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Research Article

Ex-situ Conservation of *Bulbophyllum leopardinum*, A Threatened Medicinal Orchid of Nepal

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Abstract

A successful micropropagation method was developed via the in-vitro seed germination and seedling growth of the epiphytic and/or lithophytic orchid *Bulbophyllum leopardinum*, a species having horticultural and therapeutic significance. To enhance seed germination, several quantities and combinations of naphthalene acetic acid (NAA), 6-benzyl amino purine (BAP), indole acetic acid (IAA), gibberellic acid (GA_3), and coconut water (CW) were added to 0.8% (w/v) agar-solidified MS medium. Half-strength MS medium has been experimented with alone and in combination with BAP, Kinetin (Kn), and GA_3 to promote shoot development. In-vitro-developed healthy shoots were chosen to establish roots in a half-strength MS (HMS) medium supplemented with various auxins. The best and earliest seed germination with the greenest protocorms (96.3±0.5% in 7 weeks) was achieved on HMS medium fortified with 15% CW (H15C). Further tests for the shoot as well as root development were continued with an H15C medium. H15C with 1 mg/l BAP and 1.5 mg/l Kinetin was most effective for early in vitro development and differentiation into seedlings with the many long shoots (9.3±0.1 shoots and 2.4±0.1 cm per culture) within 12 weeks of sub-culture. The most suitable rooting hormone was 1 mg/l NAA (4.2±0.26 roots per culture). This medium also produced the longest roots (1.9±0.09 cm per culture). By successfully developing a protocol for the mass propagation of *B. leopardinum*, this research has enhanced both the cultivation and the commercialization potential of this species.

Keywords: *Bulbophyllum leopardinum*, Micropropagation, PGRs, Seed germination, Shoot proliferation

Introduction

Orchidaceae, known as the "pandas of the plant world", is the second-largest family in the world and includes more than 28,000 species (Christenhusz & Byng, 2016) and 6–11% of all seed plants (Pillon & Chase, 2007). Despite being widespread, orchids are among the most fragile plants in nature (Fay, 2018). The Orchidaceae family has a significant economic impact because of its exotic beauty and long-lasting flowers, and they are especially valued in the horticulture industry. Their importance is also being acknowledged more and more in the pharmaceutical and fragrance sectors (Pant, 2013).

The tiny orchid seeds, which resemble dust, contain a tiny embryo that is encased in a single layer of protective cells. Its life cycle is complicated since it has little to no food stores. It requires at least a fungal acquaintance for germination and certain pollinators for pollination (Selosse, 2014). Because they are the most advanced blooming plants, orchids are extremely site-specific and require ideal circumstances to survive in any habitat. It makes sense that every orchid species is listed in CITES Appendix I and II.

The orchid mycorrhizal fungus (OMF) is essential for germination in nature (Pant et al., 2017; Shah et al., 2019a; Shah et al. 2019b). In contrast, an in vitro microenvironment can construct where asymbiotic orchid seed germination may occur (Knudson, 1951; Li et. al., 2018). Tissue culture is a cutting-edge method for growing plants on a large scale and may be used as a substitute source for naturally threatened species like orchids. However, it is difficult to design the procedure and methodology for mass propagation using tissue culture techniques.

The wet temperate forests of the eastern Himalayas, which lie in the nations of India, Nepal, Bhutan, Sikkim, Myanmar, and Thailand, are the natural habitat of *Bulbophyllum leopardinum* (Wall.) Lindl. It is an epiphyte and/or lithophyte, has one sharp deep-green leaf and an ovoid pseudobulb, and flourishes at elevations of 1,300-3,300 meters (eFloras, 2008). The popular name for this sympodial orchid, leopard-spotted orchid, comes from its light green blooms with red dots (Figure 1).

Figure 1: *Bulbophyllum leopardinum* in nature. **(a)** Epiphytic habitat, **(b)** Flower close-up, **(c)** Capsules close-up.

B. leopardinum is classified as a population of Least Concern by the IUCN Red List, but the ongoing decline in population and fragmentation brought on by rising rates of urbanization as well as the unauthorized and unrestrained felling, logging, and wood harvesting of its terrestrial host plants pose a threat (Cockel, 2013). Both people and animals eat this species, which is also used as herbal medicine, compost, and cow bedding (Teoh, 2016; Rajbhandari & Bhattarai, 2001; Pant & Raskoti, 2013). The present investigation has initiated the ex-situ conservation of this species by developing an efficient micropropagation protocol.

Materials and Methods

Plant material and aseptic sowing

Immature and non-dehisced pods (Figure 1c) were collected from Godawari, Lalitpur, Nepal located at 2,000 m above sea level. Collected pods were surface-sterilized by tap rinsing for 30 min, and washed with 90% ethanol for 50 sec and 1% sodium hypochlorite for 15 min before a final wash in sterile distilled water. The sterilized pods were longitudinally opened (Figure 2a) with a sterilized surgical blade and seeds were scooped out with a sterilized spatula. The seeds were spread over the surface of the medium supplemented with or without BAP and NAA or CW in culture tubes of 20 mL (Borosil Glass Works Ltd., India).

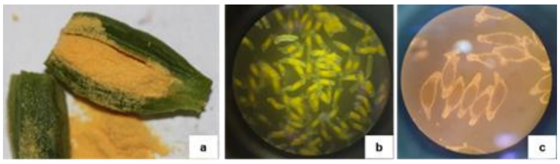

Figure 2: Microscopic seeds of *Bulbophyllum leopardinum*. **(a)** Longitudinally sectioned capsule, **(b)** Seeds under X80 of an inverted microscope exhibiting golden colour, **(c)** Seeds under X200 of an inverted microscope showing dark embryo at the centre.

Culture medium and culture conditions

MS medium of three strengths; full (FMS), half (HMS) and a quarter (QMS) were used with 3% (w/v) sucrose as a carbon source and solidified with 0.8% agar. The pH was adjusted to 5.7 in the final prepared medium, and the medium was autoclaved at 121 ˚C for 20 min at 1.05 Kg cm^2. The cultures were maintained at 25 ± 2 ˚C under a 16/8 h photoperiod (light/dark) provided by Philips white fluorescent lamps of 3,000-4,000 lux intensity.

Seed viability

A suspension of seeds in 200 µl of distilled water was observed in an inverted microscope (Olympus) under X80 magnification and seeds with and without embryos were counted. Seeds with embryos were counted as viable while those without them were considered unviable. Ten repetitions of this microscopic field reading were averaged for the final analysis.

Seed germination and protocorm development

Different strengths of MS medium supplemented with or without NAA and BAP (0, 0.5, 1, 1.5 and 2 mg/ml) and coconut water (100 and 150 ml/l) as growth additives were used for in-vitro seed germination. About 100 g of seeds were spread over the surface of each medium. Eighteen treatments, each with six replicates, were arranged in a completely randomized design (CRD).

Shoot development

For shoot multiplication, HMS medium with 15% CW (H15C) as a basal medium was fortified with cytokinins (BAP, Kn and GA_3 at concentrations of 0.5, 1, 1.5 and 2 mg/l) alone or in combination with NAA (0.25, 0.50, 0.75 and 1.0 mg/l). Shoot development data were collected every four weeks. The 17 treatments each with six independent replicates were arranged in CRD.

Root development

Similarly, for root proliferation, HMS medium as a basal medium was fortified with auxins (NAA, IBA and IAA at the concentrations of 0.5, 1 and 1.5 mg/ml). Root development data were collected every four weeks until the 12 weeks. There were ten treatments, each with six replicates, arranged in CRD.

Statistical analysis

The average percentage of seeds that germinated was calculated for in-vitro seed germination. The data for shooting and rooting were displayed as the average of their corresponding lengths and numbers with a standard error.

Results and Discussion

Seed viability

Seeds were counted at X80 magnification under an inverted microscope. After averaging the counts of six microscopic fields, it was found that 96.04±0.33% of seeds had embryos and were therefore viable (Figure 2b).

Asymbiotic seed germination

Many researchers have used immature capsules for ease, effectiveness and speed (Pant et al., 2018; Thokchom et al., 2017; Pradhan et al. 2016; De et al., 2013). Organic supplements have been used to stimulate the growth and development and promote the shoot regeneration of various orchid species by Aktar et al., 2008 (*Dendrobium*), Kaur & Bhutani, 2012 (*Cymbidium pendulum*), Obsuwan & Thepsithar, 2014, and Huh et al., 2016 (*Cypripedium macranthos*).

The percentage of germinated seeds in various strengths of MS medium (FMS, HMS and QMS) with the adjuncts BAP, NAA and CW in which the yellowish powdery seeds of *B. leopardinum* were inoculated was recorded weekly. The initial stages of germination are typical for most orchids (Arditti, 1977). The seeds started to germinate after five weeks of inoculation. The tiny yellowish seed metamorphosed into greenish spherules (Figure 3a) after seven weeks of germination. After nine weeks, the maximum number (>90%) of seeds had germinated, except for QMS which took a minimum of 13 weeks.

In this study, more than 96% of seeds were germinated in HMS medium supplemented with 15% CW (H15C) (Figure 4) and the least seeds were germinated in a QMS with 1 mg/L BAP (Q1B). After nine weeks, the photosynthetic leaves emerged from the protocorms in an FMS medium with 1 mg/L NAA (F1N) (Figure 3b).

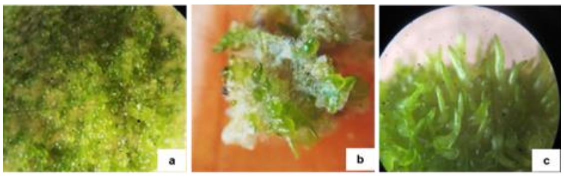

Figure 3: Diffèrent stages of *Bulbophyllum leopardinum* seeds germination. **(a)** Protocorms after 7 weeks of seeds inoculation, **(b)** Leaf primordia initiation after 9 weeks of seeds inoculation, **(c)** Shoot différentiation.

In many orchids like *Vanda pumila* (Maharjan et al., 2019), *B. nipondhii* (Pakum et al., 2016), *Bulbophyllum affine* (Maneerattanarungroj et al., 2013) and *Dendrobium nobile* (Asghar et al., 2011) introduced CW as a growth additive favoured both germination and protocorm growth. In all these species, 150 ml/l of CW was found to be optimal. Although introducing 1 mg/l NAA to FMS resulted in the good growth and development of *B. leopardinum* plantlets (Figure 3c). In 150 ml/l CW-supplemented HMS medium, protocorm development was seen early and healthy. This result indicates that the growth-promoting effect observed in *B. leopardinum* is attributable to the supply of exogenous amino acids present in the HMS medium supplemented with CW. In contrast with our result where QMS failed to germinate for a long time (13 weeks), Lee & Yeung (2010) studied *Bulbophyllum fascinator* and observed desirable germination percentages (around 90%) among three different concentrations (1/2, 1/4 and 1/10) of MS medium indicating that seed germination of this species can adapt to a wild range of inorganic salt concentrations.

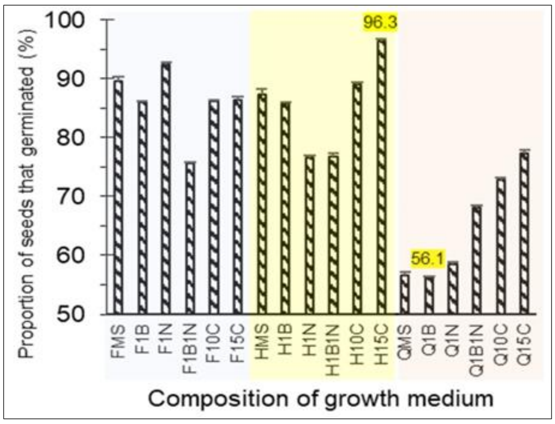

Figure 4: The percentage of seed germination in 7 weeks from inoculation.

Shoot proliferation from protocorms

H15C supplemented with GA_3 (0.5-2 mg/l) plus Kn (0.5-2 mg/l) promoted less number of shoot development than H15C supplemented with BAP (0.5-2 mg/l) after green protocorms and PLBs were transferred (Table 1). Roy & Banarjee (2001) observed that the addition of BAP to the HMS medium stimulated protocorm development and shoot bud initiation in *Geodorum densiflorum*. Watthana & Srimuang (2017) confirmed positive effect of CW along with potato extract and banana homogenate in shoot and root development.

The H15C medium with 1 mg/l BAP (H15C1B) induced protocorm multiplication and seedling growth in *B. leopardinum.* The greatest number (8.0±0.4) and longest shoots (1.8±0.3 cm) were observed (Figure 5a) in the H15C1B medium. Combined with 1.5 mg/l Kn (H15C1B1.5K), BAP induced even greater seedling development than on its own: on average there were 9.3±0.1 shoots and each was 2.4±0.1 cm long. In *Phaius tankervilleae*, MS medium supplemented with BAP stimulated protocorm formation and differentiation (Pant et al., 2011).

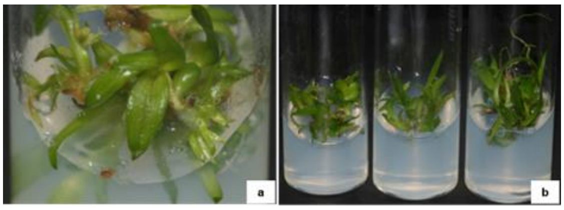

Figure 5: Shoot development in H15C1B0.75N, **(b)** Root development in H15C1N media (3rd one) compared with HMS (1st) and H15C (2nd) medium.

Root initiation and proliferation

Well-shooted plantlets were removed from the culture tubes and transferred to the rooting medium. In our study, 90% of the shoots were rooted well after being transferred to an H15C medium with or without auxins. The H15C medium supplemented with 1 mg/l NAA (Figure 5b) produced the most (4.2±0.26) and the longest (1.9±0.01 cm) roots. Rooting also took place in the two un-supplemented media but took longer than in the hormone-supplemented media.

To induce a good root system, multiple and fully developed shoot buds of *B. leopardinum* were transferred into different combinations of auxin-supplemented media. HMS medium fortified with different combinations of plant growth hormones (NAA, IAA and IBA at concentrations of 0.5 mg/l, 1 mg/l and 1.5 mg/l). HMS medium with 15% CW plus 1NAA (H15C1N) was found to be the most suitable combination for root proliferation where most (4.2 on average) and longest (1.9 cm on average) roots were found (Figure 6).

The roots of *B. leopardinum* were all thread-like; no large, thick and erect roots were observed during the research period. Mohammed et al. (2013) reported that HMS medium with auxin helps to increase the root systems of *B. lilacinum* and *Cymbidium aloifolium*. Gupta et al. (1998) and Bhadra et al. (2002) reported similar findings in *C. aloifolium* species.

Table 1: The effect of cytokinins (BAP, Kn & GA_3) with/without NAA in shoot proliferation of *Bulbophyllum leopardinum.*

Code	MS	Hormones (mg/L)				Observed after 12 weeks of subculture	
		BAP	Kn	GA3	NAA	Shoot No. ±S.E.	Shoot Length ±S.E.
H15C	H15C	0	0	0	0	4.8±0.8	1.1±0.1
H15C0.5B	H15C	0.5	0	0	0	2.4±0.1	1±0.2
H15C1B	H15C	1	0	0	0	8±0.2	1.8±0.1
H15C1.5B	H15C	1.5	0	0	0	3.3±0.3	1±0.1
H15C2B	H15C	2	0	0	0	3.6±0.1	0.8±0.2
H15C0.5K	H15C	0	0.5	0	0	3.5±0.6	1.1±0.3
H15C1K	H15C	0	1	0	0	4.5±0.3	1.3±0.2
H15C1.5K	H15C	0	1.5	0	0	3.4±0.4	1.6±0.2
H15C2K	H15C	0	2	0	0	3.3±0.2	1.5±0.2
H15C0.5G	H15C	0	0	0.5	0	5.1±0.1	1.3±0.3
H15C1G	H15C	0	0	1	0	3.8±0.1	1.7±0.2
H15C1.5G	H15C	0	0	1.5	0	6±0.1	1.4±0.1
H15C2G	H15C	0	0	2	0	6.4±0.3	1.3±0.3
H15C1B0.25N	H15C	1	0	0	0.25	5.2±0.2	1.4±0.2
H15C1B0.5N	H15C	1	0	0	0.5	5±0.1	1.8±0.1
H15C1B0.75N	**H15C**	**1**	**0**	**0**	**0.75**	**9.3±0.4**	**2.4±0.1**
H15C1B1N	H15C	1	0	0	1	4.9±0.5	1.8±0.5

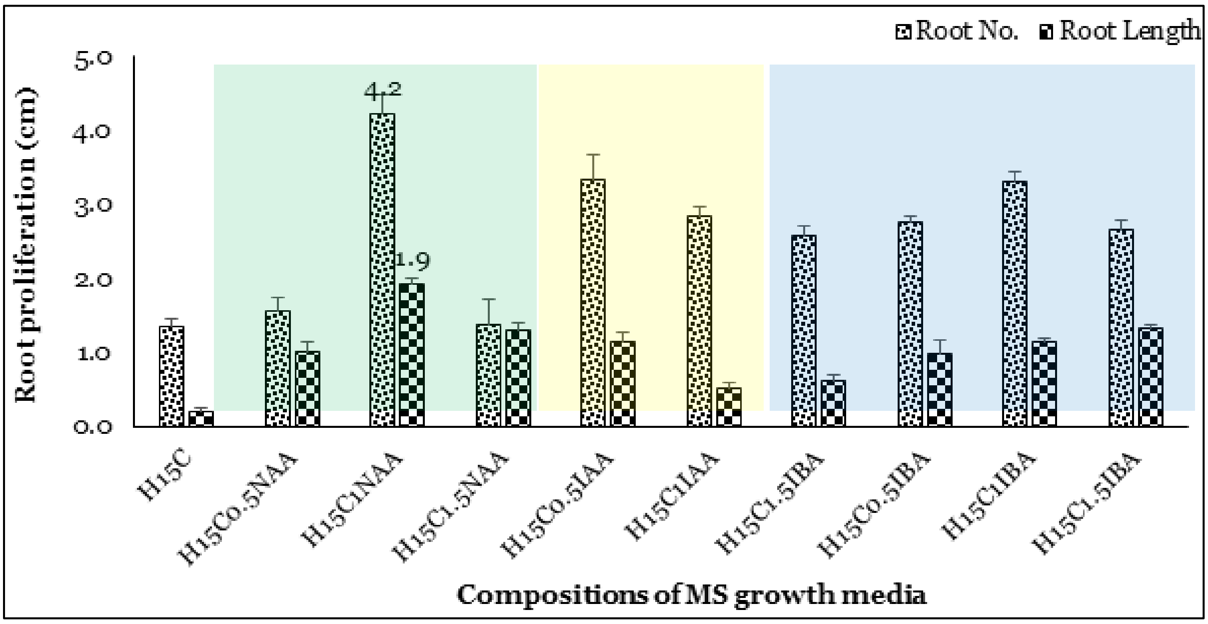

Figure 6: Root development in different root development media.

Conclusion

The results demonstrated that many cultures of the studied orchid can be produced in vitro from seeds using an asymbiotic in vitro seed germination. *B. leopardinum* (Wall.) Lindl. is a CITES-listed orchid, which has been placed on the IUCN Red List as having a decreasing and fragmented population because of the destruction and fragmentation of its habitat due, in part, to increasing urbanization. This multipurpose orchid is also traded illegally to use for compost, cattle-bedding, herbal medicine, animal feed, and food. To conserve and commercialize orchids like *B. leopardinum*, in vitro techniques of micropropagation can be a helpful tool. There is no other information regarding the in vitro seed germination of *B. leopardinum* to our knowledge, so this investigation may provide a crucial alternative to the ex-situ conservation of the horticulturally and medicinally important *B. leopardinum* species.

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Research Article

Induction, Proliferation and Differentiation of Callus in *Paris polyphylla* Sm. through Leaf Culture

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Abstract

Paris polyphylla Sm. is a vulnerable medicinal plant employed in the treatment of various ailments. This study seeks to establish a protocol for callus induction, proliferation, and differentiation of *P. polyphylla*. Immature leaf explants were cultured on MS medium with varying concentrations of plant growth regulators (PGRs), including 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kn), 6-benzylaminopurine (BAP), Thidiazuron (TDZ), α-Naphthalene acetic acid (NAA), and Gibberellic acid (GA_3), along with 10% coconut water. After 12 weeks of primary culture, the optimal callus induction was observed in MS medium supplemented with 0.25 mg/l 2,4-D + 0.5 mg/l Kn. In the secondary culture at 8 weeks, the best callus proliferation, as determined by callus weight or growth index, occurred in MS medium supplemented with 2.0 mg/l BAP alone, 2.0 mg/l Kn alone, 1.0 mg/l TDZ alone, combinations of 2.0 mg/l Kn + 1.0 mg/l BAP + 2.0 mg/l GA_3, and combinations of 0.5 mg/l NAA + 2.0 mg/l BAP + 2.0 mg/l GA_3, as well as 10% coconut water. Furthermore, callus differentiation into mini rhizomes with root primordia was successfully achieved in MS media containing 2.5 mg/l Kn and 10% coconut water. This study reports, for the first time, the formation and differentiation of callus from leaf explants in *P. polyphylla*. Large-scale callus generation from leaf explants has the potential to enhance the production of bioactive secondary metabolites for therapeutic purposes and facilitate the development of plantlets through organogenesis.

Keywords: 2,4-D, Callus, GA_3, Growth index, Primary culture

Introduction

Paris polyphylla Sm., commonly known as Paris root in English, Rhizoma Paridis in Pharmacopoeia, and Satuwa in Nepali, is a vulnerable medicinal plant found in the subtropical to subalpine forests of South Asian countries at altitudes ranging from 1800 to 3500 meters above sea level (IUCN, 2004). This plant is extensively utilized in traditional ethnomedicine, traditional Chinese medicine (TCM), Ayurveda, and Homeopathy for treating a wide array of conditions such as cuts, wounds, burns, fever, anthelmintic, scabies, diarrhoea, dysentery, liver cancer, antipyretic, pain relief, anti-inflammatory, coughing and purgative, breast cancer, fractures, convulsions, and strains, acting as an antidote, detoxicant, and soothing agent (Long et al., 2003; IUCN, 2004; Li et al., 2012). Steroidal saponin is the primary constituent of *P. polyphylla*, consisting of polyphyllin D, diosgenin, pennogenin, dioscin, and Paris saponin I, II, VI, VII, H, and polyphyllin VII (Lee et al., 2005; Zhang et al., 2014; Chen et al., 2019; Wang et al., 2019; Thapa et al., 2022).

Unfortunately, due to overutilization, habitat loss, and illegal trading for pharmaceutical and conventional medical purposes, the population of *P. polyphylla* is diminishing in its natural habitats. The process of developing callus tissue from plant cells in a nutrient medium with the use of plant growth regulators (PGRs) is known as in-vitro callus induction. The concentration of PGRs in the medium can be altered to guide the callus progression towards root formation, shoot growth, or somatic embryogenesis. Organ primordia corresponding to the callus tissues are then formed after additional cell proliferation and differentiation, contributing to the regeneration of complete plantlets and the production of bioactive compounds using precursors and elicitors. Callus culture is widely employed in both basic research and industrial applications, aiding in the establishment of cell suspensions, protoplast separation, extraction of bioactive compounds, and synthesis of significant pharmaceuticals in sizable quantities (Jhang et al., 1974; Furuya et al., 1983; Pant, 2014).

The induction of callus using various explants in culture is valuable for regenerating complete plants through organogenesis or embryogenesis, as well as for producing essential bioactive compounds. Additionally, the use of precursors and elicitors, along with chemical analysis of callus, contributes to laying the foundation for the production of natural drugs from in vitro-raised callus.

While existing literature discusses plant regeneration from rhizomes and other parts of *P. polyphylla* (Teerawatsakul et al., 2014; Raomai et al., 2014; Raomai et al., 2015; Devi et al., 2017), there is no information regarding callus induction and plant regeneration from the leaf. This study aims to fill this gap by investigating the process of callus induction, proliferation, and differentiation in MS media supplemented with various PGRs at different concentrations and combinations, utilizing leaf explants.

Materials and Methods

Plant material

P. polyphylla plants sourced from the Baglung district in western Nepal were collected in April and May of 2021 and potted. The herbarium specimen was documented, identified, and stored in the Tribhuvan University Central Herbarium (TUCH).

Preparation of explants

Immature leaves were carefully detached from the mother plants and thoroughly cleaned under running tap water for one and a half hours with the addition of a few drops of Tween-20 (Qualigens). This cleaning process was repeated three times using distilled water. The explants underwent surface sterilization with 70% ethanol for 30 seconds, followed by immersion in a solution containing 0.1% mercuric chloride ($HgCl_2$) for 3–4 minutes. To eliminate any $HgCl_2$ residue, the explants were washed three times with sterile distilled water. Using a sterile cork borer on the sterilized leaves, leaf discs (0.8–1.0 cm diameter) were created through gentle pressing.

Preparation of culture medium

Stock solutions were utilized to formulate Murashige and Skoog's (MS) medium (1962) for all leaf explant tissue cultures. The MS media were enriched with varying concentrations of plant growth regulators (PGRs), such as 2,4-dichlorophenoxyacetic acid (2,4-D) (0.25, 0.5, 1.0, 1.5, and 2.0 mg/l) and kinetin (Kn) (0.25, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/l) individually and in combinations. The primary culture included 3% (w/v) sucrose, 10% coconut water (v/v), and 0.8% (w/v) agar. The pH was adjusted to 5.6 before autoclaving. For the secondary culture, Kn, BAP, and TDZ (0.5, 1.0, 1.5, and 2.0 mg/l) were added to MS medium separately, in combination with BAP, NAA, and GA3 (0.5, 1.0, 1.5, and 2.0 mg/l), and in combination with BAP, Kn, and GA_3 (0.5, 1.0, 1.5, and 2.0 mg/l). GA_3 was added at a constant concentration (2.0 mg/l) in MS media. Controls included full, 1/2, and 1/4 strengths of MS media without added PGR supplements and 10% coconut water. Approximately 25 mL of medium were dispensed into 300 mL sterile autoclaved culture jars (78 mm×122 mm) or 15 ml of medium in sterile culture tubes (25 mm×150 mm) for culture initiation, covered with aluminum foil, and autoclaved at 121°C for 30 minutes under a pressure of 15 lb per square inch.

