Antioxidant potential of Phyllanthus fraternus Webster on cyclophosphamide induced changes in sperm characteristics and testicular oxidative damage in mice

Sangita Singh¹, Swarn Lata¹* & Kavindra Nath Tiwari²
¹Department of Zoology; ²Department of Botany, Mahila Maha Vidhyalaya, Banaras Hindu University, Varanasi, Uttar Pradesh-221005, India.

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Cyclophosphamide (CPA) is used to treat various types of cancer. It is a cytotoxic alkylating agent widely used in chemotherapeutic regimen. However, the clinical efficacy of CPA is marred by its side effects. In clinical applications of CPA, it becomes necessary to prevent the oxidative stress and reproductive toxicity induced thereby in normal cells. In the present study, we investigated the protective effect of aqueous extract of Phyllanthus fraternus (AEPF) on CPA (200 mg/kg body wt., i.p.) induced changes in sperm characteristics and testicular oxidative damage in male mice. The CPA treated group showed significant decrease in gonadosomatic index (GSI), epididymal sperm count, sperm motility and sperm viability compared to control group, while the CPA + AEPF treated group had significant increase with respect to these variables compared to the CPA-treated group. The elevated levels of lipid peroxidation by CPA were effectively reduced with AEPF. It also exhibited protective action against the CPA induced depletion of antioxidants like catalase and superoxide dismutase. DNA damage was measured by comet assay, biomonitoring with comet assay elicited significant increase in genotoxicity. Genotoxicity caused by CPA was counteracted by aqueous extract of Phyllanthus fraternus. Administration of the plant extract along with CPA restored the histopathological architecture of testis. Thus, the aqueous extract of P. fraternus by virtue of its antioxidant potential can be used as an effective agent to reduce CPA-induced oxidative stress in male mice.

Keywords: Comet assay, Genotoxicity, Gonadosomatic index, Gulf leaf-flower, Reproductive toxicity, Testis.

Cyclophosphamide (CPA) is a drug with a wide spectrum of clinical uses including cancer. The cytotoxicity of CPA is mediated by alkylation of DNA at the N7 position of guanine and the formation of DNA-DNA cross-links, DNA-protein cross links and single strand breaks. However, CPA is not absolutely free of side effects. CPA therapy to treat a variety of glomerular diseases suffers gonadal toxicity as a side effect. Adult male patients treated with CPA have demonstrated, diminished sperm counts and absence of spermatogenic cycles in their testicular tissue. Mammalian spermatozoa are particularly vulnerable to oxidative damage because of high concentration of polyunsaturated fatty acids and low antioxidant capacity.

CPA generates active oxygen species such as superoxide anions, hydroxyl radicals, etc., that induces oxidative stress and inhibit the activity of antioxidant enzymes in several tissues. This insufficiency of antioxidant system can result in oxidative stress. Oxidative stress may be defined as an imbalance between the oxidant production and oxidative capacity of the cell to prevent oxidative injury. Oxidative stress causes tissue damage and can lead to critical failure of biological functions. Combination of drug delivery together with potent and safe antioxidant may be an appropriate approach to ameliorate CPA-induced reproductive toxicity.

Natural compounds, such as Royal Jelly with antioxidant and immunomodulatory activity have been suggested useful in preventing the side effects of CPA-induced testis pathology. The Gulf leaf-flower, Phyllanthus fraternus Webster (Family: Euphorbiaceae) is employed for numerous uses by indigenous people which includes treatment of blennorrhagia, colic, diabetes, dysentery, fever, flu, tumors, jaundice, vaginitis, gonorrhea, dyspepsia, etc. Aggarwal et al. have highlighted the antilithiatic potency of P. niruri, a common component in Brazilian folk medicine. The antioxidative property of P. fraternus has not been widely elucidated as evident from the literature. Thus, here, we explored the antioxidative potential of Phyllanthus fraternus on cyclophosphamide-induced changes in sperm characteristics and testicular oxidative damage in mice.
Materials and Methods

**Drug and Chemicals**—Cyclophosphamide was obtained from Sigma Aldrich Ltd., New Delhi, India and other chemicals and solvents used were of analytical grade and the highest purity.

