OXIDATIVE STRESS BIOMARKERS IN THE EQUINE ERYTHROCYTES AFTER *IN VITRO* TREATED WITH LEAF EXTRACT OBTAINED FROM *THYMUS × PORCII* BORBÁS (LAMIACEAE)

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Antioxidants from natural products are important to protect against free radicals. The literature has documented the antioxidant activity of the *Thymus* genus (Lamiaceae) in inhibiting the formation of free radicals generation. In line with our previous study, we continue to assess the antioxidant potential of four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine on equine erythrocytes’ model. Therefore, in the present study, the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification, total antioxidant capacity (TAC)], as well as HCl-induced hemolysis in the equine erythrocytes, was used for assessing the antioxidant activity of extract obtained from the leaves of *Th. × porcii* Borbás (a hybrid between *Th. pannonicus* and *Th. pulegioides*). Leaves of *Th. × porcii* were sampled in the grass stand, on the side of the footpath of the race track (Medovoi Pechery Str., Lviv, Ukraine; N 49° 49’ 15.1”, E 24° 05’ 12.5”, 348 m a.s.l.). Equine erythrocyte aliquots were used in the study. For positive control (blank), phosphate buffer was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, samples were used for the biochemical assays. The aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered after *in vitro* incubation with an extract obtained from leaves of *Th. × porcii*. The *Th. × porcii* extract caused to increase of TBARS content as a biomarker of lipid peroxidation in the extract-treated erythrocytes, and these results were statistically significant. Total antioxidant capacity was non-significantly increased. When *Th. × porcii* extract (5 mg/mL) was added to the erythrocyte suspension, the maximum level of hemolysis occurred after 8.5 min of incubation with 0,1M HCl (17.91 ±1.87%). The total duration of hemolysis after *Th. × porcii* extract (5 mg/mL) incubation was 11.5 min. Our results showed that HCl-induced hemolysis in a typical time-dependent manner. the extract obtained from leaves of *Th. × porcii* (5 mg/mL) has a mild cytotoxic activity on the equine erythrocytes increasing the level of lipid peroxidation biomarker and hemolysis rate. Investigation of the mechanism of action revealed that this extract has hemolytic activity. Therefore, *Th. × porcii* extract at a concentration of 5 mg/mL induced the increase of hemolyzed erythrocytes and caused to decrease in hemolysis duration. Screening of *Thymus*...
species for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

**Keywords:** Thymus, leaf extract, equine erythrocytes, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity, hemolysis

**Introduction**

In recent years, the use of plants and food supplements containing botanical ingredients, as an alternative therapy for various diseases, has been intensified due to their high content of chemical agents such as polyphenols, i.e. flavonoids, tannins, and alkaloids (Nabavi et al., 2015). The genus *Thymus* consists of approximately 215 species currently recognized (Morales, 2002). These herbaceous perennials and sub-shrubs distributed in Europe, Northwest Africa, Ethiopia, Asia and Greenland (Morales, 2002; Bartolucci et al., 2013). It is one of the most widely used genera in folk medicine, where it is popular for its stimulatory action on all organism functions (Viuda-Martos et al., 2011).

These plants possess a variety of activities including antimicrobial, antioxidant, anti-inflammatory, cytotoxic, analgesic, and anti-diabetic due to the presence of second metabolites, i.e. flavonoids, phenylpropanoids, tannins, organic acids, terpenoids, and phytosterols (Li et al., 2019). Thymol (10–64%) is one of the major constituents of the essential oils of thyme (*Thymus vulgaris* L.), a medicinal plant with several therapeutic properties (Salehi et al., 2018). These multiple therapeutic effects of thymol are largely attributed to its anti-inflammatory (via inhibiting recruitment of cytokines and chemokines), antioxidant (via scavenging of free radicals, enhancing the endogenous enzymatic and non-enzymatic antioxidants and chelation of metal ions), antihyperlipidemic (via increasing the levels of high density lipoprotein cholesterol and decreasing the levels of low density lipoprotein cholesterol in the circulation and membrane stabilization) (via maintaining ionic homeostasis) effects (Nagoor Meeran et al., 2017).

