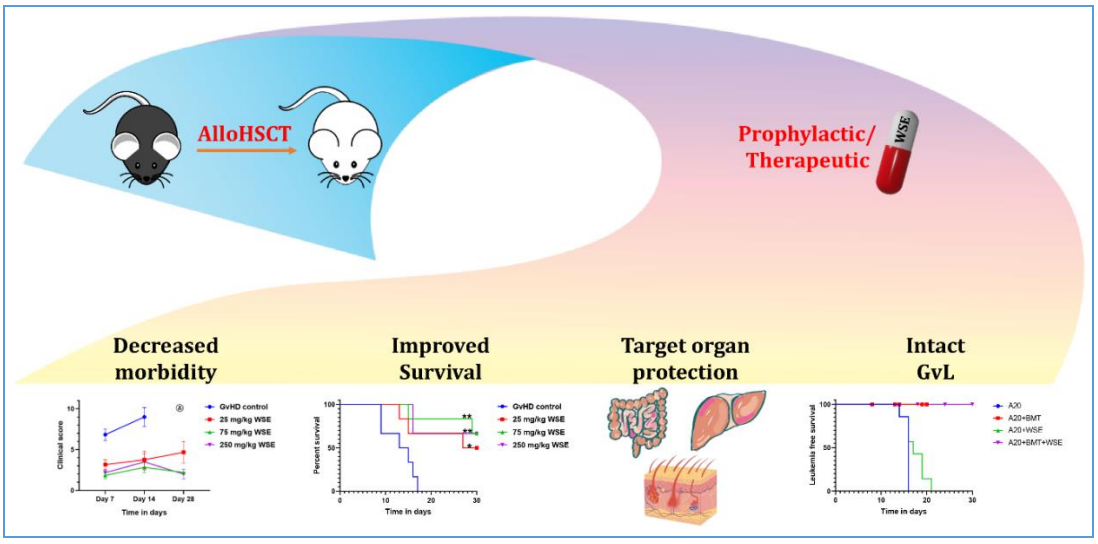


Figure 6.4: Effect of WSE on aGvHD and GvL response.

6.2 Limitations of the study: This study has two possible limitations. Firstly, we could not inject intravenous CSA beyond five doses unlike in the clinics where it is given for longer duration. We had to limit the duration of CSA use in our study due to the risk of tail vein phlebitis. Secondly, the spleen of GvHD control mice were severely destructed due to alloreactivity as shown in section 3.5.2 (fig 3.10A-C). With increasing severity of GvHD the spleen becomes smaller in size, with significant decrease in weight corresponding to low cell counts. Thus, comprehensive in-vivo mechanistic investigations could not be undertaken due to limited cell number in GvHD control group, forcing us to use hPBMCs to gain mechanistic insight. However, the efficacy of WA in this animal model coupled with a sound mechanistic understanding, and the safety and pharmacokinetics established, has prompted us to initiate two phase 2 clinical trials at our center for aGvHD prophylaxis and therapy.

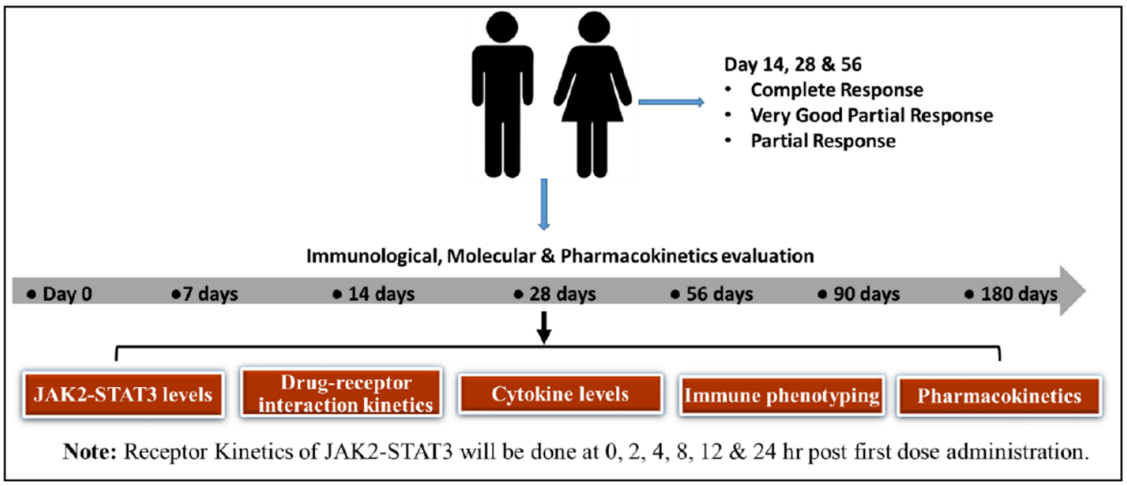
6.3 Way forward:

Based on the findings of current study, two clinical trial have been proposed at our center. A brief trial design is shown below.

Trial 1. (CTRI/2023/07/055361)

Title: A Phase II Clinical Trial of Standardized Withaferin-A for the treatment of Steroid Refractory acute Graft versus Host Disease.

