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The figure in the cover page showing : Flower buds of *Clerodendrum indicum* showing the initiation of flower opening at various stages of adaptations to higher degree of out-breeding in the article by Arijit Ghosh, *J. Botan. Soc. Bengal,* 77(2): 35-45, 2023

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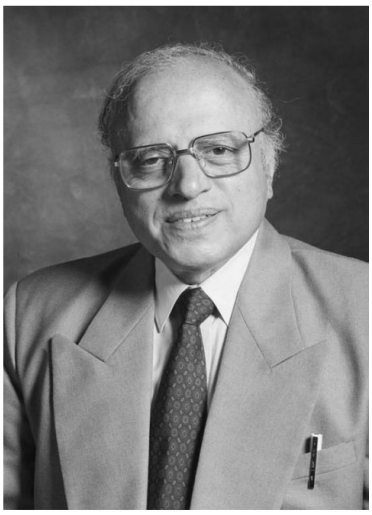
Editorial

M.S. Swaminathan – 'Godfather of India's Green Revolution'

Monkombu Sambasivan (MS) Swaminathan, the man who fed India and leader of India's 'green revolution' passed away at the age of 98, on September 28, 2023. Dr. Swaminathan was born in an agricultural family on August 7, 1925 at Kumbakonam, Tamil Nadu, India. His family wanted him to become a doctor. But the devastation of the Bengal famine of 1943, put Swaminathan on a different path. This tragic event left a profound impact on him and stirred his passion for improving India's agricultural sector. He took a leading role in India's "Green Revolution", a transformative phase in Indian agriculture that significantly increased **crop yields**, making India self-sufficient in food production and averting the looming threat of famine. Swaminathan's groundbreaking work with **Norman Borlaug** in developing high-yielding wheat and rice varieties, notably the **semi-dwarf wheat varieties**, revolutionized agriculture in India. For his outstanding contributions to agriculture in increasing agricultural self-sufficiency he was honored as the **first World Food Prize Laureate in 1987**. He was also conferred with the Padma Shri (1967), Padma Bhushan (1972) and Padma Vibhushan (1989) by the government of India. International honors including **the Ramon Magsaysay Award** (1971) and **the Albert Einstein World Science Award** (1986) are also to his credit. Despite the various accolades, he was humble and soft-spoken, and dreamt that every other member of the human family will go to bed after a nourishing meal. His resolution was that "Until such a wholly attainable world becomes a reality, our task remains unfinished."



Agriculture has traditionally been India's most important economic sector. Farmers in India had long suffered from a lack of food security. In the 1960s, it was widely predicted by demographers and economists that population would outstrip food production in developing nations, leading to famine in India and throughout the rest of Asia. At the time, massive shipments of imported grain were the only means by which the continent was averting famine. As a young scientist at the Indian Agricultural Research Institute in the 1950s, Swaminathan learned of Dr. Norman Borlaug's newly developed Mexican dwarf wheat variety and invited him to India. The two scientists worked side by side to develop wheat varieties that would yield higher levels of grain. In addition to this scientific breakthrough, Swaminathan also created new methods to teach Indian farmers how to effectively increase production by employing a combination of the high-yielding wheat varieties, fertilizers, and more efficient farming techniques. By this method the total crop yield of wheat rose from 12 million tons to 23 million tons in four crop seasons, ending India's reliance on grain imports. His pioneering work in agriculture and specific sectors like wheat breeding led to a significant increase in wheat production, thus turning India from a food-deficient country into a self-sufficient nation. This tremendous achievement earned him the well-

deserved title of, "Father of the Indian Green Revolution." He sounded the alarm by saying that if farmers didn't focus on soil fertility, desertification of land would occur. Rampant use of pesticides and sucking out groundwater could lead to a doomsday scenario. He played a key role in shaping the sixth Five Year Plan (1980-1985), where, for the first time in the history of planning, he introduced two new chapters, one on 'Women and development' and another on 'Environment and development'. He was also a pioneer in advocating for climate-resilient agriculture through advanced science and farmers' participation. He was of the opinion that with climate change and rising sea levels, there is a possibility of salinisation of coastal soils, where rice is a major crop. His diverse roles in leadership, coupled with his pioneering work in crop improvement, continue to influence agricultural practices and environmental stewardship both in India and abroad. Swaminathan's legacy serves as a beacon of inspiration for those striving to address the complex challenges of agriculture and conservation in the modern era.

His demise is almost like an end of an era in the field of agriculture. His knowledge became a boon for countless people and he will be remembered forever as the **Father of Indian Agriculture**.

Subir Bera
Ashalata D'Rozario

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REVIEW

Grasses and bamboos in north-eastern India – an overview

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Members of the family Poaceae, commonly known as grasses and bamboos, are one of the interesting floral components among the monocots owing to their economical and ecological importance. They are also taxonomically very significant due to their distinctive and curious vegetative and floral morphology. Being cosmopolitan in distribution, the family is represented by more than about 12000 species under 771 genera. In India, Poaceae is the largest family with about 1225 species. Earlier contributions on grasses and bamboos from northeastern India, along with current information are reviewed here.

Key words: Diversity, Distribution, Exomorphology, Poaceae, North-East India.

INTRODUCTION

Grasses and bamboos (Poaceae, *nom. alt.* Gramineae) are very distinctive among the monocotyledons for their complex vegetative and floral morphology. Globally, Poaceae is one of the most economically important plant families; not only as the major sources of food grains but also for their use as fodder, house building materials, paper pulps, thatching materials, etc. Both urban and rural economies are largely dependent on this plant family. Bamboos, on the other hand, are used as raw materials for different small and large-scale industries such as, for the making of handicraft products, paper, alcohol industry, textile industry, construction, furniture, biofuel, food, ornamental garden plantation and beautification. They are characteristically different from other families of monocotyledons, particularly for their spikelets with unique floral bracts, caryopsis or bacca fruit types and presence of silica (phytolith). Shape, size and abundance of phytoliths are one of the tools for identification of taxa at different hierarchical levels. Ascrobiculate pollen wall is also a significant feature of the family (GPWG, 2001).

In addition to its immense economic value, the family also contributes ecologically to a greater extent (Thomasson, 1987). Grasslands are important components in the development and stabilization of soils (Stanley, 1999). They are cosmopolitan in distribution, accounting for about 20% of the

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vegetation cover on the earth (Cronquist, 1981). In India, grassland occupies about 3.9% of the total land area of the country (Nair and Thomas, 2001). Since they are cosmopolitan, they can withstand wide range of climatic conditions, such as, warm, humid, sultry tropical climate to chilling-cold polar region. Marshy and swampy wetland habitats also support a good number of species.

The family comprises *c.*12074 species under *c.*771 genera (Soreng *et al*., 2015) distributed worldwide, abundant in the tropical and north-temperate semi-arid regions (Cronquist, 1981). Clayton and Renvoize (1986) estimated more than 10,000 species under 700 genera worldwide; whereas, Mabberley (2008) enumerated 10,550 species under 715 genera in the world. It ranks third in the number of genera, after Asteraceae and Orchidaceae, and fifth in number of species, after Asteraceae, Orchidaceae, Fabaceae and Rubiaceae (Good, 1953). In India, Poaceae is the largest family with *c.* 268 genera and *c.* 1225 species (Karthikeyan, *et al*., 1989; Moulik, 1997). Nair and Thomas (2001) reduced the number of grass genera in India to 249, of which, 15 genera are endemic to this country. Northeastern part of India harbours *c.* 146 genera and 475 species (Shukla, 1996) and is one of richest parts of India next to peninsular part in terms of grass diversity (Nair and Thomas, 2001).

NOTEWORTHY CONTRIBUTIONS ON THE FAMILY: brief history

The utilization of grasses as agricultural crops and the naming of grasses date back to at least before 2000 years (Gibson, 2009). In ancient Greece, Theophrastus (370-287 BC), in his *Enquiry into Plants*, comprehended at least 19 different grasses including two bamboos (*Bambusa* and *Dendrocalamus*) and three species of wheat (*Triticum aestivum, T. dicoccum* and *T. monococcum*) (Chapman, 1996). Taxonomic study on the family Graminae existed since *c.* 250-300 years ago. Among the earliest monumental works, Rheede (1678) illustrated 20 plates of grasses in *Hortus Malabaricus*. However, publication of '*Agrostographiae Helveticae Prodromus*' by Johann Scheuchzer in 1708 is considered as the first published paper dealing with grasses (Gould and Shaw, 1983; Roy, 1984; Gibson, 2009; Ambasta, 2016). In 1753, Carl Linnaeus with the introduction of binomial nomenclature for flowering plants in his book *Species Plantarum*, presented and listed *c.* 40 grass genera. Subsequently, Adanson (1763) divided the grass family into several natural groups on the basis of both vegetative and floral characters. The most important groups recognized by him were 'Poae' and 'Panica' which are presently recognized as tribes Poeae and Paniceae respectively (Calderón and Soderstrom, 1980). Robert Brown (1810) was the first to describe the grass spikelet by recognizing it as a reduced inflorescence branch. He gave diagnostic description of 32 genera and *c.* 200 grass species while studying Australian flora in his *Prodromus Florae Novae Hollandiae et Insulae Van-Diemen*. Subsequently in 1814, he divided the grass family into two tribes, Paniceae and Poaceae. Later, many authors treated these two tribes as present day subfamilies Panicoideae and Pooideae (Calderón and Soderstrom, 1980). In the same year Roxburgh (1814) enumerated seven species of bamboos in his book *Hortus Bengalensis*. In the year 1833, Kunth distinguished 13 tribes of grasses, but without assigning them into subfamilies. His system of classification was adopted by Endlicher, Palatore and Steudel (Gould and Shaw, 1983). Griffith (1834, 1836) inventorized the grasses of *jheels* (wetlands) of Sylhet district of present day Bangladesh. Beddome (1873) and Kurz (1878) were some of the early contributors on the taxonomy of bamboos in India. In 1883, Bentham and Hooker presented the family Gramineae in two series viz., Panicaceae and Poaceae with a total of 13 tribes. The classification was further published in *Genera Plantarum* and was adopted with modifications by Hackel (1887, 1889), Stapf (1917-1934), Hitchcock (1920, 1935) and Bews (1929). A detailed account on the members of the family *Gramineae* for the first time in India was discussed by Hooker in 1897 in his largest systematic outcome *Flora of British India* which covered 700 species under 130 genera. According to the author, there were a few Indo-Chinese and Burmese components in the flora of the region. Hooker's work was followed by the publication of several regional floras and accounts of grasses at different periods by Prain (1903), Cooke (1908) and many more. Clayton and Renvoize (1986) have made a grand commitment on world grasses; they identified around 651 genera with 10,000 species under 40 tribes

with 34 sub-tribes under six subfamilies of grasses. They also developed a three-level classification for *Gramineae* i.e., into six subfamilies, 40 tribes and 34 subtribes. In Indian subcontinent, Dassanayake (1995) presented 345 species under 136 genera of Poaceae from Sri Lanka. Noltie (2000) treated 112 genera and 389 native and introduced species in the book '*The Grasses of Bhutan*' in the series '*Flora of Bhutan*'. In 2006, Chen *et al.* presented the family Poaceae as a part of the series '*Flora of China*' in volume 22 with 28 tribes, 226 genera and 1795 (accepted) species. Significant contributions on the family were also made time to time in the form of regional floras.

In India, the earliest monumental work begun with the works of Rheede's *Hortus Malabaricus* (1678 -1703), in which he illustrated 20 plates of grasses including three bamboos. Afterward, in 1814, Roxburgh mentioned seven species of bamboos in his book *Hortus Bengalensis*. The early works on the taxonomy of bamboos in India include those of Beddome (1873) and Kurz (1878). Duthie (1883) checklisted the indigenous and cultivated grasses of Northwestern India and published the book '*Illustrations of the indigenous fodder grasses of the plains of N. W. India*' in the year 1886. Later, in 1888, he attempted a short account of the important grasses used in the plains of Northern India, either as fodder or forage and published a book titled '*The fodder grasses of northern India*'. This was followed by William (1889) who published '*Grasses of the Southern Punjab*' which became the milestone in the field of Indian grasses resulting filling of further research and expeditions. The author dealt with grasses of the semi-arid lands in the southern part of the undivided Punjab with an illustrated account of about 40 grass species. Further, Hooker's (1897) '*Flora of British India*' added significant contribution towards the family Graminae of India. The twentieth century grass systematics in the country were initiated by Prain (1903), Cooke (1908) and Hole (1911) where they incorporated a few grass species from their study area. Achariar and Mudaliyar (1921) provided an excellent account of the grasses of the plains of South India with illustrated descriptions of a 100 species under 42 genera and eight tribes of two series *viz*., Panicaceae and Poaeae. Blatter and McCann (1935) contributed detailed information on the grasses of Bombay enumerating 311 species under 110 genera of 18 tribes belonging to two subfamilies *Panicoideae* and *Pooideae*. They also provided 189 plates for 189 species. Caius (1936) documented a few medicinal and poisonous grasses of India. The monumental work on grasses in India was done by Bor (1941) who published a monograph on grasses of the State Uttar Pradesh, which gave a breakthrough on systematics and ecology of grasses in the other parts of the country as well. In addition, Hooker's work was brought up-to-date by the monumental work of Bor (1960) on grasses with a total of 1274 species under 242 genera (excluding bamboos). Majumdar (1973) made an account on the genus *Panicum* L. in India where he listed 29 species from the country. Jagdishchandra (1975) compiled the genus *Cymbopogon* Spreng. for the flora of India. Prakash *et al.* (1978) enumerated 94 species of grasses for Indian flora. Mehrotra and Jain (1980) worked on the endemicity of the tribe Andropogoneae in India and they enumerated 151 endemic species from the tribe. Deshpande and Singh (1986) enumerated 397 taxa under l09 genera of grasses in their work entitled '*Grasses of Maharashtra: An Annotated Inventory'* along with their up-to-date nomenclature, generic and specific keys for easy identification, local names, if any and district wise distribution within the state. Karthikeyan *et al.*, in 1989, published '*A checklist of monocots in India*' where they enumerated *c.* 1225 species of grasses under 262 genera with updated nomenclature and notes on distribution. In this book, R.B. Majumdar worked on the subfamily Bambusoideae and he described one new genus and seven new species from the country. Sreekumar and Nair (1991) enumerated 296 species of grasses belonging to 103 genera from Kerala with two new genera and 26 new species. The first monograph on bamboos of India was done by Tewari (1992) who enumerated 129 species under 23 genera with description, distributions, flowering records and uses. Ashalatha and Nair (1993) revised the genera *Brachiaria* Griesb. and *Urochloa* P. Beauv. in India. Later, Pal and Thothathri (1996) worked on *c*. 30 species of threatened and endangered grasses of India. Moulik (1997) comprehended grasses and bamboos of India and enumerated 1274 species under 250 genera from the country. This observation was made mainly on the basis of herbarium specimens housed at different herbaria. Seethalakshmi and Kumar (1998)

contributed towards bamboos of India with updated nomenclature; they enumerated 128 species under 18 genera from the country. Noltie (2000) in his work '*Flora of Bhutan*' covered grasses of Sikkim, Darjeeling and Chumbi. Muktesh Kumar *et al.* (2000) worked on the tribe Bambuseae and recorded the representation of 128 species of grasses under 18 genera. Nair and Sharmila (2001) recorded 1291 grass taxa in India with 318 taxa as endemic to the country. Reddy (2002) enumerated 252 wild and naturalized grasses from the Eastern Ghats. Later, Joshi and Janarthanam (2004) compiled a list of 113 endemic species from Goa. Salam and Pongen (2008) illustrated 13 species of bamboos for easy identification. Kabeer and Nair (2009) worked on grasses of Tamil Nadu where they reported 447 species under 136 genera. Negi (2009) enumerated 113 important bamboos in India along with colour plates. Yadav (2010) published a book entitled '*Know your grass genera through hand lens*' aimed at familiarizing grasses and hence popularizing grass taxonomy amongst botanists, amateurs, forest divisions, etc. This book is a pictorial depiction of 115 genera of grasses belonging to 2 subfamilies and 26 tribes occurring mainly in Maharashtra and / or Peninsular India. Benjamin *et al.* (2012) made a bibliographic account of the family Poaceae for Indian region. Kumar (2014) enlisted 212 species from Punjab out of which taxonomic descriptions of 105 species had been provided. SijiMol *et al.* (2016) documented a review of the ecological functions of reed bamboo genus *Ochlandra* in the Western Ghats of India giving emphasis on their conservation. Veldkamp *et al.* (2017), very recently, reported *Eragrostis paniciformis* (A. Braun) Steud. as a new record for Asia based on the collection made from Kerala, India. Recently, Kellogg *et al.* (2020) compiled a comprehensive list of the family in India.

The first regional flora for northeastern India by Kanjilal *et al.* (1934 – 1940) included floristic accounts of all the seven states of North East India. In this series, N. L. Bor, a forest officer, collected and studied the grasses of the then Assam and published a list of grasses (1938) and a comprehensive account of grasses in the 5th volume of Kanjial's series (1940). He enumerated about 74 Genera with 174 species under 16 Tribes of the sub-family Pooideae and 72 Genera with 219 species under three Tribes of the sub-family Panicoideae from the then Assam. The detailed floristic study of Manipur was made by Deb (1957, 1961a, b) who reported 117 species belonging to 72 Genera of the family Gramineae from the state. Gupta (1972) and Nath (1959, 1962, 1968, 1971) contributed on the flowering of certain bamboo species from the Southern Assam. Jain and Shukla (1979) reported about 44 species of grasses from Manipur among which 28 grasses were new reports for the state. Dr. D.B. Deb did immense work on the floristics of Tripura and published his monumental book '*The Flora of Tripura State*' -Vol. 1 & 2 during 1981– 1983. He enumerated 1,463 taxa of angiosperms out of which 79 species belonging to 49 genera were of grasses. Afterwards, Shukla (1996) enumerated approximately 475 grass taxa from Northeastern India in his volume '*The Grasses of North Eastern India*'. Recently, in 2007, Khan *et al.* supplemented 10 species of grasses belonging to 8 genera from Manipur. A comprehensive work on bamboos of Manipur was contributed by Naithani *et al.* (2010) where they enumerated 35 species (with two varieties) from Manipur belonging to 9 genera. Devi and Bhattacharyya (2013a) reported *Steinchisma laxa* (Sw.) Zuloaga as *Panicum laxum* Sw. for the first time in India from northeastern part of India (Assam and Tripura) with a detailed description, illustration and photograph. Rajkumari and Gupta (2013) enumerated 45 species of bamboos from Manipur with their uses. Barbhuiya *et al*. (2013) published a checklist of grasses in Southern Assam and reported a total of 98 species under 49 genera from the region. Kumari and Singh (2014) enumerated about 47 species and 2 varieties of native and naturalized bamboos from the state Meghalaya with updated nomenclature. Some important contributions were made on grass flora of North-East India by Pathak and Singh (2012, 2013, 2013a), Kandwal *et al*. (2013), Pathak (2013, 2013a), Pathak *et al.* (2013, 2015) mainly concentrating on the state Mizoram. In 2015, Devi and Bhattacharyya rediscovered a rare grass *Isachne dimyloides* from southern Assam after a gap of 130 years since its type collection. *Themeda quadrivalvis* (L.) Kuntze was also reported as new record for Assam state (Devi and Bhattacharyya, 2016). Other significant contributions on the family in NE India were made from time to time by Deb (1961, 1981), Rao (1970), Shukla (1978,

1978a, 1982, 1983, 1993, 1993a), Deb and Dutta (1987), Biswas (1993), Barooah and Borthakur (2001, 2003), Bujarbarua and Sarma (2006), Baruah *et al.* (2007), Kumari and Singh (2007, 2008), Sharma and Borthakur (2008), Naithani *et al.* (2010), Pathak and Singh (2012, 2013a, 2013b), Barbhuiya *et al*. (2013), Kandwal *et al*. (2013), Pathak (2013a, 2013b), Pathak *et al.* (2013, 2015).

Present observations

Studies on the diversity of grasses and bamboos in northeastern India were attempted since 2010. Contemporary morpho-taxonomic method of research was employed for the studies. North-eastern region lies both in the Eastern Himalaya under Himalayan and Indo-Burma Biodiversity Hotspots. Owing to its varied physiographical and climatic conditions, occurrence of numerous river basins and wetland habitats, huge diversity of grasses were recorded.

It shows a heterogeneous assemblage of species starting from small erect to creeping herbs, small to medium-sized reeds, tall, straight tufted bamboos of thin to thick culms. Climbing bamboos were also recorded from the region. From southern part of Assam (Barak Valley), we recorded a total of 121 species with one subspecies and nine varieties distributed under 59 genera; from Manipur, 109 species under 51 genera; and from Tripura, a total of 120 species under 67 genera.

All the six subfamilies of the family, *viz*., Bambusoideae, Pooideae, Centothecoideae, Arundinoideae, Chloridoideae and Panicoideae delineated by Clayton and Renvoize (1986) were found to occur in north-east. Among the subfamilies, Panicoideae was found as the most dominant followed by Bambusoideae.

Dominant genera recorded from the region are *Bambusa*, *Dendrocalamus*, *Digitaria*, *Eragrostis*, *Panicum* etc. Many new records were made; many species were found rare in the region. Continuous monitoring is required for the conservation and sustenance of the species.

Although true kind of savannas are not prevalent in the study area, but some kinds of association of true grass species have been found to occur in the study area in small patches. *Imperata-Saccharum-Themeda* association, *Saccharum-Thysanolaena* association, *Arundinella-Imperata* association, *Thysanolaena-Saccharum-Imperata* association and *Miscanthus-Imperata* association were found to occur in different habitats of the study area. Dominance of some wild bamboo species in patches was also recognized in the study area. Association of *Melocalamus compactiflorus* and *M. indicus* was seen in southern part of Assam.

Two bamboo species, endemic to North-Eastern India were also noticed *viz*., *Bambusa mizorameana H. B. Naithani and Dendrocalamus manipureanus* H. B. Naithani & N. S. Bish. *Bambusa mizorameana is endemic to Mizoram and Manipur; whereas, Dendrocalamus manipureanus* is endemic to Manipur. *Jhum* cultivation practiced in NE India is one of the major threats for grass vegetation since this practice wipe out largely the ground vegetation.

The family is monophyletic [Grass Phylogeny Working Group (GPWG), 2001; Michelangeli *et al.* 2003; Duvall *et al.,* 2007; Soreng *et al.*, 2015] and the taxonomic organization of the family into subfamilies and tribes is still in a state of flux. Prat's work (1936) is considered as a turning point in grass taxonomy. Many micro-anatomical as well as chromosome number have been proved to be of immense importance in assessing relationships among different infrageneric categories (GPWG, 2001). Some of the traditional tribes are now appeared to be heterogenous and a minimum of three subfamilies are recognized presently *viz*. Bambusoideae, Pooideae and Panicoideae. Previously, Hutchinson (1934) divided the family Poaceae into two subfamilies, Pooideae (having 24 Tribes) and Panicoideae (having 3 Tribes), however, Clayton and Renvoize (1986) divided the family into 6 subfamilies viz. (i) Bambusoideae (13 Tribes), (ii) Pooideae (10 3 Tribes), (iii) Centothecoideae (1 Tribe), (iv) Arundineae (4 Tribes), (v) Chloridoideae (5 Tribes) and (vi) Panicoideae (5 Tribes). On the basis of the morphological and molecular markers, GPWG (2001) and Soreng *et al.* (2015) recognized 12 subfamilies.

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FULL LENGTH ARTICLE

New records of parasitic *Curvularia* species from Indian Sundarbans and their phenetic studies

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Sundarbans is the largest mangrove ecosystem of the world. Almost all the plants reported here are economically and medicinally important. Majority of the mangrove plants are alternate host of various crop pathogenic fungi i.e. the reservoir of fungal inocula. Most of the mangrove plants are now the targets of various fungal attacks which may create conservation problems in future. Different species of the genus *Curvularia* viz. *C. clavata*, *C. eragrostidis, C. lunata, C. pallescens, C. senegalensis, C. tuberculata* and *C. verruculosa* have been isolated from a number of mangrove plants of Indian Sundarbans. Most of them are endophytes and parasites. These endophytic fungi are potential sources of many bioactive phytochemicals. Thus, characterization and relationship among these species is worthwhile. In this study, we have made a comparative morphological study of these species. A phenetic tree has also been developed to understand the taxonomic relationship among these species. This tree placed *C. eragrostidis, C. lunata* and *C. pallescens* in a single clade. These species are indeed morphologically more similar than other species. The Principal co-ordinate analysis also supports the phenetic tree. This study is expected to provide valuable information in the field of fungal taxonomy.

Keywords : endophytic fungi, parasitic fungi, alternate host, conidia, inocula, phenetic analysis.

INTRODUCTION

The fungal genus *Curvularia* includes numerous plant pathogens and some emerging opportunistic pathogens of humans (Madrit *et al*., 2014). *Curvularia*, typified by *C. lunata*, is a species-rich genus, which includes numerous grass pathogens and saprobes occurring on plant material, dung and soil (Faurel and Schotter, 1965; Sivanesan, 1987; Jiang and Zhang, 2007). *Curvularia* is closely related to *Bipolaris, Drechslera* and *Helminthosporium*. Several new species added to the genus *Curvularia* were originally placed under *Helminthosporium*. Morphologically *Curvularia* is characterised by the production of sympodial conidiophores with tretic, terminal and intercalary conidiogenous cells and elongate, transversely septate conidia with a dark basal scar. Conidia are often curved at an asymmetrically swollen intermediate cell, but species with straight conidia have also been described (Sivanesan, 1987). Authors such as Ellis (1971, 1976), de Hoog *et al*. (2000) and Revankar and Sutton (2010) have described the conidia as truly septate or 'euseptate'. A similar genus is *Bipolaris* which traditionally has been distinguished from *Curvularia*

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by producing conidia which lack an asymmetrically swollen intermediate cell and are 'distoseptate' (Domsch *et al*., 2007; Revankar and Sutton, 2010). The separation of the two genera has been a matter of controversy and many authors have stated that *Curvularia* species also have distoseptate conidia (Alcorn, 1983; Sivanesan, 1987; Seifert *et al*., 2011). Sexual stages of *Bipolaris* and *Curvularia* were traditionally placed in *Cochliobolus.* Typically, they feature thick-walled, ostiolate ascomata with pseudoparaphyses, and bitunicate asci that give rise to filiform, multiseptate ascospores (Sivanesan, 1987; Zhang *et al*., 2012). The ascospores often appear more or less helically coiled within the ascus. A similar genus, *Pseudocochliobolus* was segregated from *Cochliobolus* to accommodate species producing ascomata on columnar stromata, with ascospores appearing linearly parallel or loosely coiled within the asci. The asexual stages of *Pseudocochliobolus* species were *Curvularia* and *Bipolaris* species with short, rather straight conidia (Tsuda *et al*., 1977; Tsuda and Ueyama, 1981). Most authors have not accepted *Pseudocochliobolus* as a separate genus because the degree of coiling of the ascospores can vary greatly within a species. Also, the addition of a second genus with *Curvularia* and *Bipolaris* asexual stages would introduce unnecessary complexity into the taxonomy of this group of fungi instead of clarifying it (Alcorn, 1983, 1988; Sivanesan, 1987). Berbee *et al*. (1999) performed a phylogenetic study to assess the evolutionary relationships of *Cochliobolus, Pseudocochliobolus, Curvularia* and *Bipolaris.* Their phylogenetic trees revealed that isolates were distributed mainly in two clades which were named '*Cochliobolus* groups 1 and 2'. Group 1 exclusively encompassed species with *Bipolaris* asexual morphs and Group 2 included mostly plant pathogens and saprobes with *Bipolaris* and *Curvularia* asexual morphs, including the type species of the latter genus, *C. lunata* and all species of *Pseudocochliobolus.* Later *Cochliobolus* and *Pseudocochliobolus* were synonymized with the more commonly used generic names *Bipolaris* and *Curvularia*, respectively, and the generic concept of the latter genus was expanded to accommodate some species with rather straight conidia formerly placed in *Bipolaris* (Manamgoda *et al.*, 2012).

Mangroves are specialised forest ecosystem found at the land-sea interface of the tropical and subtropical regions of the world bordering the sheltered sea coasts and estuaries. These forest systems are dominated by the salt tolerant halophytic seed plants that ranged in size from tall trees to shrubs and being restricted to the intertidal belts are exposed to the high and low tides twice in 24 hours. This very vibrating ecosystem supports numerous terrestrial, benthic and aquatic organisms forming a complex association of species, exchanging materials and energy within the system and between the systems, and the adjoining coastal waters. The Sundarbans are the largest mangrove forest in the world, covering about one million hectares of the Ganges-Brahmaputra delta in India and is home to a large number of mangrove plants (Mandal and Naskar, 2008). In the present investigation a comparative morphological study between seven species of *Curvularia* from Indian Sundarbans has been done along with phenetic analysis.

MATERIALS AND METHODS

Isolation of fungi from infected plant parts

Both healthy and infected leaves were collected at random from living trees, shrubs and herbs from different places of Indian Sundarbans in different seasons, placed in polythene bags and brought to the laboratory for examination. The organisms were isolated from infected leaves following the standard method (0.1% $HgCl_2$ and sterile distilled water), identified and maintained in Potato-dextrose-agar medium at 25± 1°C. Dried infected plant parts were finally mounted on herbarium sheets.

Microscopic Examination

For microscopic examination of infected leaf materials, both dissecting and compound microscopes were used to study the vegetative and reproductive structures of fungi. A small portion of the sporing tissue was first placed under a dissecting microscope, mounted in lactophenol, teased carefully, covered with a cover glass and observed under a microscope. Vertical sections (V.S.) of pycnidia or acervuli or uredinia were also examined under compound

microscope. In most cases, cotton blue lactophenol was used for staining purpose. The slides were finally sealed with nail varnish, labelled and stored for further examination, if necessary.

Microscopic measurements of reproductive structures were made with an ocular and a stage micrometer. For spore measurements, a number of isolated spores were chosen for micrometric measurements. Camera lucida drawings were also made under necessary magnifications depicting almost all the details.

Identification of fungi

The fungi were identified with the help of different monographs, books, authentic papers and available stock cultures in the Department of Botany, University of Calcutta. The identity of each fungus was confirmed by the experts of the International Mycological Institute (IMI), Kew, Surrey, England.

Maintenance of stock cultures

Fungal cultures were finally stored under three different conditions. Two sets of cultures were maintained at 5°C and 20°C respectively. The third set of culture was preserved in sterilized liquid paraffin and kept at 25°C. Subculture was accomplished at a regular interval of time. To avoid mite infestation in culture, the technique described by Smith (1967) was followed. Sometime fungal cultures were also fumigated with 'Pyridine' for 24 hr. for killing mites. The above techniques were followed as a precautionary measure as and when necessary.

Pathogenicity test

For pathogenicity tests, 10 healthy detached leaves of each host species were thoroughly washed with sterile distilled water, allowed to dry, placed on sterilized moist filter papers in disinfected plastic trays and inoculated with drops (0.02 ml/drop) of spore suspension of test fungus. The trays were covered with glass plates and sealed with petroleum jelly to prevent evaporation of water during incubation. Control set was maintained in each case with drops of sterile distilled water instead of spore suspension. Inoculated leaves were incubated for 72 hours at room temperature (30-32°C) and in diffused light. The percentage of lesion production was calculated on the basis of total number of inoculum drops placed on leaves of test species. The organisms were reisolated from leaves and compared with stock cultures.

Assessment of mycelia growth on solid medium

For the preparation of inocula, desired fungus was grown in a Petri dish (100 mm diam.) containing 0.5% Dextrose Agar medium. Usually a block (4 mm diam.) of agar containing mycelia was cut out with the help of a sterilized cork borer from the advancing zone of the mycelial mat (4-day-old culture) and transferred to PDA medium in a Petri dish (100 mm diam.), incubated at 26-28°C and under diffused light. The growth characteristics were noted after 5 and 30 days of inoculation. Rayner's (Rayner, 1970) colour chart was used for description of colour of mycelial mats.

Construction of Phenetic Tree

To construct a phenetic tree by analyzing the morphological data, the NTSYS-pc version 2.2 statistical package (Rohlf, 2009) was used. The statistical method took into account the presence or absence of each character state as differential features. The binary qualitative data matrix was then used to construct similarity matrix using the Jaccard similarity coefficient (Jaccard, 1908). The similarity matrix was then used to construct dendrogram using Unweighted Pair Group Method with Arithmetic Average (UPGMA) and Sequential Agglomerative Hierarical Nested (SAHN) cluster analysis. Cophenetic matrix was derived from the dendrogram using the COPH (cophenetic values) program, and the goodness-of-fit of the clustering of the data matrices were calculated by comparing the original similarity matrix with the cophenetic value matrix using the Mantel matrix correspondence test (Mantel, 1967) in the MXCOMP program. Principal co-ordinate analysis (PCOORDA) was performed based on the similarity coefficient using DCENTER module to transform the symmetric similarity matrix to scalar product form and then EIGEN module was used to extract eigenvectors resulting into a three-dimensional plot showing the taxa in a three-dimensional space. This is a multivariate approach which is very informative

regarding distances among major groups (Hauser and Crovello, 1982). This can complement the cluster analysis and identify patterns of association among taxa in a three-dimensional space.

RESULTS AND DISCUSSION

In course of this study, 7 species of *Curvularia* were isolated from infected leaves of 6 hosts of mangrove plant species of Sundarbans from different areas (Table 1). Hand drawing of some of the *Curvularia* species and infected leaves of their respective host plants are shown in Fig. 1 and Fig. 2. Photographs of infected leaves of *Xylocarpus moluccensis, Acanthus ilicifolius, Avicennia alba* and *Bruguiera cylindrica* are shown in Fig.3. Microphotographs of conidiophores and conidia of some *Curvularia* species are shown in Fig. 4.

The spectrum of fungal diversity in Sundarbans appears to be very wide and hence frequent exploration is needed. It is not unreasonable to assume

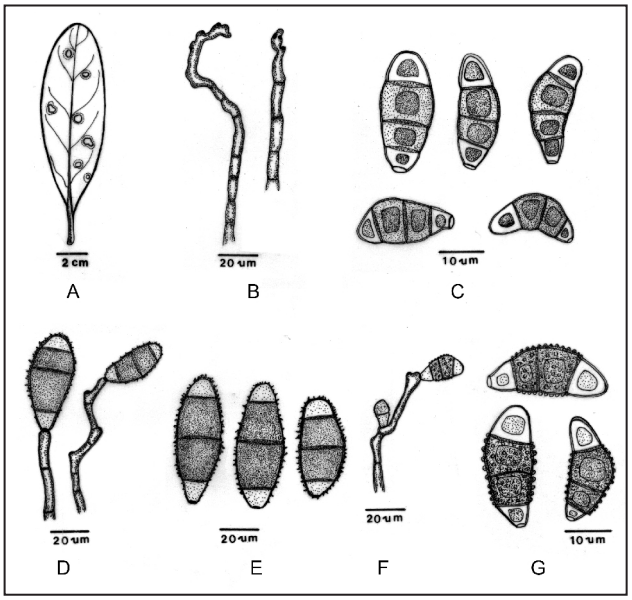

Fig. 2. A-D. *Curvularia senegalensis.* **A.** *C. senegalensis* – infected leaf of *Kandelia candel.* **B.** Conidiophores. **C.** Conidia. **D & E.** *C. tuberculata.* **D.** Conidiophores with conidia. **E.** Conidia; **F-G.** *Curvularia verruculosa.* **F** Conidiophore with conidia. **G.** Conidia.

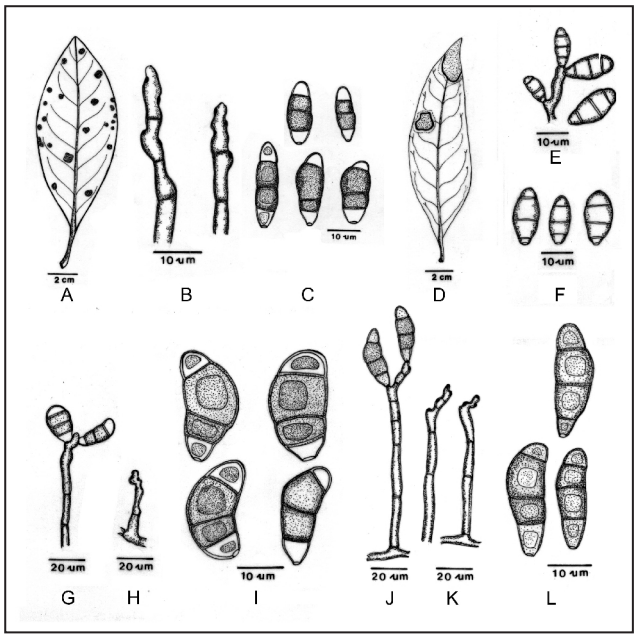

Fig. 1. A-C *Curvularia clavata.* **A.** *C. clavata* – infected leaf of *Bruguiera parviflora.* **B.** Conidiophores. **C.** Conidia; **D-F.** *Curvularia eragrostidis*. **D.** *C. eragrostridis* – infected leaf of *Avicennia alba.* **E.** Conidiophore with conidia. **F.** Conidia; **G-I.** *Curvularia lunata.* **G.** Conidiophore with conidia. **H.** Conidiophore. **I.** Conidia; **J-M.** *Curvularia pallescens.* **J.** Conidiophore with conidia. **K.** Conidiophore. **L.** Conidia.

that diversity in parasitic fungi is related to parasitism since increase in diversity leads to enhance parasitism. It is of common occurrence in the field that a fungal parasite having different strains may attack a wide range of host species. Genetic diversity in all types of organisms is increasing with time due to natural and/or unnatural causes. Besides, evolutionary changes also take place in both hosts and parasites which is probably necessary for balancing the changes in resistance of the host and virulence of the pathogen.

Curvularia is distributed worldwide and includes saprobes and pathogens of a wide range of crop plants like rice, maize etc. They are also found in other plants belonging to Aizoaceae, Caricaceae, Lamiaceae, Fabaceae and so on (Marin-Felix *et al*., 2020). This study reports several species of this fungal genus from plants of Indian Sundarbans. As mangrove is a unique ecosystem, characterization of these species of *Curvularia* is needed.

