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Protective Effect of Pomegranate Juice Extract Against Antitubercular Drugs Induced Hepatic Fibrosis

J. Ramesh[1], Anilkumar Banothu[1*], Kala Kumar Bharani[1], P. Shivakumar[1], M. Jeevanalatha[2] and Y. Ravikumar[3]

[1]*Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Rajendranagar, Hyderabad - 500030, Telangana, India; anilvetpharma@gmail.com*

[2]*Department of Pathology, College of Veterinary Science, Mamanoor, Warangal - 506166, Telangana, India*

[3]*Department of Pathology, College of Veterinary Science, Rajendranagar, Hyderabad - 500030, Telangana, India*

Abstract

The current study aimed to know the therapeutic efficacy of *Punica granatum* (fruit juice extract) against anti-TB drug-induced hepatic fibrosis in rats. 24 male *Wistar albino* rats were randomly divided into four groups, with six rats in each. INH at 27 mg/kg B.W., RIF at 54 mg/kg B.W., and PZA at 135 mg/kg B.W. were administered orally to Groups 2, 3 and 4 from days 1 to 28. Group 1 was maintained as normal control, and group 2 was a toxic control (administered anti-TB drugs, p/o). Groups 3 and 4 were administered (p/o) with Enalapril at 5mg/kg B.W. and *Punica granatum* (fresh juice extract) at 1 ml/rat, respectively, from day 1 to 28. Serum samples were collected on the 14th and 28th to assess various biochemical parameters, and liver samples were collected at the end of the experiment to analyze antioxidant parameters, TGF β1, and histopathological examination. The biochemical parameters showed significant ($p<0.05$) alterations in AST, ALT, GGT, antioxidant profile (TBARS GSH, GST, GPx and SOD), and expression of TGF β1 levels in group 2 along with collagen deposition and alterations in the histopathology when compared with group 1. There was a significant improvement in treatment groups 3 and 4 at different time intervals. These changes were reversed in groups 3 and 4, which were administered Enalapril and *Punica granatum* juice extract, respectively.

Keywords: Antioxidants, Antitubercular Drugs, Hepatotoxicity, Pomegranate Juice, TGF Beta

1. Introduction

Tuberculosis (TB) is an infectious and highly contagious disease caused by Mycobacterium Tuberculosis (MTB). It is an essential and notifiable disease of animals and has zoonotic importance[1]. Isoniazid (INH), Rifampicin (RIF), Ethambutol, and Pyrazinamide (PZA) are the first-line drugs used for the treatment of tuberculosis. A standard anti-TB therapy contains a three-drug regimen of INH, RIF, and PZA for eight weeks, followed by INH and RIF for another 4-7 months[2]. Anti-TB drugs, such as INH, RIF, and PZA, were toxic to the liver[3]. Animal models are significant for most biomedical research and to test critical hypotheses of participation of reactive metabolites. Rats

**Author for correspondence*

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were successfully used as laboratory models for the study of hepatotoxicity caused by INH, RIF, and PZA[4].

Numerous antioxidant studies of pomegranate parts (including peel, juice, and seeds) have proved that a high level of phenolic compounds is responsible for its antioxidant properties[5,6]. Therefore, despite effective INH, RIF, and PZA chemoprophylaxis, there is a need to either stop treatment due to liver damage caused by them or amelioration attempts to overcome the liver damage resulting from INH, RIF, and PZA is essential to continue the use of the above drugs indiscriminately to combat tuberculosis. Considering these observations, an experimental study was framed to evaluate the hepatoprotective effect of Pomegranate Juice (PJ) against INH, RIF, and PZA-induced hepatic fibrosis in male *Wistar albino* rats.

2. Materials and Methods

2.1 Drugs

The drugs like isoniazid (Macleods Pharmaceuticals Pvt. Ltd., Kachigam, Daman), rifampicin and pyrazinamide (Lupin Ltd., Chikalthana, Aurangabad) and enalapril (Dr. Reddy's Laboratories Ltd., Khol, Baddi, H.P) were obtained and used in the study. The pomegranate fruit were purchased from the local market.

