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In Vitro Hepatoprotective Activity of *Terminalia arjuna* Stem Bark and Its Flavonoids Against CCl_4 Induced Hepatotoxicity in Goat Liver Slice Culture

Gunjan M Chaudhari[1] and Raghunath T Mahajan[2]

[1]*Department of Biochemistry, Moolji Jaitha College, Jalgaon-425002 (MS), India*
[2]*Post Graduate College of Science Technology and Research, Moolji Jaitha College Campus, Jalgaon-425002 (MS) India*

ABSTRACT

Terminalia arjuna (Roxb) stem bark has folkloric use in the treatment of hepatic disorders. In order to validate this folkloric claim experimentally, it is planned to evaluate hepatoprotective activity of methanolic extract of Terminalia arjuna stem bark (MeOH-TASB) and its extracted flavonoids baicalein (Bai) and quercetin (Que) by using a simple in vitro goat liver slice culture model. Carbon tetrachloride (CCl_4) was used to induce hepatotoxicity in liver slice of goat. The cytotoxicity induced by CCl_4 was estimated by quantifying the release of marker enzymes such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). Also the degree of hepatic damage measured by analyzing the levels of lipid peroxidation (LPO) of hepatocytes membrane lipids. The treatment of liver cells with CCl_4 caused twice increase in LPO of cells besides release of ALT, AST, ALP and LDH was 4.26, 4.88, 2.89 and 3.66 times, respectively as compared to untreated liver cells. Thus, a toxic effect of CCl_4 was significantly reduced by the treatment of MeOH-TASB, Bai and Que. Moreover, the protective effect was a dose dependent manner, not only for MeOH-TASB extract but also for its phyto-ingredients. These results indicate that all MeOH-TASB and its extracted flavonoids protect the liver cells from CCl_4 induced oxidative/free radical mediated damage in vitro. Que shows more protective effect than Bai. Evidently, it may be stated that flavonoids of Terminalia arjuna stem bark have hepatoprotective effect against CCl_4 induced hepatic damage.

Keywords: *Terminalia arjuna,* Stem bark, Flavonoids, Hepatoprotective activity, *In vitro* goat liver slice culture assay

INTRODUCTION

The liver is the main organ involved in the metabolism. It has a major role in detoxification and excretion of various substances like xenobiotics. This physiological process through several biochemical reactions results in the generation of highly reactive free radicals. These free radicals attack the membrane lipids causing lipid peroxidation which alters the membrane permeability and causes tissue injury [1]. Hepatic tissue damage causes impairment to liver function which may have deleterious effect on human health. Management of liver diseases is still a challenge to the modern scientific community [2]. However, the use of antioxidants has been proposed as therapeutic agents to counteract liver damage [3]. Although, inherent antioxidant systems such as catalase (CAT), superoxide dismutase (SOD) and tissue glutathione (GSH), etc., protect the tissues from free radical attack [4], the excessive release of reactive oxygen species/free radicals overcome this system leading to liver damage. Reinforcing the inbuilt protective antioxidant system or external supplementation of antioxidants may help in protecting the organs [5]. However, conventional drugs do not have satisfactory results in protection of liver during serious hepatic disorders. In contrast to this herbal drugs are known to have protective effect against liver damage in various liver disorders. Most of the herbal medicines speed up the natural healing processes of the liver mediated through their antioxidative potential with almost negligible side effects [6].

Several medicinal plants are practiced for their traditional use in hepatic disorders/jaundice since long time [7]. Selectively, *Terminalia arjuna* (Family: Combretaceae) is one of the most versatile medicinal plant having several folkloric uses such as cardiotonic, wound healing agent, urinary disorders, broken bones, sex stimulant and skin

disorders, etc. [8]. The methanolic extract of *Terminalia arjuna* stem bark is rich in polyphenols and flavonoids [9]. The stem bark of *Terminalia arjuna* is known to contain many flavonoids (arjunone, apigenin, baicalein, kaempherol, luteolin, quercetin [10]. Among them, baicalein (Bai) and quercetin (Que) have been extracted in our laboratory and evaluated for their pharmacological potential as antivirulence, antimicrobial, antibiofilm and antioxidant agent [11].

