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Conservation Status, Anticancer Compounds and Pharmacological Aspects of *Podophyllum hexandrum* Royle: A Review

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Abstract: *Podophyllum hexandrum* or Himalayan may apple is an endangered medicinal plant. The plant is a major source of anticancer agent, podophyllotoxin. Podophyllotoxin content in *Podophyllum hexandrum* is more (7-15%) as compared with other species, notably *Podophyllum peltatum* (4-8%), the most common species in the American subcontinent. To meet pharmaceutical industry's demand in India, the rhizomes of *Podophyllum hexandrum* are being harvested haphazardly in enormous quantities. Subsequently, the plant is reported as an endangered species in the Himalayan region. This review highlighted various conservation initiatives, and importance of podophyllotoxin and its derivatives. In addition, the plant is reported to be antioxidant, anti-tumerogenic, antimutagenic and radioprotective. Therefore, immediate action should be taken for its conservation through *in vitro* and *in vivo* techniques, and also the genetic diversity of this valuable therapeutic plant must be understood exactly and conserved as early as possible.

Keywords: *Podophyllum hexandrum,* Podophyllotoxin, Conservation, Himalayan may apple, Pharmacology

Plants and their bioactive ingredients are forerunners in the discovery of novel medications (Kumari et al 2018, Balkrishna et al 2021, Sharma et al 2021, Sonam et al 2021, Dhatwalia et al 2021). *Podophyllum hexandrum* is commonly known as 'Aindri', in the Indian Ayurveda. Additionally, it also finds use in traditional Chinese medicine (Wong et al 2000). Further, it is also called as Indian may apple, which is regarded as an endangered medicinal herb of the family *Berberidaceae*. It is mostly found in the Himalayan region, which is the richest source of aromatic and medicinal plant diversity. *P. hexandrum*, an upright, glabrous, succulent herb enjoying moisture and shade, thrives from the Himalayan region at an elevation between 1300-4300 m above sea level. A variety of compounds such as podophyllin, podophyllotoxin, quercetin, 4-dimethypodophyllotoxin, kaempferol, picropodophyllotoxin are present in comprehensive chemical analysis of the *Podophyllum* community (Singh and Shah 1994). The Indian *Podophyllum hexandrum* contains more podophyllotoxin (7-15%) than *Podophyllum peltatum* (the American) with 4-8% (Pandey et al 2007, Qazi et al 2011) adding more importance to the *Podophyllum hexandrum*. The highest content of podophyllotoxin is found in rhizomes. However, it must be considered that *Podophyllum hexandrum*'s podophyllotoxin yield varies greatly with the place of cultivation and the collection season. In addition, the yield is optimum when the plant has entered the stage of flowering (Chatterjee 1952). Due to its efficacy as an anti-mitotic, anticancer, and immunostimulant, podophyllotoxin has gained great importance and high medicinal status (Pugh et al 2001) especially for curing uterine tumors (Macrae and Towers 1984, Richter et al 1987). Semi-synthetic derivatives of podophyllotoxin such as etoposide, etopophos, and teniposide are effective for treating lung cancer, leukemia, and tumors (Schacter 1996, Pandey et al 2007).

The plant has been successfully used in modern allopathic medicine to treat numerous diseases, monocytoid leukemia, and Hodgkin's lymphoma, warts, AIDS-associated Kaposis sarcoma, and cancer of the brain, lung as well as the bladder (Kar 2008, Kokate et al 2009, Shah and Seth 2010). The number of plant products were explored including *P. hexandrum* derived compounds in the search for novel, reliable and non-toxic radio protectants (Goel et al 1998). Interestingly, pre-radiation administration of *P. hexandrum* extracts was found to reduce radiation-induced alternations (Goel et al 2002). Besides this, *P. hexandrum* fruits are used as a cough remedy. While, its resin is a blood purifier, antibiotic medication, and hepatic stimulant, and used to alleviate constipation, skin diseases, and tumors, in addition to its use in the treatment of cancer. Further, it is used to