Culture establishment

Aseptically, small leaf discs (0.8–1.0 cm in diameter) were inoculated onto the MS medium. Following a 12-week primary culture, the regenerated callus was subcultured on MS medium. Calli for subculture were weighed in the airflow chamber to prevent contamination. The culture tubes and dishes were then transferred to the culture room and maintained at 25±2°C with 16-hour photoperiods. The experiment was conducted three times, with each treatment having three replicates. Eight-week-old calli were collected, and their growth index (%) and moisture contents (%) were determined according to Adhikari & Pant (2013) for further analysis.

"The moisture content of callus (%) = (Fresh callus wt. - Dry callus wt.) / Fresh callus wt. × 100"

"(Growth index) Increase in callus wt. (%) = (Fresh callus wt. - Fresh callus explant wt.) / Fresh callus explant wt. × 100"

Statistical analysis

Microsoft Excel 2010 was employed to calculate the percentages of moisture content and callus growth index, along with their average values and standard deviation. Additionally, the two-way ANOVA test was conducted in Microsoft Excel 2010.

Results and Discussion

Callus induction in MS medium

Inoculation of leaf discs onto MS media containing varying concentrations of 2,4-D and Kn (0.25, 0.5, 1.0, 1.5, 2.0, & 3.0 mg/l) along with 10% coconut water resulted in the induction of callus (Figure 1). As stated by Trigiano & Gray (2000), many tissues in plant tissue culture require a specific combination of plant growth regulators (PGRs) to initiate the appropriate growth response. Notably, MS media at full, half, and quarter strength without PGR supplements and 10% coconut water failed to induce callus formation. Similarly, employing different concentrations of Kn alone, 2,4-D alone, and a combination of NAA+Kn in the MS medium did not induce callus in *P. polyphylla*. Soniya and Das (2002) found that kinetin alone in MS medium did not induce callus in *Piper longum* leaf explants, but when enriched with 2,4-D+Kn, callus formation from leaf explants was successful. While kinetin is not always essential, exogenous application of auxin is crucial for initiating callus development (Okazawa et al., 1967). In tissue culture, kinetin is often used to induce callus production (in combination with auxin) and to develop shoots from the callus (with reduced auxin concentrations) (Duszka et al., 2009).

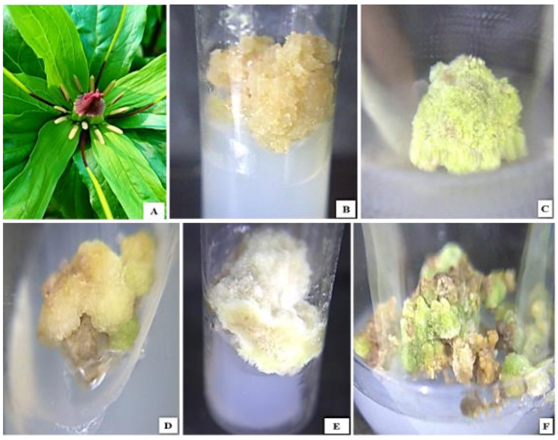

Figure 1: Induction of callus from leaf explant. Plant: *Paris polyphylla* (A), Callus: MS+0.25 mg/l 2,4-D+0.5 mg/l Kn (B), MS +2.0 mg/l Kn (C), MS+1.0 mg/l TDZ (D), MS+2.0 mg/l BAP+0.5 mg/l NAA (E), MS+1.0 mg/l BAP+2.0 mg/l Kn (F).

The most effective callus-inducing media in this study were MS media fortified with 0.25 mg/l 2,4-D+0.5 mg/l Kn (85%), followed by 0.25 mg/l 2,4-D+0.25 mg/l Kn (70%), 0.25 mg/l 2,4-D+1.0 mg/l Kn (60%), 0.25 mg/l 2,4-D+1.5 mg/l Kn (55%), and 0.5 mg/l 2,4-D+0.26 mg/l Kn (50%) after 12 weeks of initial culture (primary culture) (Table 1). The induction of callus was favoured when the concentrations of 2,4-D and Kn were nearly equal or when the Kn concentration exceeded the 2,4-D concentration. In nature, calli form on plants due to wounds, tumour-inducing bacteria (the Ti gene), and genetic tumours (Bhatiya, 2015). However, callus can also be artificially induced using a leaf or another explant and the appropriate nutritive medium. Generally, auxin alone or in combination with cytokinin induces callus formation in various plant species. The ratio of auxin to cytokinin determines callus induction (at an intermediate ratio), root development (at a high auxin to cytokinin ratio), and shoot regeneration (at a high cytokinin to auxin ratio). In some species, callus induction may occur without auxin or cytokinin, as abscisic acid and brassinosteroid hormones can induce it (Goren et al., 1979; Hu et al., 2000).

This study also revealed that callus induction and growth initially increased, then decreased, as Kn concentration was raised from 0.25 mg/l to 1.0 mg/l in MS media with a constant 0.25 mg/l of 2,4-D (Table 1). Similarly, callus induction and growth decreased as 2,4-D concentrations in MS media increased from 0.25 mg/l to 1.5 mg/l, while Kn concentration remained constant. All calli were compact and light white in the primary culture. The morphology of the callus changed from compact to friable when subcultured in the same concentrations of 2,4-D and Kn. However, when subcultured in cytokinins alone, such as BAP, Kn, and TDZ, the callus morphology shifted from friable to more compact and green. This change may indicate the initiation of organogenesis in the callus. Previous researchers successfully induced callus from leaf segments in MS media fortified with 2,4-D alone, NAA alone, indole-3-butyric acid (IBA) alone, or a combination of 2,4-D+Kn in *Melaleuca alternifolia* (Kiong et al., 2007), a combination of IAA+BAP in *Piper longum* (Sathelly et al., 2016), a combination of 2,4-D+KN in *Piper auritum* (Domínguez et al., 2006), and a combination of NAA+BAP in *Bergenia ciliata* (Shrestha & Pant, 2011). The cumulative data indicate that callus induction from leaf explants in MS media enriched with auxins, either alone or in combination with cytokinins, is genotype-dependent. Additionally, callus induction within a plant species is influenced by factors such as explant type, orientation, plant growth regulators, plant and explant age, medium composition, plant metabolic state, temperature, growing conditions, and source plant type (Klimek-Chodacka et al., 2020).

Table 1: Induction of callus in different concentrations of Kinetin and 2,4-Dichlorophenoxy acetic acid.

Concentration of 2,4-D (mg/l)	**Concentration of Kinetin (mg/l)**					
	0.25	0.5	1.0	1.5	2.0	3.0
0.25	L	L	L	M	M	S
0.5	M	M	M	M	M	-
1.0	M	M	S	S	S	-
1.5	S	S	S	S	-	-
2.0	-	-	-	-	-	-

S=Small quantity (fresh wt. <0.5 g), M=Moderate quantity (fresh wt. 0.5-2.0 g), L=Large quantity (fresh wt. >2.0 g)

Callus proliferation in MS medium

The calli derived from MS medium, containing a combination of 2,4-D+Kn and 10% coconut water, were maintained by subculturing in the same concentration of 2,4-D and Kn. Subsequently, the calli were further subcultured in MS medium supplemented with different concentrations of BAP, Kn and TDZ alone, and in combinations such as NAA+BAP+GA_3, BAP+Kn+GA_3, and 10% coconut water. The proliferation of callus increased with the elevated concentrations of all PGRs, including BAP, Kn and TDZ alone, in the MS medium. Although callus proliferation occurred in all concentrations and combinations of PGRs, the most effective proliferation was observed in MS medium enriched with 2.0 mg/l Kn alone, as evidenced by the calli's fresh weight and dry weight after 8 weeks of subculture (Table 2). At 2.0 mg/l of Kn, there was a remarkable 647.33% increase in callus growth (growth index), with the initial fresh weight of the callus explant at 0.33 g yielding 6.52 g of callus. Similarly, it was observed that callus developed in MS medium in combination with BAP+Kn+GA_3 exhibited a higher growth index compared to callus grown in MS medium in combination with NAA+BAP+GA_3 (Tables 3 and 4). The maximum callus growth index (589.03%) was observed in 1.0 mg/l BAP + 2.0 mg/l Kn + 2.0 mg/l GA_3 from an initial callus explant weight of 0.35 g (Table 4), while the maximum callus growth index (570.58%) was observed in 2.0 mg/l BAP + 0.5 mg/l NAA + 2.0 mg/l GA_3 from an initial callus explant weight of 0.37 (Table 3). This suggests that the synergistic action of two cytokinins, BAP and Kn, played a role in promoting callus proliferation and development.

Furthermore, callus proliferation and development decreased when NAA (auxin) concentrations were increased from 0.5 mg/l to 2.0 mg/l while BAP (cytokinin) concentration remained constant (Table 3). This indicates that a higher concentration of auxins combined with cytokinins is not favourable for callus proliferation. Similarly, when the concentrations of BAP and Kn were increased, the proliferation of calli grown in MS medium supplemented with BAP+Kn+GA_3 increased in terms of fresh weight or growth index but slightly decreased after reaching a concentration of 1.0 mg/l Kn (Table 4). This may be attributed to the saturation concentration of BAP+Kn for callus proliferation.

The callus morphology underwent changes after subcultures, transitioning from slightly compact light white to compact white, compact yellowish, and compact green. Calli produced in combination with cytokinin and auxin (BAP+NAA) exhibited compact light yellow and compact white morphologies, whereas calli in MS media treated with cytokinins, either alone or in combinations, displayed compact light green and compact white morphologies. Genetic variability in the callus, metabolite release, and PGR content in the media could be contributing factors to the alteration of callus morphology following sub-culture. In tissue culture, explants with different genotypes respond diversely to the same growth conditions (Nehara et al., 1989, 1990), and PGRs influence the synthesis of metabolites, organogenesis, and cell development (Liang et al., 1991). The results of the two-way ANOVA test, examining the p-values of plant growth regulators such as BAP, Kn and TDZ alone at different concentrations (0.5, 1.0, 1.5, and 2.0 mg/l) versus callus growth (g) or growth index, demonstrated a significant difference at the <0.005 level (p = 7.91×e-26 and 6.55×e-16). This indicates that callus proliferation, in terms of fresh weight (g) or growth index, was dependent on the types of plant growth regulators used and their various concentrations added to the MS medium. The successful induction of callus in leaf explants was achieved with the addition of an organic supplement (10% coconut water) in the MS medium. In MS media treated with 2,4-D+Kn and 10% coconut water, callus induction occurred, while media without 10% coconut water did not induce callus. According to Gnasekaran et al. (2009), coconut water stimulates cell division and growth in culture by acting as a cytokinin-like plant growth regulator and containing various nutritional and hormonal components.

Callus differentiation in MS medium

The callus, initially induced in MS medium enriched with 2,4-D and Kn, underwent subculturing in MS medium containing varying concentrations of Kn (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/l) along with 10% coconut water. In the presence of 2.5 mg/l Kn+10% coconut water, mini rhizomes with root primordia were successfully formed, and subsequent adventitious roots developed from these mini rhizomes (Figure 2). Kinetin, as a synthetic plant growth regulator, primarily facilitates shoot initiation and development. While rooting in the callus may have been initiated by the application of exogenous auxins (2,4-D) in combination with kinetin and 10% coconut water during the initial callus induction from the leaf explant. In tissue culture, the synthetic auxin 2,4-D is commonly used to stimulate somatic embryogenesis, but it is rarely employed commercially for root induction. Kinetin's role in supporting callus development suggests that the exogenous auxin-induced activation of callus growth is likely assisted by the inclusion of kinetin in the medium. Cytokinins like kinetin can induce the differentiation and regeneration of axillary buds (Le Bris, 2017). For roots to form in culture, the medium must contain a relatively low amount of auxin (Okazawa et al., 1967).

Table 2: Independent impacts of BAP, Kn, & TDZ on the proliferation and growth of callus following secondary culture.

BAP (mg/l)	Kn (mg/l)	TDZ (mg/l)	Callus explant fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5			0.33±0.03	1.19±0.14 CW	0.38±0.04	91.19
1.0			0.30±0.07	2.42±0.23 CW	0.88±0.12	229.65
1.5			0.30±0.01	3.71±0.13 CY	1.54±0.06	362.67
2.0			0.33±0.04	4.57±0.26 CG	2.10±0.16	449.95
	0.5		0.32±0.04	2.56±0.38 CY	0.79±0.09	243.98
	1.0		0.35±0.03	3.76±0.17 CW	1.30±0.06	366.98
	1.5		0.31±0.03	5.24±0.28 CG	2.15±0.08	518.42
	2.0		0.33±0.04	6.52±0.35 CG	2.96±0.15	647.33
		0.5	0.34±0.06	3.55±0.17 CW	1.12±0.04	345.22
		1.0	0.31±0.06	5.88±0.01 CG	2.83±0.09	582.30
		1.5	0.32±0.04	4.33±0.38 CG	1.04±0.16	425.77
		2.0	0.37±0.05	3.44±0.18 CY	0.83±0.07	334.77

CW: compact light white, CG: compact light green, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Table 3: Cumulative impacts of BAP+NAA+GA_3 on the proliferation and growth of callus following secondary culture (2.0 mg/l GA_3).

BAP (mg/l)	NAA (mg/l)	Callus explants fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5	0.5	0.33±0.07	1.96±0.67 CY	0.71±0.26	179.68
1.0	0.5	0.37±0.08	2.53±0.11 CW	0.98±0.01	238.59
1.5	0.5	0.36±0.06	2.85±0.07 CW	1.31±0.01	273.08
2.0	0.5	0.36±0.06	5.76±0.18 CW	2.74±0.05	570.58
0.5	1.0	0.34±0.06	2.69±0.22 CW	0.99±0.10	256.41
1.0	1.0	0.35±0.05	3.42±0.29 CY	1.47±0.02	332.54
1.5	1.0	0.37±0.04	3.73±0.10 CY	1.71±0.01	363.87
2.0	1.0	0.35±0.07	4.66±0.15 CW	2.33±0.10	458.68
0.5	1.5	0.39±0.03	1.30±0.06 CY	0.46±0.03	100.32
1.0	1.5	0.36±0.07	2.54±0.09 CW	1.01±0.01	240.66
1.5	1.5	0.37±0.03	2.79±0.02 CY	1.28±0.03	266.36
2.0	1.5	0.33±0.03	3.28±0.15 CY	1.38±0.27	318.41
0.5	2.0	0.35±0.03	1.01±0.11 CY	0.37±0.03	67.15
1.0	2.0	0.37±0.04	1.44±0.10 CY	0.56±0.05	118.55
1.5	2.0	0.35±0.03	2.43±0.10 CW	1.12±0.06	229.52
2.0	2.0	0.36±0.06	2.74±0.11 CW	1.30±0.08	261.13

CW: compact light white, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Table 4: Cumulative impacts of BAP+Kn+GA_3 on the proliferation and growth of callus following secondary culture (2.0 mg/l GA_3).

BAP (mg/l)	Kn (mg/l)	Callus explants fresh wt. (g)	Callus fresh wt. at 8 weeks (g)	Callus dry wt. at 8 weeks (g)	Growth index (%) at 8 weeks
0.5	0.5	0.37±0.04	2.09±0.13 CW	0.75±0.05	191.27
1.0	0.5	0.39±0.04	2.33±0.05 CW	0.93±0.03	216.46
1.5	0.5	0.33±0.05	2.40±0.02 CG	0.98±0.01	226.65
2.0	0.5	0.39±0.03	2.52±0.04 CG	1.26±0.02	236.33
0.5	1.0	0.37±0.08	2.62±0.04 CW	0.94±0.01	247.59
1.0	1.0	0.33±0.04	2.74±0.05 CY	1.13±0.02	262.73
1.5	1.0	0.35±0.05	2.82±0.05 CG	1.21±0.02	270.31
2.0	1.0	0.32±0.07	2.89±0.01 CG	1.33±0.02	278.59
0.5	1.5	0.35±0.07	3.21±0.03 CW	1.14±0.02	310.98
1.0	1.5	0.36±0.06	3.34±0.04 CY	1.35±0.04	323.26
1.5	1.5	0.36±0.06	3.59±0.01 CG	1.67±0.05	348.95
2.0	1.5	0.34±0.03	3.67±0.03 CY	1.81±0.02	358.19
0.5	2.0	0.45±0.20	5.18±0.06 CG	1.79±0.02	509.48
1.0	2.0	0.35±0.08	5.94±0.04 CG	2.84±0.13	589.03
1.5	2.0	0.36±0.05	5.80±0.01 CG	2.61±0.01	573.96
2.0	2.0	0.34±0.05	5.74±0.09 CY	2.74±0.17	568.63

CW: compact light white, CG: compact light green, CY: compact light yellow. A total of 36 explants were used, and experiments were repeated three times to collect data.

Figure 2: Various stages of mini rhizomes and root initiation in MS media supplemented with 2.5 mg/l Kn from callus.

Conclusion

In MS medium supplemented with 2,4-D+Kn and 10% coconut water, callus formation can be initiated from leaf segments. The most effective plant growth regulators for callus proliferation in MS medium are kinetin and the combination of BAP+Kn+GA_3, surpassing BAP and TDZ alone, and the combination of BAP+NAA+GA_3. Kinetin alone also supports the differentiation of callus into mini rhizomes with adventitious roots. While increased concentrations of BAP, TDZ and Kn alone, as well as the combination of BAP+Kn+GA_3 in MS medium, elevate callus proliferation, no distinct pattern of proliferation is observed following subculture in MS medium with NAA+BAP+GA_3. The protocol devised for in-vitro callus induction and growth from leaf segments, utilizing elicitors and precursors, holds promise for the synthesis and enhancement of crucial bioactive compounds. Furthermore, the callus can serve as a source for organogenesis and somatic embryogenesis, facilitating plant regeneration.

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Research Article

Chemical and Morphological Characterization of Crinis Carbonisatus

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Abstract

Crinis Carbonisatus, prepared by pyrolysis of human hair, is known as a traditional Chinese medicine used for increasing blood clotting and wound healing. Its uses have been explored in literature but no detailed structural study is yet reported. This work is aimed at studying the chemical and morphological variation of Crinis Carbonisatus under given heating conditions. Crinis Carbonisatus was obtained after pyrolyzed of human hair at 300 °C in a sealed ceramic pot. The obtained samples were characterized in terms of their physicochemical properties by scanning electron microscopy (SEM), Fourier Transform Infrared (FTIR) spectroscopy and X-ray Diffraction (XRD). Distinct morphology with nanoparticulate structure was observed on the SEM micrograph. FTIR spectroscopy of the samples revealed the presence of functional groups like –OH, -COO$^-$, and -NH as well as methyl (-CH_3) and methylene (-CH_2-) groups. The nanoparticulate graphitic form was confirmed by XRD. It has been found that with the increase in pyrolysis time; the amorphous nature of the Crinis Carbonisatus materials increases while their particle size decreases.

Keywords: Crinis Carbonisatus, FTIR, Human hair, Pyrolysis, SEM, XRD

Introduction

Hair is a keratin filament having three major compartments; cortex medulla and cuticle. It is a characteristic feature of mammals and usually grows to cover a body part or the whole. The cuticle is the outer covering of hair fibre composed of overlapping flattened cells (Bhushan, 2010; Dawber, 1996). The cortex having cortical cells, comprises the middle layer of the hair which contains macro fibrils of the keratin and matrix proteins. The medulla, the innermost layer has cells with air spaces and amorphous materials. This layer is responsible for the hardness of hair filaments. Overall, hair fibres are keratinized dead cells (Swiff, 1977). Biomolecules of the hair include keratin (alpha and beta) protein, matrix protein, lipid, melanin and water. In addition to basic organic elements carbon, oxygen, hydrogen, nitrogen and sulphur these biomolecules of hair contain trace metals like zinc, magnesium, copper, cobalt, chromium, and nickel (Rouse, 2010). The concentration of some of these metals may vary with age and sex as well as colour (Henry, 1969). Matrix protein contains 20 to 50 amino acids which are structured in helical form. Serine, cystine, methionine, threonine, glutamic acid, citrulline, cysteine, proline, tryptophan, glycine, alanine, valine etc. are the major amino acids. Functional groups like carboxylic acid (-COOH) and amino (-NH_2) group are present as peptide (CONH) group along with carbonyl (=CO) and imino (NH) group in amino acids. Among these, cystine is the most abundant amino acid in the hair filaments. Cystine imparts disulphide linkage between two nearby helical chains of protein. The matrix proteins having helical chains that are responsible for disulphide bonding are termed keratin-associated proteins. Although other binding structures like salt bond and hydrogen bonding are also present between two helices, disulphide bonding has the most remarkable effect (Rogers, 2006; Schweizer, 2006).

Despite having the same composition, the thickness and morphology of hair can vary with the geographical origin of the people. Human hair of Asian, African and Caucasian origin has diameters of 100 µm, 80 µm, and 50 µm respectively. Also, the outer cuticle of hair from an Asian has a more folded structure than that of others (Bhushan, 2010). Moreover, even a single strand of hair has a different structure on its tip, middle and near scalp region. Diameter and cuticle deposition gradually decrease from root to tip. Cuticle may even be absent in the tip due to mechanical stress, friction and combing (Wei et al., 2005).

Human hair is not bio-degradable and is considered a waste. Burning of hair in an open environment generates harmful gases like ammonia, carbonyl sulphide, hydrogen sulphide, sulphur dioxide, phenols, nitriles, pyrrole and pyridines. This also imparts unpleasant and foul odours on the locality (Brehu & Spiridon, 2011; Gupta, 2014; Robbin, 2012). However, controlled heat treatment of such municipal waste provides an alternative way to minimize pollution. Heating rate, temperature and quenching phenomenon decide the resultant products. Pyrolysis and gasification are used to produce carbon products from waste such as fuels, chemicals, and solvents (Chen et al., 2015; Sorum et al., 2001). Pyrolysis is a method of changing any organic matter to obtain an array of solid, liquid and gas products by controlled heating. Based on the temperature range, pyrolysis is of three types; low temperature (< 550 °C) pyrolysis, moderate temperature (550 °C to 800 °C) pyrolysis and high temperature (> 800 °C) pyrolysis. Char, gas and tar are the final product of pyrolysis. Char from the waste has a large surface area and porosity which can be used for the manufacturing of active carbon (Klass, 1988; Savova et al., 2001).

Pyrolysis of human hair yields a black, shiny solid product at low and moderate temperatures. The use of pyrolyzed hair was first reported by a Chinese herbalist Li Shi-Zhen in his book Ben Cao Gang in the 16th century as a medicine. It was named *Xue Yu Tan* and termed *Crinis Carbonisatus* in English (Rause, 2010). This is still used in some parts of South Asian countries for fast relief as well as long-term recovery of wounds (Chaudhari et al., 2014; Saurez et al., 2002; Qian et al., 2014)

Chemically modified pyrolyzed hair has been reported in the literature. Guo et al. (2016) prepared samples at 200°C by hydrothermal treatment and reported the presence of carbon, nitrogen and oxygen by X-ray photoelectron spectroscopy. Fourier Transform Infrared (FTIR) results of the same work revealed the presence of carboxylic, alkyl, aryl, and amino functional groups (Altuntaş et al., 2019, Shaikh, 2009). Chaudhari et al. (2014), and Qian et al. (2014), reported the crystalline nature of the pyrolyzed sample at 800 °C as confirmed by XRD. The crystalline behaviour of the sample increased with an increase in carbonization temperature (Akhtar et al., 1997).

However, Altuntaş et al. (2020) found that the crystalline nature decreases with an increase in pyrolysis temperature when the hair sample is activated by $ZnCl_2$. Transmission Electron Microscopy (TEM) images of hydrothermally prepared carbonized samples showed a quantum dot of size 2-10 nm. However, TEM images of the pyrolyzed sample (800 °C) revealed the presence of the micro/mesoporous channel-like texture. While investigating the same sample by Scanning Electron Microscopy (SEM), images displayed graphite-like carbon flakes structure with a high level of disorder. This heteroatom-doped carbon material had dimensions in the nanometer range with high surface area and better porosity (Shaikh, 2009). Due to these properties, it is useful for intensifying oxygen reduction reactions in fuel cells and increasing the electrochemical performance in supercapacitors (Chaudhari, 2014; Qian et al., 2014).

Rodriguez et al. (2020) obtained the hair char from the Tannery process at 300 °C, 350 °C, 400 °C and 450 °C and used this for the removal of drugs amoxicillin (AMOX) and diclofenac (DFC) from wastewater. It has been found that physicochemically processed char at 450 °C removes more than 90% of AMOX and more than 80% of DFC. Anom & Lombok (2022) studied the reaction kinetics in the pyrolysis of human hair waste and found the activation energy values $81.769x10^3$ kJ/mol and $30.487x10^3$ kJ/mol for unwashed and washed samples, respectively.

The works so far reported have been carried out on chemically modified pyrolyzed human hair. However, works related to the structure and properties of *Crinis Carbonisatus* have not been reported in the literature so far. This work is focused on the pyrolysis of hair under different heating conditions to obtain the *Crinis Carbonisatus* without any chemical modification and hence on the investigation of the physicochemical properties of the substance.

Materials and Methods

Materials

Black-coloured human hair was collected from the donor (a seventeen-year-old young girl). A hot air oven, muffle furnace, narrow mouth ceramics pot with lid, mortar and pestle were used to pyrolyzed hair. Chemicals used in this work were acetone, ethanol and distilled water which were purchased from Thermo Fisher Scientific, India, Ltd.

Characterization techniques

FTIR spectroscopy: To study the vibrational nature of different bonding and functional groups of the sample, we carried out FTIR spectroscopic measurements. For this, we utilized the IRTracer-100 spectrometer of the Shimandzu Company with the serial number A217053.

SEM: SEM experiments were carried out for both fresh neat hairs as well as pyrolyzed samples to study their morphology using an FEI NanoSem 200 (FEI, Eindhoven/The Netherlands) at 2 kV and a working distance of approximately 9.5 mm.

XRD: XRD was done to study the morphology, structure and crystalline behaviour of the samples. This was done by D_2 phaser Bruker X-ray Diffractometer with 0.154 nm X-ray.

Sample preparation

The hair sample was washed with double distilled water and then soaked in a mixture of ethanol and acetone (1:1) for 24 hours to remove any dye if present. After the cleansing process hair sample was dried at 60 °C in a hot air oven and cut into small pieces of size about 3 mm.

The hair sample was then pyrolyzed using a muffle furnace at controlled conditions. At first narrow-mouth ceramic pot with a tight lid was collected from the local market. The pot was heated on the burner to remove gaseous matter if any inside the pot. Then, the dried sample was kept in the pot and the lid was sealed using cement. At first, the sample was prepared by a gradual increase of temperature up to 600 °C in the presence of oxygen. It was carefully observed and found that there was no gas evolution at 200 °C. So, the temperature was further increased and the fume started at 240 °C and stopped at 400 °C. When the sample was heated for 5 hours at 600 °C and then cooled gradually to room temperature, the resultant material was a white powder.

