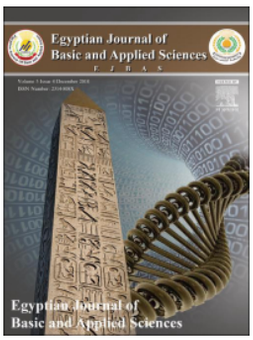


Egyptian Journal of Basic and Applied Sciences

Sl. No. 1 ISSN 2314- 808X

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/teba20

Neuroprotective role of herbal alternatives in circumventing Alzheimer's disease through multi-targeting approach - a review

Sunil K Ravi, Balenahalli Narasingappa Ramesh, Shilpa Kj, Jagadesha Poyya, Jyothsna Karanth, N.G Raju & Chandrashekhar G Joshi

To cite this article: Sunil K Ravi, Balenahalli Narasingappa Ramesh, Shilpa Kj, Jagadesha Poyya, Jyothsna Karanth, N.G Raju & Chandrashekhar G Joshi (2022) Neuroprotective role of herbal alternatives in circumventing Alzheimer's disease through multi-targeting approach - a review, Egyptian Journal of Basic and Applied Sciences, 9:1, 91-124, DOI: 10.1080/2314808X.2021.2021749

To link to this article: https://doi.org/10.1080/2314808X.2021.2021749

Published online: 24 Feb 2022.

EGYPTIAN JOURNAL OF BASIC AND APPLIED SCIENCES
2022, VOL. 9, NO. 1, 91–124
https://doi.org/10.1080/2314808X.2021.2021749

Neuroprotective role of herbal alternatives in circumventing Alzheimer's disease through multi-targeting approach - a review

Sunil K Ravi[a,b], Balenahalli Narasingappa Ramesh[b], Shilpa Kj[a,c], Jagadesha Poyya[a], Jyothsna Karanth[d], N.G Raju[e] and Chandrashekhar G Joshi[a]

[a]Department of Research & Studies in Biochemistry, Mangalore University, P.G. Centre, Chikka Aluvara, Kodagu, India; [b]Department of Biotechnology, College of Agriculture, University of Agriculture Sciences, Hassan, Bangalore, India; [c]Department of Applied Botany, Mangalore University, Mangalagangothri, Mangalore, India; [d]Department of Biochemistry, Governement Women's Sceince College, Mandya, India; [e]Department of Biotechnology, Karnataka State Open University, Mukthagangothri, Mysore, India

ABSTRACT

Alzheimer's disease (AD) is a common form of dementia affecting the elderly worldwide. It is a multifactorial neurodegenerative disorder with no known preventive therapy. Many of the drugs used in the treatment of AD, such as galantamine, rivastigmine, and donepezil, have unpleasant side effects, and hence physicians are keen to find alternatives. Research has shown that plants and their phytochemicals can alleviate AD. These plant products can act through various modes, such as inhibition of amyloid β, acetylcholine, and γ-secretase, modulation of antioxidants, and α-secretase activation, which are known to involve in the improvement of brain functions. A recent approach that has garnered the attention of many researchers in designing a drug against AD is the multi-target-directed ligand (MTDL), wherein the same molecule act on multiple targets. Many studies have reported the potential of herbs to act on multiple targets and display biological properties. The current review summarizes the ongoing evidence on the use of herbs and their derived bioactive molecules in the treatment of AD and in relieving disease-associated pathological events. Currently available plant-derived MTDLs for the treatment or slowing down of the progression of AD are also discussed.

ARTICLE HISTORY
Received 2 September 2021
Accepted 20 December 2021

KEYWORDS
Alzheimer's disease; herbs; multi-target-directed ligands; acetylcholinesterase; natural products

Introduction

Alzheimer's disease (AD) is an irreversible, chronic neurodegenerative disorder characterized by deterioration of cognitive functions and behavioral disturbances [1]. Globally, AD is the most common cause of dementia, affecting approximately 46.8 million people and expected to increase up to 131.5 million by 2050 [2]. The probability of AD aggressively increases with age, more particularly after the age of 65. Thus, age is the primary risk factor for AD development [3]. AD developed after 65 years of age is referred to as 'sporadic' (or late-onset), whereas AD developed before 65 is classified as 'familial' (or early-onset). Several complex pathogenic pathways are involved in the progression and development of the disease, including plaque formation, inflammatory cascade, oxidative stress, and cholinergic deficit [4]. These cognitive deficits lead to memory-related clinical symptoms, such as loss of episodic and newly learned memories [5] .

CONTACT Chandrashekhar G Joshi josheejoshee@gmail.com Department of Research & Studies in Biochemistry, Mangalore University, P.G. Centre, Chikka Aluvara, Kodagu, India

Sl. No. 2 ISSN 2071-5773

Herpetology Notes, volume 14: 77-81 (2021) (published online on 09 January 2021)

First record of leucism in the amphibian order Gymnophiona: *Ichthyophis kodaguensis* Wilkinson et al., 2007 from the southern Western Ghats, India

Govindappa Venu[1,*], Gandlahalli N. Balakrishna[1], Robert K. Browne[2], Narayanappa G. Raju[3], Kulkarni Varadh[1], Sompalem Ramakrishna[1], and Govindaiah Venkatachalaiah[1]

Caecilians are an order of eel-like amphibians that comprises 214 extant species. They are a small radiation compared to the 7236 species of frogs and toads, and the 757 species of salamanders (Frost, 2020). They are restricted in distribution to the tropics and sub-tropics, and most are fossorial in moist and humus-rich soil as adults. Because of their hidden life they are unfamiliar to most herpetologists, and their biology is perhaps the least known among all amphibians and reptiles (Taylor, 1968; Nussbaum and Wilkinson, 1989; Gower and Wilkinson, 2005).

The Western Ghats of India are part of a global biodiversity hotspot (Myers et al., 2000). Over the past decade the targeted sampling of caecilians by herpetologists has resulted in the discovery of many new species and has revealed other significant aspects of caecilian biodiversity and autecology in the Western Ghats (Bhatta et al., 2007, 2011; Wilkinson et al., 2007; Kotharambath et al., 2012; Sreekumar and Dinesh, 2020). The genus *Ichthyophis* Fitzinger, 1826 includes fifty currently recognized species, the greatest number in any caecilian genus (Frost, 2020). Post-metamorphosis, *Ichthyophis* species categorise into two morphs: those with a yellow or cream lateral stripe, commonly known as "striped," and those without a stripe, known as "unstriped" (Taylor, 1968).

[1] Department of Zoology, Centre for Applied Genetics, Jnana Bharathi Campus, Bangalore University, Bengaluru, Karnataka, 560056, India.

[2] Sustainability America, La Isla Road, Sarteneja, Corozal District, Belize.

[3] Department of Biotechnology, Karnataka State Open University, Mukthagangothri, Mysuru, Karnataka, 570006, India.

[*] Corresponding author. E-mail: venugcaecilian@gmail.com

*Ichthyophis kodaguensis*is is a striped Western Ghats species that was described by Wilkinson et al. (2007) based on six specimens collected at the Venkidds Valley Estate (elevation 1143 m), located about 20 km south of Mercara, Coorg District, southern Karnataka State, India, and on a seventh specimen from an uncertain location. Bhatta et al. (2011) reported two additional specimens of *I. kodaguensis* from the Western Ghats region, from Basarekatte, Koppa Taluk, Chikkamagalur District, Karnataka State. That report extended the range of *I. kodaguensis* by approximately 125 km to the north from the type locality.

During the late monsoon season in 2004–05, we collected two adult caecilians at the Hegde Coffee Plantation (12.4730°N, 75.7808°E) that resembled *I. kodaguensis*. These animals were vouchered and deposited in the collection of the Department of Zoology, Bangalore University, Bengaluru, Karnataka, India (specimen numbers BUB 1141 and BUB 1179). Hegde Plantation is a 5-ha mixed orchard producing coffee, areca, bananas, oranges, and cardamom, and it lies about 0.5 km north of the *I. kodaguensis* type locality. Specimen BUB 1141 (Fig. 1A, B) is an adult male, which was collected from rotting vegetation in agricultural trenches. Trenching is a general agricultural practice in the coffee estates of the Western Ghats. The trenches are small depression dug between adjacent coffee plants and used as artificial repositories for weeds and other cultivation refuse that when rotted are used as compost. These trenches are left undisturbed for 4–5 months before the compost is used, and they provide good habitats for endogeic herpetofauna, such as caecilians and shieldtail snakes (family Uropeltidae), who may use them as shelter and to forage for the abundant earthworms (Venu and Venkatachalaiah, pers. obs.). Specimen BUB 1179 (Fig. 1C, D) is an adult female, collected from the surroundings of an artificial pond, situated in the lowland area of the estate. Ponds

Figure 1. *Ichthyophis kodaguensis* from Hegde Coffee Plantation, Coorg District, Karnataka State, India. (A) Dorsolateral view of a male (BUB 1141) with a striped body colouration considered normal for the species. (B) The male specimen in ventral view. (C) Leucistic female (BUB 1179) collected from the area surrounding a pond in dorsal view. Note the black eyes. (D) Ventral view of the female.

in these estates serve as water storage bodies during the monsoon seasons and the stored water is pumped out during the drier seasons of the year (Fig. 2).

