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Biochemical and Antimicrobial Characterization of an Underexploited Medicinal Plant - *Verbesina encelioides*

C.K. Divya Ramakrishnan[1*], Dayal Doss[2] and A. Vijayabharathi[3]

[1]Department. of Biotechnology, Sri Narayanaguru College, Coimbatore–641 105, Tamil Nadu, India
[2]Department of Plant Biotechnology, GKVK, UAS, Bangalore – 560065, Karnataka, India
[3]Department Genetics and Plant Breeding, University of Agricultural Sciences, Bangalore – 560065, Karnataka, India
**Corresponding author*

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A B S T R A C T

Verbesina encelioides is an exotic medicinal plant widely used in traditional medicine. Antimicrobial proteins are low molecular weight compounds involved in constitutive or induced resistance to microbial attack. The search for new antimicrobial substances exhibiting minimal side effect is warranted. The study was conducted to estimate some of the biochemical parameters and characterize the antimicrobial proteins from the young leaves of *Verbesina encelioides*. Plant was surveyed in Eastern dry zones of Karnataka and sample from each places were collected. Among them sample collected from Kolar (Thavarekere village) was found to have maximum total protein, sugar, phenol, tannin and micro-nutrients. SDS-PAGE analysis estimated the low molecular weight protein found to be of 14 k Da which is responsible for the antimicrobial activity. Bioassays were done to test the antibacterial and antifungal activity. Antibacterial activity was determined against selected bacterial pathogens like *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus coagulance*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*. Later antifungal activity was determined by *Aspergillus flavus*, *Rhizactonia solani*, *Fusarium oxysporum* and *Sclerotium rolfsii*. The result confirmed that the protein exhibits moderate antimicrobial activity. This determines the relevance of antimicrobial activity of *Verbesina encelioides.*

Introduction

All organisms have evolved several defense systems in order to protect themselves against bacteria, fungi and viruses. Plants constitute an excellent ecosystem for microorganisms. Microbes interact with plant tissues and cells with different degree of dependence. Plants are the tremendous source for the discovery of new products of medicinal value for new drug development. Many of the drugs sold today are simple synthetic modifications of naturally obtained substances. Much work has been done on ethno-medicinal plants in India. *Verbesina encelioides*, a member of the *Asteraceae* (sunflower) family, is an erect annual commonly seen to heights of 1 to 5 feet. Its common names are golden crown beard, crown beard, wild sunflower and yellow top. The native range of *Verbesina encelioides* is generally considered to be North and South America, specifically

Mexico and the Southwestern United States of Texas, Arizona Open areas appear to be ideal habitat for the plant (Robbins *et al.*, 1951 and Parker 1972).

United leaves of *Verbesina encelioides* are toothed or lobed and have two distinct growth patterns like the lower leaves are opposite and triangular, while the upper leaves are alternate and lance shaped. Both upper and lower leaves featured with fine white hairs on the undersides, which are also present on the stem of *Verbesina encelioides*, which grows from a taproot system.

Flower heads are found on elongated stalks, and resemble small flowers, 1-2 inches in length. Flower heads can either be solitary, or in clusters of up to three heads. Seeds of *Verbesina encelioides* are greyish-brown, flat and winged along the margins and are considered as drought tolerant plant. This propagates by seeds. The plant demonstrates an efficient ability for both self and cross pollination (Parker, 1972).

Medicinal uses of *Verbesina encelioides* appear to be limited and not widely documented. However, M. Moore, the Director of the Southwest School of Botanical Medicine refers that the plant is primarily an anti-inflammatory for redness and swelling of the orifices.

The paste is applied directly to hemorrhoids, labial inflammations and sore gums. Earlier medicinal uses are thought to have been practiced by the Indian tribe, utilizing *Verbesina encelioides* for the treatment of spider bite symptoms. Galegine and ferulic acid have been reported as key phytochemicals, present in this plant.

