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CARDIOPROTECTIVE ACTIVITY OF SYNTHETIC GUGGULSTERONE (E AND Z - ISOMERS) IN ISOPROTERENOL INDUCED MYOCARDIAL ISCHEMIA IN RATS: A COMPARATIVE STUDY.

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ABSTRACT

Guggulsterone, a mixture of cis (E) and trans (Z) isomers (7:3 w/w) was synthesized from 16-DPA. The isomers were separated by column chromatography and evaluated for cardioprotective and antioxidant activities. Myocardial necrosis induced by isoproterenol in rats caused marked increase in serum creatine phosphokinase and glutamate pyruvate transaminase. Simultaneously in ischemic heart, phospholipase, xanthine oxidase and lipid peroxides were enhanced following depletion of glycogen, phospholipids and cholesterol. Treatment with guggulsterone and its both isomers at the dose of 50 mg/kg po., significantly protected cardiac damage as assessed by the reversal of blood and heart biochemical parameters in ischemic rats. The cardioprotective activity of guggulsterone and of both the isomers were compared with that of gemfibrozil at the same doses. Guggulsterone and both the isomers at tested concentrations (5-20mM) inhibited oxidative degradation of lipids in human low-density lipoprotein and rat liver microsomes induced by metal ions *in vitro*. The drug counteracted against the generation of superoxide anions (O_2) and hydroxyl radicals ($OH^{\cdot}$) in non-enzymic test systems. It is suggested that cardioprotective and antioxidant activities of synthetic guggulsterone and guggulsterone obtained from gum resin *Commiphora mukul* that contains isomers E & Z in the ratio of 46:54w/w are the same.

KEY WORDS

Cis and trans-guggulsterone, cardiprotective activity, antioxidant property, Isoproterenol cardiac ischemia.

INTRODUCTION

The plant *Commiphora weightii* (Syn. C. mukul ,Burrieceac,English name: Indian bdellium : Hindi : Guggul; Bengali : Guggul; Gujarati: Gugara ; Kannad: Guggulu; Tamil: Kukkulu guggulu; Telgu: Gukkulu guggulu) grows abundantly in the states of Karnataka , Gujarat and Rajasthan in India. The oleoresin secreted by this plant, known as guggul is one of the most reputed drugs in ayurveda and has been used for treatment of gout, arthritis, rheumatism, obesity and inflamation etc in traditional system of medicine (1). The lipid lowering activity of guggul however, was first reported by Satyavati in her thesis (2) and this was further confirmed in many experimental models (3). Simultaneously, lipid lowering effect of different extracts and fractions of gum-guggul was evaluated. The active lipid lowering agent, a standardized fraction from ethyl acetate extract of guggul gum containing guggulesterone mixed with some other steroids, diterpenes, esters and higher alcohols named as 'guggulipid' was developed in our institute (4, 5). CIPLA INDIA has now been marketing guggulipid as hypolipidemic agent under the trade name Guglip.

Pharmacological studies showed that guggulipid lowered blood lipids in patients of obesity (6), increased the coagulation and prothrombin time in hyperlipemic subjects (7), increased fibrinolytic activity (8) and decreased the platelet adhesive index (9), Guggulipid is effective against myocardial infarction (10) and known to cause thyrogenic effect (11)

The hyperlipemic activity of guggulipid is mainly due to guggulsterone, as the other components appear to exert significant synergistic effects with regard to lipid lowering action (5,12). As guggulipid,

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gugguisterone also inhibited platelets aggregation (13) and provide protection against myocardial ischemia induced by isoproterenol (14). The protective action of guggulsterone is due to antioxidant property because it inhibits the generation of oxygen free radicals (15).

The present study is an attempt to examine the cardio protective and lipid lowering activity of synthetic guggulsterone and its two isomers [E; 4,17(21) Cis-pregnandiene-3, 16- di – one] and [Z; 4,17(20) trans-pregnandiene-3, 16- di – one] in isoproterenol induced myocardial ischemia in rats in relation to gemfibrozil. Secondly we have studied the antioxidant activity of guggulsterone *in vitro*.

