Phytopharmacology

Inforesights Publishing

Fagaricine, a new immunorestorative phytomedicine from *Zanthoxylum heitzii*: Preclinical and multicenter cohort clinical studies based on HIV-infected patients in six countries

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Received: 13 October 2011, **Revised**: 17 November 2011 **Accepted**: 21 November 2011

Abstract

The present investigation was carried out to evaluate the safety of Fagaricine, an aqueous extract of *Zanthoxylum heitzii* by determining its potential toxicity after acute and subchronic oral administration in rodents, and his clinical benefits on HIV-infected patients. For the acute study, Fagaricine was administered to mice in orally single doses of 0-10 g/kg. General behaviour adverse effects and mortality were determined for 7 days. In the subchronic dose study, the extract was administered at doses of 0-1275 mg/kg daily for 35 days. Biochemical and haematological parameters were determined at the end of 35 days of daily administration. For the clinical benefits of Fagaricine, multicentre cohort observations were realised in six countries, on 75 patients. All patients received two tablets of the fixed-dose containing 100 mg of Fagaricine twice daily for 24 weeks. Clinical benefits were evaluated over active control on CD4 counts. In the In the acute study in mice, no adverse effects and mortality was observed. In the subchronic study, variations were observed with biological, heamatologic-

al and biochemical parameters but were significant as they were not dose-related and/or because values remained within historical control ranges, except urea nitrogen, creatinine in groups 850-1275 mg/kg. In clinical study, at baseline, 12% of patients (n = 9) had AIDS; median CD4 count was 217,5 cells per µL (IQR 118.8 – 484.3). After 16 weeks of treatment, CD4 count increase of 90 cells per µL (IQR 22-107) and 133 (IQR 45-215) after 24 weeks. The bodyweight increase in patients treated with Fagaricine after 8 weeks 4.15 kg (95% CI 2.11-6.19). In conclusion, Fagaricine appears to be safe and non-toxic in these studies and no-observed adverse effect level in mice was established less to 850 mg/kg/day. In view of the dose of Fagaricine consumed in traditional medicine (10 mg/kg/day), there is a wide margin of safety for the therapeutic use of the aqueous extract of *Zanthoxylum heitzii*. In addition our findings lend support to use of Fagaricine as an immunorestorative phytomedicine to treat immunodeficiency.

Keywords: Subchronic toxicity, *Zanthoxylum heitzii*, Fagaricine, F-352, medicinal plants, immunotherapy, immunostimulant, phytomedicine, HIV, CD4, immunodeficiency

Introduction

Zanthoxylum heitzii (Aubrev. & Pellegr.) is a medicinal plant widely used in central Africa for the treatment of many diseases such as cancer, syphilis, malaria, cardiac palpitate-ions, urogenital affections (Zirihi et al., 2005; Mbaze et al., 2009). Phytochemical investing-ations reported that *Zanthoxylum heitzii* instead of contents many components including amides, lignanes (Mbaze et al., 2009), alkaloids such as benzophenanthridines (nitidine, methylnitidine etc.) and steroids and terpenes (Ngouela, 1994; Bongui, 2005).

It is well know that many of those components instead of induced biological effects such as antiviral (Cheng et al., 2005), cytotoxic (Nakanishi et al., 2000), antimalaria (Kassim et al., 2009; Gansane et al., 2010; Iwasaki et al., 2010), antifungal and antibacterial (Hanawa et al., 2004; Tabuti et al., 2010), anti inflammatory effects (Prempeh & Mensah-Attipoe, 2008). One active principle Flindersine (2,6-dihydro-2,2-dimethyl-5H-pyrano [3,2-c] quinol-ine-5-one-9cl) was isolated from the ethyl acetate extract which exhibits antibacterial effect against bacteria *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Pseudomonas aeruginosa, Acinetobacter baumannii* and *fungi Trichophyton rubrum 57, Trichophyton mentagrophytes, Trichophyton simii, Epidermophy-ton floccosum, Magnaporthe grisea* and *Candida albicans* (Duraipandiyan & Ignacimuthu, 2009).

The aim of this study was to evaluate the safety of natural immunoreconstituant Fagaricine, an aqueous extract of *Zanthoxylum heitzii* bark by determining its potential toxicity after acute and subchronic oral administration in rodents, and his clinical benefits on HIV-infected patients.

Material and methods

Preclinical study

Plant resources and preparation of crude extract

The plant was prepared according to the method recommended traditionally for oral administration. The stem bark of *Zanthoxylum heitzii* was collected at Ambam locality, South forest region of Cameroon and identified by National Herbarium, Yaoundé, Cameroon, where the voucher specimen (Ref. No. 1482/SRFK) has been deposited. Briefly, Fagaricine (crude aqueous extract) was obtained by the maceration of the powder of dried bark in distilled water (1kg/5L) for 24 hours at 60 °C. The aqueous extract was filtered, concentrated and dried in a spray dryer (Plantex, France), to obtain powder 8% (w/w) kept at 4°C until use.

Animals

Mice Swiss-Swiss were chosen to determine the potential of Fagaricine to produce toxic effects. Healthy mice (60 males and 60 females) were obtained from Laboratory of Animals physiology (Yaoundé 1 University, Cameroon). They were housed under standard environmental conditions of temperature at 25 ± 20°C under a 12-hour light-and-dark natural cycle and allowed free access to drinking water containing vitamin (A, D3, E, B1, B6, B12, PP and C) and a standard pelleted diet (containing powder of maize 60%, corn 10%, fish 12%, soya beans 15% and palm oil 3%). Throughout the experiments, all the animals were processed according to the suggested ethical guidelines for the care of laboratory animals. After 1 week of an acclimation period, animals were equally distributed in groups (5 males and 5 females per group). Assignment was random with the constraint that the mean body weight of three dosing groups of the same sex was not statistically different. At initiation of dosing, the animals' body weights ranged from 23 ± 3 g. All animals housed in individual cages.

Dose preparation and administration

Individual doses were calculated based on the most recent weekly body weights and were adjusted each week to maintain the targeted dose level for all mice (i.e., mg/kg/day). All doses were administered volumetrically, by oral gavage at a constant volume of 10 mL/kg, after correcting for concentration of the Fagaricine (Laboratoires TBC, France). The control animals received the vehicle only by the oral gavage at the same volume as the test groups. The whole study was performed according to GLP by a laboratory with GLP accreditation.

