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Review Article

Callus Culture: A Sustainable Approach for Preserving and Enhancing Cholistan's Endangered Medicinal Plants for the Herbal Industry

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ABSTRACT

Sustainable preservation techniques are necessary due to the rapid depletion of medicinal plants, which are invaluable resources for herbal products. This review focuses on callus culture as a sustainable approach for conserving and enhancing endangered medicinal plants in the Cholistan desert, Pakistan. The Cholistan desert harbours a rich diversity of medicinally important plant species, but agricultural expansion and urbanization threaten their existence. Traditional preservation methods are often tedious and inefficient, emphasizing the need for alternative approaches. Callus culture offers a promising solution for conserving endangered species and enhancing their bioactive compounds. This technique involves the regeneration of plant tissues from explants under controlled conditions. The review discusses the challenges in preserving medicinal plants, the significance of Cholistan's flora, and the role of callus culture in pharmaceutical industries. It also highlights the importance of research and development in Pakistan to harness the potential of medicinal plants. Furthermore, the review emphasizes the significance of tissue culture techniques in producing bioactive substances and conserving endangered plant species. It discusses the conservation of callus to ensure the sustainable supply of medicinal plant materials to the industry. In summary, the review highlights the significance of callus culture for the sustainable growth of the herbal sector in Cholistan's endangered medicinal plants.

Keywords: *Bioactive compounds, callus culture, Cholistan, conservation, endangered plant species, herbal industry, medicinal plants.*

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INTRODUCTION

Medicinal plants are a vital source of herbal products throughout the world, but they are vanishing at a rapid rate (Chen et al., 2016). About 80% of individuals in developing countries rely solely on herbal medicines for primary health care (Chacko et al., 2010). Therefore, their sustainable supply to the herbal and pharmaceutical industry is considered one of the hot issues in the world (Aziz et al., 2018). This is because most desert plants are in danger of going extinct due to various factors, like climate change, habitat degradation and water scarcity. The Cholistan desert in Pakistan is the world's seventh largest desert, covering 26,000 km^2 (Hameed et al., 2011). A variety of medicinally important chemical compounds can be isolated from the flora of Cholistan desert, including antioxidants, anthocyanidins, flavonoids, phenolics, fatty acids, sterols and steroids, and terpenes and triterpenoids, which will give a breakthrough in terms of improvement in quality of herbal medicines, sustainable marketability and increased profitability (Alamgeer et al., 2018). The increased urbanization and crop cultivation posed a threat to desert flora, resulting in the extinction of various medicinal plant species. The germplasm of endangered plant species could be conserved through seed, field, tissue culture and cryopreservation. Most medicinal plant seeds are recalcitrant, losing viability in long-term storage; field conservation is costly, requires large areas and is damaged by adverse climates. Medicinal plant species which produce recalcitrant seed or do not produce viable seed can be selected for *in-vitro* storage through the production of callus.

Challenges in the preservation of medicinal plants

It has always been challenging for researchers to preserve the extracted bioactive compounds from plants. Traditional

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methods of extracting natural compounds from medicinal plants for use in the herbal, pharmaceutical, and chemical industries are tedious, time-consuming, and costly. Moreover, the quantity and quality of extract is also not of good standard. In developed countries, 25% of medicines are made from wild plant species; yet, in developing countries, nearly 80% of people use herbal medicines for primary healthcare (Ekor, 2013). Moreover, reduction in desert plant species and destruction of their habitat has increased the risk of loss of medicinal plants in deserts of China and India (Srujana et al., 2012), likewise in Pakistan. This was primarily due to deforestation and the severity of the climate. Plant extinction rates are currently 100 to 1000 times higher than natural extinction rates, with at least one major medicine being removed from our planet every year (Uhlenbrock et al., 2018). Due to these circumstances, botanicals from the Cholistan deserts were unavailable. World Wildlife Fund (WWF) has listed 15,000 indigenous flora as endangered due to habitat degradation and overgrazing. Likewise, the increasing human population already exhausted 20% of wild resources (Chen et al., 2016).

