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In vitro Effect of *Terminalia arjuna* Bark Extract on Antioxidant Enzyme Catalase

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Abstract: The bark extract of *Terminalia arjuna* is an age old Ayurvedic prescription for cardiac ailments and has been reported to be particularly beneficial in improving cardiac muscle function. However the specific biological activity of individual components of *T. arjuna* bark is not yet clearly understood. In this study, bark extract of *Terminalia arjuna* in six different solvents i.e., ethanol, acetone, water, ethyl acetate, chloroform and hexane were evaluated for free radical scavenging property, total phenols and reducing properties. Among all the extracts of *T. arjuna*, crude ethanolic bark extract had high phenolics, high reducing power and high free radical scavenging activity indicating that it is the best extract to isolate antioxidant compounds which could be used for further studies. Maximum phenol content (894.95 GAE/mg extract) and radical scavenging property (~88%) was observed for ethanol extract. *In vitro* studies on the effect of the bark extract of *T. arjuna* on endogenous antioxidant enzyme catalase showed inhibition of catalase activity. Among all the extracts used, ethanolic extract showed significantly higher levels of inhibition of catalase activity. Dose dependent studies showed a concentration dependent linear increase in inhibition of catalase activity and further assessment of the kinetic parameters showed a specific and rare competitive-non competitive" kind of inhibition. Although it has been shown in earlier studies on animal models that the bark extract increases the catalase expression levels, in this study we observed that the bark extract does not enhance the catalase activity *in vitro*. This suggests the possible role of compounds in ethanolic bark extract of *T. arjuna* in catalase gene expression.

Key words: *T. arjuna*, total phenolics, reducing property, antioxidant property, ethanolic bark extract, catalase, kinetics, inhibition

INTRODUCTION

Terminalia arjuna, a deciduous tree belonging to *Combretaceae* family, is of 20-30 m height and is found ubiquitously in India. The vernacular names of this tree in Indian languages include white marudah, arjuna, arjunam, kakubha and kahu. The bark powder from *T. arjuna* tree has been used in Ayurvedic medicine for over 2,500 years, primarily as a cardiac tonic. Improvement of cardiac muscle function and subsequent improved pumping activity of the heart seem to be the primary benefits of the bark powder.

It has been documented that bark extract from *T. arjuna* contains following compounds: acids such as arjundic acid, terminic acid, glycosides-argentine arjunosides I-IV, strong antioxidants such as, flavones, tannins, oligomeric proanthocyanidins and minerals (Kalola and Rajani, 2006). However, not much is known about the specific biological activity of individual constituents. Of these constituents

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some active compounds from *T. arjuna* bark have been isolated and shown to possess antimutagenic and anticarcinoginic activity (Kaur *et al.*, 2002). Arjunolic acid, a triterpene has been reported to have cardioprotective activity (Gauthaman *et al.*, 2004). It has been proposed that the saponin glycosides might be responsible for the inotropic effects of *Terminalia*, while the flavonoids and oligomeric proanthocynadins (OPCs) provide free radical antioxidant activity and vascular strengthening.

A number of experimental and clinical studies have proved that dried bark powder of *T. arjuna* has potent hypolipidemic and cardioprotective activity (Gauthaman *et al.*, 2001; Dwivedi and Gupta, 2002; Ramesh *et al.*, 2004). Animal studies showed augmentation of myocardial antioxidants; superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) along with induction of heat shock protein72 (HSP72) was observed (Gauthaman *et al.*, 2004). Ethanolic extract is particularly useful agent, as it has been shown to enhance myocardial endogenous antioxidants without producing any cytotoxic effects (Karthikeyan *et al.*, 2003). It has been hypothesized that antioxidants exert a protective role against cardiovascular disease by inhibiting the damaging activities of free radicals (Manson *et al.*, 1993). In this context, the present study was undertaken to evaluate the antioxidant properties of different solvent extracts of *T. arjuna* bark and its effect on antioxidant enzyme catalase *in vitro*. In addition, the kinetics of catalase in the presence and absence of ethanolic extract was studied to determine the nature of inhibition.