The specimens were transported in plastic bags to Bangalore University. Following euthanisia in a 0.5% MS-222 solution, total length of each specimen was measured with a string and a ruler, and other measurements were made to the nearest 0.1 mm on formalin-fixed specimens using dial calipers. Numbers of teeth in both upper and lower jaws were counted using a dissecting microscope. Morphometric and meristic data collected for both BUB 1141 and BUB 1179, with values listed in that order, are as follows: total length 289 mm, 324 mm; total number of annuli 302, 321; ratio of tentacle-nostril and tentacle-eye distance 1.39, 1.56; number of premaxillary + maxillary teeth 39, 42; number of vomeropalatine teeth 51, 44; number of dentary teeth 34, 43; number of inner mandibular teeth 28, 31. Mitotic chromosomes from liver and meiotic chromosomal stages from testes and ovary were prepared using the protocol of Venkatachalaiah and Venu (2002). Localization of heterochromatin was carried out on meiotic chromosomes following the protocols of Sumner (1972) and Venu (2014). All experiments were conducted in accordance with the ethical committee guidelines of the Department of Zoology, Bangalore University.

Morphometric and meristic data obtained for the two specimens were in agreement with the diagnostic features detailed for *I. kodaguensis* by Wilkinson et al. (2007). Mitotic and meiotic chromosomal analyses of both specimens revealed a diploid number of 2n = 42 chromosomes with fundamental number (FN) 60 (Fig. 3A) and n = 21 bivalents (Fig. 3B). Presence of a submetacentric Chromosome Pair 3 in the meiotic complement (Fig. 3B) and a prominent heterochromatic band at the telomeric end on the long arm of Chromosome Pair 1 (Fig. 3C, D) provide a cytogenetic identity of both specimens as *I. kodaguensis* (Venu, 2013).

Sl. No. 3 ISSN 2766- 1520

Ichthyology & Herpetology 109, No. 2, 2021, 443–448

First Report of B Chromosomes in Caecilians (Amphibia: Gymnophiona)

Govindappa Venu[1], Albert Rajendran[2], Narayanappa Govinda Raju[3], Robert Kenneth Browne[4], Sompalem Ramakrishna[5], and Govindaiah Venkatachalaiah[6]

Gymnophion amphibians (caecilians) are less well studied than the other two orders of extant amphibians: Anura and Caudata. We describe the first recorded presence of supernumerary (B) chromosomes in the order Gymnophiona, where a lone intensely stained B chromosome was observed in the somatic metaphase and meiotic complements of the Indian ichthyophiid, *Uraeotyphlus gansi*. Given the rarity of B chromosomes in caecilians and their sporadic presence within *U. gansi*, it is likely that these represent aneuploidy and are a recent acquisition derived from centromeric drive.

B chromosomes are one of the most widespread numerical polymorphisms, or a type of aneuploidy, in the karyotypes of many eukaryotic organisms (Green, 1988; Camacho, 2005; Jones, 2017). B chromosomes occur in approximately 15% of all extant eukaryotes, fungi, animals, and plants that are karyologically known (Melo et al., 2020). B chromosomes do not pair meiotically with A chromosomes but are instead inherited in a non-Mendelian mode and typically composed of constitutive heterochromatin, consisting of highly repetitive DNA sequences characteristic of satellite DNA, ribosomal DNA, and transposable elements (Douglas and Birchler, 2017). B chromosomes are inferred to originate either from intragenomic fragmentations as segmental duplications that acquire the characteristics of a B chromosome or through interspecific hybridization processes with the acquisition of foreign DNA from a closely related species that eventually evolves into a supernumerary chromosome (Camacho et al., 2000; Dhar et al., 2002; Camacho, 2005; Houben et al., 2013).

Over time, B chromosomes diverge quantitatively through sequential evolution and qualitatively through rearrangements. In the absence of selective pressure and reduced recombinational progression, B chromosomes tend to acquire and accommodate mutations. Thus, for the most part, B chromosomes are composed of amplified DNA from either single or several genomic regions. It is now apparent that B chromosomes are transmitted at a higher than expected frequency, increasing in number from one generation to the next, due to acquisition capacity or 'drive' mechanisms (Jones, 1995; Houben et al., 2014).

The advent of next generation sequencing (NGS) technology and associated bioinformatics will potentially improve our knowledge of B chromosome architecture (Ahmad and Martins, 2019); for example, the analysis of flow-sorted or micro-dissected B chromosomes has shown they contain substantial amounts of A chromosome-derived DNA sequences including organelle DNA sequences (Leach et al., 2005; Banaei-Moghaddam et al., 2015; Ruban et al., 2017). These sequential acquisitions of particular DNA sequences by B chromosomes eventually create a multi-chromosomal mosaicism (Palestis et al., 2004a; Borisov, 2014; Houben, 2017; Valente et al., 2017).

Caecilians are elongate, limbless, superficially worm-like organisms mostly inhabiting moist soils in tropical and subtropical regions. The biology of caecilians is less well studied than frogs and salamanders because of their fossorial nature, restricted faunal distribution, difficulty in collection, and difficulty in captive breeding (Taylor, 1968; Gower and Wilkinson, 2005; Wilkinson, 2012). As for other aspects of caecilian biology, there is a paucity of information concerning their cytogenetics (Wen and Pang, 1990; Matsui et al., 2006; Venkatachalaiah et al., 2006; Venu et al., 2011, 2012; Venu and Venkatachalaiah, 2012, 2013; Venu, 2013, 2014a, 2014b, 2014c, 2014d, 2014e, 2014f; Patawang et al., 2016). Cytogenetic investigation of caecilians based on conventional and differential staining protocols revealed their diploid (2n) numbers range between 20–44 (Barrio and Rinaldi De Chieri, 1970; Barrio et al., 1971; Wake and Case, 1975; Wake et al., 1980; Nussbaum and Ducey, 1988; Venu, 2008).

The genus *Uraeotyphlus* with seven nominate species is endemic to southern Western Ghats, India (Wilkinson and Nussbaum, 1996; Gower et al., 2008). It is partitioned into two species groups, viz., the *oxyurus* group (*U. interruptus*, *U. menoni*, *U. narayani*, and *U. oxyurus*) and the *malabaricus* group (*U. gansi*, *U. malabaricus*, and *U. oommeni*; Gower and Wilkinson, 2007). The chromosome numbers for the genus *Uraeotyphlus* range from 36–42 with all the *oxyurus* group species sharing the same diploid number of 36 chromosomes (Seshachar, 1939; Elayidom et al., 1963; Venu and Venkatachalaiah, 2013) and *U. gansi*, the lone cytogenetic representative for the *malabaricus* group with 2n = 42 chromosomes (Venu et al., 2011). The present paper describes the novel finding of B chromosomes in the Gymnophiona based on conventional and differential staining techniques.

[1] Centre for Applied Genetics, Department of Zoology, Jnana Bharathi Campus, Bangalore University, Bengaluru, Karnataka, 560056, India; Email: venugcaecilian@gmail.com. Send reprint requests to this address.
[2] Research Department of Zoology, St. John's College, Tirunelveli, India; Email: alrajendran@gmail.com.
[3] Department of Biotechnology, Karnataka State Open University, Mukthagangothri, Mysuru, Karnataka, 570006, India; Email: rajungdr@gmail.com.
[4] Sustainability America, La Isla Road, Sarteneja, Corozal District, Belize; Email: robert.browne@gmail.com.
[5] Department of Zoology, Jnana Bharathi Campus, Bangalore University, Bengaluru, Karnataka, 560056, India; Email: srkbuz@gmail.com.
[6] Centre for Applied Genetics, Department of Zoology, Jnana Bharathi Campus, Bangalore University, Bengaluru, Karnataka, 560056, India; Email: sondekoppa9@yahoomail.com.
Submitted: 22 August 2020. Accepted: 2 December 2020. Associate Editor: D. S. Siegel.
 DOI: 10.1643/h2020119 Published online: 31 May 2021

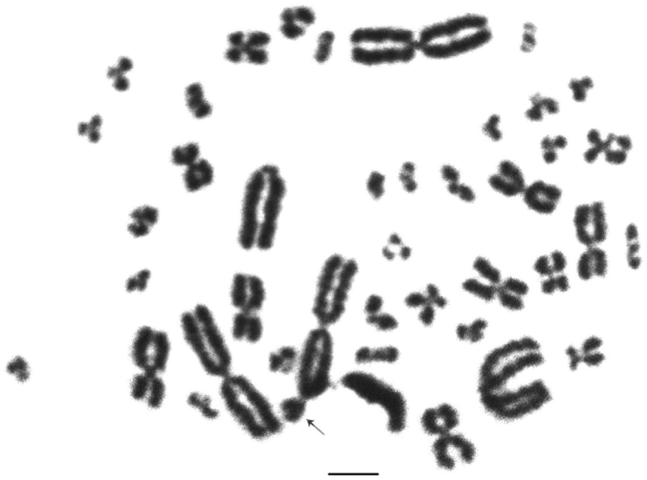


Fig. 1. Mitotic male (BUB1202) metaphase complement obtained from intestinal epithelial cells depicting a diploid number (2n) of 42 chromosomes along with a B (arrow) chromosome. Scale bar: 10 μm.

