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Callus Induction and Antibacterial Activity of Verbesina Encelioides Leaves

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ABSTRACT

Verbesina encelioides belongs to the Asteraceae family and also known as golden crown beard and wild sunflower. In present study callus was raised from the leaf explant of *V. encelioides*. Callus was obtained on MS medium formed using 3% sucrose and 0.8% agar incorporated with 2mg/L BAP and 0.5 mg/L IAA. The callus was used for antibacterial activity identification. The results indicated Callus was brownish yellow in color and compact. *E. coli* and *B. subtilis* which showed almost similar activities. *B. subtilis* was most responsive in methanol extract (14mm), *E. coli* was inhibited best by ethyl acetate. This study proves that *V. encelioides* callus extracts were more prominent and showed much efficient activity in comparison to that of leaf extract.

Keywords: *Verbesina encelioides,* Asteraceae, Callus culture, antibacterial activity,

INTRODUCTION

Plants have always been important components of both conventional and modern medicine, for their utility in health and wellness. Around 80% of the world's population relies on plant-derived ingredients (**Winter *et al*., 2012; Yuan *et al*., 2016**).

Plant-based compounds like taxol, colchicine, artemisinin, forkolin, vincristine, vinblastine, are widely used in both biology and medicine. Growing and exporting of medicinal plants is on the rise in globe market for trade. The herbal market is expanding rapidly. A seven percent annual growth rate, or $60 billion, is now by 2050, it's anticipated to produce more than $5 trillion (**Denbath *et al*., 2006**). India and China, two of the biggest nations in Asia, have the widest selections of officially recognized and well-known medicinal plants (**Raven *et al*., 1998**).

The traditional plant-based medical systems of Ayurveda, Siddha, Unani, Tibetan, Chinese, and Naturopathy continue to be very important in the treatment of disease. An ancient text on herbal medicine known as the Charak Samhita describes the creation of 340 herbal medicines for the treatment of ailments (**Nadkarni and Nadkarni, 1908; Kala *et al*., 2006**).

Chemicals produced by plants through primary or secondary metabolism are known as phytochemicals (**Molyneux *et al*., 2007; Horborne *et al*., 1999**). The antimicrobial properties of phytochemicals have been stimulated by the high demand on the pharmaceutical and food industries to develop new food preservatives to avoid synthetic preservatives and majorly to novel therapies for the treatment of various microbial infections to combat microbial resistance against conventional antibiotics (**Alamgir *et al*., 2017; O'Connor *et al*., 2015; Katz *et al*., 2016**).

Callus cultures now have the potential to be used commercially for the production of therapeutically important secondary metabolites (**Ogita *et al*., 2015; Wu *et al*., 2016**).

The Asteraceae family is one of the largest flowering plant families, with over 23,600 species and approximately 1620 genera **(Funk *et al.*, 2009)**. *Verbesina encelioides*, also known as wild sunflower or golden crown beard, is an exotic invasive weed that is thought to have originated in the United States and Mexico. Golden crown beard is an erect, annual, wild herb with a wide range of tolerance to climatic conditions and a competitive growth habit **(Singh *et al.*, 2017)**.

Micropropagation is a crucial component of the plant propagation industry, complementing or replacing traditional clonal vegetative propagation techniques (cutting, grafting, division, and separation), as well as seed propagation in some circumstances. Micropropagation refers to the aseptic cultivation of specific explants of tissues and organs in closed tubes using a specific culture medium and in a regulated environment. In addition to genetic engineering, micropropagation is now the most commercially effective and practically focused plant biotechnology. It produces a huge number of clonal plants of many plant species quickly, many of which are also virus- or other pathogen-free. Additionally, the technical link in the production of transgenic plants and other somatically altered plants is now micropropagation (**Murashige and Skoog, 1973**).