Acute oral toxicity study

A single-dose oral study was conducted in mice to evaluate the potential toxicity of high exposure to Fagaricine. Ten (10) healthy mice per group (5 males and 5 females) were administered 0, 85, 170, 340, 680, 1360, 2720, 4440 and 10000 mg/kg body weight by oral gavage. Fagaricine was administered as a 50% suspension w/w in distilled water. Each anim-

al was fasted for 18 h prior to dosing and later provided with water and food *ad libitum*. Mice were observed for mortality and changes in behaviour for 14 days after treatment. Body weights were taken before treatment and on days 7. This GLP study was performed in accordance with World health organization general guideline for methodologies on research and evaluation of traditional medicine (WHO, 2000).

35-Day repeat dose oral toxicity study

In subchronic study, Fagaricine was administrated per group of ten animals (5 males and 5 females) as 0, 425, 850, and 1275 mg/kg/day. On each day of dosing, for each concentration, an appropriate amount of the Fagaricine was accurately weighed into a container and dissolved in distilled water. Each animal was dosed by oral gavage, using a stainless steel ball-tipped gavage needle attached to an appropriate syringe. Dose administration was daily via oral gavage to each mouse for 35 consecutive days.

General observations

All animals were observed twice daily for mortality. Cage side observations were made daily during the study and any abnormal findings recorded. Detailed observations were recorded on Day 1 (prior to administration of Fagaricine) and weekly thereafter on all animals. These observations were conducted both while handling the animal and with the animal placed in an open field. Observations were not limited to changes in skin, gait, posture and response to handling as well as the presence of tonic movements, sterotypies. Aberrant behaviours were also recorded.

Body weight

Individual body weights were recorded twice during the acclimation period, at study initiation (Day 1) and weekly thereafter. Mean body weight gains were calculated for each group at each interval and for the overall (Days 1–35) testing interval.

Food and water consumption

Individual food and water consumption was measured, adjusting for spillage, and recorded weekly to coincide with body weight measurements. Mean food and water consumption were calculated for each dose level during each weekly interval and overall (Days 1–35) testing interval. Animals were allowed *ad libitum* access to food and water throughout the study

Clinical pathology examinations

All mice were fasted approximately 18 hours prior to each blood collection. Blood samples were collected on day 36 of the test period. An anticoagulant (ethylenediamine tetra acetic acid; EDTA) was used for the haematology tests. Clinical chemistry samples were collected without an anticoagulant. The haematology examinations included haemoglobin concentration, erythrocyte count, and total and differential leukocyte count. The clinical

biochemistry examinations included aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, creatinine, urea, nitrogen and cholesterol measurements.

Histopathology

At scheduled sacrifice, all mice were euthanized by exsanguinations after cervical dislocation. All male and female mice from each group were sacrificed on Day 36. The liver, kidneys, heart, lungs, spleen, testes or ovaries were weighed wet as soon as possible after dissection to avoid drying. The above vital organs were preserved in neutral buffered 10% formalin (NBF) for possible future histopathological examination. Histological examination was performed on the preserved organs of the animals from the control and high dose test group. The fixed tissues were trimmed, processed, embedded in paraffin, sectioned, placed on glass microscope slides, and stained with haematoxylin and eosin.

Clinical study

Methods

Our study is multicentre cohort. Six hospitals in five countries in which Fagaricine was approved and allowed to be marketed, Republic of Congo (RC), Democratic Republic of Congo (DRC), Gabon, and Libya, except Ivory Coast. All patients gave their informed consent to participate in cohort in real situation of prescription.

Patients

Study enrolment was between, 2007, and 2009. Patients were eligible if they had confirmed HIV-1 infection, were older than 18 years, and had not taken antiretrovirals before apart 9 patients with clinical AIDS (except for pulmonary tuberculosis) according to the 1993 revised Centers for Disease Control classification (Centers for Disease Control and Prevention, 1992) (CDC group C); mild symptoms (CDC group B) or pulmonary tuberculosis and a CD4 count less than 350 cells per μL or no symptoms (CDC group A) and a CD4 count less than 200 cells per μL and a Karnofsky score of at least 50%. The following biological criteria also applied: serum transaminases, Bilirubin, amounts less than three times the upper limit of normal; serum creatinine less than 200 μmol/L; haemoglobin more than 80 g/L; white bloods more than 0·75×109/L; and platelets more than 50×109/L. For patients with clinical AIDS, they were ineligible if they had N infection, active or uncontrolled opportunistic infections, peripheral neuropathy, active malignant disease (except for mucocutaneous Kaposi's sarcoma), active psychiatric disorders, were pregnant, breastfeeding, or had hepatocellular insufficiency, or if they were receiving anticancer chemotherapy, corticosteroids, immunomodulators, or other trial drugs.

Procedures

All patients received two tablets of Fagaricine (Laboartoires TBC France) twice daily. Patients attended study visits at weeks 0, 2 and 4, and every 4 weeks thereafter until week 24. Patients were divided in two groups. The first group or VT group content’s data from Gaboneses patients (n=39, 52%). In this group, during every visit the patient's medical history

Table 1. Regression method: summary of treatment effects on CD4 cells count and relative hazards of progression to AIDS/death from historical clinical endpoint trials (Hill et al., 2007).

Study name [reference]	Number per arm	Treatment arm	Control arm	Progression hazard ratio	16-week change in CD4 count (cells/μL)
Delta 1 [17]	738	ZDV / ddI	ZDV	0.63	55
Delta 1 [17]	738	ZDV/ddC	ZDV	0,77	35
Delta 1 [17]	361	ZDV/ddI	ZDV	0,87	25
Delta 1 [17]	361	ZDV/ddC	ZDV	0,95	20
ACTG 175 N [18]	266	ddI	ZDV	0,65	35
ACTG 175 N [18]	266	ZDV/ddC	ZDV	0,49	60
ACTG 175 N [18]	266	ZDV/ddI	ZDV	0,61	30
ACTG 175 E [19]	350	ddI	ZDV	0,72	50
ACTG 175 E [19]	350	ZDV/ddI	ZDV	0,91	75
ACTG 175 E [19]	350	ZDV/ddI	ZDV	0,65	35
ACTG 116b/117 [20]	304	ddI(500mg)	ZDV	0,72	14
ACTG 116b/117 [20]	304	ddI(750mg)	ZDV	0,91	12
ACTG 116a [21]	205	ddI(500mg)	ZDV	1,02	0
ACTG 116a [21]	205	ddI(750mg)	ZDV	1,04	0
CAESAR [22]	613	CT+3TC	CT	0,42	40
CAESAR [22]	613	CT+3TC /LOV	CT	0,41	40
ACTG 241 [23]	119	ZDV/ddI/NVP	ZDV/ddI	1,24	17
ACTG 241 [23]	323	SQV ddC	0,83	0,83	19
NV 14256 [24]	323	ddC/SQV	ddC	0,47	44
NV 14604 [24]	940	ddC/SQV	ZDV	0,85	8
NV 14604 [24]	940	ZDV/ddC	ZDV	0,69	16
NV 14604 [24]	940	SQV/ddC	ZDV	0,47	37
ACTG 326 [26]	578	ZDV/3TC/IDV	ZDV/3TC	0,50	54
ABT247 [27]	545	CT+RTV	CT	0,50	45
MX-028 [28, 29]	332	IDV	ZDV	0,39	82
MX-028 [28, 29]	332	ZDV/IDV	ZDV	0,24	91