MATERIALS AND METHODS

Source of material.—Eight adult individuals (3 females and 5 males) of *Uraeotyphlus gansi* were collected from the type locality at Nalamukku tea estate, Tirunelveli District, Tamil Nadu, India, from July to December, 2007 to 2010. The specimens were intraperitoneally injected with 2 mg/ml colchicine solution (0.1 ml/g body mass), euthanized using MS-222 24 hrs after injection, and then dissected. The specimens were deposited in the museum of Department of Zoology, Bangalore University, Bengaluru, Karnataka, India, bearing the vouchers BUB1202, 1206, 1208, 1209, 1211, 1212, 1226, and 1227. All experiments were carried out according to the guidelines of the ethical committee of the Department of Zoology, Bangalore University, Karnataka, India.

Chromosome preparation.—Somatic metaphase chromosomes from the intestinal epithelia and liver, and male meiotic stage chromosomes from the testes of euthanized specimens prior to fixation in formalin were obtained using modified methods from Venkatachalaiah and Venu (2002), Venu (2008), and Venu et al. (2011). Conventional staining with the help of a diluted Giemsa solution (5%) was used for chromosomal preparations (Venu and Venkatachalaiah, 2005, 2006; Venu et al., 2011). C-banding was accomplished using a slightly modified version of Sumner's (1972) BSG technique, in which air-dried chromosome preparations were hydrolyzed in 0.2 N HCl for a few seconds at room temperature, treated with 10% $Ba(OH)_2$ for 10 min, renatured in 2X SSC for 1 h at 60°C, and stained with 10% Giemsa solution for 25 min (Venu, 2013, 2014d).

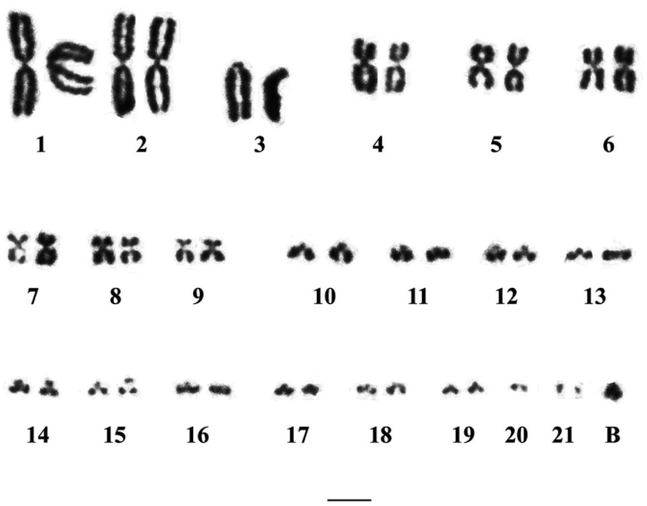


Fig. 2. Somatic male (BUB1202) metaphase karyotype with the karyotypic formula 2n = 42+1B and FN = 62. The lone B chromosome is the darkest in the karyotype and is placed at the end of the karyotype. Scale bar: 10 μm.

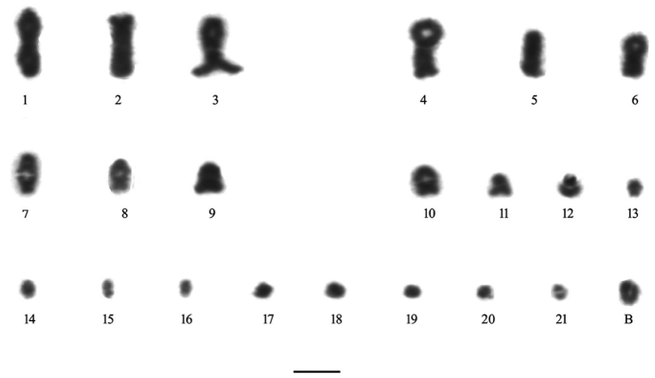


Fig. 3. Male (BUB1202) meiotic diplotene karyotype with 21 bivalents and a B chromosome. Scale bar: 10 μm.

Microphotography and karyotype construction.—Chromosome preparations were observed using a Zeiss Axioskop 2 plus microscope, and well-spread complements were photographed. Karyotypes were prepared in accordance with Levan et al. (1964), Venkatachalaiah and Venu (2002), and Venu et al. (2011, 2012).

RESULTS

Karyotypic details.—The karyotypes of *U. gansi* constructed from approximately 60 well-spread complements obtained from the females (BUB1206, 1211) and males (BUB1208, 1209, 1212, 1226) had a diploid (2n) number of 42 and a fundamental number (FN) of 58. The 21 pairs of homologous chromosomes in the somatic metaphase sets were arranged into groups (A–D) in a karyotype (Venu et al., 2011). No morphologically identifiable sex chromosomes were observed in either sex in the metaphase karyotypes.

Meiotic chromosomes prepared from testes revealed pachytene, diplotene, diakinesis, and second meiotic metaphase stages. The diplotene complements comprised 21 individually identifiable bivalents, with the number of chiasmata per bivalent ranging from 5–6 in the largest, and 2–3 in the medium-small bivalents, and a single chiasma in the smallest acrocentrics. These observations of mitotic and meiotic chromosome karyotypes are in agreement with previously published data for *U. gansi* (Venu et al., 2011).

Mitotic and meiotic chromosomes prepared from specimens bearing voucher number BUB1202 (Figs. 1, 2, 3), and mitotic chromosomes from BUB1227 (Figs. 4, 5), also had karyotypic details matching those described above. In addition, 4–5 complements obtained from mitotic and

Sl. No. 4 ISSN: 2676-685X

Journal of Animal Diversity

Online ISSN 2676-685X

Volume 2, Issue 3 (2020)

Research Article

http://dx.doi.org/10.29252/JAD.2020.2.3.2

First records of the Long-headed Caecilian, *Ichthyophis longicephalus* Pillai, 1986 (Gymnophiona: Ichthyophiidae) from the states of Karnataka and Tamil Nadu, India with comments on its conservation status

Govindappa Venu[1*], Narayanappa Govinda Raju[2], Mark Wilkinson[3], Robert Kenneth Browne[4], Kulkarni Varadh[1], Gandlahalli Narasimaiah Balakrishna[1], Sompalem Ramakrishna[1] and Govindaiah Venkatachalaiah[1]

[1]*Department of Zoology, Centre for Applied Genetics, Jnana Bharathi Campus, Bangalore University, Bengaluru, Karnataka, 560056, India*
[2]*Department of Biotechnology, Karnataka State Open University, Mukthagangothri, Mysuru, Karnataka, 570006, India*
[3]*Department of Life Sciences, The Natural History Museum, London SW7 5BD, United Kingdom*
[4]*Sustainability America, La Isla Road, Sarteneja, Corozal District, Belize*
[*]*Corresponding author*: *venugcaecilian@gmail.com*

Received: 6 September 2020
Accepted: 27 September 2020
Published online: 9 October 2020

Abstract

In the present study, we report the discovery of the poorly known striped ichthyophiid caecilian *Ichthyophis longicephalus* from three new localities, each in three southern Indian states; Karnataka, Kerala and Tamil Nadu. Present records and available information on this species enable us to revise its IUCN Red List conservation status from Data Deficient (DD) to Least Concern (LC).

Key words: Anthropogenic influence, coffee and tea plantations, Kodagu, new site record, Peninsular India, range extension

Introduction

Caecilians are elongate, limbless amphibians, and although some species, including *Ichthyophis* Fitzinger, 1826, have an aquatic larval stage, most are burrowers or surface cryptic species as adults and are consequently inconspicuous. These apodan amphibians are often mistaken for giant earthworms and are relatively poorly known (Nussbaum and Wilkinson, 1989; Gower and Wilkinson, 2005; Wake and Koo, 2018; Mailho-Fontana et al., 2020).

The caecilian fauna of Peninsular India comprises 27 species, more than 10% of the global diversity for the group (Wilkinson, 2012), in four genera in the two families Ichthyophiidae Taylor, 1968 and Indotyphlidae Lescure, Renous and Gasc, 1986 (Frost, 2020). Of these, three genera, *Uraeotyphlus* Peters, 1880, *Indotyphlus* Taylor, 1960, and *Gegeneophis* Peters, 1880 are endemic to Peninsular India (Taylor, 1968). Whereas, the genus *Ichthyophis* includes 50 species with broad distribution across South and South East Asia, of which six, including one unicolored species and five species with yellow lateral stripes, are endemic to peninsular India, occurring mainly in the Western Ghats and surrounding areas (Gower et al., 2007; Wilkinson et al., 2007; Bhatta et al., 2011; Frost, 2020).

