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Full Length Research Paper

Antimutagenic extract from *Tinospora cordifolia* and its chemical composition

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Chemical constituents of *Tinospora cordifolia* *n*-hexane extract were characterized by Gas Chromatography-Mass Spectrometry (GC-MS) and column chromatography. 14 constituents were characterised by GC-MS analysis and column chromatography led to the isolation of β-sitosterol. The immunomodulatory activity of the extract was investigated by polymorphonuclear leucocytes function test and antimutagenic activity by using *Salmonella* histidine tester strain TA 98. The extract exhibited potent antimutagenic activity and the IC_{50} value was found to be 1033.98 µg/ 0.1 ml in co-incubation mode and 298.57 µg/ 0.1 ml in pre-incubation mode.

Key words: *Tinospora cordifolia*, *n*-hexane extract, gas chromatography-mass spectrometry, *β*-sitosterol, antimutagenicity, immunomodulatory activity.

INTRODUCTION

Tinospora cordifolia (Willd.) Miers ex Hook F and Thoms (Menispermaceae), a large glabrous deciduous climbing shrub, is distributed throughout the tropical and subtropical parts of India (Chadha, 1948). It is categorized as "rasayana" in traditional Indian System of Medicine "Ayurveda" and is used as general tonic because of its anti-inflammatory, anti-arthritic, anti-allergic, anti-malarial and immunomodulatory properties (Kirtikar and Basu, 1975; Chopra et al., 1982; Chopra et al., 1956; Nadkarni et al., 1976; Rawal et al., 2009). Its general adaptogenic and prohost immunomodulatory activity was implicated in fighting infections. The *n*-hexane extract of *T. cordifolia* showed antitumor activity in mice (Thippeswamy and Salimat, 2007). Numbers of reports are available in the literature regarding the chemical investigation of *T. cordifolia*. The chemical constituents reported from this shrub belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides (Singh et al., 2003).

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Moreover, the petroleum ether extract of *T. cordifolia* also showed the presence of β-sitosterol, octacosanol, heptacosanol, nonacosan-15-one and lignan 3-(α 4-dihydroxy-3-methoxy benzyl)-4-(4-hydroxy-3-methoxy benzyl) tetrahydrofuran (Dixit et al., 1971; Hanuman et al., 1986; Khaleque et al., 1970). It has been realized recently that number of plants generates various secondary metabolites having potent antimutagenic activity against environmental mutagens (Sangwan et al., 1998; Minakata et al., 1983). These mutagenic agents are omnipresent in the human environment and it seems impossible to eliminate all of them. Moreover, several well-known mutagenic risk factors are closely connected with a modern lifestyle, and their entire eradication appears to be very burdensome, even unattainable. Therefore, there is a need to reduce genotoxic effects of mutagenic factors by the regular intake of antimutagenic agents. Similarly, the immune system is responsible for the eradication of pathogens and other non-self (foreign) substances. Many of the disorders today are based on the imbalances of our immunological processes. This necessitates the search for newer and safer immunomodulators. Therefore, the objective of present study was to investigate antimutagenic and immunomodulatory potential of *n*-hexane extract of *T. cordifolia*

