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Antioxidant Activity of Ethyl Acetate Extract/Fractions of *Terminalia chebula*

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ABSTRACT

In the present study, the Ames *Salmonella* histidine reversion assay was used to assess the antioxidant activity of the ethyl acetate extract/fractions of fruits of *Terminalia chebula* using hydroxyl radical sensitive, TA 102 strain of *Salmonella typhimurium* against hydrogen peroxide which is a directly acting oxidant. The production of reactive oxygen species by hydrogen peroxide leads to the induction of mutagenicity via oxidants. The ethyl acetate extract was prepared by the maceration method and further partitioned with ethyl acetate and water to get respective fractions. Both fractions were more effective than the crude ethyl acetate extract, which exhibited moderate inhibition of 57.98 and 55.92% during pre and co-incubation, respectively at the maximum tested dose of 2.5×10^3 µg/0.1 ml/plate. The water fraction also showed moderate inhibition at the same concentration i.e. 51.82 and 49.80% during pre and co-incubation, respectively while the crude ethyl acetate extract showed weak inhibition in general. Both, ethyl acetate and water fractions were rich in polyphenolics and were more effective than the crude extract with less phenolic.

Keywords: Ames assay, medicinal plants, natural remedies, TA 102

INTRODUCTION

The human body is continuously and unavoidably exposed to a plethora of structurally diverse chemicals with established carcinogenic activity in animal models and/or mutagenic activity in short-term tests (Maron and Ames 1983; Kriebel 2009). Reactive oxygen species are continuously produced in the human body and can give rise to mutagenic changes in DNA. Damage to DNA is likely to be one of the major causes of cancer and other degenerative diseases (Chen *et al.* 2009; Kim *et al.* 2009). The use of dietary antimutagens and anticarcinogens from plants sources like fruits and vegetables has been seen as a promising approach to the protection of human health. Recent research has underlined the chemopreventive activity of several secondary plant metabolites (Chen *et al*. 2009; Huang *et al*. 2010). Many investigators have mapped out specific compounds viz. polyphenols, triterpenoids or plant extracts with antimutagenic properties. Medicinal plants are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals (Shih *et al*. 2000; Ramos *et al*. 2003; Nicholas and Katiyar 2010).

Medicinal plants and their various extracts have been occasionally used in the treatment of many diseases. The rich diversity of medicinal plants has not yet systematically been screened for antimutagenic activities (Kaur *et al*. 2003). *Terminalia chebula*, an important medicinal plant, is distributed in the sub Himalayan tracks from Ravi to West Bengal, Assam and in all deciduous forests of India, specifically in Madhya Pradesh, Bihar, Assam and Maharashtra. Its fruits are extensively used as an adjuvant in medicines for various diseases with special reference to Ayurvedic preparations. The pericarp of the dried ripe fruit is used in the preparation of many Ayurvedic formulations for infectious diseases (Sharma and Dash 1998). *T. chebula* is reported to promote digestive power, wound healing, and is a curative of ulcers, local swelling, anemia, diabetes and chronic and recurrent fever. The fruits are astringent, purgative, laxative, gastroprotective and are used to alleviate asthma, piles and coughing (Chatterjee and Pakrasi 2000). The various extracts of *T. chebula* has been reported to show a broad spectrum antibacterial, antifungal, antimutagenic and antiviral activities (Malckzadeh *et al*. 2001; Suguna *et al*. 2002; Vonshak *et al*. 2003; Bonjar 2004; Walia *et al*. 2007, 2009).

MATERIALS AND METHODS

Extraction/fractionation procedure

The fruits of *T. chebula* were purchased from a local market and were authenticated by comparing them with the samples already available in the herbarium of the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar (Voucher No. 5980). The fruits were washed, dried and ground to a fine powder. To 500 g of fruit powder, 1000 ml of ethyl acetate was added. The supernatant was collected and filtered by using Whatman no. 1 filter paper and evaporated in a rotary evaporator (Rotavapor® R-210/R-215) to get the dry crude ethyl acetate extract (CE).

The CE was redissolved in methanol and after some time precipitates (PP) formed. The PP was separated from the supernatant (SP) and both were dried separately at room temperature. The dried SP was further redissolved first in water and then in ethyl acetate, partitioned into an ethyl acetate fraction (EAF) and a water fraction (WF). Both fractions were separated in separation funnel and dried at room temperature (**Fig. 1**).

