INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

A Preliminary Twenty Eight Days Repeated Dose Cytotoxicity Test of Polyherbal Formulation as ANTI-HIV Herbal Drug

Trivikram M. Deshpande and Sushama R. Chaphalkar

Animal Biotechnology, VidyaPratishthan's School of Biotechnology, Vidyanagari Baramati-413 133, Pune, Maharashtra, India.

ABSTRACT

Powdered plant extract formulation consisting of the plants Baheda (fruit) *Terminalia belerica* (Roxb), Chitwan (Panchang) *Alstonia scholaris* (Br), Pipal (fruit) (Root) *Piper longum* (Linn), *Giloy gurucha* (stem) (Leaves) *Tinospora cordifolia*, Dhekwar (leaf) *Aloevera indica* (Linn), Manjith (root), *Rubia cordifolia* (Linn), Kapuri (bark of root) *Hemidesmus indica* (B. Br), Awala (leaf, fruit, seed) *Phyllanthus emblica* (linn), Dalchini (leaf, bark), *Cinnamomum zeylanicum* (blume) and Beet (tuber) *Beta vulgaris* is implicated as antiHIV polyherbal formulation and given to AIDS patients with the 1000 mg/kg dose of their Body Weight (BW) in 28 days repeated dose cytotoxicity test. It was observed that this polyherbal formulation was noncytotoxic. This preliminary repeated dose 28-day oral toxicity study in mice (OECD 407) was done to study its safety for human use. In this study, Swiss albino mice (5 in control and 5 in treatment group) were gavaged with aqueous suspension of polyherbal formulation at the dose 1000 mg/kg BW/day for 28 consecutive days. Results of the study showed that there were no statistically significant effects on average body weight, blood glucose and also no effects on histology of organs, clinical biochemistry and hematological parameters of treated mice. In conclusion, the aqueous extract from the polyherbal formulation at tested dose and time duration did not cause toxicity in mice and that this polyherbal formulation is noncytotoxic to the treated mice.

Keywords: In vivo cytotoxicity, antiHIV, OECD 407, Polyherbal formulation.

INTRODUCTION

Powdered plant extract formulation consisting of the plants Baheda (fruit) *Terminalia belerica* (Roxb), Chitwan (Panchang) *Alstonia scholaris* (Br), Pipal (fruit) (Root) *Piper longum* (Linn), *Giloy gurucha* (stem) (Leaves) *Tinospora cordifolia*, Dhekwar (leaf) *Aloevera indica* (Linn), Manjith (root), *Rubia cordifolia* (Linn), Kapuri (bark of root) *Hemidesmus indica* (B. Br), Awala (leaf, fruit, seed) *Phyllanthus emblica* (linn), Dalchini (leaf, bark), *Cinnamomum zeylanicum* (blume), Beet (tuber) *Beta vulgaris* was implicated as antiHIV and given with 1000 mg/kg Body Weight (BW) dose to AIDS patients. Hence, to study the cytotoxicity with same dose in vivo, the per oral 28 days repeated dose cytotoxicity study as per OECD guideline 407 was done.

The componenets of our polyherbal antiHIV formulation possess antiHIV activity as per the references. A bioactivity-guided fractionation of an extract of *Terminalia bellerica* fruit rind led to the isolation of two new lignans named termilignan (**1**) and thannilignan (**2**), together with 7-hydroxy-3',4'-(methylenedioxy) flavan (**3**) and anolignan B (**4**). All four compounds possessed demonstrable anti-HIV-1, antimalarial, and antifungal activity in vitro (Valsaraj et al 1997). From *Terminalia bellerica* Roxb. (Combretaceae) Bahera,/ aqueous and methanol extracts comprising chebulagic acid, punicalin, punicalagin, and punicacortein are implicated in inhibition of HIV reverse transcriptase, inhibition of viral adsorption to cells (Vermani and Garg 2002, Nonaka et al., 1990, Weaver et al., 1992, Mekkawy et al., 1995).