Table II makes a comparison of different *Curvularia* species based on their morphological characteristics. Highest diameter of mycelial mat was observed in *C.*

Table 1. Names of different species of *Curvularia* (Fungal Class: Hyphomycetes) isolated from infected leaves of different plant species from Sundarbans

Sl No.	Fungus	Plant Species (Host)	Vernacular Name of the Plant	Plant Family	Characteristics of Leaf Spots	Place of Collection	Fungal Holotype	Fungal Isotype
1	*Curvularia clavata* B.L. Jain	*Bruguiera parviflora W. & A.*	Champa	Rhizophoraceae	Amphigenous, distinct, small, circular to irregular, light brown, often replaced by shot holes, discrete, frequency high, concentrated toward the margin of lamina, 0.5 to 5 mm wide (Fig. 1A)	Saznekhali, 24 PGS(S), W.B., India	CUPH 506A	IMI 364264
2	*C. eragrostidis* (P. Henn.) J.A. Meyer	*Avicennia alba* Bl.	Sada-Bain	Avicenniaceae	Epiphyllous, distinct, large, irregular, brown, blackish brown border, sometimes with yellowish halo, gradually extending from the apical portion of the lamina, 10-40 mm wide, present in older leaf, frequency very low (Fig. 1D)	Netidhopani, 24 PGS(S), W.B., India	CUPH 271	—
3	*C. lunata* (Wakker) Boed.	*Acanthus ilicifolius* L.	Hargoza	Acanthaceae	Epiphyllous, distinct, discrete, initially small specks, later coalescing forming irregular patches at the margin, brown center with sa dark brown border, leaf margin show necrotic symptoms, frequency moderate, more on older leaves, 3-15 mm long & 2-8 mm wide (Fig. 3B)	Bhagbatpur, 24 PGS(S), W.B., India	CUPH 57	IMI 348455
4	*C. pallescens* Boed.	*Xylocarpus moluccensis* (L.) Roem	Pasur	Meliaceae	Epiphyllous, distinct, small, circular, greyish white center with a black border, often replaced by shot holes, discrete, occasionally on mid-veins, sometime coalescing forming small patches, frequency high, more on older leaves, 1-5 mm diameter (Fig. 3A)	Kalas Island, 24 PGS(S), W.B., India	CUPH 566	—
5	*C. senegalensis* (Speg.) Subram	*Kandelia candel* (L.) Druce	Goria	Rhizophoraceae	Amphigenous, distinct, minute, more or less circular, greyish white center with a reddish brown border, sometimes with yellowish halo, discrete, frequency moderate, young spot 1-5 mm diam. (Fig. 2A)	Prentice Island, 24 PGS(S), W.B., India	CUPH 795	IMI 369943
6	*C. tuberculata* Jain	*Bruguiera cylindrica* (L.) Bl.	Bakul Kankra	Rhizophoraceae	Epiphyllous, distinct, small, more or less rounded, brownish to black, sometimes coalescing forming patches, discrete, 1-5 mm diam. Frequency moderate, more on older leaves (Fig. 3D)	Bak-khali, 24 PGS(S), W.B., India	CUPH 136	IMI 351337
7	*C. verruculosa* Tandon Bilgrami ex M.B. Ellis	*Avicennia alba* Bl.	Sada-Bain	Avicenniaceae	Epiphyllous, distinct, small to large, irregular, often coalescing forming large patches, grey to light brown, sometimes replaced by shot holes, concentrated towards apical portion of lamina, frequency low, 0.5-20 mm wide (Fig. 3C)	Bak-khali, 24 PGS(S), W.B., India	CUPH 175A	IMI 355120

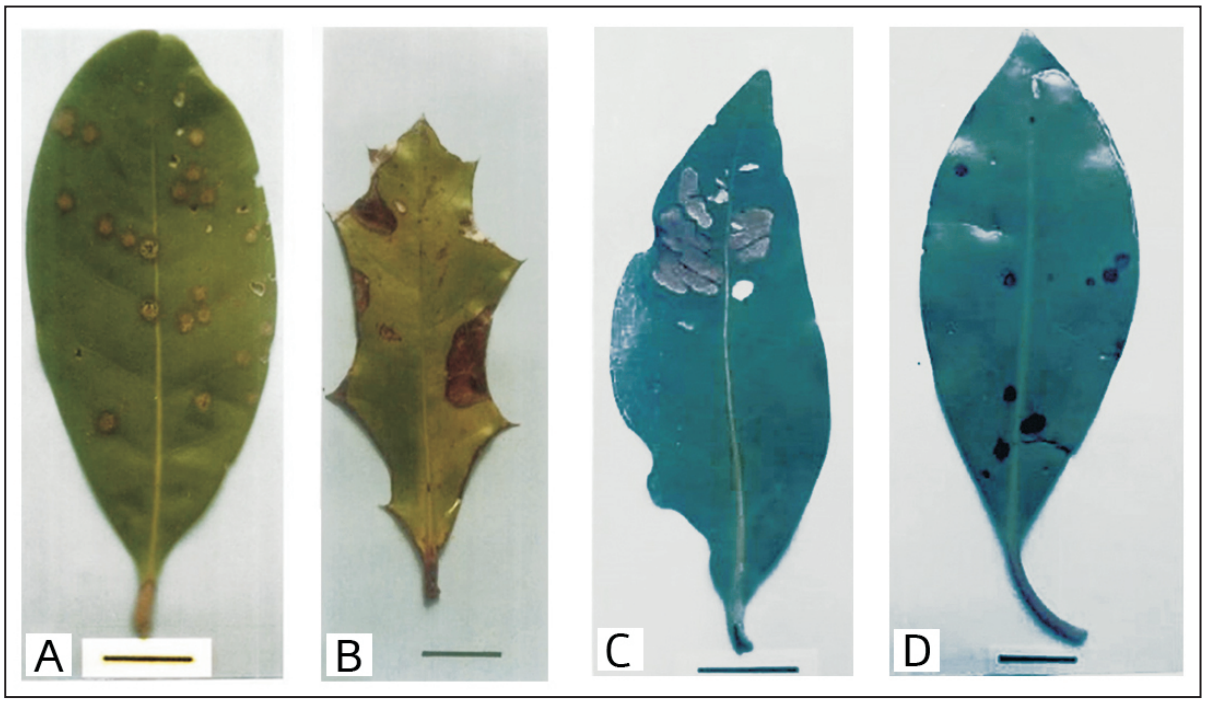

Fig. 3. A. *Curvularia pallescens* – infected leaf of *Xylocarpus moluccensis*. **B.** *Carvularia lunata* – infected leaf of *Acanthus ilicifolius.* **C.** *Carvularia verruculosa* – infected leaf of *Avicennia alba.* **D.** *Curvularia tuberculata* – infected leaf of *Bruguiera cylindrica.* **(Bar = 2 cm).**

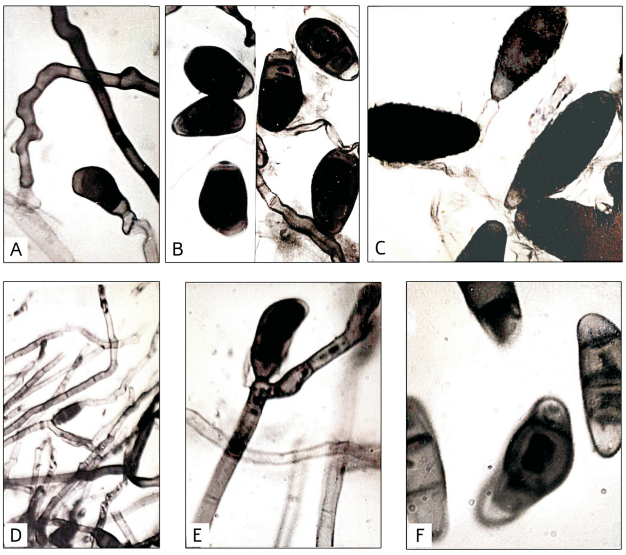

Fig. 4. A. Conidiophores & conidium (X 1000) of *Curvularia lunata*. **B.** Conidia (X1000) of *C. lunata*; **C.** Conidiophores & conidia (X 900) of *Curvularia tuberculata*; **D.** Conidiophores & conidia (X 400) of *Curvularia pallescens.* **E.** Conidiophore with conidium (X 1000) of *Curvularia pallescens.* **F.** Conidia (X 1070) of *Curvularia pallescens.*

clavata. Majority of the species showed velvety texture. Only the conidiophore of *C. tuberculata* was marked tuberculate at the swollen nodes. *C. tuberculata* was found to have the highest size variation of conidia.

Although a number of spices of *Curvularia* were isolated and identified, it was not possible to differentiate among saprophytes, epiphytes and parasites in the foliar environment. Hence it was considered worthwhile to test the pathogenicities of the isolated fungi on their host species. From the pathogenicity test results (Table III), lowest and highest percentage growth of leisons are observed in *C. verruculosa* and *C. tuberculata*, respectively. *C. senegalensis* has shown a percentage difference of 5 between 42 and 72 hours.

Phenetic analysis

The UPGMA based phenetic tree placed *C. eragrostidis, C. lunata* and *C. pallescens* in a single clade. This clade is joined to *C. senegalensis*. This

Table II. Comparison of *C. clavata, C. eragrostidis, C. lunata, C. pallescens, C. senegalensis, C. tuberculata* and *C. verruculosa*

Characters	***C. clavata***	***C. eragrostidis***	***C. lunata***	***C. pallescens***	***C. senegalensis***	***C. tuberculata***	***C. verruculosa***
Conidiophores Size (range) and peculiarity, if any	70.2-175.5 x 3.9-7.8 µm, geniculate (Fig. 1B)	58.5-130 x 3-4.5 µ, geniculate (Fig. 1E)	19.5-66.3 x 3.1-5.8 µm, geniculate (Figs. 1G,H, Fig.4A)	39-234 x 3.9-5.8 µm (Figs. 1J,K, Figs. 4D,E)	66.3-253.5 x 3.1-5 µm, geniculate (Fig. 2B)	66.3-97.5 x 3.9-7.8 µm, geniculate and marked tuberculate at the swollen nodes (Fig.2D, Fig.4C)	39-187 x 3.9-5.8 µm Slightly geniculate near the tip (Fig. 2F)
Conidia Shape and peculiarity, if any	Clavate (Fig. 1C)	Somewhat ellipsoidal (Fig. 1F)	Boat-shaped, curved or somewhat straight (Fig. 1I, Fig. 4B)	Somewhat ellipsoidal, fusiform to slightly unequally ventricose (Fig. 1L, Fig. 4F)	Unequally ventricose to fusiform (Fig. 2C)	Fusiform or ellipsoidal or cylindrical, usually 3-septate, sometimes tuberculate (Fig. 2E, Fig. 4C)	Fusiform or curved, two middle cells verruculose (Fig. 2G)
Size (range)	13.6-22.9 x 5.1-8.5 µm	9.7-18.7 x 5-9.7 µm	17.5-23.4 x 11.7-13.6 µm	17.5-31.2 x 7-12.4 µm	31.2-50.7 x 13.6-23.4 µm	19.5-66.3 x 3.9-19.5 µm	11.7-27.3 x 7.4-11.7 µm
Mycelial mat							
Diameter (in 5 days)	87.6 mm	50 mm	72 mm	75 mm	65 mm	70.33 mm	81.66 mm
Texture	Felty to floccose	Velvety	Velvety	Initially floccose, later becoming velvety	Velvety	Woolly	Velvety
Colour	Initially brown, becoming olivaceous grey	Grey	Grey to blackish brown	Grey to pale mouse grey	Black	Brown to blackish brown	Olivaceous black to greenish black

Table III. Pathogenicity tests of different fungi on detached leaves of living mangrove plants

Host	Pathogen	*% production of lesions	
		48 hr.	72 hr.
Bruguiera parviflora	*C. clavata*	32.00	32.50
Avicennia alba	*C. eragrostidis*	17.50	20.00
Acanthus ilicifolius	*C. lunata*	12.50	12.50
Xylocarpus molucensis	*C. pallescens*	20.00	20.00
Kandelia candel	*C. senegalensis*	30.00	35.00
Bruguiera cylindrica	*C. tuberculata*	45.00	45.00
Avicennia alba	*C. verruculosa*	10.00	12.50

*% based on the total number of spore suspension drops placed on leaves of each host species (4 drops/leaf; 10 leaves/host species) Temp. 30-32ºC.

combined clade is joined to *C. clavata*. This combined clade joined to *C. verruculosa*. These six species then joined to *C. tuberculata* (Fig. 5A). Indeed, *C. eragrostidis, C. lunata, C. pallescens* showed somewhat grey mycelial mats and geniculate conidiophores justifying their clustering. *C. senegalensis* and *C. clavata* also share geniculate conidiophore with these three species justifying their positions in the phenetic tree. The other two species are morphologically more different than these species and thus, formed different clades. The goodness-of-fit test of the similarity matrix with the corresponding dendrogram revealed high correlation (r = 0.93, Mantel matrix correspondence test) showing that the tree correctly reflects the similarities among different species. However, more morphological characters are needed for better understanding of their relationship.

The results of the Principal Coordinate Analysis *i.e.* PCOORDA (Fig. 5B) were comparable with the UPGMA based cluster analysis. The first three most informative PC components explained 63.1% of the total variation. Here also, *C. eragrostidis, C. lunata* and *C. pallescens* are placed closely while C. tuberculata is placed distantly from the other species. So, from both UPGMA and PCOORDA analysis, the placement of different *Curvularia* species is well resolved.

In conclusion, the present study reports seven species of the fungal genus *Curvularia* from mangroves of Indian Sundarbans. The pathogenicity test confirms

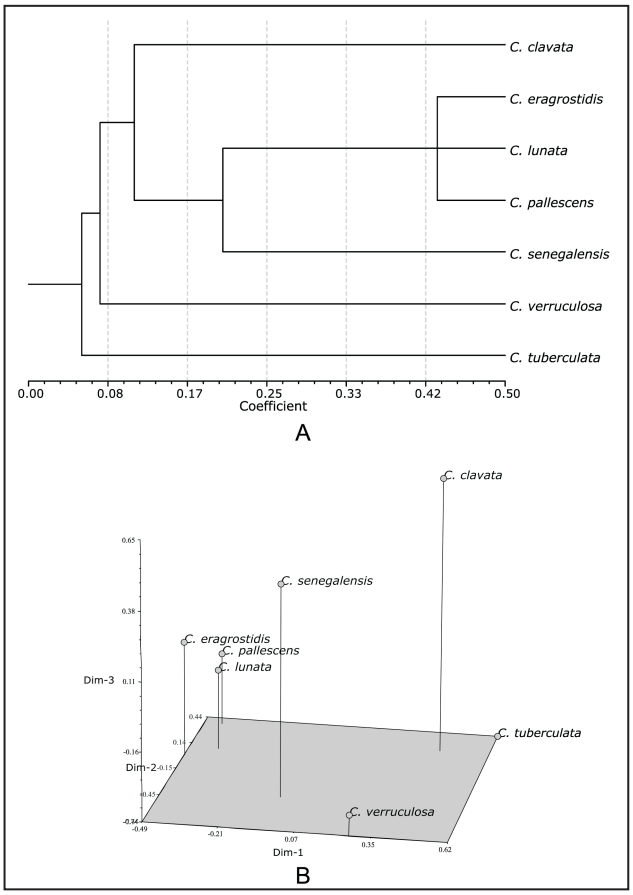

Fig. 5. A. UPGMA-based phenetic tree of seven species of *Curvularia*. The scale represents the value of Jaccard similarity coefficient (increasing value represents more similarity between taxa). **B.** Three-dimensional plot obtained from Principal coordinate analysis (PCOORDA) of seven species of *Curvularia*.

their parasitic relationship with their respective host plants. They showed ample morphological variations among them and phenetic study revealed their taxonomic relationships. Knowledge gathered from the present study can create a new avenue for future conservation of mangroves of Sundarbans.

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We are grateful to Late Prof. R.P. Purkayastha (former Rashbehari Ghosh Professor, Dept. of Botany, University of Calcutta) for giving us the idea of this work. Financial assistance received from the Ministry of Environment & Forests, Government of India is gratefully acknowledged.

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FULL LENGTH ARTICLE

Biogenic silver nanoparticles at higher concentrations inhibit seed germination and seedling development in brown Indian mustard *Brassica juncea*

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The ever-increasing production and application of biogenic silver nanoparticles in agro-nanotechnology increases the possibilities of uncontrolled release into environment which is ultimately becoming a growing ecotoxicological problem. As plants are an important part of the ecosystem and are constantly exposed to these pollutants, it is of paramount importance to assess the toxic impacts of silver nanoparticles on plants. Plants also serve a good model to observe detrimental effects of stable noble metal nanoparticles. The objective of this study was to assess the concentration dependent effects of green synthesized nanoparticles on seed germination and seedling development in Indian mustard. Field emission-scanning electron microscopy revealed dispersed silver nanoparticles below 40 nm in diameter. The green synthesized silver nanoparticles exhibited inhibition of seed germination, root length and vigour index of *Brassica juncea* seedlings but only at higher concentrations while lower concentrations of the silver nanoparticles did not affect the germination of seeds or development of seedlings.

Keywords: *Brassica juncea*, green synthesis, silver nanoparticles, edible mushroom, vigour index, seed germination, seedling development.

INTRODUCTION

In recent years, the agricultural sector is exploring the beneficial role green synthesized nanoparticles as an agent to enhance crop production through modulation in plant growth, soil fertility, enhancement of vigor, control of phytopathogens and plant diseases (Jiang *et al*., 2022). Nanoparticles can act as a direct inhibitory agent against plant pathogens and be used in nanoformulation of fungicides, herbicides, fertilizers, pesticides etc. (Ali *et al*., 2020; Ndaba *et al*., 2022). Having exceptional antimicrobial properties, silver nanoparticles (AgNPs) have been used as major plant protective agents against phytopathogens both in *in vitro* and *in vivo* studies (Danish *et al*., 2021; Ashraf *et al*., 2020). In spite of their beneficial applications, AgNPs have been associated with several detrimental effects on plant system. Due to their small size and high surface to volume ratio, AgNPs can easily get entrapped inside plant system primarily via root from rhizospheric region (Tripathi *et al*., 2017) or through foliar uptake in case of foliar exposure (Li *et al*., 2017). After exposure, AgNPs can cause phytotoxicity at several levels viz. morphological, physiological, cellular and molecular.

An earlier study reported that engineered AgNPs, accumulated in *Arabidopsis* leaves (Qian *et al*., 2013). This work was done on a model plant and not on a crop

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plant. Stampoulis *et al.* (2009) reported commercially purchased AgNPs had inhibitory effect on seed germination at higher concentration in *Cucurbita pepo*. Mirzajani *et al*. (2013) revealed the cell damaging activity of chemically synthesized AgNPs on rice. Vishwakarma *et al.* (2017) compared the detrimental effects of biogenic AgNPs made from *Aloe vera* extract on *Brassica sp.* under hydroponics system. In another report Vishwakarma *et al.* (2020) showed that silicon and Rhizobacteria differentially regulate AgNP toxicity in mustard through nitric oxide. Pandey et al., 2014 observed the effect of AgNP made from Alpha-Amylase enzyme on *Brassica juncea* seedling. Baskar *et al*. (2015) reported concentration dependent effects of biogenic AgNPs synthesized from *Vitex negundo* plant extract on *Brassica rapa* at morphological and physiological level. Marchiol *et al*. (2014) showed formation of AgNP inside *Brassica* plants when provided with $AgNO_3$ solution. Thus the concentration dependent effect of green synthesized AgNPs on seed germination and vigour index of *B. juncea* seedlings has thus far not been reported.

In this context, our present study explored the concentration dependent impact of biogenic AgNPs synthesized from edible mushroom *Pleurotus pulmonarius* on seed germination and seedlings development of Indian brown mustard, *Brassica juncea*.

MATERIALS AND METHODS

Plant sample and growth conditions

Seeds of *Brassica juncea* (L.) Czern. and Coss. obtained from Berhampur Pulse Research Station, West Bengal, India was used in this study. Plants were grown in commercial soilrite (a mixture of horticulture grade perlite, Irish peat moss and exfoliated vermiculite in 1:1:1 ratio) and were maintained in a plant growth chamber with 16/8 hr light/dark cycle at 25°$\pm$ 1°C. Four week old plants were used for further experiments.

Source of the silver nanoparticles (AgNPs)

Nanoparticles were synthesized according to our previous method (Chowdhury *et al*., 2014). The edible mushroom *Pleurotus pulmonarius* (Fr.) Quel. was used for biosynthesis of AgNPs. 1 mM solution of silver nitrate ($AgNO_3$) was added to cell free filtrate (CFF) of the *P. pulmonarius* for AgNPs synthesis. The AgNPs biosynthesis was indicated by regular visual observation for change of color of the reaction mix. The final nanoparticle stock suspension (100% stock suspension) contained approximately 85 µg/ml of nanoparticles, calculated based on TEM analyses (TEM data not presented).

Field emission-scanning electron microscopy (FE-SEM) of the AgNPs

Analysis of the biosynthesized AgNPs was done using FE-SEM. A drop of nanoparticles was used to make a thin film on a glass stub and was vacuum dried. It was subjected to FE-SEM using JEOL JSM-7600F (JEOL Ltd., Japan).

Assay of effect of the AgNPs on seeds germination and seedlings development in *B. juncea*

Seeds of *B. juncea* were washed with sterile water to remove surface dirt from seed coat. Then, four different petri-dishes with *B.juncea* soaked seeds in sterile water (control) and in three different concentration of AgNPs suspension (viz. 25%, 50% and 100% v/v of stock suspension of AgNP) were kept in dark at 25±1 °C for 3 days. All the dilutions of the AgNPs were prepared from the above stock suspension of the AgNPs with sterilized double distilled water. After the incubation period, germination percentage of seeds, root length and vigour index of seedlings were calculated for each experimental setup.

Calculation of germination percentage and vigour index

Germination percentage (%) and vigour index were calculated using the following formulas.
Germination percentage=(Number of germinated seeds/Total number of tested seeds) x100 %
Vigour index (VI) = Germination percentage (%) x

(shoot + root) length [mm].

Statistical analysis

All experimental data were analyzed according to our previous publication (Chowdhury *et al*., 2017). All analyses were performed with three independent experiments with at least three replicates each and values represented as the mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA) with different letters indicating significant difference between treatments at $p < 0.05$, according to Duncan's multiple range test (DMRT), using a software package, SPPS (version 16, 2007).

RESULTS AND DISCUSSION

Field emission-scanning electron microscopic (FE-SEM) study of the AgNPs

AgNPs as observed under FE-SEM revealed that the particles were small in size and without any aggregation (Fig.1).

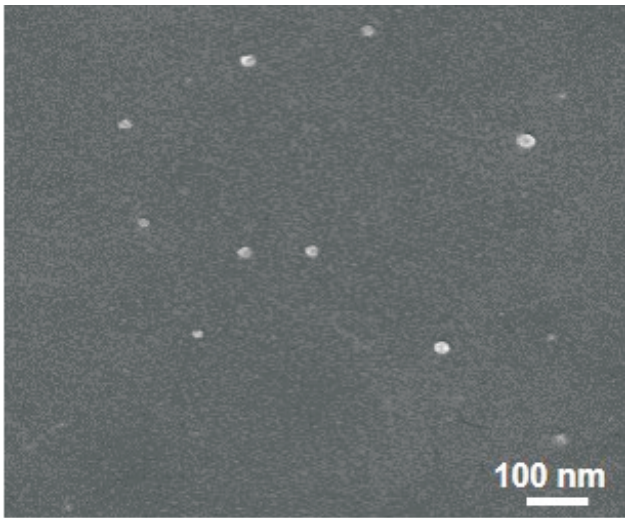

Fig. 1. Field emission-scanning electron micrograph (FE-SEM) of the biogenic silver nanoparticles. FE-SEM micrograph showing distribution of biosynthesized silver nanoparticles in a filed.

Effects of the AgNPs on germination of *B. juncea* seeds

In this assay (Fig. 2A), seeds were considered as germinated when the radicle emerged. Germination of *B. juncea* seeds soaked in low concentration of the AgNPs, 25% and 50% of stock suspension did not significantly differ in respect to the water soaked control seeds, which was in the range of 90%. However at high concentration of the AgNPs i.e. 100% of stock suspension seeds showed significantly lower germination percentage, showing its inhibitory effect on seed germination. The germination percentage decreased to 0.81 fold in 100% of stock suspension of AgNPs compared to the control (Fig. 2B). Thus the AgNPs acted in a concentration dependent manner where certain threshold level severely affected the normal physiology of seeds and inhibit their germination. Handayani *et al*. (2023) reported phytotoxic activity of biosynthesized AgNPs made with leaf extract of *Diospyros discolour*, where it significantly decreased the germination percentage of *Brassica rapa* seeds at a much lower concentration compared to the inhibitory concentration of the AgNPs used in the present study. Thus seed germination inhibition activity of AgNPs vary from plant to plant depending on the source and nature of AgNPs.

Effects of the AgNPs on root length of *B.juncea* seedling

More significant effect of the AgNPs was observed on roots of seedlings of *B. juncea* (Fig. 3A). Roots lengths (Fig. 3B) of seedlings treated with low concentration (25% and 50% of stock suspension) of the AgNPs were relatively not much different from the control treatment. However, significant retardation of 0.7 fold of root length was observed in the seeds treated with high concentration i.e. 100% of the AgNP stock suspension with respect to the control. As the roots are the first tissue which come in direct contact with the AgNPs, it became the most prominently effected organ. Retardation of root length at higher concentration of the AgNPs indicated dose dependent activity of the AgNPs. Similar observation was reported by Mujeeb *et al*. 2018, where root length of *Cassia occidentalis* was negatively regulated by high concentration of AgNPs synthesized using leaf extract of *Vitex negundo,* although low concentration exhibited growth stimulatory activity. Similar observation reported by Baskar *et al.* (2015), where

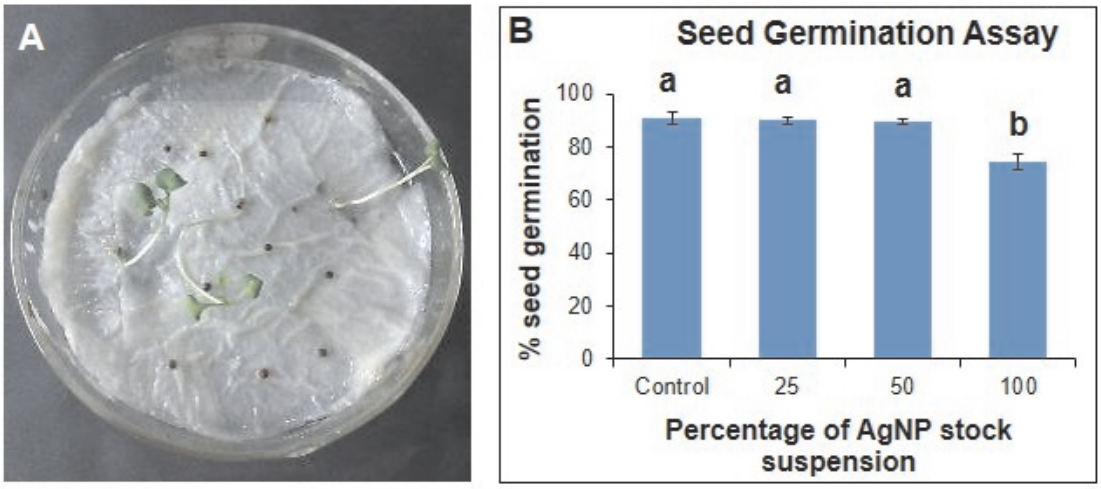

Fig. 2. Inhibitory effect of the silver nanoparticles on germination of *B. juncea* seeds. (**A**) Representative petriplate of an experimental set up. (**B**) Graphical representation of seed germination percentage with increasing concentration of the silver nanoparticles. Different letters above the bar indicate significant different ($p < 0.05$), using Duncan's multiple range test. Same letter above the bar denotes no significant difference between the groups.

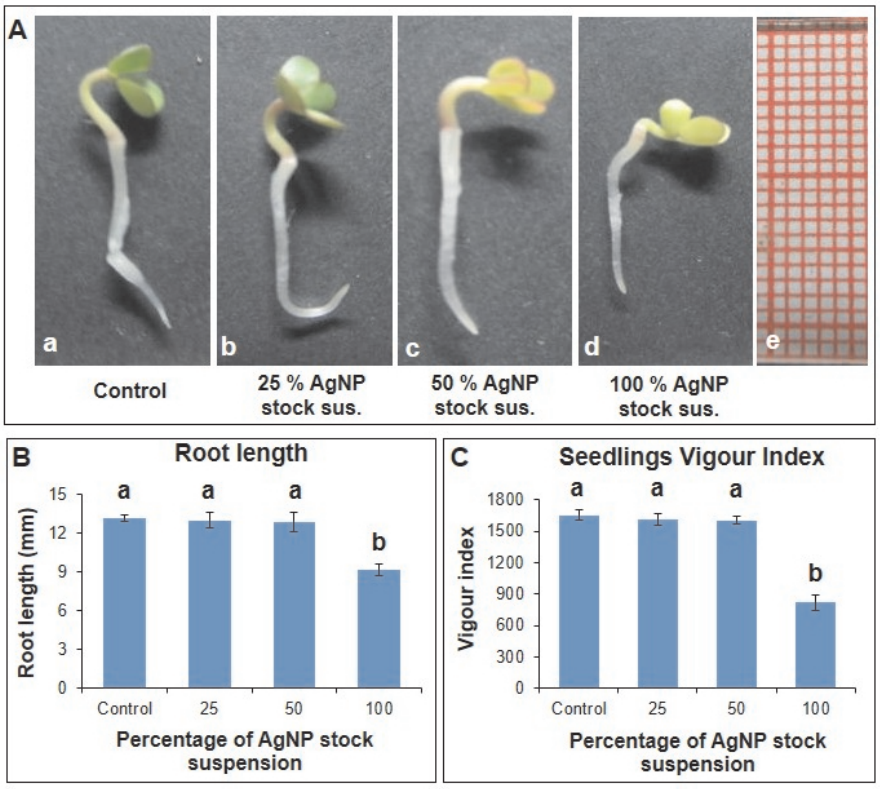

Fig. 3. Effect of the silver nanoparticles on root length and vigour index of *B. junceae* seedlings. (**A**) Representative seedling samples after 3 days of germination in a: water; b: 25%; c: 50%; d: 100% stock suspension of nanoparticles (Stock suspension AgNPs concentration: Approximately 85 µg/ml); e: Scale: each small segment = 1 mm. (**B**) Graphical representation of effect of increasing concentrations of the AgNPs on root length and (C) vigour index of *B. juncea* seedlings. Differnet letters above the bar indicate significant difference ($p < 0.05$), using Duncan's multiple range test. Same letter above the bar denotes no significant difference between the groups.

biogenic AgNPs synthesized from *Vitex negundo* plant extract showed stimulatory activity on *Brassica rapa* seedlings growth at 100 mg/L concentration while higher concentrations (200 and 500 mg/L) exhibited growth inhibition activity. Dimkpa *et al.* (2013) reported that commercial AgNPs reduced the length of shoot and root length in wheat in a dose-dependent manner.

Effects of the AgNPs on vigour index of *B. juncea* seedlings

Vigour index represents the viability of seeds along with its ability to produce normal seedlings under adverse growing conditions. In the present study, the biogenic AgNPs significantly reduced the vigour index of *B.juncea* seedlings at higher concentration (100% stock suspension). However at lower concentration (25% and 50% stock suspension), the AgNPs did not significantly affect the vigour index of Indian mustard seedlings (Fig. 3C).

CONCLUSION

Analysing the results, it can be concluded that the biogenic AgNPs made using *P. pulmonarius* up to 50% of the stock suspension, is not detrimental to germination and development of *B. juncea* seedlings after single continuous exposure. Exposure above this concentration of AgNPs cannot be considered safe for *B. juncea* seedlings.

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FULL LENGTH ARTICLE

Antibacterial and antioxidant activity of green synthesized silver nanoparticles using seeds of *Bixa orellana* L.

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Biosynthesis of nanoparticles using plant extract is an eco-friendly process. In this present study, the synthesis of silver nanoparticles (AgNPs) was performed using aqueous extract of *Bixa orellana* seeds. The synthesis of nanoparticles was confirmed and characterized through UV-Vis spectroscopy, XRD, SEM-EDX and FTIR. The view of synthesized nanoparticles through SEM indicates a high density of nanoparticles in the solution. The crystal size of the AgNPs was calculated through XRD analysis and the average size was found to be 21.28 nm. Qualitative phytochemical analysis of the aqueous seed extract of the *Bixa orellana* was also performed. Diverse phytoconstituents like carbohydrates, reducing sugars, glycosides, proteins, amino acids, flavonoids, phenolics, tannins, saponins, terpenoids, coumarins, and alkaloids were detected. FTIR analysis indicates the involvement of carboxyl, hydroxyl, nitrile, and amine groups in nanoparticle formation. The antibacterial activity of the synthesized nanoparticles was tested against four gram-negative and one gram-positive bacteria. AgNPs exhibit potent antibacterial activity and the highest activity was shown against *Escherichia coli*. The antioxidant activity of the synthesized nanoparticles was assayed through the DPPH free radical scavenging assay. Different concentrations (100-600 µg/ml) of the nanoparticles were used for the assay and significant increase of antioxidant activity with the increase in concentration. The IC_{50} value was calculated and found to be 218.22 µg/ml.

Keywords : *Bixa orellana*, silver nanoparticles, phytochemicals, antibacterial, antioxidant.

INTRODUCTION

The vegetational diversity of India is a cheap source of medicine and widely exploited in Ayurveda since ancient times. Nanobiotechnology is now one of the versatile disciplines mainly focused on the synthesis of nanoparticles by the imperative employing of different plants or plant parts. Basically, the size of particles below 100 nm is designated as nanoparticles (Banerjee *et al*., 2014). Green synthesis of nanoparticles opens a new window in the research of nanotechnology. In the modern scenario, silver nanoparticles draw special attention for their exclusive features and wide applications (Galdiero *et al*., 2011) in food, medical and agricultural technologies (Masum *et al*., 2019). Silver nanoparticles (AgNPs) have significant use in human daily life in the form of soaps, shampoos, detergents, toothpastes, cosmetics as well as pharmaceutical products (Bhattacharya and Murkherjee, 2008). Outcome products from nanobiotechnology have unique characteristics like size and properties both physical and chemical (Banerjee *et al.*, 2014). In

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addition to newly route of green synthesis, several methods are also employed for the synthesis of AgNPs like microemulsions, chemical reduction, photochemical reduction, microwave-based systems etc. (Iravani *et al*., 2014). Green synthesis way of nanoparticles follows the nontoxic route and AgNP synthesis with plants, microorganisms and algae is natural as well as environmentally friendly (Bhattacharya and Gupta, 2005; Mohanpuria *et al*., 2008). Synthesis of nanosilver using plants is much safer than bacteria and toxic chemicals; since there is always a possibility of contaminating the environment with bacteria and chemicals when using them for synthesis (Masum *et al*., 2019). Synthesis of plant-based nanoparticles also diminishes the cost of microbes-based nanoparticles by reducing the isolation, culture and maintenance cost of microorganisms (Singhal *et al.*, 2011). Additionally, plants possess different biomolecules including secondary metabolites like phenols, flavonoids, terpenoids, and other metabolites like enzymes, amides, carboxylic acids and aldehydes (Prabhu and Poulose, 2012); are responsible for attenuating metal ions (Rai and Ingle, 2012). Although in higher concentrations silver exerts toxicity, however, lower concentrations of silver are harmless and show better stability, catalytic activity, and therapeutic potentiality (Fahimirad *et al*., 2019). Actually, the notable advantage of silver nanoparticles is the slow as well as regulated release of silver from nanoparticles in comparison to metals in vastness (Totaro and Rambaldini, 2009).

Bixa orellana belongs to the family Bixaceae and is commonly known as lipstick tree, annatto, or achiote (Giorgi *et al*., 2013). *Bixa orellana* is an evergreen, short-height tree of Northern South American origin but nowadays also cultivated in tropical countries for its colour of seeds and medicinal properties (Thilagam *et al*., 2013). The important pigments present in the aril of the seeds are two carotenoids - bixin and norbixin (Stohs, 2014). These coloured pigments of seeds of lipstick trees are used in traditional body paint or to shield from sunburn (Patani *et al*., 2013). Being its nontoxicity, globally the demand for annatto pigment is next to saffron as a natural food colourant. *Bixa orellana* is well known for its use as traditional medicine in the treatment of gonorrhea, fever, dysentery, blood disorders, and jaundice (Srineeraja, 2017). In Cameroon, people traditionally use *Bixa orellana* to get relief from joint pain, abdominal pain, jaundice, and fever (Fokam Tagne *et al*., 2019). Antinociceptive, anti-inflammatory (Pacheco et al., 2019), antiproliferative effect in human lung cancer, breast cancer (Kusmita *et al*., 2022) and antimyeloma effect (Tibodeau *et al*., 2010) of this plant have been observed and well documented.

MATERIALS AND METHODS

Collection of fruits

Fruits of *Bixa orellana* L. were collected from the plants grown in the medicinal plant garden of Sidho-Kanho-Birsha University, Purulia, West Bengal. The seeds were removed from the matured fruits and allowed to shade dry.

Preparation of aqueous seed extract

Seeds extract was prepared following the method of Mohanta *et al.* (2017) with slight modification. Shade dried seeds were ground in a mixer grinder followed by sieving through 40 µm pore size. 10 g seed powder was mixed with 100 ml sterile distilled water and boiled at 60°C in the water bath. The aqueous extract was then sonicated for 15 minutes and followed by centrifugation at 5000 rpm for 15 minutes. The supernatant then passed through Whatman No 1 filter paper and stored at 4°C for further experiments.

Qualitative analysis of phytochemical composition

Alkaloids: 1 ml of seed extract was taken and 1-2 ml of Dragendorff's reagent was mixed with it. Red precipitation indicates the presence of alkaloids (Singh and Kumar, 2017).

Carbohydrates: One ml Barfoed's reagent was added to 1 ml of seed extract. The mixture was heated for 2 minutes. The appearance of red colour precipitation confirms the presence of carbohydrates (Shaikh and Patil, 2020).

Reducing sugars: One ml of each of Fehling's A and Fehling's B solutions was taken in a test tube. One ml

seed extract was mixed with Fehling's solution and boiled in the water bath. Changing colour to reddish brown with precipitation indicates the presence of reducing sugar (Singh and Kumar, 2017).

Glycosides: With 2 ml filtrated seed extract, 3 ml chloroform was mixed and shaken well. The layer of chloroform was separated from the mixture. 10% ammonia solution was then added to the solution. The appearance of pink colour indicates the existence of glycosides (Shaikh and Patil, 2020).

Flavonoids: One ml of seed extract was taken in a test tube and a few drops of 10% ferric chloride solution was added. The appearance of green precipitate confirms the existence of flavonoids (Audu *et al*., 2007).

Phenolics: One ml of ethanol was mixed with seed extract. Six to seven drops of 1% ferric chloride were mixed with it. The appearance of green, blue, or purple colour indicates the existence of phenol (Soloway and Wilen, 1952).

Tannins: Three ml distilled water was added with 1 ml seed extract. Three drops of 10% ferric chloride solution were added to the solution. The development of blue or green colour indicates the existence of tannins (Uma *et al.*, 2017).

Saponins: Two ml of distilled water was added to 1 ml of seed extract. The mixture was then shaken vigorously for 5 minutes. The appearance of 1 cm thick foam for the duration of 10 minutes concludes the presence of saponins (Kumar *et al*., 2009).

Terpenoids: Five ml seed extract was added with 2 ml chloroform. Then 3 ml of concentrated H_2SO_4 was added and allowed to boil at water bath. The development of grey colour determines the presence of terpenoids (Singh and Kumar, 2017).

Quinones: One ml of seed extract was taken in a test tube and 1 ml of concentrated sulfuric acid (H_2SO_4) was mixed with it. The appearance of green colour indicates the presence of quinones (Maria *et al*., 2018).

Coumarins: One ml of extract was taken in a test tube and 1.5 ml of 10% sodium hydroxide (NaOH) was added to it. The appearance of red colour indicates the existence of coumarins (Ugochukwu *et al*., 2013).

Synthesis of silver nanoparticles

1 mM silver nitrate ($AgNO_3$) solution using autoclaved distilled water was taken for synthesis of nanoparticles. The synthesis process was carried out by following the method of Rautela *et al*. (2019). Here 90 ml of seed extract was mixed with 10 ml 1 mM $AgNO_3$ solution and the final concentration of $AgNO_3$ became 0.1 mM. The solution was heated below its boiling point with continuous stirring by a magnetic stirrer. The entire process was carried out in the dark. The mixture was changed to brown colour within 1 hr and the characterization of the synthesized nanoparticles with their properties was studied through the following procedures.

UV-Vis spectroscopic characterization of synthesized nanoparticles

UV-Vis spectrum of synthesized nanoparticles with aqueous Bixa seed extract was recorded for monitoring the bio-reduction from the ionic form of silver (Ag^+) to silver nanoparticles (Ag^0) following the method of Banerjee *et al*. (2014). The scanning was performed using UV-Vis spectrophotometer (Shimadzu UV-1800) at the wavelength ranges from 800 nm to 300 nm with 1 nm resolution.

X-Ray Diffraction (XRD) study

Synthesized Bixa-AgNPs were centrifuged at 10000 rpm for 30 minutes followed by washing with ethanol for two times. The precipitated nanoparticles were dried in hot air oven. Oven-dried powdered of synthesized nanomaterial was used for XRD analysis in X-ray diffractometer (Rigaku, Smart Lab). The powdered X-ray diffraction analysis of the synthesized nanoparticles was carried out following Thiruvengadam and Bansod (2020). The phase transformation identification was made by using Cu-Kα radiation. The pattern of XRD was recorded in the range of 2θ in between 10° to 80°. Crystalline size of

the synthesized Bixa-AgNPs was calculated following the formula of Debye-Scherer $D=0.94\lambda/\beta\cos\theta$ (Thilagam *et al*., 2013).

Scanning Electron Microscope with Energy-Dispersive X-ray (SEM-EDX) Analysis

SEM (JEOL JCM-6000PLUS) integrated with EDX analysis was carried out to visualize the density of nanoparticles with the detection of the atomic content of the metal present in the synthesized Bixa-Ag nanoparticles. The surface structure was visualized by using scanning electron microscope under high vacuum and 15 kV accelerating voltage. The sample was fixed above the stub on carbon tape and coated with gold for two minutes.

Fourier-transform infrared spectroscopy (FTIR) based analysis of AgNPs

The FTIR analysis was carried out following the method of Farsi and Farokhi (2018) in FTIR spectrometer (JASCO 4700). The solution of synthesized silver nanoparticles was centrifuged at 10000 rpm for 10 minutes to concentrate the nanomaterial in the form of pellet. The concentrated nanoparticles were then washed with sterile distilled water by again centrifuging at 10000 rpm for 10 minutes. The obtained pellet was then air-dried and used for FTIR analysis in KBr pellets.

Antibacterial assay of synthesized nanoparticles

Antibacterial activity was conducted of the AgNPs synthesized from *Bixa orellana* seeds extract through agar well diffusion following the method of Mohanta *et al.* (2017). The antibacterial activity was tested against gram-negative bacteria *Enterobacter cloacae* (MCC 3111), *Pseudomonas aeruginosa* (MCC 4242), *Escherichia coli* (MCC 3099), *Proteus mirabilis* (MCC 3895) and one gram-positive bacterium *Enterococcus faecalis* (MCC 3037). Nutrient agar was used as media for the antibacterial assay. 100 µl solution of AgNPs was applied to the well and plant extract of the same amount was used as control.

Free radical scavenging assay

Free radical scavenging assay of biosynthesized silver nanoparticles from *Bixa orellana* aqueous seeds extract was evaluated through 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging following the method of Kabir *et al.* (2016). Different concentrations of silver nanoparticles 100, 200, 300, 400, 500, and 600 µg/ml were used for scavenging potential with 3 ml of 0.004% DPPH (w/v) in methanol. After 30 minutes of incubation in the dark, the absorbance was taken in spectrophotometer at 517 nm of wavelength. The percentage of inhibition was calculated using the formula $[(A0-A1)/A0]\times100$. Three ml of methanol was used as blank. The curve of inhibition was prepared and half of the maximal inhibitory concentration (IC50) was calculated.

A0 = Absorbance of control (DPPH Solution)
A1 = Absorbance of sample (DPPH + Nanoparticles)

RESULTS AND DISCUSSION

Phytochemical analysis

Qualitative analysis of phytochemicals from the aqueous seed extract of *Bixa orellana* revealed the presence of different primary and secondary metabolites (Table 1). This result is supported by the findings of Ahmed *et al.* (2020) who reported the presence of carbohydrates, alkaloids, glycosides, coumarins, tannins, phenols, flavonoids, saponins and proteins.

