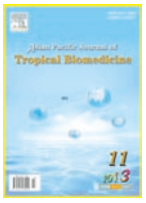
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading doi:10.1016/S2221-1691(13)60176-8

Evaluation of genotoxicity of Trois through Ames and *in vitro* chromosomal aberration tests

Manu Chaudhary, Anurag Payasi*

Venus Medicine Research Centre, Hill Top Industrial Estate, Bhatoli Kalan, Baddi, H.P. – 173205 India

PEER REVIEW

Peer reviewer

Dr. N.D. Chaurasiya, National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, MS 38677, USA.
Tel: 662-202-6317
E-mail: narayan.chaurasiya@gmail.com

Comments

This is a very useful study in which authors have evaluated the genotoxicty profile of Trois, a nano-technology based herbal tropical formulation. The results obtained in this study clearly suggested that Trois, which has been designed to treat various types of arthritis, is non-mutagenic.
Details on Page 905

ABSTRACT

Objective: To investigate the mutagenic potential of Trois using the bacterial reverse mutation assay (Ames test) and *in vitro* chromosomal aberration test.
Methods: The ability of Trois to induce reverse mutations was evaluated in *Salmonella typhimurium* (TA 98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2 *uvrA*) with and without metabolic activation system (S9 mix) at the dose range of 313 to 5000 μg/plate. Chromosomal aberrations were evaluated in Chinese hamster lung (CHL) cell line at the dose levels of 15, 7.5, 3.7, 1.9 and 0.9 mg/mL in the absence and presence of S9 mix.
Results: There were no increases in the number of revertant colonies at any concentrations of Trois used in the study with and without S9 mix in all tester strains. Trois did not produce any structural aberration in CHL cells in the presence or absence of S9 mix.
Conclusions: Results of this study suggest that Trois is non-mutagenic.

KEYWORDS
Trois, Ames test, Chromosomal aberration test

1. Introduction

Medicinal plants have been used widely around the world over many centuries for the treatment of various diseases and nearly 80% of the world populations rely on medicinal herbs for their primary health care, including 71% of the population in Canada and 90% in Kenya[1-3]. WHO strongly encourages the use of traditional herbal medicines in primary health care system[4].

Despite the wide use of herbal medicines, safety data of plants, their extracts and active ingredients and preparations containing them are very scanty[1]. Medicinal plant may produce several biological activities in human including toxicity[5].

In view of increasing demand of herbal medicine, we have developed a nano-technology based topical herbal formulation for the treatment of pain and inflammation associated with different types of arthritis such as osteoarthritis, gout, rheumatoid arthritis, septic arthritis, juvenile idiopathic arthritis, spondyloarthritis, gonococcal arthritis, psoriatic arthritis, reactive arthritis (Reider syndrome), ankylosing spondlitis, scleroderma, systemic

*Corresponding author: Anurag Payasi, Venus Medicine Research Centre, Hill Top Industrial Estate, Bhatoli Kalan, Baddi, H.P. – 173205 India.
Tel: 91-1795-302065
Fax: 91-1795-302133
E-mail: ccmb@venusremedies.com
Foundation Project: This work is supported by Venus Medicine Research Centre, Werne, Germany(Grant No. VPG-115-2012).

Article history:
Received 24 Aug 2013
Received in revised form 2 Sep, 2nd revised form 13 Sep, 3rd revised form 19 Sep 2013
Accepted 3 Nov 2013
Available online 28 Nov 2013

lupus erythematosus *etc*[6-11]. Every year, arthritis caused nearly 1 million hospitalizations and around 45 million outpatient visits to health care centers and the total cost of arthritis cases is close to be approximately $100 billion (The Arthritis Society. Retrieved on 2010-02-05). The herbal formulation contains wintergreen oil (*Gaultheria precumbens*), eucalyptus oil (*Eucalyptus globulus*), pudina ka satva (Menthol), nirgundi (*Vitex negundo*) and ajmoda (*Apium graveolens*) as major ingredients.