Determination of total phenolic content

The total phenolic content of the CE and fractions was determined by using the Folin-Ciocalteu method (Yu *et al*. 2002). To 100 µl of CE and fractions, 900 µl of water and 500 µl of Folin-Ciocalteu reagent were added. This was followed by the addition of 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and allowed to stand for 2 hrs. The volume of mixture was made up to 10 ml with distilled water and absorbance was read at 765 nm (Systronic 2202). The phenolic content was calculated as gallic acid (mg/g) equivalents.

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Research Note

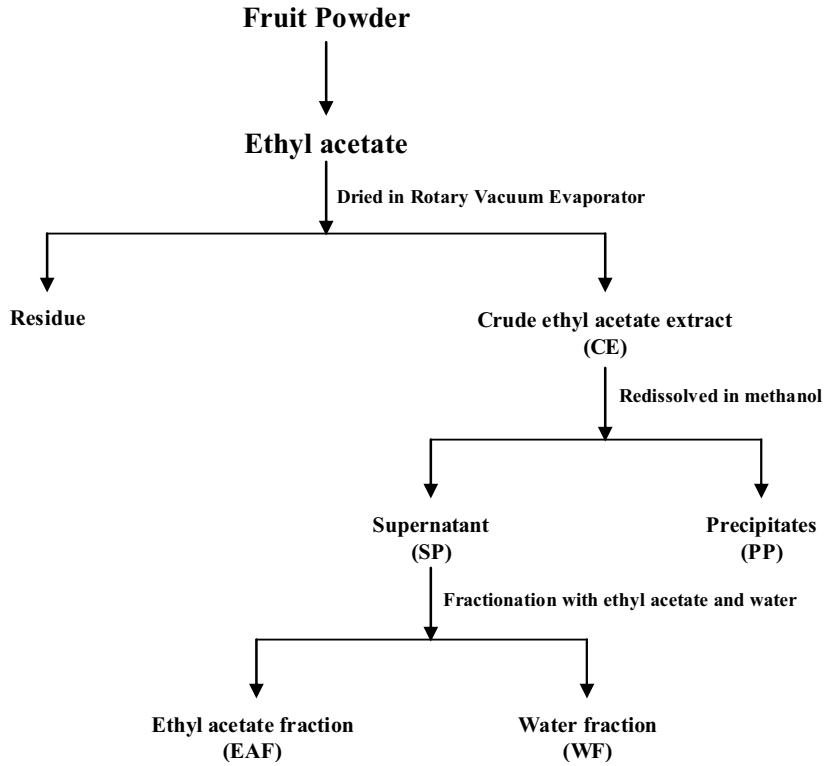

Fig. 1 Extraction/fractionation procedure.

Antioxidant testing

The ethyl acetate extract/fractions were tested for their antioxidant potential by using the Ames assay (Maron and Ames 1983). Two sets of experiments were designed i.e. co-incubation and pre-incubation mode as explained below.

Co-incubation

In the co-incubation method, 0.1 ml of freshly grown bacterial culture (1-2 × 10^9 cells/ml), 0.1 ml of direct-acting oxidant i.e. hydrogen peroxide (H_2O_2; 45 µg/0.1 ml) and 0.1 ml of non-toxic concentrations of CE and fractions were added into test tubes containing 2 ml of soft agar, which was then poured onto minimal agar plates. The poured soft agar was allowed to solidify and after that the plates were placed in incubator in an inverted position at 37°C for 72 hrs. The reverted colonies were counted.

Pre-incubation

For pre-incubation, a mixture of ethyl acetate extract/fractions of the desired concentrations and oxidant was pre-incubated at 37°C for 30 min prior pouring into Petri dishes. After incubation 2 ml of molten agar and 0.1 ml of bacterial culture was added. The contents were mixed and poured onto minimal agar plates and evenly spread on the minimal plate by revolving the Petri dish. The soft agar was allowed to solidify and after that the plates were placed in incubator in inverted position at 37°C for 72 hrs. The experiment was conducted in triplicates to estimate the variation in the number of revertants. The number of reverted colonies was counted.

The toxicity of test samples was also verified. For this, the different concentration of ethyl acetate extract/fractions in DMSO was added in the test tubes containing 0.1 ml of bacterial culture and 2 ml of soft agar supplemented with histidine. The mixture was poured onto minimal agar plates, which were then incubated at 37°C for 72 hrs. The numbers of revertants colonies were counted. The mean number of colonies per plate (triplicate) at each concentration of the extract was compared. Non-toxic concentrations were considered to be those when the number of spontaneous revertant colonies, size of the colonies and the intensity of background lawn were found to be similar to the control where no extract/fraction is added.

