

# Delineation of Antioxidant Activity of Acetone Extract/ Fractions of Fruits of *Terminalia chebula* Using TA 102 Strain of *Salmonella typhimurium*

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## ABSTRACT

Antimutagenic or antioxidant properties elicited by plant species have many prospective applications in human healthcare. The fruits of *Terminalia chebula* are a rich source of tannins and other bioactive constituents. In Ayurveda, the plant is known as the “wonder healer” due to its extraordinary power of healing. In the present study, the Ames *Salmonella* histidine reversion assay was used to assess the antioxidant activity of the acetone extract of *T. chebula* fruits in the TA 102 strain of *Salmonella typhimurium* using hydrogen peroxide ( $H_2O_2$ ) as a direct acting oxidant. The acetone extract was prepared by maceration and further fractionated with ethyl acetate and water. The antioxidant activity of ethyl acetate and water fractions was comparatively higher than that of the crude extract. The ethyl acetate fraction inhibited reduction of  $H_2O_2$  by 72.48 and 72.83% in co and pre-incubation modes, respectively although there was no difference in co- and pre-incubation modes of experimentation.

**Keywords:** Ames assay, chemoprevention, free radical, herbal remedies, hydrogen peroxide

**Abbreviations:** CE, crude acetone extract; EAF, ethyl acetate fraction; PP, precipitate; SP, supernatant; WF, water fraction

## INTRODUCTION

Cancer is still a pressing and ongoing health issue all over the world, yet it is still a major cause of mortality and morbidity in developing as well as in developed countries (Burger *et al.* 2012). Overall survival rate has only improved slightly despite advances in surgery, radiotherapy and chemotherapy. Molecular targeted agents are currently being studied in all treatment settings including that of chemoprevention (Shukla and Pal 2004; Weng and Yen 2012). Much development has been made in this field until now but much needs to be done for complete eradication of this disease (Baliga 2011). One of the convincing studies showed that free radicals has major role in carcinogenesis (Klaunig and Kamendulis 2004; Trushina and McMurray 2007; Sindhu *et al.* 2010). Therefore Bioprospection of plants with antioxidant activity has been a major focus in the search for plant-based cures. Overwhelming scientific data assure that herbal remedies and phytotherapy drugs containing active principles are currently developed to protect against electrophile e.g. free radical attack to DNA and its widespread outcomes such as ageing and cancer. Many medicinal plants are reported to treat the ailments, yet undoubtedly the plant kingdom still holds many species of plants containing substances of medicinal value which have yet to be discovered.

*Terminalia chebula* is a legendary plant used for treatment of wound healing in ayurveda. *T. chebula* a native plant in India and Southeast Asia is extensively cultivated in Taiwan. Since, it is rich in polyphenolic compounds and such compounds exhibit various biological activities, it can be suggested that this plant may play a role in the prevention of cancer and other age related diseases (Kaur *et al.* 2002; Srivastava *et al.* 2012). This plant is commonly known as “Harar” in ayurveda and has power of protection against various degenerative diseases. Its principle constituents are chebulagic, chebulinic acid and corilagin (Juang

*et al.* 2004; Manosroi *et al.* 2010). Its fruits have laxative, stomachic, tonic and alterative properties (Jagtap and Karkera 1999). It is also known as an adaptogen and hepatoprotective drug (Tasduq *et al.* 2006). The plant has been studied for its antimicrobial, antiviral, antifungal and antimutagenic activities (Malekzadeh *et al.* 2001; Jeong *et al.* 2002; Kaur *et al.* 2002; Sabu and Kuttan 2002; Saleem *et al.* 2002; Chen *et al.* 2003; Vonshak *et al.* 2003). Keeping in view the immense medicinal importance of polyphenolic compounds, the present study was intended to scrutinize the antioxidant activity of acetone extract/fractions of fruits of *T. chebula* by employing the Ames assay.

## MATERIALS AND METHODS

### Extraction/fractionation procedure

The fruits of *T. chebula* were purchased locally from the market and were authenticated by comparing them with the samples already available in the herbarium of Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar (Voucher No. 5980). It was washed with tap water, dried in oven at 40°C and ground to a fine powder. To 1000 g of fruit powder 1500 ml of acetone was added. The supernatant was collected, filtered by using Whatman paper no. 1 and evaporated through rotary evaporator to have the dry crude acetone extract. This dry crude acetone extract was further fractionated with ethyl acetate and water. For the fractionation, the crude acetone extract (CE) was redissolved in acetone and after some time the precipitates were formed. The precipitates (PP) and supernatant (SP) were separated and dried at room temperature separately. The dried supernatant (SP) was dissolved first in water and then in ethyl acetate, resulted in formation of two layers namely ethyl acetate fraction (EAF) and water fraction (WF). These layers were separated and dried at room temperature separately. The process is outlined in Fig. 1.

