



EVALUATION OF IN VITRO MUTAGENIC AND ANTIMUTAGENIC ACTIVITY OF VITAE ELIXIR BY AMES SALMONELLA MICROSOME ASSAY

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Abstract: The Ames *Salmonella* / microsome mutagenicity assay (Ames test) is a short-term bacterial reverse mutation test specifically designed to detect a wide range of chemical substances that can produce genetic damage leading to gene mutations. Vitae Elixir is a mixture of nine herbal extracts with Chlorophyllin and Allicin. All herbs from Vitae Elixir are medicinally very important with variety of roles in the different disorders or diseases, when studied individually by researchers. In spite of clinical use of Vitae Elixir, no information is available on its toxicity, mutagenicity, antimutagenicity or anticarcinogenic potential. In present study, mutagenic and antimutagenic potential of Vitae Elixir, was evaluated using plate incorporation assay, preincubation assay and modulation assay. The positive controls such as 4-NQNO, 2-AF, Danthron and MMS were used with and without S9. Ames *Salmonella* histidine reversion assay was studied using standard strains of *Salmonella typhimurium* TA97a, TA98, TA100 and TA102 with and without S9. In plate incorporation assay, Vitae Elixir was not mutagenic at and up to 500 µg/plate. It exhibits the anti-mutagenic potential at 500 to 1000µg/plate in preincubation test. Vitae Elixir is an extremely potent inhibitor of the mutagenicity at 1000µg and 500µg/plate and significantly dose dependent anti-mutagenic activity in TA98 and TA100 with and without metabolic activation. Vitae Elixir would be beneficial for prevention of malignancy.

Keywords: Vitae Elixir, Ames assay, *Salmonella typhimurium*, preincubation, Antimutagenic

INTRODUCTION

Cancer is a disease entity, in which the fundamental rules of cell behavior break down, leading to uncontrolled cell division, causing formation of undesired growth of cells and tissues. Cancer cells usually have abnormal number or arrangements of chromosomes (aneuploidy), indicating a genetic instability that plays an important role in tumor development. Currently, natural plant products are being favored against the synthetic drugs for the treatment of cancer due to less side effects^(1, 4, 25, 47). It is also believed that the mixture of herbs is better alternative than single chemical drug for the treatment of cancers as they have the advantage of synergy & potentiation^(4, 5, 6). In vivo carcinogenesis has been shown to be correlated to in vitro mutagenesis⁽⁵²⁾. Approximately 25000 plant species are used for medicinal purpose around the globe⁽⁴¹⁾. Ames *Salmonella* test was first validated in a study of 300 chemicals most of which were known carcinogens^(6, 21, 32, 33, 34). No herbal medicine is safe. There are some adverse effects such as allergic reactions and/or reactions due to interaction of herbal drugs⁽⁴⁷⁾.

Vitae Elixir a combination of nine herbs is being used under “alternate medicine therapy” as an anticancer agent against different types of human cancers. This study was performed to identify the

antimutagenic and antitumorigenic properties of Vitae Elixir. Most of the herbs from Vitae Elixir preparation were used since centuries ago. Ancient literature irrespective of religion quoted the significance and its use either single or mixture of ingredients of Vitae elixir. *Sanguinaria canadensis* is used against inflammations of eyes, throat and alimentary canal and also increases appetite⁽²⁸⁾. *Impatiens pallida* was used against hemorrhoids, warts and corns⁽¹⁰⁾. *Hydrastis Canadensis* is used in the treatment of breast cancer and antibiotic⁽⁷⁾. *Ferula galbaniflua* has tumericidal potency against in vitro malignant neuroblastoma⁽⁹⁾. *Hypericum perforatum* and *Fumaria officinalis* are used as antitumerogenic medicines^(8, 12, 36, 50). *Rubus villosus* has astringent and tonic properties. *Frasera caroliensis* helps in improving appetite and resolves problems of digestion, constipation and colitis pain. Allicin enhances the activity of phagocytic cells, natural killer cells and certain cancer cells⁽¹¹⁾. Regular intake of garlic reduces the risk of oesophageal, stomach and colon cancer⁽²⁵⁾. Garlic acts as anticancer and antimutagenic agent^(1, 13, 48). It has also been shown that many plant originated products such as alkaloids, resins, flavonoids, pigments, tannins, polyphenols, carotenes and vitamins have an antimutagenic property and can be used to prevent cancer^(9, 14, 18). In modulation / antimutagenic study the Vitae Elixir was found to suppress the number of

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revertants produced by known chemical mutagens such as 2 Aminofluorene, 4NQNO and MMS in TA98 and TA100 of *S. typhimurium*.