2.2 Chemicals and Kits

All chemicals used in the biochemical analysis were of analytical grade. All the chemicals (for preparation of reagents and buffers) were procured from Qualigens Pvt. Ltd. Mumbai, Himedia Pvt. Ltd., and SRL Pvt. Ltd., Mumbai. whereas as Kits for AST, ALT, GGT, were procured from Erba diagnostics Ltd, Surat, India, and Coral diagnostics, Goa, and ELISA kit for TGF-β1 was procured from Krishgen Biosystems., Mumbai.

2.3 Animals

A total of 24 male *Wistar albino* rats three months old were procured and acclimatized for at least one week for the study. The rats were randomly divided into four groups consisting of 6 rats in each and kept in polypropylene cages and maintained with a 12-hour dark/light cycle at the college animal house. All the rats were provided with feed and water *ad libitum* throughout the experiment. The Institutional Animal Ethics Committee approved the experimental protocol (I/2018-31/IAEC/CVSc., Hyd., Dated 16/07/2018). All the groups were maintained as per the following treatment schedule for 28 days.

2.4 Experimental Design

Group 1:	Normal control
Group 2:	INH at 27 mg/kg body weight (BW) + RIF at 54mg/kg BW + PZA at 135 mg/kg BW once daily orally.
Group 3:	INH at 27 mg/kg BW + RIF at 54mg/kg BW + PZA at 135 mg/kg BW + Enalapril at 5 mg/kg BW once daily
Group 4:	INH at 27 mg/kg BW + RIF at 54 mg/kg BW + PZA at 135 mg/kg BW + Pomegranate juice at 1 ml/Rat once daily orally Treatment scheduled followed for 28 days.

2.5 Blood Collection

Blood collection was carried out on day 14th and 28th in vacutainers vials from the rats. Sera samples were separated from the blood to estimate AST, ALT and GGT. On the 28th day, after blood collection, rats were euthanized by carbon dioxide exposure, and the liver tissues were collected and homogenized for the assay of GSH, GPX, GST, SOD, TBARS and TGF β1. A histopathological examination of liver tissues was conducted to draw possible conclusions at the end of the experiment.

2.6 Antioxidant Defense Profile

The concentration of antioxidant parameters in the liver tissue homogenate was estimated as per the standard protocol described by the authors. Reduced glutathione[7], Superoxide Dismutase (SOD)[8], Thiobarbituric Acid Reacting Substances (TBARS)[9] and Glutathione Peroxidase (GPx)[10], glutathione S-transferase[11] and estimation of protein[12].

2.7 Immunoassay

The procedure followed as per the manufacturer guidelines for the ELISA for Transforming Growth Factor (TGF) β1.

2.8 Histopathological Studies

Tissue pieces of liver were collected from the rats that were sacrificed at the end and fixed in 10% Neutral Buffered Formalin (NBF). The fixed tissues were processed and stained with Masson's trichrome stain as described by the procedure[13].

2.9 Statistical Analysis

The data were subjected to statistical analysis by applying one-way ANOVA using the Statistical Package for Social Sciences (SPSS) latest version 25. Differences between means were tested using Duncan's multiple comparison tests and significance was set at $p<0.05$.

3. Results

3.1 Liver Biomarkers

The serum AST ALT and GGT activity (IU/L) in Group 2 was significantly ($p<0.05$) increased when compared to Group 1 during the 14th and 28th days of the experiment. Treatment Groups 3 and 4 showed significant improvement compared to Group 2 at respective time intervals. There was a time-related increase in the value of toxic control Group 2 as there was a significant ($p<0.05$) difference on day 28 compared to day 14. The values of Groups 3 and 4 were comparable without any significant difference at different time intervals. Still, the values were significantly ($p<0.05$) reduced on day 28 when compared to their corresponding values on day 14 (values are enclosed in Table 1).

3.2 Antioxidant Profile in Liver Tissue

The concentration of GSH (µ moles/mg protein), the activity of SOD (U /mg protein), the activity of GPX (U/mg protein), and the activity of GST (µ moles/min/mg protein) were significant ($p<0.05$) decrease and significant ($p<0.05$) increase in TBARS (n moles of MDA released/mg protein) in the toxic control Group 2 when compared to Group 1 after 28th day of the experiment. Their concentrations were significantly ($p<0.05$) improved in the treated Groups 3 and 4 as compared to toxic control Group 2, and the values were comparable to Group 1. The values of Groups 3 and 4 were comparable without any significant difference (values are presented in Table 2).