The unstriped ichthyophiid caecilian, *Ichthyophis bombayensis* Taylor, 1960 is distributed across the 1600 km expanse of the Western Ghats. But the striped ichthyophiids viz., *Ichthyophis beddomei* Peters, 1880, *I. davidi* Bhatta, Dinesh, Prashanth, Kulkarni, and Radhakrishnan, 2011, *I. kodaguensis* Wilkinson, Gower, Govindappa, and Venkatachalaiah, 2007, *I. longicephalus* Pillai, 1986, and *I. tricolor* Annandale, 1909 are restricted to the Southern states of Peninsular India (Gower et al., 2007; Wilkinson et al., 2007; Bhatta et al., 2011; Frost, 2020).

The Long-headed Caecilian, *Ichthyophis longicephalus* was described based on a single adult specimen collected in 1979 from the Silent Valley National

Park (Nilgiri Biosphere Reserve), Palghat district, Kerala, India. Subsequently in 1985, a second specimen collected from the Kalakkad Wildlife Sanctuary, Tirunelveli District, Tamil Nadu at the southern tip of the Western Ghats, about 320 km away from the type locality was referred to *I. longicephalus* by Pillai and Ravichandran (1999). This taxonomic referral was questioned first by Dutta et al. (2004) and again by Wilkinson et al. (2007) who noted the poor condition of both the specimens (including the Holotype).

In the subsequent review of the taxonomy and natural history of *I. longicephalus*, Kotharambath et al. (2012) reported nine additional specimens from several localities in Kerala, near the type locality, and concluded that the specimen from Kalakkad is not *I. longicephalus* but an undescribed species. Kotharambath et al. (2012) suggested that additional surveys to search for *I. longicephalus* beyond the border of Kerala and into adjacent Western Ghats regions of Karnataka and Tamil Nadu were needed to further assess the true distribution range of the species. During our surveys in the Karnataka, Kerala and Tamil Nadu have yielded three additional localities for the species, including the first reports for the states of Karnataka and Tamil Nadu.

Material and Methods

Field surveys were conducted during the Monsoon seasons of 2004 and 2017–2018 in plantations in the south Western Ghats located in the districts of Kodagu (=Coorg), Karnataka; Nilgiris, Tamil Nadu and Wayanad, Kerala (Fig. 1). Specimen sampling was a part of cytogenetic analysis of caecilians of Western Ghats (Venu, 2008; Venu and Venkatachalaiah, 2012). Surveys were conducted in daylight with the assistance of local people, mostly by digging soft, humus rich soil especially along the sides of streams and other water bodies in the coffee and tea plantations. Collected specimens were transported to Bangalore University (Jnana Bharathi Campus, Bengaluru, Karnataka) in polythene bags along with soil and earthworms (as food source) collected from the sampling sites.

Specimens of *I. longicephalus* were euthanized with MS222, photographed in broad daylight using a Canon EOS 1200D DSLR camera and the lengths of the fresh specimens were measured using thread and ruler. Euthanized specimens were fixed in 10% formalin solution, followed by thorough washing under tap water overnight to remove formalin, and then stored in 70% alcohol. All other measurements were taken using Mitutoyo Digimatic caliper to the nearest 0.1 mm. Sexes of the specimens were identified by making a midventral incision and observing the presence of testes and ovaries under a Nikon SMZ-10 binocular Stereo microscope. Voucher specimens (BUB1175, BUB1379, BUB1442, BUB1587 and BUB1617) are deposited in the collections of the Museum of Department of Zoology, Bangalore University, Bengaluru (BUB).

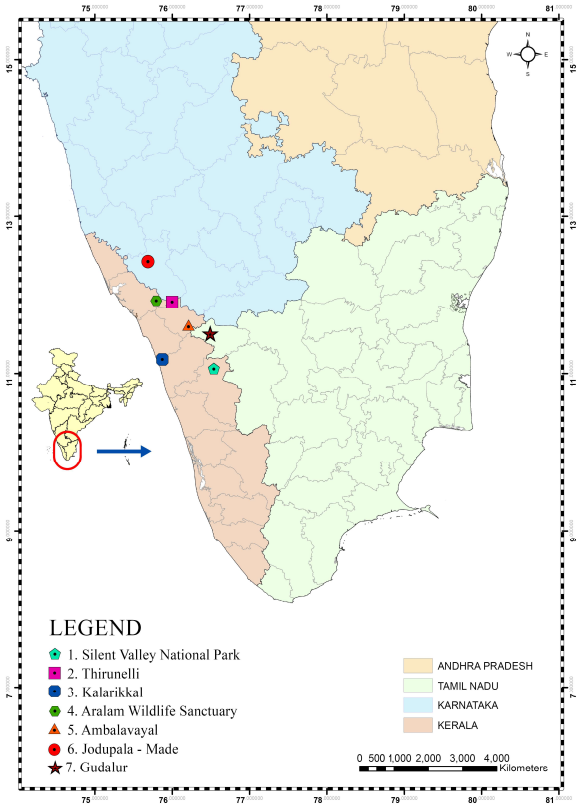


Figure 1: Map of peninsular India showing the past and currently described locality data for *Ichthyophis longicephalus*.

Five female specimens referable to *I. longicephalus* (Fig. 2) were collected. Two specimens (BUB1587 and BUB1617) (Fig. 2A) were collected between 10.00–11.00 hrs on 21st July, 2018, from the Regional Agricultural Research Station (RARS), Ambalavayal (11°36'45.84"N 76°12'35.64"E), Wayanad District, Kerala, in a heap of soil, about 974 m a.s.l., lying about 30 m from a stream (Fig. 3).

Four specimens of *I. beddomei*, two *Uraeotyphlus narayani* Seshachar, 1939 and a few uropeltid snakes (*Rhinophis* Hemprich, 1820 and *Uropeltis* Cuvier, 1829) were also found during our field work. RARS was established in 1983 by the Kerala government to provide research support for agriculture in the high range zone that includes Wayanad, Idukki and Nelliyampathy area of Palakkad district. The station is spread over 87.3 ha, has a mild tropical climate and grows a wide variety of crops including coffee, pepper, rice, ginger, turmeric, clove and cinnamon.

Two specimens (BUB1175 and BUB1442) (Fig. 2B) were collected on 28th August, 2004; between 15.00–16.00 hrs in soil adjoining a small stream in Kuchikunnel tea plantations (11°30'10.78"N 76°29'30.21"E) situated about 1,072 m a.s.l., and 12 km north of Gudalur, a municipality and a taluk of Nilgiris, Tamil Nadu.

A single specimen (BUB1379) (Fig. 2C) of *I. longicephalus* was collected from Himakshama estate, Jodupala-Made village (12°25'1.33"N 75°40'57.25"E),

Sl. No 5 ISSN 0970 - 2067 Biomedicine: 2018; 38(3): 378-383

Proteinaceous Amylase Inhibitors from Plants: A potential Anti-Diabetic Drug in *Mucuna pruriens*

Rajiv Bharadwaj P[1], Raju NG[2] and ChandrashekharaiahKS[1]

[1]Department of Biochemistry, Mangalore University PG Centre, Jnana Kaveri Campus, Kodagu,[2]Department of Biotechnology, Karnataka State Open University, Mukhtagangothri, Mysore, Karnataka, India

(Received: May 2018 Revised: Jun 2018 Accepted: Aug 2018)

Corresponding Author

Chandrashekharaiah KS. E-mail: *kschandraks@gmail.com*

ABSTRACT

Introduction and Aim: Introduction and Aim: Proteinaceous α-Amylase inhibitors (AI) are naturally occurring proteins, widely distributed in various organisms, including micro-organisms, plants, and animals. Amylase inhibitors play a key role in blocking dietary starch from being metabolized in the body for which they are also synonymic to starch blockers. Due to this function, amylase inhibitors are also drug-design targets for the development of drugs for the treatment of obesity and, diabetes mellitus. *Mucuna is* an under-utilized legume rich in proteins, extensively used as cover and to control weeds and pests in agriculture. Since the reports on the amylase inhibitors from the seeds of plants were scanty and no work has been carried out on amylase inhibitors from the seeds of *Mucuna ,* an attempt was made to characterize amylase inhibitor from the soaked seeds of *Mucuna pruriens.*

Materials and Methods: The seeds of different varieties of genus *Mucuna were* processed for preparing 10% acetone powder. Differential extraction using various buffers was processed for the presence of amylase inhibitory activity. The selected seeds were germinated with different time points to monitor the inhibitory activity.

Results: The maximum protein and amylase inhibitory activity were observed in 0.05 M sodium phosphate buffer. The seeds of *Mucuna pruriens* showed higher protein and amylase inhibitory activity. Germination studies on these seeds show an increase in the protein content and amylase inhibitory activity confirmed by protein and substrate stained cationic gel electrophoresis.

Conclusion: Amylase inhibitor was isolated and purified from the soaked seeds of *Mucuna* pruriens. The α-amylase inhibitor is known to act against mammalian amylases which find its potential in the treatment of diabetes and cure of nutritional problems, which result in obesity.

