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Chrysin Prevents Deregulation of Collagen Expression and Renal Renin-Angiotensin System in L-Name Induced Hypertensive Rats

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Abstract

Aim: Hypertension is a major risk factor for cardiovascular mortality and morbidity through its effects on target organs like the brain, heart and kidney. To evaluate the effect of chrysin, a natural biologically active compound extracted from many plants, mushrooms, honey and propolis on the blood pressure, collagen type I and III gene expression and Mg levels in N^{ω}-nitro-L-ariginine methyl ester (L-NAME)-induced hypertensive rats. **Materials and Methods**: Hypertension was induced in adult male albino rats of the Wistar strain, weighing 180-220 g, by oral administration of L-NAME (40 mg/kg BWT/day) in drinking water for 4 weeks. Rats were treated with chrysin (25 mg/kg BWT/day) for 4 weeks. **Results**: In our results shows Collagen type I and III gene expression, Mg levels in plasma and brain, and renal fold changes of Angiotension Converting Enzyme (ACE) increased significantly in the plasma of hypertensive rats. Supplementation of chrysin restore collagen type I and III gene expression, Mg and renal fold changes in ACE in Group IV compare to group I. **Conclusion**: These results suggest that chrysin has enough potential to attenuate hypertension and renal damage in nitric oxide deficiency induced hypertension.

Keywords
Angiotensin converting enzyme, Chrysin, Magnesium, Nitric oxide, Renin-angiotensin system.

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Abbreviations

ACE - Angiotensin converting enzyme, BP – Blood pressure, L-NAME -N$^{\omega}$-nitro-l-arginine methyl ester, NO - nitric oxide, NOS- nitric oxide synthase.

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INTRODUCTION:

Hypertension is considered to be a multi-factorial disease in which genetic and environmental factors and humoral and neural systems play a role. [1] It remains a major risk factor in cardiovascular mortality and morbidity, through its effects on important target organs such as the heart, liver, and kidney. [2] It is mainly caused by endothelial dysfunction which is caused by nitric oxide (NO) deficiency. An inhibitor of nitric oxide synthase (NOS) *in vitro*, N$^{\omega}$-nitro-l-arginine methyl ester (L-NAME) also inhibits the release of NO from endothelial cells and aortic rings. NO has been demonstrated and proved to have important role in the maintenance of normal blood pressure and body fluid homeostasis. Several disease conditions including essential hypertension have been linked to impaired synthesis or action of NO, which makes chronic inhibition of basal NO with an orally active NOS inhibitor (L-NAME), a particularly interesting model of hypertension. The chronic administration of L-NAME causes significant increase in blood pressure in experimental animals. [3]

In this model, an activated or unopposed renin angiotensin system (RAS) is a major pathogenic player, since both hypertension and organ damage can be prevented or reversed by angiotensin converting enzyme (ACE) inhibition or angiotensin II type 1 (AT1)-receptor blockades. [4] The RAS regulates blood pressure via angiotensin release and blood electrolyte content through release of aldosterone. [5] The ACE is a carboxypeptidase is involved in the conversion of angiotensin I (Ang I) into the biologically active angiotensin I (Ang II). [6] ACE is important in the production of Ang II. Treatment with ACE inhibitors in rats with L-NAME induced hypertension was reported not only to reduce BP but also to prevent the progression of cardio renal remodelling. [5]

Plant polyphenolic compounds the flavanoids consist of number of classes, as flavanols, flavones and flavans. A naturally occurring flavones, Chrysin (5, 7-dihydroxy flavones structure shown in Fig. 1) contained in flowers blue passion flower (Passiflora caerulea), Indian trumpet flower, as well as in edible of mushrooms, [7] honey and propolis. [8] At the same time it possess antioxidant capacity, anti-inflammatory activity, anti-allergic, anti-cancer, antiestrogenic, anxiolytic, [9] antihypertensive properties.[10] Chrysin having tyrosinase inhibitory activity, moderate aromatase inhibitory activity, and another important role are inhibits estradiol-induced DNA synthesis. C-iso-prenylated hydrophobic derivatives of chrysin are potential P-glycoprotein modulators in tumour cells. [11] The earlier study showed that chrysin has antihypertensive effects, and reduces hepatic, renal damages and endothelial dysfunction in L-NAME induced hypertensive rats. [12] The present study aimed to evaluate the effect of chrysin on blood pressure, detection of collagen type I and III gene expression, measurement of Magnesium levels in Plasma and Brain and Relative expression fold changes of renin and ACE. In the L-NAME induced hypertensive rats against the control and unsupplemented groups.