The antimicrobial activity of *Thymus* species, as well as thymol, has been well studied. In our previous study (Honcharenko et al., 2018b, c), the ethanolic extracts obtained from leaves of various *Thymus* representatives exhibited intermediate activity against β-lactamase-producing *Pseudomonas aeruginosa* and *Salmonella enteritidis* strains locally isolated. The effects varied significantly according to the *Thymus* taxa. It should be noted that the most antimicrobial effective plant against β-lactamase-producing *P. aeruginosa* was *Th. alpestris* Tausch ex A. Kern., being highly active with the ethanolic extract (mean diameter of inhibition zone was 12.8 ±0.8 mm). The antibacterial activity of extracts was greatest for *Th. alpestris* followed by *Th. pannonicus* All. followed by *Th. serpyllum* L. and then by *Th. pulegioides* L. (Honcharenko et al., 2018c). The ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against *S. enteritidis*. The antibacterial activity of extracts was greatest for *Th. pulegioides* followed by *Th. pannonicus* followed by *Th. alpestris*, *Th. × porcii*, and then by *Th. serpyllum*. These plant extracts could be a potential source of new antibacterial agents (Honcharenko et al., 2018b).
Thymol also increases the activity of endogenous antioxidant enzymes, such as superoxide
dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and the level of other
non-enzymatic antioxidants such as vitamin C, vitamin E, and reduced glutathione (Nagoor
Meeran and Prince, 2012), and thereby the total antioxidant status in vivo (Youdim and Deans,
2000). In our previous study (Honcharenko et al., 2018a), we have assessed the oxidative
stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives
content of protein oxidative modification, total antioxidant capacity] in the equine erythrocytes
after treatment with Thymus serpyllum L. extract. Lipid peroxidation biomarker, aldehydic and
ketonic derivatives of oxidatively modified proteins, total antioxidant capacity was non-
significantly altered after in vitro incubation with an extract obtained from Th. serpyllum.

In line with our previous study, we continue to assess the antioxidant potential of four species
and one interspecific hybrid of Thymus genus sampled in the western part of Ukraine on
equine erythrocytes' model. Therefore, in the present study, the oxidative stress biomarkers
[2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein
oxidative modification, total antioxidant capacity (TAC)], as well as HCl-induced hemolysis in
the equine erythrocytes, was used for assessing the antioxidant activity of extract obtained
from the leaves of Th. × porcii Borbás (a hybrid between Th. pannonicus and Th. pulegioides).

Materials and methodology

Collection of Plant Materials
Leaves of Th. × porcii (a hybrid between Th. pannonicus and Th. pulegioides) were sampled
in the grass stand, on the side of the footpath of the race track (Medovoi Pechery Str., Lviv,
Ukraine; N 49° 49' 15.1", E 24° 05' 12.5", 348 m a.s.l.) (Figure 1). Identification of this species
was made according to Nachychko (2014, 2015) and Nachychko and Honcharenko (2016). The
voucher herbarium specimens of plants used in this study were deposited at the Herbarium of
M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Plant
samples were thoroughly washed to remove all the attached material and used to prepare the
extract.

Preparation of Plant Extract
Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate
buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then
filtered and used for analysis. The extract was stored at -20 °C until use.

Horses
Eighteen healthy adult horses from the central Pomeranian region in Poland (village
Strzelinko, N 54° 30’ 48.0” E 16° 57’ 44.9”), aged 8.9 ±1.3 years old, including 6 Hucul pony,
5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used
in this study. All horses participated in recreational horseback riding. Horses were housed in
individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and
water available ad libitum. All horses were thoroughly examined clinically and screened for
hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

**Collection of blood samples**

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma. The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes. For positive control (phosphate buffer) was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Erythrocytes aliquots were used in the study.

**2-Thiobarbituric Acid Reactive Substances (TBARS) assay**

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamysnshikov (2004) method for

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**Figure 1**  *Thymus × porcii* plant (Photo: Viktor Nachychko, Vitaliy Honcharenko)
determining the malondialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The µmol of MDA per L was calculated using 1.56·10⁵ mM/cm as the extinction coefficient.

**The carbonyl derivatives content of protein oxidative modification (OMP) assay**

To evaluate the protective effects of extracts obtained from leaves of *Th. × porcii* against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine and co-workers (1990) and as modified by Dubinina and co-workers (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP₃₇₀) and 430 nm (ketonic derivatives, OMP₄₃₀).