MATERIALS AND METHODS

This study was conducted between August 2006 to April 2007.

Plant Material and Chemicals

The dry bark of *T. arjuna* was obtained from Center of traditional medicine and research, Chennai, India. Bovine serum catalase was obtained from Sigma, USA. All the chemicals and solvents used were of anylitical grade, procured in India.

Preparation of the Crude Extract

The dry bark was first powdered using an electric grinder. Powdered bark was filled onto a cellulose thimble and refluxed using various solvents for 12 h in a Soxhlet apparatus as reported earlier (Wang and Curtis, 2006). The order of the solvents used was as follows: hexane, chloroform, ethyl acetate, acetone and water ethanol. The extracts obtained were then dried using rotavapour. Upon drying the extracts resulted in crystalline form of various colors indicating purification of different molecular entities. These were stored at 30°C and dissolved in ethanol whenever required.

Evaluation of Antioxidant Properties
Determination of Total Phenolics

Amount of total phenolics was evaluated by Folin-Ciocalteau method as described earlier (Oboh, 2006; Singleton *et al.*, 1999). Fifty microgram of sample was taken and made upto 0.5 mL with ethanol and equal volume of distilled water was added. To this 0.5 mL of Folin reagent was added along with 2.5 mL of 20% (w/v) sodium carbonate. Then the absorbance was taken at 725 nm after 40 min of incubation. Total phenols were calculated using standard curve of gallic acid. The results were expressed in Gallic Acid Equivalents (GAE).

Determination of Reducing Property

Reducing property of each sample was determined by potassium ferricyanide method as described earlier (Pulido *et al.*, 2000). Fifty microgram of each extract (by weight) was taken and made

up to 2.5 mL with solvent. To this 2.5 mL of 200 mM of sodium phosphate buffer (pH 6.6), 1% (w/v) potassium ferricyanide and 2.5 mL of TCA (trichloro acetic acid) were added. The reaction was allowed to heat at 50°C for 20 min and then centrifuged at 5000 rpm for 10 min (if precipitate occurs). Five milliliter of supernatant was taken and made upto 10 mL with distilled water. Finally, 0.1% (w/v) of $FeCl_3$ (freshly prepared) was added and the green color observed was measured at 700 nm. Reducing property was expressed in terms of $OD_{700\ nm}$ and higher OD is indicative of high reducing power.

Free Radical Scavenging Assay Using DPPH

DPPH radical scavenging spectrophotometric assay was used in order to determine the inhibition concentration (IC_{50}) which is described as the amount of antioxidant necessary to decrease the initial concentration of DPPH radical by 50% and Inhibition Percentage (IP) which is described as the percentage of total DPPH radical which reacted with the antioxidant at the steady state of all the extracts. The experiments were performed using a Perkin-Elmer Lambda 35 UV-VIS spectrophotometer as described earlier (Moure *et al.*, 2001; Farrukh *et al.*, 2006). An aliquot of 50 µg each extract (1 mg mL^{-1}) was mixed separately with 100 mM ethanolic solution of DPPH radical and the final volume was of 3 mL. An equal volume of pure ethanol was taken as control. The decrease in the color of the solution which is indicative of decrease in the concentration of DPPH radical was monitored by the decrease in absorbance at 516 nm, for a period of 30 min during which time the radical was stable (Urisni *et al.*, 1994). The percentage of remaining DPPH (%$DPPH_{REM}$) was calculated according as follows:

$$DPPH_{REM}\ (\%) = (DPPH)_t/(DPPH)_{t0} \times 100,$$

where, $(DPPH)_t$ is the time when absorbance was measured at any time t and $(DPPH)_{t0}$ is the absorbance at time zero. IC_{50} was calculated by plotting the %$DPPH_{REM}$ at the steady state against various concentrations of each extract. The results were expressed as mg antioxidant g^{-1} DPPH (Argolo *et al.*, 2003).