Green synthesis of silver nanoparticles

Preliminarily, the silver nanoparticles formation was identified by observing the change of colour due to the reduction of silver ions by seed extract. The colour intensity was increased with the progression time of reduction of silver ion and finally reached to stable upon completion of reduction. The colour of the seed extract mixed with silver nitrate solution was changed to dark brown after complete bio-reduction. A similar kind of colour change was observed by Akintola *et al.* (2020) after the complete reduction of silver, which supports this bio-reduction of silver ions by *Bixa orellana* seed extract.

Table 1. Qualitative analysis of phytochmicals of aqueous extract of *Bixa* seeds

Sl. No.	Phytochemicals	Observation results
1	Carbohydrate	+
2	Reducing Sugars	+
3	Glycosides	+
4	Proteins and Amino acids	+
5	Flavonoids	+
6	Phenolics	+
7	Tannins	+
8	Saponins	+
9	Terpenoids	+
10	Quinones	-
11	Coumarins	+
12	Alkaloids	+
(+) indicates present and (-) indicates absent		

Characterization of biosynthesized nanoparticles through UV-Vis spectrophotometer

The bio-reduction of Ag to AgNPs was confirmed by observing the absorbance peak after scanning from 800 nm to 300 nm of wavelength through UV-Vis spectrophotometer. The maximum absorbance spectrum of the synthesized silver nanoparticles was observed in the range from 450 nm to 470 nm with a peak around 464 nm (Fig. 1) as a result of the characteristics of surface plasmon resonance. This finding has been supported by the earlier study where the absorbance maximum of silver nanoparticles was found to range from 425 nm to 475 nm (Banerjee *et al*., 2014).

XRD based characterization of synthesized AgNPs

XRD pattern of the synthesized nanoparticles (Fig. 2) has confirmed the colloids of silver in the sample. Braggs reflections were found at 2θ=38.07º, 44.19º, 64.44º, and 77.4° in the pattern of XRD in correspondence to the silver crystal plane (111), (200), (220) and (311) similar to the XRD pattern of Masum *et al.* (2019). The calculated size of the synthesized AgNPs nanocrystal ranges from 14.61 nm to 29.58 nm with the average value of 21.28 nm.

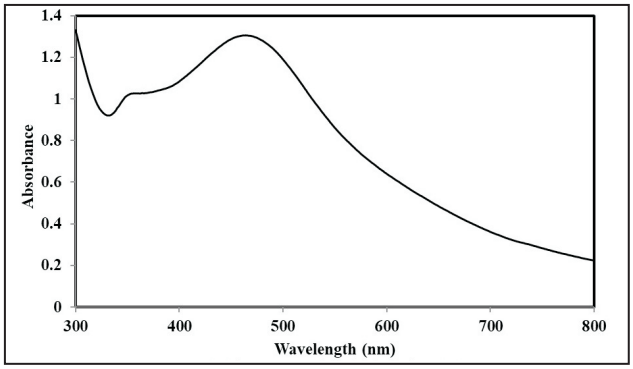

Fig. 1. UV-Vis spectrum of synthesized Bixa-AgNPs

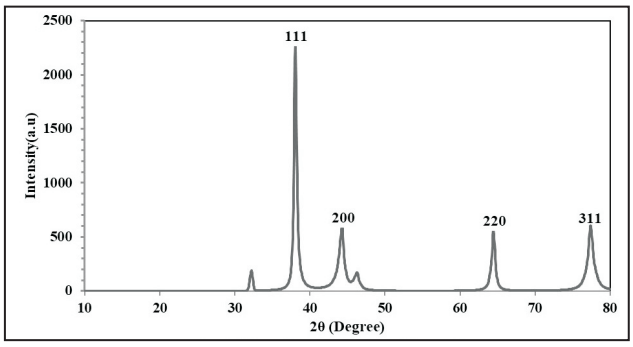

Fig. 2. XRD pattern of synthesized Bixa-AgNPs

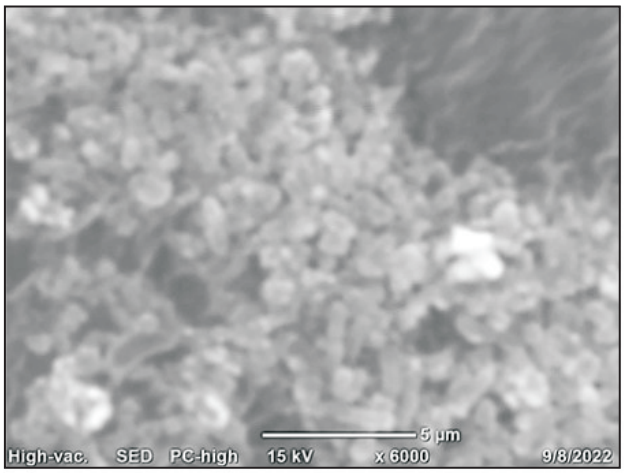

Fig. 3. Scanning Electron Microscopic view of synthesiszed AgNPs

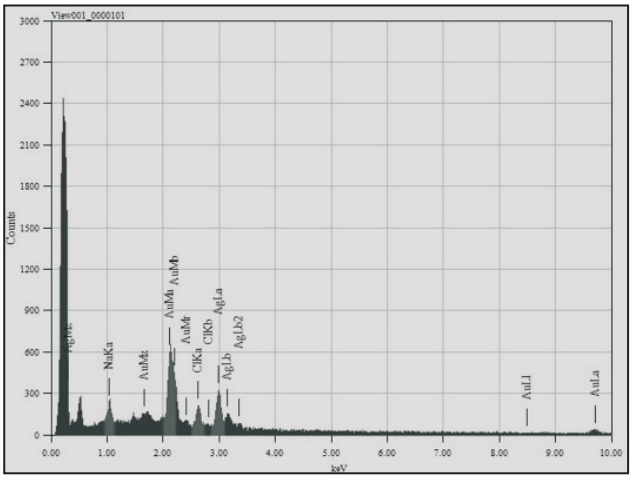

Fig. 4. Energy Dispersive X-ray (EDX) pattern of synthesized AgNPs

SEM-EDX study of synthesized AgNPs

Scanning Electron Microscopic (SEM) observation has revealed high density of the synthesized silver nanoparticles with Bixa seed extract (Fig. 3). Further Energy Dispersive X-ray (EDX) pattern with absorbance peak at 3 keV (Fig. 4) has confirmed the presence of silver in the nanoparticles (Femi-Adepoju *et al*., 2019).

FTIR spectrum of synthesized nanoparticles

Fourier transform infrared spectroscopy (FTIR) mediated analysis was carried out for the identification of the possible biomolecules responsible for the reduction, capping and proficient stabilization of AgNPs (Fig. 5). The weak peaks found at 3862 cm^{-1} and 3740 cm^{-1} were attributed to -OH stretching and –H stretching of the functional group of phenol and alcohol (Thilagam *et al*., 2013). The band appeared between 3400-2400 cm^{-1} (2922 cm^{-1} and 2851 cm^{-1}) due to stretching vibrations of N-H of amines, amine salts, sulfonamides, C-H of alkenes and alkanes, and C=O of carboxylic acids (Bhagat *et al*., 2015). However, the peak around the wavenumber 2922 cm^{-1} corresponds to the stretching vibration of protein nanoparticles (Farsi and Farokhi, 2018). The band appeared at 2361 cm^{-1} and 2344 cm^{-1} indicating the presence of nitrile group. The band in the wavenumber 1644 cm^{-1}, 1630 cm^{-1} and 1514 cm^{-1} are caused by bending vibrations of amide I, stretching of C=O group of aldehydes and ketones and bending vibrations of amide II (Paulkumar *et al*., 2017; Farsi and Farokhi, 2018). Peak observed at 1374 cm^{-1}, 1253 cm^{-1}, 1224 cm^{-1}, 1115 cm^{-1}, 1035 cm^{-1}, 1024 cm^{-1}, and 1008 cm^{-1} for the stretching vibrations of C-O groups of anhydrides, ethers, esters, phenols and alcohols, as well as C-O-H of alcohols and phenols, and of C-N of

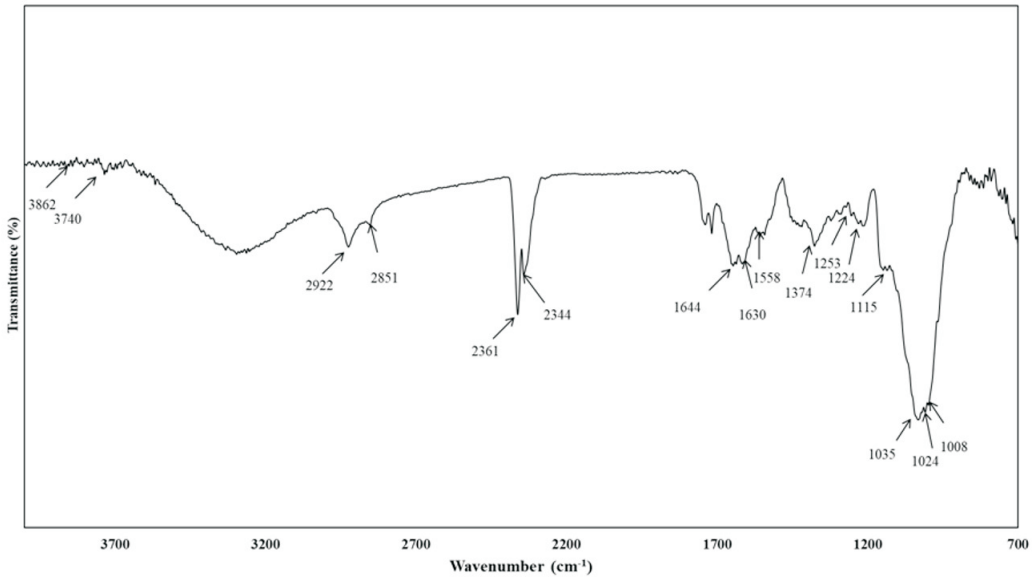

Fig. 5. FTIR Spectrum of Bixa-AgNPs

amines (Bhagat *et al*., 2015). Proteins present in the plant extract bind to the silver nanoparticles through free amino acids or by the carboxyl groups present in the protein (Gole *et al*., 2001). Different functional groups like carboxyl (–C=O), hydroxyl (–OH), and amine (–NH) in the plant extract are primarily involved in silver nanoparticle fabrication (Prasad *et al*., 2011).

Antibacterial activity of the Bixa-AgNPs

All the tested bacterial strains have shown the inhibition zone against the synthesized AgNPs. The inhibitory effect was found in both selected gram-positive bacterium and gram-negative bacteria (Fig. 6). The maximum inhibitory effect was found against *Escherichia coli* (19±0.58 mm) followed by *Enterobacter cloacae* (18±0.58 mm), *Enterococcus faecalis* (17±1.53 mm), *Proteus mirabilis* (17±0.58 mm), and *Pseudomonas aeruginosa* (16±0.58 mm). The zone of inhibition was measured after 24 hours of bacterial growth at 37°C. The aqueous extract of Bixa seed does not exhibit any inhibitory effect against the test pathogens. Antibacterial efficacy was influenced by the shape, size, and formulation of silver nanoparticles (Franci *et al.*, 2015). Bixa-AgNPs were also found with antibacterial potentiality against other gram-positive *Staphylococcus aureus* (ATCC 25956), and gram-negative *Escherichia coli* (ATCC 27853), *Shigella dysenteriae* (ATCC 26131) and *Shigella boydii* (ATCC 27890) bacterial strains (Maitra *et al*., 2023).

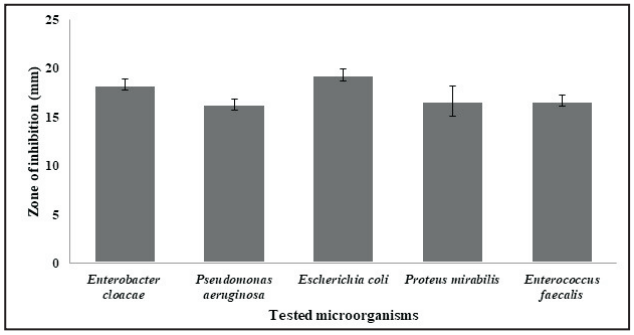

Fig. 6. Antibacterial activity of the synthesized AgNPs (100 µg) using agar well diffusion method

Antioxidant activity study of the synthesized nanoparticles

DPPH is organic free radical, generally, used for analysis in the antioxidant assay of different natural as well as synthesized products. Different dose-dependent scavenging activities of synthesized AgNPs were determined. Different concentrations of AgNPs (100, 200, 300, 400, 500, and 600 µg/ml) were shown potential DPPH free radical scavenging activity of 47.70%, 49.71%, 50.85%, 52.14%, 57.87%, and 61.03% respectively (Fig. 7). The inhibition percentages of free radical generation were found to be increased with the increasing of concentration of AgNPs. IC_{50} of DPPH free radical scavenging assay using synthesized AgNPs was found 218.22 µg/ml. The aqueous extract of seeds has been shown to lower antioxidant potentiality in comparison to Bixa-AgNPs. At the concentration of 800 µg/ml aqueous extract of Bixa seeds only shows 42.60% free radical scavenging potentiality (Ahmed *et al*., 2020).

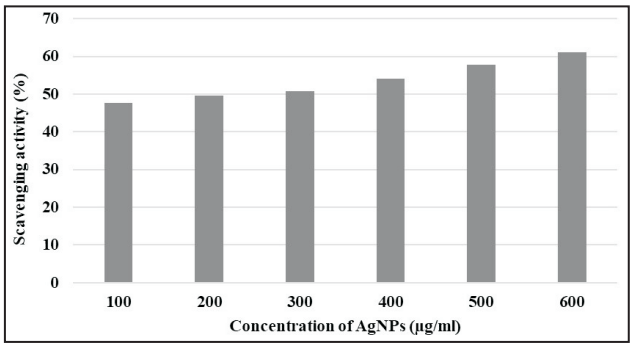

Fig. 7. DPPH free radical scavenging activity of synthesized AgNPs

CONCLUSION

Silver nanoparticles of *Bixa orellana* seed extracts were successfully synthesized. The first confirmation of AgNPs synthesis was achieved by observing the changing of colour to brown followed by the spectrum in UV-Vis with a single peak of absorbance around 464 nm. The synthesized AgNPs were characterized by XRD, SEM, EDX and FTIR. The calculated average size from the XRD data of the nanocrystal was found 21.28 nm. The image generated in SEM of AgNPs

indicated the higher density of the synthesized nanoparticles. The intense peak at 3 keV was found in EDX analysis corresponding to the presence of Ag in the synthesized nanoparticles. Synthesized Bixa-AgNPs had shown good antibacterial activity against both gram-positive and gram-negative bacteria and were found to be a good antioxidant source with the IC50 value 218.22 µg/ml proving its huge pharmacological potential.

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FULL LENGTH ARTICLE

Partial self-incompatibility in *Clerodendrum indicum* (L.) O. Kuntze with reference to its different levels of adaptations for higher degree of out-breeding

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The breeding system and the reproductive success of the ethno-medicinally important shrub *Clerodendrum indicum* have been investigated. Individual flowers of the species are of four-day longevity exhibit different levels of adaptations for higher degree of out-breeding. Structurally, the progressive backward curvature of the filaments of stamens simultaneous with the periodical shifting of style through the open flower phase imparts a herkogamous disposition between the male and female organs. Thereby, autogamous pollen transfer becomes difficult in the species. Functionally the flowers are completely dichogamous and protandrous with a pause of more than 12 hrs between the male and female phases. Existence of such a complete dichogamy totally eliminates the chance of autogamy. Furthermore, flowers are partially self-incompatible, as deduced from controlled pollination experiments. The index of self-incompatibility (ISI) has been calculated as 0.22. The study reveals that *C. indicum* is a predominantly xenogamous species and exhibits 4.381 ± 0.27 % reproductive success in its natural habitats.

Keywords : Dichogamy, herkogamy, reproductive success, xenogamy.

INTRODUCTION

Various types of pollination with respect to the breeding system are noticed in flowering plants. Autogamy involves the pollen transfer from the anther to the stigma of the same flower and effects exclusively inbreeding. On the other hand, pollen transfer from the anther to the stigma of a different flower is referred to as allogamy. Allogamous pollination in turn may either be geitonogamous, i.e. pollen from one flower's anther is transferred to another's stigma belonging to the same plant, or xenogamous, i.e. pollen from one flower's anther is transferred to another's stigma belonging to a different plant. Like autogamy, geitonogamy also gives rise to inbreeding, while xenogamy effects exclusively out-breeding.

In case of bisexual flowers, to avoid inbreeding with simultaneous adaptation for higher degree of out-breeding, various types of structural and functional modifications are noticed among different species of flowering plants. Spatial separation between anther and stigma, referred to as herkogamy, or the temporal separation between male and female phases, known as dichogamy, are generally regarded as the means to promote allogamous pollination by hindering autogamy. The efficacy of such adaptations as effective barriers to self-fertilization has been questioned from time to time (Muller, 1883; Nyman, 1992; Ortega-Olivencia and Devesa, 1993). Though

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the chance of xenogamy is increased in cases of both herkogamous and dichogamous flowers, some degrees of inbreeding in such flowers through geitonogamous pollination cannot be ruled out. Adaptation of self- incompatibility is the means for exclusively xenogamous pollination leading to total out-breeding (Kolreuter, 1761; Sprengel, 1793; Faegri and van der Pijl, 1979; Webb and Lloyd, 1986; Bertin and Newman, 1993).

Clerodendrum indicum (L.) O. Kuntze, belonging to the family Lamiaceae, is an ethno-medicinally important plant (Kirtikar and Basu, 1935; Chopra *et al*., 1958). The present paper deals with the breeding system and reproductive success of the species, which had been hitherto unknown. The work reveals that the species represents a unique combination of structural as well as functional modifications to ensure higher degree of out-breeding.

MATERIALS AND METHODS

Clerodendrum indicum is a sparsely branched shrub up to 4.5 m tall (Fig. 1A) and flowers twice a year, in May-June and again in September-October. Flowers are produced in relatively large terminal panicles (Fig. 1B). Individual flowers of typical tube construction are bisexual and pentamerous. The campanulate, gamosepalous, 5-lobbed calyx is persistent in nature. The creamish white corolla is comprised of a proximal long corolla tube and distal five ovate-lanceolate, free lobes. Stamens are four in number, epipetalous and didynamous. The dorsifixed anthers of shorter pair of stamens remain projected 14-16 mm beyond the corolla throat and those of the longer pair remain projected 20-22 mm beyond the corolla throat. The syncarpous gynoecium is constituted by a tetralocular superior ovary, a slender style and a bifid stigma with spathulate lobes. Each of the four chambers of the

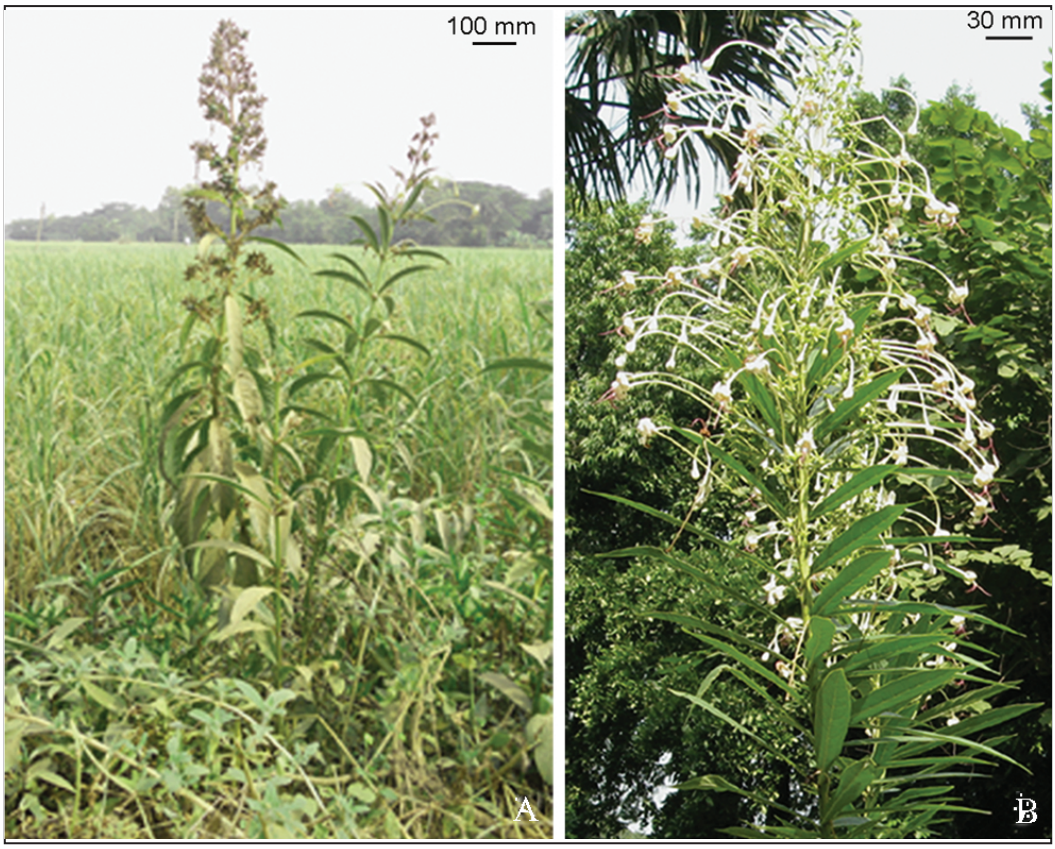

Fig. 1. A, Few Individuals of *Clerodendrum indicum* in natural habitat at Rambhadrapur Village during the end of the flowering season exhibiting predominantly the fruiting stage; **B,** a typical terminal panicle of *C. indicum* bearing flowers of tube-construction in dichasial cyme.

ovary contains a single ovule on axile placentum. Individual flowers are of four-day longevity. Flowers open in the late afternoon (5.10 pm ± 40 min) and persist till early morning (5.00 am ± 20 min) of the 5th day of flower opening. The species is monophilic, being pollinated by a species belonging to the hymenopteran genus *Tetragonula*.

The present work is based on 102 plants occurring in two widely separated wild populations at Amarun Village (23°25′10″N lat.: 87°55′01″ E long.) and Rambhadrapur Village (22°20′16″ N lat.: 87°42′84″ E long.) respectively of Burdwan and Paschim Midnapur districts of the state of West Bengal in India, together with 15 individuals grown in the experimental plot of Botany Department, Burdwan University (Fig. 2).

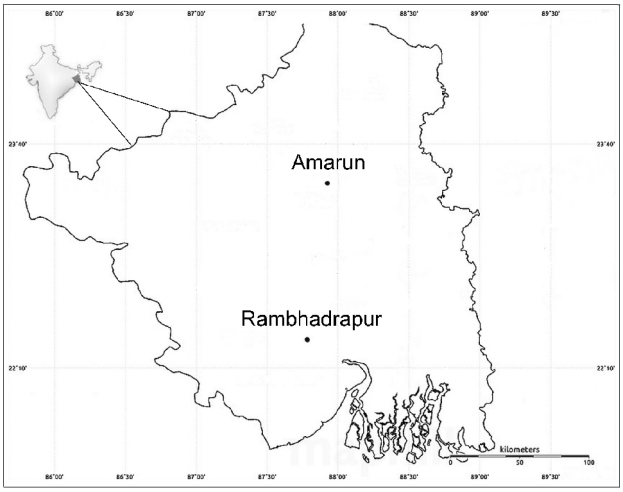

Fig. 2. Map of southern part of the state of West Bengal in India showing the sites [●] of natural habitats of *Clerodendrum indicum* at Amarun and Rambhadrapur villages.

Observations on floral morphological details and floral events were made using a 10x hand lens and a WILD M3B (Switzerland) Stereobinocular microscope. Each observation was based on randomly selected 100 flowers belonging to 10 different individuals in each of the two populations and the study was repeated for three consecutive years (2015-2017).

Pollen production was estimated by determining the number of pollen grains produced in an anther. One mature anther was gently squashed in a centrifuge tube and the volume was made up to 10 ml. Then 0.5 ml of the homogeneous solution was taken on a slide, covered with a 22 mm x 50 mm cover slip and the pollen grains were counted under the 10x objective of a bright-field microscope. Calculation of the mean value was based on 10 such observations each of10 different anthers. The determination of relative frequencies of fertile and sterile pollen grains in a flower was based on staining the pollen grains of an anther with a solution of 0.08% Cotton blue-lactophenol, as per the method of Darlington and La Cour (1960), soon after anther dehiscence. Readings were taken after allowing a staining time of 1hour. In each microscopic field, the total number of pollen grains, the number of pollen grains with uniformly stained cytoplasm and the number of pollen grains that remained unstained were recorded. Mean value was deduced from 10 such observations each of 10 different anthers.

The onset of stigma receptivity was detected with the help of in vivo pollen germination experiments. Flowers were bagged in bud condition and emasculated prior to anther dehiscence. With the help of a soft fine brush moistened with sterile distilled water, fresh pollen grains from a flower of different plant were brushed onto the stigmas at various stages of development followed by bagging. Allowing an interval 2 hrs, the stigmas were excised and pollen germination was studied under the compound microscope by the usual Cotton blue-lactophenol staining. Simultaneously, stigma receptivity was also verified through the Hydrogen Peroxide Test ascertaining the dehydrogenase activity (Zeisler, 1933; Galen and Plowright, 1987). The stigma with terminal part of the style was carefully excised, taken on a clean slide, a few drops of H2O2 solution (6% v/v) were applied to it and the appearance of bubbles (if any) was observed and recorded under the 10x objective of the compound microscope.

Studies in self-incompatibility were based on percentage of in vivo pollen germination, growth of pollen tube and percentage of successful fruit-set obtained in geitonogamous and xenogamous

pollination experiments done artificially. Mature flower buds, when starts to open, were emasculated carefully without injuring any other floral organ and then bagged with fine silken cloth. On the 3rd day morning, when the stigmas of the emasculated flowers became receptive, those were pollinated artificially by the pollen grains from the dehisced anthers of 2nd day flowers belonging to the same plant and as well as a different plant. The pollinated flowers were then kept bagged all through the flower longevity. Such artificially pollinated flowers were left allowing fruit-set in them. The observations were based on 30 tagged flowers belonging to 8 different individuals in each of the two flowering seasons. Mean values (± SE) of the percentages of fruit-set obtained through geitonogamous and xenogamous pollinations were determined. Finally, significance of the results so obtained was evaluated by 't-test'. In order to determine the degree of self-incompatibility, the index of self-incompatibility (ISI) of the species was deduced, by dividing the number of the fruit-set through geitonogamy by the number of fruit-set through xenogamy (Ruiz-Zapata and Arroyo 1978). As per the index, ISI value >1 represents self-compatible, between 0.2 and 1 partially self-incompatible, <0.2 mostly self-incompatible and 0 completely self-incompatible.

To ascertain the reproductive success of the plant, the number of flowers, number of fruits and number of seeds produced per plant in open pollinated condition were recorded in each of 30 different individuals. As each of the four ovules present in a flower seldom matures into healthy seed and fruits are variously 1, 2, 3 or 4- seeded, reproductive success of the species was determined from the percentage of seed-set instead of percentage of fruit-set.

RESULTS

Herkogamy

In a mature floral bud of *Clerodendrum indicum*, the filaments bearing anthers and the style bearing stigma remain coiled inside. Flower opening is gradual. Commencement of flower opening, marked by the initiation of divergence of corolla lobes (Fig. 3A), takes place in the afternoon at 5.10 pm ± 40 min and complete opening is achieved by 25–35 min giving rise to a bowl-shaped configuration of the terminal part of the flower (Fig. 3B). Simultaneous with corolla opening, gradual uncoiling of the filaments and the style takes place (Fig. 3B). By 20-25 min of complete flower opening, the filaments and the style become straight, thereby disposed parallelly with the long axis of the flower (Fig. 3C, 4A). Subsequently, a gradual downward shift of the alignment of the style is noticed and finally at 8.10 pm ± 25 min, it reaches to an angle of ± 45° with the floral axis (Fig. 3D, 4B). During the course of such a stylar movement, the filaments exhibit no movement at all and remain straight with the floral axis. This bent configuration of the style persists till 9.40 pm ± 20 min. At 11.40 pm ± 30 min the filaments of stamens bearing anthers start curving downward. Simultaneous with the downward curvature of the filaments the style starts to move upward and at 12.00 am ± 20 min it attains its previous straight configuration (Fig. 3E). Also, the downward coiling of filaments proceeds with progress of time and finally reaches to the maximum of 180° (semicircular) by 2.20 am ± 40 min (Fig. 3F, 4C).

By such a downward curvature of the filaments and the periodical shift in the alignment of the style, a spatial separation between the anther and the stigma is maintained all through the open-flower phase of the species.

Duration of anthesis and dichogamy

In *C. indicum* commencement of anther dehiscence, marked by the appearance of a small slit, takes place at 5.00 pm ± 35 min, 5-10 min before the initiation of corolla opening, while the flower is still in bud condition. By the time when flower opening is complete, the dehiscence slit becomes wide open and masses of yellowish pollen grains become visible through the dehiscence slits of the partially dehisced anthers. Finally, at around 45-50 minutes after flower opening, the dehiscence slits of anthers become widely gaping, bringing about full exposure of the loose masses of yellow pollen grains (Fig. 3C). Although anther dehiscence is achieved more or less simultaneously with flower opening in the late afternoon of the 1st day, a very small amount of pollen (7.7 ± 0.41 %) is seen to be dispersed from the anthers

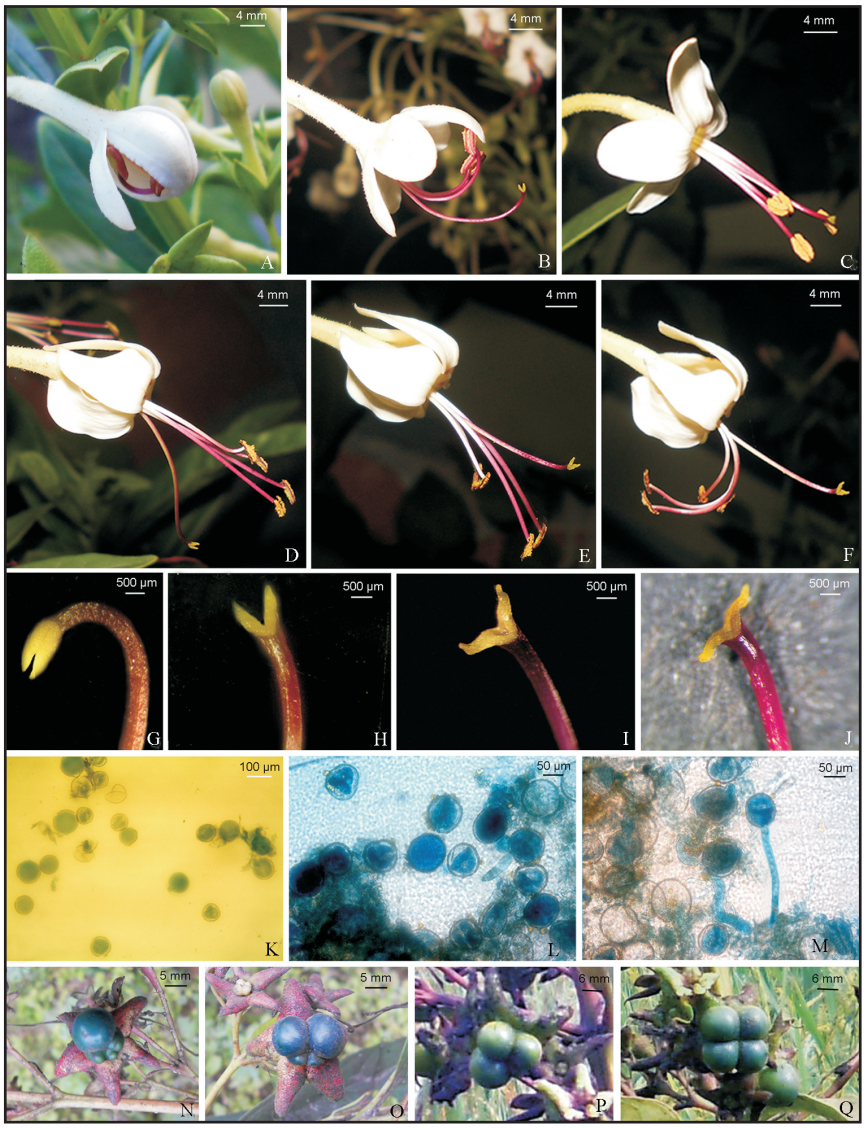

Fig. 3. A, A mature flower bud of *Clerodendrum indicum* showing the initiation of flower opening by divergence of one of the corolla-lobes; **B,** a freshly open flower of *C. indicum* showing partial uncoiling of filaments and style; **C,** a flower of *C. indicum*, soon after completion of its opening exhibiting the straight configuration of filaments and style; loose masses of yellow pollen grains are fully exposed through the widely gaping dehiscence-slits of anther; **D,** a 1st day flower of *C. indicum*, in the evening, nearly two and a half hour after flower opening, showing the backward movement of style reaching an angle of ± 45° with the long axis of the flower, while the filaments retaining their straight configuration; **E, a**flower of *C. indicum*, nearly at midnight of the 1st day of flower opening, depicting the forward movement of the style to its original straight configuration, simultaneous with the progressive backward curvature of the filaments; **F,a** flower of *C. indicum*, in the late-night of the 1st day of flower opening, backward coiling of filaments reaching the maximum of 180º (semicircular); **G-I,** gradual divergence of the two lobes of the non-receptive stigma of *C. indicum* since flower opening [**G,** stigma of a freshly open flower, the two light green lobes remaining almost adpressed with each other; **H,** stigma of a flower, nearly at midnight of the 1stday of flower opening, the two light green lobes forming an angle of divergence of ± 45°; **I,** stigma of a flower, in afternoon of the 2ndday of flower opening, the lobes turning yellowish and reaching an angle of divergence of ± 90-100°]; **J,** the receptive stigma of a flower *C. indicum*, in the morning of the 3rd day of flower opening, with lobes forming an angle of ± 180° and attaining yellow, shiny and lustrous look; **K,** staining reaction of pollen grains with a solution of 0.08% Cotton blue-lactophenol showing pollen fertility; **L-M,** *in vivo* pollen germination on geitonogamous and xenogamous pollinated stigmas of *C. indicum* [**L,** geitonogamous pollens, exhibiting relatively low frequency of germination and short pollen tubes; **M,** xenogamous pollens, exhibiting relatively high frequency of germination and long pollen tubes]; **N-Q,** 1 to 4-seeded fruits of *C. indicum* [**N,** 1-seeded fruit; **O,** 2-seeded fruit; **P,** 3-seeded fruit; **Q,** 4-seeded fruit].

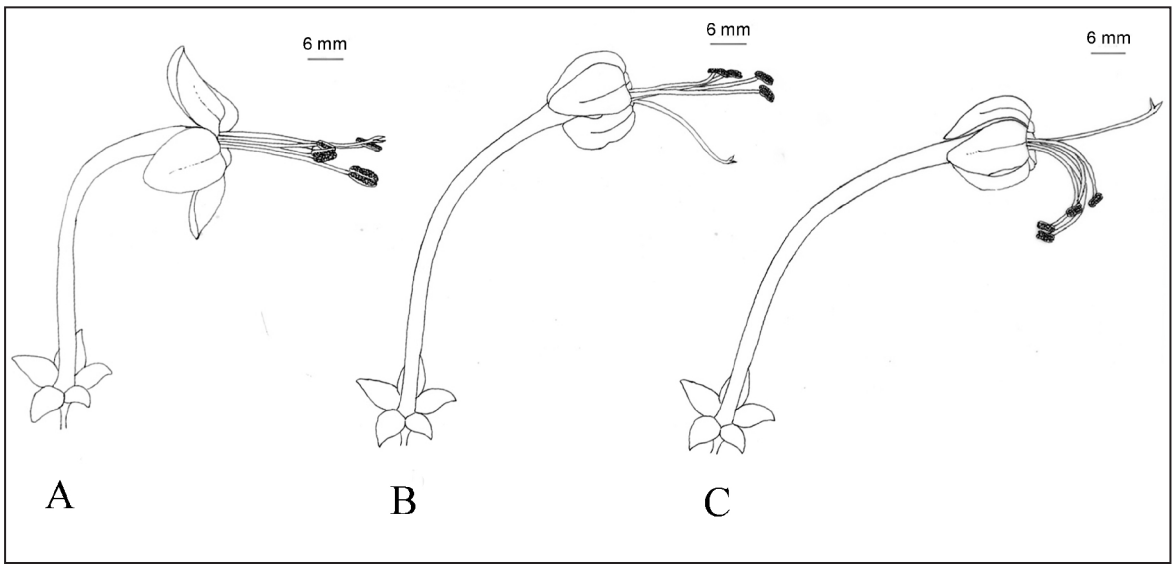

Fig. 4. Different stages of the flower of *Clerodendrum indicum* showing herkogamous disposition between anthers and stigma. **A,** Freshly open flower with straight configuration of filaments and style, cf. Fig. 3c; **B,** flower, nearly two and a half hour after its opening, exhibiting the downwardly shifted style forming an angle of ± 45° with the floral axis, cf. Fig. 3d; **C,** flower, nearly nine hour after its opening, showing the maximum backward coiling of the filaments (180°) and straight style, cf. Fig. 3f.

on that day. However, pollinator activity is never seen in a 1st day flower and this minor fraction of pollen, in all probabilities, is removed from the anther in the evening and night of the 1st day, by wind. Pollinator activity begins in the early morning of the 2nd day. Dispersal of the bulk (89.64 ± 0.91 %) of the pollen grains takes place during the 2nd day, simultaneous with the pollinator activity. Bagging experiments reveal that pollen dispersal of the species is primarily entomophilous. In flowers bagged by nets of appropriate mesh, restricting pollinator visit but allowing free flow of wind, major part of the pollen masses were seen to be retained in the dehisced anthers, while in open-pollinated condition, allowing free pollinator activity, the anthers become almost empty by the end of the 2nd day of flower opening (Fig. 5).

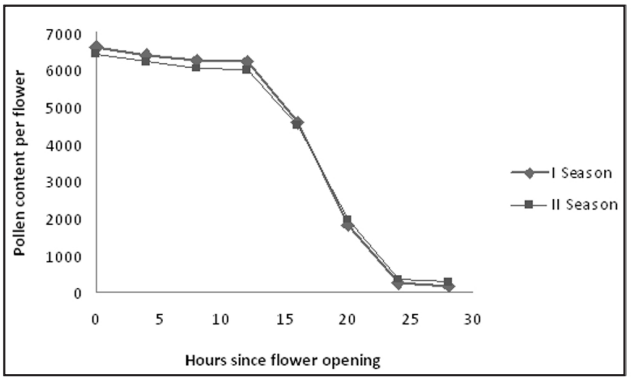

Fig. 5. Gradual loss of pollen content per flower due to pollen dispersal through the floral longevity in *Clerodendrum indicum.*

In a freshly bloomed flower the two light green, non-receptive stigmatic lobes remain almost adpressed with each other (Fig. 3G). By 20-30 min of flower opening, a slow divergence between the two stigmatic lobes is noticed. By 12.15 am ± 15 min, the stigmatic lobes reach to a divergence of ± 45° (Fig. 3H). At this stage stigma still remains non-receptive, as indicated by its light green look. The non-receptivity of the stigma at this stage was confirmed by its inability to support pollen germination experiments with such stigmas pollinated artificially. By 5.25 pm ± 15 min of the 2nd day of flower opening when the divergence of the two stigmatic lobes reaches to an angle of 90-100°, the inner surface of the lobes starts becoming light

yellow (Fig. 3I). At this stage the stigma does not support any in vivo pollen germination, however, possesses a limited dehydrogenase activity as revealed from the emission of a few bubbles in H_2O_2 solution. Thus, the stigma at this stage probably represents a metabolic preparatory phase for attainment of the receptivity. On the 3rd day morning, at 5.20 am ± 20 min, the stigmatic lobes reach to an angle of ± 180°, simultaneously attain a shiny, lustrous look (Fig. 3J). The stigma acquires its true receptivity at around this time because it supports in vivo pollen germination and also produces profuse bubbles on application of H_2O_2 solution exhibiting high level of dehydrogenase activity. This configuration of the stigma persists throughout the forenoon and early afternoon of the 3rd day of flower opening. At around 4.10 pm ± 20 min of that day, the stigmatic lobes start losing their lustrous look and appear somewhat dry, indicating the cessation of stigma receptivity and onset of stigmatic senescence.

The overall male phase (pollen dispersal) of *C. indicum* stands from 5.00 pm ± 35 min of the 1st day of flower opening to 4.30 pm ± 20 min of the 2nd day of flower opening. However, the minor fraction of pollen which is dispersed anemophilously from the dehisced anthers during the evening and night of the 1st day, cannot play any role in pollination, because during that time no receptive stigma remains available in the plant, Therefore, this 1st day's pollen dispersal can be regarded as a mere anemophilous loss. Effective commencement of anthesis in the species, as marked by the initiation of pollen transfer from dehisced anther to receptive stigma, is limited by the time of first arrival of pollinators in the flower i.e., 5.30 am ± 20 min of the 2nd day of flower opening. Pollen presentation from anthers to the stigma by the activity of pollinators continues throughout the 2nd day and the anthers become almost empty by 4.30 pm ± 20 min of that day. Stigma, on the other hand, acquires its receptivity on the 3rd day morning, at 5.20 am ± 20 min and loses its receptivity at around 4.10 pm ± 20 min of the same day. Loss of the receptivity of stigma limits the cessation of anthesis. Thus, the duration of anthesis in the species spans from 5.30 am ± 20 min of the 2nd day of flower opening, till ± 4.10 pm ± 20 min of the 3rd day of flower opening (Fig. 6).