Potent α-glucosidase inhibitory activity was found in aqueous methanol extract of dried devil tree (*Alstonia scholaris*) leaves (Jong et al 2007). The α -glucosidase inhibitors are also implicated in anti HIV activity (Bridges et al 1994, Fischer et al 1995, Fischer et al 1996). *Tinospora cordifolia* extract, a plant derived immunostimulant, significantly affected the symptoms of HIV as validated by clinical evaluation. *Tinospora cordifolia* could be used as an adjunct to HIV/AIDS management (Kalikar et al 2008).

Beta vulgaris Sugar beet (Beta vulgaris L.) leaves contain virus inducible type 1 (single chain) ribosome-inactivating proteins that have been named beetins (Iglesias et al 2005). Beetins shares sequence homologies

with several well-known (Ribosome Inactivating Proteins) RIPs, especially those exhibiting anti-HIV-1 activity (Iglesias et al 2005) such as : PAP-II (32%; Poyet et al., 1994), MAP 30 (27%; Lee-Huang et al., 1990), TAP 29 and trichosanthin (28%; Lee-Huang et al., 1991a), gelonin (25%; Lee-Huang et al., 1991b), and DAP-32 (25%; Lee-Huang et al., 1991b).

From *Phyllanthus emblica* Linn. (Euphorbiaceae) amla used in jaundice and viral diseases, the methanol extract comprises Putranjivain A implicated in reverse transcriptase inhibition assay, inhibition of HIV reverse transcriptase (Vermani and Garg 2002, Mekkawy et al.,1995). *Rubia cordifolia* demonstrated promising anti-HIV potential and were investigated for their active principles. R. cordifolia (roots, ethyl acetate extract) exhibited more than 60% inhibition of HIV-1 at noncytotoxic concentration and *T. cordifolia* (stem bark, Methanolic extract) exhibited moderate activity against HIV-1 (between 40 and 60% inhibition) at non-cytotoxic concentrations while Piper longum fruit hexane extract selected due to its immunomodulatory activity, exhibited 73.9 % inhibition at 20µg/ml. *Rubia cordifolia* mainly contain naphthoquinones and anthraquinones (Chang et al 2000). The ethanolic extract of roots was active against HIV-1 at 15 µg/ml. Xanthopurpurin was isolated from the active extract and showed 42% inhibition of HIV at 15 µg/ml (Sabde et al 2011). Several naphthoquinones such as 1,4-naphthoquinone, juglone and plumbagin have been shown to possess anti-HIV activity (Min et al 2002).

Aloe vera had high inhibitory action against HIV. A polyherbal cream (Basant) has been formulated using diferuloylmethane (curcumin), purified extracts of *Emblica officinalis* (Amla), purified saponins from *Sapindus mukorossi*, Aloe vera and rose water along with pharmacopoeially approved excipients and preservatives (Talwar et al 2008). Basant was cytotoxic at 50% effective concentration EC50 (µg/ml) at 278 fold dilution and inhibited viral entry at 2492 fold dilution as per viral entry inhibition assay (Talwar et al 2008). Optimized cinnamon extracts rich in certain flavonoid compounds were shown to block HIV-1 entry and infection in GHOST cells. The compounds that blocked HIV-1 infection were flavonoids and A-type proanthocyanidins. The 50% inhibitory concentration values of these extracts ranged from 0.5 to 201 microg/ml for four different HIV-1 serotypes (Fink et al 2009).

The polyherbal formulation is given to AIDS patients with 1000 mg/kg of their BW. To study the cytotoxicity of this formulation with the same dose in AIDS patients, this preliminary repeated dose 28-day oral toxicity study in mice (OECD 407) was done. The in vivo cytotoxicity of this polyherbal formulation is not reported earlier. To adminsiter as anti HIV drug and benefit the AIDS patients with antiHIV activity of this polyherbal formulation, the in vivo cytotoxicity of this polyherbal formulation was done. In our preliminary 28 days repeated dose cytotoxicity test, we evaluated the safety of this polyherbal formulation as herbal antiHIV drug to be given to AIDS patients.