Therefore, it was decided to prepare a pyrolyzed sample at 300 °C to get sample properties like *Crinis Carbonisatus* as mentioned in Ben Cao Gang. Three different samples named PH-1, PH-2, and PH-24 were prepared at 300 °C with different time intervals. PH-1 was heated for 1 hour, PH-2 was heated for 2 hours and PH-24 was heated for 24 hours to study the structural variation with different time frames. Thus, we used a ceramic pot to prepare the samples, instead of the closed iron vessel used to prepare *Crinis Carbonisatus.* These samples were then characterized.

The yield of the substance was calculated using the formula:

“% yield = wt. of product / wt. of hair sample × 100”

After pyrolysis, samples named PH-1, PH-2, and PH-24 were obtained. All samples were solid, black and glassy in texture. They were porous with some blisters beneath the surface. The average yield of the sample was found 35 %. It was found that the sample was insoluble in water but stable dispersion formed in glycerol.

Results and Discussion

The SEM image in Figure 1 shows the morphology of the hair filament (PH-0) at different magnifications. The low-magnification image shows that the hair has a diameter of 100 µm. This is similar to the diameter of other Asian hair used by Wei et al. (2005). When the same filament was observed at higher magnification, the well-developed folded structure of the cuticle surrounding the whole filament was seen.

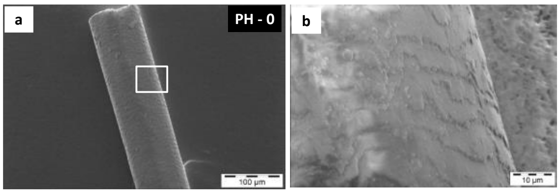

Figure 1: SEM images of the neat hair filament (PH-0) showing its morphology (a) at low magnification and (b) at high magnification.

Further, SEM was carried out on samples PH-1, PH-2 and PH-24 to study the structural variations on samples as the function of pyrolysis time. SEM images of these samples at low magnification reveal that all samples have similar morphological arrangements with irregular, wrinkled and uneven patterns consistent with previous reports (Qian et al., 2014). SEM micrographs also indicate that human hair changes into a crystal-like form after pyrolysis with some porous structure embedded in it. The morphological structures as well as arrangement are similar for PH-1 and PH-2 as shown in Figure 2.

However, the sample PH-24 at high resolution shows some crystal-like behaviour having a lamellar flake-like structure (Figure 3). These flakes are similar to cuticle flakes of untreated hair filaments whose structure may not have been lost during pyrolysis or the sample might have converted to graphitic form with the increasing time duration of pyrolysis. This indicates the trend of irregular structure of the sample gradually becoming regular with an increase in the heating time interval from sample PH-1 to PH-24. Similar results were reported in earlier work (Saurez et al., 2002).

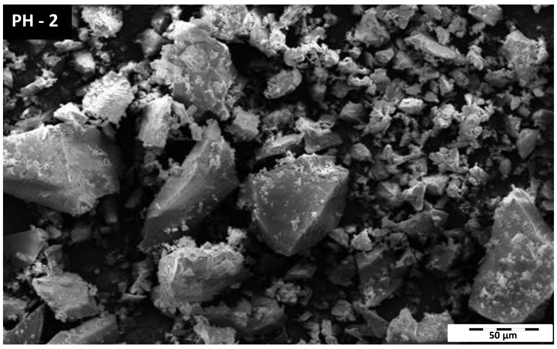

Figure 2: SEM image of PH-2 shows a crystalline-like appearance with some irregular crystal-like structure.

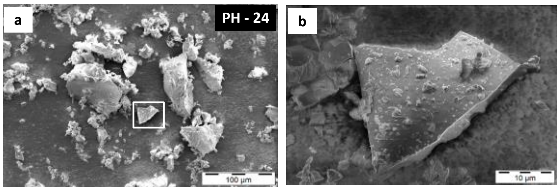

Figure 3: SEM images of PH-24 in Fig. 3a show the lamellar flake-like morphology which in magnified form in Fig. 3b shows the graphite-like flake structure.

FTIR spectroscopy is generally used to characterize the functional group of organic materials. Different bands are associated with the stretching and bending vibration of the different functional groups as can be seen in FTIR spectra in Figure 4. Three FTIR spectra of PH-1, PH-2 and PH-24 have many common peaks with different transmittance levels. The absorption band at 3750 cm^{-1} is associated with the N-H stretching which may be due to improper pyrolysis of the hair fiber. All the carbon samples have a wide band around 3250 cm^{-1} due to -OH stretching in hydroxyl functional groups (Liu et al., 2005).

Asymmetric and symmetric aliphatic methyl CH$_3$ can be reported by the band around 2924 cm^{-1} while the absorption peak at 2862 cm^{-1} is due to the aliphatic methylene **-**CH$_2$**-(**group (Sharma et al., (2002). The bands from 2800 cm^{-1} to 3000 cm^{-1} are due to the C-H stretching vibration and bands due to their deformation vibration generally appear from 1350 cm^{-1} to 1500 cm^{-1} (Grierson et al., 2011, Yorgum et al., 2001).

The absorption band at 2360 cm^{-1} is associated with the stretching of -C=C- bonding and 1604 cm^{-1} reveals the presence of -COO $^{-}$group, the aromatic and olefinic -C=C- vibrations in the aromatic region as well as carbonyl group -C=O**-** can be noted by the absorption band from 1600 cm^{-1} to 1800 cm^{-1} (Grierson et al., 2011; Jindo, 2014, Yorgum et al., 2001). Aromatic -C=C**-**C- and -C**-**H functional groups with plane bends in a ring stretching are justified by a 1454 cm^{-1} band. The OH stretching vibration of the phenol is reported between 1401 cm^{-1} to 1310 cm^{-1} (Sharma et al., 2002). The band due to functional groups -OH, -COO^{-} at 3749 cm^{-1}, 1605 cm^{-1} and 1365 cm^{-1} ascertains the presence of the carboxylic acid and its derivatives in the pyrolyzed sample (Altuntaş et al., 2019, Grierson et al., 2011). A peak around 750 cm^{-1} confirmed the presence of the sulphides along with monocyclic and polycyclic aromatic groups in the carbonized samples (Sharma et al., 2002) and Si-O-Si can be assigned to the 769cm^{-1} (Apaydin & Putun, 2012) the peak 671 cm^{-1} marked the presence of bending peak of -COO^{-} group in all samples (Herrera & Voladez, 2005).

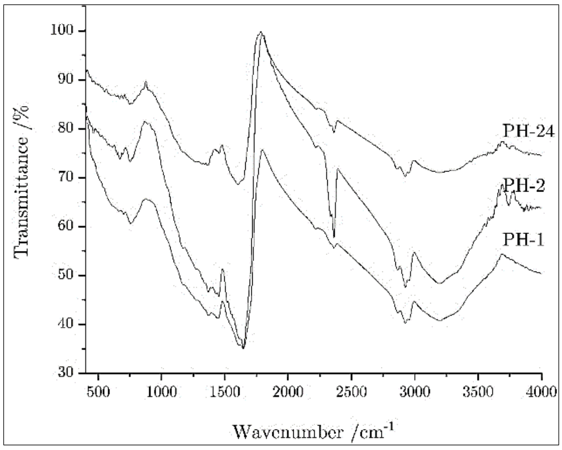

Figure 4: FTIR spectra of the Crinis Carbonisatus samples PH-1, PH-2 and PH-24 showing the presence of different functional groups.

The presence of peaks for sulphur, nitrogen, oxygen and hydrogen shows that although pyrolysis has brought a physical and chemical change in hair, the sample is not completely carbonized. The band of the N-H bond indicates that some parts of the protein moiety are still intact. In addition, the S-S bond between the cysteine residues shows that the footprint of the secondary structure of keratin is retained. In this regard, the presence of more stable groups such as hydroxyl, carbonyl and carboxyl in the pyrolyzed sample is expected. The peaks for these groups do not change significantly for all the samples; suggesting that at 300 °C, energy is not sufficient to onset the decomposition and the process is not rate controlled.

XRD spectra of the samples are given in Figure 5. Both samples PH-2 and PH-24 show one distinct peak. The peak at 26.8° for PH-2 slightly shifts to 25.8° for PH-24; this corresponds to an increase in the d spacing from 0.332 nm to 0.344 nm. This is in the range of graphite interlayer spacing distance (Sgriccia et al., 2008). The increase in interlayer spacing is associated with the addition of defects in the graphite lattice which leads to increased amorphous nature by pushing the layers away from each other (Bacon, 1951). This is also evident from the change of the peak profile from sharp and narrow to weak and broad. Assuming that the Scherrer relation holds, the particle size for PH-24 has decreased to 2.45 nm from 142.47 nm of PH-2. XRD results for increasing pyrolysis time show that the amorphous nature does not change significantly, similar results were also found by Altuntaş et al. (2020) for different pyrolysis temperatures.

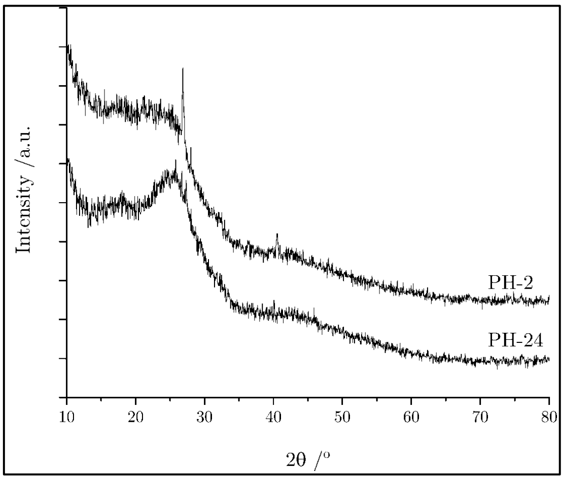

Figure 5: XRD spectra showing graphite interlayer spacing peaks at 26.8 °C and 25.8° as indicated.

Conclusion

In this study, we characterized the human hair as well as its pyrolyzed form (that is, the Crinis Carbonisatus). Morphology of the Asian hair showed the laminar, folded structure of the cuticle with a diameter of 100 µm which in pyrolysis form shows the laminar structural appearance with distinct fracture. FTIR showed the presence of N-H, C=O, O-H, C-H, $-CH_2$ and other functional groups in the pyrolyzed form of hair. XRD peaks confirmed the presence of the graphitic form which can be seen in SEM. Moreover, pyrolysis was found to convert the micron-sized particles into nanometric entities as evidenced in SEM as well as XRD results. Although Crinis Carbonisatus is mentioned as traditional Chinese medicine, here we cannot refer to its use as medicine. However, for pharmaceutical purposes, further elemental analysis as well as different biological tests are needed.

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Research Article

Pharmacological Activities of Six Species of *Hedychium* J. Koenig from Nepal

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Abstract

Species of *Hedychium* Koenig are perennial herbs. Some of the species like *H. ellipticum*, *H. spicatum*, *H. coronarium* are traditionally used as medicinal plants. In the present study, methanolic rhizome extracts of six different species of *Hedychium* namely *H. spicatum*, *H. ellipticum*, *H. thyrsiforme*, *H. coccineum*, *H. gardnerianum* and *H. coronarium* were analysed for their antioxidant, antidiabetic and antibacterial activities. The antioxidant activity analysed by DPPH assay showed the highest potential (lowest IC$_{50}$ value) in *H. coccineum* (148.82±2.83 µg/ml) and lowest potential (maximum IC$_{50}$ value) in *H. thyrsiforme* (996.55±9.42 µg/ml). The rhizome extracts of different species showed moderate α-amylase inhibition activity *in vitro*. The highest α-amylase inhibition (79.67%) was observed for *H. coronarium* while the lowest inhibition (64.0%) was observed in *H. thyrsiforme.* However, these values were found lower than the value (92.37%) obtained for positive control, i.e., Acarbose. The antibacterial activity was determined against two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) bacterial strains by agar well diffusion method. Except for *H. ellipticum* the extracts of all other species showed antibacterial activity against all the bacterial strains tested. The extracts of *H. ellipticum* showed antibacterial activity only against *B. subtilis* and *K. pneumoniae*. The extract of *H. coronarium* showed the highest zone of inhibition (16.67±1.15 mm) against *B. subtilis*. However, the antibacterial activity was weak compared to standard antibiotics for all the extracts and at all concentration tested. These results show that rhizomes of other species can also be used in the same manner as that of *H. coronarium* and *H. spicatum*, two species most used in various ethnomedicinal applications.

Keywords: Biological activity, Rhizome extract, *Hedychium*, Himalaya

Introduction

The species of *Hedychium* J. Koenig belongs to the family Zingiberaceae (Ginger family). These plants are native to Asia and distributed throughout tropical Africa and America (Chang, 2017). Out of around 65 species of *Hedychium* worldwide, 10 species are reported from Nepal (Rajbhandari & Rai, 2017). These species are distributed from tropical (150 m) to sub-alpine (3500 m) regions. Among the species reported from Nepal, five species namely *H. coccineum*, *H. coronarium*, *H. ellipticum*, *H. gardnerianum* and *H. spicatum* are reported to have been used in traditional medicine.

The rhizome of *Hedychium* has ethnomedicinal applications. Juice from the rhizomes of species like *H. ellipticum*, *H. gardnerianum* and *H. spicatum* is used in fever in Nepal (Manandhar, 2002; DPR, 2016). Decoction of rhizomes of *H. spicatum* is used in cough and cold by the Newar communities in Pharping, Nepal (Balami, 2004). Similarly, rhizomes of *H. spicatum* are used in stomachache, indigestion, loss of appetite, constipation, etc. by Raji communities in far western Nepal (Thapa et al., 2014). Additionally, it is also used in the treatment of dyspepsia, nausea and pain, tuberculosis, asthma, foul breath, bronchitis, hiccoughs, blood disease, and poor circulation (Rawat et al., 2018). Likewise, the rhizome of *H. coronarium* is also very popular in traditional medicine practice and used in diabetes and diphtheria (Bhandary et al., 1995), in headaches (Pattanaik et al., 2008), and in vomiting (Devi et al., 2014). The rhizomes of *H. coccineum* are used as medicine for swelling caused by bruises and wounds (Tushar et al., 2010, Basak et al., 2010). The rhizomes of *H. spicatum*, have been used in liver complaints, diarrhoea, inflammation, pain, snake bites, etc. (Tushar et al., 2010).

Rhizome extracts or essential oils from *H. coronarium*, *H. gardnerianum* and *S. spicatum* have been reported to show antioxidant, antidiabetic and antibacterial activity in various studies. Rhizome extracts and essential oils of *H. coronarium* and *H. gardnerianum* have antioxidant and antibacterial (Zhao et al., 2017; Ray et al., 2018). Antioxidant activity has also been reported in rhizome extracts of *H. spicatum* (Bag et al., 2015). Essential oil of *H. spicatum* (Reddy et al., 2009; Kaur & Richa, 2017) and rhizome extract of *H. coronarium* (Panigrahy et al., 2020) have also been reported to show antidiabetic activity. Similarly, the rhizome extract of *H. spicatum* (Lamichhane et al., 2014; Arora & Mazumder, 2017) and *H. coronarium* (Ho, 2011; Sah et al., 2012) has antibacterial activity. Antibacterial activity has also been reported in the essential oil of *H. spicatum* (Prakash et al., 2010), *H. coronarium* (Ray et al., 2018) and *H. gardnerianum* (Prakash et al., 2010).

Earlier studies have been confined mainly to selected medicinally known species such as *H. spicatum* and *H. coronarium* and focused more on the activities of their essential oils. Therefore, the present study is aimed to screen methanolic rhizome extracts of different species of *Hedychium* from Nepal for antioxidant, antidiabetic and antibacterial activities, and to compare such activities between ethnomedicinally important species and others.

Materials and Methods

Plant materials

Plant samples of five species were collected from Kathmandu and Parbat. Voucher specimens were deposited at TUCH for future reference. The details about the collection of *Hedychium* species are mentioned in Table 1.

Preparation of plant extracts and extract dilution

Rhizomes of *Hedychium* species (Figure 1) were cleaned, peeled, chopped and shade-dried separately. Rhizome powder (4 gm) of each plant sample was taken separately in Falcone tubes and 40 ml of methanol was poured into it. The mixture was sonicated in an Ultrasonicator (E-Chrom Tech, Taiwan UC-7240BDT) for 2 hours at 40°C. Each mixture was filtered by using filter paper (Whatman No. 1). The filtrate was collected, and the residue was sonicated again for 1 and ½ hours. The mixture was filtered and collected filtrates were mixed and then concentrated under reduced pressure by using a rota-evaporator (RE 100 PRO, DRAGON Lab, China). The concentrated filtrate was poured into a pre-weighed petriplate and left for drying under aseptic conditions. The dried extract was weighed and kept in 2 ml polypropylene tubes at -20°C.

Table 1: Different species of *Hedychium* used in the study and their locality.

S.N.	Plant Name	Locality	Altitude (m)	GPS coordinates
1.	*H. coccineum* Buch.-Ham. ex Sm.	Chalnakhel, Kathmandu	1415	27°37'57"N, 85°16'49"E
2.	*H. coronarium* J. Koenig	Kirtipur, Kathmandu	1311	27°40'56"N, 85°16'38"E
3.	*H. ellipticum* Buch.-Ham. ex Sm.	Chalnakhel, Kathmandu	1415	27°37'57"N, 85°16'49"E
4.	*H. gardnerianum* Shepperd ex Ker Gawl.	Chalnakhel, Kathmandu	1355	27°38'00"N, 85°16'45"E
5.	*H. spicatum* Sm.	Kyang, Parbat	1768	28°17'59" N, 83°41'8"E
6.	*H. thyrsiforme* Buch.-Ham. ex Sm.	Chalnakhel, Kathmandu	1415	27°37'57"N, 85°16'49"E

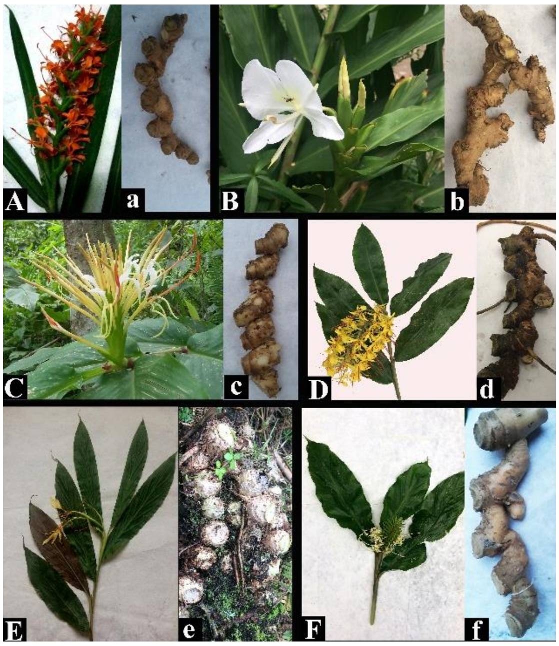

Figure 1: Plant specimen and rhizomes of selected *Hedychium* species: *H. coccineum* (A, a), *H. coronarium* (B, b), *H. ellipticum* (C, c), *H. gardnerianum* (D, d), *H. spicatum* (E, e), H. *thyrsiforme* (F, f). Uppercase: aerial part, and Lowercase: rhizome.

Antioxidant activity

For evaluating the antioxidant activity, the radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used following Blois (1958) with modification. A fresh solution of 0.2 mM DPPH in methanol was prepared. Different concentrations of ascorbic acid (10-100 µg/ml) or rhizome extract (25-200 µg/ml) were prepared in methanol. Then, 500 µl of sample (ascorbic acid or plant extract) was mixed with 500 µl of DPPH solution. The mixture was shaken well and placed in the dark for 30 minutes at room temperature. Then absorbance was measured at 517 nm. The blank was prepared by replacing plant extract or ascorbic acid with methanol. The percentage of free radical scavenging activity (RSA) of the plant samples was calculated by using the following formula:

% RSA = (control absorbance — sample absorbance) / control absorbance × 100

A curve was obtained by plotting the percentage RSA against concentration. Based on the standard curve, IC_{50} was calculated by using a linear equation of the curve obtained.

$IC_{50} = (0.5 - b)/a$

Where, X = Concentration, Y = % RSA, a and b are the coefficient and constant, respectively of the linear equation

α-amylase inhibition assay

The antidiabetic activity was measured by using a standard α-amylase assay based on increasing the reducing power of starch under the influence of the enzyme (Bernfeld, 1955) with modification. The reaction medium was prepared by dissolving porcine pancreatic amylase (Sigma Aldrich, Germany) in 0.1M potassium phosphate buffer (pH 6.8) to a final concentration of 0.1 unit/ml. Then 390 µl of reaction medium was added to 10 µl of either pure solvent (control/no inhibition) or acarbose (ARISTO Pharmaceutical Pvt. Ltd., India) or plant extracts solution in methanol (1 mg/ml) in two separate test tubes. In one of the test tubes (blank) 200 µl of DNS reagent (Sigma Aldrich, Germany) was added to the above reaction mixture. The tubes were pre-incubated at 37°C for 10 minutes. Then 200 µl of 1% soluble starch (Fisher Scientific, India) was added in all the tubes and incubated at 37°C for another 20 minutes. Then, 200 µl of DNS reagent was added to all the remaining tubes. The tubes were kept in a boiling water bath for 10 minutes and then allowed to cool. Then, 4 ml of distilled water was added to each tube and absorbance was taken at 540 nm in a UV/Vis spectrophotometer (E-Chrom Tech, Taiwan) using respective blanks. Inhibition of amylase activity was calculated by using the following formula.

% inhibition of amylase activity = (control absorbance - sample absorbance) / (control absorbance) × 100

Antibacterial activity

Bacterial strains: Various bacterial strains were obtained from Madhyapur Hospital, Thimi, Bhaktapur and the National Public Health Laboratory (GoN), Teku Kathmandu.

Gram-negative bacterial strains: *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 700603)

Gram-positive bacterial strains: *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (clinical sample)

Agar well diffusion method: The antibacterial test was performed by the modified agar well diffusion method (Perez et al., 1990). The rhizome extracts of each species were dissolved in DMSO to make a stock of 100 mg/ml. The stock was diluted to 50 mg/ml, 25 mg/ml and 12.5 mg/ml by serial dilution. The name of the bacterial strain, the name and concentration of plant extracts and the date were labelled on Muller Hinton Agar (MHA) plates (HiMedia Laboratories, Mumbai India). Then, six wells were prepared by using a cork borer in each petriplate; four wells for different concentrations of plant extracts, 1 each for DMSO (negative control) and 10 µg gentamicin (HiMedia Laboratory Pvt. Ltd., India) as a positive control. The sterilized filter paper discs and 1 gentamicin disc were placed in wells by using sterilized forceps. Then 30 µl extract of each concentration was poured into wells. The cotton swab was dipped in the fresh suspension culture in Nutrient Broth (HiMedia Laboratories, Mumbai India) adjusted to 0.5 Mcfarland standard, and swabbed on the labelled MHA petriplates under aseptic conditions. The petriplates were allowed to dry and closed tightly by using Parafilm (Bemis, USA). The plates were incubated on the microbial incubator overnight at 37°C and the zone of inhibition was measured by using a scale.

Data analysis

All data were taken in triplicate and statistically analyzed using Microsoft Excel.

Results and Discussion

Antioxidant activity

The antioxidant activity of ascorbic acid and plant extracts of different concentrations is presented in Figure 2 and Figure 3 respectively. Among the plant extracts, free radical scavenging activity was reported to range from 8.92±0.48% (*H. thyrsiforme*) to 62.32±3.05% (*H. coccineum*) at 200 µg/ml (Fig. 3). The free radical scavenging activity was increased with increasing concentration of the crude extracts.

The IC$_{50}$ value of selected species of *Hedychium* is shown in Figure 4. The IC$_{50}$ value was reported to range from 148.82± 2.83 µg/ml (*H. coccineum*) to 996.55±9.42 µg/ml (*H. thyrsiforme*). The extract with the lowest IC$_{50}$ value for *H. conninuem* was found to show the best antioxidant activity.

The antioxidants can reduce oxidative stress and help in the prevention and treatment of related diseases (Sun et al., 2018). The phenolic compounds (flavonoids, phenolic acids, stilbenes, tocopherols, tocotrienols), ascorbic acid, carotenoids and terpenoids are naturally occurring antioxidants in plants (Grassmann, 2005; Dubey et al., 2015). The free radical scavenging activity of *H. spicatum* was comparable to the study of Lock et al. (2005) at 50 µg/ml. A lower IC$_{50}$ value was reported in the present study for methanolic extract of *H. spicatum* rhizomes than in the study of Sravani & Paarakh (2012). The higher free radical scavenging activity of *H. spicatum* than *H. coronarium* in the present study supports the findings of Bag et al. (2015). Variation in radical scavenging activity in the present study was not comparable with previous findings due to the different concentrations of samples and solvents used (Ho, 2011; Zhao et al., 2017). The variation in IC$_{50}$ value among different species of *Hedychium* may be due to variations in phenolic compounds (Grassmann, 2005; Dubey et al., 2015; Bag et al., 2015).

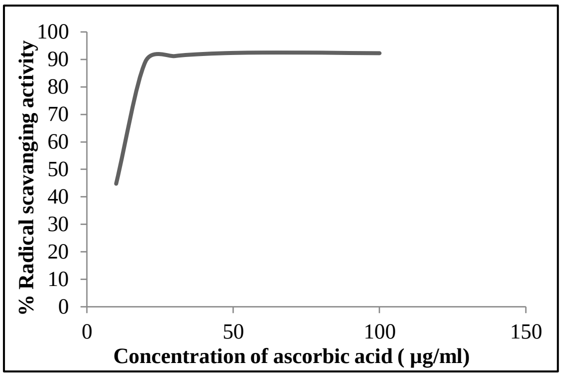

Figure 2: Standard curve of ascorbic acid.