Catalase Assay

Catalase decomposes hydrogen peroxide molecules into water and oxygen. The principle of the assay being measurement of the loss of the substrate measured at 238 nm until the substrate concentration becomes limiting. The assay was performed at 25°C using 50 mM sodium phosphate buffer of pH 7.0 containing 12 µM of H_2O_2 as substrate and 0.2 µg mL^{-1} of bovine liver catalase. The absorbance was monitored at 238 nm as a function of time for 5 min. One unit of catalase activity is defined as the amount of enzyme required to break one micromole of substrate per minute.

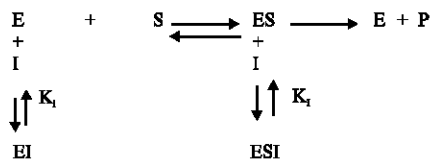## Enzymatic Assay With Different Extracts

The assay procedure was followed with 20 µg of each extract and the activity of catalase was measured as mentioned above. The decrease in the activtity of the enzyme was compared with a control reaction containing solvent alone i.e., ethanol and in absence of extract. In order to find out the dose dependent effects, enzymatic assay was done with increasing concentrations of ethanolic bark extract. (concentration range between 5-50 µg) and compared to control.

Catalase Kinetics With and Without Ethanolic Extracts

Kinetic experiments were performed by varying substrate concentrations in the presence and absence of fixed amounts inhibitor at constant enzyme concentration. Seven substrate concentrations (between 11.88-53.46 mM) in the absence of inhibitor and six substrate concentrations (between 2.97-29.7 mM) in the presence of inhibitor were considered. Three different concentrations of

inhibitors used in this study were 2, 5 and 10 µg, respectively and catalse activity was measured as described earlier.

Since ethanolic extract inhibits the catalase activity, the following kinetic mechanism was considered:

$$
\begin{array}{ccccccc}
\text{E} & + & \text{S} & \longrightarrow & \text{ES} & \longrightarrow & \text{E} + \text{P} \\
+ & & & & + & & \\
\text{I} & & & & \text{I} & & \\
\updownarrow \text{K}_\text{I} & & & & \updownarrow \text{K}_\text{I} & & \\
\text{EI} & & & & \text{ESI} & &
\end{array}
$$

where, EI is enzyme-inhibitor complex; ES, the enzyme substrate complex; and ESI, the enzyme-substrate inhibitor complex.

In the absence of inhibitor, kinetics of catalase is described by Michaelis-menten equation

$$V_0 = V_{max}[S]/[S] + K_m \tag{1}$$

In the presence of inhibitor, Michaelis-Menten equation is modified as

$$V_0 = V_{max}[S_0]/[S_0](1+I_0/K_I) + K_m(1+I_0/K_i) \ (10) \tag{2}$$

It can be written as

$$V_0 = V'_{max}[S_0]/[S_0] + K'_m \tag{3}$$

where

$$V'_{max} = V_{max}/(1+I_0/K_I)$$

$$K'_m = K_m \{(1+I_0/K_i)\}/(1+I_0/K_I) \tag{4}$$

Lineweaver Burk form of Eq. 3 is

$$1/V_0 = (K'_m/V'_{max})[1/S_0] + 1/V'_{max} \tag{5}$$

From the plot of $1/V_0$ versus $1/S_0$, slope is given by K'_m/V'_{max} and intercept is $1//V'_{max}$, where V_0 and S_0 are initial rate and initial substrate concentration, K_m and V_{max} are kinetic constants and Io is the initial inhibitor concentration.

From the plot of intercepts of primary plot $(1//V'_{max})$ versus $[I_0]$, the intercept on the $[I_0]$ axis gives $(-K_I)$ and substituting K_I in Eq. 6 K_i is calculated and the nature of inhibition was determined.

