STUDIES ON POTENTIAL BIOLOGICAL IMMUNOMODULATORS IN HIV INFECTED INDIVIDUALS

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BY

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UNDER THE GUIDANCE

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I hereby declare that the thesis entitled "Studies on potential biological immunomodulators in HIV infected individuals" submitted by me to the Bharati Vidyapeeth Deemed University, Pune for the degree of 'Doctor of Philosophy' (Ph.D.) in the subject of Microbiology under the 'Faculty of Science' is an original piece of work carried out by me under the supervision of Dr. M. G. Bodhankar and Dr. P. M. Bulakh. I further declare that it has not been submitted to this or any other university or institution for the award of any degree or diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

Place: Pune **Bharat Bajarang Ballal**

Date: 13.12.2016 Research student

Even in the darkness light dawns for those who believe,

hence dedicated

To

'The Pasteur Institute', Paris, France

For their service to the humanity

&

As all great things always have a humble beginning

Hence also dedicated to

Mr. Jaywant Devkar

He who knows much more than the letters…

And has become an example of

the institutionalized staff.

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ABBREVIATIONS AND ACRONYMS

Abbreviation	Full form
AFB	Antibody Forming B Lymphocytes
APAF1	Apoptosis Activator Factor 1
APC	Antigen Presenting Cells
ART	Anti Retroviral Treatment
CAF	$CD8^+$ Cell Antiviral Factor
CD	Cluster of Differentiation
CDC	Centers For Disease Control and Prevention
Cyt-C	Cytochrome –C
ddH_2O	double distilled H_2O
DEPC	Diethyl Poly Carbonate
DNA	Deoxyribo Nucleic acid
DTH	Delayed Type Hypersensitivity
Fas	Death receptor domain of Apoptosis (CD95)
Fas L	Ligand for Fas Domain
Fox P3	Fork Head Transcription Factor P3
GITR	Glucocorticoid induced TNF Family Related Receptor
Gp	Glycoprotein
HAART	Highly Active Anti Retroviral Therapy
HIV	Human Immunodeficiency Virus
HRD	Human Resource Development
HTLV	Human T Lymphotropic Virus
IAEC	Institutional Animal Ethics Committee
ICAM	Intercellular Adhesion Molecules
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon

Abbreviation	Full form
IL	Interleukins
LFA	Leukocyte Functioning Antigens
MDR	Multiple Drug Resistance
MIP	Macrophage Inflammatory Protein
NACO	National Aids Control Organization
NK	Natural Killer Cells
OECD	Organization For Economic Cooperation and Development Guidelines
PBS	Phosphate Buffer Saline
PGL	Persistent Generalized Lymphadenopathy
QS	Quality Standard Probe of RT PCR
RES	Reticulo Endothelial System
RNA	Ribo Nucleic Acid
Tc	T Cytotoxic Cells
TGF	T cell Growth Factor
T_H	T Helper Cells
T_r	T Regulatory Cells
TRAIL	TNF Related Apoptosis Inducing Ligand
UNG	Uracil N – Glycosylase enzyme
VLPs	Virus Like Particles
WHO	World Health Organization

INTRODUCTION

INTRODUCTION

The Human Immunodeficiency Virus (HIV) belongs to the Genus *Lentivirus* and Family *Retroviridae* [(68)]. HIV is an etiological agent of 'Acquired Immunodeficiency Syndrome' (AIDS) [(4)]. Retroviruses are well known for their ability to infect birds and rodents [(58)]. The name Retroviruses is attributed towards their ability to form a copy of DNA from RNA. The enzyme 'reverse transcriptase' responsible for the conversion was independently detected by Howard Temin and David Baltimore. First Human Retrovirus, 'Human T cell Leukaemia Virus' (HTLV) was isolated by Robert Gallo and Poisez B.J. [(141)]. Later, Japanese scientist isolated a virus and described it as an 'Adult T Cell Leukaemia Virus' (ATLV) [(118)]. Luc Montagnier described the virus as 'HTLV-III' and discriminated the type on the basis of its antagonistic behavior to other types by destroying T_4 lymphocytes. In 1985, Robert Gallo presented phylogenetic tree of *Retroviruses* and recommended inclusion of the virus in the subfamily- *Lentivirinae* [(159)]. In 1986, the 'International Committee on the Taxonomy of viruses (ICTV) organized the subcommittee for nomenclature of the Retrovirus. The committee was headed by Dr. Harold Varmus and the name HIV (Human Immunodeficiency Virus) was declared as an official name for the virus [(70)]**.** The virus was officially declared as an etiological agent of the disease 'AIDS'. Due to complexity in the molecular structure and poor response to antiviral drugs, the disease had remained as the one of the biggest challenge to human race.

GLOBAL HIV STATISTICS:

Up to June 2015, about 36.9 million people were living with HIV [(153)]. In a single calendar year of 2014, about 1.2 million people have died of AIDS related diseases. Out of total figure, only about 15.8 million people are on 'antiretroviral therapy'. About 41 % infected adults, 32 % infected children and 73 % infected pregnant women had access to 'antiretroviral therapy'. Since 2000, about 25 million people have died of AIDS related complex. In 2014, about 2 million new cases of HIV infection have been reported [(153)]. HIV status in major parts of world is summarized in Table-1.

Geographical Area	Average No. of HIV infected individuals (Up to Dec. 2014)	Average No. of Deaths due to AIDS related diseases.
Sub Saharan Africa	25.8 million	790000
Asia with Pacific	5.0 million	240000
Latin America	1.7 million	41000
Caribbean	280000	8800
Middle East and North Africa	240000	12000
Eastern Europe and Central Asia	1.5 million	62000
Western and Central Europe with North America	2.4 million	26000
Total	36.9 million	1.2 million

Table-1: HIV statistics in 2014 (Source: www.unaids.org)

NATIONAL HIV STATISTICS:

India is the country having third largest number of HIV infected individuals in the world. In India, at the end of December 2015, about 21.17 lakhs people were living with HIV. The disease statistics in the major states of India is summarized in table-2 **(127)**.

State	Average No. of HIV infected individuals as recorded at the end of Dec. 2015
Andhra Pradesh and Telangana	3.95 lakhs
Maharashtra	3.01 lakhs
Karnataka	1.99 lakhs
Gujarat	1.66 lakhs
Bihar	1.51 lakhs
Uttar Pradesh	1.50 lakhs
Rajasthan	1.03 lakhs
Tamil Nadu	1.43 lakhs
West Bengal	1.29 lakhs
Other States	$\geq$ 1 lakhs

Table 2: HIV status in the major states of India

Out of total infected individuals, 40.5 % are females and 6.54 % are children. About 86 thousand new HIV infections and 67612 AIDS related deaths were recorded in 2015. Maximum mortality in 2015 was recorded in Andhra Pradesh and Telangana (9249) while minimum was in Daman and Diu (12). From 2000 to 2015, number of new HIV cases was dropped from 2.51 lakhs to 86 thousands reflecting net decline in the infection rate by 66 % against a global figure of 35 % [153, 127].

Pune is located in the western part of Maharashtra in India. Population of Pune is around 9426959 out of which about 61% lives in urban area. Cumulative literacy rate is 87.19% with male to female ratio as 1000:910. Till Oct. 31 in the year 2014, the city recorded 1718 new cases of HIV infection [169]. HIV prevalence rate in the city as on December 2014 was 0.67 %. The city recorded prevalence rate of 4.63 % in males, 4.46 % in females, 3.91 % in referred visitors and 5.63 % as who visited voluntarily without medical recommendations and 21 % prevalence rate in widowed or divorced [127].

CHALLENGES IN THE CONTROL OF HIV/AIDS:

Food and Drug Administration had approved the drugs for the treatment of HIV infection/AIDS. The drugs have been categorized as,

i) NRTIs (Nucleoside analogue Reverse Transcriptase Inhibitors),

ii) NNRTIs (Non Nucleoside analogue Reverse Transcriptase Inhibitors),

iii) Protease Inhibitors and

iv) Fusion Inhibitors.

Zidovudin, Lamivudin, Tenofovir, Emtricitabine, Stavudin, Abacavir, Zalcitabina, Didanosin, Dideoxyinosine are the examples of NRTIs. Nevirapine, Efavirehza, Delavirdine are the examples of NNRTIs. Protease inhibitors include Indinavir, Ritonavir, Saquinavir, Amprenavir, Tipranavir, Lopinavir, Fosamprenavir, Darunavir, Atazanavir sulphate and Nelfinavir mesylate [68]. Prime problem in the treatment of HIV infection or AIDS is of the emergence of resistant viruses [151]. Study of the molecular biology of the HIV revealed about five genetic alterations associated with AZT resistance [78]. Lack of reliable animal model and high genetic variability of HIV made vaccine development programs challenging for the researchers. Prolonged treatment of ART and HAART has been reported for serious side effects. Prime side effects have shortlisted in table-3

Chemotherapeutic Agent	Side Effects
Zidovudine	Aplastic anaemia, Damage to bone marrow cells
Nevirapine, Efavirenz	Hypersensitivity
Protease Inhibitors	Atherosclerosis, Nausea, Vomiting, diarrhea
Efavirenz	Headache, Dizziness, Psychiatric disorders
Indinavir, Atazanavir	Hyperbilirubinaemia
Tipranavir, Nevirapine, Efavirenz	Liver Damage
ddI, D_4T	Cytotoxicity to mitochondria, Lactic acidosis
Protease inhibitars, d_4T, Efavirenz	Lipid metabolism disorders
d_4T, Zidovudine	Metabolism disorders
ddI, d_4T,	Inflammation of pancreas
d_4T, ddI	Nervous system disorders
Tenofavir	Renal System disorders
Nevirapine	Autoimmune Disease (Steven Jhonsan Syndrome)
Efavirenz	Birth Defects

Table-3: Prime side effects of antiretroviral therapy (Source: http://www.hivatis.org)

Microbicides are topical substances used for reduction of HIV transmission and are applied either to vagina or rectum before sexual intercourse. Phase III clinical trials of many microbicide candidate substances like nonoxynol-9, savvy, cellulose sulphate resulted in ineffective prevention of transmission of 'Human Immunodeficiency Virus' [(49)].

Overall picture reveal the limitation of anti-HIV drugs, vaccines and microbicides. Hence, it is keen need to develop safe and effective antiretroviral therapy or immunomodulating therapy which can curb the spread of HIV infection. With this view, the present research is planned with specific objective of 'Studies on potential Biological Immunomodulators in HIV infected individuals'.

OBJECTIVES

OBJECTIVES

In India, at the end of December 2011, cumulative figure of HIV infection was 2.3 millions. Reported HIV/AIDS cases in Maharashtra were 65,409. In between January 2011 to October 2011, about 3,228 HIV positive cases were detected at Pune Municipal Corporation run hospitals. Drug toxicity of antiretroviral therapy, development of drug resistance, lack of reliable vaccine revealed the need of exploring supportive safer therapy. The present research is planned on 'Studies on potential Biological Immunomodulators in HIV infected individuals' with objectives as:

1. To screen the potential biological immunomodulators.

2. To study the antibacterial and antifungal properties of selected biological immunomodulators.

3. To study the immunomodulating properties of selected biological immunomodulators.

REVIEW
OF
LITERATURE

REVIEW OF LITERATURE

In early 1980s, the cause of AIDS was not known and the scientists were looking for Herpes viruses and Parvovirus as candidate etiological agents. In 1983 Barre Sinoussi from Pasteur Institute and Dr. Chermann from Urrma Biopharma Canada detected the reverse transcriptase activity in the patient with PGL and it was the first clue for the possible role of Retroviruses in the development of AIDS [(68)]. It was the prejudice that, 'Human T lymphotropic Viruses' (HTLV) immortalizes CD_4 cells but Luc Montagnier and coworkers in 1984 explained the unusual property of 'Human T lymphotropic Viruses' (HTLV) and established their relationship with the immunodeficiency. The virus was described as 'Lymphydenophathy Associated Virus' (LAV). In the same year Robert Gallo and coworkers isolated the virus from AIDS patients and called them as HTLV-III. Similar Retrovirus was described independently by Levy et al. as 'AIDS associated Retrovirus' (ARV). Later with genomic resemblance HTLV-III, LAV and ARV were described as members of *Retroviridae* family and placed under the genus *Lentivirus* [(68)]. In 1986 'International Committee on Taxonomy of Viruses' (ICTV) organized the subcommittee for nomenclature of the virus. The committee was chaired by Dr. Harold Varmus and the name HIV (Human Immunodeficiency Virus) was declared as an official name for the virus.

SEROLOGICAL EVIDENCES FOR PRE EXISTENCE OF HIV:

Serum samples collected and stored from 1959 endemic of haemorrhagic fever in Zaire, Africa and serum samples of British seaman who died of idiopathic immunodeficiency in 1976 showed the cross reactivity with HIV-1 antigens. These evidences suggested the existence of HIV before 1981[(86)].

CLASSIFICATION OF HIV:

HIV is classified as HIV-1 and HIV-2. Full length HIV genome sequencing has generated sequence subtypes of HIV and those are called as 'clades'. HIV-1 comprise four different groups of viruses such as M (Major), O (Outlier), N (which is not from M group or O group) [(161)] and P [(139)]. Clades of M Group HIV are A, B, C, D, F, G, H. J and K. [(137)]. Subtype E is omitted and renamed as CRF-01-AE (Circulating Recombinant Forms), while I is omitted and renamed as CRF-04-cpx [(44)]. M Group has about 16 CRFs. Out of which one more aggressive CRF is CRF-02 AG [(68)]. Group O HIV do not require cyclophilin-A for their replication [(16)]. Group N viruses are

genetically different than that of Group M and Group O HIV [172]. Group P virus was isolated from a Cameroonian female. The virus showed the resemblance with 'Gorilla Simian Immunodeficiency virus' and do not show genetic similarity with other clades of HIV-1[139]. HIV-2 is classified into eight different clades such as A, B, C, D, E, F, G and H [148]. Virulence of HIV-2 is less than that of HIV-1[197]. Percentage infection for HIV-1 is 99.6 for Group M, 10.0 for Group N, 0.22 for Group O while for all HIV-2 clades it is less that 1% [68]. Proposed workshop sub classification of clades A & F from HIV-1 is A1 & A2 for A and F1 & F2 for F [45, 187]. About 10 clades have been claimed for Group N viruses [68]. Major population of newly infected HIV patients is due to clade A, C & CRF-02 AG. India, Africa, Ithiopia and China have showed the prevalence of HIV-1 (C-clade) [153]. Group O, N & P HIV are common in Cameroon, Gabon and Guinea [154]. In America and Western Europe, B clade is common [154]. In case of HIV-2, its origin is assumed as nonhuman primates [4] and it is very common in Africa [68].

STRUCTURE OF HIV:

HIV is made up of nucleoprotein core around which other protein coat exist [178]. Envelope of the virus shows gp-120 and gp-41 proteins. Both the proteins are essential to set the infection in host cell [103, 143]. It is spherical in shape and measures about 90 n.m. to 120 n.m. in diameter. Morphological shapes other than sphere have also reported [88]. Nucleocapsid of the virus shows icosahedral shell enclosing cone shaped core [145]. Icosahedral shell shows P-17/P-18 proteins while cone shaped core shows P-24, P-15 and P-55 proteins. P-17 is myristoylated protein [47] (Fatty acid group addition). Core shows presence of two strands of positive sense single stranded RNA [145]. The nucleic acid is assisted with viral RNA dependent 'DNA Polymerase' and 'Ribonuclease', together are called as 'Reverse Transcriptase' [198]. Nucleocapsid also shows presence of protenious subunits P_7, P_6, P_9 and protease P_{10}. Significant genes of HIV with their activities are enlisted in Table-4.

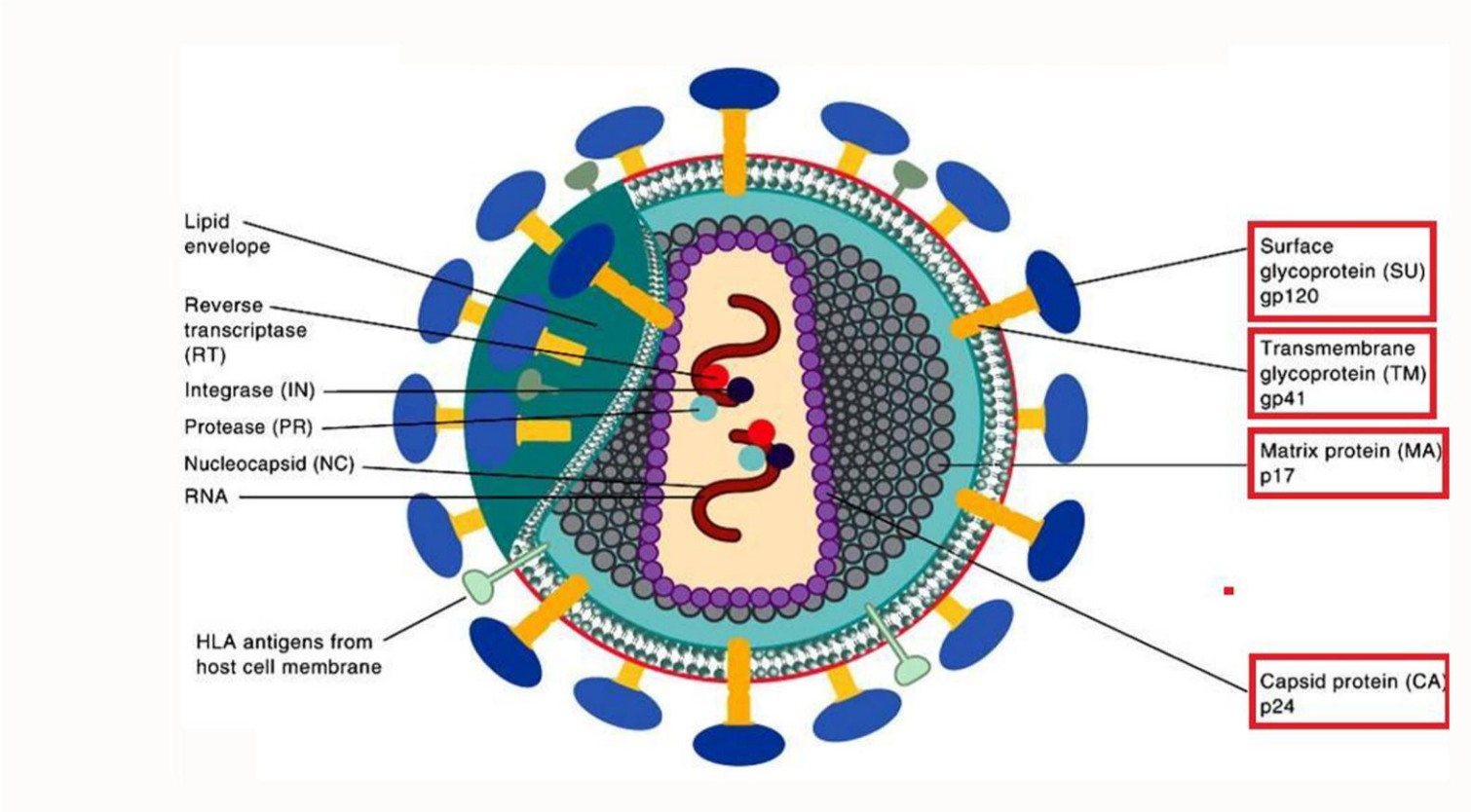


Fig: 1 Structure of HIV 1

(Source: McGraw Hill Companies Inc.)

Gene Title	Activity
Gag	Expression results in formation of core proteins including P-24, P-17 and P7
Pol	Formation of P-66 & P-51 (Reverse Transcriptase Enzyme)
Env	Formation of envelope proteins gp-120 & gp-41
PR	Formation of P-10 that is protease which involve in post translational processing of HIV proteins.
IN	Formation of P-32 (Integrase enzyme) which regulates c-DNA integration of HIV
Tat	Formation of P-14 which acts as positive regulator involving trans activation
Rev (ART, TRS)	Formation of P-19 which involves in differential regulation of HIV mRNAexpression
VIF	Formation of P-23 which is essential for infectivity of HIV
Nef (3′ ORF, B, E, F)	Formation of P-27 which is multi regulator of HIV replication (When required keeps potential to increase or decrease the rate of HIV replication)
VPU	Detected only in HIV-1, which forms P-16. It augments disruption of 'gp-160 and T Helper interaction' & facilitate the release of HIV-1
VPX	Detected only in HIV-2. It forms P-15 & facilitates entry of HIV-2 in host cell.
VPR	Also forms P-15. Prime function is to enhance the replication rate of HIV-RNA.

Table-4: Significant genes of HIV and their activities [68, 198]

Genomic size of HIV is about 9749 nucleotides [198] with open reading frames. Reverse Transcriptase of HIV commits mistake for every replication cycle resulting in changes up to 10 nitrogen bases [23]. Proviral genome of HIV-1 within the class shows variable sensitivity to restriction enzymes thus is useful for detecting source of HIV-transmission. The sensitivity pattern remains same in a donor & recipient [10, 82].

DISTRIBUTION OF HIV IN INFECTED INDIVIDUALS:

Though the blood of HIV infected individuals is a prime source of transmission, HIV has been detected in different cells, organs and fluids of the infected individuals. It is isolated from prostate gland [135], spermatocytes [126], pre ejaculatory fluid [144], vaginal fluid [199], cervix [142], anal cells & rectal secretions [81]. Mother's milk is a significant source of HIV responsible for transovarial transmission to new born baby [37, 204]. HIV though at low titer, has been detected in saliva [96]. HIV has been detected in sweat [200] but its role in transmission is dubious. The virus has been detected in tears [165].

MODE OF TRANSMISSION:

Contaminated needles and syringes shared by intravenous drug users have been reported for transmission of HIV [34]. Other means include contaminated blood and blood products used by haemophiliacs [50], sexual intercourse [98] including homosexual activity [130] and from untreated HIV infected mother to fetus [124].

VIRULANCE INCREASING FACTORS:

Virulence increasing factors during HIV infection includes,

- Survival of HIV in fridge dried blood coagulating factors [97],
- Sexual intercourse during menstrual cycle of HIV infected women [132],
- Sexual activity of HIV infected individual with uncircumcised men [158] having exposed DC-SIGN receptors,
- Presence of ulcers in genital tract [85],
- Presence of IL-8 in the genital tract of females which elevates the rate of HIV replication in T-cells [123],
- Increased rate of removal of infected cervical cells during contraceptive therapy in HIV infected women [194],
- 'Human Leukocyte Antigen – G' associated polymorphism which reduces cellular immunity in women [115],

- Oral wounds and ejaculation in the mouth during sexual intercourse in immunodeficient state [(162)],
- Increased secretion of IL-4 and TGF-β resulting in over expression of CXCR-4 receptors facilitating HIV infection to CD_4 cells [(205)].

CELL ENTRY OF HIV:

CD_4 cells or T_4 cells are key cells which directly regulates 'cellular immunity' and indirectly humoral immunity. Functional aspects of many phagocytic cells like 'Macrophages' and 'Natural killer cells' are regulated by the cytokines secreted by CD_4 cells. Gamma Interferons (γ-IFN) produced by CD_4 Cells are the antiviral elements, while IL-2, 4, 5 and 6 secreted by CD_4 cells are essential for maturation of plasma cells or antibody forming B-lymphocytes (AFB). In all sense a decline in the count of absolute CD_4 cells develops immunodeficiency and invites infections of opportunistic pathogens.

CD_4 cells carry many receptors out of which CD_4 receptors and CXCR-4 receptors are essential for interactions with HIV. 'Phenyl Alanine-43' present at CDR-2 domain of CD_4 is an actual site of HIV-binding on CD_4 receptor [(67, 120)]. The part of gp-120 of HIV which interacts with CD_4 is a folded complex structure carrying two hydrophobic domains from C_2 and C_4 and hydrophilic domains from C_3 and C_4 [(129)]. 'Aspartic acid' residues from C_4 and C_5 domains of gp-120 also play significant role in the binding of HIV [(129)]. Glycosylated gp-120 shows more binding forces than that of non glycosylated during infectivity of HIV [(180)]. After binding of viral gp-120 with CD_4 receptors of T_4 cells, the gp-120 get displaced to expose fusion domain of viral gp-41[(167)]. This distribution is attributed towards conformational change in the gp-120 following enzymatic cleavage in V_3 loop. Chemokine coreceptor CXCR4 present on CD_4 Cells/T_4 cells is essential for infectivity of HIV [(11)]. HIV strain showing affinity to CD_4 cells and following this route of entry, is called as 'X4 phenotype' [(12)]. Macrophage tropic HIV requires additional coreceptor CCR5 along with CD_4 [(18, 111)] and the phenotype of the virus is called as 'R 5' [(12)]. Macrophages also reported for presence of CXCR4 but rarely get infected by 'X4 phenotype' [(174)]. Inability to express CCR5 receptors confirms resistant to 'X4 phenotype' and 'R5 phenotype' [(146)].

Beside the above mechanism entry of HIV within the cell has been reported through cyrstallizable fragment of antibody raised against gp-120 [(62)] and through CR-2

receptor of the complements [150, 186]. This mechanism resembles the antibody mediated phagocytosis of HIV.

OUTLINE OF HIV REPLICATION:

Upon binding to the target cell surface, HIV injects its core. The core carries two strands of ssRNA, structural proteins and enzymes [198]. 'DNA polymerase' converts viral ssRNA to ssDNA. Following this original viral ssRNA is destroyed by 'Ribonuclease'. Later 'DNA polymerase' makes another copy of DNA. The 'Polymerase' and 'Ribonuclease' are collectively called as 'Reverse Transcriptase' [198]. Next enzyme 'Integrase' make integration of viral genome to host cell genome and infection become permanent as far the cell cycle continues. 'Cyclophilin A' (Peptidyl prolyl isomerase) augments HIV replication [59]. For reverse transcription of HIV genome, protein P_7 acts as 'Chaperone Protein' [107]. The molecule increases cDNA transcripts [40] and facilitate packaging of viral RNA [107]. Envelope protein 'Emerin' is essential for integration of viral cDNA into host genome [65]. LEDGE (Growth Factor) and P75 (Co activator) helps the integration of HIV at host genome 'target sites' which remain deficient in GC sequences [20]. Elevated expression of Tat gene augments viral replication [32]. Role of Nef gene as a positive regulator is allele dependent [54]. REV enhances the transportation of unspliced mRNAand enzymes into cytoplasm [177]. Two different models have been proposed for budding of HIV [51]. One is 'Lipid Rafts Model' and another is 'Trojan Exosome Hypothesis'. As per 'Lipid Rafts Model', Gag and Pol precursors remain with host membrane located lipid rafts which are rich in sphingolipids and cholesterol and later HIV buds from such rafts [52].

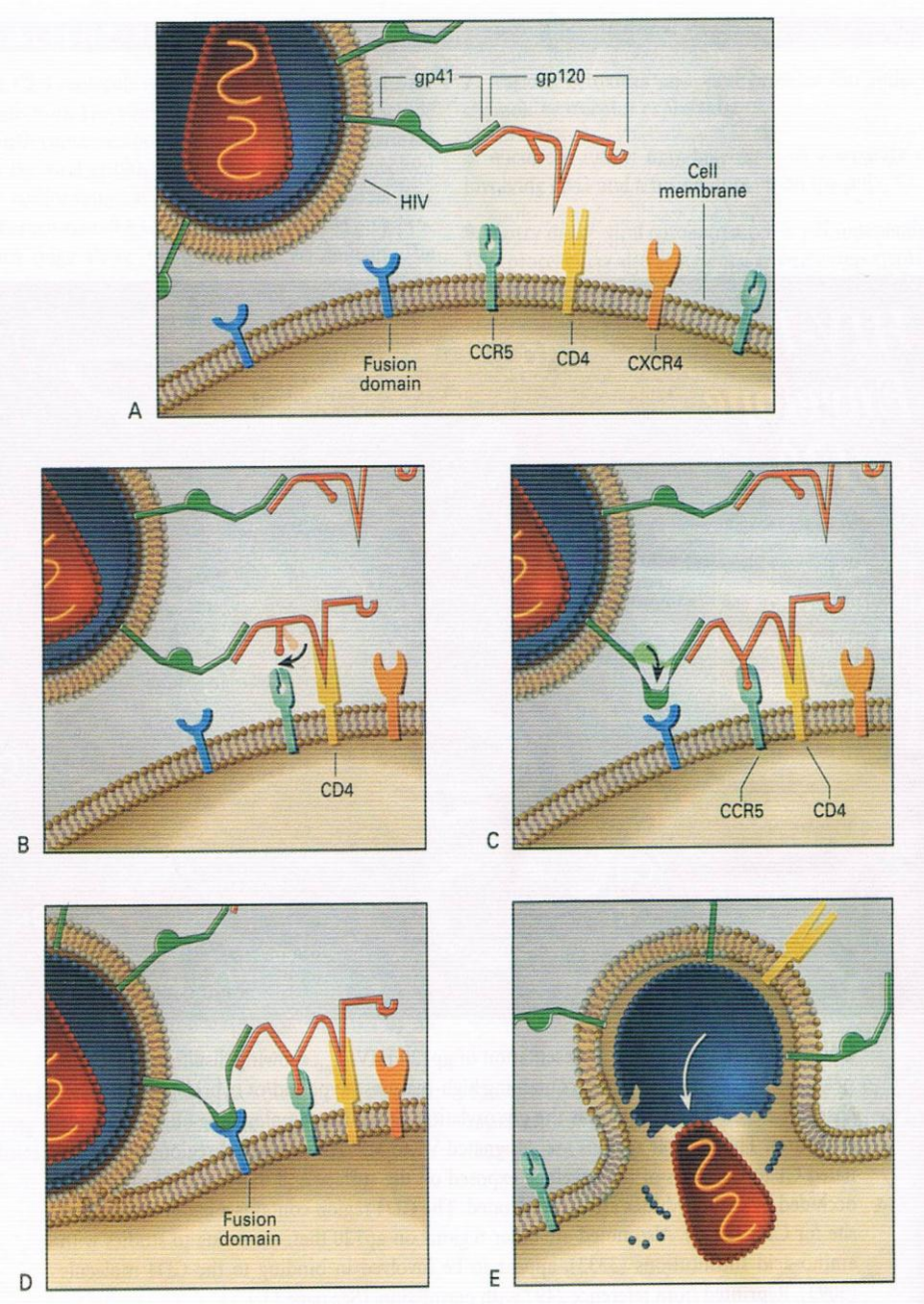


Fig: 2 Interactions of HIV 1 with host cell

Source: Jay A. Levy (2007), "HIV and the Pathogenesis of AIDS", American Society For Microbiology, Washington USA, Third Edition, ISBN: 978-1-55581-393-2

'Trojan Exosome Hypothesis' specially explains the assembly and budding of virus within macrophages [(52)]. According to the hypothesis, assembly of HIV takes place within the multivesicular bodies in the cytoplasm. Later such bodies fuse with cell membrane of the macrophage and release the virus in the form of exosome. Gene product of human host Tsg 101 facilitates the rupture of cell membrane during budding. Product of VPU gene & P2 protein participate in the eventual stage of

assembly [41]. Within T-Lymphocytes, replication cycle gets completed within 24 Hrs. [79], while in macrophages it takes long time of 36 Hrs. to 48 Hrs. [121]

MECHANISM OF DEVELOPMENT OF IMMUNODEFICIENCY BY HIV:

CD_4 cells / T_4 cells regulate various aspects of cellular immunity and humoral immunity. Hence the depletion in the count of absolute CD_4 cells in response to HIV infection is a prime cause of development of immunodeficiency. Depletion in the absolute CD_4 cell count takes place through various mechanisms. It includes apoptosis or programmed cell death of 'absolute CD_4 cells' [46, 116, 101], single cell lysis by necrosis [140] and cell lysis without forming syncytium [61]. Virally infected CD_4 cells and uninfected CD_4 cells fuse together to form multinucleated giant cell called 'syncytium' [104]. Fusion process takes place through surface carbohydrates [56], glycolipids [36], LFA-1 and CD7 [166], Few times upon binding of gp-120 to CD_4 cells, complement derived lysis takes place [181]. HIV infected cells have also reported for change in cell membrane permeability following accumulation of cations to toxic level [21, 110] and this could be one of the reasons for lethal effects of HIV. Virally infected CD_4 cells may express soluble Nef on cellular surfaces and this when comes in contact with uninfected CD_4 cells, induces lethal effect [42]. Besides these, HIV also induces programmed cell death or apoptosis in CD_4 cells. This again takes place through two different and independent mechanisms, one is 'Death receptor mediated apoptosis of CD_4 cells' and another is 'Bcl-2 mediated apoptosis of CD_4 cells'. 'Death receptor mediated apoptosis' takes place through Fas/CD-95 receptor, TNF-α receptor and 'TNF Related Apoptosis Inducing Ligand' (TRAIL) receptors [94]. Interactions of receptors with their complementary ligand activates caspases in a cascade where initially caspases-2, 8, 9 and 10 get involved and later caspases-3, 6 and 7 get involved [93]. Caspase-3 has been reported for host nucleic acid damage resulting into the death of infected CD_4 cells [9]. Granulolysin produced by 'T cytotoxic (Tc) cells' and 'Natural Killer (NK) cells reported for activation of Caspase-3 cascade and eventually results in the apoptosis of CD_4 cells [76]. Effector caspases like caspase-3, 6 and 7 activates deoxyribonuclease inhibitors. Also they block the activity of anti-apoptic proteins like Bcl-2 / Bcl-XL [1,191]. Eventually caspases bring out DNA fragmentation, shrinking of target cell, condensation of chromatin, membrane blebbing and formation of apoptic bodies which later are removed by the cells of reticuloendothelial system [39]. 'Death receptor mediated apoptosis' takes place

through external signals and generally shows activation of caspase-8 followed by activation of effector caspases. HIV protease has been reported to cleave procaspase 8 to form a fragment 'Casp8p41'. This further binds with BAK and induces apoptosis [27]. 'Bcl-2 mediated apoptosis' takes place through 'internal signals' and generally shows activation of caspase-9 followed by activation of effector caspases [39]. As a result of change in membrane potential and permeability during HIV infection, mitochondria releases 'cytochrome-c' (Cyt-c). This Cyt-c combines with APAF-1 (Apoptosis Activator Factor-1) and procaspase-9 to form a structure called 'apoptosome'. Binding of APAF-1 with procaspase-9, leads to dimerization of procaspase-9 by which it become enzymatically active. Procaspase-9 acts on Caspase-9 & further in cascade leads to formation of effector caspases. 'Effector caspases' eventually shows programmed cell death of absolute CD_4 cells. Anergy of 'Tc cells' contribute the progression of the disease. This anergy is attributed towards reduced MHC-I expression of target cells [64, 168] and expression of programmed death protein (PD-1) on 'Tc cells [31, 185]. This eventually leads to non recognition of target cell and autolysis respectively. During HIV infection, death is observed of 'Effector CD_4 cells' and not of the 'Memory CD_4 cells'. In 'Memory CD_4 cells', Casp8p41 binds with anti apoptotic protein Bcl-2. Upon binding, Casp8p41 become enzymatically inactive and fails to show apoptosis [27]. It is the reason for elevated expression of Bcl-2 on 'Memory CD_4 cells' and not on the 'Effector CD_4 cells'. During disease progression, 'CD_4 cells', 'CD_8 cells' and 'B cells' shows over expression of Fas receptors while NK cells, monocytes & macrophages shows over expression of FasL [84]. 'Bystander Effect Hypothesis' states that, "common mean of apoptosis in HIV infection is interaction of uninfected cells with infected lymphoid cells". For example HIV infected macrophages shows over expression of TRAIL (a member of TNF super family) which later induces apoptosis in non-infected CD_4 cells [102]. HIV-1 envelope glycoprotein has significant 'apoptosis inducing potential' and contributes to disease progression [72].