ddI, didanosine; ZDV, zidovudine; ddC, zalcitabine; CT, current treatment; 3TC, lamivudine; LOV, loviride; NVP, nevirapine; SQV, saquinavir; IDV, indinavir; RTV, ritonavir

was reviewed. A physical examination (including bodyweight), the combined antigen and antibody HIV screening assay (VIDAS HIV DUO, BioMérieux, Marcy l'Etoile, France), and if necessary, complementary laboratory tests. Basically the HIV DUO combines an antibody test for HIV-1 and HIV-2 with an HIV p24 antigen test (the ELFA test does test for antigens and antibodies so is a duo test). At week 0 and every 12 weeks CD4 cells counts were assessed with FACSCount apparatus. The second group or CD4 group content's data from Gabon and data from others countries. During every visit, the patient's medical history was reviewed, a physical examination (including bodyweight) and CD4 counts were assessed at week 0 and every 12 weeks. Biological assessments of tolerability of Fagaricine included measurement of serum transaminase activities, amount of haemoglobin, and white blood cell and platelet counts at weeks 0, 4, 8, 12, and 24.

Evaluation of clinical benefit of treatment by Fagaricine was expressed by two methods (regression and categorization). In regression method, the clinical benefit of treatment was expressed as the relative hazard of progression to AIDS or death for Fagaricine treatment relative to the control represented by data from historical clinical endpoint trials(Hill et al., 2007) (table 1).

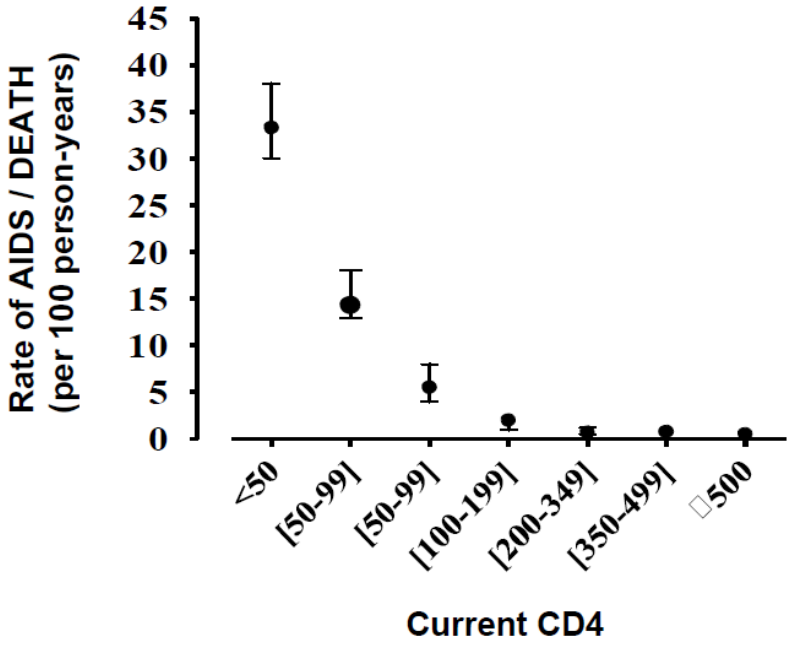

Figure 1. Rates of progression to AIDS or death (per 100 patient-years) by CD4 category, with 95% confidence intervals, from the EuroSIDA cohort (Olsen et al., 2005).

The CD4 treatment benefit was the mean CD4 rise for the treatment by Fagaricine compared to that obtained by antiretroviral drugs therapy during historical clinical endpoint trials of highly active antiretroviral therapy (HAART) (Hill et al., 2007). In categorization method we compared our result with that of EuroSIDA cohort data (Olsen et al., 2005). From this cohort, the 12-month incidence of AIDS or death during HAART treatment was determined within the CD4 categories [0–49], [50–99], [100–199], [200–349], [350–499] and >500 cells/mm3. A weighted average of the published rates for different regimens was used to produce an overall expected rate of progression to AIDS or death for patients receiving HAART, with CD4 counts in the different categories (Figure 1).

Statistical analysis

Preclinical study

All data are presented as mean ± standard deviation of the indicated number of experiments. Statistical analysis was performed with one-way analysis of variance, followed by comparisons between treated group and control (Dunnett's test) using Graphpad program for Windows (Graphpad, San Diego, CA, USA). $P < 0.05$ was considered as significant.

2.3.2. Clinical study

In clinical studies, all analyses and graphical representation in VT group were released using Graphpad program for Windows (Graphpad, San Diego, CA, USA). The effect of Fagaricine on bodyweight, VT index, was assessed by one way ANOVA followed by Bartlett's test, and multiple comparisons by Dunnett's test. We estimated 95% CIs of percentages by the binomial exact method. In CD4 group, increases in CD4 cells count from

Table 2, Mean body weights variation of Fagaricine treated mice in acute toxicity

Doses (mg/kg body weight)	Δ body weight (g)
0	1.4±0.3
85	-0.3±0.1
170	1.0±0.1
340	0.2±0.2
680	0.7±0.1
1360	0.6±0.1
2720	0.5±0.0
5440	1.2±0.1
10 000	-2.2±0.2*****

Mean body weights variation of Fagaricine treated mice in acute toxicity Data are expressed as mean ± standard deviation (g± SD); $n = 10$. No statistical difference between control and Fagaricine treated groups by Dunnett's test except with 10 000 mg/kg body weight. The loss of body weight represented 11.36%.

baseline and median changes are reported with the IQR. Comparison of the effect of Fagaricine on increase in CD4 cells count according to different categorization was realized by Bonferroni's test.

The clinical benefit of Fagaricine treatment was expressed as the relative hazard of progression to AIDS or death (HR) relative to the control (EuroSida cohort), and by interpolation on regression between Fagaricine effect on CD4 count and clinical benefit from historical clinical endpoint trials (Hill et al., 2007).

Results

Preclinical study

The 35-day subchronic oral toxicity study was conducted with a daily administration of Fagaricine at concentrations of 0, 425, 850, and 1275 mg/kg body weight whereas in acute toxicity, one single dose of Fagaricine at concentration of 0, 85, 170, 340, 680, 1360, 2720, 4440 et 10 000 mg/kg body weight was administrated in each group. Since the animals were gavaged, they received the intended dose. Homogeneity of distribution, stability, and concentration measurements was performed to ensure that the gavage amounts were correct.