RESULTS AND DISCUSSION

In ischemic heart disease condition, the endogenous antioxidant enzyme levels are lower than normal conditions. The levels of endogenous antioxidants enzymes were brought back to normal by injecting the rats with the ethanolic bark extract of *T. arjuna* and provides significant cardiac protection (Sumitra *et al.*, 2001). Till today reports are available on the ethanolic bark extracts on catalase *in vivo* and no reports are available on the activity of catalase *in vitro*. In the present study, the effects of extract from the bark of *T. arjuna* on catalase activity *in vitro* was reported.

Extraction of compounds from the bark with different solvents yielded extracts of different color and weight. This indicated that different kind of compounds extracted based on polarity of solvents used. The phytochemical tests for the different extracts showed that alkaloids were present in high number in non polar fractions, tannins and reducing sugars were highly present in polar fractions, whereas moderate levels of saponins were found in acetone and ethyl acetate fractions (Table 1). This is in accordance with prior study done on other *Terminalia* species (Farrukh *et al.*, 2006).

Evaluation of Antioxidant Properties
Total Phenolics by Folins-Ciocalteau Method

The antioxidant properties were determined with different extracts of *T. arjuna* bark. It has been found that the total phenolics in the extract varied from 11.2-895 GAE/mg extract, where highest was found in ethanolic extract (895 GAE/mg extract) and lowest in hexane extract (11.2 GAE/mg extract) as shown in Fig. 1a. Further, dose dependent studies were done for ethanolic extract, which showed increase in the amount of phenols as the concentration of the crude ethanolic extract increased (Fig. 1b).

Table 1: Phytochemical tests of different extracts of *Terminalia arjuna*

Phytochemicals	Hexane fraction	Chloroform fraction	Ethylacetate fraction	Acetone fraction	Ethanol fraction	Water fraction
Alkaloids	+++	+++	+++	+	-	-
Tannins	-	+	+++	+++	+++	+++
Saponins	-	-	+	++	-	-
Reducing sugar	+++	+++	+++	++	++	++
Flavanoids	-	+	-	-	-	-

-: Negative result; +: Small amount, ++: Average; +++: High; All the experiments were performed in triplicates
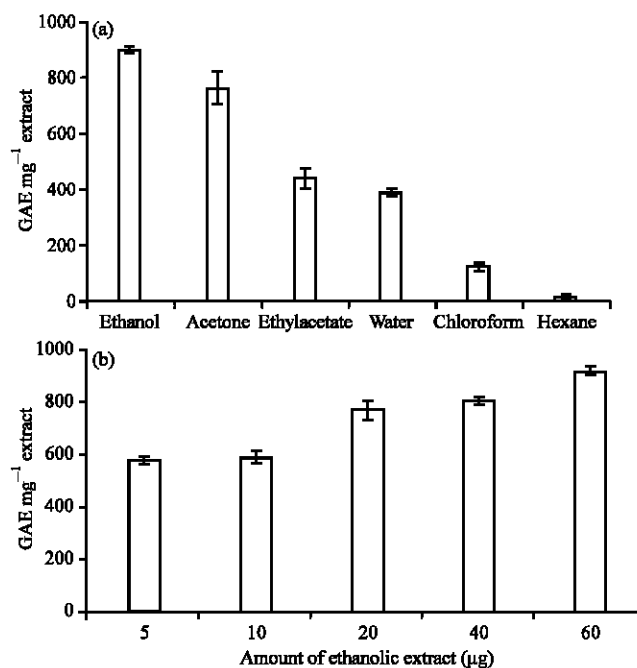

Fig. 1: Study on phenolic content of *T. arjuna bark* extracts, (a) Total phenolics determination of various extracts of *T. arjuna* using Folins-Ciocalteau method and (b) Dose dependent determination of phenolics of ethanolic extract of *T. arjuna* using Folins-Ciocalteau method. All the values are the mean of triplicate determination