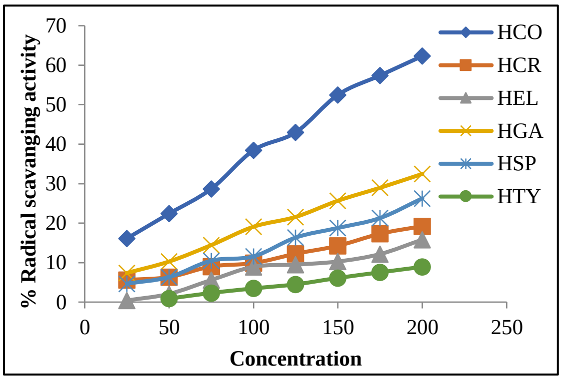

Figure 3: Percentage of free radical scavenging activity of extracts of *Hedychium* species. **Legend:** HCO- *H. coccineum*, HCR- *H. coronarium*, HEL- *H. ellipticum*, HGA- *H. gardnerianum*, HSP- *H. spicatum*, HTY- *H. thyrsiforme*.

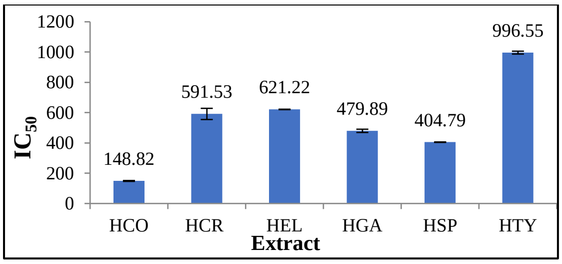

Figure 4: IC_{50} values of extracts of *Hedychium* species. **Legend:** HCO- *H. coccineum*, HCR- *H. coronarium*, HEL- *H. ellipticum*, HGA- *H. gardnerianum*, HSP- *H. spicatum*, HTY- *H. thyrsiforme*.

α-amylase inhibition activity

In general, all the tested species of *Hedychium* possessed moderate α-amylase inhibition activity (Figure 5). *H. coronarium* extract showed the highest (79.67%) while *H. thyrsiforme* extracts showed the lowest (64.0%) inhibition of α-amylase. Acarbose showed 96.19±1.28% at the same concentration. The percentage inhibition of α-amylase in extracts of all selected species was lower than that of acarbose, the standard antidiabetic drug.

Although the antidiabetic activity of *H. spicatum* and *H. coronarium* was studied earlier, the present study is not comparable due to differences in assay protocols and the use of bioactive compounds instead of crude extract (Reddy et al., 2009; Panighrhy et al., 2020). In the present study, moderate inhibition of in vitro α-amylase activity suggests that inhibition of enzymes of carbohydrate metabolism is possibly one of the mechanisms through which these plants show antidiabetic activities in vivo. This study supports the traditional use of *H. coronarium* rhizomes in diabetes. The result of the present study suggests that the rhizomes of other species can also be used in the same manner as that of *H. coronarium* for antidiabetic activity.

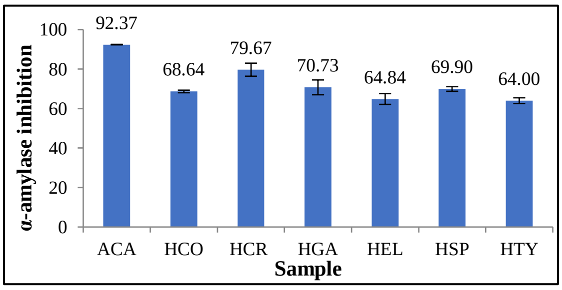

Figure 5: α-amylase inhibition activity of acarbose and extracts of *Hedychium* species (10 µg each). **Legend:** ACA- Acarbose, HCO- *H. coccineum*, HCR- *H. coronarium*, HEL- *H. ellipticum*, HGA- *H. gardnerianum*, HSP- *H. spicatum*, HTY- *H. thyrsiforme*.

Antibacterial activity

The antibacterial activity of methanolic extracts of rhizome of selected species of *Hedychium* against Gram-negative (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacterial strains are shown in Table 2. The extract of *H. coronarium* at 100 mg/ml showed the highest zone of inhibition against *S. aureus*, *B. subtilis* and *P. aeruginosa* i.e., 14.7±1.15 mm, 16.7±1.15 mm and 17.6±0.58 mm, respectively. Extracts of *H. ellipticum* did not even show inhibition of bacterial growth except in *B. subtilis*. Extracts of other species showed less inhibition zone against tested bacteria. In comparison to the positive control (10 µg of gentamicin), all extracts were found less effective. The antibacterial activity of extracts of tested species of *Hedychium* was much weaker compared to positive control even at very high concentrations of the extract.

The plants synthesize several classes of secondary metabolites, to defend against pathogens like fungus, bacteria, viruses and nematodes (Taiz & Zeiger, 2010). Some of these can be used as potent antibiotics to treat various infections. The agar well diffusion test method is one of the most practical methods routinely used to find out the antimicrobial potential of plant extracts. The same method has also been used in determining the antimicrobial potential of various species of *Hedychium*. However, the antimicrobial activities of rhizome extracts of *Hedychium* species have been carried out in different solvents like methanol, ethyl acetate, petroleum ether, dichloromethane, ethanol and water (Aziz et al., 2009; Bisht et al., 2006; Ho, 2011; Sah et al., 2012; Lamichhane et al., 2014) with methanol being the solvent of choice in most of the studies possibly due to its low cost and ability to dissolve most of the secondary metabolites. The zone of inhibition of different bacterial strains in rhizome extracts of *Hedychium* species have been reported in range of 11 to 18 mm for *S. aureus*, 15 to 18 mm for *B. subtilis* (Sah et al., 2012; Aziz et al., 2009; Ho, 2011), 10 to 19 mm for *P. aeruginosa* (Chen et al., 2008; Aziz et al., 2009; Ho, 2011) and 13 mm for *Klebsiella pneumoniae* (Sah et al., 2012). The findings of the present study are comparable to those of Aziz et al. (2009), Ho (2011) and Sah et al. (2012). Arora & Mazumder (2017), however, have reported higher antibacterial activity of *H. spicatum* extract. Since the secondary metabolites content in plant extracts is affected by several factors such as genotype, physiological status of the plant, harvesting period, extraction methods, any of these factors might have been the reason behind such discrepancies in the antibacterial activities of extracts of even the same species.

Table 2: Antibacterial activity of *Hedychium* extracts in different bacterial strains.

Rhizome extract	**Bacterial strain**	**Zone of inhibition in mm including diameter of a well (5 mm)**				
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	Gentamicin (10 µg)
H. coccineum	*K. pneumoniae*	9.3±0.58	7.7±1.15	5	5	23±0
	P. aeruginosa	9.7±1.15	8.0±0	7.0±0	6±0	22±0
	B. subtilis	10.7±0.58	9.7±0.58	7.0±0	7.0±0	23.0±0
	S. aureus	12±1.73	9.3±0.58	8.0±0	7.0±0	15.0±0
H. coronarium	*K. pneumoniae*	15±1	13±1	12±1.73	10.7±2.08	23±0
	P. aeruginosa	17.6±0.58	16.7±0.58	15.7±0.58	14.7±0.58	22±0
	B. subtilis	16.7±1.15	15.7±1.15	14.3±0.58	13.00	22.3±1.15
	S. aureus	14.7±1.15	13.0±0	11.0±0	10.0±0	15.7±1.15
H. ellipticum	*K. pneumoniae*	7±0	5	5	5	22.7±0.58
	P. aeruginosa	5	5	5	5	21±0
	B. subtilis	9.3±0.58	8.3±0.58	7.3±0.58	7±1	22±0
	S. aureus	5	5	5	5	15±0
H. gardnerianum	*K. pneumoniae*	11±1	9±0	8±0	7±0	23±0
	P. aeruginosa	10.3±0.58	9.3±0.58	8.3±0.58	7.3±0.58	22±0
	B. subtilis	10.7±1.15	9.7±1.15	8.7±1.15	7.7±1.15	22.3±0.58
	S. aureus	8.7±0.58	7.7±0.58	7.0±0	5	15.0±0
H. spicatum	*K. pneumoniae*	15.3±0.58	13±0.57	10±0	9±0.58	22.7±0.58
	P. aeruginosa	12±0	10.3±0.68	9.33±0.58	7±0	21.3±0.58
	B. subtilis	11.7± 0.57	10.7±0.58	9.0±1	5	22±1
	S. aureus	10±0	9±0	5	5	15±0
H. thyrsiforme	*K. pneumoniae*	11±0	9±0	8±0	6±0	23±0
	P. aeruginosa	11.3±0.58	9±0	8±0	5	21.7±0.58
	B. subtilis	11.0±0	10.0±0	9.0±0	8.0±0	19.5±0.58
	S. aureus	10.3±0.58	9.0±0	8.0±0	5	15.0±0

Conclusion

The present investigation of rhizome extracts of six species of *Hedychium* reveals weak antioxidant and antibacterial activity, and moderate α-amylase inhibition activity. Among the species, *H. coccineum* was the best in terms of antioxidant activity while *H. coronarium* was best in terms of antidiabetic (α-amylase inhibition) and antibacterial activity. Since the species showed antioxidant, antibacterial and antidiabetic activities, there is a possibility of using them as substitutes in traditional medicine.

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Research Article

In vitro Callus Regeneration and Chlorophyll Content Estimation in *Glycine max (L.)* variety from Uttarakhand, India

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Abstract

Soybean (*Glycine max)* is considered one of the most substantial produced crops across the globe because of its high nutritional value. It is a great alternative for lactose-intolerant patients and vegans to fulfil their daily protein requirements. Soybean has various varieties depending upon the colour of their seed coat. In the past few years, the consumption of Soybean has increased which demands higher production, better yield, and better seed quality. Conventional propagation methods fail to fulfil such demands. The alternate method of plant tissue culture ensures rapid mass propagation, better yield, and quality of the plants. However, the technique is often beset with challenges of low-field performance of tissue culture-raised plants due to defective chloroplast machinery. The present study investigates the effect of various plant growth regulators (PGRs) on *in vitro* propagation of soybean cultivars from different regions of Uttarakhand, India, and their effect on chlorophyll content in the regenerated tissues.

Keywords: Callus induction, Chlorophyll content, Carotenoids, *Glycine max*, Mass propagation

Introduction

Soybean is a major food crop belonging to East Asia. Soybean scientifically called *Glycine max (L.)* Merr. falls under the family Fabaceae and subfamily Papilionaceae. The seed coat colour of the Soybean seeds ranges from light/pale yellow to dark/black colour. The colour difference is considered because of the presence of anthocyanins, chlorophyll, and various pigments (Naresh et al., 2019).

Soybean was introduced by China in India in the tenth century past Himalayan routes as well as by Indonesia via Burma (now Myanmar). The Kumaon region of Uttarakhand, Himachal Pradesh, the Naga Hills, Eastern Bengal, Madhya Pradesh, and the Khasi Hills are known to cultivate Soybean ages on a very small scale (Hymowitz, 1990; Khoshoo, 1995; Agarwal et al., 2013). According to Parmar & Devi (2021) during the year 2018-19, India produced approximately 11 metric tons of Soybean, ranking fifth in the worldwide ranking of soybean production after China.

Earlier soybean was essentially used for its high oil content (18-22%) (Naresh et al., 2019) but now apart from high oil content, soybean is also a high protein source (40-42%) (Naresh et al., 2019). Nowadays, vegan products are high in demand and soybean products like Soy milk, “Tofu”, “Miso”, and Soy sauce are in great demand for being an alternative to dairy products also, soybean and their leftovers can be utilized in multiple ways such as leftovers of soy after oil extraction are used to feed animals. Soybean oil has been used in cooking for ages, apart from that various uses of soybean oil are in cosmetics, varnishes, pharmaceuticals, paper, paints, inks, and pesticides (Pratap et al., 2012), and the oil can also be used as biodiesel, a renewable energy source in industries (Pratap et al., 2015). Soybean has high antioxidant properties, possesses anti-hypersensitive effects, aid in diabetes, maintain cholesterol levels, and prevent cancer.

Need for tissue culture of soybean

The demand for Soybean has increased rapidly in the past several decades leading it to become the fourth-largest oil crop in the world and it has been estimated that the demand will increase in the upcoming years for food, fuel, and feed (Raza et al., 2017). Several measures are being taken to improve the quality of the crop to curb the demand of rising global hunger via conventional breeding but the efforts are not as fruitful.

Plant tissue culture is one such biotechnological tool that has helped in refining the quality of Soybean plants. Rigorous research has helped in successfully establishing the tissue culture of Soybean via different explants such as seed/embryo, hypocotyl/epicotyl, cotyledons, leaf disc culture, etc.

Presence of chlorophyll in soybean callus

Chlorophyll is a green pigment found in plants that is essential for carrying out photosynthesis in plants by absorbing sunlight. Chlorophyll is found in five different forms among various organisms. Chlorophyll a and b are majorly seen in ferns, mosses, and higher plants while chlorophyll c, d, and e are found mostly in some bacteria and algae. Another pigment commonly known as carotenoid is also present in plants and fruits and imparts red, yellow, or orange colour. Carotenoids aid in passing absorbed light to chlorophyll and protect the chlorophyll from photo-oxidative destruction (Sudhakar et al., 2016). Since the plant tissue culture technique involves growing plantlets under controlled physicochemical conditions over a long period, more often the regenerated plantlets exhibit problems in chlorophyll synthesis. It, therefore, becomes important to establish a method that ensures improving chlorophyll content in regenerated plants.

The present study focuses on establishing contamination-free seed cultures of *Glycine max* varieties collected from different villages of Uttarakhand and the assessment of various pigments present in the *in vitro* generated callus.

Materials and Methods

Explant collection and treatment

Soybean seeds (Figure 1) were collected from different districts of Uttarakhand, India, and annotated as SC1 and SC2 from the Chamoli district, SP1 and SP2 from Pauri (Garhwal) district, SR1 and SR2 from Rudraprayag district, and ST1 and ST2 from Tehri district.

Figure 1: *Glycine max* seeds. Seeds with yellow coat (a), seeds with black coat (b).

Seeds were washed underneath tap water for 3-5 minutes to ensure that seeds were dust and dirt-free. Alcohol (70%) treatment was given to the seeds for 5 minutes, then rinsed with tap water 3-5 times. Liquid detergent (Teepol) treatment was given to the seeds for 10-15 minutes and then rinsed underneath tap water, seeds were then treated with Tween-20 for 15-20 minutes and rinsed thoroughly under running tap water. Fungicide (1% Bavistin) treatment was given to the seeds for 20-25 minutes and then rinsed under tap water. Seeds were surface sterilized thoroughly with 0.1% mercuric chloride ($HgCl_2$) for different durations to achieve contamination-free cultures and finally were rinsed with autoclaved distilled water.

Culture initiation and conditions

After surface sterilization, seeds cultures were established on a culture medium (MS medium) consisting of a carbon source (3% sucrose), gelling agent (6 g w/v agar), and various PGRs at different concentrations such as cytokinin (0.5-1.0 mg/l) BAP for culture establishment. The pH of the media was set at 5.8 with NaOH (1N) or HCl (1N). The nutrient medium was autoclaved at 121℃ temperature, 15 psi pressure for 15 minutes. Cultures were maintained at 25±2℃ with 60-65% relative humidity (RH) under 16/8 light-dark conditions in the culture room.

To initiate callus, *in vitro* cotyledonary leaves of generated seeds (SC1, SC2, SP1, SP2, SR1, SR2, ST1, and ST2) were inoculated on MS medium supplemented with 3% sucrose, 6g w/v agar, and different amount of PGRs. PGRs used in callus induction were BAP (0.5-2.0 mg/l), Kn (0.5-1.0 mg/l), and 2,4-D (0.5-1.0 mg/l).

Callus multiplication

Callus initiated from *in vitro* cotyledonary leaf was further multiplied by transferring it onto MS medium consisting of 3% sucrose, 6 g w/v agar, and different PGRs at various concentrations and various amalgamations. PGRs included in the media were BAP (0.5-1.0 mg/l), IAA (0.5-1.0 mg/l), Kn (0.5-1.0 mg/l), TDZ (0.5-1.5 mg/l), NAA (0.5-1.0 mg/l), and 2,4-D (0.5-1.0 mg/l).

Quantification of chlorophyll and carotenoids

Multiplied callus of the seed varieties generated *in vitro* were tested for the presence and concentration of chlorophyll a, b, and carotenoids. Chlorophyll is loosely bound to proteins and thus, can be extracted easily in the presence of organic solvents such as DMSO (Dimethyl sulfoxide), acetone, or ether. The samples were macerated and 80% acetone was added to the macerated sample so that chlorophyll would dissolve in it. For maximum absorption of chlorophyll(a) the suitable wavelength is 663nm and for chlorophyll(b) is 645 nm. The OD (optical density) of the samples was taken at these wavelengths for the estimation of chlorophyll(a) and (b) content in the samples (Arnon, 1949). For carotenoids, the suitable wavelength is 663 nm, 645 nm, and 480 nm (Price & Hendry, 1991; Kamble et al., 2015). The formulas used to estimate the content of pigment are shown in Figure 2.

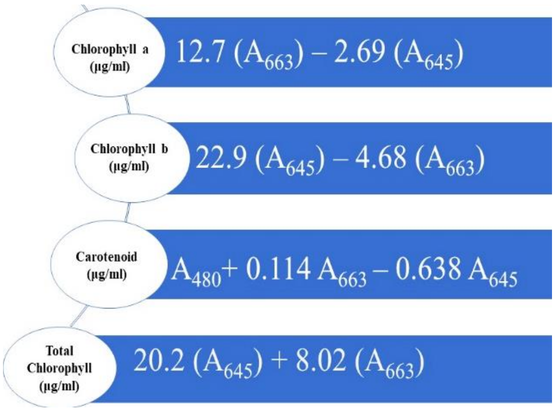

Figure 2: Formulas for the estimation of pigment content in the callus.

Results and Discussion

Callus induction

In the current study, seed samples of different varieties of Soybean were collected from various districts of Uttarakhand, India. The seed samples were inoculated on the nutrient medium to obtain cotyledonary shoots and leaves from the seeds. Surface sterilized seeds were inoculated on a nutrient medium. The best media for cotyledonary shoot induction was found to be MS + BAP (1.0 mg/l) as shown in Figure 3a, 3b and 3c. Raza et al. (2017) reported that the best seed germination medium for *Glycine max* was Gamborg B5 basal medium supplemented with BAP (1 mg/l).

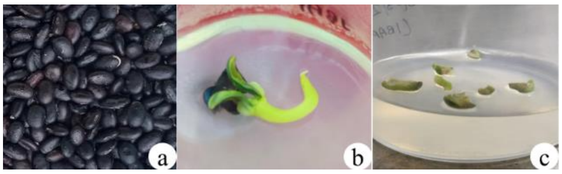

Figure 3: Cotyledonary shoot induction and inoculation for callus induction. *Glycine max* seeds (a), cotyledonary shoot formation from seed (b) and cotyledonary leaves inoculation (c).

In vitro cotyledonary leaves obtained from seed samples SC1, SP1, SP2, and ST1 were inoculated on a culture medium to induce callus. It was concluded from the present study that various PGRs have different effects on callus induction as well as on the colour of the callus.

Table 1: Different media combinations for callus induction and its properties.

BAP (mg/l)	Kn (mg/l)	TDZ (mg/l)	2,4-D (mg/l)	IAA (mg/l)	NAA (mg/l)	Degree of callus				Colour of callus			
						SC1	**SP1**	**SP2**	**ST1**	**SC1**	**SP1**	**SP2**	**ST1**
-	-	1	-	-	-	+				Pale green			
1	-	-	-	0.5	-	++				Light green			
-	-	-	0.5	-	-	+++				Dark green			
1	-	0.5	-	-	-	++				Light green			
1	-	1	-	-	-	+				Green			
1	-	-	1	-	-	+++				Dark green			
0.5	-	-	-	-	-		++				Light green		
1	-	-	-	-	-		+				Pale green		
1	-	-	-	0.5	-		+++				Dark green		
-	-	-	0.5	-	-		++				Light green		
1	-	1	-	-	-		+				Pale green		
-	-	0.5	-	-	-			++				Light green	
-	-	1	-	-	-			++				Light green	
0.5	-	-	-	-	-			+				Pale green	
1	-	-	-	-	-			++				Light green	
1	-	-	-	0.5	-			+				Pale green	
-	-	-	0.5	-	-			++				Light green	
1	-	1	-	-	-			+++				Dark green	
1	-	0.5	-	-	-			++				Green	
1	-	-	1	-	-			++				Green	
1	0.5	-	-	-	-				+				Pale green
1	1	-	-	-	-				+				Pale green
-	-	1	-	-	-				+				Pale green
0.5	-	-	-	-	-				+				Pale green
1	-	-	-	0.5	-				+				Green
1	-	1	-	-	-				++				Green
1	-	-	1	-	-				++				Green
1	-	-	-	-	-				+++				Dark green
1	-	1	-	-	-				+++				Dark green

For the cotyledonary shoot induction, various combinations of PGRs were tried as shown in Table 1, the best media was found to be MS medium containing BAP (1.0 mg/l) + 2,4-D (1.0 mg/l) for SC1, BAP (1.0 mg/l) + IAA (0.5 mg/l) for SP1, BAP (1.0 mg/l) + TDZ (1.0 mg/l) for SP2, and BAP (1.0 mg/l) for ST1.

The various PGRs had different effects on the colour of the callus, the callus obtained had varying shades of green colour from dark green to pale green as shown in Figure 4a, 4b and 4c. In a study conducted by Kim & LaMotte (1987), 77% of embryoids from cotyledon explant were generated in the presence of 4.5 µM Dicamba when cultured on MS medium, consisting of 2% sucrose. It was reported by Radhakrishnan & Ranjithakumari (2007) that callus induction from the half-seed of Soybean when cultured on B_5 medium consisting of a variety of PGRs such as 2, 4-D, BAP, Kn, NAA, and IAA. B_5 medium supplemented with 13.3 µM BAP+ 13.5 µM 2, 4-D was proven best to induce callus, and B_5 medium containing 13.3 µM BAP gave the best results for plant regeneration. Ether et al. (2013) reported the best callus induction in *Glycine max* from half seed explants on MS medium augmented with BAP (2 mg/l) + 2,4-D (5 mg/l). In a study conducted by Faraz et al. (2019), callus was successfully induced from the cotyledonary node and leaves of *G. max* when cultured on MS medium containing 2,4-D (0.5-4.5 mg/l) with Kn (0.2-2.5 mg/l), NAA (0.1-5 mg/l) and BAP (1-2 mg/l). Maximum callus weight was observed in 2,4-D (4 mg/l) in the leaves (0.73 g) and cotyledonary node (0.8 g). Apart from this medium supplemented with BAP + NAA at 2 mg/l and 3 mg/l concentration also resulted in the highest callus weight. Furthermore, callus culture has shown positive results in the development of somaclonal variations in the Soybean for the development of a better-quality variety that can be stress-tolerant or might have raised nutrient content (Singh et al., 2020). Callus was multiplied by sub-culturing on MS medium after every 15 days. Cytokinin alone or in combination with auxins can be used to induce callus in *Glycine max* explants for mass propagation.

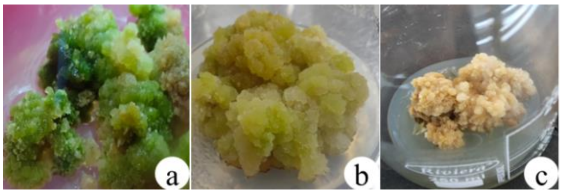

Figure 4: Callus induction from the cotyledonary explant. Dark green callus (a), light green callus (b) and pale green callus (c) of SP1, SP2 and SC samples.

Chlorophyll content

Different PGRs have different effects on the callus thus giving it a range of light to dark green color. Kresnawati (2006) reported that growth regulators play a major role in the callus colour of the explant. The colours of the different calluses obtained from various seed samples collected were quantified to calculate the concentration of chlorophyll(a) and (b) as well as carotenoids present in the seed samples. The estimation of pigments (chlorophyll a, b, and carotenoids) was done as shown in Table 2 after preparing an extract from the callus as shown in Figure 5a, 5b and 5c. The dark green and light green callus extract from each sample was taken for the estimation and pale green callus was found unsuitable for the estimation. The highest content of chlorophyll(a) (1.51 µg/ml), (b) (2.75 µg/ml), total chlorophyll (4.26 µg/ml), and carotenoids (2.03 µg/ml) were found in the sample ST1 which was collected from the Tehri region of Uttarakhand.

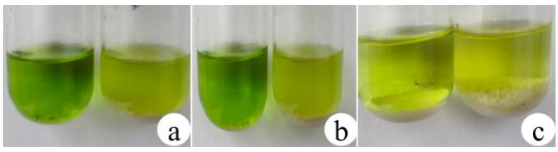

Figure 5: Extract of callus for pigment content estimation. Dark and light green of SP1, SP2 and SC samples (a, b and c).

Several studies have found a relationship between PGRs and chlorophyll content in plants. In an experiment conducted by Aldesuquy & Gaber (1993), it was found that pre-soaking soybean seeds in Kn resulted in enhanced chlorophyll(a) while soaking in other PGRs such as IAA or GA_3 did not show any significant outcome on the chlorophyll(a) content while presoaking in IAA decreased chlorophyll(b). The addition of cytokinin in the culture medium often shows a green colour because of cytokinin in the formation of chlorophylls (Widyawati, 2010). Dobránszki & Mendler-Drienyovszki (2014) reported that the type of cytokinin affects the content of chlorophyll(a) and (b). *In vitro*-raised apple leaves when treated with BA reported the highest chlorophyll content (1846-2176 µg/1g fresh weight of the leaf). Leupin et al. (2000) and Sari et al. (2018) stated high concentrations of cytokinin and light exposure play an active role in chlorophyll initiation. Sari et al. (2018) also reported that pale-coloured callus depicts the degradation of chlorophyll.

Table 2: Estimation of Chlorophyll(a), (b), total chlorophyll and carotenoids content of samples.