Many *in vitro* and *in vivo* studies have been reported regarding the assessment of hepatoprotective activity of *Terminalia arjuna* [12-24]. But till date there is no evidence regarding the evaluation of hepatoprotective activity of the methanolic extract of *Terminalia arjuna* stem bark and its constituent flavonoids against CCl_4 induced hepatotoxicity in goat liver slice culture. The liver slice is a microcosm of the intact liver consisting of highly organized cellular communities in which the different cell types are subject to mutual contact. Therefore, liver slice culture is an *in vitro* technique that offers the advantages of *in vivo* situation over maintained cell lines cultures. It is a simple and suitable model for the experimental analysis of hepatotoxic conditions [25]. Therefore, the present study was undertaken to assess the hepatoprotective activity of *Terminalia arjuna* stem bark extracts in goat liver slice culture against CCl_4 to prove its efficacy against liver disorders and to justify previously published *in vivo* studies also.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals used were of analytical grade having high purity and purchased from either Sigma chemicals (Bangalore, India) or HiMedia Ltd. (Mumbai, India).

Preparation of extract and purification of flavonoids

The methanolic extract of *Terminalia arjuna* stem bark (MeOH-TASB) was prepared by Soxhlet extraction method [26]. The twenty gram of finely ground plant part powder was placed in porous bag made of muslin cloth, which was loaded into the main chamber of the Soxhlet extractor. The extraction was carried out with methanol as extraction solvent in 1: 10 powder to solvent ratio at temperature 65ºC for 8 h. This methanolic extract was filtered through Whatman filter paper No. 1, then the filtrate were concentrated using a rotary vacuum evaporator (R-124, Buchi, Switzerland) at 45ºC. The concentrated methanolic extract was stored in dessicator until further use. Isolation of abundant flavonoids viz. baicalein (Bai) and quercetin (Que) was achieved by silica gel column chromatography and rechromatography with Sephadex LH-20 column (data communicated).

In vitro hepatoprotective activity

The goat liver was selected as the mammalian tissue to determine the *in vitro* effect of methanolic extract of *Terminalia arjuna* stem bark (MeOH-TASB) and its purified flavonoids baicalein (Bai) and quercetin (Que) using cytotoxicant CCl_4.

In vitro lipid peroxidation assay

The degree of lipid peroxidation was assayed by estimating the TBARS by using the standard method [27] and expressed in terms of µM of malondialdehyde formed/100 mg tissue. In brief, freshly excised goat liver (100 mg) was processed to get 10% homogenate in ice cold phosphate buffered saline, pH 7.4 using glass Teflon homogenizer and centrifuged to get clear homogenate. Different concentrations of the MeOH-TASB (25, 50 and 100 µg/mL), Bai (2.5, 5.0 and 10 µg/mL) and Que (2.5, 5.0 and 10 µg/mL) in DMSO were added to the liver homogenate. Lipid peroxidation was initiated by adding CCl_4 (Final concentration 15.5 mM) to the tissue homogenate. After 30 min, 100 µL of this reaction mixture was taken in a tube containing 1.5 mL of 0.67% (thiobarbituric acid) TBA in 50% acetic acid. The mixture was heated for 30 min in a boiling water bath. The intensity of the pink complex formed was measured at 535 nm. The malondialdehyde concentration of the sample was calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$. Different treatment sets for this assay are normal (untreated), CCl_4 treated, CCl_4 (15.5 mM)+MeOH-TASB (E-1: 25 µg/mL, E-2: 50 µg/mL and E-3: 100 µg/mL); CCl_4 (15.5 mM)+Bai (B-1: 2.5 µg/mL, B-2: 5.0 µg/mL and B-3: 10 µg/mL) and CCl_4 (15.5 mM)+Que (Q-1: 2.5 µg/mL, Q-2: 5.0 µg/mL and Q-3: 10 µg/mL).