Acute oral toxicity study

Survival and clinical observations

There were no Fagaricine related mortalities and no behavioural signs of toxicity, such as convulsion, vomiting, diarrhoea, paralysis, breathing difficulties, bleeding, restless, irritation, and abnormal posture, in either sex of control or treated groups.

Body weights

Average overall (test days 1–7) body-weight data indicated that Fagaricine treated mice, regardless of dose level, were comparable to the controls except in male mice which the body weight was decreased of 11.36% on Day 7 in group 10 000 mg/kg (Table 2).

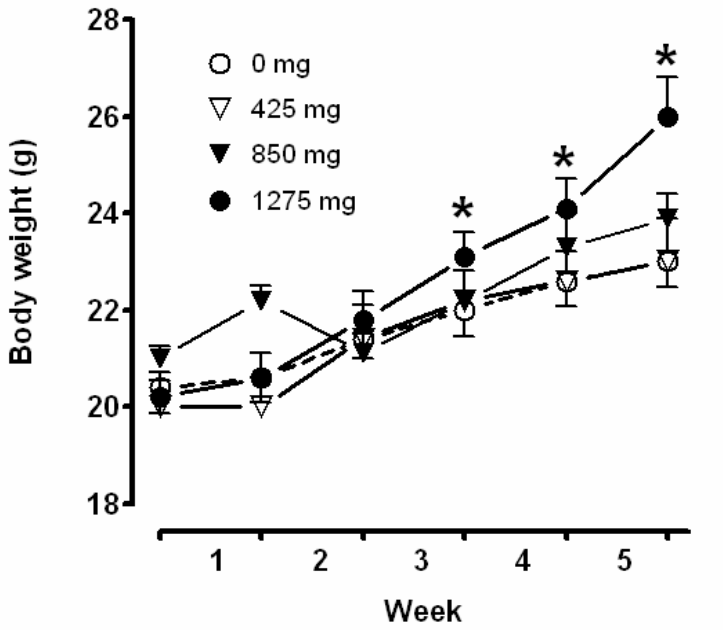

Figure 2. Body weight of male mice treated with different concentration of Fagaricine in a subchronic toxicity. Data are expressed as mean ± standard deviation; n = 5. *p<0.05; statistical difference between control and Fagaricine treated groups by Dunnett's test.

Subchronic oral toxicity study

The 35-day repeat dose study was conducted with daily administration of Fagaricine at concentrations of 0, 425, 850, and 1275 mg/kg body weight. Since the animals were gavaged, they received the intended dose.

Body weights

Average overall (test days 1–35) body-weight data indicated that the Fagaricine treated mice, regardless of dose level, were comparable to the controls excepts of males mice. The body weight was increased ($p<0.05$) on week 3, 4 and 5 in group 1275 mg of males mice (Fig.2), but no significant difference was observed in females.

Food and water consumption

Average overall (Test days 1–35) food and water consumption data indicated that there were no statistically significant differences among treated groups compared with the controls (data not shown for brevity but available upon request).

Organ weights

Absolute organ weights of treated male and female rats are shown in table 3. There were statistically significant reduction in absolute liver weights in males at the 850 mg/kg/day dose (-21.42 %; $p<0.05$) and at 1275 mg/kg/day dose (-32.14 %; $p<0.01$) compared with controls. In addition, statistically significant increases in lungs weight were noted in females at the 1275 mg/kg/day dose (+27% $p<0.05$) but remain in normal range weight (≤0.56 g). There were no statistically significant differences between treated and control ani-

Table 3. Mean organ weights (g± SD) of Fagaricine treated mice.

Organs (g)	Daily dosage of Fagaricine (in mg/kg body weight)			
	Control	425	850	1275
Males				
Liver	1.40±0.10	1.25±0.07	1.10±0.07*	0.95±0.07**
Kidneys	0.19±0.02	0.20±0.02	0.16±0.01	0.16±0.01
Lung	0.19±0.01	0.22±0.04	0.17±0.03	0.19±0.01
Heart	0.13±0.01	0.12±0.01	0.12±0.00	0.11±0.01
Spleen	0.12±0.04	0.14±0.03	0.14±0.02	0.06±0.01
Testes	0.07±0.01	0.07±0.01	0.07±0.01	0.08±0.01
Females				
Liver	1.16±0.03	1.17±0.14	1.01±0.07	1.08±0.07
Kidneys	0.13±0.01	0.14±0.02	0.12±0.01	0.36±0.24
Lungs	0.16±0.01	0.15±0.01	0.19±0.05	0.22±0.01*
Heart	0.11±0.01	0.10±0.01	0.10±0.01	0.12±0.01
Spleen	0.10±0.01	0.12±0.00	0.08±0.01	0.08±0.01
Ovaries	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.00

Data are expressed as mean ± SD, n = 5 (animals per group). *P<0.05, **P<0.01 statistical difference between control and Fagaricine treated groups by Dunnett's test.

mals at the endpoint (day 35) on any of the other weighed organs.

Haematological observations

The haematological analysis (Table 4) showed no significant changes of haematological parameters in females mice. In males mice, haematological analysis showed significant increase (11%, $p<0.05$) in red blood cell (RBC) of Fagaricine treated group with 425 mg/kg/day dose, compared to the control group. This augmentation is accompanied with reduction (-2.9 %; $p<0.05$) of mean cell volume (MCV), and mean cell haemoglobin (MCH) with 850, and 1275 mg/kg body weight ($p<0.05$). We obtained also the reduction in platelets (PLT) (-25,3% $p<0.05$), thrombocytocrit (Tct), red blood cell distribution width (RDW) and granulocytes with 850 mg/kg body weight. Although the significant variations were observed with those parameters, these results are considered not to be of toxicological significance because they were isolated, not dose related and remained in normal range variation.

Biochemical observations

The biochemical analysis (Tables 5) showed no significant differences in any of the parameters examined in either the control or Fagaricine treated groups of the male and female mice with 425 mg/kg/body weight. In females mice Fagaricine treated groups with 850 mg/kg body weight, reduction of cholesterol was observed ($p<0.01$), whereas increased in total protein was observed in treated group with 850 mg/kg body weight ($p<0.05$), increased of Urea nitrogen and creatinine in Fagaricine treated group 850 and 1275 mg/kg body weight. In males mice Fagaricine treated groups with 850 and 1275 mg/kg body weight significant increased of urea nitrogen was observed.