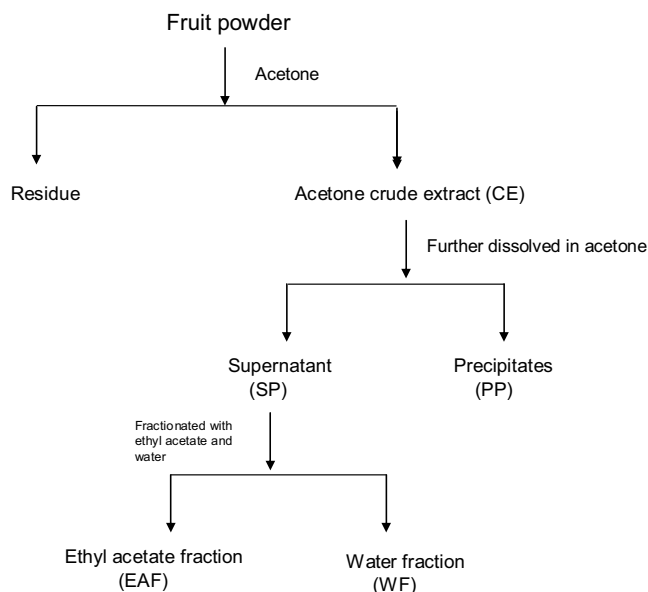


Fig. 1 Extraction/fractionation procedure.

### Determination of total phenolic content

The total phenolic content of the extract was determined using Folin-Ciocalteu (FC) method (Yu *et al.* 2002). To 100  $\mu$ l of extract/fraction, 900  $\mu$ l of water was added. To this, 500  $\mu$ l of Folin-Ciocalteu reagent was 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 h. The volume of mixture was made up to 10 ml with distilled water and absorbance was observed at 765 nm. The phenolic content was calculated as gallic acid (mg/g) equivalents.

### Antioxidant testing

The acetone crude extract/fractions were tested for their antioxidant potential by using Ames assay with little modifications (Maron and Ames 1983). In the present study two sets of experiments were designed i.e. co-incubation and pre-incubation.

#### 1. Co-incubation

In co-incubation method, 0.1ml of freshly grown bacterial culture ( $1-2 \times 10^9$  cells/ml), 0.1 ml of direct-acting oxidant i.e.  $H_2O_2$  (45  $\mu$ g/0.1 ml) and 0.1 ml of non-toxic concentrations of acetone extract/fractions were added into the sterile 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 inverted position at 37°C for 72 h and then the reverted colonies were counted.

#### 2. Pre-incubation

For pre-incubation, a mixture of acetone extract/fractions of the desired concentrations and oxidant was pre-incubated at 37°C for 30 min prior to pouring into Petri dishes. After incubation 2 ml of molten agar and 0.1 ml of culture was added in this. The contents were mixed and poured onto minimal agar plates. The top agar poured onto minimal agar plates was evenly spread on the minimal plate by revolving and tilting 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 h and the number of reverted colonies were counted. The experiment was conducted in triplicates to estimate the variation in the number of revertants.

The toxicity of test samples was verified. For this, the different concentration of acetone extract/fractions in DMSO (dimethyl sulfoxide) 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 h. The numbers of revertants colo-

