Influence of aqueous extract of Arjuna (*Terminalia arjuna*) on growth and antioxidant defense system of human hepatoma cell line (HepG2)

Mahesh Mysore Shivananjappa and Manoj Kumar Joshi*

Unilever Research Centre, 64, Main Road, Whitefield, Bangalore-560 066, India.

Accepted 22 December, 2010

**Arjuna** (*Terminalia arjuna*) is a common medicinal plant used in the ayurvedic system of medicine to treat various ailments and is one of the active ingredients in many polyherbal hepatoprotective formulations currently used in India. Despite its extensive usage, data on its ability to modulate basal oxidative markers in cell models are limited. Hence in the present study we have addressed whether aqueous extract of Arjuna possess the propensity to modulate endogenous oxidative markers in HepG2 cells. Cells were incubated with aqueous extract of Arjuna (1, 5, 10, 25 and 100 µg/ml) for varied time points (4, 8, 12, 16, 20 and 24 h) and biochemical markers of oxidative stress in cell lysate were determined. Cells incubated with Arjuna showed no significant effect in terms of cytotoxicity or cell proliferation upto 100 µg/ml concentrations. However, incubation with Arjuna for 24 h showed diminution in the levels of lipid hydroperoxide (18 -42%) and reactive oxygen species (11-29%) in cell cytosol. The antioxidant capacity (19-31%) of cells and levels of reduced glutathione (18-32%) was also found to be significantly increased. Interestingly, aqueous extract of Arjuna also enhanced activities of endogenous antioxidant enzymes (superoxide dismutase, 25-41%; catalase, 39-50%; glutathione peroxidase, 20-35%; glutathione reductase, 26-35% and glutathione transferase, 12-30%). Taken together, these data suggest that Arjuna has the propensity to improve endogenous antioxidant levels and reduce basal oxidative stress in HepG2 cells and indicates its potential as antioxidant and hepatoprotective adjuvant to combat oxidative stress *in vivo*.

**Key words:** Arjuna, *Terminalia arjuna*, oxidative stress, antioxidant, HepG2, Hepatoprotection

**INTRODUCTION**

Arjuna (*Terminalia arjuna*, Wight and Arnott, Family: Combretaceae) is well known in the traditional Indian health care system (Ayurveda) for prevention and treatment of cardiovascular diseases. Several studies support its benefits against cardiac failure, angina pectoris, ischemic cardiomyopathy, coronary artery disease and arteriosclerosis (Dwidvedi, 2007). Experiments conducted with the bark of Arjuna have been shown to possess hypolipidemic, hypocholesterolemic, hypotensive, antidiabetic and anti-inflammatory activities (Dwivedi, 2007). In addition, the thick, white to pinkish gray bark has been shown to posses’ anticancer, antiulcer, antimutagenic and wound healing activities (Warrier et al., 1996).

Oxidative stress (OS) due to increased reactive oxygen species (ROS) generation or impaired endogenous antioxidant mechanism is an important factor implicated in metabolic syndrome (MS). Accumulating evidences suggest that the changes in the cellular redox state with increased ROS plays a crucial role in various steps that initiate and regulate progression of liver diseases, independently of the type of etiologic agents.

ROS are involved in the liver damage induced by alcohol, virus, alteration of lipid and carbohydrate metabolism and xenobiotics. OS is also prominent in non alcoholic fatty liver diseases (NAFLD) closely associated with MS and occurs in 14-30% of general populations across all age groups and ethnicities (Kotronen and...
The liver being major regulator of metabolite flow, internal chemical environment and detoxification in the body, any damage to the liver inflicted by OS influences the transcription of several biochemical mediators (principally cytokines) which are able to modulate tissue and cellular events – apoptosis fibrosis, cholestasis and regeneration - which characterize the different types of liver injury (Morisco et al., 2008). There is an ever increasing need for the therapeutic agent to protect liver from such damage and reduce OS and improve liver functions (Videla et al., 2006).

It has been demonstrated that natural antioxidants prevent hepatotoxicity by inhibiting lipid peroxidation, suppressing hepatocellular damage and enhancing antioxidant enzyme activity. Earlier studies report that patients with liver cirrhosis were found responding to Arjuna powder therapy which gave symptomatic relief, and improved general health of patients (Colabawala, 1951).