Table 4. Terminal hematology for mice in subchronic toxicity

Organs (g)	Daily dosage of Fagaricine (in mg/kg body weight)			
	Control	425	850	1275
Males				
RBC (x106 cells/µL)	8.1±0.3	9.0±0.1*	8.8±0.3	8.7±0.3
Hb (g/dL)	14.0±0.5	14.1±0.6	14.5±0.5	14.3±0.5
Hct (%)	47.5±1.4	45.3±1.7	45.1±1.8	45.1±1.8
WBC (x103 cells/µL)	5.0±0.1	5.1±0.3	4.8±0.2	4.7±0.1
MCV (fL)	52.6±1.9	50.4±1.5*	52.2±2.1	51.6±2.1
MCH (pg)	18.5±0.5	16.7±0.7	16.8±0.4*	16.4±0.4*
MCHC (g/dL)	32.1±0.9	30.9±1.5	33.0±1.2	31.8±0.7
Lymphocyte (%)	76.9±2.3	66.0±6.8	77.3±1.5	40.4±5.7
Granulocyte (%)	2.2±0.1	2.2±0.5	1.4±0.2*	1.6±0.2
MID cells (%)	24.9±0.7	37.6±6.6	21.3±1.2	28.0±5.6
PLT (x103 cells/µL)	1193.4±33.2	891.4±33.1**	881.6±57.3**	891.8±58.0**
Tct (%)	0.7±0.0	0.5±0.0**	0.5±0.0**	0.5±0.0**
RDW (%)	12.0±0.4	12.5±0.2**	12.5±0.3*	12.3±0.2*
Females				
RBC (x106 cells/µL)	8.8±0.1	8.9±0.1	8.6±0.4	8.7±0.4
Hb (g/dL)	14.3±0.2	14.8±0.2	14.1±0.5	14.0±0.5
Hct (%)	43.4±0.9	46.3±1.9	43.6±2.4	42.6±1.5
WBC (x103 cells/µL)	5.0±0.1	6.4±1.3	5.4±0.3	5.5±0.3
MCV (fL)	50.1±1.4	55.5±4.0	52.7±3.4	49.6±0.3
MCH (pg)	16.5±0.3	17.7±0.7	17.1±0.7	16.3±0.3
MCHC (g/dL)	33.7±0.5	34.2±0.3	33.9±0.3	33.3±0.5
Lymphocyte (%)	76.2±0.6	74.8±1.9	68.7±7.9	60.0±8.7
Granulocyte (%)	1.2±0.2	1.5±0.3	1.9±0.4	2.2±0.4
MID cells (%)	23.2±0.3	24.3±1.1	30.3±6.2	37.8±7.9
PLT (x103 cells/µL)	814.4±6.8	957.7±125.4	931.3±86.3	968.8±94.5
Tct (%)	0.5±0.0	0.5±0.1	0.5±0.1	0.5±0.1
RDW (%)	12.3±0.1	13.4±0.5	12.8±0.1	12.1±0.2

Data are expressed as mean ± SD; n = 5 (animals per group). *P<0.05; **P<0.01 statistical difference between control and Fagaricine treated groups by Dunnett's test. RBC: Red blood cell count, WBC: White blood cell count, Hb: Hemoglobin concentration, MCV: mean corpuscular hemoglobin, MCH: mean cell hemoglobin, MCHC: mean corpuscular hemoglobin concentration, Hct: Hematocrit, RDW: Red blood cell distribution width, PLT: Platelet count, MID: Mid cells total count, Tct: Thrombocytocrit (volume % of thrombocytes).

Necropsy and histopathology

Gross necropsy findings did not show any adverse effects in both male and female mice of any organs in treated groups, as compared to control group. In the histopathological examination, no microscopic findings were observed that could be attributed to the administration of the Fagaricine. Liver, kidney, spleen and stomach were showed normal histology in treated groups, as compared to the control group (data not shown for brevity but available upon request).

Baseline characteristics of study participants

According to clinical data, 87 patients were enrolled, 20 (22.98%) in DRC (Kinshasa), 12 (7.25 %) in RC (Brazzaville), 47 (54.02 %) in Gabon (Libreville), 2 (2.29 %) in Ivory-Coast (Cerba, Abidjan), 6 (6.89 %) in Libya (Tripoli). Eight patients were lost to follow-up

Table 5. Serum biochemical parameters of mice treated with Fagaricine in a subchronic toxicity.

Organs (g)	Daily dosage of Fagaricine (in mg/kg body weight)			
	Control	425	850	1275
		Males		
Creatinine (mg/dL)	0.12±0.03	0.10±0.01	0.10±0.02	0.18±0.01
Cholesterol (mg/dL)	83.4±11.9	74.2±7.8	72.2±16.3	86.4±12.8
Urea nitrogen (mg/dL)	18.8±8	31.6±2.4	32.0±5.2*	41.2±4.3**
ALT (UI/L)	14.0±3.0	13.0±4.0	14.0±4.0	12.0±5.0
AST (UI/L)	32.0±2.0	32.0±1.0	34.0±2.0	32.0±4.0
Total protein (g/L)	1.4±0.06	1.1±0.07	0.9±0.1*	1.28±0.1
		Females		
Creatinine (mg/dL)	0.10±0.05	0.10±0.02	0.13±0.02*	0.18±0.01***
Cholesterol (mg/dL)	82.2±2.8	90.2±4.0	79.6±2.5**	73.6±5.6
Urea nitrogen (mg/dL)	18.4±2.6	24.6±3.0	29.8±3.4**	30.4±1.8**
ALT (IU/L)	16.0±2.0	14.0±2.0	14.0±3.0	15.0±2.0
AST (IU/L)	32.0±2.0	28.0±3.0	30.0±2.0	34.0±4.0
Total protein (g/L)	0.8±0.08	1.08±0.1	1.1±0.07*	0.9±0.1

Data are expressed as mean ± SD; n = 5 (animals per group). *P<0.05; **P<0.01, ***P<0.001 statistical difference between control and Fagaricine treated groups by Dunnett's test. AST: aspartate aminotransferase, ALT: alanine aminotransferase

after 12 weeks in Gabon, 3 in DRC and one in Congo. The table 6 shows their baseline charcteristics. 9 had AIDS (12%), on the basis of clinical and biological criteria. 2 patients (Ivory coast) were stopped to take antiretroviral drugs after long lasting treatment (therapeutic vacancies). Only 4 women (without AIDS) had taken antiretroviral for the prevention of mother-child transmission.

Bodyweight

The cohort study shows that Fagaricine can induce the increase of bodyweight. This augmentation of the bodyweight was significant after 8 weeks of treatment 4.15 kg (95% CI2.11-6.19) and 7.24 kg (95% CI 4.96-9.51) after 24 weeks (Fig. 3). In addition, general heal-th was ameliorated and food intake increased in all patients.