MATERIAL AND METHOD

Plant Materials

The experimental plant material of *Verbesina encelioides* leaf was collected from Campus of University of Rajasthan, Jaipur and voucher specimen was deposited to the herbarium of Department of Botany, University of Rajasthan, Jaipur for authentication (RUBL no. 211480). Leaves, nodal and inter-nodal segments were taken from young juvenile plant. MS medium was used for all tissue culture studies **(Murashige and Skoog, 1962).** Composition of MS medium is given in **Table-1.**

Table1: Composition of MS medium and preparation of stock solutions (Murashige and Skoog, 1962)

Stock Solutions	**Group**	**1X (mg/l)**	**Stock Ratio**	**Required in 1 L**
[A]	**MS- Sulphates**		**1000X1L (mg)**	**=10 ml**
	1. $MgSO_4.7H_2O$	370	37000	
	2. $MnSO_4.4H_2O$	22.3	2230	
	3. $ZnSO_4.7H_2O$	16.9	1690	
	4. $CuSO_4.5H_2O$	8.6	860	
[B]	**MS non- sulphates**		**20X-1L (mg)**	**=50 ml**
	1. NH_4NO_3	1650	33000	
	2. KNO_3	1900	38000	
	3. KH_2PO_4	1700	34000	
	4. $CaCl_2.2H_2O$	440	8800	
	5. KI	0.83	16.6	
	6. H_3BO_3	6.2	1240	
	7. $CoCl_2.6H_2O$	0.025	0.5	
	8. $Na_2MoO_4.2H_2O$	0.25	5	

[C]	**Iron- EDTA** 1. $FeSO_4.7H_2O$ 2. Na_2EDTA	 27.8 37.3	**200X-500 ml (mg)** 2785 3725	**=5 ml**
[D]	**Vitamins** 1. Glycine 2. Meso-inositol 3. Nicotinic acid 4. Pyridoxine HCl 5.Thymine HCl	 2.0 100 0.5 0.5 0.1	**100X-500 ml (mg)** 100 5000 25 25 5	**=10 ml**

Sucrose : 3% (w/v), Agar-agar 0.8-1%, pH 5.8

The medium was sterilized by autoclaving at 15 lbs per square inch pressure for 25-30 min. Solution A-G was prepared separately and stored in a refrigerator (for 4-6 weeks).

Aseptic Technique

1. **Surface Sterilization-** Using a locally available bleach solution (0.1% $HgCl_2$) for 2 min. and rinsed three times in SDW.
2. **Inoculation of explants on nutrient medium-** Explants were inoculated aseptically in culture flasks containing MS medium supplemented with BAP (1 mg/l) and IAA (0.5 mg/l) in combination form. Inoculation of explants was done under laminar airflow hood fitted with UV lamp.
3. **Incubation-** Cultured flasks were incubated in a growth chamber, provided with the suitable growth conditions. The temperature of the chamber was maintained at 26± 2°C with 50-60 % relative humidity and light intensity of 2000-2500 lux was provided by fluorescent incandescent tubes (40 watts). A photoperiod of 16h and a dark period of 8 hr. were provided per day.
4. **Maintenance of callus-** Primary callus was initiated from *in-vitro* grown explants when media was supplemented with various growth hormones.

Sub-culturing procedure- The primary cultures were obtained having callus which were cut in small pieces and then transferred to subculture medium after every 4-6 weeks of culture initiation and maintained till 32 weeks.

Antimicrobial Activities from callus in plant extracts- The antimicrobial activity of *Verbesina encelioides* leaves and callus extracts was studied with methanol, ethyl acetate and hexane extracts. Two bacterial strains were selected for the antimicrobial activity.

Preparation of Extracts - The leaves and callus of *Verbesina encelioides* have been washed thoroughly with tap water and are subjected to air-drying in the shade at room temperature (32-37°C) for about 2-3 weeks. The dried samples were ground into powder form by using a homogenizer. About 50gm of sample (50gm/250ml) were extracted in a Soxhlet extractor for 8 to 10 hours, sequentially with methanol, ethyl acetate and hexane. The extracts obtained were then concentrated and finally dried to a constant weight. Dried extracts were kept at 20°C until further tests were carried out.

Microorganisms Used- Clinical laboratory bacterial isolates of *Bacillus subtilis* (Gram positive), *Escherichia coli* (Gram negative) were collected from the stock cultures of Microbiology Laboratory, SMS Medical College Jaipur, India.

Antibacterial Assay

In vitro antibacterial activity of the sample extract was studied against Gram positive and Gram negative bacterial strains by the agar well diffusion method **(Perez *et al.*, 1990).** Mueller Hinton agar no. 2 (Hi Media, India) was used as the bacteriological medium. The extracts were diluted in 100% Dimethylsulphoxide (DMSO) at different concentrations. The Mueller Hinton agar was melted and cooled to 48 - 50ºC and a standardized inoculum (1.5×108 CFU/mL, 0.5 McFarland) was then added aseptically to the molten agar and poured into sterile petri dishes to give a solid plate. Wells (6 mm diameter) were prepared in the seeded agar plates. The test compound (20, 40, 60 and 80 µl) was introduced in the well. The plates were incubated overnight at 37ºC. The antimicrobial spectrum of the extract was determined for the bacterial species in terms of zone sizes around each well. The diameters of zone of inhibition produced by the agent were compared with those produced by the commercial control antibiotics, Ciprofloxacin (40 µl). The experiment was performed three times to minimize the error and the mean values are presented.

