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Original Article

Antimutagenicity of Chyawanprash in Ames test

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ABSTRACT

Some medicinal plants are known to contain natural mutagenic and antimutagenic substances. Chyawanprash is an Ayurvedic preparation most widely used as general tonic or health supplement. The present study was undertaken to evaluate the mutagenic/antimutagenic potential of Chyawanprash using Salmonella mutagenicity test. *Salmonella typhimurium* tester strains TA1537, TA1538, TA100 and TA102 were used for mutagenicity testing. Antimutagenicity study was carried out in tester strains TA1538 and TA100 against various standard mutagens with and without metabolic activation. Chyawanprash was found to be non-mutagenic, whereas exhibited anti-mutagenic potential in this assay as it inhibited the mutagenicity induced by a direct mutagen 4-nitro-o-phenylenediamine and indirect mutagens such as 2-Aminofluorene, 2-Anthramine, Benzo(a)pyrene and Cigarette Smoke Condensate. The antimutagenic activity was more potent in the presence of metabolic activation than without the same. The exact mechanism of antimutagenic potential of Chyawanprash is not known. The enhanced anti-mutagenic activity of Chyawanprash on pre-incubation indicates the antimutagenic factor(s) may be desmutagenic in nature. The possibility of having diverse antimutagenic factor(s) in Chyawanprash which act by different mechanisms is strongly indicated. Chyawanprash by having antimutagenic potential may play an important role in neutralization of various dietary mutagens and acts as a prophylactic agent against human ill health attributable to mutation. Further study in this aspect using some other *in vivo* and *in vitro* tests is warranted before any final conclusion.

Keywords: Chyawanprash, *Salmonella* mutagenicity test, Ames test

1. INTRODUCTION

Cancer is a disease or disorder in which a single normal body cell undergoes genetic transformation into a cancer cell and will begin to reproduce uncontrollably. There is a high correlation between carcinogenicity and mutagenicity. Most studies related to antimutagenic activity has been focused on identifying naturally occurring chemo-preventing substances capable of inhibiting, retarding or reversing the multistage carcinogenesis[1].

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Mutations are the cause of innate metabolic defects in cellular system, triggering the morbidity and mortality in living organisms. A plethora of synthetic and natural substances, apart from various genotoxic physical and biological agents, are known to act as mutagenic, co-carcinogenic and/or carcinogenic agents[2,3]. Since, the mutagens are involved in the initiation and promotion of several human diseases including cancer, the significance of novel bioactive phytocompounds in counteracting the promutagenic and carcinogenic effects are gaining credence. Such chemicals that reduce the mutagenicity of physical and chemical mutagens are called as antimutagens. The antimutagens have been first reported almost four decades ago, and since then numerous studies have been carried out in order to identify compounds, which might protect humans against DNA-damage and its consequences. Antimutagenic and anticarcinogenic properties of a wide variety of dietary constituents and plant secondary metabolites have been reported[3].

The antimutagenic or protective effect has been attributed to many classes of phytocompounds mainly flavonoids and phenolic compounds present in foods[4]. However, such compounds have also been reported to exhibit a wide range of other biological activities such as antimicrobial, anti-inflammatory, anti-allergic, antioxidant and free radical scavenging[5,6]. Natural antimutagens from edible and medicinal plants are of particular importance because they may be useful for human cancer prevention and have no undesirable xenobiotic effects on living organisms. The rich diversity of Indian medicinal plants have not yet systematically screened for antimutagenic activity.More than 800 plants are used in the treatment of various ailments in the traditional systems of Indian medicine (Ayurveda, Siddha and Unani). Ayurvedic preparations are mainly polyherbal preparations sometime with other minerals[7]. Based on the chemical diversity of known active phytoantimutagens, many traditionally used Indian medicinal plants may exhibit such desired properties due to similarity in the major class of phytocompounds[7,8].

Chyawanprash, a well-known Ayurvedic health food can alleviate general complaints and combat the so called civilization disease. The wide use of Chyawanprash by all the age group categories has drawn the attention of scientists in various fields. Chyawanprash as a whole and its different ingredient herbs individually have been subjected to phytochemical investigations. Different isolated fractions and active principles have been tested for their biological activities on experimental animals[9]. In one of the clinical studies, Chyawanprash was shown as an adjunct in the treatment of pulmonary tuberculosis[10]. In an analytical and antitumor study on Chyawanprash there was a very moderate anticancer activity in albino rats against early stages of chemical induced fibrosarcoma[11]. Even though lot of medicinal properties were identified, determined and proved by testing Chyawanprash, no publishing literature is available regarding the genotoxicity. Thus this present study was carried out to determine the possible antimutagenic or anticarcinogenic potential of Chyawanprash.