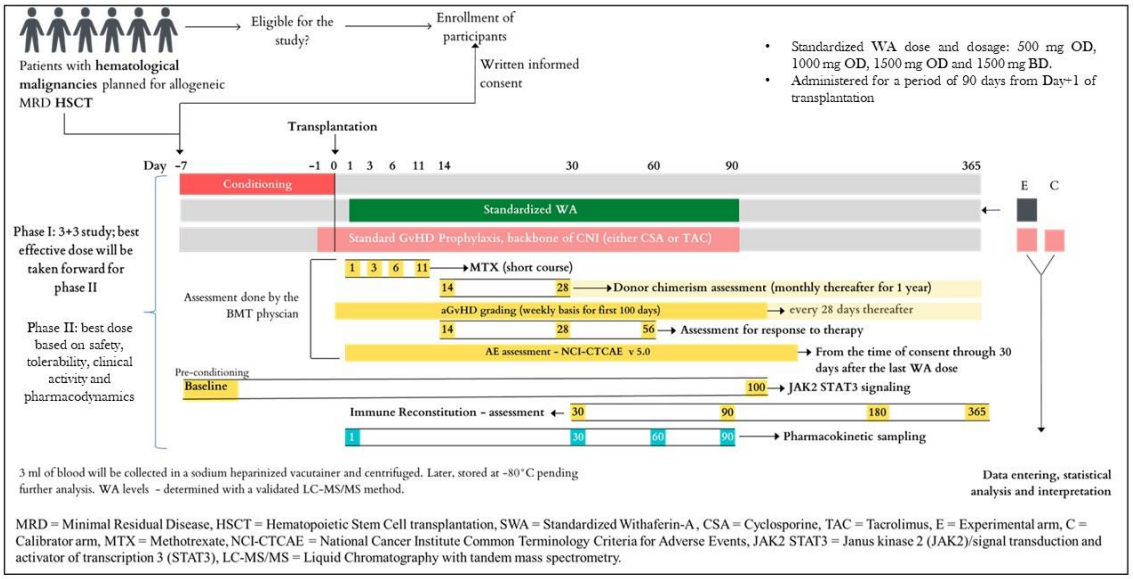
Study design:



Trial 2: Under IEC consideration

Title: A Phase I/II trial to assess Safety and Activity of Standardized Withaferin A as GvHD prophylaxis in patients undergoing Matched related donor Hematopoietic stem cell transplant

Study design:



MRD = Minimal Residual Disease, HSCT = Hematopoietic Stem Cell transplantation, SWA = Standardized Withaferin-A , CSA = Cyclosporine, TAC = Tacrolimus, E = Experimental arm, C = Calibrator arm, MTX = Methotrexate, NCI-CTCAE = National Cancer Institute Common Terminology Criteria for Adverse Events, JAK2 STAT3 = Janus kinase 2 (JAK2)/signal transduction and activator of transcription 3 (STAT3), LC-MS/MS = Liquid Chromatography with tandem mass spectrometry.

6.4 Conclusion of the study: In the current study, we aimed systemic development of WA from bench to bedside for the prevention and treatment of aGvHD. We first evaluated the safety and pharmacokinetics of WA. Wherein, WA found to be extremely safe and orally bioavailable. We further established efficacy of WA for the prophylaxis and therapy of aGvHD. WA also showed superior efficacy compared to standard prophylactic regimens of CSA+MTX. Mechanistically, WA regulates immune cell proliferation, differentiation, cytokine storm and protects GvHD target organ damage via inhibition of JAK2-STAT3 signaling. Additionally, WA do not compromised with beneficial GvL effect of the graft. These findings has prompted us to initiate two phase 2 clinical trials at our center for aGvHD prophylaxis and therapy (fig 5.5).

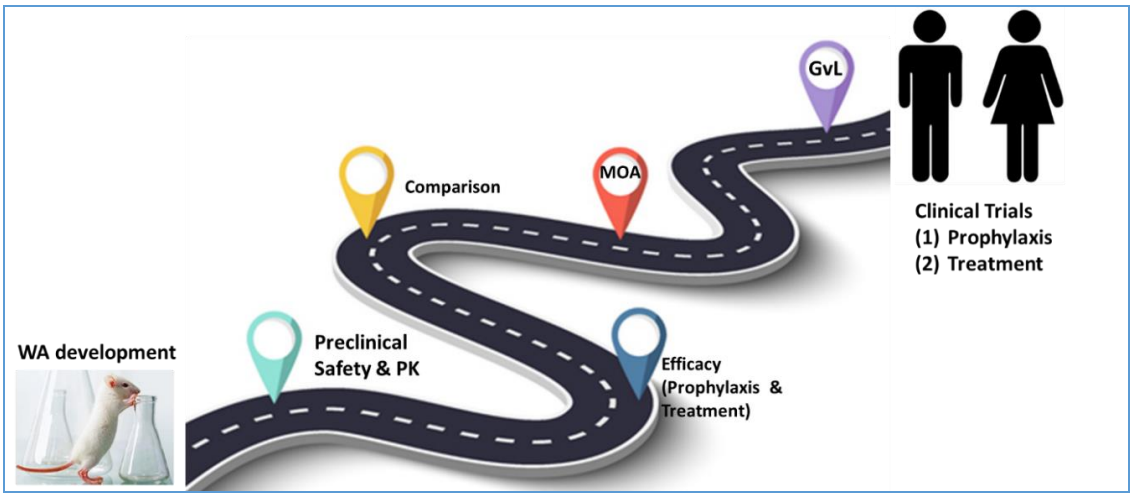


Figure 6.5: Conclusion of the current study. MOA; mechanism of action, GvL; graft versus leukemia response.

References

1. Lai HY, Chou TY, Tzeng CH, Lee OKS. Cytokine profiles in various graft-versus-host disease target organs following hematopoietic stem cell transplantation. Cell Transplant. 2012;21(9):2033–45.

2. Malard F, Huang XJ, Sim JPY. Treatment and unmet needs in steroid-refractory acute graft-versus-host disease. Leukemia. 2020;34(5):1229–40.

3. Negrin RS. Graft-versus-host disease versus graft-versus-leukemia. Hematol. 2015;2015(1):225–30.

4. Hill GR, Betts BC, Tkachev V, Kean LS, Blazar BR. Current Concepts and Advances in Graft-Versus-Host Disease Immunology. Annu Rev Immunol. 2021;39:19–49.

5. DW B, JF L, FE N. Treatment of murine leukaemia with X rays and homologous bone marrow; preliminary communication. British Medical Journal. 1956 Sep 1;2(4993):626-7.

6. Thomas ED. A history of haemopoietic cell transplantation. Br J Haematol. 1999;105(2):330–9.

7. Reddy P. Pathophysiology of acute graft-versus-host disease. Hematol Oncol. 2003;21(4):149–61.

8. Mehta RS, Alousi AM. Graft-Versus-Host Disease. Man Hematop Cell Transplant Cell Ther. 2023;453–79.

9. Apperley J, Masszi T. Chapter 13 - Graft-versus-host disease. ESH-EBMT Handb. 2008;216–33.

10. Sung AD, Chao NJ. Concise Review: Acute Graft-Versus-Host Disease: Immunobiology, Prevention, and Treatment. Stem Cells Transl Med. 2013;2(1):25–32.

