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EVALUATION OF CARDIOPROTECTIVE ACTIVITY OF LIMONIUM WRIGHTII IN MYOCARDIAL ISCHEMIC REPERFUSED RATS

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ABSTRACT

The beneficial effects of reperfusion the myocardium can be obtained by pre-treating the animals with methanolic leaf extract of limonium wrightii which increases the levels of anti-oxiadants and protect the heart from reperfusion injury. Oxidative stress was suggested to be implicated in the pathogenesis of ischemia-reperfusion (IR) injury. Many antioxidative plants were shown to be cardioprotective in experimental models of myocardial ischemia-reperfusion (IR) injury. The present study was designed to investigate the effects of pretreatment with methanolic extract of Limonium wrightii in an in vivo rat model. The model adopted was that of surgically-induced myocardial ischemia, performed by means of left anterior descending coronary artery occlusion (LDCA) for 30 min followed by reperfusion for another 4 h. Infarct size was measured by using the staining agent TTC (2,3,5-triphenyl tetrazolium chloride). A dose dependent rise in the end products of myocardial lipid peroxides (malondialdehydes [MDAs]), loss of antioxidative enzymes (superoxide dismutase, catalase) was observed with the prior treatment of L.wrightii with various doses for 7 days compared to animals treated 1 hour prior and in the control group. Hence, the present study suggests the cardioprotective activity of L.wrightii in limiting ischemia-reperfusion induced myocardial infarction.

***Key words**: Infarct size, Myocardial infarction, Reperfusion injury, antioxidants, Limonium wrightii*

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INTRODUCTION

When a disease deprives a tissue of oxygen, re-establishing blood flow (reperfusion) and therefore reintroducing oxygen can result in tissue damage. Myocardial ischemia-reperfusion (IR) can occur, resulting in damage to the myocardium following blood restoration after a critical period of blood occlusion. IR is associated with procedures such as thrombolysis, angioplasty, and coronary bypass surgery, procedures commonly used to re-establish blood and minimize damage to the heart due to severe myocardial ischemia. Reperfusionof the ischemic myocardium is accompanied by the generation of reactive oxygen species (ROS) that can cause vascular and microvascular injury, endothelial cell dysfunction, myocyte edema, increased myocyte apoptosis, increased myocyte necrosis, and cardiac contractile dysfunction. Among the molecules attacked by oxygen free radicals are proteins, carbohydrates, neurotransmitters, nucleic acids, and cell membrane phospholipids. Free radicals have been implicated in many conditions, including heart disease, diabetes, cancer, Alzheimer's disease, Parkinson's disease, cataracts, rheumatoid arthritis, and aging.Oxidative stress has also been to produce an intracellular Ca2+ overload and vice versa. The degree of oxidative stress and the magnitude of intracellular Ca2+ overload in cardiomyocytes seem to be dependent upon the duration of ischemia. In fact, oxidative stress and intracellular Ca2+overload are considered to be the major mechanisms for the development of ischemic injury, and reperfusion appears to exacerbate the impact of this pathological process. Antioxidants are widely used ingredients in dietary supplements for maintaining the health and prevention from diseases like coronary heart disease and cancer. Antioxidant compounds are exogenous or endogenous in nature which either prevents the generation of toxic oxidants or inactivate them when generated thereby blocking the chain propagation reaction produced by the oxidants.In Yamashiro S, et al.,2003 evaluated the effects of the aqueous extracts from *Psidium guajava* L. And *Limonium wrightii* on myocardial injury produced by global ischemia followed by reperfusion and

were further compared with those of quercetin and Gallic acid, major antioxidative components of *P. guajava* L. And *L. wrightii*, respectively. And reported that, both extracts significantly attenuated ischemic contracture during ischemia and improved myocardial dysfunction after reperfusion and both have cardio protective effects against myocardial ischemia-reperfusion injury in isolated rat hearts, primarily through their radical-scavenging actions. In **Garima Shakyan et al.,2012** Limonium wrightii whole plant extract has a potent antioxidant efficacy and has been used as a health drink in everyday's life and is used to cure DM (Diabetes mellitus) in folk medicine

MATERIALS AND METHODS

Albino Wistar rats (n=30) of either sex weighing 145- 185 GMs were used in the study, which were purchased from Mahavir Enterprises, Hyd, INDIA. Animals were maintained under photo period (12 h dark/12h light) and were used for the experiment. Commercial pellet diet (Rayon's biotechnologies Pvt Ltd., INDIA) and water were provided ad libitum.The experimental protocol has been approved by the Institutional Animal Ethics Committee and by the Animal Regulatory Body of the Government (Regd. No. 1722/PO/A/13/IAEC/CPCSEA).