**Measurement of Total Antioxidant Capacity (TAC)**

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe²⁺/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

**Assay of Acid Resistance of Erythrocytes**

The acid resistance of erythrocytes was measured spectrophotometrically with 0.1M HCl (Terskov and Gitelson, 1957). The assay is based on the measuring of the dynamics of erythrocytes disintegration into hemolytic reagent action. The time of hemolytic reagent action serves as the measure of erythrocyte resistance. The assay mixture contained 5 mL of 1% erythrocyte suspension and 0.05 mL of 0.1M HCl. The absorbance was read at 540 nm every 30 seconds after HCl addition till the end of hemolysis. The difference of absorbance at the beginning and at the end of hemolysis was determined as 100% (total hemolysis). The disintegration of erythrocytes (%) at every 30 seconds was expressed as a curve.

**Statistical analysis**

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (*p* >0.05). The significance of differences between the values (significance level, *p* <0.05) was examined using the Mann-Whitney U test (Zar, 1999). All statistical calculation was performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).
Results and discussion

After incubation of equine erythrocytes with a leaf extract obtained from *Th. × porcii*, the aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered. The *Th. × porcii* extract caused to increase of TBARS content as a biomarker of lipid peroxidation in the extract-treated erythrocytes, and these results were statistically significant (by 57.4%, *p* < 0.05). The aldehydic and ketonic derivatives level was non-significantly decreased by 1.8 and 1.7% (*p* > 0.05), respectively (Figure 2).

**Figure 2** The TBARS content as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes suspension after *in vitro* incubation with leaf extract obtained from *Thymus × porcii* (M ± m, n = 18)

Many *in vitro* studies confirmed the antioxidant properties of thyme extracts. Many results also clearly suggest that treatment by *Thymus* extracts *in vivo* and *in vitro* prevents organ damage via protection of the antioxidant defense system and scavenging of hydroxyl free radicals by producing of phenoxy radicals, major transient species (Nagoor Meeran et al., 2017). For example, the phenolic constituent profiles and the antioxidant, anti-proliferative, neuroprotective, anti-aging and anti-diabetic activities of both *Th. pulegioides* aqueous decoctions (AD) and hydro-ethanolic extracts (HE) were studied by Taghouti et al. (2018). Rosmarinic acid was the main phenolic compound, accounting for 35.2 or 47.8% of total identified phenols in AD or HE, respectively. Furthermore, large amounts of luteolin-6-O-hexuronide, eriodictyol-6-O-hexuronide, and chrysoeriol hexoside were found. Both extracts showed significant *in vitro* antioxidant activity and anti-proliferative activity against Caco-2 cells and reduced hepatotoxicity (HepG2 cells). In general, both *Th. pulegioides* extracts showed poor anti-diabetic activity, moderate anti-aging effects and high neuroprotective activity with both AD and HE extracts, at 0.5 mg/mL, showing 80% inhibition of the acetylcholinesterase activity and 94% inhibition of the tyrosinase activity (Taghouti et al., 2018).
Six different assays were employed in study of Kindl et al. (2015) in order to evaluate the antioxidant properties of the ethanolic extracts of selected *Thymus* species growing in Croatia (*Th. longicaulis*, *Th. praecox* subsp. *polytrichus*, *Th. pulegioides*, *Th. serpyllum* subsp. *serpyllum*, *Th. striatus*, and *Th. vulgaris*) as well as elucidate its mode of action. The tested *Thymus* extracts and pure compounds at different concentrations (0.4–25 μg/mL) significantly inhibited DPPH• in a concentration-dependent manner: The activities of plant extracts were 11–28%, 23–52%, and 52–85% at 1.56 μg/mL, 3.13 μg/mL, and 6.25 μg/mL, respectively. At the mentioned concentrations, *Th. serpyllum* subsp. *serpyllum* as well as a commercial sample of *Th. vulgaris* were the least effective (Kindl et al., 2015).

The erythrocytes of mammals represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure (Pagano and Faggio, 2015). Erythrocytes are especially vulnerable since they have no membrane repair and regenerative capacity (Webster and Toothill, 1987) and red cell damages by free radicals would probably be associated with hemolysis (Farag and Alagawany, 2018). Therefore, the next purpose of our study was to evaluate the effect of *Th. × porcii* extract (5 mg/mL) on the *in vitro* HCl-induced hemolysis of the equine erythrocytes (Figure 3).