2. MATERIALS AND METHODS

Salmonella tester strains

Salmonella typhimurium tester strains TA1537, TA1538, TA100 and TA102 were used for this study to perform all the experimental part of Ames test. These tester strains were kindly supplied by Prof. B N Ames, California University, USA.

Preparation of water extract of Chyawanprash

Chyawanprash was purchased from Ayurvedic pharmacy at Karnataka. The thick paste of 20 grams was taken and dispensed thoroughly in distilled water using tissue homogenizer at room temperature for 15

minutes. Later 180ml of distilled water was also added and boiled at 100°C using heating mantle till the volume reduced to half of the original quantity. After the mixture reached room temperature, it was centrifuged at 10000rpm for 10 minutes. The supernatant was separated and poured into a sterile petri dish and the same was kept in hot air oven at 100°C for 3 days. Finally 1.8 grams of thick syrup was obtained from 100ml of supernatant and further dissolved in DMSO and stored at -20°C until use.

Salmonella mutagenicity test

An optimum association of standard tester strains for maximum detection of mutagens was used including *Salmonella typhimurium* TA1537, TA1538, TA100 and TA102[12,]. Further plate incorporation assay and pre-incubation modification method using tester strains with and without metabolic activation were carried out essentially[13].

Metabolic activation

Rat liver S9 fraction was used where induction of liver enzymes was done[14] using phenobarbitone and beta naphthoflavone[15]. S9 mixture (8mM $MgCl_2$, 33mM KCl, 5 mM glucose 6 phosphate, 4mM NADP at the pH 7.4) was prepared freshly before the test starts by adding cofactors and 50µl of S9 was used[13].

Mutagenicity assay

All the tester strains were used for mutagenicity assay with and without S9 mix. Molten soft agar of 2ml, various concentrations of test compound (50 to 5000 µg), 0.1ml of overnight bacterial culture and 0.5ml of S9 mix (wherever used)were mixed aseptically and poured over minimal glucose agar medium and incubated at 37°C for 48 hours. After incubation, the plates were counted for $Hist^+$ revertants, same procedure was done for all concentrations and all the experiments were performed in duplicate. The results were determined as mean of 6 plates per point with standard deviation and viable cell count as approximately 1–2X10^9cfu/ml. The positive mutagenic controls including 4-Nitro-O-phenylenediamine (-S9) for TA1538 and TA100; Aminoacridine (-S9) for TA1537; Cumenehydroperoxide (-S9) for TA102, 2-Aminofluorne, 2-Antramine and Benzo(a)pyrene (+S9) for TA1538 and TA100, Emodin (+S9) for TA1537 and danthron (+S9) for TA102 were used. All the solutions were prepared freshly by dissolving in DMSO.

Antimutagenicity assay

For antimutagenicity assay, plate incorporation method and pre-incubation method were used by incorporating the tester strains TA1538 and TA100. For plate incorporation assay, 20ml of molten soft agar, various concentrations of test compound (50 to 5000 µg), 0.1ml of overnight bacterial culture, 0.1ml of known appropriate positive mutagen control and 0.5ml of S9 mix were mixed aseptically and poured over minimal glucose agar medium and incubated at 37°C for 48 hours. For antimutagen control, butylated hydroxyanesone and Dl tocopherol were used.

For pre-incubation method, 0.1ml of overnight culture, various concentrations of test compounds, optimal concentration of appropriate known mutagen control, 0.5ml of S9 mix were added in a sterile tubes, mixed thoroughly using vortex mixture and incubated at 37°C for 20 to 30 minutes. This mixture was added with top agar and poured on the surface of minimal glucose agar and incubated at 37°C for 48 hours. Results were expressed as the mean of 6 plates from two independent experiments with standard deviation. Antimutagenicity of percentage of inhibition of mutagenicity by Chyawanprash was calculated by

Antimutagencity = (a-b) / (a-c) x 100

Where a = number of $hist^+$ revertants induced by positive mutagen, b = number of $hist^+$ revertants induced by the positive mutagen in the presence of test

Compound, c = number of hist$^+$ revertants induced by the test compound alone.

3. RESULTS AND DISCUSSION

The test preparation was found to be nontoxic to the tester strains at different doses with or without S9 mix. The characteristics Hist$^+$ revertants patterns of the standard tester strains to various standard mutagens were depicted in table 1.