PLANT MATERIAL:

The fresh plant (whole plant) of Limonium wrightii was purchased from an herbalist. The plant was identified and authenticated by Dr. K. Madhava Chetty (Plant Taxonomist) of the Department of Botany, Sri Venkateshwara University, A.P. State, India with Voucher number 791. The plant was shade-dried for 5 days and pulverized, using a pestle and mortar. The pulverized part was stored in cellophane bags at room temperature.

SOLVENT EXTRACTION

The World Health Organization (WHO, 1992) procedure of extraction was adopted for this study.A total of one hundred grams of the powder of Limonium wrightii leaves were subjected to exhaustive soxhlet extraction in 500 ml of methanol for 72hrs. The extract obtained was concentrated, until a constant, dark sticky residue was obtained, this was further oven dried and maintained in a desiccator until a constant weight was obtained.The dried extract obtained was stored in a tightly stoppered container in a refrigerator at -4°C until required.Stock solution of the extract was prepared by dissolving 5g weight of the powdered extract in 50 ml of normal saline and the concentration used was 0.1 g/ Ml

ACUTE TOXICITY STUDY

This study was conducted according to the Organization for Economic Cooperation and Development (OECD) revised up and down procedure for acute toxicity testing (OECD, 2001). A limit dose of 2000 mg kg-1 of the methanol extract of the Limonium wrightii leaf was used for this study. The limit dose was performed using 5 healthy adult Albino Wistar rats. The rats were fasted overnight from food, but not water prior to dosing and then weighed before the extract was administered in a single dose by gavage. The limit dose of 2000 mg/kg of the aqueous extract was given to the first rat orally and the rat was observed for mortality and clinical signs for the first hour, then hourly for three hours and then periodically for 72hours. Other rats were subsequently dosed sequentially at 48 hours interval. The LD50 was predicted to be above 2000 mg/kg three or more rats survived.

None of the 5 rats died or showed any sign of toxicity at the limit dose of 2000mg/kg/oral in the first 48 hours and no evidence of toxicity was noted during the period of observation. The LD50 in rats was therefore taken as above 2000mg/kg/oral.

EXPERIMENTAL DESIGN AND SURGICAL PREPARATION:

In the present study, rats were anesthetized with thiopentone sodium (30mg/kg, intraperitonially) and were ventilated with room air using (Techno artificial respirator) positive pressure ventilator. Ventilator parameters were adjusted to maintain normal pH and satisfactory oxygenation. The chest was opened through the fourth intercostal space at the left side and the heart was exposed. The pericardium was removed and the left descending coronary artery (LDCA) was located. Left descending coronary artery (LDCA) was dissected free above the first diagonal branch and above the origin of the left circumflex artery. A surgical thread was passed below the LDCA artery and the LDCA was occluded with that surgical thread for 30 minutes. In control group animals treated with saline, the saline was administered orally 10 minutes before ligation of LDCA.The Limonium wrightii leaf extract was also administered orally 1 hour before ligation of LDCA at a dose of 200 mg/kg; 400mg/kg was administered according to body weights of rats. Another group which was pre-treated with Limonium wrightii whole plant extract for 1week at a dose of 200mg/kg, 400mg/kg was administered according to body weights of rats.

PARAMETERS ESTIMATED:

Quantification of Percentage Left Ventricular Necrosis:

Measurement of percent left ventricular necrosis provides information regarding viable myocardium, which is related to the functional capacity of the heart. Hence, in the present study, percent left ventricular necrosis was measured by using 2, 3, 5 – triphenyl tetrazolium chloride (TTC) stain method.In all the groups after sacrificing the animal by injecting 2.56 M molar potassium chloride directly into the left ventricle, the heart was excised from the thorax rapidly and the great vessels were removed. The left ventricle was separated from the heart and was weighed. The left ventricle was sliced parallel to the atrioventricular groove into 2-3mm thick sections and the slices were incubated in 1 % TTC solution prepared in phosphate buffer (pH 7.4) for 30 minutes at 37ºC.In viable myocardium TTC is converted by dehydrogenases to a red formation pigment that stains tissue dark red. The infracted myocardium that does not take TTC stain where the dehydrogenases are drained off remains pale in color.The pale necrotic tissue was separated from the stained portions and weighed on an electronic balance. Infarct size was calculated as a percentage fraction of non-viable myocardium of the left ventricle.