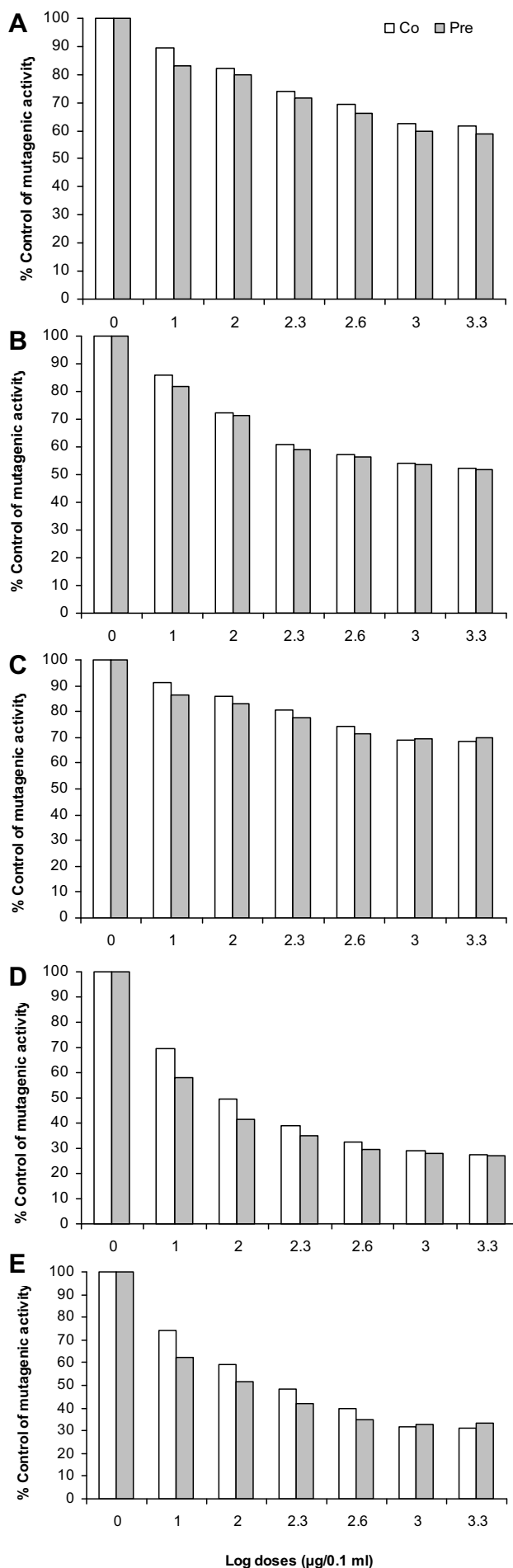


Fig. 2 Effect of (A) crude acetone extract, (B) supernatant, (C) precipitates, (D) water fraction and (E) ethyl acetate fraction of *Terminalia chebula* in Ames assay. Co = co-incubation; Pre = pre-incubation

**Table 1** Effect of acetone extract/fractions of *T. chebula* on the mutagenicity of H<sub>2</sub>O<sub>2</sub> in TA 102 tester strain of *S. typhimurium*.

Treatment	Dose (µg/0.1 ml)	CE		SP		PP	
		His <sup>+</sup> revertants/Plate Mean ± S.E.	% Inhibition	His <sup>+</sup> revertants/Plate Mean ± S.E.	% Inhibition	His <sup>+</sup> revertants/Plate Mean ± S.E.	% Inhibition
Spontaneous		283.9 ± 1.91	-	283.9 ± 1.91	-	283.9 ± 1.91	-
H <sub>2</sub> O <sub>2</sub>	45	1429.1 ± 51.22	-	1429.1 ± 51.22	-	1429.1 ± 51.22	-
Co-incubation	0.01 × 10 <sup>3</sup>	1309.2 ± 71.24	10.48	1269.5 ± 91.25	13.88	1330.2 ± 70.00	8.65
	0.10 × 10 <sup>3</sup>	1227.6 ± 68.31	17.64	1111.5 ± 71.45	27.70	1270.5 ± 66.24	13.90
	0.25 × 10 <sup>3</sup>	1130.9 ± 44.23	25.98	983.8 ± 84.71	38.99	1210.6 ± 57.12	19.18
	0.50 × 10 <sup>3</sup>	1077.5 ± 54.21	30.71	938.5 ± 69.23	42.84	1132.9 ± 67.78	25.94
	1.00 × 10 <sup>3</sup>	999.4 ± 36.85	37.50	906.4 ± 58.21	45.69	1074.2 ± 60.15	31.10
Pre-incubation	2.50 × 10 <sup>3</sup>	990.5 ± 47.48	38.47	879.5 ± 48.54	47.84	1065.9 ± 85.40	31.61
	0.01 × 10 <sup>3</sup>	1237.6 ± 29.85	16.74	1220.6 ± 38.16	18.14	1271.1 ± 22.23	13.82
	0.10 × 10 <sup>3</sup>	1198.9 ± 32.15	20.16	1102.3 ± 49.02	28.51	1235.1 ± 15.96	17.00
	0.25 × 10 <sup>3</sup>	1103.7 ± 41.26	28.35	964.5 ± 35.71	40.69	1176.7 ± 29.32	22.16
	0.50 × 10 <sup>3</sup>	1040.1 ± 50.23	33.97	930.7 ± 38.13	43.52	1099.8 ± 42.23	28.84
	1.00 × 10 <sup>3</sup>	969.9 ± 49.21	40.08	899.9 ± 43.66	46.25	1080.5 ± 18.99	30.47
	2.50 × 10 <sup>3</sup>	960.5 ± 63.87	41.10	875.1 ± 51.22	48.23	1085.2 ± 21.56	29.93