Calculation of antioxidant activity

The antioxidant activity was calculated as percent inhibition decrease of reverse mutation (Kaur *et al.* 2003).

Percent inhibition = [(x-y)/(x-z)] x 100

where x is number of histidine revertants induced by oxidant alone; y is number of histidine revertants induced by oxidant in the presence of ethyl acetate extract/fraction; z is the number of revertants induced in negative control.

RESULTS AND DISCUSSION

The tester strain TA 102 of *S. typhimurium* has a different specificity from other Salmonella strains routinely used in mutagenicity screening. This strain can detect a variety of oxidative mutagens including H_2O_2 and other hydroperoxides that react preferentially with AT base pairs (Levin *et al*. 1982). TA 102 contains ochre mutations in the gene *hisG428* and DNA repair system is intact in this. It also contains a deep rough mutation increasing the permeability of the cells to large molecules and contains plasmid pKM101 and pAQ1 that approximately equal to 30 copies of the mutant gene are available for back mutation. Oxidative damage to DNA is mutagenic and thus considered to play a role in carcinogenesis (Halliwell and Arouma 1991; Gilbert *et al*. 2005). In the present study it was observed that at the dose of 45 µg/plate of H_2O_2 was found to be mutagenic. According to the literature, the spontaneous frequency of revertants in TA102 is about 240-320 revertants/plate. The results of the present study confirm those of previous reports on the antioxidant potential of various medicinal plants checked by Ames *Salmonella* TA 102 strain (Boubaker *et al.* 2010; Mošovská *et al.* 2010; Zahin *et al.* 2010).

Ethyl acetate extract/fractions depicted moderate, albeit a dose-dependent responses as it reduced the number of his^+ revertants in both the co-incubation and pre-incubation mode of testing (**Table 1**). The non-toxic concentration evaluated was 2.5 × 10^3 µg/0.1 ml. EAF and WF were more effective than CE. The antioxidant effect of CE was 37.19% and 37.96 at 2.5 × 10^3 µg/0.1 ml of dose in co-incubation

Table 1 Effect of ethyl acetate extract/fractions of *Terminalia chebula* on the mutagenicity of H_2O_2 in Ames assay.

Treatment	Dose	CE		SP		PP	
	(μg/0.1ml)	His^+revertants/Plate Mean ± S.E.	% Inhibition	His^+revertants/Plate Mean ± S.E.	% Inhibition	His^+revertants/Plate Mean ± S.E.	% Inhibition
Spontaneous	-	0325.9 ± 01.76	-	0325.9 ± 01.76	-	0325.9 ± 01.76	-
H_2O_2	45	1398.8 ± 56.28	-	1398.8 ± 56.28	-	1398.8 ± 56.28	-
Co-incubation	0.01×10^3	1317.5 ± 40.02	07.56	1301.2 ± 55.54	09.02	1320.0 ± 61.58	07.23
	0.10×10^3	1213.2 ± 56.32	17.23	1160.3 ± 56.93	22.04	1193.1 ± 74.16	19.06
	0.25×10^3	1103.9 ± 43.81	27.27	1059.3 ± 69.82	31.48	1084.2 ± 88.54	29.19
	0.50×10^3	1059.3 ± 50.11	31.54	0979.6 ± 76.67	38.93	1019.2 ± 61.35	35.28
	1.00×10^3	1000.6 ± 32.69	37.04	0930.5 ± 78.99	43.56	0969.5 ± 92.87	40.20
	2.50×10^3	0998.1 ± 85.65	37.19	0926.6 ± 64.21	43.92	0965.9 ± 66.24	40.25
Pre-incubation	0.01×10^3	1309.5 ± 25.98	08.31	1263.4 ± 65.23	12.52	1288.2 ± 45.85	10.20
	0.10×10^3	1204.3 ± 17.25	18.06	1138.6 ± 59.23	24.04	1157.3 ± 39.96	22.38
	0.25×10^3	1097.3 ± 20.96	27.88	1021.3 ± 71.45	35.00	1074.2 ± 55.45	30.12
	0.50×10^3	1042.6 ± 29.85	33.09	0956.5 ± 81.23	41.08	1002.7 ± 87.52	36.81
	1.00×10^3	0993.3 ± 27.65	37.72	0910.5 ± 55.98	45.42	0944.3 ± 82.36	42.25
	2.50×10^3	0989.8 ± 36.96	37.96	0908.3 ± 45.58	45.64	0940.2 ± 70.23	42.55