11. Mahdi BM. Introductory chapter: concept of human leukocyte antigen (HLA). human leukocyte antigen (HLA). 2019 Jan 18:1-8.12.

12. Kanda J. Effect of HLA mismatch on acute graft-versus-host disease. Int J Hematol. 2013;98(3):300–8.

13. Fuji S, Hakoda A, Kanda J, Murata M, Terakura S, Inamoto Y, et al. Impact of HLA disparity on the risk of overall mortality in patients with grade II–IV acute GVHD on behalf of the HLA Working Group of Japan Society for Hematopoietic Cell Transplantation. Bone Marrow Transplant. 2021;56(12):2990–6.

14. Ghimire S, Weber D, Mavin E, Wang XN, Dickinson AM, Holler E. Pathophysiology of GvHD and other HSCT-related major complications. Front Immunol. 2017;8(MAR).

15. Flowers MED, Inamoto Y, Carpenter PA, Lee SJ, Kiem HP, Petersdorf EW, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. Blood. 2011;117(11):3214–9.

16. Bryson JS, Jennings CD, Caywood BE, Dix AR, Lowery DM, Kaplan AM. Enhanced graft-versus-host disease in older recipient mice following allogeneic bone marrow transplantation. Bone Marrow Transplant. 1997;19(7):721–8.

17. Ratanatharathorn BV, Nash RA, Przepiorka D, Devine SM, Klein JL, Weisdorf D, et al. Prophylaxis After HLA-Identical Sibling Bone Marrow Transplantation. Blood. 1998;92(7):2303–14.

18. Nassereddine S, Rafei H, Elbahesh E, Tabbara I. Acute graft versus host disease: A comprehensive review. Anticancer Res. 2017;37(4):1547–55.

19. Randolph SSB, Gooley TA, Warren EH, Appelbaum FR, Riddell SR. Female donors contribute to a selective graft-versus-leukemia effect in male recipients of HLA-matched, related hematopoietic stem cell transplants. Blood. 2004;103(1):347–52.

20. Kim HT, Zhang MJ, Woolfrey AE, Martin AS, Chen J, Saber W, et al. Donor

and recipient sex in allogeneic stem cell transplantation: What really matters. Haematologica. 2016;101(10):1260–6.

21. Jamy O, Zeiser R, Chen Y Bin. Novel developments in the prophylaxis and treatment of acute GVHD. Blood. 2023;

22. Watkins B, Qayed M, McCracken C, Bratrude B, Betz K, Suessmuth Y, Yu A, Sinclair S, Furlan S, Bosinger S, Tkachev V. Phase II trial of costimulation blockade with abatacept for prevention of acute GVHD. Journal of clinical oncology. 2021 Jun 6;39(17):1865.

23. Anasetti C, Logan BR, Lee SJ, Waller EK, Weisdorf DJ, Wingard JR, et al. Peripheral-Blood Stem Cells versus Bone Marrow from Unrelated Donors. N Engl J Med. 2012;367(16):1487–96.

24. Gupta A, Punatar S, Gawande J, Mathew L, Bagal B, Kannan S, et al. Risk Factors, Pattern and Clinical Outcome of Acute Graft Versus Host Disease in Acute Leukemia Patients Undergoing Allogeneic Stem Cell Transplant. Indian J Hematol Blood Transfus. 2015;31(4):404–12.

25. Chen YB, Wang T, Hemmer MT, Brady C, Couriel DR, Alousi A, et al. GvHD after umbilical cord blood transplantation for acute leukemia: An analysis of risk factors and effect on outcomes. Bone Marrow Transplant. 2017;52(3):400–8.

26. Flinn AM, Gennery AR. Recent advances in graft-versus-host disease. 2023;

27. Malard F, Holler E, Sandmaier BM, Huang H, Mohty M. Acute graft-versus-host disease. Nat Rev Dis Prim. 2023;9(1).

28. Oudin C, Chevallier P, Furst S, Guillaume T, El Cheikh J, Delaunay J, et al. Reduced-toxicity conditioning prior to allogeneic stem cell transplantation improves outcome in patients with myeloid malignancies. Haematologica. 2014;99(11):1762–8.

29. Wang Y, Wu DP, Liu QF, Xu LP, Liu KY, Zhang XH, et al. Low-dose post-

transplant cyclophosphamide and anti-thymocyte globulin as an effective strategy for GVHD prevention in haploidentical patients. J Hematol Oncol. 2019;12(1):1–9.

30. Ordemann R, Hutchinson R, Friedman J, Burakoff SJ, Reddy P, Duffner U, et al. Enhanced allostimulatory activity of host antigen-presenting cells in old mice intensifies acute graft-versus-host disease. J Clin Invest. 2002;109(9):1249–56.

31. Goker H, Haznedaroglu IC, Chao NJ. Acute graft-vs-host disease: Pathobiology and management. Exp Hematol. 2001;29(3):259–77.

32. Kataoka Y, Iwasaki T, Kuroiwa T, Seto Y, Iwata N, Hashimoto N, et al. The role of donor T cells for target organ injuries in acute and chronic graft-versus-host disease. Immunology. 2001;103(3):310–8.

33. Baker MB, Altman NH, Podack ER, Levy RB. The role of cell-mediated cytotoxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. J Exp Med. 1996;183(6):2645–56.

34. Graubert TA, DiPersio JF, Russell JH, Ley TJ. Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. J Clin Invest. 1997;100(4):904–11.

35. Van den Brink MRM, Burakoff SJ. Cytolytic pathways in haematopoietic stem-cell transplantation. Nat Rev Immunol. 2002;2(4):273–81.