![Effect of *Thymus × porcii* extract (5 mg/mL) on the HCl-induced hemolysis *in vitro* of the equine erythrocytes (M ±m, n = 18)](image)

In the control group (erythrocyte suspension), erythrocytes incubated with 0,1M HCl remained stable and demonstrated slight hemolysis. The maximum level of hemolysis was (10.6 ±0.91%); the total duration of hemolysis was 19 min. When *Th. × porcii* extract (5 mg/mL) was added to the erythrocyte suspension, the maximum level of hemolysis occurred after 8.5 min of incubation with 0,1M HCl (17.91 ±1.87%). The total duration of hemolysis after *Th. × porcii* extract (5 mg/mL) incubation was 11.5 min. Our results showed that HCl-induced hemolysis in a typical time-dependent manner. Therefore, *Th. × porcii* extract at
a concentration of 5 mg/mL induced the increase of hemolyzed erythrocytes and caused to decrease in hemolysis duration (Figure 3).

Many both in vitro and in vivo studies have shown that plants belonging to the Thymus genus possess antioxidant properties. In the study of Afonso et al. (2018), Th. zygis, Th. pulegioides, and Th. fragrantissimus has grown under organic cultivation regime were characterized regarding nutrients and phenolic compounds, as well as the antioxidant and antibacterial properties of these species, were screened. The plants were particularly notable for their high K/Na ratio, polyunsaturated fatty acids content and low omega-6/omega-3 fatty acids ratios, which are valuable features of a healthy diet. Caffeic acid and/or its derivatives, mainly rosmarinic acid and caffeoyl rosmarinic acid, represented the majority of the phenolic constituents of these plants, although they were less representative in Th. pulegioides, which in turn was the richest in flavones. These species also exhibited the highest antioxidant capacity while Th. zygis was the most active towards Gram-positive and Gram-negative bacteria (Afonso et al., 2018).

Other plants belonging to the Thymus genus also possess antioxidant activity against high-fat-diet-induced hyperlipidemia and atherosclerosis (Yu et al., 2016), cisplatin-induced nephrotoxicity (El-Sayed et al., 2015), doxorubicin-induced cardiotoxicity (El-Sayed et al., 2016), UV radiation-induced oxidative stress-mediated skin damages (Sun et al., 2017). For example, Th. vulgaris is a potential botanical agent for use against UV radiation-induced oxidative stress-mediated skin damages. In the study of Sun et al. (2017), UVB-induced skin damages have been shown to be ameliorated by treatment with Th. vulgaris in hairless mice (HR-1) skin, demonstrated by decreased matrix metalloproteinases (MMPs) and increased collagen production. These researchers also have examined the photoprotective effects of Th. vulgaris against UVB and elucidated the molecular mechanism in normal human dermal fibroblasts. Th. vulgaris remarkably prevented the UVB-induced reactive oxygen species and lactate dehydrogenase. A dose-dependent increase in glutathione, NAD(P)H: quinone oxidoreductase-1 and heme oxygenase-1, by Th. vulgaris was confirmed by increased nuclear accumulation of Nrf2. Furthermore, 5-Methoxyindole-2-carboxylic acid was introduced as a specific inhibitor of dihydrolipoyl dehydrogenase (DLD). Moreover, Nrf2 expression was regulated by DLD, which was a tricarboxylic acid cycle-associated protein that decreased after UVB exposure. Besides, Th. vulgaris significantly diminished UVB induced phosphorylation of mitogen-activated protein kinases pathway, containing extracellular signal-regulated kinase, Jun N-terminal kinase, and p38, which consequently reduced phosphorylated c-fos and c-jun (Sun et al., 2017).

The in vitro antioxidant activity of thymol and investigated the effect of thymol on high-fat-diet-induced hyperlipidemia and atherosclerosis was studied by Yu et al. (2016). New Zealand white rabbits were fed with regular chow, high-fat and high-cholesterol diet (HC), T3, or T6 (HC with thymol supplementation at 3 mg/kg/d or 6 mg/kg/d, respectively) for 8 weeks. Aortic intimal thickening, serum lipid parameters, multiple inflammatory markers, proinflammatory cytokines, and atherosclerosis-associated indicators were significantly increased in the HC group but decreased upon thymol supplementation. In the study of Yu et al. (2016), thymol exhibits antioxidant activity and may suppress the progression of high-
fat-diet-induced hyperlipidemia and atherosclerosis by reducing aortic intimal lipid lesion, lowering serum lipids and oxidative stress, and alleviating inflammation-related responses.