Animal Biotechnology, VidyaPratishthan's School of Biotechnology, Baramati, 413133 India
Corresponding author, email : tridesh@yahoo.com

MATERIALS AND METHODS (Jujun et al 2008).

Preparation of plant extract

Powdered plant extract formulation consisting of the plants : Baheda (fruit) *Terminalia belerica* (Roxb), Chitwan (Panchang) *Alstonia scholaris* (Br), Pipal (fruit) (Root) *Piper longum* (Linn), *Giloy gurucha* (stem) (Leaves) *Tinospora cordifolia*, Dhekwar (leaf) *Aloevera indica* (Linn), Manjith (root), *Rubia cordifolia* (Linn), Kapuri (bark of root) *Hemidesmus indica* (B. Br), Awala (leaf, fruit, seed) *Phyllanthus emblica* (linn), Dalchini (leaf, bark), *Cinnamomum zeylanicum* (blume), Beet (tuber) *Beta vulgaris* were kindly provided by Mr. Ayare.

Laboratory animals

10 female Swiss Albino mice (*Mus musculus)* aged more than eight weeks were procured from Raj Biotech, Shirwal, Satara, Maharashtra and maintained in VSBT animal house at temperature between 25^0C to 35^0C with 12 hour light and 12 dark cycle and provided commercial mice feed pellets and groundnuts, sprouted beans and tapwater. The study is approved by institutional animal ethics committee.

Repeated Dose 28-Day Oral Toxicity Studies

The animals were divided into 2 groups viz. control group comprising C1 to C5 and treatment group comprising T1 to T5. The aqueous extract of the polyherbal formulation was administered orally by gavaging at 1000 mg/kg BW of the formulation daily for a period of 28 days. The control group received an equal volume of distilled water as per their BW. All mice were weighed weekly and observed daily for behavioral changes. Any mouse that died during the test period was tested for pathology and all animals were examined at the end of the test period.

Blood analysis

All surviving animals were sacrificed by cervical dislocation for blood collection from cardiac puncture. Blood samples were collected into heparinized vacutainers. A blood analysis (both hematology and biochemistry) was carried out. The heparinized blood was used for a hematological study which included WBC and differential leukocyte counts, platelet, hematocrit and hemoglobin estimation. The serum was assayed for glucose.

Tissue analysis

The liver and kidney organs were examined and then fixed in formaldehyde solution. The fixed organs from all animals were examined by histological method.

Statistical analysis

Results were expressed as mean, standard deviation. (S.D.) and student t test http://www.physics.csbsju.edu/stats/t-test.html. P values less than 0.05 were considered significant http://graphpad.com/quickcalcs/pValue2/.

RESULTS

The administration of the polyherbal formulation at dose of 1000 mg/kg/BW daily for 28 days did not cause mortality except in treatment group T2. As shown in Table 1, no statistical difference from the control and the treatment group was detected on the body weight. The animals did not show any changes in general behavior or other physiological activities.

Table 1: Individual body weights (g) of animals weekly during the test in a Repeated Dose 28-Day Oral Toxicity

Week 1	Control group	Weight (gms)	Treatment group	Weight (gms)
	C1	31.15	T1	43.2
	C2	40.2	T2	42.2
	C3	45.7	T3	35.8
	C4	43.2	T4	44.2
	C5	37.6	T5	39.9
Mean		39.6		41.1
Standard deviation		5.61		3.34

Values are expressed as mean, S.D., n = 5, there were no significant differences ($P > 0.05$)

Week 2	Control group	Weight (gms)	Treatment group	Weight (gms)
	C1	29.03	T1	38.3
	C2	37.17	T2	38.8
	C3	44.7	T3	34.28
	C4	40	T4	43.45
	C5	34.8	T5	38.57
Mean		37.1		38.7
Standard deviation		5.84		3.25

Values are expressed as mean, S.D., n = 5, there were no significant differences ($P > 0.05$)