CLINICAL PROGRESSION OF HIV:

Clinical progression of the HIV is monitored through 'CDC Classification system' and 'WHO Clinical staging system.' CDC is the 'US Centre for disease control and prevention'. The system was introduced in 1986 and was further revised in 1993. CDC system monitors the progression of disease on the basis of 'absolute CD_4 count'

and specific disease related conditions. When the 'absolute CD_4 count' falls below 200/µl with persistent secondary infections, the condition is described as AIDS. The system is widely used for the study of epidemiology of AIDS. Also it is used as a reference tool for clinical management of HIV infected individuals. On other side W.H.O. clinical staging system do not take account of CD_4 cells and is based on resource constrained setting.

Absolute CD_4 Count	**Clinical Category**		
	A **No symptoms but persistent lymphadenopathy**	**B** **Symptoms Excluding A & C**	**C** **AIDS indicator conditions**
> 500 cells / µl	A1	B1	C1
200- 499 cells / µl	A2	B2	C2
< 200 cell/ µl	A3	B3	C3

Table-5: CDC classification system: (Source: www.cdc.gov)

Category B should meet at least one of the following criteria:

- They should be related to HIV infection as justified by defect in cellular immunity.
- Involvement of HIV in the clinical course or management must be there. For example Herpes Type-1 infection can be observed in HIV infected as well as non infected individuals. But when it appears as a co infection with HIV resulting in multiple episodes, then only can be categorized as B [155, 152].

CATEGORY 'C' INFECTIONS:

Category C infections are secondary infections or complications caused due to HIV induced immunocomprised state. Complications include 'wasting syndrome' in which involuntary weight loss of > 10% baseline body weight is observed. No single reason can be correlated with 'wasting syndrome' and it may be due to impairment of carbohydrate metabolism, calorie loss or elevated rate of calorie use [134]. Many patients during ART therapy have reported for anorexia. Few are showing drug induced diarrhoea or mal absorption. TNF-α and IL-6 also contributes the 'wasting syndrome' as these cytokines have reported for increased rate of catabolism. Fat and

vitamin B_{12} deficiency is common in HIV patients and it is related to mal absorption or may be due to loss of intrinsic factors / receptors or enzymatic deficiencies. Microbial infection leads to electrolyte loss during diarrhoea and one of the reasons for development of 'wasting syndrome'. Uncontrolled candidiasis or oral herpetic lesions leads to dysphagia and contributes the 'Wasting Syndrome'. Other metabolic factors include lactose intolerance and protein loss. Overall picture is involuntary weight loss of > 10% baseline body weight.

Fever persisting for more than one month is another symptom and this is attributed to secondary infections, release of microbial toxins or products acting as endogenous pyrogen. The fever development is also related to increased secretion of proinflammatory cytokines like IL-1 and TNF-α.

Gastrointestinal complications in HIV patients includes difficulty in swallowing (dysphagia), painful swallowing (odynophagia). And these conditions are mostly observed due to oesophagal ulcers. *Candida spp*. develops white curdy patches. 'Herpes simplex' and 'Herpes zoster virus' generates small multiple patches resembling spray sprinkles, 'Cytomegalovirus' develops large sized ulcers. Ulcers due to *Mycobacterium* are also reported. Cause of diarrhoea includes protozoal infections, viral infections, bacterial infections, fungal infections and neoplasms **(95)**. Many HIV patients are showing frequent bowel movements with unknown or unidentified reasons. Microbial colitis is also recognized as 'Gay Bowel Syndrome'.

Neurological complications includes mononucleosis, encephalopathy, myelopathy, angitis, 'Guillain-Barre Syndrome', mononeuritis multiplex, late sensory neuropathy, progressive multifocal leukoencephalopathy, lymphoma, cranial neuropathy, anxiety, psychoses, dementia, vacuolar myelopathy, predominantly sensory neuropathy, headache due to space occupying lesions, meningitis, metabolic encephalopathy, development of focal cerebral lesions, spinal lymphoma, spinal abscess, CNS toxoplasmosis etc. **(86)**.

W.H.O. CLINICAL STAGING CLASSIFICATION SYSTEM:

It was introduced in the year 1990 and was revised in 2007. It decides the eligibility for ART where flow cytometric analysis of CD_4 count is not available. The Classification system is divided into 4 stages indicating gradual progression of the disease from initial HIV infection to AIDS.

Clinical Stage	Symptoms
Initial HIV infection	-Asymptomatic -Acute Retroviral Syndrome
Clinical Stage 1	-Persistent Generalized Lymphadenopathy (PGL)
Clinical Stage 2	-Weight Loss (≥ 10% of body weight) -Relapsing respiratory infections including tonsillitis, otitis media, sinusitis, pharyngitis) -Herpes virus infections -Cheilitis (Angular) -Recurrent ulcers in mouth -Maculopapular lesions -Seborrheic inflammation of skin -Fungal infections of nail
Clinical Stage 3	-Chronic diarrhoea which last for 1 month or more and which is not IBS -Unexplained pyrexia which persist for > 1 month -Persistent oral thrush -Hairy leukoplakia in mouth -Secondary bacterial infections -Tuberculosis (Pulmonary) -Ulcerative stomatitis -Unexplained anaemia -Neutropenia (< 500 cells/µl) -Thrombocytopenia (< 50000 platelets / µl)
Clinical Stage 4	-Wasting Syndrome -*Pneumocystis carinii* induced pneumonia -Herpes simplex infections which generally last for more than one month -Candidiasis -Tuberculosis -Cytomegalovirus infections -Kaposi's sarcoma -*Cryptococcal* meningitis -Secondary but recurrent chronic infections -Lymphoma

Table-6: W.H.O. clinical staging of HIV / AIDS

COMMAN SECONDARY INFECTIONS IN HIV INFECTED INDIVIDUALS:

HIV infected individuals always shows prevalence of bacterial infections. *Mycobacterium avium, Mycobacterium tuberculosis, Salmonella, Listeria* are commonly observed with deficiency or dysfunction of 'T lymphocytes'. While the common organisms associated with 'B lymphocyte' dysfunction are *Streptococcus pneumonia*e, *Haemophilus influenzae*, *Branhamella catarrhalis, Campylobacter jejuni, Shigella*. Mycobacterial infections which are commonly observed in HIV patients are *Mycobacterium tuberculosis, Mycobacterium avium-intracellulare, Mycobacterium kansasii*. *Mycobacteria* are reported for ulceration of gastroduodenum. *Mycobacterium kansasii* causes pulmonary and mandibular infections in HIV patients. *Mycobacterium gordonae* a non pathogen become opportunistic pathogen in HIV infected people. *Mycobacterium kansasii* and *Mycobacterium avium* develop general weakness in AIDS patients. *Mycobacterium xenopi* reported for respiratory infections in Canada. *Mycobacterium fortuitum* also detected in sputum sample of HIV infected individuals.

Non typhoidal *Salmonella* infections are common in AIDS patients. *Salmonella enteritidis, Salmonella dublin* and *Salmonella copenhagen* have been reported for subdural cerebral empyema. *Salmonella typhimurium* develop pyelonephritis. *Salmonella enteritidis* is established for cystitis, lung abscess, empyema, cutaneous abscess and pyomyositis. *Salmonella cholera-suis* causes meningitis. *Salmonella enteritidis* also develop lymphangioma. 'Gay Bowel syndrome' (diarrhea) in HIV infected individuals reported *Shigella* as one of the etiological agents.

Staphylococcus aureus is the third most common etiological agent for respiratory infections in HIV infected individuals. *Staphylococcus aureus* develop carbuncles, furuncles, tonsillitis, otitis media and sepsis with recurrent infections. *Staphylococcus cohnii* causes primary septic arthritis, *Staphylococcus xylosus* forms pancreatic pseudocyst. *Staphylococci* were also reported for malacoplakia. *Staphylococcus epidermidis* has been isolated from 'pneumatosis intestinalis' the condition distinguished by multiple gas filled cysts in wall of gastrointestinal tract. *Staphylococcus* specifically methicillin resistant *Staphylocci* develop nosocomial infections. *Pseudomonas aeruginosa* is one more common pathogen in HIV infected patients. It causes pyogenic pneumonia, urinary tract infections and blastomycosis like pyoderma in AIDS patients. *Trichomonas vaginalis* develop pelvic inflammatory disease in HIV infected women. *Xanthomonas maltophilia*, a gram negative bacterium

is a MDR organism reported for nosocomial infections in HIV patients. About 80% of strains of the organism are resistant towards Ampicillin, Mezlocillin, Aztreonam and Imipenem. *Klebsiella pneumoniae* causes pneumonia, neutopenia and malacoplakia in majority of the HIV infected persons. *Enterobacter cloacae* develop oral mucositis. *Clostridium difficile* causes diarrhoea and documented as MDR organism.

Campylobacter jejuni, Campylobacter coli and Campylobacter lari develop bloody diarrhoea, nausea and fever in HIV infected persons. Keeping close interactions with cats increases risk of *Bartonella* infection in HIV infected persons. *Bartonella quintana* and *Bartonella henselae* causes 'Bacillary angiomatosis' a vascular proliferative lesion with intra abdominal mass, lymphadenopathy and acute psychiatric symptoms. *Bartonella henselae* also reported for bacillary neuroretinitis, endocarditis and 'cat scratch diseases' (CSD). *Helicobacter pylori* cause gastroduodenal ulcers. *Prevotella bivia* develop blastomycosis like pyoderma. HIV infected pet owners requires special care and concern as animal's excreta remain rich in *Cryptosporidium, Salmonella* and *Campylobacter*.

Streptococcus pneumoniae is a common opportunistic pathogen in HIV infected patients causing upper respiratory tract infections, lung disorders. It is characterized by bilateral patchy lower lobe infiltrates with fever, cough and dyspnoea. *Streptococci* have been isolated from malacoplakia.

Other common bacteria are *E. coli*, *Coxiella burnetti*, *Rhodococcus equi*, *Enterococcus faecium*, *Proteus* species which frequently occurs in HIV infected individuals and reported for various complications.

Hymenolepis nana and *Strogyloides stercoralis* develop diarrhoea in HIV infected persons.

Demodex folliculorum and Demodex bravis lives in human pilosebaceous duct and sebaceous gland respectively with predilection to colonize facial part. It causes pruritus, erythema, papules, pustules, nodules or scales on face, neck, upper thorax in HIV infected patients.

Co infection of *Trypanosoma cruzi* is also reported. HIV seropositive women are more susceptible to *Candidiasis* than that of HIV seronegative women. *Candida albicans* causes mucocutaneous oral or oesophageal candidiasis with large yellow white plaques. It also causes enlargement of cystic parotid gland. Rarely it develop 'yellow nail syndrome'. Majority of HIV infected patients show microbial resistance towards the drug flucanazole. 'Flucanazole resistant *Candida*' isolated from the

patients include *Candida inconspicua, Candida parapsilosis, Candida tropicalis, Candida kruseii* and *Candida glabrata*. Candidemia is a lethal nosocomial complication. Candidal vulvovaginitis and meningitis have been frequently reported in the patients.

Penicillium marneffei is a significant opportunistic fungal pathogen reported from 'South East Asia'. It causes fatal systemic disease with the symptoms fever, emaciation, skin lesions and haemolysis. It is reported for high mortality rate.

Cryptococcus neoformans, encapsulated yeast is a common cause of meningitis and disseminated disease in AIDS patients. Pigeon droppings act as a common source of *Cryptococcus*.

Pneumocystis carinii is a fungus causing pneumonia in AIDS patients and HIV infected children (age group 2-24 months). The *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis* and *Cryptosporidium parvum* reported for diarrhoea. *Toxoplasma gondii* causes encephalitis, retinochorditis or lymphadenoapthy. *Isospora belli* reported for diarrhea and *Histoplasma capsulatum* for 'Fibro cavitatory pneumonia'.

Co infection with Cytomegalovirus is a prime cause of mortality in eventual stages of AIDS. The virus causes retinitis, encephalitis, ulcerative oesophagitis and polyradiculitis. Other common viral isolates from HIV infected individuals include Hepatitis-C virus, Hepatitis-B virus, Epstein-Barr virus (lymphoma and other lymphoproliferative disorders, Herpes Simplex virus (either HSV-1, HSV-2 or both), Human Herpes virus-8 (associated with Kaposi's sarcoma), Varicella zoster and BK virus (haemorrhagic cystitis).

LYMPHOCYTE RESPONSE TO HIV INFECTION DURING CLINICAL PROGRESSION:

As the lymphocytes are prime target of the virus, their response decides the rate of the disease progression. In the due course of the disease, 'Absolute CD_4 cell' count declines at the rate of 25 to 60 cells / µl / year [(91)]. In clinical stage 3, CD_4 cell count declines below 350 cells / µl [(90)]. AIDS which is the eventual phase of the disease characterized by CD_4 cells as a prime source of the HIV and not the macrophages. In this phase 'absolute CD_4 cells' shows higher rate of expression of CCR5 receptors [(131)]. Hyperactive cytotoxic T lymphocytes augment the disease progression [(117)]. Cytotoxic T lymphocytes interferes the cellular immunity by destroying 'Antigen

Presenting Cells (APCs)' [99]. Cytotoxic T lymphocytes may destroy 'Antibody Forming B lymphocytes (AFBs)' and thus may interfere the humoral immunity by ceasing the production neutralizing antibodies against HIV [184]. During HIV infection loss of memory cells has been observed which seriously impair the 'secondary immune response' against most of the virulent microorganisms. T cytotoxic cells or CD_8 cells which shows anti-HIV activity are '$CD_8^+ CD_{28}^-$ cells', while those do not show anti-HIV activity are CD_8^+, CD_{28}^+ cells [13, 90]. Expression of CD-57 markers and CCR-7 markers on T_c cells with concurrent IL-2 and gamma interferon production is taken as a criterion for monitoring T_c activity during HIV infection [133]. 'T cytotoxic cells' produce perforin (a protein). Perforin along with granzymes kills HIV infected cells. Tc cells also produces MIP 1-α, MIP 1-β and chemokine 'RANTES'. These cytokines of T_c cells binds with CCR-5 receptors of HIV prone cells and prevents subsequent binding of HIV [22]. T regulatory cells (T_r cells) express 'Fox P3' markers (Fork head transcription factor P3), GITR receptors (Glucocorticoid induced TNF family related receptor), and CTLA-4 [170]. T_r cells have been reported for production of TGF-β and IL-10 [164]. TGF-β converts CD_4^+ cells into regulatory cells and during clinical progression of the disease show suppression of HIV specific activity [196]. IL-2 produced by 'absolute CD_4 cells' (T_H cells) is accepted by 'Tc cells', which thereafter maintains the sufficient production of 'CD_8^+ cell antiviral factor' (CAF) and prolongs the disease progression.

VACCINES FOR HIV

Vaccine trials against HIV infection were aimed towards induction of cellular / humoral or mucosal immunity. Following problems have encountered during vaccine development program.

- Competence of animal models,
- Ability of HIV to get integrated into cellular genome,
- Release of progeny viruses from infected cells,
- Differential features of different quasi species of HIV,
- Unpredictable MHC / HLA dependent cellular immune responses to vaccines.

Broad categories of HIV vaccines includes,

- Whole killed virus vaccines,
- Live attenuated vaccines,

- Subunit vaccines,
- Vector vaccines,
- Pseudo virion vaccines,
- Sequence derived peptide vaccines and
- Plant derived protein vaccines.

Inactivated 'Simian Immunodeficiency Virus' (SIV) was used as 'whole killed vaccine' but reported for limited success [179, 35]. Most of the clinical trials with 'whole killed vaccines' have reported cellular toxicity with limited antiviral effects [26, 92]. New inactivating agents include psoralen, aldrithiol-2 while other conventional agents also were used. Nef deleted SIV was used as 'live attenuated virus' and reported for development of anti-HIV antibodies [29]. Using pathogenic virus along with live attenuated SIV failed to develop protective immunity [105]. Use of original or recombinant 'Env proteins', 'Tat proteins', 'Recombinant proteins' expressed by different viruses /bacteria, 'Virus Like Particles (VLPs) resembling core proteins of HIV' as vaccines are under trial. Peptides of HIV-1 including Gag, CpG have been used for induction of mucosal immunity. Human clinical trials with gp-160, gp-120, recombinant 'HIV1-SF2 -gp-160', 'HIV1-MN-Env like proteins', 'Clade B HIV gp-120 recombinant adenovirus 5 vector vaccines' have failed to established either safety parameters or efficacy.

BIOLOGICAL IMMUNOMODULATORS:

Immune system has two prime elements such as 'cellular immunity' and 'humoral immunity' [14,2,188,30,122,138]. The 'cellular immunity' is multifaceted system comprised of phagocytic cells like Eosinophils, Neutrophils, Monocytes, Macrophages, Natural killer cells, Killer cells, 'Lymphokine Activated Killer' cells and the Lymphocytes (T and B Cells) with their cytokines. Humoral immunity is antibody mediated immunity in which 'primary immune response' is regulated by IgM and 'secondary immune response' by IgG, Mucosal immunity is regulated by IgA, process of self and non self discrimination by IgD and IgE participates in various allergic reactions. Complements regulate various significant aspects of the immune response towards non self antigens through 'Classical Pathway' and 'Alternate Pathway'.

Immunomodulators can be defined as biological or synthetic compound in pure or crude form which keeps potential to enhance, suppress or modulate either of the components of immune system [3].

TYPES OF IMMUNOMODULATORS:

'Immunostimulant', 'Immunosuppressant' and 'Adjuvants' are the three broad categories of immunomodulators. The diseases like cancer, AIDS are characterized by dysfunction or deficiency of one or several components of immune system. This deficiency or dysfunction can be restored by the use of immune enhancing compounds called 'Immunostimulant'. During organ transplants and autoimmune diseases hyperactivity of immune system required to be regulated and compounds used to suppress the immune response are called as 'Immunosuppressant'. 'Adjuvants' are the compounds used along with antigenic preparations like vaccines to increase the degree of immunogenicity. Plants are the precious source of such immunomodulators which can be effectively used for the control of diseases or disorders [55, 74].

India has an antecedent of using plant based medicines [125]. Many research scholars gave experimental evidences for immunomodulating features of plant derived substances. *Allium sativum* has been reported for NK cell activation, T cell activation [66, 43] and augmentation of IL-2 response [182]. *Aloe vera* serves as biological immunomodulator by enhancing IL-1 production, TNF-α activation and tumor suppression [19]. *Azadirachta indica* reported for activation of macrophages, phagocytosis, expression of MHC-II, activation of primary and secondary immune response [3, 190]. *Curcuma longa, Oscimum sanctum, Caesalpinia bonducella, Ephorbia tirucalli, Withania somnifera, Tinospora cordifolia, Panax ginsengs, Capparis zeylanica, Rhododendron spiciferum, Arnica montena, Nelumbo nucifera, Asparagus racemosus, Boerhaavia diffusa, Celendula officinalis, Echinacea purpurea, Luffa cylindrical* have been reported for immunomodulating properties [176]. *Euphorbia hirta L.* was reported for stimulation of phagocytosis [73]. *Cirsium bracteosum* has been reported for apoptosis in human lymphocytes. *Tinospora cordifolia* has been reported for various immunomodulating activities including control of microbial infection [183], obstructive jaundice [149], minimizing stress [136]. Use of *Emblica officinalis* was reported in recovery of pancreatitis in experimental animals [60]. Various Chinese herbs including *Captis chinensis (Franch.), Codonopsis Pilosula (Franch.), Lonicera japonica Thunb., Dioscorea opposita Thunb., Isatis tinctoria L., Smilax glabra Roxb., Chrysanthemum morifollium* have documental as immunomodulatory plants [193]. Plant metabolites have been studied in details for their chemical properties and potential chemotherapeutic properties. Still many plants are

left to get explored for their potential antiviral and immunomodulating properties. The branch of 'Allopathy' still insists on the use of active & known chemical agent for the treatment of diseases or disorders. Most of the times the plant kingdom mimics the modern experimental tools like F.T.I.R., which simply notes unknown peaks or overlapping peaks during isolation. Such peaks are often neglected as those do not match with the peaks of standard and known chemical compounds. Also the size of particle matters a lot as on reduction of particle size, better chemotherapeutic properties are observed of plant derived products. Conventional extraction methods using chemicals like ethanol often changes the properties of active chemical agents in plants which few times results in the loss of activity. All these observations now give the inkling of potential synergistic activities of plant metabolites which can prolong the progression of HIV infection to AIDS. Many plants have studied for their potential anti-HIV properties. Those include *Ancistrocladus abbreviatus* [(112)], *Anogeissus acuminate* [(7)], *Ancistrocladus korupensis* [(15)], *Areca catechu* [(87)] *Arnebia euchronia* [(75)], *Annona squamosa* [(201)], *Aspalanthus lineraris* [(38)], *Astragalus membranaceus* [(171,195)], *Aqularia agallocha* [(195)], *Angelica sinensis* [(195)], *Atractylodes macrocephala* [(195)], *Amomum villosum* [(195)], *Brucea javancia* [(128)], *Allium sativum* [(5, 173)], *Artemisia afra* [(108)], *Artemisia annua* [(108)], *Aspilia pluriseta* [(24)], *Albizia amara* [(113)], *Azadirachta indica* [(163)], *Argemone mexicana* [(17)], *Buchnavia capitata* [(69)], *Bersama abyssinica* [(8)], *C. teysmanii* [(25)], *Camellia sinensis* [(57)], *Calophyllus spp.* [(38)], *Crinum macowani* [(83)], *Carica papaya* [(147)], *Cleome gynandra* [(119)], *Combretum hartmannianum* [(80)], *Cucurbita maxima* [(28)], *Croton lechleri* [(106)], *Citrus limon* [(89)], *Dodonaea viscosa* [(8)], *Stepherdia argentea* [(203)]. Inhibition of binding sites of HIV-1 by *Pelargonium sidoides* has raised therapeutic potential of plants [(114)]. *Copparis tomentosa, Syzygium guineense, Lannea stuhlmannii, Oncoba spinosa, Combretum collinum, Euclea divinorum* are commonly used in the treatment of HIV related complications in Livingstone, Zambia [(77)]. Tethrin / BST-2 is considered as immunomodulator which increases cell mediated immune response in HIV infected individuals [(100)].

COUNTING OF ABSOLUTE CD_4 CELLS BY 'FLOW-CYTOMETRY':

Absolute CD_4 cells are the key cells which regulates various vital aspects of immune system. As these cells are the target for HIV; their periodical counting becomes essential to check disease progression and also the response of the patient towards

antiretroviral therapy. 'Flow Cytometry' analyses various cytological and morphological parameters of cells including T lymphocytes and thus gives precise and less error prone qualitative and quantitative information of the T-lymphocytes. Basic components of 'Flow Cytometer' include light source, flow cell (flow chamber), optics, light detectors, electronics and computer. Flow chamber is designed to pass the biological cells in single file at the particular point of measurement. Biological sample reacted with fluorescent monoclonal antibodies or absorption dyes is mixed with physiological solution and injected under controlled pressure through a centre of stream of water or buffer (Sheath Fluid). Sheath fluid confines the biological cells to be studied to the core, where those get exposed to laser beam. Delivery rate of flow chamber often remains around 1000 cells / sec. Laser or arc lamp can be used as a light source. The arc lamps require optical filters for choosing appropriate wavelength, hence are not popular. 'Air cooled argon ion laser' (λ- 488 nm), 'Helium-Neon air cooled laser' (λ -633 nm) are preferred for 'Flow Cytometry'. Solid state laser producing power of 10 to 25 mW are least expensive but error prone while diode lasers or water cooled lasers produces higher power (> 200 mW) are highly expensive but gives errorless information during cell sorting. A specific arrangement of optics is required for 'Flow Cytometry'. Dichromic mirrors or beam splitters are used to choose different wavelengths of light. Dichromic mirror no. 1, 2 and 3 selects the wavelengths of <500 nm, < 540 nm and <590 nm respectively. Every separate dichromic mirror is assisted with separate photomultiplier tube. Additional photomultiplier tube only collects the wavelength of > 600 nm and is called as 'red sensitive photomultiplier tube'. 'Flow Cytometer' is set for desired cellular parameters and these parameters are called as 'threshold discriminators'. Forward scatter mirror is situated in a prime part of laser beam and its intensity is a measure of only cellular size and not the refractive index. Side scatter detecting lenses collects refracted and reflected light rays. These rays are proportional to refractive index that is granularity of a biological cell. It is detected at 90^0 to incident light beam. Photomultiplier tubes receive photons and generate electric signals. These signals are processed by a data processor. 'Analog to Digital Converter (ADC)', which facilitates the plotting of events on graphical scale. 'Forward Scatter' and 'Side Scatter' cellular signals are interpreted using 'linear amplification', while cellular fluorescence is interpreted as 'logarithmic amplification'. Flurochromes which are used for labeling protein for exposure to 'argon ion laser' includes 'fluorescein isothiocyanate' (FITC)

whose isothiocyanate group binds with NH_2 group of lysine within the target protein, Phycoerythrin Texas Red (PE-TEXAS Red), PE-Cyanine -5 (PC5), PE-Cyanine 7 (PE7), Peridinin chlorophyll (Per CP), Alexa 488. When exposure is to 'He-Ne laser; 'Allophycocyanin (APC-Cyanine 5.5 or 7) is used. Flurochromes used for staining nucleic acid includes acridine orange (AO), Ethidium bromide (EB), Thiazole orange (TO), Propidium iodide (PI), Vibrant orange etc. Two different methods are in use for measurement of 'absolute CD_4 cells'.

1. DUAL PLATFORM METHOD:

Here 'absolute CD_4 count' is calculated by using a formula,

Absolute CD_4 T cell count = Percentage CD_4 T cell count x Absolute lymphocyte count

Using appropriate fluorescent labeled monoclonal antibodies through forward scatter (FSC) and side scatter (SSC); percentage of CD_4^+ T lymphocytes is obtained. Total lymphocyte count obtained by blood cell analyzer also can be used for the formula. Better and errorless count can be obtained by using fluorescent monoclonal antibodies against CD_{45} and CD_{14}. All CD_{45} bright positive cells with CD_{14} negative gives 'absolute lymphocyte count'.

2. SINGLE PLATFORM METHOD:

This method does not need blood cell analyzer and instead a finite number of fluorescent microbeads are mixed with finite volume of CD_4^+ blood sample. The method is handy and requires two reagent tubes. One tube uses 'anti human CD_3 monoclonal antibody' tagged with 'Phycoerythrin Cyanine-5' along with 'antihuman CD_4 monoclonal antibody' tagged with 'Phycoerythrin'. Known number of fluorescent labeled reference beads are added in the same tube. Another reagent tube uses monoclonal fluorescent labeled monoclonal antibodies to CD_8^+ T cells ('CD_8^+-Phycoerythrin Cyanine 5' and 'CD_3^+ Phycoerythrin') along with reference beads. Advantage of using two different tubes is that of getting separate count of CD_4^+ T cells and CD_8^+ T cells. This count can be summed to get absolute no. of total T lymphocytes and also to express the ratio of CD_4 cells to CD_8 cells which has prognostic value during antiretroviral treatment. The system is supported by built in software program. The method requires minimum laboratory skills [63, 71].

MATERIALS

AND

METHODS

MATERIALS AND METHODS

1. Source:

i) *Chlorophytum borivilianum, Withania somnifera* were collected from Pavan Agro Farm, Pune, *Wagatia spicata Dalz.* was collected from hilly regions of Malakapur (Dist. Kolhapur), *Picrorrhiza kurroa* was collected from licenced ayurvedic supplier and *Spilanthes paniculata Wall. ex. DC.* was collected from hilly regions of Mulashi (Pune). All plant derivatives were authenticated by 'Botanical Survey of India'. These plant derivatives form one of the sources for the study.

ii) Albino mice were made available by 'National Toxicology Center, Pune'.

iii) HIV positive patients who volutarially participated in the study.

iv) Clinical trials conducted under the supervision of registered medical physician as approved by 'Institutional Ethics Committee, Bharati Vidyapeeth Deemed University, Pune' (BVDU/YM/Ethics/2012-13/452 Dt. 30/10/2012).