Callus colour	**Absorbance**			**Chlorophyll(a) (µg/ml)**	**Chlorophyll(b) (µg/ml)**	**Total Chlorophyll (µg/ml)**	**Carotenoids (µg/ml)**
	480 nm	645 nm	663 nm				
Light Green	0.096	0.098	0.099	0.99	1.78	2.77	0.044
Dark Green	0.144	0.145	0.145	1.45	2.64	4.13	0.068
Light Green	0.148	0.150	0.150	1.50	2.73	4.23	0.69
Dark Green	0.099	0.100	0.101	1.01	1.81	2.83	0.04
Light Green	0.025	0.023	0.025	0.25	0.40	0.66	0.01
Dark Green	0.140	0.141	0.141	1.41	2.56	3.97	0.06
Light Green	0.098	0.100	0.101	1.01	1.81	2.83	0.04
Dark Green	0.149	0.151	0.151	1.51	2.75	4.26	2.03

Conclusion

Conventionally, Soybean is propagated through seeds, and for better quality classical breeding is practised but it does not help in achieving the goal of mass production along with better quality. For this purpose, Soybean is raised via tissue culture. In the present study, various seed samples of Soybean were collected from different regions of Uttarakhand and were inoculated on MS+ BAP (1mg/l) for *in vitro* cotyledonary shoot induction and afterwards, *in vitro* leaves were inoculated on MS medium augmented with cytokinin with auxin or cytokinin alone for callus induction. PGRs also showed an effect on the colour of the callus which varied from dark green to light green in the presence of various cytokinins and auxins. Furthermore, chlorophyll(a) and (b), total chlorophyll and carotenoid content were estimated from the different colored callus extracts of seed samples and seed sample annotated as ST1 from Tehri region of Uttarakhand showed the highest chlorophyll(a) (1.51 µg/ml), chlorophyll(b) (2.75 µg/ml), total chlorophyll (4.26 µg/ml) and carotenoid content (2.03 µg/ml).

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Research Article

Association between Vitamin D Receptor FokI and BsmI Gene Polymorphism and Diabetes Mellitus in Nepalese Population

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Abstract

Vitamin D being involved in the secretion of insulin is a known fact. Moreover, studies have shown that steroids might be a factor in influencing insulin sensitivity. Vitamin D receptor (VDR), a factor required for genetic regulation involving vitamin D, thus can be regarded as a good candidate for Diabetes Mellitus (DM). Several studies have been conducted on the association between VDR polymorphism and the risk of DM but did not provide clear-cut answers. This study was conducted to search for the involvement of FokI and BsmI polymorphisms of the VDR gene with DM in a Nepalese population. A total of 200 blood samples were collected; 100 from clinically diagnosed DM patients and 100 from healthy controls. DNA was extracted from blood by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, where FokI and BsmI primers as well as restriction enzymes were used. After restriction digestion, SNPs of FokI (T/C) [rs2228570] and BsmI (A/G) [rs1544410] were assayed using agarose gel electrophoresis. As patients and controls were likened for genotype distribution and allelic frequencies, it was found that the frequency of VDR gene BsmI rs1544410 differed significantly ($p < 0.05$, each) between cases and control whereas A allele was dominant (91%) in healthy controls with Odd ratio (OR) of 0.55, unlike VDR FokI were not significantly associated between subjects and control. The data obtained from this research suggests that the VDR gene (especially BsmI) is associated with the risk of DM.

Keywords: Diabetes Mellitus, PCR-RFLP, Vitamin D Receptor

Introduction

Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to the heart, blood vessels, eyes, kidneys, and nerves. It is not limited to a single factor, studies have revealed genetics, obesity, heredity, lifestyle, high blood pressure, autoantibodies, age, abnormal cholesterol, triglyceride level, race, etc. as contributing factors for Diabetes Mellitus (DM) (Angel et al., 2018; Bonakdaran et al., 2012). Recently Vitamin D has gained popularity as a risk modifier for DM. Vitamin D acts by stimulating insulin receptor expression. It is involved in the Transcription activation of the Insulin receptor gene along with the vitamin D receptor (VDR) as a transcription factor. VDR is a nuclear, ligand-dependent transcription factor that is in complex with active Vitamin D, $1,25(OH)_2D_3$, controlling the synthesis of numerous different proteins involved in calcium transport and utilization (Kjalarsdottir, 2012). More than 25 polymorphisms of VDR have been reported to date. The most common polymorphisms of the VDR gene are the BsmI, FokI, TaqI and ApaI. FokI is located in exon 2 of the VDR gene and its polymorphism involves alteration in the start codon leading to the shortening of the VDR protein (van Etten et al., 2007). BsmI polymorphism is located in intron 8 at the 3′ end of the VDR gene and does not change the amino acid sequence of the VDR protein. However, there might be changes in gene expression through changes in intronic regulatory elements, alteration of mRNA stability, or disruption of splice sites (Vogel et al., 2002; Wang et al., 2017). Single nucleotide polymorphisms (SNP) in the vitamin D receptor (VDR) gene affect vitamin D synthesis, transportation and action and might hinder insulin secretion, causing insulin resistance (Sung et al., 2012). Different studies have described the significance of BsmI and/or FokI polymorphism association with DM (Angel et al., 2018; Sarma et al., 2018; Wang et al., 2017; El Gendy et al., 2019; Jia et al., 2013; Mukhtar et al., 2017; Iyer et al., 2017; Mohammadnejad et al., 2012).

It is a known fact that Nepal shares a central origin with India as well as China. Even lifestyle of the people of Nepal has been more or less similar to these countries for a very long period; it was relevant to check the occurrence of similar mutation in the diabetic population of Nepal. Moreover, no such molecular-level studies have been performed in Nepal which is why the study was designed to investigate the association between VDR gene polymorphism (Bsml and Fokl) with the occurrence of diabetes in Nepal. Furthermore, knowing the genetic aetiology of the problem to predict the progression of mutation, screening of family members and genetic counselling regarding the risk was the objective of the study. Diabetes is both hereditary as well as lifestyle-based disease but the exact connection of the disease with a gene is still unknown. Thus, this study takes limitations to identify whether VDR Bsml and VDR fokl were associated with diabetes or not to take a step further to relate diabetes with DNA.

Materials and Methods

A cross-sectional study was conducted at Annapurna Research Center, Annapurna Neurological Institute and Allied Sciences (ANIAS), and Om Hospital and Research Center, Kathmandu from July 2019 to February 2020. The study was undertaken only after obtaining approval from the Institutional Ethics Committee of ANIAS and written informed consent was obtained from all the participants. The study populations were both inpatients and outpatients with DM cases from different hospitals in Kathmandu Valley as well as healthy people not attending any hospitals. A total number of 200 people were enrolled in the study; among them 100 were diabetic patients and 100 were healthy controls.

Inclusion and exclusion factors for sample selection

Diabetes mellitus patients with elevated glucose levels or those with normal levels of glucose but were under medication were taken as DM cases. Similarly, healthy age and sex-matched controls with no history of T2DM and normal oral glucose tolerance test as per ADA criteria of diabetes and without any family history of diabetes mellitus in first-degree relatives were enrolled as controls. People with vitamin D supplementation within the past 3 months, patients on drugs altering Vitamin D levels (anticonvulsants, estrogen, cholestyramine, and orlistat), chronic liver disease and chronic kidney disease stages 3-5 were excluded.

DNA extraction and thrombophilic mutation

Blood samples from both normal and DM subjects were collected and DNA was extracted using a Promega kit (Promega Corporation, USA). Spectrophotometric analysis was carried out to check both the quality and quantity of DNA samples. Genotype screening was performed for the identification of the VDR gene among 200 patients and healthy controls. The VDR Bsml (A/G) polymorphism (rs1544410) gene of 825bp was amplified by polymerase chain reaction (PCR). During PCR, 25 µl reaction mixture contained 0.5 µl of oligonucleotide forward primer: 5′-CAACCAAGACTACAAGTACCGCGTCAGTGA-3′, 0.5 µl of oligonucleotides reverse primer: 5′-AACCAGCGGGAAGAGGTCAAGGG-3′ (Macrogen, Korea), 12.5 µl master mix (Solis Bio Dyne, Estonia), 3 µl of DNA template and the rest volume was adjusted by PCR grade water. As for negative control, a similar mixture was prepared but DNA was replaced by PCR grade water. A similar method was applied for the amplification of 265bp VDR Fokl (T/C) polymorphism (rs2228570) gene using forward primer: 5′-AGCTGGCCCTGGCACTGACTCTTGCTCT-3′ and reverse primer: 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′ (Macrogen, Korea).

The PCR tubes were placed on the tube holder of the thermo-cycler (ProFlex PCR System, Lifescience, Thermofisher, USA). For BsmI gene initial denaturation was performed at 94˚C for 3 minutes followed by 35 cycles at 94˚C for 20 seconds, 62˚C for 40 seconds, 72˚C for 1 minute and a final extension at 72˚C for 6 minutes (ProFlex PCR System, Thermofisher, USA). Likewise, for Fokl gene initial denaturation was carried out at 94˚C for 10 minutes followed by 35 cycles at 94˚C for 30 seconds, 60˚C for 30 seconds, 72˚C for 30 seconds and final extension at 72˚C for 10 minutes (ProFlex PCR System, Thermofisher, USA). For the confirmation of PCR amplification, 1.5% agarose gel was prepared in TBE (1X) buffer by adding ethidium bromide (EtBr-10mg/ml). The agarose gel was run at 60V for 90 min. Then, agarose gel was visualized under the gel doc system (UV Cambridge, USA).

Restriction fragment length polymorphism (RFLP)

RFLP of Bsm1 (rs1544410) gene: Restriction fragment length polymorphism (RFLP-PCR) was used to identify VDR genotypes. The amplified PCR product using Bsml primers was subjected to restriction digestion using Mva1269I (Thermofisher, USA) restriction endonuclease in 13 µl reaction volume contained: PCR product (1 µg/µl) 5 µl, buffer R (10X) 2 µl, restriction enzyme (Mva1269I) (100U/µl) 1 µl, nuclease-free water 5 µl. The mixture was incubated at 37˚C for 75 minutes followed by incubation at room temperature for 10 minutes. The digested DNA fragments were separated by agarose gel-electrophoresis in 2% agarose gel, run at 60 volts for 1 hour and 30 minutes and the bands were then examined under a UV light of gel documentation system (UV Cambridge, USA) as shown in Figure 1. Genotype was determined according to fragment length i.e. homozygote GG (BB) subjects = 650 and 172bp product; heterozygote GA (Bb) subjects = 825, 650 and 172bp products and homozygote AA (bb) subjects = 825bp product. SNP resulting in G to A substitution in VDR gene intron 8 leads to degeneration of a BsmI restriction site. Homozygous subjects with alleles containing nucleotide A show one band at 825bp, designated as having AA BsmI genotype. Homozygous subjects with alleles containing G show 2 bands of 650 and 172 bp were designated as GG. Subjects with heterozygote status showing 3 bands: 825, 650 and 172bp were denoted as GA (Tobón-Arroyave et al., 2017).

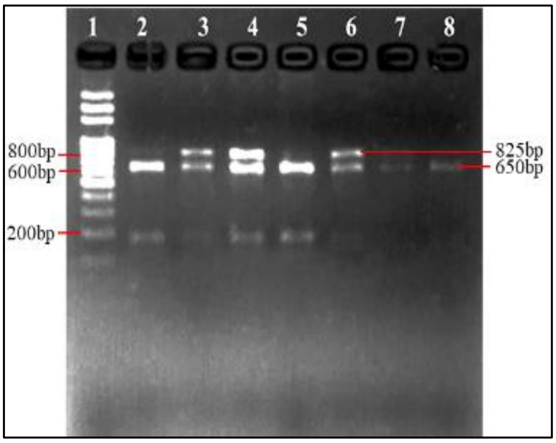

Figure 1: PCR reaction for VDR gene SNP BsmI with gene products size 825bp and the ladder marker were resolved on 2% agarose gel. RFLP analysis after digestion with BsmI restriction enzyme lane 1 in gel shows DNA ladder, lane 8 shows homozygous GG (B/B) wild type which is normal, lanes 2,3,4,5,6,7 showing heterozygous GA (B/b) mutation.

RFLP of Fok1 (rs2228570) gene: The PCR product after amplification using Fokl primers was digested by fast digest restriction enzyme (FOKL) (Thermofisher, USA) in 14.5 µl reaction mixture containing PCR product (1 µg/µl) 5 µl, green buffer (10X) 2 µl, FOKL 1 µl, nuclease-free water 7.5 µl. The mixture was incubated at 37˚C for 90 minutes followed by incubation at room temperature for 10 minutes. The digested DNA fragments were separated by agarose gel-electrophoresis in 2% agarose gel, run at 60 volts for 1 hour and 30 minutes and the bands were examined under a UV light of gel-doc system (Figure 2).

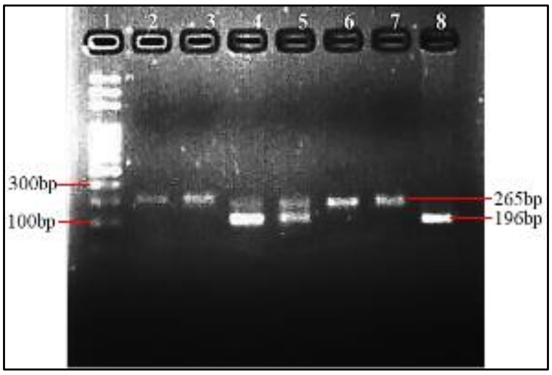

Figure 2: PCR reaction for VDR gene SNP Fokl with gene products size 265bp and the ladder marker were resolved on 2% Agarose gel. RFLP analysis after digestion with Fokl restriction enzyme lane 1 in gel shows DNA ladder, lane 8 shows homozygous TT (F/F) wild type, lanes 2,3,6,7 show homozygous CC (f/f) mutant, lanes 4,5 shows heterozygous TC (F/f) mutation.

VDR Fokl site is located in exon 2. After RFLP using Fok1 endonuclease fragments, 3 different lengths can be obtained. Fok1 genotype was determined according to fragment length, homozygote TT (FF) subject = 196, 69bp product; heterozygote TC (Ff) = 265, 196 and 69bp product and homozygote CC (ff) subject = 265bp product. SNP resulting in T-C substitution in exon 2 of the VDR gene leads to the degeneration of a Fok1 restriction site. Homozygous subjects with alleles containing nucleotide T at this position showed an intact 265bp band and were designated as having CC Fok1 genotype. Likewise, homozygous subjects with alleles containing C at this position show 2 bands of 196 and 69bp (TT subjects). Similarly, products with all 3 bands: 265, 196 and 69bp were assigned as TC (Vandevyver et al., 1997).

Statistical analysis

The percentage genotype distribution of polymorphism and frequency of heterozygous and homozygous were compared between cases and control with RFLP using SPSS version 20. The chi-square (χ2) test was used to evaluate the Hardy-Weinberg equilibrium for the genotype distribution of patients and controls. Allele and genotype frequencies were compared between different study groups using the χ2, Odds ratio (OR) and the Z-test were calculated using Statcalc program (Epi info version 6.0.4, Atlanta, GA, July 1996).

Results and Discussion

The descriptive characteristics of the 100 diabetic mellites (DM) patients and 100 healthy controls are shown in Table 1. The gender-wise distribution of the case participants included 54 (54%) males, and 46 (46%) females and the control were 58 (58%) and 42 (42%) (p=>0.050). The overall mean age of participants was 57.6 and 55.49 years old in the case and control groups (p=<0.05). There was no significant difference between mean age, weight, BMI, systolic, diastolic, hypertension, glucose (F), glucose (PP), cholesterol, LDL, HDL, serum TG and Vitamin D (p<0.05), whereas height and cholesterol were significantly difference with the subjects taken for cases and control (p>0.05). Similarly, the occupation and alcohol consumption were not significantly different whereas age group and sex were significantly different with case and control groups (Table 1). Since the mean weight for cases were higher BMI than that of controls. In the case of blood pressure, mean systolic pressure case was high, controls were found to have normal systolic pressure. The mean diastolic pressure however was at normal range for both cases and controls. The variants like glucose level (F/PP), Vitamin D level, serum Triglycerides level, and LDL were in the normal range for controls and higher than the normal range for cases. However, other variants like cholesterol level and HDL were in the normal range for both cases and controls (Table 1).

Out of 100 cases, 36% of DM subjects were found to have the GG genotype, 57% carried heterozygous GA genotype and only 7% had mutant AA genome with 13.51, 11.93 and 0.012 Odds ratio (OR) respectively. Furthermore, allele-A was dominant (91%) in healthy control and 35.5% in DM with an OR of 0.55 whereas the G allele was dominant (64.5%) in DM and 9% in healthy control with an OR of 18.37. The frequency of VDR gene BsmI rs1544410 was not significant ($p < 0.05$, each) between cases and control (Table 2). Similarly, 64% of DM subjects were found to have the TT genotype, 27% carried heterozygous TC genotype and only 9% had mutant CC genome with 1.39, 0.60 and 1.54 Odds ratio (OR) respectively. Furthermore, the T allele was dominant (77.5%) in DM and 75% in healthy controls with an OR of 0.56. The frequency of VDR gene FokI rs2228570 was significantly different between cases and control ($p > 0.05$, each) (Table 3).

Table 1: Socio-demographic and biochemical indices of diabetic Mellitus and healthy subjects.

Characteristics	**Group**	**Case-control study**		**Z-test / χ^2-test**	***p*-value**
		Diabetes Mellitus	Control		
Age (Mean±**SD,**Years)	Age (Years)	57.6±12.93	55.49±17.68	3.81	0.00
Age Groups	30-49	25	28		
	50-69	51	46	1.03	0.79
	70-89	22	24		
	>90	2	2		
Sex	Male	54	58	0.32	0.56
	Female	46	42		
Height (Mean±**SD,** ft)	Height	5.26±0.41	5.19±0.33	0.81	0.41
Weight (Mean±**SD,** Kg)	Weight	66.49±11.40	48.29±5.85	43.82	0.00
BMI (Mean±**SD,** Kg/m^2)	BMI	26.88±4.30	20.06±2.62	25.92	0.00
Occupation	Housewife	32	24		
	Government officer	8	14		
	Non-Government officer	16	16	34.62	0.00
	Retired	28	14		
	Farmer	9	6		
	Businessman	7	26		
Alcohol Consumption	Yes	3	63		
	No	69	15	272.48	0.00
	Occasional	13	17		
	Stopped	15	5		
Systolic (Mean±**SD,** mmHg)	Systolic	129.85±17.07	121.29±5.9	17.86	0.00
Diastolic (Mean±**SD,** mmHg)	Diastolic	81.40±11.04	80.51±4.09	0.28	0.02
Hypertension	Yes	59	5	67.0	0.00
	No	41	95		
Glucose (F) (Mean±**SD,** mg/dl)	Glucose (F)	141.91±55.01	89.70±12.07	63.74	0.00
Glucose (PP) (Mean±**SD,** mg/dl)	Glucose (PP)	201.03±79.48	90.30±6.5	119.41	0.00
Family Diabetes History	Yes	24	4	16.61	0.00
	No	76	96		
Cholesterol (Mean±**SD,** mg/dl)	Cholesterol	157.53 ±34.19	158.4±31.61	1.07	0.28
LDL (Mean±**SD,** mg/dl)	LDL	93.40±38.60	86.72±6.97	9.89	0.00
HDL (Mean±**SD,** mg/dl)	HDL	41.90±19	39.94±2.11	4.26	0.00
Serum TG (Mean±SD, mg/dl)	Serum	175.52±62.73	133.4±24.70	45.04	0.00
Vitamin D (Mean±SD, ng/ml)	Vitamin D	17.54±2.66	24.40 ±9.41	19.74	0.00

Diabetes is associated with numerous factors like age, heredity, viruses, obesity, blood pressure, cholesterol, physical activity etc. Our study also linked diabetes with hypertension, serum triglyceride level and low-density lipoprotein. These parameters were found to be normal in non-diabetic subjects, but their mean was higher than the normal range in DM subjects. DM is also linked with high alcohol consumption, but we found that a greater number of non-diabetic controls were involved in the activity rather than cases. This was probably due to the reason that alcohol consumption may lead to severe consequences for DM patients rather than healthy ones. Moreover, the stoppage rate of alcohol consumption was high in some cases.

Despite the various factors involved in the onset of DM, the VDR gene plays one of the major parts as the gene is involved in the secretion, sensitivity as well as protection of insulin-producing B-cell from inflammation (Issa, 2017). Bsml polymorphism of the VDR gene occurs in intron 8 where removal of the restriction site occurs as the G allele gets replaced by A creating 3 different lengths of gene (825bp, 650 bp and 172 bp) (Tobón-Arroyave et al., 2017) after restriction digestion of the product with the longest size indicating the mutation, AA genotype.

Table 2: Vitamin D Receptor (VDR) genotype and alleles at BSML rs1544410 position in T2DM patients and healthy control.

Genotype	**DM**	**Control**	**OR**	**95% Cl**	**p-value**
GG	36	4	13.71	4.65-40.41	0.0001
GA	57	10	11.93	5.55-25.61	0.0001
AA	7	86	0.012	0.007-0.03	0.0001
Alleles					
G	129	18	18.37	10.45-32.30	0.0001
A	71	182	0.55	0.03-0.11	0.0001

The AA genotype is associated with increased production of VDR protein leading to a decrease in the risk of DM whereas the presence of the GG genotype is associated with lower lever Vitamin D as well as the different affiliated diseases (Sinharay et al., 2018; Ferrara et al., 2002; Morrison et al., 1994).

Table 3: Vitamin D Receptor (VDR) genotype and alleles at Fok1 rs2228570 position in T2DM patients and healthy control.

Genotype	DM	Control	OR	95% Cl	p-value
TT	64	56	1.39	0.79-2.46	0.24
TC	27	38	0.60	0.33-1.09	0.09
CC	9	6	1.54	0.53-4.52	0.42
Alleles					
T	155	150	1.19	0.73-1.82	0.56
C	45	50	0.87	0.55-1.38	0.56

Our study showed that for the VDR-Bsml polymorphism, the A allele was dominant (91%) in healthy controls, and the VDR-Bsml genotype distribution in this group was 4% with GG 10% with GA and 86% with AA. For DM subjects G allele was dominant (64.5%) and VDR-Bsml genotype distribution was 36% with GG, 57% with GA and 7% with AA. The odds ratio was 13.71, 11.93 and 0.012 with respective GG, GA and AA genotypes between cases and controls. These results were consistent with other research as reported by a meta-analysis conducted by Zhang et al. (2012) where populations of different regions were analyzed including Asian, European and Latino populations indicating that the subjects included in the current study represented the group well.

In comparison with the controls, the frequency of the GG and GA genotypes was significantly higher in subjects with T2D, suggesting an association between the VDR-FokI genotypes “GG and GA” and T2D patients of the Nepalese population. A similar result was obtained in the study conducted in Northeast India (Sarma et al., 2018) and China as well (Xu et al., 2014). However, Santos et al. observed conflicting results in the Brazilian population (Santos et al., 2012). Similar results were obtained for VDR-BsmI, a report by García et al., where three polymorphisms in the VDR gene were studied, focusing on their influence on the immune response in Chilean children with type 1 diabetes. As in the publication by García et al., the data obtained in this investigation do not suggest an association of VDR-BsmI with T2D (García et al., 2007). Likewise, Fokl is located in exon 2 of the VDR gene and its polymorphism involves alteration in the start codon leading to the shortening of the VDR protein (van Etten et al., 2007). In the N-terminal region of the VDR gene, a mutation when occurs at the 1st of the two start codons, ATG changes the nucleotide sequence to ACG leading to the translation of 3 additional codons downstream, resulting in a protein with an additional 3 amino acid chain (Harris et al., 1997). However, the reason for the difference in the activity of the two proteins, for example, if they bind to 1,25-hydroxyvitamin D differently, is not yet clear; therefore, the molecular mechanism that marks the association between individuals carrying the T allele of VDR-Fokl and T2D has not yet been defined.

Like Bsml, Fokl polymorphism, the change from T to C allele, leads to the disappearance of the restriction site. However, the formed longer mutant genotype CC (625bp) is found to decrease the secretion of VDR compared to wild-type TT (196bp, 69bp) (Tobón-Arroyave et al., 2017). Specific signs of disc generation in Turkish (Eser et al., 2010), Brazilian (Nunes et al., 2007), and Finnish (Videman et al., 1998) population subjects with TC and CC genotypes showed a predisposition towards worst genotype. Our study showed that for the VDR-FokI polymorphism, the distribution of both T and C alleles was similar for cases and control. The VDR-FokI genotype distribution in case and control was 65% and 56% with TT, 27% and 38% with TC and 9% and 6% with CC respectively. The OR ratio for respective TT, TC and CC genotypes were 1.39, 0.60 and 1.64. This proves that Fokl Polymorphism resulted to be insignificant ($p<0.05$) with DM for Nepalese population. Similar to our results, the findings on two subpopulations of Spain and Iran reported no association of Fokl ($p<0.05$) polymorphism with T1D. Moreover, the frequency of the CT allele was reported to be higher in cases as compared to controls in the Iranian population (Bonakdaran et al., 2012; Audi et al., 2004). In contrast, the current study reported that Fokl polymorphism significantly increased the chances of disease development in the Pakistani population. Further, the frequency of the Fokl “C” allele was higher in cases in Hungary (Maahs et al., 2010). The findings on Dalmatian as well as Japanese populations were in line with the results of our study that the Fokl restriction site was not significantly associated with T1D (Ban et al., 2001).

Conclusion

The findings of this study showed a significant association (p≤0.05) of the GG+GA genotype with DM cases compared to controls but the case is not the same for the Fokl genotype. Furthermore, other variables such as gender, age, occupation, BMI, family history, blood pressure, alcohol consumption and serum triglyceride level were also not associated with VDR gene polymorphism except for LDL level. Thus, Bsml polymorphism can be used as a risk marker for DM in the Nepalese population.

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Research Article

Oral Microbial Diversity Among Nepalese Individuals Across Various Geographical Regions

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Abstract

A comprehensive study encompassed the collection of 153 samples derived from oral patients across 17 diverse locations throughout Nepal. The assortment of samples included extracted teeth, dental plaque, and dental calculus, procured from dental clinics, dental hospitals, and dental camps. Employing six distinct culture media, namely nutrient agar (NA), Muller Hilton agar (MHA), mannitol salt agar (MSA), blood agar (BA), brain heart infusion agar (BHA), and potato dextrose agar (PDA) for potential fungal strains, plates were meticulously incubated at 37℃ for 5-7 days. The ensuing bacterial colonies were judiciously isolated, and their morphological and biochemical traits were scrutinized. The microscopic structures of the bacterial cells were examined, considering shape, size, colour, opacity, and texture. Gram-staining was employed, and each colony's biochemical attributes were assessed for protease, pectinase, cellulase, and lipase enzymes. From the 1200 colonies isolated from dental samples, 300 diverse colonies, distinguished by morphological and biochemical characteristics, were chosen for further taxonomic identification. Subsequent sequencing revealed the identification of 60 distinct species within 21 genera of bacterial isolates, including *Achromobacter*, *Bacillus*, *Chryseobacterium*, *Citrobacter*, *Curtobacterium*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Flavobacterium*, *Klebsiella*, *Kocuria*, *Lyinibacillus*, *Novosphingobium*, *Ochrobactrum*, *Proteus*, *Pseudomonas*, *Sporosarcina*, *Staphylococcus*, *Stenotrophomonas*, *Serratia* and *Streptococcus*. The research underscored the presence of various pathogenic bacterial species in oral samples.