36. Braun MY, Lowin B, French L, Acha-Orbea H, Tschopp J. Cytotoxic T cells deficient in both functional Fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. J Exp Med. 1996;183(2):657–61.

37. Arvindam US, Aguilar EG, Felices M, Murphy W, Miller J. Natural Killer Cells in GvHD and GvL. Immune Biology of Allogeneic Hematopoietic Stem Cell

Transplantation. Elsevier Inc.; 2019. 275–292 p.

38. Betts BC, Bastian D, Iamsawat S, Nguyen H, Heinrichs JL, Wu Y, et al. Targeting JAK2 reduces GVHD and xenograft rejection through regulation of T cell differentiation. Proc Natl Acad Sci U S A. 2018;115(7):1582–7.

39. Bartalucci N, Calabresi L, Balliu M, Martinelli S, Rossi MC, Villeval JL, et al. Inhibitors of the PI3K/mTOR pathway prevent STAT5 phosphorylation in JAK2V617F mutated cells through PP2A/ CIP2A axis. Oncotarget. 2017;8(57):96710–24.

40. Hu X, li J, Fu M, Zhao X, Wang W. The JAK/STAT signaling pathway: from bench to clinic. Signal Transduct Target Ther. 2021;6(1).

41. Sarah S, Huarte E, Peel M, Juvekar A, Dub P. ameliorates acute and chronic steroid-refractory GvHD mouse models. 2021;13:977–87.

42. Jia H, Cui J, Jia X, Zhao J, Feng Y, Zhao P, et al. Therapeutic effects of STAT3 inhibition by nifuroxazide on murine acute graft graft-vs.-host disease: Old drug, new use. Mol Med Rep. 2017;16(6):9480–6.

43. Sun X, He Q, Yang J, Wang A, Zhang F, Qiu H, et al. Preventive and Therapeutic Effects of a Novel JAK Inhibitor SHR0302 in Acute Graft-Versus-Host Disease. Cell Transplant. 2021;30(100):1–16.

44. Okiyama N, Furumoto Y, Villarroel VA, Linton JT, Tsai WL, Gutermuth J, et al. Reversal of CD8 T-cell-mediated mucocutaneous graft-versus-host-like disease by the JAK inhibitor tofacitinib. J Invest Dermatol. 2014;134(4):992–1000.

45. Zeiser R, von Bubnoff N, Butler J, Mohty M, Niederwieser D, Or R, et al. Ruxolitinib for Glucocorticoid-Refractory Acute Graft-versus-Host Disease. N Engl J Med. 2020;382(19):1800–10.

46. Przepiorka D, Luo L, Subramaniam S, Qiu J, Gudi R, Cunningham LC, et al. FDA Approval Summary: Ruxolitinib for Treatment of Steroid-Refractory Acute Graft-

Versus-Host Disease. Oncologist. 2020;25(2):e328–34.

47. Zhang MY, Zhao P, Zhang Y, Wang JS. Efficacy and safety of ruxolitinib for steroidrefractory graft-versus-host disease: Systematic review and meta-analysis of randomised and non-randomised studies. PLoS One. 2022;17(7 July):1–17.

48. Gooptu M, Antin JH. GVHD Prophylaxis 2020. Front Immunol. 2021;12(April):1–13.

49. Couriel D, Caldera H, Champlin R, Komanduri K. Acute graft-versus-host disease: Pathophysiology, clinical manifestations, and management. Cancer. 2004;101(9):1936–46.

50. Hamilton BK. Current approaches to prevent and treat GVHD after allogeneic stem cell transplantation. Hematol (United States). 2018;2018(1):228–35.

51. Sabry W, Le Blanc R, Labbé AC, Sauvageau G, Couban S, Kiss T, et al. Graft-versus-Host Disease Prophylaxis with Tacrolimus and Mycophenolate Mofetil in HLA-Matched Nonmyeloablative Transplant Recipients Is Associated with Very Low Incidence of GVHD and Nonrelapse Mortality. Biol Blood Marrow Transplant. 2009;15(8):919–29.

52. Chang Y, Zhao X, Huang X. Strategies for Enhancing and Preserving Anti-leukemia Effects Without Aggravating Graft-Versus-Host Disease. 2018;9(December):1–17.

53. Lee IC, Choi BY. Withaferin-A—A natural anticancer agent with pleitropic mechanisms of action. Int J Mol Sci. 2016;17(3).

54. Atteeq M. Evaluating anticancer properties of Withaferin A—a potent phytochemical. Front Pharmacol. 2022;13(October):1–13.

55. Lee DH, Lim IH, Sung EG, Kim JY, Song IH, Park YK, et al. Withaferin A inhibits matrix metalloproteinase-9 activity by suppressing the Akt signaling pathway.

Oncol Rep. 2013;30(2):933–8.

56. Kakar SS, Ratajczak MZ, Powell KS, Moghadamfalahi M, Miller DM, Batra SK, et al. Withaferin a alone and in combination with cisplatin suppresses growth and metastasis of ovarian cancer by targeting putative cancer stem cells. PLoS One. 2014;9(9).

57. Suttana W, Mankhetkorn S, Poompimon W, Palagani A, Zhokhov S, Gerlo S, et al. Differential chemosensitization of P-glycoprotein overexpressing K562/Adr cells by withaferin A and Siamois polyphenols. Mol Cancer. 2010;9(Il):1–22.

58. Ram N, Peak S, Perez A, Jinwal U. Implications of Withaferin A in neurological disorders. Neural Regen Res. 2021;16(2):304–5.

59. Raziya Banu M, Ibrahim M, Prabhu K, Rajasankar S. Ameliorative effect of withaferin a on ageing-mediated impairment in the dopamine system and its associated behavior of wistar albino rat. Pharmacology. 2019;103(3–4):114–9.

60. Palliyaguru DL, Chartoumpekis D V., Wakabayashi N, Skoko JJ, Yagishita Y, Singh S V., et al. Withaferin A induces Nrf2-dependent protection against liver injury: Role of Keap1-independent mechanisms. Free Radic Biol Med. 2016;101:116–28.