MATERIAL AND METHODS

Vitae Elixir is a mixture of Chlorophyllin, *Sanguinaria canadensis*, *Impatiens pallida*, *Hydrastis canadensis*, *Ferula galbaniflua*, *Hypericum perforatum*, *Rubus villosus*, *Fumaria officinalis*, *Frasera caroliensis*, Allicin and Garlic.

The test employs histidine dependent strains of *Salmonella* each carrying different mutations in various genes in the histidine operon^(27, 29, 34, 37, 49, 51). These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (*his*⁺) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner^(2, 3, 15).

Table.1: Characters of the *Salmonella typhimurium* strains

Strain	His-mutation	LPs	Repair	PKM101	Nature of mutation	Reference
TA97a	his O 1242	rfa	ΔuvrB	+	+ 4 near CCC	27, 34, 37.
TA98	his D 3052	rfa	ΔuvrB	+	- 1 near CG	49.
TA100	his G 46	rfa	ΔuvrB	+	AT→GC	29, 34.
TA102	PAQ 1 his G 428 / Δ his	rfa		+	GC→ AT ochre	26, 34, 51.

Strains, except TA102, are defective in DNA repair capacity (*uvrB*) and have a defective lipopolysaccharide barrier on the cell wall (*rfa*). These two properties confer extra sensitivity to DNA damage and also increase permeability to large molecules that do not penetrate the normal cell wall.

Strains TA97a, TA98, TA100 and TA102 also contain resistant transfer factor (Plasmid pKM 101). This factor, which confers resistant to ampicillin, enhances the operation of an error prone repair system. In case of TA102, it contains Plasmid pAQ1, which confers resistant to tetracycline. TA100 is highly sensitive to base – pair substitution mutation and TA97a, TA98 are highly sensitive to frame – shift mutation. The strain TA102 contains ochre mutation (Table 1).

Genetic markers of the test strain and the degree of its spontaneous reversion were checked each time before testing the samples. Average numbers of revertants formed spontaneously were close to those given by Maron and Ames^(29, 31, 32). The strain sensitivity check was based on a positive response and performed

by exposing the bacteria to diagnostic mutagens. The positive controls used in the study are listed in table 2.

Table.2: Positive controls for *Salmonella typhimurium* strains

Strain	Positive Control		Reference
	With S9	Without S9	
TA97a	2AF	4NQNO	39.
TA98	2AF	4NQNO	39.
TA100	2AF	MMS	19, 39.
TA102	Danthron	MMS	19, 39.

Quantitative evaluation of Vitae Elixir was performed by using plate incorporation assay. It was designed to establish relationship between the number of induced revertants and the doses of test substance used. Vitae Elixir under study was tested using four tester strains viz. TA97a, TA98, TA100, TA102. Procedure for *Salmonella* microsome assay described by Maron and Ames (1983) was adopted in this study^(3, 6, 15).

Suspensions of bacterial cells were exposed to the test article in the presence and in the absence of an exogenous metabolic activation system. These suspensions were mixed with an overlay agar and plated immediately onto minimal medium. After 48 hours incubation period revertant colonies were counted and compared to the number of spontaneous revertant colonies on control plates. The entire study was carried out in triplicates.

In preincubation method TA97a, TA98, TA100 and TA102 cultures along with Vitae Elixir were incubated for 30 minutes at room temperature along with and without S9 metabolic activation and poured in the agar plates along with top agar^(9, 35, 43).

Vitae Elixir was assessed by one step modification in the antimutagenicity assay. *Salmonella* strains TA98 and TA100 were treated with Vitae Elixir and respective positive mutagen with and without microsomal fraction. Equal volumes of mutagens and test substance were mixed and allowed to stand for 30 minutes at 37°C under continuous shaking. This was added to 2ml of top agar with 0.1ml of fresh bacterial culture^(30, 35, 44). The effect of Vitae Elixir on the indirectly acting mutagen (2AF, 4NQNO and MMS) was studied by 0.5ml of S9 mix directly added into soft agar containing 0.1ml of bacterial culture and 0.1ml of S9 dependent mutagen^(16, 23, 29, 44). The plates were observed for a uniform lawn of auxotrophs (*his*⁻) and the colonies for histidine revertants as the prototrophs (*his*⁺). Histidine revertant colonies per plate were counted and the mean number of colonies at each test point was calculated.