Liver slice culture

Liver slice culture was performed according to method described by Wormser et al [28]. The fresh liver was collected from local slaughter house immediately after the sacrifice of the animal. Liver was transferred to sterilized Krebs Ringer Hepes medium (KRH 2.5 mM Hepes, pH 7.4, 118 mM NaCl, 2.85 mM KCl, 2.5 mM $CaCl_2$, 1.15 mM KH_2PO_4, 1.18 mM $MgSO_4$ and 4.0 mM glucose). The liver was cut into thin slices using sharp blade, and slices ranging from 4 and 6 mg were used for this study. Each set of experiments contains 20 to 25 slice tissues weighing 100 mg. These slices were washed with 10 mL KRH medium every 10 min over a period of 1 h and pre-incubated for 60 min in small plugged beakers containing 10 mL KRH on a shaker water bath at 37°C.

Experiment design

The liver slices were further divided into individual culture for respective treatment as shown in Table 1. All the cultures were incubated in a constant temperature water bath at 37°C for 2 h. At the end of incubation, the culture medium of each set was centrifuged at 3,000 rpm for 10 min at 4°C, to remove cells and supernatants were assayed for leakage of biochemical markers such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH).

Biochemical studies

In culture medium supernatant, the marker enzymes like alanine transaminase, aspartate transaminase and alkaline phosphatase were estimated using reagents and kits (Nirmal laboratory, Jalgaon, India).

LDH activity assay

Lactate dehydrogenase (LDH) activity was assayed by the method adopted from Sigma Aldrich. Briefly, to the suitable cuvette 2.8 mL of β-NADH (0.13 mM) and 0.1 mL of sodium pyruvate (34 mM) both prepared in sodium phosphate buffer (100 mM, pH 7.4) mixed well by inversion. After adding 0.1 mL of properly diluted medium supernatant again mixed by inversion, immediately the decrease in the absorbance at 340 nm for 5 min at 30 s intervals was recorded. The blank was run by using 1% BSA in phosphate buffer instead of culture medium supernatant in the above reaction. The change in absorbance per minute for both test and blank was calculated and the results are expressed as LDH units/mL of culture medium supernatant.

LDH activity unit was defined as amount of enzyme required to reduce 1.0 µM of pyruvate to L-lactate per minute at pH 7.4 at 25°C.

LDH units/mL= (T-B) × 3 × df/6.22 × 0.1

Where, T=ΔA_{340nm} per min of test; B=ΔA_{340nm} per min of Blank; 3=total volume of reaction mixture in mL; df=dilution factor; 6.22=mM extinction coefficient of β-NADH at 340 nm; 0.1=Volume of culture supernatant (enzyme) solution used in ml

Table 1: Experimental set up for evaluation of hepatoprotective activity

Set No	Treatment
Set 1	Control
Set 2	CCl_4
Set 3a	CCl_4+MeOH-TASB (25)
Set 3b	CCl_4+MeOH-TASB (50)
Set 3c	CCl_4+MeOH-TASB (100)
Set 4a	CCl_4+Bai (2.5)
Set 4b	CCl_4+Bai (5)
Set 4c	CCl_4+Bai (10)
Set 5a	CCl_4+Que (2.5)
Set 5b	CCl_4+Que (5)
Set 5c	CCl_4+Que (10)
Set 6	MeOH-TASB (100)
Set 7	Bai (10)
Set 8	Que (10)
15.5 mM concentration of CCl_4 used for experiment MeOH-TASB, Bai and Que expressed in µg/mL	

RESULTS AND DISCUSSION

Terminalia arjuna is sacred plant having various uses in Indian traditional medicinal system, Ayurveda. A compiled data on this plant regarding its assessment of hepatoprotective activity is summarized in Table 2. Earlier researchers used mostly water and alcohol for extraction of stem bark, leaves and fruits of *Terminalia arjuna* as a drug in both *in vivo* and *in vitro* assay method, where they used different toxicants to induce hepatotoxicity [16-28].