control spindle formation and disperse chromosomes like colchicines (Purohit et al 1998). The plant contains alkaloids, carbohydrates, phenols, glycosides, flavonoids, saponins, steroids, terpenes, and volatile oil (Kumar and Dhillon 2015). Podophyllotoxin is in an increased demand worldwide due to its anti-cancer properties. Complete podophyllotoxin synthesis is an excessive procedure and the availability of natural resource compounds is a key challenge for pharmaceutical players producing these products (Canel et al 2000). At present, the annual supply is projected to be 50-80 tonnes as compared to demand (>100 tonnes). To meet the demand for crude drugs*, P. hexandrum* rhizomes are being harvested on a large scale. As a result, the plant is identified in the Himalayan region as an endangered species. The importance of this valuable plant endeavored us to review the salient morphological features, conservation status concerns, anticancer components, and pharmacological potential of *P. hexandrum*.

Geographical Distribution and Botanical Description

Podophyllum hexandrum is a native to Afghanistan, China, East and West Himalayas, India, Nepal, Pakistan, Tibet and has been introduced into Czechoslovakia as shown in Figure 1 (KewScience-Plants of the World online). *Podophyllum hexandrum* is herbaceous, 15-60 cm tall, erect, glabrous, succulent, with creeping rootstock. Leaves alternate, long petiolate; lamina 6-15 × 3.5-l0 cm, orbicular-reniform, base cuneate, margins serrate, apex acute, pubescent beneath, brownish, palmately divided into 3 broadly elliptic or obovate segments. Flower large, 3.8-5 cm in diameter, white or rose coloured, bisexual; calyx 3-6, petaloid; corolla 6-9; stamen 6; ovules many. Fruit berry, 2.5-5 cm in diameter, elliptic or ovoid, orange or red, with numerous seed as shown in Figure 2 (Grierson and Long 1984, Sharma et al 2012).

Conservation status: *Podophyllum hexandrum* is one of the Indian Himalaya's most threatened plants as per a reported study of 113 taxa for population dynamics in the Western Himalaya (Samant and Pant 2006). According to new IUCN standards, it has been designated as an endangered species (Chaurasia et al 2012). Approximately, 37.3 tonnes of *P. hexandrum* rhizomes were uprooted in the Himachal Pradesh region of India during 1995-2000. The export of *Podophyllum*, its derivatives, and extracts obtained from the wild is prohibited under Schedule 2-Appendix 2 of Export and Import Policy 1997-2002 (Bhardwaj et al 2019). The State Medicinal Plant Board has been formed in every state to stop the illegal collection of medicinal plants from forests (Sharma et al 2012). In addition, forest departments are responsible for maintaining a record of the harvesting, and shipment of endangered species (Bhardwaj et al 2019).

Some policies regarding the promotion, cultivation, and propagation of medicinal plants from forests have been

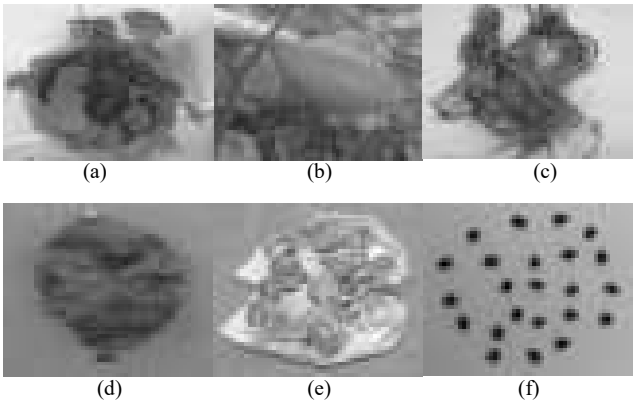

Fig. 2. Salient botanical features of *Podophyllum hexandrum* a) Plant b) Fruit c) Roots d) Dried fruit e) seeds inside fruit f) Separated seeds