Research and development in Pakistan

In Pakistan, research and development on medicinal plants is often ignored, even though there are numerous avenues for research in this sector (Asghar et al., 2022). A thriving government policy and regulation, public awareness, and modern biotechnological interventions can result in mass production of these important herbs with long-term metabolite profiles, paving the way for the establishment of a medicinal plant industry in Pakistan and thus supporting the emerging economy (Sher et al., 2014). Storage of medicinal plants as seeds is more difficult because their reproduction biology is not under control as they are wild in nature. Moreover, the bioactive substances are found to be higher in the callus than in field-grown plants (Altemimi et al., 2017). Therefore, clonal propagation is preferred to conserve elite plant species due to the high level of heterozygosity. However, such conserved material is at risk of destruction by natural disasters. It is therefore recommended that endangered medicinal plant materials be conserved through advanced tissue culture approaches (Scherwinski-Pereira et al., 2010), and it is critically required in medicinal plant species (Panis and Lambardi, 2006). Pakistan urgently needs to create a national narrative around medicinal plant preservation and propagation.

The tissue culture techniques have been effectively used in developed countries to regenerate, multiply, and conserve highly valued wild medicinal plants through callus formation (Chokheli et al., 2020). This approach offers a sustainable supply of bioactive compounds regardless of the season, environment, or location (Coelho et al., 2020). Furthermore, it provides a long-term, environment-friendly basis for the industrial production of plant bioactive compounds (Donno et al., 2020).

Tissue culture techniques for medicinal plants

The production of bioactive substances from callus can further be increased through advancements in cell line selection, cell permeabilization, and biotransformation (Marisol et al., 2016). Bahawalpur's arid region encompasses a wide area of hot Cholistan desert with great seasonal changes, contributing to the extinction of the region high-value medicinal plant species (Malik et al., 2015). Therefore, understanding *in-vitro* conservation techniques is imperative to preserve the enriched medicinal flora of Cholistan and increase the sustainable production of antioxidants, phenolic and other natural products through callus production to boost the pharmaceutical and herbal industry of Pakistan (Fig. 1). Moreover, the outcome of the project will promote research and development activities in academics, researchers and other stakeholders of the industry. The present study intends to identify the endangered species of Cholistan medicinal plants and then compare the production of bioactive substances in vitro and in vivo. For that purpose, the optimization of culture media for callus production, as well as its multiplication and storage, were reviewed (Fig. 2). Masses of

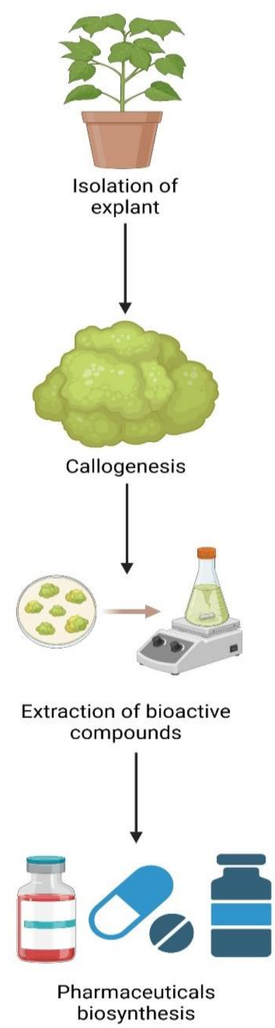

Figure 1: Schematic representation of the process of callus culture for preserving and enhancing endangered medicinal plants. The process begins with the isolation of explants from target plants, followed by callogenesis (formation of callus tissue under controlled conditions). Bioactive compounds are then extracted from the callus tissue for further pharmaceutical biosynthesis, contributing to the production of high-value herbal medicines and products.

callus were used to extract bioactive compounds. These optimized techniques of callus induction and multiplication will boost the pharmaceutical industry.