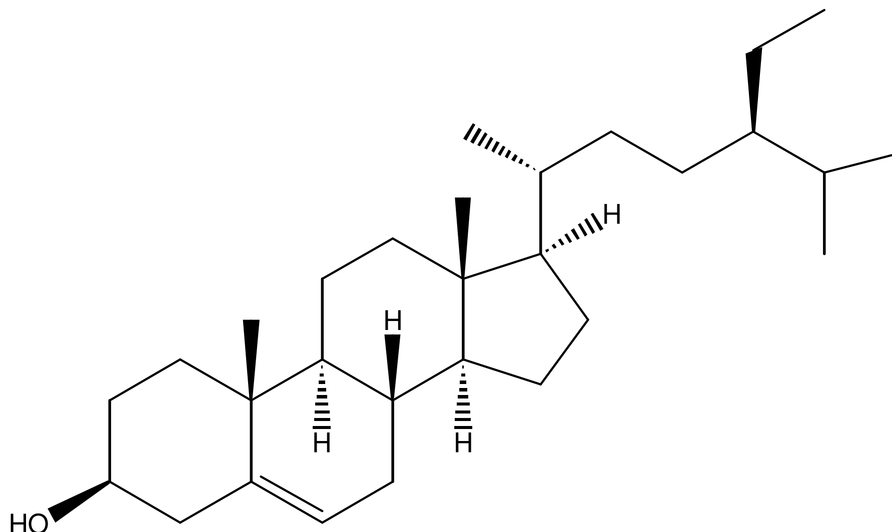

Figure 1. Structure of β-sitosterol (1).

and to characterize its constituents by Gas Chromatography-Mass Spectrometry (GC-MS) analysis and column chromatography.

EXPERIMENTAL

Chemicals

4-Nitro-o-phenylenediamine was purchased from Sisco-Chem Industries, Mumbai. Phorbol myristate acetate was purchased from Sigma Aldrich and Sabouraud's agar, for maintaining *Candida albicans*, was purchased from HiMedia. Silica gel 60-120 mesh was purchased from Merck India Ltd. Different solvents (analytical grade) and all other chemicals were purchased from Merck India Ltd. Mumbai.

Plant material

Plant material was collected from Mandi, Himachal Pradesh, India in June, 2009. The plant was identified from the herbarium of IHBT, Palampur, India where a voucher specimen (PLP-12994) has been deposited.

Preparation of *n*-hexane extract

The dried and powdered stem (1 kg) of the plant was extracted with (2.5 L) of *n*-hexane using a soxhlet extractor for 7 h at 60°C. The mixture was filtered using Whatman filter paper No.1 and dried under vacuum at 40°C using a rotary evaporator to give 3.2 g of extract.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis was carried out on a Shimadzu (QP 2010) series GC-MS (Tokyo, Japan) equipped with a FID, AOC-20i auto-sampler coupled, and a DB-5 capillary column, (30 m x 0.25 mm i.d., 0.25 µm). The initial temperature of column was 40°C held for 5 min and was programmed to 230°C at 4°C/min, then held for 5 min at 230°C; the sample injection volume was 2 µl in High Performance Liquid chromatography (HPLC) grade dichloromethane. Helium was used as carrier gas at a flow rate of 1.1 ml min^{-1} on split mode (1:50). A standard solution of *n*-alkanes (C_8-C_{23}) was used to obtain the retention indices. Individual components were identified by retention indices (RI) with those reported in literature and also by comparison of their mass spectra (MS) with NIST data base and Adams libraries (NIST/EPA/NIH, 1998; Adams, 2004).

Isolation of β-sitosterol from *n*-hexane extract

Hexane extract (2 g) was subjected to silica gel (60 - 120) column chromatography. Column was packed in *n*-hexane and eluted by increasing polarity with ethyl acetate. A single compound was obtained at polarity 5% ethyl acetate in *n*-hexane. Spectral analysis by using ^{1}H NMR and ^{13}C NMR and a melting point of 132 - 133°C showed that the compound is β-sitosterol (Figure 1).

Characterization of β-sitosterol

^{1}H NMR and ^{13}C NMR experiments were performed on Bruker Avance-300 spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from an internal standard. The melting point is uncorrected and was determined on a Barnstead Electrothermal 9100 capillary melting point apparatus.