The possible protective effects of thymol and carvacrol against cisplatin (CP)-induced nephrotoxicity was assessed by El-Sayed et al. (2015). Administration of thymol [20 mg/kg, orally (p.o.)] and/or carvacrol (15 mg/kg, p.o.) for 14 days before CP injection and for 7 days after CP administration restored the kidney function and examined oxidative stress parameters. Thymol was more effective nephroprotective than carvacrol. Moreover, a combination of thymol and carvacrol had a synergistic nephroprotective effect that might be attributed to antioxidant, anti-inflammatory, and anti-apoptotic activities (El-Sayed et al., 2015). Moreover, El-Sayed et al. (2016) also demonstrated that thymol and carvacrol prevent doxorubicin-induced cardiotoxicity by abrogation of oxidative stress, inflammation, and apoptosis in rats. The administration of thymol (20 mg/kg p.o.) and/or carvacrol (25 mg/kg p.o.) for 14 days before doxorubicin administration and for 2 days after doxorubicin injection ameliorated the heart function and oxidative stress parameters. Summarily, thymol was more cardioprotective than carvacrol. Moreover, a combination of thymol and carvacrol had a synergistic cardioprotective effect that might be attributed to antioxidant, anti-inflammatory, and anti-apoptotic activities (El-Sayed et al., 2016).

Thymol and carvacrol administration also decreased the oxidative damage and increased the antioxidant levels and improved the sperm quality parameters Güvenç et al. (2019). However, the combined use of these two active ingredients had a limited therapeutic effect on the mentioned parameters. These researchers have investigated the effects of different doses of thymol and carvacrol on sperm quality oxidative stress and the antioxidant system. During the study, sperm quality parameters (motility, concentration, abnormal spermatozoa, and live-dead sperm ratio), biochemical parameters [malondialdehyde (MDA), reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), AST, ALT, GGT, urea, and creatinine] were analyzed, and histopathological examination was performed. The study results showed that monotherapies of thymol and carvacrol significantly decreased MDA levels in testicles, liver and kidney tissues compared to the control group. GSH levels increased only with the thymol administration and GSH-Px and catalase activity increased only with the carvacrol administration compared to the control group. The combined administration of these two agents did not cause any significant change in any parameter. Regarding the sperm quality parameters, only the spermatozoa concentration and motility increased significantly in the thymol and carvacrol groups compared to the control group. In respect of spermatozoon anomaly, there was a significant decrease in thymol and carvacrol monotherapy groups. The histopathological analysis of the testicle, liver and kidney tissues of the animals showed no difference between the groups (Güvenç et al., 2019).

In conclusion, the extract obtained from leaves of Th. × porcii (5 mg/mL) has a mild cytotoxic activity on the equine erythrocytes increasing the level of lipid peroxidation biomarker and hemolysis rate. Investigation of the mechanism of action revealed that this extract has hemolytic activity. These findings suggest the use of Th. × porcii extract as a source of prooxidant compounds and warrant further studies to evaluate their therapeutic potential.
Conclusions

The aldehydic and ketonic derivatives level, as well as total antioxidant capacity, was non-significantly altered after in vitro incubation with an extract obtained from leaves of Th. × porcii. The Th. × porcii extract caused to increase of TBARS content as a biomarker of lipid peroxidation in the extract-treated erythrocytes, and these results were statistically significant. Total antioxidant capacity was non-significantly increased. When Th. × porcii extract (5 mg/mL) was added to the erythrocyte suspension, the maximum level of hemolysis occurred after 8.5 min of incubation with 0,1M HCl (17.91 ±1.87%). The total duration of hemolysis after Th. × porcii extract (5 mg/mL) incubation was 11.5 min. Our results showed that HCl-induced hemolysis in a typical time-dependent manner. Therefore, Th. × porcii extract at a concentration of 5 mg/mL induced the increase of hemolyzed erythrocytes and caused to decrease in hemolysis duration. Screening of Thymus species for other biological activities including antioxidant activities is essential and may be effective for searching the preventive agents in the pathogenesis of some metabolic diseases.

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References


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