Wintergreen oil contains natural methyl salicylate (upto 98%) which Inhibits platelet aggregation locally and acts as an analgesic by exerting anaesthetic effect in the nerves present in that area[12]. Eucalyptus oil contains 70%-85% 1,8-cineole (eucalyptol), which significantly reduces the myeloperoxidase activity, and causes repletion of glutathione which confirmed the anti-inflammatory action of 1,8-cineole[13]. Pudina ka satva causes a feeling of coolness due to stimulation of 'cold' receptors by inhibiting Ca++ currents of neuronal membranes. Since Ca++ channel blockers are endowed with analgesic properties, it is endowed with analgesic properties mediated through a selective activation of kappa-opioid receptors[14]. Nirgundi has anti-inflammatory and pain suppressing activities possibly mediated via prostaglandin synthesis inhibition, antihistamine, membrane stabilizing and antioxidant activities[15]. Apiin is a major constituent of Ajmoda which has anti-inflammatory property[16]. However, there is a little information about its general toxicity, acute and sub-acute and there is no information about Trois genotoxicity. Therefore, the present study was undertaken to assess whether Trois is genotoxic. Bacterial reverse mutation test (Ames test) and *in vitro* chromosomal aberration test were conducted.

2. Materials and methods

2.1. Test substance

Trois was obtained from Venus Remedies Limited, Baddi, H.P India. It is a kind of clear, transparent, pink to red slight viscous liquid. It was stored at room temperature. Aseptic precautions were taken while handling. This study was done in Venus Medicine Research Centre, Baddi, India from 24 December to 21 January, 2013.

2.2. Ames test

The Ames test was carried out using the plate incorporation method as described previously[17,18]. The potential to induce reverse mutation of Trois was studied using *Salmonella typhimurium (S. typhimurium)* (TA100, TA98, TA1535, TA1537) and *Escherichia coli (E. coli)* (WP2 *uvrA*). All of the strains were procured from Institute of Microbial Technology, Chandigarh, India. The test was carried out at the highest dose of 5000 μg/plate and 4 doses of 2500, 1250, 625 and 313 μg/plate as the dose range, and finding of the study revealed that no antibacterial effect (cytotoxicity) was noted at 5000 μg/plate in the presence or absence of S9 Mix.

The negative control used in this study was sterile distilled water (solvent). Specific positive controls were used in order to confirm the reversion properties and the specificity of each tester strain and the efficacy of the metabolic activation system. Five types of positive controls were used and these include sodium azide, 9-aminoacridine, (9AA), 2-nitrofluorene (NF), benzo[a]pyrene, 2-aminoanthracene (2-AAN), methyl methansulfonate (MMS). All of these positive controls were supplied by Sigma Aldrich Chemical Co. Ltd., USA.

2.3. In vitro chromosomal aberration test in Chinese hamster lung (CHL) cells

To explore the potential of Trois to damage chromosomes, an *in vitro* assay was conducted in CHL cells according to the method and guidelines of Organization for Economic Co-operation and Development[5,19]. The cell lines, CHL was procured from National Centre For Cell Science, Pune, India. The cells have been kept and passaged at our laboratory using the Eagle's minimum essential medium (MEM, Himedia, Mumbai, India) supplemented with 10% calf serum containing 0.12% sodium bicarbonate. A dose range-finding study was done to determine the highest concentration of drug to be used in the study. The result of the dose finding study revealed that 50% inhibition of cell growth was estimated to be around 10 mg/mL in the direct method, but could not be obtained in the metabolic activation method because the cell growth was inhibited by only 9% at the dose of 15 mg/mL. Therefore, the testing doses of Trois on both methods were decided to be 15 mg/mL (the maximum dose), 7.5, 3.7, 1.9, and 0.9 mg/mL with common ratio of 2. The mitomycin C (MMC) and benzopyrine (BP) were used as a positive control.