Treatment	Dose	WF		EAF	
	(μg/0.1ml)	His^+revertants/Plate Mean ± S.E.	% Inhibition	His^+revertants/Plate Mean ± S.E.	% Inhibition
Spontaneous	-	0325.9 ± 01.76	-	0325.9 ± 01.76	-
H_2O_2	45	1398.8 ± 56.28	-	1398.8 ± 56.28	-
Co-incubation	0.01×10^3	1270.4 ± 56.32	11.93	1256.3 ± 44.12	13.21
	0.10×10^3	1179.5 ± 22.96	20.41	1145.1 ± 31.74	23.46
	0.25×10^3	0972.7 ± 34.52	39.61	0980.3 ± 30.21	38.78
	0.50×10^3	0933.3 ± 74.32	43.05	0870.2 ± 17.56	49.23
	1.00×10^3	0867.1 ± 88.75	49.32	0803.3 ± 25.54	55.45
	2.50×10^3	0861.3 ± 96.35	49.80	0798.2 ± 22.87	55.92
Pre-incubation	0.01×10^3	1241.4 ± 54.87	14.62	1232.7 ± 31.48	15.40
	0.10×10^3	1129.2 ± 65.32	25.09	1109.3 ± 40.12	26.78
	0.25×10^3	0931.5 ± 54.12	43.44	0965.8 ± 52.32	40.13
	0.50×10^3	0898.7 ± 60.21	46.25	0837.2 ± 49.85	52.30
	1.00×10^3	0842.1 ± 71.25	51.64	0780.8 ± 50.32	57.54
	2.50×10^3	0839.6 ± 78.88	51.82	0775.9 ± 55.23	57.98

and pre-incubation respectively (**Table 1**). The antioxidant effect of CE increased upon fractionation, which implies that the fractions can strongly arrest the oxidation of oxidant. The EAF showed a modest inhibition of 57.98 and 55.92% in the pre-incubation and co-incubation, respectively at the dose of 2.5×10^3 μg/0.1 ml/plate (**Table 1**). There is no significant difference in the activities as obtained in the co-incubation and pre-incubation studies. The increasing order of reduction in the number of his^+ revertants induced by H_2O_2 is: EAF > WF > SP > PP > CE with the values of 57.98, 51.82, 45.64, 42.55 and 37.96%, respectively in the pre-incubation mode (**Figs. 2A-E**). The co-incubation mode of experimentation followed the similar inhibition pattern but the values were slightly less than pre-incubation. This indicates that the pre-incubation of ethyl acetate extract/fractions with H_2O_2 prior to plating does not cause any significant increase in inhibition.

The amount of total phenolics in ethyl acetate extract/fractions ranged from 168-235 mg/g of gallic acid. The total phenolic content was maximum in EAF i.e. 235 mg/g of gallic acid, which signify its highest antioxidant activity. The fractionation of CE also increases the phenolic content. The order of total phenolic content was EAF (235 mg/g) > WF (214 mg/g) > SP (207 mg/g) > PP (179 mg/g) > CE (168 mg/g). Phenolics are chemical compounds characterized by the presence of at least one aromatic ring (C_6) with one or more hydroxyl groups (Sakihama *et al*. 2002). These compounds tend to be potent free radical scavengers and their abilities to act as antioxidants depend on their chemical structure, capability to donate/accept electrons, thus delocalizing the unpaired electron within the aromatic structure. The ethyl acetate extract/fractions are rich in phenolic compounds so, it is presumed that these phenolic compounds are responsible for their activity. Nikaidou *et al*. (2005) reported the radical scavenging effect of catechins which is major polyphenolic component of green tea using TA102 strain of *S. typhimurium*. Catechin significantly reduced mutagenesis or genotoxicity caused by hydroxyl radical. This radical-scavenging action of catechins may indeed contribute to the anticarcinogenic activity of green tea as has been proposed (Unachukwu *et al*. 2010). So, it can presume that phenolic compounds in extract/fraction might be responsible for antioxidant activity.