2. Sample Collection:

Blood samples were collected by following W.H.O. norms. Written consent of respective HIV infected individuals was obtained.

2.1 Identification of Potential Biological Immunomodulators:

All plant derivatives were identified using standard identification keys and authenticated by 'Botanical Survey of India.'

2.1.1 *Chlorophytum borivilianum* (Vernacular Name: Safed Musli)

Family- *Agavaceae*

Genus- *Chlorophytum*

Species- *borivilianum*

Medicinal part- *Root*

2.1.2 *Withania somnifera* (Vernacular Name: Ashwagandha)

Family- *Solanaceae*

Genus- *Withania*

Species- *somnifera*

Medicinal part- Root

2.1.3 *Wagatia spicata Dalz. {Caesalpinia spp.}* (Vernacular Name: Wakeri)

Family- *Leguminoceae*

Subfamily- *Caesalpinaceae*

Genus- *Wagatia*

Species- *spicata*

Medicinal part- Stem

2.1.4 *Picrorrhiza kurroa* (Vernacular Name: Kutki)

Family- *Scrofulariaceae*

Genus- *Picrorrhiza*

Species- *kurroa*

Medicinal part- Root

2.1.5 *Spilanthus paniculata Wall. ex. DC.* (Vernacular Name: Akkarkara)

Family- *Asteraceae*

Genus- *Spilanthus*

Species- *paniculata*

Medicinal part- Root

2.2 Heavy Metal Analysis of selected immunomodulators *Chlorophytum borivilianum* (Root), *Withania somnifera* (Root), *Wagatia spicata Dalz* (Stem), *Picrorrhiza kurroa* (Root), *Spilanthus paniculata Wall. ex. DC.* (Root):

2.2.1 Determination of Lead and Cadmium by 'Graphite furnace atomic absorption spectroscopy':

Principle: Finely ground powders of biological immunomodulators were dried and ashed at 500º C with slow increase in the temperature at the rate of 50º C / hour and in that 6 N HCl was added. The solution was allowed to evaporate and 0.1 N HNO_3 was added in the residual sample. The contents were subjected to 'graphite furnace atomic absorption spectroscopy'.

Method:

- 25 gm of dried powder of each biological immunomodulator was separately collected in a clean silica dish. In this 25 ml of 20% H_2SO_4 was added and mixed with glass rod. Adhered sample to the glass rod was removed by small quantity of distilled water and the contents were dried at 110º C in oven.

- Silica dish was transferred to furnace set at 250º C and it was gradually increased to 500º C. The samples were ashed for 6 to 8 hours. Samples were removed and cooled. Clean white / brownish red carbon free ash was ensured.
- In the ash 1 ml of HNO_3 and 10 ml of water was added.
- Contents were dissolved in hot plate.
- Contents were transferred to 50 ml capacity volumetric flask. Residual from dish was removed by heating the dish with 10 ml of HCl and transferring the solution to same volumetric flask. The contents were diluted to 50 ml with distilled water.
- Similarly blank was prepared using same reagents and procedure.
- Instrument was set to the optimum conditions as per the instructions given in the manual.
- Absorption values of sample and blank were recorded.
- Lead and Cadmium contents were calculated from respective standard curves.
- Lead requires 217 nm with 'air-acetylene mixture' while Cadmium requires 228.8 nm with 'air-acetylene mixture'.

2.2.2 Estimation of Mercury by 'Flameless Atomic Absorption Spectroscopy':

Method:

- Biological immunomodulators were digested using 'microwave method'. 5 gm of each sample was separately mixed with 25 ml of 18 N H_2SO_4. It was further mixed with 20 ml of 7 N HNO_3 and finally 1 ml of 2 % of sodium molybdate solution was added.
- Condenser was connected and heat treatment was given for one hour followed by 15 min of cooling period.
- 20 ml of the mixture of HNO_3 and H_2ClO_4 was added through condenser, taking precaution of preventing water circulation through condenser.
- The contents were boiled for 10 min and cooled.
- 10 ml of distilled water was added through condenser and again the contents were boiled for 10 min.
- The condenser was rinsed with 15 ml distilled water and the procedure was repeated for two times. The contents were collected in volumetric flask and were diluted to 100 ml distilled water.
- 25 ml of this digest solution was transferred to another flask and was diluted to 100 ml with diluting solution.

- Output pump was adjusted to 2 lit. Air/min.
- Apparatus was mounted and 20 ml of reducing solution was added to diluted sample.
- Gas inlet adaptor was connected and the sample was aerated for 3 minutes.
- Absorbance was recorded.
- Standard curve was plotted using 0, 0.2, 0.5, 1.0, 1.5, 2.0 µgm of Hg. Same procedure is followed as for samples.
- From standard curve, the quantity of Hg was estimated.

2.2.3 Estimation of Arsenic by 'Colorimetric molybdenum-blue method':

Method:

- 35 gm of each biological immunomodulator was separately collected in Kjeldahl flask and in that 10 ml of distilled water, 25 ml of HNO_3, and 20 ml of H_2SO_4 was added. The mixture was digested using standard protocol.
- 20 ml of digested sample and 20 ml of blank digested solution were added to 'generated bottles'.
- In every 'generated bottle', 10 ml of water, 5 ml of dil. HCl, 5 ml of potassium iodide solution and 4 drops of $SnCl_2$ solution were added. The contents were kept for 15 min.
- Using 4 gm of sand over 'glass wool wad' in sulphide absorption tube, 3 ml of sodium hypobromide solution was added.
- Apparatus was assembled and 4 gm of Zn was added.
- Generator bottles were attached and allowed to react for 30 min.
- 25 ml of contents from each bottle was separately collected in volumetric flask and in that 0.5 ml of 'Ammonium molybdate - Sulphuric acid' mixture solution was added.
- Finally 1 ml of hydrazane sulphate solution was added.
- The sample was kept for1.5 hours and absorption was recorded at 845 nm.
- Standard curve was plotted using 0.1, 1.0, 2.0, 3.0, 5.0, 6.0 and 10 ml of standard solution of 2 µgm AS_2O_3 / ml
- Absorption of the test was compared with the standard curve and arsenic content was estimated.

3.1 Study of Antibacterial, Antifungal and Antiviral Activity of the selected biological immunomodulators:

Antibacterial and antifungal activity of selected biological immunomodulators was tested by using 'Agar Cup Diffusion Method'.

3.1.1 Study of Antibacterial activity:

Test Microorganisms Used:

Bacillus marscescens NCIM 5041 and *Klebsiella pneumoniae NCIM 2719* were selected for the study. *Bacillus marscescens* has been reported for septicaemia, cellulitis and pneumonia in HIV infected individuals. *Klebsiella pneumoniae* is established etiological agent of pneumonia in immunocomprised individuals. The cultures were collected from 'National Collection of Industrial Microorganisms (NCIM)', Pune.

Materials:

Chlorophytum borivilianum (Root), *Withania somnifera* (Root), *Wagatia spicata Dalz.* (Stem), *Picrorrhiza kurroa* (Root), *Spilanthes paniculata Wall. ex. Dc.* (Root) were selected for the study. Respective parts of biological immunomodulators were selected on the basis of prior search indicating medicinal properties in Ayurveda [125].

Extraction of selected biological immunomodulators:

i) Aqueous extracts were obtained of each biological immunomodulator separately using Soxhlet extraction.
ii) 33 gm of each biological immunomodulator was soaked in 'Dimethyl Sulphoxide (DMSO)'. Soaking was done at room temperature for 3 days in sterile bottles.

Method:

- Saline suspension was prepared of ***Bacillus marscescens NCIM 5041*** and ***Klebsiella pneumoniae NCIM 2719*** in the concentration of 10^6 /ml. Sterility of the saline was ensured by transferring it on sterile nutrient agar petriplates and subsequent incubation.
- For the method, HiMedia 'Muller Hinton Agar Medium' was used. Sterility of the 'Muller Hinton Agar Medium' was ensured by incubating Muller Hinton Agar Medium petriplates at 37º C for 48 hours. One ml of suspension of each test microorganism was aseptically transferred to 20 ml of autoclaved 'Muller Hinton Agar Medium' and the medium was poured in sterile petriplate. It was allowed to

solidify. Separate sets were prepared for *Bacillus marscescens NCIM 5041* and *Klebsiella pneumoniae NCIM 2719* as a test organism.

- With the help of 6 mm sterile 'gel puncher', microwells were developed on every 'Muller Hinton Agar Medium' petriplate.
- Aqueous and DMSO extracts of biological immunomodulators were adjusted at neutral pH and aseptically filled in microwells separately for both the test organisms.
- Plates were kept for diffusion at 4^0 C for 15 minutes. This was done to avoid growth of the test bacteria before complete diffusion of the aqueous / DMSO extracts of selected biological immunomodulators.
- After diffusion, the plates were kept at 37^0 C for 24 hours and results were recorded.

3.1.2 Study of Antifungal activity:

Test Fungi Used:

Candida albicans NCIM 3466 and *Hansenula capsulata NCIM 3439* as common representatives of fungal pathogens in immunocomprised individuals were selected for the study. The fungal cultures were collected from 'National Collection of Industrial Microorganisms (NCIM)', Pune.

Method:

- Saline suspension was prepared of *Candida albicans NCIM 3466* and *Hansenula capsulata NCIM 3439.* Sterility of the saline was ensured before use.
- For the method, HiMedia 'Sabouraud Agar Medium' was used. Sterility of the 'Sabouraud Agar' was ensured before use.
- Antifungal activity was tested using 'Agar Diffussion Method'.

3.1.3 Study of anti viral activity of blended mixtures of selected biological immunomodulators by 'HIV-1 P-24 Antigen Inhibition Assay'.

Materials:

a) Mixture no. 1: Blended mixture of *Wagatia spicata Dalz. (Caesalpinia spp.)* (stem) -100 mg and *Picrorrhiza kurroa* (root) – 50 mg

b) Mixture no. 2: Blended mixture of *Withania somnifera* (root)-250 mg, *Wagatia spicata Dalz., (Caesalpinia spp.)* (stem)–50 mg, *Chlorophytum borivilianum* (root)-30 mg. *Spilanthes paniculata Wall. ex. DC.* (root) - 20 mg

c) Cell Line- PHA activated Peripheral Blood Mononuclear Cells (PBMCs; PHA Blasts)

d) Clinical HIV-1 (C-Strain)

Experimental Conditions:

i) Test temperature- 37º C ± 1º C

ii) Contact time- 30 minutes

iii) Period of observation- 4 days

Method:

HIV-1 p-24 Antigen Inhibition Assay was performed as mentioned in the protocol.

Protocol:

- 0.5 ml of diluted immunomodulator aqueous extract was added to 0.5 ml of HIV-1 (C-Strain) dilution.
- The mixture of immunomodulators was allowed to interact with the viral strain for 10 minutes.
- After the incubation, mixture no. 1 and 2 were separately inoculated on PHA stimulated 'Peripheral Blood Mononuclear Cells' (approximately 10^4 cells/ml)
- The viruses were allowed to adhere for 60 minutes at 37º C and then the cells were centrifuged to remove free viruses.
- The cells were incubated at 37º C in presence of diluted aqueous mixture no.1 and 2 separately for 4 days, after which they were removed and 450 µl of the cell suspension from each mixture was separately used for HIV-1 p-24 antigen ELISA.
- 450 µl of the removed cell suspension was added to 50 µl of lysing buffer and mixed.
- Out of total volume, 200 µl of mixture was added to the microtitre wells.
- Positive and negative controls along with p-24 antigen standards were added and the microtitre was incubated for 2 hours at 37º C.
- Following incubation, the microtitre was aspirated and washed with buffer.
- 100 µl antibody solution was added to each well. The plate was incubated for 60 minutes at 37º C.
- The microtitre plate was aspirated, washed with buffer and 100 µl 'Streptavidine-Peroxidase' conjugated enzyme was added.
- The microtitre plate was incubated at 37º C for 30 minutes.

- The microtitre plate was aspirated and washed with buffer as before and 100 µl of substrate was added to each well.
- The microtitre plate was incubated for 30 minutes at room temperature.
- 100 µl of 'Stop solution' was added
- The absorbance (optical density) of each well was measured at 450 nm using automated microplate reader (ELISA Reader).
- The concentration of p-24 antigen (picograms/ml) was calculated from the standard graph plotted using OD readings obtained from the standard wells.
- The percent inhibition (reduction) of p-24 antigen was calculated using the levels of p-24 antigen in the immunomodulator treated wells and controlled wells.

4.1 Fourier Transformer Infra Red Spectroscopy (F.T.I.R.) of selected biological immunomodulators:

Principle:

For a particular molecule an internal energy is the sum of rotational, vibrational and electronic energy.

F.T.I.R. Spectroscopy provides information about interactions between molecules and electromagnetic fields in the 'Infra Red' region.

Absorption peaks obtained during F.T.I.R. analysis correspond to the frequencies of vibrations between the bonds of the atoms present in a particular biological immunomodulator and thus can explore the reactive groups of the particular biological immunomodulators.

Every biological immunomodulator is a unique combination of atoms, hence produce unique infrared spectrum. Hence, F.T.I.R. can be used for identification and qualitative analysis. Size of the peak is directly proportional to the concentration of biochemical compounds.

Method:

- Background spectrum was taken by placing KBr in horse shoe shaped KBr plate.
- Biological immunomodulator under test was mixed with KBr in proportion of 9 parts KBr : 1 part of test sample.
- The plate was placed in F.T.I.R. unit. 'Mychleson Interferometer' was used for the analysis.
- Spectrum was computed from interferogram by performing a computer assisted 'Fourier Transform'.

5.1 Acute toxicity testing of biological immunomodulators:

Rationale of the Test:

Before starting clinical trials, all the target biological immunomodulators i.e. *Chlorophytum barrivilianum, Wagatia spicata Dalz, Withania somnifera, Picrorrhiza kurroa, Spilanthus paniculata Wall. ex. DC.* were tested for potential toxicity.

Method:

- The test was performed in 03 different groups of albino mice as per OECD guidelines (Organization for Economic Cooperation and Development Guidelines) and using 'Litchfield and Wilcoxon method'.

Sr. No.	Character	Description of Characters and environmental conditions
1.	Species	Albino mice
2.	Strain	Swiss albino
3.	Weight range	20-24 gm
4.	Age	6-8 weeks
5.	Sex	Females
6.	Number	03 Female/ Groups
7.	Housing	03/ cage
8.	Diet	Pelleted feed supplied by Nav Maharashtra Chakan Oil Mills Ltd. Pune
9.	Water	Community tap water
10.	Room temperature	$20\text{-}24^0$ C.
11.	Relative humidity	40-60%
12.	Light cycle	12 hours light & 12 hours darks
13.	Vehicle for dosaging	Water
14.	Dose volume	1.0 ml / 100 gm

Table-7: Details of test animals and environmental conditions

- For group no. I of the experimental animals, herbal extracts were administered through oral route at the dose of 50 mg/kg. Similarly for group no. II and group no. III, standardized doses were 300 mg/kg and 2000 mg/kg.

- The test materials were administrated by gavages (a special sterile feeding instrument resembling needle of disposable syringe) to mice. The mice were deprived of feed 3-4 hours before and two hours after administration of herbal extracts (This is done for avoiding possible interfearence of dietary nutrients or growth inhibiting substances). Signs and symptoms were recorded at 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours & 24 hours and later twice a day thereafter up to 14 days. Acute toxicity testing was performed as per protocols standardized by Roll R. (1986), Diener W. (1994) and Schlede E (1994). For performing the test OECD monograph series on Testing and Assessment No.19 was followed.

6. In vivo study of immunomodulating activities of biological immunomodulators:

6.1.1 Study of 'Carbon Clearance Test':

(The study was conducted as approved by 'Institutional Animal Ethics Committee'.)

Rationale of the Test:

Phagocytic efficacy of phagocytic cells can be measured in terms of 'Carbon Clearance Test' which is often expressed in terms of phagocytic index.

Method:

- The method of Biozzi *et al.* as modified by Mayank Thakur *et al.* was used for the study.
- Accordingly, three different groups each consisting of three males and three females of Albino mice were selected for the study as;

Group I: Control.

Group II: Standard, in which all the animals were treated with FDA approved drugs as 'Cytonini' and 'Cytomaw'.

Group III: Test and were treated with preparation number 1 and 2 of selected biological immunomodulators.

Preparation no.- 1

Wagatia spicata Dalz. (crude stem powder) – 100 mg

Picrorrhiza kurroa (crude root powder) – 50 mg

(**Dosage – One blended animal dose in the morning**)

Preparation no. (2)

Withania somnifera (crude root powder) – 250 mg

Wagatia spicata Dalz (crude stem powder) – 50 mg

Spilanthes paniculata Wall. ex. DC. (crude root powder) – 20 mg

Chlorophytum borivilianum (crude root powder) – 30 mg

(Dosage: One blended animal dose in the evening)

Animal dose was calculated as:

The blended dose of final product as mentioned in process of making standardized product was determined for 'Albino Mice' using formula

X x 50=Y mg/kg (Ref: OECD monograph series on testing and assessment)

Where X=Blended human dose x 0.0026

Y value was dissolved in 10 ml of sterile water = Z

Body weight values of mice were used for animal dose conversion. For every 10 gm of body weight, 0.1 ml of Z was used.

- The dosing was continued for 15 days.
- On fifteenth day, 25 µl of blood sample from each animal, from each group was collected from 'retro orbital plexus'. These samples were treated as a 'zero time sample'. Subsequently each animal was injected with 0.1 ml of 1% carbon suspension.
- Keeping the time intervals of 4 min, 8 min, 12 min, and 16 minutes after injection, 0.25 µl of blood sample from every member animal of respective groups was collected by 'retro orbital plexus' puncture.
- All the blood samples were lysed with 2 ml of 0.1% acetic acid and absorbance value was recorded at 675 nm.
- The graph of Absorbance Vs. Time was plotted for each animal in Group I, II, III
- Slope values for every graph and phagocytic indices were calculated using the formula: K test / K control and K standard / K control.

6.1.2 Study of 'Delayed Type of Hypersensitivity Test':

Rational of the Test:

'Type IV Hypersensitivity Test' which is also called as 'Delayed Type of Hypersensitivity' is routinely used for assessment of T lymphocyte function. Anergy of T lymphocyte is very common in immunocomprised condition. Difference between right and left foot pad erythema observed in albino mice is the measure of delayed type of hypersensitivity and its comparative study within the group indicates efficacy of T lymphocytes.

Method:

- The method suggested by Lagrange *et al.* (1974) was used for assessment of 'Type IV Hypersensitivity Test'.
- Three different groups of Swiss albino mice, each carrying 3 males and 3 females were selected for the study as **control**, **standard** (the group treated with FDA approved drugs 'Cytonini' 1 O.D. Morning and 'Cytomaw' 1 O.D. Evening) and **test** (treated with blended dose of preparation no.1as 1 O.D. morning and blended dose of preparation no. 2 as 1 O.D. in evening).
- Preparation no.1 carried 100 mg of *Wagatia spicata Dalz* (stem), 50 mg of *Picrorrhiza kurroa* (root) as a single human dose and was converted to animal dose considering weight fluctuations in Swiss albino mice.
- Preparation no.2 was the blended dose of 250 mg of *Withania somnifera* (root), 50 mg of *Wagatia spicata Dalz* (stem), 20 mg of *Spilanthes paniculata Wal. ex. DC.* (root), 30 mg of *Chlorophytum borivilianum* as a single human dose and was similarly converted to animal dose considering weight fluctuations in Swiss albino mice.
- In standard and test groups, respective treatment was continued for 14 days.
- 20 µl of washed saline suspension of sheep RBC containing 5 x 10^9 cells/ml was injected in right hind foot pad while 20µl of plain pyrogen free saline was injected in left hind foot pad of every animal from every group.
- Foot pad erythema was measured by 'Mitutoyo Digimatic Caliper'after 2, 4, 6 and 8 hours.
- Difference between right and left foot pad erythema was taken as a measure of DTH

6.1.3 Study of 'Neutrophil Adhesion Test':

Method:

- 'Neutrophil Adhesion Test' was conducted by the method described by Wilkonson (1978).
- Three different groups of Swiss albino mice, each carrying 3 males and 3 females were selected for the study as **control**, **standard** (the group treated with FDA approved Cytonini 1 O.D. morning and Cytomaw 1 O.D. evening) and **test** (treated with blended dose of preparation no.1as 1 O.D. morning and blended dose of preparation no.2 as 1 O.D. in evening).

- Preparation no.1 carried 100 mg of *Wagatia spicata Dalz* (stem), 50 mg of *Picrorrhiza kurroa* (root) as a single human dose and was converted to animal dose considering weight fluctuations in Swiss albino mice.
- Preparation no.2 was the blended dose of 250 mg of *Withania somnifera* (root), 50 mg of *Wagatia spicata Dalz* (stem), 20 mg of *Spilanthes paniculata Wall. ex. DC.* (root), 30 mg of *Chlorophytum borivilianum* as a single human dose and was similarly converted to animal dose considering weight fluctuations in Swiss albino mice.
- In standard and test groups, respective treatment was continued for 14 days.
- After fourteen days, 0.5 ml blood sample was collected from all the three groups of animals, in heparinized vials by 'retro orbital puncture'.
- In each vial, 20 mg of nylon fiber was added and vials were incubated at 37° C for 15 min. Using 'Coulter Type Blood Cell Counter' haematological parameters were studied. Count of fiber bound neutrophil was taken by dispensing the fibers in fresh 1 ml saline.

7.1 Human Clinical Trials - Phase I Study:

Rational of the study:

Phase I study was conducted to evaluate drug safety and tolerance in human being.

Method:

- Phase I clinical trials were conducted as per the cosmetics Amendment Bill LV II of 2007.
- Fifteen HIV infected individuals and five healthy individuals were selected for phase I trials.
- Study was performed in two groups. Group I was of HIV reactive patients and Group II was of healthy volunteers.
- Blended doses of biological immunomodulators were administered through oral route and the dose administration was followed under the supervision of registered medical practitioner and continued for 60 days (Average).

Morning dose:

Wagatia spicata Dalz. (crude stem powder)– 100 mg

Picrorrhiza kurroa (crude root powder) – 50 mg

(Dosing – 1 capsule in the morning after breakfast)

Evening dose:

Withania somnifera (crude root powder) – 250 mg

Wagatia spicata Dalz. (crude stem powder)– 50 mg.

Spilanthes paniculata Wall. ex. DC. (crude root powder) – 20 mg.

Chlorophytum borivilianum (crude root powder) – 30 mg

(Dosing: 1 capsule after dinner)

- Patients were observed for drug toxicity and other parameters like pyrexia, hypersensitivity, body weight and secondary infections.

7.2 Human Clinical Trials- Phase II Study:

Rational of the study:

Phase II study was conducted to evaluate the efficacy of biological immunomodulators in human being.

Method:

- Phase II clinical trials were conducted as per the cosmetics Amendment Bill LVII of 2007 and recommendations and amendments of World Health Organization.
- By taking written consent, effect of biological immunomodulators was studied in 18 HIV reactive males and 14 HIV reactive females (age group 20-47 yrs).
- Blended doses of biological immunomodulators were advised through oral route and the dose administration was followed under the supervision of registered medical practitioner and continued for average 11 months.

Morning dose:

Wagatia spicata Dalz. (crude stem powder) – 100 mg

Picrorrhiza kurroa (crude root powder) – 50 mg

(Dosing – 1 capsule in the morning after breakfast)

Evening dose:

Withania somnifera (crude root powder) – 250 mg

Wagatia spicata Dalz. (crude stem powder) – 50 mg

Spilanthes paniculata Wall. ex. DC. (crude root powder) – 20 mg

Chlorophytum borivilianum (crude root powder) – 30 mg

(Dosing: 1 capsule after dinner)

Record of absolute CD_4 count and viral load was kept.

7.2.1 Counting of absolute CD_4 cells:

Instrument used:

'Beckman Coulter Flow Cytometer'.

Method:

- 100 µl of the blood sample with anticoagulant was added in dry reagent tube of the 'T cell subset kit'.
- 5 µl of each monoclonal antibody solution (antihuman CD-95, antihuman CD-25 and antihuman CD-127) was added in the tube.
- The tube was agitated for 8 seconds followed by 15 min incubation in dark at room temperature.
- 2 ml of lysing solution was added for lysis of erythrocytes.
- 50 µl of IO test fixative solution was added.
- The tube was centrifuged at 2000 rpm for 5 minutes.
- By removing supernatant, the pallet was collected and added in 3 ml of PBS.
- The contents were loaded in the 'Flow Cytometer' to run the analysis.
- 'Absolute CD_4 count' was calculated by using a formula,
- Absolute CD_4 T cell count = Percentage CD_4 T cell count as reflected through software calculations x Absolute lymphocyte count as reflected through software calculations.

7.2.2 Viral load testing of HIV:

Rationale of the Test:

As 'polymerase chain Reaction' detects specific nucleic acid sequence by logarithmic amplification of oligonucleotide primers annealed to 5′ and 3′ DNA strands; the technique provides maximum sensitivity for quantitative estimation of HIV nucleic acid copies. The technique facilitates the detection of HIV infection before seroconversion. When HIV infected mother gives birth to baby, the baby may have maternal anti HIV antibodies or the antibodies of real infection. Such false seropositive cases can be identified using PCR test. Common primers / probes for detection of HIV-1 are enlisted in table - 8.

Material:

Region on HIV Genome	Primer / Probe	Sequence
gag	SK-38 Primer	ATAATCCACCTATCCCAGTAGGAG AAAT
gag	SK-39 Primer	TTTGGTCCTTGTCTTATGTCCAGAA TGC
ltr	SK-29 Primer	ACTAGGGAACCCACTGCT
gag	SK-19 Probe	ATCCTGGGATTAAATAAAATAGTA AGAATGTATAGCCCTAC
gag	SK-145 Primer	AGTGGGGGGACATCAAGCAGCCA TGCAAAT
gag	SKCC1B Primer	TACTAGTAGTTCCTGCTATGTCACT TCC
gag	SK102 Probe	GAGACCATCAATGAGGAAGCTGC AGAATGGGAT

Table-8: Common primers / probes for detection of HIV-1

Method:

- Blood was collected in the tubes with EDTA or ACD as anticoagulants. Heparin is inhibitory to PCR; hence should not be used as an anticoagulant.
- The tube was centrifuged at 1200 rpm for 10 min.
- Plasma was removed into 'Sarstedt tube'.
- The contents were centrifuged at 1200 rpm for 10 min. This step removed residual cells and small sized cells like platelets.
- Centrifuged plasma was collected into fresh 'Sarstedt tube'.

- **Extraction of HIV-1 RNA:**

- Quantitation standard (100 copies / µl) was added to the 600 µl of lysis buffer.
- The prepared lysis buffer (600 µl) was collected in 'Sarstedt tube' X in that 140 µl of patients plasma is added.
- The tube was capped and vortexed for 3-5 sec.
- The tube was incubated at room temperature for 10 min.
- Cap was removed and 800 µl of absolute isopropanol was added. The tube was vortexed for 3-5 sec.

- The sample was collected in a specially designed collection tube with filter probe and was centrifuged at 12000 rpm for 15 min at room temperature.
- Filter probe of the collection tube removed pellet and inner small tube carried the filtrate. Collection tube was discarded and filtrate collected from inner small tube was used for further processing.
- One ml of 70% ethanol was added in the inner tube and was vortexed for 3 to 5 seconds with capping.
- The contents were recollected in specially designed collection tube, centrifugation was repeated at 12000 rpm for 15 min at room temperature. Pellet was removed using mounted filter in the collection tube and it was discarded. Inner small tube with supernatant was used for further processing.
- 500 µl of 'Wash Buffer No.1' was added and centrifuged at 8000 rpm for 3 minutes.
- 500 µl of 'Wash Buffer No.2' was added and centrifuged at 15000 rpm for 5 minutes.
- Pellet was suspended in 50 µl of sterile 'ddH_2O' treated with diethylpolycarbonate (DEPC).

Polymerase Chain Reaction of HIV-1 RNA:

1. 50 µl of mastermix solution was collected in a reaction tube and in that 50 µl of the extracted HIV-1 RNA was added.
2. Thermal cycler is programmed as:
   a. One cycle of 2 Min. at 50^0 C
   b. One cycle of 30 Min. at 60^0 C
   c. 4 different cycles were operated of 10 seconds interval each at 95^0 C, 55^0 C and 72^0 C.
   d. 26 different cycles were operated of 10 seconds interval each at 90^0 C, 60^0 C and 72^0 C
   e. One cycle was operated of 15 min at 72^0 C
3. 100 µl of denaturation solution (0.4 M NaOH) was added in reaction tube.

QUANTITATIVE ANALYSIS:

- Quantitative analysis of HIV copies was conducted in a plate having multiple microwells in which sample and known standards were loaded. Rows No. 1 to 6 were coated with HIV probes and Row No.7 and 8 were with QS probes.
- 100 µl of hybridization buffer is added to each microwell and was mixed with multichannel pipettor.
- The contents were serially transferred in 25 µl aliquots starting from row no. 1 to 2, 2 to 3, 3 to 4, 4 to 5 and 5 to 6. From row No.6, 25 µl was collected and discarded to keep volume standards.
- In row no.7, 25 µl of denatured amplicon was added and mixed for 10 times with multichannel pipettor.
- 25 µl from row no.7 was transferred to row no.8 and mixed for 10 times with multichannel pipettor. Eventually 25 µl from row no. 8 was collected and discarded.
- Covered microwell plate was incubated at 37^0 C for 60 min.
- In each microwell 100 µl of 'Avidin horseradish peroxidase conjugate' was added and the plate was incubated at 37^0 C for 15 min.
- Microwell plate was washed with 'working wash solution' using automated washer for 5 times.
- Just before use 'substrate solution' was freshly prepared by adding "0.001% H_2O_2" and 0.01% "3, 3′, 5-5′ tetramethylbenzidine in 40% dimethylformamide" in the ratio of 4:1 respectively.
- 100 µl of freshly prepared 'working substrate solution' was added into well no.1, 2, 3, 4, 5, 6, 7 and 8. The plate was incubated at room temperature for 10 min in dark. This developed the coloured product.
- 100µl of 1M H_2SO_4 was added to stop the reaction.
- O.D. readings were recorded of each microwell at 450 nm.

Calculations:

- Row No.1 to 6 from microwell plate represented the serial dilution of HIV-1 amplicon as 1:5, 1:25, 1:125, 1:625 and 1:3125 respectively.
- Only one microwell was selected having lowest OD at 450 nm and the value was > 0.20 and < 2.00 O.D. units. This value was used for calculation.

- O.D. reading of selected HIV microwell should be corrected for background O.D. and thus 0.07 background O.D was subtracted from the chosen reading. This value was recoreded as X value.
- Total HIV OD = X value x dilution factor
- Similarly one QS well was selected. Tube No. 7 and Tube No. 8 represented baseline dilution and 1:5 dilutions respectively. QS microwell was selected having lowest O.D. at 450 nm and it was > 0.30 and < 2.00 O.D units.
- OD reading of selected QS microwell was corrected for 0.07 background OD (OD-0.07) and this reading was recorded as Y.
- Total QS OD = Y Value X dilutions factor.
- Final Calculation for HIV-1 RNA copies / ml at plasma
  = (Total HIV OD / Total QS OD) x Added QS copies for every PCR (75 but may vary from kit to kit) x Conversion Factor (40)

RESULTS

AND

DISCUSSION

RESULTS AND DISCUSSION

'Human Immunodeficiency Virus' adversely hampers the immune system developing immunodeficient state. Also the drug toxicity, development of drug resistance limits the efficacy of antiretroviral chemotherapy. On this background the present study explored few of the potential biological immunomodulators. Considering heavy metal toxicity and possible interference, the selected biological immunomodulators were analyzed for lead, cadmium, mercury and arsenic. The study also tested the antibacterial, antifungal and anti-HIV activity of selected biological immunomodulators. In the present study the selected biological immunomodulators were subjected to 'Fourier Transformer Infra Red Spectroscopy' which enabled to explore the active chemical groups within the immunomodulators. This could establish the relationship between active chemical groups and efficacy of the selected biological immunomodulators. Observations may further refine the existing regime of 'Ayurveda' or 'Herbal therapy'. By performing 'Acute Toxicity Testing', the study also ensured the safety parameters for human use. During the study efficacy of phagocytic cells was studied by conducting 'Carbon Clearance Test'. Effect of biological immunomodulators for improving T cell function was studied using 'Delayed Type of Hypersensitivity Test'. Role of the immunomodulators for enhancing phagocytic ability of Neutrophils was studied during 'Neutrophil Adhesion Test'. For establishing human safety, 'Phase I Human Clinical Trial' was conducted. Absolute CD_4 cells are the targets for HIV. Present study also completed the 'Phase II Human Clinical Trials' during which effect of the selected biological immunomodulators on absolute CD_4 cells and viral load of HIV was carefully assessed. The studies were conducted under the supervision of registered medical practitioner and with the permission of 'Institutional Ethics Committee', Bharati Vidyapeeth Deemed University Pune, vide Ref.:BVDU/YM/Ethics 2012-13/452 dt. 30/10/2012.