3.3 Transforming Growth Factor (TGF)-β1 Expression

The expression of TGF-β1 (ng/ml) in liver homogenate revealed a significant ($p<0.05$) increase in the toxic control Group 2 when compared to Group 1. Conversely, the expression of TGF-β1 in the treated Groups 3 and 4 showed a significant ($p<0.05$) decrease as compared to Group 2. There was no statistically significant difference

Table 1. Liver biomarkers in different groups of rats

GROUP	AST activity (IU/L)		ALT activity (IU/L)		GGT activity (IU/L)	
	14th DAY	28th DAY	14th DAY	28th DAY	14th DAY	28th DAY
1. NORMAL CONTROL	60.26±3.03^{a}	59.95±2.06^{a}	34.08±1.10^{a}	32.01±1.06^{a}	2.24±0.26^{a}	2.11±0.15^{a}
2. INH+RIF+PZA	88.17±1.14^{cA}	96.54±2.51^{cB}	56.69±2.64^{cA}	69.50±2.30^{cB}	4.69±0.42^{cA}	6.47±0.32^{cB}
3. INH+RIF+PZA+ENA	75.36±1.02^{bA}	64.55±1.37^{bB}	48.38±1.66^{bA}	43.33±1.58^{bB}	3.64±0.45^{b}	3.07±0.51^{b}
4. INH+RIF+PZA+PMJ	78.97±1.75^{bA}	66.29±1.26^{bB}	45.04±1.16^{aA}	41.27±1.10^{bB}	3.12±0.14^{b}	3.00±0.25^{b}

Values are Mean ± Standard Error (S.E.) (n = 6); One way ANOVA (SPSS) Means with different alphabets as superscripts differ significantly ($p<0.05$) among the groups at respective time intervals. Alphabets with lowercase represent vertical comparison. Alphabets with uppercase represent the horizontal comparison (AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase and GGT: Gamma-Glutamyl Transferase)

Table 2. Antioxidant profile and TGF-β1expressions in liver homogenate in different groups of rats

GROUP	GSH (n moles/mg protein)	SOD activity (U/mg protein)	TBARS (n moles of MDA released/mg protein)	GPX activity (U/mg protein)	GST activity (U/mg protein	TGF-β1 (ng/ml)
1. NORMAL CONTROL	8.95±0.05 ^{c}	5.72±0.11 ^{bc}	38.02±0.96 ^{a}	18.32±0.21 ^{bc}	5.48±0.56 ^{bc}	0.50±0.03 ^{a}
2. INH+RIF+PZA	3.05±0.17 ^{a}	2.62±0.08 ^{a}	85.18±1.07 ^{c}	10.74±0.15 ^{a}	2.76±0.06 ^{a}	1.52±0.12 ^{c}
3. INH+RIF+PZA+ENA	5.56±0.11 ^{b}	4.01±0.08 ^{b}	46.33±2.62 ^{b}	16.11±0.15 ^{c}	3.66±0.15 ^{b}	0.80±0.05 ^{b}
4. INH+RIF+PZA+PMJ	5.83±0.09 ^{b}	4.86±0.37 ^{b}	48.40±1.71 ^{b}	16.41±0.33 ^{c}	3.87±0.06 ^{b}	0.93±0.09 ^{b}

Values are Mean ± Standard Error (S.E.) (n = 6); One way ANOVA (SPSS) Means with different alphabets as superscripts differ significantly ($p<0.05$) among the groups at respective time intervals. Alphabets with lowercase represent vertical comparison. (GSH: Glutathione, SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reactive substances, GPX:Glutathione peroxidase, GST:Glutathione S-transferases and TGF-β1: Transforming growth factor beta).

among the treated Groups 3 and 4 (values are enclosed in Table 2).

3.4 Histopathology of the Liver

The histopathological examination of liver tissues in toxic control (Group 2) revealed moderate necrotic degeneration and pericellular fibrosis (Masson's trichrome staining exhibited deposition of collagen) (Figure 2). Groups 3 and 4 showed mild pericellular fibrosis, degenerative changes, and normal architecture of hepatocytes (Masson's trichrome staining) (Figure 3 and Figure 4, respectively) compared to Group 2. The sections of group 1 (Figure 1) did not reveal any significant lesions, no evidence of collagen deposition and the histoarchitecture was normal.