Arjuna is also used in the many formulations of drugs for liver diseases (Shastri, 1962). Earlier, few studies in rodent models have investigated the effect of various extracts of Arjuna on modulating xenobiotics and pathophysiological conditions induced OS viz., CCL4 (Manna et al., 2007), alloxan-diabetes (Raghavan and Kumari, 2006) and n-nitrosodiethylamine induced hepatotoxicity (Sivalokanan et al., 2006). Although, antioxidant effect is attributed to benefits of Arjuna against xenobiotics induced toxicity and in various cardiac ailments, antioxidant effect of Arjuna per se is not well studied.

To the best of our knowledge this is the first study which investigates the antioxidant effect of aqueous extract of Arjuna and its propensity to enhance endogenous antioxidant enzymes at cellular levels. Therefore, in this study we addressed two issues: (a) Effect of Arjuna on basal oxidative stress markers in HepG2 cells (b) propensity of Arjuna to modulate the activities of endogenous antioxidant enzymes and thereby potentiate cells to counter the oxidative stress.

**MATERIALS AND METHODS**

**Materials**

Aqueous extract of Arjuna (AEA) was purchased from Natural Remedies; Bangalore, India, which was prepared as follows. The sun dried bark of Arjuna was powdered and extracted in water (1:8, after passing through 80 mesh) by boiling (4 h). The extracts were subsequently filtered through muslin cloth and filtrate was spray dried. For preparation of water solution of Arjuna extracts, dried powder (500 mg), was boiled in distilled water (10 ml, 10 min) at 100°C and then centrifuged (500 Xg, 15 min). The supernatants were transferred to micro-centrifuge tubes and stored (-20°C).

The amount of total soluble solids in supernatant was measured using gravimetric analysis which served as the basis to define Arjuna concentrations.

**Characterization of Arjuna extracts**

The extracts of Arjuna were checked for compliance to monograph in Indian Pharmacopoeia (2007). The consistency of composition of Arjuna extracts, across four separate preparations, were determined in terms of their spectral [infra-red (IR) and H NMR] and chemical (HPLC) profile and found to be similar in terms of concentration of gallic and ellagic acid (supplementary Figures A-C). The Arjuna extract used in this study contained total polyphenols equivalent to 33.6% w/w gallic acid and 10.8% w/w total tannins.

**Cell line**

The human hepatocyte carcinoma (HepG2) cells were purchased from American type cell culture (ATCC, Manassas, VA) and cultured in DMEM (Sigma, Bangalore India) containing 10% heat-inactivated fetal bovine serum (Gibco, NJ, USA), 100 IU/ml of penicillin and 100 µg/ml of streptomycin. Cells were grown at 37°C in humidified air/CO2 (19:1) atmosphere and harvested at logarithmic growth. The culture medium was changed alternate days and were sub-cultured (1:3) twice/wk using 0.5% trypsin and 0.2% EDTA in PBS (0.1 M phosphate buffer with 0.9% saline, pH 7.4).

**Treating cells with AEA and preparation of cytosolic extracts**

HepG2 cells (10^5 cells/well) were grown to confluence (70-80%) in 6-well plates. Confluent cells were incubated with different concentrations of AEA (0, 5, 10, 20, 50 and 100 µg/ml) with fresh media. Each treatment was conducted in three replicates and repeated thrice.

Cells were harvested in lysis buffer (PBS with 0.1% Triton X-100) using a cell scraper at varied time points (4, 8, 12, 16, 20 and 24 h) after washing twice with ice-cold PBS. Subsequently, cells were sonicated (5 sec, pulse-10; power-30, Bandelin Sono plus, Berlin, Germany) keeping the vials in ice. Cells were then centrifuged (10,000 Xg, 15 min) and supernatants were stored at -80°C for further analysis. For each parameter, separate aliquots were used to avoid freeze thaw effect; aliquots were discarded after single use. Protein concentration was determined using “Protein determination Kit” from Cayman chemical company (CCC) (Bradford, 1976).

