Chapter – II

Studies on Terminalia arjuna
CHAPTER – 2
STUDIES ON TERMINIALIA ARJUNA

2.1 INTRODUCTION

*Terminalia arjuna* [Roxb. Ex DC] Wight & Arnott belongs to the family Combretaceae and is known as *Arjuna* in Sanskrit and *Marudam* in Tamil. It is used in the indigenous system of medicine. The other vernacular names are given below.

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*Terminalia arjuna* [TA] (Photograph 1a – 1c) is found all over the country in deciduous forests. It is found in Andhra Pradesh, Assam, Bihar, Gujarat, Jammu and Kashmir, Maharashtra, Karnataka, Tamil Nadu, Uttar Pradesh and West Bengal.

It is a large tree with huge, often buttressed trunk and horizontally spreading branches; bark smooth grey, flaking-off in large flat pieces. Leaves usually sub-opposite, 10 – 15 by 4 – 7 cm; oblong or elliptic oblong, obtuse or sub-acute, pale dull green
Photograph 1. *Terminalia arjuna*

A. Tree

B. Shoot with flower

C. Bark, Leaf and Fruit
above, pale brown beneath, shallowly crenate-serrate in the upper part or sometimes throughout, base rounded or chordate, often unequal sided; main nerves actuate, 10 – 15 pairs, veins reticulate, pellucid; petioles 6 – 10 mm long, with 1 or usually 2 prominent glands at the top immediately below the leaves. Flowers sessile, in short auxiliary spikes or in terminal panicles, bracteoles linear-lanceolate, shorter than the flowers, caduceus, calyx glabrous. Teeth triangular. Ovary quite glabrous, disk cothed with yellowish or reddish hairs, drupe 2.5 – 5 cm, ovoid or obovoid-oblung, fibrous-woody, glabrous, dark brown with 5 hard projecting wings striated with numerous curved veins. Bark smooth, pinkish grey, sapwood, reddish white, heartwood brown, variegated with darker coloured streaks.

2.2 REVIEW OF LITERATURE

2.2.1 Medicinal properties

The bark is medicinally used in the indigenous system of medicine. It is astringent, sweet, cooling, aphrodisiac, demulcent, cardio tonic, styptic, antidysentric, urinary astringent, expectorant, alexiteric, lithontriptic and tonic. It is useful in fractures, ulcers, ureathorrhoea, spermatorrhoea, leucorrhoea, diabetes, anaemia, cardiac disorders, excessive perspiration, fatigue, asthma, bronchitis, cough, intrinsic haemorrhages, tumor, otalgia, inflammations, skin diseases like freckles, wound healing, haemorrhoids, cirrhosis of liver and hypertension. The fruit is tonic and deobstruent. The juice of the fresh leaves is used in ear-ache. Reviews on TA have been published.
2.2.2 Phytochemical Review on *T. arjuna*

The chemical constituents isolated from the plant include tannins (15 - 25%), triterpenoids, terpenoid saponins, flavonoids, flavonoid glycosides, mannitol, phytosterols, magnesium, zinc, copper, sodium and large amounts of calcium.

2.2.2.1 Tannins

The bark contains 15-25% tannins, both pyrogallol and catechol tannins, gallic acid, ethyl gallate, catechol, gallocatechol, epicatechol, epigallo catechol and ellagic acid showed to be present in the bark\textsuperscript{31-33}. Chemical investigation of the bark of TA has led to the isolation and characterization of rare complex type tannin in addition to nine known hydrolysable tannins\textsuperscript{33}.

2.2.2.2 Flavonoids

Leucodelphinidine has been isolated and reported from the heart wood of TA\textsuperscript{34}. Cerasidine (5, 7, 2’, 4’ tetra methoxy flavone) – a new flavone was isolated from the ether soluble portion of the methanolic extract of the fruits\textsuperscript{35}. Another new flavone – arjunolone was isolated along with baicalin from the stem bark of TA and its structure was elucidated as 6, 4’ dihydroxy, 7 methoxy flavone\textsuperscript{36}. From the root extract of TA, leucocyanidine was isolated and reported\textsuperscript{37}. Quercetin – 7 – O – rhamnoside and Aformisin had been reported from the fruits the fruits of the plant\textsuperscript{38}. Luteolin\textsuperscript{39} and apigenin-7-o-neohesperidoside\textsuperscript{40} have been reported from the methanolic extract of the leaves.
2.2.2.3 Triterpene aglycones

Arjunolic acid (2α, 3β, 23α - trihydroxy olean-12-en-28-oic acid) has been first reported from the bark of the plant. Tomentosic acid and arjunic acid (2α, 3β, 19α - trihydroxy olean-12-en-28-oic acid) has been reported from the fruits and bark respectively. Arjunenin another new triterpene aglycone (2α, 3β, 23-tetra hydroxy olean 12-en-28-oic acid) had been reported from the bark. Oleanolic acid, methyl oleanolate and friedelin, maslinic acid were also reported from the bark of TA. Terminic acid (3β, 13β dihydroxy lup-20 (29)-en-28-oic acid) - a lupeol derivative and terminoic acid (2α, 3β, 19α-trihydroxy olean-12-en-29 oic acid) and were also reported from the plant. The design and synthesis of the first arjunolic acid derived 18-crown-6 and its binding studies with metal and tertiary butyl ammonium ions are reported.

2.2.2.4 Triterpene glycosides

Arjunetin whose structure was elucidated as 28-carboxy-β-D (+) glycopyronoside of arjunic acid has been reported from the root bark as well a stem bark of the plant. Arjunoglucoside I (β-D- glycopyronosyl-2α, 3β, 19α, 23-tetra hydroxy olean-12-en-28-oate) and arjunoglucoside II (β-D- glycopyronosyl-2α, 3β, 23-tri hydroxy olean-12-en-28-oate) were reported from the bark. Arjunoglucoside III (β-D-glycopyronosly -2α, 3β, 19α trihydroxy - 11-oxo-olean 12-en-28-oate has also reported from the plant. From the root bark of the plant, the following new arjunic acid glycosides viz., arjunoside III (28-β-D (+)-glucuropuronoside of arjunic acid) and arjunoside IV (3-O-α-L(-) rhamnopyranoside of arjunic acid) were reported. Arjunoside I (Arjunic acid – 3-O-β-D-galactopyranose) and arjunoside II (Arjunic acid – 3-O-(β-D-galactopyranosyl) - α-L- 2-deoxy rhamnoside were also reported from TA. Arjunolotin, which was earlier
reported from the plant, was shown to be 3-O- (\(\beta\)-D-glucopyranosyl -2\(\alpha\), 3\(\beta\), 23-trihydroxy olean-12-en-28-oic acid-28-O-\(\beta\) glucopyranoside\(^{52}\). A new triterpene glycoside, terminolitin has been isolated from the fruits of the plant and the structure was elucidated as 23-deoxy arjunolotin\(^{53}\). Arjunetoside – a new triterpene glycoside has been isolated from the root bark of TA and its structure has been established as 3-O-\(\beta\)-D glycopyranosyl-2\(\alpha\), 3\(\beta\), 19\(\alpha\) - trihydroxy-olean-12-en-28-oic acid 28-O - \(\beta\)-D glucopyranoside\(^{54}\). Another new triterenoid glycoside 2\(\alpha\), 19\(\alpha\) dihydroxy 3-oxo-olean-12-ene-28-oic acid-28-O -\(\beta\)-D glucopyranoside was isolated from the roots TA and has been reported\(^{55}\). A rapid sensitive and reproducible reversed phase HPLC method for the simultaneous quantification of major oleane derivatives has been reported\(^{56}\). HPTLC method of simultaneous quantitative determination of five oleane derivatives has been reported\(^{57}\). Terminoside A (Olean-1\(\alpha\), 3\(\beta\), 22\(\beta\)-triol-12-en-28oic acid-3-\(\beta\)-D glucopyranoside) – a new oleanane type triterpene was isolated from the stem bark of TA\(^{58}\). Arjunaphthanoloside – a novel naphthol glycoside was isolated form the stem bark of TA and its structure was established as 2, 3, 6, 7, 8, 9 - hexa hydroxy naphthalene-2-O- \(\alpha\) - L (-) rhamnoside by means of spectroscopical and chemical methods\(^{59}\).

2.2.2.5 Steroids

\(\beta\)-Sitosterol\(^{37, 41, 42, 43, 46 \& 47}\) and \(\beta\)-Sitosterol-\(\beta\)-D-glucoside\(^{38}\) have been isolated and reported from the plant.
Structures of phytoconstituents isolated from *Terminalia arjuna*

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Arjunoglucoside III
2.2.2.6 Cardionolides

From the ethyl acetate soluble fraction of the methanolic extract of the seeds, a new cardinolide 14, 16-dianhydro gitoxigenin-3-β-D-xylopyranosyl (1 → 2) O-β-D-glucopyranoside\(^60\) and another cardionolide - 16, 17 dihydroneridienone-3-β-D-glucopyranosyl (1→6) O-β-D-glucopyranoside was isolated from the seeds and been reported\(^61\).

2.2.2.7 Other Compounds

8 hydroxy hexadecanoic acid has been reported from the root bark\(^46\). Hentriacontane, myrisyl oleate and arachidic stearate were reported from the fruits\(^62\).
2.2.3 Pharmacological Review on T. arjuna

2.2.3.1 Cardiovascular related activities

2.2.3.1.1 Experimental Reports

Hypotensive activity

Intravenous administration of the methanolic extract of TA was found to induce dose dependent decrease in blood pressure and heart rate in dogs. The possible mechanism involved in the hypotensive response of the extract was studied on pressor responses induced by bilateral carotid occlusion, norepinephrine injection and electrical stimulation of preganglionic fibers of abdominal splanchnic nerve. TA extract in graded doses (1-5 mg/kg, I.V.) neither modified norepinephrine response nor the splanchnic nerve stimulation response. This rule out the possible blockade of autonomic, adrenergic neuron and peripheral adrenergic receptors. But the extract inhibited the carotid occlusion response there by indicating involvement of the central nervous system in the hypotensive effect. The cardiovascular effects of methanolic extracts of TA, Terminalia chebula and Terminalia bellerica were studied both in-vivo and in-vitro methods using isolated frog, rat atria, perfused frog, rabbit hearts, isolated rabbit aortic rings and in dogs. All the plant extracts (9 mg/kg to 36 mg/kg by I.V.) produced a hypotensive effect. This hypotensive response of the plant extract was abolished by pretreatment with atropine (0.5 mg/kg/IV) suggesting a muscarinic action. An aqueous extract of TA produced a transient decrease in blood pressure accompanied by a slight decrease in heart rate. The hypotensive effect was observed with a fraction (F2) containing tannin related compounds separated from the aqueous extract of TA. The hypotensive effect of F2 was not affected by pretreatment of rats with propranolol but was attenuated by pretreatment with atropine. The effects on blood pressure of acetylcholine, epinephrine, isoprenaline and bilateral carotid occlusion were not affected by treatment with F2. These results
suggest that an aqueous fraction of TA containing tannin related compounds has hypotensive effect which may be mediated by cholinergic mechanisms. On contrary to both of the above studies, the hypotensive activity through cholinergic action was ruled out. Intra venous administration of TA produced dose dependent hypotension in anaesthetized dogs. The hypotension produced by the extract was blocked by propranolol but not by atropine or mepyramine maleate. This ruled out the muscarinic or histaminergic mediated hypotension. The blockade by propranolol indicated that the extract might have adrenergic $\alpha_2$ receptor agonist action and/or act directly on the heart muscle.

**Myocardial infarction studies**

TA bark powder (500 mg/b.d. daily) along with *Inula racemosa, Saussurea lappa* administered for 90 days to rabbits and then challenged with isoproterenol sulphate (2 mg/kg) to induce myocardial ischaemia. The aorta was removed and subjected for bioassay of PG E$_2$ like activity. There was an enhancement of aortic PG E$_2$ like activity in all the drug treated rabbits. PG E$_2$ by inducing coronary vasodilatation and inhibiting platelet aggregation thought to prevent myocardial ischemia.

TA bark powder [250 and 500 mg/kg] when administered to isoproteranol induced infarcted rats, reduced circulating cholesterol levels and the catecholamine content in the brain and blood, which prove it to be anti-thrombotic, anti-arrhythmic and anti-hypertensive. A marked increase in the catecholamine content of the adrenal gland along with the significant decrease in circulating catecholamine levels in infarcted rats are due to the inhibition of catecholamine release into the circulation from the adrenal gland, which may further protect the heart from the catecholamine cardiotoxicity.
Abana a herbomineral preparation contains TA 30 mg along with other herbal drugs, showed significant protection against the biochemical changes in isoproterenol induced myocardial necrosis. The drug reversed the increased levels of serum creatinine phosphokinase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and γ glutamyl transpeptidase, succinate dehydrogenase. Mitochondrial oxygen uptake in heart were also significantly protected. This showed that Abana treatment could contribute in restoring myocardial integrity and cardiac function disturbed by isoproterenal induced ischemia.

The aqueous extract of the bark of TA, in a dose range of 1-1024 µg was injected in the isolated perfused rabbit heart preparation and the effect of each dose at the end of the 1st 3rd, and 5th min of its administration on the cardiac rate, amplitude of contraction and coronary flow was recorded. TA produced dose dependent bradycardia and inotropic effect and also enhanced the coronary flow at and above the doses of 64 µg and 256 µg respectively. The maximal responses for all the parameters were observed at 1024 µg in the 1st min. The cardio protective effect of TA was also evaluated in wistar rats. It indicated that the aqueous extract of TA enhanced coronary flow, decreased blood pressure and also reduces the intensity of isoproteranol induced myocardial necrosis in experimental animals.

Positive ionotropic activity

Aqueous, ethanolic, chloroform and petroleum ether extracts of TA were studied on isolated atria of rats. The aqueous extract produced a substantial positive ionotropic effect but no change in the heart rate. The ethanol, chloroform and petroleum ether extracts all decreased the rate of contraction. A slight increase in force was seen with ethanol and chloroform extracts. Higher concentrations of the chloroform extract had a
negative inotropic effect. The increase in the force of contraction was most evident with the water extract progressively smaller effects were found with the ethanol and chloroform extracts and petroleum ether extract was inactive. This suggests that the increase in force is produced by a polar compound. Reduction in rate of contraction was most produced in chloroform extract, less activity was found in the ethanol and petroleum ether extracts. This suggested that the negative inotropic action is produced by a compound or group of compounds having medium polarity.

The aqueous extract of TA produced positive inotropic effect of the rat atria and the maximum contraction was comparable as that of isoprenaline. The positive inotropic effect was completely blocked by a β-adreno receptor blocker propranolol and an uptake-1 blocker cocaine. In precontracted aorta, the aqueous extract produced a contraction followed by relaxation. Propranolol did not block the relaxant effect of the aqueous extract. It is concluded that the positive inotropic effect was mediated via an action on β1 adrenoceptor and was likely due to the release of noradrenaline from the sympathetic nerve endings. The vasorelaxant effect, however, was not mediated via an action on β2 adrenoceptor.

**Ischaemic Reperfusion Studies**

Coronary flow was measured on the isolated perfused rabbit heart after administration of different doses of the TA extract (1 to 1024 µg) at the time intervals of 1st, 3rd and 5th min. The results showed coronary flow was significantly enhanced with higher doses of the extract. The report supported the antianginal activity of TA. TA bark powder was administered orally to wistar albino rats in two doses (500 & 750 mg/kg in 2% SCMC) 6 days per week for 12 week. At the end of the study, various biochemical parameters were measured. There was a significant increase in the contents of TBARS.
and loss of SOD, Catalase and GSH occurred in the vehicle treated hearts subjected to ischaemic reperfusion injury (IRI), which is an indication of increased oxidative stress. But the hearts harvested from 500 mg/kg treated rats were significantly protected from oxidative stress when subjected to *in-vitro* IRI. These findings suggested that the crude bark of TA augments endogenous antioxidant compounds of rat heart.\(^{74-76}\)

TA bark powder (500 & 750 mg/kg in 2% SCMC suspension), when orally administered for 12 weeks to rabbits, caused augmentation of myocardial antioxidants; superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) along with the induction of heat shock protein 72 (HSP 72). *In-vivo* ischaemic reperfusion injury induced oxidative stress, tissue injury of heart and haemodynamic effects were prevented in the TA treated rabbit heart. This study provided scientific basis for the therapeutic effect of TA in ischaemic rabbit hearts.\(^{77}\)

2.2.3.1.2 Clinical reports

**Antihypertensive studies**

A report on the therapeutic evolution of TA in fifty one cases of coronary heart diseases has been presented. One month trial revealed significant regulation of blood pressure and lipid profile of the treated patients was observed.\(^{78}\)

**Myocardial infarction studies**

The role of TA bark powder in ischaemic mitral regurgitation (IMR) following acute myocardial infarction was studied. Forty patients with fresh acute myocardial infarction showing ischaemic mitral regurgitation were randomly divided into two groups of twenty each. They were given placebo or 500 mg of TA in addition to anti-ischaemic treatment. After one and three months of follow-up, patients receiving
adjuvant TA showed significant decrease in IMR and considerable reduction in anginal frequency.\textsuperscript{79}

**Congestive Heart Failure [CHF]**

A decoction of TA was administered to patients of CHF, essential hypertension and cirrhosis of liver. It showed clinical improvement in 42%, 62% and 40% patients respectively. Improvement in CHF substantiated earlier claims of its cardio tonic property.\textsuperscript{80} In a double blind study in thirty patients of decompensate rheumatic valvular heart disease, treatment with 200 mg TA improved left ventricular [LV] fraction significantly.\textsuperscript{81} A double blind, placebo controlled, two phase trial of TA extract treatment in twelve patients with severe refractory heart failure [New York Heart Association (NYHA) Class IV] was conducted. Either 500 mg of TA bark extract or placebo was given every 8 hourly for two weeks, in addition to the patient’s current medication (dioxin, diuretics, angiotensin converting enzyme inhibitors, vasodilators and potassium supplementation). All patients experienced dyspnea at rest or after minimal activity at the start of the trial. Dyspnea, fatigue, edema and walking tolerance all improved when patients were on TA therapy. TA treatment was also associated with significant improvement in stroke volume and left ventricular ejection fraction, as well as decreases in end diastolic and end systolic left ventricular volumes compared to placebo. In second phase of the study, patients from phase I continued on TA extract for approximately 2 years. Improvements were also noted in the ensuing 2-3 months, and were maintained throughout the study. After four months treatment nine patients had improved NYHA class II and three improved in Class III. The study concluded that adjuvant TA therapy in the patients with refractory CHF, mostly related to idiopathic dilated cardiomyopathy, appeared safe and caused long lasting improvement in
symptoms and signs of heart failure along with improvement in left ventricular ejection phase indices with definite improvement in quality of life\textsuperscript{82}.

**Antianginal studies**

TA bark powder alleviates anginal pain in ischaemic heart diseased patients. It is also effective in those patients who have rhythmic disturbances, particularly ventricular premature beats. This drug appears to modify known coronary risk factors such as body weight, blood pressure, blood sugar and circulating catecholamine. In view of this, TA could be used in primary preventive measure in ischaemic heart diseases\textsuperscript{83}. A clinical trial involving twenty five patients of angina pectoris were administered TA bark extract (500 mg/kg, b.d.) along with other antianginal drugs for 3 months. Following 3 months therapy, reduction in grade of positivity of treat mill test (TMT) response was observed in six patients. Also improvement in exercise tolerance was also observed\textsuperscript{84}. In yet another similar study, 500 mg/kg, b.d., daily was administered to twenty patients, of which fifteen patients with stable angina pectoris (Group A) and five patients with unstable angina pectoris (Group B). In both these groups, patients experienced reduction in anginal frequency and increase in LV ejection fraction. Group A cases also exhibited decline mean systolic blood pressure and body mass index. TMT on ten patients of stable angina pectoris showed reversion from moderate to mild changes after three months of therapy\textsuperscript{85}. Effect of TA on angina pectoris, CHF and left ventricular mass was studied in patients of MI with angina and/or ischaemic cardiomyopathy. Bark stem powder of TA 500 mg, 8 hourly was administered to ten patients of post myocardial infarction angina and 2 patients of ischaemic cardiomyopathy for a period of 3 months. TA therapy has been found to improve left ventricular ejection fraction and reduced left ventricular mass in coronary artery\textsuperscript{86}. An open comparative trial of safety and efficacy of *Hartone* (herbal product containing TA) in stable angina pectoris was done. In this study,
ten patients with stable angina pectoris were given *Hartone* two capsules twice daily 6 weeks. Haematological and biochemical investigations to assess safety were carried out on 0, 4th, 42nd and 84th day. Serum lipid profile was done before and after therapy. Efficacy had assessed by considering the reduction in the number of anginal episodes and improvement in stress test. These results were compared with ten patients of stable angina pectoris on isosorbide mononitrate (ISMN) 20 mg twice daily. In this study *Hartone* afforded symptomatic relief in 80% of patients and ISMN in 70%. The number of anginal attacks was reduced from 79 per week to 24 per week by *Hartone* and 26 per week to 7 per week by ISMN. *Hartone* treatment also improved blood pressure response stress test in 2 patients and ejection in one patient \(^8^7\). A double blind placebo controlled cross over studies comparing TA with ISMN in chronic stable angina pectoris was conducted. Fifty eight males with chronic stable angina (NYHA class II & III) with evidence of provokable ischaemia on TMT received TA (500 mg 8 hourly), ISMN (40 mg/kg/daily) or a matching placebo for one week each, separated by a wash out period of at least three days in a randomized, double-blind, crossover design. They underwent clinical, biochemical and tread mill exercise evaluation at the end of each therapy, which were compared during the three therapy periods. TA therapy was associated with significant decrease in the frequency of angina and the need for isosorbide. The TMT parameters improved significantly during therapy with TA compared with placebo. The total duration of exercise increased, maximal ST depression during the longest equivalent stages of sub-maxal exercise decreased, time to recovery decreased. These benefits were similar to those observed with ISMN (40 mg/day) therapy and the extract was well tolerated \(^8^8\).
2.2.3.2 Hypolipidamic activity

TA bark powder and cholestyramine, when fed to male rabbits, the serum total cholesterol, triglycerides and VLDL – cholesterol were reduced significantly in TA treated group. Reduction in total cholesterol/high density lipoprotein cholesterol ratio was significantly more in TA treated groups\textsuperscript{89}. In another study, to one group of rabbits TA bark powder suspension along with high density lipoprotein cholesterol was administered and to the other group of rabbits, only high cholesterol diet was administered. Hypercholesterolemic rabbits receiving TA treatment showed a more marked reduction in total cholesterol and triglycerides and elevation in high density lipoprotein cholesterol than the hypercholesterolemic control rabbits\textsuperscript{90}. TA bark powder, when administered in rabbits showed significant reduction in total cholesterol and LDL cholesterol. HDL cholesterol did not alter. There was slight rise in triglycerides and VLDL cholesterol and significant reduction in total cholesterol and HDL cholesterol ratio\textsuperscript{91}. Chronic feeding of TA (100 mg/kg, p.o.) to rats, simultaneously fed with cholesterol (25 mg/kg) for 30 days, caused lowering of β-lipoproteins level followed by an increase in HDL compared with the cholesterol fed groups. The lipid lowering action of TA found to be mediated though inhibition of hepatic cholesterol biosynthesis, increased faecal bile acid excretion, enhanced plasma lecithin, cholesterol acyltransferase activity and stimulation of receptor mediated catabolism of LDL\textsuperscript{92}. The finely powdered bark in oral suspension (500 mg/kg), 5 ml was administered to hypercholesterolemic rabbits for 90 days. The drug treatment raised the HDL cholesterol and lowered the LDL cholesterol levels\textsuperscript{93}.

In another study, diet induced hyperlipidaemic rabbits were given 50% ethanolic extract of the bark in doses of 100 mg/kg and 500 mg/kg. The extract decreased total and LDL cholesterol level. No adverse effect was observed on liver or kidney and haematological parameters\textsuperscript{94}. Oral administration of TA stem bark (500 mg/kg) has
reduced cholesterol, phospholipids and triglyceride serum level in rabbits. It also showed a significant decrease in heart cholesterol, triglycerides and aorta phospholipids values. TA pretreated rabbits showed normal aorta with no atherosclerotic changes, indicating that TA can reduce the deposition of cholesterol in aorta of the animals. In another study, the effect of orally administered indigenous drugs such as TA, *Terminalia belerica* and *Terminalia chebula* were investigated on experimental atherosclerosis. Rabbits were fed a cholesterol rich diet to induce atherosclerosis. The three drugs were fed along with cholesterol. At the end of the study, plasma and lipid components analysis revealed that TA was found to be most potent hypolipidemic agent and induced partial inhibition of rabbit atheroma. These results concluded that TA might play an antiatherogenic role.