Treatment	Dose (µg/0.1 ml)	WF		EAF	
		His <sup>+</sup> revertants/Plate Mean ± S.E.	% Inhibition	His <sup>+</sup> revertants/Plate Mean ± S.E.	% Inhibition
Spontaneous		283.9 ± 1.91	-	283.9 ± 1.91	-
H <sub>2</sub> O <sub>2</sub>	45	1429.1 ± 51.22	-	1429.1 ± 51.22	-
Co-incubation	0.01 × 10 <sup>3</sup>	1131.4 ± 14.65	25.91	1074.3 ± 19.63	30.74
	0.10 × 10 <sup>3</sup>	961.1 ± 12.85	40.88	849.2 ± 15.26	50.42
	0.25 × 10 <sup>3</sup>	841.3 ± 21.85	51.48	726.3 ± 11.26	61.18
	0.50 × 10 <sup>3</sup>	737.9 ± 17.26	60.47	655.3 ± 10.32	67.47
	1.00 × 10 <sup>3</sup>	645.1 ± 11.02	68.47	611.1 ± 28.54	71.24
Pre-incubation	2.50 × 10 <sup>3</sup>	639.7 ± 6.58	69.02	601.5 ± 29.99	72.48
	0.01 × 10 <sup>3</sup>	996.9 ± 19.63	37.61	941.6 ± 19.55	42.21
	0.10 × 10 <sup>3</sup>	872.6 ± 45.63	48.61	757.7 ± 28.47	58.38
	0.25 × 10 <sup>3</sup>	763.7 ± 50.12	58.28	680.7 ± 12.98	65.15
	0.50 × 10 <sup>3</sup>	687.9 ± 28.26	64.85	620.7 ± 17.54	70.49
	1.00 × 10 <sup>3</sup>	659.2 ± 27.40	67.24	602.3 ± 45.62	72.03
	2.50 × 10 <sup>3</sup>	666.1 ± 30.30	66.71	597.5 ± 36.36	72.83

Data shown is Mean ± SE of triplicates.

CE: crude extract; SP: supernatant; PP: precipitates; WF: water fraction; EAF: ethyl acetate fraction

nies 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.

### 3. Calculation of antioxidant activity

The antioxidant activity was calculated as percent inhibition decrease of reverse mutation.

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

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

## RESULTS

Hydrogen peroxide was shown to be mutagenic in different strains of *S. typhimurium* in the Ames test (Shakra and Zeiger 1990). It readily diffuses into bacterial cells and induces oxidative DNA damage (Storz and Toledano 1994; Asad *et al.* 1997). Oxidative damage to DNA is mutagenic and thus considered to play a role in carcinogenesis (Halliwell and Aruoma 1991; Gilbert 2005). In the present study Ames tester strain TA 102 was examined for H<sub>2</sub>O<sub>2</sub> induced oxidation. The TA 102 strain has a unique sensitivity for reversion by chemical oxidants (Levin *et al.* 1982). It was observed that at the concentration of 45 µg/plate hydrogen peroxide was found to be mutagenic, which was further used for the investigation of the antioxidant activity of acetone extract/fractions. The antioxidant potential was cate-

gorized as "Very strong" (75% antioxidant potential), "Strong" (>50% antioxidant activity), "Moderate" (between 25% and 50% antioxidant activity) and "Weak" (<25% antioxidant activity) (Hour 1999).

**Table 1** and **Fig. 2** exhibited the antioxidant efficacy of acetone extract/fractions against the oxidative effect of H<sub>2</sub>O<sub>2</sub>. There is a gradual increase in inhibition with the increasing concentration and the maximum non-toxic dose evaluated was 2.5 × 10<sup>3</sup> µg/0.1 ml. The acetone extract/fractions showed "strong" to "moderate" effect against direct acting mutagen i.e. H<sub>2</sub>O<sub>2</sub>. It is clear from the results that there was no remarkable difference in co and pre-incubation approach. The maximum suppression of oxidation was observed with ethyl acetate fraction (EAF) which was 72.48% and 72.83% in co-incubation and pre-incubation modes respectively (**Fig. 2E**). The inhibitory effect of crude acetone extract (CE) was observed to be 38.4% and 41.1% at 2.5 × 10<sup>3</sup> µg/0.1 ml/plate in co and pre-incubation mode of experimentation (**Fig. 1**). The results clearly showed that the inhibitory activity increases with the fractionation of crude extract. The precipitates (PP) showed the lowest activity of 29.93% in pre-incubation method at maximum dose tested (**Fig. 2C**).