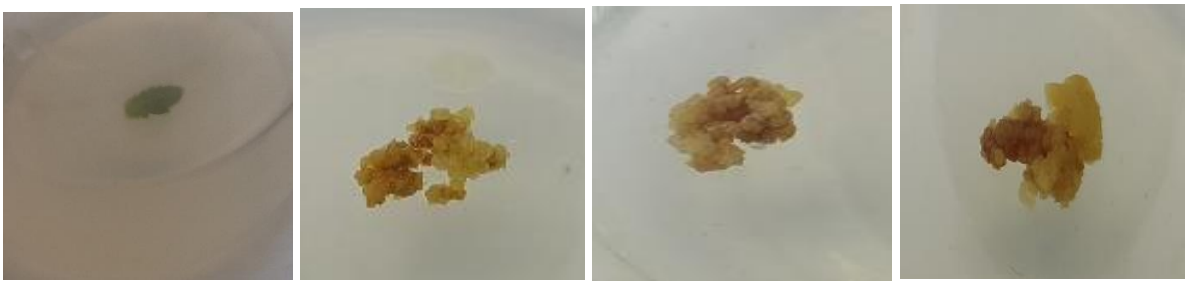
Result and observation

1) Callus induction

MS medium supplemented with BAP + IAA for callus induction in three different concentrations which were 1.50+0.25mg/L, 1.75+0.75 mg/L and 2+0.5 mg /L. The leaf showed significant callus formation on MS medium with highest concentration 2mg/L BAP and 0.5 mg/L IAA. Callus was compact and brownish yellow in colour. Callus obtained after 8 weeks of culture from MS medium was further evaluated for primary metabolites after 4 weeks (Figure 1)

Table 1. Callus production from leaf segment of *Verbesina encelioides* MS medium supplemented with varied concentrations of growth hormones.

Growth hormone	Concentration (mg/l)	Callus Formation (%)
BAP+IAA	2+0.5	48.2 + 0.07
	1.75+0.75	21.1 + 0.4
	1.50+0.25	13.5 + 0.23



(a) **(b)** **(c)** **(d)**

Figure 1. Leaf derived callus of *Verbesina encelioides* with various concentration of BAP+IAA: a) Inoculation, b) 1.5 + 0.25 mg/L, c) 1.75 + 0.75mg/L, and d) 2 + 0.5 mg/L (after 4 weeks)

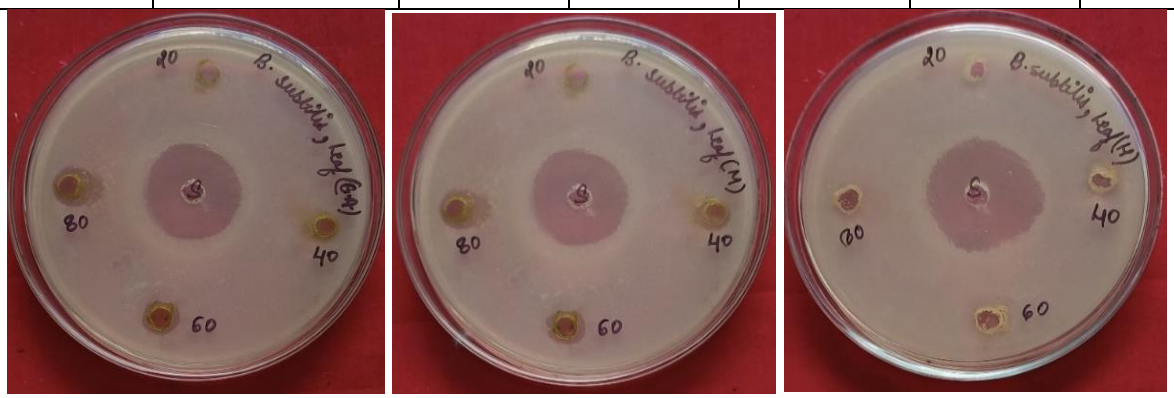
2) Antibacterial activities

a) Anti-bacterial activity of leaf extracts

In-vitro study of anti-bacterial activity of *Verbesina encelioides* leaves (Table 2) with the standard ciprofloxacin was conducted during the research. *B. subtilis* has shown prominent activity in methanol and ethyl acetate extract with inhibition zone (IZ) being 11 mm at 80 µL whereas hexane extract displayed no activity at all. *E. coli* gave best IZ (13mm) at ethyl acetate extract followed by methanol and hexane which gave equal IZ of 9 mm at 80 µL.