**Plant material**—The aerial part of *Phyllanthus fraternus* collected from the campus of Banaras Hindu University, Varanasi, India was authenticated and identified by Prof. NK Dubey, Department of Botany, Banaras Hindu University, Varanasi. A voucher specimen (Euphor./14/2013) has been kept in the herbarium for future reference.

**Preparation of aqueous extract of *P. fraternus* (AEPF)**—Fresh aerial parts of plants were washed under running tap water, dried in shade at room temperature (25 ± 2°C) for a wk and powdered mechanically. The powder (100 g) was dissolved overnight in 400 ml deionized water under stirring at room temperature. Faremi *et al.* was followed for aqueous extraction. The mixture was then centrifuged at 5000 rpm to separate the supernatant. The filtrate was evaporated to dryness at 45°C with a rotary evaporator. The dry extract was collected and stored in a refrigerator at 4°C for further use.

**Phytochemical screening**

The aqueous extract obtained was subjected to preliminary phytochemical screening for identification of its chemical constituents as described by Evans.

**Preparation of extract for GC-MS analysis**—The plants were dried and pulverized to powder in a mechanical grinder. Required quantity of the plant powder of *P. fraternus* was treated with the methanol until the powder was fully immersed, incubated over night and filtered through a filter paper. About 2 µl sample solution was employed in GC-MS for analysis of different compounds.

**GC-MS analysis**—The GC-MS analysis was carried out using a Clarus 500 Perkin-Elmer (Auto system XL) Gas Chromatograph coupled to a mass detector Turbo mass gold-Perkin Elmer Turbomass 5.1 spectrometer with an Elite-1 (100% Dimethyl poly siloxane), 30 m x 0.25 mm ID x 1 µm of capillary column. The instrument was set to an initial temperature of 100°C, and maintained at this temperature for 4 min. At the end, the oven temperature was raised up to 320°C, at the rate of an increase of 5°C/min, and maintained for 13 min. Injection port temperature was ensured at 260°C and Helium flow rate as 1 ml/min. The ionization voltage was 70eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 40-600 (m/z).

Using computer search on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS, compounds present in the plants sample were identified.

**Identification of phytocompounds**—Interpretation on Mass-Spectrum GC-MS was conducted using the database of National institute Standard and Technology (NIST) having more than 62000 patterns. The spectrums of the unknown components were compared with the spectrum of known components stored in the NIST library. The name, molecular weight and molecular formula of the components of the test materials were ascertained.

**Test system**—All the experiments were performed in accordance with institutional practice and within the framework of revised animals (Committee for the Purpose of Control and Supervision of Experiments on Animals; CPCSEA) Act of 2007 of Govt. of India on animal welfare. The study was conducted on adult male Parke’s strain mice (30 ± 3 g), which was obtained from Department of Zoology, Banaras Hindu University, Varanasi, India. Animals were fed with commercially available standard mice pellet feed and water, ad libitum. Mice were housed under conditions of controlled temperature (25 ± 2°C) and acclimatized to 12 h light:dark cycles.

**Experimental design**—Animals were divided into six groups of six mice each. Treatment of various groups followed is as Group I (Control) received distilled water (i.p.) once a week for 5 wk; Group II (AEPF) received the aqueous extract of *Phyllanthus fraternus* @ 400 mg/kg body wt., orally) once a week for 5 wk; Group III (CPA) received cyclophosphamide @ 200 mg/kg body wt., i.p.) once a week for 5 wk; Group IV (CPA+AEPF) received CPA (as that of Gr III) and AEPF @ 200 mg/kg body wt. orally once a week for 5 wk; Group V (CPA+AEPF) received CPA as that of Gr III but AEPF @ 300 mg/kg body wt. orally once a week for 5 wk; and Group VI (CPA+AEPF) received CPA as that of Gr III but AEPF @ 400 mg/kg body wt. orally once a week for 5 wk.

**Preparations of tissues**—At the end of the treatment period, the mice were sacrificed by cervical dislocation. The testis were removed, cleared off the adhering tissues, weighed and frozen for the antioxidant assays. The epididymis was removed and used for sperm analysis and comet assay.