Fig. 1. Global distribution of *Podophyllum hexandrum* (Created with mapchart.net)

introduced by the government of Himachal Pradesh. These policies are being implemented by government departments/agencies like the department of rural development, Forest department, and Horticulture department by supporting the farmers financially, making them familiar with the market value of medicinal plants, propagation, and transportation of plant materials. The Himachal state government has started herbal gardening in their state, which is also known as 'Vanaspati vanas' with the assistance of the Union Health Ministry. The farmers are trained to cultivate medicinal plants as cash crops through these herbal gardens. Himachal Pradesh has biodiversity conservation biosphere reserves, national parks, and wildlife sanctuaries, and they are spread across all altitudinal zones within the state (HPFD, 2021).The government of Himachal Pradesh has taken steps to conserve "medicinal and aromatic plants" with the desire to become the largest herbal state in the country by 2025 (H.P. Forestry Sector Medicinal Plant Policy 2006). In this regard, so many schemes have been introduced, including Vanaspati Van, Sanjhi Van Yojna, conservation of Green Gold, production of agro techniques, and establishment of medicinal plant nurseries (Ved and Goraya 2008). Several research institutes/universities contribute to the protection and distribution of endangered and threatened species (Samant et al 2007).

In Uttar Pradesh (India) government constituted a committee of experts in 1985 to ban the processing and marketing of all endangered species, *P. hexandrum* was one among them. In addition, it was banned in the Himalayan area of Uttaranchal vide State Govt. Order no. 535/1-9-20 dated January 1986. It was the government's first substantial step toward preserving several valuable plants, including *Podophyllum*, that were under threat of extinction (Shah 1997). In 1994, a major step was taken by the Govt. of India, Public Notice No. 47(PN)/92-97 (www.envfor.nic.in/legis/ wildlife/ wildlife9.html) on 30 March 1994. As a result, the export of 56 Himalayan species, including *P. hexandrum*, should be strictly prohibited. The list of these important species has been further amended with notification no. 24(RE-98)/1997-2002 (dgftcom.nic.in/exim/2000/ not/not98/not2498.html). A total of 29 species were barred from export, including *P. hexandrum* again (Shah 1997).

In 1997, a market survey was conducted by The Wild Life Trade Monitoring Network India 1997 and found that the endangered medicinal, as well as aromatic plants were being traded at Delhi, Mumbai, Calcutta (Kolkata), and Amritsar (Misra and Jain 1998). *P. hexandrum* is procured only on particular demand from Himachal Pradesh and Jammu and Kashmir in the Delhi and Amritsar markets. In Kolkata trade is quite limited and supplies are made from Sikkim and also illegally from Nepal and Bhutan. According to the 1997-2002 Export and Import Policy, only Kolkata, Cochin, Mumbai, Chennai, Delhi, Tuticorin, and Amritsar ports are allowed to export medicinal plants. Various regulations exist in India to protect endangered plant species, but only a small percentage of the population is aware of them. The important legislation includes the Foreign Trade Act 1992, the export-import policy, the plant fruit and seeds order 1989, and the convention on biological diversity (CBD). There is a separate provision enlisted in schedule VI, in "wildlife protection act 1972" by Govt. of India for endangered and threatened plant species. The Government of India has established a specific provision in Schedule VI of the Wildlife Protection Act, 1972 for endangered and threatened plant species.

Podophyllotoxin and Its Derivatives

Podophyllotoxin, a natural lignan is currently used as an anticancer drug. Podwyssotzki was the first to isolate it from *Podophyllum peltatum* in 1880 (American Podophyllum) (Podwyssotzki 1880). After that, it was isolated from *P. hexandrum* Royle and *P. pleianthum* (Taiwanese *Podophyllum*). The search for natural anticancer drugs stretches back to *Ebers papyrus* in 1550 BC, but the scientific phase of this search was started in the 1950s with the discovery and production of vinca alkaloids, vinblastine, vincristine, and cytotoxic podophyllotoxin isolation (Srivastava et al 2005). Podophyllotoxin from *Podophyllum* sp. is among world's best-known lead anti-neoplastic agents (Wink et al 2005). For the semi-synthesis of etoposide, teniposide, and etopophos (anticancer drugs), podophyllotoxin is the starting material. These compounds have been employed against lung, testicular cancer, as well against some cases of leukemia (Moraes et al 2002). The chemical structures of some of the representative compounds have been depicted in Figure 3. *P. hexandrum* and *P. peltatum* are the plant species that are currently used to extract podophyllotoxin. Indian introduced podophyllin, is a

Structure source: PubChem https://pubch em.ncbi.nlm.nih.gov; Created using KingDraw http://www.kingdraw.cn/en/index.html