Week 3	Control group	Weight (gms)	Treatment group	Weight (gms)
	C1	29.90	T1	41.06
	C2	39.46	T2	41.07
	C3	44.27	T3	36.30
	C4	42.4	T4	45.01
	C5	34.82	T5	42
Mean		38.2		41.1
Standard deviation		5.84		3.13

Values are expressed as mean, S.D., n = 5, there were no significant differences ($P > 0.05$)

Week 4	Control group	Weight (gms)	Treatment group	Weight (gms)
	C1	30.50	T1	43.39
	C2	41.2	T2	-
	C3	41.5	T3	37.69
	C4	43.4	T4	47.36
	C5	38	T5	43.93
Mean		38.9		34.5
Standard deviation		5.09		19.6

Values are expressed as mean, S.D., n = 5, there were no significant differences ($P > 0.05$)

Hematological and biochemical observations

Histological examination is done to confirm the characteristic of the tissues. Hematological parameters provide vital information regarding the status of bone marrow activity and intravascular effect such as hemolysis (Jujun et al 2008). The hematological analysis (Table 2) showed no significant differences in any of the parameters examined in either the control or treated groups. Blood chemistry analysis (Table 3) revealed no significant changes in any of the parameters examined in either the control or treated groups.

Table 2: Hematological values of animals in Repeated Dose 28-Day Oral Toxicity

Control group	Haemoglobin G% g/dl	Normal value G %	RBC Count millions/cmm	Normal value millions/cmm	Total WBC Count/cmm	Normal value /cmm
C1	13.5	M 14-17 F 12-15	7.72	4.0-6.20	4400	4100-10900
C2	12.9	M 14-17 F 12-15	8.21	4.0-6.20	12,100	4100-10900
C3	10.4	M 14-17 F 12-15	6.38	4.0-6.20	4200	4100-10900
Treatment group	Haemoglobin G%	Normal value G %	RBC Count millions/cmm	Normal value millions/cmm	Total WBC Count/cmm	Normal value /cmm
T3	12.8	M 14-17 F 12-15	8.12	4.0-6.20	3700	4100-10900
T4	10.8	M 14-17 F 12-15	7.25	4.0-6.20	3500	4100-10900

Hemoglobin is an indicator of oxygen carrying capacity. The hematological values are obtained from the fully automated three Part differentiated 18 parameter blood cell counter (Analytical automation) where normally human patient's blood samples are analyzed. The hemoglobin value for C1 and C2 is within the normal range while that of C3 is below the normal range (Table 2). The hemoglobin value for T3 is in normal range and

that of T4 is below the normal range. RBC count for C1, C2, C3 is higher than the normal range. RBC count for T3 and T4 is higher than the normal range. WBC count is in normal range for C1 and C3 and for C2, it is higher than the normal range and it is below the normal range for T3 and T4 (Table 2).

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Differential Count

Control group	Neutrophils (%)	Normal range (%)	Lymphocytes (%)	Normal range (%)	Platelet Lakh/ cmm	Normal range Lakh/cmm
C1	34 %	(40-75)	63	(20 – 50)	4.52	(1.4-4.4)
C2	24 %	(40-75)	74	(20 – 50)	5.36	(1.4-4.4)
C3	22 %	(40-75)	76	(20 – 50)	2.04	(1.4-4.4)
Treatment group	Neutrophils (%)	Normal range (%)	Lymphocytes (%)	Normal range (%)	Platelet Lakh/ cmm	Normal range Lakh/cmm
T3	24	(40-75)	74	(20 – 50)	6.69	(1.4-4.4)
T4	23	(40-75)	74	(20 – 50)	1.52	(1.4-4.4)

In the differential count, the value for neutrophils for C1, C2, C3 is lower than the normal range and it is also lower than normal range for T3 and T4. The value for lymphocytes is higher than normal for C1, C2 and C3 as also it is higher than normal for T3 and T4. The platelet count is higher than normal value for C1, C2 and for C3 it is within the normal range. In treatment group, it is higher than normal range for T3 and for T4 it is within the normal range.