As expected the concentration of total phenolics were high in ethanolic extract since the solubility of phenolic compound is high in polar solvents (Mavi *et al.*, 2003). This is in agreement with other reports where ethanolic extracts showed high polyphenol contents when compared with other extracts from dry plant material of *Maydis stigma* (Zoran *et al.*, 2005). In another study, 114.01 mg GAE g^{-1} dry weight was reported for ethanolic extracts of black tea whereas low phenolic content was observed in hexane (Turkmen *et al.*, 2007). This is in agreement with the results reported in this study.

Determination of Reducing Power by Ferric Reducing Assay

The reducing property was determined by ferric reducing assay. It was observed that ethanolic bark extract had maximum reducing power among the tested extracts and hexane had the least (Fig. 2a). The reducing power is expected to be high in polar fractions of extracts of medicinal plants as they principally contain effective H^+ donors, reducing agents and H_2O_2 scavengers which act as antioxidants and scavenge free radicals (Repetto and Llesuy, 2002). As the ethanolic extract had maximum reducing property, dose dependent studies were done which showed linear increase in reducing property shown in Fig. 2b.

Free Radical Scavenging Assay Using DPPH (2,2 Di Phenyl Picryl Hydrazine)

IC_{50} and IP values are considered to be a good measure of the antioxidant efficiency of pure compounds and extracts (Argolo *et al.*, 2003). Free radical scavenging capacity of all the extracts (50 µg dry wt.) was evaluated and the results are shown in Fig. 3a. The DPPH radical scavenging activity was found to be in the range of 0.66-88.69 IP for various extracts of *T. arjuna*. Ethanolic and acetone extract gave highest scavenging activity (~88 IP) and hexane extract gave the least. This was
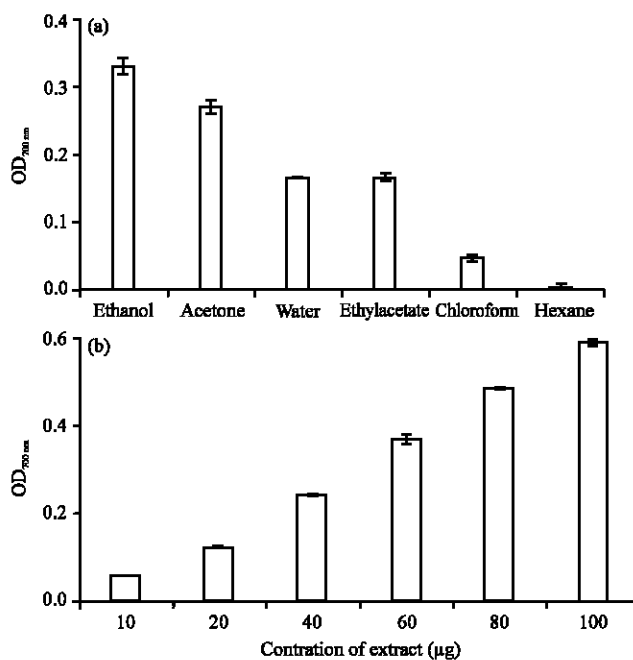