β-sitosterol (1)

Mp 132-133°C
^{1}H NMR (300 MHz, $CDCl_3$): δ 5.37 (1H, *m*, H-6), δ 3.51 (1H, *m*, H-3), δ 1.03 (3H, *s*, Me-19), δ 0.92 (3H, *m*, Me-21), δ 0.88 (3H, *m*, Me-29), δ 0.88 (3H, *m*, Me-26), δ 0.83 (3H, *m*, Me-27), δ 0.71 (3H, *s*, Me-18).
^{13}C NMR (75 MHz, $CDCl_3$): δ 12.2 (C-29), 12.4 (C-18), 19.2 (C-21), 19.4 (C-27), 19.7 (C-19), 20.2 (C-26), 21.4 (C-11), 23.1 (C-28), 24.7 (C-15), 25.8 (C-23), 28.6 (C-16), 29.7 (C-25), 32.1 (C-7), 32.3 (C-2), 32.3 (C-8), 34.4 (C-22), 36.5 (C-20), 36.9 (C-10), 37.7 (C-1), 40.2 (C-12), 42.7 (C-4), 42.7 (C-13), 46.3 (C-24), 50.6 (C-9), 56.5 (C-17), 57.2 (C-14), 72.2 (C-3), 122.1 (C-6), 141.2 (C-5).

Immunomodulatory activity

Separation of polymorphonuclear leucocytes

Venous blood (10 ml) was collected from each volunteer, using aseptic condition in a sterile heparinised tube. The blood was subjected to Ficoll hypaque density gradient centrifugation for the separation of polymorphonuclear (PMN) leucocytes. The RBC-PMN pellet was separated and mixed with 1 ml each of autologous plasma and 5% dextran. The mixture was allowed to stand at 37°C for 1 h for sedimentation of RBCs. The supernatant layer, which contains more than 90% of PMN, was collected. Cell density (count/ml) of PMN was calculated using Neubaeur's chamber.

Viability studies

The viability of the PMN leucocytes was assessed using the Trypan blue dye exclusion test over extract concentrations ranging from 5 to 100 µg/ml. Appropriate controls were also assessed for effects on cell viability, viz., cell control, DMSO. All sets were assessed in duplicates. The concentrations of the *n*-hexane extract showing a viability of 90% and more were selected for the actual assay.

Preparation of C. albicans

C. albicans was used as the test organism for the phagocytosis assays. *C. albicans* was maintained on Sabouraud's agar. For the assay, 18 h old culture was washed using saline and the cells were counted using Neubaur's chamber. The count was adjusted to $1x10^6$ cells/ml using Minimum Essential Medium (MEM).

PMN function test

For the bioassay, the count of the isolated PMNs from individual volunteers was adjusted to 1×10^6 cells/ml using MEM. A method described by Lehrer and Cline (1969) as "specific cell assay" applying *C. albicans* for assessing phagocytic activity of neutrophil was used with few modifications. In brief, 1×10^6 neutrophils (in MEM) were suspended with 1×10^6 cells of *C. albicans* in absence or presence of varying concentrations of the samples. The concentrations tested were 0.1, 0.5, 1, 5, 10, 25 and 50 µg/ml of the samples. 5 µl of the test suspension prepared was added along with 245 µl of MEM to obtain the desired concentration of the test drug *in vitro*. As control, the PMN were incubated with MEM and the respective vehicle that was used to dissolve the test agents. Phorbol Myristate Acetate (PMA), a known immunostimulant, at three different concentrations viz., 10^{-4} M, 10^{-6} M and 10^{-8} M was used as standard. The tubes containing the systems were incubated at 37°C for 1 h in 5% CO_2. Cytosmers were prepared after the incubation. They were fixed with methanol, stained with Giemsa stain and observed under 100X oil immersion objective to determine the phagocytic activity of neutrophils. 100 neutrophils were scanned and the cells with ingested organisms and the numbers of organisms engulfed in each phagocytic neutrophil were counted.

Antimutagenic activity

Bacterial strain: Tester strain TA 98 was procured from IMTech (CSIR), Chandigarh, India. This strain has frame shift mutation in his D gene encoding histidinol dehydrogenase, an enzyme which is required for histidine biosynthesis. Therefore, bacteria with this mutation are unable to synthesize their own histidine. This is used as marker for antimutagenic studies.