CONCLUSION

Keeping the results of the present study in view, it is concluded that antioxidant activity of the ethyl acetate extract/fractions may be due to the enrichment with phenols and polyphenols. The partitioning of the crude extract into the fractions leads to enhancing the antioxidant activity of the extract. The studies to isolate the active principles and other biological activities of the extracts/fractions are on the way.

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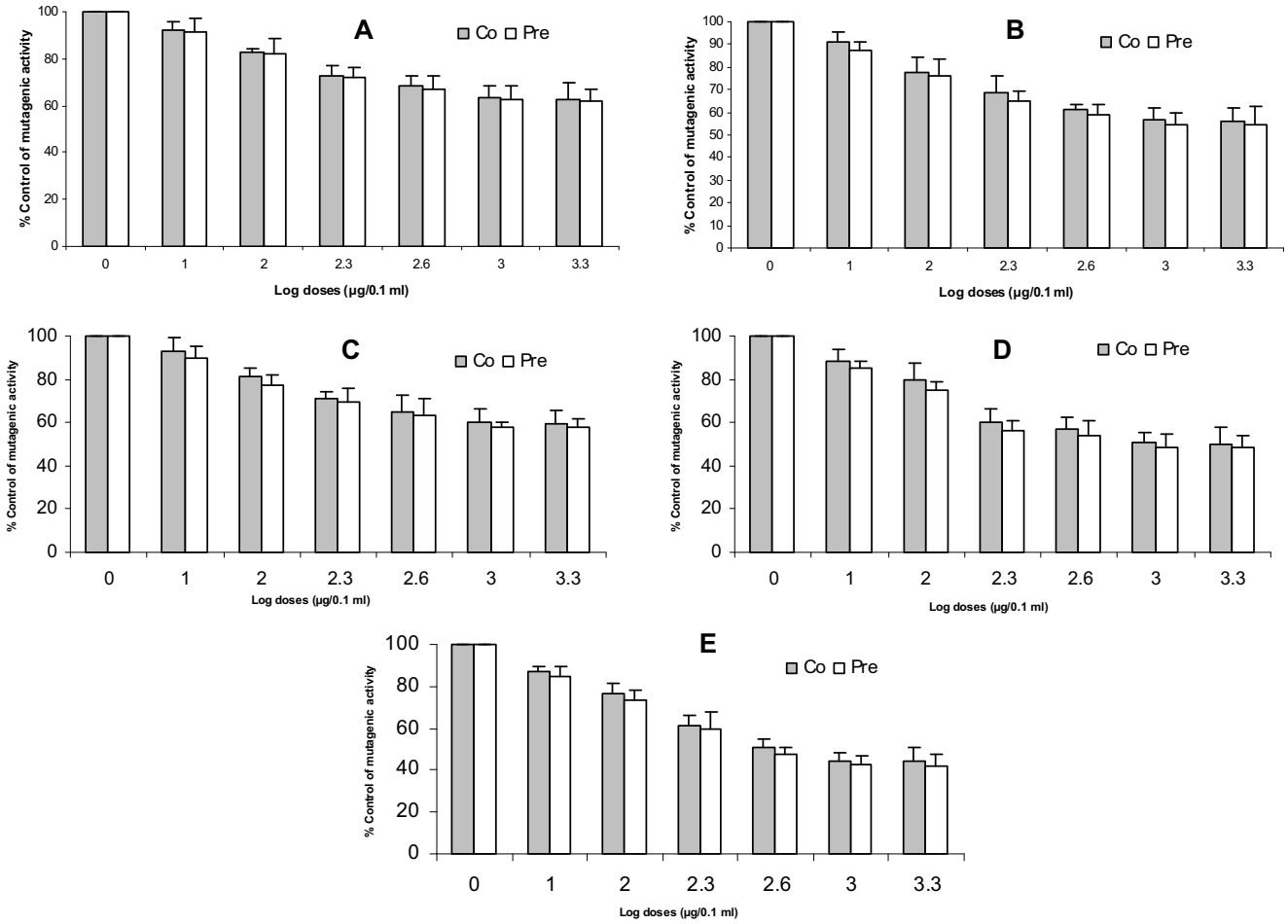

Fig. 2 Effect of crude ethyl acetate extract (**A**), supernatant (**B**), precipitates (**C**), water fraction (**D**) and ethyl acetate fraction (**E**) of *Terminalia chebula* in the Ames assay.

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