61. Shiragannavar VD, Sannappa Gowda NG, Puttahanumantharayappa LD, Karunakara SH, Bhat S, Prasad SK, et al. The ameliorating effect of withaferin A on high-fat diet-induced non-alcoholic fatty liver disease by acting as an LXR/FXR dual receptor activator. Front Pharmacol. 2023;14(February):1–12.

62. Kim G, Kim TH, Kang MJ, Choi JA, Pack DY, Lee IR, et al. Inhibitory effect of withaferin A on Helicobacter pylori-induced IL-8 production and NF-κB activation in gastric epithelial cells. Mol Med Rep. 2016;13(1):967–72.

63. Kumar S, Mathew SO, Aharwal RP, Tulli HS, Mohan CD, Sethi G, et al. Withaferin A: A Pleiotropic Anticancer Agent from the Indian Medicinal Plant

Withania somnifera (L.) Dunal. Pharmaceuticals. 2023;16(2).

64. Gambhir L, Checker R, Sharma D, Thoh M, Patil A, Degani M, et al. Thiol dependent NF-κB suppression and inhibition of T-cell mediated adaptive immune responses by a naturally occurring steroidal lactone Withaferin A. Toxicol Appl Pharmacol. 2015;289(2):297–312.

65. Mehta M, Gohil D, Khattry N, Kumar R, Sandur S, Sharma D. International Immunopharmacology Prevention of acute graft-versus-host-disease by Withaferin a via suppression of AKT / mTOR pathway. Int Immunopharmacol. 2020;84(April):106575.

66. Kumano K, Kanak MA, Saravanan PB, Blanck JP, Liu Y, Vasu S, et al. Withaferin A inhibits lymphocyte proliferation, dendritic cell maturation in vitro and prolongs islet allograft survival. Sci Rep. 2021;11(1):1–11.

67. S G. India as the Heritage of Medicinal Plant and their Use. Curr Trends Biomed Eng Biosci. 2017;4(4):50–1.

68. Mendoza N, Silva EME. Introduction to Phytochemicals: Secondary Metabolites from Plants with Active Principles for Pharmacological Importance. Phytochem - Source Antioxidants Role Dis Prev. 2018;

69. Tekula S, Khurana A, Anchi P, Godugu C. Withaferin-A attenuates multiple low doses of Streptozotocin (MLD-STZ) induced type 1 diabetes. Biomed Pharmacother. 2018;106(July):1428–40.

70. Bale S, Pulivendala G, Godugu C. Withaferin A attenuates bleomycin-induced scleroderma by targeting FoxO3a and NF-κβ signaling: Connecting fibrosis and inflammation. BioFactors. 2018;44(6):507–17.

71. Baker JT, Borris RP, Carté B, Cordell GA, Soejarto DD, Cragg GM, Gupta MP, Iwu MM, Madulid DR, Tyler VE. Natural product drug discovery and development: new perspectives on international collaboration. Journal of natural

products. 1995 Sep;58(9):1325-57.

72. Haq I. Special Supplement on The Importance of Herbs. Pak J Med Res. 2004;43(4):203–10.

73. Singh N, Bhalla M, de Jager P, Gilca M. An overview on Ashwagandha: A Rasayana (Rejuvenator) of Ayurveda. African J Tradit Complement Altern Med. 2011;8(5 SUPPL.):208–13.

74. Pires N, Gota V, Gulia A, Hingorani L, Agarwal M, Puri A. Safety and pharmacokinetics of Withaferin-A in advanced stage high grade osteosarcoma: A phase I trial. J Ayurveda Integr Med. 2020;11(1):68–72.

75. Jana SN, Charan SM. Review Article Health Benefits and Medicinal Potency of Withania somnifera : A Review. 2018;48(08):22–9.

76. Mishra LC, Singh BB, Dagenais S. Scientific basis for the therapeutic use of Withania somnifera (ashwagandha): A review. Altern Med Rev. 2000;5(4):334–46.

77. Modi SJ, Tiwari A, Ghule C, Pawar S, Saste G, Jagtap S, et al. Pharmacokinetic Study of Withanosides and Withanolides from Withania somnifera Using Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS). Molecules. 2022;27(5):1–24.

78. Shohat B, Gitter S, Lave D. Effect of withaferin a on ehrlich ascites tumor cells — cytological observations. Int J Cancer. 1970;5(2):244–52.

79. Patel K, Singh RB, Patel DK. Pharmacological and analytical aspects of withaferin A: A concise report of current scientific literature. Asian Pacific J Reprod. 2013;2(3):238–43.

80. Straughn AR, Kakar SS. Withaferin A: A potential therapeutic agent against COVID-19 infection. J Ovarian Res. 2020;13(1):1–5.

81. Hahm E-R, Singh S. Autophagy Fails to Alter Withaferin A-Mediated

Lethality in Human Breast Cancer Cells. Curr Cancer Drug Targets. 2013;13(6):640–50.

82. Vedi M, Sabina EP. Assessment of hepatoprotective and nephroprotective potential of withaferin A on bromobenzene-induced injury in Swiss albino mice: possible involvement of mitochondrial dysfunction and inflammation. Cell Biol Toxicol. 2016;32(5):373–90.

83. Prabu PC, Panchapakesan S, Raj CD. Acute and Sub-Acute Oral Toxicity Assessment of the Hydroalcoholic Extract of Withania somnifera Roots in Wistar Rats. 2012;(August).

84. Patel SB, Rao NJ, Hingorani LL. Safety assessment of Withania somnifera extract standardized for Withaferin A: Acute and sub-acute toxicity study. J Ayurveda Integr Med. 2016;7(1):30–7.

85. Hoppe BR, Lassen ED. Blood Smears and the Use of Wright' s Stain. Iowa State Univ Vet. 1978;40(3):113–6.

86. Yoo S, Kim K, Nam H, Lee D. Discovering health benefits of phytochemicals with integrated analysis of the molecular network, chemical properties and ethnopharmacological evidence. Nutrients. 2018;10(8).