Fig. 3. Chemical structures of representative compounds from *P. hexandrum.* a) Podophyllotoxin; b) Teniposide; c) Etoposide

resin obtained from roots and rhizomes of *Podophyllum* spp. by ethanolic extraction. Podophyllin is comprised of the lignans of podophyllotoxin, α, β-peltatin, and 4'-demethylpodophyllotoxin. In 1820, podophyllin was included in the first Pharmacopoeia of the U.S. as a cathartic and cholagogue. The medicine was excluded from the 12th edition of this Pharmacopoeia that appeared in 1942 because of its extreme toxicity (Horwitz and Lokie 1977). However, in the same year, it was confirmed that the topical application of podophyllin could selectively destroy venereal warts. Further, instead of resin, purified podophyllotoxin is applied against the same (Frega et al 1997, Gross 2001). Pure podophyllotoxin is more effective than podophyllin, quercetin and kaempferol are absent in pure form which is associated with many adverse effects (Von et al 2000, Wiley et al 2002).

Genital warts are very frequent sexually transmitted diseases in the Netherlands that may lead to cervical cancer, and other malignancies (Wiley et al 2002). Podophyllotoxin was found to be an essential component to mitigate sexually transmitted diseases. After that, several glycoside derivatives were developed by Sandoz (Switzerland) to produce more active and less toxic anticancer agents, eventually, etoposide was discovered as a result of this in 1966, which further obtained FDA approval in 1983. To tackle etoposide's poor water solubility, etopophos, or etoposide phosphate, was introduced which gets FDA approval in 1996. Another important podophyllotoxin derivative is teniposide (Imbert 1998). Podophyllotoxin is, therefore, an essential precursor with enormous therapeutic potential.

Mode of Action

The first big move was Kaplan's publication (Kaplan, 1942), explaining the effective use of topically applied podophylline to treat venereal warts (*Condylomata acuminata*). This success resulted in the use of podophyllin against tumor tissues, and the chemical analysis of its components was performed on a broad front at the same time. The next major achievement was the 1946 publication of King and Sullivan (King and Sullivan 1946) revealed the mechanistic basis of podophyllotoxin's action, its cytostatic potential at cell level was close to colchicine, which is a process of suppressing the development of the mitotic spindle that interrupts metaphase during cell division and chromosome clumping (c-mitosis). During the cell cycle (late S or early G2 phases) podophyllotoxin arrested cells and single-strand breaks were observed, which are associated with the interaction of the drug and DNA in HeLa cells. Topoisomerases (Topo) are ubiquitous enzymes that are responsible for solving topological problems that occur during several DNA metabolism activities, such as transcription, recombination, replication, and chromosome division during cell division. The relaxing of supercoiled chromosomal DNA replication is catalyzed by these enzymes, which are classed as Topo I and II. Topo II relaxes DNA by causing transitory double-strand breaks, strand passage, and relegation. Further, this activity necessitates ATP and leads to a two-fold increase in linking number. Topo I's DNA relaxing mechanism requires temporary cleavage of a single strand, unwinding, and relegation. As per existing literature, the primary mechanism of action of the anti-tumor potential of etoposide or teniposide is attributed to their association with Topo II. Etoposide is a semisynthetic derivative of podophyllotoxin, a plant alkaloid. In the late S to G2 phase, this agent blocks cells, and topoisomerase II is the main target. Its attachment with the enzyme-DNA complex

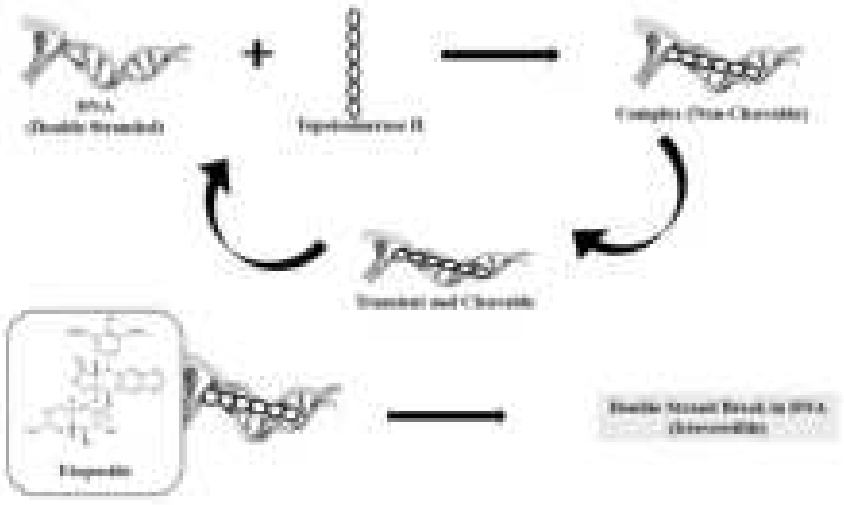

Fig. 4. Mechanism of action of etoposide (Created using biorender.com)

