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**Abstract**

*Terminalia arjuna* is an important medicinal plant widely used in the preparation of Ayurvedic formulations used in cardioprotection. Only one sapogenin acid i.e. arjunolic acid has been shown to provide cardiac protection in induced necrotic rats but no other report for the biological activity of any of the isolated components is available. The present work is to rationalize all saponins for their biological effects using oxidative mechanisms as reactive oxygen species (ROS) implicated in many pathogenic processes including the cardiovascular system. Two sapogenin namely arjungenin and arjunic acid and their respective glucosides arjunetin and arjunglucoside II, isolated from the alcoholic extract of bark of *Terminalia arjuna* were tested for their action on the free radical scavenging action using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, effect on the superoxide release from PMN cells using NBT (Nitroblue tetrazolium) reduction assay, and hypochlorous acid release from human neutrophils using luminol enhanced chemiluminescence assay. Arjungenin was found to be most active as direct free radical scavenger and inhibitor for the hypochlorous acid production followed by its glucoside that was almost 50% active.

**INTRODUCTION**

The damage due to excess ROS during the oxidative stress is known to play an important role in the pathogenesis of various human diseases (Finkel and Holbrook, 2000). Reactive oxygen species (ROS) are implicated in many pathogenic processes including the cardiovascular system. The research in the cardiovascular disease implies that oxidants play an important role in the atherogenesis and in epidemiological studies these diseases are associated with lower plasma concentrations of antioxidant enzymes. The reperfusion injury in ischemic tissue is the result of radicals produced by enzyme xanthine oxidase and is reported to be reduced by agents that decrease the formation or scavenge free radicals (Halliwell, 1987). There are increasing evidences where these diseases exponentially increase with age. One common feature of many of these conditions is the recruitment of inflammatory cells that leads to oxidative stress (Finkel and Holbrook, 2000). Further, there is a growing body of evidence suggesting that antioxidants contribute to cardioprotection (Maxwell, 1995).

*Terminalia arjuna* (TA) is an Ayurvedic medicine for over three centuries, primarily used as cardiac tonic. The bark is reported to contain tannins, triterpenoid saponins, flavonoids, gallic acid, ellagic acid, oligomeric proanthocyanidins, and phytosterols. In in vitro studies the aqueous extract of TA showed significant DPPH radical scavenging activity that was similar to L-ascorbic acid (Munasinghe et al., 2001). Further the bark is also reported to augments endogenous antioxidant compounds of rat heart like SOD, GSH and CAT and also prevents oxidative stress associated with IRI of the heart (Gauthaman et al., 2001). In a randomized controlled trial the bark powder had shown to exhibit significant hypcholesterolaemic effect (Gupta et al., 2001). In rabbits TA showed hypolipidemic activity and induced partial inhibition of rabbit atheroma (Shaila et al., 1998). In another clinical study, TA significantly reduced the anginal...
frequency and provided symptomatic relief in coronary heart failures. There is only one report in which an isolated chemical constituent was ascribed for the activity. Arjunolic acid treatment has been shown to provide significant cardiac protection in isoproterenol induced myocardial necrosis in rats by preventing the decrease in the levels of superoxide dismutase, catalase, glutathione peroxidase, ceruloplasmin, α-tocopherol, reduced glutathione (GSH), ascorbic acid, lipid peroxide, MPO (Sumitra et al., 2001). The above reports confirm that TA is a cardioprotective drug having traditional origin.

The present investigation aims to evaluate sapogenins and saponins, isolated form TA, for their action on the release of reactive oxygen species from the phagocytic cells. Sapogenins arjunic acid and arjungenin and their respective glucosides arjunetin and arjunglucoside II were isolated from the methanolic extract of bark of TA. These compounds were tested for direct free radical scavenging activity in the DPPH assay, their action on the superoxide release from the human PMN cells in the NBT reduction assay and hypochlorous acid production from human neutrophils measured in the luminol enhanced chemiluminescence assay. The results indicate that arjungein and its glucoside are moderately active in the above assays.

MATERIALS AND METHODS

Plant Material
The dried bark of the *Terminalia arjuna* was purchased from local market at Chandigarh. The corresponding author identified the material and the voucher specimen is preserved in the Herbarium of our institute.

Chemicals and Reagents
Diphenyl picryl hydrazyl, luminol, Nitroblue tetrazolium were procured from Sigma chemicals, USA, and the solvents were obtained from Ranbaxy, India. Fresh human blood was collected from healthy human volunteers by venipuncture.