87. Sajid M, Cameotra SS, Ahmad Khan MS, Ahmad I. Nanoparticle-Based Delivery of Phytomedicines: Challenges and Opportunities. New Look to Phytomedicine: Advancements in Herbal Products as Novel Drug Leads. Elsevier Inc.; 2018. 597–623 p.

88. Pandey V, Ansari WA, Misra P, Atri N. Withania somnifera: Advances and implementation of molecular and tissue culture techniques to enhance its application. Front Plant Sci. 2017;8(August):1–11.

89. Hassannia B, Logie E, Vandenabeele P, Vanden Berghe T, Vanden Berghe W. Withaferin A: From ayurvedic folk medicine to preclinical anti-cancer drug. Biochem

Pharmacol. 2020;173(August):0–1.

90. Dutta R, Khalil R, Green R, Mohapatra SS, Mohapatra S. Withania somnifera (Ashwagandha) and withaferin a: Potential in integrative oncology. Int J Mol Sci. 2019;20(21).

91. Xia Y, Wang P, Yan N, Gonzalez FJ. Withaferin A alleviates fulminant hepatitis by targeting macrophage and NLRP3. Cell Death Dis. 2021;

92. Das R, Rauf A, Akhter S, Islam MN, Emran T Bin. Role of Withaferin A and Its Derivatives in the Management of Alzheimer ' s Disease : Recent Trends and Future Perspectives. 2021;

93. Olayode OA, Daniyan MO, Olayiwola G. Biochemical, hematological and histopathological evaluation of the toxicity potential of the leaf extract of Stachytarpheta cayennensis in rats. J Tradit Complement Med. 2020;10(6):544–54.

94. Sharada AC, Solomon FE, Devi PU, Udupa N, Srinivasan KK. Antitumor and radiosensitizing effects of withaferin a on mouse ehrlich ascites carcinoma in vivo. Acta Oncol (Madr). 1996;35(1):95–100.

95. Shohat B, Gitter S, Abraham A, Lavie D. Antitumor activity of withaferin A (NSC-101088). Cancer chemotherapy reports. 1967 Sep 1;51(5):271-6.

96. Dai T, Jiang W, Guo Z, Wang Z, Huang M, Zhong G, et al. Studies on oral bioavailability and first-pass metabolism of withaferin A in rats using LC–MS/MS and Q-TRAP. Biomed Chromatogr. 2019;33(9).

97. Patil D, Gautam M, Mishra S, Karupothula S, Gairola S, Jadhav S, et al. Determination of withaferin A and withanolide A in mice plasma using high-performance liquid chromatography-tandem mass spectrometry: Application to pharmacokinetics after oral administration of Withania somnifera aqueous extract. J Pharm Biomed Anal. 2013;80:203–12.

98. Jacobsohn DA, Vogelsang GB. Acute graft versus host disease. Orphanet J Rare Dis. 2007;2(1):1–9.

99. Zeiser R, Blazar BR. Acute Graft-versus-Host Disease — Biologic Process, Prevention, and Therapy. N Engl J Med. 2017;377(22):2167–79.

100. Ferrara JL, Chaudhry MS. GVHD: biology matters. Blood advances. 2018 Nov 27;2(22):3411-7.

101. Hill GR, Betts BC, Tkachev V, Kean LS, Blazar BR. Current concepts and advances in graft-versus-host disease immunology. Annual review of immunology. 2021 Apr 26;39:19-49

102. Kuba A, Raida L. Graft versus host disease: from basic pathogenic principles to DNA damage response and cellular senescence. Mediators Inflamm. 2018;2018(1).

103. Wolff D, Fatobene G, Rocha V, Kröger N, Flowers ME. Steroid-refractory chronic graft-versus-host disease: treatment options and patient management. Bone Marrow Transplant. 2021;56(9):2079–87.

104. Chen YB. New treatment options for the management of acute graft-versus-host disease. Clinical Advances in Hematology & Oncology: H&O. 2019 Sep 1;17(9):482-5.

105. Dubey S, Yoon H, Cohen MS, Nagarkatti P, Nagarkatti M, Karan D. Withaferin a associated differential regulation of inflammatory cytokines. Front Immunol. 2018;9.

106. Sorelle JA, Itoh T, Peng H, Kanak MA, Sugimoto K, Matsumoto S, et al. Withaferin A inhibits pro-inflammatory cytokine-induced damage to islets in culture and following transplantation. Diabetologia. 2013;56(4):814–24.

107. Cooke KR, Jr JD, Ferrara JL, Cooke KR, Hill GR, Crawford JM, et al. Tumor necrosis factor- alpha production to lipopolysaccharide stimulation by donor cells

predicts the severity of experimental acute graft-versus-host disease . Find the latest version : Tumor Necrosis Factor – Production to Lipopolysaccharide Stimulati. 1998;102(10):1882–91.

108. Mondor HH, Créteil UP, Marne V De, Oncologie- S. Simple, Reproducible, and Efficient Clinical Grading System for Murine Models of Acute Graft-versus-Host Disease. 2018;9(January):1–9.

109. Cooke BKR, Kobzik L, Martin TR, Brewer J, Jr JD, Crawford JM, et al. An Experimental Model of Idiopathic Pneumonia Syndrome After Bone Marrow Transplantation: I. The Roles of Minor H Antigens and Endotoxin. Blood. 1996;88(8):3230–9.

110. Tembhare PR, Sriram H, Chatterjee G, Khanka T, Gokarn A, Mirgh S, et al. Comprehensive immune cell profiling depicts an early immune response associated with severe coronavirus disease 2019 in cancer patients. Immunol Cell Biol. 2022;100(1):61–73.

111. Yamanouchi S, Adachi Y, Shimo T, Umezawa K, Okigaki M, Tsuji S, et al. A nuclear factor-κB inhibitor, dehydroxymethylepoxyquinomicin, ameliorates GVHD in allogeneic bone marrow transplantation. Immunobiology. 2015;

112. Fajgenbaum DC, June CH. Cytokine Storm. N Engl J Med. 2020;383(23):2255–73.

113. Maitra R, Porter MA, Huang S, Gilmour BP. Inhibition of NFB by the natural product withaferin a in cellular models of cystic fibrosis inflammation. J Inflamm. 2009;6:1–5.