**Cytotoxicity (Lactate dehydrogenase, LDH leakage) and cell proliferation assay (MTT)**

The CCC’s LDH and MTT assay kits were used to measure cytotoxicity and cell proliferation effect of AEA in HepG2 cells. For both the assay cells (10^5 cells/well) were seeded in 96-well plate (outer wells not used) in serum free culture medium (120 µl) with or without Arjuna (0.1-4 mg/ml) for various time points (2-48 h). Experiments were done in quadruplets for each concentration and time points.

In cytotoxicity assay, LDH released into medium catalyzes the reduction of NAD+ to NADH and H+ by oxidation of lactate to pyruvate. Later diaphorase uses NADH and H+ to catalyze the reduction of INT (tetrazolium salt) to formazan (490-520 nm) which is proportional to LDH released (Wolterbeek and van der Meer, 2005). For cell proliferation assay MTT (10 µl) was added to each well at the desired endpoint and incubated for 4 h. After the incubation, culture medium was carefully aspirated out and formazan formed was dissolved in crystal dissolving solution (100 µl) and resultant purple colour was read at 570 nm (Francoeur and Assalian, 1996).
**Figure A.** Infra red spectral profile Arjuna extract.

**Figure B.** NMR spectral profile Arjuna extract.

**Measurement of basal oxidative stress parameters**

Lipid hydroperoxides (LHP) were measured with CCC's LHP Assay Kit which is based on redox reactions of hydroperoxides with ferrous ions to produce ferric ions detected using thiocyanate ions at 500 nm (Mihaljevic et al., 1996). Total antioxidant capacity (TAC) was measured as the ability of antioxidants in the cell lysate to inhibit the oxidation of ABTS (2,2-Azino-di[3-Ethylbenzthiazoline sulphonate]) to ABTS⁺ by metmyoglobin, monitored at 750 nm, compared to Trolox and quantified as mM Trolox equivalents.
Determination of ROS was based on the modified method of Lebel (1992). Non-fluorescent dye DCFH-DA (dichlorofluorescein di-acetate) is pre-incubated (15 min) to allow its incorporation and cleavage by esterases in the membrane-bound vesicles. The conversion of DCFH to DCF (fluorescent) due to ROS was measured after 30 min (Ex-485, Em-530nm) and was quantified from a DCF standard curve (Lebel et al., 1992).

**Measurement of activities of antioxidant enzymes**

Activities of antioxidant enzymes were analysed using CCC’s kit based on standard protocol for the same. Catalase and SOD assays were based on method described by Johansson and Borg (1988) and Flohe and Otting (1984) respectively. Similarly activities of glutathione dependent enzymes based on methods described by Flohe and Gunzler (1984) for GPX, Carlberg and Mannervik (1985) for GR and Warholm et al., (1985) for GST were assayed using CCC kits.

**Measurement of Glutathione (GSH)**

The sulfhydryl group of GSH reacts with DTNB (Elman’s reagent, 5,5-dithiobis-2-nitrobenzoic acid) producing TNB (5-thio-2-nitrobenzoic acid) which is reduced by glutathione reductase (GR) to recycle the GSH. The rate of TNB (measured at 405 nm) production is directly proportional to this recycling reaction which in turn directly proportional to the concentration of GSH in sample measured using CCC’s kit (Eyer and Podhradsky, 1986).

**Statistical analysis**

All values expressed as mean±SD of 9 measurements. Statistical analysis was performed using Holm-Sidak test. This test being more powerful than Turkey and Bonferroni test is recommended as the first line procedure for most multiple comparisons (Sigma Stat 3.5, Systat Software, Inc., CA). A P<0.05 value was considered statistically significant.
RESULTS

Effect of Arjuna on cell proliferation and cytotoxicity

Arjuna upto 100 µg/ml did not affect the cell viability (Figure 1a). However, Arjuna at 250 µg/ml and above caused a significant decrease in viability of HepG2 cells (21-79%). At the highest concentration (4 mg/ml) of Arjuna tested, the % viability was reduced to 21%. A 24 h treatment with 10, 25, 50, 100 and 250 µg/ml of Arjuna did not change LDH leakage into culture medium indicating that these doses are not cytotoxic (Figure 1b). However, Arjuna at the doses above 250 µg/ml caused a significant and dose dependent increase in LDH leakage. Considering these results, the non-cytotoxic doses of Arjuna were selected for further study.