Viability assays and determination of test concentrations: Different concentrations of extract were first tested for their toxicity against bacteria employing negative control (100 µl bacterial culture + 100 µl different concentrations of *n*-hexane extract) as proposed by Maron and Ames (1983). It was confirmed that there was normal growth of the background lawn, spontaneous colony numbers within the regular range and no significant reduction in cell survival. Thus, for the concentrations and conditions reported here, no toxicity or other adverse effects were observed.

Antimutagenicity test: The '*Salmonella* histidine point mutation assay' proposed by Maron and Ames (1983) was followed with little modifications to check the inhibitory activity of the extract. Two methodologies were followed, that is co-incubation and pre-incubation. In co-incubation method, 0.1 ml each of bacterial culture, mutagen and extract was added to 2 ml of top agar. In pre-incubation method, equal volume of mutagen and extract was mixed and allowed to stand at 37 °C for 30 min under continuous shaking. 0.2 ml of this was added to 2 ml of soft agar with 0.1 ml of fresh bacterial culture. After pouring the soft agar on minimal agar plates, the plates were tilted and rotated very quickly to ensure speedy and uniform spreading, and then incubated at 37°C for 48 h. The histidine revertant colonies were counted against a background lawn. To verify the toxicity of the test sample, parallel controls were run with extract alone at all concentrations tested with mutagens. The concentrations of extract used for antimutagenicity assay were 50, 100, 250, 500, 1000 and 2500 µg/0.1 ml. The test samples were tested against direct acting mutagen, NPD (20 µg/0.1 ml/plate). The inhibitory activity is expressed as percentage decrease of reverse mutation. The percent inhibition was calculated as follows:

$$\text{Percent inhibition} = [(x - y)\text{-}(x - z)] \times 100$$

Where 'x' is the number of histidine revertants induced by mutagen alone, 'y' the number of his+ revertants induced by mutagen in the presence of extract and 'z' is the number of revertants induced in negative control. The antimutagenic potency of extract was categorized as 'strong' (> 50% inhibition of mutagenic activity), 'moderate' (between 50 and 25% inhibition of mutagenic activity) and 'weak' (< 25% inhibition of mutagenic activity).

Statistical analysis:The results are presented as the average and standard error of three experiments with triplicate plates/dose experiment. The data were further analyzed for statistical significance using analysis of variance (ANOVA), and the difference among means was compared by high-range statistical domain using Tukey's test. A level of probability ≤ 0.05 was taken as level of statistical significance.

RESULTS AND DISCUSSION

Chemical composition of *n*-hexane extract

Total 14 constituents were characterized by GC-MS analysis (Table 1), where the major constituents were methyl-9,12-octadecadienoate (23.17%), methyl 9-octadecenoate (19.74%), methyl hexadecanoate (16.28%) and Methyl octadecanoate (5.52%). One major compound (19.56%), a derivative of amino-furanose sugar, was not fully characterized by GC-MS. To characterize this compound *n*-hexane extract was subjected to column chromatography. But column chromatography led to the isolation of one pure compound which is characterized as β-sitosterol (Figure 1) by comparing its ^1H and

Table 1. Chemical composition of *n*-hexane extract from *T. cordifolia* determined by GC-MS.