To ensure sterility of the vehicle, test article and equipments, tests for evaluation of contamination were performed along with the assay. The solvent, overlay

agar, S9 mix and the highest employed dose of the test article were evaluated at the same volume that was used in the assay, but in the absence of *S. typhimurium*. Plates were observed after incubation period of 48 hours for contamination if any.

Mutagenicity ratio (MR) was calculated as the ratio of the number of *Salmonella typhimurium* revertants grown in the presence of the tested sample to the number of spontaneously appeared revertants. The sample was considered mutagenic when $MR \geq 2$ (42).

RESULTS

Plate incorporation assay (With and without S9): -

In plate incorporation assay the colony counts of all 4 strains were found non mutagenic at and up to 500µg/plate. It was strongly mutagenic in TA98 and TA102 at the 1000µg /plate dose. The mutation ratio at 1000µg/plate in TA98 was 4.20 without microsome fraction (-S9) and 3.29 with microsome fraction (+S9). In TA102 the mutation ratio was 2.27 without microsome fraction (-S9) and 1.91 with microsome fraction (+S9).

Table.3: Mutagenicity ratio of Plate incorporation assay

Treatment	Dose µg / plate	MUTAGENICITY RATIO							
		TA97a		TA98		TA100		TA102	
		- S9	+S9	- S9	+S9	- S9	+S9	- S9	+S9
Vitae Elixixir	10.0	1.00	0.92	0.99	0.95	1.07	1.12	1.36	1.13
	50.0	1.03	1.02	1.01	0.87	0.99	1.05	1.25	1.04
	100.0	1.02	1.11	0.99	0.79	1.03	1.15	1.15	1.06
	500.0	1.08	1.19	0.83	1.13	1.12	1.16	1.18	1.21
	1000.0	1.82	1.74	4.20	3.29	1.26	1.25	2.27	1.91
DMSO (µl)	10.0	-	-	-	-	-	-	-	-
POSITIVE CONTROL									
4-NQNO	0.50	3.19	-	10.33	-	-	-	-	-
2- AF	10.0	-	2.95	-	8.06	-	3.22	-	-
MMS (µl)	1.00	-	-	-	-	3.01	-	2.80	-
DANTHRON	30.0	-	-	-	-	-	-	-	2.87

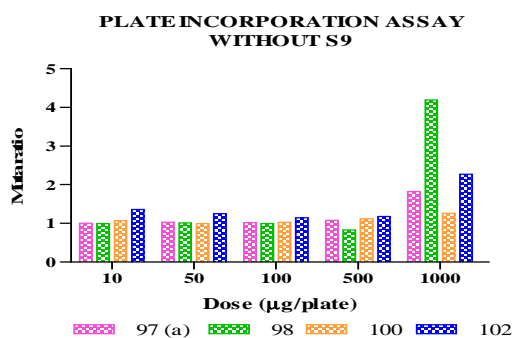


Fig. 1: Plate incorporation assay without S9

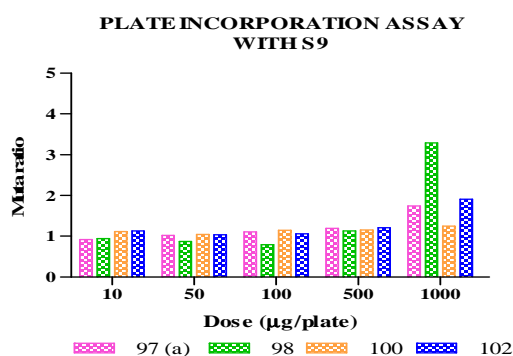


Fig. 2: Plate incorporation assay with S9

Table.4: Mutagenicity ratio of Preincubation test

Treatment	Dose µg / plate	MUTAGENICITY RATIO							
		TA97a		TA98		TA100		TA102	
		- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
Vitae Elixixir	10.0	1.29	1.29	0.85	1.00	1.10	0.97	1.00	0.99
	50.0	1.32	1.26	0.96	1.05	1.10	1.04	0.99	0.92
	100.0	1.38	1.30	0.93	1.03	1.09	1.12	0.95	1.03
	500.0	1.41	1.27	1.05	1.10	1.16	1.09	1.16	1.08
	1000.0	1.59	1.81	2.25	2.26	1.18	1.13	1.28	1.20
DMSO (µl)	10.0	-	-	-	-	-	-	-	-
POSITIVE CONTROL									
4-NQNO	0.50	2.83	-	9.54	-	-	-	-	-
2- AF	10.0	-	2.78	-	9.86	-	2.82	-	-
MMS (µl)	1.00	-	-	-	-	2.47	-	4.14	-
DANTHRON	30.0	-	-	-	-	-	-	-	3.67