Effect of Arjuna on basal OS parameters

Baseline LHP’s (Figure 2a) varied according to time of sampling. In general, there was concentration and time dependent decrease in the basal LHP’s in cytosol of HepG2 cells incubated with different concentrations and length of exposure to Arjuna. Intermediate and higher concentrations (50 and 100 µg/ml) of Arjuna offered significant protection (12-48%) at all time points. In our study, low and moderate doses (5, 10 and 15 µg/ml) did not alter any biochemical parameters of OS considerably at any time point tested. Further, the modulatory effects of Arjuna did not vary much between 12 and 16 and 20 and 24 h for all parameters. Hence data at these time points are not shown. The basal ROS levels at 4 h were 246±19 which increased to 276.69±13 at 24 h.
Figure 2. Effect of Arjuna on a) levels of lipid hydroperoxides (LHPs) and b) pattern of generation of reactive oxygen species (ROS) and c) total antioxidant capacity of cells in HepG2 cells incubated for various time points. Values are mean±SD; Data analyzed by Holm Sidak aP<0.05. TA denotes Arjuna. The number against it denotes concentration in µg/ml.

(Figure 2b). All doses significantly reduced the basal ROS levels (20-46%). The highest protection (46%) was observed at 24 h with 100 µg/ml Arjuna. Data on the basal TAC is presented in the Figure 2c. The basal TAC varied from 2.52±0.21 (4 h) to 3.52±0.47 (24 h). Even at intermediate concentrations, Arjuna enhanced TAC of
Alterations in the activity of antioxidant enzymes by Arjuna

Modulation in the SOD activity by Arjuna in HepG2 cells is depicted in Figure 3a. Basal SOD activity did not vary over the time and ranged between 31.05±0.38 (4 h) and 35.86±0.65 (24 h) U/min/mg protein. While lower conc. of Arjuna enhanced SOD activity only at 8 h, intermediate concentration increased the activity at all time points (19-35%). Further, it was found to be increased by 34 and 56% at 4 and 24 h, respectively with higher concentrations of Arjuna. The catalase activities in cell cytosol of HepG2 incubated with Arjuna and that of controls are illustrated in Figure 3b. The basal enzyme activity varied from 91.05±9.4 at 4 h to 105.86±10.6 at 24 h. Even with 8 h of incubation, all the three tested dose of Arjuna enhanced the catalytic activity by 20-36%. The increase in catalytic activity was more discernible at intermediate and higher concentration at all time points ranging between 21-73%.

Alterations in the GSH and GSH based enzymes

Lower dose of Arjuna did not alter the GSH levels appreciably at any time points (Figure 4a). However, intermediate concentrations increased the GSH levels moderately (20-35%) at 4, 12 and 24 h. At higher concentrations, Arjuna enhanced the levels of GSH at all the time points. While at 8 and 12 h, the increase was 22 and 37%, at 24 h the increase was 62%.

Data on the effect of Arjuna on the activities of glutathione based antioxidant enzymes are illustrated in Figure 4b, c and d. In general, lower dose (25 mg/ml) of Arjuna failed to evoke a significant increase in any of the
Figure 4. Effect of Arjuna on (a) levels of reduced glutathione (GSH) and activities of glutathione based antioxidant enzymes b) Glutathione peroxidase (GPX) c) Glutathione reductase (GR) and d) Glutathione S Transferase (GST) in HepG2 cells incubated for various time points. Values are mean±SD; Data analyzed by Holm Sidak aP<0.05. TA denotes Arjuna. The number against it denotes concentration in µg/ml.
Arjuna. Arjuna acid was found to decrease the sodium-oxidative damage to cells has not been studied using shown in different experimental models such as cell-free evaluation of intracellular ROS which is good indicator of in vitro Arjuna extracts determined by other methods has been efficiently. The antioxidant and free radical activity of cells to counter the basal ROS production more points indicate that active ingredients in Arjuna has ROS with 50 and 100 µg of Arjuna at all the tested time.