S/No.	Compounds name	(%)	RI	Mass fragments
1	Methyl Dodecanoate (Methyl laurate)	0.51	1423	214[M+], 183, 171, 143, 129, 115, 101, 87, 74, 69,55
2	Dimethyl nonanedioate (Dimethyl Azelate)	0.21	1445	216[M+], 185,156, 152, 128, 124, 111, 97, 87, 83, 74, 59, 55
3	Methyl tetradecanoate (Methyl myristate)	0.89	1623	242[M+], 211, 199, 157, 143, 129, 101, 87, 74, 55
4	Amino-furanose sugar derivative	19.56	1709	313[M+], 271, 259, 242, 211, 200, 193, 169, 156, 152, 139, 114, 96, 72, 60, 55
5	Methyl Pentadecanoate	0.80	1724	256[M+], 255, 213, 199, 157, 143, 129, 115, 101, 87, 74, 57, 55
6	Methyl-9-Hexadecenoate (Methyl palmitoleate)	0.24	1796	268[M+], 236, 194, 152, 115, 110, 98, 84, 74, 69, 55
7	Methyl Hexadecanoate (Methyl Palmitate)	16.28	1825	270[M+], 227, 199, 185, 171, 143, 129, 101, 87, 74, 57,55
8	Methyl-9,12-Heptadecadienoate	0.4	1890	280[M+], 248, 164, 150, 135, 123, 109, 95, 81, 67, 55
9	Methyl 9,10,-methylene hexadecanoate	0.52	1899	250[M+], 208, 166, 152, 138, 123, 97, 83, 74, 69, 55
10	Methyl Heptadecanoate	1.89	1924	284[M+], 253, 241, 199, 185, 143, 129, 101, 87, 74, 55
11	Hexadecanoic acid	2.4	1966	256[M+], 226, 212, 186, 172, 156, 128, 114, 96, 74, 57, 43
12	Pregnan-20-one, (5 α,7 α)	0.75	1972	302[M+], 287, 233, 209, 194, 175, 149, 121, 109, 95, 85, 71, 55
13	Methyl 9, 12 octadecadienoate	23.17	1991	294M+], 263, 164, 150, 136, 123, 109, 95, 81, 67, 55
14	Methyl 9-Octadecenoate	19.74	1998	296[M+], 264, 222, 180, 166, 152, 137, 123, 97, 83, 74, 69, 55
15	Methyl Octadecanoate (Methyl Stearate)	5.52	2023	298[M+], 255, 199, 185, 143, 129, 101, 87, 74, 55

^{13}C values with literature data (Moghaddam et al., 2007).

Immunomodulatory activity

In case of immunomodulatory activity the viability was affected at higher concentrations that is from 50 µg/ml onwards (Table 2). Hence, a concentration range of 1 to 50 µg/ml was selected for the test samples. Dose dependent decrease in the percent phagocytosis with the maximal effect at 0.1 µg/ml was observed which was not significant when compared to controls (Table 3). In case of phagocytic index, the maximal response was observed at 10 µg/ml.

Antimutagenic activity

The mutagenic effect of *n*-hexane extract was not observed when mutagenicity assays was performed with *Salmonella* histidine tester strain TA 98 at six different concentrations including 50, 100, 250, 500, 1000 and 2500 µg/0.1 ml which confirms that extract is genotoxically safe at tested concentrations. The possible anti-mutagenic potential of *n*-hexane extract was examined against 4-nitro-*o*-phenylenediamine (NPD) in *Salmonella* histidine tester strain TA 98 by using two methodologies, that is co-incubation and pre-incubation. The extract noticeably showed antimutagenic activity against the standard mutagen that is NPD, as evaluated by standard plate incorporation method (Table 4). A dose dependent increase in the antimutagenic activity was observed (Figure 2).

The maximum inhibitory effect was observed at highest concentration that is 88.0 and 80.9% at 2500 µg/ 0.1 ml in pre-incubation and co-incubation mode, respectively (Figure 2). The observation of higher anti-mutagenic

Table 2. *In vitro* effect of the *n*-hexane extract of *T. cordifolia* on viability of neutrophils.

Conc. (µg/ml)	Hexane extract
Control	100
C. DMSO (vehicle)	85.35 ± 4.99
5	100
10	100
25	100
50	90.9
75	85.91± 5.79
100	80

Table 3. *In-vitro* effect of *n*-hexane extract of *T. cordifolia* on phagocytic function of neutrophils (n = 6).