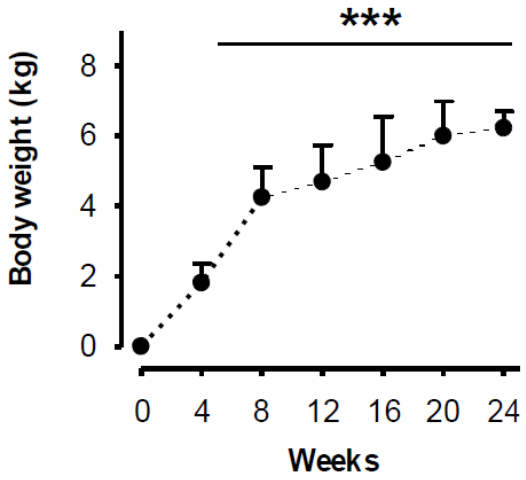

Figure 3. Variation of bodyweight after 24 weeks of Fagaricine treatment. ***p<0.0001

Table 2**.** Baseline characteristics of Study Participants

Number of Patients (n = 75)	
Demography	
Women	38 (51%)
Men	37 (49 %)
Age (years)	37 (28 - 42)
Anthropometry	
Bodyweight (kg)	71 (51.25 - 82)
HIV infection	
Time since diagnosis of HIV seropositivity (months)	12 (4 - 12)
CDC clinical stage	
A	32 (42.66 %)
B	34 (45.33 %)
C	09 (12 %)
CD4 count (cells per µL)	217.5 (118.8 – 484.3)
(VIDAS HIV DUO test) or VT	10.47 (8.27 – 16.20)
Haematology	
Total lymphocytes count (mm3)	1890 (1425 - 2350)
Haemoglobin concentration (g/L)	109.0 (99 - 157)
White blood cells count (×109/L)	4.4 (1.8 – 12.9)
Platelet count (×109/L)	187.5 (137.5 - 352)
Biochemistry	
Alanine aminotransferase (U/L)	21 (12 - 30)
Aspartate aminotransferase (U/L)	24 (14.5 – 29.5)
Gamma aminotransferase (U/L)	28 (13 – 45)
Bilirubin (mg/L)	1 (1 – 3.25)
Serum creatinine (µmol/L)	72.5 (38.5 – 89.25)
Serum creatine phosphokinase	89 (30 – 114)
History of antiretroviral therapy	
Naive	66 (88 %)
Antiretroviral therapy	9 (12%)

Data are median (IQR) or number of patients (%).

VT index

In VT group (n=39, 52 %), simultaneous testing of p24 antigen plus HIV antibodies (VT index) clearly indicate that, treatment with Fagaricine can reduced the presence of p24 antigen in blood. This reduction is effective after 8 weeks of treatment 3.27 (95% CI 1.46-5.08), and remained lower after 24 weeks of treatment 9.60 (95% CI 5.79-13.40) (Fig.4).

Clinical benefits of Fagaricine

In CD4 group, the treatment with Fagaricine had no effect on CD4 restoration when baseline of CD4 counts was lower than 10 cells/µL (n=3, 4%), even after 24 weeks of treatment. The restoration effect began when baseline of CD4 count is close to 25 cells/µL. with the baseline CD4 counts of [25-50[cells /µL, the percentage of the CD4 restoration was close to 50 % and became 100% when baseline of CD4 count is 350 cells/µL. (see fig.5). The

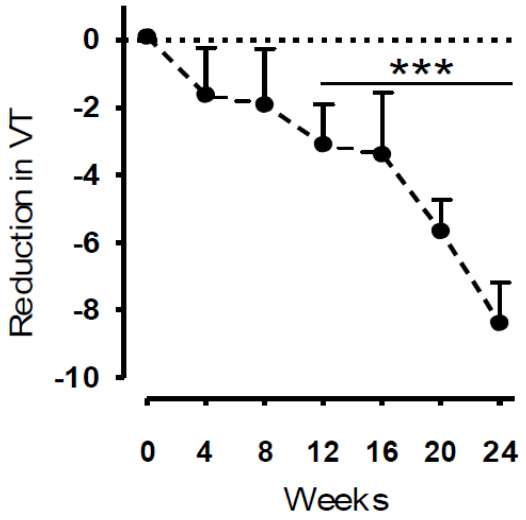

Figure 4. Time course of antibody test for HIV-1 and HIV-2 with an HIV p24 antigen test (VIDAS HIV DUO) on patients treated with Fagaricine. ***p<0.0001.

real benefit of utilisation of Fagaricine on immunodeficiency patients was found, when baseline of CD4 counts is close to 200 cells/µL then, the prediction of CD4 restoration is 100 cells/µL after 24 weeks of treatment. The figure 6 shows the rise of CD4 with the categorization of baseline CD4 count. After 24 weeks of treatment the CD4 count rise of 135 cells/µL from baseline ([200-300[cells/µL) and was correlated with a hazard ratio of progression to AIDS/death (HR) of 0.0, a 100 % reduction in hazard of progression to AIDS or death (Fig.7). The TMC114 trial led to a 80 cells/µL rise from baseline by week 16, whereas Fagaricine (F-532) led to 90 cells/µL rise from baseline during the same time. This treatment benefit in CD4 cell counts was correlated with a hazard ratio of 0.45, a 55% reduction in the hazard of progression to AIDS or death for TMC114 versus 0.40, a 60% reduction in the hazard of progression to AIDS or death for Fagaricine. Predicted rates of pr-

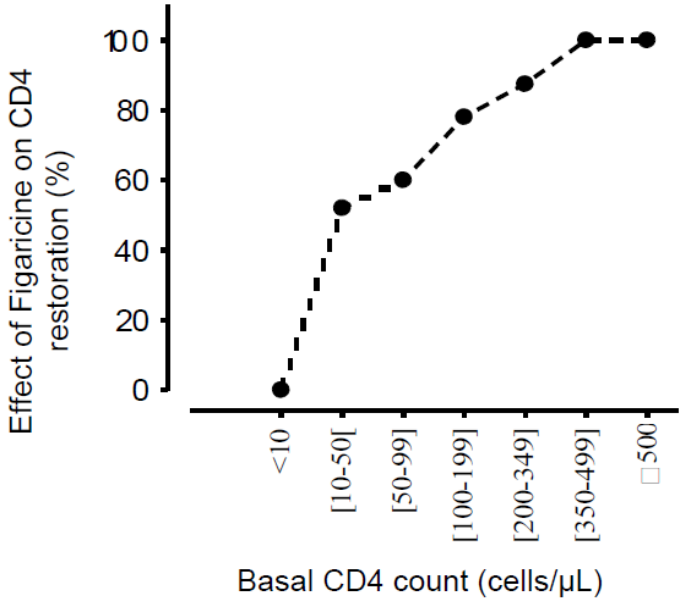

Figure 5. Effect of Fagaricine on CD4 counts restoration according to categorization of baseline CD4