1. Identification of Biological Immunomodulators:

Chlorophytum borivilianum, Withania somnifera were collected from Pavan Agro Farm, Pune. *Wagatia spicata Dalz.* was collected from hilly regions of Malakapur (Dist. Kolhapur) , *Picrorrhiza kurroa* was collected from licenced ayurvedic supplier. *Spilanthes paniculata Wall. ex. DC.* was collected from hilly regions of Mulashi

(Pune). All plant derivatives were identified using standard identification keys and authenticated by 'Botanical Survey of India'.

Fig: 3 *Chlorophytum borivilianum* (Safed Musli)

Fig: 4 *Withania somnifera* (Ashwagandha)

Fig: 5 *Wagatia spicata Dalz.* (Wakeri)

Fig: 6 *Picrorrhiza kurroa* (Kutki)

Fig: 7 *Spilanthus paniculata Wall. ex. DC.* (Akkarkara)

Discussion:

AIDS is a pandemic disease and the fear of HIV infection has affected the major global population than that of clinical infection of the virus. Due to this many people have started believing that, HIV is one of the deadliest infections that have so far been invented. Many scholars of Ayurveda have claimed that the disease "Saha" described in 'Sushruthsamhitha' is nothing but the AIDS. Beyond the geographical and cultural boundries, the infection appeared on global scale including India with high mortality and deeply impacted social and cultural interactions of human beings. At the end of June 2015 about 36.9 million people were living with HIV [153]. Since 2000 about 25 million people have died of AIDS related complications. In a single year of 2014 about 2 million new cases of HIV infection were documented [153]. India is the country having third largest number of HIV infected individuals in the world. In India at the end of December 2015, about 21.17 lakh people were living with HIV [127]. In a single year of 2015 about 86,000 new HIV infections and 67614 HIV related deaths were recorded in India. In Maharashtra about 3.01 lakhs HIV cases were documented in 2015 [156, 157]. In present study five different plants were selected as potential biological immunomodulators. *Withania somnifera* has been utilized in Ayurveda as a base of different medicines. Seena *et al.* (1993) reported anticancer effects of *Withania somnifera* [3]. Effect on cytokine secretion of the plant was studied by Duley *et al.* (1997). Antimalignant properties of Withaferin isolated from *Withania somnifera* were reported by Devi *et al.* (1995) [3]. Immunomodulating properties of *Picrorrhiza kurroa* were reported by Atal *et al.* (1986). DTH response of the leaves of *Picrorrhiza kurroa* was studied by Sharma et al. (1994). Anti *Leishmania* property of the plant was reported by the Puri *et al.* (1992). *Chlorophytum borivilianum* is a popular ingradient of many Ayurvedic medicines. Triveni (2003) has described herb as excellent rejuvenator. Mayank Thakur *et al.* (2006) have reported the effect of the plant on cellular immunity. Bodhankar *et al.* (2015) documented immunomodulating activity of *Wagatia spicata Dalz.* Activation of macrophages in experimental animals by *Spilanthus acmella* was reported by Savadi R.V. (2010). No other documentation consolidated the use of *Spilanthus paniculata* as an immunomodulator. Previous studies as described were conducted by selecting an individual plant or herb. Present study differs by focusing on synergistic action of selected biological immunomodulators than that of single one. The study also claims for anti HIV properties of the formulations derived from selected biological immunomodulators.

2. Heavy metal analysis of selected biological immunomodulators:

Heavy Metal	Concentration in *Chlorophytum borivilianum* (ppm)	Acceptable limit as per W.H.O. (ppm)
Lead	1.5	2.0
Arsenic	0.5	2.0
Cadmium	0.1	2.0
Mercury	0.5	2.0

Table – 9: Heavy metal analysis of *Chlorophytum borivilianum*

Heavy Metal	Concentration in *Withania somnifera* (ppm)	Acceptable limit as per W.H.O. (ppm)
Lead	1.1	2.0
Arsenic	0.4	2.0
Cadmium	0.1	2.0
Mercury	0.5	2.0

Table – 10: Heavy metal analysis of *Withania somnifera*

Heavy Metal	Concentration in *Wagatia spicata Dalz.* (ppm)	Acceptable limit as per W.H.O. (ppm)
Lead	1.4	2.0
Arsenic	0.3	2.0
Cadmium	0.1	2.0
Mercury	0.5	2.0

Table -11: Heavy metal analysis of *Wagatia spicata Dalz.*

Heavy Metal	Concentration in *Picrorrhiza kurroa* (ppm)	Acceptable limit as per W.H.O. (ppm)
Lead	1.6	2.0
Arsenic	0.5	2.0
Cadmium	0.1	2.0
Mercury	0.5	2.0

Table-12: Heavy metal analysis of *Picrorrhiza kurroa*

Heavy Metal	Concentration in *Spilanthes paniculata Wall.ex Dc.* (ppm)	Acceptable limit as per W.H.O. (ppm)
Lead	1.2	2.0
Arsenic	0.4	2.0
Cadmium	0.1	2.0
Mercury	0.5	2.0

Table-13: Heavy metal analysis of *Spilanthes paniculata Wall. ex. DC.*

Discussion:

Lead (Pb), Mercury (Hg), Cadmium (Cd) and Arsenic (As) have documented as non vital heavy metals by Chang L.W. (1996). In nature mercury exist in elemental, inorganic and organic form. Tchounwou P. B. (2003) reported gastrointestinal toxicity, neurotoxicity and nephrotoxicity of Mercury. CDC guidelines (2001) documented dementia and central nervous system degeneration by Lead. Lead induced premature apoptosis was reported by Yedjou C.G. (2010). In soil, cadmium remains in high concentration which plants may readily absorb to toxic level. Davison A.G. (1995) reported for pulmonary dysfunction while Schutte R. (2008) documented osteoporosis by Cadmium. Arsenic is found in inorganic and organic forms. Trivalent arsenite and pentavalent arsenate are inorganic forms while monomethyl arsonic acid, dimethyl arsinic acid and trimethyl arsine oxide are organic forms of Arsenic. Toxicity by Arsenic has been described in terms of neurobehavioral disorders, portal fibrosis, and leucopenia. 'National Research Council' has described Arsenic as carcinogen. W.H.O. norm for safer concentration of lead, arsenic, cadmium and mercury is 2.00 p.p.m.

In present study *Chlorophytum borivilianum, Withania somnifera, Wagatia spicata Dalz., Picrorrhiza kurroa, Spilanthus paniculata Wall. ex. DC.* were selected for animal clinical trials and human clinical trials. For all selected biological immunomodulators, concentration of Lead, Arsenic, Cadmium and Mercury was found below 2.00 ppm.

3. Study of antibacterial activity:

Sr. No	Name of the Plant	Zone diameter against *Bacillus marscescens NCIM 5041* (mm)	Zone diameter against *Klebsiella pneumoniae NCIM 2719* (mm)
1.	*Chlorophytum borivilianum*	Nil	Nil
2.	*Withania somnifera*	20	Nil
3.	*Wagatia spicata Dalz.*	24	Nil
4.	*Picrorrhiza kurroa*	43	17
5.	*Spilanthes paniculata Wall. ex. Dc.*	29	Nil

Table-14: Antibacterial Activity of Aqueous extract of selected biological immunomodulators at the conc. of 0.33 gm/ml. (Medium- Muller Hinton Agar)

Sr. No	Name of the Plant	Zone diameter *Bacillus marscescens NCIM 5041* (mm)	Zone diameter against *Klebsiella pneumoniae NCIM 2719* (mm)
1	*Chlorophytum borivilianum*	Nil	Nil
2	*Withania somnifera*	23	17
3	*Wagatia spicata Dalz.*	21	17
4	*Picrorrhiza kurroa*	39	14
5	*Spilanthes paniculata Wall. ex. Dc.*	27	15

Table-15: Antibacterial Activity of DMSO extract of selected biological immunomodulators at the conc. of 0.33 gm/ml. (Medium: Muller Hinton Agar)

Results:

a) Aqueous extracts of *Chlorophytum borivilianum, Withania somnifera, Wagatia spicata Dalz., Picrorrhiza kurroa, Spilanthes paniculata Wall. ex. DC.* showed antimicrobial activity against *Bacillus marscescens NCIM 5041*.

b) Maximum antibacterial property was exhibited by aqueous extract of *Picrorrhiza kurroa* against *Bacillus marscescens NCIM 5041*. *Picrorrhiza kurroa* also showed antibacterial activity against bacterium *Klebsiella pneumoniae NCIM 2719*, but not the other immunomodulators.

c) No antibacterial activity was observed of aqueus extract of *Chlorophytum borivilianum.*

d) DMSO extracts of selected biological immunomodulators except *Chlorophytum borivilianum* showed antibacterial activity against *Bacillus marscescens NCIM 5041* and *Klebsiella pneumoniae NCIM 2719.* Maximum activity was showed by DMSO extract of *Picrorrhiza kurroa.*

e) Better antibacterial activity was observed of aqueous extract of *Picrorrhiza kurroa* than that of DMSO extract.

4. Study of antifungal activity:

Sr. No.	Name of the Biological Immunomodulator	Zone diameter in mm against *Candida albicans NCIM 3466*		Zone diameter in mm against *Hansenula capsulata NCIM 3439*	
		Aqueous	**DMSO**	**Aqueous**	**DMSO**
1.	*Chlorophytum borivilianum*	13	18	Nil	13
2.	*Withania somnifera*	Nil	Nil	Nil	18
3.	*Wagatia spicata Dalz.*	Nil	15	Nil	20
4.	*Picrorrhiza kurroa*	Nil	Nil	Nil	Nil
5.	*Spilanthes paniculata Wall. ex. Dc.*	Nil	Nil	Nil	Nil

Table-16: Antifungal Activity of Aqueous and DMSO extracts of selected biological immunomodulators at the conc. of 0.33 gm/ml. (Medium- Sabouraud Agar)

Results:

a) Both Aqueous and DMSO extracts of *Chlorophytum borivilianum* showed antifungal activity against *Candida albicans NCIM 3466*.

b) DMSO extract of *Chlorophytum borivilianum* inhibited the growth of *Hansenula capsulata NCIM 3439,* but not the aqueous extract.

c) Aqueous and DMSO extracts of *Withania somnifera* failed to exhibit antifungal activity against *Candida albicans NCIM 3466*.

d) Aqueous extract of *Withania somnifera* did not show the activity against *Hansenula capsulata NCIM 3439.*

e) DMSO extract of *Withania somnifera* inhibited the growth of *Hansenula capsulata NCIM 3439.*

f) DMSO extract of *Wagatia spicata Dalz.* inhibited the growth of both *Candida albicans NCIM 3466* and *Hansenula capsulata NCIM 3439* but not the aqueous extract.

g) Aqueous and DMSO extracts of *Picrorrhiza kurroa* and *Spilanthes paniculata Wall. ex. DC.* failed to show antifungal activity.

Discussion:

Antibacterial properties of *Chlorophytum borivilianum* were described by Syed Ahmad et al. (2014). Vidhi Mehrotra (2011) reported antimicrobial activities of *Withania somnifera*. No convincing documents are available for antimicrobial activity of *Wagatia spicata Dalz*. Deepti Rathee et al. (2012) reported antimicrobial properties of *Picrorrhiza kurroa*. Noor Jahan (2013) documented antimicrobial properties of *Spilanthes spp*.

In the present study *Bacillus marscescens* NCIM 5041, *Klebsiella pneumoniae* NCIM 2719, *Candida albicans* NCIM 3466, *Hansenula capsulata* NCIM 3439 were used as test microorganisms. *Bacillus marscescens,* the catalouged bacterium now is recognized as *Serratia marcescens* with noticeable difference in the spelling of species. It has been reported for cellulitis, septicaemia and pneumonia in HIV infected individuals. *Klebsiella pneumoniae* is documented for development of pneumonia as HIV/AIDS related complication. *Candida albicans* develop oral thrush in HIV infected individuals. *Hansenula capsulata* is rich in 'phospomannans' and the component reported for prime role in fungal pathogenicity. Jim E. Cutler (2002) vide European patent number '1165614 A1' elaborated the use of antibodies against phosphomennan for preventing fungal disease. Both DMSO and aqueous extracts of biological immunomodulators were separately tested for potential antimicrobial properties. Zones of growth inhibition > 10 mm were reported as positive. Maximum antibacterial property against *Bacillus marscescens* NCIM 5041 was exhibited by aqueous extract *Picrorrhiza kurroa*. At the concentration of 0.33 gm/ml zone diameter was 43 mm. DMSO extract of *Wagatia spicata Dalz.* and *Withania somnifera* exhibited maximum activity against *Klebsiella pneumoniae* NCIM 2719. At the concentration of 0.33gm/ml, both the extracts exhibited zone diameter of 17 mm separately. Maximum antifungal activity against *Candida albicans* NCIM 3466 was showed by DMSO extract of *Chlorophytum borivilianum*. Maximum antifungal activity against *Hansenula capsulata* NCIM 3439 was showed by DMSO extract of

Wagatia spicata Dalz. At the concentration of 0.33gm/ml of DMSO, the extract showed zone diameter of 20 mm. As the study also included known fungal pathogens, it may give new perspectives for the development of chemotherapeutic regime against fungi.

5. Study of Antiviral activity: (HIV-1 P-24 Antigen Inhibition Assay)

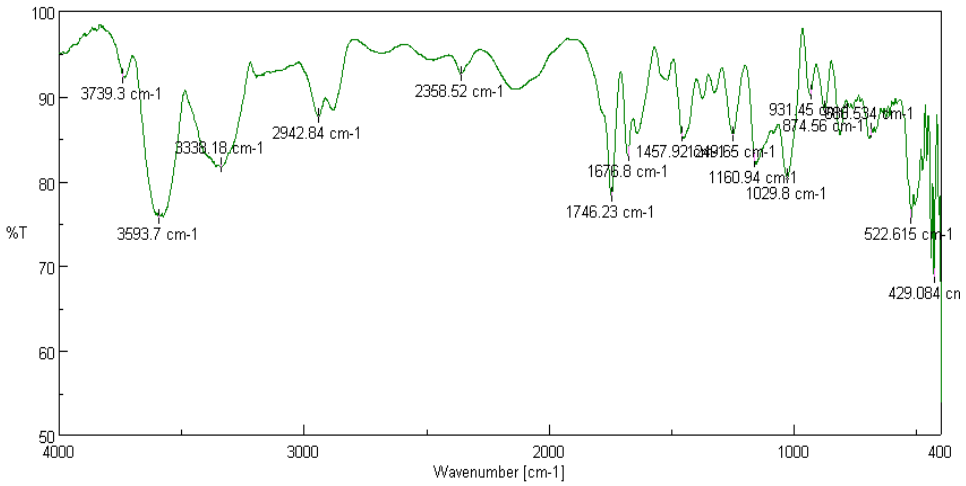
Observation: The percentage inhibition reduction of p-24 antigen on PHA activated PBMCs cell line was about 20%.

Result: As the percentage inhibition reduction of p-24 antigen was about 20%, results as per standard protocols were inconclusive.

Discussion: AIDS is a pandemic disease and the fear of HIV infection has affected the major global population than that of clinical infection of the virus. Due to this many people have started believing that, HIV is one of the deadliest infections that have so far been invented. Many scholars of Ayurveda have claimed that the disease "Saha" described in 'Sushruthsamhitha' is nothing but the AIDS. Beyond the geographical and cultural boundries, the infection appeared on global scale including India with high mortality and deeply impacted social and cultural interactions of human beings. At the end of June 2015 about 36.9 million people were living with HIV [(153)]. Since 2000 about 25 million people have died of AIDS related complications. In a single year of 2014 about 2 million new cases of HIV infection were documented [(153)]**.** India is the country having third largest number of HIV infected individuals in the world. In India at the end of December 2015, about 21.17 lakh people were living with HIV [(127)]. In a single year of 2015 about 86,000 new HIV infections and 67614 HIV related deaths were recorded in India. In Maharashtra about 3.01 lakhs HIV cases were documented in 2015 [(156, 157)]. In the present study percentage inhibition reduction of p-24 antigen was about 20%.

6. Fourier Transformer Infra Red Spectroscopy of selected biological immunomodulators:

i) FTIR analysis of DMSO extract of *Chlorophytum borivilianum*:



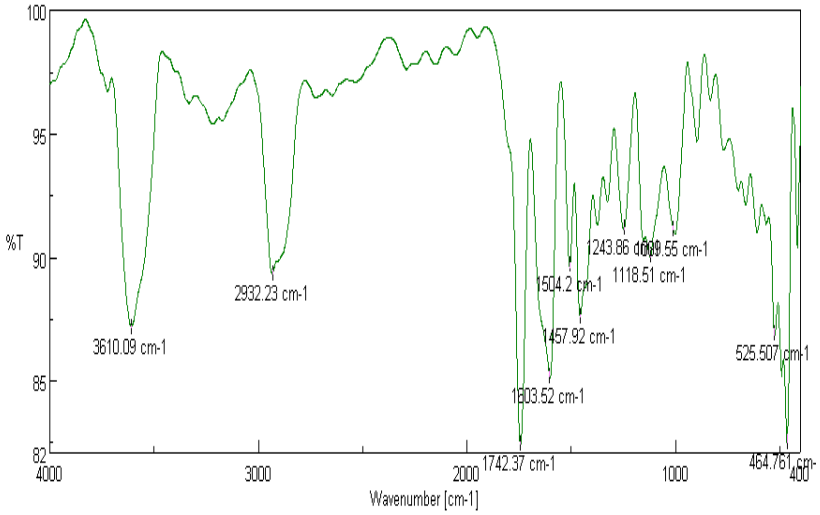
Graph: 1 FTIR analysis of DMSO extract of *Chlorophytum borivilianum.*

Observations: Analysis of peaks showed correlation with functional groups as mentioned in Table 17.

Sr. No.	Prime Peak Wavelength cm^{-1}	Description of Functional Groups
1.	429.084	Alkyl halides C-I stretch
2.	522.615	Bromo alkane, Iodoalkane,alkyl halides C-Br stretch
3.	888.534	Alkene, ≡CH out of plane
4.	931.45	Alkene, ≡CH out of plane
5.	874.56	Benzenes (weak)1,2,3,4,5 pentasubstituted =CH out of plane
6.	1029.8	Amines (C-N Stretch)
7.	1160.94	Ethers (C-O stretch)
8.	1457.921	Aromatic compound,(C=C)Alkanes ($CH_2$and CH_3)
9.	1240.165	Ethers (C-O bond), Alkyl halides (C-F stretch)
10.	1676.8	Amides (C=O stretch),Ketones (C=O stretch) (C=CCOR)
11.	1746.23	Amides (4 ring) (C=O stretch)
12.	2358.52	Triple bonds, P-H bonds
13.	2942.84	Alkanes (C-H stretch), Carboxylic acid (dimer O-H)
14.	3338.18	Carboxylic acid (monomer O-H)
15.	3593.7	Alcohol (O-H stretch)
16.	3739.3	Amide (N-H stretch)

Table-17: Functional group analysis of DMSO extract of *Chlorophytum borivilianum.*

ii) FTIR analysis of DMSO extract of *Wagatia spicata Dalz*:



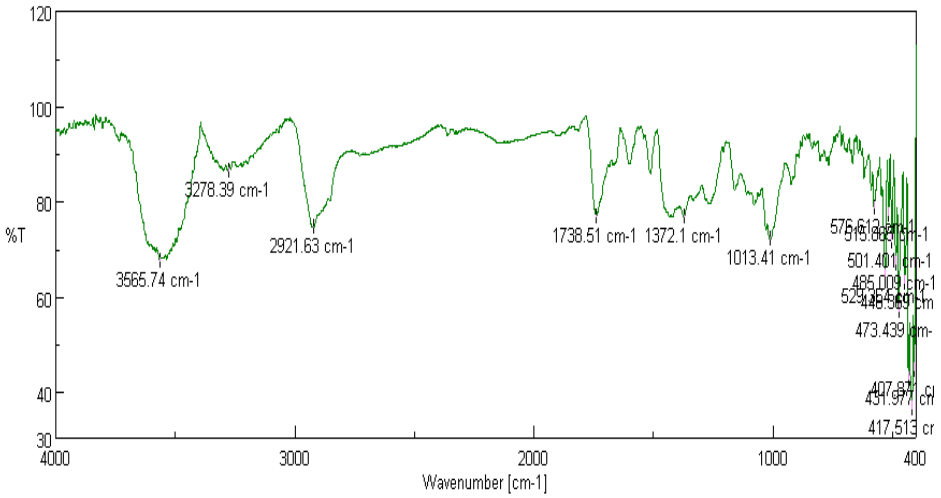
Graph: 2 FTIR analysis of DMSO extract of *Wagatia spicata Dalz.*

Observatins: Analysis of peaks showed correlation with functional groups as mentioned in Table 18.

Sr. No.	Prime Peak Wavelength cm^{-1}	Description of Functional Groups
1.	464.761	Alkyl halides C-I stretch
2.	525.507	Bromo alkane, Iodoalkane, alkyl halides C-Br stretch
3.	1118.51	Ethers (C-O stretch), Alcohol (C-O stretch)
4.	243.86	Alkyl halides C-I stretch, out of range
5.	1504.2	Aromatic (C=C) (usually 3 or 4 bonds)
6.	1457.92	Alkanes ($CH_2$and CH_3)
7.	1803.52	Acyl halides
8.	1742.37	Amides (4 ring, C=O stretch) , Ketone 5 ring (C=O stretch)
9.	2932.23	Alkanes (CH stretch), Carboxilic acid (dimer OH)
10.	3610.09	Alcohol with OH stretch, Carboxylates

Table-18: Functional group analysis of DMSO extract of *Wagatia spicata Dalz.*

iii) FTIR analysis of DMSO extract of *Picrorrhiza kurroa*



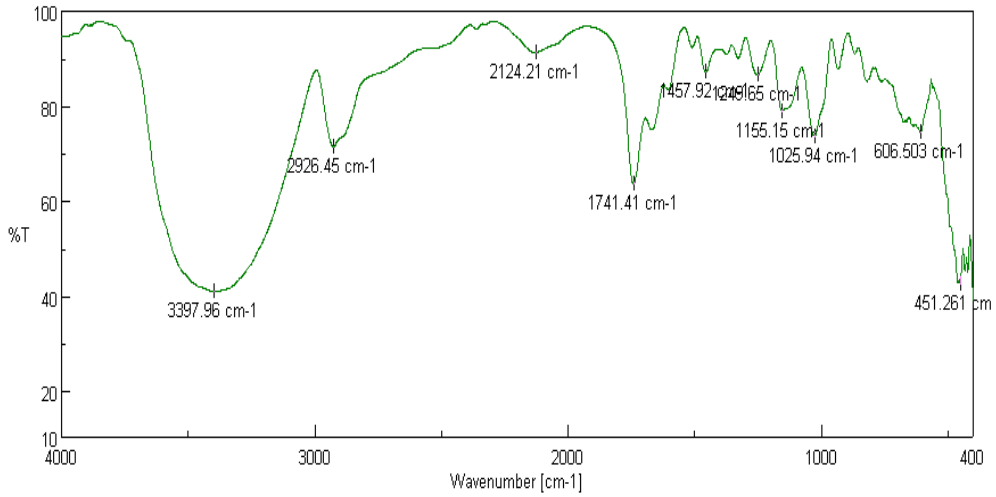
Graph: 3 FTIR analysis of DMSO extract of *Picrorrhiza kurroa*

Observations: Analysis of peaks showed correlation with functional groups as mentioned in Table 19.

Sr. No.	Prime Peak Wavelength cm^{-1}	Description of Functional Groups
1.	417.513	Alkyl Halides, C-I stretch
2.	431.977	Alkyl Halides, C-I stretch
3.	407.87	Alkyl Halides, C-I stretch
4.	473.439	Alkyl Halides, C-I stretch
5.	448.369	Alkyl Halides, C-I stretch
6.	529.364	Alkyl Halides, C-Br stretch, Bromo alkane, Iodoalkane
7.	485.009	Alkyl Halides, C-Br stretch, Bromo alkane, Iodoalkane
8.	501.401	Alkyl Halides, C-Br stretch
9.	515.865	Alkyl Halides, C-Br stretch,
10.	576.613	Alkyl Halides, C-Br stretch,Chloroalaknes
11.	1013.41	Alkyl Halides, C-F stretch,stretching vibrations of (C-OH) side groups and C-O-C glycosidic bond vibrations
12.	1372.1	Nitrates, Nitramines, Carboxyalte ions, Alkanes (CH_2 and CH_3)
13.	1738.51	Esters (C=O stretch) 6 rings, amides 4 rings
14.	2921.63	Alkanes (C-H stretch), Carboxylic acid dimer O-H
15.	3278.39	Acetylenic C-H stretching, Carboxylic acid with dimer O-H
16.	3565.74	O-H and N-H stretching, alcohol (O-H stertch)

Table-19: Functional group analysis of DMSO extract of *Picrorrhiza kurroa*

iv) FTIR analysis of DMSO extract of *Spilanthes paniculata Wall. ex. DC.*:



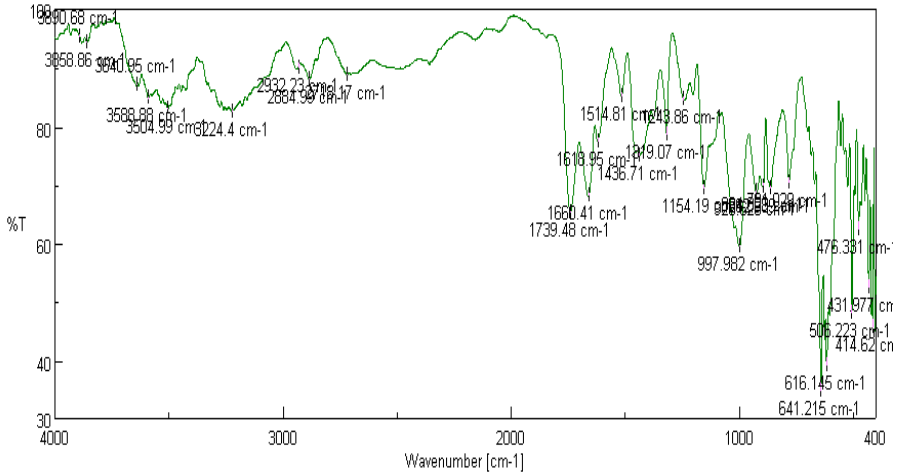
Graph: 4 FTIR analysis of DMSO extract of *Spilanthes paniculata Wall. ex. DC.*

Observations: Analysis of peaks showed correlation with functional groups as mentioned in Table 20

Sr. No.	Prime Peak Wavelength cm^{-1}	Description of Functional Groups
1.	451.261	Alkyl Halides, C-I stretch
2.	606.503	Chloroalkanes, Bromoalkanes, (C-Br stretch)
3.	1025.94	Amines (C-N stretch)
4.	1155.15	Ethers (C-O stretch)
5.	1457.92	Alkanes (CH_2 and CH_3)
6.	1741.41	Ketones 5 rings (C=O stretch)
7.	2124.21	Alkynes (RC≡CH) C≡C stretch
8.	2926.45	Alkanes (C-H stretch), Carboxylic acid (dimer OH)
9.	3397.96	Amides NH stretch (H bond)

Table-20: Functional group analysis of DMSO extract of *Spilanthes paniculata Wall. ex. DC.*

v) FTIR analysis of DMSO extract of *Withania somnifera*:



Graph: 5 FTIR analysis of DMSO extract of *Withania somnifera*

Observations: Analysis of peaks showed correlation with functional groups as mentioned in Table 21.

Sr. No.	Prime Peak Wavelength cm^{-1}	Description of Functional Groups
1.	641.215	Alkyl halides, C-Br stretch
2.	616.145	Alkyl halides, C-Br stretch
3.	414.62	Alkyl halides, C-I stretch
4.	506.223	Alkyl halides, C-Br stretch
5.	431.977	Alkyl halides, C-I stretch
6.	476.331	Alkyl halides, C-I stretch
7.	997.982	Alkenes, =C-H out of plane
8.	1154.19	Alkyl halides, C-F stretch
9.	1243.86	Alkyl halides, C-F stretch
10.	1319.07	Alkyl halides, C-F stretch
11.	1514.81	Nitro compounds
12.	1618.95	Alkenes, 5 ring, C=C stretch
13.	1660.41	Alkenes C=C stretch
14.	2884.90	Alkanes CH stretch
15.	2932.23	Alkanes CH stretch
16.	3224.4	Carboxyliic acid, dimer OH
17.	3504.99	Alcohols OH stretch, Amides NH stretch
18.	3588.88	Amides NH stretch

Table-21: Functional group analysis of *Withania somnifera*

Result:

Functional groups of *Chlorophytum borivilianum*, *Withania somnifera*, *Wagatia spicata Dalz.*, *Picrorrhiza kurroa*, *Spilanthes paniculata Wall. ex. DC.* were studied by using 'Fourier Transformer Infrared Spectroscopy' and their structural relevance is as:

Sr. No.	Name of the biological Immunomodulator	Structural relevance of the functional groups
1.	*Chlorophytum borivilianum*	Alkaloides, Glycosides, Saponine glycosides, Steroides, Tannins, Phenolic compounds.
2.	*Wagatia spicata Dalz*	Flavanoides, Quercitin, Gallic acid.
3.	*Picrorrhiza kurroa*	Irridoid glycosides (Picroside –I , Picroside –II, Kutkoside) and Pikoroside , Veronicoside, Nninecoside, Picein , Androsin, Cucurbitacin glycosides and 4-hydroxy-3-methoxyacetophenone.
4.	*Spilanthes paniculata Wall. ex. DC.*	Alkaloides, Glycosides, Flavanoides, Tannines, Arthraquinones, Saponines, Spilantho (alkamide).
5.	*Withania somnifera*	Withanolides (steroidal lactones with ergostane skeleton) and alkaloides (Withanone,Withaferin A, Withanolides I,II,III), Withasomodienone, Cuscohygrine, Anahygrine, Aanaferine, Isopellatierine.

Table-22: F.T.I.R. Interpretation

Discussion:

Many research scholars including Ramamoorthi et al. (2007), Kareru (2008), Gaurav Kumar (2010), Thangarajan Starlin et al. (2012), Bhanu Raman (2013), R. Ashok Kumar (2014) have reported F.T.I.R. analysis of medicinal plants. Medicinal properties of plants including antimicrobial and immunomodulating are regulated by different functional groups of phytochemical compounds. 'Fourier Transformer Infra Red Spectroscopy' helps to explore the chemical nature of functional groups present in medicinal plants.