Table 1: Characteristic reversion patterns of standard tester strains to diagnostic mutagens

Diagnostic mutagens	Hist$^+$ revertants/ plate							
	TA1537		TA1538		TA100		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Mutagen control	8±1	15±2	15±1	23±2	143±11	161±9	386±29	409±33
Aminoacridine	858±51	-	-	-	-	-	-	-
Cumene hydroperoxide	-	-	-	-	-	-	1060±93	-
4-nitro-o-phenylene diamine	-	-	671±41	-	539±31	-	-	-
Sodium azide	-	-	-	-	725±56	-	-	-
2-Anthramine	-	331±16	-	608±42	-	721±50	-	-
2-amino fluorine	-	-	-	633±39	-	526±38	-	-
Benzo(a)pyrene	-	-	-	-	-	621±40	-	-
7,12-dimethyl benzanthracene	-	-	-	-	-	783±56	-	-
Danthron	-	-	-	-	-	-	-	1108±83
Cigarette smoke condensate	-	-	-	201±9	-	433±26	-	-

[Aminoacridine-100µg; Cumene hydroperoxide-100µg; 4-nitro-o-phenylene diamine-5µg; Sodium azide-1µg; 2-Anthramine-1µg; 2-amino fluorine-5µg; Benzo(a)pyrene-2.5µg; 7,12-dimethyl benzanthracene-20µg; Danthron-30µg and Cigarette smoke condensate-100µg]

[Viable cell count is approximately 1-2X10^9 cells/ml]

The water extract of Chyawanprash was failed to induce hist$^+$ revertants in all the four tester strains with in the presence or absence of metabolic activation by plate incorporation method (Table 2). Also there was no change in the result of preincubation modification method.

Table 2: Non mutagenicity of Chyawanprash water extract in tester strains TA1537, TA1538, TA100 and TA102 with and without S9 mix by plate incorporation method.

CWE concentrations	Hist$^+$ revertants/ plate							
	TA1537		TA1538		TA100		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Spontaneous revertants	12±1	15±1	14±2	24±2	134±16	151±16	380±27	419±47
50µg/ plate of CWE	9±2	13±1	13±2	26±3	136±11	158±10	392±32	427±38
100µg	9±1	18±2	19±2	31±3	146±13	162±14	361±31	426±31
150µg	8±2	16±1	16±2	24±1	139±9	150±18	394±42	432±20
200µg	13±2	21±3	12±1	20±1	141±13	152±12	406±39	438±30
250µg	11±1	16±2	15±1	26±2	137±15	147±10	372±29	427±51
500µg	10±2	13±2	15±2	28±2	140±15	158±9	386±36	420±31

[CWE – water extract of Chyawanprash]

[Viable cell count is approximately 1-2X10^9 cells/ml]

It was very clearly defined and proved that Chyawanprash inhibited NPD induced hist$^+$ revertants significantly in both the strains that was dose dependent. In TA1538, the highest inhibitory effect was 36.2% and in TA100 it was 45.7% at the dose of 200µg per plate. Figure 1 highlighted the effects of Chyawanprash water extract on NPD induced mutation frequency in TA1538 and TA100 by preincubation method. Further there is a significant enhanced inhibitory effect on preincubation (49% in TA1538 and 61.6% in TA100).

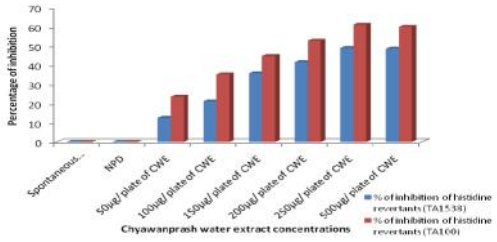

Fig 1: Antimutagenicity of Chyawanprash water extract against NPD induced mutagenesis in TA1538 and TA100 without S9 mix by pre incubation method

The effect of CWE on 2-aminofluorene, 2-anthramine and cigarette smoke condensate induced mutation frequency in TA1538 was interpreted in figure 2. From these data, it was evident that, in general CWE inhibited 2-aminofluorene, 2-anthramine and cigarette smoke condensate induced hist$^+$ revertants significantly and this was in a dose dependent manner. At the dose of 250µg per plate, CWE inhibited maximum of 53.7%, 41.7% and 42.8% of 2-aminofluorene, 2-anthramine and cigarette smoke condensate induced hist$^+$ revertants respectively. Further increase of the test

compound in the bioassay did not make significant difference in the inhibitory effect.

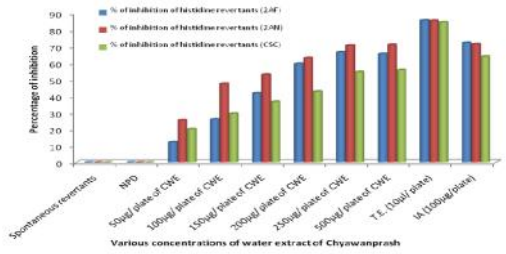

Fig 2: Antimutagenicity of Chyawanprash water extract against 2-AF, 2-AN, CSC induced mutagenesis in TA1538 with S9 mix by pre-incubation method

The effect of CWE on 2-aminofluorene, 2-anthramine, benzo(a)pyrene and cigarette smoke condensate induced mutation frequency in TA100 was well defined in this study. From this data it is obvious that in TA100 also CWE reduced hist$^+$ revertants induced by the above said indirect mutagens, in a dose dependent manner. For the dose of test compound 250µg per plate the percentage inhibitions of hist$^+$ revertants were highest of 101.3 for 2-AF, 100 for 2-AN, 99.7 for B[a]P and 105 for CSC (Figure 3).