Galegine, an alkaloid, has both antimicrobial and anti-tumor properties. Ferulic acid has been claimed to reduce the side effects of chemo- and radiotherapy of carcinomas by increasing natural immune defenses. So keeping this in mind, an attempt has been made to study the biochemical, antimicrobial and antifungal characters of the medicinal plant *Verbesina encelioides.*

Materials and Methods

Geographical survey of *Verbesina encelioides*

The plant material was surveyed in different regions of Eastern dry districts of Karnataka, India. Sample 1 was collected from Marathalli, Bangalore. Sample 2 was collected from Bangarapet Kolar district. Sample 3 was also collected from Thavarekere, Kolar. Sample 4 was collected from Chintamani. Sample 5 was collected from Mulabagal and Sample 6 was also from Kolar-Tavarekere village.

Biochemical analysis

Preparation of acetone powder

Five gram of fresh plant leaves were transferred to a blender. Pre-chilled acetone (kept at–20°C) was added sufficiently to cover the sample and then blended for 2-3 minutes at low speed followed by high speed for 3 to 5 minutes with an intermittent break. Then the mixture was filtered through a Buchner funnel with Whattman Grade 1 filter paper. Finally, the powder was spread on filter paper and air dried and kept at –20°C till further use.

Estimation of total soluble protein content

100 mg of acetone powder was extracted with 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) for one hour on a magnetic stirrer at room temperature. The extract was centrifuged at 10,000 rpm for 20 minutes and the supernatant was used for the estimation of total soluble protein content (Lowry *et al.*, 1951).

Reagents

Solution A

20 g of anhydrous sodium carbonate ($Na_2CO_3.2H_2O$) and 4 g of sodium hydroxide were dissolved in 1000 ml of distilled water.

Solution B

1 ml of 1.35% sodium potassium tartarate and 0.1 ml of 5.5% $CuSO_4.5H_2O$ solutions were mixed together.

Solution C

50 ml of solution A was mixed with 1 ml of solution B just before use.

Folin–Ciocalteau reagent (FCR)

The commercial FCR was diluted in 1:1 before use.

Standard bovine serum albumin (BSA) solution: A stock BSA solution was prepared containing 2 mg BSA/ ml in water. This solution was diluted 1:10 to obtain 200 µg BSA/ ml of working standard solution.

Estimation

A known volume of aliquot sample was made up to 1 ml with distilled water. To this, 5 ml of solution C was added and mixed well. After 10 minutes, 0.5 ml of FCR was added and mixed immediately. The blue colour developed was read at 660 nm after 30 minutes against a reagent blank in a colorimeter.

Preparation of leaf sample

The leaf samples collected from plants were dried in oven and then made into fine powder using mortar and pestle. The powdered samples were preserved in butter paper/ polythene cover and used for biochemical analysis.

Estimation of total soluble sugars

Phenol reagent

5 g of re-distilled phenol is dissolved in 95 ml of distilled water.

Sample extraction

100 mg of dry sample was extracted with 10 ml of 80% warm ethanol in a pestle and mortar.

Then the extract was centrifuged at 10,000 rpm for ten minutes and the extract was evaporated to dryness in a hot water bath and the residue was dissolved in 5 ml of distilled water. Interfering coloured pigments were removed by using activated charcoal by keeping it overnight.

The extract was centrifuged and clear supernatant was used for the estimation of total sugars (Dubios, 1951).

Estimation

Known aliquot extracts were made up to 1 ml with distilled water and 0.5 ml of phenol reagent was added and mixed well. 5 ml of 96% sulphuric acid and placed in a hot water bath at 30°C for 20 minutes. Absorbance was read at 490 nm. The amount of total sugars in the sample was estimated by comparing the results with a standard glucose curve.

Estimation of total phenol content

Reagents: Folin–Ciocalteau reagent

Commercial grade reagent was diluted 1:1 ratio with distilled water.

20% Sodium carbonate solution

20 g of Na_2CO_3 was dissolved in distilled water and made up to 100 ml.

Acidified methanol

10 ml of hydrochloric acid (HCl) was mixed with 90 ml of methanol.