Determination of MDA levels in Heart: Malondialdehyde (MDA) levels in the heart were measured by the method developed by Ohkawa et al 1979

Determination of Superoxide Dismutase (SOD) levels in the Heart: Superoxide dismutase (SOD) activity was determined by the method developed by Kakkar et al. 1984.

Determination of heart tissue Catalase (CAT): Catalase activity wasmeasuredby the method Aebi et al., 1974.

Statistical Analysis:

All the values were expressed as mean ± SD. The data were analyzed using One way repeated measure ANOVA. A level of $P<0.05$ was considered as statistically significant. Tukey's test was performed to find the significant difference at $P<0.05$.

RESULTS AND DISCUSSIONS:

With Sham control animals percentage left ventricular necrosis (PLVN) was found to be **0.99±0.11 %**. With normal control treated with vehicle (sodium CMC), percentage left ventricular necrosis was found to be **30.8±0.83 %**. So all the values of other groups were compared with those of control animals treated with vehicle. When administered orally at the dose of 200mg/kg, 1hr prior to the ischemic reperfusion, the PLVN was found to be **31±1.00 %,** which was not found to be significant. With administration of 200mg/kg 1week daily dose prior to the ischemic reperfusion, the PLVN was significantly reduced to **22.4±1.14 %**.When administered orally at the dose of 400mg/kg, 1hr prior to the ischemic reperfusion, PLVN was found to be **30.6±0.89 %**, which was not found to be significant.With administration of extract 400mg/kg 1week daily dose prior to the ischemic reperfusion, the PLVN was significantly reduced to **16.2±0.83 %.** MM 400mg/kg 1week daily dose offered more degree of cardio protection when compared to low dose (200mg/kg 1hr and 1week prior administration.Results were shown in figure-1).

TISSUE MDA LEVELS

The effect of *Limonium wrightii* leaf extract on MDA levels **(n moles/ml)** of ischemia reperfusion induced myocardium infarction in rats shown in figure-2.

With Sham control animals MDA levels were found to be **5.82±0.50NM/ml** with normal control treated with vehicle (sodium CMC), MDA levels were found to be **13.73±0.91 NM/ml**. So all the values of other groups were compared with those of control animals treated with sodium CMC. When administered orally at the dose of 200mg/kg, 1hr prior to the ischemic reperfusion, the MDA levels were found to be **13.14±0.14 NM/ml,** which was not found to be significant. With 200mg/kg 1week daily dose prior to the ischemic reperfusion, the MDA levels were significantly reduced to **9.83±1.28 NM/ml.** When the Limonium wrightii leaf extract was administered orally at the dose of 400mg/kg, 1hr prior to the ischemic reperfusion, MDA levels were found to be **13.88±0.38 NM/ml**, which were not found to be significant. With administration of 400mg/kg 1week daily dose prior to the ischemic reperfusion, the MDA levels were significantly reduced to **7.78±0.25 NM/ml.** 400mg/kg 1week daily dose offered more degree of cardio protection when compared to low dose (200mg/kg 1hr and 1week prior administration).

TISSUE SOD LEVELS:

The effect of *Limonium wrightii* leaf extract on SOD levels **(U/mg protein)** of ischemia reperfusion induced myocardium infarction in rats shown in fiure-3.