Carbon tetrachloride-induced hepatic injury is commonly used as an experimental method for the study of hepatoprotective effects of medicinal plants and drugs [1]. CCl_4 is metabolized by Cytochrome P-450 enzyme system to trichloromethyl radical (CCl_3). This in turn reacts with molecular oxygen and converted to highly reactive trichloromethyl peroxy radical [5]. The polyunsaturated fatty acids of cell membranes are predominantly vulnerable to this oxidative attack leading to membrane lesions and loss of cellular homeostasis [29]. Moreover, these radical forms covalent bonds with sulfhydryl groups of various membrane molecules like GSH leading to depletion and causes lipid peroxidation [30].

The Figure 1 shows the degree of lipid peroxidation in different treatments. The level of LPO was significantly increased by 2.25 fold ($P<0.001$) in the tissue homogenates incubated with CCl_4 as compared to control. Such increased level of lipid peroxidation has been documented earlier in the liver of rat after oral administration of CCl_4 [16] and in liver cells treated with CCl_4 *in vitro* [31].

However, the level of LPO was significantly reduced ($p<0.001$) in liver tissue homogenate incubated with CCl_4 with the presence of MeOH-TASB, Bai and Que in concentration dependent manner. The peroxidation of lipids in liver tissue homogenate due to CCl_4 is reduced by 55%, 48% and 51% with the treatment of 100 µg/mL concentration of MeOH-TASB and 10 µg/mL of Bai and Que, respectively. The maximum restoration of LPO to the basal value was achieved at 100 µg/mL concentration of MeOH-TASB. Since lipid peroxidation is caused by free radicals/oxidative stress, MeOH-TASB, Bai and Que appear to reduce the amount of free radicals or oxidative stress substantially. The Que was more potent in inhibition of lipid peroxidation than Bai. However our results are in contrast with result obtained by Chang and Yang [32]. They showed that baicalein inhibit lipid peroxidation due to ferric chloride by 95% at a concentration of 50 µg/mL whereas, at the same concentration quercetin inhibit lipid peroxidation by 80%.

The lipid peroxidation initiates a cascade of reactions leading to liver necrosis [1,4]. Liver damage is detected by measuring the activities of liver function marker enzymes like AST, ALT and ALP, which are released into the blood from damaged cells. The result of the present study shows that level of AST, ALT and ALP in CCl_4 treated liver slices raised in comparison to the control liver tissues. The liver tissue treated with CCl_4 in presence of MeOH-TASB (100 µg/mL), Bai (10 µg/mL) and Que (10 µg/mL) significantly reduced ($p<0.05$) the AST, ALT, ALP levels compared to the CCl_4 induced set. Overall depend on the concentration of test samples, MeOH-TASB, Bai and Que, reduces the elevated AST, ALT and ALP levels (Table 3).

Table 2: References on assessment of hepatoprotective activity of medicinal plant *Terminalia arjuna*

S.no.	*In vivo/vitro*	Plant part	Extraction solvent	Toxicant	References
1	Swiss albino mice	SB	H_2O	Carbon tetrachloride	[12]
2	Wistar albino rats	SB	EtOH and H_2O	Carbon tetrachloride	[13]
3	Swiss albino mice	SB	H_2O	Sodium-fluoride	[14]
4	Mice	Fr	EtOH and H_2O	Cadmium	[15]
5	Wistar rats.	Lf	MeOH	Paracetamol	[16]
6	Swiss mice	SB	Powder	Carbon tetrachloride	[17]
7	Albino rats	SB	H_2O	Isoniazid	[18]
8	Wistar albino rats	SB	H_2O	Paracetamol	[19]
9	Wistar albino rats	SB	H_2O	Carbon tetrachloride	[19]
10	Wistar albino rats	SB	EtOH	Paracetamol	[19]
11	Wistar albino rats	SB	EtOH	Carbon tetrachloride	[19]
12	*In vitro*, Goat liver slice*	SB	H_2O	Copper-ascorbate	[20]
13	*In vitro*, HepG2 cell line*	SB	H_2O	Tert-butylhydroperoxide	[21]
14	Albino Rats	SB	H_2O	Cadmium	[22]
15	Adult rats	Lf	MeOH	Paracetamol	[23]
16	Wistar albino rats	SB	H_2O	Adrenaline	[24]