The hypocholesterolaemic activity of different fractions from TA was tested on rats fed an atherogenic diet. In the control group the feeding of cholesterol led to a 97% increase in serum cholesterol levels at the end of three weeks. The methanolic and aqueous fractions inhibited this raise in serum cholesterol. The possible mechanism of action of cholesterol reducing activity was correlated with rapid excretion of the bile acids.

Administration of TA bark extracts (250 & 500 mg/kg) in cholesterol fed rabbits preserved endothelial vasodilator function. These values might have important clinical implications regarding the treatment of abnormal vasoconstrictor phenomenon encountered in patients with hypercholesterolemia and atherosclerosis. The efficacy of TA along with *vacha* (*Acorus calamus*) and *Mrugashrunga bhasma* in the treatment of *Dhaminipratichaya* (Atherosclerosis) was reported. The drug combination, when administered to albino rats, reduced the elevated levels of LDL, VLDL, serum cholesterol and triglycerides both in high cholesterol fed as well as estrogen administered male albino rats.
Antioxidant and hypocholesterolaemic effects of TA tree was studied through a randomized controlled trial involving 105 patients with coronary heart disease, who are administered TA bark powder 500 mg capsules daily for 30 days and the results showed that the bark powder has significant antioxidant action that is comparable with Vitamin E and also has a significant hypocholesterolemic effect.

### 2.2.3.3 Anticancer activity

TA along with various plants, were shown to reduce the genetic hazards of mutagens. By means of bio-assay guided separation of the methanol extracts of the bark, stem and leaves of the plant, gallic acid, ethyl gallate and luteolin were found to be the main anticancer constituents against tested cell-lines in-vitro. Antimutagenic potential of ellagic acid isolated from TA has been evaluated and reported. The antimutagenic activity of various extracts of TA performed by assessing the inhibition of genotoxicity of direct acting mutagen 4-nitroquinoline N-oxide (4 NQO) using the comet assay and the micro nucleus (MN) test. The results suggested that TA bark contains some polar as well as nonpolar compounds with antimutagenic activity against 4 NPQ.

In another study, the acetone and methanolic extracts of TA tested against the growth of normal fibroblasts, osteosarcoma and glioblastoma cells in-vitro. It was observed that both extracts at 30 µg and 60 µg/ml concentration inhibited growth of transformed cells; the growth of normal cells was least affected and proposed mechanism of anticancer effect is due to the interference with p53 protein dependent and independent pathways.

A tannin fraction isolated from TA was studied for its antimutagnic effect against 4-nitro-O-phenylene diamine (NPD), sodium azide and 2-aminofluorene (2AF) using the Ames assay. The antimutagenic effect of benzene, chloroform, acetone and methanol fractions of TA were determined against acid black dye, 2-aminofluorene and 4-nitro-O-
phenylene diamine. Among the different fractions studied, the acetone and methanol fractions exhibited better activity profile than other fractions. Casuarinin – a hydrolysable tannin isolated from the bark of TA inhibits human non-small cell lung cancer cells by blocking cell cycle progression in the G₀/G₁ phase arrest. Casuarinin was also investigated for its antiproliferative activity in human breast adenocarcinoma cells. The results showed that casuarinin inhibited the proliferation of adenocarcinoma cells by blocking cell cycle progression in the G₀/G₁ phase and inducing apoptosis. TA when administered to rats, reduced the elevated liver glycolytic enzymes such as hexokinase, phosphoglucoisomerase and aldolase in N-nitroso diethylamine induced hepatocellular carcinoma.

### 2.2.3.4 Hepatoprotective activity

TA is one of the ingredients in the popular Ayurvedic medicine *Liv 52*. The hepatoprotective property of *Liv 52* has been extensively studied and reported. Suppression of Hepatitis B virus surface antigen secretion by the TA bark extract was observed *in-vitro*. The secretion of hepatitis B surface antigen (HBsAg) in the cell culture was observed and reported.

### 2.2.3.5 Antiviral activity

Efficacy of traditional herbal medicines including TA against herpes simplex virus-1 has been reported. Screening of various plant extracts used in Ayurvedic medicine for inhibitory effect on HIV-1 protease has been reported. Casuarinin – a hydrolysable tannin isolated from the TA was investigated for its antiviral activity on herpes simplex type 2 virus.
2.2.3.6 Antioxidant activity

Antiradical and anti-lipoperoxidative effects of some plant extracts including TA used by Srilankan traditional medicinal practitioners for cardio protection has been reported\textsuperscript{123}. Antioxidant property of TA bark was studied by measuring thiobarbituric acid reacting substances (TBARS) and the results are compared with Vitamin E. The results showed that TA significantly decreased the lipid peroxide levels\textsuperscript{100}. Arjunaphthanoloside\textsuperscript{59} – a novel naphthanol glycoside and Terminoside A\textsuperscript{58} – a new triterpene glycoside from the bark of TA showed antioxidant activity and inhibits nitric oxide (NO) production in macrophages. Casuarinin isolated from TA protected cultured Madin-Darby Canine Kidney (MDCK) cells against hydrogen peroxide mediated oxidative stress. The results are compared with Trolox – a vitamin E analogue. The study showed that casuarinin is more effective against hydrogen peroxide induced oxidative stress, decreased DNA oxidative damage and prevented the depletion of intracellular GSH in MDCK cells\textsuperscript{124}. In another study, ethanolic extract of TA (25 mg/kg, p.o.) exerted significant lipid lowering effect as assessed by reversal of plasma levels of total cholesterol, triglycerides and phospholipids. It also exhibited lowering of plasma levels of lipids and glucose in diabetic and high fat diet induced dyslipidemic hamster\textsuperscript{125}. The oleanane triterpenes such as arjunic acid, arjungenin and their glucosides arjunetin and arjunglucosides II isolated from the bark of TA and were subjected to free radical scavenging activity. Arjungenin and its glucoside exhibited a moderate free radical scavenging activity while all the compounds showed no effect on the superoxide release from poly morpho nucleus [PMN] cells. Arjungenin also exhibited greater inhibitory action on the hypochlorous acid production from human neutrophills\textsuperscript{126}. The antioxidant activity of TA bark extract on N-nitrosodiethylamine [DEN] induced hepatocellular carcinoma in rats was reported. Enzymic antioxidants such as superoxide dismutase
[SOD], catalase, glutathione peroxidase and non-enzymic anti-oxidants like vitamin C and E levels were determined in all the groups of animals. A significant increase in lipid peroxides was observed while the levels of enzymic and non-enzymic antioxidants were decreased when subjected to DEN induction. These altered enzyme levels were ameliorated significantly by administration of ethanolic extract of TA$^{127}$.

### 2.2.3.7 Anti-fertility activity

Screening of Indian medicinal plants including TA for anti-fertility activity was reported$^{128}$. The benzene and 50% ethanolic extracts of TA bark showed no anti-implantation activity. Only resorptive activity was observed at a dose level of 200 mg/kg$^{129}$. In another study, hexane, 50% aqueous ethanol and butanol extracts together with two flavonoids viz., arjunolone and baicaline all showed resorptive activity but devoid of anti-implantation activity$^{130}$. Crude powder and ethanolic extract of TA administered to the pregnant rats showed significant anti-implantation and abortifacient/absorptive activity$^{131}$.

### 2.2.3.8 Anti-asthmatic activity

The methanolic extract of TA and arjunolic acid has shown significant protection against mast cell disruption in rats induced by compound 48/80. TA and AA also protected the guinea pig against histamine as well as acetylcholine induced bronchospasm. The anti-asthmatic and anaphylactic activity was correlated with the membrane stabilizing potential and inhibition of antigen induced histamine and acetylcholine release$^{132}$.
2.2.3.9 Anti-diabetic activity

Free radicals associated oxidative stress has been implicated in eliciting pathological changes in diabetes mellitus. The effect of chloroform extract of TA has studied in alloxan induced diabetic rats and its antilipid peroxidation activity was investigated in the cardiac and renal tissues. The extract produced a significant reduction in the peroxide products such as thio barbituric acid reactive substances (TBARS) and conjugated dienes with simultaneous increase in the levels of antioxidant enzymes viz., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione s-transferase (GST) and glutathione reductase (GR). The study indicated that, the TA exhibits anti-oxidant activity through the correction of oxidative stress.

2.2.3.10 Wound healing activity

50% ethanolic extract of the bark of TA reported to have wound healing activity in both incision and excision wound models. The proposed mechanism of action by increasing hydroxy proline content of the granulation tissue of the excision wounds which causes rapid collagen turnover and thus leaning to rapid healing of the wounds.

2.2.3.11 Antiplatelete and anticoagulant activity

An emulsion of TA bark powder when fed to rabbits for seven days at the dose of 10 g/kg body weight showed significant increase in prothrombin time (20 sec vs 10.01 sec) and decrease in platelet count (44 vs. 473 per thousand). With a further increase in dose to 15 g/kg the response was not much affected. Further investigation proved that petroleum ether extract possessed the fraction responsible for its anticoagulant action. TA (250 & 500 mg/kg) was fed to hypercholesterolamic rabbits for 60 days. On 61st day, circulating platelet aggregates and thrombogenic responses was tested. The results...
showed that treatment of TA bark reduced the circulatory platelets aggregates and incidence of ECG abnormalities in hypercholesterolaemic rabbits, as compared to untreated cholesterol fed rabbits. The results of the study indicated the usefulness of TA in prevention of thrombogenic disorders in atherosclerotic subjects. A significant reduction in platelet count was observed in experimental dogs on oral administration of 70% methanolic extract for 15 days. The other parameters were unaffected.

2.2.3.12 Endothelial physiology

Eighteen healthy male smokers and equal number of age matched non smoker control were employed in the study. The baseline brachial artery reactivity studies were performed using high frequency ultrasound, to determine endothelium-dependent, flow mediated dilation and endothelium-independent nitroglycerine mediated dilation. While flow mediated dilation was significantly impaired amongst smokers compared to controls, the nitroglycerine mediated dilation was similar in the two groups. Subsequently the smokers were given TA (500 mg/kg, 8 hourly) or matching placebo randomly in a double blind cross over design for two weeks each, followed by repetition of brachial arterial reactivity studies to determine various parameters including flow-mediated dilatation after each period. There was no significant difference between vessel diameter and flow velocities among the two therapies. However, the flow-mediated dilation showed significant improvement from baseline values after TA therapy but not with placebo. This study revealed that TA therapy for 2 weeks leads to significant regression of the endothelial abnormality among smokers.
2.2.3.13 Anti-bacterial activity

Screening of some Indian medicinal plants including TA for their antimicrobial properties has been reported\textsuperscript{139,140}. In another study, a total of 34 plant species belonging to 18 different families, selected on the basis of folklore medicinal reports practiced by the tribal people of Western Ghats, were assayed for antibacterial activity against \textit{Escherichia coli}, \textit{Klebsiella aerogenes}, \textit{Proteus vulgaris} and \textit{Pseudomonas aerogenes} at 1000-5000 ppm using the disc diffusion method. Of the plants studied, \textit{Cassia fistula} and \textit{Vitex negundo} showed significant antibacterial activity against the tested bacteria\textsuperscript{141}. Methanolic and aqueous extracts of about 54 plant extracts (including TA) were assayed for their activity against multi-drug resistant \textit{Salmonella typhi}. Strong antibacterial activity was shown by the methanolic extracts of \textit{Aegle marmelols}, \textit{Salmalia malabarica}, \textit{Punica granatum}, \textit{Myristica fragrans}, \textit{Holarrheana antidysenterica}, and \textit{Triphal} (mixture of \textit{Emblica officinalis}, \textit{Terminalia chebula} and \textit{Terminalia beilerica})\textsuperscript{142}.

2.2.3.14 Anti-fungal activity

Antifungal properties of some Indian medicinal plants including TA were reported\textsuperscript{143-145}. A new triterpenoid glycoside 2\(\alpha\), 19\(\alpha\)-dihydroxy-3-oxo-olean-12-en-28 oic acid-28-O-beta D-glucopyranoside has been reported to have antifungal activity\textsuperscript{55}.

2.2.4 Review literature on arjunolic acid

2.2.4.1 Anti-cancer activity

Arjunolic acid [AA] isolated from the rhizome of \textit{Cochlospermum tinctorium}, its acetate derivative and their methyl ester were tested using the short term \textit{in-vitro} assay on \textit{Epstein – Barr virus early antigen (EBV-EA)} activation in Raji Cells induced by 12-O-
tetradecanoyl phorbol-13-acetate (TPA). Their inhibitory effects of skin tumor promoters were found to be greater than those of previously studied natural products\textsuperscript{146}.

2.2.4.2 Anti-platelet and anti-coagulant activity

Arjunolic acid was isolated from the stem bark of \textit{T. arjuna}. When AA administered to isoproteranol induced myocardial necrotic rats, it is shown to prevent the decrease in the levels of superoxide dismutase, glutathione peroxidase, ceruloplasmin, \textalpha\text{-}tocopherol, reduced glutathione, ascorbic acid, lipid peroxide and myeloperoxidase. It also inhibited the platelet aggregation and showed anticoagulant activity by its ability to prolong the thrombin time, prothrombin time and activated partial thromboplastin time assays\textsuperscript{147}.

2.2.4.3 Insecticidal activity

Arjunolic acid isolated from the stem of \textit{Cornus capitata} (Cornaceae) exhibited significant inhibitory activity towards 4\textsuperscript{th} instar larvae of \textit{Spilarctia oblique}. The effective concentration (EC\textsubscript{50}) to reduce feeding and growth of the larvae of \textit{S. oblique} was found to be 617.8 ppm\textsuperscript{148}.

2.2.4.4 Anti-asthmatic activity

Arjunolic acid isolated from the stem bark of \textit{T. arjuna} and the methanolic extract of TA and has shown significant protection against mast cell disruption in rats induced by compound 48/80. AA and TA also protected the guinea pig against histamine as well as acetylcholine induced bronchospasm. The anti-asthmatic and anaphylactic activities of AA and TA were correlated with the membrane stabilizing potential and inhibition of antigen induced histamine and acetylcholine release\textsuperscript{132}. 

\newpage
2.2.4.5 Anti-bacterial activity

Arjunolic acid was isolated from the leaves of *Syzygium guineense* and the antibacterial activity was performed by disc diffusion against *Escherichia coli*, *Bacillus subtilis* and *Shigella sonnei*. AA exhibited significant antibacterial activity against all the tested micro-organisms\(^{149}\).

2.2.4.6 Anti-inflammatory activity

Arjunolic acid isolated from the leaves of *Combretum leprosum*. AA, ethanolic extracts of roots and leaves of *C. leprosum* were able to inhibit significantly the carrageenan-induced paw edema in rats. The ethanolic extract of root and AA inhibited the acetic acid induced writhing. AA and ethanolic extract of root also inhibited acetyl and butyryl cholinesterase enzymes in modified Ellman’s method in TLC. Since AA has close structural similarity with asiatic acid, it was suggested that AA may be promising model for the development of innovative multifunctional drugs for Alzheimer drug treatment\(^{150}\).

2.2.5 Review of literature on arjunolic acid acetate

Arjunolic acid was isolated from the bark of *Mitragyna ciliate* and it was derivatized into its tri-acetate (arjunolic acid acetate AAc). Both AA and AAc were tested *in vivo* on a two-stage carcinogenesis assay in mouse skin, using dimethylbenz[a]anthracene as initiator and 12-O-tetradecanoylphorbol-13-acetate as promoter. The activities were evaluated by both rate (%) of papilloma-bearing mice and average number of papillomas per mouse and compared with the control. The results suggest that acetylation of hydroxyl groups increased the tumour inhibitory effect of arjunolic acid\(^{151}\).
2.3 AIM AND OBJECTIVE OF THE STUDY

*Terminalia arjuna* (Combretaceae), known as *Marudam* in Tamil. It is well known in the traditional system of medicines such as Ayurveda and Siddha. Several medicinal properties have been attributed for the plant. Several phytoconstituents particularly triterpenoids and their glycosides have been reported (vide section 2.2.2).

**The aim of the present work is as follows**

- To document the HPTLC finger printing of the methanolic extract of the stem bark of *T. arjuna* with marker compounds.
- To isolate new phytoconstituents hitherto not reported.

The extensive literature survey has revealed that majority of the pharmacological studies in general and cardiac activity in particular has been reported for the alcoholic extract of *Terminalia arjuna*. Arjunolic acid is the major triterpenoid for which only few pharmacological activities have been reported (vide section 2.2.4).

Diallo et al\(^{151}\) acetylated arjunolic acid to arjunolic acid acetate and reported that Arjunolic acid acetate has better anticancer activity than arjunolic acid due to increased lipophilic property.

In the present study, the following pharmacological activities have been studied for total methanolic extract, arjunolic acid and arjunolic acid acetate.

- Cardiac activity
- Hepatoprotective activity
- Anti-inflammatory activity
- *In-vitro* antioxidant activity
• Analgesic activity

2.4 PHYTOCHEMICAL STUDIES

2.4.1 Materials and Methods

2.4.1.1 Plant material

The stem bark of *Terminalia arjuna* was collected from Palayamkottai, Tamil Nadu, India, during October 2001 and was authenticated by Dr. S. Usman Ali, Central Research Institute for Siddha, Arumbakkam, Chennai, India. A voucher specimen was deposited at the herbarium of C.L. Baid Metha College of Pharmacy, Thorapakkam, Chennai, India. The stem bark was shade dried and powdered.

2.4.1.2 Solvents and Chemicals

All the chemicals and solvents used were of AR grade. Silica gel used for column chromatography was Acme’s silica gel (100 – 200 mesh) or finer than 200 mesh. It was activated by heating 120°C for 1 h. Silica gel G containing 13% gypsum as binder was used for preparing TLC plates (20 x 5 cm), layer thickness 0.5 mm. The plates were activated by heating at 120°C for half an hour before use. Visualization was done either by exposure to iodine vapour or by spraying with 1:1 aqueous sulphuric acid and by heating at 110°C for 5 min. Precoated aluminium plates coated with silica gel 60 F_{254} (E-Merck), layer thickness 0.2 mm were used for HPTLC analysis.

2.4.1.3 Extractive values

The extractive values for the stem bark *T. arjuna* was carried out by successive extraction with solvents viz., hexane, chloroform, ethyl acetate and methanol.
2.4.1.4 Preliminary phytochemical screening

All the extracts viz., hexane, chloroform, ethyl acetate and methanol of *T. arjuna*, were subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents present in it.

2.4.1.5 High performance thin layer liquid chromatography (HPTLC) finger printing

HPTLC finger printing was performed on CAMAG TLC scanner – 3 instrument, equipped with Linomat IV applicator and CATS 3.1 software. Precoated aluminium silica gel 60 F_{254} (E. Merck) plates, layer thickness 0.2 mm were used. Finger prints were obtained by development in CAMAG twin chamber and was scanned at 200 nm. The HPTLC chromatogram for the methanolic extract of *T. arjuna* was recorded along with the marker compounds viz., hederagenin, maslinic acid and arjunolic acid.

2.4.1.6 Spectral Analysis

IR Spectroscopy

IR spectra were taken on FT-IR, Perkin – Elmer instrument in potassium bromide disc or chloroform, in the range of 4000 – 600 cm\(^{-1}\).

\(^1\)H NMR spectroscopy

\(^1\)H NMR spectra were recorded on Bruker instrument (AV300 MHz) or JEOL instrument (GSX 400 MHz) in CDCl\(_3\) or DMSO – d\(_6\) with tetra methyl silane (TMS) as the internal standard. Chemical shifts were recorded in d scale.
\(^{13}\)C NMR spectroscopy

\(^{13}\)C NMR spectra were recorded on Bruker instrument (75 MHz) or JOEL instrument (100 MHz) in CDCl\(_3\) or DMSO – d\(_6\) with tetra methyl silane (TMS) as the internal standard. Chemical shifts were recorded in d scale.

2.4.2 Experimental Studies

2.4.2.1 Extraction of the plant material

The shade dried and powdered stem bark of *T. arjuna* (4 kg) was extracted with methanol in the cold (48 h). The extract was filtered and distilled on a water bath and finally dried in vacuum to get a dark reddish brown mass (300 g). This extract was used for phytochemical investigations.

2.4.2.2 Extractive value\(^{52}\)

**Procedure**

Air dried coarsely powdered *T. arjuna* (5 g) was macerated with 100 ml of hexane in a closed flask for 24 h, shaking frequently during 6 h and allowed to stand for 18 h. Then the solvent was filtered and the drug is air dried for further successive extraction with other solvents. 25 ml of the filtrate was evaporated to dryness in a flat bottomed shallow dish and dried at 105°C and weighted. The % of hexane soluble extractive value was calculated.

The air dried drug (after 18 h of hexane extraction) was subjected to the successive extraction procedure as described above with solvents such as chloroform, ethyl acetate and methanol. Their respective extractive values are also calculated.
2.4.2.3 Preliminary phytochemical screening

The preliminary phytochemical screening of various extracts viz., hexane, chloroform, ethyl acetate and methanol were performed for the presence of various phyto constituents namely steroid, triterpenoid, phenol, flavonoid, tannins, glycoside/sugar, alkaloid, furanoid/indole, quinonoid, cumarin and lignan according to the literature\textsuperscript{153,154} and the results are presented in the table 2.

2.4.2.4 HPTLC finger printing

**Preparation of the extract and marker compounds**

The dried stem bark of *T. arjuna* was powdered and passed through 50 mesh. 5 g of the powder was extracted with 50 ml of methanol in soxhlet apparatus. The methanolic extract was concentrated and made up to 50 ml in a standard flask. All the marker compounds viz., hederagenin, maslinic acid and arjunolic acid each 10 mg was dissolved in 10 ml of methanol. 10 µl of the extract solution and marker compound solutions were spotted for the chromatogram. The scanning wavelength was 200 nm.

**HPTLC finger printing procedure**

The HPTLC finger print profile of methanolic extract of *T. arjuna* and the marker compounds such as hederagenin, maslinic acid and arjunolic acid were performed using pre coated silica gel 60 F\textsubscript{254} TLC plate (E. Merck) by employing CAMAG Linomat IV automatic sample spotter. The plate was developed by using chloroform - methanol (9:1) as the developing solvent system and was dried at room temperature and scanned using CAMAG TLC scanner 3 at UV 200 nm as the scanning wavelength. The R\textsubscript{f} value and HPTLC spectrum were recorded and the results are presented in the fig 1.
2.4.2.5 Isolation of phytoconstituents from methanolic extract of *T. arjuna*

The methanolic extract (300 g) obtained from 4 kg (dry weight) of *T. arjuna* stem bark was chromatographed over silica gel (100 – 200 mesh) in benzene and eluted with solvents of increasing polarity viz., benzene, chloroform and methanol. The results are presented in the scheme 1.
Scheme 1

Terminalia arjuna
(stem bark, 4 kg)

Methanol (6 L)

Methanolic extract
(300g)

Column Chromatography / Silica gel /
100 – 200 mesh

Chloroform (100 %)

Chloroform - Methanol

Compound I
(150 mg)

(3-oxo-olean-12-ene-28-oic acid)

Compound II
(200 mg)

(Methyl maslinate)

Compound III
(180 mg)

(Hederagenin Methyl ester)

Compound IV
(210 mg)

(Hederagenin)

Compound V
(350 mg)

(Maslinic acid)

Compound VI
(4.1 g)

(Arjunolic acid)
Compound I

Elution of the column with chloroform gave compound I and was crystallized from acetone (mp 184°C). It gave a single spot on TLC over silica gel with chloroform – methanol (19:1) as the developing system (Rf 0.73). It answered for the Noller’s test for triterpenoid by giving pink colour with tin and thionyl chloride.

IR ν<sub>max</sub> cm<sup>−1</sup>

(Fig 2) 2943, 2864, 1733 (carbonyl), 1638 (trisubstituted double bond), 1458, 1385 (gem dimethyl), 1210, 1030, 886, 823 (trisubstituted double bond).

<sup>1</sup>H NMR (d, CDCl<sub>3</sub>, 400 MHz)

(Fig 3a – 3c)

0.80 (3H, s, H-26), 0.91 (3H, s, H-29), 0.93 (3H, s, H-30), 1.03 (3H, s, H-24), 1.08 (3H, s, H-23), 1.14 (3H, s, H-25), 1.25 (3H, s, H-27), 2.39 (1H, brm, H-2a), 2.52 (1H, brm, H-2b), 2.85 (1H, dd, J=12 & 4 Hz, H-18), 5.29 (1H, t, J=4 Hz, H-12).