With Sham control animals SOD levels were found to be **24.75±0.19 U/mg protein** with normal control treated with vehicle (sodium CMC), SOD levels were found to be **17.76±0.20 U/mg protein**. So all the values of other groups were compared with those of control animals treated with sodium CMC.When Limonium wrightii leaf extract was administered orally at the dose of 200mg/kg, 1hr prior to the ischemic reperfusion, the SOD levels was found to be **16.81±0.28 U/mg protein,** which was not found to be significant.With administration of 200mg/kg 1week daily dose prior to the ischemic reperfusion, the SOD level was significantly reduced to **19.26±0.23 U/mg protein**.When the dose of 400mg/kg was administered, 1hr prior to the ischemic reperfusion, SOD levels was found to be **15.76±0.24 U/mg protein**, which was not found to be significant.With administration of 400mg/kg 1week daily dose prior to the ischemic reperfusion, the SOD was significantly reduced to **22.1±0.19 U/mg protein.**400mg/kg 1week daily dose offered more degree of cardio protection when compared to low dose (200mg/kg 1hr and 1week prior administration).

TISSUE CATALASE LEVELS:

Effect of *Limonium wrightii* leaf extract on CATALASE levels of ischemia reperfusion induced myocardium infarction in rats. Measured as **nM of H_2O_2 decomposed/mg protein/min** units shown in figure-4. With Sham control animals CATALASE levels was found to be **25.17±0.26 nM** with normal control treated with vehicle (sodium CMC), SOD levels was found to be **18.11±0.22 nM**. So all the values of other groups were compared with those of control animals treated with sodium CMC. When administered orally at the dose of 200 mg/kg, 1hr prior to the ischemic reperfusion, the CATALASE levels was found to be **17.46±0.12 nM**, which was not found to be significant.With administration of 200 mg/kg 1week daily dose prior to the ischemic reperfusion, the CATALASE level was significantly increased

to **19.71±0.12 nM**.When administered orally at the dose of 400mg/kg, 1hr prior to the ischemic reperfusion, CATALASE levels was found to be **16.90±0.19 nM**, which was not found to be significant.With administration of 400mg/kg 1week daily dose prior to the ischemic reperfusion, the CATALASE was significantly increased to **22.98±0.30 nM.** Limonium wrightii leaf plant extract 400mg/kg 1week daily dose offered more degree of cardio protection when compared to low dose (200mg/kg 1hr and 1week prior administration) data was given in table-1.

In present study SOD and Catalase levels in the 1 week pre-treatment group were significantly increased and MDA levels were significantly decreased when compared with 1 hour pre-treatment group and ischemia reperfusion group.

Ischemia and reperfusion cause myocardial injury via multiple pathways. Previous studies demonstrate that reactive oxygen species are elevated during myocardial ischemia and reperfusion which plays a major role in the pathophysiology of ischemia reperfusion related injury (Angela Maria Vicente Tavares et al., 2010). In order to investigate the mechanism of protection induced by Limonium wrightii powder against ischemia reperfusion injury, lipid peroxidation and antioxidant defenses including SOD and CAT in the infarcted myocardium of rats were measured.

The present study demonstrated that the myocardial damage was observed in ischemia reperfused myocardium as it is evidenced by increased percentage left ventricular necrosis in I/R control group rats subjected to ischemic reperfusion injury when compared to sham control rats.The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation (reperfusion) through the ischemic tissue results in set of reaction that can cause injury to vascular and parenchymal cells (Aslan et al.,2010). This can be triggered by a variety of mechanisms, including calcium overload, endothelial dysfunction free radical damage and inflammatory injury. Most extensively evaluated cardio protective agents include β- blockers, vasodilators, nitrates, anticoagulants.One of the leading causes of reperfusion injury is oxidative stress. Thus, there is a strong basis to expect that antioxidant agents might be valuable in myocardial damage resulting from ischemia reperfusion injury and act as cardio protective agents.Limonium wrightii leaf extract have offered significant cardio protection in terms of reduction in the infract size in myocardial ischemia reperfusion injury. It contains antioxidant vitamins like vit-E, vit-C and also β-carotene (Charles Schnabel et al., 1940) these compounds might be valuable in decreasing the oxidative stress induced during myocardial ischemia reperfusion injury.Antioxidants of natural and synthetic origin had shown significant amelioration of myocardial injury and hence improve myocardial function (Hearse DJ et al., 1991). Free radicals are difficult to estimate directly because of its high reactivity and short half life therefore the level of product of lipid peroxidation MDA, was used to measure the ROS generation (Shreesh Ohja et al., 2010). The increased oxidative stress was measured in terms of increased MDA levels and decreased SOD and Catalase levels.