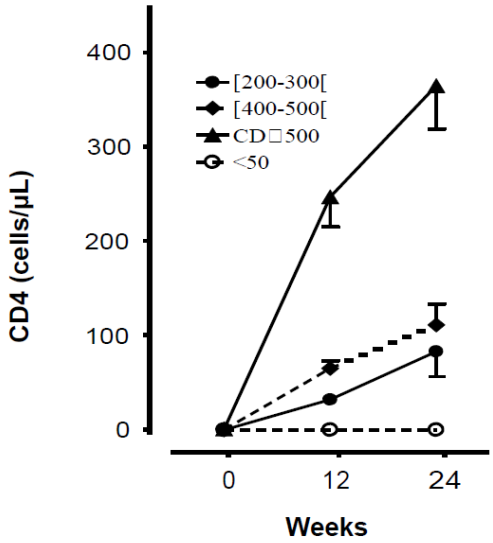

Figure 6: Time course effect of Fagaricine on CD4 restoration according of baseline categorisation of CD4 before treatment

ogression to AIDS/death by CD4 categorization method (Olsen et al., 2005) was 53,6 % (data not shown) reduction in clinical progression to AIDS/death for Fagaricine versus control treatment, using historical data for CD4 (Hill et al., 2007). The biochemical analysis showed no significant differences in any of the parameters examined in both groups. A few variations were observed with biological, haematological and biochemical parameters but values remained within biological normal control ranges after 24 weeks of treatment.

Discussion

In preclinical studies, bodyweight changes are the indication of adverse effects of drugs and chemicals, and will be significant if the bodyweight losses were more than 10% from the initial value (Tofovic & Jackson, 1999; Teo et al., 2002). In the present study bodyweights were all comparable to the control group but decreased of 11.36% on Day 7 in acute toxicity study with 10 000 mg/kg bodyweight. This reduction may be considered as adverse effect of drugs on mice (table 1).

In subchronic study significant increase in bodyweight was observed in Fagaricine treated group with 1 275 mg/kg body weight in male mice compared to the control. No statistical significance was observed in all the groups of female mice. This result is different to that observed in human. It is well know that, the traditional used of decoction of an aqueous extract of *Zanthoxylum heitzii* bark (75 mL/day orally) induced food intake and increased of bodyweight close to 2 kg/month in both men and women (see clinical studies). The determination of food and water consumption parameters is important in the study of the safety of a product with therapeutic purpose, as proper intake of nutrients and water are essential to the physiological status of the animals and to the accomplishment of the proper response to the drug tested, instead of a "false" response due to improper nutritional conditions (Iversen & Nicolaysen, 2003). In this study, no significant change was found in water and food consumption of the treated groups, as compared to the control group. Slightly (but not significant) in food consumption was observed on week 1 in all the groups and then remained

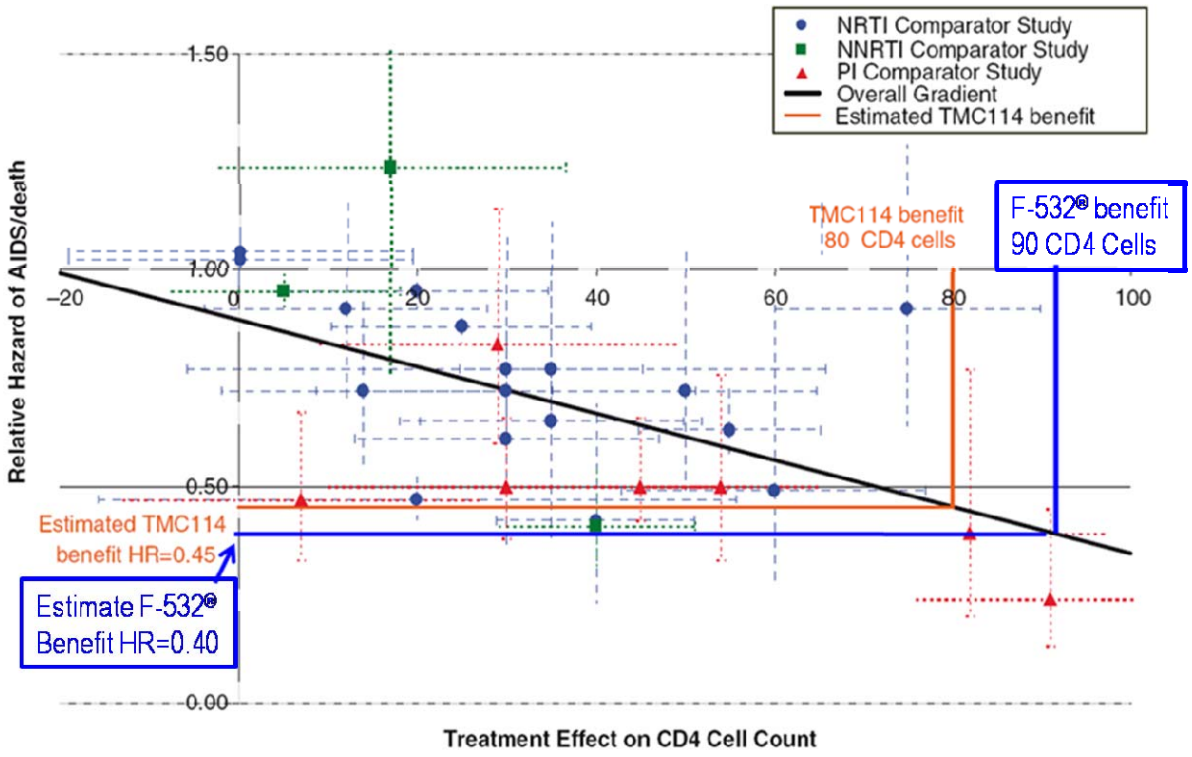

Figure 7. Regression between treatment effect on CD4 count and clinical benefit, from clinical endpoint trials and Fagaricine (F-532) treatment. Each data point is the comparison of two treatment groups in a randomized clinical trial. The x-axis is the difference between the treatment arms in CD4 counts at weeks 16–24. For the y-axis, relative hazard of progression to AIDS/death (HR) is shown on a scale of 1.25 - 0, where a hazard ratio of 1 corresponds to no clinical benefit for the treatment arm relative to the control arm, and 0.5 corresponds to a 50% lower hazard of clinical progression for the treatment arm. The HR = 0.40 (after 16 weeks of treatment) of F-532 was obtained by interpolation. NRTI is Nucleoside reverse transcriptase inhibitor. NNRTIs is non nucleoside reverse transcriptase inhibitors and PI protease inhibitor

stable until the end of the study. Perhaps administration of the extract was seen as an additional food intake, partially explaining the lowered food consumption in week 1. The food-consumption fluctuation did not reflect in the bodyweight in Fagaricine treated group with 1 275 mg/kg. Hence, these results are considered to be adverse effect in this group. Blood is an important index of physiological and pathological status in man and animals, and the parameters usually measured are Hb, RBC count, WBC count and differential leukocyte count (Krishnaraju et al., 2010). The normal range of these parameters can be altered by the ingestion of some toxic plants (Kumar et al., 2005). These blood indices were all measured in the present study after 35 days of oral administration with alterations of some haematological parameters in male mice.