C. indicum is dichogamous and protandrous. The dichogamy in the species is complete having exclusive male (5.30 am ± 20 min - 4.30 pm ± 20 min of the 2nd day of flower opening) and female (5.20 am ± 20 min-4.10 pm ± 20 min of the 3rd day of flower opening) phases with the pause by a neuter phase of more than 12 hrs in between (Fig. 6).

Pollen production, pollen fertility and pollen-ovule ratio

An anther produces 1620 ± 74.182 pollen grains. As there are four anthers in a single flower, the estimated number of pollen grains produced per flower is 1620 x 4 = 6480. Cotton blue-lactophenol staining and *in vivo* germination experiments performed with fresh pollen grains revealed that 74.85 ± 11.737 % of the pollen grains of a flower are fertile and the rest i.e. 25.15 ± 11.737 % are sterile or abortive (Fig. 3K). As the estimated number of pollen grains produced by a flower is 6480 and the ovary contains typically four ovules, the pollen-ovule ratio of the species is 6480:4 = 1620:1.

Partial self-incompatibility

C. indicum produces dry, fibrous, more or less globose drupes of 11-20 mm in diameter. In open pollinated condition, 10.33 ± 0.622 % fruit-set was noticed. Artificial geitonogamous pollination gives rise to merely 6.84 ± 0.166 % fruit-set. On the other hand, controlled xenogamous pollination yields as much as 31.8 ± 0.109 % fruit-set (Table1, Fig. 7). Based on the results of pollination experiments, the index of self-incompatibility (ISI) was calculated as 0.22. As the self-incompatibility index is between 0.2 and 1, the species is to be regarded as partially self-incompatible. Also, much higher degree of pollen germination was met with in stigmas pollinated with xenogamous pollens (34.132 ± 4.281 %) than those pollinated with geitonogamous pollens (7.25 ± 2.191 %). Moreover, xenogamous pollens exhibited faster growth of pollen tube than the geitonogamous ones (Fig. 3L, M). Significance of the results of controlled geitonogamous and xenogamous pollination experiments each with respect to open-pollinated condition when evaluated by 't-test', the 't-value' of

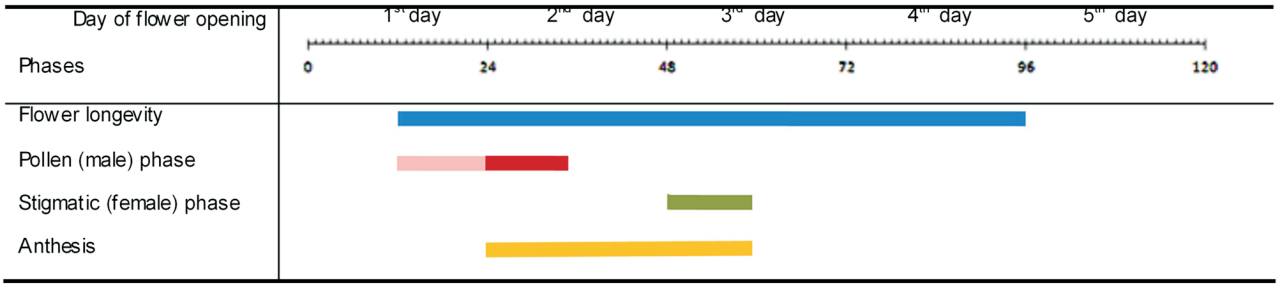

Fig. 6. Diagramatic representation of the duration of anthesis of *Clerodendrum indicum*, exhibiting complete dichogamy with a pause of >12 hrs between the male and the female phases.

xenogamy with open-pollinated condition exhibits high significance (33.991) in comparison to that of geitonogamy with open-pollinated condition (5.42) at $P_{0.05}$.

Reproductive success

In its natural habitats, *C. indicum* is obligatorily entomophilous and monophilic. The four-chambered ovary of a flower contains altogether four ovules, one in each chamber. However, all of the four ovules of an ovary very rarely mature as seed. Majority of the fruits are 1-seeded. Often a fruit is 2-seeded, less often 3-seeded and rarely 4-seeded (Fig. 3N-Q). The overall seed-ovule ratio of the species is 1:23. Thereby, its seed-set percentage i.e. the reproductive success is 4.381 ± 0.27 % (Table2).

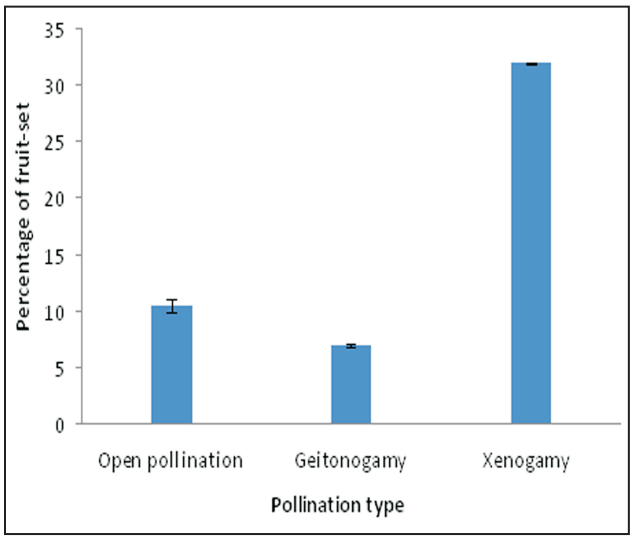

Fig. 7. Diagrammatic representation of relative percentages of fruit-set in open and controlled geitonogamous and xenogamous pollination experiments in *Clerodendrum indicum*.

Table 1. Percentage of fruit-set of *Clerodendrum indicum* in open and controlled pollination experiments

Types of pollination	No. of flowers (Mean ± SE)	No. of fruit-set (Mean ± SE)	Percentage of fruit-set (Mean ± SE)
Open pollination	198 ± 20.067	20.63 ± 2.639	10.33 ± 0.622
Geitonogamy	88.13 ± 2.12	6 ± 0.505	6.84 ± 0.166
Xenogamy	100.25 ± 2.707	31.88 ± 0.833	31.8 ± 0.109
Apomixis	92 ± 0.845	0	0

Table 2. Seed-set percentage and seed-ovule ratio of *Clerodendrum indicum* in open pollinated condition in each of its two wild populations

Locality	No. of flowers per plant (Mean ± SE)	No. of 1-seeded fruits (Mean ± SE)	No. of 2-seeded fruits (Mean ± SE)	No. of 3-seeded fruits (Mean ± SE)	No. of 4-seeded fruits (Mean ± SE)	No. of healthy seeds (Mean ± SE)	Seed-set percentage (Mean ± SE)	Seed- ovule ratio
Amarun	190 ± 23.452	10.25 ± 2.097	5.5 ± 0.601	2.5 ± 0.82	1 ± 0.289	32.75 ± 6.969	4.367 ± 0.424	01:23.2
Rambhadrapur	206 ± 35.849	12.25 ± 1.887	6 ± 1.472	3 ± 0.913	0.75 ± 0.125	36.25 ± 6.836	4.4 ± 0.401	01:22.7
Mean	198 ± 20.067	11.25 ± 1.306	5.75 ± 0.834	2.75 ± 0.522	0.88 ± 0.198	34.5 ± 4.52	4.381 ± 0.27	01:23.0

DISCUSSION

In *Clerodendrum indicum*, the spatial separation, i.e. herkogamous disposition between the male and female organs, by backward curvature of filaments of the stamens with simultaneous shifting of style, is fully established sufficiently before the commencement of pollinator activity. Also, the species is distinctly dichogamous with an interruption of neuter phase of more than 12 hrs between the male and female phases. Because of the presence of such a strong dichogamy, there is no chance of herkogamy with respect to its breeding system. From evolutionary point of view, in all probabilities, the herkogamy in the species represents the retention of a relictual feature of some homogamous ancestors. Further achievement of complete dichogamy, though makes the autogamy impossible in the species, but cannot rule out the chance of inbreeding by geitonogamy. Presence of a sort of self-incompatibility in the species is reflected by a lesser percentage of fruit-set in geitonogamous pollination (6.84±0.166 %) than that of the xenogamous one (31.8 ± 0.109 %). However, the self-incompatibility is not complete, rather partial, because successful fruit-set, though in appreciably lesser frequency, is imparted by geitonogamous pollen transfer. High significance of 't-value' of xenogamy with open-pollinated condition (33.991) in comparison to that of geitonogamy with open-pollinated condition (5.42) at $P_{0.05}$ also corroborates the partial self-incompatibility of the species. Existence of such a partial self-incompatibility is a further evolutionary achievement in the species, because it acts as a rider for geitonogamous pollination which may take place among the dichogamous flowers. The relatively low reproductive success (4.381 ± 0.27 %) of the species can be attributed to its partial self-incompatibility. Partial self-incompatibility has been noticed earlier in a number of taxa belonging to different unrelated families, viz., Medicago sativa (Cooper and Brink, 1940), Solanum carolinense (Mena-Ali and Stephenson, 2007).

C. indicum is a unique representation of structural as well as functional modifications in its floral biology for higher degree of out-breeding. Adaptation for out-breeding by the species has been achieved by modifications at more than one levels: i) a herkogamous disposition of anthers and stigma, creating hindrance for autogamy, ii) complete dichogamy, imparting the pollination as obligatorily allogamous, iii) partial self-incompatibility, giving rise to predominantly xenogamous pollination. The species is characterized by a quite high pollen-ovule ratio (1620:1). High pollen-ovule ratio is in general regarded as a characteristic of out-breeding (Cruden, 1977). Therefore, the high pollen-ovule ratio *C. indicum* corroborates the predominance of out-breeding in the species.

Occurrence of either herkogamy or dichogamy or both are known in some other species of *Clerodendrum*. Flowers of *C. inerme* are herkogamous (Ramesha *et al.*, 2011), while those of *C. trichotomum*, C. izuinsulare (Miyake and Inoue, 2003), *C. splendens*

(Rohitash and Jain, 2010), *C. infortunatum* (Rajurkar, 2010), *C. molle* (McMullen, 2011) and *C. trichotomum* (Sakamoto *et al*., 2012) are dichogamous (protandrous). Dichogamy (protandry) together with herkogamy is observed in *C. serratum* (Singh *et al.,* 2012), *C. thomsoniae* and *C. trichotomum* (Yuan *et al*., 2010). However, in none of the species exhibiting herkogamy and/or dichogamy, any degree of self-incompatibility is known. Also, in several pollination studies conducted earlier on the members of the family Lamiaceae, it has been shown that the plants belonging to the family are in general self-compatible (Owens and Ubere-Jimenez, 1992). In view of that, *C. indicum*, by virtue of its partial self-incompatibility, is an uncommon member of Lamiaceae. Only *Salvia smyrnaea* is another such member of the family which is also known to be partially self-incompatible (Subasi and Guvensen, 2011).

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FULL LENGTH ARTICLE

Estimation of cyanogenic glycosides in processed and unprocessed plant parts of *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst.(chayamansa)

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The estimation of cyanogenic glycosides in different processed and unprocessed plant parts (leaf, petiole, root, and stem) of *Cnidoscolus aconitifolius* (Chayamansa) involved both qualitative and quantitative methods using the modified alkaline picrate method (Onwuka, 2005) and the picrate-impregnated paper method (Harborne, 1972) respectively. Notably, all processing techniques do not equally reduce cyanogenic glycosides in plant parts, which is significant ($p < 0.05$). The quantity of cyanogenic glycosides (CG) in plant parts can be reduced more effectively by boiling in water than by other processing techniques (such as sun drying, water soaking, or freezing). However, the duration of boiling also plays a significant role. Boiling for 25 minutes proves to be much more effective than boiling for 15 or 20 minutes. Extending the boiling process further reduces the amount of cyanogenic material in Chayamansa plant parts, enhancing safety for consumption. This study highlights significant discrepancies in cyanogenic glycoside levels across plant components, with roots containing the highest concentrations and leaves exhibiting the lowest. Qualitative analysis through picrate-impregnated paper shows varying colour changes within two to six hours, indicating the presence of cyanogenic compounds in roots, leaves, petioles, and stems. However, freezing increases cyanogenic glycoside levels in Chayamansa. The study emphasises the importance of proper processing methods to alleviate potential health risks associated with consuming Chayamansa. Also recommending the discarding of the filtrate post-boiling due to residual hydrogen cyanide. It also underscores that consumption of leaves and tender petioles is comparatively safer than using highly cyanogenic roots. Further research is needed on the cyanogenic content of Chayamansa latex. Overall, this research work emphasises the critical role of appropriate processing techniques in reducing cyanide-related health risks linked to Chayamansa consumption.

Keywords : Chayamansa, *Cnidoscolus*, Cyanogenic glycosides, Processing, Linamarin.

INTRODUCTION

In the pre-Cambrian Maya region of Guatemala, Belize, and Southeast Mexico, domestication of wild varieties of *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst. (chaya,tree spinach) results in the sprouting out of a newly cultivated variety called chayamansa (Ibarra and Cruz, 2002). McVaugh's designation of *Cnidoscolus chayamansa* is still occasionally used (McVaugh,1944). Today, Chayamansa is widely practised in Kerala as well. Chaya has been brought to Africa and Asia due to its many beneficial nutraceutical properties (Porres and Cifuentes, 2014). According to Ranhotra *et al*.,1998, chaya contains sizeable levels of mineral nutrients that contribute to a healthy diet. Chaya has about three times iron and twice the calcium content of spinach.

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(Ebel *et al*., 2019). Many biological activities are asscociated with *Cnidoscolus aconitifolius*. The methanolic leaf extract of chayamansa has been reported to exhibit antibacterial and antifunal activity against *P. vulgaris, S. aureus*, and *Aspergillus* sp. (Kiran *et al*., 2021) while the aquatic extract showed activity against *S. typhi, S. pyrogens, E. coli, K. pneumoniae* (Lennox and John, 2018). *C. aconitifolius* extract can elevate the activity of several antioxidant enzymes and alleviate the oxidative stress and stopping lipid peroxidation-induced cell death (Ezebuiro *et al*., 2020). According to Moura *et al*. (2019) many other properties are associated with the genus *Cnidoscolus* including analgesic, anti-inflammatory, antibiotic, diuretic effects, as well as anticancer due to the presence secondary metabolites such as alkaloids, phenolic compounds, terpenoids, saponins etc.

Chayamansa is an underutilised crop due to incomplete understanding of it (Galluzi, 2014). According to Ibarra and Cruz (2002), Chayamansa can be linked to Manihot species both physically and in terms of their cyanogenic ability.

Several cyanogenic plants contain cyanogenic glycosides, which are organic plant poisons. Many plants store them in inactive forms that are activated by hydrolysing enzymes (-glucosidases), which are compartmentalised (Britto, 2022). When cyanogenic plants are chewed by herbivores or when the plants are broken down by procedures like grinding, pounding, or in the presence of water, such as during soaking or fermentation, the cell walls break, and cyanogenic glycosides (CG) come into contact with hydrolysing enzymes and undergo hydrolysis (CFS, 2007). The enzyme linamarase, which detoxifies the cyanogenic glycosides in cassava, makes up a significant portion of the protein in the leaves (Bokanga *et al*., 1994). When plant tissues are damaged, the enzyme linamarase converts cyanogenic glycosides (CG) to acetone cyanohydrin, which reacts with hydroxyl nitrile lyase to form poisonous hydrocyanic acid (HCN) (Ndam *et al.*, 2019). Cyanogenic glycoside itself is not poisonous. Active phytochemicals are currently being used to create non-toxic, anti-hazardous, and cost-effective management for a variety of treatment techniques (Suraj and Monojit, 2023). Plant extracts act as a natural remedy for many pathophysiological diseases due to the presence of various metabolites in it.(Banerjee *et al*., 2023). Lack of scientific knowledge about actual phytochemical properties of a medicinal plant will affect the efficiency of a crude drug developed from that plant.(Sultana *et al*., 2023). Cyanogens are a collective term for cyanogenic glycosides, cyanohydrins, and hydrogen cyanide (Onojah and Odin, 2015).

In the digestive systems of animals and humans, there's an enzyme called β-glycosidase that breaks down these harmful CGs, preventing the release of HCN. However, some plants don't have this enzyme naturally. When animals or humans consume the plants with out β-glycosidase, the CGs can still be broken down in their digestive systems – but only if their gut contains gut microflora that produce β-glycosidase (Siegie, 2007). Numerous cyanogenic food plants include various cyanogenic glycosides (CG), such as taxiphyllin in bamboo shoots and linamarin in cassava and chayamansa (Kutti and Konuru, 2006; WHO, 1993). Using high-performance thin-layer chromatography, linamarin was found to be the main form of cyanogenic glycosides in edible *Cnidoscolus* leaves (Kutti and Konuru, 2006). These glycosides will be hydrolysed during cooking or other heat-related processes , such as boiling in water, steaming, sun drying, frying and oven heating etc. reducing the risk of poisoning (Ranhotra *et al.*, 1998). According to WHO, 1993, the safe cyanogen intake limit is 10 mg/kg of food. (Geller *et al*., 2006) pointed out that acute cyanide poisoning can include rapid breathing, a reduction in blood pressure, a rapid heartbeat, headaches, dizziness, vomiting, diarrhoea, mental confusion, cyanosis, twitching, and convulsions.

Due to widespread misconceptions about its cyanide level, nobody in Kerala pays much attention to the Chayamansa variety, which is introduced into India in 2006 and around 2000 plants were thriving there (Kumar *et al*., 2011). Heat treatment is the most widely used approach for properly treating edible plants to minimise their HCN concentration (Kumar, 1992). The goal of the current research is to assess the amount

of cyanogenic material contained in processed and unprocessed Chayamansa plant parts.

MATERIALS AND METHODS

Plant Materials

Since 2021, *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst. (Chayamansa) cultivars have been continuously grown in Edakulam, a village in the Thrissur district of Kerala, India. The plant is cultivated in well-aerated, well-irrigated alluvial soil. For each analysis, gathered healthy plant parts such as the leaves, petioles, roots, and stem s individually. Both processed and unprocessed plant components were selected for the estimation of cyanogenic glycosides. Processing techniques include freezing, sun drying, water soaking for 12 hours, boiling for 15 minutes, 20 minutes, and 25 minutes, as well as testing the filtrate left after boiling. Fresh, mature, and tender plant portions were used as raw samples for the assessment of cyanogenic glycosides.

Methods

Detection of cyanogenic glycosides:

The picrate-impregnated paper method was used to detect the cyanogenic glycosides (Harborne, 1972). Cut-up leaves, petioles, roots, and stems were placed in test tubes with 1.5 ml of water and 6 drops of chloroform, and then simply crushed with a glass rod. Filter paper strips, approximately 4.0 x 1 cm in size, were first neutralised with sodium bicarbonate before being exposed to an aqueous solution of 0.05 M picric acid for 20 to 25 seconds. After allowing the impregnated paper to dry, and it was stored in a freezer at 4°C until the time we want it for keep up its stability. Soaked it with an aqueous picric acid solution when needed. The test tube containing the test sample should be attached to a plastic strip, sealed with a cork, and allowed to stand at room temperature. The release of HCN by the plant was indicated by a change in the colour of the paper, from yellow to brown-red. If there was no colour shift, HCN was not present. A brown-red colouration that appeared after 24 hours indicated the presence of cyanogenic glycoside. According to Franscisco and Pimenta (2000), the absence of a colour change after 48 hours indicated a negative test result for cyanogenic glycosides. Changes in colour were photographed using a camera phone and compared to the (Bradbury, 1999) *Manihot esculenta* (cassava) HCN colour chart (0-800 ppm). A negative control (picrate-impregnated paper without a test sample) and a positive control (Picrate paper treated with Linamarin instead of plant parts) were used.

Quantitative Estimation of Cyanogenic Glycosides:

A modified approach (Onwuka, 2005) was employed for the quantitative determination of cyanogenic glycosides, utilizing a UV-Visible spectrophotometer. Five grams of Chayamansa leaves, petiole, root, and stem were separately mashed using a mortar and pestle, centrifuged, and 1 ml of the supernatant was transferred into test tubes. These test samples were mixed with 4 ml of a yellow-colored alkaline picrate solution and allowed to stand for 15 minutes in a 50°C water bath. The absorbance of the test tubes was measured at 490 nm using a UV-visible spectrophotometer 119 (systronics). The colour change from yellow to reddish-brown or dark yellow was compared to a blank (1 ml of distilled water combined with 4 ml of alkaline picrate solution). The analyses were conducted in triplicate.

A standard graph of linamarin (positive control) (Fig.1) with varying concentrations ranging from 0.005 mg/ml to 0.025 mg/ml was generated using a stock solution prepared by diluting linamarin with water (1 mg/ml). Linear regression analysis was carried out using the equation $y = mx + c$, ($y = 0.0156 x -0.05$, $R^2 = 0.9971$), where 'y' represents the absorbance of the test sample, 'm' is its slope, 'x' is its sample concentration(mg/ml) 'c' is its intercept, and 'R^2' indicates the Coefficient of determination Analysis of variance (ANOVA) was employed to validate the test's significance.

RESULTS AND DISCUSSION

Qualitative Analysis of Cyanogenic Glycosides

In the roots, the picrate-impregnated paper began to change from its original yellow colour to a yellowish-

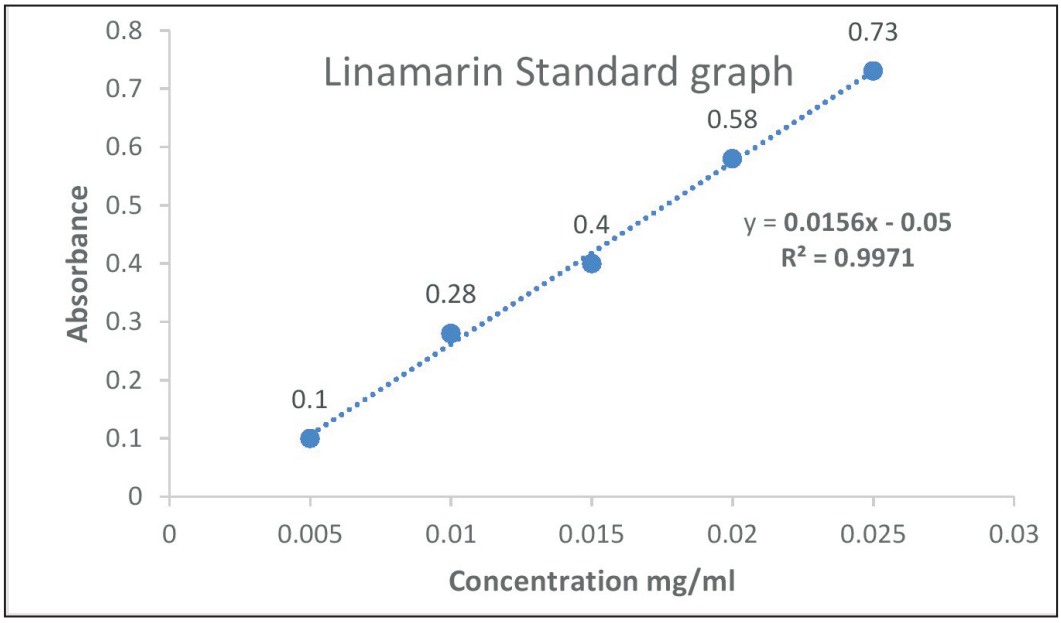

Fig.1. Shows standard calibration curve for Linamarin. Linear regression analysis were done by using equation y=mx+c (y=0.0156x-0.05, R^2=0.0071) here 'y' is absorbance of test sample; 'm' is the slope; 'x' is the sample concentration and 'c' is intercept and R^2 is the coefficient of determination.

brown shade within two hours. The leaves, petiole, and stem displayed varying hues on the paper, ranging from two to six hours. According to (Bradbury, 1999) charts for cassava HCN, plant sections (leaves, petiole, root, and stem) showed 20 ppm, while linamarin displayed 100 ppm (Fig. 2).

Quantitative Analysis of Cyanogenic Glycosides

The cyanogenic content in the plant parts of Chayamansa resulting from various processing and unprocessed methods is recorded in (Tables 1 and 2) respectively. The CG content of the filtrate remaining

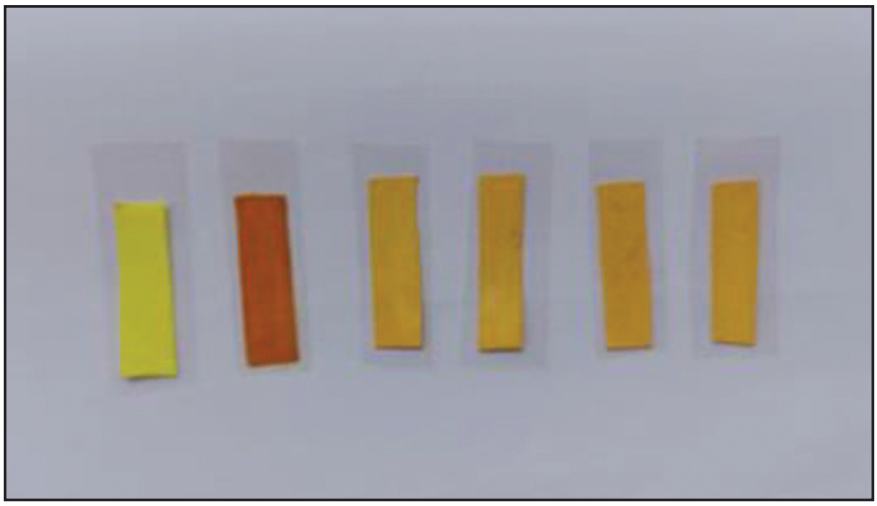

Fig.2. Picrate impregnate paper method for qualitative cyanogen analysis of chayamansa plant parts. Figure showing as (left to right) 1) Blank, 2) Standard (100 ppm), 3) Leaves (20 ppm), 4) Petiole (20 ppm), 5) Root (20 ppm), 6) Stem (20 ppm).

Table 1. HCN content (mgHCN equivalent/g fresh weight) in the processed plant parts of chayamansa plant parts by using UV-Visible spectrophotometer. Values are mean±SEM of triplicate determinations.

HCN content in the Processed plant parts (Leaves, Petiole, Root, Stem) of chayamansa

Sl. No.	Type of Processing	Plant Parts	HCN Content (mg/g)
1.	Frozen	Leaves	73.8±1.01
		Petiole	94.4±1.30
		Root	98.8±2.09
		Stem	95.2±1.15
2.	Water-soaked	Leaves	58.4±0.32
		Petiole	66.2±0.15
		Root	95.0±0.34
		Stem	86.5±0.46
3.	Sun dried	Leaves	51.8±1.35
		Petiole	55.8±0.74
		Root	91.0±0.37
		Stem	62.0±0.20
4.	Boiled in 15 min	Leaves	23.9±1.13
		Petiole	46.8±0.74
		Root	48.5±1.50
		Stem	40.4±0.98
5.	Boiled in 20 min	Leaves	18.5±0.17
		Petiole	26.3±0.37
		Root	33.0±1.74
		Stem	21.9±0.07
6.	Boiled in 25 min	Leaves	10.3±0.74
		Petiole	16.2±0.77
		Root	17.5±0.77
		Stem	11.5±0.37

Table 2. HCN content (mgHCN equivalent/g fresh weight) in the unprocessed plant parts of chayamansa plant parts by using UV-Visible spectrophotometer. Values are mean±SEM of triplicate determinations.

HCN content in the Unprocessed fresh mature and tender plant parts (Leaves, Petiole, Root, Stem) of chayamansa

Sl. No.	Unprocessed Methods	Plant Parts	HCN Content (mg/g)
1.	Fresh	Mature Leaves	50.3±0.42
2.		Mature Petiole	54.0±0.11
3.		Mature Root	89.8±0.20
4.		Mature Stem	52.7±0.25
5.		Tender Leaves	20.0±0.28
6.		Tender Petiole	45.5±0.36
7.		Tender Root	46.2±0.19
8.		Tender Stem	40.0±0.08

after plant parts were boiled is also analysed (Table 3). In all cases, both processed and unprocessed roots showed a higher CG content than all other selected plant materials (leaves, petiole, and stem) because the distribution of cyanogen may vary in each part (Kutti and Konuru, 2006).

The toxicity of cyanogenic glycosides is determined by the ability of CG to hydrolyse either spontaneously or in the presence of an enzyme to produce cyanide as the end product. Consequently, the amount of free cyanide produced after hydrolysis is used to assess the hazardous levels of cyanogenic glycosides (Bolarinwa *et al.*, 2016). Both qualitative and quantitative estimations revealed that roots are more cyanogenic than other parts (leaves, petiole, and stem). Picrate impregnated method is a primary analytical tool for determining whether a plant is cyanogenic or not. The hue of the picrate paper and the rate at which the colour changed determine how strong the cyanogenic feature is. If the colour of the picrate paper changes within two hours, it is considered cyanogenic (Franscisco and Pimenta, 2000). The picrate paper used in our investigation changed colour in all plant sections between 2 and 6 hours, indicating that they are all cyanogenic (Table 4).

Cyanogenic glycosides are present throughout plants, building up in various above- and below-ground plant parts. This pattern of variation is also present in the plant's reproductive and vegetative tissues, with HCN levels often higher in plants growing in suboptimal temperatures, such as below 15°C or above 25°C (Gleadow and Moller, 2014). In comparison to unprocessed plant parts, none of the processing techniques (except boiling) reduced the concentration of cyanogenic components. In contrast to other processed and unprocessed samples, all frozen plant samples had higher concentrations of cy*anogen (leaf* 73.8±1.01, petiole 94.4±1.30, root 98.8±2.09, stem

Table 3. HCN content (mgHCN equivalent/g fresh weight) in the filtrate of boiled plant pants of chayamansa by using UV-Visible spectrophotometer. Values are mean±SEM of triplicate determinations.

HCN content in the filtrate of boiled plant parts of chayamansa

Sl. No.	Filtrate of Boiled Plant Parts	Plant Parts	HCN Content (mg/g)
1.	Boiled in 15 min	Leaves	20.9±0.21
		Petiole	18.5±0.39
		Root	16.8±0.11
		Stem	18.4±0.28
2.	Boiled in 20 min	Leaves	23.0±0.29
		Petiole	16.9±0.25
		Root	19.2±0.37
		Stem	20.6±0.15
3.	Boiled in 25 min	Leaves	28.6±0.21
		Petiole	15.4±1.01
		Root	24.9±1.35
		Stem	23.7±0.97

Table 4. Picrate impregnated method showing time taken for colour change and cyanogenic type of the various plant parts of chayamansa.

Sl. No.	Plant parts (Analysed for picrate impregnated method	Time taken for colour change	Cyanogenic type
1.	Leaves	Within 6 hour	Moderate
2.	Petiole	6 hour	Moderate
3.	Root	Within 2 hour	Intense
4.	Stem	6 hour	Moderate

95.2±1.15 mg HCN equivalent/g fresh weight). This result aligns with a study conducted on frozen leaf and petiole samples of Chayamansa (Kutti and Konuru, 2006) to estimate its cyanogenic glycoside concentration, which revealed higher CG content in frozen leaf 4.93±2.3 µg HCN equivalent/g fresh weight and petiole 6.25±0.2 µg HCN equivalent/g fresh weight when compared to fresh.

Many people choose to freeze food to preserve it for later use and prevent spoilage. However, freezing can cause harm due to the plant's physiological reactions to freezing temperatures when plant tissues are cut (Kutti and Konuru, 2006). Freezing can lead to de novo protein synthesis, changes in intracellular concentrations of carbohydrates, and structural alterations in lipid membranes (Pearce, 2001). Therefore, not all plant portions of Chayamansa can have their CG content reduced by freezing.

Among the other processing techniques in this study, water soaking and sun drying did not decrease the CG content of unprocessed parts, but their amounts were less than that of frozen samples (leaf 58.4±0.32, petiole 66.2±0.15, root 95.0±0.34, stem 86.5±0.46 mg HCN equivalent/g fresh weight), and sun-dried samples had CG content as (leaf 51.8±1.35, petiole 55.8±0.74, root 91.0±0.37, stem 62.0±0.20 mg HCN equivalent/g fresh weight). Many plants may undergo cyanogenesis as a result of temperature variations (Jones and Ramnani, 1985). Genetic variability, enzyme activity, plant age, and variations in leaf nitrogen can all influence the amount of cyanogen present in plant leaf tissues (Gleadow and Woodrow, 2000).

According to our research, the only processing technique that significantly reduces the cyanogenic content is boiling in water, although the duration of boiling also plays a significant role. This aligns with two research studies as the amount of cyanogenic glycosides in Chayamansa leaves is unknown, although heat treatment can diminish or remove these compounds, reducing the risk of poisoning (Martin and Ruberte, 1978). Chaya leaves in their raw form had a dry weight of 1121.72 mg/kg. HCN was significantly ($p \leq 0.05$) reduced from cooked leaves by more than 99% (1.72 mg/kg dry weight) (Kongapa et al., 2021).

Boiling for 25 minutes is much more effective than boiling for 15 or 20 minutes. The amount of cyanogenic material in Chayamansa plant parts can be further reduced by extending the boiling process. Plant parts boiled for 15 minutes have values close to unprocessed tender plant parts (leaves 23.9±1.13, petiole 46.8±0.74, root 48.5±1.50, stem 40.4±0.98 mg

HCN equivalent/g fresh weight), but their cyanogenic content is less than unprocessed mature plant parts (leaf 50.3±0.42, petiole 54.0±0.11, root 89.8±0.20, stem 52.7±0.25 mg HCN equivalent/g fresh weight). So there is a decrease in the cyanogenic content after 15 minutes of boiling. On boiling for 20 and 25 minutes, a further reduction in the quantity of cyanogenic glycosides is observed. The cyanogenic glycosides in plant parts boiled for 20 minutes were leaves 18.5±0.17, petiole 26.3±0.37, root 33.0±1.74, stem 21.9±0.07 mg HCN equivalent/g fresh weight. In 25 minutes of boiling, leaves (10.3±0.74, petiole 16.2±0.77, root 17.5±0.77, and stem 11.5±0.37 mg HCN equivalent/g fresh weight) had the lowest amount of cyanogenic content compared to all other methods. So an increase in the boiling time will decrease the amount of cyanogenic glycosides.

We examined the CG content in the filtrate to determine whether it was safe to consume after being removed from boiled plant parts. The highest concentration of cyanogen was found in the filtrate from boiling for 25 minutes (leaves 28.6±0.21, petiole 15.4±1.01, root 24.9±1.35, stem 23.7±0.97 mg HCN equivalent/g fresh weight), and the lowest was in the 15-minute boiling filtrate (leaves 20.9±0.21, petiole 18.5±0.39, root 16.8±0.11, and stem 18.4±0.28 mg HCN equivalent/g fresh weight). The filtrate from 20 minutes of boiling had CG content as follows (leaves 23.0±0.29, petiole 16.9±0.25, root 19.2±0.37, and stem 20.6±0.15 mg HCN equivalent/g fresh weight).

Cooking and boiling significantly reduce antinutrients in fruits and vegetables. During boiling or heating, cell walls break down, allowing harmful and antinutritional substances to leach out of the cells (Ogbadoyi *et al*., 2006). The volume of water used for boiling and the duration of boiling play a crucial role in reducing cyanogenic glycosides (Montagnac *et al*., 2009). Increased boiling time enhances the ability of HCN to evaporate, leach out from the leaves into the water, and have contact with heat generated during boiling, leading to the reduction of CG (Bokanga *et al*., 1994). As the heating process progresses, the HCN gas evaporates more rapidly, resulting in a quicker reduction of HCN in the processed leaves (Dolor Estrellana *et al.*, 2023). Therefore, as the boiling time increases, more CG is leached out into the filtrate. Different processing methods, such as fermentation, heating, drying, and grinding, decrease the amount of HCN in plant tissue while releasing it into the air or cooking water (Teels, 2002). According to earlier research, chaya leaves should only be cooked for 20 minutes, and the water used to prepare them still contained traces of cyanogenic substances due to cyanide residues that became trapped in the pot.

Female albino rats become intoxicated by cyanide and nitrite when they consume fresh *Cnidoscolus* leaves (Chukwuemeka *et al.*, 2015). According to Nigerian Industrial Standards (NIS) and the Codex Alimentarius Commission (CAC) standards, the maximum safe limit for cassava varieties for human consumption is 10 mg/kg (Sanni *et al*., 2005; Ubwa *et al*., 2015). Therefore, it is safe to consume Chayamansa parts without the filtrate after boiling for 25 minutes or longer. According to Ross-Ibara and Molina-Cruz (2002), boiling Chaya leaves for 15 minutes reduces the HCN concentration, which aligns with our current findings.

Cyanide intoxication typically affects those who are deficient in proteins and amino acids, particularly sulfur-containing amino acids. In humans, the conversion of cyanide to thiocyanate is carried out by rhodanase with the help of sulfur-containing amino acids, which is then eliminated in the urine (Banea, 2012). However, the human body's detoxication system for cyanide can only handle modest amounts of cyanide ingested from cyanogenic plants. Raw Chaya leaves have less HCN compared to cassava (Teels, 2002). Boiling of chayamansa leaves will increase the phenolic compounds and its antioxidant power than raw leaves (Godínez-Santillán *et al*., 2019).

According to Vetter (2000) and JEFCA (1993), the amount of cyanogenic glycosides in plants is influenced by endogenous development, exogenous ecology, genetics, the environment, location, season, and soil types.

The root of the Chayamansa plant is the component that contains the most cyanide, while the leaves contain the least, as per a quantitative comparison of processed and unprocessed plant parts. Therefore, consuming leaves is safer than consuming other parts,

and cooking or boiling is necessary to prevent health problems. When given orally, the LD50 (lethal dose 50, the amount of chemical or poison which is lethal to 50% of the experimental animals exposed to it under controlled condition) for dogs and rats has been reported to be between 2 and 6 mg HCN/kg (WHO, 1993). We can safely consume 0.02 mg of HCN equivalents per kg of body weight every day. According to the FAO/WHO Expert Committee on Food Additives 2012, an adult weighing 70 kg can consume 1.4 mg/day of HCN equivalents, while a child weighing 12 kg can consume 0.24 mg/day.

Our research indicates that the best safety measure to follow when consuming Chayamansa is to boil it for at least 25 minutes. Cyanide can lead to respiratory difficulty by suppressing the mitochondrial enzyme cytochrome oxidase (Onwuka, 2005).

Various processing techniques can be applied to Chayamansa, such as chopping, drying, LPC (Leaf protein concentrate), and wilting, to observe the variance in cyanogenic glycoside (CG) content. Since latex can cause irritation upon contact, there have been no further studies on the HCN concentration in latex from *Cnidoscolus* species. Therefore, caution should be exercised when coming into contact with latex from *Cnidoscolus* species. After removing the intact leaves, ground leaves can retain their toxicity for up to 3 days with fresh latex, and intact leaves can retain their toxicity for up to 30 days (Moura *et al*., 2019). According to Agneswari *et al*. (2020) *Cnidoscolus* latex exhibit anti-arthritic activity and petroleum ether extract of *Cnidoscolus* latex shows better antimicrobial activity (Jayasree and Gopakumar, 2018). But there is limited published research on Chayamansa latex, so a comprehensive study on the HCN and bioactive content of latex is wanted, as latex is present in all parts of Chayamansa.

CONCLUSION

According to our study, processing methods, except boiling, did not reduce the cyanogenic content in all parts of Chayamansa. Boiling for 25 minutes is safe for consumption. It is advisable to discard the filtrate that remains after boiling chayamansa plant parts due to the presence of HCN in it. Leaves, as well as tender petioles, can be safely consumed, while proper evaluation is required when consuming the root, which is the most cyanogenic part in both processed and unprocessed states.

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FULL LENGTH ARTICLE

Phytochemical profiling and bioactivity of methanolic stem extract of *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh: A comprehensive study

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This study explores the phytochemical profiling and bioactivity of the methanolic stem extract of *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh an indigenous plant from the Southern Western Ghats known as 'Kametti valli' in Malayalam. The research aims to validate the therapeutic properties and address the rising demand for natural remedies with minimal side effects. The physicochemical characteristics of the plant align with established quality standards, ensuring its suitability for herbal drug development and applications. The preliminary phytochemical analysis identified the presence of alkaloids, phenolics, flavonoids, terpenoids, and glycosides in the plant extract. quantitative analysis revealed substantial alkaloids, phenolics, and notably high flavonoid content. GC-MS analysis identified 12 bioactive compounds, including therapeutic agents like lupeol and β-Amyrin. Antioxidant activity was assessed through the DPPH scavenging method. The extract displayed notable antioxidant activity, making it a potential agent against oxidative stress. Antimicrobial activity was determined using the Agar Well Diffusion method against five pathogenic microorganisms. The extract exhibited varying degrees of antimicrobial activity against selected pathogens, with *Candida albicans* and *Pseudomonas aeruginosa* being the most susceptible. At the same time, *Escherichia coli, Staphylococcus aureus*, and *Klebsiella pneumonia* show varying degrees of resistance. In conclusion, this study underscores the value of *Kamettia caryophyllata* methanolic stem extract in traditional medicine and its potential for further research and development in herbal remedies and pharmaceutical products.

Keywords : *Kamettia caryophyllata*, Physicochemical analysis, phytochemical screening, GC-MS analysis, phytochemical groups, antioxidant property, antimicrobial property.

INTRODUCTION

People have been using medicinal plants to treat various diseases since ancient times. Screening plant extracts has garnered keen interest among scientists in the quest to discover new drugs for the efficient treatment of various diseases. This is due to the presence of secondary metabolites, a broad class of phytochemicals present in plants (Dimayuga and Garcia, 1991). The demand for plants with therapeutic value has been steadily increasing, owing to their comparatively less or negligible negative effects on the host. The world's one-fourth population is said to be dependent on traditional medicine to treat various ailments (Payum, 2017).