## DISCUSSION

From the results it is clearly indicated that the acetone extract/fractions showed 'moderate' to 'strong' suppression of oxidation in all the cases. The crude extract showed less activity as compared to water and ethyl acetate fractions. It is assumed that the polyphenols present in acetone extract/fractions confirmed by determining total phenolic content can be the foremost cause of its activity. A direct linear relationship between the total phenolic content and total antioxidant activity of fruit extract was observed in pub-

lished literature, indicating that the phenolic compounds might be the major contributors to the antioxidant activities (Banerjee *et al.* 2005; Rudnicki *et al.* 2007). Phenolics are chemical compounds characterized by the presence of at least one aromatic ring (C<sub>6</sub>) 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 acetone extract/fractions are rich in phenolic compounds so it is presumed that these phenolic compounds are responsible for their activity. The total phenolic content was determined by using Folin-Ciocalteu method and it was observed that the acetone extract/fractions are rich in them. The amount of total phenolics in extract/fractions ranged from 340-780 mg/g of gallic acid. The total phenolic content was maximum in ethyl acetate fraction (EAF) i.e. 780mg/g of gallic acid, which signify its highest antioxidant activity. The order of total phenolic content was EAF (780 mg/g) > WF (647 mg/g) > SP (557 mg/g) > CE (437 mg/g) > PP (340 mg/g). The results are consistent with those of Lira and co-workers who investigated the antimutagenic potential of methanol and chloroform extracts of *Byrsonima basiloba* using TA102 of *Salmonella typhimurium* against direct and indirect mutagenic agents viz. hydrogen peroxide and mitomycin C (Lira *et al.* 2008). Both the extracts showed antimutagenic activity, but the highest value of inhibition (89%) was obtained with the MeOH extract. Phytochemical analysis of the extracts revealed the presence of *n*-alkanes, lupool, ursolic and oleanolic acid, (+)-catechin, quercetin-3-*O*- $\alpha$ -L-arabinopyranoside, gallic acid, methyl gallate, amentoflavone, quercetin, quercetin-3-*O*-(2"-*O*-galloyl)- $\beta$ -D-galactopyranoside and quercetin-3-*O*-(2"-*O*-galloyl)- $\alpha$ -L-arabinopyranoside which are known phenolic compounds. Likewise, Lakshmi *et al.* (2006) and co-workers evaluated the antimutagenic activity of the methanolic extract of the fruiting bodies of *Ganoderma lucidum*. The activity was assayed by Ames test using histidine mutants of *Salmonella typhimurium* TA102 strain. The methanolic extract of the mushroom significantly inhibited ( $P < 0.001$ ) the *in vitro* benzo[a]pyrene induced his (+) revertants in a dose-dependent manner. Similarly, Nikaidou and coworkers reported the effect of catechins and caffeine from green tea on mutagenesis elicited by *tert*-butyl hydroperoxide that is sensitive to hydroxyl radicals in TA102 strain of *Salmonella typhimurium*. The results revealed that the caffeine significantly reduced the mutagenesis caused by hydroxyl radicals (Nikaidou *et al.* 2005). Keeping in view a number of reports, it can be concluded that antioxidant activity of the acetone extract/fractions in the present work may be due to phenolic compounds. However, the study is underway to identify the compounds responsible for their activity. Boubaker *et al.* (2010) reported antioxidant activity of four leaf extract viz. methanol, ethyl acetate, chloroform and hexane of *Nitraria retus* by using *Salmonella typhimurium* TA102 and TA 104 strains. The highest protection against methylmethanesulfonate induced mutagenicity was observed with chloroform and methanol extracts with 44.93% in TA102 strain and 38% in TA104 strain, respectively. Whereas Hexane and chloroform extracts reduced the mutagenicity induced by 2-aminoanthracene by 83.4% in TA104 and 65.3% in TA 102, respectively. The studies indicated that phenolic compounds may be responsible for the activity.

## CONCLUSION

It may be concluded that antioxidant activity of acetone extract/fractions may be due to the presence of polyphenolic compounds. The fractionation of crude extract leads to enhance the antioxidant activity of the extract.

## ACKNOWLEDGEMENTS

We are grateful to CSIR, New Delhi, for financial support in the form of major research project (grant number 38(1044)/02/EMR-II).

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