results in the continuity of the complex's transient, cleavable shape and thus makes it vulnerable to irreversible double-strand breaks (Fig. 4). The etoposide is used for the treatment of lung cancer alone and in combination with bleomycin and cisplatin for treating testicular carcinoma (Yousefzadi et al 2010, Nagar et al 2011).

Pharmacological potential of *Podophyllum hexandrum*

Antioxidant activity: The antioxidant activity of ethyl acetate (EAP) and ethanol (EP) extracts of *Podophyllum hexandrum* rhizome and petiole (10 mg mL^{-1}) was investigated using DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Ferric reducing antioxidant power) assay, with ascorbic acid (88.25% scavenging at 500 µM, and FRAP value 1267.5 µM, respectively) as a positive control. It was observed that DPPH scavenging of EAP and EP of rhizome (90.17 and 94.52%, respectively) was higher than the petiole (68.75 and 77.23%, respectively). Similarly, the FRAP values of EAP and EP rhizome were 1784.09 and 2079.55 µM as compared with the petiole (420.45 and 886.36 µM respectively) (Li et al 2012). The aqueous extract of *P. hexandrum* rhizome was evaluated for antioxidant activity using in vitro assay methods and CCl4 induced toxicity model in mice (in vivo). In the DPPH assay, 90% scavenging was observed at 800 µg/mL, which was comparable to standard vitamin E. Similarly, hydroxyl radical scavenging activity and reducing power of the extract are dose-dependent and are comparable to that of standard catechin and butylated hydroxytoluene, respectively. *In vivo* study revealed that elevated levels of the serum aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase were normalized with *P. hexandrum* treatment in rats. *P. hexandrum* aqueous extract decreased the malondialdehyde level to 1.42 from 7.75 nmol/mg protein, and this effect was comparable to vitamin E. Pre-treatment with the extract for 15 days before CCl_4 intoxication enhanced catalase activity significantly. Adding on, the glutathione transferase (GST) activity in the group pre-treated with vitamin E and water extract (50 mg/Kg) was equivalent (Ganie et al 2012). Additionally, methanol and ethyl acetate extracts of *P. hexandrum* along with their isolated active fractionated parts were evaluated for antioxidant activity by using DPPH assay. The results showed that free radical scavenging activity of methanol fraction (40 µg/mL) was 81%, the effect was equivalent to rutoside (85%). However, the activity of the ethyl acetate fraction was lower (45%) (Dar et al 2017).

Similarly, the antioxidant activity of the *P. hexandrum* rhizome water extract was examined using DPPH, superoxide, nitric oxide, and hydroxyl radical scavenging assay. The DPPH scavenging was >75% at 20 µg mL^{-1}, while 57.56% superoxide scavenging was observed at 1 mg mL^{-1}. In addition, >30% inhibition of nitric oxide radicals was observed (0.5 mg mL^{-1}), while in hydroxyl free radical scavenging assay, the extract showed a dose-dependent increase (100-600 µg mL^{-1}) (Arora et al 2010).

Antitumor activity: The water extract of *P. hexandrum* (34.5 mg/Kg body weight, for 15 days) showed anti-tumour effect in Ehrlich ascites tumor (EAT) mouse model evident from tumor doubling time from 1.94-19.1 days (Goel et al 1988).

Anti- mutagenicity: The hexane, methanol, ethyl acetate, chloroform, and water extracts of *P. hexandrum* as well as the fractions of methanol and ethyl acetate extract were evaluated for anti-mutagenicity effect against endosulfan-induced clastogenicity in a piscine model using micronucleus (MN) and chromosomal aberration (CA) test. Endosulfan significantly induced CA frequency and reached 12% at 96 h (Dar et al 2017).