REVIEW OF LITERATURE

A large number of individuals have been using herbal remedies as primary healthcare in developing countries (Myers et al., 2000). However, rapid urbanization, habitat destruction, ruthless herb collection, pollution, and other anthropogenic activities diminished the ecosystem's medicinal plants (Shaik et al., 2011), and most wild plant species in desert zones of the world, including Cholistan are on the verge of extinction To prevent the future extinction of endangered medicinal plants, the conservation of plant genetic resources has long been recognized as an essential component of biodiversity conservation (Khan et al., 2021). As a result, it is proposed that callus be conserved not only from various medicinal plant species but also utilized for quantitative and qualitative enhancement of antioxidant and phenolic extraction on a long-term basis. The advancement in plant tissue culture provides enormous potential for the preservation and multiplication of rare medicinal plants (Robert et al., 2012). *In-vitro* propagation is the process of propagating chosen genotypes in a laboratory setting (Govarthanan et al., 2015). Several types of explants, like roots or leaves pieces, can be subjected to a growing medium with essential nutrients to produce calli. Recent studies have reported the *in vitro* growth of various medicinal plants (Table 1). As a result, attempts have been made to improve the *in-vitro* culture of valuable medicinal plants that are highly threatened.

Role of cell culture in pharmaceutical industry

Plants have been used for therapeutic purposes worldwide since prehistoric times (Chandran et al., 2020). Most tropical and subtropical medicinal plant species have recalcitrant seeds, which reduces their viability in storage and makes them unsuitable for traditional preservation (Dhaniya and Parihar, 2019). Furthermore, in situ conservation (field gene banks) is a simple technique, it is always vulnerable to natural disasters, insects/pests, and diseases (Zair, 2020). So, it is critical to have access to a coordinated system (tissue culture techniques) to preserve plant germplasm that can serve as the foundation for global agriculture (Scherwinski-Pereira et al., 2010). Rapid multiplication and production of medicinal plants under disease-free conditions can be accomplished using cell culture, nodal culture, and other tissue culture techniques (Cruz-Cruz et al., 2013). *In vitro* propagation of medicinal plant species offers the safe exchange of plant material, the establishment of a large collection of calli through a multiplication procedure that takes up little space, and the facilitation of molecular and ecological investigations in various parts of the world (Tandon and Kumaria, 2005).

Plant organs like shoots, leaves, and roots are produced through calluses and the differentiation of meristems by adjusting the concentrations of plant growth hormones in a nutrient medium through organogenesis (Oseni et al., 2018). Worldwide, a lot of research success has been achieved in vitro propagation and conservation of various endangered medicinal plant species like *Mandevilla velutina*, used as an anti-inflammatory agent and snake bites. Explant (nodal segments) of this crop was