<sup>13</sup>C NMR (d, CDCl<sub>3</sub>, 100 MHz)

(Fig 4)

40.8 (C-1), 36.9 (C-2), 220.3 (C-3), 47.3 (C-4), 55.1 (C-5), 19.4 (C-6), 33.4 (C-7), 39.1 (C-8), 47.3 (C-9), 36.6 (C-10), 23.4 (C-11), 122.2 (C-12), 143.4 (C-13), 46.3 (C-14), 27.4 (C-15), 23.3(C-16), 48.5 (C-17), 43.0 (C-18), 46.3 (C-19), 30.9 (C-20), 33.9(C-21), 33.7 (C-22), 25.7 (C-23), 21.3 (C-24), 14.8 (C-25), 16.8 (C-26), 26.3 (C-27), 184.2 (C-28), 33.9 (C-29), 23.4 (C-30).
Oxidation of Oleanolic acid

Oleanolic acid was obtained by hydrolysis of the methanolic extract of *Randia dumetorium* fruits with 5% ethanolic hydrochloride and crystalized from acetone (mp 272°C). 50 mg of oleanolic acid was dissolved in 25 ml of acetone, cooled in ice and 10 drops of Joan’s reagent\(^{155}\) (cromic acid in concentrated sulphuric acid) were added. The solution was stirred for half an hour with mechanical stirrer and the solution turned green. It was poured into water and extracted with chloroform (2 x 100 ml). The combined chloroform extracts was washed with water and dried over anhydrous sodium sulphate. Then it was distilled to dryness and crystallized from the ether. (mp 184°C). The compound was found to be identical with compound I (mp, mmp, Co TLC and super imposable IR).

Compound II

Elution of the column with chloroform – methanol (19:1) (Fr 1-9), gave compound II and was crystallized from acetone (mp 226°C). It gave a single spot on TLC over silica gel with chloroform – methanol (19:1) as the developing system (R\(_f\) 0.59). It answered for the triterpenoid.

\[
\text{IR } V_{\text{KBr}}^{\text{max}} \text{ cm}^{-1}
\]

(Fig 5) 3420 & 1039 (hydroxyl), 2922, 2851,1736 (ester carbonyl), 1620 & 830 (trisubstituted double bond). 1465, 1375 (gem dimethyl), 1219 (ester bending), 1174, 969.
$^1$H NMR (d, CDCl$_3$, 400 MHz)

(Fig 6a – 6b) 0.66 (3H, s, H-26), 0.81 (3H, s, H-24), 0.96 (9H, brs, H-23, H-29 & H-30), 1.02 (3H, s, H-25), 1.25 (3H, s, H-27), 3.13 (1H, brs, H-18), 3.33 (1H, d, J=11 Hz, H-3a), 3.67 (1H, brm, H-28), 5.43 (1H, brs, H-12), 3.70 (3H, s, COOCH$_3$)

$^{13}$C NMR (d, CDCl$_3$, 100 MHz)

(Fig 7) 46.1 (C-1), 68.7 (C-2), 81.6 (C-3), 38.3 (C-4), 55.3(C-5), 18.4 (C-6), 29.2 (C-7), 38.3 (C-8), 47.7 (C-9), 38.3 (C-10), 24.4 (C-11), 124.8 (C-12), 142.7 (C-13), 41.2 (C-14), 28.5 (C-15), 24.9 (C-16), 45.1 (C-17), 43.8 (C-18), 45.4 (C-19), 34.6 (C-20), 32.4 (C-21), 31.9 (C-22), 29.4 (C-23), 16.7 (C-24), 16.3 (C-25), 16.9 (C-26), 27.9 (C-27), 178.5 (C-28), 32.4 (C-29), 24.6 (C-30), 58.2 (COOCH$_3$).

**Compound III**

Elution of the column with latter fraction (Fr 10 – 21) of chloroform – methanol (19:1) gave compound III and was crystallized from ether (mp 221°C). It gave a single spot on TLC over silica gel with chloroform – methanol (19:1) as the developing system (R$_f$ 0.71). It answered for the triterpenoid.
\( \text{IR } V_{\text{max}} \text{ cm}^{-1} \)

(Fig 8)

3400 & 1032 (hydroxyl), 2925, 2862, 1721 & 1260 (ester carbonyl), 1655 & 821 (Trisubstituted double bond) 1453, 1385 (gem dimethyl).

\(^1\text{H NMR (d, CDCl}_3, 400 \text{ MHz)}\)

(Fig 9a - 9b)

0.72 (3H, s, H-26), 0.90 (3H, s, H-29), 0.93 (3H, s, H-30), 1.03 (3H, s, H-24), 1.13 (3H, s, H-25), 1.25 (3H, s, H-27), 2.86 (1H, dd, \( J =11.0 \) & \( 4.0 \text{ Hz} \), H-18), 3.62 (3H, s, -COOCH\(_3\)), 3.60 – 3.66 (1H, brm, H-3a), 5.28 (1H, brs, H-12).

\(^{13}\text{C NMR (d, CDCl}_3, 100 \text{ MHz)}\)

(Fig 10)

38.2 (C-1), 27.5 (C-2), 76.7 (C-3), 41.6 (C-4), 51.5 (C-5), 18.4 (C-6), 32.5 (C-7), 39.3(C-8), 47.6 (C-9), 36.9 (C-10), 23.4(C-11), 122.1 (C-12), 143.8 (C-13), 41.6 (C-14), 27.5 (C-15), 23.0 (C-16), 46.3 (C-17), 41.2 (C-18), 45.8 (C-19), 30.6(C-20), 33.8 (C-21), 32.3 (C-22), 68.9 (C-23), 11.4 (C-24), 16.8 (C-25), 16.8(C-26), 25.9 (C-27), 178.2 (C-28), 33.8(C-29), 23.6 (C-30), 55.2 (COOCH\(_3\)).
Methylation of Hederagenin

Fifty milligram of hederagenin (compound IV), was dissolved in 25 ml of methanol and 25 ml of ethereal solution of diazomethane was added. The solution was allowed to stand over night and distilled to dryness and crystallised from acetone (mp 221°C). This compound was found to be identical with compound III. (mp, mmp, Co TLC and super imposable IR).

Compound IV

Elution of the column with latter fraction (Fr 1 – 8) of chloroform – methanol (9:1) gave compound IV and was crystallized from ethyl acetate (mp 330°C). It gave a single spot on TLC over silica gel with chloroform – methanol (19:1) as the developing system (Rf 0.48). It answered for the triterpenoid.

IR $\nu_{\text{KBr}}$ cm$^{-1}$

(Fig 11) 3455 & 1047 (hydroxyl), 2983, 2870, 1694 (acid carbonyl), 1638 & 824 (trisubstituted double bond), 1459, 1377 (gem dimethyl), 1271, 750.

$^1$H NMR (d, CDCl$_3$, 400 MHz)

(Fig 12a – 12c) 0.72 – 1.20 (18H, 6 x C – CH$_3$), 3.30 (1H, brd, J=9.0 Hz, H-3a), 2.80 (1H, dd, J=12 & 4Hz, H-18), 5.17 (1H, brs, H-12).
$^{13}$C NMR (d, CDCl$_3$, 100 MHz)

(Fig 13) 38.2 (C-1), 26.1 (C-2), 76.2 (C-3), 41.7 (C-4), 49.5 (C-5),
18.3 (C-6), 32.5 (C-7), 39.2 (C-8), 47.5 (C-9), 36.8 (C-10),
23.4 (C-11), 122.2 (C-12), 143.8 (C-13), 42.0 (C-14), 27.6
(C-15), 23.5 (C-16), 46.3 (C-17), 41.1 (C-18), 45.9 (C-19),
30.6 (C-20), 33.8 (C-21), 32.5 (C-22), 71.0 (C-23), 11.4 (C-
24), 15.7 (C-25), 16.8 (C-26), 25.8 (C-27), 181.1 (C-28),
33.0 (C-29), 23.5 (C-30).

Acetylation of hederagenin

Fifty milligram of hederagenin (Compound IV), was treated with 20 ml of acetic
anhydride and 1 ml of pyridine and the mixture was allowed to stand over night at room
temperature. It was poured into crushed ice and extracted with chloroform (2 x 100 ml).
The combined chloroform extracts was washed with 50 ml of water and dried over
anhydrous sodium sulphate. The solution was distilled to dryness and recrystallized from
chloroform – ether (1:1) to get hederagenin acetate (mp 286°C).

IR $^{\nu}_{\text{KBr}}$ cm$^{-1}$

(Fig 14) 2946, 2867, 1741 (acetate carbonyl), 1647 & 824
(trisubstituted double bond), 1463, 1370 (gem dimethyl),
1249, (acetate bending), 1043, 1033.
$^1$H NMR (d, CDCl$_3$, 400 MHz)

(Fig 15a – 15d) 0.77 – 1.17 (18H, 6 x C – CH$_3$), 2.05 & 2.02 (each 3H, s, 2 x OCOCH$_3$), 2.85 (1H, brd, J=10 Hz, H-18), 3.72 and 3.89 (ABq J=11.6 Hz, H-23), 4.35 (1H, t, J=7.0 Hz, H-3), 5.25 (1H, brs, H-12).

$^{13}$C NMR (d, CDCl$_3$, 100 MHz)

(Fig 16) 36.7 (C-1), 22.7(C-2), 80.6 (C-3), 41.5 (C-4), 55.0(C-5), 18.1 (C-6), 33.1 (C-7), 39.4(C-8), 47.7 (C-9), 38.2 (C-10), 23.6(C-11), 122.5 (C-12), 140.0 (C-13), 41.5(C-14), 27.6 (C-15), 23.4 (C-16), 46.5(C-17), 43.9 (C-18), 45.8 (C-19), 30.7(C-20), 33.8 (C-21), 32.4 (C-22), 65.4(C-23), 11.0 (C-24), 15.8 (C-25), 17.2(C-26), 25.9 (C-27), 183.3 (C-28), 33.2(C-29), 23.6 (C-30), 170.9, 2 x O COCH$_3$, 21.0, 21.2 : 2 x OCOCH$_3$.

**Compound V**

Elution of the column with latter fraction (Fr 9 -17) of chloroform – methanol (9:1) gave compound V and was crystallized from ethyl acetate – ethanol (8:2) (mp 270° C). It gave a single spot on TLC over silica gel with chloroform – methanol (9:1) as the developing system (R$_f$ 0.46). It answered for the triterpenoid.
(Fig 17) 3420 & 1076 (hydroxyl), 2922, 2851, 1687 (carboxylic acid carbonyl), 1620 & 867 (trisubstituted double bond), 1461, 1372 (gem dimethyl), 1271, 1051, 1034.

$^1$H NMR (d, CDCl$_3$, 400 MHz)

(Fig 18a – 18b) 0.69 – 1.20 (21H, 7 x C – CH$_3$), 2.80 (1H, brd, J=12Hz, H-18), 4.50 (1H, m, H-2ß), 3.20 (1H, d, J = 9.5 Hz, H-3a), 5.25 (1H, brs, H-12).

$^{13}$C NMR (d, CDCl$_3$, 100 MHz)

(Fig 19) 47.4 (C-1), 63.7 (C-2), 80.2 (C-3), 39.0 (C-4), 55.0 (C-5), 18.2 (C-6), 31.6 (C-7), 37.9 (C-8), 47.4 (C-9), 37.9 (C-10), 23.3 (C-11), 122.3 (C-12), 143.6 (C-13), 41.3 (C-14), 29.3 (C-15), 24.4 (C-16), 46.8 (C-17), 41.3 (C-18), 46.8 (C-19), 34.9 (C-20), 32.5 (C-21), 32.5 (C-22), 28.5 (C-23), 16.4 (C-24), 16.3 (C-25), 17.1 (C-26), 27.3 (C-27), 179.3 (C-28), 32.5 (C-29), 24.6 (C-30).

Compound VI

Elution of the column with latter fraction (Fr 18 -22) of chloroform – methanol (9:1) gave compound VI and was crystallized from methanol (mp 338° C). It gave a
single spot on TLC over silica gel with chloroform – methanol (9:1) as the developing system ($R_f$ 0.30). It answered for the triterpenoid.

<table>
<thead>
<tr>
<th>IR $\nu_{\text{KBr}}^{\text{max}}$ cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Fig 20) 3467 &amp; 1063 (hydroxyl), 2926, 2877, 1708 (acid carbonyl), 1640 &amp; 818 (trisubstituted double bond), 1455, 1377 (gem dimethyl), 1267, 1168, 1048, 1010, 767.</td>
</tr>
</tbody>
</table>

$^1$H NMR (d, CDCl$_3$, 400 MHz)

| 0.74 (3H, s, H-26), 0.90 (3H, s, H-29), 1.02 (6H, s, H-24, H-30), 1.12 (3H, s, H-25), 1.26 (3H, s, H-2), 2.82 (1H, dd, $J$=11 & 4Hz, H-18), 3.25 (1H, d, $J$=10.75, Hz, H-23), 3.55 (1H, d, $J$=10.75, Hz, H-23), 3.40 (1H, d, $J$=9.41, Hz, H-3a), 3.65 (1H, m, H-28), 5.36 (1H, brs, H-12). |

$^{13}$C NMR (d, CDCl$_3$, 100 MHz)

| 46.9 (C-1), 67.8 (C-2), 75.8 (C-3), 42.8 (C-4), 47.4 (C-5), 17.8 (C-6), 32.2 (C-7), 41.7 (C-8), 48.9 (C-9), 37.7 (C-10), 23.3(C-11), 121.8 (C-12), 144.3 (C-13), 46.8 (C-14), 27.5 (C-15), 22.9 (C-16), 46.3 (C-17), 41.1 (C-18), 45.8 (C-19), 30.7 (C-20), 33.6 (C-21), 32.4 (C-22), 64.2 (C-23), 14.1 (C-24), 17.2 (C-25), 17.1 (C-26), 26.0 (C-27), 179.0 (C-28), 33.2 (C-29), 23.7 (C-30). |
Acetylation of arjunolic acid

Fifty milligram arjunolic acid (Compound VI), was treated with 20 ml of acetic anhydride and 1 ml of pyridine and the mixture was allowed to stand over night at room temperature. It was poured into crushed ice and extracted with chloroform (2 x 100 ml). The combined chloroform extracts was washed with 50 ml of water and dried over anhydrous sodium sulphate. The solution was distilled to dryness and recrystallized from acetone to get arjunolic acid acetate (mp 352° C).

\[ \text{IR } \nu_{\text{KBr}} \text{ cm}^{-1} \]

(Fig 23) 2949, 2870, 1730 (acetate carbonyl), 1646 & 825 (trisubstituted double bond), 1455, 1377 (gem dimethyl), 1233, 1044 (hydroxyl), 969, 803.

\[ ^1\text{H NMR (d, CDCl}_3, 400 \text{ MHz)} \]

(Fig 24a – 24b) 0.76 (3H, s, H-26), 0.88 (3H, s, H-29), 0.91 (3H, s, H-30), 0.93 (3H, s, H-24), 1.09 (3H, s, H-25), 1.25 (3H, s, H-2), 2.82 (1H, brd, J=12.7 Hz, H-18), 3.58 (1H, d, J=11.9 Hz, H-23), 3.85 (1H, d, J=11.9 Hz, H-23), 5.08 (1H, d, J=10.3 Hz, H-3a), 5.14 (1H, m, H-28), 5.28 (1H, m, H-12), 1.98, 2.02 (3H each, s, C-2 & C-3, OCOCH\text{3}), 2.09 (3H, s, -CH\text{2}, OCOCH\text{3}).
$^{13}$C NMR (d, CDCl$_3$, 100 MHz)

(Fig 25) 42.5 (C-1), 68.7 (C-2), 73.8 (C-3), 42.5 (C-4), 46.6 (C-5), 19.8 (C-6), 32.9 (C-7), 39.1 (C-8), 46.5 (C-9), 36.8 (C-10), 124.0 (C-12), 143.3 (C-13), 40.9 (C-14), 28.6 (C-15), 24.8 (C-16), 46.5 (C-17), 40.7 (C-18), 46.6 (C-19), 29.7 (C-20), 31.1 (C-21), 30.8 (C-22), 13.2 (C-23), 64.2 (C-24), 16.9 (C-25), 16.0 (C-26), 26.6 (C-27), 178.8 (C-28), 31.4 (C-29), 25.3 (C-30), 169.2, 2 x OCOCH$_3$, 169.5, 1 x OCOCH$_3$, 19.9, 21.7, 22.6, 3 x OCOCH$_3$.

2.4.3 RESULTS AND DISCUSSION

2.4.3.1 Introduction

As mentioned in the review of literature, the following phytoconstituents viz., triterpenoids, triterpenoid glycosides, steroids, flavonoids, tannins and mannitol have been reported from the plant *T. arjuna*. In this section, we report the isolation and characterization of 3 oxo olean-12-ene-28-oic acid, methyl maslinate, hederagenin methyl ester, hederagenin, maslinic acid and arjunolic acid by the application of spectroscopic data as IR, $^1$H NMR and $^{13}$C NMR. Preliminary phytochemical screening and HPTLC fingerprinting with marker compounds are also reported.

2.4.3.2 Extractive value (Successive extraction)

The results of the successive extraction of the stem bark powder of the *T. arjuna* with hexane, chloroform, ethyl acetate and methanol were found to be 2.2, 4.7, 6.4 and 7.5 w/w respectively.
2.4.3.3 Preliminary qualitative analysis

The preliminary qualitative analysis of hexane, chloroform, ethyl acetate and methanol extracts (successive extraction) of the stem barks of *T. arjuna* was carried out and their results are presented in the table 2. The following phytoconstituents viz, steroids, triterpenoids, glycosides, flavonoids, tannins and phenolic compounds were answered for the qualitative tests.

2.4.3.4 HPTLC finger printing

The HPTLC finger printing analysis is shown in the fig 1. The methanolic extract of *T. arjuna* stem bark was developed with chloroform - methanol (9:1) as the developing system. The scanning wavelength was 200 nm.

The chromatogram showed 13 peaks (Tract ‘a’). Track ‘b’ shows the marker hederagenin peak at R_f value 0.35. Tract ‘c’ shows the marker compound maslinic acid peak at R_f value 0.25. Track ‘d’ shows the marker compound arjunolic acid peak at R_f value 0.15.

From the chromatogram of the extract, peak 7 of track ‘a’ coincides with that of hederagenin (track ‘b’). Peak ‘6’ of track ‘a’ corresponds with maslinic acid (track ‘c’) and peak 4 of track ‘a’ corresponds to arjunolic acid (track ‘d’). Thus the methanolic extract of *T. arjuna* can be standardized by employing these three marker compounds.

2.4.3.5 Characterization of phytoconstituents

**Compound I** (mp 184°C, lit mp 183-185°C) analyzed for C_{30} H_{46} O_{3} was eluted with chloroform. It answered the Noller’s test for triterpenoid. The IR spectrum (Fig 2) showed peaks for ketone carbonyl (1733 cm$^{-1}$), trisubstituted double bond (1638
Gemm dimethyl (1385 cm\(^{-1}\)) and the IR spectrum did not show any hydroxyl absorption.

The \(^1\)H NMR spectrum (Fig 3a – 3c) showed the absence of the usual 3 hydroxyl group. The H\textsubscript{3} a signal appearing around d 3.5 was absent, instead the 3 keto carbonyl carbon appeared at d 220.3 in the \(^{13}\)C NMR spectrum. The \(^1\)H NMR spectrum also showed the H-2 protons adjacent to the carbonyl group. H-2 a appeared as broad multiplet at d 2.39 and H-2\textbeta\ appeared at d 2.52. No other proton was found adjacent to the carbonyl group and thus confirming the position of the keto at C-3. H-24 and H-25 appeared at d 1.03 and d 1.14 respectively, slightly downfield when compared to oleanolic acid\(^{158}\), thus showing the presence of 3 keto group. H-18 appeared at double doublet at d 2.85 (J=12 and 4 Hz) showing the presence of oleanane skeleton. H-12 appeared as triplet at d 5.29 (J= 4Hz).

The \(^{13}\)C NMR also confirmed the structure of the compound I to be \textbf{3 oxo olean-12-ene-28-oic acid}. The 3 keto carbonyl carbon, as stated before appeared at d 220.3. The other signals were C-12 and C-13 appeared at d 122.2 and d 143.4 which are characteristic of olean-12-ene H-18\textbeta\ skeleton. C-28 of the COOH appeared at d 184.2.

![3 Oxo olean-12-ene-28-oic acid](image-url)
The identity of the compound I as 3-oxo olean-12ene-28-oic acid was finally confirmed by comparison with the reported data.\textsuperscript{158}

**Compound II** (mp 226°C, lit mp 225-227°C\textsuperscript{159}) analyzed for C\textsubscript{31} H\textsubscript{50} O\textsubscript{4}, was eluted with chloroform methanol (19:1). It answered Noller’s test for triterpenoid.

The IR spectrum (Fig 5) showed the presence of hydroxyl (3420, 1039 cm\textsuperscript{-1}), ester carbonyl (1736 and 1219 cm\textsuperscript{-1}), gem dimethyl (1375 cm\textsuperscript{-1}) and trisubstituted double bond (1620 and 830 cm\textsuperscript{-1}).

The \textsuperscript{1}H NMR spectrum (Fig 6a – 6b) was also characteristic of an oleanane triterpene methyl ester. Seven tertiary methyls appeared in the region \textit{d} 0.66-1.25. H-2ß appeared as the broad multiplet at \textit{d} 3.67. H-3a appeared as a doublet \textit{d} 3.33 (J=11.0 Hz). These two signals being broad showed diaxial interactions. The olefinic proton H-12 appeared at \textit{d} 5.43 as broad singlet. H-18 of the oleanane skeleton appeared at \textit{d} 3.13 as broad singlet. The presence of methyl ester at C-28 was shown by a three proton singlet \textit{d} 3.70.

The \textsuperscript{13}C NMR spectrum (Fig 7) showed the olefinic carbons C-12 and C-13 appeared at \textit{d} 124.8 and \textit{d} 142.7 respectively. C-2 and C-3 bearing the hydroxyl appeared at \textit{d} 68.7 and 81.6 respectively. The presence of methyl ester at C-28 was revealed by two carbons signal at \textit{d} 178.5 and 58.2 corresponds to ester carbonyl and ester methyl groups.
The identity of the compound II as methyl masline (2a, 3ß dihydroxy olean-12-ene-28-oic acid methyl ester) was confirmed by comparison with reported data\textsuperscript{160}.

**Compound III** (mp 221°C, lit mp 219 - 222°C\textsuperscript{159}) analyzed for C\textsubscript{31} H\textsubscript{50} O\textsubscript{4} was eluted with latter fraction of chloroform – methanol (19:1). It answered for triterpenoid.

The IR spectrum (Fig 8) showed the presence of hydroxyl (3400, 1032 \textsuperscript{cm}\textsuperscript{-1}), ester (1721 \textsuperscript{cm}\textsuperscript{-1} and 1260 \textsuperscript{cm}\textsuperscript{-1}), trisubstituted double bond (1655, 821 \textsuperscript{cm}\textsuperscript{-1}) and gem dimethyl group (1385 \textsuperscript{cm}\textsuperscript{-1}).

\textsuperscript{1}H NMR spectrum (Fig 9a – 9b) contained six tertiary methyls in the region d 0.72 – 1.25. H-3a appeared as multiplet at d 3.60 - d 3.65 (J=10.8 Hz). H-18 appeared at d 2.86 (dd, J=11.0 and 4.0 Hz). The olefinic proton H-12 appeared as broad singlet d 5.28. The methyl ester function at C-28 was revealed by a three proton singlet at d 3.62.

The \textsuperscript{13}C NMR spectrum (Fig 10) also confirmed the structure of compound III as hederagenin methyl ester (Methyl 3ß, 23α - dihydroxy olean-12-ene-28-oate). C-12 and C-13 appeared at d 122.1 and d 143.8 respectively, characteristic of oleanane skeleton. C-3 and C-23 bearing hydroxyl groups appeared at d 76.7 and d 68.9 respectively. The
presence of methyl ester at C-28 shown by ester carbonyl at d 178.2 and ester methyl at d 55.2 respectively.

Hederagenin methyl ester

The identify was confirmed by comparison with an authentic sample prepared by methylation of hederagenin using diazomethane as the methylating agent (mp, mmp, superimposable IR and CO-TLC) and also by comparison with reported values\textsuperscript{161}. Compound IV (mp 330°C, lit mp 330-334°C\textsuperscript{162}) analyzed for C\textsubscript{30} H\textsubscript{48} O\textsubscript{4}, was eluted with chloroform-methanol (9:1). It answered for triterpenoid.

The IR spectrum (Fig 11) showed the presence of the hydroxyl (3455 and 1047 cm\textsuperscript{-1}), trisubstituted double bond (1638 and 824 cm\textsuperscript{-1}) and gem dimethyl group (1377 cm\textsuperscript{-1}).