Key Words: Amylase inhibitor, *Mucuna* seeds, Purification, Physicochemical properties

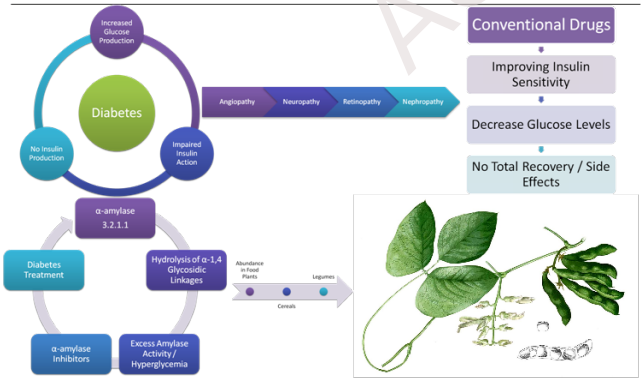
INTRODUCTION

Proteinaceous α-Amylase inhibitors (AI) are widely distributed in various organisms including plants, animals, and micro-organisms. They occur in multiple forms and are present as antinutritional factors in the seeds of plants. They are most abundantly present in food plants, particularly in cereals (1, 4, 19) and legumes (9, 15, 20, 6). They inhibit the activity of carbohydrate-digesting enzymes, amylases, thereby preventing the digestion and absorption of dietary starches in the digestive tract. They also inhibit the activities of amylases of bacteria, insect and fungi (18, 13, 14, 15). Plant amylase inhibitors also regulate the activities of endogenous enzymes and are active components of the plant defense system. They also have been used as bioinsecticide or biopesticide to prevent target harmful insects and pests (17). This approach can be used to design and develop these inhibitory proteins as biotechnological tools to control insects, pests, and pathogens (16, 5, 7, 21). Amylase inhibitors are also known as starch blockers, delays postprandial carbohydrate digestion and absorption thereby lowering plasma glucose levels without disturbing pancreatic growth. Therefore, amylase inhibitors can also be drug design targets for the treatment of type-II diabetes, obesity and related cardiovascular disease and hypertension (22) and diabetes mellitus, since they slow down the

degradation and absorption of dietary starch by the body (12, 10). Figure 1 illustrates the manifestation of onset of diabetes mellitus and the role of alpha-amylases and necessity for an alternate method of disorder management.

The genus *Mucuna* belongs to the fabaceae (leguminoceae) which form an important part of annual and perennial legumes. *Mucuna* is an underutilized legume rich in proteins and has great demand in food and pharmaceutical industries (8, 2). Since the reports on the amylase inhibitors from the seeds of plants were scanty and no work has been carried out on amylase inhibitors from the seeds of *Mucuna* , an attempt was made to study amylase inhibitor from the soaked seeds of *Mucuna* pruriens. In the present study, amylase inhibitor profile of soaked seeds and germinated seedlings of *Mucuna* pruriens and their action on human salivary amylase is described.

Figure 1: Illustration of events manifested by the onset of diabetes (factors responsible) into disorders (types) and availability of conventional treatment along with its progression. Due to limitations in conventional treatment, α-amylase inhibitors known to inhibit the α-amylase activity which can be a potential treatment option for diabetes. These inhibitors are known to be present in abundance in food plants like cereals and legumes. *Mucuna* , a tropical under-utilized legume is one such plant considered in this study for investigating inhibitor activities.



MATERIALS AND METHODS

Different varieties of seeds of genus *Mucuna* (*Mucuna* pruriens, *Mucuna* pruriens var. utilis, *Mucuna* pruriens var. hirsuta, *Mucuna* monosperma and *Mucuna* gigantea) were collected from Tropical Botanical Garden and Research Institute (TBGRI), Palode, Kerala. Acrylamide, N, N, Methylene bisacrylamide, Coomassie Brilliant Blue G-250, and Bovine serum albumin were obtained from Sigma Chemical Company, USA. All other chemicals used were of analytical grade.

Methods

Preparation of acetone powder

The acetone powder (10 %) of soaked seeds of *Mucuna* pruriens was prepared according to the method of Wetter. Seeds were blended in a homogenizer with chilled acetone and filtered using the suction pump. The cake obtained was dried at 37°C, powdered and stored at 4°C until further use.

Preparation of crude amylase inhibitor extracts using different solvents

A 10 % extracts of acetone powders of all the varieties/species of genus *Mucuna* were prepared as described by Chandrashekharaiah*et al*. (3) using 0.1 N HCl, 0.05 M Sodium Acetate Buffer pH 4.0, 0.05 M Sodium Acetate Buffer pH 5.0, 0.05 M Sodium Citrate Buffer pH 6.0, 0.05 M Sodium Phosphate Buffer pH 7.0, 0.05 M Sodium Phosphate Buffer pH 7.5, 0.05 M Tris HCl pH 8.0 and 0.1 N NaOH. The extract was then centrifuged at 12,000 rpm for 15 min at 0 - 4°C. The supernatants were collected and then used for qualitative and quantitative analysis of proteins and amylase inhibitory activity.

Germination studies of amylase inhibitor from the seeds of *Mucuna* pruriens

The seeds of *Mucuna* pruriens were germinated for 7 days in the sterile sand (acid washed), and seedlings were collected for every 24 hours interval. The acetone powder of seedlings of all the 6 days was prepared as described above. The amylase inhibitor extracts of seedlings of all the 6 days were prepared as described above using 0.05 M sodium phosphate buffer, pH 7.0. The extract was then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant obtained was collected, used as a crude extract of amylase inhibitor and used for qualitative and quantitative analysis of proteins and amylase inhibitory activity.

Protein assay

The protein content was determined according to a method of Lowry *et al*. 1951 (24) using bovine

Received: 29 May 2018 | Revised: 2 August 2018 | Accepted: 27 August 2018

DOI: 10.1111/jfbc.12686

FULL ARTICLE Sl.No 6 ISSN 1745 - 4514 WILEY Journal of Food Biochemistry

Purification and characterization of alpha-amylase inhibitor from the seeds of underutilized legume, *Mucuna pruriens*

Rajiv Pranesh Bharadwaj[1] | Narayanappa Govinda Raju[2] | Kempohalli Sayanna Chandrashekharaiah[1]

[1]Department of Biochemistry, Mangalore University PG Centre, Chikka Aluvara, India

[2]Department of Biotechnology, Karnataka State Open University, Mysuru, India

Correspondence
Kempohalli Sayanna Chandrashekharaiah, Department of Biochemistry, Mangalore University PG Centre, Chikka Aluvara, Kodagu-571232, India.
Email: kschandraks@gmail.com

Abstract

An α-amylase inhibitor (EC 3.2.1.1) was purified by buffer extraction, ammonium sulfate fractionation, CM-cellulose, and sephadex G-75 chromatography from the soaked seeds of *Mucuna pruriens*. The molecular weight determined by gel permeation chromatography on Sephadex G-100 and SDS-PAGE, both in the presence and absence of 2-mercaptoethanol, was found to be 27.24 kDa and 25.6 kDa, respectively. The purified *Mucuna pruriens* amylase inhibitor showed a specific inhibitor activity of 61.18, fold purity of 36.68, and the yield obtained was 14.01%. The purified amylase inhibitor was found to be heat-stable and retained 80.50% activity at 65°C. Inhibitor was found to have pH optima of 6.9. Hundred percent zone of inhibition was observed when added on the plated organisms of purified inhibitor. Purified amylase inhibitor was found to inhibit the activity of human salivary α-amylase. Inhibitory activity of α-amylase inhibitor against mammalian amylases could suggest its potential in treatment of diabetes and cure of nutritional problems, which results in obesity.

Practical applications

Purified amylase inhibitor was found to inhibit the activity of human salivary α-amylase. The potential of this inhibitory activity from α-amylase inhibitors, especially in the mammalian α-amylase, could play an important role in the management of nutritional and diabetes-related disorders. *Mucuna*, an underutilized legume found in tropical region and also cultivated as food by various tribal's in Asia and Africa can be used as a potential source for extraction of these beneficiary protease inhibitors, which in turn finds its applications in various human therapeutic and/or disorder management.

KEYWORDS

amylase inhibitor, *Mucuna* seeds, physicochemical properties, purification

1 | INTRODUCTION

Carbohydrate metabolism is one of the important aspects for balancing both endocrine- and nutrition-related activities in the living systems. The glucose–fatty acid cycle is the determining factor in prevention of abnormalities related to this metabolism. Some of the common abnormalities observed are impaired sensitivity to insulin, glucose intolerance, and variation in insulin levels (Randle, Garland, Hales, & Newsholme, 1963). Glycoside hydrolases like amylases has profound roles in hydrolyzing dietary starch into saccharide units. α-Amylase in particular is one such classification of amylases which acts as digestive enzyme and found as both salivary and pancreatic

amylases in humans. α-Amylases are actively involved in the breakdown of carbohydrates at physiological pH leading to accumulation of maltose/glucose in blood. This condition is amplified by the intolerance/insensitivity to insulin or variation of insulin levels leading to diabetes mellitus. Various studies indicate the positive role of amylase inhibitors in addressing the abnormalities resulting in excessive amylase activity. Reduction in postprandial glucose levels by inhibition of α-Amylase can be a potential treatment option for diabetes mellitus (Funke & Melzig, 2006; Layer, Rizza, Zinsmeister, Carlson, & Dimagno, 1986). Plants were and are a pandora box of components with therapeutic value. Studies on various plant sources indicate the presence of inhibitors for proteases (Chagolla-Lopez, Blanco-Labra, Patthy, Sánchez, & Pongor, 1994; García Olmedo et al., 1987).