Fig. 2: Determination of reducing power of *T. arjuna*, (a) Reducing power determination of various extracts of using ferric reducing assay and (b) Dose dependent determination of reducing power of ethanolic extract of *T. arjuna* using ferric reducing assay. All values are the mean of triplicate experiments
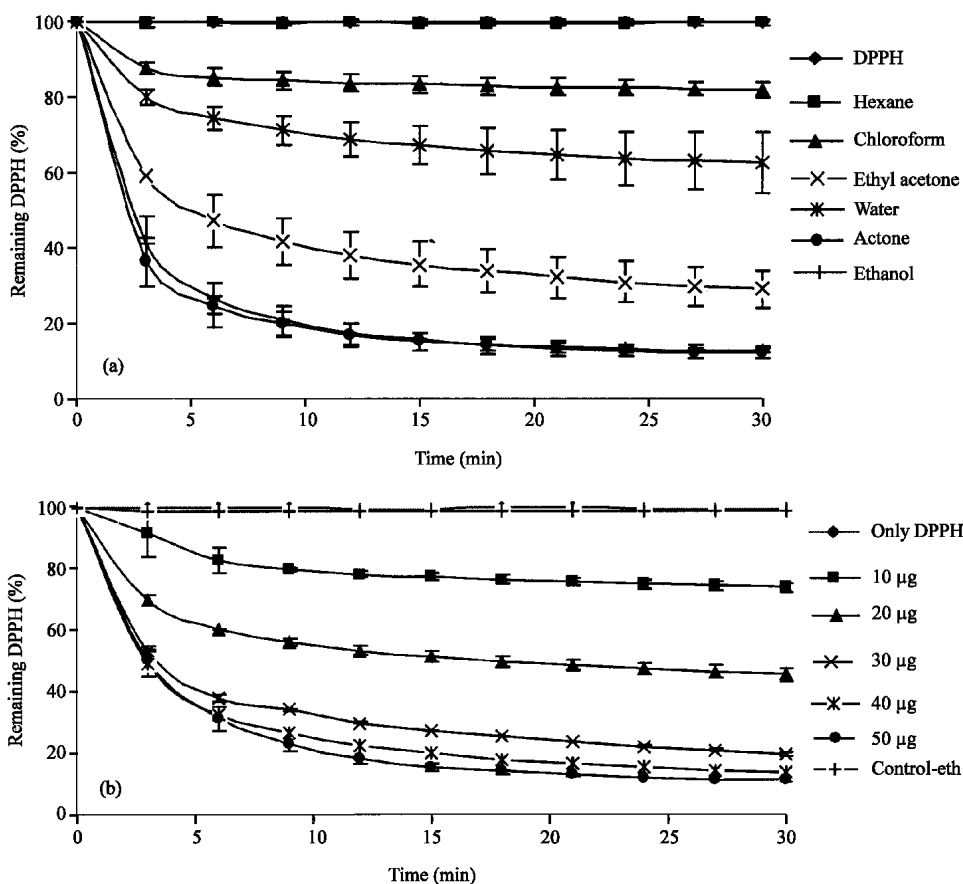
Fig. 3: Free radical scavenging assay using DPPH, (a) Determination of free radical scavenging property of all extracts of *T. arjuna* using DPPH and (b) Free radical scavenging assay of ethanolic extract (dose dependent studies) using DPPH. All the values are the mean of triplicate determination

Table 2: IC $_{50}$ values of different extracts of *Terminalia arjuna* using DPPH assay

Extracts	IC$_{50}$ value (mg extract/g DPPH)	IP (Inhibition %)
Ethanol	0.45±0.021	87.49±0.45
Acetone	0.60±0.150	88.69±1.48
Ethyl acetate	0.75±0.023	74.55±5.01
Water	1.13±0.087	8.11±3.28
Chloroform	3.77±0.087	1.92±1.54
Hexane	59.38±2.650	0.66±0.62

Data are mean (n = 3)

further confirmed by IC$_{50}$ values, the concentration of antioxidant needed to decrease by 50% the initial concentration of DPPH radical (Table 2).

The time taken for reaching a steady state for free radical scavenging was faster for acetone and ethanolic extract, whereas chloroform and water extracts relatively took longer time. This is in accordance with the study done on black tea by Turkmen *et al.* (2007) which showed that polar fractions like ethanol and acetone gave highest free radical scavenging activity (around 93.75-95.42 IP). Similarly, highest IP values for ethanol (64.4 IP and IC$_{50}$2.25 mg/DPPH) and least IP for hexane

(11.2 IP and IC_{50} 17.0 mg/DPPH) have been already shown with leaf extracts of *Bauhinia monandra* (Argolo *et al.*, 2003). Since ethanolic extract commonly showed high free radical scavenging property it was taken for dose dependent studies using DPPH (Fig. 3b). It has been found that the bark extract showed increased free radical scavenging property with the increase in concentration.