The \textsuperscript{1}H NMR spectrum (Fig 12a – 12c) also supported the structure of the compound IV as hederagenin (3β, 23α - dihydroxy olean-12-ene-28-oic acid). The six tertiary methyls appeared as singlet in the region d 0.72 - d 1.20. H-3β appeared as broad doublet at d 3.30 (J= 9.0 Hz). H-18 appeared as double doublet at d 2.80 (J=12.0 & 4.0 Hz) showing the presence of oleanane skeleton. H-12 appeared as broad singlet at d 5.17.
H-23 signal however was not detected since it was merged with solvent peak in the region δ 3.60 - δ 3.85.

The $^{13}$C NMR spectrum (Fig 13) also confirmed the structure of the compound IV as hederagenin. C-12 and C-13 appeared at δ 122.2 and δ 143.8 respectively. C-3 and C-23 carbon bearing hydroxyls appeared at δ 76.2 and δ 71.00 respectively. C-28 of the carboxylic acid appeared at δ 181.1.

![Hederagenin](image)

The compound IV was assigned as hederagenin by comparison with reported data$^{163-165}$. The identity of the compound was also confirmed by comparison with an authentic sample (mp, mmp, CO-TLC and superimposable IR) and also by preparing its diacetate derivative.

**Compound V** (mp 270°C, lit mp 269-271°C$^{166}$) analyzed for C$_{30}$ H$_{48}$ O$_{4}$, was eluted in the latter for fraction of chloroform-methanol (9:1). It also answered for the Noller’s tests for triterpenoid.
The IR spectrum (Fig 17) showed the presence of hydroxyl (3420, 1076 cm\(^{-1}\)), carboxylic acid (1687 cm\(^{-1}\)), trisubstituted double bond (1620 and 867 cm\(^{-1}\)) and gem dimethyl group (1372 cm\(^{-1}\)).

The \(^1\)H NMR spectrum (Fig 18a – 18b) showed seven tertiary methyls in the region d 0.69 – d 1.2. The presence of vicinal hydroxyl system in ring ‘A’ was shown by H-2 \(\beta\) (d 4.5, 1H, multiplet) and H-3a (d 3.2, 1H doublet, J= 9.5 Hz). H-18 appeared as broad doublet (J=12.0 Hz) and H-12 appeared as broad singlet at \(d\) 5.25. These data corresponded to maslinic acid (2a, 3\(\beta\) - dihydroxy olean-12-ene-28-oic acid) for compound V.

The \(^13\)C NMR data (Fig 19) also confirmed the structure. C-2 and C-3 bearing hydroxyls appeared at \(d\) 63.7 and 80.2 respectively. C-12 and C-13 appeared at \(d\) 122.3 and \(d\) 143.6 respectively, which corresponding to olean-12-ene skeleton. The carboxylic acid at C-28 was shown by the peak at \(d\) 179.3.

![Maslinic acid](image)

Maslinic acid

The observed data corresponded to the reported of maslinic acid\(^{167 - 169}\). Maslinic acid, however been reported already from the stem park of \(T.\ arjuna\)^{40}.

**Compound VI.** (mp 338°C, lit mp 335 - 338°C\(^{170}\)), analyzed for, C\(_{30}\) H\(_{48}\) O\(_{5}\), was eluted in the latter fraction of chloroform-methanol (9:1). It answered for triterpenoid.
The IR spectrum (Fig 20) showed the presence of hydroxyl (3467, 1063 cm\(^{-1}\)), acid carbonyl (1708 cm\(^{-1}\)), tri substituted double bond (1640, 818 cm\(^{-1}\)) and gem dimethyl group (1377 cm\(^{-1}\)). The IR spectrum did not show any absorption for ester function.

The \(^1\)H NMR spectrum (Fig 21a – 21c) showed the presence of six tertiary methyls, thereby showing that one tertiary methyl has been converted into hydroxy methylene function. This was confirmed by the H-23 signal appearing as doublets (J=10.7Hz at d 3.25 and d 3.55). The presence of vicinal dihydroxy system with 2a – OH and 3ß – OH in the ring ‘A’ was shown by the H-2 ß and H-3a signals appearing at d 2.65 (1H multiplet) and d 3.40 (1H, d, J= 9.4 Hz). The H-18 of the olean-12-ene skeleton appeared at d 2.82 as one proton double doublet (J=11.0, 4.0 Hz). The olefinic proton at H-12 appeared as broad singlet at d 5.36. Thus the compound was identified as arjunolic acid (2a, 3ß, 23α - tri hydroxy olean-12-ene-28-oic acid).

The \(^{13}\)C NMR spectrum (Fig 22) also confirmed the structure. The hydroxylated carbons at C-2, C-3, and C-23 appeared at d 67.8, d 75.8 and d 64.2 respectively. C-12 and C-13 appeared at d 121.9 and d 144.3 respectively. The C-28 appeared at d 179. The above data corresponded to arjunolic acid which is the major constituent of T. arjuna.
The identity was confirmed by compared with the reported data\textsuperscript{171}. The identity was further confirmed by the preparation of the triacetate (acetic anhydride/pyridine).

2.5 PHARMACOLOGICAL STUDIES

2.5.1 Materials and Methods

2.5.1.1 Plant Material

The stem bark of *Terminalia arjuna* was collected from Palayamkottai, Tamil Nadu, India. It was shade dried and powdered. The powdered stem bark (4 kg) was extracted with methanol in the cold (48 h). The extract was filtered and distilled on a water bath and finally dried in vacuum to get a dark reddish brown mass (300 g).

2.5.1.2 Isolation of arjunolic acid

The shade dried and coarsely powdered heart wood of *Terminalia arjuna* (8 kg) was extracted with ethyl acetate in the cold (48 h). The extract was filtered and distilled on a water bath and concentrated about 500 ml, when cold arjunolic acid was precipitated. The solution was allowed to stand at room temperature and the precipitated crude arjunolic acid was filtered under suction and washed with ethyl acetate when
arjunolic acid was obtained as white powder (80 g). It was purified by crystallization from acetone. The procedure was repeated for thrice to get pure arjunolic acid (338°C, 78 g).

2.5.1.3 Acetylation of arjunolic acid

Arjunolic acid (40 g) was converted into its tri acetate by acetylating with acetic anhydride/pyridine method. The detailed procedure is described in the page 51 (mp 352°C, yield 42 g).

2.5.1.4 Preparation of the drug for the pharmacological study

Methanolic extract of *T. arjuna* (TA), arjunolic acid (AA), arjunolic acid acetate (AAC) and other standard drugs (silymarin, diclofenac sodium and paracetemol) were administered orally in the form of suspension in water with 1% w/v sodium carboxy methyl cellulose (SCMC) as the suspending agent.

2.5.1.5 Animals

Wistar albino mice (25-30 g), wistar albino rats of either sex (140-200 g) were obtained from the inbred colony of department of pharmacology, C.L. Baid Metha College of Pharmacy, Thorapakkam, Chennai-96. The animals were kept in polypropylene cages at 25 ± 2°C with relative humidity 45-55% under 12 h light and 12 h dark cycles. They were fed with standard laboratory animal fed (Poultry Research Station, Tamil Nadu Veterinary and Animal Sciences University, Chennai, India) and tap water *ad libitum*.

All the pharmacological and toxicological experimental protocols were approved by the Institutional Animals Ethics Committee [IAEC] of Committee for the Purpose of
Control and Supervision on Experimentation on Animals [CPCSEA]. Vide sanction letter no.

2.5.1.6 Chemicals

All fine chemicals were purchased from Sigma and other chemicals from SRL (Mumbai), Aldrich (USA) and CDH (New Delhi).

2.5.1.7 Acute toxicity

Acute oral toxicity study\textsuperscript{172} was performed as per Organization for Economic Co-operation and Development [OECD] 423 guidelines (acute toxic class method). Wistar rats (n=6) of either sex was selected by random sampling technique. The animals were kept fasting for overnight, had access only to water. TA, AA and AAC were administered orally at the initial dose 5mg/kg body weight by intra gastric tube and observed for 14 days. The animals are observed individually after dosing once during the first 30 min, periodically during the first 24 h with special attention given during the first 4 h, and daily thereafter, for a total of 14 days. Since there was no mortality with 5 mg/kg for 14 days, the procedure was repeated for next higher doses such as 50, 500 and 2000 mg/kg for all the test compounds such as TA, AA and AAC.

2.5.1.8 Dose fixation

All the pharmacological activities of test as well as standard drugs are performed at two dose levels. In case of the methanolic extract of \textit{T. arjuna} [TA], the dose selected was 250 mg /kg and 500mg/kg based upon the literature\textsuperscript{68} \& \textsuperscript{132}. Based on the literature\textsuperscript{147},
the dose for arjunolic acid [AA] is fixed as 20mg/kg and 40mg/kg and for the standard drug captopril, the dose is fixed as 12.5 mg/kg and 25 mg/kg based on the literature.  

2.5.2 Experimental Studies  
2.5.2.1 Cardiac activity  
Introduction  

Heart muscle is largely dependent on uninterrupted blood flow, which guarantees delivery of oxygen and substrates and washesout the harmful products of metabolism. Ischaemia, i.e., decrease or cessation of myocardial blood flow, leads to rapid changes in myocardial metabolism. Ischaemic Heart Diseases (IHD) is associated with an imbalance between myocardial oxygen supply and demand.  

At the beginning of the 20th century, cardiovascular diseases (CVD) were responsible for fewer than 10% of all the deaths worldwide. Today, the figure is about 30% with approximately 80% of the burden now occurring in developing countries. In 2001, CVD was the number one cause of death worldwide and IHD is the predominant form of CVD.  

With the advent of improved technology, effective health care measures and public awareness about CVD, the mortality rates of IHD in USA has declined by more than 50%. According to the American National Center for Health statistics, from 1970 to 1995, the case fatality rate associated with acute myocardial infarction (AMI) decreased from 37% to 13% among persons 45 to 64 years of age. Similar pattern of reduction in % of mortality was observed in developed countries.  

On contrary, the epidemiological status of IHD in developing countries is alarmingly increasing. Between 1990 and 2020, IHD alone anticipated to increase by
120% for women and 137% for men in developing countries, compared with age related increases of between 30% and 60% in developed countries\textsuperscript{176 - 178}.

It has been predicted that cardiovascular disease will be the most important cause of mortality in India by the year 2015. Recent studies showed a high prevalence of IHD in both urban and rural populations of India. The incidence of IHD among the Indian urban population has multiplied by 9 times between 1960’s to 1990’s. The disturbing trend of steadily increasing incidences of IHD in younger age group (20-39 yrs) warrants urgent remedial measures\textsuperscript{179}.

The treatment of acute myocardial ischaemia involves the use of the thrombolytic agents (i.e., tissue plasminogen activator, streptokinase) or percutaneous transluminal coronary ballonet angioplasty (PTCA), which effectively restores the blood flow to the ischaemic myocardium\textsuperscript{180}.

Timely reperfusion has clearly been shown to be a more effective means to prevent the progression of ischaemic cells necrosis after coronary artery occlusion and it is now widely accepted that prompt reopening of the occluded vessel either by thrombolytic therapy or by primary angioplasty must be pursued whenever possible in patients with AMI\textsuperscript{181}.

**Method**

Ischaemic reperfusion method\textsuperscript{75}.

**Principle**

Reduction in the blood flow to myocardium leads to IHD and reperfusion by drug/surgery is essential for tissue/organ survival. However, reperfusion itself exacerbates myocardial injury commonly known as ischaemic reperfusion injury \textsuperscript{182 & 183}. 

85
Reperfusion studies on the isolated heart can be performed by Langendorff’s technique. The aorta is cannulated. The heart is perfused in retrograde direction from the aorta at constant pressure. Retrograde perfusion closes the aortic valves, just as in the in-situ heart during diastole. The perfusate is displaced through the coronary arteries, flowing of the coronary sinus and opened right atrium.

**Animals**

Male albino rats (150 to 200 g)

**Materials**

All the drugs TA, AA, AAc and Captopril (CA) were prepared in the form of suspension with 1% w/v SCMC.

**Chemicals**

All the chemicals were of analytical grade and the chemicals required for biochemical assays were obtained from Sigma chemical CO., USA. Double distilled water was used in all biochemical assays.

**Equipments**

1. Langendorff’s heart perfusion apparatus (Experimenta, Hungary)
2. Spectrophotometer (Shimadzu, Japan)
3. Refrigerated super – speed centrifuge (Model RC-5B, Sorvall Inc., USA)
4. Tissue homogenizer (Normal Scientifics, New Delhi, India)
5. Electronic Metler balance (Metler AE240, Switzerland)
6. pH analyzer (Model LI 612, Elico Ltd., India.)
**Treatment duration**

All the test drugs as well standard drug are administered daily by oral gavage, once a day for 30 days.

**Experimental Protocol**

After 48 h of the last dose, rats were heparinized (375 units/200g, i.p.) and of an hour later rates were anaesthetized with anaesthetic ether and subjected to any one of the protocols as presented in table 3.
Table - 3. Groups studied in *in-vitro* experiments.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>BL</td>
<td>Vehicle treated hearts</td>
</tr>
<tr>
<td>2.</td>
<td>C</td>
<td>Vehicle treated hearts subjected to 26 min. of reperfusion</td>
</tr>
<tr>
<td>3.</td>
<td>I</td>
<td>Vehicle treated hearts subjected to 5 min. perfusion + 9 min. of ischaemia</td>
</tr>
<tr>
<td>4.</td>
<td>IR</td>
<td>Vehicle treated hearts subjected to 5 min. perfusion + 9 min. of ischaemia + 12 min. reperfusion</td>
</tr>
<tr>
<td>5.</td>
<td>TA&lt;sub&gt;1&lt;/sub&gt; BL</td>
<td>Rats treated with 250 mg/kg of TA</td>
</tr>
<tr>
<td>6.</td>
<td>TA&lt;sub&gt;1&lt;/sub&gt; IR</td>
<td>Rats treated with 250 mg/kg of TA and subjected to IR</td>
</tr>
<tr>
<td>7.</td>
<td>TA&lt;sub&gt;2&lt;/sub&gt; BL</td>
<td>Rats treated with 500 mg/kg of TA</td>
</tr>
<tr>
<td>8.</td>
<td>TA&lt;sub&gt;2&lt;/sub&gt; IR</td>
<td>Rats treated with 500 mg/kg of TA and subjected to IR</td>
</tr>
<tr>
<td>9.</td>
<td>AA&lt;sub&gt;1&lt;/sub&gt; BL</td>
<td>Rats treated with 20 mg/kg of AA</td>
</tr>
<tr>
<td>10.</td>
<td>AA&lt;sub&gt;1&lt;/sub&gt; IR</td>
<td>Rats treated with 20 mg/kg of AA and subjected to IR</td>
</tr>
<tr>
<td>11.</td>
<td>AA&lt;sub&gt;2&lt;/sub&gt; BL</td>
<td>Rats treated with 40 mg/kg of AA</td>
</tr>
<tr>
<td>12.</td>
<td>AA&lt;sub&gt;2&lt;/sub&gt; IR</td>
<td>Rats treated with 40 mg/kg of AA and subjected to IR</td>
</tr>
<tr>
<td>13.</td>
<td>AAc&lt;sub&gt;1&lt;/sub&gt; BL</td>
<td>Rats treated with 20 mg/kg of AAc&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>14.</td>
<td>AAc&lt;sub&gt;1&lt;/sub&gt; IR</td>
<td>Rats treated with 20 mg/kg of AAc&lt;sub&gt;1&lt;/sub&gt; and subjected to IR</td>
</tr>
<tr>
<td>15.</td>
<td>AAc&lt;sub&gt;2&lt;/sub&gt; BL</td>
<td>Rats treated with 40 mg/kg of AAc&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>16.</td>
<td>AAc&lt;sub&gt;2&lt;/sub&gt; IR</td>
<td>Rats treated with 40 mg/kg of AAc&lt;sub&gt;2&lt;/sub&gt; and subjected to IR</td>
</tr>
<tr>
<td>17.</td>
<td>CA&lt;sub&gt;1&lt;/sub&gt; BL</td>
<td>Rats treated with 12.5 mg/kg of CA</td>
</tr>
<tr>
<td>18.</td>
<td>CA&lt;sub&gt;1&lt;/sub&gt; IR</td>
<td>Rats treated with 12.5 mg/kg of CA and subjected to IR</td>
</tr>
<tr>
<td>19.</td>
<td>CA&lt;sub&gt;2&lt;/sub&gt; BL</td>
<td>Rats treated with 25 mg/kg of CA</td>
</tr>
<tr>
<td>20.</td>
<td>CA&lt;sub&gt;2&lt;/sub&gt; IR</td>
<td>Rats treated with 25 mg/kg of CA and subjected to IR</td>
</tr>
</tbody>
</table>
Protocol I (Estimation of basal (BL) endogenous antioxidants levels)

Hearts from BL groups were harvested and stored in liquid nitrogen for estimations of basal (BL) endogenous antioxidants and in 10% buffered formalin for histological studies.

Protocol II (Estimation of endogenous antioxidant levels after Ischaemic Reperfusion (IR) Process)

Hearts from IR group were anaesthetized with ether, the chest opened and the heart along with one cm of ascending aorta attached was quickly removed and dipped in ice-cold saline. The hearts were then mounted on Langendorff’s apparatus and perfused with modified Kreb’s-Hensleitt’s (K-H) buffer\textsuperscript{185} at a constant pressure of 60-70 mm Hg at 37°C, and aerated with a mixture of O\textsubscript{2} (95%) and CO\textsubscript{2} (5%).

Composition of modified Kreb’s – Hensleitt’S

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>118.5</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>2.8</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>1.2</td>
</tr>
<tr>
<td>Magnesium Sulphate</td>
<td>0.6</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>25</td>
</tr>
<tr>
<td>Potassium hydrogen phosphate</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.1</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Production of *In-vitro* Ishaemic Reperfusion Injury

Following an initial period of 5 min of stabilization, the flow was stopped for 9 min (ischaemia) followed by perfusion with K-H buffer for 12 min (reperfusion). At the end of ischaemic reperfusion, the hearts were cut into two parts and one part was kept at -20°C for TBARS (Thio Barbituric Acid Reactive Substances) estimation and the other part was kept at -80°C in liquid nitrogen, till they were taken up for the estimation of myocardial SOD (Super Oxide Dismutase), Catalase (CAT) GSH (reduced glutathione) and lactate.

*Langendorff’s perfusion apparatus*

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**Biochemical Estimations**

In order to obtain tissue samples for biochemical estimation, roughly 0.5-1.0 g of tissue was quickly cut out from the previously marked out area after reperfusion and immediately frozen in liquid nitrogen. The whole process was done in 8-10 sec. The
samples for biochemical estimations were weighed in an electronic pan balance and subsequently processed for the estimation of following parameters.

**Estimation of TBARS**

Lipid peroxidation is a well established mechanism of cellular injury associated with ischaemic reperfusion and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from poly unsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds such as malon dialdehyde (MDA).

**Principle**

The most common method of measuring MDA is based on the reaction with thiobarbituric acid (TBA). The thiobarbituric acid reactive substances (TBARS) assay is the colourimetric method, widely used for the detection of lipid peroxidation in biological fluids. MDA is formed as a result of lipid peroxidation and reacts with thiobarbituric acid under high temperature (90-100°C) and acidic condition. The reaction yields a pink MDA – TBA adduct the product of two mole of TBA plus one mole of MDA. The coloured complex can be extracted into organic solvents such as butanol and measured by spectrophotometer using wavelength 532 nm.
Reagents

Sodium lauryl sulphate - 8.1% (w/v)
Acetic acid - 20% v/v (pH-3.5)
Thiobarbituric acid - 0.8% (w/v)
Butanol: Pyridine - (15:1) (v/v)
Trichloracetic acid (TCA) - 10% (v/v)

Procedure

Tissue was homogenized with 2 ml of Trichloroacetic acid (TCA). 0.2 ml of whole homogenate was taken to which 0.2 ml of 8.1% w/v sodium lauryl sulphate, 1.5 ml of 20% v/v acetic acid and 1.5 ml 0.8% w/v thiobarbituric acid were added. The volume was made up to 4 ml with double distilled water. It was heated at 95°C for 60 min. After cooling, 1 ml of double distilled H\textsubscript{2}O and 5 ml of butanol - pyridine mixture was added. The solution was shaken vigorously in a vortex and centrifuged at 4000 rpm for 10 min in a cold centrifuge. The organic layer was separated and absorbance was observed at 532 nm in a spectrophotometer.

Standard curve

Various concentrations of 1, 1, 3, 3-Tetra methoxy propane (TMP) were used as external standard (1-10 nm) and were subjected to the steps mentioned in the procedure section. The readings of the absorbance were plotted against the concentration of TMP to derive a linear standard graph. Data expressed as n mole/g wet wt tissue.

Calculation

The concentration of TBARS was determined from the linear standard graph. The results of TBARS data are presented in the table 4 and fig 26.
**Myocardial Reduced Glutathione [GSH]**

**Principle**

Bis (p-nitro phenyl) disulphide reacts with aliphatic thiol compounds at pH 8.0 to produce one mole of p-nitrothiophenol anion per mole thiol. Since this anion is highly coloured (\(\lambda_m \sim 13,6000 \text{ at } 412 \text{ nm}\)), it can be used to measure the thiol concentration.

**Reagents**

- Phosphate buffer (\(\text{K}_2\text{HPO}_4\)) - 0.3 M (pH-8.4)
- 5, 5 – Dithiobis (2-nitrobenzoic acid) (DTNB) - 0.2% w/v
- Sulphosalicylic acid - 10% w/v

**Procedure**

The hearts were homogenized with 10% v/v TCA and centrifuged at 3000 rpm for 10 min. The reaction mixture contained 0.1 ml of supernatant, 2.0 ml of 0.3 M phosphate buffer (pH-8.4), 0.4 ml of double distilled water and 0.5 ml of DTNB [5,5’ dithiobis (2-nitrobenzoic acid)]. The reaction mixture was incubated for 10 min and the absorbance was measured at 412 nm in the spectrophotometer.

**Standard Curve**

Various concentrations of the standard glutathione (1-10 µg) were subjected to the steps mentioned above. The readings of absorbance were plotted against the concentration of GSH to produce a linear standard graph.
Calculation

The concentration of GSH was determined from the linear standard graph. Data are expressed as µg / g wet wt.

Results

The results of myocardial GSH data are presented in the table 5 and fig 27.

Estimation of SOD\textsuperscript{189} & \textsuperscript{190}

Principle

Superoxide anions were generated in a system comprising of NADH and phenazine methosulphate. This superoxide anion reduced into blue tetrazolium forming a blue formozan, which was measured at 560 nm. SOD inhibited the reduction of nitroblue tetrazolium and thus the enzyme activity was measured by monitoring the rate of decrease in optical density at 560 nm.

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pyrophosphate</td>
<td>0.052 M, pH 8.3</td>
</tr>
<tr>
<td>Phenazine methosulphate</td>
<td>186 mM</td>
</tr>
<tr>
<td>Nitroblue tetrazolium [NBT]</td>
<td>300 mM</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>60.05 M</td>
</tr>
<tr>
<td>Tris buffer with sucrose</td>
<td>0.25 M</td>
</tr>
<tr>
<td>Tris HCl buffer</td>
<td>0.0025 M, pH 7.4</td>
</tr>
</tbody>
</table>
Procedure

The tissue was homogenized in 0.25M Tris-sucrose buffer. Then the homogenized tissue was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected, to which 50% w/v ammonium sulphate was added, vortexed, and the reaction mixture was kept for incubation at 4°C for 4 h. After the incubation period it was again centrifuged at 12,000 rpm for 30 min at 4°C. The samples were then kept overnight for dialysis in 0.0025 Tris HCl buffer.

The next day, appropriate volumes of samples to a maximum of 1.2 ml were taken. To it, 1.2 ml sodium pyrophosphate, 0.1 ml of phenazine methosulphate, 0.3 ml of NBT and 0.2 ml NADH were added. The final volume was made up to 3 ml with distilled water. After adding NADH, it was immediately incubated for 90 sec at 30°C and the reaction was stopped by adding 1 ml acetic acid to the reaction mixture. 4 ml of butanol was added and after 10 min, centrifuged at 3000 rpm, for 10 min. The organic layer was separated and absorbance was observed at 560 nm in the spectrophotometer.

Standard curve

Various concentrations of standard SOD (0.1 to 2.0 µg) were subjected to the same steps at the samples mentioned in the above procedure. The reading of absorbance was plotted against the concentrations of SOD to derive a linear standard graph.

Calculation

The concentration of SOD was determined from the linear standard graph. Data expressed as IU/mg protein.
Results

The results of myocardial SOD data are presented in the table 6 and fig 28.