While natural plant-based medicines have many advantages over synthetic alternatives, they also encounter challenges including adulteration,

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substitution, and misidentification during manufacturing (Chanda, 2014). Local people frequently gather medicinal plants from the outdoors, occasionally from polluted places. The absence of proper documentation and standardization can impact the efficacy of herbal medicine. In response, the World Health Organization has published three volumes of WHO monographs on selected medicinal plants since 1999. Consequently, research in herbal medicine has become a prominent area of global scientific exploration.

This study is an endeavour to assess the phytochemical and bioactivity potential of *Kamettia caryophyllata* (Roxb.) Nicolson & Suresh, a member of the Apocynaceae family known locally as 'Kametti valli' in Malayalam. Indigenous to the Southern Western Ghats, it thrives in evergreen, semi-evergreen forests, and sacred groves. *Kamettia caryophyllata* is a large climber with a historical record of traditional use in India for treating various conditions, including spasm, epilepsy, anodyne poison, arthritic pains, itches, scabies, cachexia, lichen, and leprosy, as documented in Hortus Malabaricus (Manilal and Remesh, 2010). This unexplored plant offers an intriguing opportunity for standardization and validation of its pharmacological potential through scientific analysis.

The study focuses on physicochemical analysis, and preliminary and quantitative analysis of phytochemicals in *Kamettia caryophyllata* stem extract using methanol as solvent. It also aims to identify bioactive compounds using GC-MS and assess the antioxidant and antimicrobial properties of the methanolic extracts.

MATERIALS AND METHODS

Collection and authentication of plant material

The plant material of *Kamettia caryophyllata* was collected in a fresh condition from Sankulangara sacred grove, in S.N. Puram, Kerala, precisely located at 10.520 N and 76.210 E, with an average altitude of 2.83m. Taxonomic verification was conducted by Dr. Sunil Kumar, an experienced botanist and Associate Professor in the Department of Botany at SNM College, Maliankara, Ernakulum District. A voucher specimen of the plant was deposited in the SNMH! International Herbarium Collection, Department of Botany, SNM College, Maliankara, accredited by the New York Botanical Garden and is assigned accession numbers 2002 and 2003 for future reference.

Extraction of plant sample

Fresh and healthy stems of *K. caryophyllata* are cleaned thoroughly with running tap water, air-dried in the shade, and then ground into a powder. This powdered plant material, weighing 1000 grams, is placed in a Soxhlet apparatus for extraction using methanol as the solvent. The resulting extract is filtered, and the filtrate is evaporated under reduced pressure, maintaining a temperature below 40°C until a consistent weight is attained. The final product, a crude dried extract, is stored in desiccators for further analysis.

Physicochemical analysis

Parameters used for this analysis were loss on drying, total ash content, acid-insoluble ash content, water-soluble ash content, pH value in 10% solution, aqueous, and alcoholic extractive values were carried out according to the methods recommended by API (2016) and WHO recommendations regarding quality control methods for medicinal plant materials.

Loss on drying

Ten grams of *K. caryophyllata* stem powder, without prior drying, were placed in a pre-weighed dish. Drying at 105°C for 5 hours, weight measurements were taken hourly until the change between successive readings was less than 0.25 percent. The constant weight point was determined after consistent measurements following 30 minutes of drying and cooling in a desiccator, allowing the calculation of moisture percentage in the air-dried sample.

Total Ash Content

To determine the ash content of *K. caryophyllata* stem powder, one gram was placed in a pre-weighed silica crucible. After initial drying at 100-105°C for an hour,

the sample was ignited in a muffle furnace at 600°C for five hours. If carbon remained, 15 ml of 95% ethanol was added, and the mixture was incinerated at 675°C. After cooling, the ash was weighed, and the percentage of ash content was calculated based on the air-dried crude drug.

Acid-Insoluble Ash

To determine acid-insoluble ash, the total ash from *K. caryophyllata* stem was boiled with 25 ml of 2 N HCl for 5 minutes, and the insoluble matter was collected on ashless filter paper, washed, and ignited at 600°C for 3 hours. The resulting residue was weighed to calculate the percentage of acid-insoluble ash based on dried plant powder.

Water-soluble ash content

The total ash was boiled with distilled water, and the insoluble ash was collected, washed, and ignited at 450°C for 15 minutes to achieve constant weight. The percentage of water-insoluble ash was calculated based on dried plant material.

pH Value at 10% Dilution

10 grams of dried stem powder was dissolved in 100 ml of water, filtered, and the pH of the filtrate was measured using a standardized glass electrode.

Determination of Alcohol and Water-soluble Extractive

To determine alcohol and water-soluble extractives, five grams of air-dried *K. caryophyllata* stem powder was macerated with 100 ml of solvents (alcohol and chloroform-water) for 24 hours. After filtration, 25 ml of the filtrate was evaporated to dryness, dried at 105°C until reaching a constant weight, and the weight of the dried extract was recorded. The percentage of soluble extractives was calculated based on the initial weight of the air-dried plant material.

Preliminary phytochemical analysis

Different biochemical tests were performed to establish a preliminary and qualitative profile of various bioactive phytochemical groups present in the methanolic stem extracts of *Kamettia caryophyllata*. Qualitative chemical tests were conducted using the standard procedures described in Experimental Phytopharmacognosy (Khadabadi *et al*., 2013).

Detection of alkaloids

The plant extract was dissolved in 2 N HCl and filtered.

Mayer's test: Another portion was mixed with 2ml of Mayer's reagent. The appearance of a creamish precipitate showed the presence of alkaloids.

Dragendroff's test: A portion was combined with 2ml of Dragendroff's reagent. The formation of a reddish-brown precipitate indicated the presence of alkaloids.

Detection of phenolic compounds

Ferric Chloride Test: Introduced a few drops of neutral 5% ferric chloride solution to the plant extract, blue-black to blue-green colour showed the presence of phenolic compounds.

Lead Acetate Test: 3 ml of 10% lead acetate solution was added to the plant extract, and the formation of a bulky white precipitate confirmed the presence of phenolic compounds.

Detection of flavonoids

Shinoda test: Magnesium powder and a few drops of concentrated H_2SO_4 were added to 2 ml of the test solution. The appearance of colours ranging from orange, red, purple, and pink to magenta indicated the presence of flavonoids.

Alkaline reagent test: A few drops of sodium hydroxide solution were added to the test solution. The appearance of a yellow colouration, which turned colourless upon the addition of a few drops of dilute acid, indicated the presence of flavonoids.

Detection of Glycosides

Legal's test: 2 ml of pyridine and sodium nitroprusside were mixed with 1 ml of the plant extract. The appearance of a pink or red colour indicated the presence of glycosides.

Detection of Terpenoids

Salkowski test: 1-2 mg of the test sample was dissolved in 1 ml of $CHCl_3$ and 1 ml of concentrated H_2SO_4 was added. The chloroform layer showed a red colour, and the acid layer showed green fluorescence, indicating the presence of terpenoids.

Detection of Saponins

Foam test: The aqueous solution of the test sample was shaken vigorously. The stable foam produced for 15 minutes or more indicated the presence of saponins.

Detection of carbohydrates

Benedict's test: 2 ml of Benedict's reagent was mixed with 2 ml of the extract solution and boiled in a water bath. The formation of a red, yellow, or green colour or precipitate indicated the presence of carbohydrates.

Molisch's test: 1 ml of Molisch's reagent was mixed with 2 ml of the extract solution, and 1 ml of concentrated H_2SO_4 was added. The appearance of a red-to-violet ring at the junction of the two liquids indicated the presence of carbohydrates.

Detection of Proteins

Biuret Test: 2 ml of the extract solution was mixed with 2 ml of Biuret reagent, and the appearance of a violet-to-pink colour indicated the presence of proteins.

Millon's Test: 2 ml of the extract solution was mixed with 2 ml of Millon's reagent and then boiled. The appearance of a red colour indicated the presence of proteins.

Quantitative determination of secondary metabolites

Total estimation of Alkaloid

Total alkaloid content was determined using the acid dye colorimetric method following Trease and Evans (2019). A 1 mg plant extract was dissolved in methanol and mixed with 1 ml of 2 N HCl. After filtration, the solution was transferred to a separating funnel. Bromocresol green solution, phosphate buffer, and chloroform were added and mixed. A range of atropine standard (2, 4, 6, 8,10 and 12 µg/ml) solutions was prepared. Using a UV-Visible Spectrophotometer, the absorbance of test and standard solutions was measured at 470 nm. The total alkaloid content was expressed as atropine equivalent per gram of extract using a standard curve. The percentage yield was calculated and recorded.

Total estimation of Phenolics

The quantification of total phenolics in the methanolic stem extract of *K. caryophyllata* was done using the Folin-Ciocalteu reagent (Singleton and Rossi,1965) with gallic acid as the standard reference at various concentrations (2, 4, 6, 8,10 and 12 µg/ml). A 1 mg/ml methanolic stem extract was prepared, and 0.05 ml of each sample was mixed with a 10-fold diluted Folin-Ciocalteu reagent and sodium carbonate. After a 30-minute incubation at room temperature, absorbance was measured at 760 nm. A calibration curve relating gallic acid concentration to absorbance was established using linear regression. The total phenol content in the extract was then calculated in milligrams of gallic acid equivalent per gram. The percentage yield was also recorded.

Total estimation of Flavonoids

Flavonoid content was determined using the aluminum chloride colorimetric method (Marinova *et al*.,2005) with quercetin as the standard. Ten milligrams of quercetin were dissolved in 1 ml of methanol and diluted to create standard solutions ranging from 2 to 12 µg/ml. For both the standards and the plant extracts, 20 µl of the sample was mixed with methanol, 10% aluminum chloride, 1M sodium

acetate, and distilled water. After 30 minutes at room temperature, the absorbance at 415 nm was measured using a spectrophotometer. Methanol served as a blank reference for the analysis. This method provided an assessment of flavonoid content in the plant extract.

GC-MS screening for volatile bioactive compounds

The GC-MS screening of methanolic extracts of *K. caryophyllata* stems were performed using an Agilent Technologies (Model–5975C) Gas chromatographic system connected to a mass spectrometer (GC-MS) instrument (MS 7890A) using the following conditions like column: DB5-MS fused silica capillary column (30 X 0.25 mm ID X 0.25 mm film thickness), composed of 5% Phenyl, 95% Dimethyl Polysiloxane, conducting in electron impact mode at 70 eV, helium (99.999%) was employed as the carrier gas at a constant flow of 1 mL/min, ion-source temperature was set to 150°C and injector temperature was 250°C. The oven was set to begin at a temperature of 40°C for 5 minutes, then rise 5°C/min, to 280°C hold for 10 Minutes. Mass spectra were taken at 70 eV, a scan interval of 0.2 s and fragments were scanned from 50 to 550 Da. The total GC took 57 minutes to complete. The constituents were recognised and reported after being compared to those found in the Computer Library (NIST ver. 2.1) attached to the GC-MS instrument and reported.

Antioxidant Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

The DPPH free radical scavenging capacity of the extract was assessed using the methodology outlined by Cheng *et al*., with slight modifications. DPPH in its free radical form absorbs at 517 nm, but when reduced by an antioxidant, its absorption decreases. Briefly, 0.5 ml of a 0.1 mM solution of DPPH in methanol was added to 1.5 ml of stem extract of *K. caryophyllata* in methanol at different concentrations (2-12 µg/ml) and incubated. Absorbance was measured at 517 nm after 30 minutes. Lower absorbance indicated greater DPPH free radical scavenging activity. The assay was calculated as indicated.

The assay was calculated as : Scavenging activity = [(Abs. of control - Abs. of sample)/(Abs. of control] × 100

where control represents the absorbance of the control sample and test represents the absorbance of the sample being tested. The result is expressed as a percentage and The IC50 value was also recorded.

Antimicrobial assay

Collection of test organisms and preparation of stock culture

Cultures of 5 pathogenic microorganisms were used for in vitro analysis. All the microorganisms namely *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were collected from the Microbiology section, Jubilee Mission Medical College & Research Institute, Thrissur, Kerala, India.

Determination of antimicrobial activity

Antimicrobial activity tests were conducted following the Agar Well Diffusion Method (Magaldi *et al*., 2004). DMSO (dimethyl sulfoxide) was used to dissolve Stem extract of *K. caryophyllata* and filtered through a 0.2 µm Nylon filter. For the assay 200 µl culture suspension of the tested microorganisms (carrying 106 colony-forming units /ml of bacteria cells and 108 spores/ml of fungal strains) were evenly dispensed on Muller-Hinton Agar medium and PDA medium, respectively. Wells were then created using a sterile borer (3 mm depth and 4 mm diameter), and each well was loaded with 100 µl of the sample extract. DMSO, without any extract, served as the negative control. Additionally, 100 µl of various concentrations of plant solvent extracts (25 µg, 50 µg, 100 µg) were added using a sterile syringe into the agar wells, allowing them to diffuse at room temperature for 24 hours at 37°C for bacteria and 72 hours at 30°C for fungal strains.

In the assessment of antimicrobial activity, the diameter of the growth inhibition zones was measured in millimetres, including the 4 mm diameter of the well. triplicates were maintained, and readings were taken in three different fixed directions, with the

average values recorded. Chloramphenicol was used as the positive standard for bacteria, and Clotrimazole served as the positive standard for fungi.

Statistical analysis

All the determinations were replicated in three independent assays, and the results were reported as a mean±standard deviation. Data analysis was performed using GraphPad Prism software 8.0.2.

RESULTS

Physicochemical analysis

The physicochemical analysis of the plant powder is detailed in Table 1. These parameters for *K. caryophyllata* are essential for quality control and assessing drug purity.

Table 1. Physicochemical parameters of *Kamettia caryophyllata* stem

Test parameters	Stem (in %)
Loss on drying 105ºC	7.62±0.50
Total ash	3.03±0.40
Acid insoluble ash	0.02±0.004
Water soluble ash	1.89±0.22
pH value of 10% solution	6.35
Alcohol Soluble extractive	7.98±0.78
Water soluble extractive	11.76±0.43

Values are represented as mean±Standard deviation –3

Preliminary phytochemical analysis

To identify the existence of various bioactive chemicals, we performed a preliminary phytochemical investigation on the methanolic stem extract of *Kamettia caryophyllata* for this study. Several significant phytochemical elements that have been uncovered from this investigation are listed in Table 2. These extracts demonstrated the presence of active compounds like alkaloids, phenolics, flavonoids, terpenes, and glycosides which are essential medicinal compounds are present.

Total quantitative determination of secondary metabolites

Total quantitative analysis focused on three crucial classes of secondary metabolites: alkaloids, phenolics and flavonoids (Fig. 1). The total alkaloid content in the methanolic stem extract of *K. caryophyllata* was found to be 13.77 ± 1.81%. The percentage yield of phenolic content was determined to be 3.03 ± 0.11%. Additionally, flavonoid content in the extract was estimated to be 19.05 ± 3.160%.

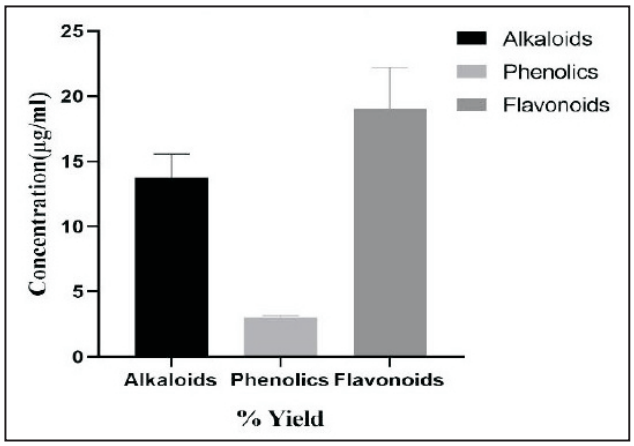

Fig. 1. Total Quantitative determination of secondary metabolites in methanolic stem extract of *Kamettia caryophyllata* (% Yield).

GC-MS screening for volatile bioactive compounds

The identification of bioactive compounds in the methanolic stem extract of *Kamettia caryophyllata* was carried out using Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Figure 2 presents the chromatogram illustrating the identified peaks.The GC-MS analysis performed in the methanolic stem extract of *K. caryophyllata* in the present study showed the presence of 12 bioactive compounds that might contribute to the medicinal property of the plant. The name of bioactive compounds with their retention time (RT), percentage of peak area, molecular formula and molecular weight are depicted in Table 3. The major compounds that were identified in the stem component of *K. caryophyllata* based on peak area percentage include 3-O-methyl-d-glucose (33.505%);

Table 2. Preliminary phytochemical screening of methanolic stem extact of *Kamettia caryophyllata*

Sl. No.	Compounds	Text	Methanol extract		
			R1	R2	R3
1.	Alkaloid	Mayers test Dragendroffs test	+ +	+ +	+ +
2.	Phenolics	Ferric chloride test Lead acetate test	+ +	+ +	+ +
3.	Flavanoid	Alkaline reagent Test Shinoda Test	+ +	+ +	+ +
4.	Terpenes	Salkowski test	+	+	+
5.	Glycosides	Legal test	+	+	+
6.	Saponins	Foam test	–	–	–
7.	Carbohydrates	Benedict's test Molisch's Test	+ +	+ +	+ +
8.	Proteins	Biuret Test Millon's Test	– –	– –	– –

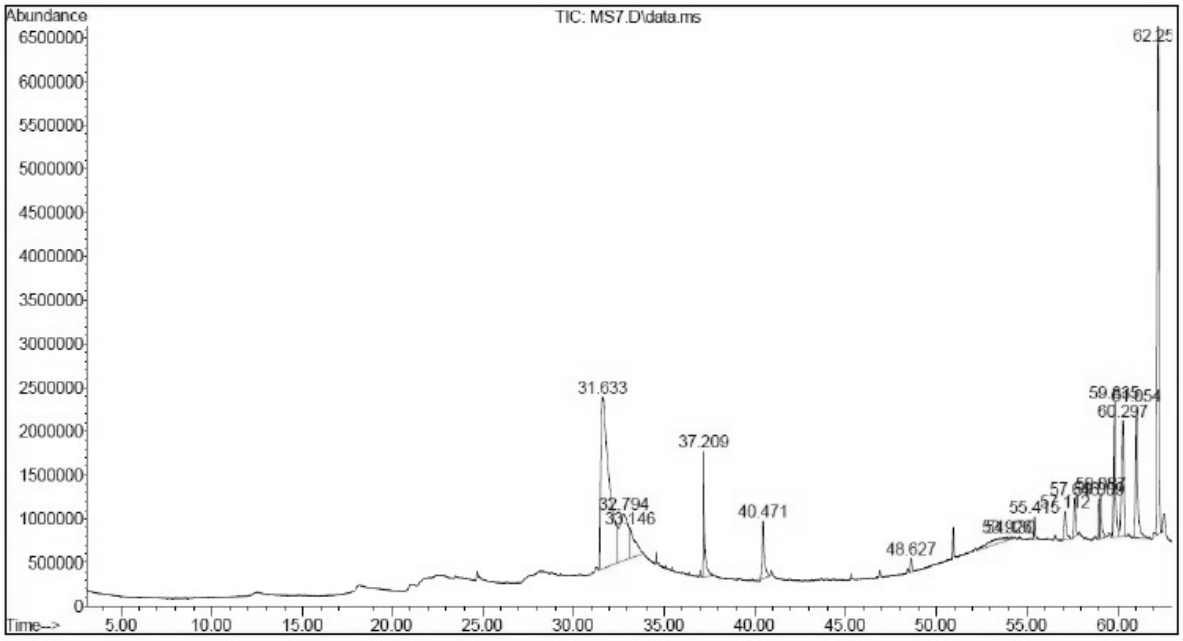

Fig. 2. GC-MS chromatogram of methanolic stem extract of *Kamettia caryophyllata.*

Table 3. GC-MS analysis of methanolic stem extract of *Kamettia caryophyllata* showing the details of specific bioactive constituents

Peak	Retention Time (min)	Compound name	Molecular formula	Molecular weight (g/mol)	Peak area %
1	31.63	3 O Methyl d glucose	$C_7H_{14}O_6$	194.18	33.505
2	37.21	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	3.766
3	40.47	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282.5	2.212
4	48.62	Squalane	$C_{30}H_{50}$	410.73	0.385
5	55.42	dl-α-Tocopherol	$C_{29}H_{50}O_2$	430.717	0.628
6	57.11	Campesterol	$C_{28}H_{48}O$	400.691	1.363
7	57.64	Stigmasterol	$C_{29}H_{48}O$	412.702	1.333
8	59.01	γ-Sitosterol	$C_{29}H_{50}O$	414.707	1.164
9	59.84	β-Amyrin	$C_{30}H_{50}O$	426.72	5.638
10	60.29	Lup-20(29)-en-3-one	$C_{30}H_{48}O$	424.7015	5.956
11	61.05	Lupeol	$C_{30}H_{50}O$	426.729	6.704
12	62.25	12-Oleanen-3-yl acetate, (3α)-	$C_{32}H_{52}O_2$	468.766	20.732

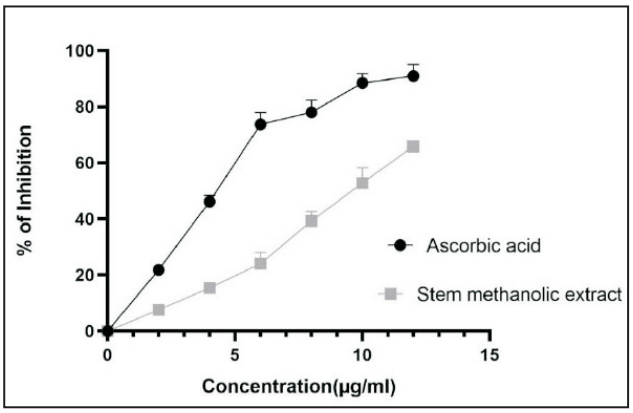

Fig. 3. DPPH free radical scavenging assay of the methanolic stem extacts of *Kamettia caryophylata*

12-Oleanen-3-yl acetate, (3α)-(20.732%); lupeol (6.704%); Lup-20(29)-en-3-one (5.956%); β-Amyrin (5.638%). Besides there are some other compounds that are also shown in this analysis. 3-O-Methyl-d-glucose,n Hexadecanoic acid, Trans-13-Octadecenoic acid, Squalane, dl-α-Tocopherol, Campesterol, Stigmasterol, γ-Sitosterol are the other compounds analyzed. Another important observation of the study is that the majority of the specific compounds identified in the GC-MS analysis are fatty acids and terpenoids.

Antioxidant Activity

The widely accepted DPPH radical has been employed to assess the antioxidant activities of plant extracts. As shown in Fig. 3, the scavenging effect on the DPPH radical was notably higher for ascorbic acid when compared to the stem extract. The half maximal inhibitory concentration (IC50) value for the methanolic extract was found to be 9.84 ± 0.54 µg/ml, whereas the standard (ascorbic acid) displayed an IC50 value of 5.42 ± 0.24 µg/ml. These results highlight the varying antioxidant capacities of the methanolic stem extract of *Kamettia caryophyllata* in contrast to the well-established standard, ascorbic acid, and emphasize the utility of the DPPH radical assay in evaluating these differences.

Antimicrobial activity

The results of antimicrobial activity tested against the selected pathogenic microorganisms using methanolic stem extract of *K. caryophyllata* are depicted in the Table 4. The treatment using 25µg dilution of the stem extract showed no activity against the pathogenic organisms such as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans*. Further increasing the concentration of stem extract to 50µg dilution, a moderate level of inhibition zone is found to be recorded against *Candida albicans* (13.33±1.53mm) and *Pseudomonas aeruginosa* (14.00±1.00mm). While low or poor activity recorded as zone of inhibition (ZOI) against *Escherichia coli* (1.33±2.31mm), and no activity against *Staphylococcus aureus, Klebsiella pneumoniae*. The maximum antimicrobial activity and the ZOI are found recorded against all the selected pathogenic organisms with the highest concentration of 100µg dilution selected for the study. The result shows that the negative control used in the investigation did not exhibit antimicrobial activity against any of the test pathogenic bacteria or the fungi while, the inhibition zone formed by the positive control either chloramphenicol against pathogenic bacteria or clotrimazole against the fungal pathogen even in 25µg dilution.

DISCUSSION

The physicochemical characteristics and extractive values obtained were according to WHO and the Indian pharmacopoeia recommendations for ensuring the purity and quality of herbal drugs. The moisture content in the stem powder of *K. caryophyllata* is indicative of the water content in the sample and is crucial for storing and calculating the shelf life of herbal items (Swathi Priya and Rajasekaran, 2022). Total ash content provides insights into the inorganic substances within the plant, including minerals and other inorganic matter (Barus *et al*., 2018). The water-soluble ash content is a subset of the total ash and represents the portion that dissolves in water. The low acid-insoluble ash content in the stem suggests minimal impurities or non-dissolvable matter like silica (Jeevitha *et al*., 2021). The pH values of the plant parts, particularly the slightly acidic nature of the stem at pH 6.35, can be relevant in understanding the

Table 4. Antimicrobial activity of methanolic stem extract of *Kamettia caryophyllata*

ORGANISM	ZONE OF INHIBITION (mm)				
	25 µg	**50 µg**	**100 µg**	**Negative control**	**Chloramphenicol/ Clotrimazole (25 µg)**
Candida albicans	0.00 ± 0.00	13.33 ± 1.53	15.00 ± 1.00	0.00 ± 0.00	17.67 ± 1.16
Escherichia coli	0.00 ± 0.00	1.33 ± 2.31	11.33 ± 1.15	0.00 ± 0.00	29.67 ± 2.52
Staphylococcus aureus	0.00 ± 0.00	0.00 ± 0.00	9.00 ± 1.00	0.00 ± 0.00	14.67 ± 1.53
Klebsiella pneumoniae	0.00 ± 0.00	0.00 ± 0.00	17.67 ± 1.53	0.00 ± 0.00	27.33 ± 1.53
Pseudomonas aeruginosa	0.00 ± 0.00	14.00 ± 1.00	16.33 ± 1.53	0.00 ± 0.00	28.67 ± 1.53

Values are represented as mean ± Standard deviation n=3

chemical environment and stability of constituents within these parts (Sulana *et al*., 2022). This information is valuable when considering extraction and formulation processes (Nguyen *et al*., 2017).

The presence of water-soluble extracts in the stem, observed the highest values than alcohol-soluble extracts signify the solubility of bioactive compounds.

Phytochemical screening of *K. caryophyllata* methanolic stem extract is a valuable method for screening the medicinal properties of these plants and facilitating the development of medical formulations. For instance, alkaloids have been found to exhibit various essential biological properties, including muscle relaxant, analgesic, and antioxidant effects (Heinrich *et al*., 2021). Phenolic compounds are known for their ability to prevent oxidative damage in living systems (Kumar and Goel, 2019). Flavonoids have shown effective antimicrobial activity against pathogenic microorganisms, along with anti-inflammatory, antiallergic, and anti-tumour activities. They also provide protection against damage from free radicals (Krishnamoorthy and Subramaniam, 2014). A class of substances known as terpenoids is important pharmacologically and is valued for its medical benefits, which include anti-carcinogenic, anti-malarial, anti-microbial, anti-inflammatory, and diuretic effects (Masyita *et al*., 2022). Glycosides, another important phytoconstituent, contain both carbohydrate and non-carbohydrate residues in the same molecule and have been associated with various biological effects such as antioxidant, anti-diabetic, anti-viral, anticancer, anti-allergic, and anti-inflammatory activities (Jaradat *et al*., 2015).

The quantitative assessment of bioactive compounds like alkaloids, phenolics and flavonoids provide valuable insights into the potential medicinal properties of this methanolic stem extract. The total alkaloid content in the stem methanolic extract of *K. caryophyllata* was determined to be 13.77 ± 1.81%. This finding indicates a substantial presence of alkaloids in the plant which suggests that this plant extract may have the potential for various medicinal applications. Moreover, we found that the phenolic content in the extract was 3.03±0.11%. Phenolic compounds are recognized for their antioxidant properties, which are essential for protecting living systems from oxidative damage (Sampaio *et al.*, 2016). While the phenolic content is relatively lower compared to alkaloids and flavonoids in this extract, it still contributes to the overall medicinal potential of the plant. Remarkably, the flavonoid content in the extract was estimated to be 19.05±3.16%. Flavonoids are known for their antimicrobial, anti-inflammatory, antiallergic, and anti-tumour activities. They are also important for protecting cells from the harmful effects of free radicals. The relatively high flavonoid content in the extract suggests that it may have significant therapeutic applications, particularly in combating microbial infections and inflammation.

The results of the Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the methanolic stem extract of *Kamettia caryophyllata* provide valuable insights into the bioactive compounds present in the plant and their potential contributions to its medicinal properties. The presence of 12 bioactive compounds in the extract underscores the complexity of its chemical composition and suggests a diverse range of potential pharmacological activities.

Among the identified compounds, several significant compounds stand out due to their elevated peak area percentages. These include 3-O-Methyl-d-glucose, 12-Oleanen-3-yl acetate, lupeol, Lup-20(29)-en-3-one, and β-Amyrin. Each of these elements has been associated with a variety of bioactivities. For instance, the terpenoids lupeol and β-Amyrin are both known for their antioxidant and anti-inflammatory effects (Ramos-Hernández *et al*., 2018). 3-O-Methyl-d-glucose is a sugar derivative, and its presence may contribute to the plant's sweetness and potential health benefits (Holman, 2020). An interesting observation is that the majority of the identified compounds fall into the categories of fatty acids and terpenoids. Fatty acids, such as n-Hexadecanoic acid and Trans-13-Octadecenoic acid, are often associated with metabolic and energy-related processes in living organisms (Oteng and Kersten, 2020). Terpenoids, like lupeol and Lup-20(29)-en-3-one, have well-documented pharmacological activities, including anti-inflammatory and antimicrobial effects (Nistor *et al.,* 2022). The identification of these bioactive compounds is a promising sign that *Kamettia*

caryophyllata may have a wide range of potential medicinal applications.

In the evaluation of antioxidant activity,the study utilized the widely accepted DPPH radical assay to assess the antioxidant activities of the plant extract and ascorbic acid. The results show that ascorbic acid had a notably higher scavenging effect on the DPPH radical compared to the stem extract. The findings illustrate that the methanolic stem extract of *Kamettia caryophyllata* possesses antioxidant properties, but slightly less potent when compared to ascorbic acid. These findings underscore the potential health benefits of the plant extract in terms of its antioxidant capacity.

The antimicrobial efficacy of the methanolic stem extract of *K. caryophyllata* against a number of pathogenic pathogens was also examined. At a dosage of 25µg, the stem extract exhibited no activity against pathogenic microorganisms such as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans*. On the other hand, when the concentration of the stem extract was increased to 50µg, a moderate level of inhibition was seen against *Candida albicans* and *Pseudomonas aeruginosa*. It showed low or poor activity against *Escherichia coli* and no activity against *Staphylococcus aureus* and *Klebsiella pneumoniae*. The highest concentration of 100µg of the stem extract exhibited the maximum antimicrobial activity, producing inhibition zones against all selected pathogenic organisms. Notably, the negative control used in the study did not show antimicrobial activity against any of the test pathogenic bacteria or fungi. Conversely, the positive control, either chloramphenicol against pathogenic bacteria or clotrimazole against the fungal pathogen, exhibited inhibition zones even at a concentration of 25µg.

These results suggest that the methanolic stem extract of *Kamettia caryophyllata* possesses antimicrobial activity, with the highest concentration demonstrating the most significant effect against the tested pathogens. However, the effectiveness of the extract varies among different microorganisms, with *Candida albicans* and *Pseudomonas aeruginosa* being the most susceptible, while *Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumoniae* show varying degrees of resistance. These findings highlight the potential of the plant extract as an antimicrobial agent, and further studies can elucidate the mechanisms and specific applications of this activity in medical and pharmaceutical context.

CONCLUSION

In summary, the detailed examination of the methanolic stem extract of *Kamettia caryophyllata* has provided significant insights into its potent therapeutic properties. The plant's physicochemical characteristics align with quality criteria ensuring its purity and suitability for herbal drug applications. Phytochemical screening has uncovered a rich array of bioactive compounds, including alkaloids, phenolics, flavonoids, terpenoids, and glycosides, renowned for their diverse pharmacological properties. Our total quantitative investigation of alkaloids, phenolics and flavonoids present in the methanolic stem extract of *K. caryophyllata* has revealed substantial levels of alkaloids, phenolics, and significantly high levels of flavonoids. GC-MS analysis identified 12 bioactive compounds, including therapeutic agents like lupeol and β-Amyrin highlighting the plant's therapeutic potential. While the extract exhibited remarkable antioxidant activity, ascorbic acid demonstrated slightly higher efficacy Moreover, the plant extract displayed varying degrees of antimicrobial activity against selected pathogens, showcasing its potential as an antimicrobial agent. These findings highlight the potential medicinal values of *Kamettia caryophyllata* methanolic stem extract and call for further investigation into its specific biological activities and potential applications in the field of natural medicine and drug development. Further studies can explore deeper into the specific applications and mechanisms of these bioactive compounds for medical and therapeutic purposes.

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FULL LENGTH ARTICLE

Ethnomedicinally important plants used in orthopedic treatment by Hill Tribes of North Bengal, India

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The North Bengal Hilly regions of India are rich in multicultural diversity and are home to various ethno-medicinal traditions. The purpose of this study is to document and analyze traditional knowledge on the practice and utilization of plants in treating bone fracture. It is an attempt to conserve the disappearing wealth of knowledge. The key informants were identified in pre-survey and collected information through open-ended interviews. Information on age, location, experience of key informant, and practice, and disease treatment, method of diagnosis, storage, and use of plants was collected. The identification of the plants and their information were verified through repeated guided transects in different seasons with key informants and focussed group discussions. The identified plants were deposited at the herbaria of Lloyd Botanical Garden Darjeeling. In this study, forty-four plant species belonging to thirty-two families were identified. Forty-six key informants providing treatment of bone fracture in the region were identified. Highest number of species representation is found under families Fabaceae and Zingiberaceae The habit of the species showed that 48% of the herbal drugs were obtained from herbs, followed by trees, shrubs and climbers and majority of plants used were collected from wild (74%). The stem or stem bark (33%) was most commonly used plant part to prepare medicine. 16 formulations of 32 plant species were directly used in treating bone fracture, where Urtica dioica L. has mostly used. Twelve plant species belonging to 11 families were found to be used for bone strengthening, pain relieving, inflammation reduction and speedy recovery. The findings demonstrated the significance of the traditional herbal practices in the community for their health requirements. Both conservation practices and additional validation studies are necessary in order to better utilize and sustain the documented knowledge.

Keywords : Bone fracture, Medicinal plants, Traditional knowledge, Hill tribes, North Bengal.

INTRODUCTION

India is renowned for having a long history of natural product expertise, especially with regard to herbal medicine. Since the beginning of time, Indians have used medicinal plants (Singh and Lahiri, 2010). Tribes, who reside primarily in isolated forest locations, continue to rely heavily on the local medical system. Throughout the entire evolution of human civilization and the environment, indigenous healing methods have been embraced. Approximately 85% of conventional medications come from plants (Farnsworth, 1988; Prasad and Bhattacharya, 2003; Phondani *et al*., 2014). Traditional medicines, especially herbal preparations, are increasingly being incorporated into local healthcare systems in underdeveloped nations, and numerous contemporary

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researchers are working to uncover the immense potential of ethno botanical knowledge for treating a wide range of disorders (Cox and Balick, 1994; Jain *et al.*, 2010; Jeyaprakash *et al*.; 2011).

The tribal communities, who have historically been the main occupants of natural environments, possess a vast quantity of traditional knowledge on the usage of different biotic resources (Acharya and Acharya 2001), which may be more significant to ongoing study and discoveries in the field. It is widely acknowledged in the literature (Anonymous, 1976) that their long-standing custom of employing plants to treat a variety of maladies paved the way for the subsequent discovery of numerous life-saving medications. Despite the rapid growth in medicinal plant utility, there is still very little knowledge about its usage patterns. This knowledge needs to be documented, analyzed and evaluated not only for cultural reasons but also for commercial reasons. Ethno medicinal use of plants is among the most effective criteria used in the pharmaceutical industry to identify new therapeutic agents (Cox *et al*.,1994; Abat *et al*., 2017; Bandyopadhyay and Dey, 2022). The current state of medicine in India is a curious amalgamation of traditional and conventional, and has often been utilised for a variety of historical, cultural, ecological and socio-economic purposes (Khan, 2006; Kunwar *et al*., 2010). The present work was to survey and to document the ethno medicinal practices against bone fracture followed by the hill tribes of North Bengal thereby protecting their ethnic knowledge from being extinct.

MATERIALS AND METHODS

Study area

The District of Darjeeling, the queen of hills covers an area of about 2436.55 km², lies between 26º27'05″ to 27º13'10″ N latitudes and between 87º59'30″ to 88º53'22″ E longitudes (O'Malley, 1999). The district is bordered by Sikkim in the north, Terai and Dooars in the south, Bhutan in the east and Nepal in the west. The altitudinal variation ranges from 150 m (at Sukna) to 3636m (at Sandakphu) presenting diverse topographical conditions (Das, 1995, 2004; Acharya and Acharya, 2001). The region is significantly rich in floral and faunal diversity. Total population of the area is 1,846,823 (Census, 2011), which comprises of several communities, tribes and castes such as Lepcha, Rai, Tamang, Bhutia, Limboo, Sherpa, Gurung, Thami, Dewan, Newar, Magar, Sunuwar, Khas and Gallay. More than half of the population is residing in rural areas, which has an easy access to traditional herbal medicine than the modern medicine facilities.

Selection of key informants

The traditional practitioners, as recommended by WHO (Anonymous, 1976) were considered as the 'Key Informants'. As the present work is confined to bone fracture, the traditional practitioners offering treatment for bone fracture and related complications, who are ready to share their practicing knowledge, have been considered as 'Key Informants'.

Ethnomedicinal survey

The contact of key informants in the area have been collected from various sources like local Non-Government Organizations, village chiefs, crude drug venders, school and primary health center staff and patients attending to traditional practices. Once the rapport with key informants has been built up, the aim and objectives of the study has been explained to them in local languages to obtain their consent. The collection of information was through semi-structured, open-ended interviews as suggested by (Martin, 1995). The details on age, place of practice, learning of practice, disease they treat and mode of diagnosis, storage and usage of plants were collected during interviews. The identification and confirmation of the medicinal plant species used by key informants were made through specimen display method (Bhattarai *et al*., 2006). The identity and information was confirmed through repeated guided transect walks in different seasons with the informants and focus group discussions (Martin, 1995; Maundu, 1995).

Plant identification and herbaria

Information about the local healers was gathered and the personal interviews were conducted by taking their

prior permission to share their traditional knowledge. The questionnaire was designed that included information on name, age and profession of medicine knowledge, local name of medicinal plants, parts used, dose composition, methods of preparation of dose, time taken to cure. All the data were recorded in the field note book and properly photographed for further use and for efficacy rate each patient of different areas were also interviewed with prior permission of respective healers and the patients. With few exceptions most of the healers and patients gave their consent to share their ethnic knowledge regarding the bone fracture treatment and also allowed us to record the data. Different formulations from different herbalists have been collected and useful plants that were spotted by the medicine men were photographed, distinguished characters were observed, carefully tagged, uses were noted in field note book and collected specimens were processed following conventional herbarium techniques (Jain and Rao, 1977). The specimens were identified by referring local floras (Prain, 1903; Grierson and Long, 1983-1987 and 1991, 1999, 2001). Identifications of processed specimens were done step wise one after other in the Taxonomy of Angiosperms and Ecology Laboratory of the PG Department of Botany, Darjeeling Government College under North Bengal University. Identification will be also confirmed by matching specimens with the pre identified and authenticated specimens available in Lloyd Botanical Garden Herbarium, Darjeeling, West Bengal and at PG Department of Botany Darjeeling Government College Herbarium.

RESULTS

Knowledge of informants

In this study, 46 out of 235 identified traditional practitioners were considered key informants who provided treatment for bone fractures. The low number of women in traditional medicinal practice from different regions was observed in previous studies (Teklehaymanot *et al*., 2007; Muthee *et al*., 2011). The same is true in this study, as only 3 out of 46 key informants were female. The average age of the informants ranged from 55 years to 85 years with a minimum age of 28 and a maximum age of 75. The transmission of knowledge from generation to generation was verbal, possibly in the same family or same community or to an interested outsider as a global phenomenon. According to the informants, learning of the practice starts from age 15–20 and takes around 10–15 years.

It was observed that some of the informants evaluated the x-ray data prior to or after the fixation of the fractured bone. Additionally, it was noted that both fresh plant material and stored drugs were employed for treatment. The majority of the informants employed bamboo sticks to support and immobilize the bone after the administration of herbal preparation.

Quantitative study of the plants used

In all, 44 plant species belonging to 32 families have been documented in the present study (Tables 1 and 2). Highest number of species representation was found in families Fabaceae and Zingiberaceae with four and three respectively. This deviation from the existing general trend of medicinal plants, where Asteraceae leads with more medicinal species (Mac *et al*., 2005; Mia *et al*., 2009; Namsa et al., 2011) may be due to the easy availability of perennial trees and shrubs rather than annual, seasonal Asteraceae members for the usage. The value of the botanical family was highest for Verbenaceae, though represented by only one species, followed by Urticaceae, Saxifragaceae, Asteraceae, Lamiaceae, Piperaceae, Malvacee and Poaceae with two species each. Forty four genera were represented in the study where Tinospora were represented by two species. The habit of the species showed that 48% of the herbal drugs were obtained from herbs, followed by trees (34%), shrubs (14%) and climbers (4%). Majority of plants used were collected from wild (74%) indicating the species diversity and abundance in the study area. This also indicates the need for conservation measures, especially for those plants with high use value. The stem or stem bark (28%) was most commonly used plant part to prepare the medicine, followed by leaf (22%), root or rhizome (18%), whole plant (15%), fruit or seed (12%) and gum or latex (5%). However, in few preparations it was observed that more than one part of the plant was used for medicinal preparation.