Based on the IP and IC_{50} values (Table 2), it was inferred that antioxidant properties increases with increase in polarity of the solvent used for extraction. High IP values are obtained as expected for ethanolic fraction as observed in previous studies done on various ethanolic plant extracts (Badami *et al.*, 2005). The IC_{50} and IP values obtained from the crude extracts allow us to categorize the antioxidant sources and help us in selection of the extracts for the isolation of the pure antioxidant compounds. The present results show that ethanol and acetone extracts of *T. arjuna* bark is preferable sources for extracting pure antioxidant compounds as they show high IP values.

Catalase Assay

All the extracts of *T. arjuna* showed inhibition of catalase activity when added to the reaction mixture (Fig. 4a). Among the tested extracts, chloroform showed the least inhibition (15%) while the polar fractions, i.e., acetone, ethanol and ethyl acetate showed almost similar levels of inhibition (around 60%). Hexane extract showed maximum inhibition (99%) of catalase activity (Fig. 4a). The present study shows that the extract does not augment the catalase activity *in vitro*, whereas previous studies showed that ethanolic bark extracts enhanced the catalase levels *in vivo* under in ischemic heart
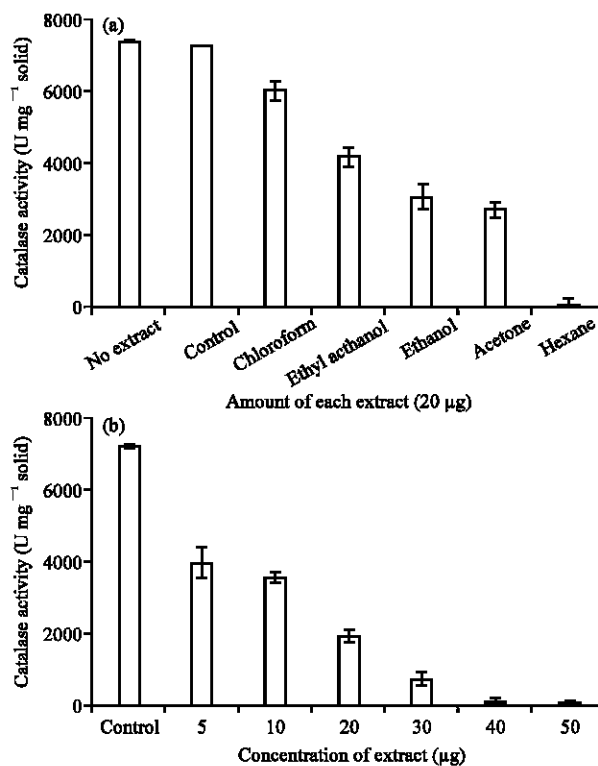

Fig. 4: Effect of *T. arjuna bark extracts* on catalase activity, (a) Effect of various extracts on catalase activity and (b) Dose dependent studies of ethanolic extract on catalase activity. (Reaction without extract and in presence of solvent i.e., ethanol serves as control). All the values are the mean of triplicate determination

(Karthikeyan *et al.*, 2003; Mary *et al.*, 2003; Gauthaman *et al.*, 2001). To test further, dose dependent study of ethanolic bark extract on catalase activity was studied and inhibition effect increase with increase in ethanolic bark extract concentration and inhibition gets saturated at 40-50 μg dry weight of extract (Fig. 4b). In order to determine the nature of inhibition of catalase activity, further studies were carried out with ethanolic extracts.