**DISCUSSION**

Terminalia Arjuna is a major constituent of several herbal formulations for cardio-protection and is shown to have cardioprotective properties (Dwivedi, 2007). While several studies have investigated protective effect of various extracts of Arjuna against xenobiotics and pathophysiological conditions, and attributed it to its potential antioxidant property, the evidence on antioxidant effect of Arjuna is lacking. Therefore, in this study we investigated the effect of Arjuna on basal OS markers in HepG2 cells.

HepG2 is a well differentiated transformed cell line in which steady-state functioning of the antioxidant defenses is relatively higher than that in hepatocytes and other non-transformed cells which makes it better and more sensitive system for studying the variations of responses to different conditions (Alia et al., 2006). Arjuna, in the present study was not cytotoxic till 100 µg/ml which deviates from the earlier studies reporting significant cytotoxicity and growth suppression of HepG2 (Sivalokanathan et al., 2006), osteosarcoma and glioblastoma cells (Nagpal et al., 2000) in vitro with ethanolic, methanolic and acetone extracts between 24 and 48 h.

Difference in extracts (aqueous v/s ethanolic/methanolic/acetone) and incubation time (24 v/s 48 h) may partly explain for this variation. Also, Arjuna has been used in amounts as high as 500 mg/kg body weight in rodent models and reported no hepatic (Devi et al., 2007), renal or hematological perturbations (Raghavan et al., 2006). Significant protection against generation of ROS with 50 and 100 µg of Arjuna at all the tested time points indicate that active ingredients in Arjuna has stoichiometrically scavenged the ROS or potentiate the cells to counter the basal ROS production more efficiently. The antioxidant and free radical activity of Arjuna extracts determined by other methods has been shown in different experimental models such as cell-free in vitro assays (Munasinghe et al., 2001). However, direct evaluation of intracellular ROS which is good indicator of oxidative damage to cells has not been studied using Arjuna. Arjunic acid was found to decrease the sodium-azide induced ROS levels in rat liver mitochondria in vitro (Sun et al., 2008). Here we report for the first time the inhibition of ROS generation in a dose dependent manner by Arjuna using DCF based assay system in cell model. In a study involving bark of Arjuna, cinnamon, tea leaves, capsicum and turmeric showed that Arjuna contains 12, 4.5, and 3.0 times more flavonoids, than turmeric (rhizome), tea (leaves) and cinnamon (bark). A higher amount of flavonoids present in Arjuna compared to other plant food items may be responsible for its high ROS scavenging effect (Nair and Nagar, 1997). LHP’s has not been estimated in any reported studies and hence could not be compared. Quantification of LPO in terms of TBARS has been done in several studies and all these studies have consistently shown that Arjuna reduced prooxidant induced lipid peroxidation in various tissues (Manna et al., 2007; Sivalokanathan et al., 2006). However, very few studies have reported the reduction in the basal LPO. While one study reports increase in basal LPO in rat heart, other studies found no change in basal LPO in gastric mucosa (Devi et al., 2007), hepatic, renal (Raghavan and Kumari, 2006) and cardiac tissues (Manna et al., 2007; Singh et al., 2008) of rat administered methanolic, hydroalcoholic and ethanolic extracts. However administration of whole bark powder (500 mg/kg body weight, as 2% Carboxymethyl cellulose for 12 weeks) significantly decreased LPO in heart of rats (Gauthaman et al., 2001). Our data show that formation of basal lipid hydroperoxide diminished significantly in presence of Arjuna. The efficacy may be arising due to flavonoids, which by virtue of their free radical scavenging ability prevent oxidation of lipids.

The TAC may provide more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in the cell cytosol (Rice-Evans and Miller, 1994). Although, TAC has been used as valuable marker to study the effects of many other phytonutrients in cell models, there are no studies which utilized this parameter to study the antioxidant effect of Arjuna in any tissues or cells. Here we report that Arjuna at higher concentration (50 and 100 µg) elevated the TAC of cells.