Table 1. Formulation used against orthopedic treatment by the tribal people of hilly areas, North Bengal

Formulations, Healers Name, Age and Address	Botanical name	Local name	Parts used	Mode of administration
1. Dikpal Rai Age- 52 Address- Mungpoo, Cinchona plantation	*Urtica dioica* L	Sishnu	Roots (2-3 pieces, 2 inches)	All the plants were washed, mixed and crushed to make paste. paste applied to the fractured area and tied with cotton cloth. Stem of bamboo is used for supporting structure in most cases.
	Lepidium sativum L	Chausoor	Seeds (4g)	
	Peperomia pellucida (L.) Kunth	Luchipati	Leafy twigs (4-5 pieces)	
	Cissus quadrangularis L	Harjora	Stems (2-3 inches, 2 pieces)	
2. Nauraj Sharma Age- 39 Address- Singhmuli, Mirik	*Viscum articulatum* Burm.f.		Stems (10g)	All the plant materials were washed and crushed to make a paste, along with white layer of local egg, small quantity of kachosimrik added and the mixture applied on fracture and bandaged with cloth.
	Gonostegia hirta (Blume ex Hassk. Miq.	Chipley	Whole plant (2-3 pieces)	
	Curcuma longa Linn.	Hardi	Rhizome (15-20 g)	
	Moringa oleifera Lam.	Sajana	Leaves (10 g)	
	Urtica dioica L	Sishnu	Roots (One, 4 inches)	
3. Chandra Bahadur Chettri Age - 71 Address- Dhargaon Busty, Darjeeling	*Bergenia ciliata* (Haw.) Sternb	Pakhambed	Leaves (1-2 pieces)	Plant materials are crushed finely, made into paste and applied as poultice along with ratomato. In the rib's bone, hands and leg bones after the application of paste tied with cloth.
	Bixa orellana L	Simrik	Seeds (3-5 pieces)	
	Sonchus arvensis L	Bankapasi	Roots (2 pieces, 4 inches)	
	Kaempferia rotunda L	Vuichampa	Rhizomes (5 pieces)	
4. Champa Sewa Age-65 Address- Pound Road, Near Sadar Thana, Darjeeling	*Abroma augusta* Linn.	Kapasey	Leaves (4-5 pieces)	Paste made out of plant parts with Ghee and applied and tied over the fractured bones. 6-follow ups set the fractured bones.
	Urtica dioica L	Sishnu	Roots (two, 3 inches)	
	Barleria prionitis L	Jhinili	Leaves (10-12 pieces)	
	Clinopodium umbrosum (M. Bieb.) Kuntze	Furkejhar	Roots/Leafy twigs	
5. T.K. Pradhan Age- 52 Address- Singhmari, Darjeeling	*Kaempferia rotunda* L.	Vuichampa	Rhizomes (5-7 pieces)	Ingredients are mixed in equal amounts and crushed. White layer of local hen's egg is mixed thoroughly and paste is prepared. It is applied on the fractured part and tied with cotton cloth.
	Acacia catechu (L.f.) Wild.	Khair	Barks (5 g)	
	Prunus cerasoides Buch.-Ham.ex D.Don	Painyu	Barks (4 g)	
	Bergenia ciliata (Haw.) Sternb	Pakhambed	Leaves (one piece)	
6. Dilip Subba Age- 32 Address- Kharkidara, Sukhia pokhari bazaar, Darjeeling	*Cissus quadrangularis* L *Urtica dioica* L *Senna tora* (L.) Roxb. *Heynea trijuga* Roxb. ex Sims.	Harjora Sishnu Tapray Anakhataruwa	Stems (2-3 inches, 3 pieces) Roots (2-3 pieces, 2 inches) Leaves (10 g) Barks (12-15 g)	Required plant materials are crushed and paste applied over the fracture region and applied a cloth bandage with kachosimrik.

Contd....

Table 1. (Contd.)

Formulations, Healers Name, Age and Address	Botanical name	Local name	Parts used	Mode of administration
7. Bikash Tamang Age- 55 Address- Labdha, Chowrata dhura, Mungpoo	*Viscum articulatum* Burm.f.	Harchur	Stems (15-20 g)	Plant materials are crushed separately and mixed in equal amounts of chaussor seeds powder then the paste is applied in the fractured part externally and tied with clean cotton cloth.
	Tinospora cordifolia (Thunb.) Miers	Gurjo	Stem (one-foot-long)	
	Urtica dioica L	Sishnu	Roots (2-3 pieces, 2 inches)	
	Lepidium sativum L	Chausoor	Seeds (3 g)	
8. Nilam Sewa Age- 38 Address- H.L. Ghosh Road, Darjeeling	*Kaempferia rotunda* L.	Vuichampa	Rhizomes (5-7 pieces)	All the plant materials were washed and crushed to make a paste, along with albumin of local hen's egg, small quantity of kachosimrik and ratomato. Applied on fracture portion and tied with cloth.
	Urtica dioica L	Sishnu	Roots (2-3 pieces, 2 inches)	
	Fraxinus floribunda Wall.	Lakuri	Bark (4 g)	
	Ocimum basilicum L.	Babari Ful	Leaves (25-30 pieces)	
9. Chandra Kumar Gurung Age- 58 Address- Lower RabekLadam Khasmal, Kalimpong, P.O.- KageyBazar- 734301	*Viscum articulatum* Burm.f.	Harchur	Stem (7 g)	Ingredients are mixed in equal amounts and crushed. Then mixed with ratomato and bandage and affected area with it, till it loosens. The treatment is carried on for 20-30 days.
	Prunus cerasoides Buch.-Ham.ex D.Don	Painyu	Barks (10 g)	
	Kaempferia rotunda L.	Vuichampa	Rhizomes (5-7 pieces)	
	Saraca asoca (Roxb.) Willd	Ashok	Barks (15 g)	
10. Sukman Rai Age- 62 Address- Singla	*Urtica dioica* L	Sishnu	Roots (2-3 pieces, 2 inches)	Plant material is collected and washed, mixed in equal proportions and crushed then boiled until a thick broth was visible and was left to cool down. The herbal broth applies to the fractured part and covers it with Nepali kagaj (paper). Similarly second layer of broth and Nepali kagaj is applied followed by third. Finally the whole affected portin is tied with soft cotton gauge. It should be kept for 10-15 days until it loosens by itself and falls off. Similar process is repeated for the second time which heals the fracture. In case of serious injury the process is repeated for 3rd and 4th time respectively until it is fully cured.
	Cissus quadrangularis L	Harjora	Stems (2-3 pieces, 2 inches)	
	Heynea trijuga Roxb.ex Sims.	Anakhataruwa	Barks (12-15 g)	
	Zingiber officinale Roscoe	Aduwa	Rhizome (15 g)	
	Symphytum officinale L.	Confrey	Leaves (10 pieces)	

Contd....

Table 1. (Contd.)

Formulations, Healers Name, Age and Address	Botanical name	Local name	Parts used	Mode of administration
11. Mingma Bhutia Age- 60 Address- Gumba line, Sukhia Pokhari Bazar, Sukhia Pokhari, Darjeeling	*Cissus quadragularis* L	Harjora	Stems (15 g)	Plant materials are finely crushed and paste is made. The paste is applied in the fractured area and tied with cotton cloth. It is then supported by stem of Choyabas (*Dendrocalamus hamiltonii* Nees & Arn. Ex Munro) it prevents the fractured part from being bend.
	Bixa orellana L	Simrik	Seeds (4-5 pieces)	
	Curcuma longa Linn.	Hardi	Rhizome (20 g)	
	Lepidium sativum L	Chausoor	Seeds (5 g)	
12. Ruben Subba Age- 46 Address- Lebong, Ging Tea Garden, Gelongter	*Bergenia ciliata* (Haw.) Sternb	Pakhambed	Leaves (2 pieces)	Required plant materials are mixed and make a paste along with white layer of egg, kachosimrik and turmeric ground and the mixture applied on fracture and bandaged with cloth.
	Zingiber officinale Roscoe	Aduwa	Rhizome (15-20 g)	
	Clinopodium umbrosum (M.Bieb.) Kuntze	Furkejhar	Roots/Leafy twigs Small amount)	
	Tinospora cordifolia (Thunb.) Miers	Gurjo	Stem (one-foot-long)	
13. Yogesh Subba Age- 52 Address- Santi Bazar, Sonada	*Viscum articulatum* Burm.f.	Harchur	Stems (5 g)	All the materials were washed and crushed to make a paste, along with white layer of local egg and applied as poultice.
	Urtica dioica L	Sishnu	Roots (2-3 pieces, 3 inches)	
	Acacia catechu (L.f.) Wild.	Khair	Barks (5 g)	
	Sonchus arvensis L.	Bankapasi	Roots (2 pieces, 4 inches)	
14. R.K. Rai Age- 58 Address- Kothidhura, Sonada	*Kaempferia rotunda* L.	Vuichampa	Rhizomes (6-8 pieces)	Ingredients are crushed one by one and mixed thoroughly adding kachosimrik and powdered of chausoor and mixed again. The paste is applied on the fractured part and tied with cotton cloth and kept for two weeks and if injury is serious the process is repeated at the interval three to four weeks.
	Litsea glutinosa (Lour.) C.B.Rob	Brown ballygum	Barks (5 g)	
	Cissus quadrangularis L	Harjora	Stems (two, 2 inches)	
	Lepidium sativum L.	Chausoor	Seeds (2-3 g)	
15. Archana Pradhan Age- 48 Address- Rimbick, Bijanbari, Darjeeling	*Cissus quadragularis* L	Harjora	Stems (20 g)	The vine of Gurjo, Dubo, bark of Arjun and stems of *Cissus quadrangularis* L were ground into a sticky paste using a mortar and pestle or stone slab, then placed in a clean cotton cloth and tied around the fractured area for 10-15 days.
	Tinospora cordifolia (Thunb.) Miers	Gurjo	Vine (one-foot-long)	
	Terminalia arjuna (Roxb.) Wight & Arn.	Arjun	Bark (8 g)	
	Cynodon dactylon (L.) Pers.	Dubo	Whole plant	
16. Amir Mangar Age- 60 Address- Nagari Tea Estate, Magarjun	*Astilbe ruvulraris* Buch. Ham	Buro okhati	Rhizome (2-3 pieces, 2 inches)	Four ingredinets are crushed into paste with kachosimrik and deshi ghe applied in clean cloth for bandage for 30-50 days and drinks goat milk at night every day.
	Nyctanthes arbortritis L.	Parijat	Leaves (8-10 pieces)	
	Asparagus racemosus Willd.	Satamuli	Roots (2-3 inches, 2 pieces)	
	Artemisia vulgaris L.	Tiepati	Leaves (20 g)	

Table 2. List of recorded plants used in treatment for Bone setting

Sl. No.	Botanical Name	Local Name	Family	Habit/Habitat	Parts
1.	*Kaempferia rotunda* Linn.	Bhui champa	Zingiberaceae	Herb/Cultivated	Rhizome
2.	*Urtica dioica* Linn.	Lekh sisnu	Urticaceae	Herb/wild	Roots
3.	*Berginia ciliata* (Haw.) Sternb	Pakhnaved	Saxifragaceae	Herb/wild	Whole plants
4.	*Viscumarticulatum* Burm.	Harchur	Viscaceae	Shrub/wild	Whole plants
5.	*Cissus quadrangularis* L	Harjora	Vitaceae	Evergreen climber/wild	Stem
6.	*Prunus cerazoides* D.Don	Payun/Arupatey	Rosaceae	Tree/wild	Bark
7.	*Fraxinus floribunda* Wall.	Lankuri	Oleaceae	Tree/wild	Bark
8.	*Curcuma longa* Linn.	Hardi	Zingiberaceae	Herb/Cultivated	Rhizome
9.	*Gonostegia hirta* (Blume ex Hassk.) Miq.	Bhui Chiley	Urticaceae	Herb/widl	Whole plants
10.	*Lepidium sativum* L.	Chausur	Brassicaceae	Edible herb/cultivated	Seeds
11.	*Sonchus arvensis* L.	Bankapas	Asteraceae	Herb/wild	Whole plants
12.	*Clinopodium umbrosum* (M. Bieb.) Kuntze	Furkejhar	Lamiaceae	Herb/wild	Whole plants
13.	*Artemisia vulgaris* L.	Titepati	Asteraceae	Herb/wild	Leaves
14.	*Asparagus racemosus* Willd.	Satamuli	Asparagaceae	Herb/wild	Roots
15.	*Bixa orellana* L	Simrik	Bixaceae	Tree/wild	Seeds
16.	*Heynea trijuga* Roxb. Ex Sims.	Taruwa	Meliaceae	Tree/wild	Barks
17.	*Acacia catechu* (L.f.) Willd.	Khair	Fabaceae	Tree/wild	Barks
18.	*Nyctanthes arbrtitis* L.	Parijat	Oleaceae	Shrub/cultivated	Leaves
19.	*Tinospora cordifolia* (Lour.)	Gurjo	Menispermaceae	Shrub/wild	Stems
20.	*Terminalia arjuna* (Roxb.) Wight & Arn.	Arjun	Combretaceae	Tree/wild	Barks
21.	*Astilbe rivularis* Buch. Ham	Buro okhati	Saxifragaceae	Herb/wild	Rhizome
22.	*Peperomia pellucida* (L.) Kunth	Pononoa	Piperaceae	Herb/wild	Whole plants
23.	*Barleria prionitis* L	Jhinili	Acanthaceae	Shrub/wild	Leaves
24.	*Moringa oleifera* Lam.	Sajana	Moringaceae	Tree/cultivated	Gum/root
25.	*Abroma augusta* Linn.	Kapasey	Malvaceae	Tree/wild	Leaves
26.	*Senna tora* (L.) Roxb.	Tapray	Fabaceae	Herb/wild	Leaves
27.	*Ocimum basilicum* L.	Babari Ful	Lamiaceae	Herb/cultivated	Leaves
28.	*Zingiber officinale* Roscoe	Aduwa	Zingiberaceae	Herb/cultivated	Rhizome
29.	*Saraca asoca* (Roxb.) Willd	Ashok	Fabaceae	Tree/wild	Barks
30.	*Symphytum officinale* L.	Comfrey	Boraginaceae	Herb/cultivated	Root, leaves
31.	*Cynodon dactylon* (L.) Pers.	Dubo	Poaceae	Herb/wild	Whole plants
32.	*Litsea glutinosa* (Lour.) C.B.Rob.	Brown bollygum	Lauraceae	Tree/wild	Bark

Table 3. Plants used for Bone strengthening, Pain relieving and Inflammation reduction.

Sl. No.	Botanical Name	Local Name	Family	Habit/Habitat	Parts	Purpose
1.	*Dendrocalamus hamiltonii* Nees & Arn. Ex Muno	Choyabaas	Poaceae	Tree-grass/ wild	Stems	Helping in fracture healing
2.	*Sesamum indicum* L	Til	Pedaliaceae	Herb/cultivated	Seeds	Used as massage oil for Pain reliever
3.	*Calotropis procera* (Aiton) Dryand.	Anak	Apocynaceae	Tree/wild	Latex/Leaves	Bone hardening and pain reliever
4.	*Gmelina arborea* Roxb.	Gambhari	Verbenaceae	Tree/wild	Bark/Leaves	Bone strengthening after bone setting
5.	*Vitex negundo* L.	Sewali, Nirgundi	Lamiaceae	Shrub or small tree/wild	Leaves	For speedy recovery
6.	*Ricinus communis* L.	Reri	Euphorbiaceae	Shrub/wild	Barks/Twig	Pain reliever
7.	*Allium sativum* L.	Lasun	Amaryllidaceae	Herb/cultivated Shrub/wild	Bulb	Used as massage oil to set the broken bones
8.	*Tinospora sinensis* (Lour.) Merr.	Gurjo	Menispermaceae	Climber/ cultivated	Stems	Bone strengthening
9.	*Piper longum* L.	Pipla	Pipereceae	Herb/wild	Seeds	Applied and given orally for speedy recover
10.	*Sida acuta* Burm.f.	Khareto	Malvaceae	Herb/wild	Leaves	Bone strengthening
11.	*Achyranthes aspera* L.	Ankhlay Jhar	Amaranthaceae	Tree/wild	Leaves	Pain reliever
12.	*Madhuca longifolia* (J. Konig ex. L.) J.F. Macbr.	Mahuwa	Sapotaceae		Seeds	Used as massage oil to set the broken bones

Figure: A- Rhizome of *Kaemferia rotunda* Linn. **B-** Plants of *Kaempferia rotunda* Linn. **C-** Patient being ready for second round of treatment. **D-** Bark of *Prunus ceresoides* Buch.Ham. ex D.Don. **E-** The plant parts of *Prunus ceresoides* Buch.Ham. ex D.Don. from where to barks collected. **F-** Plant materials boiled in a silver container and on hand plant extract after boiling.

Among 16 formulations prepared from 32 plant species, only one formulation contained five herbal ingredients, while the rest were four herbal preparations (Table 1). All the preparations were applied externally as paste or poultice as a plaster. Application of the prepared drug depends on the portion of fracture, severity and physical appearance of an individual. The broken bones were set together to right position and were supported by bamboo sticks and tied with rope. Duration of bandage varies from 10 to 60 days with a reapplication of herbal preparation once in 14 days. Twelve plant species belonging to 11 families are found to be used for bone strengthening, pain relieving, inflammation reduction and speedy recovery (Table 3). The administration routes were both oral and external application. The external applications were for pain relieving or to reduce the inflammation, whereas oral administration was preferred for bone strengthening and speedy recovery. Decoctions and juices were the normal mode of preparations, mostly using water. Again the dosage depends on several factors. In each case, fistful of plant parts was used for preparation which accounted for about 200 mg. Gmelina arborea Roxb. has mostly used for bone strengthening, pain relieving among the recorded plants. The use of the same has previously been reported in the treatment of bone fractures in both human beings (Venkataratnam and Venkataraju, 2008) as well as in animals (Hershra *et al*., 2002, Rajakumar and Shivanna, 2010).

CONCLUSION

The documented species from the present study shows an effective property in healing and can be further considered for both in situ and ex situ conservation. Among 44 recorded species, Urtica dioica L., Kaempferia rotunda Linn., Viscum articulatum Burm. and Cisus quadrangularis L were recorded as most potent drug for bone fracture treatment in hilly regions of North Bengal because of their high use and it is quit available in this region. Sixteen formulations are prepared by using 4-5 medicinal plants along with some additive materials. These species additionally needs thorough phyto-chemical screening which may bring a novel finding in the pharmaceutical industry. Further detailed research regarding the principle component identification and scientific trial will required to evaluate for specific drug designing against bone fracture, and it also helped us preserve our precious herbal wealth by making us realize how valuable these plants are to future generations. The needs to collaborate all the healers for detail documentation of the medicinal plants similarly arises for long term sustainability and affordable medication especially in the rural areas for the benefit of control and management of diseases and also provides the employment and improve the economical status of the society.

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FULL LENGTH ARTICLE

Phenotypic characterization of some pigmented rice landraces (*O. sativa* L.) and their *in vitro* response

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Characterization based on yield attributing parameters was done on eight coloured rice landraces collected from Zonal Adaptive Research Station, Krishnagar, Nadia. Remarkable variability has been found in respect to the traits *viz.,* grain length, grain length/breadth ratio in the cultivars like Chandrakanta, Kalamkathi, Badsabhog and Sitasal which showed extra long feature with maximum grain length feature. Among the varieties examined, the chalkiness was present very occasionally in the cultivars like Suakalma, Chandrakanta and in Sarkele aman. Moreover, finding a promising *in vitro* protocol for selection of some promising rice germplasm with efficient regeneration capability is now keenly essential for any biotechnological research of rice. With this aim, this study aims at induction of calli on callus induction media followed by regeneration taking mature seeds of rice as explants. Treatment of 2,4-D with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.5mg/l) showed a remarkable callus induction in the cultivars Sarkele aman and Kalamkathi under light period of 16h. To observe the frequency of plant regeneration from calli derived from explants by sub culturing, a combination of 2,4-D (0.5mg)/l) and BAP (0.5-1.5mg/l) revealed maximum regeneration frequency in the cultivars *viz*., Sarkele aman, Kalamkathi and Badsabhog. This study provides efficient means of finding *in vitro* protocol and response by the cultivars which may be useful for future research and eco-friendly conservation of rice landraces.

Keywords : Characterization, conservation, BAP, MS media, 2,4-D, rice landrace regeneration.

INTRODUCTION

Pigmented rice is rich in antioxidants and polyphenols and has two or three times as much zinc and iron as white rice (Ramaiah,1953) which possess the desirable quality to boost, strengthen, regenerate and energize the body (Sensarma, 1989). Ninety percent of Rice (*Oryza sativa* L.), the staple food crop of more than half of the world's population (Anonymous, 2009), is grown and consumed in South and South East Asia. In this sub continent, thousands of locally adapted cultivars of rice have evolved by nature and human (Singh *et al*., 2000), many of which are either fine grain with pigmentated or aromatic types. Land races genotypes, wild and weedy relatives which serve as a treasure trove of beneficial gene pool (Chakravorty *et al*., 2013; Chakravorty and Ghosh, 2014) which can be used for further exploitation and necessary to check their *in vitro* response. Development of efficient plant regeneration *in vitro* is essential for the successful utilization of

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biotechnology in rice crop improvement (Ghosh *et. al.*, 1997). Variability pattern in some *in vitro* developed somaclones in rice *landraces* of Gangetic Alluvial Zone was studied by Chakravorty (2016). To accomplish the need of *in vitro* conservation, there is a primary and urgent need to establish a challenging *in vitro* regeneration method because this work is still limited to a number of genotypes (Tagichi-Shiobara *et al*., 1997). For *in vitro* regeneration, use of seed embryo is more advantageous as a starting material over other explants and has mature seed embryos are more efficient to response in callus induction and regeneration (Din *et al*., 2015). Keeping this in view, present investigation aims at an attempt to develop a protocol for the induction and regeneration of some traditional pigmented rice landraces using mature seed derived calli as explants.

MATERIALS AND METHODS

Phenotypic Characterization

The experimental materials consist of a diverse group of eight coloured traditional rice germplasms (Table 1) collected from the gene bank of Zonal Adaptive Research Station, Krishnagar, Nadia, West Bengal (23˚24´N latitude and 88˚31´E longitude with an altitude of 9.75 meters above mean sea level). The investigation was done during kharif season in the research farm following the distinctiveness, uniformity and stability (DUS) test in a randomized block design with three replications. Twenty one days old seedlings of each entry were transplanted in $3.0\times2.85m^2$ plot with plant to plant spacing 15 cm within a row and row to row spacing of 20 cm. Plot to plot distance was 60 cm. A random sample of five competitive plants was used for observations on different traits under study. The crop was growing following recommended package of practices. Observations were made on grain quality characteristics at different stages following DUS test guidelines of PPV and FR Authority. At the time of maturity the data on five competitive plants from each genotype in each replication were recorded for different characters like grain length, grain breadth, panicle length, grain length/ breadth ratio, grain weight, kernel weight and number of grains $panicle^{-1}$. The methodology given by Steel *et al*., 1997 was used for statistical analysis. The individual genotypic means were compared by Duncan's Multiple Range Test (DMRT) and the total variance was partitioned into genotypic and phenotypic components. Grain size and shape are among the first criteria of rice quality that breeders consider in developing new varieties for releasing for commercial production (Adair *et al*. 1973). A comparative study of the physical quality characteristics comprising eight landraces of rice was completed at the Research Station of Krishnagar and Sripat Singh College. (Table 1). Eight samples were milled at 10% moisture content, after milling, the obtained brown rice was polished. Head and broken rice were separated through a rice grader. The graded samples comprising of full shape grains were used to proceed for the study. The length, breadth and thickness of milled rice (50 grains per sample) were taken with the help of micrometer. Size and shape was determined according to scale of FAO standards given below. For the determination of Chalkiness of endosperm, Milled rice was observed under a stereo-zoom microscope and based on the orientation of chalkiness, the rice grains were classified into white belly, white centre and white back (Anonymous 2004).

In vitro callus induction and regeneration

Surface sterilization of seed and Inoculation in MS medium

Manually mature dehusked seeds were collected from the gene bank of Zonal Adaptive Research Station, Krishnagar Nadia, West Bengal and their pre and post agro-morphological evaluation were done followed by *in vitro* culture of these cultivars. Laboratory works were done at the plant tissue culture laboratory of Sripat Singh College, Jiaganj in assistance of tissue culture laboratory of Dept. of Botany, University of Kalyani, Nadia. The collected seeds were surface sterilized at first at 70% alcohol (v/v), vigorously using detergent and with 1%(v/v) Tween 20 and rinsed many times with distilled water. This was followed by washing with bavistin (20min) and rinsing repeatedly with distilled water. After that the seeds were treated with 4% (v/v) sodium hypochlorite solution for 3mins.

Table 1. Mean values of the physical characteristics of coloured rice grain

Sl.No.	Cultivars	Grain length (mm)	Grain breadth (mm)	Grain L/B ratio	Grain size	Grain shape	Chalkiness		
							Frequency	Kernel area	Type
1	Kalamkathi	9.90^{kl}	2.20b	4.50^{kl}	EL	Sl	OP	M	WB
2	Suakalma	8.30^{e-j}	3.50n	2.37^{a-g}	EL	M	VOP	S	WB
3	Daharnagra	8.80^{f-l}	3.50m	2.51^{a-g}	EL	M	OP	M	WC
4	Badsabhog	8.90^{f-l}	2.20b	4.04^{i-l}	EL	Sl	OP	M	WB
5	Chandrakanta	9.00^{f-l}	4.10r	2.19^{a-e}	EL	M	VOP	S	WC
6	Sitasal	8.90^{f-l}	3.00hi	2.96^{a-j}	EL	M	OP	M	WB
7	Danaguri	8.90^{f-l}	4.20s	2.11^{a-d}	EL	M	OP	M	WB
8	Sarkele aman	7.30^{c-e}	4.20s	1.73^{a}	L	D	VOP	S	WB

Mean associated with common letters, in a column are not significantly different at 5% level of significance using DMRT : EL – Extra Long; L – Long; Sl – Slender; M – Medium; B – Bold; S – Small
OP = Occasionally Present, VOP = Very Occasionally Present, P = Present, WB = White Belly, WC = White Centre

Completing the surface sterilization, the seeds were then dried and inoculated to culture media of MS (Murashige and Skoog, 1962) as a basal media with different concentration of 2,4-D (0.5, 1.0, 1.5, 1.5, 2.0, 2.5 and 3, 3.5 and 4.0mg/l) (Table 2) for induction of callus. For rice *in vitro* study, MS has been found promising (Niroula *et al*., 2005) along with N6 medium (Rashid, 2004). Then, thorough washing of seeds under tap water along with their surface sterilization with 70% ethanol for 5 minutes was done followed by washing with five drops of extran. They were then washed thrice in distilled water and surface-dried with tissue papers. The sterilized seeds were next cultured on modified MS medium (Murashigee and Skoog, 1962) containing $30mgL^{-1}$ of sucrose supplemented with $2mgL^{-1}$ of 2,4-D and $2gL^{-1}$ of gellen gum. Cultures were incubated in the culture room at 28℃ under white illumination with a 16h photoperiod of 2,000lux. Each treatment contained 10 seeds with 3 replicates and the experiments were repeated twice after 7 days, seed sterilization (%), seed germination (%) and callus induction(%) ensured using the equation: Callus induction frequency = (No. of seeds producing calli)/(Total no. of seeds)×100.

For the study of regeneration frequency, high quality embryogenic callus was visually selected by microscopic observations. Study of regeneration was done in regeneration culture room at 25℃ with a 16/8 h (light/dark) photoperiod duration at 2000lux light at sterile condition selecting the regeneration media supplemented with 2,4-D (0.5, 1.0, 1.5mg/l) along with BAP (0.5, 1.0, 1.5mg/l) for 4-5 week. Every treatment contained three replications with four clumps of callus each. For the production of root, regenerated *in vitro* plants were transferred to MS media supplemented with cytokinine (0.3-4.5mg/l) for seven days. Regular and thorough minute observations were done and visually recorded data was put for the statistical analysis. Regeneration frequency was calculated depending on the number of plantlet regenerated per embryogenic callus divided by number of callus inoculated. Analysis of variance (ANOVA) was done and differences of mean were calculated following Duncan's Multiple Range Test (DMRT) of SPSS statistical software.

RESULTS AND DISCUSSION

Phenotypic characterization of eight pigmented landraces of *Oryza sativa* L.

Results showed that size of the grain of Sarkele aman showed shortest size having maximum grain breadth followed by Danaguri, and Chandrakanta significantly different from the remaining ones. (Table 1). Variety Kalamkathi and Badsabhog are *at par* and are statistically significant. Cultivar Sarkele aman had minimum grain length/breadth ratio and which is statistically very significant. The chalkiness of the rice grain was classified into white belly, white centre and white back. Among the varieties examined, the chalkiness was present very occasionally in the cultivars like Suakalma, Chandrakanta and in Sarkele aman (Table 1). In maximum cultivars chalkiness was present occasionally while centre type of chalkiness was found in the cultivars like Chandrakanta. The rest of the cultivars had white belly type of chalkiness. The chalky grains reduce the palatability of cooked products, thus the presence of more than 20% chalkiness in rice kernels is not acceptable in world markets (Cheng *et al.,* 2005). For any crop improvement programme, selection of traits is a prerequisite to characterize the germplasm for further exploitation (Chakravorty & Ghosh, 2011, 2013).

Studies on *in vitro* response of eight pigmented rice landraces

The maximum callus formation ability for these eight traditional coloured rice landraces was found out in MS medium supplemented with 2mgL^{-1} of 2,4-D. The results presented that the increasing 2,4-D concentrations greatly induced callus induction from 55-87%, when explants were cultured under 16h photoperiod, but in dark condition, showed that callus induction was reduced to 55% (Table-2). The most significant step of this study is to obtain high numbers of calli, masses of undifferentiated cells which provide

Table 2. *In vitro* response of eight coloured rice landraces in callus induction, regeneration and its comparative study.

Sl. No.	Name of the cultivars	Callus production (%)			Callus regeneration frequency (%)
		MS-Media (2,4D; 2 mgL^{-1})			MS Media 2,4D:BAP (1:3 mgL^{-1})
		Total	Necrotic	No response	
1	Kalamkathi	69.23^{a-g}	19.1^{a}	7.2^{a}	23.2±32^{a-e}
2	Suakalma	68.67^{a-e}	12.5^{e}	7.8^{e}	32.09±14^{a-g}
3	Daharnagra	60.23^{e-f}	25.6^{e}	15.7^{f}	19.2±11.2^{i-l}
4	Badsabhog	58.76^{a-e}	19.8^{e}	8.0^{e}	19.5±14.25^{i-l}
5	Chandrakanta	55.21^{a-g}	18.2^{f}	7.9^{f}	22.2±12^{a-c}
6	Sitasal	62.4^{e-g}	21.2^{e}	6.8^{e}	32.11±11^{a-g}
7	Danaguri	58.76^{a-g}	19.8^{a}	8.0^{g}	19.5±08.55^{e-g}
8	Sarkele aman	87.17^{e-l}	9.5^{a}	5.3^{a}	58.2±12^{a-e}
	Total	527.1^{a-l}			234.69±59^{a-f}
	Mean	65.89^{a-f}			29.33±18^{a-l}

Values are means of replicates ± SD. Means in the same column with different superscripts are significantly different ($P<0.05$)

a good deal of starting material for *in vitro* manipulation (Wani *et al*., 2011). The ability of mature seeds (Jiang *et al*.,2000) makes them suitable as explants; thus, they were used in the present study. Different 2,4-D concentrations induced a fair amount of calli and also promoted callus growth.. In Poaceae, the presence of green spots in cultures has been considered as predictors of potential shoot formation (Nabors *et al*.,1982). Nature of calli, their growth rate, texture and colour varied on variety or cultivar. The mature seeds derived calli were creamy, white to creamy in MS medium. The natures of mature seeds calli were compact. It was also found that highest callus induction was observed in Sarkele aman and Kalamkathi cultivars. Thus, this result indicated the possibility of acceptability and cultivation of Sarkele aman and Kalamkathi as potent material in tissue culture or biotechnological research for crop improvement programme. The results also revealed that the genotype exhibited great variability for early induction and high production of callus. Effect of genotype and explants age was also found to be important factor in callus induction in indica rice (Hoque and Mansfield, 2004 and Doah and Anzai, 2006). It was very interesting to observe that the factors like, sucrose (25-30gmL^{-1}), hormones (Auxin:Cytokine ratio) and BAP affected the callus formation and regeneration more deeply. It has been found that the frequency of plant regeneration from calli derived from explants by sub culturing, a combination of 2,4-D (0.5mg)/l) and BAP (0.5-1.5mg/l) revealed maximum regeneration frequency in the cultivars *viz*., Sarkele aman, Kalamkathi and Badsabhog. It was also observed that 2,4D was better than NAA for its mode of action. Some problems like browning of callus, appearance of albino plants made the study more challenging.

CONCLUSION

Pigmented rice is the treasure trove of beneficial gene for future crop improvement programme. So, there is an urgent need for variety development and this *in vitro* technique has been found to be quick and better efficient than the conventional hybridization. The choice of optimal medium compositions was done on the basis that yields a high percentage of callus induction and growth. Our experiment showed that culturing mature seeds of eight pigmented rice landraces on MS medium supplemented with 2mgL^{-1} 2,4-D under a 16 h photoperiod could induce the highest percentage of callus induction (88%) along with moderate to high regeneration capability. This paper also concentrates on the physical characteristics of rice grain with consumer preference of traditionally cultivated rice varieties. Thus, it can be concluded that this study will help the breeder, researcher and the farmers to choose the desirable traits and the parents which are the reservoir of desired gene pool for the exploitation in future research and breeding program in developing the grain quality rice and in developing the high yielding varieties.

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FULL LENGTH ARTICLE

An enumeration of Lejeuneaceae (Marchantiophyta) in Assam, India

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An enumeration of the family Lejeuneaceae (Marchantiophyta) in the state Assam, India reveals the occurrence of 43 species distributed under 12 genera with new records of *Lejeunea minutiloba* A. Evans, *Leptolejeunea foliicola* Steph. and *Leptolejeunea latifolia* Herzog for the state.

Keywords : Lejeuneaceae; Liverwort; Bryophyte; Diversity; Assam; North-eastern India.

INTRODUCTION

The family Lejeuneaceae Cavers (Marchantiophyta) is one of the advanced groups which mainly grows in humid tropical-subtropical forests and constitutes dominant composition of epiphytic liverworts. The state Assam is a forest dominated state of north-eastern India lying in the rain shadow of Himalayan ranges between the coordinates 89°41' E to 96°02' E longitudes and 24°07' N to 28°02' N latitudes. It covers an area of ca 78,438 sq km (www.wikipedia.org) with a forest cover of ca 26,832 sq. km, which is approximately 34% of the state's geographical area. The study area covers a vast range in its topography ranging from hills and hillocks to wide plains and low lying water-logged areas or flood plains with an altitudinal variation of less than 100 m to more than 1850 m. The climate is characterized by moderate temperature and high humidity with an average annual rainfall of *ca* 2177 mm. This tropical-subtropical monsoon rainforest climate and dense tropical wet evergreen, semi evergreen and moist deciduous forests have favoured luxuriant growth of various members of the family Lejeuneaceae growing as terrestrials as well as epiphytes on various ferns, bamboos and other angiosperms. But, till date, no comprehensive systematic account on the family Lejeuneaceae is available from the study area as well as North-eastern India. Various workers (Barukial *et al*., 2002; Barukial, 2011; Das and Sharma, 2012; Singh and Barbhuiya, 2012; Verma *et al.*, 2012; Das and Sharma, 2013, 2016; Verma *et al.,* 2018) reported/enlisted *ca* 50 species of the family from different areas of the state within their works done on the Hepaticae and Anthocerotae of Assam. The reports are based upon authors own collections and/or previous reports/literature survey. Present communication is the first attempt for enumeration of taxa under the family Lejeuneaceae in Assam based completely upon field collections. This study reveals the occurrence of 43 species under 12 genera of Lejeuneaceae from the region including new records of *Lejeunea minutiloba* A. Evans, *Leptolejeunea foliicola* Steph. and *Leptolejeunea latifolia* Herzog for the state Assam.

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MATERIAL AND METHODS

Collection of fresh plant materials of the family Lejeuneaceae was done during 2012 – 2016 by extensive field visits to different localities of the study area. Field numbers were noted in the field book along with other field details of the specimens such as locality, habitat, altitude, etc. Plant specimens were preserved in blotting paper packets and/or in 70% alcohol solution. Morpho-anatomical analysis of the specimens was done under SZ61 zoom dissecting and Olympus CH20i compound microscope. Characters like presence or absence of underleaves, shape of underleaves where present, thickness of cell walls in stem and leaves, presence or absence of trigones, structure and number of tooth in leaf lobules, number of ocellus, etc. were considerd for identification of the taxa. Identification was done following standard literature (Das, 2009; Das and Singh, 2012; Das and Sharma, 2013, 2016; Dey and Singh, 2012; Gradstein, 1994; Zhu and So, 2001). Photographs were taken in the field and/or laboratory along with the habitat of the epiphytic taxa. The specimens were deposited in the Herbarium of Assam University, Silchar (AUSCH)..

RESULT AND DISCUSSION

Salient Findings

A total of 43 species under 12 genera of the family Lejeuneaceae are identified. Among the 12 genera found, genus *Cololejeunea* (Spruce) Schiffn. is found to be the dominant one with 13 species followed by the genera *Lejeunea* Lib. with seven species and *Leptolejeunea* (Spruce) Schiffn. and *Cheilolejeunea* (Spruce) Schiffn. both with five species each. Other genera are: *Acrolejeunea* Schiffn. (1 sp.), *Archilejeunea* (Spruce) Schiffn. (2 spp.), *Caudalejeuna* (Steph.) Schiffn. (1 sp), *Colura* (Dumort.) Dumort. (1 sp.), *Drepanolejeunea* (Spruce) Schiffn.(1 sp.), *Lopholejeunea* (Spruce) Schiffn. (4 spp.), *Microlejeunea* Steph. (2 spp.) and *Ptychanthus* Nees (1 sp.). Graphical representation of the percentage of the genera is depicted in Fig. 1. Photographs of some species along with their natural habitat are given in Fig. 3. Among the taxa identified, three species are found as new to the state of Assam viz. *Lejeunea minutiloba* A. Evans, *Leptolejeunea foliicola* Steph. and *Leptolejeunea latifolia* Herzog. *Lejeunea minutiloba* was earlier reported from Arunachal Pradesh and Tamil Nadu in India and also from North America (Stotler and Crandall-Stotler, 1977; Das and Singh, 2012; Singh *et al.*, 2016). *Leptolejeunea foliicola* was hitherto known from Andaman & Nicobar Islands, Arunachal Pradesh, Kerala, Meghalaya, and Tamil Nadu in India and from Bangladesh, Cambodia, China, Indonesia, Malaysia, Philippines and Thailand (Singh, 1996; Das, 2009; Dey and Singh, 2012; Singh *et al*., 2016). *Leptolejeunea latifolia* was so far known from Sikkim and West Bengal (Darjeeling) in India and from Bhutan, China, Nepal and Thailand (Singh *et al*., 2016). Three species viz. *Archilejeunea minutilobula* Udar & U.S. Awasthi, *Cololejeunea mizutaniana* Udar & G. Srivast. and *C. siangensis*are G. Asthana & S.C. Srivast. are endemic to India among which *C. mizutaniana* is found only in the state Assam within the Eastern Himalayan region other than Karnataka-Kerala of Southern India (Singh and Barbhuiya, 2012; Singh *et al*., 2016). *C. siangensis* is confined within the Eastern Himalayan territory only in the states of Arunachal Pradesh and Assam (Das and Sharma, 2012, 2013, 2016; Singh *et al*., 2016). *Cololejeunea denticulata* (Horik.) S. Hatt., a taxon found in Bangladesh, China, India, Japan, Korea and Taiwan, is found only in the state Assam within the Indian subcontinent (Banu-Fattah, 2001; Zhu and So, 2001; Singh and Barbhuiya, 2012; Singh *et al*., 2016). Within the Indian Eastern Himalayan zone, two species, *Colura ari* (Steph.) Steph. and *Lopholejeunea eulopha* (Taylor) Schiffn. are found to occur in state Assam only apart from their occurrence in other countries. Another taxon, *Leptolejeunea epiphylla* (Mitt.) Steph., in addition to its distribution in many Asian countries, is also found in India. Apart from its occurrence in Andaman & Nicobar Islands, Kerala and Tamil Nadu, within the Eastern Himalayan bryogeograpical region, it is found only in the state Assam (Verma *et al*., 2012; Singh *et al*., 2016).