Keywords: 16S rRNA, Oral bacteria, Oral disease, Oral hygiene

Introduction

The human body serves as a diverse habitat for a multitude of microorganisms, with certain microbes playing pivotal roles in influencing human health and contributing to various diseases. The oral cavity, in particular, provides a conducive environment for the thriving of numerous bacteria and fungi. These microorganisms establish colonies on oral surfaces, giving rise to dental plaque or oral biofilm (Takahashi, 2005). This biofilm becomes a breeding ground for common oral infections such as dental caries, gingivitis, periodontitis, and peri-implantitis, posing significant health risks not only locally in the mouth but also contributing to systemic diseases.

The dental biofilm forms a microbial community on tooth and tissue surfaces within the oral cavity, encompassing the tongue, mucosa, and other variant surfaces. The implications of such biofilms extend beyond oral health, impacting global health concerns. Dental caries and periodontal diseases are recognized as critical global oral health issues (Bhardwaj & Bhardwaj, 2012). Furthermore, the link between poor oral health, the invasive potential of oral microbes in the body, and their impact on cardiac health and cognitive function has been explored (Noble et al., 2013). The far-reaching consequences of oral health also extend to an association with various cancers, including pancreatic, gastrointestinal, and oral/pharyngeal cancers like oral squamous cell carcinomas (Chakraborty et al., 2014). Despite being a pervasive concern affecting individuals of all ages and societies worldwide, oral health problems seem to be escalating due to the presence of hard surfaces facilitating bacterial colonization, leading to the development of dental biofilms and the onset of dental caries (Kirby et al., 2014).

In developing countries, particularly in rural areas, maintaining oral hygiene poses a significant challenge due to limited awareness and practice. While oral health is a major concern in Nepal, research in this field is scarce. The geographical and cultural diversity in Nepal suggests potential variations in oral microorganisms, emphasizing the need for more extensive research in this underexplored domain.

Materials and Methods

Sampling

A total of 153 samples were gathered from oral patients residing in diverse locations across the country, spanning 17 different places within 16 districts (Table 1). The selection for sampling was conducted randomly, contingent upon the availability of oral samples. The collected samples comprised extracted teeth, dental plaque, and dental calculus, sourced from a variety of settings including dental clinics, dental hospitals, and dental camps.

Table 1: The list of geographical locations for oral sample collection.

SN	Location	Number of samples
1	Baglung	2
2	Baitadi	5
3	Bhairahawa	8
4	Butwal	11
5	Dang	3
6	Dharan	7
7	Ilam	5
8	Janakpur	9
9	Jhapa	9
10	Kathmandu	8
11	Biratnagar	12
12	Myagdi	3
13	Nuwakot	14
14	Palpa	5
15	Pokhara	6
16	Sindhupalchowk	46
17	Udaypur	1

Microbial organism culture

Standard microbial techniques were employed to isolate all cultivable microorganisms. The isolation process utilized six different culture media, namely nutrient agar (NA), Muller Hilton agar (MHA), mannitol salt agar (MSA), blood agar (BA), brain heart infusion agar (BHA), and, for potential fungal strains, potato dextrose agar (PDA). The grown colonies underwent careful observation and purification through repeated streaking until a pure colony was achieved. Incubation took place at 37°C for 5-6 days, with most samples exhibiting growth in the nutrient agar medium.

The morphological characteristics of the microorganisms were scrutinized, encompassing considerations of their shape, size, colour, margin, opacity, and texture. This comprehensive approach facilitated the thorough examination and categorization of isolated microorganisms, enhancing the understanding of their diverse traits and behaviours.

Identification of bacteria

Gram staining: The gram staining procedure involves the execution of four fundamental and crucial steps. Initial staining employed crystal violet, binding with grams of iodine to entrap the CV-I complex within the cell. This was succeeded by alcohol decolourization and, ultimately, a counter-stain application of safranin. Following decolourization, gram-negative cells lost their purple colour, retaining the safranin counter-stain, resulting in a pink hue. In contrast, gram-positive cells retained the CV-I complex, maintaining their purple colour throughout the subsequent stages. Gram-variable bacteria exhibited a distinct behaviour, deviating from the aforementioned mechanisms, leading to a mixture of pink and purple cells in the staining process (Davies et al., 1983).

16S ribosomal RNA sequencing: Genomic DNA extraction was carried out using the Labo Pass Mini Tissue Genomic DNA Isolation Kit from Cosmogentech Inc., Korea. Subsequently, the 16S rRNA genes were PCR-amplified utilizing universal primers, 27F (5’-AGA GTT TGA TCM TGG CTC AG-3’) and 1492R (5’-GGT TAC CTT GTT ACG ACT T-3’). The resulting PCR products underwent purification with the Labo Pass PCR Purification Kit from Cosmogenetech, Seoul, Korea, and were subsequently sequenced using the same primers employed for amplification. To identify closely related species, the obtained 16S rRNA gene sequences were compared with those of type strains accessible in the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon) as outlined by Kim et al. (2012). This comparative analysis facilitated the determination of the genetic similarities and relationships between the sequenced 16S rRNA genes and the reference strains in the EzTaxon-e database.

Phylogenetic analysis: Two online tools, ClustalW with the Clustal Omega multiple alignment tool, and a robust phylogenetic analysis tool on Phylogeny.fr, were employed in this study. Both tools share a common underlying principle: the extracted sequences were initially aligned using the Clustal Omega multiple alignment tool. Subsequent steps included curation, phylogenetic analysis, and tree rendering to ultimately generate a comprehensive phylogenetic tree. The construction of a rooted phylogenetic tree was facilitated by utilizing ribosomal RNA partial sequences extracted from NCBI for all identified bacteria. Additional sequences, particularly those associated with pathogenically significant oral bacteria, were also incorporated into the analysis. These tools, as described by Dereeper et al. (2010), played a crucial role in the alignment, curation, and subsequent generation of a phylogenetic tree, offering insights into the evolutionary relationships among the identified bacterial species.

Enzymatic assay of the colonies

Isolated colonies were cultivated on agar plates containing compounds that activate target enzymes. To screen for protease-producing colonies, they were grown on skim milk agar plates. The presence of a clear zone around the grown colony served as an indicator of protease activity. Similarly, cellulase enzyme activity was assessed by cultivating colonies in Carboxymethylcellulose (CMC) media. Positive cellulase activity was confirmed by the appearance of a clear halo zone after pouring a 1% congo red indicator and subsequent washing with 1N NaCl. For amylase enzyme screening, starch plates were employed, and 0.1% iodine solution was applied. Additionally, the same 0.1% iodine solution was utilized to screen pectinase enzymes on plates containing pectin. These screening methods allowed for the identification of colonies exhibiting amylase and pectinase enzyme activities, respectively, based on the visual cues provided by the iodine solution on the respective agar plates.

Results and Discussion

From 153 oral samples collected across 17 different locations in Nepal, a total of 1200 colonies were isolated. Six diverse culture media were employed to isolate bacterial colonies. Among these, a meticulous selection process was undertaken, choosing only 300 colonies based on criteria such as colony morphology, colour, cellular morphology, biochemical characteristics, and gram-staining properties for further taxonomic identification.

A considerable portion of the isolated colonies, numbering 186, exhibited a moist texture, while a substantial amount displayed dry and mucoid characteristics. The majority of the colonies, specifically 213, demonstrated irregular shapes, with only 6 showcasing filamentous attributes. Nearly 90% of the colonies appeared opaque, contrasting with the few that were transparent. Smooth colonies accounted for 112 instances, while 121 were categorized as rough, and a small number exhibited wrinkled and shiny features.

Approximately half of the colonies displayed undulated patterns, while the remainder exhibited entirely marginal or lobulated structures, with 5 colonies adopting filiform shapes. None of the colonies displayed concave attributes, but approximately 60% were raised. This detailed analysis provides a comprehensive overview of the varied morphological characteristics observed among the isolated colonies, adding depth to the understanding of their diverse traits.

Within the isolated bacterial genera, those identified as gram-negative included *Chryseobacterium*, *Flavobacterium*, *Pseudomonas*, *Stenotrophomonas*, *Proteus*, *Escherichia*, *Ochrobactrum*, *Serratia*, *Novosphingobium*, *Achromobacter*, *Klebsiella*, *Enterobacter* and *Citrobacter*. Conversely, the gram-positive genera encompassed *Bacillus*, *Enterococcus*, *Staphylococcus*, *Lysinibacillus*, *Kocuria*, *Streptococcus* and *Curtobacterium*. This categorization sheds light on the diverse bacterial composition within the isolated samples, offering insights into the distribution of both gram-negative and gram-positive genera in the examined oral microbiome.

Subsequent to the sequencing of the 16S rRNA of 300 colonies, a noteworthy outcome emerged: the identification of 60 different species (Table 2) spanning 22 distinct genera. The breakdown of these genera is as follows: *Achromobacter* (3 species), *Bacillus* (14 species), *Chryseobacterium* (1 species), *Citrobacter* (3 species), *Curtobacterium* (1 species), *Enterobacter* (4 species), *Enterococcus* (2 species), *Escherichia* (1 species), *Flavobacterium* (1 species), *Klebsiella* (6 species), *Kocuria* (1 species), *Lyinibacillus* (1 species), *Novosphingobium* (1 species), *Proteus* (1 species), *Obesumbacterum* (1 species), *Serratia* (4 species), *Ochrobactrum* (1 species), *Pseudomonas* (5 species), *Sporosarcina* (1 species), *Streptococcus* (1 species), *Stenotrophomonas* (2 species) and *Staphylococcus* (5 species). This comprehensive analysis (Figure 1) provides valuable insights into the diversity and distribution of bacterial species within the oral microbiome in the context of Nepalese populations.

Table 2: Identified bacterial species by using 16S ribosomal RNA sequencing.

No.	Name of the identified species	No.	Name of the identified species
1.	*Achromobacter marplatensis* B2(T)	31.	*Klebsiella michiganensis* W14
2.	*Achromobacter pulmonis* LMG 26696	32.	*Klebsiella pneumoniae* subsp. *ozaenae*ATCC 1196
3.	*Achromobacter spiritinus* LMG 26692	33.	*Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104(T)
4.	*Bacillus aerophilus 28K*(T	34.	*Klebsiella quasipneumoniae* subsp.*quasipneumoniae* 01A030
5.	*Bacillus altitudinis* 41KF2b(T)	35.	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044
6.	*Bacillus amyloliquefaciens* subsp. *plantarum* FZB42(T)	36.	*Klebsiella variicola* DSM 15968
7.	*Bacillus aryabhattai* B8W22	37.	*Kocuriarosea* DSM 20447
8.	*Bacillus cereus* ATCC 14579(T)	38.	*Lysinibacillus macroides* LMG 18474(T)
9.	*Bacillus circulans* ATCC 4513	39.	*Novosphingobium capsulatum*GIFU11526
10.	*Bacillus flexus* IFO 15715	40.	*Obesumbacterium proteus* DSM 2777
11.	*Bacillus licheniformis* ATCC 14580(T)	41.	*Ochrobactrum anthropi* ATCC 49188
12.	*Bacillus methylotrophicus* KACC 1310	42.	*Proteus mirabilis* ATCC 29906(T)
13.	*Bacillus paralicheniformis* KJ-16(T)	43.	*Pseudomonas aeruginosa* JCM 5962
14.	*Bacillus safensis* FO-36	44.	*Pseudomonas geniculata* ATCC 19374
15.	*Bacillus siamensis* KCTC 13613(T)	45.	*Pseudomonas hibiscicola* ATCC 19867
16.	*Bacillus subtilis* subsp. *inaquosorum* KCTC 13429	46.	*Pseudomonas japonica* NBRC 103040
17.	*Bacillus tequilensis*KCTC 13622(T)	47.	*Pseudomonas taiwanensis* BCRC 17751
18.	*Chryseobacter iumvietnamense* GIMN1 .005	48.	*Serratia glossinae* C1(T)
19.	*Citrobacter farmeri* CDC 2991-81	49.	*Serratia grimesii* DSM 30063(T)
20.	*Citrobacter freundii* ATCC 8090	50.	*Serratia liquefaciena* ATCC 27592(T)
21.	*Citrobacter koseri* CDC3613-63	51.	*Serratia marcescens* subsp.*marcescen* ATCC 13880
22.	*Curtobacter iumoceanosedimentum* ATCC31317(T)	52.	*Sporosarcina contaminans* CCUG 53915
23.	*Enterobacter aerogenes* KCTC 2190	53.	*Staphylococcus epidermidis*ATCC 14990
24.	*Enterobacter ludwigii* EN-119	54.	*Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305
25.	*Enterobacter tabaci* YIM Hb-3(T)	55.	*Staphylococcus sciuri* DSM 20345(T)
26.	*Enterobacter xiangfangensis*10-17(T)	56.	*Staphylococcus sciuri* subsp. *sciuri* DSM 20345(T)
27.	*Enterococcus faecalis* ATCC 19433(T)	57.	*Staphylococcus xylosus* ATCC 29971(T)
28.	*Enterococcus faecium* CGMCC 1.2136(T)	58.	*Stenotrophomonas chelatiphaga* LPM-5
29.	*Escherichia marmotae* HT07301	59.	*Stenotrophomonas maltophilia* MTCC 434
30.	*Flavobacter iumoceanosedimentum* ATCC 31317	60.	*Streptococcus mutans* NCTC 10449

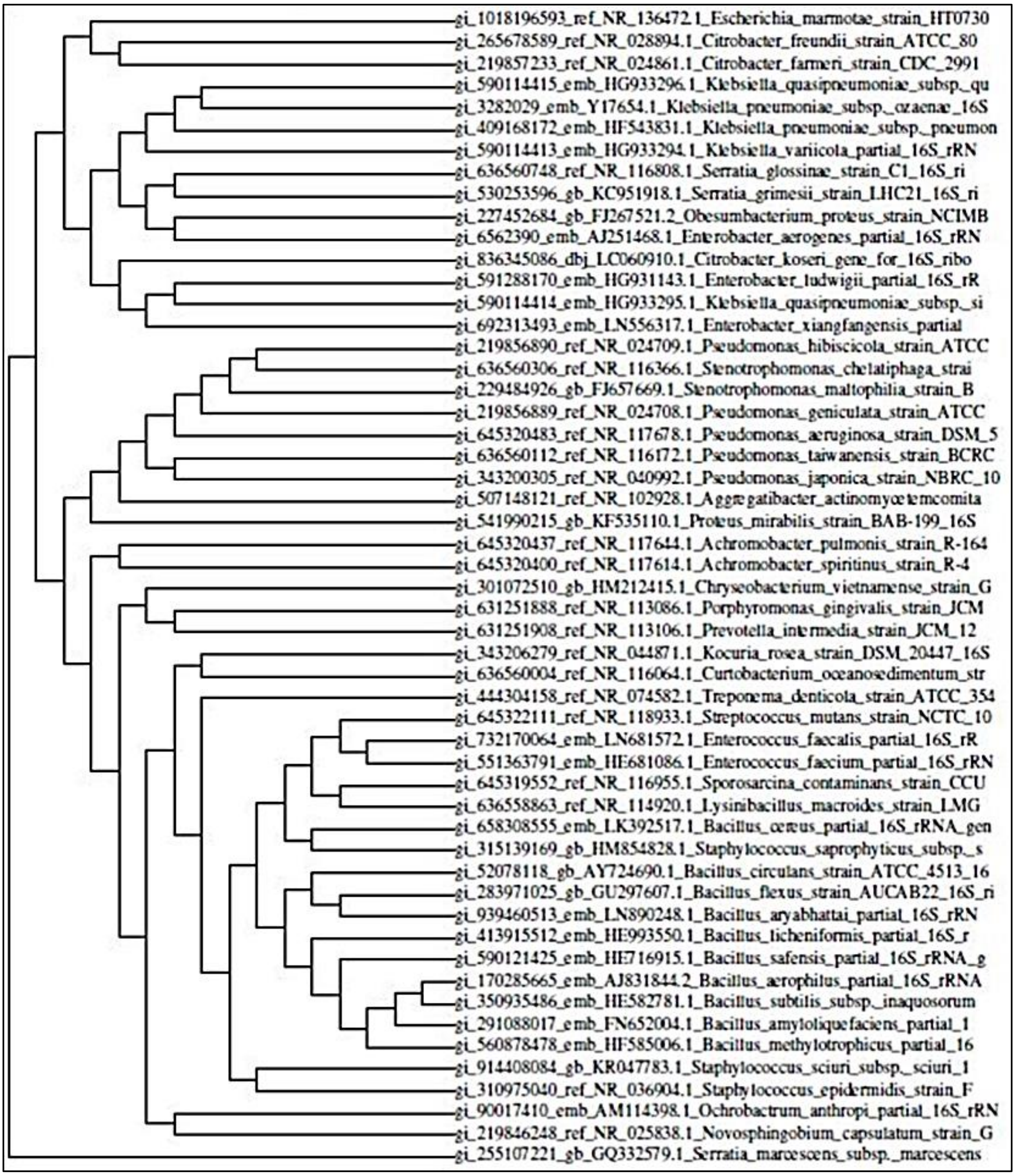

Figure 1: Phylogenetic tree of the isolated bacterial strains.

Figure 2 illustrates that over 50% of the organisms (173) demonstrated at least one enzyme activity, while 127 colonies exhibited no enzyme activities. Specifically, 27 colonies exhibited amylase activity, 81 colonies showcased protease activity, and 18 isolates displayed cellulase activity. Furthermore, 21 colonies demonstrated both cellulase and protease activities, 19 colonies exhibited both protease and cellulase activities, and 4 colonies displayed all three enzyme activities (cellulase, amylase, and protease). This detailed enzymatic analysis on respective agar plates provided clear evidence of the prevalence of three major enzymes.

Notably, strains of *Klebsiella pneumonia*e and *Achromobacter pulmonis* were observed to produce all three enzymes (amylase, cellulase, and protease). Additionally, strains of *Enterobacter xiangfangensis*, *Bacillus cereus*, *Bacillus aerophilus*, *Bacillus amyloliquefaciens*, and *Pseudomonas taiwanensis* exhibited positive results for two enzymes, namely cellulase and protease.

Escherichia marmotae, *Ochrobactrum anthropi* and *Obesumbacterium proteus* demonstrated single enzyme activity, specifically protease. Conversely, *Staphylococcus epidermidis*, *Citrobacter koseri*, *Serratia grimesii*, *Chryseobacterium vietnamense* and *Sporosarcina contaminans* did not exhibit any enzyme activity. This comprehensive enzymatic analysis provides valuable insights into the diverse enzyme production capabilities of isolated bacteria. Salivary microbiota richness was strongly correlated with poor oral health hygiene, with *Prevotella* and *Veillonella* dominance associated with an increased risk of periodontal disease. Conversely, *Neisseria* dominance was linked to a healthy periodontal condition (Yamashita and Takeshita, 2017). Additionally, *Staphylococcus* colonization in the oral cavity was identified as a potential cause of endocarditis, a severe heart infection (Ohara-Nemoto et al., 2008), while *Escherichia faecalis* was reported as the primary causative organism for root canal treatment failures (Mahmoudpour et al., 2007). This underscores the need for further research to correlate oral microbial specimens with specific oral health symptoms and conditions.

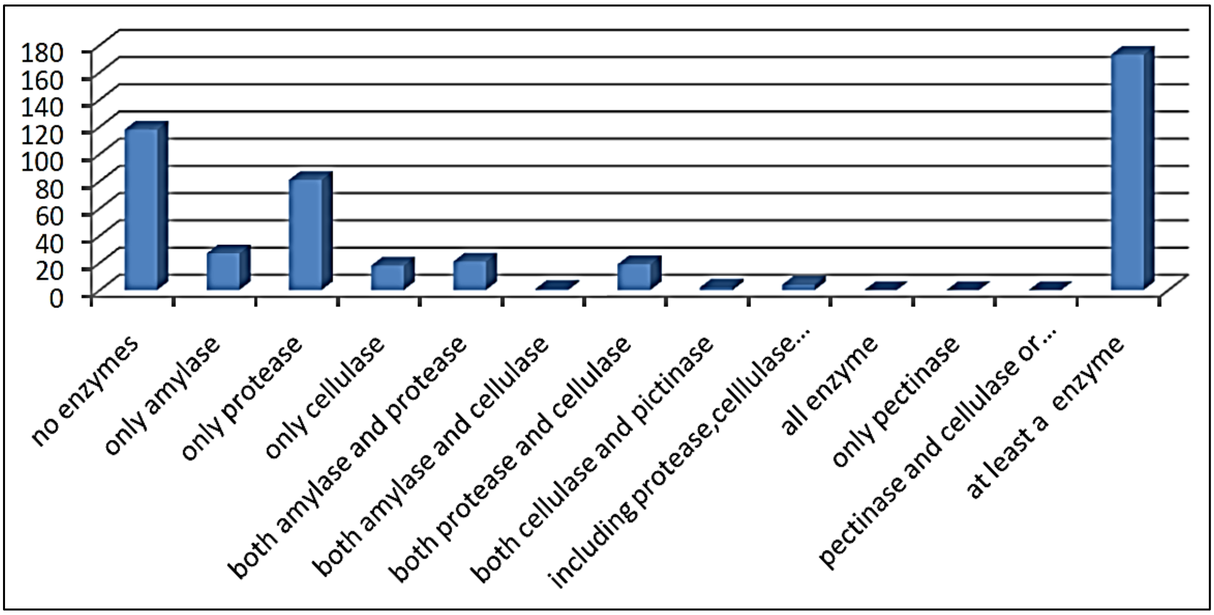

Figure 2: The enzymatic activity of 300 bacterial colonies.

Conclusion

The predominant bacteria associated with dental diseases, *Streptococcus mutans* and *Porphyromonas gingivalis*, were noted, though their prevalence varied in different stages of oral disease lesions. *Streptococcus*, *Porphyromonas* and *Actinomyces* species were identified as predominant in the supragingival area, but as our main samples consisted of extracted teeth, only a subset of these species was identified in our sequencing.

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Research Article

Impact of Invasive *Ageratina adenophora* on Soil Fungi in Native Plant-Grown Soils in Nepal

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Abstract

Invasion of alien plants impacts both the above- and below-ground communities. The specific response of soil microbes to alien plants may depend upon the presence of native inhabitants and the origin of the byproducts released from different parts of invasive plants. This study evaluated the response of common soil fungi against *Ageratina adenophora* byproducts (extracts) in the presence of two native shrubs, *Elsholtzia blanda* and *Osbeckia stellata*, in Nepal. A total of eight fungal species were isolated from soils where these two native species were grown separately, and the occurrence of the fungi was evaluated. The occurrence and frequency of fungus species varied with extracts of *A. adenophora* leaves, litter and roots as well as with the presence of specific native plants. Particularly, *A. adenophora* further inhibits the fungi that are naturally less frequent in soil, like *Hormodendrum* sp. and fresh leaves and litter of *A. adenophora* were responsible for inhibiting antagonistic fungi like *Trichoderma harzianum.* Influence in below-ground fungal communities by *A. adenophora* is one of the reasons for poor growth and development of native seedlings and the mechanism could be a strategy of invasion of *A. adenohora* in novel areas.

Keywords: Antagonists, Frequency, Invasive plant, Native shrubs, Soil fungi

Introduction

Ageratina adenophora (Spreng.) R.King. & H.Rob., one of the invasive alien species native to Mexico and Central America, has been extensively spread all over the world, including Nepal (Wang & Wang, 2006; Cronk & Fuller, 2014; Shrestha, 2016). Its priority areas of invasion are roadsides, fallow and degraded lands, disturbed forests and agro-ecosystems from tropical to sub-tropical regions (Tiwari et al., 2005; Thapa et al., 2016a). Most of the studies on *A. adenophora* are confined to its ecology, distribution and impacts on native vegetation (Tererai & Wood, 2014; Fu et al., 2018; Wu et al., 2020). Negative impacts of the weed, such as altering native plants' species composition and inhibition of seed germination and growth of native plants as well as crops, have been reported by several previous studies (Thapa et al., 2020b; Darji et al., 2021; Khatri et al., 2023). Besides the impacts of *A. adenophora* on above-ground vegetation, some of the work has been concentrated to impact on the below-ground communities. For example, Niu et al. (2007) found that the weed is responsible for increasing the abundance of soil vesicular-arbuscular mycorrhizal fungi. Similarly, the modification of soil microbial communities and functions by *A. adenophora* is expected as one of the mechanisms for successful invasion in novel areas (Bo et al., 2014; Balami & Thapa, 2017; Xia et al., 2021).

Soil microbes, especially pathogenic and symbiotic microbes, will have a strong influence on above-ground plant communities and ecosystem functioning, and alien plant invasion can have major effects on these microbial activities in soil (Van der Putten et al., 2007). Invasive plant, *A. adenophora* can have a range of impacts on microbial communities, as explored previously (Niu et al., 2007; Kong et al., 2017), but specific interactions between invasive species and particular microbes could be a complex phenomenon. Moreover, the interaction mechanisms between soil microbes and invasive species might depend on the presence of particular native species.

Ageratina adenophora has been invading degraded forests where *Elsholtzia blanda* (Benth.) Benth. and *Osbeckia stellata* Buch.-Ham. ex D.Don. are the frequent native shrub species in the Chitlang area of Makwanpur district, Nepal. Darji et al. (2021) found that these two native species were negatively affected by *A. adenphora.* Allelopathic inhibition of *A. adenophora* has been observed as one of the causes of the diminished growth and development of natives and on the other hand, the soil microbes might have been impacted by *A. adenophora* as well. Hence, this study aims to evaluate the response of common soil fungi to invasive *A. adenophora* in the presence of native shrubs *E. blanda* and *O. stellata*.

Materials and Methods

Pot experiment

Seeds of the native plant species, *Elsholtzia blanda* and *Osbeckia stellata,* growing in Takhtar Community Forest (27°24′59.99″N and 85°01′60.00″E) nearby Chitlang village, Makwanpur district, Nepal were collected. The seeds were spread on moist filter paper to allow germination in the dark at a temperature of 25±5°C. Polyethene pots were filled with homogeneously mixed garden soil, then soil in each pot was saturated by distilled water. The native plant seedlings of homogeneous size were transplanted into the pots. Each pot had six seedlings of each native plant.