Estimation of Catalase

Principle

In the ultraviolet range, $H_2O_2$ shows a continual increase in absorption with decreasing wavelength. The decomposition of $H_2O_2$ can be followed directly by the decrease in extinction at 240 nm. The difference in extinction at 240 nm was measured. The difference in extinction per unit time is a measure of the catalase activity.

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>50 mM at pH 7.0 (0.05 M KH$_2$PO$_4$) and 0.053M Na$_2$HPO$_4$ mixed in a ratio of 1:1.55</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>30 mM solution</td>
</tr>
<tr>
<td>Isotonic buffer</td>
<td>pH 7.4 (0.9% NaCl and 0.01 M phosphate buffer)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>99.99% v/v</td>
</tr>
<tr>
<td>Triton X</td>
<td>10% w/v</td>
</tr>
</tbody>
</table>

Procedure

The tissue was homogenized with isotonic buffer and centrifuged at 3000 rpm for 10 min. The supernatant liquid was collected and 0.01 ml of ethanol per ml of supernatant liquid was added. Then the samples were incubated for 30 min in an ice water bath. At the end of the incubation period, 10% w/v Triton X was added to 0.1ml of supernatant and used for catalase estimation. 2 ml of sample was appropriately diluted in
phosphate buffer and 1 ml H$_2$O$_2$ was added and absorbance was read at 240 nm at 15 sec interval for a total of 30 sec.

**Standard curve**

Various concentrations of standard catalase (1-20 µg) were subjected to the steps mentioned above. The readings of absorbance were plotted against the concentrations of catalase to derive a linear standard graph.

**Calculation**

The concentrations of catalase in the samples were determined from the linear standard graph. Data expressed as IU / mg protein.

**Results**

The results of myocardial catalase data are presented in the table 7 and 29.

**Estimation of Myocardial Lactate**

**Procedure**

Tissue was homogenized with 1 ml 0.6 mol/L perchloric acid and centrifuged at 3000 rpm for 10 min. 0.5 ml of the supernatant was taken for the estimation of lactate and was estimated using commercial kit (Sigma chemicals, USA). Data expressed as µmole/g wet wt.

**Results**

The results of myocardial lactate data are presented in the table 8 and fig 30.
Histopathology

The hearts were removed, washed immediately with saline and then fixed in 10% w/v buffered formalin, were embedded in paraffin; sections were cut at 5 µm and stained with haematoxylin and eosin. These sections were then examined under a light microscope for histological changes.

Statistical Analysis

One way analysis of variance (ANOVA) was carried out to test the significance of the biochemical data of the different groups in myocardial tissue samples. Values are expressed as mean ± SEM. Significance is set at p<0.05.

2.5.2.2 HEPATOPROTECTIVE ACTIVITY

Introduction

Liver is the largest and most complex internal organ in the body. It plays an important role in the maintenance of internal environment through its multiple and diverse functions. It is involved in the intermediary metabolism of proteins, fats and carbohydrates and in the synthesis of number of plasma proteins such as albumin, fibrinogen and clotting factors. It also plays an important role in the production of various enzymes, formation and excretion of bile. It acts as storage depot for proteins, glycogen, various vitamin and metals. It also plays central role in detoxification and excretion of many endogenous and exogenous compounds. Hence, any injury to it or impairment of its functions has grave implication for the health of the affected person.

According to WHO report\textsuperscript{193}, more than 2000 million persons or 2 out of every 5 people on earth, are infected with the hepatitis B virus. About 300 million of them are
chronically infected carriers, one-quarter of who are at high risk of serious illness and eventual death from cirrhosis of the liver and primary liver cancer. It is estimated that more than 1 million deaths per year occur from the sequelae of these infections.

Modern drugs do not provide any remedies for hepatitis, cirrhosis, liver damage by toxins or for bilary tract disorders. However, herbal drugs have a long history in treating such disorders. The promising hepatoprotective effect of silymarin - a flavonolignan from *Silybum marianum* has renewed the interest and drew the attention of research works throughout the world towards the research on medicinal plants.

**Method**

 Carbon tetrachloride toxic model$^{194}$.

**Principle**

 A toxic dose or repeated dose of a known hepatotoxins such as carbon tetra chloride, paracetamol, thiocetamide, alcohol, D-galactosamine, allyl alcohol etc., is administered to induce the liver damage in experimental animals. Animals can then be treated or pretreated with plant drugs. The liver damage and recovery are assessed by measuring many parameters such as serum marker enzymes, bilirubin level and histopathological changes in liver.

 When the liver is damaged, the liver enzymes such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) are enter into the circulation. An increase in the levels of these marker enzymes in the serum is an indication of liver damage.
Animals

Rats of six per group were used.

Materials

Methanolic extract of TA, AA, AAc and silymarin (SM) were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals. For inducing acute hepatic damage, carbon tetrachloride in olive oil (1:1) was employed.

Serum aspartate transaminase (AST), serum alanine transaminase (ALT), serum alkaline phosphatase (ALP), serum bilirubin (BILN), total proteins (TPN) and total albumin (TAL), were estimated by using standard kits from M/S Ranbaxy Laboratories Ltd., New Delhi, India. All the reagents used were of analytical grade. Silymarin (Silybon, M/S Microlabs, Bangalore) was used as standard drug.

Experimental Protocol

Rats (n=6) are randomly divided in to nine groups. I group served as vehicle control and received only 1% w/v SCMC (10 ml/kg, p.o.) for 5 days. II group served as toxic control and received carbon tetrachloride - olive oil (1:1) (2 ml/kg, s.c.). III group animals served as standard drug control and received SM 25 mg/kg, p.o., in 1% w/v SCMC for 5 days. IV and IV group animals received methanolic extract of TA 250 and 500 mg/kg, p.o., in 1% w/v SCMC for 5 days. VI and VII group animals received AA 20 and 40 mg/kg, p.o., in 1% w/v SCMC respectively for 5 days. VIII and IX group animals received AAc 20 and 40 mg/kg, p.o., in 1% respectively for 5 days. All groups except I group animals received carbon tetrachloride - olive oil (1:1) (2ml/kg, s.c.) on 2nd and 3rd days, 30 min after the administration of the test compounds. On the fifth day, after 4 h of drug administration, animals were sacrificed by decapitation. Blood was collected by
excising the jugular vein. It is allowed to clot and then centrifuged at 3000 rpm for 15 min to separate the serum for various biochemical estimations like AST, ALT, ALP, total albumin (TAL), total protein (TPN) and bilirubin (BILN).

Estimation serum aspartate transaminase [AST] $^{195}$

Reagents required

1. Standard Pyruate

   Sodium pyruate (10 mg) was dissolved in 100 ml of phosphate buffer (0.1M), pH 7.4.

2. Substrate

   DL – Aspartate (2.66 g) and 38 mg of α-ketoglutarate were dissolved in 20.5 ml of 1N sodium hydroxide with heating and the final volume was made up to 100 ml with distilled water.

3. 2, 4 – dinitoro phenyl hydrazine reagent (DNPH)

   Dinitro phenyl hydrazine (1 mM) in 2N hydrochloric acid.

4. Phosphate buffer (0.1 M), pH 7.4

5. Sodium hydroxide (0.4 N solution)

Procedure adopted

The buffered substrate (1 ml) was added to 0.1 ml of serum in different test tubes and were incubated at 37°C for an hour. 1 ml of DNPH reagent was added to stop the reaction. To the blank tubes, after the addition of DNPH reagent, 0.1 ml of enzyme was
added. The tubes were kept aside for 15 min, then 10 ml of 0.4 N sodium hydroxide was added and the absorbance measured at 520 nm in a Shimadzu UV spectrophotometer. The enzyme activity was expressed as IU/Litre in serum.

**Estimation of serum alanine transaminase (ALT)**

The reagents and the method employed are similar to that of the assay of aspartate transaminase except for the substrate solution and the incubation time was reduced to 30 min.

**Assay of serum alkaline phosphatase (ALP)**

**Reagents**

1. Carbonate – bicarbonate buffer (0.1M), pH 10.0
2. Disodium phenyl phosphate solution (0.01M)
3. Magnesium chloride solution (0.1M)
4. Folin’s phenol reagent

Sodium tungstate (100 g), 25 g of sodium molybdate, 700 ml of water, and 50 ml of 85% v/v ortho phosphoric acid were taken in 2 litres round bottomed flask and refluxed for 10 h. 150 g of lithium sulphate, 50 ml of distilled water and few drops of bromine were added. The mixture was boiled to remove excess bromine. It was then cooled and diluted to one litre with water. The reagent was diluted 1:2 with distilled water before use.

5. Sodium carbonate solution (15% w/v)
6. Standard phenol solution. (0.1% w/v) solution in water
Experimental detail

Carbonate-biocarbonate buffer (1.5 ml), 1ml of substrate and 1.5 ml of enzyme sources were taken. The above reaction mixture was incubated at 37ºC for 15 min. The reaction was arrested by the addition of 1.0 ml of Folin’s phenol reagent. The control tubes received the enzyme after arresting the reaction. The contents were centrifuged and to the supernatant, 1.0 ml of 15% w/v sodium carbonate solution, 1 ml of substrate and 0.1 ml of magnesium chloride were added and the mixture was incubated for 10 min at 37ºC. The absorbance was measured at 640nm against a blank in UV spectrophotometer. The standard solution of phenol of varying concentrations was also treated similarly. The enzyme activity was expressed as IU/litre in serum.

Estimation of serum bilirubin (BILN)\textsuperscript{97}

Reagents

1. Diazo-reagent
   Solution A: 0.1 g of sulphanilic acid is dissolved in 1.5 ml of concentrated hydrochloric acid and the volume was made up to 100 ml with distilled water.
   Solution B: 0.5g of sodium nitrite is dissolved in water and the volume was made up to 100 ml with distilled water.
   Freshly prepared solutions B (0.8 ml) was added to 10 ml of freshly prepared solution A.

2. Absolute methanol

3. Hydrochloric acid 1.5% v/v in water

4. Standard solution of bilirubin (0.1% w/v solution in chloroform)
**Experimental**

In one test tube, 0.2 ml of serum and in another test tube, 1.8 ml of distilled water were added. To the serum test tube, added 0.5 ml of the diazo reagent and to another test tube, 0.5 ml of 1.5% v/v hydrochloric acid was added. Finally to each of the test tubes, 2.5 ml of methanol was added. After 5 min, the colour developed was measured at 540 nm against the blank containing chloroform.

The values are expressed as mg/dl.

**Estimation of total protein [TPN]**

**Reagents**

1. Alkaline copper reagent
   - Solution A: 2% w/v sodium carbonate in 0.1N sodium hydroxide solution.
   - Solution B: 0.5% w/v copper sulphate in water.
   - Solution C: 1% w/v sodium potassium tartarate in water
     - Solution A (50 ml) is mixed with 0.5 ml of solution B and 1 ml of solution C.

2. Folin’s phenol reagent C
   - Preparation as described in ALP.

3. Standard bovine serum albumin (BSA)
   - Crystalline bovine serum albumin (100mg) is dissolved in 100 ml of distilled water.

**Experimental detail**

An aliquot of the suitably diluted sample (0.1 ml to 10 ml by two serial dilutions) is made up to 1 ml with water. 4.5 ml of alkaline copper reagent is added to each of the tubes including blank. Blank containing 1.0 ml of water and standard containing aliquots of BSA were also similarly treated. The contents of the test tubes were set aside for 10 min...
in room temperature. 0.5 ml diluted Folin’s – phenol reagent is added to all the the tubes. The blue colour developed is measure at 640 nm after 20 min in UV spectrophotometer. The values are expressed as g/dl in serum.

**Estimation on of total albumin [TAL]**

**Reagents**

1. Anhydrous sodium sulphite solution (25% w/v)
2. Diethyl ether
3. Biuret reagent

   Freshly prepared 25% w/v sodium hydroxide solution (5 parts) is mixed with 1 part of freshly prepared 2% w/v copper sulphate solution.

**Experimental detail**

Serum (0.04 ml), 4 ml of sodium sulphite solution and 4 ml of diethyl ether were added and centrifuged at 3000 rpm for 5 min. Then 2 ml of lower layer is taken and added 2 ml of water. The blank contained 2ml of sodium sulphite solution and 2ml of Biuret reagent was added to all the tubes and kept aside for 15 min. The colour developed is measured at 540nm in UV spectrophotometer.

**Results**

The results of hepatoprotective activity is presented in the table 9 and fig 31

**Histopathological examination**

Small fragments of liver is washed in ice-cold saline, fixed in 10% w/v formalin solution, dehydrated in the ethanol (50% v/v) embedded in paraffin and cut into 5 µm
thick sections using a microtome. The sections were stained with eosin-haemotoxylin dye for photo microscopical observations.

**Statistics**

The statistical analysis was performed by one way ANOVA followed by Dunnet’s ‘t’ test. The results were expressed as the mean ± SEM (standard error mean) to show variations in a group. Differences are considered significant when p<0.05.

**2.5.2.3 ANTI-INFLAMMATORY ACTIVITY**

**Introduction**

The term inflammation covers a complex series of reparative and protective responses to tissue injury, whether caused by infection, auto immune stimuli or mechanical injury. Anti-inflammatory drugs are used to treat disorders which lead to inflammation, pyrexia and pain of whatever cause e.g., rheumatoid conditions, gout, dysmenorrhoea, neoplastic diseases etc.

Most currently used anti-inflammatory agents inhibit cyclo-oxyganase and therefore prostaglandin synthesis. Free radical scavenging agents also play a role in the treatment of inflammation, because liberation of free radicals causes tissue damage during the inflammatory process. Triterpenoids, flavonoids and other phenolics are thought to act by preventing the generation or action of free radicals.

**Method**

Carrageenan induced oedema method\textsuperscript{200}. 
Principle

The inflammatory reaction is readily produced in rats in the form of paw oedema with the help of irritants or inflammagens. Substances such as carrageenan, formalin, bradykin, histamine, 5 hydroxy tryptamine, mustard and egg white, when injected in the dorsam of the foot in rats produce acute paw oedema within a few min of the injection.

Animals

Wistar albino rats.

Materials

Methanolic extract of TA, AA, AAc and Diclofenac sodium (DS) prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals.

Plethysmograph

Indigenously prepared graduated plethysmograph was used for the study. The mercury displacement due to dipping of the paw was directly read form the scale attached to the mercury column.

Experimental protocol

Rats (six per group) were randomly divided in to eight groups. I Group animals received 1% w/v SCMC (10 ml/kg, p.o.), Group II animals received DS 5 mg/kg, p.o.) in 1% w/v SCMC. III and IV Group animals received methanolic extract of TA 250 and 500 mg/kg, p.o., in 1% w/v SCMC respectively. V and VI Group animals received AA 20 & 40 mg/kg, p.o., in 1% w/v SCMC respectively. VII and VIII Group animals received
AAc 20 and 40 mg/kg, p.o., in 1% w/v SCMC respectively. Diclofenac sodium was employed as the standard drug.

A mark was made on both the hind paw (right and left) just beyond tibio tarsal joint, so that every time the paw is dipped in the mercury column upto the fixed mark, to ensure constant paw volume. The initial paw volume (both right & left) of each rat was measured by mercury displacement.

After 30 min of drug administration, 0.1 ml of 1% w/v carrageenan is injected in the right hind paw sub-plantar region of each rat. The left paw served as reference (non-inflammatory paw) for comparison. The paw volumes of both legs of control and test compound treated rats were measured at 1 h, 2 h, 3 h, 4 h and 5 h after carrageenan administration.

The results of the anti-inflammatory activity data are presented in table 10 and fig 32. The percentage inhibition for each rat and each group was obtained by using the formula C-T/C x 100 where C is the oedema rate of control group and T is treated group.

**Statistics**

The statistical analysis was performed by one way ANOVA followed by Dunnet’s ’t’ test. The results were expressed as the mean ± SEM to show variations in a group. Differences are considered significant when p<0.05.

**2.5.2.4 IN-VITRO ANTI OXIDANT ACTIVITY**

**Introduction**

Chemical compounds and reactions capable of generating potential toxic oxygen species free radicals are referred to as pro-oxidants. On the other hand, compounds and
reactions disposing off these species, scavenging them, suppressing their formation or opposing their actions are called anti-oxidants.

In a normal cell, there is an appropriate pro-oxidant: anti-oxidant balance. However, this balance can be shifted towards the pro-oxidant, when the production of oxygen species is increased or when the levels of anti-oxidants are diminished. This state is called oxidative stress and can result in serious cell damage, if the stress is massive or prolonged\textsuperscript{201}.

The oxidants/free radicals are species with very short half life, high reactivity and damaging activity towards macro molecules like proteins, DNA and lipids. These species may be either oxygen derived (Reactive oxygen species - ROS) or Nitrogen derived (Reactive nitrogen species - RNS). The oxygen derived species include $O_2^-$ (superoxide), HO (hydroxyl), $HO_2$ (hydroperoxyl), $ROO^-$ (peroxyl), $RO^-$ (alkoxyl) as free radicals and $H_2O_2$ (hydrogen peroxide) HOCl (hypochlorous acid), $O_3$ (ozone). Similarly nitrogen derived oxidant species are mainly NO (nitric oxide) ONOO (peroxynitrite), $NO_2^-$ (nitrogen dioxide) and $N_2O_3$ (dintrogen trioxide)\textsuperscript{201}.

Oxygen free radicals have been implicated in variety of diseases such as ischaemia, atherosclerosis, heart failure, cancer, diabetes, alzheimer's disease, parkinsonism, inflammation, rheumatoid arthritis, vascular disease, hypertension, cataract formation, emphysema, immune system decline, lung diseases such as asthma, lung fibrosis, kidney diseases, gene activation, brain dysfunction as well as aging\textsuperscript{202 - 206}.

There is an increasing interest in anti-oxidants, which specifically intended to prevent the presumed deleterious effects of free radicals in the human body and also to prevent the deterioration of fats and other constituents of food stuffs. In both cases, there is a preference of anti-oxidants from natural rather than synthetic source\textsuperscript{207}. There is,
therefore a parallel increase in the use of methods for estimating the efficacy of such substances as anti-oxidants.

**Types of Anti-oxidants Studies**

Anti-oxidant activity can be measured both by *in-vivo* and *in-vitro* methods. The *in-vivo* anti-oxidant activity involves the measurement of the anti-oxidant enzymes such as super-oxide dismutase (SOD), reduced glutathione (GSH), peroxidase and catalase. The *in-vitro* anti-oxidant activity can be studied by following methods viz., DPPH (1,1-diphenyl, 1-2 picryl hydrazyl) method, nitric oxide inhibition method, FTC (Ferric Thio Cyanate) method, TRAP (Total Radical Trapping Antioxidant Parameter) assay, ORAC (Oxygen Radical Absorbance Capacity) assay, ABTS (2,2 Azino Bis – (3 – ethyl benzo Thiazoline – 6- Sulphonic acid) assay, DMPD (N,N Di Methyl p- Phenylene Diamine) assay etc.  

**In-vitro anti-oxidant methods**

**DPPH Assay**

**Principle**

2,2 diphenyl-1- picryl hydrazyl (DPPH) is a stable free radical, shows deep violet colour, characterized by an absorption band in ethanol solution at 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, the free radical DPPH is reduced to corresponding hydrazine.
Basically this technique is the decolouration assay which evaluates the absorbance decrease at 517 nm, produced by the addition of the anti-oxidant to a DPPH solution in ethanol. DPPH assay is considered a valid and easy assay to evaluate scavenging activity of anti-oxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays\textsuperscript{211} & \textsuperscript{212}.

**Chemicals and Instrumentation**

DPPH (Aldrich, USA), Naphthylene diamine dichloride (Loba Chemie, Mumbai). All other reagents used were of analytical grade. UV spectra used is Shimadzu UV-1601 model.

**Experimental details:**

The free radical scavenging activity of the methanolic extract of TA, AA and AAc at different concentrations were examined using DPPH radical. The reaction mixture consisted of 1 ml of 0.1 Mm DPPH in ethanol, 0.95 ml of tris-HCl buffer (pH 7.4), 1 ml of ethanol and 0.05 ml of different concentrations of TA, AA and AAc.
The absorbance of the mixture was measured at 517 nm exactly 30 sec after adding the extract. The experiment was performed in triplicate and % scavenging was calculated using the formula 100-[100/blank absorbance x sample absorbance]. The antioxidant activity was compared with Vitamin C which was used as the standard antioxidant.

The results are presented in the table 11 – 14 and fig 33 – 36.

Statistics

The statistical analysis was performed by Students’ t test. The results were expressed as the mean ± SEM. Differences in the absorbance between the blank solutions and the test solutions are considered significant when p<0.05.

Nitric Oxide radical Inhibition Assay

Introduction

Nitric oxide (NO) is a hydrophobic molecule and a highly diffusible free radical, generated through the oxidation of l-arginine to l-citrulline by NO synthases. Low concentrations of NO acts as a signaling molecule with dichotomous regulatory roles in many physiological processes, whereas high levels of NO produced by inflammatory cells can damage DNA, RNA, lipids and proteins, leading to increased mutations and altered enzyme and protein function important to multistage carcinogenesis process.

Effects of NO on cells ultimately dependent on a complex of factors including existing biological milieu; rate of NO production and its rate of diffusion; interaction with other free radicals, metal ions and proteins; levels of protective enzymes such as catalase and superoxide dismutase; levels of anti-oxidants such as glutathione.
However, the fate of NO in biological systems is broadly governed by three main reaction processes: diffusion and intra-cellular consumption, auto oxidation to form nitrous alhydride ($N_2O_3$) and reaction with superoxide to form peroxynitrite ($ONOO^-$).\textsuperscript{216}

Damage to cellular macromolecules is produced by mixtures of ultimate reactants derived from nitrous anhydride, peroxynitrite and nitrogen dioxide radical. The diversity of reactions in which NO participates correspondingly increases the complexity of mechanisms underlying its role in various pathological conditions, especially with regard to cell death and carcinogenesis.\textsuperscript{215}

**Principle**

The assay is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions and the same can be estimated using Griess Illosvoy reaction.\textsuperscript{217}

In the present investigation, Griess Illosvoy reagent is modified by using 0.1% w/v naphthyl ethylene diamine (NED) dichloride, instead of 5% w/v 1-naphthylamine. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions.\textsuperscript{218}

**Chemicals required**

Sodium nitroprusside and naphthyl ethylene diamine (Acros organics, Belgium) and sulphanilamide (Suvchem Chemicals, Mumbai).
Experimental details

Sodium nitroprusside (5 µM) prepared in standard phosphate buffer solution was incubated with different concentrations of the methanolic extract of TA, AA and AAC, dissolved in standard phosphate buffer (0.025 M, pH4) and the tubes were incubated at 25°C for 5 h. After 5 h, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess reagent (prepared by mixing equal volume of 1% w/v sulphanilamide in 2% v/v phosphoric acid and 0.1% w/v naphthyl ethylene diamine dihydrochloride in water). The absorbance of chromophore formed was read at 546 nm. The control experiment was also carried out in similar manner. The experiment was performed in triplicate and % scavenging was calculated using the formula 100-[100/blank absorbance x sample]. The activity was compared with Vitamin C, which was used as a standard anti-oxidant.

The results are presented in the table 15 – 18 and fig 37 – 40.

Statistics

The statistical analysis was performed by Students’t’ test. The results were expressed as the mean ± SEM. Differences in the absorbance between the blank solutions and the test solutions are considered significant when p<0.05.

2.5.2.5 ANALGESIC ACTIVITY

Introduction

There are two types of analgesic drugs i.e., narcotic and non-narcotic agents. Narcotic analgesic drugs act through their interaction with opioid receptors and at spinal level, they inhibit the transmission nociceptive impulses through the dorsal horn and suppress nociceptive spinal reflexes. They also inhibit the release of substance P from the
dorsal horn neurons in-vitro and in-vivo by a pre-synaptic inhibitory effect on the central terminals of nociceptive afferent neurons.

The non-narcotic analgesic agents (NSAID’S), act through the inhibition of arachidonate cyclo-oxygenase, which in turn leads to the decreased production of prostaglandins. Prostaglandins sensitize nociceptive nerve endings to inflammatory mediators such as bradykinin and 5 hydroxy tryptamine.

**Acetic acid induced writhing reflex method**

**Principle**

Painful reactions in animals may be produced by chemicals. Intraperitoneal injection of phenylquinone, bradykinin or acetic acid produces painful reactions, which are characterized as a writhing response. Constriction of abdomen, turning of trunk (twist) and extension of hind legs are considered as the reaction to chemically induced pain. Compounds exhibiting narcotic as well as non-narcotic analgesic activity inhibit the writhing reflex.

**Animals**

Wistar albino mice (25 – 30 g) of either sex.

**Materials**

Methanolic extract of TA, AA, AAc and Paracetamol (PA) were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals.