**Sperm parameters**—The motility, viability, and count of spermatozoa in the cauda epididymis were assessed by the WHO Laboratory Manual (World Health Organization, 1999). Data were obtained from all the six animals of each group.
Antioxidants in testis—In testis, the level of lipid peroxidation in all tissue were evaluated by the method of Ohkawa et al.\textsuperscript{15}. SOD was assayed by the method of Das et al.\textsuperscript{16} and catalase (CAT) by Aebi\textsuperscript{17} in testis.

Sperm comet assay—The DNA damage (single strand breaks) was measured by comet assay or single cell gel electrophoresis\textsuperscript{18}. It was quantified by comet length, tail length, tail DNA (\%) and tail moment measurement.

Histopathological studies—The testicle of each mice fixed in aqueous Bouin’s solution for 12 h, dehydrated through a gradual series of alcohol and then processed for paraffin embedding. Sections of 5 μm thickness were made and stained with hematoxylin and eosin according to the standard method. Histological assessment was performed under light microscope in terms of the changes in different groups as compared to control group.

Statistical evaluation—The results were expressed as standard error of mean (SEM), analyzed through one way ANOVA, followed by the post hoc Dunnett’s test for comparison of various treatments using the SPSS 16.0. Differences were considered statistically significant at $P < 0.05$.

Results

Phytochemical analysis—The aqueous extract of aerial part of Phyllanthus fraternus (AEPF) was subjected to preliminary phytochemical screening test for presence of various constituents such as alkaloids, tannins, saponins, flavonoids, etc. (Table 1). Presence of flavanoids, phenolics, tannins, etc., confirmed strong antioxidant potential of the extract.

The studies on the active principles in the aerial part of $P.$ fraternus methanolic extract\textsuperscript{13} by GC-MS analysis clearly showed the presence of compounds. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (\%) are presented in Table 2. The

<table>
<thead>
<tr>
<th>Retention Time (RT)</th>
<th>Molecular Formula</th>
<th>Molecular Weight</th>
<th>Area (%)</th>
<th>Name of compounds</th>
<th>Uses</th>
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GC-MS chromatogram of the different peaks of the compounds detected are shown in Fig. 1. The compounds identified by mass spectroscopy are presented. The major components in the methanol extract were: carissanol (24.31%), cerosol (6.11%), chromane (8.68%), eugenol (4.72%), oleic acid (10.86%), pinane (30.62%), pyrogallol (7.76%).

Organ weight changes—The gonadosomatic index (GSI) is the calculation of the gonad mass as a proportion of the total body mass. It is a tool for measuring the sexual maturity of animals in correlation to testis development. As shown in Fig. 2, the treatment of male mice with CPA for 5 wk caused a significant ($P <0.001$) loss in the GSI compared...
with the control group. There is a percent increase in GSI in a dose dependent manner with CPA + AEPF treated groups. GSI showed significant increase ($P < 0.001$) at different doses of AEPF (200, 300 and 400 mg/kg body wt.) as compared to the CPA-treated group. Administration of AEPF alone did not change the GSI as compared with that of the control group.

Sperm parameters—The effect of CPA and co-administration of AEPF on epididymal sperm count, sperm motility and sperm viability are presented in Fig. 2. No significant changes in sperm parameters were observed in group II (AEPF alone) when compared to the control group. On the other hand, CPA-treated group showed significant decrease ($P < 0.001$) in epididymal sperm count, sperm motility and sperm viability exposed to as compared to the control. Co-administration of AEPF to CPA treated animals resulted in significant increase ($P < 0.001$) in sperm parameters (sperm count, motile sperm and viable sperm) when compared with CPA-treated mice in a dose dependent manner. Co-administration of AEPF caused a significant increase in semen quality and minimized toxic effects of CPA.

Antioxidants in testis—Intoxication of mice with CPA was followed by a significant decrease in the activities of all antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Exposure to CPA caused a significant decrease ($P < 0.001$) in activity of SOD and CAT in the testis (Fig. 3B & C). Administration of AEPF restored these enzymes to near normalcy compared to the CPA-treated group. Fig. 3A depicts significantly higher ($P < 0.001$) level of malondialdehyde (MDA) in CPA-treated group as compared to the normal mice. The CPA-induced increased level of MDA was significantly compensated by administration of AEPF in dose dependent manner. Group II (AEPF alone) showed no significant changes when compared to the control group indicating that it did not have any adverse effect. The results demonstrated that AEPF treatment provided significant protection against the LPO production induced by CPA in the mice testis.