114. Abboud R, Choi J, Ruminski P, Schroeder MA, Kim S, Abboud CN, DiPersio JF. Insights into the role of the JAK/STAT signaling pathway in graft-versus-host disease. Therapeutic Advances in Hematology. 2020 Jun;11:2040620720914489.

115. Um HJ, Min K jin, Kim DE, Kwon TK. Withaferin A inhibits JAK/STAT3 signaling and induces apoptosis of human renal carcinoma Caki cells. Biochem Biophys Res Commun. 2012;427(1):24–9.

116. Yco LP, Mocz G, Opoku-Ansah J, Bachmann AS. Withaferin A Inhibits STAT3 and Induces Tumor Cell Death in Neuroblastoma and Multiple Myeloma. Biochem Insights. 2014;7:BCI.S18863.

117. Narasimhan PB, Marcovecchio P, Hamers AAJ, Hedrick CC. Nonclassical Monocytes in Health and Disease. Annu Rev Immunol. 2019;37:439–56.

118. Long J, Chang L, Shen Y, Gao W, Wu Y, Dou H, et al. Valproic Acid Ameliorates Graft-versus-Host Disease by Downregulating Th1 and Th17 Cells. 2022;

119. Rafei H, Elbahesh E. Acute Graft Versus Host Disease : A Comprehensive Review. 2017;1555:1547–55.

120. Kumar S, Jadhav S, Gohil D, Ch G. Safety , toxicity and pharmacokinetic assessment of oral Withaferin-A in mice. Toxicol Reports. 2022;9(May):1204–12.

121. Saraiva M, Saraiva M, Vieira P, Vieira P, Vieira P, O'Garra A, et al. Biology and therapeutic potential of interleukin-10. J Exp Med. 2020;217(1):1–19.

122. Steen EH, Wang X, Balaji S, Butte MJ, Bollyky PL, Keswani SG. The Role of the Anti-Inflammatory Cytokine Interleukin-10 in Tissue Fibrosis. 2020;9(4):184–98.

123. Calabrese LH, Lenfant T, Calabrese C. Cytokine storm release syndrome and the prospects for immunotherapy with COVID-19, part 4: The role of JAK inhibition. Cleve Clin J Med. 2020;1–6.

124. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer. 2004;4(5):371–80.

125. Mathe G, Amiel JL, Schwarzenberg L, Cattan A, Schneider M. Haematopoietic Chimera in Man After Allogenic (Homologous) Bone-Marrow

Transplantation. (Control of the Secondary Syndrome. Specific Tolerance Due To the Chimerism). Br Med J. 1963;2(5373):1633–5.

126. Sweeney C, Vyas P. The Graft-Versus-Leukemia Effect in AML. Front Oncol. 2019;9(November):1–19.

127. Handgretinger R, Schilbach K. The potential role of gd T cells after allogeneic HCT for leukemia. Blood. 2018;131(10):1063–72.

128. Song Y, Zhu Y, Hu B, Liu Y, Lin D, Jin Z, et al. Donor γδT Cells Promote GVL Effect and Mitigate aGVHD in Allogeneic Hematopoietic Stem Cell Transplantation. Front Immunol. 2020;11(October):1–12.

129. Harris R, Karimi M. Dissecting the regulatory network of transcription factors in T cell phenotype/functioning during GVHD and GVT. Front Immunol. 2023;14(June):1–17.

130. Pan B, Shang L, Liu C, Gao J, Zhang F, Xu M, et al. PD-1 antibody and ruxolitinib enhances graft-versus-lymphoma effect without increasing acute graft-versus-host disease in mice. Am J Transplant. 2021;21(2):503–14.

131. Snyder KJ, Choe HK, Gao Y, Sell NE, Braunreiter KM, Zitzer NC, Neidemire-Colley L, Kalyan S, Dorrance AM, Keller A, Mihaylova MM. Inhibition of bromodomain and extra terminal (BET) domain activity modulates the IL-23R/IL-17 axis and suppresses acute graft-Versus-Host disease. Frontiers in oncology. 2021 Oct 15;11:760789.

132. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, Abhyankar S, et al. Long term disease-free survival in acute leukemia patients recovering with increased γδ T cells after partially mismatched related donor bone marrow transplantation. Bone Marrow Transplant. 2007;39(12):751–7.

133. Wolf Y, Anderson AC, Kuchroo VK. TIM3 comes of age as an inhibitory

receptor. Nat Rev Immunol. 2020;20(March).

134. Oikawa T, Kamimura Y, Akiba H, Yagita H, Okumura K, Takahashi H, Zeniya M, Tajiri H, Azuma M. Preferential involvement of Tim-3 in the regulation of hepatic CD8+ T cells in murine acute graft-versus-host disease. The Journal of Immunology. 2006 Oct 1;177(7):4281-7.

135. Das M, Zhu C, Kuchroo VK. Tim-3 and its role in regulating anti-tumor immunity. Immunol Rev. 2017;276(1):97–111.

136. Lamb LS, Gee AP, Hazlett LJ, Musk P, Parrish RS, O'Hanlon TP, et al. Influence of T cell depletion method on circulating γδ T cell reconstitution and potential role in the graft-versus-leukemia effect. Cytotherapy. 1999;1(1):7–19.

137. Harris R, August A, Gentile T, Henty-ridilla J, Waickman AT, August A, et al. Targeting SLP76 : ITK interaction separates GVHD from GVL in allo-HSCT GVHD from GVL in allo-HSCT. ISCIENCE. 24(4):102286.

138. Orhan IE. Pharmacognosy: Science of natural products in drug discovery. 2014;4(3):109–10.

139. Moreira DDL, Teixeira SS, Monteiro MHD, De-oliveira ACAX, Paumgartten FJR. Original article Traditional use and safety of herbal medicines. 2014;24:248–57.

140. Mohamed I, Shuid A, Borhanuddin B, Fozi N. The Application of Phytomedicine in Modern Drug Development. 2012;1(2):1–9.