**Experimental protocol**

Mice of either sex were randomly divided into eight groups, each group containing six animals. Group I animals received only 1% w/v SCMC (10 ml/kg, p.o.).
Group II animals received PA 100 ml/kg, p.o., in 1% w/v SCMC; Group III & IV animals received methanolic extract of TA 250 and 500 mg/kg, p.o., in 1% w/v SCMC respectively. Group V & VI animals received AA 20 & 40 mg/kg, p.o., in 1% w/v SCMC respectively. Group VII & VIII animals received AAc 20 & 40 mg/kg, p.o., in 1% w/v SCMC respectively.

All the animals received intraperitoneal (i.p.) injection of 3% v/v of acetic acid (1 ml/100 g) 30 min after the administration of drugs viz., TA (250 & 500 mg/kg), AA(20 & 40 mg/kg), AAc(20 & 40 mg/kg) and PA (100 mg/kg). Paracetamol was used as the standard drug.

The number of writhings produced by each animal was observed individually under a glass jar for a period of 20 min and the same was counted. The % protection of analgesic activity was calculated by using the formula C -T/C x 100, where C is the number writhings in control group and T is the number of writhings in the treated group.

The results of the analgesic activity data are presented in table 19 and fig 41.

ii] Tail immersion model

Principle

Exposing heat to rat tail is one way of stimulating pain in cutaneous receptors, which in turn excites the thermoselective and nociceptive fibers. Immersion of rat tail in hot water provokes an abrupt withdrawal of the tail from the heat source. The time difference in the withdrawal of tail of opioid and other test drug treated animal was monitored. Tail immersion method is most widely and reliably used for revealing the potency of opioid analgesics.
Animals

Wistar albino female rats.

Materials

Methanolic extract of TA, AA, and AAc were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals. Morphine (MO) injection used as the standard drug.

Experimental protocol

Rats (six per group) were randomly divided into eight groups. I Group animals served vehicle control group and received only 1% w/v SCMC (10 ml/kg, p.o.) II Group animals received MO (50 mg/kg, s.c.). III and IV Group animals received methanolic extract of TA 250 & 500 mg/kg, p.o., in 1% w/v SCMC respectively. V and VI Group animals received AA 20 & 40 mg/kg, p.o., in 1% w/v SCMC respectively. VII and VIII Group animals received AAc 20 & 40 mg/kg, p.o., in 1% w/v SCMC respectively.

The animals were screened for the sensitivity test by immersing 3 cm of the tail of the rat gently in hot water maintained at 55 ± 0.5°C. Within a few sec, the rats reacted by withdrawing the tail. The reaction time was recoded with a stopwatch. Each animal served as its own control and two readings were obtained for the control at 0 and 10 min interval. The average of the two values was taken as the initial reaction time. After 30 min, tail withdrawal time of each group animals was noted and the % protection of analgesia was calculated by using the formula C·T/C x 100 where ‘C’ represents the tail withdrawal (in sec) of control and ‘T’ to that of treated groups.

The results of the analgesic activity data are presented in table 20 and fig 42.
Statistics

The statistical analysis was performed by one way ANOVA followed by Dunnet’s ‘t’ test. The results were expressed as the mean ± SEM to show variations in a group. Differences are considered significant when p<0.05.

2.5.3 RESULTS AND DISCUSSION

2.5.3.1 Acute Toxicity Studies

Methanolic extract of TA showed no marked sign of toxicity upto 2000 mg / kg and was considered as safe (OECD – 423 guidelines unclassified). On the other hand, arjunolic acid (AA) and arjunolic acid acetate (AAc) showed motor incoordination, tremor, tachycardia and dyspnea in two animals at 2000 mg / kg. Hence the experiment was repeated again at 2000 mg / kg and found the same observation but no mortality was observed in both the tests. Then the LD_{50} for AA and AAc was fixed with the lower dose i.e., 500 mg / kg (toxic category 4 as per OECD) where no sign of toxicity was observed.

2.5.3.2 CARDIAC ACTIVITY

2.5.3.2.1 Results

Base line changes

The baseline changes brought about by the pretreatment of TA_{1} & TA_{2} BL (250 & 500 mg/kg), AA_{1} & AA_{2} BL (20 & 40 mg/kg), AAc_{1} & AAc_{2} BL (20 & 40 mg/kg) and CA_{1} & CA_{2} BL (12.5 & 25 mg/kg) respectively or with vehicle (1% w/v SCMC) (BL group), for 30 days and these animals are not subjected to ischaemic reperfusion process.
Mycardial TBARS

The myocardial TBARS values were significantly high [p<0.001] in all the test drug as well as standard drug treated groups when compared with TBARS value of BL group and their values are as follows. TA₁ BL & TA₂ BL [71.31 ± 4.97 and 99.49 ± 2.46 nmole/g wet wt], AA₁ BL & AA₂ BL [86.34 ± 4.00 and 102.71 ± 2.46 nmole/g wet wt], AAc₁ BL & AAc₂ BL [98.6 ± 2.08 and 121.95 ± 2.05 nmole/g wet wt] and CA₁ BL & CA₂ BL [48.40 ± 1.97 and 59.42 ± 2.05 nmole/g wet wt] respectively in comparison to BL group [44.6 ± 3.40 nmole/g wet wt].

The results are presented in the Table 4 and fig 26.

Mycardial GSH

The myocardial baseline GSH values were significantly increased [p<0.001] in TA₁ BL & TA₂ BL [395.60 ± 2.87 and 449.26 ± 4.04 µg/g wet wt], AA₁ BL & AA₂ BL [470.47 ± 4.47 and 496.26 ± 9.21 µg/g wet wt], AAc₁ BL & AAc₂ BL [488.51 ± 6.73 and 532.37 ± 6.07 µg/g wet wt] and CA₁ BL & CA₂ BL [824.21 ± 7.38 and 968.79 ± 6.81 µg/g wet wt] respectively in comparison with that of GSH value of BL group [331.05 ± 6.31 µg/g wet wt].

The results are presented in the Table 5 and fig 27.

Mycardial SOD

The myocardial baseline SOD values were significantly [p<0.01] increased in TA₁ BL & TA₂ BL [6.5 ± 0.2 and 8.4 ± 0.2 IU/mg protein], AA₁ BL & AA₂ BL [7.4 ± 0.2 and 10.9 ± 0.2 IU/mg protein], AAc₁ BL & AAc₂ BL [7.8 ±0.2 and 11.9 ±0.2 IU/mg protein].
protein] and CA$_1$ BL & CA$_2$ BL [12.8 ± 0.2 and 18.3 ± 0.1 IU/mg protein] respectively in comparison with SOD value of BL group [3.5 ± 0.1 IU/mg protein].

The results are presented in the Table 6 and fig 28.

Myocardial catalase [CAT]

The myocardial baseline CAT values were significantly increased [p<0.001] in TA$_1$ BL & TA$_2$ BL [129.52 ± 4.19 and 145.5 ± 4.32 IU/mg protein], AA$_1$ BL & AA$_2$ BL [75.43 ± 1.81 and 83.40 ± 2.91 IU/mg protein], AAc$_1$ BL & AAc$_2$ BL [99.02 ± 4.41 and 105.49 ± 3.56 IU/mg protein] respectively and CA$_2$ BL [62.25 ± 2.04 IU/mg protein]. The myocardial CAT value of BL group is 46.21 ± 1.87 IU/mg protein.

The results are presented in the Table 7 and 29.

Myocardial Lactate

The myocardial baseline lactate value of ‘I’ (Ischaemic) group was significantly elevated [P<0.001] [28.4 ± 0.17 µmole/g wet wt] when compared with myocardial lactate level of BL group i.e., 11.9 ± 0.22 µmole/g wet wt. There are no significant alterations in the myocardial lactate levels in other treated groups.

The results are presented in the Table 8 and 30.

Ischaemic Reperfusion (IR) induced changes

The ischaemic reperfusion induced changes are brought by the pretreatment of TA$_1$ IR & TA$_2$ IR (250 & 500 mg/kg), AA$_1$ IR & AA$_2$ IR (20 & 40 mg/kg), AAc$_1$ IR & AAc$_2$ IR (20 & 40 mg/kg) and CA$_1$ IR & CA$_2$ IR (12.5 & 25 mg/kg) respectively for 30 days and are subjected to ischaemic reperfusion procedure as per the protocol in table 3.
Myocardial TBARS

The myocardial TBARS value of IR group [61.98 ± 1.66 nmole/g wet wt] was significantly [P<0.001] higher than in C group [45.8 ± 0.77 nmole / g wet wt]. Whereas, the myocardial TBARS levels in the treated group were significantly lowered when compared with the TBARS value of IR group and the details are as follows. TA₂ IR [54.14 ± 1.24 nmole /g  wet wt], AA₂ IR [49.67 ± 1.24 nmole/g wet wt], AAc₁ IR & AAc₂ IR [55.57 ± 3.35 and 46.52 ± 3.28 nmole/g wet wt], CA₁ IR & CA₂ IR [53.4 ± 2.10 and 52.60 ± 3.28 nmole/g wet wt] respectively. The myocardial TBARS values of TA₁ IR [60.24 ± 3.20 nmole/g wet wt] and AA₁ IR [61.56 ± 3.42 nmole/g wet wt] are not significant when compared with IR group value.

The results are presented in the Table 4 and fig 26.

Myocardial GSH

The myocardial GSH value of IR group is 328.98 ± 9.50 µg/wet wt and the GSH value of C group is 335.23 ± 2.81 µg/g wet wt. In all the drug treated IR groups, such as TA₁ IR & TA₂ IR [342.55 ± 6.22 and 392.38 ± 7.39 µg/g wet wt], AA₁ IR & AA₂ IR [348.35 ± 6.16 and 373.32 ± 3.07 µg/g wet wt], AAc₁ IR & AAc₂ IR [363.64 ± 3.65 and 394.50 ± 6.82 µg/g wet wt] and CA₁ IR & CA₂ IR [412.59 ± 7.44 and 495.96 ± 6.83 µg/g wet wt] respectively. The myocardial GSH values were significantly [P<0.001] higher in comparison to IR group.

The results are presented in the Table 5 and fig 27.
Myocardial SOD

The myocardial SOD value of IR group [2.9 ± 0.2 IU/mg protein] is significantly [P<0.01] lowered when compared with C group GSH value [3.3 ± 0.1 IU/mg protein]. In all the drug treated IR groups such as TA₁ IR & TA₂ IR [4.8 ± 0.1 and 5.4 ± 0.2 IU/mg protein], AA₁ IR & AA₂ IR [5.1 ± 0.1 and 6.3 ± 0.1 IU/mg protein], AAc₁ IR & AAc₂ IR [5.3±0.3 and 7.4±0.1 IU/mg protein] and CA₁ IR & CA₂ IR [8.4±0.2 and 11.4 ± 0.1 IU/mg protein] respectively, were significantly [P<0.01] higher than IR group.

The results are presented in the Table 6 and 28.

Myocardial catalase

The myocardial catalase value of IR group [34.63 ± 0.83 IU/mg protein] is significantly [P<0.001] lowered than that of C group [44.36 ± 1.08 IU/mg protein]. In case of all the drug treated IR groups, the myocardial catalase values were significantly [P<0.001] increased when compared with IR group and are discussed as follows. TA₁ IR & TA₂ IR [64.42 ± 1.90 and 82.44 ± 2.08 IU/mg protein], AA₁ IR & AA₂ IR [44.56 ± 1.90 and 43.63 ± 2.22 IU/mg protein], AAc₁ IR & AAc₂ IR [44.38 ± 2.60 and 56.46 ± 1.73 IU/mg protein] and CA₁ IR & CA₂ IR [40.52 ± 3.30 and 43.52 ± 2.45 IU/mg protein] respectively.

The results are presented in the Table 7 and fig 29.

Myocardial Lactate

When compared with the myocardial lactate value of IR group [13.1 ± 0.13 µmole/g wet wt], there were no significant difference in myocardial lactate values among all the drug treated IR groups. There were no significant difference in the levels of the
biochemical parameters between BL group and C group, ruling out any effect of 26 min of normoxic perfusion.

The results are presented in the Table 8 and fig 30.

**Histopathological Studies**

**Group C**

Light microscopy of the tissue sections of control group showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 43)

**Group IR**

Light microscopy of the tissue sections of IR group showed edema, focal haemorrhage and leukocyte infiltration. The muscle fibers showed vascular changes like capillary congestion with fragmentation suggestive of focal necrosis. (Fig 44).

**Group I**

Light microscopy of the tissue sections of I group showed extensive cellular leukocyte infiltration, marked degeneration of muscle fibers, edema and haemorrhage. (Fig 45)

**Group TA₁ BL**

Light microscopy of the tissue sections of group TA₁ BL showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 46)

**Group TA₁ IR**

Light microscopy of the tissue sections of group TA₁ IR showed normal architecture of myofibrillar structure with striations, branched appearance and continuity
with adjacent myofibrils. The morphology of cardiac muscle fibers was relatively well preserved. (Fig 47).

**Group TA₂ BL**

Light microscopy of the tissue sections of group TA₂ BL showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 48)

**Group TA₂ IR**

Light microscopy of the tissue sections of group TA₂ IR showed normal architecture of myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. The morphology of cardiac muscle fibers was relatively well preserved. (Fig 49).

**Group AA₁ BL**

Light microscopy of the tissue sections of group AA₁ BL showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 50).

**Group AA₁ IR**

Light microscopy of the tissue sections of group AA₁ IR showed normal architecture of myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. The morphology of cardiac muscle fibers was relatively well preserved. (Fig 51).
**Group AA₂ BL**

Light microscopy of the tissue sections of group AA₂ BL showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 52)

**Group AA₂ IR**

Light microscopy of the tissue sections of group AA₂ BL showed normal architecture of myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. The morphology of cardiac muscle fibers was relatively well preserved. (Fig 53).

**Group AAc₁ BL**

Light microscopy of the tissue sections of group AAc₁ BL showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 54)

**Group AAc₁ IR**

Light microscopy of the tissue sections of group AAc₁ IR showed normal architecture of myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. The morphology of cardiac muscle fibers was relatively well preserved. (Fig 55).

**Group AAc₂ BL**

Light microscopy of the tissue sections of group AAc₂ BL showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 56).
Group AAc$_2$ IR

Light microscopy of the tissue sections of group AAc$_2$ IR showed normal architecture of myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. The morphology of cardiac muscle fibers was relatively well preserved. (Fig 57).

Group CA$_1$ BL

Light microscopy of the tissue sections of group CA$_1$ BL showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 58).

Group CA$_1$ IR

Light microscopy of the tissue sections of group AAc$_2$ IR showed normal architecture of myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. The morphology of cardiac muscle fibers was relatively well preserved. (Fig 59).

Group CA$_2$ BL

Light microscopy of the tissue sections of group CA$_1$ BL showed normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. (Fig 60).

Group CA$_2$ IR

Light microscopy of the tissue sections of group AAc$_2$ IR showed normal architecture of myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. The morphology of cardiac muscle fibers was relatively well preserved. (Fig 61).
2.5.3.2.2 DISCUSSION

Experimental model

In the present study, the isolated perfused rat heart as a model was used to investigate the cardiac activity. This model was also the most frequently used one for the development and assessment of anti-ischaemic agents. It is possible to induce ischaemia in the mammalian heart by stopping flow of the perfusate for 9 min. The isolated rat heart model allows an excellent biochemical and morphological assessment and the results are comparable with clinical correlations in ischaemic disease. Thus, keeping in view of the above feature, the rat was chosen as the experimental model for the study 222.

Myocardial TBARS

Oxygen free radicals (OFR) produce tissue damage, which greatly reduce the beneficial effects of reperfusion of the ischaemic myocardium. This hypothesis is supported by the observation that significant quantities of oxygen radicals are generated at the time of post-ischaemic reflow. OFRs cause oxidative damage to important biomolecules, e.g., lipids, proteins and DNA. Membrane phospholipids peroxidation is involved in the mechanism of OFR induced IR injury and explains the loss of structural integrity and function of cellular membranes that occur during the early phase of reperfusion. The evidence for this mechanism has been established in several studies by an indirect observation of thiobarbituric acid reactive substance production by means of thiobarbituric acid reactive substance (TBARS) assay 186 & 187. The convenience, ease and rapidity of TBARS test have attracted a lot of acceptance, despite some minor defects, such as the non-specificity of TBARS chemical reactivity 223.

There are no significant difference between the myocardial TBARS levels between BL (vehicle treated) group [44.60 ± 3.40 mole/g wet wt] and C (vehicle treated
group subjected to 26 min of reperfusion) group [45.80 ± 0.77 mole/g wet wt]. This rules out neither the vehicle treatment nor the 26 min reperfusion process altered the normal TBARS levels.

**Base line changes**

30 days oral administration of all the drug treated groups i.e., TA$_1$ BL & TA$_2$ BL, AA$_1$ BL & AA$_2$ BL, AAC$_1$ BL & AAC$_2$ BL at two doses level, produced a significant increase in base line myocardial TBARS when compared with the base line myocardial TBARS value of vehicle treated BL group. In case of the standard drug CA, such increase is observed only with higher dose i.e., CA$_2$ BL treated groups.

The increase in TBARS is suggestive of an enhanced oxidative stress, which in the absence of cellular damage (as evidenced by histological study), may be considered to be sub-lethal. On the contrary, it is possible that such sub-lethal oxidant stress might be a stimulus for the compensatory increase in the synthesis of endogenous antioxidant enzymes$^{75 - 77}$. It has been reported that even low concentrations of cytokines can stimulate the synthesis of antioxidant enzymes, without causing cellular injury$^{224}$.

**Ischaemic reperfusion (IR) injury changes**

The myocardial TBARS level of group I [52.65 ± 2.07 nmole/g wet wt] is significantly elevated than group BL value [44.60 ± 3.40 nmole/g wet wt]. The elevated level of TBARS in ‘I’ group is an indication of oxidative stress and hence increased lipid peroxidation.

When compared with myocardial TBARS levels of ‘I’ group [52.65 ± 2.07 nmole/g wet wt], the myocardial TBARS level of IR group is further elevated [61.98 ± 3.97 nmole/g wet wt].
1.66 nmole/g wet wt]. The elevated myocardial TBARS in IR group is an indication of further oxidative stress.

Although reperfusion of coronary flow is necessary to resuscitate the ischemic myocardium, reperfusion of the previously ischaemic myocardium may be entirely beneficial, as there is also evidence that it may partly counteract the beneficial effects of restoration of blood flow. This phenomenon has thus been termed “reperfusion injury”\(^{225 - 227}\). This phenomenon may have major clinical implications, because reperfusion after a period of ischaemia occurs not only during myocardial infarction but also in other frequent and important clinical conditions such as angina and cardioplegic arrest during cardiac surgery\(^{226}\).

Ischaemia followed by reperfusion favours free radical formation\(^{228}\). The main mechanisms responsible for free radical production are xanthine oxidase reaction, mitochondrial respiratory chain (oxidation/reduction reactions), neutrophil activation and auto oxidation of catecholamines\(^{229 - 231}\). The other reported mediators of reperfusion injury include intracellular calcium overload\(^{232}\), endothelial and microvascular dysfunction\(^{233}\) and altered myocardial metabolism\(^{234 & 235}\).

In spite of oxidative stress associated with ischaemic reperfusion, the results of all the IR subjected, drug treated groups (TA\(_1\) IR & TA\(_2\) IR, AA\(_1\) IR & AA\(_2\) IR, AAc\(_1\) IR & AAc\(_2\) IR and CA\(_1\) IR & CA\(_2\) IR shown decreased levels of myocardial TBARS than their corresponding drug treated BL groups (i.e., TA\(_1\) BL & TA\(_2\) BL, AA\(_1\) BL & AA\(_2\) BL, AAc\(_1\) BL & AAc\(_2\) BL and CA\(_1\) & CA\(_2\) BL).

It is evident from the above results that, all the drug treated groups, when subjected ischaemic reperfusion (IR), despite the higher baseline (BL) myocardial TBARS levels, there was better recovery profile in terms of myocardial TBARS
generation. This suggests the fact that chronic drug treatment (TA, AA, AAC and CA) imparts better oxidant stress bearing capacity to rat hearts.

In terms of myocardial TBARS recovery profile, captopril (CA) treated groups shown maximum protection, followed by arjunolic acid acetate (AAc) treated group followed by arjunolic acid (AA) treated group and finally by *T. arjuna* (TA) treated group and the same can be graded as CA > AAc > AA > TA.

**Myocardial SOD**

The enzyme superoxide dismutase (SOD) is present in the mammalian heart in two forms (i.e.,) i] cystolic form - which contains copper and zinc (Cu-Zn-SOD) and ii] mitochondrial form - which contains manganese (Mn-SOD).

Super oxide radical anion ($\text{O}_2^-$) is one of the reactive oxygen species (ROS) and plays a central role, since other reactive intermediates are formed in the reaction sequence starting with $\text{O}_2^-$.

Actually, the super oxide radical is a poorly reactive radical species, and is unable to cross lipid membranes, but its protonated form i.e., $\text{H}_2\text{O}_2$ is able to permeate through and react with biological membranes. It is currently believed that the most of the damaging effects of the super oxide radicals are due to the generation of more reactive species, such as hydrogen peroxide and the hydroxyl radical.

Super oxide dismutase specifically catalyses the dismutation of super oxide to hydrogen peroxide, and thus provides the first step in the metabolism of this radical. However the subsequent metabolism of hydrogen peroxide is essential to prevent its accumulation and the ensuring generation of other highly reactive radical species.
There is no significant difference between the myocardial SOD level of BL (vehicle treated) group (3.5±0.1 IU/mg protein) and C (vehicle treated group subjected to 26 min of perfusion) group (3.3±0.1 IU/mg protein). This rules out that neither the vehicle treatment nor the 26 min reperfusion process has altered the normal myocardial SOD level.

**Base line changes**

When compared with the myocardial SOD level of BL (vehicle treated) group [3.5 ± 0.1 IU/mg protein] the myocardial SOD level of ‘I’ group is significantly decreased (1.7 ± 0.1 IU/mg protein) which is a clear indication that, under stressful conditions such as ischaemia, the endogenous SOD level is degreased. But, the myocardial SOD level is significantly increased in IR group (2.9 ± 0.2 IU/mg protein). This is due to the fact that reperfusion process, to a certain extent, counter act the oxidative stress occurred during ischaemia.

Interestingly the basal the myocardial SOD levels in all the drug treated [TA₁ BL & TA₂ BL, AA₁ BL & AA₂ BL, AAC₁ BL & AAC₂ BL and CA₁ BL & CA₂ BL] groups significantly elevated, when compared with BL (vehicle treated) SOD level.

**In other words, chronic oral treatment of drugs such as TA, AA, AAC, and CA augments the basal endogenous SOD level.**

In terms of augmentation of myocardial SOD level captopril(CA) treated groups shown maximum level, followed arjunolic acid acetate (AAC) treated groups followed by arjunolic acid treated group and finally by *T. arjuna* (TA) treated group. The above results be graded as CA AAc>AA > TA.
Ischaemic repertusion (IR) injury changes

When compared with the myocardial SOD level of BL (vehicle treated) group, the myocardial SOD levels are increased in all the IR subjected, drug treated groups (i.e. TA_{1IR} & TA_{2IR}, AA_{1IR} & AA_{2IR}, AAc_{1IR} & AAc_{2IR} and CA_{1IR} & CA_{2IR}).

But, the degree of increment of SOD levels in drug treated IR group is lesser than their corresponding drug treated BL groups [i.e., TA_{1BL} & TA_{2BL}, AA_{1BL} & AA_{2BL}, AAc_{1BL} & AAc_{2BL} and CA_{1BL} & CA_{2BL}]. The observed difference in myocardial SOD levels between BL and IR groups may be due to the fact that reperfusion process itself generates oxygen free radicals and to scavenge such free radicals, some amount of SOD might have been utilized.

Therapeutic intervention of myocardial infarction with exogenous administration of SOD and catalase was reported to have reduced the infarct size in canine model^{236}. Other studies also reported to have the cardio protective role of SOD^{237 & 238}. However, a bell shaped dose–response relationship of the effect of SOD on infarct size shown, in that cardio protection was diminished at higher doses^{239}. This study casts some doubt on the efficacy of SOD as a therapeutic approach in treating myocardial ischaemic reperfusion injury. SOD intervention also failed in preclinical studies^{240 & 241}. To overcome the ambiguity about the efficacy of exogenously administered SOD in cardio protection, a multi-centered, randomized, placebo-controlled clinical trial was designed to test the hypothesis that free radical–medicated reperfusion injury could be reduced by intravenous administration of recombinant human SOD (hSOD) prior to percutaneous trans luminal coronary angioplasty (PTCA) in patients with acute trans mural myocardial infarction^{242}. According to this study, both the control group and the hSOD treated group associated with an improvement in the left ventricular function. There was however, no additional
left ventricular function benefit of h-SOD administration. The findings concluded the fact that exogenous administration of SOD did not protect the human heart from ischaemic reperfusion injury.