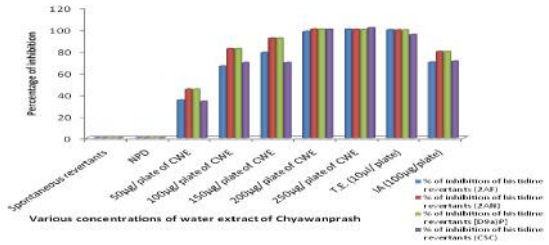

Fig 3: Antimutagenicity of Chyawanprash water extract against 2-AF, 2-AN, B[a]P and CSC induced mutagenesis in TA100 with S9 mix by pre-incubation method

Figure 2 and 3 showed the effect of CWE on the above said indirect mutagens induced mutation frequency in TA1538 and TA100 respectively in pre-incubation method. It is clear from the data that there is significant enhancement in inhibitory action by CWE on pre-incubation. It is evident that CWE is not mutagenic in the Salmonella mutagenicity assay. Lack of induction of hist$^+$ revertants in tester strains TA1537 and TA1538 indicates the absence of mutagens causing frame shift mutation whereas the same results in TA100 and TA102 indicate that the absence of mutagens causing base pair substitution and oxidative damages respectively.

Antimutagenicity of Chyawanprash was demonstrated against NPD induced mutagenesis in the absence of metabolic activation in both the tester strains TA1538 and TA100. Chyawanprash also exhibited antimutagenicity against 2-AF, 2-AN and CSC induced mutagenesis in TA1538 and 2-AF, 2-AN, B[a]P and CSC induced mutagenesis in TA100 in the presence of metabolic activation.

It was evident from the data that the antimutagenic activity of Chyawanprash was more potent in the presence of metabolic activation than in the absence of S9 mix. Further it was noticed that the antimutagenic activity of Chyawanprash against both direct and S9 dependent mutagens was comparatively more than TA100 than in TA1538. These results indicate that Chyawanprash inhibits hist$^+$ revertants arising by base pair substitution more effectively as compared to frame shift mechanisms.

The exact mechanism by which Chyawanprash exerts antimutagenic activity is not clearly understood. It is a polyherbal formulation with several ingredients. This consistently enhanced activity of antimutagenicity against all the above said mutagens in preincubation studies suggests that the antimutagenic factors may be desmutagenic in nature[16]. More over Chyawanprash exhibited antimutagenic activity against various direct and indirect mutagens and these mutagens bring mutagenesis by different mechanisms the possibility of having diverse antimutagenic factors which act by different mechanisms on Chyawanprash is strongly indicated.

Thus, due to the proven antioxidant activity of Chyawanprash, it is important to assess whether the consumption of aqueous extracts of these constituents

can assist in the prevention or repair of cellular changes caused by the exposure to potentially mutagenic agents, in addition to the proven beneficial effects for health and well-being. The protective effect of aqueous extracts of any medicinal products was determined by testing their anti-mutagenicity potential. Our results are supported by the fact that this product showed no mutagenic activity in previous studies [17, 18]. This ayurvedic product may have better antioxidant activity endow with the ability to intercept the free radicals generated by cellular metabolism or exogenous sources, such as those resulting from the action of cyclophosphamide, thereby preventing their damage to lipids, amino acids, proteins, polyunsaturated fatty acid double bonds and DNA bases [18, 19]. The aqueous extracts of AM and BF can act directly on compounds that induce mutations in DNA, chemically or enzymatically inactivating them, may inhibit the metabolic activation of promutagenic agents, or may scavenge reactive molecules, as explained [16,20]. Thus, the considerable content of flavanoids in these two plants certainly contributed to their effective antimutagenic activity.

4. CONCLUSION

We observed that the aqueous extracts of Chyawanprash, which are routinely used in Ayurvedic practice, have considerable antimutagenic activity, show no cytotoxic activity and may contribute to reducing the chromosomal damage. Chyawanprash having antimutagenic potential in it may play an important role in neutralization of various dietary mutagens and may act as a prophylactic agent against human ill health attributable to mutation. Thus, the consumption of this Ayurvedic product can bring added benefits and protection to individuals and also improving their quality of life and health. Further study in these aspects using some other *in vitro* and *in vivo* tests will warranted before finalizing the product as more successful

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