MATERIALS AND METHODS:

Chemicals:

Chrysin and L-NAME was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and obtained from E-Merck or HIMEDIA, Mumbai, India.

Animals:

All the animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee of Bharathidasan University and animals were cared for in accordance with the Indian National Law on Animal Care and Use. Male Wistar rats (180-220g) were purchased from the Indian Institute of Science, Bangalore, India. Rats were housed in plastic cages with filter tops under controlled conditions of a 12 h light-dark cycle, 50% humidity and temperature of 28ºC. All rats received a standard pellet diet (Lipton Lever Mumbai, India) and water *ad libitum*.

Induction of L-NAME induced hypertension:

L-NAME (40 mg/kg B.W) was dissolved in drinking water and given to rats at an interval of 24 h for 8 weeks. Mean arterial blood pressure (MAP) was measured using tail cuff method. MAP measurements were performed during the time of 1-8 weeks. [13]

Blood pressure measurements

Systolic and diastolic blood pressures were determined by the tail-cuff method (IITC, model 31, Woodland Hills, CA, USA). The animals were placed in a heated chamber at an ambient temperature of 30-34°C for 15 minutes and from each animal one to nine blood pressure values were recorded. The lowest three readings were averaged to obtain a mean blood pressure. All recordings and data analyses were done using a computerized data acquisition system and software.

Study design

Animals were divided into four groups of six rats each and all were fed the standard pellet diet. The rats were grouped as given below.

Group I : Control.

Group II : Normal + Chrysin (25 mg/kg of B.W) after 4th week.

Group III: L-NAME induced hypertension (40 mg/kg of B.W).

Group IV: L-NAME induced hypertension (40 mg/kg of B.W) + Chrysin (25 mg/kg of B.W).

Administered orally once in a day in the morning for 4 weeks. The compound was suspended in 2% dimethyl sulfoxide solution and fed by intubation. After 8 weeks morning, the animals were sacrificed by cervical dislocation. The blood was collected in clean dry test tubes and allowed to coagulate at ambient temperature for 30 minutes. Serum was separated by centrifugation at 2000 rpm for 10 minutes. The blood, collected in a heparinized centrifuge tube, was centrifuged at 2000 rpm for 10 minutes and the plasma separated was removed by aspiration and was used for estimations.

The Liver, Brain and kidney were immediately removed and washed in ice-cold saline to remove the blood. The tissues were sliced and homogenized in 0.1 M Tris‑HCl buffer (pH 7.0). The homogenates were centrifuged at 48 × g for 10 minutes at 4ºC in a cold centrifuge. The supernatants were separated and used for the determination of various parameters.

Detection of collagen type I and III gene expression by Quantitative Real Time PCR:

Total RNA was extracted from heart tissue using SV Total RNA Isolation system (Promega, Madison, WI, USA). The cDNA master mix was prepared according to the kit and was added (19 ll for each sample) to the 13 ll RNA-primer mixture. The last mixture was incubated in the programmed thermal cycler. Then RNA was changed into cDNA. The gene-specific forward and reverse primer pair was normalized. Each primer (forward and reverse) concentration in the mixture was 5 pmol/ll. At the end of a qPCR running with SYBR Green chemistry, the relative quantification was used according to step one+ applied biosystem software. [14]

Sequence of the primers used for real-time PCR	
Collagen type 1	F: 50-TGACCAGCCTCGCTCACAG-30
	R: 50-5 GCGGGCAGGGTTCTTTCTA-30
Collagen type 3	F: 50-TCCCAGAACATTACATACCACT-30
	R: 50-GCTATTTCCTTCAGCCTTGA-30