Standard catechol solution

A stock catechol solution was prepared containing 1 mg catechol / ml in water. This solution was diluted 1:10 ratio to obtain 100 µg catechol / ml of working standard solution.

Sample extraction

100 mg of oven-dried powdered sample was extracted in 10 ml of warm 80% ethanol for 1 hour at room temperature. The extract was centrifuged at 6000 rpm for 15 minutes. The supernatant was evaporated to dryness on a water bath and the residue was dissolved in 5 ml of water.

The alcohol free extract was used for the estimation of total phenols (Malick and Singh, 1980).

Estimation

0.1 ml of aliquot sample was diluted to 3 ml with distilled water and 0.5 ml of FCR was added and mixed thoroughly. Exactly after 3 minutes, 2 ml of 20% sodium carbonate solution was added and kept in a boiling water bath for one minute.

After cooling under running tap water, the absorbance was read at 650 nm, against the reagent blank in a colorimeter. A standard graph was constructed with catechol as a standard in the range of 20-100 µg per gram of sample.

Estimation of total tannin content

Reagents: Vanillin–HCl reagent

8% HCl in methanol and 4% vanillin in methanol were mixed in equal volumes just before use.

Standard catechin solution

A stock catechin solution was prepared containing 1 mg catechin / ml in methanol. This solution was diluted 1:10 ratio to obtain 100 µg catechin / ml of working standard solution.

Sample extraction

100 mg of oven-dried powdered sample was extracted with 5 ml of methanol for 24 hours at room temperature with occasional stirring. The extract was centrifuged at 5000 rpm for 10 minutes. The supernatant was used for the estimation of total tannins (Burns, 1971).

Extraction

To 1 ml of the aliquot sample, 5 ml of vanillin–HCl reagent was added and mixed. After incubation for 20 minutes, the absorbance was read at 500 nm against a reagent blank in a colorimeter.

A standard graph was constructed using catechin as a standard in the range of 0.2–2.0 mg/ g of oven-dried sample.

Estimation of micronutrients Fe, Mg, Zn and Cu

Powdered leaf sample is pre-digested in concentrated nitric acid, then with di-acid mixture, which gives snow white residue. This is then cooled and volume is made up to 150 ml with distilled water and filtered to remove silica precipitate. The obtained

supernatant is used to estimate micronutrients by atomic absorption spectrophotometer (Black, 1965).

Electrophoretic characterization of soluble proteins

Sample preparation

100 mg of acetone powder was extracted in 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) for 1 hour on a magnetic stirrer at room temperature.

The extract was centrifuged at 10,000 rpm for 20 minutes and the supernatant was used for characterization of soluble protein profiles.

SDS–PAGE

The SDS–PAGE of soluble proteins was performed in a slab gel by preparing 10% resolving gel and 5% stacking gel. The method followed was based on the procedure described by Laemmli (1970).

Anti-microbial activity of plant extracts of *V. encelioides*

Bioassay for anti-bacterial activity

Collection of bacterial cultures

The animal or human pathogens were collected from Ramaiah, Medical Institute, Bangalore, Karnataka. Plant pathogen, which was maintained in the Department of plant pathology, University of Agricultural Sciences (UAS), GKVK, Bangalore.

The animal pathogens (B1 - *Staphylococcus aureus***,** B2 - *Pseudomonas aeruginosa***,** B3 - *Enterococcus faecalis*, B4 - *Escherichia coli* and B5 - *Staphylococcus coagulance*) and plant pathogens were used for the zone of inhibition test as follows.

Filter disc

The filter paper was cut into 1.5 cm diameter and kept in a bottle and autoclaved at 121°C at 15 lbs. for 15 minutes. Nutrient agar medium was prepared and autoclaved after the medium was poured into the sterile petri plate. Each plate contains 15-20 ml of media.

Plant extract for zone of inhibition studies

The selected healthy plant material of *V. encelioides* was thoroughly washed with distilled water. The leaves of the plant were crushed with the help of sterile pestle and mortar and extracts were collected.