H_2O: Water, MeOH: Methanol, EtOH: Ethanol, SB: Stem Bark, Fr: Fruits, Lf: Leaves
All activities are *in vivo* except those with * are *in vitro*

Table 3: Effect of MeOH-TASB, baicalein and quercetin treatment on release of biochemical markers of hepatotoxicity induced by CCl_4 in the goat liver tissue *in vitro*

Set No.	Units of enzyme per mL of culture medium			
	LDH	AST	ALT	ALP
Set 1	11.47 ± 2.93	35.33 ± 5.51	48.67 ± 7.02	11.53 ± 2.70
Set 2	42.00 ± 3.93[a]	172.67 ± 8.50[a]	207.67 ± 9.45[a]	33.35 ± 3.60[a]
Set 3a	24.35 ± 3.07[*]	106.33 ± 6.11[**]	103.33 ± 5.03[**]	29.34 ± 3.08[ns]
Set 3b	18.69 ± 1.90[**]	66.33 ± 6.81[**]	72.67 ± 4.16[**]	21.60 ± 3.02[*]
Set 3c	14.51 ± 2.71[**]	44.67 ± 5.13[**]	59.00 ± 4.58[**]	15.06 ± 1.21[**]
Set 4a	27.70 ± 3.48[*]	97.33 ± 8.50[**]	112.67 ± 7.77[**]	30.50 ± 2.63[ns]
Set 4b	16.34 ± 1.72[**]	73.33 ± 5.69[**]	85.33 ± 4.04[**]	22.87 ± 2.91[*]
Set 4c	12.25 ± 2.26[**]	62.00 ± 4.58[**]	62.33 ± 6.43[**]	13.35 ± 1.32[**]
Set 5a	31.85 ± 3.70[*]	107.67 ± 4.04[**]	107.67 ± 4.04[**]	29.35 ± 3.10[ns]
Set 5b	21.53 ± 3.56[*]	67.33 ± 3.51[**]	72.67 ± 5.51[**]	20.65 ± 1.98[*]
Set 5c	16.28 ± 2.38[**]	42.33 ± 3.06[**]	52.33 ± 5.51[**]	13.27 ± 1.47[**]
Set 6	14.67 ± 2.26[**]	39.33 ± 3.06[**]	52.00 ± 4.58[**]	14.56 ± 0.99[**]
Set 7	13.94 ± 2.25[**]	40.33 ± 2.08[**]	55.00 ± 5.29[**]	15.40 ± 1.43[**]
Set 8	16.16 ± 2.29[**]	41.67 ± 4.16[**]	47.67 ± 4.16[**]	13.57 ± 0.94[**]

All values are expressed as Mean ± S.D. (n=3); Superscript (a) in columns indicates value differ significantly ($p<0.001$) from control mean; Superscript asterisk in columns indicate value differ significantly (*$p<0.05$, **$p<0.001$) from CCl_4 mean; Superscript (ns) in columns indicates value do not differ significantly ($p<0.001$) from control mean

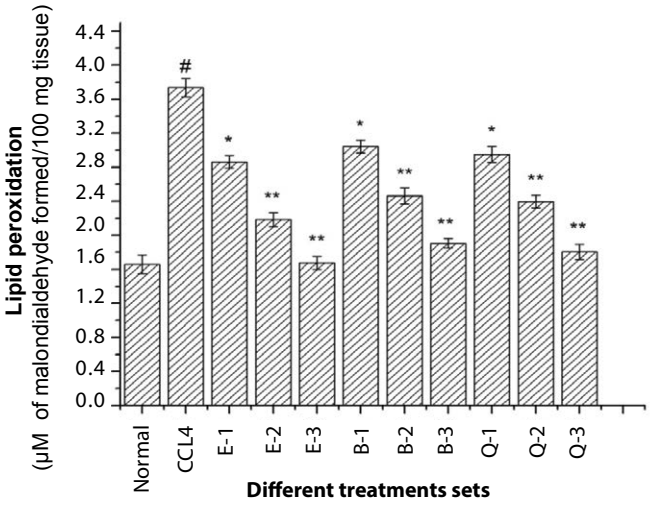

Figure 1. Inhibition of lipid peroxidation of liver tissue by MeOH-TASB, Bai and Que. Sign (#) above the bar indicates value differ significantly ($p<0.001$) from control mean (Set 1); Sign asterisk above the bar indicate value differ significantly (*$p<0.05$, **$p<0.001$) from CCl_4 mean (Set 2)