In present study 16 different peaks correlated to different chemical groups were observed in DMSO extract of *Chlorophytum borivilianum*, 10 different peaks representing different chemical groups were observed in DMSO extract of *Wagatia spicata Dalz.*, 16 different peaks noted during F.T.I.R. of DMSO extract of

Picrorrhiza kurroa, 9 peaks were observed in DMSO extract of *Spilanthus paniculata Wall.ex. DC.* and maximum of 18 different peaks were reflected through FTIR of DMSO extract of *Withania somnifera*. In case of *Chlorophytum borivilianum* structural relevance of the peaks was with alkaloides, glycosides, saponine glycosides, steroides, tannins, phenolic compounds. In case of *Wagatia spicata Dalz*. structural relevance of the peaks was with flavanoides, quercitin and gallic acid. In case of *Picrorrhiza kurroa* structural relevance of the peaks was with irridoid glycosides (picroside –I, picroside –II, kutkoside) and pikoroside, veronicoside, ninecoside, picein, androsin, cucurbitacin glycosides and 4-hydroxy-3-methoxyacetophenone. In case of *Spilanthes paniculata Wall. ex. DC.* structural relevance of the peaks was with alkaloides, glycosides, flavanoides, tannines, arthraquinones, saponines and spilantho (alkamide). In case of *Withania somnifera*, structural relevance of the peaks was observed with withanolides (steroidal lactones with ergostane skeleton) and alkaloides (withanone, withaferin A, withanolides I, II, III), withasomodienone, cuscohygrine, anahygrine, anaferine and isopellatierine. Exploring functional groups could establish the relationship between the functional groups and immunomodulating properties and may refine the existing system of 'Ayurveda' where active ingredients can be isolated and blended to achieve desired degree of immunomodulation at lower doses and with safer parameters.

7. Acute toxicity testing of selected biological immunomodulators:

Observation:

Following oral administration of blended doses for specified time period, Swiss albino mice were kept under observation for the time interval of 30 min, 1 hr, 2hr, 3hr, 4hr and 24 hr. After 24 hr, daily health record was kept and was extended up to 14 days. Over the period of 14 days, no signs and symptoms of illness were observed.

Result: LD-50 doses for both blended formulations were concluded as above 2000 mg of human dose.

Discussion:

'Acute Toxicity Testing' of selected biological immunomodulators was performed in Swiss albino mice. The test was performed in three different groups having three female animals /group. The test was conducted using oral route as per OECD guidelines 423. Acute oral toxicity refers to unwanted effects or delayed death (14 days) or moribund status following oral administration of dose. Roll R. (1986), Diener W. (1994) and Schlede E (1994) discussed different aspects of 'Acute

Toxicity Testing'. Chan (1994) discussed principles and methods of toxicology. OECD monograph series on 'Testing and Assessment No. 19' recommended the use of female animals for acute toxicity testing. Female animals are highly sensitive and accurately predict potential toxicity of a substance under study. In present study, the test was conducted to judge potential toxicity of blended formulations of biological immunomodulators. Following dosing with biological immunomodulators, test animals were observed for 14 days. No signs and symptoms of illness were observed. 'Limit Test' was also performed giving one dose of 2000 mg/kg and no toxicity was observed.

8. In vivo study of immunomodulating activities of biological immunomodulators:

8.1 Study of 'Carbon Clearance Test':

Time in minutes	Male animals with code tag			Female animals with code tag		
	C1	C2	C3	C4	C5	C6
0	0.899	1.231	0.903	0.882	0.967	0.741
4	0.765	0.777	0.766	1.119	0.745	0.649
8	1.452	1.114	1.016	1.225	0.678	0.588
12	1.215	1.049	1.044	0.995	0.428	0.249
16	0.986	1.156	0.89	Hypovolemic shock	Hypovolemic shock	Hypovolemic shock

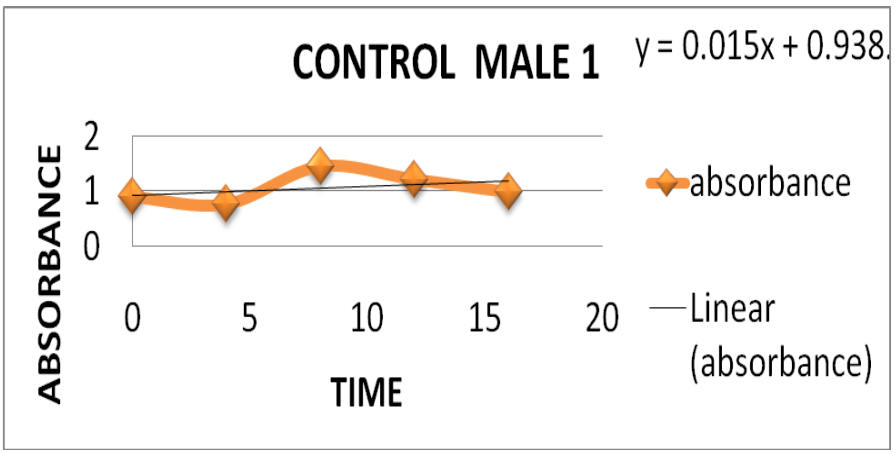
Table-23: Absorbance values for control animal group following 'Carbon Clearance Test'

Time duration in minutes	Male animals with code tag			Female animals with code tag		
	S7	S8	S9	S10	S11	S12
0	0.511	0.817	0.716	0.357	0.547	0.519
4	0.633	0.816	0.798	0.219	0.517	0.643
8	0.452	0.745	0.569	0.874	0.35	0.499
12	0.971	0.573	0.443	0.486	0.046	0.471
16	1.159	1.111	1.335	Hypovolemic shock	Hypovolemic shock	0.466

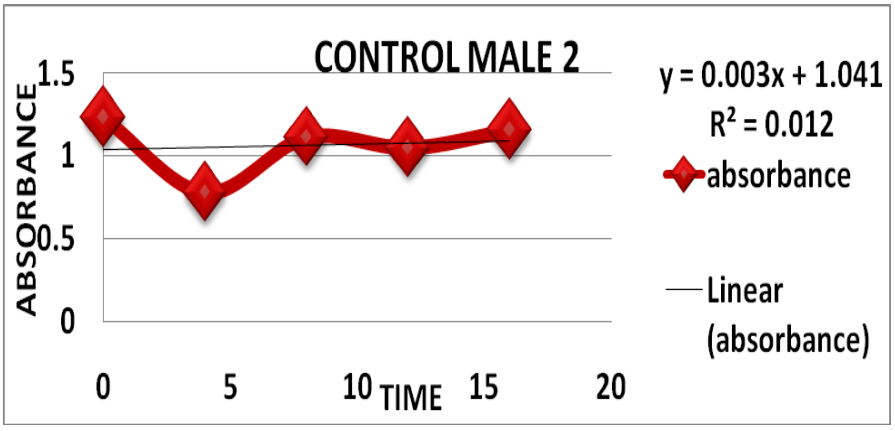
Table-24: Absorbance values for Standard animal group following 'Carbon Clearance Test'

Time duration in minutes.	Male animals with code tag.			Female animals with code tag.		
	T13	T14	T15	T16	T17	T18
0	0.757	1.02	0.963	0.408	0.854	0.838
4	0.717	0.576	1.11	1.229	0.69	0.598
8	1.05	0.78	1.045	0.823	0.411	0.261
12	1.291	1.36	1.117	1.458	Hypovolemic shock	Poor volume of sample
16	1.601	0.846	1.23	1.114	Hypovolemic shock	0.59

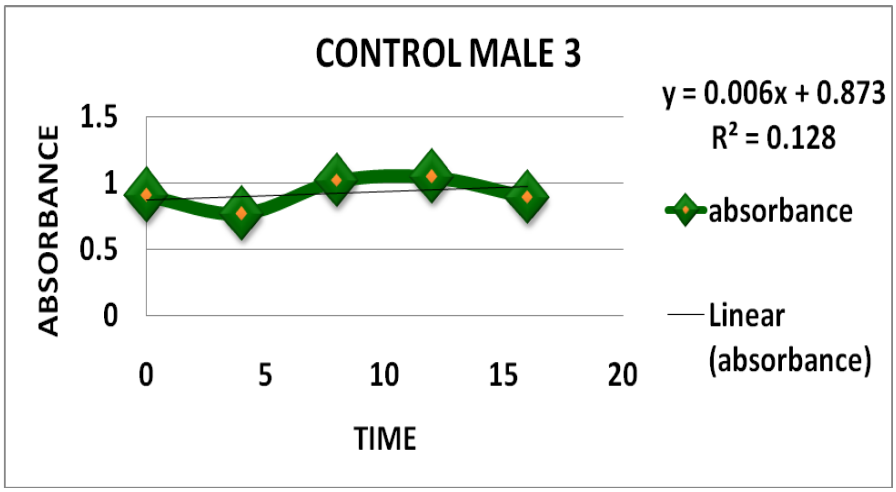
Table-25: Absorbance values for test animal group following 'Carbon Clearance Test'



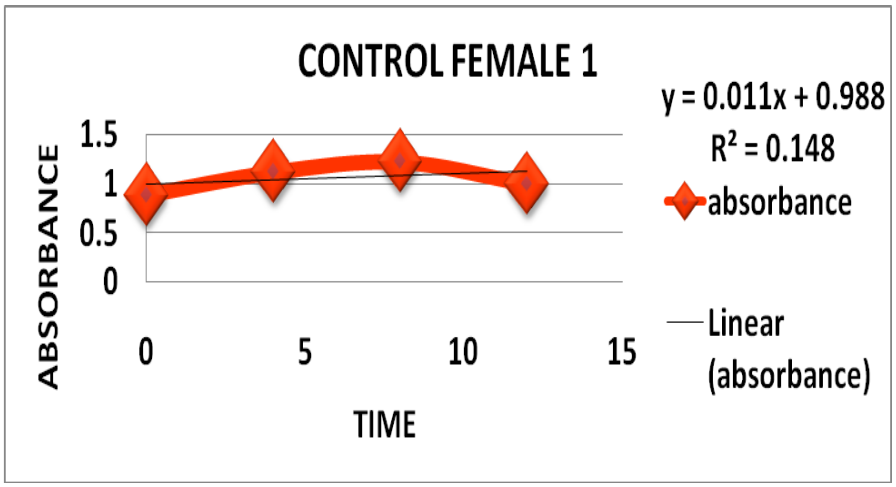
Graph: 6A Absorbance vs. Time: (Control group male no.1)



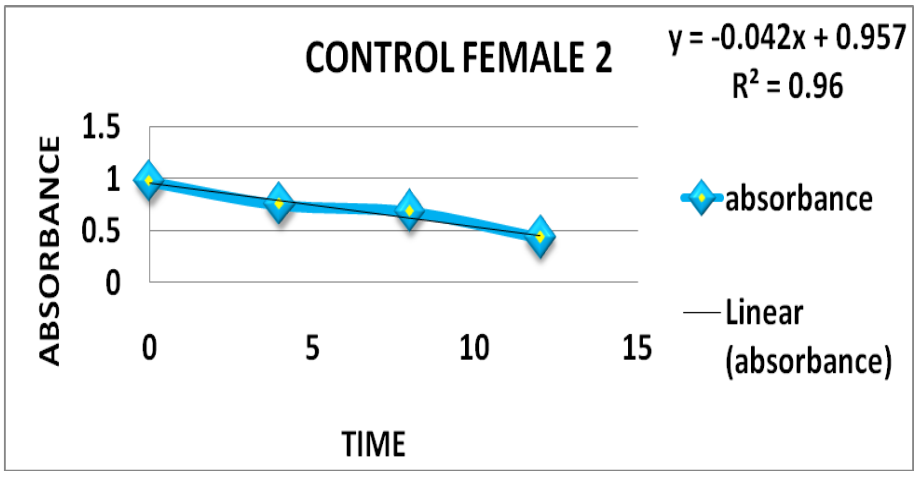
Graph: 6B Absorbance vs. Time: (Control group male no. 2)



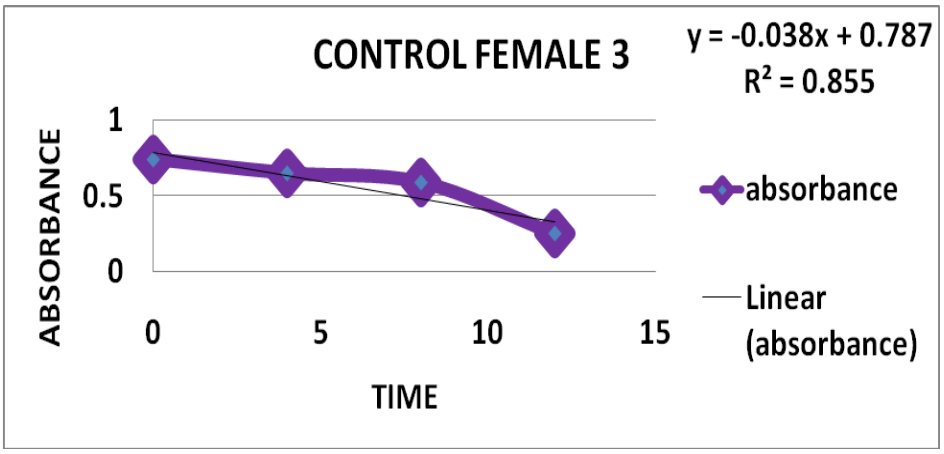
Graph: 6C Absorbance vs. Time: Control group male no. 3)



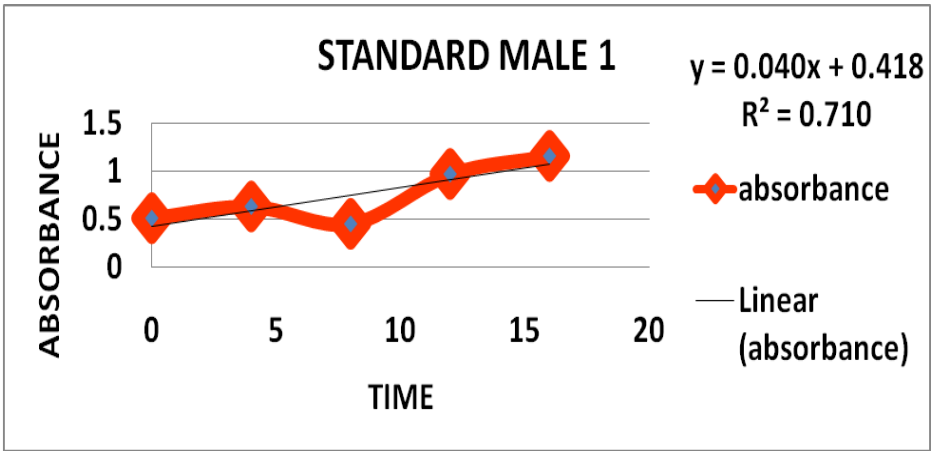
Graph: 7A Absorbance vs. Time: (Control group female no. 1)



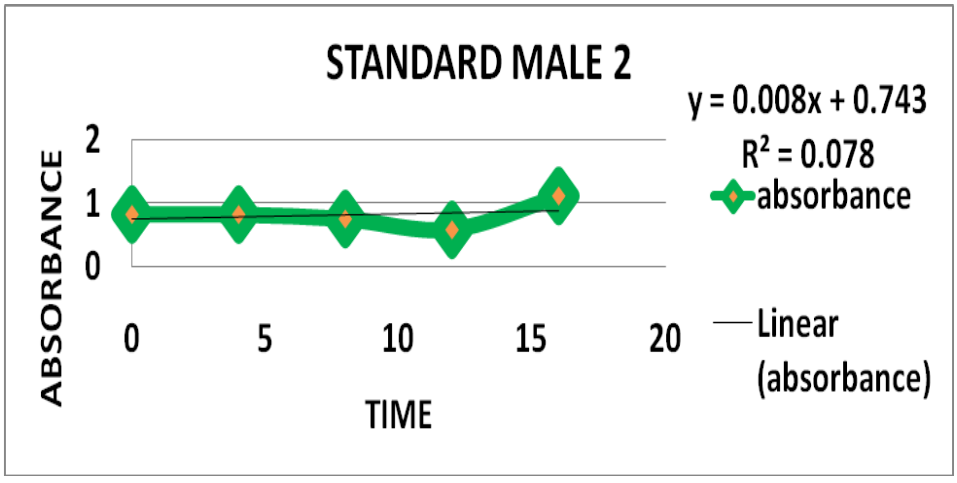
Graph: 7B Absorbance vs. Time: (Control group female no. 2)



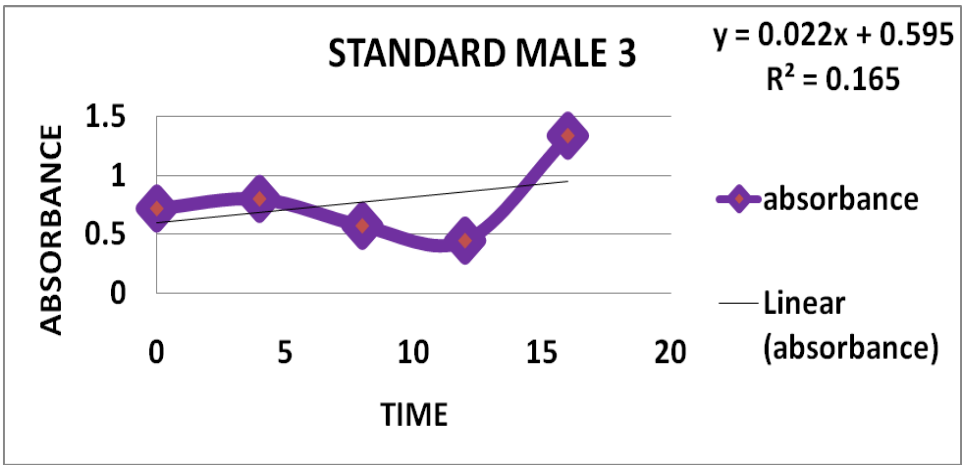
Graph: 7C Absorbance vs. Time: (Control group female no. 3)



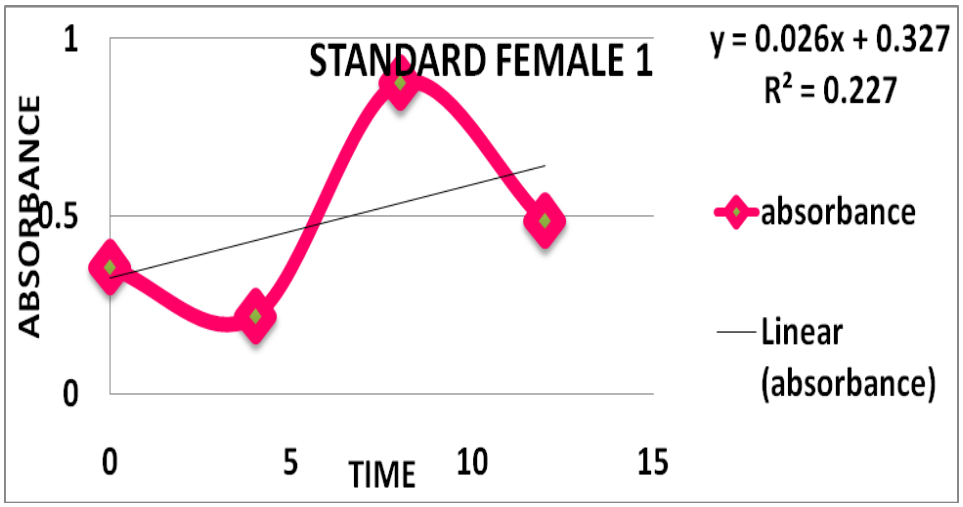
Graph: 8A Absorbance vs. Time: (Standard group male no.1)



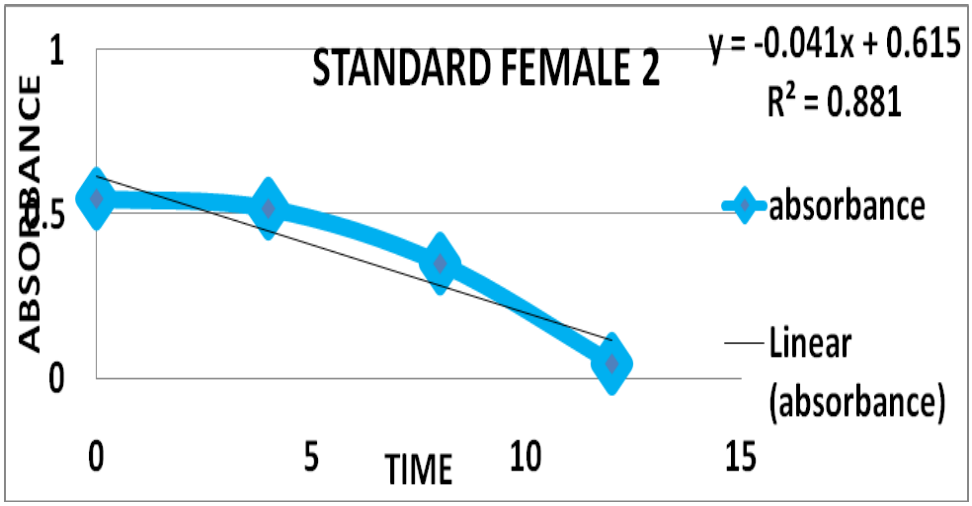
Graph: 8B Absorbance vs. Time: (Standard group male no. 2)



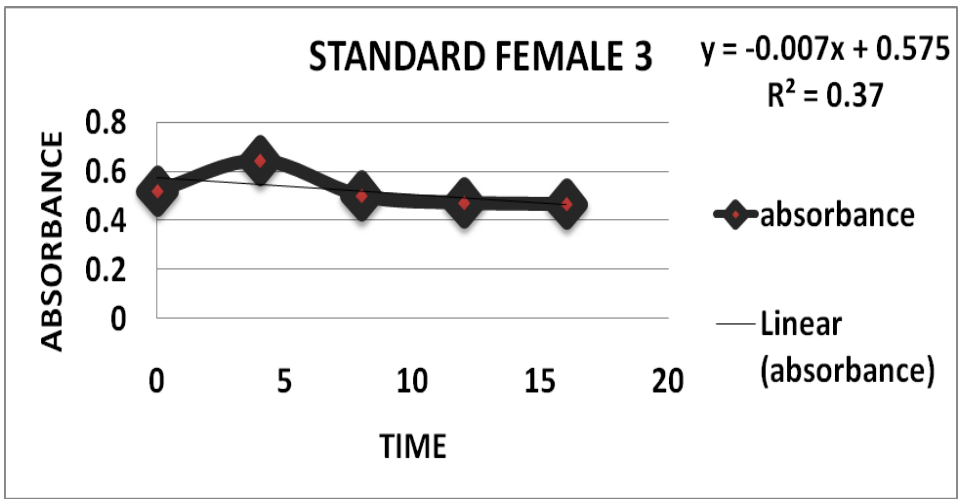
Graph: 8C Absorbance vs. Time: (Standard group male no.3)



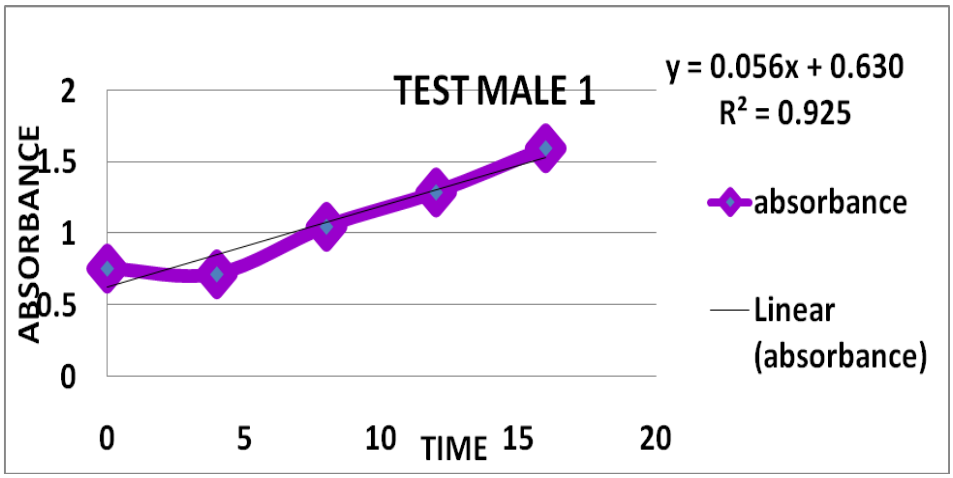
Graph: 9A Absorbance vs. Time: (Standard group female no.1)



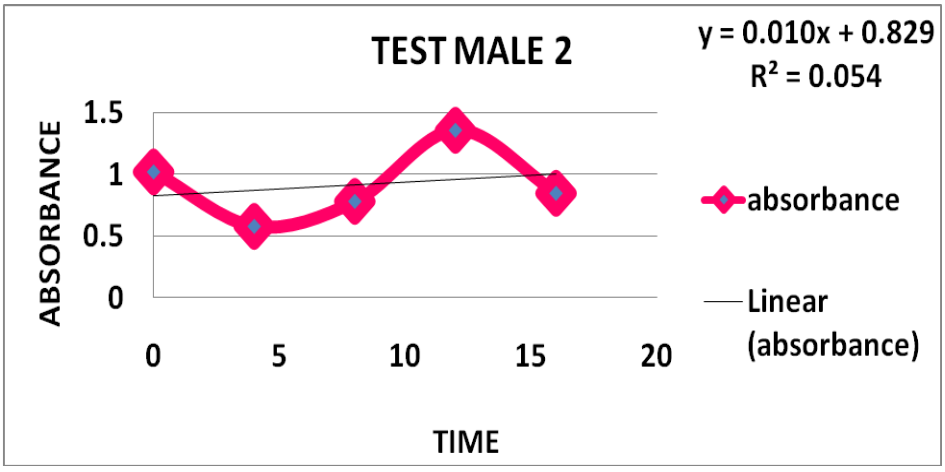
Graph: 9B Absorbance vs. Time: (Standard group female no. 2)



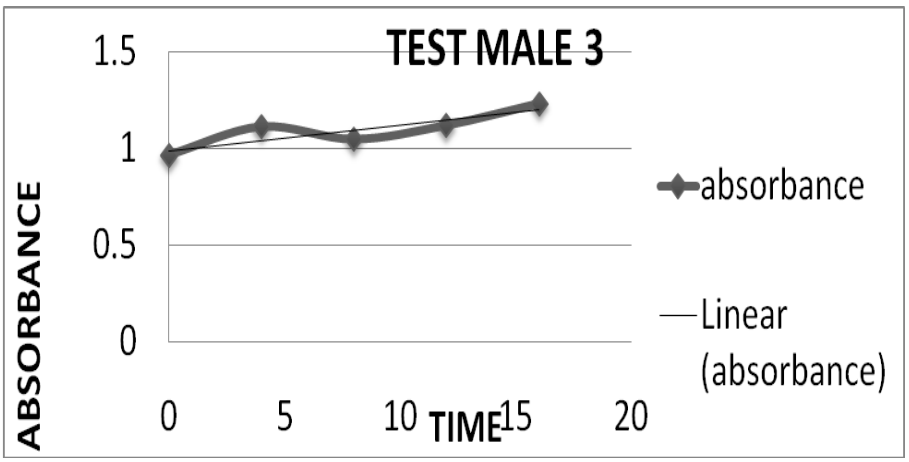
Graph: 9C Absorbance vs. Time: (Standard group female no.3)



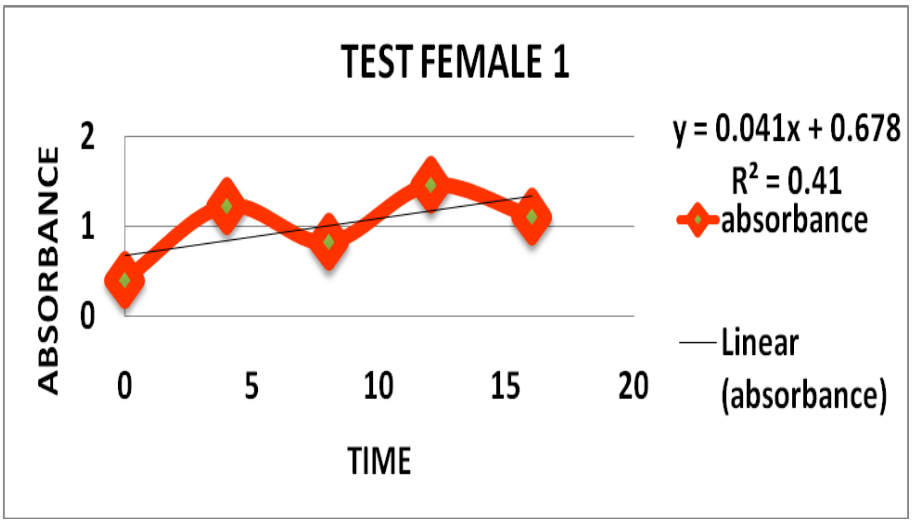
Graph: 10A Absorbance vs. Time: (Test group male no.1)



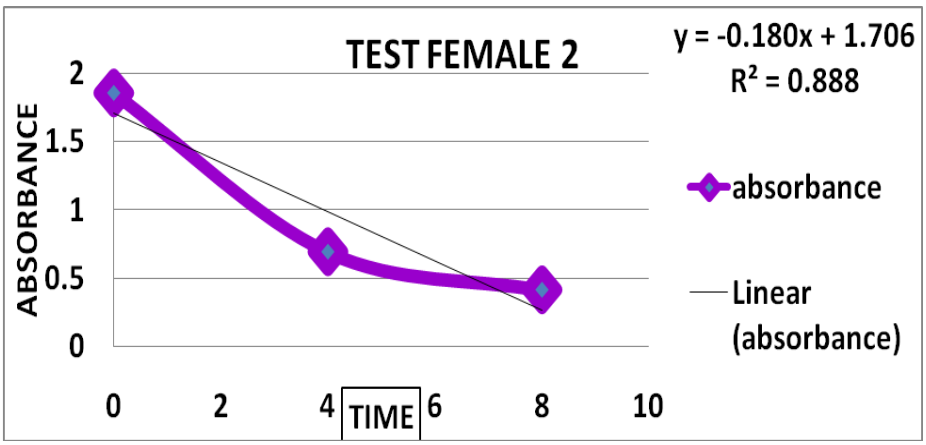
Graph: 10B Absorbance vs. Time: (Test group male no. 2)



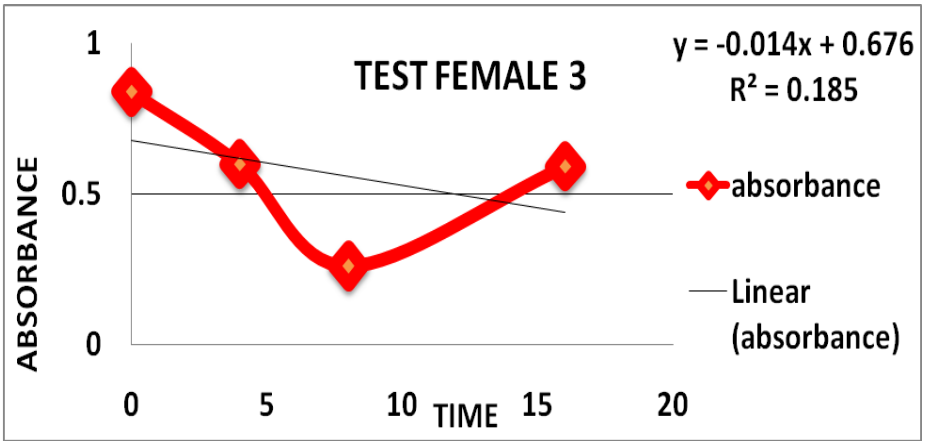
Graph: 10C Absorbance vs. Time: (Test group male no. 3)



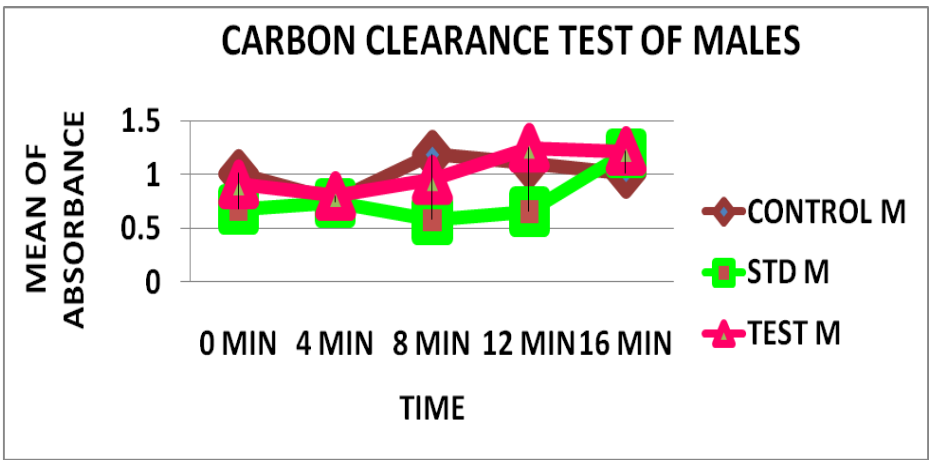
Graph: 11A Absorbance vs. Time: (Test group female no.1)



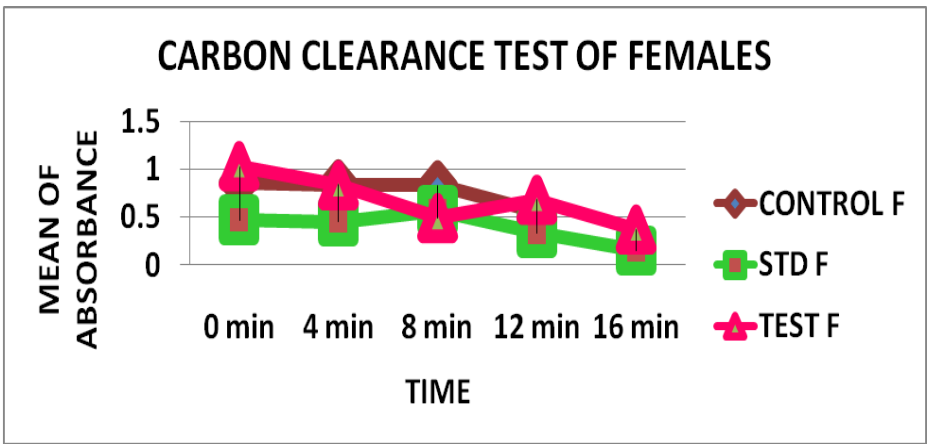
Graph: 11B Absorbance vs. Time: (Test group female no. 2)



Graph: 11C Absorbance vs. Time: (Test group female no. 3)



Graph: 12 Comparative graphs of absorbance vs. time (control, standard and test group male animals)



Graph: 13 Comparative graphs of absorption vs. time (control, standard and test group females)

Animal groups	Slope values for Males with Code Tags			Slope values for females with code tags			Mean slope values
Standard	S7= 0.04085	S8= 0.008625	S9= 0.022075	S10= 0.02605	S11= -0.04175	S12= -0.00695	0.1463
Test	T13= 0.05655	T14= 0.0109	T15= 0.013525	T16= 0.041025	T17= -0.18038	T18= -0.01492	0.3173
Control	C1= 0.0156	C2= 0.00305	C3= 0.0063	C4= 0.011125	C5= -0.0421	C6= -0.03843	0.1166

Table-26: Slope values as calculated from graph by Prism Software for the calculation of phagocytic indices

Title	Phagocytic Index
Control group	1.00
Standard group	1.25
Test group	2.72

Table-27: Result: Phagocytic indices for control, standard and test group animals.