The extracts were centrifuged at 2,000 rpm for 10 to 15 minutes. The solid particles that were settled in the bottom and the clear supernatant extracts were collected in a sterile bottle. Then the solution was filtered through Millipore membrane (0.45 µm) was stored in sterile bottle at 4^{o}C till use.

Zone of inhibition test

To make a lawn of bacterial cultures a standard spread plate method was followed. Then the sterile filter disc (1.5 cm diameter) was taken and immersed in cell free extract of *V. encelioides* and the excess extracts were removed.

Then it was transferred on lawn of bacterial culture in the center of the plate. The plates were incubated 37^{o}C for 24 hours and then observed for the zone of inhibition.

Anti-fungal activities of plant extract of *Verbesina encelioides*

Pathogenic plant fungi used were obtained from Department of Pathology, UAS, GKVK, Bangalore. The list of fungi culture was as follows.

Fungal culture

F1- *Sclerotium rolfsii*, F2- *Fusarium oxysporum*, F3 - *Aspergillus flavus*, F4 - *Aspergillus niger* and F5-*Rhizoctonia solani*.

Zone of inhibition test for fungal culture

The fully-grown fungal plates were taken. With the help of the sterile cork borer (1.5 cm in diameter) cut the culture blocks in to cubical blocks. These blocks were then aseptically transferred in to the potato dextrose agar (PDA) plates and incubated for 2 days at 28^{o}C. Then, all the fungal cultures were grown on both the sides of the culture till paper disc dipped in cell free extract were transferred. The whole set up was incubated at 28^{o}C for 2-3 days and the zone of inhibition was observed.

Results and Discussion

The plant was surveyed in Eastern dry zones of Karnataka and places like Kolar, Bangalore, Chintamani and Mulabagal. The survey yielded only five strains of the plants from the above regions. *Verbesina encelioides* plant was characterized biochemically by estimating the total soluble protein, total sugars, phenols and tannin contents.

The total soluble protein estimated results shown that the Strain-3 collected from Kolar district (near Thavarekere village) was found to have higher total protein of 10%, maximum phenol content of 3.6 mg/g, total soluble sugar content of 17.17% and higher tannin content of 4.72% compared to all other strains. Strain-3 has recorded maximum zinc content of 60.11µg/ g of dry weight of the plant sample, maximum iron content of 202.17µg/ g, manganese content of 120.60 µg/ g and copper content of 15.26 µg/ g of the dry weight of the plant sample. The results are present in the Table 1.

The standard procedure for the extraction of protein was carried out and total soluble protein banding pattern profile was also done. All strains had similar banding pattern. To determine its inhibition against various bacterial pathogens, bioassays were done. For this zone inhibition assay was carried out. The concentration of protein from *Verbesina encelioides* used in this study ranges between 5-20 µg. The radii of growth inhibition zones were measured in each case after 24 hours. The results indicated the susceptibility of *Streptococcus coagulance, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa* to anti-bacterial action of protein. *E. coli* showed better inhibition when compared to other cultures which was followed by *E. faecalis*, *S. aureus*, *P. aeruginosa* and *S. coagulance.* Similar results were shown by all strains.

Bioassays were done to determine the inhibition of protein against some of the agriculturally important fungal pathogens. For this, zone inhibition assay was carried out (Table 2). The concentration of protein ranges from 5-20 µg.

The radii of growth inhibition zones were measured in each case after 48 hours. The results indicated the susceptibility of *Aspergillus flavus, Sclerotium rolfsii, Rhizactonia solani, Fusarium oxysporum* to anti-fungal action of *Verbesina encelioides*. *Rhizactonia solani* and *Aspergillus flavus* has shown maximum inhibition compared to other cultures. *Aspergillus niger* showed no inhibition to corresponding concentrations of protein. These results were similar in all the strains. It was an advantage over the other anti-microbial plants as *Verbesina encelioides* is only effective on fungal plant pathogens. This evidence put together suggest that *Verbesina encelioides* protein was a moderate anti-fungal protein compared to anti-bacterial activity.