The chrosomal preparations were done according to the method described earlier[20]. The frequency of the cells with structural and numeric chromosomal aberrations was scored in 100 well spread metaphase for each dose. Types of structural chromosomal aberrations were classified into following groups: chromatid breaks, chromatid exchange, chromosome breaks, chromatid and chromosomal gap and chromosome exchanges including dicentric and ring chromosomes total cells which have chromosomal aberrants including chromatid and chromosomal gap, total cells which have chromosomal aberrants excluding chromatid and chromosomal gap. The final results of Trois was judged as follows: negative (-) if the frequency of aberrant cells was<5%, inconclusive (±) if the frequency of aberrant cells was≥5% but<10%, and positive (+) if the frequency of aberrant cells was≥10%.

2.4. Counting procedure and data presentation

All plates for all concentrations were counted by hand. Data were presented as the number of revertant colonies

per plate. All data represented the mean±SD of three independent experiments.

2.5. Statistical analysis

Data were analyzed using Graph Pad InStat-3 and expressed as mean±SD of three independent experiment. The continuous variables were tested with one-way analysis of variance and Dunnett's test values<0.05 was considered statistically significant.

2.6. Non statistical analysis

A compound is considered as a mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains. A minimum fold increase, usually 2 fold, in revertants (over the solvent control) is the cut-off between a mutagenic and nonmutagenic response.

A compound is considered as a weak mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains, but the number of revertants is not double of the background. A compound is considered as a nonmutagen if no dose-related increase in the number of revertant colonies is observed in at least two independent experiments.

3. Results

3.1. Bacterial reverse mutation

The results of reverse mutation test are summarized in Table 1. The data showed no significant increase in the number of revertant colonies as a result of Trois treatment in the four strains of *S. typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *E. coli* (WP2*uvrA*) at any tested concentration with those of the corresponding solvent controls in either the absence or presence of S9 mix (P>0.05). However, the number of revertant colonies in positive controls increased remarkably (>2 folds) with or without S9 mix (P<0.001). Hence, the result for Trois is negative.

3.2. In vitro chromosomal aberrations

The results of chromosomal aberrations test are presented in Table 2. The incidence of cells having aberrants (including gap) in chromosomal structure was 0%-1% and 0%-6% in solvent groups and Sanovul treated groups, respectively, and the incidence of aberrant cells excluding gap was 0% and 0%-1%, respectively. There were no significant differences in the chromosomal aberration between any concentrations of Trois and the corresponding solvent groups (P>0.05). In contrast, the incidence of aberrant cells in each positive control group increased greatly as compared with each solvent group (P<0.001).

Table 1
Mutagenicity assay for Trois with and without-metabolic activation using *S. typhimurium* and *E. coli* strains.

		Concentration of test material μg/plate	Average of revertant colonies (mean±SD)				
			Base-pair substitution			Frameshift	
			TA100	TA1535	WP2 *uvrA*	TA98	TA1537
Trois	S9 mix (-)	0[a]	171±12	41±4	100±8	44±3	12±1
		313	171±13	44±3	101±9	42±4	13±1
		625	170±12	39±5	98±11	45±4	14±1
		1250	169±13	42±4	103±8	43±3	14±1
		2500	172±12	41±4	101±9	40±3	13±1
		5000	170±12	39±5	99±10	39±4	12±2
	S9 mix (+)	0[a]	172±12	38±3	97±8	39±3	12±2
		313	169±13	37±4	96±9	38±4	12±2
		625	168±14	40±2	97±8	39±4	13±1
		1250	170±12	40±2	96±8	40±3	11±2
		2500	173±12	39±3	99±7	39±4	11±3
		5000	170±14	41±3	98±9	37±5	12±2
+ control	S9 mix (-)	Compound	NaN_3	NaN_3	MMS	2-NF	9-AA
		Concentration (μg/plate)	1.5	1.5	2.5	2.5	25
		Colony No.	710±25	692±21	473±19	418±21	74±7
	S9 mix (+)	Compound	2-AAN	2-AAN	2-AAN	BP	BP
		Concentration (μg/plate)	10	10	10	20	20
		Colony No.	787±31	691±41	458±31	417±21	74±8
Historical negative[b]			10-50	60-220	5-50	1-25	65-115

[a]Negative (solvent) control; [b]the historical negative range was formed by reference literature. Test article can be mutagenic if either a two fold increase over the spontaneous reversion rate (percent of controls >200%) or demonstration of a dose-response curve when dilutions are tested and can be a non-mutagenic if either a less than two-fold increase over spontaneous reversion rate (percent of control <200%) or no dose response curve when dilutions are tested. MMS=methyl methansulfonate; 2-NF=2-nitrofluorene; 9-AA=9-aminoacridine; 2-AAN=2-aminoanthracene; BP=benzo[a]pyrene.