An increase in GSH concentration prepares the cell against a potential oxidative injury (Alia et al., 2006). Arjuna at higher concentrations significantly enhanced the GSH in our study. Solvent extracts (ethanolic/methanolic) of Arjuna at tested doses did not alter the GSH levels in cardiac tissue (Singh et al., 2008), liver and kidney (Raghavan and Kumari, 2006) and gastric mucosa (Devi et al., 2007). However, both short term (ethanolic extract, 9.75 mg/kg bw, 4 wk) and long term (whole bark powder, 500 mg/kg bw, 12 wk) administration of Arjuna significantly enhanced the basal GSH levels in cardiac tissue (Karthikeyan et al., 2003). On the contrary, ethanolic extracts of Arjuna incubated for 48 h significantly decreased the GSH levels in HepG2 cells and was responsible for induction of apoptosis.
The variations in the effect of Arjuna in terms of modulating the basal oxidative stress parameters in rat tissues may arise due to fact that the different extracts (methanolic, aqueous, ethanolic, acetone and whole bark powder) has been tested by various authors using different dose and also the target tissues were different in which these parameters were assayed.

The amount of SOD present in cellular and extracellular environments is crucial for the prevention of diseases linked to OS as activity of this enzyme in cells dissipate superoxide $\text{O}_2^-$ keeping its amounts low (Beckman and Koppenol, 1996). In our study, Arjuna at concentrations of 50 and 10µµg raised the activity of SOD. This observation is consistent with studies which have reported the increase in SOD activity in various tissues of rat in response to Arjuna (Gauthaman et al., 2001).

However, few studies found the administration of organic solvent extracts did not alter basal SOD activity in gastric mucosa (Devi et al., 2007), cardiac (Singh et al., 2008), hepatic and renal tissue of rat (Raghavan and Kumari, 2006).

Catalase is involved in the detoxification of hydrogen peroxide and is present in highest concentrations in liver, kidney and erythrocytes, where it is believed to account for the majority of hydrogen peroxide decomposition. Earlier reports indicate that mucosal (Devi et al., 2007), hepatic and renal (Raghavan and Kumari, 2006) tissue catalase activity was unaltered whereas cardiac catalase activities was enhanced in rats administered with solvent extracts of Arjuna (Gauthaman et al., 2001). Our study demonstrates that exposure of HepG2 cells to aqueous extract of Arjuna significantly enhances activities of catalase in HepG2 cells. Increase in both SOD and catalase activity due to Arjuna has a special significance in relation to defense against OS. The most abundant oxidative free radicals generated in living cells are the superoxide anions which are inactivated by SOD. An increase in SOD activity is beneficial in the event of increased free radical generation (Yen et al., 1996). However, it has been reported that an augmented SOD activity, without a concomitant rise in the activity of catalase and/or GPX might be detrimental, since SOD generates hydrogen peroxide, which is cytotoxic and must be scavenged by catalase or GPX. Thus a simultaneous increase in catalase and/or GPX activity is required for an overall antioxidant effect in HepG2 cell. These results become even more relevant when viewed in the light of a fundamentally new approach to antioxidant therapy which emphasizes on sustained long term induction of endogenous antioxidant enzymes to be more beneficial rather than the conventional scavenging of free radicals at cellular levels (Nelson et al., 2006).

In summary, we find that aqueous extracts of Arjuna can modulate basal OS parameters in HepG2 cells. The aqueous extracts of Arjuna demonstrated significant antioxidant effect in HepG2 cells by reducing generation of ROS and LHP thus preventing or delaying conditions which favour OS in the cell. This effect may be brought about by an increase in the activity of antioxidant enzymes SOD and catalase leading to a sustained and faster dissipation of superoxide and hydrogen peroxide in addition to a stochiometric scavenging of free-radicals potentially by flavonoids. The emerging view is that antioxidant compounds could exert beneficial effects on cells not only through antioxidant potential but also through the modulation of different pathways such as signaling cascades, anti-apoptotic processes or gene expression. The effect of Arjuna on antioxidant gene expression and redox signaling pathways remains to be investigated.

**ACKNOWLEDGEMENTS**

The authors are grateful to Dr. Vilas Sinkar, for his keen interest and for critically reviewing the manuscript, Dr. Shovan Ganguly, for his support, Ms Aparna Damle, for her assistance with cell culture, Mr. Deepak Mhasavade for infrared, HPLC and NMR spectral analysis of Arjuna extracts.

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