CONCLUSION:

In myocardial ischemic reperfusion injury, a significant decrease in endogenous myocardial antioxidants
superoxidedismutase (SOD), catalase (CAT), incre ased lipid peroxidation characterized by malondialdehyde (MDA) formation along depletion of cardiomyocytes specific enzymes.Acute injury induced by ischemic and reperfusion revealed marked alterations in antioxidant enzyme activities such as low activities of antioxidant enzymes such as SOD, CAT and increased MDA levels. Limonium wrightii significantly increase catalase, superoxide dismutase activity and decrease malondialdehyde levels.

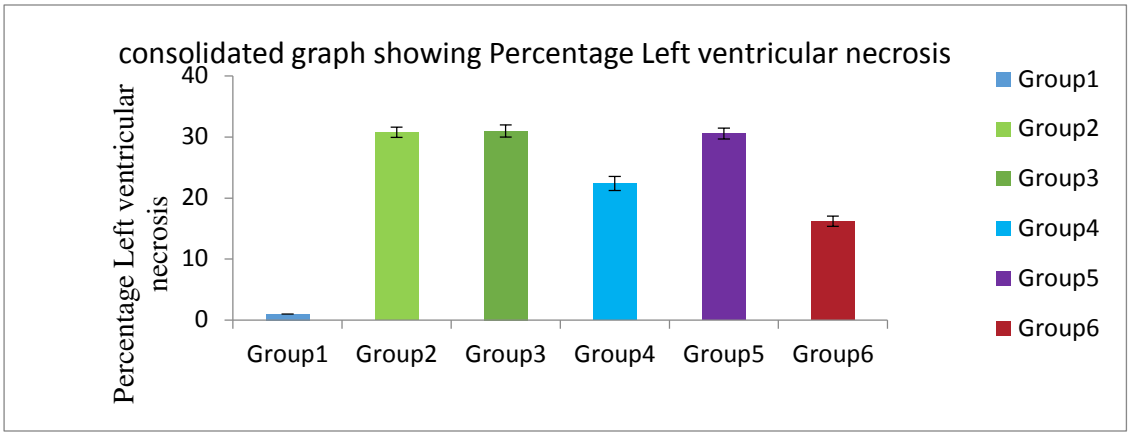

Fig-1 PERCENTAGE OF INFARCT SIZE: Consolidated graph showing Percent Left ventricular necrosis