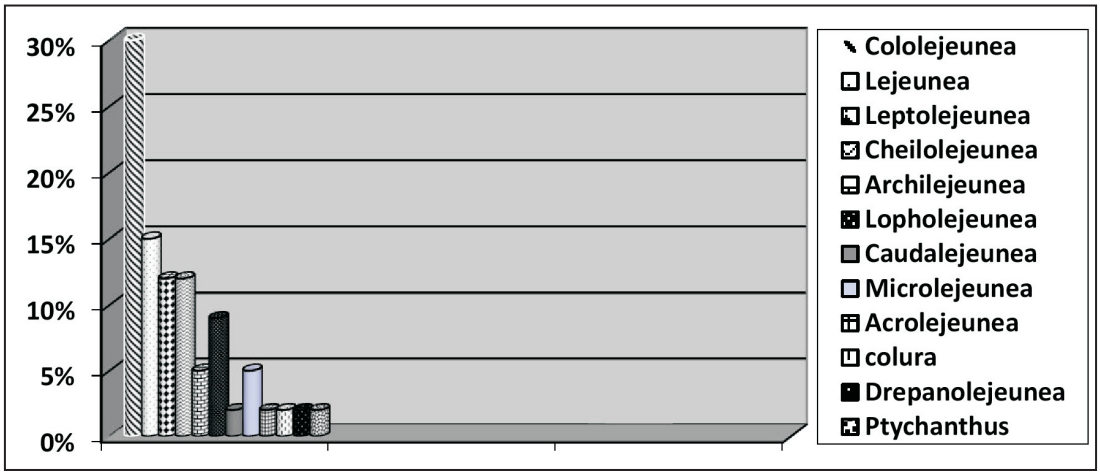

Fig. 1. Graphical representation of percentage of the genera found in the study area.

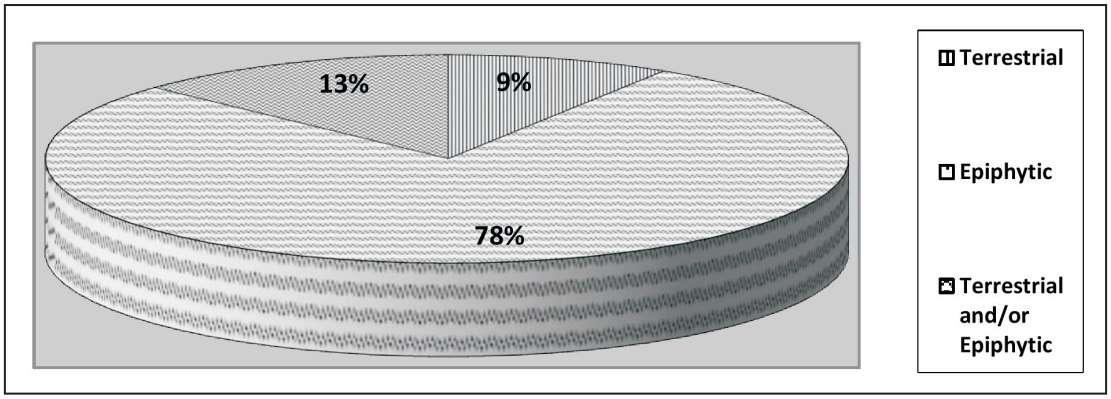

Fig. 2. Graphical representation of percentage of habitat preference.

Habitat

The habitat analysis (Fig. 2) of all the taxa identified reveals that the members of Lejeuneaceae contribute a large percentage of epiphytic bryophytes of the state. Out of the total taxa identified, only 9% are found to grow as purely terrestrials whereas, 78% are found as purely epiphytic (Corticolous and/or Foliicolous); 13% do not show any habitat specificity. Some common plants found to act as hosts for corticolous taxa are *Bambusa* sp, *Ficus* sp., *Morus* sp., *Shorea robusta*, etc; whereas plants reported as phorophytes for foliicolous taxa are Ferns, *Gnetum* sp., various *Bambusa* sp., *Canna* sp., *Cinnamomum tamala*, *Dendrocalamus* sp., *Licuala* sp., *Ophiorrhiza* sp., *Zingiber* sp., etc. It shows that the corticolous taxa grow mostly on plants having rough stem bark for ease of anchorage of the rhizoids (Das *et al*., 2023).

Plants of the family Lejeuneaceae are monoecious or dioecious, small – medium or large, pinnately branched. Stem in cross section differentiated or not. Leaves incubous, alternate, distant – imbricate; lobes oblong – ovate – obovate – quadrate – suborbicular – linear-lanceolate, margin entire – dentate – spinose, apex rounded – obtuse – acute – apiculate; lobe cells thin – thick-walled, with or without intermediate thickenings and trigones; lobule more or less inflated, sometimes ligulate or cylindrical-tubular, tooth prominent – indistinct. Underleaves distant –

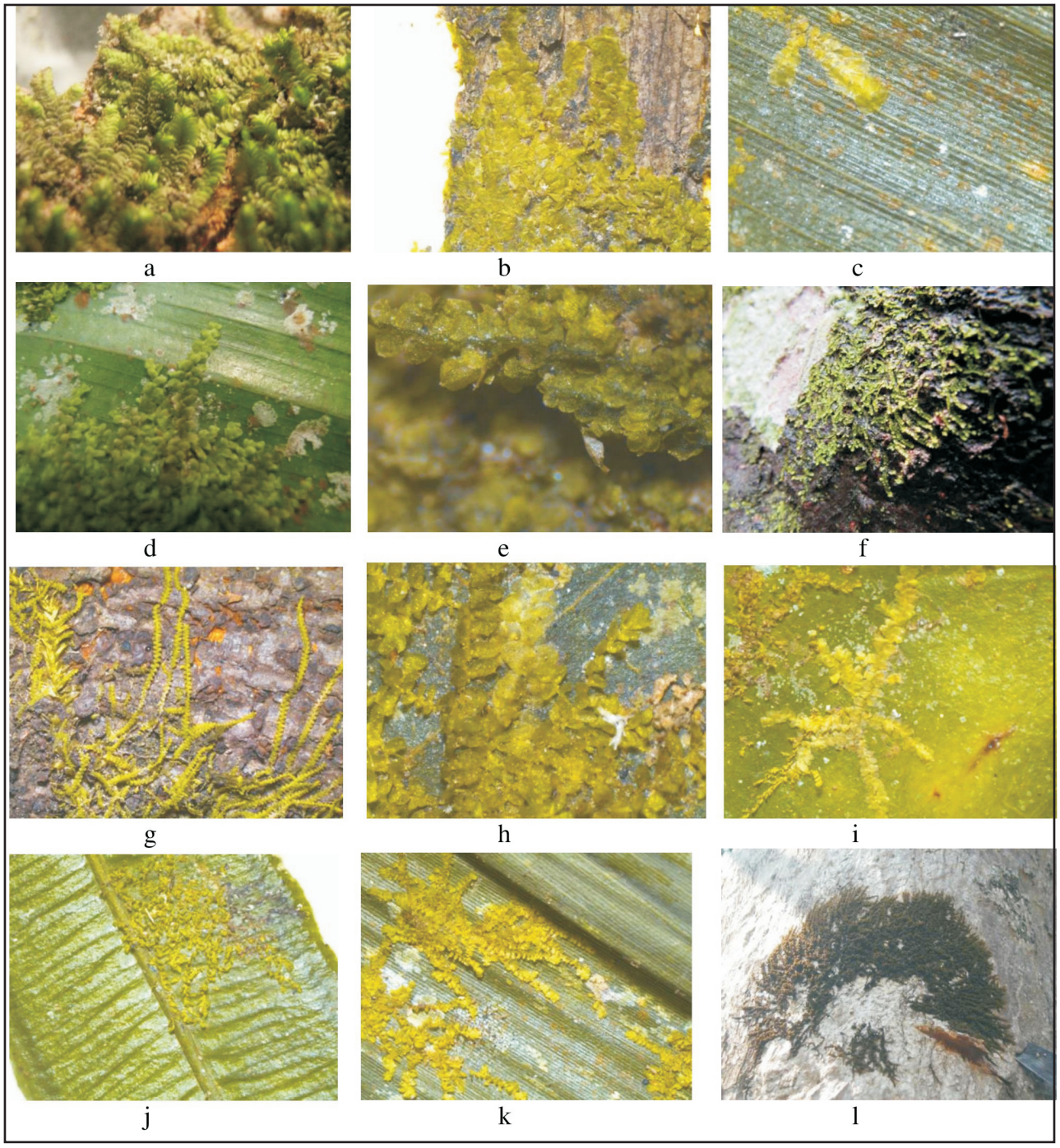

Fig. 3. Some members of the family Lejeuneaceae along-with their natural habitat; a. *Caudalejeunea reniloba*; b. *Cololejeunea furcilobulat*a; c. *Cololejeunea latilobula*; d. *Cololejeunea trichomanis*; e. *Lejeunea minutiloba*; f. *Lejeunea parva*; g. *Lejeunea tuberculosa*; h. *Leptolejeunea balansae*; *i. Leptolejeunea epiphylla*; j. *Leptolejeunea foliicola*; k. *Leptolejeunea latifolia; l. Lopholejeunea eulopha*.

contiguous – overlapping, entire – bilobed, margin entire – slightly wavy or dentate towards apex or absent. In fertile plants, androecia and gynoecia teminal on short lateral branches.

Below is the key to the genera identified in this study.

Key to the genera

1a. Underleaves absent…….....*Cololejeunea*
1b. Underleaves present…….....….2

2a. Underleaves entire…….....….3
2b. Underleaves bifurcate…….....……7

3a. Leaf cell walls with blackish pigmentation .....….....….*Lopholejeunea*
3b. Leaf cell walls without blackish pigmentation ..... ……...….....…….... 4

4a. Underleaves irregularly dentate at apex ..... …….....…….. 5
4b. Underleaves without any dentation at apex ..... …….....…….. 6

5a. Underleaves entire ……....…… *Ptychanthus*
5b. Underleaves retuse or shallowly notched at apex.... …….....….. *Caudalejeunea*

6a. Stem cells thin-walled without any distinct trigones; underleaf apex recurved ..... …….....…..*Acrolejeunea*
6b. Stem cells thick-walled with distinct trigones; underleaf apex not recurved ..... …….....….*Archilejeunea*

7a. Plants minute, 0.2 – 0.3 mm wide ..... …….....……...*Microlejeunea*
7b. Plants small to medium, more than 0.5 mm wide …….....…. 8

8a. Leaf lobules tubular – cylindrical, terminating into a sac distally ……..... *Colura*
8b. Leaf lobules inflated, ovate-oblong, bidentate, not terminating into a sac …….....….. 9

9a. Ocellus present in leaf …….....….10
9b. Ocellus absent in leaves …….....…….11

10a. Usually 1 – 3 intermediate thickenings present along each side of wall of leaf cells; underleaf lamina with 6 marginal cells surrounding numerous small rhizoidal initial cells ..... ……..…….....…….. *Leptolejeunea*
10b. Intermediate thickenings absent in leaf cells or sometimes 1 along each side of wall; underleaf lamina without 6 marginal cells surrounding numerous small rhizoidal initial cells ..... …….....……... *Drepanolejeunea*

11a. First tooth of leaf lobule prominent, hyaline papillae present at proximal side of first tooth ..…….....….*Lejeunea*
11b. Second tooth of leaf lobule prominent, hyaline papillae present at distal side of second tooth …….....……. *Cheilolejeunea*

Enumeration of the taxa

Genus 1: Acrolejeunea (Spruce) Schiffn.

Acrolejeunea is represented in the study area by single species viz. *A. infuscata*.

Acrolejeunea infuscata (Mitt.) J. Wang bis & Gradst., Bry. Div. Evo. 36 (1): 38. 2014.

Habitat: Epiphytic, on bark of higher plants under moist condition.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, *ca* 50 m, 06.05.2012, *S. Das* 25067.

Distribution: India (Arunachal Pradesh, Assam, Himachal Pradesh, Manipur, Meghalaya, Sikkim, West Bengal), Bhutan, China, Myanmar, Nepal, Philippines, Sri Lanka, Taiwan, Thailand [Das, 2009; Das and Sharma, 2013, 2016 (as *Trocholejeunea infuscata*); Singh *et al*., 2016].

Genus 2: Archilejeunea (Spruce) Schiffn.

Archilejeunea is represented in the study area by two species viz. *A. minutilobula* and *A. planiuscula*.

Key to the species:

1a. Medullary cells 12 – 15 in number in cross section of stem; leaf lobes rounded obtuse at apex; lobules smaller, 1/6-1/4 of lobe length; underleaves reniform……*A. minutilobula*
1b. Medullary cells 30 – 32 in number in cross section of stem; leaf lobes apiculate or occasionally obtuse at apex; lobules larger 1/4 – 1/3 of lobe length; underleaves orbicular ..... ……..... *A. planiuscula*

Archilejeunea minutilobula Udar & U.S.Awasthi, Geophytology 11 (1): 72. 1981.

Habitat: Epiphytic; growing on the bark of angiosperms.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, 25 km away from Kumbirgram, *ca* 70 m, 23. 01.2012, *S. Das* 25010a; in association with *C. latilobula* and *C. trichomanis*.

Distribution: India (Andhra Pradesh, Arunachal Pradesh, Assam, Kerala, Maharashtra, Tamil Nadu, West Bengal), endemic to India [Das & Singh, 2007; Das, 2009; Das and Sharma, 2013, 2016; Singh *et al*., 2016].

Archilejeunea planiuscula (Mitt.) Steph., sp. Hepat. 4: 731. 1911

Habitat: Epiphytic. Growing on the stem bark of the fig tree in the vicinity of the forest, under exposed condition.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, Indranagar, *ca* 80 m, 06.05.2012, *S. Das* 25080.

Distribution: India (Andaman & Nicobar Islands, Assam, Karnataka, Kerala, Tamil Nadu), Myanmar, Papua New Guinea [Das and Sharma, 2016 as *A. apiculifolia*; Singh *et al*., 2016].

Genus 3: Caudalejeunea (Steph.) Schiffn.

Genus *Caudalejeunea* is represented in the study area by single species, *C. reniloba*.

Caudalejeunea reniloba (Gottsche) Steph., Sp. Hepat. 5:16. 1912.

Habitat: Epiphytic. Growing on the stem bark of the angiospermic plant in dense forest; epiphyllous on *Licuala* leaf.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, Indranagar, *ca* 80 m, 06.05.2012, *S. Das* 25079; Lakhichhera, *ca* 105 m, 24.04.2014, *S. Das* 25261.

Distribution: India (Andaman & Nicobar Islands, Assam, Karnataka, Kerala), Cambodia, China, Fiji, Indonesia, Laos, Papua New Guinea, Philippines, Samoa, Sri Lanka, Australia, Micronesia [Das and Sharma, 2016 ; Singh *et al*., 2016].

Genus 4: Cheilolejeunea (Spruce) Schiffn.

Cheilolejeunea is represented in the study area by five species.

Key to the species:

1a. Leaf lobes suborbicular……..…...2
1b. Leaf lobes ovate – oblong……..…3

2a. Plants monoecious…….....*C. intertexta*
2b. Plants dioecious……*C. serpentina*

3a. Leaf lobes acute to apiculate at apex..... ……..... *C. subopaca*
3b. Leaf lobes rounded-obtuse at apex……...…4

4a. Leaf lobules large, *ca.* 3/5 – 2/3 of lobe length; second tooth acute, 3 – 5 cells long, sharp ..... ……..... *C. trapezia*

4b. Leaf lobules small, *ca.* 1/4 – 1/3 of lobe length; second tooth short, 1–3 cells long, obtuse ……..……...*C. krakakammae*

Cheilolejeunea intertexta (Lindenb.) Steph., Hedwigia 29:85. 1890.

Habitat: Terrestrial; growing on loose, moist soil in rock crevices under shady condition; epiphytic on bark of higher plants.

Specimen Examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, Malidar, ca 70 m, 15.04.2012, *S. Das* 25039b; Marwachhera-Lakhichhera junction, ca 100 m, 24.04.2014, *S. Das 25222 in association with L. flava.*

Distribution: India (Andaman & Nicobar Islands, Assam, Kerala, Meghalaya, Tamil Nau), China, Fiji, Hawaii, Indonesia, Japan, Malaysia, New Caledonia, New Zealand, Papua New Guinea, Philippines, Singapore, Sri Lanka, Taiwan, Thailand, Africa, Australia, Micronesia [Das and Sharma, 2016; Singh *et al*., 2016].

Cheilolejeunea krakakammae (Lindenb.) R.M. Schust., Beih. Nova Hedwigia 9: 112. 1963.

Habitat: Terrestrial, growing on loose soil over rock in moist and shady places.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, 25 km away from Kumbirgram, ca 70 m, 23.01.2012, *S. Das* 25009b.

Distribution: India (Arunachal Pradesh, Assam, Kerala, Manipur, Meghalaya, Tamil Nadu, West Bengal), Bhutan, China, Indonesia, Japan, Nepal, New Zealand, Philippines, Sri Lanka, Africa, Australia [Das and Sharma, 2016 as *C. giraldiana*; Singh *et al*., 2016].

Cheilolejeunea serpentina (Mitt.) Mizut., J. hattori Bot. Lab. 26: 171. 1963.

Habitat: Terrestrial.

Specimen Examined: India, Assam, Guwahati, 28.06.2013, *S. Das* 23001.

Distribution: India (Andamn & Nicobar Islands, Assam, Kerala, Meghalaya, Madhya Pradesh, Sikkim, Tamil Nadu,), Indonesia, Japan, Singapore, Sri Lanka, Thailand, Africa, Australia [Singh *et al*., 2016].

Cheilolejeunea subopaca (Mitt.) Mizut., J. Hattori Bot. Lab. 26:183. 1963.

Habitat: Epiphytic, on twig of angiosperms.

Specimen Examined: Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, *ca* 50 m, 06.05.2012, *S. Das* 25070a.

Distribution: India (Arunachal Pradesh, Assam, Kerala, Meghalaya, Sikkim, West Bengal), Bhutan, China, Nepal, Sri Lanka [Das and Sharma, 2013; Singh *et al.*, 2016].

Cheilolejeunea trapezia (Nees) Kachroo & R. M. Schust., J. Linn. Soc. Bot. 56:509. 1961.

Habitat: Epiphytic on twig.

Specimen examined: India, Assam, Dima Hasao district, Jatinga, *ca* 950 m, 25º13'N & 93º01'E, 15.04.2016, *S. Das* 28031.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Karnataka, Kerala, Manipur, Meghalaya, Sikkim, Tamil Nadu, West Bengal), Bhutan, Cambodia, China, Fiji, Indonesia, Japan, Korea, Malaysia, Nepal, New Caledonia, Papua New Guinea, Philippines, Samoa, Sri Lanka, Taiwan, Thailand, Vietnam, Australia, Europe, Micronesia (Singh *et al.*, 2016).

Genus 5: Cololejeunea (Spruce) Schiffn.

Cololejeunea is the largest genus found to occur in the study area. It is represented by 13 species.

Key to the species

1a. Leaf lobules dimorphic, both ligulate and/or inflated ……..…. 2
1b. Lobules monomorphic, inflated 5

2a. Leaf margin not bordered by any specialized hyaline or sigmoid cells……....*C. furcilobulata*
2b. Leaf margins bordered by hyaline or sigmoid cells ……..…….. 3

3a. Stem, in sections, bordered by 5 vertical rows of cortical cells; leaf margins bordered by sigmoid cells; lobule lanceolate - ciliate .....…... ..... *C. ceratilobula*
3b. Stem, in sections, bordered by 6 – 10 vertical rows of cortical cells; leaf margins bordered by rectangular to polygonal hyaline cells; lobule triangular, ovate or ligulate ..... …….....……. 4

4a. Leaf lobule with single, large tooth ..... ……....……*C. latilobula*
4b. Leaf lobule with 2 teeth ……..….. *C. lanciloba*

5a. Plants strongly appressed to the substratum; vitta cells present 6
5b. Plants loosely appressed to the substratum, vitta cells absent……..…….....7

6a. First tooth of leaf lobule 2-celled, curved towards the keel……....…….*C. floccosa*
6b. First tooth of leaf lobule 1 – 2-celled, not curved ……....……...*C. appressa*

7a. Dorsal papillosity on leaf cells well developed……....…..… 8
7b. Dorsal papillosity on leaf cells absent or weakly developed ……....…….…… 9

8a. Leaf lobes with pointed apex and spinose margin; dorsal papillosity spinose, spines acute …….....…… *C. spinosa*
8b. Leaf lobes with rounded apex and entire to crenate margin; dorsal papillosity papillose …….....…….. *C. mizutaniana*

9a. Nodular intermediate thickenings present in leaf cells 10
9b. Nodular intermediate thickenings absent in leaf cells …….……..11

10a. Leaf lobes lanceolate with acute apex ..... …….. *C. longifolia*
10b. Leaf lobes spathulate with rounded apex ..... ……..…. *C. denticulata*

11a. Leaf lobes with entire margin; stylus absent ……...….. *C. siangensis*
11b. Leaf cells with crenulated or serrulate margins; stylus present …….....…. 12

12a. Leaf lobes with irregularly serrulate margins; stylus unicellular…….....….. *C. serrulata*
12b. Leaf cells with crenulated margins; stylus 4 – 6 cells long ……..……. *C. trichomanis*

Cololejeunea appressa (A. Evans) Benedix, Feddes Repert. Spec. Nov. Regni Veg. Beih. 134: 31. 1953.

Habitat: Foliicolous, on leaf of angiosperms.

Specimen Examined: India, Assam, Bhuban Hill, ca 800 m, 07.03.2016, *S. Das* 27025.

Distribution: India (Andaman & Nicobar Islands, Assam, Karnataka, Kerala, Meghalaya, Tamil Nadu, West Bengal), Cambodia, China, Indonesia, Japan, Laos, Malaysia, New Caledonia, Papua New Guinea, Philippines, Sri Lanka, Taiwan, Thailand, Vietnam, Africa, Australia, South America [Singh *et al*., 2016].

Cololejeunea ceratilobula (P.C.Chen) R. M. Schust., Nova Hedwigia 9: 179. 1963.

Habitat: Foliicolous, on leaf of angiosperms.

Specimen Examined: India, Assam, Bhuban Hill, ca 800 m, 07.03.2016, S. Das 27025a.

Distribution: India (Arunachal Pradesh, Assam, Sikkim, Tamil Nadu), Cambodia, China, Indonesia, Japan, Malaysia, Sri Lanka, Taiwan, Thailand, Vietnam [Singh *et al*., 2016].

Cololejeunea denticulata (Horik.) S. Hatt., Bull. Tokyo Sci. Mus. 11:99.1944

Habitat: Epiphyllous on angiosperm leaf.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, on way to Doorbin, *ca* 130 m, 24.04.2014, *S. Das* 25259, in association with *C. siangensis and L. latifolia.*
Distribution: India (Assam), Bangladesh, China, Japan, Korea, Taiwan [Banu-Fattah, 2001; Zhu and So, 2001; Singh and Barbhuiya, 2012; Singh *et al.*, 2016].

Cololejeunea floccosa (Lehm. & Lindenb.) Steph., Hedwigia 28: 135. 1890.

Habitat: Foliicolous, on angiosperm Leaf.

Specimen Examined: India, Assam, Dima Hasao district, Jatinga, *ca* 950 m, 15.04.2016. *S. Das* 28040.

Distribution: India (Andaman & Nicobar islands, Arunachal Pradesh, Assam, Karnataka, Meghalaya, Sikkim, Tamil Nadu), Bangladesh, Cambodia, China, Fiji, Indonesia, Japan, Laos, Malaysia, New Caledonia, Papua New Guinea, Philippines, Sri Lanka, Taiwan, Thailand, Vietnam, Africa, Australia [Singh *et al*., 2016].

Cololejeunea furcilobulata (Berrie & E. W. Jones) R. M. Schust., Beih. Nova Hedwigia 9: 178. 1963.

Habitat: Epiphytic on angiosperms.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, *ca* 50 m, 06.05.2012, *S. Das* 25070; Bhuban Hill, *ca* 800 m, 07.03.2016. *S. Das* 27017.

Distribution: India (Assam, Karnataka, Kerala, Tamil Nadu), Africa [Wigginton, 2009; Singh and Barbhuiya, 2012; Verma *et al*., 2012; Singh *et al*., 2016].

Cololejeunea lanciloba Steph., Hedwigia 34: 250. 1895.

Habitat: Epiphyllous, on angiosperm leaf.

Specimen examined: India, Assam, Dima Hasao district, Jatinga, *ca 95*0 m, 25º13'N & 93º01'E, 15.04.2016, *S. Das* 28035.

Distribution: India (Andaman & Nicobar Islands, Assam, Kerala, Maharashtra, Manipur, Meghalaya, Sikkim, Tamil Nadu), Bangladesh, Cambodia, China, Fiji, Hawaii, Indonesia, Japan, Laos, Malaysia, Nepal, New Caledonia, Papua New Guinea, Philippines, Sri Lanka, Taiwan, Thailand, Vietnam, Africa, Australia (Singh *et al*., 2016).

Cololejeunea latilobula (Herzog) Tixier, Bryophyt. Biblioth. 27:156. 1985.

Habitat: Epiphytic, growing on the bark of angiosperms; epiphyllous, on bamboo leaf.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, 25 km away from Kumbirgram, ca 70 m, 23.01.2012, *S. Das* 25010b, in association with *A. minutilobula* and *C. trichomanis*; Bhuban Hill, *ca* 800 m, 07.03.2016. *S. Das* 27014.

Distribution: India (Arunachal Pradesh, Assam, Chhattisgarh, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Manipur, Meghalaya, Sikkim, Tamil Nadu, Uttar Pradesh, Uttarakhand, West Bengal), China, Fiji, Myanmar, Nepal, Taiwan, Vietnam, Africa [Das and Sharma, 2016; Singh *et al*., 2016].

Cololejeunea longifolia (Mitt.) Benedix ex Mizut., J. Hattori Bot. Lab. 26: 184. 1963.

Habitat: Epiphyllous, on *Zingiber* leaf.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, along Marwa stream, ca 90 m, 24.04.2014, *S. Das* 25202*;* Marwachhera-Lakhichhera junction, ca 100 m, 24.04.2014, *S. Das* 25224, 25226; in association with *C. trichomanis*.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Kerala, Manipur, Sikkim, West Bengal), Bhutan, China, Fiji, Indonesia, Japan, Korea, Malaysia, New Caledonia, Papua New Guinea, Sri Lanka, Taiwan, Thailand [Das, 2009; Barukial, 2011 as *L. longifolia*; Singh *et al.*, 2016].

Cololejeunea mizutaniana Udar & G. Srivast., Misc. Bryol. Lichenol. 9: 137. 1983.

Habitat: Epiphyllous, on *Ophiorhhiza* leaf.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, Lakhichhera Valluknala, ca 105 m, 24.04.2014, *S. Das* 25248.

Distribution: India (Assam, Karnataka, Kerala), endemic to India [Singh and Barbhuiya, 2012; Singh *et al*., 2016].

Cololejeunea serrulata Steph., Hedwigia 34: 252. 1895.

Habitat: Foliicolous, on angiosperm leaf.

Specimen Examined: India, Assam, Dima Hasao district, Jatinga, *ca 95*0 m, 15.04.2016, *S. Das* 28041.

Distribution: India (Arunachal Pradesh, Assam, Sikkim, West Bengal), China, Indonesia, Malaysia, Vietnam [Singh *et al*., 2016].

Cololejeunea siangensis G. Asthana & S. C. Srivast., Bryophyt. Biblioth. 60: 57. 2003.

Habitat: Epiphytic, appressed to rough stem bark of angiosperms; Epiphyllous on angiosperm leaf.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, 25 km away from Kumbirgram, *ca* 70 m, 23. 01.2012, *S. Das* 25001; Marwacherra, on way to Doorbin, *ca* 100 m, 24.04.2014, *S. Das* 25259b, in association with *C. denticulata and L. latifolia.*

Distribution: India (Arunachal Pradesh, Assam), endemic to India [Das and Sharma, 2012, 2013, 2016; Singh *et al*., 2016].

Cololejeunea spinosa (Horik.) Pande & R. N. Misra, J. Indian Bot. Soc. 22:166. 1943.

Habitat: Epiphyllous, on fern and angiosperm leaves.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, 4 km towards Doorbin peak along Marwa Nala, ca 90 m, 08. 03. 2014, *S. Das* 25162, 25165, 25173, 25192, in association with *R. tjibodensis*.

Distribution: India (Arunachal Pradesh, Assam, Sikkim, Tamil Nadu, West Bengal), China, Japan, Korea, Nepal, Philippines, Taiwan, Thailand [Das, 2009; Singh and Barbhuiya, 2012; Singh *et al*., 2016].

Cololejeunea trichomanis (Gottsche) Steph., Hedwigia 34: 252. 1895.

Habitat: Epiphytic, on bark of angiosperm; lignicolous, on decaying twig; epiphyllous, on *Zingiber* leaf.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, 25 km away from Kumbirgram, *ca* 70 m, 23. 01.2012, *S. Das* 25003, 25010; in association with *A. minutilobula* and *C. latilobula*; along Marwa stream, ca 90 m, 24.04.2014, *S. Das 25202, 25224, in association with C. longifolia.*
Distribution: India (Arunachal Pradesh, Assam, Karnataka, Manipur, Meghalaya, Sikkim, West Bengal), Cambodia, China, Fiji, Indonesia, Japan, Korea, Laos, Malaysia, Nepal, Papua New Guinea, Philippines, Singapore, Taiwan, Thailand, Vietnam, Australia [Das, 2009; Das and Sharma, 2013; Singh *et al.*, 2016].

6: Colura (Dumort.) Dumort.

Colura is represented in the study area by single species, *C. ari*.

Colura ari (Steph.) Steph. Sp. Hepat. 5: 936. 1916.

Habitat: Epiphytic, on bark.

Specimen Examined: India, Assam, Cachar district,

Lakhichhera, ca 90 m, 25º03'N & 92º44'E, 24.04.2014, S. *Das* 25196a.

Distribution: India (Andaman & Nicobar Islands, Assam), Bangladesh, Cambodia, China, Fiji, Indonesia, Malaysia, New Caledonia, Pakistan, Papua New Guinea, Philippines, Samoa, Sri Lanka, Vietnam, Australia. [Singh *et al*., 2016].

Genus 7: Drepanolejeunea (Spruce) Schiffn.

Drepanolejeunea is represented by single species viz. *D. yunnanensis* in the study area.

Drepanolejeunea yunnanensis (P.C.Chen) Grolle & R.L. Zhu, Nova Hedwigia 70: 388. 2000.

Habitat: Epiphyllous, on leaf of angiosperms.

Specimen Examined: India, Assam, Cachar district, Lakhichhera, along Valluknala, ca 90 m, ca 25º03'N & 92º44'E, 24.04.2014, S. *Das* 25240a.

Distribution: India (Arunachal Pradesh, Assam, Karnataka, Meghalaya, Manipur, West Bengal), China, Japan, Laos, Vietnam [Singh *et al.*, 2016].

Genus 8: Lejeunea Lib.

Lejeunea is a prolific genus in the study area being next to *Cololejeunea*. It is represented by seven species.

Key to the Species:

1a. Leaf lobule very much reduced, 4– 5 cells, *ca* 1/10 of lobe length ……..... *L. minutiloba*
1b. Leaf lobule larger, 1/7–1/3 of lobe length ..... ……..... 2

2a. Intermediate thickenings absent in leaf cells ……..... *L. alata*
2b. Intermediate thickenings present in leaf cells ……..... 3

3a. Underleaf margins with 1–3 teeth ..... ……... *L. anisophylla*
3b. Underleaf margins entire to wavy ……..…… 4

4a. Underleaves imbricate to contiguous ..…. *L. flava*
4b. Underleaves distant ……..……. 5

5a. Leaf lobules very small, 1/7–1/5 of lobe length .... ……..... *L. obscura*
5b. Leaf lobules larger, 1/4 – 1/3 of lobe length ....… 6

6a. Plants minute, upto 2 mm long, 0.3 – 0.5 mm wide ……... *L. parva*
6b. Plants larger, 5–10 mm long, 0.5–1.0 mm wide ……... *L. tuberculosa*

Lejeunea alata Gottsche in Gottsche *et al*., Syn. Hepat. 406. 1845.

Habitat: Epiphytic, on bark.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, Lakhichhera, ca 90 m, S. *Das* 25196.

Distribution: India (Arunachal Pradesh, Assam, Manipur, Sikkim, Uttarakhand, West Bengal), China, Comoro Archipelago, Fiji, Indonesia, Malaysia, Papua New Guinea, Samoa, Vietnam, Africa [Das, 2009; Dey & Singh, 2008, 2012; Das & Sharma, 2016; Singh *et al*., 2016].

Lejeunea anisophylla Mont., Ann. Sci. Nat. Bot. Ser. 2, 5: 116. 1861.

Habitat: Epiphyllous, on leaf of angiosperms.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, Valluknala, ca 90 m, S. *Das* 25240.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Manipur, Meghalaya, Sikkim), Chagos Archipelago, hina, Fiji, Hawaii, Indonesia, Japan, Malaysia, New Caledonia, New Zealand, Philippines, Samoa, Sri Lanka, Tahiti, Taiwan, Thailand, Tonga, Vietnam, Africa, Australia, Micronesia [Barukial *et al*., 2002 as *L. boninen*sis; Das, 2009; Dey and Singh, 2012; Singh *et al.*, 2016].

Lejeunea flava (Sw.) Nees, Naturgesch. Eur. Leberm. 3:277. 1838.

Habitat: Epiphytic on twig and bark of higher plants; epiphyllous on *Licuala* leaf.

Specimen examined: India, Assam, Cachar district, Barail Wildlife Sanctuary, Marwachhera-Lakhichhera, ca 100 m, 24.04.2014, *S. Das* 25197, 25203; Marwachhera-Lakhichhera junction, ca 100 m, 24.04.2014, *S. Das* 25222a, in *association with C. intertexta.*

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Himachal Pradesh, Karnataka, Kerala, Manipur, Meghalaya, Sikkim, Tamil Nadu, West Bengal), Bhutan, Cambodia, China, Galapagos, Fiji, Indonesia, Japan, Korea, Laos, Malaysia, Nepal, New Caledonia, New Zealand, Papua New Guinea, Philippines, Samoa, Singapore, Sri Lanka, Taiwan, Thailand, Vietnam, Africa, Australia, Europe, Micronesia, North America, South America [Dey and Singh, 2012; Singh and Barbhuiya, 2012; Singh *et al.*, 2016].

Lejeunea minutiloba A. Evans, Bull. Torrey Bot. Club 44: 525. 1917.

Habitat: Epiphyllous, on higher plant leaf.

Specimen examined: India, Assam, Cachar district, Bhuban Hill, ca 800 m, 07.03.2016, *S. Das 27003.*

Distribution: India (Arunachal Pradesh, Assam – **present study**, Tamil Nadu), North America [Stotler and Crandall-Stotler, 1977; Das and Singh, 2012; Singh *et al.*, 2016].

N.B.: New record for the state Assam.

Lejeunea obscura Mitt., J. Proc. Linn. Soc., Bot. 5: 112. 1861.

Habitat: Epiphytic, on bark of *Alstonia scholaris* along road side.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, *ca* 50 m, 06.05.2012, *S. Das* 25061a.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Manipur, Sikkim, Tamil Nadu, West Bengal), Bhutan, China, Indonesia, Nepal, Sri Lanka, Taiwan [Das, 2009; Das and Sharma, 2013; Singh *et al*., 2016].

Lejeunea parva (S. Hatt.) Mizut., Misc. Bryol. Lichenol. 5:178. 1971.

Habitat: Epiphytic, on rough stem bark of angiosperm.

Specimen examined: India, Assam, Dima Hasao district, Jatinga, *ca* 950 m, 25º13'N & 93º01'E, 15.04.2016, *S. Das* 28030.

Distribution: India (Assam, Meghalaya), China, Japan, Korea, Singapore, Taiwan, Thailand (Singh *et al.*, 2016).

Lejeunea tuberculosa Steph., Sp. Hepat. 5: 790. 1915.

Habitat: Epiphytic on twig or bark of higher plants.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, Lakhhichhera, ca 105 m, 24.04.2014, *S. Das* 25260, 25264.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Kerala, Manipur, Meghalaya, Sikkim, Tamil Nadu, West Bengal), Bhutan, China, Indonesia, Nepal, Philippines, Sri Lanka, Thailand, Africa [Singh and Barbhuiya, 2012; Singh *et al*., 2016].

Genus 9: Leptolejeunea (Spruce) Schiffn.

Leptolejeunea is represented in the study area by five species.

Key to the Species:

1a. Ocelli arranged scatteredly in leaf lobes …..... 2
1b. Ocelli arranged in a non continuous longitudinal series in leaf lobes ..... *L. epiphylla*

2a. Ocellii 2–3 per leaf ……..... *L balansae*
2b. Ocelli 4–15 per leaf ……...…….. 3

3a. Underleaf lobes uniseriate at base .... *L. elliptica*
3b. Underleaf lobes 2 cells wide at base …....…….. 4

4a. Leaf lobes oblong-ovate – ovate, underleaf lobes always 2 cells wide at base, uniseriate at apex ..……...……... *L. latifolia*
4b. Leaf lobes oblong – obovate, underleaf lobes 2 cells wide throughout ……...…..….... *L. foliicola*

Leptolejeunea balansae Steph., Hedwigia 35: 105. 1896.

Habitat: Epiphyllous on angiosperm leaf.

Specimen examined: India, Assam, Cachar district, Bhuban Hill, ca 800 m, 07.03.2016, *S. Das* 27011.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Kerala, Manipur, Sikkim, Tamil Nadu, Wes Bengal), Cambodia, China, Indonesia, Laos, Malaysia, Thailand, Vietnam [Singh and Barbhuiya, 2012; Singh *et al*., 2016].

Leptolejeunea elliptica (Lehm. & Lindenb.) Schiffn., Hepat. (Engl.- Prantl) 126. 1895.

Habitat: Epiphyllous, on fern leaf, on Rubber plantation, on *Canna* leaf and on Fig leaf.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, *ca* 50 m, 06.05.2012, *S. Das* 25072; along bank of Marwa stream, *ca* 50 m, 24.04.2014, *S. Das* 25199, 25200, 25204.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Karnataka, Kerala, Manipur, Meghalaya, Sikkim, Tamil Nadu, West Bengal), Bangladesh, Bhutan, Cambodia, China, Fiji, Galapagos, Indonesia, Japan, Laos, Malaysia, Nepal, Philippines, Singapore, Sri Lanka, Taiwan, Thailand, Vietnam, Africa, Australia, Micronesia, North America, South America [Barukial *et al*., 2002 as *L. subacuta*; Singh and Barbhuiya, 2012; Singh *et al*., 2016].

Leptolejeunea epiphylla (Mitt.) Steph., Sp. Hepat. 5: 380. 1913.

Habitat: Epiphyllous, on higher plant leaf.

Specimen examined: India, Assam, Cachar district, Bhuban Hill, ca 800 m, 07.03.2016, *S. Das* 27007, 27008.

Distribution: India (Andaman & Nicobar Islands, Assam, Kerala, Tamil Nadu), Cambodia, China, Fiji, Indonesia, Japan, Laos, Malaysia, New Caledonia, Papua New Guinea, Philippines, Sri Lanka, Taiwan, Thailand, Vietnam, Africa [Verma *et al.*, 2012; Singh *et al.*, 2016].

Leptolejeunea foliicola Steph., Hedwigia 35: 106. 1896.

Habitat: Epiphyllous, on fern frond.

Specimen examined: India, Assam, Cachar district, Lakhichhera, ca 105 m, 24.04.2014, *S. Das* 25266.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam – **present study**, Kerala, Meghalaya, Tamil Nadu), Bangladesh, Cambodia, China, Indonesia, Malaysia, Philippines, Thailand [Singh, 1996; Das, 2009, Dey and Singh, 2012; Singh *et al.*, 2016].

N.B.: New record for the state Assam.

Leptolejeunea latifolia Herzog, Memoranda Soc. Fauna Fl. Fenn. 26: 58. 1950.

Habitat: Epiphyllous, on bamboo and other angiosperm leaf.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, on way to Doorbin, *ca* 100 m, 24.04.2014, *S. Das* 25259, in association with *C. denticulata* and *C. siangensis;* Bhuban Hill, ca 800 m, 07.03.2016, *S. Das* 27001.

Distribution: India (Assam – **present study**, Sikkim, West Bengal), Bhutan, China, Nepal, Thailand [Singh *et al*., 2016].

N.B.: New record for the state Assam.

Genua 10: Lopholejeunea (Spruce) Schiffn.

Lopholejeunea is represented in the study area by four species.

Key to the Species:

1a. Underleaves *ca*. 7 times wider than stem ……..……*L. eulopha*
1b. Underleaves 2 – 5 times wider than stem …...… 2

2a. Leaf lobes acute to apiculate at apex ..... ……...…..*L. nigricans*
2b. Leaf lobes rounded at apex ……...…… 3

3a. Stem in cross section with 10 – 18 medullary cells ……..……....*L. subfusca*
3b. Stem in cross section with 25 – 32 medullary cells ……....….*L. sikkimensis*

Lopholejeunea eulopha (Taylor) Schiffn., Hepat. (Engl.-Prantl) 129. 1895.

Habitat: Epiphytic, on bark of angiosperms.

Specimen Examined: India, Assam, Cachar district, Lakhichhera, ca 105 m, 25º03'N & 92º44'E, 24.04.2014, *S. Das* 25272.

Distribution: India (Andaman & Nicobar Islands, Assam), Cambodia, China, Fiji, Indonesia, Japan, Malaysia, New Caledonia, Papua New Guinea, Philippines, Samoa, Solomon Islands, Sri Lanka, Taiwan, Thailand, Vietnam, Africa, Australia, South America [Singh *et al.*, 2016].

Lopholejeunea nigricans (Lindenb.) Schiffn., Consp. Hepat. Arch. Ind. 293. 1898.

Habitat: Terrestrial, on loose moist soil in rock crevices.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, from the bank of river Boleswar at Malidar, *ca* 70 m, 15.04.2012, *S. Das* 25037a.