Conclusion:

As compared to control group, phagocytic index of test group showed about threefold increase. This indicated effective removal of foreign substances by phagocytic cells in the group of animals treated with selected biological immunomodulators.

STATISTICAL ANALYSIS OF PHAGOCYTIC INDICES:

A) GROUP WISE ANALYSIS:

Purpose: To study the effect of biological immunomodulators on 'Phagocytic Index' of Swiss albino mice.

H_0: The two groups i.e. standard and test groups are identical. There is no significant difference in the mean ranks of the two groups.

H_1: The two groups are not identical. There is a significant difference in the mean ranks of the two groups.

Level of significance: $\alpha = 0.05$

Statistical Test: Mann-Whitney Test

Test type: 'Non Parametric Test'. Since the distribution of data for the three groups was unknown, the non parametric counterpart of 'One Way ANOVA' was used to

examine whether the three groups are identical. A 'Mann Whitney Test' was conducted to check whether there is a significant difference between mean ranks of Standard and Test group.

'Mann Whitney Test' for standard and test groups:

Ranks				
	Groups	N	Mean Rank	Sum of Ranks
Phagocytic Index	Standard group	6	5.00	30.00
	Test group	6	8.00	48.00
	Total	12		

Test Statistics [b]	
	Phagocytic Index
Mann-Whitney U	9.000
Wilcoxon W	30.000
Z	-1.441
Asymp. Sig. (2-tailed)	0.150
Exact Sig. [2*(1-tailed Sig.)]	0.180[a]
a. Not corrected for ties.	
b. Grouping Variable: Group	

Observations:

Z = -1.441, Mann-Whitney U = 9.000,

P Value (0.150) > 0.05

Since the P value is more than the level of significance we fail to reject the null hypothesis. It is therefore concluded that, there is no significant difference in the mean ranks of 'standard group' and 'test group. Thus, the biological immunomodulators and FDA approved medicines have same effect on 'Phagocytic Index' in Swiss albino mice.

B) GENDER WISE ANALYSIS:

Purpose: Genderwise study the effect of biological immunomodulator on 'Phagocytic Index' of Swiss albino mice.

H_0: The two groups i.e. male and female groups are identical. There is no significant difference in the mean ranks of the two groups.

H_1: The two groups are not identical. There is a significant difference in the mean ranks of the two groups.

Level of significance: $\alpha = 0.05$

Statistical Test: Mann Whitney Test

Test type: 'Non Parametric Test'. Since the distribution of data for the three groups was unknown, the non- parametric counterpart of 'One Way ANOVA' was used to examine whether the three groups are identical. A 'Mann Whitney Test' was conducted to check whether there is a significant difference between mean ranks of 'standard group' and 'test group'.

'Mann Whitney Test' for male and female groups:

Ranks					
		Gender	**N**	**Mean Rank**	**Sum of Ranks**
Phagocytic Index	Dimension -1	Males	6	7.33	44.00
		Females	6	5.67	34.00
		Total	12		

Test Statistics[b]	
Parameter: Phagocytic Index	
Mann Whitney U	13.000
Wilcoxon W	34.000
Z	-0.801
Asymp. Sig. (2-tailed)	0.423
Exact Sig. [2*(1-tailed Sig.)]	0.485[a]
a. Not corrected for ties.	
b. Grouping Variable: gender	

Observations:

Z = -0.801, Mann Whitney U = 13.000,

P Value (0.423) > 0.05

Since the P value is more than the level of significance we fail to reject the null hypothesis. It is therefore concluded that, there is no significant difference in the mean ranks of 'Male group' and Female group'. Thus, the biological immunomodulators have same effect in males and females.

Discussion:

Carbon is a foreign particle hence when injected in experimental animal; the immune system starts its removal. As the injected carbon enters through venous system, it reaches to organs of reticuloendothelial system (RES). Specifically splenic macrophages and Kuffers cells remove major part of injected carbon by the process of phagocytosis. Phagocytic efficiency of the cells of RES is determined by phagocytic index. Smriti Tripathi et al. (2012), Mayank Thakur (2006) reported for 'Carbon Clearance Test' of plant derivatives. In the present study, 'method of Biozzi et al.' modified by Mayank Thakur et al. was used to study the rate of clearance of carbon by phagocytic cells of experimental animals. Control group of Swiss albino mice was not receiving any treatment, standard group was treated with F.D.A. approved medicines 'Cytonini' and 'Cytomaw' and test group was treated with blended formulations of *Chlorophytum borivilianum , Withania somnifera, Wagatia spicata Dalz., Picrorrhiza kurroa, Spilanthus paniculata Wall.ex DC*. Phagocytic index of standard group as calculated from slope values of standard / control, was 1.25. For control group, phagocytic index value was 1.00. Group treated with selected biological immunomodulators (Test group) showed phagocytic index value of 2.72. Thus about threefold increase in phagocytic index was observed in test group. This indicated that, selected biological immunomodulators significantly increased the rate of carbon clearance as reflected through phagocytic indices.

In order to compare the selected biological immunomodulators with F.D.A. approved medicines One Way ANOVA; 'Mann-Whitney' tests were performed. As p value (0.150) > 0.05, it is concluded that both F.D.A. approved medicines and selected biological immunomodulators have same effect on phagocytic index of experimental animals.

8.2 Study of 'Delayed Type of Hypersensitivity Test'

Fig: 8 Diet and water supply to animals as per standard protocol

Fig: 9 Housing of a single group of animals under study

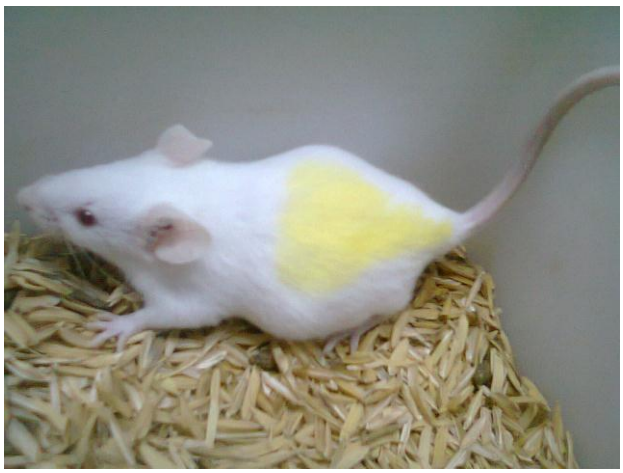


Fig: 10 Animal at the end of study indicating good health status

Fig: 11 Mitutoyo digimatic caliper

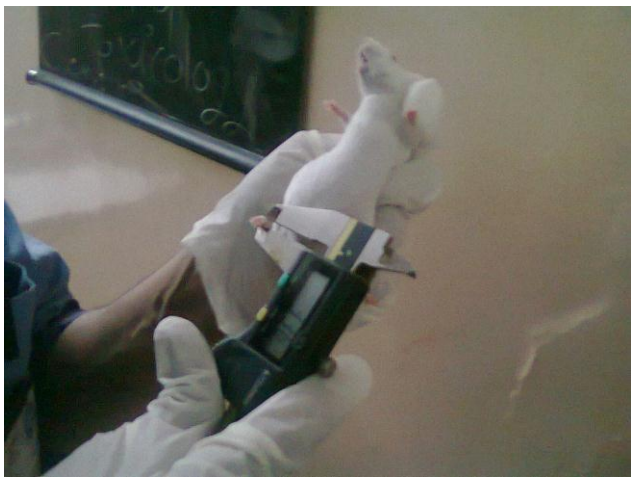


Fig: 12 Measurement of foot pad erythema of the animal under study

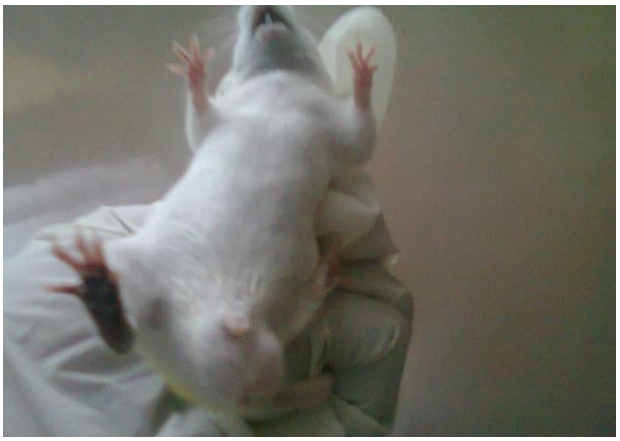


Fig: 13 Foot pad erythema of test animal under study

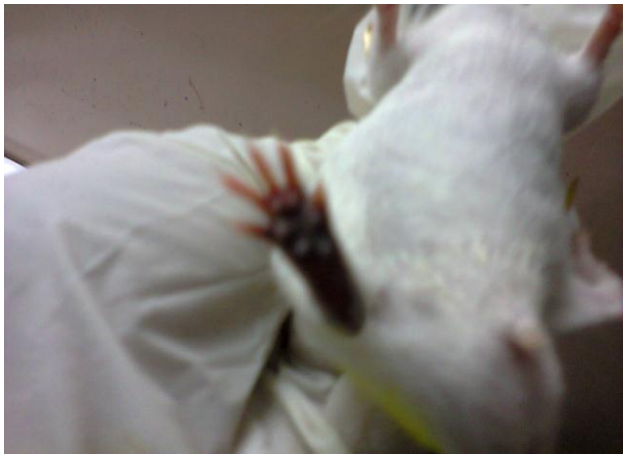


Fig: 14 DTH response in the test animal under study

8.2 Study of 'Delayed Type of Hypersensitivity Test':

Test Animal No.	0 hour		2 hour		Difference *	4 hour		Difference *	6 hour		Difference *	18 hour		Difference *
	Right *	Left *	Right *	Left *		Right *	Left *		Right *	Left *		Right *	Left *	
1	2.4	2.35	2.47	2.42	0.05	2.51	2.49	0.02	2.55	2.52	0.03	2.61	2.58	0.03
2	2.55	2.47	2.61	2.51	0.1	2.59	2.55	0.04	2.7	2.58	0.12	2.75	2.56	0.19
3	2.63	2.49	2.69	2.54	0.15	2.72	2.61	0.11	2.71	2.59	0.12	2.7	2.52	0.18
Summation					**0.3**	**Summation**		**0.17**	**Summation**		**0.27**	**Summation**		**0.4**
Mean					**0.1**	**Mean**		**0.0566**	**Mean**		**0.09**	**Mean**		**0.1333**
4	2.49	2.53	2.59	2.58	0.01	2.54	2.49	0.05	2.58	2.49	0.09	2.65	2.58	0.07
5	2.19	2.28	2.49	2.35	0.14	2.28	2.17	0.11	2.26	2.2	0.06	2.48	2.44	0.04
6	2.5	2.56	2.56	2.46	0.1	2.56	2.53	0.03	2.59	2.56	0.03	2.57	2.43	0.14
Summation					**0.25**	**Summation**		**0.19**	**Summation**		**0.18**	**Summation**		**0.25**
Mean					**0.083**	**Mean**		**0.0633**	**Mean**		**0.06**	**Mean**		**0.0833**

Table-28: DTH: Erythema in control group

*** All values indicated in the table are in mm.**

Test Animal No.	0 hour		2 hour		Diffe-rence *	4 hour		Differ-ence *	6 hour		Differ-ence *	18 hour		Differ-ence *
	Right *	Left *	Right *	Left *		Right *	Left *		Right *	Left *		Right *	Left *	
7	2.46	2.48	2.6	2.5	0.1	2.64	2.53	0.11	Note: Erythema subsided after 4 hrs.					
8	2.33	2.3	2.45	2.35	0.1	2.57	2.4	0.17						
9	2.54	2.51	2.65	2.54	0.11	2.71	2.61	0.1						
Summation					**0.31**	**Summation**		**0.38**						
Mean					**0.1033**	**Mean**		**0.1266**						
10	2.51	2.5	2.57	2.37	0.2	2.63	2.42	0.21						
11	2.27	2.3	2.36	2.33	0.03	2.45	2.39	0.06						
12	1.96	1.98	2.06	2.0	0.06	2.15	2.11	0.04						
Summation					**0.29**	**Summation**		**0.31**						
Mean					**0.096**	**Mean**		**0.1033**						

Table-29: DTH: Erythema in standard group

*** All values indicated in the table are in mm**

Test Animal No.	0 hour		2 hour		Difference *	4 hour		Difference *	6 hour		Difference *	18 hour		Difference *
	Right *	Left *	Right *	Left *		Right *	Left *		Right *	Left *		Right *	Left *	
13	2.03	2.09	2.82	2.13	0.69	2.9	2.2	0.7	3.01	2.31	0.7	3.25	2.39	0.86
14	1.9	1.87	2.01	1.95	0.06	2.42	2.12	0.3	2.56	2.24	0.32	2.89	2.28	0.61
15	1.95	1.92	2.15	2.05	0.1	2.93	2.22	0.71	3.03	2.31	0.72	3.36	2.36	1.0
Summation					**0.85**	**Summation**		**1.71**	**Summation**		**1.74**	**Summation**		**2.47**
Mean					**0.2833**	**Mean**		**0.57**	**Mean**		**0.58**	**Mean**		**0.82**
16	2.35	2.49	2.62	2.51	0.11	2.7	2.6	0.1	2.82	2.68	0.14	2.96	2.71	0.25
17	2.01	2.07	2.95	2.36	0.59	3.01	2.45	0.56	3.25	2.57	0.68	3.49	2.62	0.87
18	2.36	2.39	2.93	2.44	0.49	3.06	2.69	0.37	3.32	2.75	0.57	3.62	2.8	0.82
Summation					**1.19**	**Summation**		**1.03**	**Summation**		**1.39**	**Summation**		**1.94**
Mean					**0.396**	**Mean**		**0.343**	**Mean**		**0.463**	**Mean**		**0.646**

Table-30: DTH: Erythema in test group

STATISTICAL ANALYSIS OF D.T.H. TEST:

A) GROUP WISE ANALYSIS:

Purpose: To study the effect of 'Biological Immunomodulators' on the activity of T_4 lymphocytes in terms of 'Delayed Type of Hypersensitivity (D.T.H.)' in 'Swiss albino mice'.

H_0: The three groups i.e. control, standard and test groups are identical. There is no significant difference in the mean ranks of the three groups.

H_1: The three groups i.e. control, standard and test groups are not identical. There is a significant difference in the mean ranks of the three groups.

Level of significance: $\alpha = 0.05$

Statistical Test: 'Kruskal Wallis Test'

Test type: 'Non Parametric Test'. Since the distribution of data for the three groups was unknown, the non- parametric counterpart of **'One Way ANOVA'** was used to examine whether the three groups are identical.

Kruskal-Wallis Test for DTH:

Ranks:

Groups	No. of Animals	Mean Rank
Control group	6	6.50
Standard group	6	6.67
Test group	6	15.33
Total	18	-

Test Statistics [a, b]	
Parameter: Erythema Difference DTH	
Chi-square	10.771
Df	2
Asymp. Sig.	**0.005**
a. Kruskal Wallis Test b. Grouping Variable: Groups	

Observations:

χ^2 (2) = 10.771,

p value (0.005) < 0.05

Since the P - value is less than the level of significance we reject the null hypothesis. It is therefore concluded that, the three groups are not identical and there is a difference in mean ranks of the three groups. In order to find out where the difference lies that is, which group is affected, a 'Post-hoc Test' was performed. In order to conduct Post-hoc Test, standard group and test group were compared with the control group. We therefore get the two conditions:

A: Standard vs. Control.

B: Test vs. Control.

Since, the Post-hoc results are in two tests we no longer use α as 0.05. Instead, 0.05/2 = 0.025 is used as the level of significance.

'MANN WHITNEY TEST' FOR 'CONTROL GROUP' AND 'STANDARD GROUP':

A Mann Whitney Test was conducted to check whether there is a significant difference between mean ranks of control group and standard group.

Ranks				
Groups		**Animal No.**	**Mean Rank**	**Sum of Ranks**
1.	Control group	6	6.50	39.00
2.	Standard group	6	6.50	39.00
	Total	12		

Test Statistics[b]	
Parameter: Erythema Difference DTH	
Mann Whitney U	18.000
Wilcoxon W	39.000
Z	0.000
Asymp. Sig. (2-tailed)	**1.000**
Exact Sig. [2*(1-tailed Sig.)]	1.000[a]
a. Not corrected for ties.	
b. Grouping Variable: groups	

Observations:

Z = 0.000, Mann Whitney U = 18.000,

p value (1.000) > 0.025

Since the P value is more than the level of significance we fail to reject the null hypothesis. It is therefore concluded that, there is no significant difference in the mean ranks of control group and standard group. Thus, the standard medicine therapy has no significant effect on 'Delayed Type of Hypersensitivity' (DTH) in 'Swiss albino mice'.

'MANN-WHITNEY TEST' FOR 'CONTROL GROUP' AND 'TEST GROUP':

A Mann-Whitney Test was conducted to check whether there is a significant difference between mean ranks of 'Control group' and Test group'.

Ranks				
Parameter	**Groups**	**Animal No.**	**Mean Rank**	**Sum of Ranks**
Erythema Difference DTH	Control group	6	3.50	21.00
	Test group	6	9.50	57.00
	Total	12		

Test Statistics [b]	
Parameter	**Erythema Difference DTH**
Mann-Whitney U	0.000
Wilcoxon W	21.000
Z	-2.882
Asymp. Sig. (2-tailed)	**0.004**
Exact Sig. [2*(1-tailed Sig.)]	0.002[a]
a. Not corrected for ties.	
b. Grouping Variable: groups	

Observations:

Z = 0.000, Mann Whitney U = 0.000,

p value (0.004) < 0.025

Since the P - value is less than the level of significance we reject the null hypothesis. It is therefore concluded that, there is a significant difference in the mean ranks of control group and test group. Mean Rank of test group is 9.50 while, Mean Rank of control group is 3.50. Thus, the synergistic action of *Chlorophytum borivilianum , Withania somnifera, Wagatia spicata Dalz, Picrorrhiza kurroa and Spilanthes paniculata Wall. ex. DC.* have significant effect on population of T_4 lymphocytes in terms of 'Delayed Type of Hypersensitivity' response in 'Swiss albino mice'.

A) GENDER WISE ANALYSIS:

Purpose: Gender wise study of the effect of 'Biological Immunomodulators' on activity of T_4 lymphocytes in terms of 'Delayed Type of Hypersensitivity' (DTH) in 'Swiss albino mice'.

H_0: The two groups i.e. male and female groups are identical. There is no significant difference in the mean ranks of the two groups.

H_1: The two groups are not identical. There is a significant difference in the mean ranks of the two groups.

Level of significance: α =0.05

Statistical Test: Mann Whitney Test

Test type: 'Non Parametric Test'. Since the distribution of two groups is not known, non parametric counterpart of independent sample t-test has been conducted.

A 'Mann Whitney Test' was conducted to check whether there is a significant difference between mean ranks of male and female groups.

'Man Whitney Test' for male and female groups with respect to erythema difference observed during DTH:

Ranks				
Parameter	**Gender**	**No. of Animals**	**Mean Rank**	**Sum of Ranks**
Erythema Difference during DTH	Male	9	9.72	87.50
	Female	9	9.28	83.50
	Total	18		

Test Statistics [b]	
Parameter	**Erythema difference (DTH)**
Mann-Whitney U	38.500
Wilcoxon W	83.500
Z	-0.177
Asymp. Sig. (2-tailed)	**0.860**
Exact Sig. [2*(1-tailed Sig.)]	0.863[a]
a. Not corrected for ties.	
b. Grouping Variable: Gender	

Observations :

Z = -0.177, Mann Whitney U = 38.500,

p value (0.860) > 0.05

Since the p value is more than the level of significance we fail to reject the null hypothesis. It is therefore concluded that, there is no significant difference in the mean ranks of male group and female group. Thus, the biological immunomodulators have no significant gender wise variation with respect to effect on 'Delayed Type of Hypersensitivity' in Swiss albino mice and have same impact in males and females.

Discussion:

'Delayed Type of Hypersensitivity' or 'Type IV Hypersensitivity' is a function of cellular immunity [(2, 30, 138)] and is commonly used to assess T lymphocyte function. The hypersensitivity involves the 'Antigen presenting cells' like 'Langerhans cells' which receives the hapten from region like skin and present the same to 'T lymphocytes' of nearest 'lymph node'. It is followed by the cytokine secretion by T lymphocytes which trigger the delayed type of hypersensitivity [(2)]. Many types of human malignancies, specifically metastatic malignancies show downregulation or anergy (non responsiveness) of APCs like 'Follicular Dendritic Cell' and it is the basis of 'Follicular Dendritic Cell Therapy' for cancers. Thus the delayed type of hypersensitivity not only assesses the T lymphocytes function but also gives inkling about functional aspects of APCs. In HIV infected individuals, prime target of the virus is CD_4 cells or 'T_4 lymphocytes' and a good chemotherapy is expected to

improve the function of 'T_4 lymphocytes' and 'APCs'. This was the rationale for conducting this test in experimental animals.

In present study, selected biological immunomodulators were tested in Swiss albino mice for potential 'T_4 lymphocyte activating properties' through DTH. Present study was conducted in three groups of Swiss albino mice, each carrying three males and three females. Control group was not receiving any treatment, standard group was receiving the the therapy of FDA approved drugs and test group was treated with the biological immunomodulators under study. Using standard protocols, DTH response was tested in all groups. As the distribution data was unknown for selected group, non parametric counter part of 'One Way ANOVA' was used followed by 3 different tests such as 'Kruskal Wallis Test' (p value < 0.05), Post hoc test and Man Whitney Test (p value < 0.025). The statistical analysis revealed significant effect on 'T_4 lymphocytes' in terms of DTH response in Swiss albino mice (p value < 0.004). The study consolidated the use of plants as 'biological immunomodulators'.

8.3 Study of 'Neutrophil Adhesion Test':

Sample No.	Group	Absolute Neutrophil	% Neutrophil
1.	Control male	1,092	14
2.	Control male	1,680	16
3.	Control male	900	12
4.	Control female	406	14
5.	Control female	732	12
6.	Control female	200	05
7.	Standard male	14,457	61
8.	Standard male	Haemolysed sample	Haemolysed sample
9.	Standard male	1,456	26
10.	Standard female	520	08
11.	Standard female	1,404	54
12.	Standard female	900	30
13.	Test male	1,280	32
14.	Test male	1,421	29
15.	Test male	840	21
16.	Test female	500	20
17.	Test female	924	33
18.	Test female	1,134	14

Table-31: Absolute and percent neutrophil count in experimental animals

STATISTICAL ANALYSIS OF 'NEUTROPHIL ADHESION TEST:

Purpose: To study the effect of biological immunomodulators on 'Percent Neutrophil count' of white albino mice.

A) GROUP WISE ANALYSIS:

H_0: The three groups i.e. control, standard and test groups are identical. There is no significant difference in the mean ranks of the three groups.

H_1: The three groups are not identical. There is a significant difference in the mean ranks of the three groups.

Level of significance: α =0.05

Statistical Test: Kruskal-Wallis Test

Test type: 'Non Parametric Test.' Since the distribution of data for the three groups was unknown, the non parametric counterpart of 'One Way ANOVA' was used to examine whether the three groups are identical.

Kruskal-Wallis Test:

Ranks			
Parameter	**Groups**	**No. of Animals**	**Mean Rank**
Percent Neutrophil	Control group	6	4.67
	Standard group	5	11.80
	Test group	6	11.00
	Total	17	-

Test Statistics [a, b]	
Parameter	**Percent Neutrophil**
Chi-square	6.939
Df	2
Asymp. Sig.	**0.031**
a. Kruskal Wallis Test	
b. Grouping Variable: Groups	

Observations:

χ^2 (Z) = 6.939,

p value (0.031) < 0.05

Since the p value is less than the level of significance the null hypothesis is rejected. Thus it is concluded that, the three groups were not identical and there is a significant difference in the mean ranks of the three groups. In order to find out where the difference lies that is, which group is benefitted, we performed a 'Post-Hoc Test'. In order to conduct 'Post-Hoc Test', standard group and test group were compared with the control group. We therefore get the following two conditions

A: Standard Vs Control.

B: Test Vs Control.

Since, the 'Post-Hoc' results in two tests, we no longer use α as 0.05. Instead, 0.05/2 = 0.025 is used as the level of significance.

Mann Whitney Test for Control group and Standard group:

A 'Mann Whitney Test' was conducted to check whether there is a significant difference between mean ranks of control group and standard group.

'Mann Whitney Test' for 'Control group' and 'Standard group':

Ranks				
Parameter	**Groups**	**No. of Animals**	**Mean Rank**	**Sum of Ranks**
Percent Neutrophil	Control group	6	4.33	26.00
	Standard group	5	8.00	40.00
	Total	11		

Test Statistics [b]	
Parameter	**Percent Neutrophil**
Mann-Whitney U	5.000
Wilcoxon W	26.000
Z	-1.834
Asymp. Sig. (2-tailed)	**0.067**
Exact Sig. [2*(1-tailed Sig.)]	0.082[a]
a. Not corrected for ties.	
b. Grouping Variable: Groups	

Observations:

Z = -1.834, Mann Whitney U = 5,

p value (0.067) > 0.025

Since the p value is more than the level of significance, we fail to reject the null hypothesis. It is therefore concluded that, there is no significant difference in the mean ranks of control group and standard group. Thus, the standard medicine therapy has no significant effect on 'percent neutrophil count' in albino mice.

'Mann Whitney Test' for 'Control group' and 'Test group':

A 'Mann Whitney Test' was conducted to check whether there is a significant difference between mean ranks of control group and test group.

Ranks				
Parameter	**Groups**	**No. of animals**	**Mean rank**	**Sum of ranks**
Percent Neutrophil	Control group	6	3.83	23.00
	Test group	6	9.17	55.00
	Total	12		

Test Statistics [b]	
Parameter	**Percent Neutrophil**
Mann-Whitney U	2.000
Wilcoxon W	23.000
Z	-2.585
Asymp. Sig. (2-tailed)	**0.010**
Exact Sig. [2*(1-tailed Sig.)]	0.009[a]
a. Not corrected for ties.	
b. Grouping Variable: Groups	

Observations:

Z = -2.585, Mann-Whitney U = 2,

p value (0.010) < 0.025

Since the p value is less than the level of significance, we reject the null hypothesis. It is therefore concluded that, there is a significant difference in mean ranks of control

group and test group. Thus, biological immunomodulators have significant effect on percent neutrophil count in albino mice.

B) GENDER WISE ANALYSIS:

Purpose: Genderwise study the effect of biological immunomodulators on Percent Neutrophil count of 'Swiss albino mice'.

H_0: There is no significant difference in the mean ranks of male and female Swiss albino mice.

H_1: There is a significant difference in the mean ranks of male and female Swiss albino mice.

Level of significance: $\alpha = 0.05$

Statistical Test: Mann Whitney U Test

Test type: 'Non Parametric Test'. Since the distribution of two groups is not known, non parametric counterpart of independent sample t- test has been conducted.

Mann Whitney Test:

Ranks					
Parameter	**Gender**		**No. of animals**	**Mean rank**	**Sum of ranks**
Percent Neutrophil	Dimension 1	Male	8	10.19	81.50
		Female	9	7.94	71.50
		Total	17		

Test Statistics [b]	
Parameter	**Percent Neutrophil**
Mann-Whitney U	26.500
Wilcoxon W	71.500
Z	-0.917
Asymp. Sig. (2-tailed)	**0.359**
Exact Sig. [2*(1-tailed Sig.)]	0.370[a]
a. Not corrected for ties.	
b. Grouping Variable: Gender	

Descriptive Statistics					
	No. of Animals	Mean	Std. Deviation	Minimum	Maximum
Percent Neutrophil	17	23.59	15.350	5	61
Gender variable	18	1.50	0.514	1	2

Observations:

Z = -0.917, Mann Whitney U = 26.500,

p value (0.359) > 0.05

Since the p value is more than the level of significance, we fail to reject the null hypothesis. It is therefore concluded that there is no difference in mean ranks of both the groups. Thus, biological immunomodulators have same effect in males and females with respect to effect on 'Percent Neutrophil Count' in Swiss albino mice.