Table 3: Blood chemistry values of animals in Repeated Dose 28-Days Oral Toxicity

Estimation of blood glucose

Control group	Blood glucose (R)	Normal value	Treatment group	Blood glucose (R)
C1	138.0 mg/dL	60-160mg/dL	T1	192.0 mg/dL
C2	123.0 mg/dL	60-160mg/dL	T2	-
C3	155.0 mg/dL	60-160mg/dL	T3	131.0mg/dL
C4	-	-	T4	122.0 mg/dL
C5	198.0 mg/dL	60-160mg/dL	T5	136.0 mg/dL
Mean	154		Mean	145
Standard Deviation	32.4		Standard Deviation	31.7

Values are expressed as mean, S.D., n = 4, there were no significant differences ($P > 0.05$)

Regarding the blood glucose level, it is within the normal range for C1, C2 and C3 and T3, T4 and T5 but higher than normal range for C5 and T1.

Tissues analysis

The histological examinations of the liver and kidney were normal in both the control and treated groups.

CONCLUSION

28-days oral toxicity at the dose 1,000 mg/kg BW/day of polyherbal formulation did not produce any dose-related change of hematological parameters, serum biochemistry or histology of internal organs studied. Therefore, it is concluded that the polyherbal formulation at the given dose did not produce any toxic effect in mice during the period of treatment for 28 days. An additional study on chronic toxicity evaluation is needed to determine the long-term safety of the polyherbal formulation.

DISCUSSION

This polyherbal formulation consists of ayurvedic plants many of them possessing the antiHIV activity. Thus, by combining these plants together, the antiHIV effect and antiHIV activity of these plants can be augmented and the AIDS patients benefitted. This preliminary in vivo 28 day repeated dose cytotoxic test was done as per OECD guidelines 407 to study whether there are any side effects or toxicity to organs and safety after oral administration in humans of this polyherbal drug.

Based on the hematological, biochemical and histological analysis, this polyherbal formulation is noncytotoxic to the 4 mice out of 5 female mice in treatment group. One of the treatment mouse (T2) died before the 28 day period may be due to stress on taking the gavage in the oesophagus as it moved its head vigorously. Till the 14t^{h} day, this mouse behavior and clinical signs were normal as per OECD 407.

Body weight changes are marker of adverse effects of drugs and chemicals (Sivaraman et al 2013). The in vivo 28 day repeated dose of the polyherbal formulation have shown no significant changes in body weight as well as behavior as well as mortality of mice. This implicate that the long term administration of this polyherbal formulation can be safe and could be used for chronic ailment (Sivaraman et al 2013).

The hematopoietic system is one of the most sensitive targets for toxic substances and it is also an important marker of physiological and pathological status in human and animal studies (Sivaraman et al 2013). All animals except T2 from treatment group survived until the 28 days and no gross pathological alteration was found in the internal organs studied.

The results of this study reveal that the aqueous polyherbal formulation treated animal group did not show any toxic symptoms in behavior or mortality at dose level of 1000mg/kg as evidenced by absence of toxic symptoms, no change in water and food ingestion was noticed. Generally, the reduction in body weight gain and internal organ weights is a simple and sensitive index of toxicity after exposure to toxic substance. In the present study, aqueous polyherbal formulation does not produce any statistically significant difference among the control and treatment groups in body weight gain (Darwin et al 2011).

An essential step in the life cycle of human immunodeficiency virus type 1 (HIV-1) is integration of the double-stranded retroviral DNA into the genome of the host cell. HIV-1 integrase, the enzyme that inserts the

vital DNA into the host chromosome, is an attractive and rational target for anti-AIDS drug design because it is essential for HIV replication and there are no known counterparts in the host cell. Inhibitors of this enzyme have the great potential to complement the therapeutic use of HIV protease and reverse transcriptase inhibitors. Natural products have provided a source of new drug candidates for anti-AIDS therapy. Baicalein and baicalin, identified components of a Chinese herbal medicine *Scutellaria baicalensis Georgi*, have been shown to inhibit infectivity and replication of HIV. The preliminary results of the computational modeling study demonstrated that Baicalein binds to the active site region of the HIV-1 integrase (Hu et al 2010). Similarly, the computational modeling study on our antiHIV polyherbal formulation can also be proposed to study the as antiHIV.