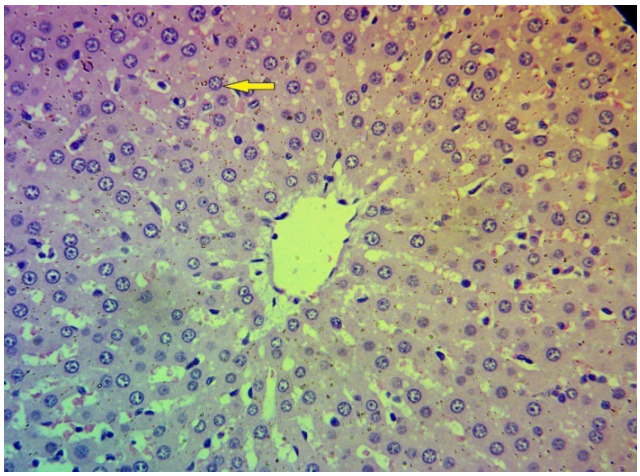


Figure 1. Photomicrograph of liver showing normal histoarchitecture of hepatocytes (marked with yellow arrow). x 200 Masson's trichrome stain (Group 1).

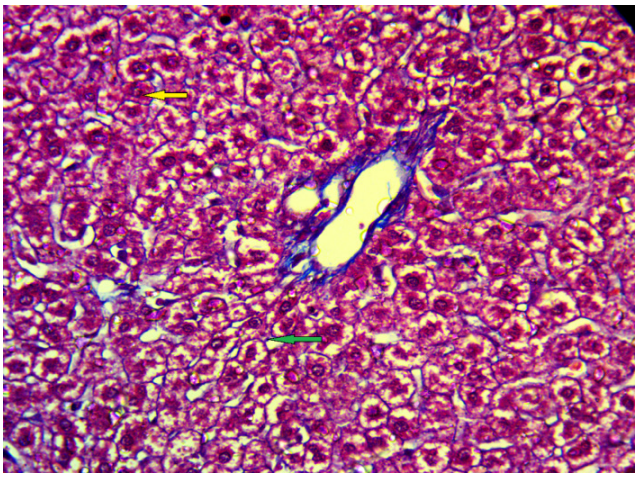


Figure 2. Photomicrograph of liver showing moderate pericellular fibrosis (marked with green arrow) and moderate hepatocyte degeneration (yellow arrow). x 200 Masson's trichrome stain (Group 2).

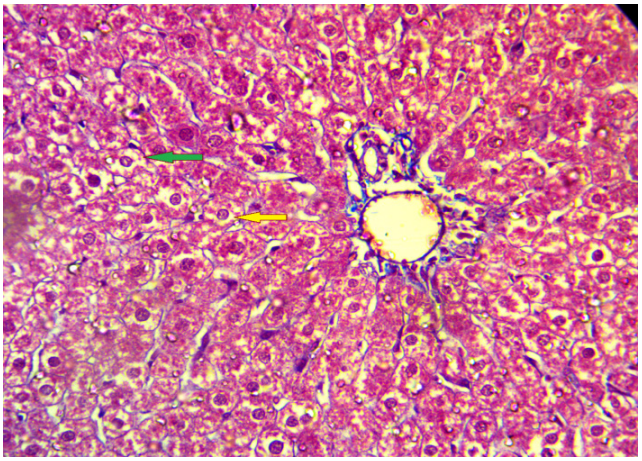


Figure 3. Photomicrograph of liver showing mild pericellular fibrosis (marked with green arrow) and mild degeneration (yellow arrow) of hepatocytes. x 200 Masson's trichrome stain (Group 3).