Extraction of plant materials and treatment of plants

Extracts from the leaves and roots of *Ageratina adenophora* were prepared by soaking 10 g (leaves and roots separately) per 100 ml of distilled water. The seedlings transplanted in the pots were irrigated with respective extracts. In the litter treatment, irrigation was done over the litter spread on the surface of the soil in the litter pots (Darji et al., 2021). The pots were exposed to the following treatments for each of the native seedlings: (i) irrigation by distilled water – control, (ii) irrigation by leaf extract, (iii) irrigation by root extract, and (iv) irrigation with litter extract.

Fungi culture condition

The plants were grown in pots in the glasshouse of the Central Department of Botany, Tribhuvan University, Kathmandu, Nepal. The temperature of the glasshouse varied from 20-38 °C and moisture ranged from 50-88%. After 48 days of seedling transplantation, the plants were harvested and soil from each pot was sampled for fungal assay. A potato dextrose agar (PDA) medium was used for fungal culture. Each soil sample (0.05 g) was mixed thoroughly with 20 ml molten PDA medium in sterile petriplates (Warcup, 1950). Altogether, 72 petriplates were used for each native species (3 plates per pot × 6 pots × 4 treatments). The plates were incubated at 25°C for 7 days. The fungi grown on PDA plates were then isolated and recultured as a pure colony. The fungi from pure culture were observed under a digital microscope and photographs were taken. They were identified based on macro- and micro-morphological characters following Watanabe (2002) and with the help of experts. The frequency of isolated fungi was calculated using the formula:

Frequency = Number of plates where the fungal species present × 100/Total number of plates incubated.

Results and Discussion

Occurrence of fungal species

The most commonly occurring 8 fungal species were isolated from pot soil where the native species *Elsholtzia blanda* and *Osbeckia stellata* were grown. They were *Rhizopus stolonifer* (Ehrenb.) Vuill, *Mucor hiemalis* Wehmer, *Trichoderma harzianum* Rifai, *Penicillium chrysogenum* Thom, *Geotrichum candidum* Link, *Trichophyton* sp., *Hormodendrum* sp. and *Aspergillus flavus* Link. Among them, *M. hiemalis* and *R. stolonifer* belong to the class Mucoromycetes; and the species *Aspergillus flavus, Penicillium chrysogenum* and *Trichophyton* sp. belong to Eurotiomycetes. *Hormodendrum* sp. and *T. harzianum* represented the class Dothiodeomycetes and Sordariomycetes, respectively. Two species *Aspergillus flavus* and *Hormodendrum* sp. were present and *Trichophyton* sp. was absent in the soil where *O. stellata* seedlings were grown (Table 1).

Interestingly, *A. flavus* has appeared and *T. harzianum* disappeared in *O. stellata* soil treated with *Ageratina adenophora* root extract. The species *G. candidum* disappeared in *E. blanda* soil treated with *Ageratina adenophora* litter extract. *Hormodendrum* sp. and *Trichophyton* sp. were found only in the control soil of *O. stellata* and in the *E. blanda* soil treated with root extract, respectively. Another species, *M. hiemalis* was present in both *E. blanda* and *O. stellata* soils except the soils treated with leaf extract. The species present in all the treatments in both the native plant species were *P. chrysogenum* and *R. stolonifer* (Table 2).

Table 1: List of fungal species isolated from soils of native species grown.

S.N.	Name of species	Class	*Elsholtzia blanda*	*Osbeckia stellata*
1	*Aspergillus flavus* Link	Eurotiomycetes	-	+
2	*Geotrichum candidum* Link	Saccharomycetes	+	+
3	*Hormodendrum* sp.	Dothiodeomycetes	-	+
4	*Mucor hiemalis* Wehmer	Mucoromycetes	+	+
5	*Penicillium chrysogenum* Thom	Eurotiomycetes	+	+
6	*Rhizopus stolonifer* (Ehrenb.) Vuill	Mucoromycetes	+	+
7	*Trichoderma harzianum* Rifai	Sordariomycetes	+	+
8	*Trichophyton* sp.	Eurotiomycetes	+	-

'+' means presence; '-' means absence

Table 2: Occurrence of fungal species in soils treated with *Ageratina adenophora* extracts.

S.N.	Name of species	*E. blanda*				*O. stellata*			
		Control	ALE	ALiE	ARE	Control	ALE	ALiE	ARE
1	*A. flavus*	-	-	-	-	-	-	-	+
2	*G. candidum*	+	+	-	+	+	+	+	+
3	*Hormodendrum* sp.	-	-	-	-	+	-	-	-
4	*M. hiemalis*	+	-	+	+	+	-	+	+
5	*P. chrysogenum*	+	+	+	+	+	+	+	+
6	*R. stolonifer*	+	+	+	+	+	+	+	+
7	*T. harzianum*	+	+	+	+	+	+	+	-
8	*Trichophyton* sp.	-	-	-	+	-	-	-	-

ALE: *Ageratina* leaf extrat, AliE: *Ageratina* litter extract, ARE: *Ageratina* root extract; '+' means presence; '-' means absence

Fungal frequency

The frequency of the soil fungi associated with *E. blanda* differed with treatments of *A. adenophora* leaves, litter and root extracts. In the control soil of *E. blanda*, the most frequent fungi were *T. harzianum* and *R. stolonifer* (100%) followed by *M. hiemalis* (60%) and *P. chrysogenum* (53%). The fungus *G. candidum* had the lowest frequency i.e., 13% in the control soil of *E. blanda*. The frequency of all of these fungi found in the control soil of *E. blanda* was reduced in the control soil of *O. stellata* (Figure 1a).

In the treatment of *A. adenophora* leaf litter, the fungus *T. harzianum* and *R. stolonifera* were 15% more frequent while the frequency of *H. hiemalis* was significantly lower in *E. blanda* than *O. stellata.* Two of the fungi *G. candidum* which was not found in *E. blanda* but its frequency was 7% in *O. stellata.* Similarly, *O. stellata* increased the frequency of *P. chrysogenum* (33%) than *E. blanda* (13%) (Figure 1b).

The fungi isolated from the soil of both *E. blanda* and *O. stellata* treated with *A. adenophora* leaf extract were common with variation in occurrence frequency (Table 2, Figure 1c). Comparing the frequencies, *G. candidum, T. harzianum* and *P. chrysogenum* showed high and *R. stolonifera* showed low frequencies towards the soil of *E. blanda* than *O. stellata* (Table 2, Figure 1c).

Among the seven fungal species, *Trichophyton* sp. and *T. harzianum* showed 13% and 20% occurrence towards the soil of *E. blanda* which were absent in *O. stellata* treated with *A. adenophora* root extract. The fungus *A. flavus* was nearly 50% frequent in the soil treated with root extract towards *O. stellata* which was absent in *E. blanda.* Two fungal species *P. chrysogenum* and *H. hiemalis* were more frequent in the soil of *O. stellata* than *E. blanda* while the frequency of *R. stolonifera* was just reverse (Figure 1d).

Most of the fungi isolated from the soils untreated and treated with *A. adenophora* leaf, litter and root extracts were common saprophytes. The fungal species *A. flavus* (Figure 2), *G. candidum, M. hiemalis* (Figure 2), *P. chrysogenum, R. stolonifera* (Figure 2), *T. harzianum* and *Hormodendrum* sp. are among the species associated with decomposing soil nutrients and contributors in the nutrient cycling and facilitate plant growth and development (Robinson, 2014; Nayak et al., 2020).

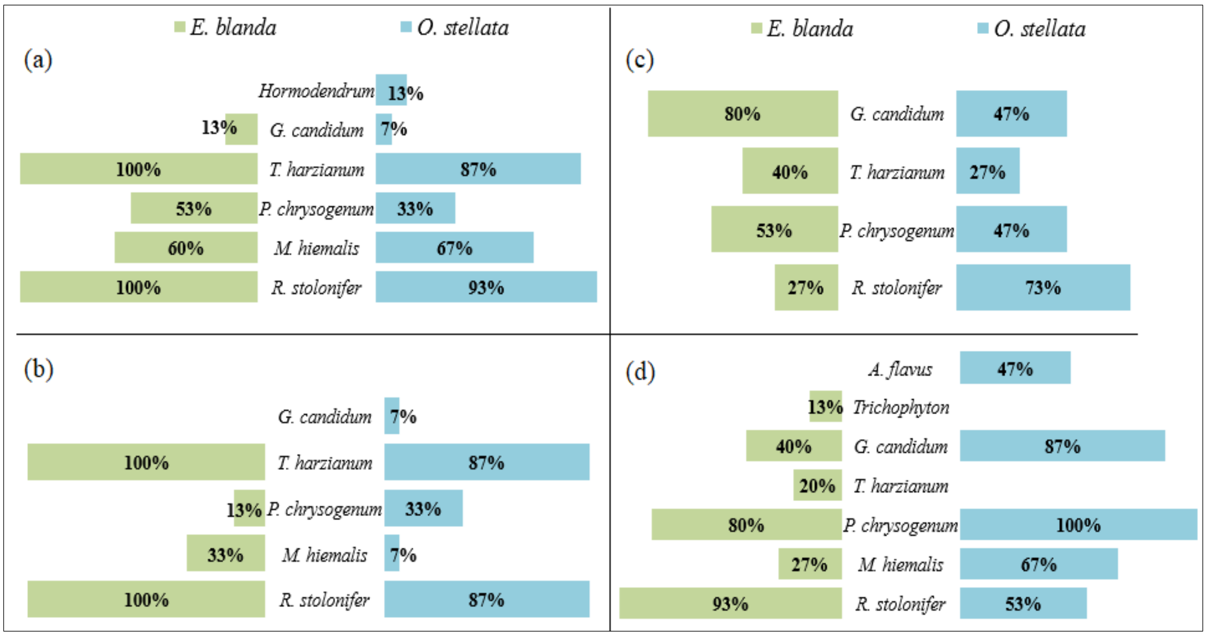

Figure 1: Frequency of fungi in soils of native species *E. blanda* and *O. stellata* treated with (a) normal water-control (b) *A. adenophora* litter extract (c) *A. adenophora* leaf extract (d) *A. adenophora* root extract.

One of the fungi *Trichophyton* sp. isolated from soil treated with *A. adenophora* root extract in *E. blanda* soil is uncommon in soil as many of the species of *Trichophyton* are explained as dermatophytes (de Hoog et al., 2021). Several species of this genus such as *T. terrestre, T. verrucosum, T. mentagrophytes* have also been isolated from soils (Papini et al., 1998; Mahmoudabadi & Zarrin, 2008). As the presence of *Trichophyton* sp. only in the potting soil treated with *A. adenophora* root extract in *E. blanda*, it cannot be said that the fungus is prevalent in our soil samples and there was an effect of native plants and root extract of *A. adenophora* particularly on this fungus. Pontes et al. (2013) conclude that dermatophytes like *Trichophyton* may be prevalent in soils of urban areas which are influenced by several non-biological factors including soil organic matter and pH.

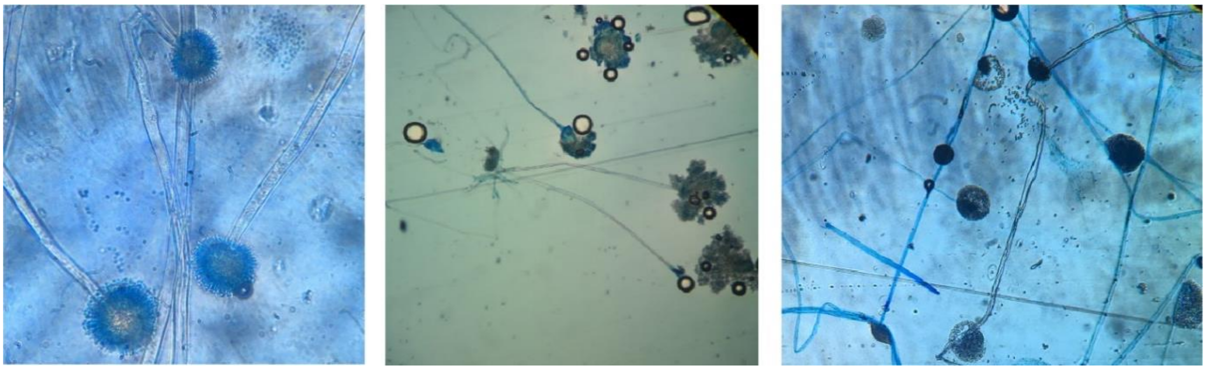

Figure 2: Isolated fungi from native plant species grown pot soil (*A. flavus* (left), *R. stolonifera* (middle) and *M. hiemalis* (right)).

Similar to *Trychophyton, A. flavus* appeared only in the soil of *O. stellata* treated with *A. adenophora* root extract with a frequency of 47% (Figure 1). *A. flavus* is a common saprophyte found in soil and sometimes behaves as an opportunistic pathogen of certain plants, mainly the crop plants (Klich, 2007). The fungus did not show its presence in control and all treatments with *A. adenophora* in the soils of *E. blanda* and *O. stellata* (except root extract). Specific interaction of the growing seedlings of native plants and the addition of extracts of invasive *A. adenophora* with the occurrence of this fungus can be expected from the result. Also, the particular components of *A. adenophora* root extract which influenced *A. flavus* to grow with *O. stellata* could be an issue of further studies.

The presence of *Hormodendrum* sp. only in the control soil of *O. stellata* shows its specific association with this native plant. The fungus grows on organically rich soil and is involved in decomposing plant products showing a high rate of cellulolytic activity (Đukić et al., 2018). Even in the control soil, its frequency was relatively lower than other fungi (13%) (Figure 1). The results indicate that *A. adenophora* can have toxic substances in its extract that may be harmful to the less frequent fungi like *Hormodendrum* sp. Also, the less frequent fungi may be influenced by plant-plant and soil interactions. Two of the fungi, *P. chrysogenum* and *R. stolonifer* were the species representing all treatments in soils of both native plants (Table 2). This indicates that these fungi are less sensitive to the extracts from *A. adenophora* but variation in their frequencies in different treatments with the native plants was evident. Figure 1 explains that the frequency of *P. chrysogenum* was high (80 to 100%) in the soils of both native plants which were treated with root extract while the frequency was low (<33%) in the soils treated with litter extract of *A. adenophora*. This indicates that the litter extract of *A. adenophora* inhibited and the root extract promoted the growth of *P. chrysogenum*. Similarly, *R. stolonifer* is less frequent towards *O. stellata* compared to *E. blanda* but its frequency was significantly low (27%) in the *A. adenophora* leaf extract with *E. blanda* (Figure 1). These results indicate that these two fungi show specific response patterns towards extract type from *A. adenophora* with particular native seedlings. A notable result is that *A. adenophora* litter extract is harmful to *P. chrysogenum* and leaf extract is toxic to *R. stolonifer.*

Three of the fungi, *G. candidum*, *T. harzianum* and *M. hiemalis* are extremely common in soil with a worldwide distribution and play a significant role as organic matter decomposers in natural ecosystems (Singh et al., 2016; Botha, 2006; Khalid et al., 2006). The leaf and root extracts of *A. adenophora* also increased the frequency of *G. candidum* (40-80% in *E. blanda* and 47-87% in *O. stellata,* respectively) while it was negligible in control and litter extract-treated soils (Figure 1). In addition, the fungus disappeared in *E. blanda* soil treated with litter extract (Figure 1). Hence, it can be highlighted that leachates from *A. adenophora* leaves and roots may colonize *G. candidum* in the soils infested by *A. adenophora.*

Just contrary to *G. candidum, T. harzianum* showed extensive occurrence in control soils with both native plants (Figure 1a). The addition of extract from *A. adenophora* litter did not reduce the frequency of this fungus with both native species (Figure 1b). However, the occurrence percentage of *T. harzianum* reduced significantly with the addition of extracts from leaves and roots. Moreover, the fungus could not grow in soil with *O. stellata* treated with root extract of *A. adenophora* (Figure 1c, d). Similarly, *M. hiemalis* occurrence was nil in soils treated with *A. adenophora* leaf extract with both of the native plants and the frequency was decreased by root and litter extracts (Figure 1c). This signifies that *T. harzianum* and *M. hiemalis* are highly sensitive to allelochemicals present in live plant materials (leaves and roots) of *A. adenophora.*

Trichoderma harzianum is considered a biological control agent against a wide range of economically important plant pathogens as it may fight pathogens through antibiosis and competition (Mukhopadhyay & Kumar, 2020). Similarly, *M. hiemalis* also suppress the growth of pathogenic fungi like *Thielaviopsis paradoxa* and acts as an entomopathogen for plant pests of Dipteran members (Zhu et al., 2022; Hammia & Bouatrous, 2023). The frequency occurrence of these two fungi in the presence of *A. adenophroa* extracts clearly illustrates that these antagonistic fungi can be diminished in *A. adenophora*-infested soils. It can be anticipated that *A. adenophora* may compete with native species by inhibiting the growth of such antagonistic fungi in natural ecosystems. Fungal antagonists play a crucial role in controlling plant pathogens and diseases (Thambugala et al., 2020). If the population antagonists get diminished, a pathogenic fungal population may increase with an increasing rate of disease incidence of native plant species.

Darji et al. (2022) have shown the presence of hydroxyl compounds, alkynes, amines and C-H stretching (aromatic) or C-O-C stretching (ethers) in extracts of *A. adenophora* leaves, roots and litters which were responsible for reducing growth and development of seedlings of native *O. stellata* and *E. blanda*. The current study has clearly shown that there is a serious alteration in fungal community composition and their frequency occurrence by *A. adenophora*. These changes in fungal communities are another reason for the poor growth and development of native seedlings in *A. adenohora*-invaded soils.

Conclusion

The fungal species *A. flavus, G. candidum, M. hiemalis, P. chrysogenum, R. stolonifer, T. harzianum* and *Hormodendrum* sp. isolated from the soils untreated and treated with *A. adenophora* leaf, litter and root extracts were common saprophytes. *A. flavus* has appeared only in the soil of *O. stellata* treated with *A. adenophora* root extract and therefore, the components of *A. adenophora* root extract influencing *A. flavus*. *A. adenophora* is harmful for the less frequent fungi like *Hormodendrum* sp. indicating they are influenced by plant-plant and soil interactions. The fungi *P. chrysogenum* and *R. stolonifer* show their presence in all types of *A. adenophora* extracts but are less frequent in the litter and leaf extracts indicating they have specific response patterns towards alien invasion with particular native seedlings. Based on the responses of *G. candidum, T. harzianum* and *M. hiemalis,* the antagonistic fungi can be diminished in *A. adenophora*-infested soils and it is predicted that *A. adenophora* may compete with native species by inhibiting the growth of such antagonistic fungi in natural ecosystems. Hence, changes in fungal communities are another reason for the poor growth and development of native seedlings in *A. adenohora*-invaded soils. It would be extremely advantageous to employ molecular techniques for identifying fungi and conducting tests on specific allelochemicals for confirming the interactions between alien plants and soil fungi.

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Review Article

Exploring Holistic Wellness: Unveiling the Probiotic Wonders of Fermented Dairy – Meet Kefir and Its Health Benefits

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Abstract

This article explores the rich historical background and emerging health advantages of kefir, a fermented dairy product renowned for its abundant probiotics and positive impact on gut health and overall well-being. Additionally, the research delves into the microbial composition of kefir, its potential probiotic benefits, and the intricate interplay of microorganisms within the kefir grains. Examining specific *Lactobacillus* strains, the study assesses their health-promoting benefits, ranging from improved digestion and immune system support to potential contributions to mental health. By scrutinizing the probiotic microorganisms found in kefir, this paper aims to offer valuable insights to researchers, food technologists, and entrepreneurs interested in the nexus of nutrition, health, and sustainable food production.

Keywords: Kefir, Kefiran, Microbes, Mutualism, Probiotics

Introduction

Kefir, a fermented dairy product, is distinguished by its unique microbial composition resulting from the symbiotic co-cultivation of lactic acid bacteria and yeasts within a matrix of polysaccharides and proteins, primarily derived from milk. Traditionally fermented at ambient temperature, this process imparts distinct sensory and nutritional attributes to kefir, making it a rich source of probiotic microorganisms, bioactive peptides, vitamins, and minerals (Marco et al., 2017).

Originating from the Caucasus Mountains and consumed for centuries, kefir has gained global recognition for its potential health benefits. These benefits include improvements in gastrointestinal health, enhanced immune function, and the modulation of lipid metabolism (Liu et al., 2006). The positive effects are primarily attributed to the live probiotic microorganisms present in kefir, which have been demonstrated to exert beneficial influences on gut microbiota and host physiology (Leite et al., 2015).

The diverse microbial community of kefir includes lactic acid bacteria like *Lactobacillus* species (e.g., *Lactobacillus kefiranofaciens*) and *Streptococcus* species, as well as various yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* (Garofalo et al., 2015). Working collaboratively, these microorganisms break down lactose and other milk constituents, producing lactic acid, acetic acid, ethanol, carbon dioxide, and various volatile chemicals that contribute to kefir's distinctive flavor and aroma while maintaining its original color (Figure 1) (Zamberi et al., 2016).

Figure 1: Kefir produced from milk (left) and kefir grain (right).

Brief History of Kefir

Kefir, the fermented milk product, is believed to have been discovered by shepherds in the Caucasus Mountains. These shepherds stored milk in leather pouches crafted from animal hides, noticing that over time, the milk would ferment, transforming into a tangy, effervescent beverage. They named this fermented milk "kefir," possibly derived from the Turkish word "keyif," meaning "feeling good" or "pleasure," reflecting both its delightful taste and potential health advantages (Ahmed et al., 2013).

The true origins of kefir remain elusive, but it is widely believed to have been consumed in the Caucasus region for over a millennium. Kefir grains, symbiotic colonies of bacteria and yeast, were passed down as invaluable artifacts through the centuries, and the kefir-making process was kept as a closely guarded secret. It wasn't until the late 19th century that kefir gained widespread attention. In 1880, Russian scientist Ilya Ilyich Mechnikov, a later Nobel Prize winner in Physiology or Medicine, conducted research on the health benefits of kefir consumption. He proposed that kefir contributed to the longevity of Caucasus region residents and hypothesized that the lactic acid bacteria in kefir played a role in promoting health (Smith et al., 2014).

Kefir's popularity surged in the early 20th century with its introduction to Europe and later North America. The production and consumption of kefir expanded globally, and today, it stands as a popular fermented dairy product enjoyed worldwide for its distinctive flavor and potential health benefits.

Kefir Grains

Kefir grains, resembling cauliflower, are comprised of hollow globular structures forming a polyhedral network with diameters ranging from 2.0 to 9.0 mm. The diverse microbiota residing within these grains play a significant role in metabolism, community interactions, and provide crucial health benefits, particularly in supporting the immune system. This becomes especially pertinent in preventing bacterial and viral infections, such as during the COVID-19 pandemic (Lu et al., 2014). The matrix of kefir grains consists of kefiran, proteins, microbial cell debris, and other unspecified materials.

The arrangement of microbiota on or within this structure is an ongoing subject of research. While some studies indicate that microorganisms occupy both interior and exterior surfaces of grains, variations in cultivation conditions and environmental factors contribute to diverse observations. Differences in cell sizes and chain lengths may result from various physiological stages or external stresses, such as cultivation conditions and nutrient limitations. For example, *Lactobacillus kefiranofaciens* exhibited two morphotypes, short rods (3.0 µm) and long rods (10.0 µm), colonizing outer or inner surfaces of kefir grains, underscoring the importance of careful interpretation of microscopy data in microbial community studies (Wang et al., 2018).

The reason behind the stable consortium of kefir microbiota, sustaining functionality indefinitely, remains unclear. All attempts to create kefir grains from pure starter cultures in fermentation mixtures have, so far, been unsuccessful (Kotova et al., 2016). Various hypotheses exist regarding the mechanisms behind grain formation in kefir. Initial auto- and co-aggregation of lactobacilli and yeasts, starting with self-aggregation of *L. kefiranofaciens* and *L. kazachstaniaturicensis*, are believed to be crucial. Biofilm-producing species like *L. kefiri* then attach to granule surfaces, co-aggregating with other microorganisms and milk components to form larger granules, potentially leading to kefir grains. Recent studies also emphasize the role of *Acetobacter orientalis*, indicating that LAB and AAB contribute to polysaccharide production and biofilm formation, while yeasts facilitate complex networks among the three microbes (Nejati et al., 2020).

Chemical Composition of Kefir

Kefiran, the primary polysaccharide found in kefir grains, is a heteropolysaccharide consisting of equal proportions of glucose and galactose. The typical composition of kefir includes 89-90% moisture, 0.2% lipid, 3.0% protein, 6.0% sugar, 0.7% ash, and 1.0% each of lactic acid and alcohol. The chemical makeup of kefir is largely influenced by factors such as the type of milk used, the composition of grains or culture mixtures, additives, and the production technology employed (Arslan, 2015).

Microbial Composition of Kefir and Kefir Grains

Kefir and kefir grains consist of a diverse symbiotic community of microbes. The composition of these microbes varies across different types of kefirs, influenced by factors such as growth conditions. Table 1 illustrates the microbial composition of some kefir varieties.

Table 1: Microbial Composition of kefir and kefir grains.