Many variations in haematological parameters in male mice, although significant, remained in physiological normal range such as red blood cell count (RBC), red cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), platelet (PLT) and Thrombocytocrit (Tct).

Biochemical parameters are an important marker to evaluate the organs and cellular functions. In the results obtained from the biochemical evaluation, significant difference between 3 doses administered and sexes were noticed. Among the evaluated parameters such as AST, ALT, total proteins are considered as liver function markers (Palmeiro et al., 2003; El Hilaly et al., 2004). The analysis of these parameters is important because several reports of liver toxicity are related to the use of phytotherapeutic products (Corns, 2003; Pittler & Ernst, 2003; Johannsson et al., 2010). It is known that many toxic plant compounds accumulate in the liver, where they are detoxified (Aboyade et al., 2009). Liver function tests may prove useful in assessing the toxic effects of medicinal plants. Any marked necrosis of the liver cells can lead to a significant change of these parameters in the blood serum. In the present study, no significant changes were observed in AST, ALT. These result shows that Fagaricine has no adverse effect on the hepatocytes.

Kidney toxicity has also been reported after use of phytotherapeutic products (Corns, 2003; Isnard Bagnis et al., 2004). In that case, urea and creatinine determinations are vital, as these substances are markers of kidney function. In the present study, no significant differences in the parameters were detected in males mice except significant increased of urea nitrogen with 850 and 1275 mg/kg body weight. In females Urea and creatinine increased with 850 and 1275 mg/kg body weight but was considered to have no toxicological significance because it was dose related although all the values are in normal range variation (0.1-0.9 mg/dL for creatinine, 8-33 mg/dL for Urea). Fagaricine can cause adverse effect on kidney function of mice when use at the higher concentration up to 850 mg/kg body weight.

Total cholesterol was determined to evaluate whether the Fagaricine has hypo or hyperlipidemic properties. There was slight decrease in serum total cholesterol in females mice with 850 mg/kg body weight but was considered to have no toxicological significance because it was an isolated, not dose related and remain in normal range (26-82 mg/dL). Collectively, these data demonstrated that Fagaricine has a high margin of drug safety when used in traditional medicine (lower than 10 mg/kg body weight).

Organ weight is a simple, sensitive index of toxicity after exposure to toxic substances. In the present study, significant changes were not observed in the weight of the organs in Fagaricine treated mice, as compared to control except in male liver with the group of 850 and 1275 mg/kg body weight. Organ weight revealed that Fagaricine, at the doses of 850 mg/kg body weight produce liver atrophy in male mice and hypertrophy of lungs of females mice at the doses up to 850 mg/kg body weight. Moreover, gross examination of internal organs of all mice revealed no detectable abnormalities. Thus, it can be suggested that Fagaricine.

Histopathology has historically been the most consistent criterion to establish the no observed adverse effect level (Dorato & Engelhardt, 2005). Histopathologic evaluation in repeated administrations of this preparation did not reveal any observable damage in all the vital organs.

In the clinical studies, the aim of the cohort was to confirmed clinical benefits effect of Fagaricine observed by clinicians and patients infected by HIV with and without AIDS. It very important to note that this study is the preliminary report and most be completed with a

large cohort. Fagaricine is the sole immunorestorative phytomedicine commercially available in some few countries in Africa. According for this raison, it was no easy to get a control arm with another immunorestorative phytomedicine. For the pertinence of our study, we decided to use historical clinical trials as control arm, to estimate the benefit of Fagaricine treatment based on its efficacy in improving CD4 counts compared to that of antiretroviral approved drugs.

Two independent prediction methods were used, including data sets from recent cohort studies of CD4 count (Hill et al., 2007) and clinical progression from highly active antiretroviral therapy (HAART) treated patients, together with a meta-analysis of randomized clinical endpoint trials conducted before the introduction of HAART.

Our clinical results confirmed the effect of Fagaricine on bodyweight gain and reduction in VT index. In addition, we found that the treatment was no efficient when baseline of CD4 counts was lower than 50 cells/μL suggesting that the capacity of Fagaricine to restore CD4 counts is effective when the immune system contains, at least 50 cells/μL of CD4 counts. The restorative capacity Fagaricine depends of baseline CD4 count before the treatment. When the baseline CD4 count is up to 200 cells/μL, the efficiency of Fagaricine is better and remains insufficient when baseline CD4 count was lower.

The regression and categorization methods were used to predict the reduction in progression to AIDS and death induced by Fagaricine treatment relative to control treatment (antiretrovirals drugs). The regression method predicted a 60% (HR=0.40) reduction in the hazard of progression to AIDS/death for Fagaricine versus control treatment, using historical data for CD4, and the CD4 categorization method (Olsen et al., 2005) predicted a 53,61% reduction in clinical progression to AIDS/death. These methods for predicting clinical benefits were based on large historical databases: a database of 22 766 patient-years of observation for the categorization method, and data from clinical trials in 17 496 patients for the regression method. The estimates of clinical benefit were consistent between the independent methods used. The categorization method is based on more contemporary data for HAART-treated patients, which may be more applicable to the HAART treatment used in recent trials and which gives an estimate of the actual progression rates for the different treatment arms. The regression method is based on randomized clinical trials data and also provides a hazard ratio but does not provide a predicted incidence of events by treatment group. It cannot adjust for baseline levels of CD4 count, which themselves may affect clinical progression rates.

The present study concludes that the oral administration of the Fagaricine at 10 000 mg/kg in single dose in acute toxicity and 450, 850, 1275 mg/kg body weight for 35 consecutive days to males and females mice induce atrophy of liver in male mice and increased of urea nitrogen in both males and females with the doses of 850 and 1275 mg/kg body weight. In subchronic study, the no observed adverse effect level (NOAEL) for Fagaricine in mice was determined to 425 mg/kg/day. This study confirms a wide margin of safety for the therapeutic use of the aqueous extract of *Zanthoxylum heitzii* bark when used as traditionally (lower than 10 mg/kg body weight). Cohort study lends support to traditional use of Fagaricine as an immunorestorative phytomedicine to treat immunodeficiency

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