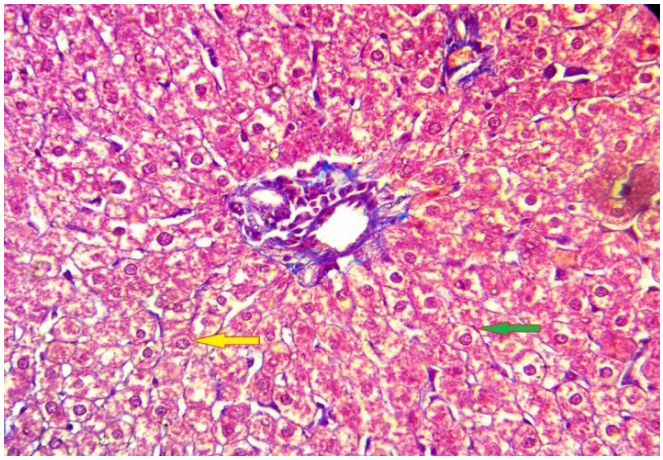


Figure 4. Photomicrograph of liver showing mild pericellular fibrosis (marked with green arrow) and mild degeneration of hepatocytes (yellow arrow). x 200 Masson's trichrome stain (Group 4).

4. Discussion

Antituberculosis drugs impose a hazardous effect on the patient's liver, leading to hepatotoxicity especially. The first three drugs are hepatotoxic agents and are causing a significant problem in tuberculosis-treated patients, and their toxic effect is more pronounced when they are used in combination. The toxicity may be due to membrane damage indicated by increased serum markers, suppression of antioxidant defense mechanism accompanied by enhanced lipid peroxidation, and stimulation of the CYP2E1 activation[14]. The present study was carried out with simultaneous supplementation of Pomegranate Juice (PJ) to mitigate the adverse effects of anti-TB drugs, and the results of the present study are discussed here.

The activities of ALT, AST and GGT were determined to assess the degree of damage to the liver and whether their levels get elevated after hepato-cellular injury[15,16]. In the present investigation, the activities of ALT, AST and GGT were significantly elevated in the toxic control group when compared to the normal control group suggesting the hepato-cellular insult, indicating cellular damage and loss of functional integrity of cell membrane in the liver following administration of antituberculous drugs[17]. These results are further substantiated by the histopathological studies of the liver using Masson's trichrome staining. There were moderate to severe degenerative changes, mild to moderate cell necrosis, and pericellular fibrosis in the liver. Further, these results were also supported by the significant increase in the relative liver weights of the toxic Group 2 in comparison to the control, which might be attributed to increased collagen synthesis. Moreover, the increased activity of AST, ALT and GGT in Group 2 might be due to the toxic effect of metabolites of INH (acetyl hydrazine and hydrazine), RIF (increases the metabolism of INH to isonicotinic acid and hydrazine), and PZA (Pyrazinoic acid, 5-hydroxy pyrazinoic acid) that cause damage to cellular macromolecules in the liver by free radical generation and oxidative stress with subsequent hepatic damage and necrosis, which leads to the discharge of the intracellular contents into the systemic circulation[18] and it was evident from the present study showing a significant alteration in the oxidative stress parameters.

Co-administration of antituberculous drugs with PJ has significantly reversed the changes recorded in serum ALT, AST and GGT activities as compared to the toxic control group. This agrees with previous studies[19-21]. Furthermore, PJ administered along with aspartame protected the rats from aspartame-induced oxidative injury and ameliorated liver function[22]. Administration of yogurt supplemented with PJ is also acceptable as functional food alleviates the harmful effect of CCl4-induced liver injury and offers a pleasant and effective route in increasing the total phenolic content and antioxidant intake in our daily diet[23].

Biomarkers of oxidative stress and antioxidant profile were studied in liver tissue homogenate to evaluate INH, RIF, and PZA - induced free radical damage in this study.

The results revealed a significant increase in SOD, GPX, and GST activity in the toxic control group 2 compared to the control group at the end of the experiment. Oxidative damage to the cells and tissues occurs when the concentration of reactive oxygen species (O^{-2}, H_2O_2, and hydroxyl radical) exceeds the antioxidant capability of the body[24,25]. The status of lipid peroxidation and endogenous antioxidant enzyme system gives direct evidence of oxidative stress[26]. Reduced Glutathione (GSH) is considered the major non-enzymatic antioxidant in the body. The biological system utilizes GSH chiefly for reducing oxidized vitamins C and E back to their reduced state to detoxify many toxins, maintain cellular redox potential and maintain erythrocyte membrane integrity[27]. In the current investigation, the reduced GSH level and GPx activity of liver tissue were significantly increased in the groups treated with Enalapril and PJ when compared to the values of the toxic control group.