Conc. (µg/ml)	% Phagocytosis	Phagocytic index
Control	27.85 ± 1.46	1.59 ± 0.14
Vehicle control	25 ± 1.82	1.66 ± 0.12
PMA 10^{-8}	29.28 ± 1.38	1.72 ± 0.11
PMA 10^{-6}	33.42 ± 3.21	1.77 ± 0.12
PMA 10^{-4}	37.67 ± 3.01	1.83 ± 0.09
0.1	32.66 ± 7.57	1.61 ± 0.12
0.5	29 ± 2.64	1.7 ± 0.06
1	26.16 ± 4.11	1.66 ± 0.11
5	27.83 ± 3.37	1.84 ± 0.12
10	26.66 ±3.77	1.88 ± 0.18
25	28 ± 0.63	1.54 ± 0.29
50	23.16 ±2.71	1.57 ± 0.31

Values are expressed as Mean ± SD.

Table 4. Antimutagenic activity of *n*-hexane extract of *T. cordifolia*.

Treatment	Dose		
	µg/ 0.1 ml	Mean ± SE	% Inhibition
Spontaneous	-	36.66± 6.173	-
Positive control	-	1399.33±6.67	-
NPD	20	-	-
Negative control	0.05×10^3	38 ± 1.62	-
	0.1×10^3	24 ± 0.15	-
	0.25×10^3	30± 2.10	-
	0.5×10^3	29± 0.17	-
	1×10^3	37 ± 1.11	-
	2.5×10^3	40± 0.52	-
Co-incubation	0.05×10^3	1115±9	20.886
	0.1×10^3	1110±10	21.037
	0.25×10^3	1090 ± 4	22.589
	0.5×10^3	857 ± 35	39.576
	1×10^3	477 ± 15	67.702
	2.5×10^3	299± 2	80.946
Pre-incubation	0.05×10^3	1199 ± 12	14.715
	0.1×10^3	1109 ± 1	21.109
	0.25×10^3	936± 18	33.836
	0.5×10^3	357± 3	76.064
	1×10^3	262 ± 9	83.484
	2.5×10^3	203± 6	88.008

effect in pre-incubation mode indicated that the direct interaction of the mutagen with *n*-hexane extract which may lead to the increase in anti-mutagenic potential. The IC_{50} value was found to be 1033.98 µg/ 0.1 ml in co-incubation mode and 298.57 µg/ 0.1 ml in pre-incubation mode.

Despite the presence of several mutagenic risk factors which are closely connected with our modern lifestyle, the complete eradication of mutagen is not possible. However, the reduction in genotoxic effects of mutagenic factors by the regular intake of antimutagenic agents is rather a viable option. Therefore, the main aim of this study was to identify the antimutagenic compounds from *n*-hexane extract of *T. cordifolia*. The choice of appropriate experimental model for anti-mutagenic assessment has a strong impact on the extrapolation of results of laboratory experiments to the human situation (Knasmuller et al., 2002). In most cases, the required tests include a microbial assay such as Ames test which is a short time assay to screen mutagens and anti-mutagens.

The GC-MS analysis of *n*-hexane extract showed the dominancy of long chain fatty acids and their ester. These types of fatty acids are widely occurring in nature being found in all vegetables and animal matter. In earlier study, one of the fatty acid, palmitic acid was found responsible for the antimutagenicity against an established mutagen (Nadathur et al., 1996). Other saturated straight chain and methyl-branched fatty acids present in yogurt were also found to have anti-mutagenic activity. Iso-palmitic acid, a natural constituent of bovine milk, was also found to show anti-mutagenic activity about five times of palmitic acid (Nadathur et al., 1996). The anti-mutagenic activity of other fatty acid such as butyric, oleic, palmitic, palmitoleic acids and conjugated linoleic acids (CLA) was also shown (Hayatsu et al., 1981, 1983; Nadathur et al., 1995; Ha et al., 1987; Rabizadeh et al., 1993; Ip et al., 1994; Miller et al., 1995; Yang et al., 2002). β-sitosterol and its glycoside are also reported to