Isolation of Constituents
The dried and powdered bark was defatted with n-hexane and extracted with methanol. The methanolic extract on de-tannification with lead acetate was subjected to column chromatography on silica gel with chloroform-methanol mixtures. Two sapogenins (1 and 2) and two saponins (3 and 4) were obtained on repeated column chromatographies. The extensive chemical and spectral studies along with comparison with the literature spectral data confirmed their chemical structures to be arjunic acid (1) (Row et al., 1970a), arjungenin (2) (Honda et al., 1976a), arjunetin (3) (Row et al., 1970b) and arjunglucoside II (4) (Honda et al., 1976 b).

DPPH Assay
Assay was performed by the method of Bouchet and Viturro (Viturro et al., 1999; Bouchet et al., 1998). DPPH solution (95 µL) was incubated in dark with methanolic solutions of the test compounds (5 µL) in microtitre plate well. After 15 min. the absorption was measured at 515 nm. Ascorbic acid was used as a standard. The IC<sub>50</sub> was calculated by SigmaPlot software.

Chemiluminescence Assay
The assay was performed and standardized according to the method prescribed in the manual of the luminometer. To cell suspension, luminol stock solution was added so as to attain the concentration of 0.5 mM. The cell suspension containing luminol was added to the microtitre plates containing test compounds in triplicate. After 5 min PMA (150 µL) was added to trigger the reaction. The microtote plate was measured in kinetic mode for 90 min. during which each well was read for 740 ms. A curve of light intensity (RLU) was plotted against time and the area under curve (AUC) was calculated as total luminescence. The percent inhibition of luminescence was calculated as
\[
\text{% inhibition of luminescence} = \frac{(\text{Control} - \text{sample})}{\text{control}} \times 100
\]

**NBT Reduction Assay**

We have followed the method of Richardson (Richardson et al., 1998), in which the cell suspension (50 µL) was incubated with different concentrations of the extract/fractions/compounds in triplicate in a microcentrifuge tube for 5 min. In case of control the cells were incubated with the medium alone. NBT (200 µL) and opsonized zymosan (100 µL) were added and incubated at 37°C. After 30 min of incubation the reaction was quenched by the addition of 1 mL of 1N HCl. To the cell pellet obtained by centrifugation, pyridine was added and heated on a water bath for 30 min at 70°C. The pyridine extract of the cell mass was transferred to microtitre plates and absorbance was measured at 515 nm on ELISA plate reader. The percentage inhibition of NBT reduction was calculated by the formula

\[
\text{% inhibition of NBT reduction} = \frac{(\text{Control} - \text{sample})}{\text{control}} \times 100
\]

**RESULTS AND DISCUSSION**

Four compounds namely, arjunic acid (1), arjungenin (2), arjunetin (3) and arjunglucoside II (4) were isolated from the methanolic extract of bark of TA and were subjected to the three bioassays. The sapogenins differ in the hydroxylation at the C-23 position. All the compounds were tested in DPPH assay. Only 2 and 4 were found to be moderately active as free radical scavengers with an IC$_{50}$ value of 290.6 and 669.9 µg/mL, respectively. Further, a maximum of 58% inhibition was achieved with 4 at the highest concentration of 1200 µg/mL. The compounds (1) and (3) were found to be inactive at the tested concentrations. In NBT reduction assay all the compounds (1-4) were found to be inactive at the tested concentrations in the assay. Therefore, it can be concluded that *T. arjuna* saponins have no action on the superoxide production from the human PMN cells. Further, in the chemiluminescence assay the saponins (3) and (4) were seen to have weak activity on the chemiluminescence response while the aglycone 1 is about half active as compared to the 2.

On reviewing the results of the DPPH and chemiluminescence assay for compound (2) and (4), it was observed that the glycoside has lower activity as compared to their aglycones. Therefore, it may be postulated that glycosylation at C-28 position does not significantly contribute to the bioactivity. A comparison of the results of compound (1) and (2) reveals that the presence of 23-hydroxyl group enhances the free radical scavenging activity and inhibits the chemiluminescence response at the tested concentrations.

From the plant material used for the present study, it was not possible to isolate arjunolic acid that have hydroxylation at 2, 3, 23 positions. It would be interesting to evaluate their biological activity and derive a relationship for the structural requirement of the 19-hydroxyl group. At our hands, arjungenin exhibited the maximum activity. This would be of interest to develop its structural analogues for enhancing the activity. The literature reports for the free radical scavenging activity of the bark is supposed to be mainly due to the higher amounts of tannins. *Terminalia arjuna* is known for its cardioprotective activity in the literature. Saponins are thought to be responsible for the activity but no reports are available for activity of isolated constituents. The present study is the first to report the bioactivity of these saponins on respiratory oxyburst. Arjungenin and its glucoside were found to be active as inhibitors in DPPH and chemiluminescence assay. Both the compounds are moderately active but warrant animal testing to confirm the in vitro finding.

**Literature Cited**


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