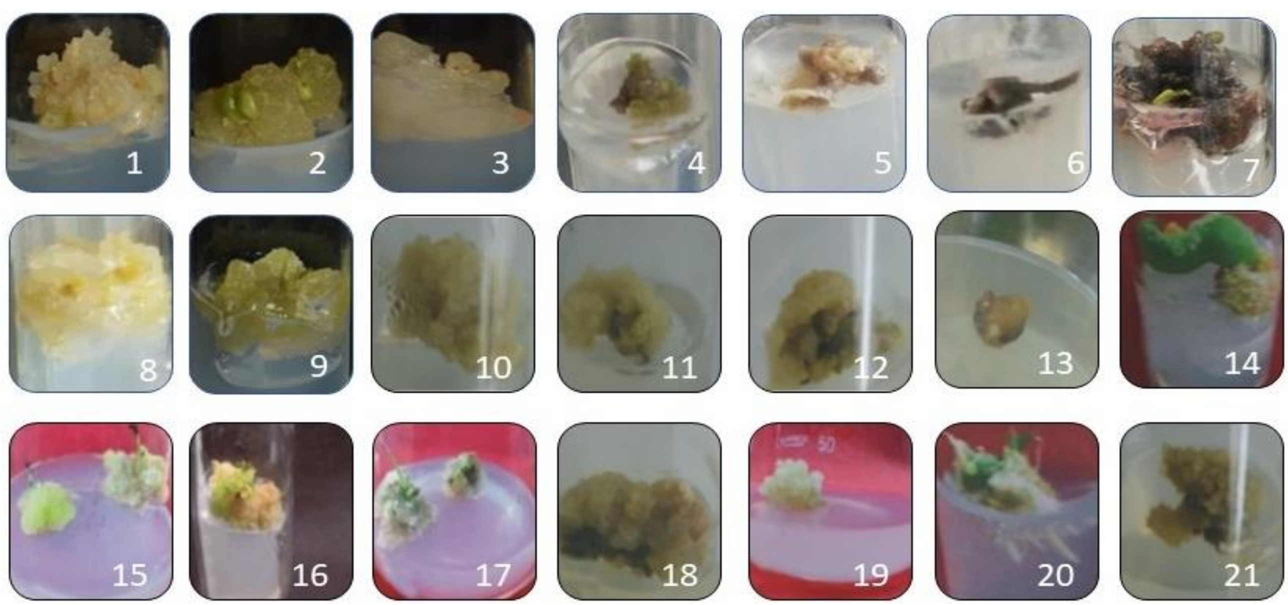

Figure 2. Medicinal plants species with successful calli induction after optimization of MS media 1, Carnation (*Dianthus caryophyllus*); 2, Goji berry (*Lycium barbarum*); 3, *Harmal (Penganum harmala*); 4, Bitter cucumber (*Cucumis callosus*); 5, *Datura (Datura stramonium)*; 6, Stevia (*Stevia rebaudiana*); 7, *Jujube* (*Ziziphus jujube*); 8, Vaccaria (*Vaccaria hispanica*); 9, Indian rennet (*Withania coagulans*); 10, Desert Pink (*Sesuvium sesuvioides*); 11, Kapok bush (*Aerva javanica*); 12, False Amaranth (*Digera muricata*); 13, Ak (*Calotropis procera*); 14, Bitter apple (*Citrullus colocynthis*); 15, Fagonia (*Fagonia bruguieri*); 16, Seetzenia (*Seetzenia lanata*); 17, Gokshur (*Tribulus terrestris*); 18, Kair (*Capparis decidua*); 19, Sand button (*Neurada procumbens*); 20, Bindweed (*Convolvulus prostrartus*); 21, Camelthorn (*Alhagi maurorum*).

successfully inoculated on MS media by supplementing different concentrations of zeatin, benzyl adenine, and thidiazuron (Biondo et al., 2007).

There is a great commercial importance of plant secondary metabolites containing anthocyanins, alkaloids and flavonoids group of organic compounds. The extraction of these compounds is limited due to various ecological and climatic conditions worldwide (Praveen and Murthy, 2011). Plant tissue culture technology can extract secondary metabolites more efficiently than conventional approaches (Praveen et al., 2010). Moreover, the production of these metabolites depends on the age of plants and is tissue-specific (Nasim et al. 2010). Commercial propagation of *Capparis decidua* in the field is difficult because of less ability to produce viable seeds; moreover, their seed germination is very poor in the soil, so there is a need to develop micropropagation approaches for this species (Sottile et al., 2021). *In vitro* regeneration of *Alhagi maurorum* (rare halophytic medicinal plant) to produce phenotypic clones to extract bioactive compounds was successfully done in tissue culture practices (Agarwal et al., 2015). In 3 to 4 weeks of culture of *Dicoma tomentosa* and *Alhagi maurorum*, calli production started from leaves in tested medium MS-5 after optimization of Auxins and cytokinin concentrations (Dhaniya and Parihar, 2019).

Conservation of cell culture products

Traditionally, the biodiversity of medicinal plants was conserved through in situ and ex-situ practices; however, there is always a threat to habitat devastation and change in the natural environment. Therefore, it is recommended that in situ conservation of endangered medicinal plant species is insufficient and that other advanced means of conservation be followed (Mir et al., 2021). Advancements in plant biotechnology and molecular studies provide effective tools to support and improve the conservation of plant diversity, done in tissue culture practices (Cruz-Cruz et al., 2013). Conservation of callus or cell culture is necessary not only for sustainable supply of medicinal plant material to industry for drug making but also to save these species from extinction. Various conservation methods and temperatures are followed depending on conservation objectives and type of plant species. *In vitro*, slow growth through extending subculture practices from several months to years, depending on plant species, is termed medium-term conservation. Conservation of callus of medicinal plant species in liquid nitrogen (-196°C) is the only safe and cost-effective technique for long-term conservation of diverse plant species. Moreover, these cryopreservation techniques have also been successfully applied to shoot tips to eliminate systemic plant pathogens (Cruz-Cruz et al., 2013). Tissue culture protocols are successfully used to preserve callus or organs as clones instead of seeds, saving the genetic background of endangered medicinal plant species due to various biotic or abiotic stresses (Shahzad et al., 2017). Biondo et al. (2007) established long-term storage of Brazilian endangered medicinal plant species (*Mandevilla velutina*) through in vitro culture of micro-cuttings and transferring it to different culture media and concluded that MS media with reduced nutrient concentration (MS/2) improved slow regeneration of explants.