Proteinaceous α-Amylase inhibitors (AI) are present in microorganisms, plants, and animals. They occur in multiple forms and are most abundantly present in food plants, particularly in cereals and (Bonavides et al., 2007; Franco, Rigden, Melo, & Grossi-de-Sá, 2002; Payan, 2004; Xiaoyan et al., 2009). They inhibit the activity of amylases of mammalian pancreas, human saliva, bacteria, insect, and fungi (Lin et al., 2007; Liu et al., 2006; Melo et al., 2002; Wijaya, Neumann, Condron, Hughes, & Polya, 2000). These inhibitors were also known as starch blockers since they prevent dietary starches from being absorbed by inhibiting the starch digesting enzymes, α-amylases in the digestive track of mammals (Shaikh et al., 2013). In plants, α-amylase inhibitors act as natural defense system against insect pests and pathogens. These inhibitors impede digestion of carbohydrates through their action on the insect gut α-amylases which play a key role in the digestion of plant starch. Therefore, these inhibitors can also be used as bioinsecticide or biopesticide to prevent target harmful insects and pests (Dias et al., 2010; Farias et al., 2007). This property creates interest among the researcher to design and develop these inhibitor proteins as biotechnological tools to control insects, pests, and pathogens (Franco et al., 2006, 2000; Pereira et al., 1999). Amylase inhibitors are also drug design targets for the treatment of obesity and diabetes mellitus since they slows down the degradation and absorption of dietary starch by the body. Certain industrial applications like baking and brewing make use of alpha amylase inhibitors for improving baking properties and starch degradation, respectively (Ali, Houghton, & Soumyanath, 2006; Barrett & Udani, 2011; Layert, Carlson, & DiMango, 1985; Obiro, Zhang, & Jiang, 2008).

The genus *Mucuna* belongs to the family fabaceae (leguminoceae) which includes many species of annual and perennial legumes. *Mucuna* is an underutilized legume rich in proteins, extensively used as cover crop to control weeds and pests in agriculture and has great demand in food and pharmaceutical industries (Adebowale & Lawal, 2003; Gilbert, 2002). Since the reports on the purification of amylase inhibitors from the seeds of plants was scanty and no work has been carried out on amylase inhibitors from the seeds of *Mucuna*, an attempt was made to purify amylase inhibitor from the soaked seeds of *Mucuna pruriens*. In the present study, purification and characterization of amylase inhibitor isolated from the soaked seeds of *Mucuna pruriens* is described.

2 | MATERIALS AND METHODS

2.1 | Materials and chemicals

Seeds of *Mucuna pruriens* were collected from IIHR, Bangalore, Karnataka, India. Acrylamide, N, N, Methylene bisacrylamide, CM-cellulose (0.84 meq/g, medium), Sephadex G-75, Sephadex G-100, ampholyte carrier (pH 3–10), Coomassie Brilliant Blue G-250, and Bovine serum albumin (BSA) were obtained from Sigma Chemical Company, USA. All other chemicals used were of analytical grade.

2.2 | Methods

2.2.1 | Germination of seeds and extraction of amylase inhibitors

The seeds of *Mucuna pruriens* were germinated for 144 hr in sterile sand (acid-washed) and seedlings were collected at 24-hr interval. The seedlings of all the 6 days were dehulled and blended with 0.1 M HCl containing 0.1 M NaCl and 1% PVP. The suspension was stirred for 2.5 hr at 0°C–4°C and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant obtained was collected, used as crude extract of amylase inhibitor, and stored at 0°C–4°C until further use.

2.2.2 | Extraction of amylase inhibitors

The seedlings of all the 6 days were dehulled and blended with 0.1 M HCl containing 0.1 M NaCl and 1% PVP. The suspension was stirred for 2.5 hr at 0°C–4°C and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant obtained was collected, used as crude extract of amylase inhibitor, and stored at 0°C–4°C until further use.

2.3 | Purification of Amylase inhibitor

All the purification procedures were performed at 0°C–4°C unless otherwise stated. The protein purification was performed as described by Chandrashekharaiah, Ramachandra Swamy, and Siddalinga Murthy (2011). To the crude amylase inhibitor extract, solid ammonium sulfate was added slowly with constant stirring over magnetic stirrer at 0°C–4°C to obtain 0%–85% saturation. The solution was allowed to stand for 1 hr at room temperature and the precipitate obtained was removed by centrifugation at 12,000 rpm for 30 min. The precipitate obtained was dissolved in 0.025 M sodium acetate buffer, pH 5.5, and dialyzed against low ionic strength of same buffer. The dialyzed fraction was loaded onto a CM-cellulose column (2.5 × 22 cm) pre-equilibrated in 0.025 M sodium acetate buffer, pH 5.5, and flow rate was adjusted to 30 ml/h. The unbound proteins were removed in start buffer and bound proteins were eluted by stepwise increase in ionic strength using start buffer containing 0.1, 0.3, and 0.5 M NaCl and 10 ml fractions were collected. The CM-cellulose fraction III containing amylase inhibitor activity were pooled, concentrated, and applied to a Sephadex G-75 column (120 × 1.2 cm) pre-equilibrated with 0.05 M sodium phosphate

Sl. No.7, ISSN 0974- 4150

Asian J. Research Chem. 11(2): March - April 2018

ISSN 0974-4169 (Print)
0974-4150 (Online)

www.ajrconline.org

RESEARCH ARTICLE

Characterization of Protease inhibitors from the seeds of *Senna alata*

K S Chandrashekharaiah[1]*, H P Shweta[1], Rajiv Bharadwaj, N G Raju[1,2], N. Ramachandra Swamy[3]

[1]Department of Biochemistry, Mangalore University, Chikka Aluvara, Kodagu-571232
[2]Department of Biotechnology, Karnataka State Open University, Mysore-570006
[3]Department of Biochemistry, Bangalore University, Bangalore-560001
*Corresponding Author E-mail: **kschandraks@gmail.com**

ABSTRACT:

Manifestations of metabolic disorders are greatly influenced by proteases in either up or down regulation of related biomarkers. Inhibition of these potential proteases results in better management of systemic disorders. Medicinal plants like *Senna alata* has been a great source of such protease inhibitors in which the current study was focused to isolate, purify and characterize inhibitors, more specifically, trypsin inhibitors. The protease inhibitors were isolated and purified employing conventional protein purification such as salt precipiataion, gel filtration chromatography on Sephadex G-10 and G-50 and RP-HPLC. Two *Senna alata* protease inhibitors (SAPI-I and SAPI-II) were purified. SAPI-I was purified to 36.11 fold with a recovery of 42.80% and showed a specific inhibitor activity of 12.16. SAPI-II was purified to 41.23 fold with a recovery of 51.26 and showed a specific inhibitor activity of 19.61. Both the purified inhibitors were found to be stable in the pH range 5 – 8 and temperature between 4 – 65^{o}C.

KEYWORDS: Protease inhibitors, *Senna* alata, purification, properties.

INTRODUCTION:

Role of proteases in the early stage tumor growth and progression followed by invasion and metastasis has been studied and the need for protease inhibitors has been highlighted (Jennifer et. al., 2000). Protease inhibitors like trypsin and chymotrypsin inhibitors are known to inhibit the enzymatic activity leading to reduced physiological event progression. These inhibitors are mostly proteinaceous and are very specific in function. Protease inhibitors are broadly studied in microorganisms, plants and animals (Hamao 1976, Caroline et. al 2013, Leiner et. al., 1980).

Received on 23.09.2017 Modified on 15.01.2018
Accepted on 19.02.2018
Asian J. Research Chem. 2018; 11(2):251-254.

Plants are known to contain abundance of protease inhibitors which can be easily isolated and purified. These inhibitors can also be commercialized for use in therapeutic applications. Two trypsin inhibitors were isolated and purified from the leaves of tomato using salt precipitation and Sephadex based gel filtration chromatography (Gregory et. al., 1982). These inhibitors were also found to inhibit chymotrypsin and subtilisin up to an extent. The molecular weights of these inhibitors were in the range of 41,000 Da and 23,000 Da. Pea is known to contain protease inhibitors which was purified on a Sephadex platform with a total activity of ~50% and molecular weight in the range of 7000 Da. These inhibitors are known to act as plant defense against insects (Amrit et. al., 2016). A protease inhibitor with molecular weight of 39,000 Da was isolated from tobacco separated on a Sephadex G-75 platform (Tsung-Min 1984).