Gene expression analysis:

After the study period the kidney tissues were subjected to total RNA extraction using RNA isolation kit (Fluka, Sigma Aldrich). The integrity, quality and quantity of the RNA were determined by nano-drop spectrometer. RNA was used for quantitative real time polymerase chain reaction (qRT-PCR) using SYBR Green qRT-PCR kit (sigma Aldrich, USA). Primer sequences used were as follows, Renin FP: TATATCTCGGGCC-CTACCAG and RP: GGTGGGTACCTGGCTACAGT; ACE FP: GTG-GTGATGTTCCAGAGCAC and RP: CTCTGCAAACTCCTGGTTGA; glyceraldehydes-3-phosphate dehydrogenase (GAPDH) FP: ACCACAGTCCATGCCAT CAC and RP: TCCACCACCCTGTTGCT-GTA. The amplification specificity of all the primers was confirmed through resolving the PCR products by agarose gel electrophoresis. The relative fold change method was employed for calculating the differential expression between samples [15]. Fold change values were averaged from six reactions.

Measurement of Magnesium Levels in Plasma and Brain:

Plasma and brain Mg levels were determined by atomic absorption spectrometer (Varian.AA-280FS, USA).

Statistical analysis:

Statistical analysis were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a commercially available Software Package for the Social Science (SPSS) software package version 11.0. Results were expressed as mean ± S.D. for six rats in each group.

For all the statistical tests, values of $P < 0.05$ were statistically significant.

RESULTS:

Table 1 and 2 shows the effect of chrysin on systolic and diastolic blood pressures in control and experimental rats for 4 weeks. The systolic and diastolic blood pressures were found significantly higher ($P < 0.05$) in L-NAME induced hypertensive rats (group III). Treatment with chrysin significantly ($P < 0.05$) reduced the systolic and diastolic blood pressure in L-NAME -induced hypertensive group (group IV). There are no significant variations between groups I and II.

Plasma and brain Mg values for the studied groups are shown in Fig. 2 and table 3. The chrysin supplementation in normotensive rats caused a significant rise only in the plasma Mg levels, but no significant change in the brain Mg levels. In L-NAME group of rats, Mg concentrations in the plasma and brain were found to be reduced; however, these levels showed no significant difference when compared with those of the control group. There was a significant increase in Mg levels of the plasma in LNAME+ chrysin group when compared with L-NAME group.

Fig. 3 shows effect of chrysin in graphical representation of collagen I and III gene expression in experimental rats. In collagen I and III gene expression levels are increased in L-NAME induced hypertension as compared to control. Treatment of chrysin significantly ($P < 0.05$) reduces the levels of collagen I and III gene expression in group IV as compared to group I. There is no significant difference between group I and II.

The quantitative PCR analysis have shown that, expression of renin and ACE was significantly ($P < 0.05$) elevated in kidney tissue of L-NAME rats (Group III) as compared to group I whereas chrysin treatment significantly reduced expression of rennin and ACE in kidney tissue at group IV.

There are no changes between group I and II. Chrysin (25 mg/kg of B.W) is effective dose for all parameters significant effect in L-NAME induced rats as compared to control rats. Chrysin in normal rats didn't show any significant.

Table 1: Effect of chrysin on systolic blood pressure in control and L-NAME-induced hypertensive rats

	Control	Control+25 mg chysin	L-NAME induced hypertension	L-NAME+25 mg chrysin
Initial	112±10	110±10	114±10	112±10
1 week	114±8^{a}	112±10^{a}	130±10^{b}	122±10^{c}
2 week	114±10^{a}	112±12^{a}	142±12^{b}	128±10^{c}
3 week	116±12^{a}	114±10^{a}	168±12^{b}	126±10^{c}
4 week	118±12^{a}	116±12^{a}	182±14^{b}	124±12^{c}

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

Table 2: Effect of chrysin on systolic blood pressure in control and L-NAME-induced hypertensive rats