Free radical scavenging enzymes like SOD and catalase protect the body from oxidative stress. The SOD distributes superoxide radicals (O^{2-}) into hydrogen peroxide (H_2O_2) and singlet oxygen[28,29]. SOD and catalase activities were significantly increased in enalapril, and pomegranate juice-treated groups compared to the untreated Group 2. Also, there was a corresponding decrease in TBARS (extent of lipid peroxidation) in the liver of treated Groups 3 and 4. It might be due to excessive utilization of these enzymes in inactivating the free radicals generated during hepatic damage.

The concentration of TBARS in liver tissue was studied to determine the extent of lipid peroxidation in this study, which demonstrated a significant increase in TBARS in the toxic control Group 2 as compared to the control group. INH, RIF and PZA trigger massive lipid peroxidation, which is responsible for the liver injury[30]. The present study also assessed the Malondialdehyde (MDA) level as an important and sensitive marker for lipid peroxidation[31]. An increased level of MDA was observed in the liver tissue of toxic control Group 2, but it was significantly decreased in the groups treated with enalapril and PJ (Groups 3 and 4, respectively). Thus, PJ showed free radical scavenging ability comparable to the enalapril-treated group. This observation coincides with the activities of endogenous antioxidants, *viz*., SOD, GSH, GST, etc., which were also significantly increased in PJ-treated groups. The expression of a biomarker for fibrosis, Transforming Growth Factor (TGF)-β1 peptide, was studied in liver tissue homogenate. In the present study, the TGF-β1 expression in the liver tissue was significantly increased in groups treated with anti-TB drugs compared to the control group. The increased levels of TGF-β1 could be attributed to liver injury and released TGF-β1 may cause necrotic hepatocytes, activated Hepatic Stellate Cells (HSCs) and macrophages (Kupffer cells)[32]. For example, Enalapril treated group 3 showed decreased TGF-β1 levels attributed to the protection of hepatocytes against oxidative stress and suppression of HSC activation by Angiotensin II type 1 receptor expressed on the surface of HSCs[33]. *In vitro* and *in vivo* studies on hepatic fibrosis suggest that the Angiotensin II Type 1 receptor antagonists suppress proliferation, collagen synthesis, and expression of pro-fibrogenic cytokines (TGF-β1 and CTGF) in activat ed HSCs[34]. Two mechanisms of action have been suggested: First, the Angiotensin II Type 1 receptor antagonist inhibits activated HSCs by blocking Angiotensin II Type 1 receptor expressed on the surface of HSCs; second, it suppresses the activation of HSCs as a result of the decrease in TGF-β1[35]. Similarly, PJ treated Group 4 also revealed a significant reduction in TGF-β1 levels, which might be due to improved antioxidant defenses (increased GPx, SOD, GST and GSH) and decreased MDA levels, thus protecting hepatocytes from oxidative damage and subsequent prevention of HSC activation, which ultimately resulted in the decreased levels of TGF-β1.

In our study, P.J. treated Group 4 showed significant improvement due to the presence of marked antioxidant potential with a high content of tannins, phenols, and flavonoids, which can directly or indirectly reduce oxidative damage by preventing the excessive generation of free radicals. P.J. is rich in polyphenols, including ellagic acid in the free form linked to glycosides, gallotannins, anthocyanins (cyanidin, delphinidin, pelargonidin glycosides), and other flavonoids (quercetin, kaempferol and luteolin glycosides)[36].

In conclusion, the results of the present investigation enunciated that PJ extract has potent hepatoprotective activity based on biochemical, antioxidants, and histopathological parameters. Further, it is also suggested that PJ extract could effectively scavenge free radicals released during the metabolism of anti-TB drugs (INH, RIF and PZA) and protect the binding of their toxic reactive metabolites with cellular macromolecules like proteins, lipids, and nucleic acids, which were evident in this study by reduced the hepatotoxic markers, restoration of antioxidant enzymes, decreased TGF-β1 protein

expression and collagen deposition. Therefore, PJ extract could be a potential source in managing drug-induced hepatocellular fibrosis.

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6. References

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