A review on *Senna alata* discusses about the medicincal properties of this plant which belongs to shrub family and found in intertropical areas. Being native of central

America, this plant also shows its presence in Caribbean area. Few of the medicinal applications to which *Senna alata* is used include digestive related, dermatologic, antiinfectious, antidiabetic and some of the inflammation and pain related conditions (Hennebelle et. al., 2009). Extracts from Senna alata has demonstrated anti-lipogenic effects (Jarinyaporn et. al., 2016). In this study, we have successfully isolated and purified two protease inhibitors showing trypsin inhibitor activity separated using Sephadex G-10 followed by Sephadex G-50 based gel filtration chromatography. Reverse phase high performance liquid chromatography was use for further purification of these inhibitors.

MATERIALS AND METHODS:

Materials: *Senna alata* seeds were collected from surroundings of Puthige panchayath of Kasaragod district of Kerala State.

Chemicals: Bovine Serum Albumin, trypsin, BAPNA, α-amylase, Starch, Sephadex G-10 and Sephadex G-50 were purchased from Sigma chemical laboratory, USA. All the other chemicals and reagents used were of technical grade.

Methods:

Extraction of protease inhibitor and purification

Crude protease inhibitor extract was prepared using sodium phosphate buffer pH 7.0 by stirring over a magnetic stirrer for 1.5 hr at 4^{o} C from acetone powder of soaked seeds of *Senna alata* which was prepared by blending with chilled acetone for 5 – 6 minutes as described by Chandrashekharaiah (2013). It was then centrifuged at 10000 rpm for 15 minutes. The supernatant obtained was used as inhibitor extract. Solid powdered ammonium sulphate was added slowly to inhibitor extract with stirring over magnetic stirrer at 4^{o}C for 30 minutes to obtain 0 - 85% saturation. After the addition of all the salt stirring was continued for 30 minutes and allowed to stand at 4^{o}C for 3 hours followed by centrifugation at 10000 rpm for 30 minutes. The pellet obtained was dissolved in small volume of extraction buffer and fractionated on sephadex G-10 chromatography which was performed as described by Rajiv Bharadwaj and Chandrashekharaiah (2017). The faction volume was 2.0 ml with a flow rate of 10 ml/hr and 0.025 M sodium phosphate buffer, pH. 7.0 as elution buffer. Among all the fractions obtained, fractions with inhibitor activity were pooled, concentrated and fractionated on Sephadex G-50 gel chromatography which was performed as described by Rajiv Bharadwaj and Chandrashekharaiah (2017) using 0.025 M sodium phosphate buffer, pH. 7.0 as elution buffer. The flow rate was calibrated to 10 ml/hour and 2.0 ml fractions were collected. The inhibitor fractions were further fractionated on RP-HPLC in Reversed-phase octadecylsilica (C18) column in binary solvent system with binary gradient capability and a UV detector. Buffer A is 0.1% (v/v) TFA in water and Buffer B is 100% acetonitrile containing 0.1% (v/v) TFA.

Determination of total protein: Total Protein was estimated from the inhibitor extract, fraction of sephadex G-10 and G-50 chromatography according to the method of Lowry et al., (1951).

Trypsin and trypsin inhibitor activity

Trypsin activity was measured according to the modified photometric method of Kakade et al., (1969) using the substrate BAPNA. BAPNA (40mg) was dissolved in 2 mL of dimethylsulfoxide (DMSO) and then diluted (1:100) in 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$, prior to enzyme assay. The enzyme assay was carried out by incubating trypsin with BAPNA for 10 min at 37 °C followed by the addition of 30 % acetic acid to arrest the reaction. The absorbance of p-nitroanilide liberated was measured at 410 nm against an appropriate blank. One trypsin (TU) unit is arbitrarily defined as an increase in absorbance of 0.01 at 410 nm under conditions of assay.

The trypsin inhibitor activity was determined by incubating trypsin with an aliquot of inhibitor inhibitor for 10 min at 37 °C. The reaction was started by the addition of BAPNA followed by incubation for 10 min and the reaction was arrested by the addition of 30 % acetic acid. The residual trypsin activity was measured 410 nm against an appropriate blank. The trypsin inhibitory unit (TIU) is defined as the number of trypsin units inhibited under the assay conditions.

Polyacrylamide gel electrophoresis:

An anionic disc gel electrophoresis was carried out essentially according to the method of Davis and Ornstein (1964). A discontinuous gel system consisting of 8% separating gel and4% spacer gel was used. The electrophoresis was carried out in cold applying a current of 20 – 25 mA for 4 hours using tris – glycine (pH 8.3) as electrode buffer and bromophenol blue as marker dye. After the electrophoresis, the proteins were stained with CBB R – 250 for 1 hour and distained using 7 % acetic acid.

Effect of pH and temperature

Determination of pH stability

The effect of pH on the activity of the partially purified *Senna alata* trypsin inhibitor was studied as described by Chandrashekharaiah (2013) using the buffers, Glycine – HCl buffer (0.2M, pH 2), Sodium acetate buffer (0.2 M, pH 4), Sodium citrate buffer (0.2 M, pH 5.5), Sodium phosphate buffer (0.2 M, pH 6.5), Tris – hydrochloride buffer (0.2 M, pH 8.0), Sodium borate buffer (0.2 M, pH

Research Journal of Pharmacognosy and Phytochemistry. 10(1): January- March, 2018

ISSN 0975-2331 (Print)
0975-4385 (Online)
DOI: 10.5958/0975-4385.2018.00020.1

Vol. 10| Issue-01|
January- March, 2018

Available online at
www.anvpublication.org
Research Journal of Pharmacognosy and Phytochemistry
Home page *www.rjpponline.org*

RESEARCH ARTICLE

Protease Inhibitors from the seeds of *Senna sophera:* Isolation and Properties

K S Chandrashekharaiah[1*], K B Sangeetha[1], Rajiv Bharadwaj[1], N G Raju[2]
N Ramachandra Swamy[3]
[1]Department of Biochemistry, Mangalore University, Chikka Aluvara, Kodagu-571232
[2]Department of Biotechnology, Karnataka State Open University, Mysore-570006
[3]Department of Biochemistry, Bangalore University, Bangalore-560001
*Corresponding Author E-mail: **kschandraks@gmail.com**

ABSTRACT:
Need for protease inhibitors in medicinal and therapeutic applications have been well acknowledged by the research community. The natural flora has always been an extensive contributor for various compounds used in disorder treatment owing to its abundance and ease of isolation and purifications methodologies. Conventional protein purification methods such as salt precipitation, gel filtration chromatography on Sephadex G-10 and G-50 and RP-HPLC have been employed for the purification of protease inhibitors from the seeds of Senna sophera. Two protease inhibitors SSPI-1 and SSPI-2 were purified. The protease inhibitor SSPI-1 was purified to 16.11 fold with a recovery of 26.20% and showed a specific inhibitor activity of 31.12. SSPI-2 was purified to 61.08 fold with a recovery of 66.23 and showed a specific inhibitor activity of 24.51. Both the purified inhibitors were found to be stable in the pH range 2 – 10 and temperature between 4 – 65^{o}C.

KEYWORDS: *Senna sophera* seeds, protease inhibitors, purification, properties.

INTRODUCTION:
Protease inhibitors are storage proteins of plants present in more amounts in the storage organs of plants and seeds. In plants, these proteinacious inhibitors involved in several functions such as regulation of proteases which hydrolyze proteins thereby preventing the unwanted proteolytic cleavage in the cell, act as defense proteins against insect pests and pathogens by binding to the active sites of proteases required for their growth and development thereby preventing crop loss or yield in agriculture[1].

Leguminous plants are the potential sources of these inhibitors and most of the inhibitors isolated and studied were serine protease inhibitors which play a significant role in many biological processes such as cell signaling, cell cycle progression, digestion, immune responses, blood coagulation, wound healing, cancer[2], apoptosis, hormone processing pathways, inflammation, hemorrhage, intracellular protein breakdown, transcription, cell invasion and also in the treatment of AIDS.[3-10]

The plant *Senna sophera* belong to leguminoseae is a shrub and popular due to its numerous medicinal values. All parts of the plant are used for various medicinal properties. The whole plant is purgative and febrifuge. It is used in homeopathic medicine. The roots are diuretic. It is also used in the treatment of osteoarthritis, asthma

Received on 24.09.2017 Modified on 19.11.2017
Accepted on 20.02.2018
Res. J. Pharmacognosy and Phytochem. 2018; 10(1): 139-140.
DOI: 10.5958/0975-4385.2018.00020.1

and allergic conditions. The pharmacological activities of the seeds of *Senna sophera* includes analgesic, anticonvulsant, antidiabetic, herbicidal and fungicidal effect. The seeds are used to reduce fevers. The boiled seeds are used for the treatment of Bright's disease. An infusion of the bark is used in the treatment of diabetes. Extracts of all plant parts are used to treat epilepsy. The dried leaves have been shown to have insect repellent and insecticidal properties. Root bark and seeds are used for preparation of medicine. It has been used by ancient Indian physicians for its efficiency in respiratory disorder. In the present study, isolation and properties of trypsin inhibitory peptides were described.