MATERIALS AND METHODS

Preparations of guggulsterone

A mixture of cis and trans (7:3w/w) guggulsterone was prepared following the method of Benn and Dodson., 1964 (16) through reduction of 16-dehydropregnenolone acetate (16-DPA). The two isomers were separated over silica gel column using toluene: ethyl acetate in the ratio of 4: 1(v/v).

Animals

Colony bred male Charles Föster rats, each animal weighing 200-225g, was divided into control, ischemic and ischemic plus drug treated groups of six animals in each group. These animals were kept in a room controlled for temperature at 25-26° humidity 60-80% and 12/12 hours light /dark cycle (light from 8.00am to 8.00 pm) under hygeinic conditions. All animals have free access to diet (Lipton India Limited, Mumbai) and water.

Cardio protective activity- Ischemia was produced by intraperitonial injections of aqueous solution of dl-isoproterenol hydrochloride (85mg/kg) for five consecutive days (14). The drug (guggulsterone isomers E or Z or mixture of guggulsterone E+Z (7:3w/w) or gemfibrozil were macerated with aqueous gum acacia and fed orally at the dose of 50mg/kg p.o., once daily for five days. The control animals received same amounts of normal saline (i.p) and vehicle p.o for five days simultaneously with isoproterenol. At the end of the experiment, blood was withdrawn after four hours of the last drug administration and serum was prepared. Rats were sacrificed and their hearts were excised immediately. Both serum and hearts were kept at –20°C till analysis.

ESTIMATION

Serum was used for the assay of creatine phosphokinase (CPK), as well as glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) by standard methods as described earlier (17).

Alkaline phosphate was assayed according to the method of Bassy et al (18). Heart homogenate was used for the estimation of Ca-ATPase, phospholipase, lipid peroxide, total cholesterol, phospholipid, glycogen and cytosolic xanthine oxidase as mentioned earlier (19,20).

ANTIOXIDANT ACTIVITY

Normal human serum LDL was isolated by sequential ultra centrifugation using Beckman ultracentrifuge; model LE-80 K (21) LDL preparation (d, 1.063) was dialyzed against 0.15 M NaCl solution containing EDTA (0.02%w/v) in cold. The purity was checked on polyacrylamide gel electrophoresis. LDL (0.71mg) and 10mM $CuCl_2.2H_2O$ in the absence and presence of guggulsterone isomers EorZ or E+Z in a dose of 5,10 and 20μM in 50mM phosphate buffer (pH 7.4) was incubated at 37°C for 90 minutes. The level of lipid peroxide in oxidized LDL with Cu^{+2} in the presence and absence of guggulsterone was assayed as thiobarbituric acid reactive substances (TBARS) as mentioned earlier (19). Lipid peroxidation in normal rat liver microsomes (3mg protein) was induced *in vitro* by both enzymic and nonenzymic reactants (22). Enzymemic system containing liver microsome(3mg protein) NADPH (100μM), ADP (500μM), FeSO4.7H2O (2mM) and EDTA (6mM) in 50 mM phosphate buffer (pH 7.4) to a final volume of 2.0 ml. Similarly in other set of nonenzymic system liver microsomes(3mg protein)were added with $FeSO_4.7H_2O$ (2mM), EDTA (6mM), sodium ascorbate (20mM) and phosphate buffer (pH 7.4). Both groups i.e. enzymic and nonenzymic were incubated at 37°C for 90 minutes in the absence or presence of guggulsterone isomers E or Z or isomer E+Z in dose of 5,10 and 20 μM. The lipid peroxides formed was estimated as TBARS.

Superoxide Anions (O^{2-}) were generated in an in vitro system comprising of NADH (160μM), phenozine methosulphate (10μM) and NBT (320μM) in the absence or presence of guggulsterone isomers Eor Z or isomer E+Z in a dose of 5, 10, and 20 μM in 50mM pyrophosphate buffer (pH 8.2). After

incubation at 37°C for 30 min, the reaction was checked by 0.5 ml glacial acetic acid and amount of formazon formed in both sets was estimated spectrophotometrically (23). In another set of experiment hydroxyl radicals (OH^-) were generated non enzymically (24) by $FeSO_4.7H_2O$ (2mM), sodium ascorbate (2mM), H_2O_2 (2.8mM), and deoxyribose (2.8mM) in 50mM KH_2PO_4-KOH buffer (pH 7.4) in the absence or presence of guggulsterone samples (5-20μM). The incubation mixture was measured for TBARS formed.