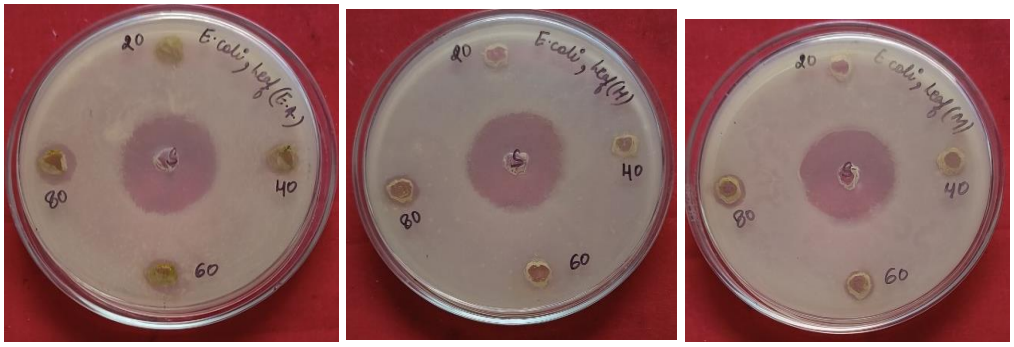
Table 2. Antibacterial activity of *Verbesina encelioides* leaves against pathogenic bacteria

Bacteria	Extract	Inhibition zone (mm)				
		Standard	20 µL	40 µL	60 µL	80 µL
B. subtilis	Ethyl acetate	30	-	-	9	11
	Methanol	30	-	8	10	11
	Hexane	30	-	-	-	-
E. coli	Ethyl acetate	30	-	8	9	13
	Methanol	30	-	-	7	9
	Hexane	30	-	-	-	9



(a) **(b)** **(c)**

Figure 1. Antibacterial activity of *Verbesina encelioides* leaves against *Bacillus subtilis* (a) Ethyl acetate extract (b) Methanol extract (c) Hexane extract



(a) **(b)** **(c)**

Figure 2. Antibacterial activity of *Verbesina encelioides* leaves against *E. coli* (a) Ethyl acetate extract (b) Hexane extract c) Methanol extract

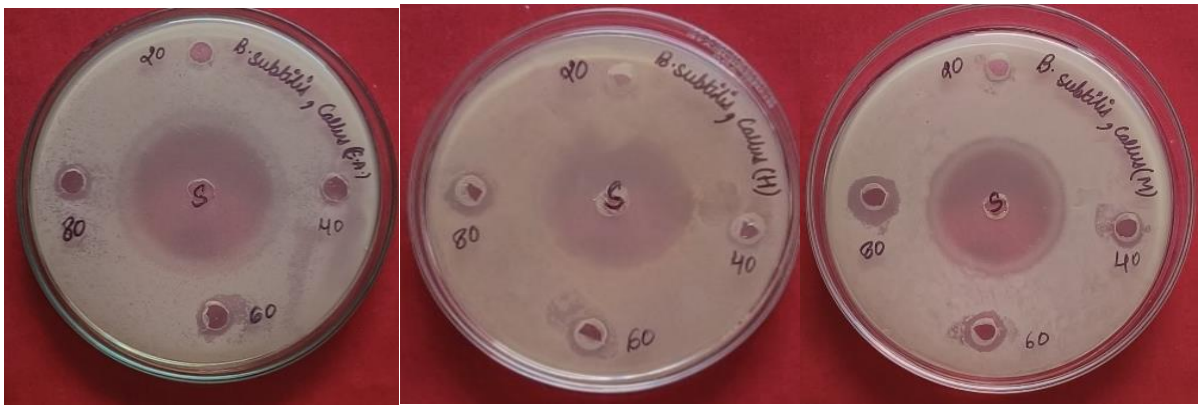
b) Anti-bacterial activity of callus extract

In the antibacterial study of *Verbesina encelioides* callus using these bacterial strains (*B. subtilis* and *E. col)* (Table 3), *B. subtilis* was most responsive in methanol extract (14mm) as compared to hexane extract (10mm) and ethyl acetate (9mm) at 80 µL. In *E. coli*, methanol gave best IZ (15mm) at 80 µL followed by ethyl acetate (11mm) and hexane extract (8mm).