Microorganisms	Source-Country	References
Lactobacillus kefir, Lactobacillus kefiranofaciens, Lactobacillus paracasei, Lactobacillus plantarum, Lactococcus lactis ssp. *lactis, Kluyveromyces marxianus, Lactobacillus parakefir, Saccharomyces cerevisiae, Saccharomyces unisporus, Leuconostoc mesenteroides, Acetobacter* sp.*, Saccharomyces* sp.*, Lactococcus lactis* ssp. *Lactis biovar diacetylactis, Lactococcus lactis, Lactobacillus kefiri, Lactobacillus parakefiri*	Kefir grains and beverage – Argentina	Garrote et al., 2001; Londero et al., 2012; Hamet et al., 2013; Diosma et al., 2014.
Lactobacillus kefiri, Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Lactobacillus paracasei, Lactobacillus helveticus, Gluconobacter japonicus, Lactobacillus uvarum, Acetobacter syzygii, Lactobacillus satsumensis, Saccharomyces cerevisiae., Leuconostoc sp.*, Streptococcus* sp.*, Acetobacter* sp.*, Bifidobacterium* sp.*, Halococcus* sp.*, Lactobacillus amylovorus, Lactobacillus buchneri, Lactobacillus crispatus, Lactobacillus kefiranofaciens* ssp. *kefiranofaciens, Lactobacillus kefiranofaciens* ssp. *kefirgranum, Lactobacillus parakefiri*	Kefir grains – Brazil	Miguel et al., 2010; Leite et al., 2012; Zanirati et al., 2015
Lactobacillus kefiri, Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Lactococcus lactis ssp. *cremoris, Gluconobacter frateurii, Acetobacter orientalis, Acetobacter lovaniensis, Kluyveromyces marxianus, Naumovozyma* sp.*, Kazachastania khefir*	Kefir grains and beverage – Belgium	Korsak et al., 2015
Lactobacillus helveticus, Lactobacillus buchneri, Lactobacillus kefiranofaciens, Lactobacillus acidophilus, Lactobacillus helveticus, Streptococcus thermophilus, Bifidobacterium bifidum, Kluyveromyces marxianus	Kefir grains- Turkey	Kok-Tas et al., 2012; Nalbantoglu et al., 2014
Acetobacter acetic, Enterococcus faecalis, Enterococcus durans, Lactococcus lactis ssp. *cremoris, Leuconostoc pseudomesenteroides, Leuconostoc paramesenteroides, Lactobacillus brevis, Lactobacillus acidophilus, Saccharomyces* sp.*, Brettanomyces* sp.*, Candida* sp.*, Saccharomycodes* sp.*, Acetobacter rancens*	Kefir beverage – China	Yang et al., 2007
Lactobacillus paracasei, Lactobacillus parabuchneri, Lactobacillus casei, Lactobacillus kefiri, Lactococcus lactis, Acetobacter lovaniensis, Kluyveromyces lactis, Kazachstania aerobia, Saccharomyces cerevisiae, Lachanceame yersii	Kefir beverage – Brazil	Magalhães et al., 2011

Symbiotic Interaction between Microorganism in the Kefir

Yeast-bacteria interaction

The interaction between yeast and bacteria plays a pivotal role in kefir and various fermented foods. This collaboration encompasses:

Assimilation of lactic acid: Yeasts that assimilate lactic acid play a crucial role in facilitating the thriving of lactobacilli. This process helps prevent the accumulation of acid, ultimately leading to an increased production of kefiran (Katakura et al., 2010).

CO_2 production / O_2 removal: Yeasts, such as *S. cerevisiae*, contribute to creating a favorable environment for the growth of *Lactobacillus* spp. by generating carbon dioxide and reducing oxygen levels (Suharja et al., 2014).

Nutrient provision: The cooperation between yeast and bacteria involves trophic interactions and the exchange of metabolites. Yeast plays a vital role in supplying essential nutrients, such as vitamins and amino acids, to bacteria. This nutrient provision supports bacterial growth, especially when resources are limited (Ponomarova et al., 2017).

Bacteria-bacteria interaction

Interactions among bacteria in food have received less exploration compared to yeast-bacteria interactions. Research focusing on yogurt bacteria, specifically *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, has unveiled protocooperative interactions. In the case of kefir bacterial species (e.g., *L. kefiranofaciens*, *L. kefiri*, *Lactococcus lactis*, *Acetobacter fabarum*, *Leuconostoc mesenteroides*), investigations have revealed dynamics of competition. *L. kefiranofaciens* was observed to suppress *L. kefiri*, promote *L. mesenteroides*, and have no impact on *L. lactis* and *A. fabarum*. These findings contribute to a broader understanding of microbiota interactions in the context of kefir (Ponomarova et al., 2017).

Yeast-yeast interaction

Exploration of Quorum Sensing (QS) communication among yeasts is relatively limited. Studies conducted in various ecosystems, such as wine and sourdough, have illuminated the impact of environmental factors, including nitrogen content, cell density, and ethanol levels, on the production of QS-related molecules by *S. cerevisiae*. For example, the secretion of aromatic alcohols is most pronounced when ammonium sulfate levels are below 50 µM, diminishing above 500 µM. These aromatic molecules, known for their antioxidant and antimicrobial properties, play a role in quality control. Additionally, certain strains of *S. cerevisiae* release peptides that inhibit non-*Saccharomyces* strains, and this trait is dependent on the specific strain. Given the significance of these yeasts in kefir, comprehending their interactions is crucial for ensuring the quality and functionality of kefir (Avbelj et al., 2016).

Importance of Kefir

Kefir is documented to have positive effects on various disorders, as illustrated in Figure 2, highlighting its diverse health benefits. Kefir exhibits anticancer, antioxidant, antimicrobial, anti-inflammatory, and antidiabetic activities. Additionally, it has the potential to offer benefits for gastrointestinal tract infections and other health-related conditions.

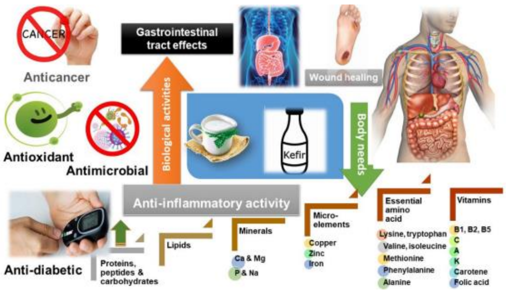

Figure 2: Kefir's biological characteristics, nutritional value, and macro- and micronutrient content (source: Farag et al., 2020).

Potential Health Benefits

In a comprehensive exploration of probiotic strains, Kakisu et al. (2013) delved into the impact of *Lactobacillus plantarum* CIDCA 83114 on *S. flexneri* invasion in Hep-2 cells. The study revealed a significant reduction in pathogen invasion by 5% during co-incubation, while pre-incubation demonstrated superior protective effects. Notably, all probiotic strains, especially *L. plantarum* CIDCA 83114, exhibited enhanced performance after pre-incubation. Dose testing of *L. plantarum* CIDCA 83114 indicated a dose-dependent effect on *S.*

flexneri, with internalization rates of 3.7% at 109 CFU/mL and 12.7% at 108 CFU/mL. However, this dose dependence was less pronounced in the presence of *S. sonnei*.

Examining the interaction with *E. coli*, the study found that pre-treating Hep-2 cells with 108 CFU of Lb. plantarum per well effectively inhibited E. coli adherence, particularly when the *E. coli* concentration was below 106 per well. At higher concentrations (107 and 108), the difference was less substantial. The researchers proposed that optimal protection required at least one Lactobacillus cell for every 100 pathogen cells (Hugo et al., 2008).

Shifting focus to *L. kefiranofaciens* M1, the bacterium demonstrated promise in reducing allergies by influencing Th1 and Th2 responses. A preliminary study indicated that mouse immune cells exposed to bacterial cells produced more IL-6, while the supernatant alone elevated cytokine levels. Another investigation with a different cell line revealed that bacterial cells induced larger amounts of cytokines. Despite challenges in cell-line analysis, *L. kefiranofaciens* M1 affected cytokine production. IL-6 generation from bacterial cells was specifically inhibited when the TLR-2 receptor was disabled, indicating a different mechanism. However, this did not diminish the amount of IL-6 generated by the supernatant, suggesting the involvement of another receptor (Hong et al., 2009).

Chen and Lee et al. (2013) explored the positive impact of treating mice with *L. kefiranofaciens* M1 at 2 × 108 CFU/mL for seven days on enterohemorrhagic *E. coli* (EHEC) infection. The pretreatment improved histology scores, indicating enhanced intestinal health, prevented a decrease in food intake, and significantly reduced intestinal hemorrhage. This treatment also decreased renal glomeruli and kidney interstitial tissue congestion, particularly severe in untreated mice. However, heat treatment diminished the efficacy of the *Lactobacillus* strain. Additionally, *E. coli* was less likely to reach the liver and spleen after pre-treatment with *L. kefiranofaciens*, as evidenced by the absence of *E. coli* in the blood of pre-treated mice compared to considerable concentrations in untreated controls.

In a follow-up trial assessing the length and amount of treatment, *L. kefiranofaciens* M1 exhibited effects on the allergic airway response in mice. Over 32 days (Course A), mice receiving a daily dose of 108 CFU/mL showed a significant decrease in inflammatory markers (IL-4, IL-13, IL-6, IL-1β, CCL20, TNF-α) and an increase in T-regulatory cells. The same dosage was given to a third group for the final three days (Course C) and a second group (Course B) for the first fourteen days. Although Courses B and C exhibited less IL-6 and TNF-α, the advantages observed in Course B did not persist, emphasizing the necessity of ongoing microbial treatment for lasting results. Course C highlighted that the observed benefits could not be achieved through short-term intake (Hong et al., 2011).

Chen & Chen et al. (2013) observed increased ileac villi length, crypt depth, and greater goblet cell levels in the stomach of mice continually administered *L. kefiranofaciens* M1. In comparison to untreated mice, the histological score of treated mice significantly decreased from 9 to 2, suggesting a beneficial therapeutic action against DSS-induced colitis. *L. kefiranofaciens* also improved the immunological response to Toll-like receptor (TLR) agonists—lipopolysaccharide (LPS) and R848. Treated animals exhibited higher levels of IFN-γ and IL-12 compared to germ-free (GF) or singly inoculated mice, suggesting a strengthened immunological response.

Jeong et al. (2017) explored the effects of *L. kefiranofaciens* DN1 given orally to mice for two weeks. The treatment improved the weight and water content of feces, indicating potential benefits for intestinal motility and constipation. Additionally, it adjusted the composition of gut bacteria by increasing beneficial bacteria like *Firmicutes*, *Bacteroidetes*, *Lactobacillus,* and *Prevotella*, while decreasing harmful bacteria like Proteobacteria, Enterobacteriaceae, and *Clostridium*. The production of a new exopolysaccharide by *L. kefiranofaciens* DN1 suggested potential benefits for gut health. In a second investigation, the same team found that the *L. kefiri* strain was a notable probiotic candidate, exhibiting encouraging probiotic traits by adhering to colon and small intestine mucus. Moreover, the *L. kefiri* strain demonstrated the ability to inhibit the growth of several pathogens, including *Salmonella enteritidis, P. aeruginosa, S. flexneri, B. cereus, L. monocytogenes, S. aureus,* and *E. faecalis*. This demonstrates that the *L. kefiri* strain may inhibit the growth of many harmful bacteria, supporting its probiotic properties (Carasie et al., 2014).

Kefir, renowned for its health benefits attributed to Lactobacilli, holds promise in various aspects such as immune system modulation, potential cancer and allergy risk reduction, oxidative stress mitigation, and cholesterol level control, along with potential aid in managing diabetes. Despite these potential advantages, the specific mechanisms and their applicability to humans remain unclear, necessitating further research, especially into the genetic factors responsible for health benefits. A comparative analysis of well-characterized kefir strains' genomes could offer valuable insights.

While studying individual strains has its limitations, the variation in effects between strains underscores the need for further investigation. Multi-species kefir models exhibit synergistic benefits, but understanding the primary contributors can be elucidated through single-strain models. The dairy industry's pursuit of artificial kefir grains underscores the potential application of such knowledge in selecting strains for specific functional properties. As personalized medicine gains prominence, such studies are poised to become increasingly significant (Slattery et al., 2019).

Examining the anti-inflammatory properties, studies involving rats' paws and cotton pellets revealed that kefir and kefiran treatments significantly reduced granuloma tissue and paw edema, akin to anti-inflammatory medications. The potential suppression of inflammatory mediators is suggested as a mechanism for these benefits. Recommendations for further research on their processes and active components highlight kefir and kefiran as potential natural treatments for inflammation (Rodrigues et al., 2005).

Huseini et al. (2012) delved into kefir's therapeutic properties, evaluating its wound healing and antimicrobial effects on *Pseudomonas aeruginosa*-infected burn injuries in rats. The 96-hour kefir gel and kefir grains 96-hour gel emerged as particularly effective in terms of antimicrobial and wound healing properties. Longer fermentation times, especially in kefir gel therapy, were deemed to improve clinical outcomes in thermal injuries.

Kurniati et al. (2020) conducted a study comparing lactic acid, protein, fat, and carbohydrate levels in curd kefir and colostrum kefir. Significant variations were observed, with good kefir exhibiting high lactic acid, proteins, and carbohydrates but low fat. Starter concentration and fermentation time notably influenced the content in both kefir types. Another investigation explored kefir's impact on gut microbiota and metabolic syndrome (MetS). While the study revealed no significant changes in body weight, lipid profile, glucose, or inflammatory markers, kefir ingestion led to a substantial reduction in fasting insulin, HOMA-IR, TNF-α, IFN-γ, and blood pressure, suggesting positive impacts on various MetS indicators, notably in gut microbiota (Belikci-Koyu et al., 2019). The resilience of lactic acid bacteria in kefir varieties to simulated digestion further emphasizes the potential benefits, with kefir grains exhibiting the highest probiotic potential (Ince et al., 2023).

Tu et al. (2020) investigated how kefir peptides derived from dairy milk proteins, produced by kefir probiotic bacteria, affected the gut microbiome in ovariectomized mice, preventing menopausal osteoporosis while having minimal impact on the gut microbiome.

Conclusion

This analysis underscores kefir's rich microbial composition, historical roots, and diverse health benefits. Recognized for enhancing digestive health, boosting immunity, and influencing fat metabolism since its introduction from the Caucasus Mountains, kefir, particularly with strains like *Lactobacillus kefiranofaciens*, holds promise for the digestive and nervous systems. Emphasizing kefir's role in health management, the study envisions potential applications in personalized medicine and sustainable food production. Despite acknowledging the need for further research, kefir captivates academia, food technologists, and health enthusiasts as a complex and advantageous agent contributing to overall well-being.

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Research Note

The Medicinal Fungus *Cordyceps militaris*: Academia to Industry in Thailand

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Abstract

The medicinal mushroom (*Cordyceps* species) is a highly valued source of useful natural products having diverse biological activities. *Cordyceps militaris* (commonly known as orange caterpillar fungus), a kind of herbal drug and food additive mushroom, were used as a tonic food in Asia from ancient times. Their products have been developed from cultivated fruit bodies and fermented mycelia of *C. militaris*. Various studies of culture techniques will be expected to reduce contamination. A traditional methodology (autoclave) to sterilize culture media was compared with a protocol that included hydrogen peroxide to sterilize culture media (without autoclaving and inoculating cultures without the laminar airflow cabinet) for growing *C. militaris* CMRU strain. The results showed that the two methods had no significant differences in cordycepin production and biomass production. Production of *C. militaris* products is now becoming a large industry in Thailand.

Keywords: *Cordyceps*, Knowledge transfer, Academia to industry

Main Body

Medicinal mushrooms (*Cordyceps* species) have received attention from the world because of their biological activities. *Cordyceps* species belong to Ascomycota, Pyrenomycetes, Hypocreales, and Clavicepitaceae, and at least 700 species are known (Das *et al.*, 2021). Cordyceps are specific macrofungi due to their characteristic parasite habit on larvae and insect pupae (Liu et al., 2015).

Cordyceps militaris belonging to the class Ascomycetes, have been used widely as a raw drug and a folk tonic food in Asia. It contains many types of active compounds (such as cordycepin, adenosine, polysaccharides, ergosterol, mannitol, etc.). Cordycepin is a unique active substance of Cordyceps, and its production in *C. militaris* is higher than that of other Cordyceps species. The production of cordycepin from *C. militaris* fruiting bodies can be enhanced by artificial cultivation (Lin et al., 2022).

Cordyceps militaris have beneficial properties such as pro-sexual, anti-inflammatory, antioxidant, anti-ageing, anti-tumour/anticancer/antileukemic, anti-proliferative, anti-metastatic, immunomodulatory, anti-microbial, anti-bacterial, anti-viral, anti-fungal, anti-protozoal, insecticidal, larvicidal, anti-fibrotic, steroidogenic, hypoglycemic, hypolipidaemic, antiangiogenetic, anti-diabetic, anti-HIV, anti-malarial, anti-fatigue, neuroprotective, liver-protective, reno-protective and pneumo-protective (Das et al., 2010). *Cordyceps militaris* helps in the improvement of learning and memory impairment in patients with Alzheimer's disease (Thakur et al., 2022). *Cordyceps militaris* extract has the potential to promote eye healthcare, especially for high-acuity vision healthcare (Chen et al., 2022).

The cultivation of *C. militaris* can be divided into 2 methods: insects and artificial media.

Insects: Artificial growth and stroma production of *C. militaris* have been studied in the laboratory on various insect pupae and larvae, most commonly on the silkworm *Bombyx mori*. Other insects used for artificial stroma production are *Antherea pernyi*, *Mamestra brassicae*, *Tenebrio molitor*, *Ostrinia nubilalis*, *Heliothis virescens*, *H. zea* and *Spodoptera frugiperda*, *Andraca bipunctata*, *Philosamia cynthia*, *Spodoptera litura* (reviewed by Shrestha *et al*., 2012). Chen and Ichida (2002) found a higher rate of infection and stroma formation in silkworm pupae compared with silkworm larvae.

Artificial media: Since insects can be difficult to handle and therefore prone to microbial contamination, alternative artificial media have been tested for commercial production of *C. militaris* fruiting bodies. *Cordyceps militaris* was grown in liquid media for mycelium harvesting and in solid media for fruiting-bodies induction. Many types of natural organic substrates can be used as artificial media. Cereals (such as rice, barley, millet, corn grain, etc.) with the addition of some organic substances turned out to be good substitutes for insects. In our laboratory, rice mixed with silkworm pupa powder has proven to be superior to other substrates and is now routinely used. The fruiting bodies of *C. militaris* have been successfully grown on a large scale. Yields were found less on insects than on cereals in cultivation (Xie et al., 2009a, b).

Not only media, but successful cultivation also requires appropriate control of temperature, humidity, and light. Some experiments have already demonstrated that the chemical components of natural and cultured *C. militaris* are similar (Jiang and Sun, 1999).

Links between universities and industries (University-Industry Linkages: UIL) are one way to develop technology and innovation to create competitiveness in the industry. There are many forms of UIL, ranging from linkage by market forces to institutional linkages. From research on UIL in the past, most of them found that institutional linkages are a more effective form of generating and transferring knowledge and technology. However, there are relatively few institutionalized UIL models in Thailand. This is due to the limitations in the technological capacity gap between universities and industry, lack of mutual trust, and policy problems at both the government and university levels. Resolving these issues is necessary if institutional linkages between universities and industry are seen as important.

Links between industry and universities found in Thailand, most of them are limited to links by market mechanisms. Market-coordinated linkages such as consulting, technical services, sale of products, and licensing. These activities will mainly focus on buying and selling knowledge and academic services. Long-term institutional linkages, such as joint labs at a company/university, joint patenting and joint research publication (joint publication), are a minority. A survey of 136 UIL projects of UIL activities in Thailand found that the major patterns of UIL were in the areas of consulting, technical services, innovation and invention trading, and licensing activities accounting for 49, 35, 17 and 8 percent, respectively (Schiller, 2006).

Cordyceps militaris were known to Thai people no more than 15 years ago, but before that, few Thai people only knew *Ophiocordyceps sinensis* (Tibetan cordyceps mushrooms). *Ophiocordyceps sinensis* is expensive, therefore, only the rich can consume it. *Cordyceps militaris* contains similar secondary metabolites in its fruiting bodies as *O. sinensis*. Therefore, *C. militaris* has been widely regarded as a substitute for *O. sinensis*. In 2010, Tapingkae *et al*. studied the effect of a cultivation method on yield and cordycepin production of *C. militaris*. A traditional methodology (autoclave) to sterilize culture media was compared with a protocol that included hydrogen peroxide to sterilize culture media (without autoclaving and inoculating cultures without the laminar airflow cabinet) for growing *C. militaris*. The results showed that the two methods had no significant differences in cordycepin production and biomass production. *Cordyceps militaris* is suitable for production in the industrial system because the trend of the world is concerned about health. Nowadays, Thailand has entered an ageing society. Quite a several people are turning to take care of their health more and see the importance of nutritional supplements. The food supplement is a huge market, and its value is constantly increasing. *Cordyceps militaris* are used as dietary supplements in humans and animals. There are many forms such as capsules, tablets, water extracts, and cosmetics can be mixed with a variety of food. However, there is very little medical research on *C. militaris* in Thailand. Most medical information is derived from research abroad.

Successful knowledge transfer can be achieved if universities do not expect too much commercial interest in their research. The training course was one of the methods that saw clear results as in the case of *C. militaris* mushroom. Many people had benefited from the research. Research related to medicinal plants or mushrooms is needed by industry. People prefer to use medical herbs rather than synthetic ones.

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Research Note

Qualitative Analysis of Adulterant Mixed in Different Food Stuffs

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Abstract

This study focuses on identifying adulterants present in items such as food, fuels, chemicals and cosmetics, known for degrading their overall quality. The escalating concern over food adulteration prompted this research, emphasizing the detection of adulterants in daily consumables. The detrimental effects of food adulteration are profound, leading to health issues such as cancers (colon and peptic ulcer diseases), chronic liver diseases, electrolyte imbalance, kidney failure, heart diseases, blood disorders, and bone marrow abnormalities. The primary objective of this research is to ensure the quality of commonly consumed food items by detecting potential adulterants. Numerous rapid detection techniques have been developed to address this problem, including the implementation of quick and straightforward DART methods (Detect Adulterant Rapid Test). In this study, we applied various DART and DIY methods to test selected food items like milk, turmeric powder, and chilli powder. Each sample underwent testing with specific chemical reagents to determine the presence of adulterants. Post-tests, the samples were analyzed for observable changes, and conclusions were drawn regarding the presence or absence of adulterants in each tested item.

Keywords: Adulterants, DART, DIY, Human health

Main Body

Food stands as a fundamental necessity for the sustenance of all living beings, playing a vital role in growth and various life processes (Foskett et al., 2021). The quality of the food we consume is paramount, as subpar products can have detrimental effects on consumer health. Unfortunately, in contemporary times, there is a prevalence of poor-quality food products, exemplified by instances such as the adulteration of milk with water or starch, the inclusion of tiny stones in rice, and the addition of chalk powder to sugar, among other adulterations like metanil yellow in pulses and lead salts in chilli powder (Rees, 2020).

Food adulteration, defined as the introduction of unwanted elements into foodstuffs, is considered a legal offence when the product fails to meet established standards or quality (Rahman et al., 2015). The intentional, unintentional, or naturally occurring adulteration of food presents challenges for consumers in detecting the extent of adulteration. The irresponsible practices of producers and sellers during production or the presence of undesirable substances in raw materials contribute to this problem, persisting across all stages of food from preparation to consumption (Wani et al., 2015).

The consequences of food adulteration extend to health risks, including toxicity, chemical contamination with substances like formalin, mycotoxins, metals, and pesticides in commonly consumed items such as milk and rice, which pose significant threats to human health (Vyralakshmi & Jayasheela, 2017). Moreover, excessive use of colouring dyes, calcium carbide, urea, lead chromate, coal tar, and prohibited colours and preservatives affects multiple organs in the human body (Banti, 2020).

This study aims to raise awareness about food adulteration and its long-term adverse effects on health. Various tests and experiments conducted during this project employ accessible and rapid methods, ensuring that individuals can perform them in their local labs using readily available chemical reagents. The simplicity of these procedures eliminates the need for specialized expertise, allowing ordinary individuals to test their daily foodstuffs and safeguard against the consumption of adulterated food to a significant extent.

Samples were gathered from various vendors in the Rupandehi district, including milk (from local farmers and packed samples), turmeric powder (open and packed samples), mustard oil (open and packed samples), chilli powder (open and packed samples), and open sugar from the local market.

Numerous techniques exist for detecting food adulterants, including chemical tests, DART, DIY, HPLC, GC-MS, and spectroscopic methods. In this study, rapid chemical tests, DART and DIY methods were employed. Starch presence in milk was identified using an iodine solution, with a blue colour indicating its presence. Detection of skim milk powder involved testing with dil. HNO_3 and an orange colour indicated its presence. Detection of lead chromate and coal tar dye in turmeric powder was carried out with conc. HCl, 1% diphenyl carbazide reagent, petroleum ether, and conc. HCl, revealing the presence through colour changes. Detection of argemone oil in mustard oil and red colour lead salt in chilli powder involved conc. HCl and 10% $FeCl_3$, as indicated by colour crystals and layers. Chalk powder and washing soda in sugar were identified using conc. HCl (He et al., 2021; Elmadfa, 2005).

Public concern and reactions significantly influence perspectives on food safety and health (Pal & Mahinder, 2020). Numerous instances of food adulteration have been recorded in products like vegetables, fruits, spices, oils, meat, fish, milk, and drinks (Schieber, 2018). Local findings reveal extensive adulteration, particularly in spices. Turmeric powder, a popular natural colouring, often contains harmful additives like coal tar colour or lead chromate, impacting its brightness (Sudershan et al., 2009). Notably, turmeric powder and mustard oil are highly adulterated, containing harmful substances like coal tar dye and prohibited colours, as indicated in the table below from the conducted tests.

Table 1 reveals the absence of lead chromate in all samples tested, while coal tar dye is present in both packed samples but absent in the open sample from a local farmer. These adulterants pose severe health risks, with lead chromate potentially causing anaemia, brain damage, paralysis, and miscarriage, especially when added to spices for vibrant colouring (Cowell et al., 2017). Coal tar dyes, by-products of hydrocarbon solvents, are considered unsafe for consumption. Heavy metal additions, like aluminium in trace levels, may harm the brain, potentially leading to conditions like Alzheimer's. Lead-contaminated foods, deadly poisonous, adversely affect various body parts, especially in children, causing issues like sleeplessness, irritability, restlessness, and mental retardation (Momtaz et al., 2023). Chilli powder in this study did not exhibit the presence of red-coloured lead salts.

Table 1: Detection of adulterants in different food items.

Adulterant in food	Open sample	Packet sample
Lead chromate in turmeric powder	Absent	Absent
Coal tar dye in turmeric powder	Absent	Present
Lead salts in chilli powder	Absent	Absent
Starch in milk	Absent	Absent
Skim milk in milk	Absent	Present
Argemone oil in mustard oil	Absent	Present
Prohibited oil in mustard oil	Absent	Present
Chalk powder/ washing soda in sugar	Absent	Absent

Food items, such as milk, sugar, and chilli powder, exhibited either minimal or no adulteration in the chosen small and local sample area. However, these findings don't necessarily extend to other regions. The limited sample area influenced the lower extent of observed adulteration. To achieve comprehensive results on food adulteration, a broader sample collection area is essential. Methodology plays a crucial role, and this study employed rapid techniques due to cost and time constraints. While the qualitative approach lacks standardized data for comparison, results may vary based on different brands, manufacturing methods, and regional disparities observed in previous research. Developed countries often show minimal adulteration, contrasting with higher levels in underdeveloped nations like Nepal, attributed to producer greed, fraud, and buyer ignorance.

Detecting sophisticated food adulteration methods demands highly efficient and reliable techniques. The conducted laboratory tests on a limited sample of daily-used food products reveal a concerning trend: while some items are safe, the majority of packed foods consumed are adulterated. The study, based on a restricted sample, concludes that approximately half of these items are adulterated, suggesting a potential rise in adulteration.

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