Augmentation of basal endogenous SOD offers a better protection against oxidative stress than exogenously administered SOD, which failed to exert the desired cardio protection.

**Myocardial GSH**

It is a tripeptide composed of three amino acids. Chemically, glutathione is glutamyl cysteine glycine. It exists in reduced or oxidized states

\[
2 \text{G-SH} \leftrightarrow \text{G-S-S-G}
\]

(Reduced) \hspace{1cm} (Oxidized)

The reversible oxidation-reduction of glutathione is important for many of its biological functions. In a steady state, the cells generally maintain a ratio of about 100/1 of GSH to G-S-S-G.

Glutathione is a major cellular redutant and its metabolism is controlled by a number of important enzymes. One of the important functions of reduced glutathione (GSH) is, it reduces hydrogen peroxide to water, and itself it is converted into oxidized glutathione (G-S-S-G). GSH is cycled back from the oxidized glutathione (GSSG) by glutathione reductase, the reduction process requiring NADPH generated by the hexose mono phosphate shunt.

There is considerable evidence that the myocardial glutathione status plays a crucial role in the defense against oxidant stress. Glutathione with maleic acid diethyl ester resulted in the impairment of recovery of contractile function of isolated rat hearts.
after 20 min of hypothermic ischemia\textsuperscript{245}. Inhibition glutathione synthesis by pretreatment with buthionine sulfoximine (BSO) also results in marked depletion of myocardial glutathione, and is associated with impairment of recovery of contractile function of both isolated blood-perfused cat heart after 40 min of ischaemia\textsuperscript{246} and isolated buffer perfused rat heart after 30 min of ischaemia\textsuperscript{247}. In the pig heart, glutathione depletion with BSO resulted is a marked increase in the extent of myocardial infarction after 45 min ischaemia\textsuperscript{248}.

These results in whole heart are complemented by a study of isolated neonatal rat cardiomyocytes where depletion of intracellular glutathione by BSO resulted in increased sensitivity to the oxidative stress in comparison to untreated cells\textsuperscript{249}. Acquired deficiency of the glutathione redox system occurs in humans where chronic selenium deficiency results in cardiomyopathy Keshans disease\textsuperscript{250}.

There was no significant difference between the myocardial GSH levels of BL (vehicle treated) group (331.05 ± 6.35 \( \mu \)g/g wet wt) and C (vehicle treated group subjected to 26 min of perfusion) group (335.23 ± 2.81 \( \mu \)g/g wet wt). This explains the fact that neither the vehicle treatment nor the 26 min of reperfusion process has altered the normal myocardial GSH level.

**Base line changes**

When compared with the myocardial GSH level of BL (vehicle treated) group (331.05 ± 6.31 \( \mu \)g/g wet wt), the myocardial GSH level of ‘I’ group is significantly decreased (317.04 ± 6.27 \( \mu \)g/g wet wt) this may be due the ‘oxidative stress’ associated with ischaemia. But the myocardial GSH level is significantly increased in IR group (328.98 ± 9.50 \( \mu \)g/g wet wt). This may be due to the influence of ischemic process, which is able to overcome the oxidative stresses to certain extent.
It is noteworthy that, the basal myocardial GSH levels in all the drug treated groups (i.e. TA1 BL & TA2 BL, AA1 BL & AA2 BL, AAc1 BL & AAc2 BL and CA1 BL & CA2 BL) are significantly elevated, when compared with vehicle treated BL group GSH level.

Chronic oral treatment of drugs viz., TA, AA, AAc and CA augments the basal endogenous GSH level.

In terms of augmentation of myocardial GSH level, captopril (CA) treated groups shown maximum level, followed by arjunolic acid acetate (AAc) treated group, followed by arjunolic acid (AA) treated group and finally by T. arjuna (TA) treated group and it can be graded as CA>AAc> AA> TA.

Ischaemic reperfusion (IR) injury changes

The myocardial GSH levels of all the IR subjected, drug treated groups (i.e. TA1 IR & TA2 IR, AA1 IR & AA2 IR, AAc1 IR & AAc2 IR and CA1 IR & CA2 IR) is significantly increased when compared with the vehicle treated BL group.

But the degree of increment of GSH levels in drug treated IR group is lesser than their corresponding drug treated BL groups (i.e. TA1 BL & TA2 BL, AA1 BL & AA2 BL, AAc1 BL & AAc2 BL and CA1 BL & CA2 BL).

The observed difference in myocardial GSH levels between BL and IR groups may be attributed due to the fact that some of the myocardial GSH might have lost in their battle field against ischaemic reperfusion induced oxidative stress.

The cardio protective potential of glutathione (GSH), was supported by the experimental findings that transgenic mice that over express glutathione peroxidase
(GSHPx) (the enzyme that catalyses the conversion of hydrogen peroxide into water in the presence of GSH) appear to be resistant to myocardial ischaemic reperfusion injury\textsuperscript{251}, whereas GSHPx knockout mice are more susceptible to myocardial reperfusion injury, compared with their wild type counterparts\textsuperscript{252}. The antioxidant property of GSHPx is also reported\textsuperscript{253-255}.

The above experimental findings indirectly support the present cardioprotective property of augmented endogenous glutathione.

**Myocardial Catalase**

Myocardial catalase is an antioxidant enzyme that along with glutathione redox system acts as a route for the metabolism of hydrogen peroxide in the heart\textsuperscript{256}.

There was no significant difference between the myocardial catalase levels of BL (vehicle treated) group (46.21 ± 1.87 IU/mg protein) and C (vehicle treated group subjected to 26 min of perfusion) group (44.36 ± 1.08 IU/mg protein). Above findings suggests that neither the vehicle treatment nor the 26 min of reperfusion process has altered the normal myocardial GSH level.

**Base line changes**

When compared with myocardial catalase level of BL (vehicle treated group) (46.21 ± 1.87 IU/mg protein) the myocardial catalase level of ‘I’ group is significantly decreased (24.62 ± 1.99 IU/mg protein) which may be due to ischaemic stress, but the myocardial catalase level is significantly increased is IR group (34.63 ± 0.83 IU/mg protein). This may be due to beneficial effect of reperfusion process.
When compared with BL (vehicle treated) catalase level, the basal myocardial catalase levels in all the drug treated groups (i.e. TA₁ & TA₂ BL, AA₁ & AA₂ BL, AAc₁ & AAc₂ BL and CA₁ & CA₂ BL) are significantly elevated.

**Chronic oral treatment of drugs TA, AA, AAc and CA augments the basal endogenous catalase level.**

In terms of augmentation of myocardial catalase level, *T. arjuna* treated groups shown maximum level, followed by arjunolic acid acetate (AAc) treated groups followed by arjunolic acid (AA) treated groups and finally captopril (CA) treated group and the same can be expressed as TA > AAc > AA > CA.

**Ischaemic reperfusion changes**

When compared with the myocardial catalase level of vehicle treated BL group, the myocardial catalase level is increased in all the IR subjected drug treated groups (i.e., TA₁ IR & TA₂ IR, AA₁ IR & AA₂ IR, AAc₁ IR & AAc₂ IR and CA₁ IR & CA₂ IR).

But the degree of increment of catalase levels in drug treated IR group is lesser than their corresponding drug treated BL groups (i.e. TA₁ BL & TA₂ BL, AA₁ BL & AA₂ BL, AAc₁ BL & AAc₂ BL and CA₁ BL & CA₂ BL).

The observed difference in myocardial catalase levels between BL and IR groups may be due to loss of myocardial catalase in the fight against reperfusion induced free radical generation.

Exogenous administration of catalase along with SOD was reported to have reduced the infarct size in canine model\(^2\). But latter study disproved the efficacy of both catalase and SOD to alter infarct size in dogs\(^2\).
Failure of exogenously administered catalase may be advantageous if the drug treatment was able to enhance/augment the endogenous catalase. The present study reveals the beneficial therapeutic property of augmenting endogenous catalase.

Myocardial lactate changes

Lactate dehydrogenase enzyme levels are elevated in myocardial ischaemia which in turn leads to increased levels of myocardial lactate. Significant elevation of myocardial lactate levels was observed only in ‘I’ group and is a clear indication that under the experimental conditions (when the perfusion was cut-off for 9 min) the animal hearts become ischaemic.

2.5.3.2.3 CONCLUSION

1. In the present work, when the flow of perfusate has been cut-off for 9 min, a significant increase in myocardial lactate level was observed in ‘I’ (Ischaemic) group. It is an evidence that ischaemia is produced under the experimental conditions.

2. When chronic drug treated animals were subjected to 12 min of reperfusion after 9 min of ischaemia, they exhibited a significant increase in the levels of myocardial TBARS. The elevated TBARS is an indication of lipid peroxidation which is due to increased oxidative stress - an undesirable phenomenon associated with ischaemic reperfusion injury.

3. This enhanced oxidative stress, in the absence of cellular damage, (as evidenced by histological study) may be considered as sub-lethal.

4. Such sub-lethal oxidant stress may be a stimulus for the compensatory increase in the endogenous antioxidants (i.e., augmentation of SOD, GSH and CAT)
5. In the present study, chronic treatment of TA, AA, AAc and CA exhibited augmentation of endogenous antioxidants.

6. Augmenting cellular endogenous antioxidant has considered as a major constituent of myocardial adaptation against oxidative stress\textsuperscript{259}.

7. Augmenting endogenous antioxidants (SOD, GSH and CAT) has been recognized as an important pharmacological property present in natural as well as many synthetic compounds\textsuperscript{260-263}. This contributes a major mechanism of cardioprotection against oxidative stress offered by them\textsuperscript{264-266}.

8. The cardioprotective effect of methanolic extract of \textit{T. arjuna} mediated through augmentation endogenous antioxidants has been reported\textsuperscript{75-77}.

9. In the present work, the cardioprotective effect of arjunolic acid (the major triterpenoid present in the \textit{T.arjuna} bark) and its tri-acetate derivative (arjunolic acid acetate) were evaluated for the first time.

10. With respect to the augmenting endogenous antioxidants, among the test drugs, such as TA, AA and AAc, AAc shown maximum activity followed by AA and finally TA (except in case of myocardial catalase profile, where TA shown maximum activity).

11. ACE inhibitors such as captopril used widely in ischaemic heart disease, have been recently shown to possess the property of augmenting endogenous antioxidants. Hence captopril was taken as standard drug\textsuperscript{267 & 268}.

\subsection*{2.5.3.3 HEPATOPROTECTIVE ACTIVITY}

\subsubsection*{2.5.3.3.1 Results}

Rats treated with carbon tetrachloride alone (Group II), developed significant liver damage as observed from elevated serum levels of hepatic marker enzymes (AST,
ALT and ALP) as well as severe alterations in other biochemical parameters. Serum bilirubin level was also significantly enhanced by carbon tetrachloride administration. The levels of total proteins and total albumin were significantly decreased when compared with the control rats (Group I). Histopathological changes also confirmed the hepatic damage induced by carbon tetrachloride. When compared to the normal animal liver tissue (Fig 62), the carbon tetra chloride treated animals showed fatty degeneration of hepatocytes (Fig 63).

Oral administration of silymarin and all other test drugs such as TA, AA, AAc, significantly (P<0.001) decreased the elevated levels of serum AST, ALT and ALP. The drug treatment also significantly (P<0.001) reduced the elevated serum bilirubin level. The drug treatment also significantly (P<0.001) raised the total albumin level.

The degree of protection was observed maximally with the silymarin treated rats. In silymarin treated group, the serum hepatic marker enzyme levels such as AST, ALT and ALP (90.16 ± 1.99, 63.16 ± 1.55 and 151.16 ± 3.52 IU/L respectively) were almost comparable with the AST, ALT and ALP levels (83.66 ± 1.31, 58.50 ± 1.65 and 125.33 ± 3.41 respectively) of vehicle treated rats (Table 9 and fig 31). Higher dose of AAc (i.e., 40 mg/kg) exhibited maximum protection among the test drugs (TA, AA and AAc). The serum hepatic marker enzyme levels such as AST, ALT and ALP in AAc (40mg/kg) treated animals values (96.5 ± 1.22, 76 ± 2.06 and 148 ± 3.97 IU/L respectively) are comparable to that of the enzyme levels of silymarin treated groups.

In terms of recovery of serum total albumin total protein and bilirubin values, among the test drugs (TA, AA and AAc), AAc (40mg/kg) shown higher degree of hepato protection. Arjunolic acid treated groups exhibited lesser degree of protection than AAc treated group and lowest degree of protection shown by *T. arjuna* (TA) treated group.
The above finding was also supported by the histopathological study. Normal lobular architect of the liver with central vein and portal tracts and cords of hepatocytes with sinusoids in between was observed in vehicle treated rats (Fig 62). Rats treated with carbon tetrachloride alone shown fatty degeneration of hepatocytes (Fig 63). In TA (250 mg/kg) treated group (Fig 65), the fatty changes are seen more in perivenular region. Larger areas of normal hepatocytes are also evident. In TA (500mg/kg) treated animals (Fig 66), relatively normal hepatocytes with minimal fatty changes were observed. Rats treated with AA (20 mg/kg) (Fig 67) exhibited patchy areas of normal hepatocytes, intermingled with map like areas of fatty changes. AA (40 mg/kg) treatment (Fig 68) showed larger areas of normal hepatocytes with foci of fatty changes. Animals treated with AAc (20 & 40 mg/kg) exhibited larger areas of normal hepatocytes. (Fig 69 and 70)

2.5.3.3.2 Discussion

Carbon tetrachloride (CCl$_4$) induced hepatotoxicity in rats represents an adequate experimental model of cirrhosis in man and hence it is used for the screening of hepatoprotective drugs. It is well established that hepatotoxicity by CCl$_4$ is due to the release of CCl$_3$? radical in the liver endoplasmic reticulum by cytochrome P-450. The CCl$_3$? radical alkylates cellular proteins and other macromolecules with a simultaneous attack on poly unsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage. Hepatotoxic compounds such as CCl$_4$ are known to cause marked elevation in serum enzymes such as AST, ALT, ALP and bilirubin levels.

In the present study also, it was seen that the administration of CCl$_4$ elevated the levels of serum marker enzymes such as ALT, AST, ALP and bilirubin. The levels of total protein and total albumin were lowered. Treatment with silymarin, TA, AA and
AAc lowered the elevated levels of AST, ALT, ALP and bilirubin when compared with groups treated with CCl₄ alone. The stabilization of serum bilirubin, AST, ALT and ALP by the drug treatment is a clear indication of the improvement of the functional status of the liver cells. These findings are further corroborated with the histopathological studies.

Arjunolic acid is an oleanane type triterpenoid. The hepatoprotective effect of oleanolic acid isolated from various plants are reported²⁷³ - ²⁷⁵. Since oleanolic acid exhibited promising hepatoprotective property in experimental studies, it has been successfully used as an oral drug in the treatment human liver disease in China²⁷⁶. The extraction of oleanolic acid from *Beta vulgaris* L (sugar beats) for the treatment of liver failure has also been patented in Japan²⁷⁷.

The proposed mechanism of hepatoprotection of oleanolic acid includes suppression of cytochrome P-450 and inhibition of lipid peroxidation²⁷⁸, enhancement of hepatic glutathione system²⁷⁹, prevention of fibrosis and stimulation of liver generation²⁸⁰. Since arjunolic acid has structure similarity with oleanolic acid, the observed hepatoprotection of arjunolic acid (and arjunolic acid acetate) may involve similar hepatoprotective mechanism as that of oleanolic acid.

It has been shown that hepatoprotective agents also exert their action against CCl₄ induced liver injury (mediated via lipid peroxidation) either through decreased production of free radicals derivatives²⁸¹ or due to the antioxidant activity of the protective agent itself²⁸².

Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense against different human diseases including cancer, ischaemia, atherosclerosis, inflammation and the ageing process²⁸³ & ²⁸⁴.
Antioxidants can interfere with the oxidation process by reacting with the free radicals, checking the free catalytic metals and also by acting as oxygen scavengers\(^{285}\).

All the test compounds such as TA, AA and AAc exhibited significant *in-vitro* antioxidant activity against DPPH and nitric oxide free radical and is discussed in detail in the section 2.5.3.5. This may also, impart, contribute the observed hepatoprotective activity of the test drugs TA, AA and AAC.

### 2.5.3.3 Conclusion

(i) *T. arjuna*, in the traditional system of medicine, is said to posses the hepatoprotective activity. *T. arjuna* is one of the ingredients in popular hepatoprotective Ayurvedic medicine *Liv 52*. But *T. arjuna*, has not been scientifically evaluated for its hepatoprotective property.

(ii) The present findings revealed that *T. arjuna* exhibited significant hepatoprotection against carbon tetrachloride induced hepatic damage.

(iii) Arjunolic acid - the major triterpoid compound isolated from the bark of *T. arjuna*, showed better hepatoprotection than methanolic extract of *T. arjuna* bark.

(iv) It may be concluded that, Arjunolic acid may be the active constituent responsible for the observed hepatoprotection due to methanolic extract of stem bark of *T. arjuna*.

(v) The degree of hepatoprotection is increased when arjunolic acid is acetylated to arjunolic acid acetate.

(vi) The hepatoprotective effect of arjunolic acetate is comparable to that of the hepatoprotection of the standard drug silymarin.
2.5.3.3 ANTI-INFLAMMATORY ACTIVITY

2.5.3.4.1 Results

In carrageenan induced paw oedema (acute model), the standard anti-inflammatory drug (Diclofenac sodium 5 mg/kg, p.o.) as well as all the test drugs (TA, AA and AAc) exhibited a significant reduction (P<0.001) in the volume of paw oedema in rats. All the drugs showed maximum inhibition of the carrageenan induced rat paw oedema at the end of 3 h (Table 10). Their % inhibition of oedema is written within parenthesis. Diclofenc sodium shown maximum of 76.92% inhibition of oedema. TA 250 & 500 mg/kg produced 42.30 & 57.39% inhibition respectively. AA 20 & 40 mg/kg has shown 48.07 & 61.53% inhibition respectively. AAc 20 & 40 mg/kg exhibited 55.76 & 67.30 respectively. With respect to the % inhibition of oedema, among the test drugs, arjunolic acid acetate, (AAc) shown maximum protection, followed by arjunolic acid (AA) and finally T. arjuna (TA) and the same can be graded as AAc > AA > TA.

The results are presented in the table 10 & Fig. 32.

2.5.3.4.2 Discussion

Among the many methods used for screening and evaluation of anti-inflammatory drugs, one of the most commonly employed models is carrageenan induced paw edema. The development of oedema in the paw of the rat after the injection of carrageenan has been reported. The initial phase seen at 1 h is attributed to the release of histamine and serotonin. The oedema maintained during the plateau phase is presumed to be due to kinine like substances. The second accelerating phase of swelling is due to the release of prostaglandins, i.e., PG E2 and nitric oxide. It has been reported that the second phase of oedema is sensitive to both clinically useful steroidal and nonstroidal anti-inflammatory agents.
During the inflammatory phase, from the macrophages, the reactive free radical nitric oxide (NO) is synthesized by inducible NO synthase (iNOS). Accumulating evidence indicates that excessive production of NO plays a pathogenic role in both acute and chronic inflammations. NO is responsible for the vasodilatation, increase in vascular permeability, edema formation, and inducing synthesis of prostaglandins at the site of inflammation. The role of NO in relation to carrageenan induced paw oedema was also reported.

Manipulation of NO free radical can be a potential and promising therapeutic area in treating inflammations. Approaches being currently used for inflammatory disorders include NO scavenges as well as NO inhibitors.

Since all the test drugs (TA, AA & AAc) exhibited significant in-vitro NO free radical scavenging activity as discussed in the section 2.5.3.5, the same may be, in part, attributed to the observed anti-inflammatory effect of these test drugs.

2.5.3.4.3 Conclusion

(i) All the test drugs (TA, AA and AAc) produced significant % protection of oedema. This findings support the traditional claims of T. arjuna in treating inflammatory diseases.

(ii) Arjunolic acid - the major triterpenoid isolated from the T. arjuna bark showed better % protection of oedema than methanolic extract of T. arjuna. It may be concluded that, the anti-inflammatory property T. arjuna bark, attributed due to its arjunolic acid content.

(iii) The % protection of oedema is increased by 6% when arjunolic acid is acetylated to its triacetate derivative (i.e. arjunolic acid acetate).
Since, all the test drugs, showed significant \textit{in-vitro} NO free radical scavenging activity, this may, in part, be responsible for the observed anti-inflammatory activity.

\textbf{2.5.3.5 IN-VITRO ANTIOXIDANT ACTIVITY}

\textbf{2.5.3.5.1 Results}

\textbf{DPPH Assay}

The methanolic extract of TA, AA, AAc were studied for its \textit{in-vitro} anti-oxidant activity by DPPH assay. Vitamin C was used as the reference standard.

The IC$_{50}$ values of TA, AA, AAc and Vitamin C were found in DPPH assay to be 386, 28, 19 and 0.64 µg/ml respectively.

The results are presented in the table 11 – 14 and Fig 33 -36.

\textbf{Nitric Oxide radical inhibition assay}

The IC$_{50}$ values of TA, AA, AAc and Vitamin C by nitric oxide radical inhibition assay found to be 394, 32, 24 and 0.72 µg/ml respectively.

The results are presented in the table 15 – 18 and Fig 37 -40.

\textbf{2.5.3.5.2 Discussion}

DPPH assay is considered a valid and easy assay to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to generated as in other radical assays$^{211, 212}$. From methodological point of view, the DPPH method is recommended as easy and accurate with regard to measuring the antioxidant activity of fruit and vegetable juices or extracts. The results are highly reproducible$^{296, 297}$. 

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Over production of NO has been correlated with many diseases such as cancer\textsuperscript{298}, diabetes\textsuperscript{299}, trauma\textsuperscript{300}, sepsis\textsuperscript{301}, atherosclerosis\textsuperscript{302}, multiple sclerosis\textsuperscript{303}, arthritis and inflammation\textsuperscript{304}. Herbal drugs having NO radical scavenging property are gaining importance\textsuperscript{305} & \textsuperscript{306}. In the present study, vitamin C exhibited strong antioxidant activity in both DPPH and NO radical inhibition methods.

Among the test compounds AAc exhibited better free radical scavenging property followed by AA and finally by TA in both DPPH and NO radical inhibition methods.

2.5.3.5.3 Conclusion

i) The role of free radical involvement in the pathophysiology of ischaemia, hepatitis and inflammation are well documented.

ii) TA, AA and AAc are found have cardioprotective, hepatoprotective and anti-inflammatory properties.

iii) The above observed, pharmacological activities of TA, AA and AAc may be, in part, be correlated with anti-oxidant property of these test drugs.

2.5.3.6 ANALGESIC ACTIVITY

2.5.3.6.1 Results

Acetic acid induced writhing response in mice

In the acetic acid induced writhing method, TA, AA, AAc and PA showed a significant reduction in the number of writhings mice. This reduction was dose related.

TA (250 & 500 mg/kg) produced 33.33 and 53.33% protection (P<0.001) respectively. AA (20 & 40 mg/kg) shown 35.55 and 57.77% protection (P<0.001) respectively, AAc (20 & 40 mg/kg) exhibited 40 and 64.44% protection (P<0.001)
respectively. The standard drug Paracetamol (PA) produced a maximum of 80% protection (P<0.001) of analgesia. (Table 19 and fig 41)

**Tail immersion model**

In tail immersion model, the standard drug morphine (5mg/kg) showed 205% protection (P<0.001) of analgesia. Other test drugs such as TA, AA and AAc did not shown significant protection of analgesia. (Table 20 and fig 42).

**2.5.3.6.2 Discussion**

In order to distinguish between the central and peripheral analgesic action of test drugs, both acetic acid induced writhing reflex as well as tail immersion methods were carried out. Acetic acid induced writhing response is a non specific model i.e., it will not distinguish between opioid and non opioid type activities. In this test, the animals react with characteristic stretching behaviour which is called writhing.

In acetic acid induced writhing model, among the test drugs AAc shown maximum % protection of analgesia followed by AA and then by TA.

The abdominal contraction is related to the sensitation of nociceptive receptors to prostaglandins. All the test drugs exert analgesic activity only in acetic acid induced writhing model, probably by inhibiting the synthesis or action of prostaglandins.

**2.5.3.6.3 Conclusion**

i) TA, AA and AAc have shown significant % protection against acetic acid induced writhing in mice.

ii) These test drugs did not show significant % protection in tail immersion model.
The above finding suggests that, the observed analgesic activity of the test drugs may be due to peripheral analgesic mechanism rather than central analgesic mechanism.
Table- 2. Preliminary qualitative analysis on various extracts of *T. arjuna*.