Table 2
Chromosome aberration test of Trois in CHL cells.

Compound	S9	Time (h)	Dose (mg/mL)	Scored cell no.	Polyploid (%)	Judge	Frequency of cells with chrosomal aberrations (%)						
							ctg	ctb	cte	csb	cse	TAG	TA
Solvent	−	24−0[a]	0	100	0	−	0	0	0	0	0	0	0
Trois	−	24−0[a]	0.9	100	0	−	2	0	0	0	0	2	0
		24−0	1.9	100	1	−	0	0	0	0	0	1	0
	−	24−0	3.7	100	0	−	0	0	0	0	0	1	0
	−	24−0	7.5	100	0	−	1	1	0	0	0	2	1
	−	24−0	15	100	0	−	1	1	0	0	0	2	1
MMC	−	24−0	0.00005	100	0	+	40	21	56	0	4	80	68
Solvent	−	48−0	0	100	0	−	1	0	0	0	0	1	0
Trois	−	48−0	0.9	100	0	−	0	0	0	0	0	0	0
		48−0	1.9	100	0	−	3	0	0	0	0	3	0
	−	48−0	3.7	100	0	−	2	0	0	0	0	2	0
	−	48−0	7.5	100	1	−	1	0	0	0	0	1	0
	−	48−0	15	100	0	−	4	0	0	0	0	4	0
MMC	−	48−0	0.0001	100	0	+	29	9	49	0	3	52	44
Solvent	−	6−18	0	100	0	−	0	0	0	0	0	0	0
Trois	−	6−18	0.9	100	0	−	0	0	0	0	0	0	0
		6−18	1.9	100	0	−	2	2	2	0	0	6	1
	−	6−18	3.7	100	0	−	3	0	0	0	0	3	0
	−	6−18	7.5	100	1	−	1	0	0	0	0	1	0
	−	6−18	15	100	0	−	2	0	0	0	0	2	0
BP	−	6−18	0.02	100	0	+	1	0	1	0	0	2	1
Solvent	+	6−18	0	100	0	−	1	0	0	0	0	1	0
Trois	+	6−18	0.9	100	0	−	0	0	0	0	0	0	0
	+	6−18	1.9	100	0	−	1	0	0	0	0	1	0
	+	6−18	3.7	100	0	−	0	0	0	0	0	0	0
	+	6−18	7.5	100	0	−	2	1	0	0	0	3	0
	+	6−18	15	100	0	−	1	1	0	0	0	2	0
BP	+	6−18	0.02	100	0	−	32	4	40	0	3	49	41

a=Treatment time; Abbreviation: ctg=chromatid and chromosome gap, ctb=chromatid break, cte=chromatid exchange, csb=chrosomal break, cse=chrosomal exchange, TAG=total cells which have chrosomal aberrants including ctg, TA=total cells which have chrosomal aberrants excluding ctg, MMC=mitomycin C, BP=benzopyrine.

4. Discussion

Despite the fact that herbal formulations have usually been used widely across the world[1], there is limited safety data of herbal formulations. Alteration in the structure of DNA of somatic and germ cells may lead a variety of genetic diseases[21]. Alteration of DNA of somatic cells has been known to cause accelerated aging, immune dysfunction, cardiovascular and neurodegenerative diseases, whereas mutations in germ cells may lead to spontaneous abortions, infertility or heritable damage to the offspring and possibly to the subsequent generations[1,22,23]. To observe the potential genotoxicity of Trois, bacterial reverse mutation and chromosomal aberration test were carried out in this study.