141. Park M, Moon S, Lee S, Yang E, Min J, Cho S, et al. Curcumin Attenuates Acute Graft-versus-Host Disease Severity via In Vivo Regulations on Th1 , Th17 and Regulatory T Cells. 2013;8(6).

142. Li Z, Wu Q, Yan Z, Li D, Pan X, Qiu T, et al. with Tripterygium hypoglaucum Hutch combined Prevention of acute GVHD in mice by treatment with Tripterygium hypoglaucum Hutch combined with cyclosporin A. 2013;8454.

143. Westphal S, Mcgeary A, Rudloff S, Wilke A, Penack O. The Green Tea Catechin Epigallocatechin Gallate Ameliorates Graft-versus-Host Disease. 2017;1–13.

144. Alvarez-laderas I, Ramos TL, Medrano M, Caracuel-garcía R, Zamora R, Alarc C, et al. Biology of Blood and Marrow Transplantation Polyphenolic Extract (PE) from Olive Oil Exerts a Potent Immunomodulatory Effect and Prevents Graft-versus-Host Disease in a Mouse Model Antonio P e Jos e. 2020;26:615–24.

145. Dar NJ, Hamid A, Ahmad M. Pharmacologic overview of Withania somnifera, the Indian Ginseng. Cell Mol Life Sci. 2015;72(23):4445–60.

146. Saleem S, Muhammad G, Hussain MA, Altaf M, Abbas Bukhari SN. Withania somnifera L.: Insights into the phytochemical profile, therapeutic potential, clinical trials, and future prospective. Iran J Basic Med Sci. 2020;23(12):1501–26.

147. Kumar Gupta S, Gohil D, Dutta D, Panigrahi GC, Gupta P, Dalvi K, et al. Withaferin-A alleviates acute graft versus host disease without compromising graft versus leukemia effect. Int Immunopharmacol. 2023;121(January):110437.

148. Abboud R, Choi J, Ruminski P, Schroeder MA, Kim S, Abboud CN, DiPersio JF. Insights into the role of the JAK/STAT signaling pathway in graft-versus-host disease. Therapeutic Advances in Hematology. 2020 Jun;11:2040620720914489.

149. Cutler CS, Koreth J, Ritz J. Mechanistic approaches for the prevention and treatment of chronic GVHD. Blood, The Journal of the American Society of Hematology. 2017 Jan 5;129(1):22-9.

150. Hooker DS, Grabe-Heyne K, Henne C, Bader P, Toumi M, Furniss SJ. Improved Therapeutic Approaches are Needed to Manage Graft-versus-Host Disease. Clin Drug Investig. 2021;41(11):929–39.

151. Gupta SK, Jadhav S, Gohil D, Panigrahi GC, Kaushal RK, Gandhi K, et al. Safety, toxicity and pharmacokinetic assessment of oral Withaferin-A in mice. Toxicol

Reports. 2022;9(February):1204–12.

152. Paul S, Chakraborty S, Anand U, Dey S, Nandy S, Ghorai M, et al. Withania somnifera (L.) Dunal (Ashwagandha): A comprehensive review on ethnopharmacology, pharmacotherapeutics, biomedicinal and toxicological aspects. Biomed Pharmacother. 2021;143(September).

153. Rai M, Jogee PS, Agarkar G, Santos CA Dos. Anticancer activities of Withania somnifera: Current research, formulations, and future perspectives. Pharm Biol. 2016;54(2):189–97.

154. Yang Z, Garcia A, Xu S, Powell DR, Vertino PM, Singh S, et al. Withania somnifera Root Extract Inhibits Mammary Cancer Metastasis and Epithelial to Mesenchymal Transition. PLoS One. 2013;8(9):1–12.

155. Turrini E, Calcabrini C, Sestili P, Catanzaro E, de Gianni E, Diaz AR, et al. Withania somnifera induces cytotoxic and cytostatic effects on human T leukemia cells. Toxins (Basel). 2016;8(5):1–15.

156. Singh D, Aggarwal A, Maurya R, Naik S. Withania somnifera inhibits NF-κB and AP-1 transcription factors in human peripheral blood and synovial fluid mononuclear cells. Phytotherapy Research. 2007 Oct;21(10):905-13..

157. Naidoo DB, Chuturgoon AA, Phulukdaree A, Guruprasad KP, Satyamoorthy K, Sewram V. Withania somnifera modulates cancer cachexia associated inflammatory cytokines and cell death in leukaemic THP-1 cells and peripheral blood mononuclear cells (PBMC's). BMC complementary and alternative medicine. 2018 Dec;18:1-1..

158. Kaur T, Kaur G. Withania somnifera as a potential candidate to ameliorate high fat diet-induced anxiety and neuroinflammation. J Neuroinflammation. 2017;14(1):1–18.

159. Pawar P, Gilda S, Sharma S, Jagtap S, Paradkar A, Mahadik K, et al. Rectal

gel application of Withania somnifera root extract expounds anti-inflammatory and muco-restorative activity in TNBS-induced Inflammatory Bowel Disease. BMC Complement Altern Med. 2011;11.

160. Vedi M, Rasool M, Sabina EP. Amelioration of bromobenzene hepatotoxicity by Withania somnifera pretreatment: Role of mitochondrial oxidative stress. Toxicol Reports. 2014;1:629–38.

161. Deeg HJ. How I treat refractory acute GVHD. Blood. 2007;109(10):4119–26.

162. Yu J, Judy JT, Parasuraman S, Sinha M, Weisdorf D. Inpatient Healthcare Resource Utilization, Costs, and Mortality in Adult Patients with Acute Graft-versus-Host Disease, Including Steroid-Refractory or High-Risk Disease, following Allogeneic Hematopoietic Cell Transplantation: HCRU, Costs, and Mortality . Biol Blood Marrow Transplant. 2020;26(3):600–5.

163. Smith T, Resetar H, Morton C. US sales of herbal supplements increase by 9.7% in 2021. HerbalGram. 2022;136:42-69.

Publications