	Control	Control+25 mg chysin	L-NAME induced hypertension	L-NAME+25 mg chrysin
Initial	76±6	76±6	78±6	78±6
1 week	78±6^{a}	76±6^{a}	98±8^{b}	88±8^{c}
2 week	80±8^{a}	78±6^{a}	106±10^{b}	86±8^{c}
3 week	82±8^{a}	78±8^{a}	116±10^{b}	86±6^{c}
4 week	82±8^{a}	76±8^{a}	126±11^{b}	84±8^{c}

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

Table 3: Effect of chrysin in brain Mg levels in various experimental groups

	Control	Control+25 mg chysin	L-NAME induced hypertension	L-NAME+25 mg chrysin
Brain Mg	0,138± 0,005 ^{a}	0,144± 0,004 ^{a}	0,069± 0,0006 ^{b}	0, 136 ± 0,005 ^{c}

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

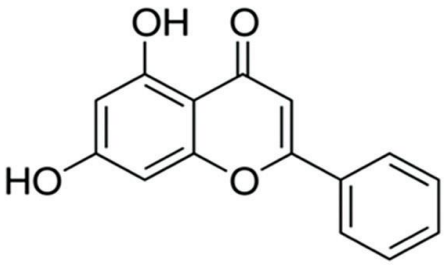
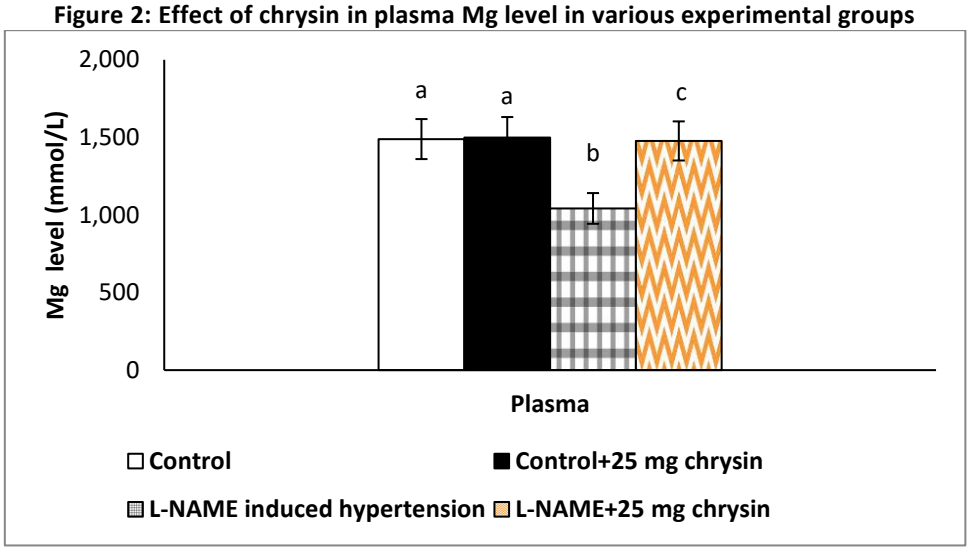


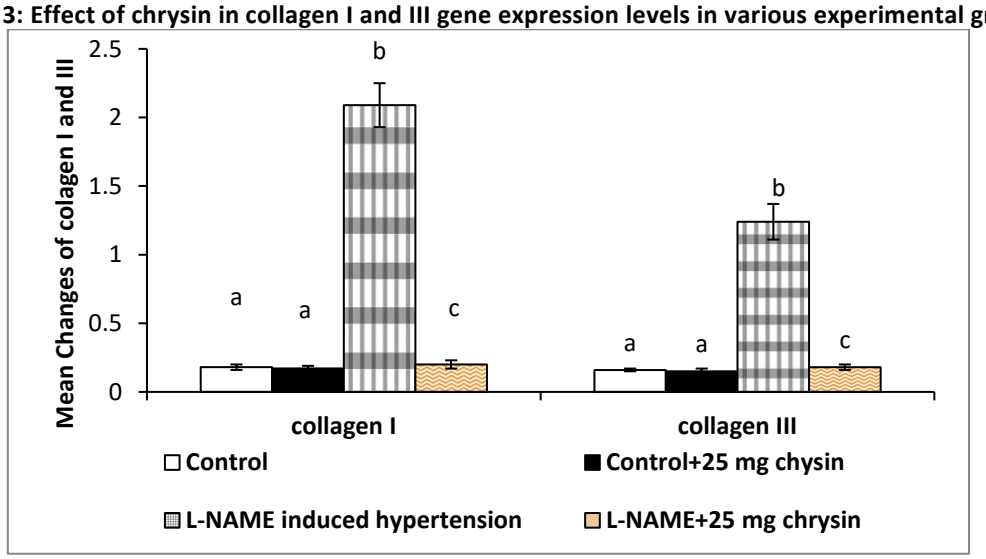
Figure 1: Chemical structure of chrysin (5, 7 dihydroxyflavone)