There were no increases in the number of revertant colonies at any concentration (5000, 2500, 1250, 625 and 313 μg/plate) with and without metabolic system in *S. typhimurium* (TA 98, TA100, TA1535 and TA1537) and *E. coli* (WP2 *uvrA*). In the chromosome aberration assay, there were no statistical significant increases in the number of structural aberrations at any dose of Trois in the presence or absence of the metabolic activation system in CHL cells. In our a previous study, Trois was safe up to 2000 mg/kg body weight on dermal toxicity in rabbits[24]. The literature of genotoxicity study of individual ingredients of Trois is not available. Trois was not mutagenic in the four strains of *S. typhimurium* and one strain of *E. coli*, and did not induced chromosomal aberrations in CHL cells. No dose-dependent effect was observed in any of the parameters suggesting the absence of a treatment related adverse effect of Trois. Our results reveal that Trois is safe in terms of genotoxicity.

In conclusion, the results of this investigation revealed that Trois is non-mutagenic.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Authors are thankful to sponsor, Venus Medicine Research Centre, Werne, Germany, for providing financial assistance (Grant no. VPG-115-2012) to carry out this study.

Comments

Background

Herbal medicines have been used from the ages in health care systems. They produce numerous beneficial as well as harmfull effects in human beings. However, despite their wide use in health care system, there is insufficient background information about its safety.

Research frontiers

Several studies are performed in order to determine the safety profile of herbal medicines. Among them, genotoxicity study is an important parameter. This study has been focused on studying the genotoxicity profile of a newly developed nano-technology based herbal medicine through Ames and chromosomal aberration test.

Related reports

There are very limited reports on the genotoxicity study of herbal plants. Ha *et al*. (2011) have studied the genotoxicity assessment of a herbal formula, Ojeok-san and have found that Ojeok-san is toxicologically safe on genotoxicity studies.

Innovations and breakthroughs

The safety data of herbal medicines are very scanty. This study has established that the Trois is a safe herbal topical formulation and can be used for the treatment of pain and inflammation associated with different types of arthritis.

Applications

There are numerous reports which have shown that synthetic drug are being used for the treatment of arthritis but they have adverse effects that can compromise the therapeutic treatment. To overcome such adverse effects Trois can be used for the treatment of arthritis which has been proved to be safe and non-genotoxic.

Peer review

This is a very useful study in which authors have evaluated the genotoxicty profile of Trois, a nano-technology based herbal tropical formulation. The results obtained in this study clearly suggested that Trois, which has been designed to treat various types of arthritis, is non-mutagenic.

References

[1] Jordan SA, Cunningham DG, Marles RJ. Assessment of herbal medicinal products: challenges, and opportunities to increase the knowledge base for safety assessment. *Toxicol Appl Pharmacol* 2010; **243**(2): 198-216.

[2] Bussmann RW, Sharon D. Traditional medicinal plant use in Northern Peru: tracking two thousand years of healing culture. *J Ethnobiol Ethnomed* 2006; **2**: 47.

[3] Njoroge GN, Kaibui IM, Njenga PK, Odhiambo PO. Utilisation of priority traditional medicinal plants and local people's knowledge on their conservation status in arid lands of Kenya (Mwingi District). *J Ethnobiol Ethnomed* 2010; **6**: 22.

[4] Atsamo AD, Nguelefack TB, Datté JY, Kamanyi A. Acute and subchronic oral toxicity assessment of the aqueous extracts from the stem bark of *Erythrina senegalensis* DC (Fabaceae) in rodents. *J Ethnopharmacol* 2011; **134**(3): 697-702.

[5] Organisation for Economic Co-operation and Development. Organisation for economic co-operation and development guidelines for the testing of chemicals. Paris: Organisation for Economic Co-operation and Development; 2007.

[6] Reynard LN, Loughlin J. The genetics and functional analysis of primary osteoarthritis susceptibility. *Expert Rev Mol Med* 2013; **15**: e2.