Table.1 Estimation of various biochemical parameters and micro nutrients content in different strains of *Verbesina encelioides*

Sample	Place of collection	Total soluble protein (%)	Total soluble sugars (%)	Total phenol content (%)	Total tannin content (%)	Zinc (µg/g)	Manganese (µg/g)	Iron (µg/g)	Copper (µg/g)
Strain 1	Bangalore (Marathalli)	5.05	7.20	0.62	2.77	41.15	74.27	121.57	9.37
Strain 2	Kolar (Bangarapet)	6.00	13.30	0.57	3.10	48.02	91.35	144.21	11.28
Strain 3	Kolar (Thavarekere)	10.00	17.17	3.60	4.72	60.11	120.60	202.17	15.26
Strain 4	Chintamani (Hadripura)	5.25	6.30	0.20	2.07	58.14	103.24	180.77	10.82
Strain 5	Mulbagal (Pethandalahalli)	5.30	15.20	1.10	3.37	55.18	78.37	165.85	8.98
Strain 6	Kolar (Thavarekere)	6.25	15.30	0.72	3.25	54.11	77.20	157.22	11.10

Table.2 Zone of inhibition study of bacterial and fungal pathogens with 14kDa protein from the medicinal plant *Verbesina encelioides*

Sl. No.	Bacterial pathogen	Zones of inhibition (diameter in mm.)with particular concentration of protein(10µg)					
		Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6
1.	*Escherichia coli*	10.0	7.0	6.0	5.0	6.0	8.0
2.	*Pseudomonas aeroginosa*	9.0	8.0	7.0	6.0	5.0	8.0
3.	*Staphylococcus aureus*	8.5	10.0	6.8	9.2	8.6	NI
4.	*Staphylococcus coagulans*	7.0	6.0	8.0	7.0	10.5	9.0
5.	*Enterococcus faecalis*	7.5	7.4	9.0	8.0	8.6	7.6
	Fungal pathogen						
1.	*Aspergillus niger*	NI*	NI	NI	NI	NI	NI
2.	*Aspergillus flavus*	7.0	7.6	9	9.0	7.0	8.0
3.	*Rhizoctonia solani*	7.8	6.6	7	6.0	9.0	8.0
4.	*Fusarium oxysporum*	7.0	8.2	6	9.0	9.0	7.6
5.	*Sclerotium rolfsii*	NI	NI	NI	NI	NI	NI

* NI – No inhibition

Verbesina encelioides is one among the under exploited medicinal plants of Indian origin. The under exploited are to be used to the full extent for unknown medicinal properties. The geographical area of Karnataka is divided in to ten agro-climatic zones. *Verbesina encelioides* is identified by surveying different dry zones like Bangalore, Kolar, Chintamani and Mulabagal etc. Among the place of collection, the strains collected from Kolar region were found to be more prominent in most of the regions, which were

identified as Strain - 3 and Strain - 2. These genotypes, which were from different zones, were identified for the strain specificity in different locations. This was done with the help of taxonomist and was confirmed as *Verbesina encelioides.* The importance of this species was earlier verified for the protein profiling and was found to have both high and low molecular weight proteins. The total soluble protein estimation results shown that Strain-3 collected from Kolar district was found to have higher total protein of 16% compared to other strains. Bhagat and Jadeja (2003) reported the protein content varied between 10.07-11.96% in Safed musli accession. Similarly, Dhan *et al.,* (2001) noticed total soluble protein for erect and spreading type of *Desmodium gangeticum* respectively. There was significant increase in the total soluble sugar content (17.17%) in the Strain-3 compared to other strains. Similar results were observed in the traditional medicinal plant namely bitter guard, fenugreek and jambu, which varied from 2.03 to 11.765 (Kochhar *et al.,* 2006). There was comparable variation in the total phenol content in the different plant strains.