STATISTICAL ANALYSIS

The present Data was analyzed using Student 't' test. The ischemic group was compared with control and various drug treated groups. Oxidized LDL was compared with guggulsterone isomers treated oxidized LDL. Similarly the generation of oxygen free radicals and lipid peroxidation in microsomes with guggulsterone isomers was compared with that of their formation without guggulsterone. $P < 0.05$ was considered to be significant.

RESULTS

Effect of Drugs on Cardiac Ischemia - Administration of isoproterenol to i.e. to induce ischemia in rats increase in serum levels of CPK, GOT, GPT and alkaline phosphates by 111, 42, 85 and 29 % respectively as compared to control (Table-1). However treatment of ischemic rats with guggulsterone isomers E or Z or E+Z or gemfibrozil at the dose of 50 mg/kg p.o. reversed the serum levels of CPK, GOT, GPT and alkaline phosphatase (Table 1). In ischemic heart (Table-2) there was a significant redution in levels of Ca-ATPase (45%), glycogen (20%), phospholipids (48%) and total cholesterol (46%) as compared to control. However there was a significant increase in Phospholipase (217%) and xanthine oxidase (56%) along with increase in lipid peroxide (66%). Where as treatment with guggulsterone isomer E or Z or mixture of E+Z significantly reversed the biochemical parameters in serum and hearts as compared to the levels of ischemic group (Table 2).

Effect of Guggulsterone on Lipid Peroxidation and Oxygen Free Radical Generation *in vitro* - Oxidation of LDL in the absence of metal ions caused the formation of lipid peroxides (0.65nmol MDA/mg protein; data not shown). However, incubation of LDL with Cu^{+2} caused a mark increase in lipid peroxide by 26 folds (Table-3). Addition of guggulsterone in the concentration of 5-20μM inhibited the Cu^{+2} mediated lipid peroxidation of LDL in concentration dependent manner. At peak level of 20 μM, guggulsterone isomer E or Z or mixture of E+Z inhibited LDL-peroxidation by 32, 41 and 36% respectively. It was also observed that normal rat liver microsomes containing 0.92 nmol MDA/mg protein, when challenged with the reactants employed in enzymic and non-enzymic system for peroxidation, increased the levels of lipid peroxides by 2 and 3 folds respectively. Addition of guggulsterone isomers E or Z or (E+Z) (5-20μM) in both enzymic and non-enzymic systems made microsomes less susceptible against oxidative degradation of their lipids and at 20mM of guggulsterone as well as its isomers (E and Z) could protect peroxidation in microsome by 16,18 & 17% and 54,69 & 68% respectively (Table-3). Furthermore, the scavenging potential of guggulsterone and two isomers at 5-20μM against formation of O^{2-} and OH^- in non-enzymic system was studied (Table-3). Guggulsterone Eor Z or (E+Z) inhibited the formation of oxygen free radicals in concentration dependant manner. The maximum decrease in super oxide anions (28,50 & 39%) and hydroxyl radicals (43,52 & 44%) was found at concentration of 20μM guggulsterone isomers E or Z or E+Z. Guggulsterone Z showed more antioxidant activity as compared to that of E or combination (E+Z).