Significance of Cholistan's medicinal plant species

There are lots of medicinal plants naturally growing in the desert zone of Bahawalpur (Naz et al., 2010). The most prevalent medicinal plants of the Cholistan region include *Peganum harmala* L., which belongs to the family (Nitrariaceae) known as Syrian rue, Wild rue (common names) and Harmal (local name). It can tolerate extensive drought by losing leaves and branches during the moisture-free months (Gouja et al., 2014). It is a perennial desert shrub that shows different activities in biological sciences, including antimicrobial activity (Alkhalifah, 2013), antioxidant and cytotoxic activities (Badria et al., 2007), anti-ulcer, anti-inflammatory activities and hypoglycemic activities (El-Hawary and Kholief, 1990). Several plant species, including *Aerva javanica, Capparis decidua, Cleome brachycarpa, Dipterygium glaucum, Gisekia pharnacioides* and *Suaeda fruticosa* are used for vermicidal and anthelmintic properties against intestinal worms or antimicrobial activities against bacteria and other microorganisms (Alkhalifah, 2013). The flora of the Cholistan desert contains several chemical compounds that have been isolated and recognized to have medicinal significance, including terpenes, triterpenoids, phenolics, and antioxidants. Allopathic medicines are often expensive and out of reach for many locals (Sharif et al., 2011).

Bathu (*Chenopodium album)* has been declared an endangered herb, native to Europe and Western Asia, as narrated in the book "Food in China". It has been a food source for different old civilizations as it was likely grown in Neolithic Europe (7000-1700 BC). It usually grows straight up to a height of around 30 cm. Its stem often has red, purple, or green stripes. The leaves in cultivated varieties vary greatly from simple, deltoid, and rhomboid to lanceolate form and length, ranging from 10-15 cm with an entire top surface and a toothed or uneven lower surface. The petioles are frequently as long as the thick blade, measuring 1 to 1.3 cm. The opposite leaves can be very different in shape. The early leaves are serrated, roughly diamond-shaped, 3-7 cm long and 3-6 cm broad. They are located close to the base of the plant. It has been spotted in a dark green colour with a smooth undersurface. The leaves are wax-coated, un-wettable and mealy in shape. Flowers are radial and symmetrical and grow in small cymes on a deeply branched inflorescence, 10-40 cm long, consisting of shining black seeds. pollen contributed to high fever-like allergies (Wiart, 2006; Pande and Pathak, 2010). It shows a source of anthelmintic agents and used medicinally in different countries for a source of many potent and strong drugs. Ethanol leaf extract of plant has been found to reveal antibacterial activity on all Gram (+) and Gram (-) bacteria (Korcan et al., 2013). It is a fast growing "weedy annual plant" with about 150 species occurring almost in different parts of the world (Colombo and Bosisio, 1996). It is used as a food crop in Northern India and is also given to the animals as their daily base feed. The leaves contain about 0.76% fats, 3.9% proteins, 8.93% carbohydrates and 3% ash, calcium, phosphorus and vitamin A (Parekh and Chanda, 2007). Iron and fibre are also usually present in very small quantities to do any harm (Rojas et al., 2003).

Haloxylon salicornicum, locally known as "Khar" or "Lana", a member of Chenopodiaceae, is a widespread shrub in different desert regions of Pakistan (Raza et al., 2014). It is a perennial,

Table 1: List of plants species cultured *in-vitro* with protocol: tap water washing → distilled water washing → Tween-20 + 5% NaClO for 2 min → 70% ethanol treatment for 2 min → distilled water washing → double distilled water washing.