Radioprotective effect: A mouse model was used to evaluate the radioprotective activity of plant extracts. The plant extract was administered (34.5 mg/Kg body weight (b.w.)) to mice before irradiation of 10 Gy. *P. hexandrum* protected mice and its dose-dependent radioprotective properties are comparable to synthetic radioprotective agents such as diltiazem (Goel et al 1998).

The radioprotective effect of semi-purified extract of *P. hexandrum* (intramuscular) in lethally irradiated mice was determined. It was found that extract provided a high survival rate (>90% at 6 mg/Kg b.w.). However, comet assay studies in peripheral blood leukocytes showed that extract administration before irradiation reduced DNA damage score and tail length when compared to the radiation-only group. Furthermore, the spleen cell count after radiation exposure decreased until day 5. In the extract-treated group, the initial count decreased dramatically, but this stage did not prolong. In addition, compared with the control group, after 5 hours of irradiation, >60% decrease was recorded in the thymocytes, and the decline further decreased at a similar rate until day 5. Further, with time thymus was completely degenerated. In the irradiated group pretreated with extract, thymocytes were reduced to day 5, but then they regained their viability within 30 days. These observations indicate that very small doses of extract provide high survival rates, protect DNA, and support rapid immune system replacement (Sankhwar et al 2011).

Similarly, the radioprotective effects of hydroalcoholic materials (HM) extracted from the rhizomes of *P. hexandrum* on gamma radiation (10 Gy) treated mice were evaluated. It was observed that HM normalized the hemoglobin (14.73 g dl^{-1}) and total leukocyte count (TLC) 4166.66 on day 15 as compared to control mice (radiation only). Whereas, the hemoglobin in the radioprotective drug + irradiation group

was 21.25% more than control on day 10. In addition, compared with the radiation group, the TLC of the drug + radiation group increased (83.33 times). Further, western blotting studies revealed hemopoietic recovery in irradiated mice evident from overexpression of heme oxygenase 1 and Bcl-2 protein. Therefore, results revealed that the biologically active ingredients of HM play a role in radioprotection by regulating the hematopoietic system (Rajesh et al 2007).

Additionally, aqueous extract (AE) from *P. hexandrum* rhizome was examined for its radioprotective effect by survival analysis, DNA protection ability, anti-hemolytic potential, anti-lipid peroxidation assay. AE application (4-20 mg/Kg b.w., intraperitoneal) before 30 minutes of radiation exposure (10 Gy) showed >80% protection at 8 mg/Kg. Also, it significantly modulated radiation-induced hemopoietic syndrome. Interestingly, 26% supercoiled form (SF) was observed in the irradiated group (250 Gy γ-radiation) as compared to untreated control (pBR322 DNA with 72% SF). The maximum DNA protective effect was noticed at 50 µg mL^{-1} of extract (i.e. 59% of the DNA in the form of SF was retained). Compared with controls, the optimal protection was at 10 µg mL^{-1}. Taking into account that even after radiation exposure extract showed significant anti-hemolytic potential (10-500 µg mL^{-1}). In addition, extract (10-1000 µg mL^{-1}) inhibited almost 90% lipid peroxidation and this effect was comparable to standard gallic acid (Arora et al 2010).

CONCLUSION

For thousands of years, plants have been an important source of medicine. To reduce the burden on the natural population, a vegetative and *in vitro* technique is therefore necessary. Due to application of podophyllotoxin, etoposide, and teniposide against particular types of cancers, the rhizome, and roots of *Podophyllum* species have gained much significance. As a result, there is a noticeable decline in plant populations. Therefore, it is appropriate to provide immediate impetus to generate the reliable traditional mass cultivation protocols of *P. hexandrum* and to urgently maintain its genetic diversity. An alternative strategy for conservation, wherein dependence of plant can be reduced and phytochemicals can be produced *in vitro*. In this regard, plant tissue culture is useful, for the multiplication and survival of species, which are difficult to regenerate and save from extinction. This study will motivate researchers to collect podophyllotoxin from plant tissue culture-derived plants in order to meet the pharmaceutical industry's need.

AUTHOR CONTRIBUTIONS

All authors have made a substantial and direct contribution to the work.

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