Sperm comet assay—The alkaline comet assay was used in mature spermatozoa collected from the cauda epididymis to detect DNA damage in response to CPA administration and the protective effect of AEPF. From comet images by fluorescent microscopy, the undamaged DNA was recognized as a fluorescent core, while the presence of strand breaks in the chain (damaged DNA) possibly caused DNA to migrate and form a tail (“comet”) during the electrophoresis Fig 4. The parameters used to detect DNA damage were; comet length, tail length, tail moment and % tail DNA. As we could see in Fig. 5A, the alkaline comet assay showed a significant increase ($P < 0.001$) in comet length in CPA-treated groups as compared to controls. Treatment with AEPF significantly restored ($P < 0.001$) the comet length thereby showing its effect in maintaining the DNA damage.

![Fig. 3](image3.png) Effect of CPA and AEPF treatment on the activities of (A) LPO; (B) SOD; and (C) CAT were carried out in testis. Data shown represent mean ± SEM (n= 6). *Level of significance $P <0.001$.

![Fig. 4](image4.png) Image of spermatozoa nuclei following the comet assay. Sections from (A) control sperm, (B) AEPF, (C) CPA exposed sperm and (D) CPA + AEPF treated sperm. All images are at 100X magnification.
The tail length significantly increased ($P < 0.001$) in the CPA-treated group (Fig. 5B). The CPA+AEPF treatment effectively decreased the tail length. Similarly, % tail DNA and tail moment also found to be higher than the level compared to the normal control mice, while in CPA+AEPF treated group, the level of % tail DNA and tail moment declined significantly ($P < 0.001$). The AEPF alone did not show any significant change in comparison with control group.

**Histopathological findings**—The spermatogonia, spermatocytes, spermatids and spermatzoa could be clearly identified in the seminiferous tubules. The well preserved sertoli cells and delineated tubular basement membrane were observed in group I (control) and group II (AEPF). The interstitial space between tubules and Leydig cells was also intact (Fig. 6 A & B). However, in the CPA-treated group, differences were observed in histology of testis. Complete atrophy of seminiferous tubules was exhibited, most of the germ cells degenerated, seminiferous tubules shrunken and widening of interstitial space (Fig. 6 C). This indicated that, the testis tissues were significantly damaged by CPA as compared with the normal architecture of the control group. There were no marked changes in testicular histology relative to the control as well as AEPF alone treated groups. In the CPA + AEPF-treated groups, toxic effects were ameliorated in a dose dependent manner and restored these changes towards normalcy (Fig. 6 D-F). Administration of various doses of AEPF partly restored the morphological structure of seminiferous tubules in the damaged testis. The results showed better protective effect on the damaged testis in the AEPF at 400 mg/kg body wt. group than the rest of AEPF groups (200 and 300 mg/kg body wt). To some extent, the morphological structure of seminiferous tubules for the AEPF (400 mg/kg body wt.) group (Fig. 6D) was close to that of control (Fig. 6A), compared with CPA-treated group (Fig. 6C).

![Fig. 5](image-url) Effect of CPA and AEPF on comet assay. (A) Comet length; (B) tail length; (C) % tail DNA; and (D) tail moment were carried out in epididymis spermatozoa. Data shown as mean ± SEM (n=6). *Level of significance $P < 0.001$.

![Fig. 6](image-url) Effect of AEPF co-treatment on the testicular damage induced by CPA. Sections from (A) control mice testis showed different stages of spermatogenesis (B) AEPF treated testis showed normal spermatogenesis. (C) CPA treated mice testis showed alterations in spermatogenesis, atrophy of seminiferous tubules, degenerated germ cells and shrunken seminiferous tubules, compared with the normal cellular content of the control group and (D-F) In the CPA + AEPF showed, the damage to the seminiferous tubules was considerably less severe, showing undamaged spermatogenesis. (H & E, X40)
Discussion

Phytochemical screening of the Phyllanthus fraternus plant extract revealed the presence of alkaloids, tannins, saponin, flavonoids, phenols and carbohydrates. Based on the lethal dose of aqueous extract of P. fraternus (AEPF), 400 mg/kg body wt. was selected to check its protective effect. Various phytochemicals have been identified from the methanolic extract of aerial part of P. fraternus through GC-MS analysis. The presence of various bioactive compounds justifies the use of plant for treating various ailments by traditional practitioners.