Figure 2: Effect of chrysin in plasma Mg level in various experimental groups



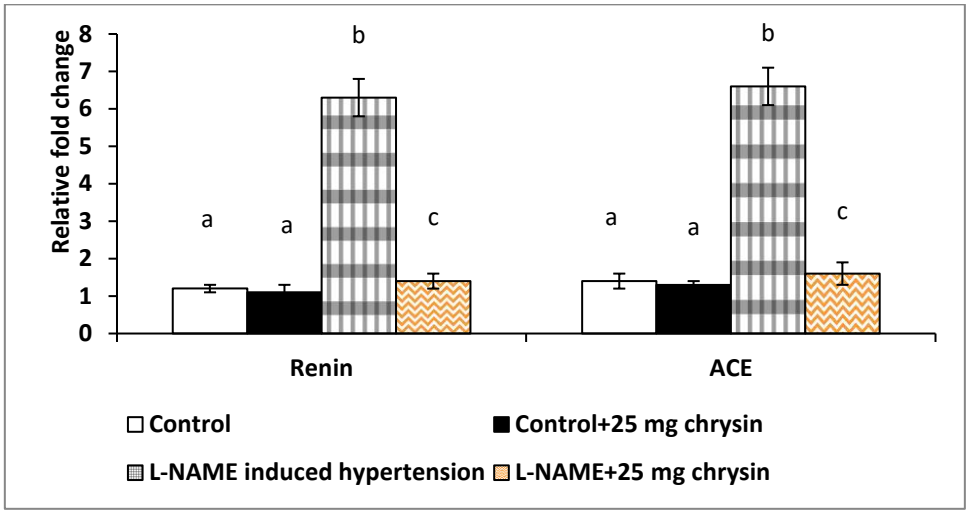
Columns are mean ± SD of six rats from each group.
Columns not sharing a common superscripts (a, b and c) differ significantly at *P* < 0.05 (DMRT).

Figure 3: Effect of chrysin in collagen I and III gene expression levels in various experimental groups



Columns are mean ± SD of six rats from each group.
Columns not sharing a common superscripts (a, b and c) differ significantly at *P* < 0.05 (DMRT).

Figure 4: Relative expression fold changes of renin and ACE.



Columns are mean ± SD of six rats from each group.
Columns not sharing a common superscripts (a, b and c) differ significantly at *P* < 0.05 (DMRT).

DISCUSSION:

The purpose of this study is to investigate the protective effects of chrysin against L‑NAME‑induced hypertension. The present experiments provide evidence that chronic inhibition of NO synthesis in rats leads to marked elevations of systemic blood pressure and peripheral vascular resistance with alteration of vascular responsiveness. These vascular alterations were associated with marked oxidative stress. Six weeks of L‑NAME administration causes a chronic increase in blood pressure in rats as previously described by other authors after shorter duration experiments. [16] Many studies have reported chronic blockade of NO synthesis by NOS inhibitors like L‑NAME leading to endothelial dysfunction, a significant increase in blood pressure and further pathological injuries to the cardiovascular system and kidneys, which may lead to aggravation of hypertension. [17] A mechanism responsible for the increase of BP during L‑NAME‑treatment is associated with NO deficiency and alterations in various blood pressure regulating systems. Several authors have observed elevation of vasoconstriction and attenuation of vasorelaxation in different parts of the vascular tree and increased sympathetic activity and alterations in renin‑angiotensin system in L‑NAME treated rats. [18] In our previous work demonstrated that a daily oral dose (25 mg/kg) of chrysin for 8 weeks reduced the elevated blood pressure and hepatoprotective effect in L‑NAME induced hypertensive rats. [10, 12] The vasodilator effect of chrysin on resistance vessels might contribute to its antihypertensive effect.