No.	Plant Name	Ex-plant	Media	Reference
1.	Carnation (*Dianthus caryophyllus*)	Meristems	MS media	Thakur et al. (2018)
2.	Goji berry (*Lycium barbarum*)	Seed	MS media	Karakas (2020)
3.	Harmal (*Peganum harmala*)	Seed	MS media	Mutasher and Attiya (2019)
4.	Bitter cucumber (*Cucumis callosus*)	Seeds	MS media	Jesmin and Mian (2016)
5.	Datura (*Datura stramonium*)	Leaf disc	MS media supplemented + 2 mg 2,4-D	Rajewski et al. (2019)
6.	Stevia (*Stevia rebaudiana*)	Leaf disc	MS media supplemented + 2 mg 2,4-D	Singh et al. (2017)
		Seeds	MS media	Keshvari et al. (2018)
7.	Jujube	Leaf disc	MS media	Ren et al. (2019)
8.	Vaccaria (*Vaccaria hispanica*)	Seeds	MS media supplemented + 1 mg 2,4-D	Bedir et al. (2022)
9.	Indian rennet (*Withania coagulans*)	Seed	MS media	Tripathi et al. (2018)
10.	Desert Pink (*Sesuvium sesuvioides*)	Leaf	MS media	Sajid-ur-Rehman et al. (2021)
11.	Kapok bush (*Aerva javanica*)	Seed	MS media supplemented + 1.5 mg NAA	Boobalan and Kamalanathan (2019)
12.	False Amaranth (*Digera muricata*)	Shoot tip	MS Media supplemented + 2,4-D	Charan and Sharma (2016)
13.	Ak (*Calotropis procera*)	Seed	MS media supplemented + 2,4-D + NAA	Tripathi et al. (2013)
14.	Bitter apple (*Citrullus colocynthis*)	Leaf	MS Media	Meena et al. (2014)
15.	Fagonia (*Fagonia bruguieri*)	Leaf	MS Media	Saleem et al. (2019)
16.	Seetzenia (*Seetzenia lanata*)	Leaf	MS Media supplemented + BA and NAA	Shamso et al. (2013)
17.	Gokshur (*Tribulus terrestris*)	Seed	MS media	Sharifi et al. (2012)
18.	Kair (*Capparis decidua*)	Leaf	MS media	Tyagi et al. (2010)
19.	Sand button (*Neurada procumbens*)	Leaf	MS media	Zareen et al. (2018)
20.	Bindweed (*Convolvulus prostrartus*)	Leaf	MS media supplemented + 1 mg 2,4-D	Malik et al. (2015)
21.	Black cumin (*Nigella sativa*)	Seeds	MS media	Alemi et al. (2013)
22.	Poppy (*Opium poppy*)	Seeds	MS media	Rostampour et al. (2010)
23.	Mint (*Mentha spicata*)	Leaf disc	MS media supplemented + 2 mg 2,4-D	Samantaray et al. (2012)
24.	*Cosmos* (*Cosmos bipinnatus*)	Seeds	MS media	Jaberi et al. (2018)
25.	Cockscomb (*Celosia cristata*)	Seeds	MS media	Bakar et al. (2014)
26.	Dahlia (*Dahlia pinnata*)	Seeds	MS media	Hetman et al. (2017)
27.	Verbena (*Verbena officinalis*)	Seeds	MS media	Kumar et al. (2019)
28.	Papaya (*Carica papaya*)	Shoot tip	MS media supplemented + 2 mg 2,4-D	Setargie et al. (2015)
29.	Sour orange (*Citrus aurantium*)	Seeds	MS media	Cai et al. (2012)