[7] Horng HC, Kuo CP, Cherng CH, Yeh CC, Wang TC, Liaw WJ, et al. The effects of collateral meridian therapy for knee osteoarthritis pain management: a pilot study. *J Manipulative Physiol Ther* 2013; **36**(1): 51-56.

[8] Reinke T. Aiming at a moving target in rheumatoid arthritis. *Manag Care* 2013; **22**(2): 23-26.

[9] Doghramji PP. Managing your patient with gout: a review of treatmentoptions. *Postgrad Med* 2011; **123**(3): 56-71.

[10] DiCarlo EF, Kahn LB. Inflammatory diseases of the bones and joints. *Semin Diagn Pathol* 2011; **28**(1): 53-64.

[11] Prakken B, Albani S, Martini A. Juvenile idiopathic arthritis. *Lancet* 2011; **377**(9783): 2138-2149.

[12] Tanen DA, Danish DC, Reardon JM, Chisholm CB, Matteucci MJ, Riffenburgh RH. Comparison of oral aspirin versus topical applied methyl salicylate for platelet inhibition. *Ann Pharmacother* 2008; **42**(10): 1396-1401.

[13] Ciftci O, Ozdemir I, Tanyildizi S, Yildiz S, Oguzturk H. Antioxidative effects of curcumin, β-myrcene and 1,8-cineole against 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced oxidative stress in rats liver. *Toxicol Ind Health* 2011; **27**(5): 447-453.

[14] Uhl GR, Walther D, Behm FM, Rose JE. Menthol preference among smokers: association with TRPA1 variants. *Nicotine Tob Res* 2011; **13**(12): 1311-1315.

[15] Zheng CJ, Tang WZ, Huang BK, Han T, Zhang QY, Zhang H, et al. Bioactivity-guided fractionation for analgesic properties and constituents of *Vitex negundo* L. seeds. *Phytomedicine* 2009; **16**(6-7): 560-567.

[16] Ram HNA, Srivastawa NK, Makhija IK, Shreedhara CS. Anti-inflammatory activity activity of *Ajmodadi churna* extract against acute inflammation in rats. *J Ayurveda Integra Med* 2012; **3**(1): 33-37.

[17] Ames BN, McCann J, Yamasaki E. Methods of detecting carcinogens and mutagens with the *Salmonella*/mammalian microsomal mutagenicity test. *Mutat Res* 1975; **31**(6): 347-364.

[18] Maron DM, Ames BN. Revised methods for the *Salmonella* mutagenicity test. *Mutat Res* 1983; **113**(3-4): 173-215.

[19] Ishidate M Jr, Sofuni T, Yoshikawa K. Chromosomal aberration tests *in vitro* as primary screening tool for environmental mutagens and/or carcinogens. In: Inui N, Kuroki T, Yamada MA, Heidelberger C, editors. *Mutation, promotion and transformation in vitro*. Tokyo, Japan: Japan Scientific Societies Press; 1981.

[20] Shin IS, Seo CS, Ha HK, Lee MY, Huang DS, Huch JI, et al. Genotoxicity assessment of Pyungwi-san (PWS), a traditional herbal prescription. *J Ethanopharmacol* 2011; **133**(2): 696-703.

[21] Erickson RP. Somatic gene mutation and human disease other than cancer: an update. *Mutat Res* 2010; **705**(2): 96-106.

[22] Slatter MA, Gennery AR. Primary immunodeficiencies associated with DNA-repair disorders. *Expert Rev Mol Med* 2010; **12**: e9.

[23] Frank SA. Evolution in health and medicine sackler colloquium: somatic evolutionary genomics: mutations during development cause highly variable genetic mosaicism with risk of cancer and neurodegeneration. *Proc Natl Acad Sci USA* 2010; **107**(Suppl 1): 1725-1730.

[24] Payasi A, Gupta A, Chaudhary M, Sehgal R, Naithani V. Acute dermal toxicity studies of Trois in Newzealand white rabbits. *Int J Drug Dev Res* 2010; **2**(2): 310-313.