DISCUSSION

Increased activities of CPK, transaminase and alkaline phosphatase in serum, 'the diagnostic markers', were due to the leakage of these enzymes as a result of necrosis induced by isoproterenol (β adrenergic stimulant) in rats (25). Creatine phosphokinase is a muscle specific enzyme mainly for heart and brain; therefore, its increase in serum is the result of myocarditis, cardiac in sufficiency, arrhythmias and myocardial infarction (26). The inhibition of glycolytic pathways of energetic in cardiac muscles of isoproterenol treated rats, lead to glycogen break down, loss of pyridine nucleotides and ATP, which resulted to an increase in intracellular Ca^{++} (27). The calcium is essential factor in phospholipase-associated degradation of membrane phospholipids, causing damage of mitochondrial membrane, which leads to impaired electron transport along with the leakage of lysosomal enzymes (28). Treatment with Calcium antagonist nifidipine, β adrenergic blocker, propanolol and lipid

lowering drug like guggulsterone partially reversed the changes in sarcolemma enzymes and stimulate the Ca^{++} uptake in the damaged heart (19). There are several reports(14,19,20) on effectiveness of guggulsterone isolated from *C. mukul* against isoproterenol induced myocardial ischemia in experimental animals and these effects on heart and blood parameters at the same doses were almost comparable to our results. The increase in xanthine oxidase and lipid peroxide in damaged rat heart was protected significantly by the treatment with guggulsterone and by both isomers in present study. It has been reported that xanthine dehydrogenase is converted into xanthine oxidase under ischemic conditions that at the moment of reoxygenation (respiratory burst) produces O^{2-} and uric acid. The O^{2-} and H_2O_2 are the main source of OH^-, which play an important role in cardiac necrosis (29). Guggulsterone protects LDL against oxidative modifications in lipid and protein components induced by Cu+2 *in vitro* (30). The protection provided by the guggulsterone and the two isomers against the cardiac damage may be due to the ability of test drugs to inhibit the generation of O^{2-} free radicals induced by isoproterenol treatment in rats. A standardized alcoholic fraction from guggulipid containing E- guggulsterone (cis isomer) is already studied and proven for its protection against free radical damage in the skin (31). It is suggested that isoproterenol may induce hypothyroidism that contributes to the development of myocardial ischemia in rats. It has been reported that (Z) guggulsterone enhanced the synthesis of thyroid hormones and tissue oxygen uptake (32,33). Pharmagological studies showed guggulsterone caused decrease in the brain levels of catecholamines and dopamine β hydroxylase activity in rats (34). Recent reports (35,36) showed that guggulsterone is a potent antagonist of bile acid receptor the famesoide- x -receptor (FXR) that is activated by bile acids. It is suggested that antioxidant property, alterations caused in the levels of brain biogenic monoamines, thyrogenic action and antagonism for FXR by guggulsterone suitably explain the lipid lowering and anti ischemic activity of the drug. Present data demonstrate that Z-isomer of guggulsterone at the same doses exerted more cardioprotective and antioxidant activity of the drug. Present data demonstrate that Z-isomer of guggulsterone at the same doses exerted more cardioprotective and antioxidant activity than E-isomer. Therefore it is suggested that Z-guggulsterone is a suitable and better replacement for the natural drug isolated from *C.mukul.*

Table 1

Effect of guggulsterone and gemfibrozil on serum biochemical parameters in isoproterenol induced ischemia in rats.

Treatments	Creatine[a] Phospho Kinase	Glutamate[b] Oxaloacetate Transaminase	Glutamate[b] Pyruvate Transaminase	Alkaline[c] Phosphatase
Control	0.73±0.11	67.16±2.17	33.42±4.56	14.73±0.26
Ischemic	1.54±0.07**	95.09±6.79**	61.86±1.90**	18.97±0.55**
Ischemic + Guggulsterone E	1.31±0.2*	85.14±2.63*	55.14±2.63*	17.22±0.53 NS
Ischemic + Guggulsterone Z	1.18±0.91**	82.98±2.21**	50.36±1.33*	16.48±0.65*
Ischemic + Guggulsterone (E + Z)	1.38±0.57*	85.28±3.08*	55.96±1.30*	17.00±0.07*
Ischemic + Gemfibrozil	1.26±0.05**	88.13±1.92*	56.50±2.50*	17.22±0.05 NS

Units: A, μ mole Pi /hr / mg protein; B, μmole Sodium pyruvate/ min/L; C, μmole p-nitro phenol /min/dl. Each value is the mean ± S.D. of 6 rats. *p<0.05, **<0.01, ***<0.001, NS=non significant. Ischemic group was compared with control, Ischemic plus drug treated with ischemic..