upright, leafless shrub with many branches. The branches and stem are jointed and pale yellow in colour. The joints split into two small triangle points, replacing leaves, flowers, and fruits. It is a fodder plant with high salt content and is mostly grazed by the camels, which is better for reclaiming the soil. Its extract is used to wash clothes. Some residents also contend that this plant is toxic due to inaccurate knowledge, and most people use it as an external remedy for local population insect stings (Ashraf et al., 2012). Its decoction is known to have anti-inflammatory and antibacterial properties in folk medicine. Traditional healers use it to treat intestinal ulcers (Fatima et al., 2019; Shafi et al., 2002). It is utilized in multiple forms as fodder, food, fuel, and medicinal plants in different countries (Singh et al., 2022). The stems and leaves of this plant are used to make animal feed, which contains various minerals and chemical components (Ashraf et al., 2012). In Oman, its stem is used for dyeing wool in traditional weaving (Abdullah et al., 2020). It is also a densely branched pale diffuse shrub. Its stem is almost leafless and glabrous, and it secretes thick fluid on the cut wounds. The inflorescence is pale greenish, like a small cup with spikes. Wings like fruits are brownish (Arora et al., 2010). It is also used as fodder (Khan and Qaiser, 2006). Other reported uses in various countries are burned plants used in washing clothes, in glass, in the soap industry, and in dyeing clothes and medicines (Baber et al., 2018). *Mollugo cerviana* is a flowering plant species, locally known as thread stem carpet weed. It grows as a weed in many dry and sandy habitats on different continents. It is an annual herb producing a thin, erect stem up to about 20 centimetres tall. The narrow, waxy leaves are up to 1.5 centimetres long, linear in shape, and arranged in whorls around the stem. The inflorescence is a loose umbel of tiny flowers, each made of whitish, petal-like sepals <2 mm long and no true petals. The annual, glabrous herb grows up to 12 cm tall, branchless glaucous, in whorls of 8 from the rootstock. It has antimicrobial and anti-inflammatory properties (Valarmathi et al., 2012). The extracts of this plant can act as a uterine stimulant and antiseptic, and it is also used to treat jaundice (Valarmathi et al., 2011). Besides this, in India, it is identified as a suppressor of stomach ache and to promote vaginal discharge in childbirth. It also clears the eyesight and reduces body odour (Aglin, 2022). Decoction of flowers and tender shoots is used to treat diaphoretic impact. C-glycolsyl flavonoids compounds are also present in this species (Dewangan et al., 2022). Its crude extract contains alkaloids, saponins, flavonoids, glycosides tannins, triterpenoids, and phenolic groups, while the ethyl acetate fraction contains active constituents like saponins, glycoside, triterpenoids and steroids (Valarmathi, 2012). This plant species belongs to the family Capparidaceae. This plant is typically found in arid environments, such as wastelands and foothills. It is highly branched, typically grows between 6-10 meters, thorny and climbing shrub with 2.4 meters of stem diameter. Delicate branches with waxy blooms; rough, corky and grey bark, covered with 3-7 mm long pointed, straight orange-yellow stipular thorns. The leaves on the young branches are linear, 1-2 cm long, caduceus with a short rigid apex that resembles a pickle, with extremely short petiole (Kirtikar and Basu, 1999). The flowers in lateral corymbs are crimson, pink, or occasionally yellow, ovoid or globose, 1-2 cm in diameter, dull red berries with a hard, woody, 1-2 mm thick brownish rind; gynophores, 1.5-2 cm long, originating from the enlarged base of the thalamus; pedicel slender and fragile; tip with a little point resembling the style scar; bitter flavour; powerful, foul-smelling scent; globose seeds, 2-5 mm in diameter (Sulakshana and Raju, 2019). This plant is used to treat lumbago, hiccough, emmenagogue cough, and asthma and as a tonic, carminative, appetizer, alexipharmic, and aphrodisiac. The young leaves and upper branches are used as powder as an antidote to poison and for boils, eruptions, and swellings. They are very effective in relieving toothache when chewed (Kirtikar and Basu, 1999). The fruits are useful in cardiac scrapes. The fruits and tender flower buds are soused. Fruits are consumed either ripe or green. Useful in treating mudpack paralysis, enlarged spleen issues, and intestinal worm infestation (Srivastava et al., 2023).

CONCLUSION

Callus culture offers a promising and sustainable approach to preserving and enhancing endangered medicinal plant species in the Cholistan region. It ensures their survival, enhances their therapeutic value, and secures their role in the herbal industry. Moreover, callus culture provides a platform for further research on metabolic engineering and secondary metabolite production, opening new avenues for pharmaceutical and nutraceutical applications.

Declaration of competing interests

The authors declare no conflict of interest.

Author contribution statement

Muhammad Wasim Haider, Muhammad Nafees: Critically reviewed the literature and prepared the manuscript draft. **Ishtiaq Ahmad, Asad Masood, Muhammad Samsam Raza, Izhar Ul Haq, Muhammad Ahmad Saeed, Aqsa Shabbir, Umar Farooq:** Performed editing and reviewing of the manuscript.

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