Cancer chemotherapy drugs are known to produce toxic side-effects in multiple organ systems including the testis. In a clinical context, testicular germ cell damage in patients exposed to chemotherapeutic drugs for a limited duration could result in long-term infertility or genetic alterations. CPA is an effective anticancer drug but its use is limited due to its physiological side effects. Its possible induction of reproductive toxicity in non-tumor cells has been documented in humans as well as in a variety of animal species. An oxidation mechanism may be responsible for reproductive toxicity, wherein CPA and its metabolite acrolein cause an inactivation of cellular antioxidants system which results in increased reactive oxygen species generation and lipid peroxidation. Our present study demonstrated marked protective effect of AEPF against CPA-induced oxidative stress and reproductive toxicity.

Sperm analysis was carried out to investigate the effect of CPA and AEPF on male fertility. Deformity of sperm in mammals was used as an important parameter to access male fertility. Although, it is not possible to find the cause of infertility in a large percentage of infertile men, an alteration of the spermatogenic parameters such as decreased sperm count, diminished motility and viability in CPA administered mice, somewhat indicates reproductive toxicity. The observed reduction in GSI in CPA treated group can be explained by the declined number of germ cells and a significant lower rate of spermatogenesis because the weight of the testis mainly depends on the mass of the differentiated spermatogenic cells. In the present study, decreased number of sperm in the epididymis may reflect less bioavailability or production of androgen in CPA treated mice as shown by Oh et al. It was established earlier that CPA causes testicular toxicity by germ cell degeneration and inhibits androgen production in adult male mice, probably by affecting pituitary luteinizing hormones and thereby inhibits Leydig cell testosterone production and serum testosterone levels, which in turn compromises spermatogenesis. Decrease in sperm count may be a consequence of reactive oxygen species (ROS) induced damage in infertile males. Hence, reduction in sperm count may be due to the generation of ROS by CPA. The oxidative stress is a critical factor that should be considered in addition to hormonal and enzymatic factors in the CPA-induced testicular alterations. Hence, the decrease in testicular sperm count in CPA treated mice reflects spermatogenic cell death.

Sperm motility and viability are important parameter in respect to the fertility of the male. Dietary magnesium has been reported to promote overall testicular morphology and function including serum testosterone level and relative sperm count. However, toxic effect of CPA on the flagellum, the important machinery for motility of sperm cells, significantly reduces sperm motility and viability. CPA treated mice showed decreased testicular tricarboxylic acid cycle enzyme activities, and thus, impaired energy metabolism. It is possible that ATP may serve as an energy source for sperm motility and decrease in energy metabolism may be one of the limiting factors responsible for loss of sperm motility in CPA administered mice. A direct toxicity of CPA to the spermatogenic compartment may be considered as one of the mechanisms of action of CPA in producing the abnormal and dead sperms. The amount of testicular antioxidant enzymes reduced significantly after CPA-treatment. It is a well known reason that antioxidant enzymes are necessary for normal differentiation and development of spermatogonial cells to mature spermatozoa, possibly via protection from ROS injury. Present study indicates the beneficial effects of AEPF against CPA induced germ cell toxicity in mice. AEPF treatment improved the GSI, sperm count, motility and viability. Thus, it may be proposed that, AEPF substantially attenuated the testicular spermatogenic cell damage induced by CPA treatment.

ROS are chemically reactive molecules containing oxygen. Examples include oxygen ion, peroxides and hydroxy radicals. Lipid peroxidation (LPO) is one of the main manifestations of oxidative damage initiated by ROS and it has been linked to altered membrane structure and enzyme inactivation. It is initiated by abstraction of a hydrogen atom from the side chain of...
polyunsaturated fatty acids in the membrane. Increased LPO could be detected by MDA production, the most frequently used biomarker.

Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are involved in counteracting the toxicity of ROS. Under normal conditions these enzymes protect the cells and tissues from oxidative damage. SOD constitutes an important link in the biological defense mechanism through dismutation of endogenous cytotoxic superoxide radicals to H₂O₂ and molecular oxygen that are deleterious to polyunsaturated fatty acids and proteins. The activity of CAT is to degrade H₂O₂ into H₂O and O₂. In the presence of inadequate CAT activity, more H₂O₂ could be converted to toxic hydroxyl radical that may contribute to the oxidative stress of CPA toxicity. Decline in the activities in these enzymes might be due to their inactivation caused by excess ROS production. Thus, the balance of this enzyme system is essential to dispose the superoxide anion and peroxides generated in testes. The present study showed the increased MDA level and decline activity of SOD and CAT in CPA-treated group. AEPF treatment significantly reversed the upsurge in the MDA level and normalizes the antioxidant activities. Almost restored the activity of SOD and CAT indicates that AEPF have some antioxidative/free radical scavenging property.

In present study, we also examined DNA damage caused by CPA through comet assay, in sperms of epididymis. The comet assay is a simple, sensitive and rapid method that can be used to estimate DNA damage at the individual cell level through strand breaks, open repair sites, cross-links and alkali labile sites caused by oxidative stress. This study showed the extent of oxidative DNA damage following the administration of CPA. The CPA treatment was associated with oxidative stress leading to oxidative DNA damage. In our study, it was found that AEPF co-treatment improved all the studied reproductive toxic parameters with sperm DNA damage. The reason behind the curative effect of AEPF might be due to its antioxidative property as similar studies have proved that plants having phytochemicals like polyphenols, flavonoids, etc., shows protective effect on DNA damage.

Histopathological examination of testicular tissue of CPA-treated group revealed disturbed spermatogenesis, severe damage of seminiferous tubules that reached to early atrophic changes with complete loss of the spermatogonial cells together with decreased number of spermatids. The increased oxidative stress results in the lipid peroxidation, which affects the membrane integrity and fluidity. Further, the ROS mediated peroxidation of critical thiol groups in protein can alter the structure and function of spermatozoa. Acrolein induced the formation of reactive oxygen species (ROS), including superoxide anion, hydroxyl radical, hydrogen peroxide and hypochlorite, which might affect the acrosome integrity of human spermatozoa.

Reactive oxygen species such as free radicals may reduce sperm count, motility and viability. Ascorbic acid has been found to help in preventing cell damage by neutralizing free radicals. It is a powerful antioxidant and has been found to increase fertilization rates significantly. It enhances sperm quality and prevents sperm agglutination thus making them more motile with more forward progression. The present study shows that AEPF may be used as herbal medicine to promote fertility in male mice as Ascorbic acid would be effective in improving male fertility. Therefore it may be concluded that Ascorbic Acid present in the P. fraternus may increase the testosterone production, sperm count, motility, viability and thus enhance male fertility. The extract also contains flavonoids, which are associated with functions related to fertility enhancement. These listed constituents are bioactive compounds which might serve as promoter of fertility in male mice.

In present study, we administered AEPF and the observations were significantly positive as it improved all studied parameters used to determine testis injury. The possible way of AEPF protection is attributed to its antioxidative and free radical scavenging property as it is clear that AEPF have some important phytochemicals like salicylate, chromane, grape seed oil, cineol, tartaric acid etc which act as antioxidants.

The present results exhibited that the AEPF could protect the testis from toxicity induced by CPA. This activity can be partially attributed to the free radical scavenging activity and enhancement of the antioxidant system effectively by AEPF since many of the active ingredients present in AEPF are potent free radical scavengers. Thus, it may be concluded that the aqueous extract of Phyllanthus fraternus (AEPF) have antioxidant potential, to reduce cyclophosphamide-induced oxidative stress and reproductive toxicity in male mice. Therefore, AEPF may be supplemented...
with CPA to improve sperm characteristics and testicular oxidative damage in men. This study therefore, supports the claim on the folkloric use of the aerial part of *P. fraternus* improves libido and reproductive function in men. Further investigations to elucidate the mechanism of protective role of AEFP in CPA-induced toxic manifestation are under way.

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**References**