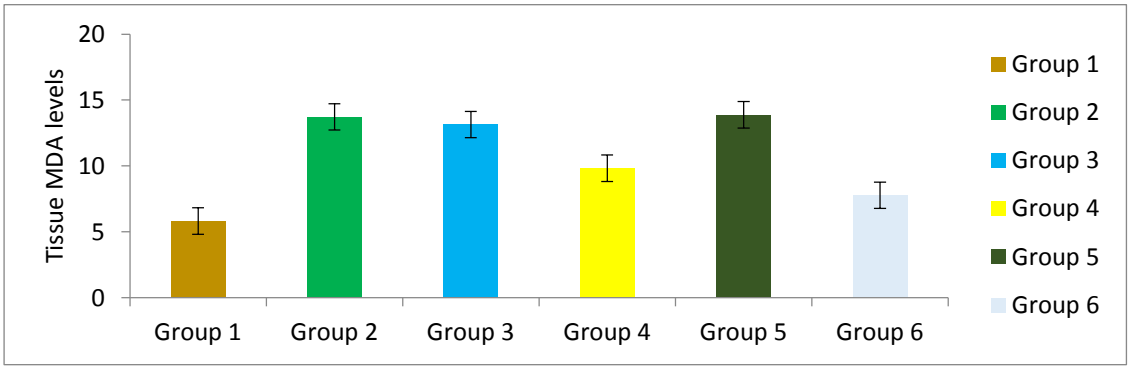

Fig-2 Consolidated data graph showing tissue MDA levels

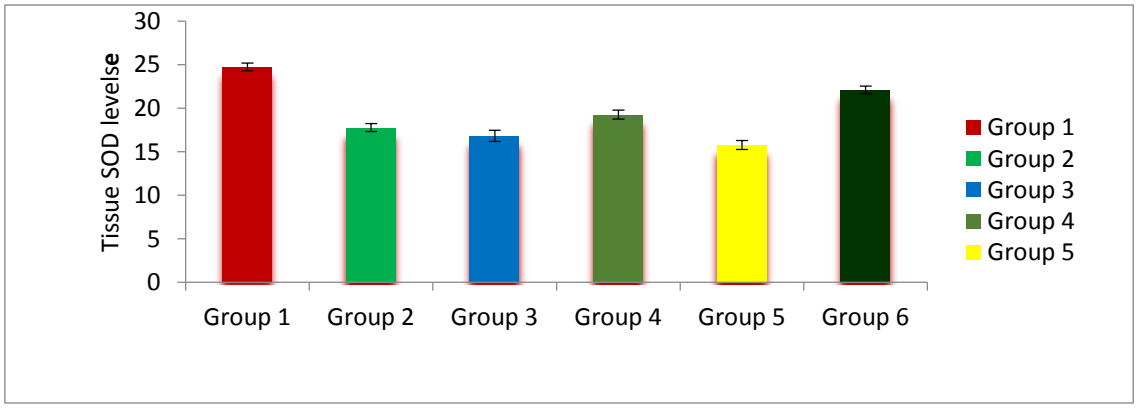

Fig-3 Consolidated graph showing Tissue SOD levels

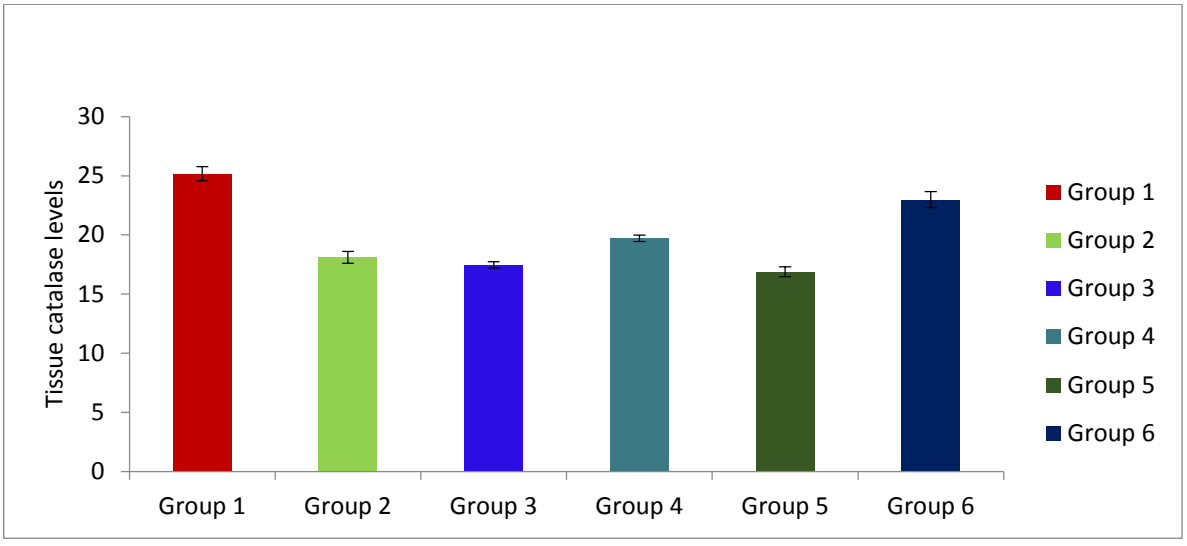

Fig-4 Consolidated Graph showing tissue CATALASE levels

Table-1 Consolidated data showing estimated parameters

L.Wrightii leaf extract dose	Infracted tissue weight (mg/kg)	PLVN (%)	MDA levels (nM/ml)	SOD levels (U/mg protein)	CATALASE levels(nM)
Control	**0**	0.99±0.11	5.82±0.50	24.75±0.19	25.17±0.26
Vehicle	68.43±0.84	30.8±0.83	13.73±0.91	17.76±0.20	18.11±0.22
200mg 1hr	65.71±0.92	31±1.00	13.14±0.14	16.81±0.28	17.46±0.12
200mg 1week	53.88±0.76	22.4±1.14	9.83±1.28	19.26±0.23	19.71±0.12
400mg 1hr	66.94±0.80	30.6±0.89	13.88±0.38	15.76±0.24	16.90±0.19
400mg 1week	38.50±0.77	16.2±0.83	7.78±0.25	22.1±0.19	22.98±0.30

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