In *Verbesina encelioides* maximum total phenol content of 3.60% was noticed in the Strain-3. Javanmarde *et al.,* (2003) reported similar observation in Basil (*Ocimum basilicum*). In betel wine fruit pulp, seeds and leaves, Maiti *et al.,* (1999) reported supporting results. Total tannin content recorded in Strain-3 was 4.78%. Similarly Kireeva *et al.,* (1999) observed maximum content of tannin of 16.20% in *Hipericum perforatum*, which is measured during intensive vegetative growth. The results of the above experiment revealed that there is difference in the collection of strains. Even though all the plants belong to same genera but seem to be difference in strain. There is strain specificity. This was further verified by other molecular characterization.

Plants unlike humans and animals become sick, but they have evolved a sophisticated defense response against microbes, on a combination of constitutive and inducible response, which can be localized or spread throughout plant organs and tissues. The response is mediated by several messenger molecules that activate pathogen responsive genes coding for enzyme or antimicrobial compounds (Montesinos *et al.,* 2002). Antimicrobial peptides are important biomolecules functioning as self-defense against infection by harmful pathogens. They are isolated from various sources among plants, animals and bacteria and have been characterized (Fugimora *et al.,* 2003). The defense strategy of plants against stress factor involves a multitude of tools, including the synthesis of various types of stress proteins with protective function. A group of plant encoded proteins included by various stress stimuli, pathogenesis related proteins are assigned important role in plant defence against pathogenic infection and in general adaptation to stressful environment.

Conventional control of crop disease depends on the use of chemically synthesized products. However repeated use of such chemicals has several drawbacks, such as their lack of specificity, increased incidence of development of resistance upon prolonged application, and the adverse impact on human health and environment. Genetic engineering of plants by transferring a gene coding for potent anti-microbial protein has now been accepted as a method of choice for directional improvement and development of disease resistant plants (Moreno *et al.,* 2005). Taking into consideration the potential of anti-microbial proteins in plant protection strategies, the relevance of this study can be very well understood.

Young leaves of *Verbesina encelioides* were used for extraction of anti-microbial protein.

In many reports, more anti-microbial activity has been found in young leaves as compared to old ones. Further it has been shown that some of the PR proteins are expressed in tissue specific manner during the development (Bol *et al.,* 1990). Hegaard *et al.,* (1992) reported several fold increase in the synthesis and extra cellular accumulation by barley leaf protein in response to *Erysiphae* infection. Three distinct basic 14k Da proteins p14a, p14b, and p14c were isolated from tomato (*Lycopersicum esculentum* Mill cv. Baby) leaves infected with phytophthora infestance both *In vitro* and *with* bioassay carried out with tomato leaf discs (Niderman *et al.,* 1995).

Zone inhibition assay was carried out for the determination of antibacterial and antifungal activity. Antibacterial activity of protein revealed that *Pseudomonas aeruginosa* showed minimum inhibition when compared to other cultures. *Enterococcus faecalis* was found to be maximum effective to corresponding concentrations of protein. Similar results were observed *Verbesina encelioides* extract, which was most effective against *Staphylococcus aureus*, *Bacillus cereus and Vibrio cholera*, whereas it was not much effective against *Shiegella dysentriae and Salmonella typhi* (Nickavar and Mojab, 2003).

Antifungal action of protein revealed that *Aspergillus flavus* and *Fusarium oxysporum* showed maximum inhibition as compared to other fungal pathogens where as *Aspergillus niger* and *Sclerotonia rolfsii* showed no inhibition. Similar results were recorded by Mahasner (2002) in *Avecennea marina*, which aqueous extract exhibited moderate antifungal activity against *Aspergillus flavus and Candida albicans*. Based on results it implies *Verbesina encelioides* is a very potential anti-bacterial agent, which may act on broad range of phytopathogens, and it is a moderate anti-fungal agent, which act on few phytopathogenic fungi. Cloning and expression of *Verbesina encelioides* anti-microbial protein gene will be of great importance in agriculture for developing transgenic plants resistant to wider range of fungal phytopathogens. The result of the protein helps to characterize genotypes collected from different localities. Further the bands have to be still analysed. For the other characters, this protein profiling in the exotic medicinal plant was done for the first time and the other molecular characterization results are awaited.

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