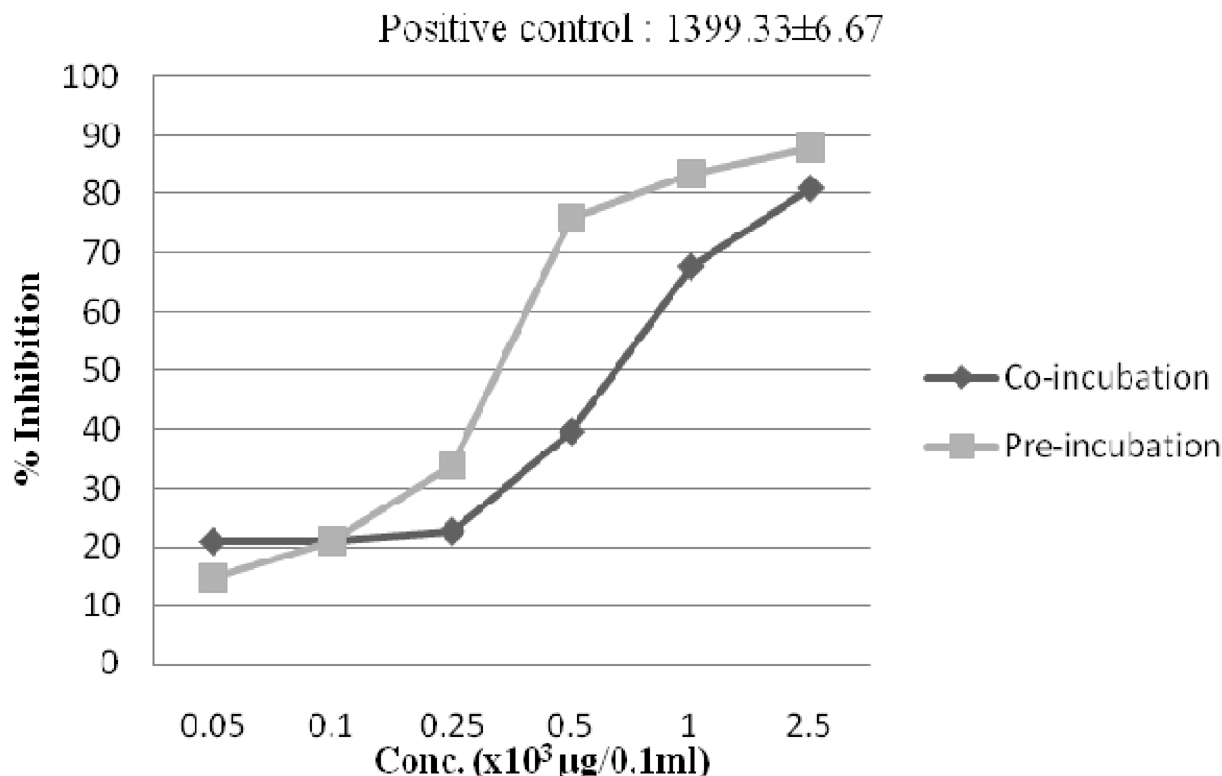

Figure 2. Effect of *n*-hexane extract on mutagenicity of NPD in *Salmonella* histidine tester strain TA 98.

have anti-mutagenic activity (Villasenor et al., 2002). Thus, on the basis of previous reports, it has been concluded that the β-sitosterol, fatty acids and their ester are mainly responsible for anti-mutagenic activity of *n*-hexane extract of *T. cordifolia*.

Conclusion

In conclusion, *n*-hexane extract of *T. cordifolia* can be considered genotoxically safe at tested concentration and can be a new source of antimutagenic fatty acids and their derivatives. These activities are valuable towards an extension of employing these drugs as new phyto-therapeutic, besides their consolidated ethnomedical use.

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