Animal Groups	Hb	Platelets / µl	RBC X 10^6 / µl	PCV	MCV	MCH	MCHC
Control male	12.30	1005000	7.30	41.90	57.30	16.90	29.50
Control male	13.10	705000	7.96	46.80	58.80	16.50	28.10
Control male	12.40	1117000	7.11	42.80	60.20	17.40	28.90
Control female	12.90	806000	7.40	45.00	60.90	17.40	28.60
Control female	13.80	743000	7.41	47.90	64.70	18.70	28.90
Control female	12.20	1000000	7.26	41.60	57.40	16.80	29.40
Standard male	10.80	761000	6.70	36.60	54.60	16.00	29.40
Standard male	*H	*H	*H	*H	*H	*H	*H
Standard male	14.00	1117000	8.87	43.00	48.50	15.80	32.70
Standard female	12.60	976000	6.96	42.40	60.90	18.10	29.70
Standard female	13.10	1128000	8.10	40.20	49.70	16.10	32.50

Animal Groups	Hb	Platelets / µl	RBC X 10^6 / µl	PCV	MCV	MCH	MCHC
Standard female	14.00	1023000	8.95	44.30	49.50	15.60	31.60
Test male	12.80	790000	8.28	42.20	50.90	15.50	30.40
Test male	14.70	945000	8.84	48.80	55.30	16.60	30.10
Test male	14	1735000	8.56	48.00	56.10	16.40	29.20
Test female	13	978000	8.10	43.80	54.10	16.10	29.70
Test female	10.30	1158000	6.82	36.30	53.30	15.10	28.30
Test female	13.50	831000	8.50	46.50	54.70	15.90	29.10

*Note: H in the observation table stands for haemolysed sample.

Table-32: Haematological parameters of experimental animals as observed during 'Neutrophil Adhesion Test'.

STATISTICAL ANALYSIS OF HAEMATOLOGICAL PARAMETERS OF EXPERIMENTAL ANIMALS FOLLOWING 'NEUTROPHIL ADHESION TEST':

A) GROUPWISE ANALYSIS OF RBCs:

Purpose: To study the effect of biological immunomodulators on RBC count of Swiss albino mice.

H_0: The three groups i.e. control, standard and test groups are identical. There is no significant difference in the mean ranks of the three groups.

H_1: The three groups are not identical. There is a significant difference in the mean ranks of the three groups.

Level of significance: α **= 0.05**

Statistical Test: Kruskal-Wallis Test

Test type: Non Parametric Test. Since the distribution of data for the three groups was unknown, the non- parametric counterpart of 'One Way ANOVA' was used to examine whether the three groups are identical.

Kruskal –Wallis Test:

Ranks (RBC in millions/µl):

Groups	No. of Animals	Mean Rank
Control group	6	6.50
Standard group	5	9.50
Test group	6	11.08
Total	17	-

Test Statistics [a, b]

RBC in millions/µl	
Chi-square	2.544
Df	2.0
Asymp. Sig.	**0.280**

a. Kruskal Wallis Test

b. Grouping variable: Groups

Observations:

χ^2 (2) = 2.544, p value (0.280) > 0.05

Since the p value is more than the level of significance we fail to reject the null hypothesis. It is therefore concluded that, the three groups are identical and there is no difference in mean ranks of the three groups. Thus the biological immunomodulators have no significant effect on RBC count of Swiss albino mice.

B) GENDER WISE ANALYSIS OF RBCs:

Purpose: Genderwise study of the effect of biological immunomodulators on RBC count of 'Swiss albino mice'.

H_0: There is no significant difference in the mean ranks of male and female 'Swiss albino mice'.

H_1: There is a significant difference in the mean ranks of male and female 'Swiss albino mice'.

Level of significance: $\alpha = 0.05$

Statistical Test: Mann Whitney U Test

Test type: 'Non Parametric Test'. Since the distribution of two groups is not known, non parametric counterpart of independent sample t-test has been conducted.

Mann Whitney Test:

Ranks:

Gender	No. of animals	Mean rank	Sum of ranks
Male	8	9.63	77.00
Female	9	8.44	76.00
Total	17		

Test Statistics [b]

RBCs in millions/µl	
Mann Whitney U	31.00
Wilcoxon W	76.00
Z	-0.481
Asymp.Sig.(2-tailed)	**0.630**

b. Grouping Variable: Gender

Descriptive Statistics:

Parameters	No. of animals	Mean	Std. deviation	Minimum	Maximum
RBCs in millions/µl	17	7.8306	0.76004	6.70	8.95
Gender	18	1.50	0.514	1	2

Observations:

Z = -0.481, Mann Whitney U = 31.00,

p value (0.630) > 0.05

Since the p value is more than the level of significance, we fail to reject the null hypothesis. It is therefore concluded that there is no difference in mean ranks of both the groups. Thus, biological immunomodulators have same effect in males and females with respect to effect on RBCs in 'Swiss albino mice'.

A) GROUPWISE ANALYSIS OF HAEMOGLOBIN:

Purpose: To study the effect of biological immunomodulators on Haemoglobin (Hb) of 'Swiss Albino mice'.

H_0: The three groups i.e. control, standard and test groups are identical. There is no significant difference in the mean ranks of the three groups.

H_1: The three groups are not identical. There is a significant difference in the mean ranks of the three groups.

Level of significance: $\alpha = 0.05$

Statistical Test: Kruskal-Wallis Test

Test type: 'Non parametric Test'. Since the distribution of data for the three groups was unknown, the non- parametric counterpart of 'One Way ANOVA' was used to examine whether the three groups are identical.

Ranks			
Parameter	**Groups**	**No. of Animals**	**Mean Rank**
Hb (Group wise)	Control group	6	7.25
	Standard group	5	9.70
	Test group	6	10.17
	Total	17	

Kruskal-Wallis Test:

Test Statistics [a, b]	
Parameter	Hb (Group wise)
Chi-square	1.144
Df	2
Asymp. Sig.	**0.564**
a. Kruskal Wallis Test	
b. Grouping Variable: Groups	

Observations:

χ^2 (2) = 1.144,

p value (0.564) > 0.05

Since the p value is more than the level of significance we fail to reject the null hypothesis. It is therefore concluded that, the three groups are identical and there is no difference in mean ranks of the three groups. Thus the biological immunomodulators have no significant effect on haemoglobin concentration of 'Swiss albino mice'.

B) GENDER WISE ANALYSIS OF HAEMOGLOBIN:

Purpose: Genderwise study of the effect of biological immunomodulators on haemoglobin of 'Swiss albino mice'.

H_0: There is no significant difference in the mean ranks of male and female 'Swiss albino mice'.

H_1: There is a significant difference in the mean ranks of male and female 'Swiss albino mice'.

Level of significance: $\alpha = 0.05$

Statistical Test: Mann Whitney U Test

Test type: 'Non Parametric Test'. Since the distribution of two groups is not known, non parametric counterpart of independent sample t-test has been conducted.

Mann Whitney Test:

Ranks					
Parameter	**Gender**		**No. of animals**	**Mean rank**	**Sum of ranks**
Hb	Groups	Male	8	9.44	75.50
		Female	9	8.61	77.50
		Total	17		

Test Statistics [b]	
Parameter	Hb
Mann-Whitney U	32.500
Wilcoxon W	77.500
Z	-0.338
Asymp. Sig. (2-tailed)	**0.735**
Exact Sig. [2*(1-tailed Sig.)]	0.743[a]
a. Not corrected for ties.	
b. Grouping Variable: Gender	

Descriptive Statistics					
Parameter	**No. of animals**	**Mean**	**Std. deviation**	**Minimum**	**Maximum**
Hb	17	12.912	1.1319	10.3	14.7
Gender	18	1.50	0.514	1	2

Observations:

Z = -0.338, Mann Whitney U = 32.500,

p value (0.735) > 0.05

Since the p value is more than the level of significance, we fail to reject the null hypothesis. It is therefore concluded that there is no difference in mean ranks of both the groups. Thus biological immunomodulators have same effect in males and females with respect to effect on haemoglobin concentration in 'Swiss albino mice'.

A) GROUPWISE ANALYSIS OF MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC):

Purpose: To study the effect of biological immunomodulators on MCHC count of 'Swiss albino mice'.

H_0: The three groups i.e. control, standard and test groups are identical. There is no significant difference in the mean ranks of the three groups.

H_1: The three groups are not identical. There is a significant difference in the mean ranks of the three groups.

Level of significance: α =0.05

Statistical Test: Kruskal Wallis Test

Test type: 'Non Parametric Test'. Since the distribution of data for the three groups was unknown, the non- parametric counterpart of 'One Way ANOVA' was used to examine whether the three groups are identical.

Kruskal-Wallis Test:

Ranks			
Parameter	**Groups**	**No. of animals**	**Mean rank**
MCHC	Control group	6	5.25
	Standard group	5	13.60
	Test group	6	8.92
	Total	17	-

Test Statistics [a, b]	
Parameter	MCHC
Chi-square	7.487
Df	2
Asymp. Sig.	**0.024**
a. Kruskal Wallis Test	
b. Grouping Variable: Groups	

Observations:

χ^2 (2) = 7.487,

p value (0.024) < 0.0

Since the p value is less than the level of significance the null hypothesis is rejected. Thus it is concluded that, the three groups were not identical and there is a significant difference in the mean ranks of the three groups. In order to find out where the difference lies that is, which group is benefitted, we performed a 'Mann Whitney Test'. In order to conduct 'Mann Whitney Test', standard group and test group were compared with the control group. We therefore get the following two conditions

A: Standard vs. Control.

B: Test vs. Control.

Mann Whitney Test for 'Standard vs. Control':

Ranks				
	Groups	**No.of animals**	**Mean rank**	**Sum of ranks**
MCHC	Control group	6	3.75	22.50
	Standard group	5	8.70	43.50
	Total	11		

Test Statistics [b]	
Parameter	MCHC
Mann-Whitney U	1.500
Wilcoxon W	22.500
Z	-2.476
Asymp. Sig. (2-tailed)	**0.013**
Exact Sig. [2*(1-tailed Sig.)]	0.009[a]
a. Not corrected for ties.	
b. Grouping Variable: Groups	

Since the 'Mann Whitney test' result in two tests, we no longer use α as 0.05. Instead, 0.05/2=0.025 is used as the level of significance.

Observations:

Z = -2.476, Mann-Whitney U = 1.500,

p value (0.013) < 0.025

Since the p value is less than the level of significance, we reject the null hypothesis. It is therefore concluded that, there is a significant difference in mean ranks of control group and standard group. Thus FDA approved standard drugs have significant effect on 'Mean corpuscular haemoglobin concentration' (MCHC) in 'Swiss albino mice'.

Mann-Whitney Test for 'Test Group vs. Control Group':

Ranks				
Parameter	**Groups**	**No. of animals**	**Mean rank**	**Sum of ranks**
MCHC	Control group	6	5.00	30.00
	Test group	6	8.00	48.00
	Total	12		

Test Statistics [b]	
Parameter	**MCHC**
Mann-Whitney U	9.000
Wilcoxon W	30.000
Z	-1.444
Asymp. Sig. (2-tailed)	**0.149**
Exact Sig. [2*(1-tailed Sig.)]	0.180[a]
a. Not corrected for ties.	
b. Grouping Variable: Groups	

Observations:

Z = -1.444, Mann Whitney U = 9.000,

p value (0.149) > 0.025

Since the p value is more than the level of significance, we fail to reject the null hypothesis. It is therefore concluded that, there is no significant difference in mean ranks of control group and test group. Thus biological immunomodulators under study have no significant effect on 'Mean corpuscular haemoglobin concentration' (MCHC) in 'Swiss albino mice'.

B) GENDER WISE ANALYSIS OF MCHC:

Purpose: Genderwise study of the effect of biological immunomodulators on MCHC of 'Swiss albino mice'.

H_0: There is no significant difference in the mean ranks of male and female 'Swiss albino mice'.

H_1: There is a significant difference in the mean ranks of male and female 'Swiss albino mice'.

Level of significance: α =0.05, **Statistical Test:** Mann Whitney U Test

Test type: 'Non Parametric Test'. Since the distribution of two groups is not known, non parametric counterpart of independent sample t-test has been conducted.

Mann Whitney Test:

Ranks					
	Gender		**No. of Animals**	**Mean Rank**	**Sum of Ranks**
MCHC	Dimension 1	Male	8	9.38	75.00
		Female	9	8.67	78.00
		Total	17		

Test Statistics [b]	
Parameter	MCHC
Mann Whitney U	33.000
Wilcoxon W	78.000
Z	-0.289
Asymp. Sig. (2-tailed)	**0.772**
Exact Sig. [2*(1-tailed Sig.)]	0.815[a]

a. a. Not corrected for ties., b. Grouping Variable: Gender

Descriptive Statistics:

Parameter	**No. of Animals**	**Mean**	**Std. Deviation**	**Minimum**	**Maximum**
MCHC	17	29.771	1.3429	28.1	32.7
Gender	18	1.50	0.514	1	2

Observations:

Z = -0.289, Mann Whitney U = 33.000,

p value (0.772) > 0.05

Since the p value is more than the level of significance, we fail to reject the null hypothesis. It is therefore concluded that there is no difference in mean ranks of both

The groups. Thus biological immunomodulators under study have same effect in males and females with respect to effect on MCHC in 'Swiss albino mice'.

A) GROUPWISE ANALYSIS OF BLOOD PLATELATES:

Purpose: To study the effect of biological immunomodulator on platelet count of 'Swiss albino mice'.

H_0: The three groups i.e. control, standard and test groups are identical. There is no significant difference in the mean ranks of the three groups of 'Swiss albino mice'.

H_1: The three groups are not identical. There is a significant difference in the mean ranks of the three groups of 'Swiss albino mice'.

Level of significance: $\alpha = 0.05$

Statistical Test: Kruskal-Wallis Test

Test type: 'Non parametric Test'. Since the distribution of data for the three groups was unknown, the non-parametric counterpart of 'One Way ANOVA' was used to examine whether the three groups are identical.

Kruskal –Wallis Test

Ranks		
Groups	**No. of Animals**	**Mean Rank**
Control group	6	7.08
Standard group	5	10.30
Test group	6	9.83
Total	17	-

Test Statistics [a, b]

Platelets	
Chi- square	1.361
Df	2.0
Asymp. Sig	0.506

a. Kruskal Wallis Test,

b. Grouping Variable: Groups

Observations:

χ^2 (2) = 1.361,

p value (0.506) > 0.05

Since the p value is more than the level of significance we fail to reject the null hypothesis. It is therefore concluded that, the three groups are identical and there is no difference in mean ranks of the three groups. Thus the biological immunomodulators therapy has no significant effect on platelet count of 'Swiss albino mice'.

B) GENDER WISE ANALYSIS OF BLOOD PLATELATES:

Purpose: Genderwise study of the effect of biological immunomodulators on platelet count of 'Swiss albino mice'.

H_0: There is no significant difference in the mean ranks of male and female 'Swiss albino mice'.

H_1: There is a significant difference in the mean ranks of male and female 'Swiss albino mice.

Level of significance: $\alpha = 0.05$

Statistical Test: Mann-Whitney U Test

Test type: 'Non Parametric Test'. Since the distribution of two groups is not known, non parametric counterpart of independent sample t- test has been conducted.

Ranks					
Parameter	**Gender**		**No. of Animals**	**Mean Rank**	**Sum of Ranks**
Platelet	Groups	Male	8	8.75	70.00
		Female	9	9.22	83.00
		Total	17		

Test Statistics [b]	
Parameter	Platelet
Mann Whitney U	34.000
Wilcoxon W	70.000
Z	-0.193
Asymp. Sig. (2-tailed)	0.847
Exact Sig. [2*(1-tailed Sig.)]	0.888[a]
a. Not corrected for ties.	
b. Grouping Variable: Gender	

Descriptive Statistics:

Parameter	No. of animals	Mean	Std. deviation	Minimum	Maximum
Platelet	17	989294.12	241367.656	705000	1735000
Gender	18	1.50	0.514	1	2

Observations:

Z = -0.193, Mann Whitney U = 34.0,

p value (0.847) > 0.05

Since the p value is more than the level of significance, we fail to reject the null hypothesis. It is therefore concluded that there is no difference in mean ranks of both the groups. Thus biological immunomodulators have same effect in males and females with respect to effect on 'platelet count' in 'Swiss albino mice'.

Blood Parameters	p-value following 'Kruskal –Wallis' Test	Interpretation	Standard Vs. Control p value following Mann-Whitney Test	Test Vs. Control p value following Mann-Whitney Test	Gender wise study p value following Mann-Whitney Test
MCHC	0.024 (< 0.05)	H_0 is rejected	0.013 (< 0.025)	0.149 (> 0.025)	0.772 (> 0.05)
Platelet Count	0.506 (> 0.05)	We fail to reject H_0	NA	NA	NA
RBC Count	0.280 (> 0.05)	We fail to reject H_0	NA	NA	NA

Table-33: Interpretation of statistical analysis of blood parameters.

Histopathological study of experimental animals:

After completion of the experiment, 'postmortem histopathological study' of the experimental animals was conducted. The study was assisted by registered animal pathologist and no abnormality was detected in the experimental animals under stud**y.**

Animal code	Liver	Kidney	Testis	Prostate gland	Seminal vesicle
HBT control male	NAD	NAD	NAD	NAD - mild dilation in prostatic acini	NAD
HBT control female	NAD	NAD	-	-	-
B standard male	NAD	NAD	NAD	NAD	NAD
B standard female	NAD	NAD	-	-	-
H test male	NAD	NAD	NAD	NAD- mild dilation in prostatic acini	NAD
H test female	NAD	NAD	-	-	-

Table-34: Histopathological study of the experimental animals

NAD: No abnormality was detected.

Discussion:

Mayank Thakur (2007) reported 'Neutrophil Adhesion Test' as one of the tests used for assessment of plant immunomodulatory activities. Neutrophil is one of the efficient phagocytic cells containing more than thirty enzymes and keeps potential to break every existing chemical bond. Viral sensitization of host cells releases 'superoxide radicals'. Pitrak E.L. *et al.* (1993) reported decline in the level of superoxide radicals by neutrophils. During extravasation process, membrane of neutrophil shows conformational changes in the LFA-1 and MAC-1 in presence of IL-1 and MIP-1β **(02, 138)** and thus increases their affinity towards ICAM-1 of endothelial cells. Nylon fiber serves as an ICAM analogue and facilitates binding of neutrophils through modified integrins. Hence, 'Neutrophil Adhesion Test' can be used for assessment of immunomodulatory activities.

In present study, 'Neutrophil Adhesion Test' was conducted in Swiss albino mice. Three different groups of Swiss albino mice such as control, standard and test, each carrying three males and three females were selected for the study. Control was no treatment group, standard group was receiving therapy of FDA approved drugs and

Test group was given the blended formulations of selected biological immunomodulators. Statistical analysis was performed using 'One Way ANOVA' followed by 'Kruskal Wallis Test' (p value < 0.05), Post hoc test and Man Whitney Test (p value < 0.025). In comparative analysis of control group, standard group and test group Kruskal Wallis p value was 0.031. Comparative study of control group and standard group showed Man Whitney p value of 0.067. Comparative study of control group and test group showed Man Whitney p value of 0.010 (p value < 0.025). Thus synergistic action of selected biological immunomodulators had a significant effect on 'Percentage Neutrophil Count'.

Similarly statistical analysis of other haematological parameters including RBCs, haemoglobin, mean corpuscular haemoglobin concentration (MCHC) and blood platelets was carried out. It was found that, 'Biological Immunomodulators' had no significant effect on RBCs, haemoglobin, MCHC and platelets. During the study FDA approved drugs showed significant effect on MCHC. Thus the study confirmed the selective immunomodulatory action of selected biological immunomodulators. The immunomodulators targeted 'cellular immunity' without disturbing other cells metabolism or function.

No histopathological abnormalities were observed during postmortem study of the animals under study.

8.4 Human Clinical Trials: Phase I study:

Observations:

Without developing adverse reactions, doses of biological immunomodulators were well tolerated by Group I and Group II human participants. No signs and symptoms of illness were observed for the period of 60 days.

Result:

Formulations of biological immunomodulators at the given doses were found to be safe for human use.

8.5 Human Clinical Trials: Phase II study:

Observations:

Patient's id no.	Absolute CD_4 count		HIV-1 RNA load	
	Initial	Final	Initial	Final
M 1	358	381	206903	< 400
F 2	509	630	222711	<400
M 3	419	459	351917	< 400
M 4	483	576	314715	32969
F 5	335	582	231821	11521
F 6	162	444	221622	10432
M 7	75	110	330614	191901
F 8	384	1040	361912	< 400
M 9	60	171	282421	98841
M 10	75	104	271332	13989
F 11	106	580	216321	11360
F 12	423	534	342694	253745
M 13	528	629	197233	38654
M 14	319	469	359872	40559
F 15	167	320	419346	219483
M 16	285	446	384572	175958
M 17	621	671	170246	30173
F 18	348	432	350173	180715
M 19	73	193	548369	379540
F 20	265	396	340633	230481
M 21	83	179	476856	254672
M 22	367	492	270410	131094
F 23	117	110	260954	291891
M 24	354	374	246090	150437
F 25	69	227	416477	290734
M 26	184	340	328140	183259
F 27	413	408	353811	381755
M 28	258	361	316723	174120
F 29	79	60	523231	571428
M 30	415	560	243846	45632
F 31	89	171	493482	290761
M 32	169	230	389651	150390

Table-35: Record of 'Absolute CD_4 count' and 'HIV-1 RNA Load' of selected HIV reactive patients during Phase II study

STATISTICAL ANALYSIS OF 'ABSOLUTE CD_4 CELLS' AND 'VIRAL LOAD' FOLLOWING PHASE-II HUMAN CLINICAL TRIALS:

Purpose: To study the effect of 'Biological Immunomodulators' on the count of 'Absolute CD_4 Cells' and 'Viral load' following phase-II human clinical trials.

Method used: **Split-Plot Analysis**

Independent Variables:

I (Within subject): Biological immunomodulators,

II (Between subjects): Gender

Dependent variables: CD_4 count and Viral load

Design: Repeat Measures Design (biological immunomodulators)

NULL AND ALTERNATIVE HYPOTHESIS FOR 'ABSOLUTE CD_4 COUNT':

H_0: Treatment of Biological Immunomodulators does not influence the 'Absolute CD_4 count' in HIV infected individuals.

H_1: Treatment of Biological Immunomodulators significantly influences the 'Absolute CD_4 count' in HIV infected individuals.

NULL AND ALTERNATIVE HYPOTHESIS FOR VIRAL LOAD:

H_0: Treatment of Immunomodulators does not influence the 'Viral load' in HIV infected individuals.

H_1: Treatment of Immunomodulators significantly influences the 'Viral load' in HIV infected individuals.

NULL AND ALTERNATIVE HYPOTHESIS FOR ABSOLUTE CD_4 COUNT AND VIRAL LOAD:

H_0: Effect of Biological Immunomodulators on 'Absolute CD_4 count' and 'Viral Load' is not same in males and females.

H_1: Effect of Biological Immunomodulators on 'Absolute CD_4 count' and 'Viral Load' is same in males and females.

NULL AND ALTERNATIVE HYPOTHESIS FOR INTERACTION EFFECTS:

H_0: Interaction Effect is Zero

H_1: Interaction Effect is not Zero

STATISTICAL TEST: Split-Plot Analysis (Mixed Design Test)

Within-Subjects Factors		
Measure	**Biological Immunomodulators**	**Dependent Variable**
CD_4	Therapy regime as mentioned in methodology	CD_4 (Initial)
	Therapy regime as mentioned in methodology	CD_4 (Final)
Viral load	Therapy regime as mentioned in methodology	Viral load (Initial)
	Therapy regime as mentioned in methodology	Viral load (Final)

Above table of within subject factors provides information of independent and dependent variables. Independent variable was biological immunomodulators with two experimental conditions like initial and final. Dependant variables are 'Absolute CD_4 count' and 'Viral load'.

Between-Subjects Factors			
		Value Label	**N**
Gender	1.00 (Male code)	Male	18
	2.00 (Female code)	Female	14

Above table of between subject factors provides information of second independent variable that is gender. Gender was nominal variable with two response options such as 1. Male, 2. Female. There were 18 males and 14 females participated in the study.

EFFECT OF BIOLOGICAL IMMUNOMODULATORS WAS STUDIED IN 18 MALES AND 14 FEMALES (AGE GROUP 20-47 YRS). THE TREATMENT REGIME WAS CONTINUED FOR AVERAGE PERIOD OF 11 MONTHS.

Descriptive Statistics				
Parameter	**Gender**	**Mean**	**Std. Deviation**	**No of participants**
Absolute CD_4 count (Initial)	Male	284.7778	173.70244	18
	Female	247.5714	151.78107	14
	Total	268.5000	162.96863	32
Absolute CD_4 count (Final)	Male	374.7222	178.33933	18
	Female	423.8571	252.60515	14
	Total	396.2188	211.69191	32
Viral load (Initial)	Male	316106.1111	95831.07584	18
	Female	339656.2857	100411.74399	14
	Total	326409.3125	96980.44605	32
Viral load (Final)	Male	116277.1111	101994.55920	18
	Female	196079.0000	172534.81659	14
	Total	151190.4375	140733.93326	32

The descriptive statistical test provides information of mean and standard deviation with respect to gender across 'Absolute CD_4 count' (Initial), 'Absolute CD_4 count' (Final), 'Viral Load' (Initial), 'Viral Load' (Final).

Box's Test of Equality of Covariance Matrices[a]	
Box's M	32.493
F	2.763
df1	10
df2	3691.430
Sig.	**0.002**

Box's Test of equivalence of covariance matrix was statistically insignificant, (p value > 0.001) indicating that the observed covariance matrices of dependent variables were equal across independent variable groups. Hence 'Wilk's Lambda' was employed to evaluate all multivariate effects.

Bartlett's Test of Sphericity [a]					
Effect		**Likelihood Ratio**	**Approx. Chi-Square**	**df**	**Sig.**
Between Subjects		0.000	345.582	2	0.000
Within subjects	Biological Immunomodulators	0.000	355.473	2	0.000

Bartlett's Test of Sphericity is significant. [p value is < 0.001] indicating that the dependent variable sufficiently correlate with each other so as to proceed with analysis.

Levene's Test of Equality of Error Variances[a]				
	F	df1	df2	Sig.
CD_4 Initial	0.123	1	30	0.728
CD_4 Final	0.602	1	30	0.444
Viral Load Initial	0.091	1	30	0.765
Viral Load Final	3.996	1	30	0.055

The Leven's test for homogeneity of variance is insignificant for all four groups (CD_4 Initial, CD_4 Final, Viral load Initial, Viral load Final). This indicates that assumption of homogeneity of variance is true and tangible.

TESTS OF WITHIN-SUBJECTS EFFECTS:

Multivariate[a,b]					
Within Subjects Effect		Value	F	Hypothesis df	Error df
Biological Immunomodulators	Pillai's Trace	0.785	52.982[c]	2.000	29.000
	Wilks' Lambda	0.215	52.982[c]	2.000	29.000
	Hotelling's Trace	3.654	52.982[c]	2.000	29.000
	Roy's Largest Root	3.654	52.982[c]	2.000	29.000
Biological Immunomodulators * Gender	Pillai's Trace	0.366	8.360[c]	2.000	29.000
	Wilks' Lambda	0.634	8.360[c]	2.000	29.000
	Hotelling's Trace	0.577	8.360[c]	2.000	29.000
	Roy's Largest Root	0.577	8.360[c]	2.000	29.000

Multivariate [a, b]				
Within Subjects Effect		Sig.	Partial Eta Squared	Non cent. Parameter
Biological Immunomodulators	Pillai's Trace	0.000	0.785[c]	105.964
	Wilks' Lambda	0.000	0.785[c]	105.964
	Hotelling's Trace	0.000	0.785[c]	105.964
	Roy's Largest Root	0.000	0.785[c]	105.964
Biological Immunomodulators * Gender	Pillai's Trace	0.001	0.366[c]	16.720
	Wilks' Lambda	0.001	0.366[c]	16.720
	Hotelling's Trace	0.001	0.366[c]	16.720
	Roy's Largest Root	0.001	0.366[c]	16.720

From the test of within subjects effects it can be seen that selected biological immunomodulators have significant impact on 'Absolute CD_4 Count' and 'Viral Load'. [Wilk's Lambda=0.215, F(2,29) =52.98, p=0.000].

Partial Eta Squared = 0.785. As per Cohen's guidelines the value is more than 0.14. This indicates very significant effect of selected biological immunomodulators on 'Absolute CD_4 Count' and 'Viral Load'. Since biological immunomodulators have significant impact on aggregate outcome immune parameters, we now look for impact of biological immunomodulators on 'Absolute CD_4 Count' and 'Viral Load' separately.

Univariate Tests				
Source	**Measure**		**Type III Sum of Squares**	**Df**
Biological Immuno modulators	CD_4	Sphericity Assumed	279084.084	1
		Greenhouse-Geisser	**279084.084**	1.000
		Huynh-Feldt	279084.084	1.000
		Lower-bound	279084.084	1.000
	Viral load	Sphericity Assumed	464341015955.572	1
		Greenhouse-Geisser	**464341015955.572**	1.000
		Huynh-Feldt	464341015955.572	1.000
		Lower-bound	464341015955.572	1.000
Biological Immuno modulators * Gender	CD_4	Sphericity Assumed	29353.334	1
		Greenhouse-Geisser	29353.334	1.000
		Huynh-Feldt	29353.334	1.000
		Lower-bound	29353.334	1.000
	Viral load	Sphericity Assumed	12459255480.321	1
		Greenhouse-Geisser	12459255480.321	1.000
		Huynh-Feldt	12459255480.321	1.000
		Lower-bound	12459255480.321	1.000
Error (Biological Immunom odulator)	CD_4	Sphericity Assumed	253590.901	30
		Greenhouse-Geisser	253590.901	30.000
		Huynh-Feldt	253590.901	30.000
		Lower-bound	253590.901	30.000
	Viral load	Sphericity Assumed	128425966191.429	30
		Greenhouse-Geisser	128425966191.429	30.000
		Huynh-Feldt	128425966191.429	30.000
		Lower-bound	128425966191.429	30.000

Univariate Tests					
Source	Measure		Mean square	F	Sig.
Biological Immunomodulators	CD_4	Sphericity Assumed	279084.084	33.016	0.000
		Greenhouse-Geisser	279084.084	33.016	0.000
		Huynh-Feldt	279084.084	33.016	0.000
		Lower-bound	279084.084	33.016	0.000
	Viral load	Sphericity Assumed	4.643E+11	108.469	0.000
		Greenhouse-Geisser	4.643E+11	108.469	0.000
		Huynh-Feldt	4.643E+11	108.469	0.000
		Lower-bound	4.643E+11	108.469	0.000
Biological Immunomodulators * Gender	CD_4	Sphericity Assumed	29353.334	3.473	0.072
		Greenhouse-Geisser	29353.334	3.473	0.072
		Huynh-Feldt	29353.334	3.473	0.072
		Lower-bound	29353.334	3.473	0.072
	Viral load	Sphericity Assumed	12459255480	2.910	0.098
		Greenhouse-Geisser	12459255480	2.910	0.098
		Huynh-Feldt	12459255480	2.910	0.098
		Lower-bound	12459255480	2.910	0.098
Error (Biological Immunomodulator)	CD_4	Sphericity Assumed	8453.030		
		Greenhouse-Geisser	8453.030		
		Huynh-Feldt	8453.030		
		Lower-bound	8453.030		
	Viral load	Sphericity Assumed	4280865540		
		Greenhouse-Geisser	4280865540		
		Huynh-Feldt	4280865540		
		Lower-bound	4280865540		

$\alpha = 0.05$

Univariate Tests					
Source	**Measure**		**Partial Eta Squared**	**Non cent. Parameter**	**Observed power [a]**
Biological Immuno modulators	CD_4	Sphericity Assumed	0.524	33.016	1.000
		Greenhouse-Geisser	**0.524**	33.016	1.000
		Huynh-Feldt	0.524	33.016	1.000
		Lower-bound	0.524	33.016	1.000
	Viral load	Sphericity Assumed	0.783	108.469	1.000
		Greenhouse-Geisser	0.783	108.469	1.000
		Huynh-Feldt	0.783	108.469	1.000
		Lower-bound	0.783	108.469	1.000
Biological Immuno modulators * Gender	CD_4	Sphericity Assumed	0.104	3.473	0.438
		Greenhouse-Geisser	0.104	3.473	0.438
		Huynh-Feldt	0.104	3.473	0.438
		Lower-bound	0.104	3.473	0.438
	Viral load	Sphericity Assumed	0.088	2.910	0.379
		Greenhouse-Geisser	0.088	2.910	0.379
		Huynh-Feldt	0.088	2.910	0.379
		Lower-bound	0.088	2.910	0.379

From Univariate test it can be seen that Biological Immunomodulators have a significant impact on 'Absolute CD_4 Cells'. [Greenhouse-Geisser = 279084.08, f(1,30)=33.016, p-value=0.000], Partial Eta Squared=0.52. According to Cohen's guideline this effect is very strong effect.