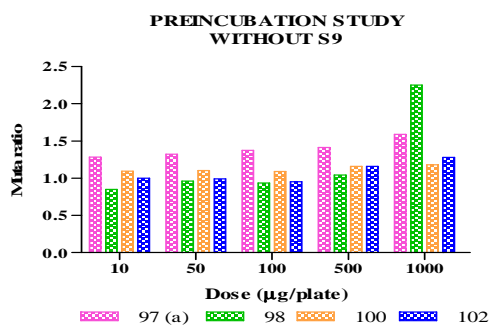


Fig. 3: Preincubation study without S9

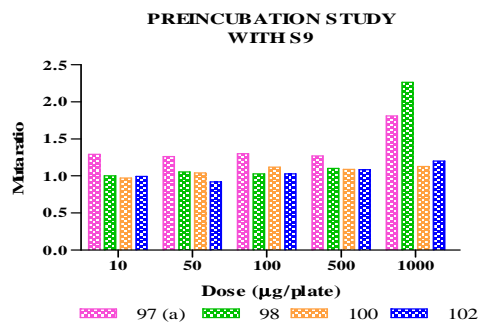


Fig. 4: Preincubation study with S9

Preincubation assay (With and without S9):-

In TA98 at 1000µg/plate mutation ratios showed 2 fold increases which were 2.25 and 2.26 without and with metabolic activation respectively. In TA98 the revertants were comparable up to 500µg/plate with spontaneous revertants. More than 2 fold increase was observed in all the groups of positive mutagens.

Modulation assay (With and without S9):

Antimutagenicity study: In modulation study results of TA98 indicate the increase in reversion colonies with respect to decrease in concentration of Vitae Elixixir against 2AF and 4NQNO with and without metabolic activations respectively. At 10µg/plate concentration of Vitae Elixixir the mutation ratio were 13.83 and 13.28 with and without metabolic activation respectively. The revertant colonies were decreased as the concentration increases. At 1000µg/plate Vitae Elixixir mutation ratio was observed 2.78 and 2.60 with and without metabolic activation respectively. As concentration of Vitae Elixixir increased the revertant colonies decreased.

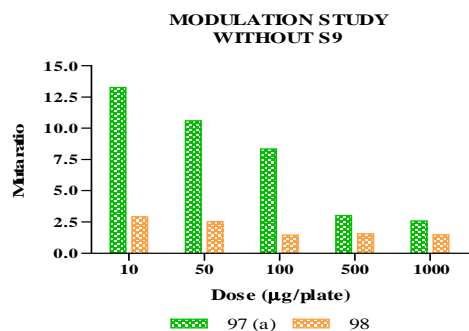


Fig. 5: Modulation study without S9

Table.5: Mutagenicity ratio of Antimutagenicity test

Treatment	Dose µg /plate	MUTAGENICITY RATIO			
		TA98		TA100	
		- S9	+ S9	- S9	+ S9
Vitae Elixixir	10.0	13.28	13.83	2.93	3.86
	50.0	10.59	10.42	2.52	2.70
	100.0	8.36	8.72	1.46	1.50
	500.0	3.03	3.32	1.56	1.53
	1000.0	2.60	2.78	1.48	1.58
DMSO (µl)	10.0	-	-	-	-
4-NQNO	0.50	13.48	-	-	-
2- AF	10.0	-	12.28	-	3.44
MMS (µl)	1.00	-	-	3.17	-

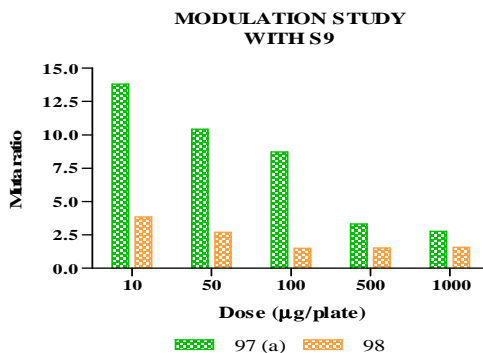


Fig. 6: Modulation study with S9

The revertants in positive mutagen showed increase by 4 folds and comparable with the other sets. The Vitae Elixixir showed moderate to high antimutagenic potential against 2AF and 4NQNO in TA98 along with and without metabolic activation.