In the study of antibacterial activity of *Verbesina encelioides* leaf extracts and *in-vitro* cultured callus extracts, the callus extracts were more prominent and efficient activity was visible in comparison to that of leaf extract.

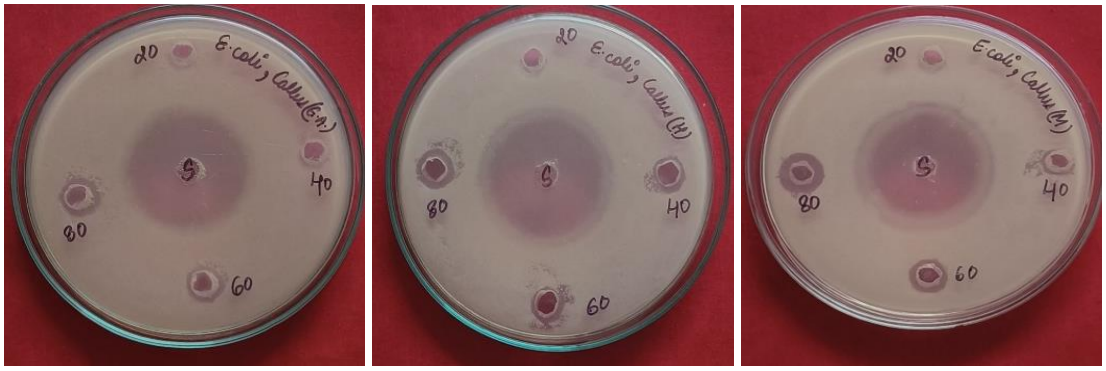
Table 3. Antibacterial activity of callus extract of *Verbesina encelioides* against pathogenic bacteria

Bacteria	Extract	Inhibition zone (mm)				
		Standard	20 µL	40 µL	60 µL	80 µL
B. subtilis	Ethyl acetate	30	-	7	8	9
	Methanol	30	7	9	12	14
	Hexane	30	-	7	9	10
E. coli	Ethyl acetate	30	-	7	9	11
	Methanol	30	-	9	11	13
	Hexane	30	7	8	9	12



(a) (b) (c)

Figure 3. Antibacterial activity of callus extract of *Verbesina encelioides* leaf explant against *Bacillus subtilis.* (a) Ethyl acetate extract (b) Hexane extract c) Methanol extract



(a) (b) (c)

Figure 4. Antibacterial activity of callus extract of *Verbesina encelioides* leaf explant against *Escherichia coli* (a) Ethyl acetate extract (b) Hexane extract c) Methanol extract

Callus induction

In the present study three different concentrations of BAP + IAA were added to MS medium to induce callus formation: a) 1.50+0.25mg/L, b) 1.75+0.75 mg/L, and c) 2+0.5 mg/L. The leaf showed significant callus formation on MS medium with the highest concentration of 2 mg/L BAP and 0.5 mg/L IAA. Callus was brownish yellow in colour and compact.

Anti-bacterial activity

In the result of *in-vitro* study of anti-bacterial activity of *Verbesina encelioides* leave with the standard ciprofloxacin being 30µL, *E. coli* and *B. subtilis* which showed almost similar activities.

Callus extracts were more prominent and showed much efficient activity in comparison to that of leaf extract. *B. subtilis* was most responsive in methanol extract (14mm) as compared to ethyl acetate and hexane extract. *E. coli* was inhibited best by ethyl acetate.

Conclusion

In the present shoot regeneration started in the sixth week. In contrast to both, the best shoot generation was seen at a concentration of 0.5 mg/l, where two prominently visible shoots were grown along with a minutely formed root. However, the lowest rate of regeneration was seen in the treatment at 2 mg/l, which only resulted in minute root growth.

Callus extracts were more prominent and showed much efficient activity in comparison to that of leaf extract. *B. subtilis* was most responsive in methanol extract (14mm) as compared to ethyl acetate and hexane extract. *E. coli* was inhibited best by ethyl acetate.

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