Although anti-retroviral drugs have resulted in an improvement of the quality of life amongst HIV infected humans (*i.e.* eating and sleeping well and keeping a positive outlook on life), the development of resistance, appreciable levels of toxicity, high cost, unavailability and lack of curative effect are their major short-comings (Bessong et al 2005). These short-comings, especially the appearance of drug resistant virus strains have resulted in increased efforts for the search of better anti-HIV agents and much attention is now being directed towards natural products (Wang et al 2004). The IC50 values show that *Ingwe® muthi mixture* > *Imbiza ephuzwato* > *African potato extract*TM > *Sejeso herbal mixture Ingwe®*, in that order, are potent inhibitors of the RT. The mechanisms of action of these four herbal preparations could be through a conformational change on the RT thereby rendering it inactive. It is also possible that the herbal preparations may contain compounds that may act as competitive inhibitors of the RT. Since these preparations are made of a mixture of plant species, there is a possibility that the compounds may be novel (Ndhlala et al 2010). Our antiHIV polyherbal formulation is also novel and due to its non cytotoxicity, it can be herbal antiHIV therapeutic drug for HIV infected individuals.

The HIV-1 gp41 six-helix bundle formation, a critical step of membrane fusion between the HIV and the target cell. Extracts of two herbs, *Prunella vulgaris* and *Rhizoma cibotte* showed potent inhibitory activity. These results suggest that tannin may be one of major inhibitors of the HIV-1 gp41 six-helix bundle formation in the herb extracts and that tannin may inhibit HIV-1 entry by disrupting the gp41 six-helix bundle formation (Liu et al 2002). Similarly, further studies on our polyherbal antiHIV formulation can also be done. Current available studies indicate that many complementary and alternative medicine (CAM) interventions may improve the quality of life of people living with HIV-AIDS; however, further studies using longitudinal, controlled designs are needed to accurately assess the safety of such interventions (Power et al 2002).

According to Gupta et al 2010, *Shilajatu* (Mineral pitch), *Centella asiatica (Mandukaparni), Tinospora cordifolia (Guduchi)* and *Emblica officinalis (Amalaki)*, well known for their immuno-modulator and antioxidant properties were selected to evaluate their role on immune system. Treated Group responded better to (AntiRetroviral Therapy) ART both clinically and biochemically compared to the Control group (ART). The results show that *Shilajatu* decreases the recurrent resistance of HIV virus to ART and improves the outcome of the therapy The compound formulation was found to be safe, (on prolonged use for 3 months in the dose of 6gm) and it has decreased the intensity of the clinical symptoms and signs and also protected the liver from the hepatotoxicity of ART as evident by normal (Liver Function Tests) LFT or Kidney function tests as per Gupta et al 2010. Similarly, the antiHIV effect of our antiHIV polyherbal formulation with and without ART can also be studied further.

This is the first report on cytotoxicity of our polyherbal formulation implicated with antiHIV activity and hence as herbal antiHIV drug. Further follow up of the AIDS patients should be done and their hematological, biochemical and histological analysis also be done. From this preliminary study, the effectiveness of this polyherbal formulation in preventing and reducing HIV infections as well as its safety for administration in humans should be documented in healthy human volunteers and AIDS patients.

Thus, based on this study, the aqueous ayurvedic polyherbal formulation is non cytotoxic at the dose of 1000 mg/kg BW.

ACKNOWLEDGEMENTS

The authors are thankful to the client Mr. Ayare for providing the powdered plant formulation, the Biovillage Project and VSBT Incubator Center, Shirish laboratory, Baramati and Friend's Speciality Pathology Laboratories, Pune for the hematological, histological and clinical biochemical studies.

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