MATERIAL AND METHODS:

Materials:

Senna alata seeds were collected from surroundings of Puthige panchayath of Kasaragod district of Kerala State. Bovine Serum Albumin, trypsin, BAPNA, acrylamide, bisacrylamide, Sephadex G-10 and Sephadex G-50 were purchased from Sigma chemical laboratory, USA. All the other chemicals and reagents used were of technical grade.

Methods:

Isolation and purification of protease inhibitors:

Crude protein extract containing inhibitor proteins was prepared acetone powder prepared from soaked seeds of Senna sophera using 0.05M sodium phosphate buffer pH 7.0 by stirring over a magnetic stirrer for 1.5 hr at 4^{o} C as described by Chandrashekharaiah (2011)[11]. It was subjected to centrifugation at 10000 rpm for 15 minutes. The supernatant obtained was collected and used as crude protein extract. The crude protein extract was subjected to 0 – 85% ammonium sulphate fractionation. The appropriate amounts of ammonium sulphate was weighed, powdered and added slowly to crude protein extract while stirring over magnetic stirrer at 4^{o}C until it dissolved completely. The solution was allowed to stand at 4^{o}C for 3 hours for precipitation followed by centrifugation at 10000 rpm for 30 minutes. The pelleted protein was dissolved in small volume of extraction buffer and subjected to separation on sephadex G-10 chromatography which was performed as described by Rajiv Bharadwaj and Chandrashekharaiah (2017)[12]. The factions with trypsin inhibitor activity were pooled, concentrated and separated on Sephadex G-50 gel chromatography which was performed as described by Rajiv Bharadwaj and Chandrashekharaiah (2017)[12] using 0.05 M sodium phosphate buffer, pH. 7.0 as elution buffer. The flow rate was calibrated to 10 ml/hour and 2.0 ml fractions were collected. The trypsin inhibitor fractions were further fractionated on RP-HPLC in Reversed-phase octadecylsilica (C18) column in binary solvent system with binary gradient capability and a UV detector. Buffer A is 0.1% (v/v) TFA in water and Buffer B is 100% acetonitrile containing 0.1% (v/v) TFA.

Determination of total protein, Trypsin and trypsin inhibitor activity:

Total Protein was determined from the crude protein extract, fraction of sephadex G-10 and G-50 chromatography according to the method of Lowry et al., (1951)[13]. Trypsin activity was measured with substrate Nα-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (BAPNA) according to the modified photometric method of Kakade et al., (1969)[14]. The substrate concentration of 0.919 mM BAPNA was prepared in dimethylsulfoxide (DMSO) followed by 1:100 dilution in 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$ (40mg BAPNA in 2 mL of dimethylsulfoxide (DMSO) and diluted to 100 ml with 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$) prior to enzyme assay. The trypsin assay was carried out by incubating enzyme with BAPNA for 10 min at 37 °C followed by the addition of 30 % acetic acid to arrest the reaction. The absorbance of p-nitroanilide liberated was measured at 410 nm against an appropriate blank. One trypsin (TU) unit is arbitrarily defined as an increase in absorbance of 0.01 at 410 nm under conditions of assay.

The trypsin inhibitor assay was performed by incubating trypsin with an aliquot of inhibitor for 10 min at 37 °C. The reaction was started by the addition of BAPNA followed by incubation for 10 min and the reaction was arrested by the addition of 30 % acetic acid. The residual trypsin activity was measured at 410 nm against an appropriate blank. The trypsin inhibitory unit (TIU) is defined as the number of trypsin units inhibited under the assay conditions.

Polyacrylamide gel electrophoresis:

An anionic disc gel electrophoresis was carried out essentially according to the method of Davis and Ornstein (1964)[15]. A discontinuous gel system consisting of 8% separating gel and4% spacer gel was used. The electrophoresis was carried out in cold applying a current of 20 – 25 mA for 4 hours using tris – glycine (pH 8.3) as electrode buffer and bromophenol blue as marker dye. After the electrophoresis, the proteins were stained with CBB R – 250 for 1 hour and distained using 7 % acetic acid.

Effect of pH and temperature:

The effect of pH on the activity of the partially purified *Senna sophera* trypsin inhibitor was studied as described by Chandrashekharaiah (2013)[3] using different buffers such as Glycine – HCl buffer (0.2M, pH 2), Sodium acetate buffer (0.2 M, pH 4), Sodium citrate buffer (0.2 M, pH 5.5), Sodium phosphate buffer (0.2 M, pH 6.5), Tris – hydrochloride buffer (0.2 M, pH 8.0), Sodium borate buffer (0.2 M, pH 10). Similarly, the pH stability was determined by preincubating the partially purified *Senna sophera* trypsin inhibitor with above buffers for 30 min. Trypsin inhibitor assay was performed as

International Journal of Biotechnology and Biochemistry
ISSN 0973-2691 Volume 13, Number 4 (2017) pp. 361-370

http://www.ripublication.com

Characterization of Protease Inhibitors from the Seeds of *Adenanthera Pavonina*

K S Chandrashekharaiah[1*], A Shashank[1], Rajiv Bharadwaj[1], N G Raju[2] and N Ramachandra Swamy[3]

[1]Department of Biochemistry, Mangalore University, Chikka Aluvara, Kodagu-571232, India.
[2]Department of Biotechnology, Karnataka State Open University, Mysore-570006, India.
[3]Department of Biochemistry, Bangalore University, Bangalore-560001, India.

**(Corresponding author)*

Abstract

Enzyme inhibitors such as protease inhibitors are widely distributed in nature inhibit the catalytic activity of proteolytic enzymes. They involved in variety of proteolytic process of biological/physiological significance. The protease inhibitors, APPI-1, APPI-2and APPI-3 have been isolated and purified from the seeds of *Adenanthera pavonina* employing salt fractionation, Sephadex G-10 and Sepahdex G-50 gel-permeation chromatography and RP-HPLC. The APPI-1, APPI-2and APPI-3 was purified to 31.24, 36.02 and 19.21 fold with a recovery of 41.10%, 51% and 43.28%, respectively. Further, APPI-1, APPI-2and APPI-3 showed a specific inhibitor activity of 16.15, 20.43 and 11.06, respectively. The purified APPI-1, APPI-2and APPI-3 showed a molecular weight of 7 - 8 kDa, 11 -12 kDa and 13 – 14 kDa (approximately) as determined by gel filtration chromatography. All the three purified inhibitors are both pH and temperature stable. The APPI-1, APPI-2and APPI-3 showed antimicrobial activity on E.coli and streptococcus species.

Key words: *Adenanthera pavonina,* Protease inhibitors, Isolation, Purification, Characterization, Antibacterial properties.

INTRODUCTION

Seeds are one of the storage organs of plants contains variety of proteins which includes carbohydrate binding proteins, ribosome activating proteins, inhibitors of various enzymes, chitin degrading enzymes etc. Among these proteins, enzyme inhibitors such as inhibitors of proteolytic enzymes which are produced in response to pest or pathogen attack attracted a great attention of researchers due to their participation in variety of biological functions. In plants, these inhibitors of proteolytic enzymes acts as storage proteins, involved in the regulation of endogenous proteases thereby preventing unwanted proteolytic degradation, act as defense proteins preventing the attack of insect pests or pathogens thereby prevents crop loss in agriculture. Pharmacologically. The inhibitors of proteolytic enzymes can also be used in the treatment of various proteolytic enzymes involved pathogenic process of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular distropy, cancer and even AIDS [1 – 6].

Adenanthera pavonina is a perennial and non-climbing species of leguminous tree. Its uses include food and drink, traditional medicine and timber. It is commonly called Red Lucky Seed and it is cultivated for forage. It is also grown as medicinal plant, an ornamental garden plant or urban tree. The young leaves and seeds can be cooked and eaten. This tree is used for making soap and a red dye can be obtained from the wood. Since the seeds are used as medicine and no research work on protease inhibitor from this plant is carried out, the present work is undertaken. In the present study, partial purification and characterization of protease inhibitor is described.

MATERIALS AND METHODS

Seeds of *Adenanthera pavonina* were collected from areas of Puthige Panchayath of Kasaragod District of Kerala state. Bovine Serum Albumin, trypsin, BAPNA, Casein, Sephadex G-10 and Sephadex G-50 were obtained from Sigma chemical company, USA. All other chemicals used were of technical grade.

METHODS:

Isolation and purification

The seeds of *Adenanthera pavonina* were soaked for overnight and dehulled. The acetone powder was prepared blended in a homogenizer with chilled acetone for 5 mins as described by chandrashekharaiah [7]. It was filtered and the cake obtained was dried at 37^{o} C which was powdered and used for the extraction of protease inhibitor. The protease inhibitor extract was prepared in sodium phosphate buffer, pH 7.0 by stirring over a magnetic stirrer for 1.5 hr at 4^{o} C. The extract was then