Table 2

Effect of guggulsterone and gemfibrozil on heart biochemical parameters in isoproterenol induced ischemic rats.

Treatments	Ca-ATPase [a]	Xanthine oxidase [b]	Phospho Lipase [c]	Phospho Lipid [d]	Total [d]Cholesterol	Glycogen[d]	Lipid Peroxide[d]
Control	18.9±1.81	7.53±0.56	3.45±0.37	7.49±0.68	4.57±0.82	14.46±1.94	1.40±0.07
Ischemic	10.09±1.96 **	11.81±0.22 **	10.92±1.52 ***	3.86±0.38 **	2.49±0.18 **	11.54±1.94 **	2.33±0.45 ***
Ischemic + Guggulsterone E	13.17±2.01 *	9.71±0.98 *	7.05±1.23 **	5.32±0.60 **	3.32±0.40 **	12.51±1.18 *	1.60±0.22 **
Ischemic + Guggulsterone Z	14.79±2.96 NS	10.15±0.64 **	6.73±1.24 **	5.72±0.33 **	4.07±0.40 NS	12.42±1.08 *	1.82±0.13 *
Ischemic + Guggulsterone (E + Z)	13.49±2.96 *	9.50±0.79 **	6.38±1.63 **	4.53±0.24 ***	4.04±0.29 NS	12.39±0.71 *	1.71±0.14 *
Ischemic + Gemfibrozil	12.54±1.88 **	9.57±0.67 **	5.86±0.79 **	4.48±0.62 **	3.09±0.36 **	12.48±0.82 *	1.68±0.11 **

Units: a, μ mole Pi/hr/mg protein; b, μmole uric acid/ min/ mg protein, C, μmole FFA/hr/mg protein; D, mg/g; E mMole MDA/g. Each value is the mean ± S.D. of 6 rats. *p<0.05, **<0.01, ***<0.001, NS=non significant. Ischemic group was compared with control, Ischemic plus drug treated with ischemic.

Table 3

Antioxidant activity of Guggulsterone *In vitro.*

Treatment	Conc. μ Mole	LDL Oxidation[a]	Microsomal Lipid Peroxidation[a]		Formation of Oxygen free radicals	
			Enzymic	Non Enzymic	Superoxide anions[b]	Hydroxyl radicals[b]
Control		42.38±5.26	2.14±0.16	2.52±0.18	63.30±0.05	37.75±3.40
Guggulsterone E	5	37.40±5.10NS	1.830.16NS	2.20±0.14*	54.80±7.0 NS	31.45±3.20NS
	10	36.65±3.45*	1.82±0.21*	1.60±0.10*	51.55±4.6NS	26.38±3.20*
	20	28.70±1.88*	1.80±0.14*	1.17±0.13**	45.35±5.0*	21.60±5.00**
Guggulsterone Z	5	35.00±4.30NS	1.94±0.15NS	1.80±0.20*	54.80±6.80NS	31.88±2.65NS
	10	30.56±3.85*	1.78±0.20*	1.42±0.12*	49.90±4.25*	24.50±3.28*
	20	24.80±3.60**	1.75±0.2**	0.77±0.10***	31.90±4.00**	18.25±1.70**
Guggulsterone (E + Z)	5	35.55±4.00*	1.98±0.17NS	2.00±0.16*	55.50±4.90NS	31.42±4.16NS
	10	34.86±4.35*	1.86±0.15*	1.46±0.16*	52.00±4.80NS	25.82±5.40*
	20	27.00±3.60**	1.78±0.18*	0.81±0.09***	38.60±3.45*	21.25±2.12**

Units: a, n mole MDA/mg protein; b, nmole formazone formed/ min/; C, nmole MDA/hr. Each value is the mean ± S.D. of 4 separate observations. *p<0.05, **<0.01, ***<0.001, NS=non significant, as compared to the systems without drug treatment.

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