Distriution: India (Assam, Arunachal Pradesh, Kerala, Manipur, Meghalaya), Bangladesh, China, Fiji, Hawaii, Indonesia, Japan, Malaysia, Myanmar, Nepal, New Caledonia, Papua new Guinea, Philippines, Samoa, Singapore, Sri Lanka, Tahiti, Taiwan, Thailand, Africa, Australia, South America [Das, 2009; Das and Sharma, 2013; Singh *et al.*, 2016].

Lopholejeunea sikkimensis Steph., Sp. Hepat. 5: 87. 1912. var. ***sikkimensis***

Habitat: Epiphytic. Growing on the stem bark of the fig tree.

Specimen examined: India, Assam, Cachar district, Indranagar, *ca* 80 m, 24º55'N & 92º54'E, 07.04.2013, *S. Das* 25080a.

Distribution: India (Andaman & Nicobar Islands, Assam, Chhattisgarh, Karnataka, Kerala, Madhya Pradesh, Manipur, Meghalaya, Sikkim, Tamil Nadu, Uttarakhand, West Bengal), Bhutan, Nepal [Singh *et al.*, 2016].

Lopholejeunea subfusca (Nees) Schiffn., Bot. Jahrb. Syst. 23: 593. 1897.

Habitat: Terrestrial, on bare rock wall and rock crevices with dripping water, in shady condition; epiphytic, on bark of *Alstonia scholaris* along road side and on twig.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, *ca* 50 m, 06.05.2012, *S. Das* 25061, 25063a; Lakhichhera, ca 105 m, 24.04.2014, *S. Das* 25271.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Bihar, Karnataka, Kerala, Meghalaya, Tamil Nadu, Sikkim, West Bengal), Bangladesh, Bhutan, Cambodia, China, Fiji, Indonesia, Japan, Korea, Malaysia, Nepal, New Caledonia, Papua New Guinea, Philippines, Samoa, Singapore, Sri Lanka, Tahiti, Taiwan, Thailand, Tonga, Vietnam, Africa, Australia, Micronesia, North America [Das, 2009; Das and Sharma, 2013; Singh *et al.*, 2016].

Genua 11: Microlejeunea Steph.

Genus *Microlejeunea* is represented in the study area by two species, viz. *M. punctiformis* and *M. ulicina*.

Key to the species:

1a. Leaf lobes sub-acute at apex; tooth of lobule slightly acute .....*M. punctiformis*
1b. Leaf lobes rounded at apex; tooth of lobule obtuse .....*M. ulicina*

Microlejeunea punctiformis (Taylor) Steph., Hedwigia 29: 90. 1890.

Habitat: Terrestrial, on loose moist soil in rock crevices under shady condition; epiphytic, on bark of *Alstonia scholaris* along road side.

Specimen examined: Assam, Cachar district, Barail Wildlife Sanctuary, Marwacherra, *ca* 50 m, 06.05.2012, *S. Das* 25061b, 25068a.

Distribution: India (Andaman & Nicobar islands, Arunachal Pradesh, Assam, Kerala, Manipur, Meghalaya, Sikkim, Tamil Nadu, West Bengal), Bhutan, China, Nepal, Singapore, Sri Lanka, Taiwan, Thailand, Vietnam, Australia [Das and Sharma, 2013; Singh *et al*., 2016].

Microlejeunea ulicina (Taylor) Steph., Hedwigia 29: 88. 1890.

Habitat: Terrestrial, on loose soil in shady places.

Specimen examined: India, Assam, Dima Hasao district, Jatinga, *ca* 950 m, 25º13'N & 93º01'E, 15.04.2016, *S. Das* 28029.

Distribution: India (Arunachal Pradsh, Assam, Kerala, Meghalaya, Tamil Nadu, West Bengal), China, Japan, Korea, Nepal, Philippines, Russia, Singapore, Taiwan, Thailand, Turkey, Vietnam, Africa, Europe, Macronesia, North America (Singh *et al*., 2016).

Genus 12. Ptychanthus Nees

Ptychanthus is represented in the study area by single species, *P. striatus*.

Ptychanthus striatus (Lehm. & Lindenb.) Nees, Naturgesch. Eur. Leberm. 3:212. 1838.

Habitat: Terrestrial, on loose moist soil; epiphytic, on twig.

Specimen examined: India, Assam, Dima Hasao district, Haflong, *ca* 900 m, 25º16'N & 93º01'E 14.04.2016, *S. Das* 28010*; Jatinga, ca* 950 m, 25º13'N & 93º01'E, 15.04.2016, *S. Das* 28032.

Distribution: India (Andaman & Nicobar Islands, Arunachal Pradesh, Assam, Goa, Himachal Pradesh, Karnataka, Kerala, Maharashtra, Manipur, Meghalaya, Madhya Pradesh, Nagaland, Sikkim, Tamil Nadu, Uttarakhand, West Bengal), Bhutan, China, Fiji, Indonesia, Japan, Laos, Malaysia, Nepal, New Zealand, Papua New Guinea, Philippines, Samoa, Sri Lanka, Taiwan, Thailand, Vietnam, Africa, Australia (Singh *et al*., 2016).

CONCLUSION

Moist tropical and subtropical rain forests are the main abode of bryophytic vegetation including Marchantiophyta. Among them, it is reported that family Lejeuneaceae often dominate the cryptogamic flora and may represent more than 70% of the species inventory (Gradstein, 1994). The present study also reveals that the study area ia an abode of Lejeuneaceae members It is noticed from this study, that some explored areas of the state are under tremendous threat in terms of their floral wealth mainly due to

anthropogenic activities. This is also creating change in the extremely sensitive microhabitat of the bryophytic taxa. As most of the members of Lejeuneaceae are epiphytic, loss of higher plants acting as hosts (Das *et al*., 2023) for those epiphytes is also exerting indirect pressure on the Lejeuneaceous taxa by destruction of their habitats. Habitat destruction due to cutting of woods, collection of bamboos, jhum cultivation, pineapple plantation, etc. is challenging survival of these extremely vulnerable taxa. Species like *Archilejeunea planiuscula, Caudalejeunea reniloba, Cheilolejeunea intertexta, Cololejeunea latilobula, Cololejeunea siangensis*, etc. were found rare and threatened within the study area. So, documentation of all the bryophyte taxa in this region is in urgent need to know the exact field status of the taxa and to opt subsequent conservation measures for them.

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The author is thankful to the Principal, Sundarban Hazi Desarat College for encouragement. She is indebted to the Head, Department of Life Science & Bioinformatics, Assam University, Silchar for providing assistance to carry out the research work. She is thankful to the Officer in-charge, Assam Forest Department for giving permission for exploration work in different areas of the state. Thanks are also due to Science & Engineering Research Board (SERB), New Delhi for financial assistance.

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SHORT COMMUNICATION

Two new distributional records of grasses from Tripura, India

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During field surveys at different localities in Tripura since 2014, as a consequence of morpho-taxonomic studies on grasses, the first author came across and collected some notable specimens from the study area. After critical morphological investigation and perusal of relevant authentic literature (Bor, 1940 and 1960; Clayton and Renvoize, 1986; Shukla, 1996; Moulik, 1997; Kabeer and Nair, 2009), the specimens were identified as *Setaria barbata* (Lam.) Kunth and *Urochloa reptans* (L.) Stapf. Further consultation of herbarium specimens at CAL and ASSAM and scrutiny of pertinent literature (Deb, 1981-83; Karthikeyan *et al.*, 1989; Datta *et al.*, 2008; Kellogg *et al.*, 2020), it has been revealed that they are unreported so far from Tripura. Therefore, these two species constitute new distributional records of grasses from the state Tripura, India. Out of the two species, *Urochloa reptans* was found abundantly in some region of the state; whereas, *Setaria barbata* was rare in the study area.

Following traditional morpho-taxonomical research methodology, the present study comprised extensive and intensive field surveys in the different localities of the study area, collection of fresh plant specimens and critical morpho-taxonomic analysis with the help of stereo-zoom dissecting microscope as well as compound microscope. Identification was carried out following keys available in different authentic literature (Shukla 1996; Moulik 1997; Kabeer and Nair, 2009). Matching of materials with the specimens housed at ASSAM and CAL and with the Type specimens available at Kew Herbarium were also done. IPNI (http://www.ipni.org) was followed for updated nomenclature. Here, in addition to detailed descriptions, illustrations are also provided for their easy identification. Voucher specimens have been deposited at the Herbarium of Assam University, Silchar (AUSCH).

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SYSTEMATIC ENUMERATION

1. Setaria barbata (Lam.) Kunth, Revis. Gramin. 1: 47. 1829; Bor, Grasses Burma, Ceylon, India & Pakistan 360. 1960; Sreek. & V.J. Nair, Fl. Kerala Grasses 303. 1991; Shukla, Grass. N.E. India 358. 1996. *Panicum barbatum* Lam., Tab. Encycl. 1: 171. 1791.

Tufted annual. Culms 20-75 cm, slender above, creeping at base with rooting at lower nodes. Leaf blades 9-25 × 0.7-4.5 cm; elliptic-lanceolate, plicate, folded basally, apically acuminate, narrowed basally, hispid, margin serrulate; ligule a row of white hairs; sheaths white tuberculate hairy. Inflorescence contracted to narrowly open panicle, 7-18 cm long, sub-whorled, branching many. Spikelets 2-2.5 mm; elliptic to oblong, acute apically, not crowded; bristles

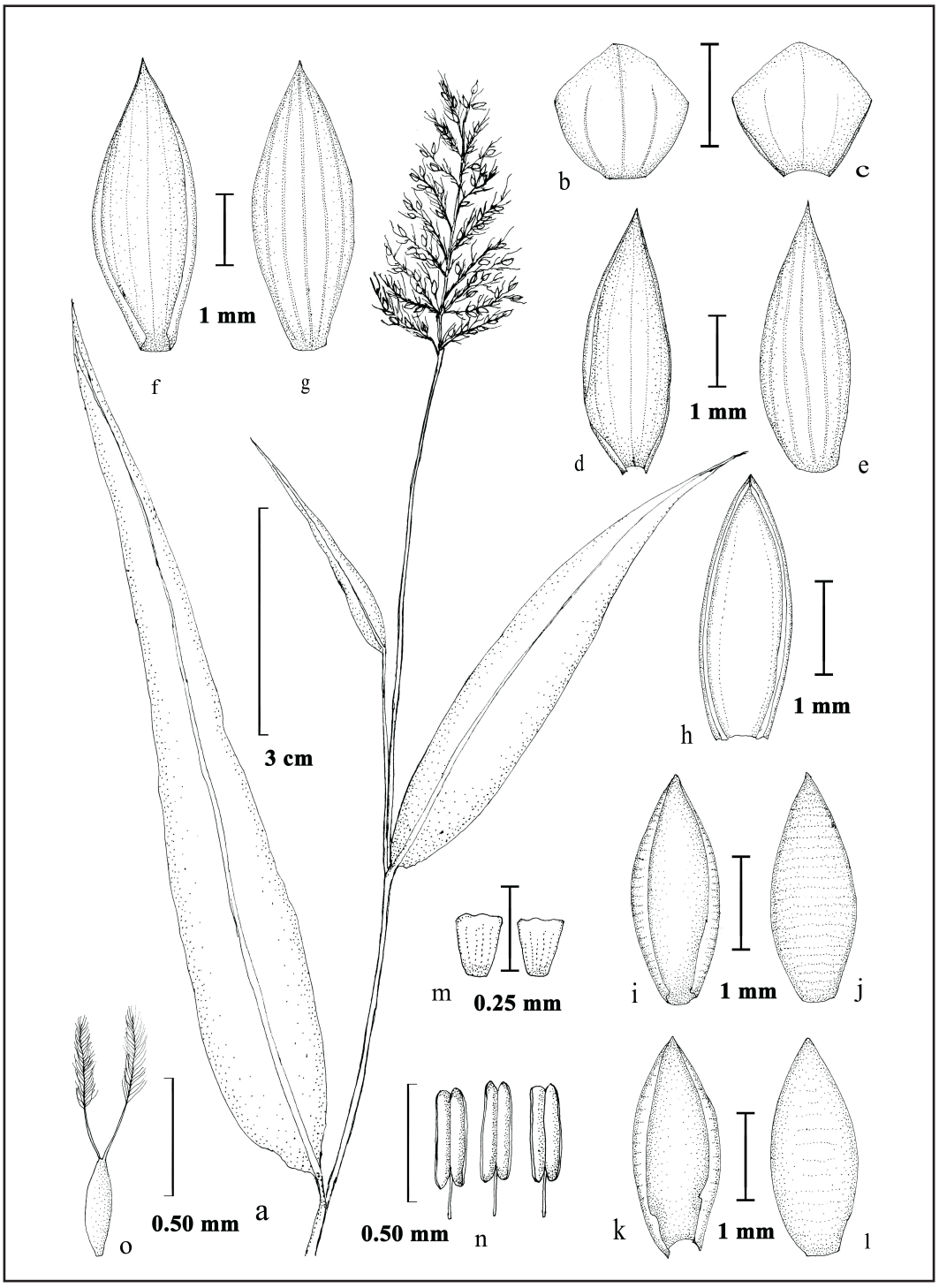

Fig. 1. *Setaria barbata* (Lam.) Kunth a. Habit; b. Lower glume (abaxial view); c. Lower glume (adaxial view); d. Upper glume (abaxial view); e. Upper glume (adaxial view); f. Lower lemma (adaxial view); g. Lower lemma (abaxial view); h. Lower Palea (abaxial view); i. Upper lemma (adaxial view); j. Upper lemma (abaxial view); k. Upper palea (adaxial view); l. Upper palea (abaxial view); m. Lodicules; n. Stamens; o. Pistil.

5 mm long, barbellate; pedicels cupuliform at tip. Lower glume 0.77-1.2 mm long; ovate, cup-shaped, acute apically, membranous, greenish, 3-veined. Upper glume 1.45-2.2 mm long, obtuse to acute apically, 5-veined, smooth, greenish. Florets 2; basal barren or male, upper hermaphrodite. Lower lemma broadly elliptic, concave, 2-2.3 mm, acute apically, chartaceous, greenish, margins inrolled, 7-veined. Lower palea elliptic, 1.7-2.5 × *c*. 1.2 mm, acute to obtuse apically, membranous, 2-veined, hyaline, keeled; keels winged. Upper lemma 1.7–2.2 × *c*. 1.4 mm, elliptic-lanceolate, apically acute or shortly mucronate, chartaceous crustaceous, surface rugose, margins inrolled, faintly 3-veined. Upper palea oblong-elliptic, acute to acuminate at apically, crustaceous, rugulose, margins inrolled, 2-veined, keeled. Stamens 3. Ovary oblong-elliptic; styles 2; stigmas 2, plumose, purple to pink.

Fl. & Fr.: July – November.

Habitat: Along roadsides, in waste lands.

Distribution: India: Andaman, Assam, Bihar, Karnataka, Kerala, Madhya Pradesh, Tamil Nadu, West Bengal (Shukla, 1996; Kabeer and Nair, 2009) and Tripura (present report); Tropical Africa, Tropical Asia, Tropical America.

Specimen examined: India: Tripura, West district, North Bardowali, 12.11.2015, *S. Ghosh* 12731, Fl. (AUSCH).

IUCN status: Not evaluated.

Uses: Fodder grass.

2. Urochloa reptans (L.) Stapf, Fl. Trop. Afr. [Oliver *et al*.] 9: 601. 1920. *Panicum reptans* L., Syst. Nat. ed. 10. 2: 870. 1759. *Brachiaria reptans* (L.) C.A. Gardner and C.E. Hubb. in Hooker's Icon. Pl. 34(3): pl. 3363. f. 3. 1938; Bor, Grasses Burma, Ceylon, India & Pakistan 285. 1960; Chandrab. ad N.C. Nair, Fl. Coimbatore 337. 1988; Karthik. *et al*., Fl. Ind. Enum. Monocot. 191. 1989; Sreek. and V.J. Nair, Fl. Kerala Grasses 224. 1991; U. Shukla, Grasses N.E. India 313. 1996; Kabeer and V.J. Nair, Fl. Tamil Nadu Grasses 222. 2009.

Tufted annual. Culm 12-40 cm high; creeping, decumbent. Leaf blades 4-6.5 cm, lanceolate, stiff, shallow cordate basally, both surfaces sparsely covered with white hairs, margins wavy; sheath loose, densely hairy on one margin; ligule a row of hairs. Inflorescence of 4-6 racemes, each raceme 2.2-4.5 cm long; rachis of the raceme triquetrous, sparsely hairy. Spikelets paired, solitary apically, secund, compact, 1-2 mm long, elliptic, yellowish green; pedicel hairy with 1-few bristles. Lower glume *c*. 0.65 mm; obovate, truncate, papery, 1-3-veined, mid-nerve prominent. Upper glume 1.6-2 × 0.5-0.7 mm; ovate to elliptic, chartaceous, strongly 5-7-veined, glabrous. Florets 2, basal floret barren, upper hermaphrodite. Lower lemma 5-veined, glabrous. Lower palea 1-1.3 × *c*. 0.5 mm, oblong, thin, hyaline, membranous, 2-veined, keeled. Upper lemma *c*. 1.3 mm; ovate, slightly mucronate, rugulose, 3-5-veined, brownish green. Upper palea elliptic, crustaceous, rugulose, faintly 2-veined. Lodicules 2, truncate. Stamens 3; anther brownish yellow. Pistil *c*. 2 mm; ovary elliptic; style 2; stigma 2, feathery, purplish green.

Fl. & Fr.: June – November.

Habitat: Shady areas of forest floors, hill slopes, along the roadsides.

Distribution: INDIA: Andaman, Andhra Pradesh, Assam, Bihar, Daman and Diu, Gujarat, Haryana, Himachal Pradesh, Kashmir, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Meghalaya, Odisha, Punjab, Rajasthan, Tamil Nadu, Uttarakhand, Uttar Pradesh, West Bengal (Shukla, 1996; Kabeer and Nair, 2009) and Tripura (present report); Pan Tropical.

Specimens Examined: INDIA: Tripura, Khowai district, Teliamura, 22.07.2015, *S. Ghosh* 12670, Fl.; Khowai district, way to Chebri, 22.06.2016, *S. Ghosh* 12750, Fl.; Dhalai district, on the way to Ambasa, 24.10.2017, *S. Ghosh* 12877, Fl.; Unakoti district, Pecharthal, 17.09.2018, *S. Ghosh* 13069, Fl.; Dhalai district, Chailengta, 02.11.2018, *S. Ghosh* 13027, Fl. (AUSCH).

IUCN status: Least concern (LC).

Uses: Fodder grass; soil binder.

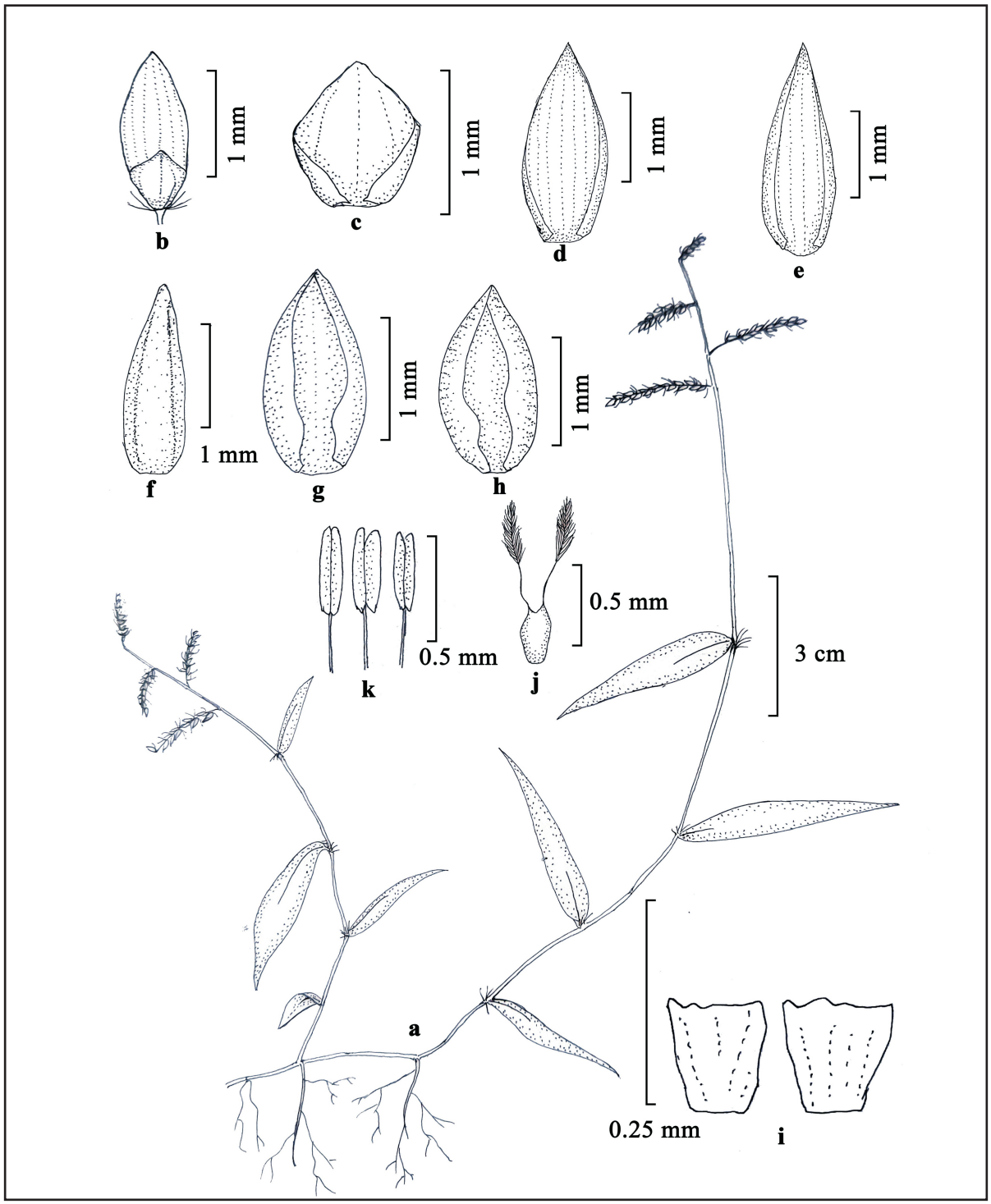

Fig. 2. *Urochloa reptans* (L.) Stapf : a. Habit; b. Single spikelet; c. Lower glume (adaxial view); d. Upper glume (adaxial view); e. Lower lemma (adaxial view); d. Upper glume (adaxial view); e. Lower lemma (adaxial view); f. Lower palea (adaxial view); g. Upper lemma (adaxial view); h. Upper palea (adaxial view); i. Lodicules, j. pistil; k. Stamens.

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IPNI. *International Plant Names Index* [Internet]. The Royal Botanic Gardens, Kew, Harvard University Herbaria & Libraries and Australian National Botanic Gardens.

Author correction: The research article entitled 'Antibacterial activity of some unifloral honeys from Eastern India' published in the volume 73(2): 81-88, December (2019) requires an author correction to an error in Figure 1 of page 84; necessary corrections are made in the photographs B and E that are provided below.

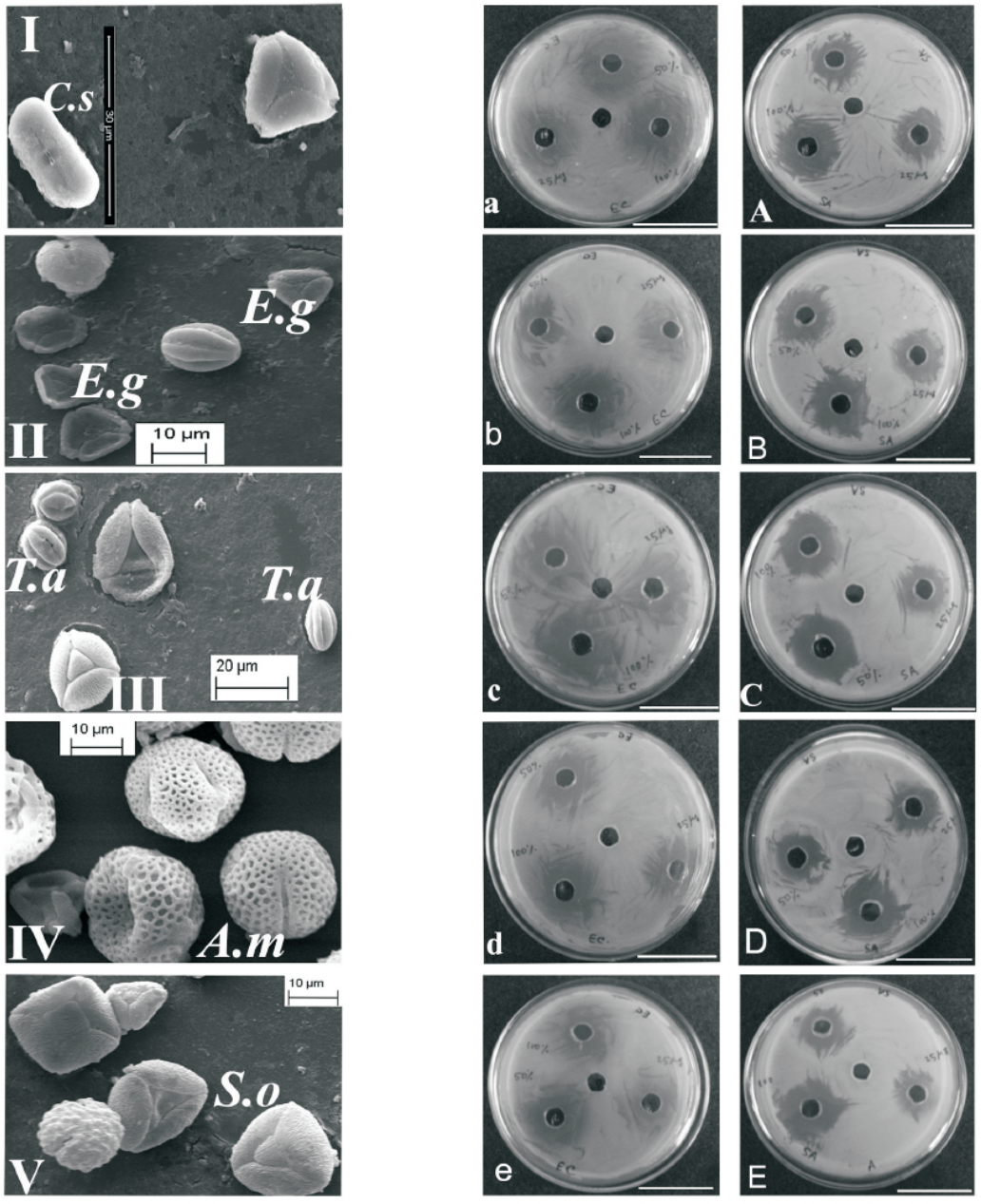

Fig.1. SEMs (I-V) of pollen assemblage of the unifloral honey samples under study and photographs of zone of inhibition in Agar cup assay (a-E) to show antibacterial activity of these honeys against the ATCC isolates of *E. coli* and *S. aureus*: I. *Coriandrum sativum* pollen grains in sample Jtrl-1; II. *Eucalyptus globulus* pollens in Jrsm-2; III. *Terminalia arjuna* pollens in Geti-2; IV. *Aegle marmelos* pollens in Gdpi-1; V. *Scleichera oleosa* in sample Ggta-1; a-e against *E. coli*; A-E against *S. aureus* (Bar = 5 cm unless stated otherwise)

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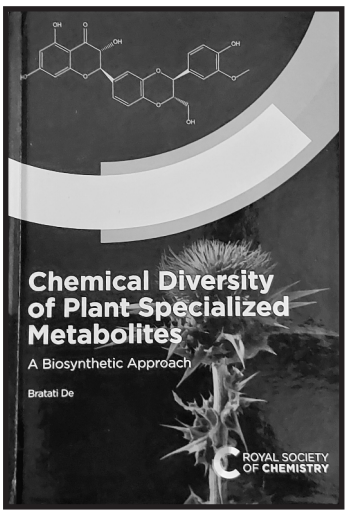
BOOK REVIEW

Chemical Diversity of Plant Specialized Metabolites: A Biosynthetic Approach. Bratati De. Royal Society of Chemistry, London, 2023. Hardback ISBN 978-1-83767-045-1, pp. i-x + 310, £ 99.00.

Plants produce a plethora of low molecular weight compounds, usually < 1500 Da. They were functionally separated into three overarching categories: primary metabolites, required for autonomous plant growth and development; secondary metabolites, which mediate plant–environment interactions; and phytohormones, which regulate organismal processes and metabolism. For many decades, this distinction shaped our thinking and research on plant metabolism. Though no clear-cut boundaries were ever established between these metabolite classes, recent research have totally blurred the distinction between this functional trichotomy[1]. For instance, we now know that some specialized metabolites (SMs) do exert control over plant tissue development. The enzymatic activity of thalianol synthase and thalianol acyltransferase 2 (involved in the biosynthesis of thalianol and other triterpenes (in *Arabidopsis thaliana*), exerting control over root development, is a case in point[2].



Several classes of so-called plant secondary metabolites have been shown to serve as both regulators and as primary metabolites, paving the way for their re-integration into primary metabolic network, or even to the suggestion, that they be considered as primary metabolites *sensu lato*. No surprises therefore, that the term 'plant secondary metabolism' itself has gradually morphed into a new epithet -- 'plant specialized metabolism' (PSM). The function of these diverse 200,000 odd, mostly lineage-specific set of diverse molecules, accumulated in only small amounts under particular conditions, does not appear to be 'secondary', at either the intuitive or at the experimental level. For, not without reason does natural selection conserve an array of biochemical pathways, through ca. 470 million years since terrestrialization of green plants. However, attempts to understand the evolutionary history of the specialized metabolome in the context of plant terrestrialization has just begun. Survival is the primary instinct of all life and plants are no exception. Their sessile habit forces them to develop, sharpen and apply diverse tricks to hoodwink, evade, resist herbivores and pathogens; to communicate, respond to specific environmental and developmental cues, abiotic stresses; manipulate and refashion their surroundings and the associated microbiome with plant-plant, plant-insect and plant-microbe interaction. Plants perform this feat by selective activation of specific metabolic pathways in a spatiotemporal manner, that interact in complex modular networks. They are also adept at switching metabolic pathways. Thus, small changes in amino acid sequence in rapidly evolving enzymes, can entirely shift the production of specialized metabolites within (e.g., monoterpenes) or between subclasses (e.g. monoterpene to sesquiterpene), or even between classes (e.g. phenolic acid to a flavonoid). Understanding their variability, biosynthetic pathway and biological role will naturally entail extensive phytochemical surveys in different taxa. However, acquisition of PSMs in diverse taxa through convergent, divergent and parallel evolution remains an enigma.

Plants constantly update their specialised metabolite repertoire, to enhance their ecological fitness. Seen in this way, PSM evolution is a virtual 'arms race' for acquiring adaptive chemical traits, not unlike those of pathogen effectors vis-à-vis plant immune response. A modular-acting complex of transcription factors-- the master regulators --lies at the

heart of the evolved combinatorial control of PSMs. Intriguingly, they have also managed to avoid autotoxicity by toxic metabolites via conjugation (e.g. acylation, glycosylation, methylation), sequestration to specialised organs, intracellular organelles, or by secretion to extracellular spaces. Apart from the emergence of new catalysts and consequent neofunctionalisation during plant metabolic evolution, optimal multifunctionality of diverse specialized metabolic processes have accrued through various adaptive mechanisms at subcellular, cellular, tissue, organ and interspecies levels. In general, however, the PSMs derive from primary metabolite precursors through the action of modifying enzymes such as hydroxylases, oxygenases, methyltransferases, and glycosyltransferases. The specialized metabolic enzymes tend to have lower catalytic efficiency and greater substrate promiscuity than primary metabolic enzymes. The genes dedicated to specialized metabolism are believed to have arisen from their counterparts in primary metabolism by gene duplication, mutation and subsequent diversification. Several biosynthetic gene clusters (BGCs) of related genes that produce several classes of specialized metabolites (terpenes, alkaloids, benzoxazinoids and cyanogenic glucosides) have even been shown to behave like prokaryotic operons. Such gene clusters include regulatory sequences, promoters, coding sequences and intergenic regions. It has been suggested that cluster arrangement may facilitate co-expression, coinheritance, assembly of metabolic channels, and regulatory chromatin changes. As more genomes are being sequenced, more BGCs are being discovered. It is now even possible to predict computationally the existence of new BGCs – leading to the discovery of novel PSM[3].

Decades of research have in fact gone into the elucidation of the genetic and biochemical basis of the PSMs. Next-generation sequencing have enabled elucidation of pathways in non-model plants. Identification of candidate genes poses a challenge, though the numbers of such genes have been effectively reduced by co-expression, phylogenetic, gene cluster, QTL and GWAS analyses. To pinpoint cell types involved in PSM, a combination of traditional methods (in-situ hybridization and immunolabeling) with novel approaches (single-cell RNA sequencing) may be contemplated. Still, we do not have much detail about the specific selective advantages conferred by the unique mix of specialized metabolites, to the survival and reproductive success of individual plants. Our ability to decrypt the chemical language of plants remains woefully inadequate. Meanwhile, concerted efforts are underway to elucidate the metabolic networks involved in relation to the evolutionary trade-off, at the heart of their occurrence in nature along with fecundity. A major objective continues to be efficient bioengineering of desirable metabolic traits in chassis organisms for producing high-value plant natural products, nutraceuticals, drugs, pharmaceuticals, insecticides, cosmetics and food additives.

The world of PSMs is enormous, with more than 200,000 different compounds now known, and surely many more to be discovered. And there is no dearth of books, monographs, protocol handbooks etcetera on plant secondary metabolites. However, after the concept of 'secondary metabolism' was replaced by 'plant specialized metabolism', only a few books or monographs have emerged, reflecting the change. One such book is *Plant Specialized Metabolism: Genomics, Biochemistry and Biological Functions*, edited by Gen-Ichiro Arimura and Massimo Maffei (CRC Press, 2016). A second one, *Plant Specialized Metabolism for plant protection: Genomics and Biotechnology* edited by Liao Z, Zhang L and Vazquez-Flota FA (Frontiers Media SA, 2022), deals with one, albeit very important, function of PSMs. Another recent addition to the list is *Natural Secondary Metabolites: From Nature, through Science to Industry*, edited by M Carocho, SA Heleno and L Barros (Springer Nature, 2023). All these are edited volumes with their primary focus on niche areas, which is perfectly understandable, given the vast and rapidly developing genomic landscape of specialized metabolites and their industrial potential. But the book under review has a text-book style comprehensive coverage of the entire corpus of PSMs. The Royal Society of Chemistry, London, has done a great service to the plant science fraternity by publishing this book, which covers almost all the chemical diversity and their occurrence in plants. Advanced students and researchers will now have a book to fall back upon, whenever they tend to miss the proverbial forest of chemodiversity for the trees.

The author, Professor Bratati De, brings out her more than three decade-long experience of teaching and research in phytochemistry, pharmacognosy and the plant metabolome, at the University of Calcutta, to cover in considerable detail, the entire arsenal of PSMs. The editors of the book describe the coverage of the material to include classification of PSMs according to their chemical structures their "sources and distribution in plant families …their

biosynthetic pathways … (and) important and notable uses in phytochemistry and pharmacology." The intended readership mentions plant scientists, pharmacognosists, chemical ecologists, bioengineers, synthetic biologists, synthetic organic chemists and medicinal chemists among others. Apart from the introductory chapter 1, which presents the subject-matter in proper perspective, the author covers all the PSMs by their broad chemical categories and their pathways, in six chapters. Chapter 2 deals with carbohydrates and glycosides. Their role in nutrition, human health, pharmaceutical and non-pharmaceutical applications are all dealt with in extensive details. The important role of carbohydrates in plant immunity has been covered in a succinct manner (p.20-21). The discussion on glycosides and glucosinolates is comprehensive. Chapter 3 covers the acetate-malonate pathway and derived fatty acids and polyketides. The detailed chemistry of fatty acids and their derivatives as well as the polyketides has been presented with rare clarity. I found the discussion on polyunsaturated fatty acids (PUFAs) particularly illuminating. A table listing almost the entire spectrum of macrolide antibiotics with their sources and clinical applications (p. 61) should prove helpful. Chapter 4 discusses the mevalonate and methylerythritol phosphate pathways and their terpenoid and steroid derivatives. Incidentally, terpenes constitute the largest class of (>50 000) PSMs which are at the forefront in interaction of plants with the environment. The discussion on plant steroids embraces all their diverse categories, their biosynthesis, role and myriad applications. A discussion on the analogues of vertebrate sex hormones in plants is also included. Even hopanoids, adjudged 'molecular fossils' with their role in cell membrane integrity and stress tolerance, finds a place (p. 105-6) in this volume. Chapter 5 deals with the shikimic acid pathway and the diverse array of phenolic compounds, including ubiquitous polyphenols such as flavonoids, which number more than 9000. In chapter 6, the author discusses the entire range of plant-derived alkaloids, the second largest class of (>21 000) PSMs. Finally, in chapter 7, the essential oils and resins are covered. The structure and biosynthesis of major essential oil (aka ethereal oil / volatile oil) constituents, terpenes (monoterpenes and sesquiterpenes) and phenylpropenes have been described separately in chapters 3 and 5, respectively. Their spectacular role in plants including the role of herbivore-induced plant volatiles (HIPVs), modulation of systemic acquired resistance (SAR), allelopathy (the "Salvia-phenomena") are mentioned, along with their burgeoning use in industry and human welfare. A brief idea of resins and their myriad uses are also discussed. A table which I find very useful lists no less than 50 essential oils and resins with their plant source, major chemical components and uses. All chapters contain useful tables. A list of tables at the beginning could have been a useful feature. However, the extensive chapter-end reference and unfailing mention of all plant (even microbial) sources with their current family names should prove extremely useful to teachers, students and researchers. The coverage of the diverse PSMs, their structure, mention of almost all plant sources and uses is succinct and exhaustive. Biosynthetic pathways in plants are typically distributed across multiple cell types, organelles and phase-separated bodies. The concept of metabolons (physical assembly of sequential enzymes of a pathway in large multi-subunit structures)[4] underlines the importance of spatial organisation within the cell. The author has painstakingly depicted this compartmentalization with the aid of self-explanatory diagrams. Even a cursory glance at the book reveals the pains the author has taken to bring out in meticulous details, the vast panoply of SMs and their myriad plant life-sustaining functions. One would hope that the author brings out a companion volume that covers rapidly emerging facets of PSMs such as the role of biosynthetic gene clusters (BGCs), miRNAs, metabolons and the network modules constituting a systems approach to this fascinating field.

Reference

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Shyamal K Chakraborty
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Numbers up to and including ten should be spelt out unless they are measurements. All numbers above ten are to be in numerals except at the beginning of sentences. Appropriate position of tables and figures should be indicated in the text. The journal style prohibits the use of footnotes within articles. If required, such notes may be merged parenthetically in the text, or as a separate paragraph.

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b) Books and book chapters

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4] Sharma AK, Sharma A. 1994. *Chromosome Techniques: A manual.* Reading, UK: Harwood Academic Publishers.

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5] Samajpati N, Chattopadhyay SB. 1984. (Eds.) *Interaction of plant pathogens in the host.* Calcutta: Oxford & IBH.

iii) A chapter in an edited book

6] Arora RK. 1981. Native food plants of north-eastern India. *In* Jain SK (Ed.), *Glimpses of Indian ethnobotany* New Delhi: Oxford & IBH, 91-106.
c) Doctoral dissertation
7] Ghosh M 1964. Cytogenetical and embryocultural work in rice (*O. sativa* L.) and related species. PhD thesis. University of Calcutta (unpublished).
d) Dataset with persistent identifier
8] Bennett MD, Leitch IJ. 2012. Plant DNA C-values database. (Release 6.0, Dec 2012) http://data.kew.org/cvalues[accessed 8 July, 2017].
e) Online-only journals

9] Behera PK, Kumar V, Sharma SS, Lenka SK, Panda D. 2023. Genotypic diversity and abiotic stress response profiling of short-grain aromatic landraces of rice (*Oryza sativa* L. *indica*. *Current Plant Biology* 33: 100269 https://doi.org/10.1016/j.cpb.2022.100269.

f) Pre-prints

10] Dolzhenko E,English A, Dashnow H *et al*. 2023. Resolving the unsolved: comprehensive assessment of tandem repeats at scale. Preprint at *Bio Rxiv.* doi: ttps://doi.org/10.1101/2023.05.12.540470.
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h) Scientific & Technical Reports:
11] Tomato Genome Consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485: 635-641.
12] Cohen JT, Duggar K, Gray GM, *et al.* 2001. Evaluation of the potential for bovine spongiform encephalopathy in the United States. Boston: Harvard School of Public Health, Center for Risk Analysis, Report No. PB2002-108684, p.116. Supported by the US Department of Agriculture.
i) Software:
13] Felsenstein J. 2022. PHYLIP, v3.698, Dep. Genome Sciences & Dep. Biology, University of Washington https://doi.org/10.12345/ABC000999
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giga (10^{9})	G	gram	g	millimolar	mM
mega (10^{6})	M	milligram	mg	micromolar	µM
kilo (10^{3})	k	microgram	µg	parts per million	ppm
deca (10)	da				
deci (10^{-1})	d	***Units of time***		***Units of temperature***	
centi (10^{-2})	c	hour	h	Kelvin	K
milli (10^{-3})	m	minute	min	Celsius	ºC
micro (10^{-6})	µ	second	s		
nano (10^{-9})	n	day	day		
pico (10^{-12})	p	month	month		
femto (10^{-15})	f	year	yr		
atto (10^{-18})	a				
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