The findings of increased expression of types I and III of collagen genes in L-NAME induced hypertensive rats when compared with normotensive ones, are in harmony with the results of Kobayashi *et al*., [19] and Zambrano *et al*. [20] Data of the present study showed a cardio protective effect of chrysin in L-NAME treated rats as evident by significant decrease in systolic and diastolic blood pressure compared to L-NAME group. Since the quality rather than the quantity of the hypertrophied myocardium is important, while fibrosis is one of the decisive negative prognostic factors. [21] This antifibrotic effect of chrysin may be of importance in patients with hypertensive heart. That might be due to chrysin having antihypertensive effects in L-NAME induced hypertensive rats.

Decreased plasma and/or tissue Mg levels have been shown in different experimental models of hypertension. [22] In the present study, plasma Mg levels in L-NAME group were not statistically different compared to control (Fig. 2). However, chrysin-enriched diet lead to higher plasma Mg levels in L-NAME+ chrysin group compared to L-NAME group. This increment of plasma Mg level and decrement of SBP in Mg-supplemented hypertensive rats (Table 1) are in accordance with the view concerning the inverse correlation between Mg ingestion and blood pressure in hypertension. [23] Our findings indicated that there was no significant difference of brain Mg levels between the control and chrysin treated control (Table 3). Previously, it was observed that chronic hypermagnesemia did not significantly increase brain Mg concentration despite a threefold increase in plasma. However, a dose-dependent increment of brain Mg levels was

achieved by intra cerebro ventricular infusion of $MgSO_4$ in the same study. [24] The central nervous system normally maintains higher Mg in the cerebrospinal fluid than plasma, suggesting an active transport process to maintain this gradient [25] and limit the amount of Mg that can be loaded into the brain. [26] However, different states can be achieved under conditions of altered blood–brain permeability. It is well known that hypertension induces blood–brain barrier disruption. [27]

Previously, it was demonstrated that hypertension caused by L-NAME leads to lower Mg levels in the cerebral cortex. [28] In the present study, although there was a decrease tendency in L-NAME group and an increase tendency in L-NAME+ chrysin group, we could not find a statistically significant difference between groups in the means of Mg levels of the brains (Table 3). The limitation of our study was measuring Mg levels in the whole brain. Because of this, we were unable to show the effect of chrysin treated on specific brain regions such as the cerebral cortex. It is likely that there might be variations below the threshold of assay detection in our study. Besides, lack of significant changes could be related with the insufficiency of Mg treatment schedule. Recently, a highly bioavailable Mg compound (magnesium-L-threonate, MgT) has been developed which increases Mg levels in the brain by dietary supplementation. [29]

In this study the deregulated expression of RAS components was restored by chrysin treatment. Recent experimental studies in rats have shown that chronic administration of L-NAME caused systemic hypertension and renal parenchymal injury. In these models, it was also reported that the RAS was stimulated and that the RAS inhibition with either ACE inhibitor or angiotensin II type I receptor antagonist prevented both the onset of hypertension and renal damage. [30] Previous studies suggest the possibility of the early inhibition of RAS as an effective strategy to prevent chronic kidney disease development. [31] Likewise the renoprotective mechanism of chrysin may be partially mediated by its role on expression of RAS components.

CONCLUSION:

It is clear that treatment with chrysin significantly reduced the BP and improved Mg level in brain and plasma in L-NAME–induced hypertensive rats. Collagen I and III was significantly decreased and highest percentage of inhibition was in chyrsin treated group compared to L-NAME. The present biochemical findings showed that chrysin possesses an antihypertensive effect which is evidenced by lowered blood pressure, collagen I and III expression. That might be evidence to chrysin is having antihypertensive effect.

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CONFLICT OF INTEREST STATEMENT

None declared

SOURCES OF SUPPORT

Nil

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