The normalization of the above enzyme levels in goat liver with the plant drugs establishes the hepatoprotective effect of plant extract which may be due to the prevention of the leakage of intracellular enzymes by their membrane stabilizing activity [33]. This is in good agreement with the commonly accepted view that serum levels of the marker enzymes return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [34]. Many studies proved hepatoprotective efficacy of *Terminalia arjuna* stem bark in Swiss albino mice and Wistar albino rats [12-19]. In previous studies, researchers used ethanolic and aqueous extracts of *Terminalia arjuna* against hepatotoxicity induced by different toxicant such as carbon tetrachloride, cadmium, sodium fluoride, isoniazid and paracetamol. Both solvents are highly polar which is very close to methanol used in the present study for extraction. This clearly indicates that polar phytoingredients like flavonoids are responsible for hepatoprotective activity. Thus, the hepatoprotective effect of *Terminalia arjuna* stem bark is in accordance of previously published studies [12-24].

Release of LDH in the liver slice culture medium was used as cytotoxicity marker. CCl_4 was highly toxic to the treated liver cells, which increased LDH concentration four times higher (40.67 ± 2.16 units/mL) as compared to control. The MeOH-TASB (100 µg/mL), Bai (10 µg/mL) and Que (10 µg/mL) were nontoxic as it showed release of LDH in the

medium similar to that of control untreated liver slices. The amount of LDH release in medium reduced after addition of MeOH-TASB, Bai and Que along with CCl_4 cytotoxicant and the activity was concentration-dependent (Table 3).

The results of treatment of liver slices with CCl_4 along with MeOH-TASB, Bai and Que showed decrease in the elevated LDH and hepatic marker enzyme (AST, ALT, ALP) levels in the medium indicating the hepatoprotective action which may be a consequence of membrane stabilization as well as free radical scavenging effect of flavonoids present in the plant extracts. The flavonoids may have interrupted the reaction of ROS with cellular proteins and nucleic acids and preventing the formation of adducts by acting as scavengers there by stabilizing the cell membrane resulting in decreased leakage of hepatic biomarkers [35]. The hepatotoxicity induced by CCl_4 increases level of biochemical markers, LDH, ALT, AST and ALP by 3.66, 4.26, 4.88 and 2.89 times in culture medium as compared to control. The hepatoxicity of CCl_4 in terms of biochemical markers ALT, AST and ALP is remarkably reduced by Que followed by MEOH-TASB and Bai. However, the level of LDH elevated by CCl_4 toxicity in culture medium is highly reduced by Bai as compared to Que presenting its role in cardio protection [36]. Here again the Que was more potent hepatoprotective than Bai. The hepatoprotective activity of methanolic extract of *Terminalia arjuna* extract is due to presence of high amount of flavonoids which have remarkable antioxidant and anti-inflammatory activity [7,9]. Thus, the hepatoprotective effect of *Terminalia arjuna* stem bark were coherent to previously published data [12-24].

CONCLUSION

The present investigation indicates that, the methanolic extract of *Terminalia arjuna* stem bark and its purified flavonoids (baicalein and quercetin) exhibit hepatoprotective effect against CCl_4 induced hepatic damage. The hepatoprotective role of *Terminalia arjuna* stem bark is due to its flavonoids. Evidently, flavonoids produce antioxidant activity so this mechanism signifies that the extract of plant may be useful to prevent the oxidative stress induced damage in liver. Our observations of *in vitro* hepatoprotection support and enrich the findings of earlier researchers. Thus this plant should be recorded for its global acceptance as herbal drug.

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