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<th>Hexane extract</th>
<th>Chloroform extract</th>
<th>Ethyl acetate extract</th>
<th>Methanolic extract</th>
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+ indicates positive test  -  indicates negative test
HPTLC finger print of the methanolic extract of T. arjuna bark with marker compounds

(d) Arjunolic acid
(c) Maslinic acid
(b) Hederagenin
(a) TA methanolic Ext

Developing systems: CHCl₃: CH₃OH (9:1)
Scanning Wave Length: 200 nm.
Instrument: CAMAG - SCANNER 3
Software: CATS V 4.05 Version
Fig. 3a ¹H NMR Spectrum of Compound I (δ, CDCl₃, 400 MHz)
Fig. 3b ¹H NMR Spectrum of Compound I  (δ, CDCl₃ , 400 MHz)
Fig. 3c 'H NMR Spectrum of Compound 1 (δ, CDCl₃, 400 MHz)
Fig. 4 $^{13}$C NMR Spectrum of Compound I (5, CDCl$_3$, 100 MHz)
Fig. 5a $^1$H NMR Spectrum of Compound II (5, CDCl$_3$, 400 MHz)
Fig. 6b $^1$H NMR Spectrum of Compound II (δ, CDCl$_3$, 400 MHz)
Fig. 7 $^{13}$C NMR Spectrum of Compound II (δ, CDCl$_3$, 100 MHz)
Fig. 8 | R Spectrum of Compound III

Transmittance / Wavenumber (cm⁻¹)
Fig. 9a $^1$H NMR Spectrum of Compound III  ($\delta$, CDCl$_3$, 400 MHz)
Fig. 9b ¹H NMR Spectrum of Compound III  (δ, CDCl₃, 400 MHz)
Fig. 12a ¹H NMR Spectrum of Compound IV (δ, CDCl₃ + CDOD₃, 400 MHz)
Fig. 12c ¹H NMR Spectrum of Compound IV  \((\delta, \text{CDCl}_3 + \text{CDOD}_2, 400 \text{ MHz})\)
Fig. 13 $^1$H NMR Spectrum of Compound IV ($\delta$ CDCl$_3$ + CDOD$_3$, 100 MHz)
Fig. 16a. $^1$H NMR Spectrum of Acetate of compound IV (δ, CDCl$_3$, 400 MHz)
Fig. 15b. $^1$H NMR Spectrum of Acetate of compound IV (δ, CDCl$_3$, 400 MHz)
Fig. 15c. $^1$H NMR Spectrum of Acetate of compound IV (δ, CDCl₃, 400 MHz)
Fig. 15d. 'H NMR Spectrum of Acetate of compound IV (δ, CDCl₃, 400 MHz)
Fig. 16. $^{13}$C NMR Spectrum of Acetate of Compound IV ($\delta$, CDCl$_3$, 100 MHz)
Fig. 10b. $^1$H NMR Spectrum of Compound V (DMSO - $d_6$, 400 MHz)
Fig. 21a. $^1$H NMR Spectrum of Compound VI (5. DMSO - d$_6$, 400 MHz)
Fig. 21b. $^1$H NMR Spectrum of Compound VI (δ, DMSO - $d_6$, 400 MHz)
Fig. 22. $^1$H NMR Spectrum of Compound VI (DMSO-d$_6$, 100 MHz)
Fig. 23. IR Spectrum of acetate of Compound VI
Fig. 24b. $^1$H NMR Spectrum of acetate of Compound VI ($\delta$, CDCl$_3$, 400 MHz)
Fig. 25. $^1$H NMR Spectrum of acetate of Compound VI (δ, CDCl$_3$, 100 MHz)
Table – 4. Myocardial TBARS levels of methanolic extract of *T. arjuna* [TA], Arjunolic acid [AA] and Arjunolic acid acetate [AAc] and Captopril [CA] treated groups.

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All values are expressed as Mean ± SEM (n=6), *p < 0.001 vs BL, #p < 0.001 vs IR. (One way ANOVA)
Table – 5. Myocardial GSH levels of methanolic extract of *T. arjuna* [TA], Arjunolic acid [AA] and Arjunolic acid acetate [AAc] and Captopril [CA] treated groups.

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<td>532.37 ± 6.07*</td>
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All values are expressed as Mean ± SEM (n=6), *p < 0.001 vs BL, #p < 0.001 vs IR. (One way ANOVA)
Fig 26. Myocardial TBARS levels of TA/AA/AAc/CA treated groups

Fig 27. Myocardial GSH levels of TA/AA/AAc/CA treated groups
Table 6. Myocardial SOD levels of methanolic extract of T. arjuna [TA], Arjunolic acid [AA] and Arjunolic acid acetate [AAC] and Captopril [CA] treated groups.

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<th>CA1IR</th>
<th>CA2BL</th>
<th>CA2IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(IU/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.9 ± 0.2*</td>
<td>6.3 ± 0.1*</td>
<td>7.8 ± 0.2*</td>
<td>5.3 ± 0.1*</td>
<td>11.9 ± 0.2*</td>
<td>7.4 ± 0.1*</td>
<td>12.8 ± 0.2*</td>
<td>8.4 ± 0.2*</td>
<td>18.3 ± 0.1*</td>
<td>11.4 ± 0.1*</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± SEM (n=6), *p < 0.001 vs BL, #p < 0.001 vs IR. (One way ANOVA)
Table 7. Myocardial Catalase levels of methanolic extract of *T. arjuna* [TA], Arjunolic acid [AA] and Arjunolic acid acetate [AAC] and Captopril [CA] treated groups.

<table>
<thead>
<tr>
<th></th>
<th>BL</th>
<th>C</th>
<th>I</th>
<th>IR</th>
<th>TA&lt;sub&gt;1&lt;/sub&gt;BL</th>
<th>TA&lt;sub&gt;1&lt;/sub&gt;IR</th>
<th>TA&lt;sub&gt;2&lt;/sub&gt;BL</th>
<th>TA&lt;sub&gt;2&lt;/sub&gt;IR</th>
<th>AA&lt;sub&gt;1&lt;/sub&gt;BL</th>
<th>AA&lt;sub&gt;1&lt;/sub&gt;IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(IU/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>46.21 ± 1.87</td>
<td>44.36 ± 1.08</td>
<td>24.62 ± 1.99</td>
<td>34.63 ± 0.83*</td>
<td>129.52 ± 4.19*</td>
<td>64.42 ± 1.90*</td>
<td>145.50 ± 4.32*</td>
<td>82.44 ± 2.08*</td>
<td>75.43 ± 1.81*</td>
<td>44.56 ± 1.90*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>AA&lt;sub&gt;2&lt;/sub&gt;BL</th>
<th>AA&lt;sub&gt;2&lt;/sub&gt;IR</th>
<th>AA&lt;sub&gt;C1&lt;/sub&gt;BL</th>
<th>AA&lt;sub&gt;C1&lt;/sub&gt;IR</th>
<th>AA&lt;sub&gt;C2&lt;/sub&gt;BL</th>
<th>AA&lt;sub&gt;C2&lt;/sub&gt;IR</th>
<th>CA&lt;sub&gt;1&lt;/sub&gt;BL</th>
<th>CA&lt;sub&gt;1&lt;/sub&gt;IR</th>
<th>CA&lt;sub&gt;2&lt;/sub&gt;BL</th>
<th>CA&lt;sub&gt;2&lt;/sub&gt;IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μmole /g wet wt.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>83.40 ± 2.91*</td>
<td>43.63 ± 2.22*</td>
<td>99.02 ± 4.41*</td>
<td>44.38 ± 2.60*</td>
<td>105.49 ± 3.56*</td>
<td>56.46 ± 1.73*</td>
<td>47.59 ± 1.31</td>
<td>40.52 ± 3.30*</td>
<td>62.25 ± 2.04*</td>
<td>43.52 ± 2.45</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± SEM (n=6), *p < 0.001 vs BL, #p < 0.001 vs IR. (One way ANOVA)
Fig 28. Myocardial SOD levels of TA/AA/AAc/CA treated groups

Fig 29. Myocardial Catalase levels of TA/AA/AAc/CA treated groups
Table – 8. Myocardial lactate levels of methanolic extract of *T. arjuna* [TA], Arjunolic acid [AA] and Arjunolic acid acetate [AAc] and Captopril [CA] treated groups.

<table>
<thead>
<tr>
<th></th>
<th>BL</th>
<th>C</th>
<th>I</th>
<th>IR</th>
<th>TA₁BL</th>
<th>TA₁IR</th>
<th>TA₂BL</th>
<th>TA₂IR</th>
<th>AA₁BL</th>
<th>AA₁IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11.9±0.22</td>
<td>12.4±0.10</td>
<td>28.4±0.17*</td>
<td>13.1±0.13</td>
<td>11.4±0.10</td>
<td>10.8±0.32</td>
<td>11.9±0.24</td>
<td>11.2±0.11</td>
<td>13.8±0.19</td>
<td>13.8±0.28</td>
</tr>
</tbody>
</table>

(μ mole/g wet wt.)

<table>
<thead>
<tr>
<th></th>
<th>AA₂BL</th>
<th>AA₂IR</th>
<th>AA₃BL</th>
<th>AA₃IR</th>
<th>AA₄BL</th>
<th>AA₄IR</th>
<th>CA₁BL</th>
<th>CA₁IR</th>
<th>CA₂BL</th>
<th>CA₂IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14.0±0.23</td>
<td>13.6±0.12</td>
<td>12.8±0.20</td>
<td>13.4±0.21</td>
<td>14.2±0.25</td>
<td>13.2±0.20</td>
<td>12.3±0.50</td>
<td>11.2±0.38</td>
<td>11.8±0.38</td>
<td>11.2±0.38</td>
</tr>
</tbody>
</table>

(IU/mg protein)

All values are expressed as Mean ± SEM (n=6), *p < 0.001 vs BL, #p < 0.001 vs IR. (One way ANOVA)
Fig 30. Myocardial Lactate levels of TA/AA/AAc/CA treated groups
Table – 9. Hepatoprotective activity of methanolic extract of T. arjuna [TA], Arjunolic acid [AA] and Arjunolic acid acetate [AAC] and Silymarin [SM] on CCl₄ induced hepatoxicity in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>AST [IU/L]</th>
<th>ALT [IU/L]</th>
<th>ALP [IU/L]</th>
<th>TAL [g/dl]</th>
<th>TP [g/dl]</th>
<th>BILN [mg/dl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% w/v SCMC</td>
<td>10 ml/kg</td>
<td>83.66±1.31</td>
<td>58.50±1.65</td>
<td>125.33±3.41</td>
<td>3.62±0.13</td>
<td>6.43±0.14</td>
<td>0.64±0.07</td>
</tr>
<tr>
<td>CCl₄</td>
<td>2 ml/kg</td>
<td>242±5.74</td>
<td>184±3.51</td>
<td>347.50±4.64</td>
<td>1.28±0.08</td>
<td>4.67±0.14</td>
<td>3.89±0.19</td>
</tr>
<tr>
<td>Silymarin</td>
<td>25 mg/kg</td>
<td>90.16±1.99*</td>
<td>63.16±1.55*</td>
<td>151.16±3.52*</td>
<td>5.24±0.19*</td>
<td>7.57±0.11*</td>
<td>0.91±0.05*</td>
</tr>
<tr>
<td></td>
<td>250 mg/kg</td>
<td>169.50±1.60*</td>
<td>132±1.94**</td>
<td>251.00±6.54</td>
<td>3.71±0.08</td>
<td>5.48±0.06</td>
<td>2.36±0.06*</td>
</tr>
<tr>
<td></td>
<td>500 mg/kg</td>
<td>118.16±3.35*</td>
<td>97.17±3.35*</td>
<td>180.00±4.61*</td>
<td>4.42±0.09*</td>
<td>6.60±0.16</td>
<td>1.55±0.09*</td>
</tr>
<tr>
<td>TA</td>
<td>20 mg/kg</td>
<td>189.16±2.42*</td>
<td>146.66±2.48***</td>
<td>270.17±4.45</td>
<td>4.26±0.9*</td>
<td>5.58±0.12</td>
<td>2.04±0.13*</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>111.33±3.30*</td>
<td>87.16±2.70</td>
<td>164.50±2.95*</td>
<td>5.11±0.12*</td>
<td>7.07±0.10</td>
<td>1.23±0.06*</td>
</tr>
<tr>
<td>AA</td>
<td>20 mg/kg</td>
<td>180.83±5.55*</td>
<td>138±6.24**</td>
<td>254.17±6.40*</td>
<td>4.35±0.10*</td>
<td>5.94±0.11</td>
<td>1.73±0.06*</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>96.5±1.22*</td>
<td>76±2.06*</td>
<td>148.00±3.97*</td>
<td>5.02±0.06*</td>
<td>7.19±0.09*</td>
<td>1.20±0.10*</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± SEM. The statistical difference between control and treated groups were tested by one way ANOVA followed by Dunnett’s ‘t’ test. * P<0.0001, **P<0.01, ***P<0.05.
Fig. 31. Hepatoprotective activity of TA/AA/AAc/SM on CCl₄ induced hepatotoxicity in rats
Table – 10. Anti-inflammatory activity of methanolic extract of *T. arjuna* [TA], Arjunolic acid [AA] and Arjunolic acid acetate [AAc] and Diclofenac sodium [DS] on rat hind paw oedema induced by carrageenan model.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Increase in paw volume ± SEM (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>1% w/v SCMC</td>
<td>10 ml/kg</td>
<td>0.21±0.013</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>5 mg/kg</td>
<td>0.12±0.009 [42.85]*</td>
</tr>
<tr>
<td>TA</td>
<td>250 mg/kg</td>
<td>0.19±0.013 [9.52]*</td>
</tr>
<tr>
<td></td>
<td>500 mg/kg</td>
<td>0.17±0.013 [19.04]*</td>
</tr>
<tr>
<td>AA</td>
<td>20 mg/kg</td>
<td>0.18±0.025 [14.28]*</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>0.14±0.008 [33.33]*</td>
</tr>
<tr>
<td>AAc</td>
<td>20 mg/kg</td>
<td>0.17±0.007 [19.04]*</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>0.13±0.012 [38.09]*</td>
</tr>
</tbody>
</table>

Results are expressed as Mean±SEM and are in seconds. Percentage of protection against carrageenan induced paw oedema are in parenthesis. The statistical difference between control and treated groups were tested by one way ANOVA followed by Dunnett’s ‘t’ test. * P<0.0001, **P<0.01, ***P<0.05.
Fig. 32. Anti-inflammatory activity of TA/AA/AAc/DS after 3rd hour of carrageenan administration

% Protection

Diclofenac sodium 5mg/kg
TA 250 mg/kg
TA 500 mg/kg
AA 20 mg/kg
AA 40 mg/kg
AAc 20 mg/kg
AAc 40 mg/kg
Table – 11. *In-vitro* free radical scavenging effect of methanolic extract of *T. arjuna* by DPPH method

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Concentration (µg/ml)</th>
<th>% Scavenging (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract of TA</td>
<td>4</td>
<td>23.87 ± 0.0037*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>24.03 ± 0.0020*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>24.48 ± 0.0033*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>25.54 ± 0.0049*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>28.18 ± 0.0018*</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>33.90 ± 0.0018*</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>47.10 ± 0.0020*</td>
</tr>
<tr>
<td></td>
<td>720</td>
<td>72.63 ± 0.0024*</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>33.90 ± 0.0018*</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>47.10 ± 0.0020*</td>
</tr>
<tr>
<td></td>
<td>720</td>
<td>72.63 ± 0.0024*</td>
</tr>
</tbody>
</table>

IC$_{50}$ of TA is 386 µg/ml (r=0.99). Results are expressed Mean ± SEM. *P<0.001 compared to reagent blank. (Student’s ‘t’ test).

Table – 12. *In-vitro* free radical scavenging effect of Arjunolic acid by DPPH method

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Concentration of the test substance (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arjunolic acid</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>125</td>
</tr>
</tbody>
</table>

IC$_{50}$ of Arjunolic acid is 28 µg/ml (r=0.99) P<0.001 compared to reagent blank. (Student’s ‘t’ test).
Table – 13. *In-vitro* free radical scavenging effect of Arjunolic acid acetate by DPPH method

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Concentration (µg/ml)</th>
<th>% Scavenging (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arjunolic acid acetate</td>
<td>1</td>
<td>43.41 ± 0.0034*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>43.30 ± 0.0028*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>43.51 ± 0.0012*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>44.13 ± 0.0004*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>45.17 ± 0.0004*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>48.90 ± 0.0043*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>63.03 ± 0.0050</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>83.80 ± 0.0038*</td>
</tr>
</tbody>
</table>

IC$_{50}$ of AAc is 19 µg/ml (r=0.99), Results are expressed Mean ± SEM. *P<0.001 compared to reagent blank. (Student’s ‘t’ test)

Table – 14. *In-vitro* free radical scavenging effect of Vitamin C by DPPH method

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Concentration (µg/ml)</th>
<th>% Scavenging (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>0.1</td>
<td>5.86 ± 0.002*</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>14.28 ± 0.002*</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>30.06 ± 0.001*</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>48.04 ± 0.002*</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>60.82 ± 0.002*</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>79.62 ± 0.002*</td>
</tr>
</tbody>
</table>

IC$_{50}$ of Vitamin C is 0.68 µg/ml (r=0.99). Results are expressed Mean ± SEM. *P<0.001 compared to reagent blank (Student’s ‘t’ test)
Fig 33. *In-vitro* free radical scavenging effect of TA by DPPH Method

Fig 34. *In-vitro* free radical scavenging effect of AA by DPPH method
Fig 35. *In-vitro* free radical scavenging effect of AAc by DPPH method

![Graph showing the free radical scavenging effect of Arjunolic acid acetate (AAc) by DPPH method.](image)

- **Concentration of Arjunolic acid acetate AAc (micro g/ml)**

Fig 36. *In-vitro* free radical scavenging effect of Vitamin C by DPPH method

![Graph showing the free radical scavenging effect of Vitamin C by DPPH method.](image)

- **Concentration of Vitamin C**

204
Table – 15. *In-vitro* free radical scavenging effect of methanolic extract of *T. arjuna* by nitric oxide method

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Concentration (µg/ml)</th>
<th>% Scavenging (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract of TA</td>
<td>5 10 20 40 80 160 320 640</td>
<td>18.03 ± 0.0067* 18.39 ± 0.0042* 19.20 ± 0.0028* 20.65 ± 0.0014* 24.09 ± 0.0033* 30.98 ± 0.0007* 43.84 ± 0.0082* 70.10 ± 0.0037*</td>
</tr>
</tbody>
</table>

IC<sub>50</sub> of TA is 394 µg/ml (r=0.99). Results are expressed Mean ± SEM. *P<0.001 compared to reagent blank. (Student’s ‘t’ test)

Table – 16. *In-vitro* free radical scavenging effect of Arjunolic acid by nitric oxide method

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Concentration of the test substance (µg/ml)</th>
<th>% Scavenging (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arjunolic acid</td>
<td>1 2 4 8 15 30 60 125</td>
<td>40.19 ± 0.001* 40.68 ± 0.001 * 41.35 ± 0.003 * 42.44 ± 0.002* 44.70 ± 0.002* 50.60 ± 0.002* 58.83 ± 0.002* 79.17 ± 0.001*</td>
</tr>
</tbody>
</table>

IC<sub>50</sub> of Arjunolic acid is 32 µg/ml (r=0.99), P<0.001 compared to reagent blank. (Student’s ‘t’ test).
### Table – 17. *In-vitro* free radical scavenging effect of Arjunolic acid acetate by nitric oxide method

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Concentration (µg/ml)</th>
<th>% Scavenging (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arjunolic acid acetate</td>
<td>1</td>
<td>43.41 ± 0.0034*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>43.30 ± 0.0028*</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>43.51 ± 0.0012*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>44.13 ± 0.0004*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>45.17 ± 0.0004*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>48.90 ± 0.0043*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>63.03 ± 0.0050</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>83.80 ± 0.0038*</td>
</tr>
</tbody>
</table>

IC$_{50}$ of AAc is 24 µg/ml (r=0.99). Results are expressed Mean ± SEM. *P<0.001 compared to reagent blank. (Student’s ‘t’ test).

### Table – 18. *In-vitro* free radical scavenging effect of Vitamin C by nitric oxide method

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>Concentration (µg/ml)</th>
<th>% Scavenging (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>0.1</td>
<td>5.40 ± 0.0017*</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>14.00 ± 0.0015*</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>25.00 ± 0.0005*</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>34.90 ± 0.0017*</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>57.90 ± 0.0015*</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>76.92 ± 0.0017*</td>
</tr>
</tbody>
</table>

IC$_{50}$ of Vitamin C is 0.72 µg/ml (r=0.99). Results are expressed Mean ± SEM. *P<0.001 compared to reagent blank. (Student’s ‘t’ test).
Fig 37. In-vitro free radical scavenging effect of TA by NO method

Fig 38. In-vitro free radical scavenging effect of AA by NO method
Fig 39. *In-vitro* free radical scavenging effect of AAc by NO method

![Graph showing the free radical scavenging effect of AAc by NO method.](image)

Fig 40. *In-vitro* free radical scavenging effect of Vitamin C by NO method

![Graph showing the free radical scavenging effect of Vitamin C by NO method.](image)
Table 19. Analgesic activity of methanolic extract of *T. arjuna* [TA], Arjunolic acid [AA], Arjunolic acid acetate [AAc] and Paracetamol [PA] by Acetic acid induced writhing model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>No. of writhings</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% w/v SCMC</td>
<td>10 ml/kg</td>
<td>45.33 ± 0.77</td>
<td>---</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>100 mg/kg</td>
<td>9.16 ± 0.83</td>
<td>80.00*</td>
</tr>
<tr>
<td>TA</td>
<td>250 mg/kg</td>
<td>30.16 ± 1.58</td>
<td>33.33*</td>
</tr>
<tr>
<td></td>
<td>500 mg/kg</td>
<td>21.16 ± 1.55</td>
<td>53.33*</td>
</tr>
<tr>
<td>AA</td>
<td>20 mg/kg</td>
<td>29.00 ± 0.02</td>
<td>35.55*</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>19.33 ± 1.87</td>
<td>57.77*</td>
</tr>
<tr>
<td>AAc</td>
<td>20 mg/kg</td>
<td>27.16 ± 1.44</td>
<td>40.00*</td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>16.16 ± 1.96</td>
<td>64.44*</td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± SEM and are in seconds. The statistical difference between control and treated groups were tested by one way ANOVA followed by Dunnett’s ‘t’ test. * P<0.0001, **P<0.01, ***P<0.05.
Table – 20. Analgesic activity of methanolic extract of *T.arjuna* [TA], Arjunolic acid [AA] and Arjunolic acid acetate [AAc] and Morphine [MO] by tail immersion model in mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Reaction time in seconds</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>After 60 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% w/v SCMC</td>
<td>10 ml/kg</td>
<td>1.77±0.049</td>
<td>1.90 ± 0.040</td>
<td></td>
</tr>
<tr>
<td>Morphine</td>
<td>5 mg/kg</td>
<td>1.61± 0.042</td>
<td>4.92± 0.031 (205.59)*</td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>250 mg/kg</td>
<td>1.68± 0.040</td>
<td>1.82 ± 0.080 (8.33)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500 mg/kg</td>
<td>1.66 ± 0.039</td>
<td>1.98 ± 0.110 (19.27)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>20 mg/kg</td>
<td>1.95± 0.070</td>
<td>1.90 ± 0.120 (2.63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>1.76± 0.060</td>
<td>1.81 ± 0.080 (2.84)</td>
<td></td>
</tr>
<tr>
<td>AAc</td>
<td>20 mg/kg</td>
<td>2.03± 0.110</td>
<td>1.80 ± 0.010 (11.33)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40 mg/kg</td>
<td>1.76 ± 0.080</td>
<td>1.91 ± 0.080 (8.52)</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as Mean ± SEM and are in seconds. Percentage of protection against thermally induced pain by warm water are in parenthesis. The statistical difference between control groups were tested by one way ANOVA followed by Dunnett’s ‘t’ test. * P <0.001, **P<0.001, *** P<0.05.
Fig 41. Analgesic activity of TA/AA/AAc/PA by Acetic acid induced writhing model

Paracetamol 100 mg/kg
TA 250 mg/kg
TA 500 mg/kg
AA 20 mg/kg
AA 40 mg/kg
Aac 20 mg/kg
Aac 40 mg/kg

Fig 42. Analgesic activity of T/TA/AA/AAc/MO by tail immersion model

Morphine 5 mg/kg
TA 250 mg/kg
TA 500 mg/kg
AA 20 mg/kg
AA 40 mg/kg
Aac 20 mg/kg
Aac 40 mg/kg