Biological Immunomodulators also have significant impact on 'Viral Load'. [Greenhouse-Geisser = 4.643, E+11, f(1,30)=108.46, p-value=0.000], Partial Eta Squared=0.783. According to Cohen's guideline this effect is strong effect.

Estimates					
Measure	**Biological Immuno modulators**	**Mean**	**Std. Error**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
Absolute CD_4 count	1 (Initial)	266.175	29.321	206.294	326.055
	2 (Final)	399.290	38.078	321.524	477.056
Viral load	1 (Initial)	327881.198	17432.981	292278.301	363484.096
	2 (Final)	156178.056	24426.470	106292.549	206063.562

Table – 36: Comparative statistics of absolute CD_4 count and viral load

The impact on gender as aggregate outcome variable is insignificant.

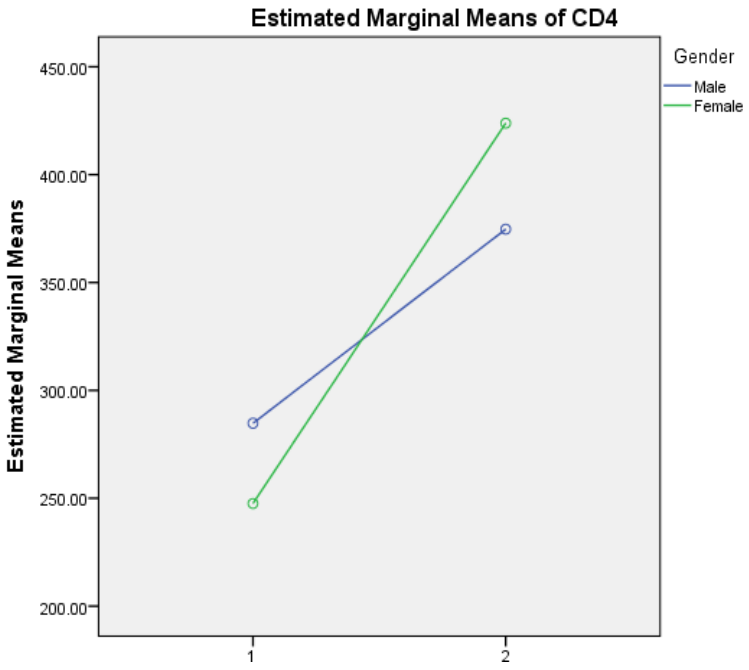
Tests of Between-Subjects Effects						
Transformed Variable: Average						
Source	**Measure**	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept	CD_4	6974772.770	1	6974772.770	108.470	0.00
	Viral load	3690435441298.767	1	3690435441298.767	153.214	0.00
Gender	CD_4	560.270	1	560.270	0.009	0.926
	Viral load	42058993048.016	1	42058993048.016	1.746	0.196
Error	CD_4	1929034.964	30	64301.165		
	Viral load	722604438710.984	30	24086814623.699		

Tests of Between-Subjects Effects				
Transformed Variable: Average				
Source	**Measure**	**Partial Eta Squared**	**Noncent. Parameter**	**Observed Power**
Intercept	Absolute CD_4 count	0.783	108.470	1.000
	Viral load	0.836	153.214	1.000
Gender	Absolute CD_4 count	0.000	0.009	0.051
	Viral load	0.055	1.746	0.249
Error	Absolute CD_4 count	0	0	0
	Viral load	0	0	0

- With respect to absolute CD_4 count as a biological parameter, males and females have same response towards selected biological immunomodulators. f(1,30)=0.009, p=0.926
- With respect to viral load as a biological parameter, males and females have same response towards selected biological immunomodulators. f(1,30)=1.746, p=0.196

Statistical Parameters:					
Measure	**Gender**	**Mean**	**Std. Error**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
Absolute CD_4 count	Male	329.750	42.263	243.438	416.062
	Female	335.714	47.922	237.846	433.583
Viral load	Male	216191.611	25866.546	163365.077	269018.145
	Female	267867.643	29329.906	207967.984	327767.302

ABSOLUTE CD_4 CELLS:



Therapy of Biological Immunomodulators

1. Initial Count

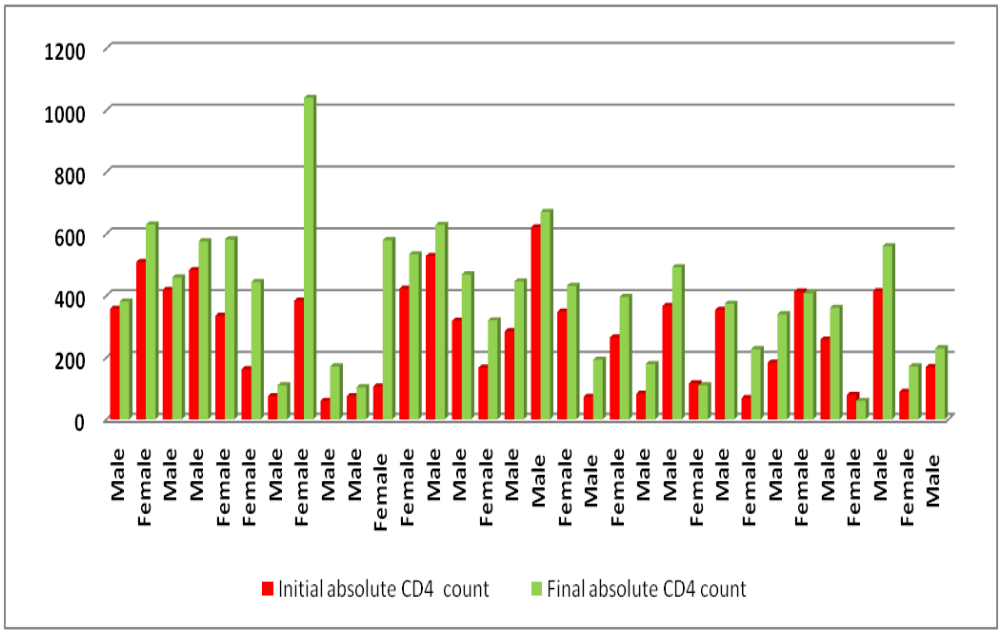
2. Final Count

Graph: 14A Effect of 'Biological Immunomodulators' on CD_4 Count of HIV infected individuals

The graph shows that, absolute CD_4 count for males before the treatment is lower as compared to females. There is a positive effect of the immunomodulators on absolute CD_4 count both in males and females. There is a considerable increase in CD_4 count after treatment of biological immunomodulators both in males and females. Thus biological immunomodulators acts as positive immunomodulators by increasing degree of immunity in terms of CD_4 count.

From the Univariate Tests Table, it can be seen that, for CD_4 count, Greenhouse-Geisser = 279084.084, F[1,30]=33.016, p value = 0.000. Since p value is < 0.05, it is concluded that, there is a significant difference between initial CD_4 count (Before Treatment) and Final CD_4 count (After Treatment). To find out whether difference lies we refer to descriptive table.

Descriptive table of Absolute CD_4 Count:					
Measure	**Gender**	**Mean**	**Std. Error**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
CD_4	Male	329.750	42.263	243.438	416.062
	Female	335.714	47.922	237.846	433.583



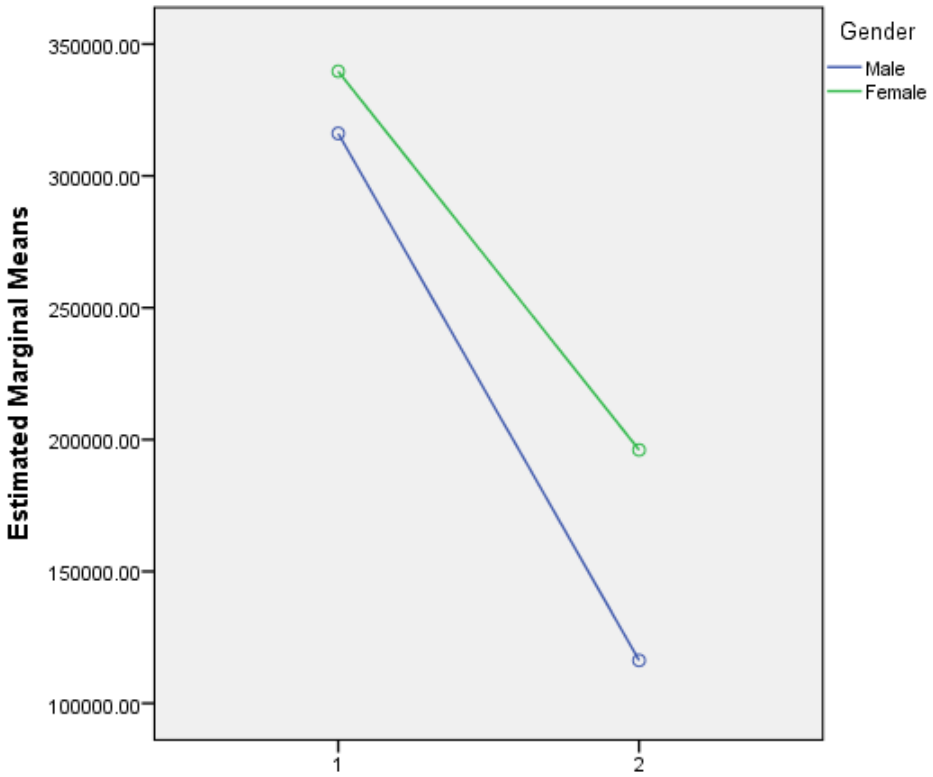
Graph: 14B Effect of 'Biological Immunomodulators' on Absolute CD_4 count of HIV infected individuals

Interaction effect for Absolute CD_4 count:

Interaction effect for CD_4 is zero. From the Table, it can be seen that, for CD_4 count, Greenhouse-Geisser = 29353.33, f[1,30]=3.473, p value=0.072. Since p value is < 0.05, it is concluded that, the impact of biological immunomodulators on CD_4 count is same for men and women.

VIRAL LOAD OF HIV 1 RNA COPIES.

Estimated Marginal Means of Viral Load



Therapy of Biological Immunomodulators

1. **Initial Count**
2. **Final Count**

Graph: 15 Effect of 'Biological Immunomodulators' on Viral load of HIV infected individuals

The graph shows that, viral load for males before the treatment is higher as compared to females. There is a considerable decrease in viral load after treatment of biological immunomodulators both in males and females. Thus biological immunomodulators also acts as antiviral agents by decreasing titer of HIV-1 RNA load in peripheral blood of the infected individuals.

Interaction effect for viral load:

Interaction effect for viral load is zero. From the Table, it can be seen that, for viral load, Greenhouse-Geisser = 12459255480.0, f[1,30]=2.910, p value=0.098. Since p value is < 0.05, it is concluded that, the impact of biological immunomodulators on viral load is same for men and women.

From Univariate test table, it is observed that, for 'viral load', Greenhouse-Geisser =4.643, E+11, f[1,30]=108.65, p = 0.000. Since p value < 0.05, it is concluded that, there is significant difference in the 'Initial viral load' (Before Treatment) and 'Final viral load' (After treatment).

Estimates					
Measure	**Gender**	**Mean**	**Std. Error**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
Viral load	Male	216191.611	25866.546	163365.077	269018.145
	Female	267867.643	29329.906	207967.984	327767.302

From Descriptive statistical Table, it can be also observed that, viral load mean of HIV 1 RNA copies before treatment of biological immunomodulators was 327881.198 and after treatment was 156178.056. Thus it can be concluded that, there was a significant decrease in viral load of HIV 1 RNA copies in the peripheral blood of the infected individuals after treatment of biological immunomodulators.

Discussion:

Human clinical trials were conducted in groups vide IEC approval BVDU/YM/Ethics 2012-13/452 dt. 30/06/2012. The trials were conducted under supervision of registered medical practitioner (M. D. Ayurveda). During the clinical trials all legal norms regarding written consent of the selected patients and non exposure of names were followed. As T- lymphocyte is a prime target of HIV; their response decides the rate of disease progression. Clinical progression of HIV infection reported decline in 'Absolute CD_4 cells' at the rate of 25 to 60 cells/µl/year **[91].** Differentiation and maturation of microphages and macrophages requires cytokines secreted by CD_4 cells (eg. GM-CSF). Activation of human macrophages and subsequent distribution of virally infected cells requires ɣ-IFN produced by CD_4 cells. Even the formation of 'antibody forming B lymphocytes'requires cytokines produced by CD_4 cells **[2,122,138]**. Thus CD_4 cells indirectly regulates the humoral immune response including secondary immune response. During HIV infection as the count of CD_4 cells declines, many functional aspects of cellular immunity and humoral immunity get badly hampered to the extent to develop immunodeficient state. Hence immunomodulating

therapy in HIV infected individuals is expected to improve the 'absolute CD_4 cells count' along with proportional decline in the viral load of HIV-1 RNA copies.

In the present study, phase I human clinical trial was conducted in 15 HIV infected individuals and 5 healthy individuals. Blended human doses as found safe and non toxic during animal clinical trials were selected as mentioned in the methodology. Under the supervision of registered medical practitioner the treatment regime was continued for 60 days (Average). No signs and symptoms of illness were observed in Group I and Group II human participants. During Phase I clinical trials, formulations of biological immunomodulators were standardized keeping safety of the participants. Phase –II clinical trials were conducted in 18 HIV reactive males and 14 HIV reactive females in the age group of 20-47 yrs. Under the supervision of registered medical practitioner (M. D. Ayurveda), the doses of blended formulations of selected biological immunomodulators were given through oral route. The treatment regime in every patient under study was continued for the average period of 11 months. Before and during treatment, record of 'absolute CD_4 count' was kept by using 'Beckman Coulter Flow Cytometer'. Before and after treatment record of HIV-1 viral load was kept using 'RT-PCR'. Statistical analysis was carried out using 'Split –plot Analysis' (Mixed Design Test) followed by 'Bartlett's Test'. It indicated the significant impact of selected biological immunomodulators on 'Absolute CD_4 cells' and 'Viral load of HIV-1 RNA'. [Wilk's Lambda = 0.215, F (2, 29) 52.98, P=0.000]. Partial Eta Squared was 0.785. As per Cohen's guidelines the value is more than 0.14. This consolidated the significant effect of selected biological immunomodulators on 'Absolute CD_4 cells' and 'Viral load of HIV-1 RNA'. Effect of selected biological immunomodulators on both the parameters that is 'Absolute CD_4 cells' and 'Viral load' was found same in males and females. Interaction effect was zero. Since the Wilk's Lambda was significant 'Univariate's Test' was conducted on each dependent variable separately to determine the locus of statistically significant multivariate effects. From 'Univariate Test' for CD_4 count, Greenhouse-Geisser was found to be 279084.084, F [1, 30] =33.016, p- value= 0.000. For viral load it was 4.643, E+11, F [1, 30] =108.65, p-value=0.000. This indicated significant impact of biological immunomodulators on 'Absolute CD_4 cells' and 'Viral load of HIV-1 RNA'. From descriptive table it was observed that, viral load mean of HIV-1 RNA copies before treatment of biological immunomodulators was 327881.198 and after treatment was 156178.056. Thus it can be concluded that there was significant decrease in viral load

of HIV-1 RNA copies in the peripheral blood of the HIV-1 infected individuals following therapy of biological immunomodulators. Tests between the subjects for viral load reveal same effect in males and females. Overall picture is the selected biological immunomodulators have significantly elevated the count of CD_4 cells and also exhibited promising anti HIV properties. Separate cell line study was carried out using 'HIV-1 P-24 Antigen Inhibition Assay'. Percentage inhibition reduction of P-24 antigen on 'PHA activated PBMC cell line' was 20%. Thus the study claimed the immunomodulating and anti – HIV properties of the blended formulations of selected biological immunomodulators.

SUMMARY

SUMMARY

AIDS is a pandemic disease for which causative agent is 'Human Immuno Deficiency Virus (HIV)'. At the end of June 2015, about 36.9 million people were reported for HIV infection. Since 2000, twenty five million people were died of HIV related complications. Though the decline in new infected cases is reported, India has third largest number of HIV infected individuals in the world. In India about 21.17 lakhs people were living with HIV [127].

Present study was aimed at the study of antimicrobial and immunomodulating properties of selected biological immunomodulators. In the present study, animal and human clinical trials were conducted along with conventional laboratory methods. The study was conducted as per guidelines framed by 'Institutional Ethics Committee'. The study is summarized as:

1) Biological Immunomodulators under study were *Chlorophytum borivilianum, Withania somnifera, Wagatia spicata Dalz., Picrorrhiza kurroa, Spilanthus paniculata Wall. ex. DC.* and as per WHO norms the contents of heavy metals (Pb, Hg, Cd and As) were within normal limits (below 2 ppm). This indicated non hazardous level of heavy metals in the selected biological immunomodulators and confirmed their safe use in animals and humans under study.

2) Maximum antibacterial activity against bacterium *Bacillus marscescens* NCIM 5041 was exhibited by aqueus extract of *Picrorrhiza kurroa*. *Bacillus marscescens* has been documented for septicaemia, cellulitis and pneumonia in HIV infected individuals and hence was selected for the study. At the concentration of 0.33gm/ml, zone of growth of inhibition was 43 mm.

3) DMSO extracts of both *Wagatia spicata Dalz.,* and *Withania somnifera* showed maximum activity against bacterium *Klebsiella pneumoniae* NCIM 2719. The organism is capsulated bacterium and reported for development of pneumonia in immunocomprised individuals and hence, selected for the study. At the concentration of 0.33gm/ml of DMSO, both the immunomodulators showed 17 mm zone of growth inhibition.

4) *Candida albicans* develops oral thrush in HIV infected individuals hence was selected for the present study. Maximum antifungal activity against *Candida*

albicans NCIM 3466 was exhibited by DMSO extract of *Chlorophytum borivilianum* (zone diameter of growth inhibition = 18mm). *Hansenula capsulata* is rich in phosphomannans and its role in the fungal pathogenicity is wel established (Jim E. Cutler et. al. 2002 Europian patent number 1165614 A1) and hence was selected for the study. DMSO extract of *Wagatia spicata Dalz.* showed maximum antifungal activity against *Hansenula capsulata NCIM 3439*(zone diameter of growth inhibition = 20 mm).

5) Spectral peaks revealed during F.T.I.R. analysis of DMSO extract of root of *Chlorophytum borivilianum* had a close structural relevance with Alkaloides, Glycosides, Saponine glycosides, Steroides, Tannins, Phenolic compounds.

6) Spectral peaks revealed during F.T.I.R. analysis of DMSO extract of stem of *Wagatia spicata Dalz.* had a close structural relevance with Flavanoides, Quercitin, Gallic acid.

7) In case of DMSO extract of root of *Picrorrhiza kurroa*, structural relevance of the peaks was with Irridoid glycosides (Picroside–I, Picroside–II, Kutkoside) and Pikoroside , Veronicoside, Ninecoside, Picein, Androsin, Cucurbitacin glycosides and 4-hydroxy-3-methoxyacetophenone.

8) In case of DMSO extract of root of *Spilanthes paniculata Wall. ex. DC.*, structural relevance of the peaks was with Alkaloides, Glycosides, Flavanoides, Tannines, Arthraquinones, Saponines, Spilantho (alkamide).

9) Spectreal peaks revealed during F.T.I.R. analysis of DMSO extract of root of *Withania somnifera* had a close structural relevance with Withanolides (steroidal lactones with ergostane skeleton) and alkaloides (Withanone, Withaferin A,Withanolides I, II, III), Withasomodienone, Cuscohygrine, Anahygrine, Aanaferine, Isopellatierine.

10) 'Acute Toxicity Testing' of blended formulations of *Chlorophytum borivilianum, Withania somnifera, Wagatia spicata Dalz., Picrorrhiza kurroa, Spilanthus paniculata Wall.ex. DC.* in Swiss albino mice was negative and the 'Limit Test' showed the drug tolerance of 2000 mg/kg.

11) 'Carbon Clearance Test' conducted in Swiss albino mice showed 1.0 as a phagocytic index of non treated (control) animals. It was observed as 1.25 for standard and 2.72 for the test group animals. Thus animals treated with selected biological immunomodulators showed considerable increase in the phagocytic activity in terms of 'Carbon Clearance Test'.

12) ‘Delayed Type of Hypersensitivity’ (DTH) response was tested in control, standard and test group animals. The statistical analysis revealed significant effect of selected biological immunomodulators on ‘T_4 lymphocytes’ in terms of DTH response. Thus the biological immunomodulators reported for enhancing T lymphocyte function.

13) ‘Neutrophil Adhesion Test’ was conducted in Swiss albino mice. In comparative analysis, test group Kruskal Wallis p value was 0.031. Comparative study of standard group animals and test group animals showed Man Whitney p value 0.067 for standard and 0.010 for test group animals. This indicated the improvement of ‘Neutrophil Function’ by selected biological immunomodulators in terms of ‘Percentage Neutrophil Count’.

14) Haematological parameters tested during ‘Neutrophil Adhesion Test’ showed no significant effect of selected biological immunomodulators on RBC count, percentage of haemoglobin, MCHC and platelets.

15) No histopathological abnormalities were observed during postmortem study of Swiss albino mice under study and these observations confirmed the safety level of selected biological immunomodulators.

16) No signs and symptoms of illness were observed in the human participants during phase I clinical trials. The blended doses of selected biological immunomodulators were wel tolerated by the participants of test group individuals.

17) Phase II clinical trials were conducted in 32 HIV reactive patients out of which 18 were males and 14 were females. The treatment of selected biological immunomodulators was given for average period of 11 months in each participant. Readings of ‘absolute CD_4 count’ were recorded using ‘Flow Cytometry’. Similarly readings of ‘HIV1 RNA viral load’ were obtained using RT-PCR. Statistical analysis was carried out using ‘Split-Plot Analysis’ (Mixed Design Test) followed by ‘Bartlett’s Test’ of Sphericity (p value < 0.001) and the Leven’s test for homogeneity of varience. Test of within subject effects showed that selected biological immunomodulators have significant impact on ‘Absolute CD_4 Count’ and ‘Viral load’. [Wilk’s Lambda=0.215, F(2,29) =52.98, p=0.000].Partial Eta Squared = 0.785. As per Cohen’s guidelines the value is more than 0.14. This indicated very significant effect of selected biological immunomodulators on both ‘Absolute CD_4 count’

and ‘Viral load’. Univariate test, for CD_4 count indicated, Greenhouse-Geisser = 279084.084, f [1, 30] = 33.016, p value=0.000. Since p value is < 0.05, it is concluded that, there is a significant difference between initial absolute CD_4 count (Before Treatment) and Final absolute CD_4 count (After Treatment). Univariate test, for ‘viral load’, indicated Greenhouse-Geisser =4.643, E+11, f [1, 30] = 108.65, p = 0.000. Since p- value < 0.05, it is concluded that, there is significant difference in the ‘Initial viral load’ (Before Treatment) and ‘Final viral load’ (After treatment). Descriptive statistics showed that, viral load mean of HIV 1 RNA copies before treatment of biological immunomodulators was 327881.198 and after treatment was 156178.056. Thus it can be concluded that, there was a significant decrease in viral load of HIV 1 RNA copies in the peripheral blood of the infected individuals after treatment of biological immunomodulators.

18) The study will help to design the therapeutic regime for HIV infected individuals including drug resistant cases. The study also will help to maintain the cellular immunity of HIV infected individuals at affordable prise.

CONCLUSIONS

CONCLUSIONS

1) Potential biological immunomodulators were screened.

2) Antibacterial and antifungal properties of selected biological immunomodulators were studied. *Picrorrhiza kurroa, Wagatia spicata Dalz., Withania somnifera and Spilanthes paniculata Wall. ex. Dc.* showed antibacterial properties. *Chlorophytum borivilianum, and Wagatia spicata Dalz.* exhibited antifungal properties. Thus selected biological immunomodulators may control secondary infections in HIV infected individuals.

3) Immunomodulating properties of selected biological immunomodulators were studied

   A) In Swiss albino mice, administration of morning dose and evening dose of biological immunomodulators increased phagocytic index, enhanced T lymphocyte function and improved the function of 'Neutrophil'

   B) Administration of morning dose and evening dose of biological immunomodulators in HIV infected individuals for the average period of 11 months reduced viral load of HIV-1 RNA and increased the count of 'Absolute CD_4 cells'. Thus synergistic action of biological immunomodulators can be used to curb HIV infection and also to increase the immunity of the patients.

4) Blended dose of *Wagatia spicata Dalz.* and *Picrorrhiza kurroa* was well tolerated by animals and human beings and can be administered as O.D. dose through oral route.

5) Blended dose of *Withania somnifera, Wagatia spicata Dalz., Spilanthes paniculata Wall. ex. DC.,* and *Chlorophytum borivilianum* was well tolerated by animals as well as human beings.The dose can be administered as O.D. dose through oral route.

6) During human clinical trials, no participant was reported for side effects. Hence the blended formulations when used at described doses do not exert toxicity or side effects. This observation may help to plan treatment regime for HIV infected individuals who fail to tolerate the antiretroviral therapy.

7) Still scope is there to test immunomodulating properties of selected biological immunomodulators at different combinations than that have used in the present study.

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ANNEXURES

ANNEXURE – I

STAINS

STAINS USED FOR TESTING GRAM CHARACTERISTICS OF TEST BACTERIA DURING STUDY OF ANTIBACTERIAL ACTION OF BIOLOGICAL IMMUNOMODULATORS:

A) Hucker's Crystal Violet Solution:

Crystal Violet Chloride Powder	2 gm
Ethanol	20 ml
Dissolve Crystal Violet in ethanol and mix it with,	
Ammonium oxalate solution (1%) solution	80 ml

B) Gram's Iodine:

Iodine powder	1 gm
Potassium iodide	2 gm
Distilled water	10 ml
Dilute 1:3 before use.	

C) Safranin:

Safranin powder	25 gm
Absolute ethanol	10 ml
Dissolve safranin in ethanol and dilute to	
Distilled water	100 ml

REAGENTS

1) Reagents for Determination of Lead and Cadmium :

A) Standard solution of Lead :

Lead powder	1gm
Conc. HNO_3	7 ml
Distilled water	1000 ml

B) Standard solution of Cadmium :

Cadmium powder	1gm
Conc. HNO_3	7 ml
Distilled water	1000 ml

C) Working standard solution for graphite furnace analysis:
Standard solution of lead and cadmium diluted with 0.1 M HNO_3 to range of standards as described in methodology

2) Reagents for Estimation of Mercury by 'Flameless Atomic Absorption Spectroscopy':

A) Mercury Standard Solution: 1000 mg Mercury / lit

B) Mercury Stock Solution:

$HgCl_2$	0.1354 gm
Distilled water	100 ml

C) Mercury Working Solution :
1 ml of stock solution is diluted to 1000 ml with 1N H_2SO_4 and should be freshly prepared.

D) Diluting Solution:

HNO_3	58 ml
H_2SO_4	67 ml
Distilled water	300 ml

3) Reagents for estimation for Arsenic by 'Colorimetric molybdenum – blue method':
A) Bromine water

Saturated Bromine water	75 ml
Distilled water	75 ml

B) Sodium hypobromite solution

0.5N NaOH	25 ml
Bromine water	75 ml

C) Ammonium molybdate sulphuric acid solution:

$(NH_4)_6MO_7O_{24}.H_2O$	5 gm
H_2SO_4 (sp. Grade 1.84)	42.8 M
Distilled water	100 ml

D) Hydrazine Sulphate reagent:

Hydrazine Sulphate powder	1.5 gm
Distilled water	100 ml

E) Potassium Iodide Solution:

KI Powder	15 gm
Distilled water	100 ml

F) Stannous Chloride Reagent:

$SnCl_2$ powder	40 gm
Conc.HCl	100 ml

G) Dil HCl Solution:

Conc.HCl (Sp. Grade 1.18)	180 ml
Distilled water	70 ml

H) Lead Acetate Solution:

Pb (OAC) $2.3H_2O$	10 gm
Distilled water	100 ml

4) Reagents for absolute CD_4 counting:

Flow Cytometer Kit:

Dura Clone IM 'T cell subsets kit'.

Brilliant Violet antihuman CD-127

Brilliant Violet antihuman CD-95

Brilliant Violet antihuman CD-25

IO Test 3 fixative solution

'Versa Lyse', lysing solution

5) Reagents for viral load testing:

A) 'Lysis buffer':

Guanidium thiocyanate	5.75 M
Tris (pH 7.5)	50 mM
2 Mercaptoethanol	100 mM
Poly (rA)	1 µg/ml

B) Working Mastermix Solution:

Tris Buffer (pH 8.3)	20 mM
KCl	180 mM
d ATP	300 µM
d CTP	300 µM
d GTP	300 µM
d TTP	300 µM
d UTP	400 µM
'r Tth DNA polymerase'	10 U
UNG	2U
Glycerol	30 %
Primers	20 p-moles each

C) Substrate Solution:

H_2O_2 (0.001%) and 0.01% 3, 3', 5-5' tetramethylbenzidine in 40% in dimethylformamide in the ratio of 4:1 respectively.

6) Reagents for HIV 1, P-24 Antigen Inhibition Assay:
P-24 Antigen Inhibition Assay kit containing,
A) HIV 1, P-24, Immunoglobulin coated microplate
B) HIV 1, P-24 biotin labeled monoclonal antibody
C) Monoclonal antibody diluents
D) HIV 1, P-24 Standard antigen
E) Lysing Buffer containing triton-100 in PBS and 2-Chloroacetamide.
F) Streptavidin conjugated with horseradish peroxidase
G) Wash Buffer Kit
H) Tetramethylbenzidine (TMB) in DMSO
I) Stop solution

ANNEXURE – II

PUBLICATIONS AND PRESENTATIONS

JOURNAL PUBLICATIONS:

1. Ballal B.B., P.M.Bulakh, M.G.Bodhankar (2015), **"**Neutrophil Adhesion Test' as a tool for assessment of immunomodulatory activities of blended formulation of *Chlorophytum borivilianum, Withania somnifera, Wagatia spicata Dalz, Picrorrhiza kurroa and Spilanthes paniculata*", International Journal of Advanced Research in Biological Sciences 2**(11):** 97-201, **(ISBN 2348-8069), IF 5.142. Index Copernicus (5.01)**

2. Ballal B.B., P.M.Bulakh, M.G.Bodhankar (2015), **"**Immunomodulatory activities of blended formulation of *Chlorophytum borivilianum, Withania somnifera, Wagatia spicata Dalz, Picrorrhiza kurroa and Spilanthes paniculata*", Asian Journal of Multidisciplinary Studies 3**(11):**26-28**, (ISBN 2321-8819), IF 1.498**

CONFERENCE PRESENTATIONS:

1. "Effect of herbal extracts on CD_4 count in HIV infected individuals", at New Trends in Biotechnology, Osmania University Hydrabad, 26-28 Feb.2015

2. "Exploring Anti HIV principles of blended formulation of *Chlorophytum borivilianum, Withania somnifera, Wagatia spicata Dalz ,Picrorrhiza kurroa and Spilanthes paniculata*", at 3rd International Conference on Herbal and Synthetic Drug Studies (HSDS-2016), Pune, sponsored by University of Kansas Cancer Center, USA 07-09 Jan, 2016.