Kinetics of Catalase With and Without Ethanolic Bark Extracts (Inhibitor)

The kinetic parameters were determined both in the absence and in the presence of different concentrations of inhibitor. From Lineweaver-Burk plot ($1/V_0$ Vs $1/[S_0]$), it has been observed that both the slope (K_m/V_{max}) and intercept ($1/V_{max}$) varied indicating that the type of inhibition is non-competitive mixed inhibition (Table 3 and Fig. 5). From the secondary plots ($1/V_{max}$ Vs [I]), the K_I was determined (Fig. 6). Based the equations Eq. 4, Ki was calculated. It was inferred that K_I (14.99 mM) was greater than K_i (5.49 mM) indicating that the type of inhibition is competitive-

Table 3: Kinetic parameters of the enzyme in absence and in presence different concentrations of inhibitor

Concentration of Inhibitor (μg)	Slope	Intercept	K_m (mM)	V_{max} (U mg^{-1})
0	0.0009	0.0001	15.0	16666.67
2	0.0016	8E-05	22.8	14285.7
5	0.0019	7E-05	21.7	12500.0
10	0.0019	6E-05	19.0	10000.0

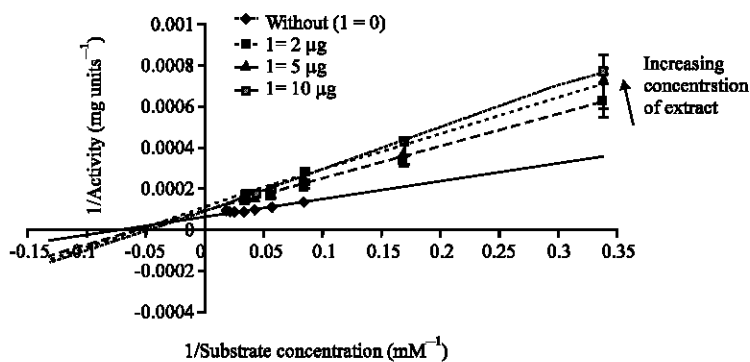All the experiments were performed in triplicates

Fig. 5: Lineweaver-Burk plot in the absence and presence of various concentrations (2, 5 and 10 μg) of inhibitors. All the experiments performed in triplicates
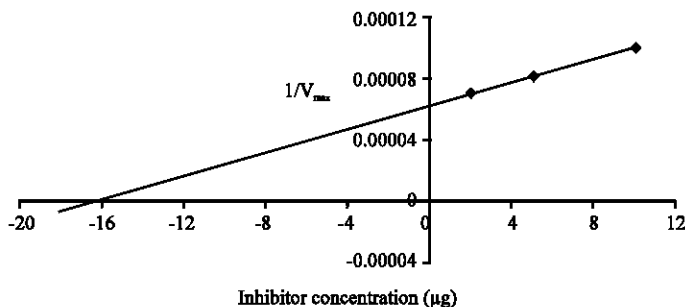

Fig. 6: Secondary plot for the determination of K_i. All the experiments were performed in triplicates

noncompetitive inhibition. Similar kind of inhibition has been reported for β-1,3-glucanase (Rana *et al.*, 2003). Even though kinetic experiments were performed with crude extract, the results in this study showed that catalase activity is inhibited by ethanolic bark extract of *T. arjuna in vitro*. This is the first report on effect of *T. arjuna* bark extract on catalase activity *in vitro*.

In conclusion, among all the extracts of *T. arjuna*, crude ethanolic bark extract had high phenolics, high reducing power and high free radical scavenging activity indicating that it is the best extract to isolate antioxidant compounds which could be used for further studies. Although it has been shown in earlier studies on animal models that the bark extract increases the catalase expression levels, in our study we observed that the bark extract does not enhance the catalase activity *in vitro*. This suggests the possible role of compounds in ethanolic bark extract of *T. arjuna* in catalase gene expression.

ACKNOWLEDGMENTS

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