In TA100 antimutagenic response was found to be dose dependant. There was decrease in number of revertants as the concentration of Vitae Elixixir increases. The number of revertants was approximately 3 fold than the spontaneous revertants at 10µg/plate in with and without S9 and a ratio was absolutely normal at 1000µg/plate. The activity of Vitae Elixixir in TA100 along with 2AF was found to be dose dependant from 10 to 1000µg/plate. Vitae Elixixir in TA00 showed weak to moderate antimutagenic potential against 2AF and MMS with and without microsomal fraction. As shown in table 5 and Fig. 5 & 6, the dose response with TA98 and TA100 with respective mutagen is seen suppressed. The observations showed moderate inhibitory effect due to Vitae Elixixir.

DISCUSSION

Emphasis in this discussion was now being focused on each investigation. The mutagenicity of Vitae Elixixir when tested in *Salmonella* / microsome assay 5000 µg/disc was found to be cytotoxic to TA98 and TA102 strains (2, 3, 15).

The doses selected were 1000 to 10 µg/plate. Plate incorporation assay with and without S9 was found to be non-mutagenic at and up to 500µg/plate in TA 97a, TA98 and TA102 where the mutation ratio was 2.0 to 3.94 and 1.8 to 3.29 with and without metabolic activation respectively⁽³²⁾. In pre-incubation study each tester strain along with the Vitae Elixir were found to be non-mutagenic at and up to 1000 µg/plate^(38, 40).

Antimutagenicity was studied in TA98 and TA100 with metabolic activation by 2AF and without metabolic activation by 4 NQNO and MMS respectively. Both strains with respective positive controls showed antimutagenic property. There was an interference of Vitae Elixir in the process of mutation. Anti-mutagenic activity governed by interacting with known mutagen and Vitae Elixir which protects the site of DNA interaction with mutagen⁽²⁴⁾. It may be due to free radical effect and polyphenols generally present in plants. This property of both strains found to be most beneficial in prevention of cancer by blocking the site of DNA where the interaction of mutagen is possible^(8, 19, 36, 37, 46, 50, 53). Hydroethanolic extract of *H. Perforatum* showed mutagenic findings in Ames test^(45, 46). Garlic and allicin inhibits *S. typhimurium*, the DNA and protein synthesis. The results were extensively acknowledged that the components of Vitae Elixir possess antimutagenic potential^(4, 14, 22, 23, 24). The mixture, Vitae Elixir has cumulative effect as antimutagenic. Anti-tumor activity in the mouse which is in progress also supports the Ames test.

Mutagenicity and antimutagenicity tests are synergistic to each other. The cause of genetic damage by interaction with herbal extract with known and unknown ingredients is interesting because they play significant role in the repair mechanism of genetic material^(10, 11). Cumulative anticancer properties of the herbs included in the extract have become main focus of the study. Most of the cancer patients along with the allopathic drugs use traditional medicine^(1, 4, 7, 9) prepared from plant parts. It may be supplementary or remedial against cancer. Thus the study of mutagenic and antimutagenic activity of Vitae Elixir is more important than the individual plant extract.

In mutagenicity tests (Fig. 1 & 2) results showed that the Vitae Elixir is mutagenic at 1000 µg/plate per plate in TA98. In preincubation study (Fig. 3 and 4) only with Vitae Elixir and strains after 30 minutes of incubation, 1000 µg/plate showed 4 fold mutagenic ratio in TA98 and approximately 2 fold increase in TA102 of the spontaneous reversion. It is weak to moderate mutagenic in TA102 and TA98 respectively. As shown in table 5 and Fig. 5 & 6, the dose response with TA98 and TA100 with respective mutagen is seen suppressed. The observations showed moderate inhibitory effect due to Vitae Elixir.

As Bhatia *et al.*,⁽⁸⁾ and Schwarz *et al.*,⁽⁴⁶⁾ suggested the antimutagenic property of *H. perforatum*^(12, 13) which is one of the ingredients of Vitae Elixir, can be beneficial for prevention of cancer. Present study was carried out on mixture of anticancerous plants and some plants which are routinely used to avoid secondary and side effects during cancer therapy.

Individual plant extracts^(8, 12, 18, 35, 46) may be effective but the extracts of herbal mixtures^(23, 28) are proven more effective in cancer treatment. Most of the plants from this mixture also have antioxidant property. The antimutagenic effect showed by the Vitae Elixir needs to be assigned for further studies of its mechanism responsible for reversion.

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