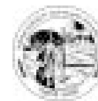

BIOPOTENTIALITIES OF *VERBESINA ENCELIOIDES* CELL CULTURES

S.C. JAIN*, R. SINGH AND R. JAIN+

Medicinal Plants and Biotechnology Research Laboratory, Department of Botany, University of Rajasthan, Jaipur-302 004
+Department of Chemistry, University of Rajasthan, Jaipur-302 004

Received on 21 July, 2008, Revised on 30 Sept., 2008

SUMMARY

Verbesina encelioides **(Cav.) Benth. & Hook. fil ex Gray is regarded as ornamental garden plant and there are many reports of its use in folk medicine as analgesic, emetic, febrifuge, insecticide and anti-inflammatory. It is even used to treat cancer, gastrointestinal disturbance, skin ailments, and snake bite. The aim of present investigation is to evaluate the biosynthetic and bioefficacy potentials of the cell cultures of *V. encelioides*. For this purpose, cell cultures were established from seeds on MS basal medium in the absence or presence of IAA, NAA, Kn and BAP singly or in various combinations. Initiation of callus was observed after 20 days of inoculation and callus was successfully established on MS medium supplemented with 10 mg/L NAA and 0.4 mg/L Kn. The callus was whitish brown in colour and friable in nature. Various compounds viz. friedelin, epifriedelin, lupeol, α-, β- amyrin, stigmasterol, betulin and β-sitosterol have been isolated and identified using spectral studies. Different bioefficacies like antibacterial, antifungal followed by disc diffusion method and antioxidant using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were studied and compared with *in vivo* system.**

Key words**: Antimicrobial, antioxidant, cell cultures, phytochemical analysis, *Verbesina encelioides***

INTRODUCTION

Verbesina encelioides (Cav.) Benth. & Hook. fil ex Gray (Vern. Golden Crownbeard, Fam. Asteraceae) a plant with bright yellow flowers, is an excellent example of an exotic invasive plant. It is a well known weed which has extended its range within the United States, including Hawaii, and to several Latin American countries, as also to South Africa, Australia and India. This plant species is generally used in traditional medicines and possesses analgesic, emetic, febrifuge and insecticidal action. It is also found to be important in folk remedies for cancer, snake-bite, gastrointestinal disturbance, skin ailments and hemorrhoids treatment. Toxicity of this plant is attributed to galegine, which is an active antihyperglycaemic agent, and is used ethnomedicinally for treatment of diabetes (Petricic and Kalodera 1982). Jain *et al.* (1988) demonstrated antibacterial, antifungal, antiviral, antitumor, hypoglycaemic and antiimplantation activities of different extracts/fractions of this plant species, where the root extract was found to be active against *Streptococcus faecalis* while essential oil- and flavonoid - rich fractions exhibited maximal inhibitory activity against *S. faecalis* and *Curvularia lunata*, respectively. Likewise, aqueous infusion of roots demonstrated significant antitumor inhibitory activity (11-40%) and effectively lowered the implantation sites and corpus luteum. According to Toribio *et al.* (2005), methanolic extract of the flowers demonstrated higher antimicrobial activity against Gram +ve bacteria and *Candida albicans*. Phytochemically, primary metabolites (Jain and Purohit 1985, 1989),

*Corresponding author, E-mail: jainnatpro3@rediffmail.com

terpenoids (β-sitosterol, β-sitosterol-D-(+)glucoside, hentriacontanol, pseudotaraxasterol, pseudotaraxastenone and pseudotaraxasteryl acetate) from the whole plant (Tiwari *et al*. 1978) and three rare flavonol glycosides (quercetin-3-galactoside, quercetin-3-galactoside-7-glucoside and quercetin-3-xyloside-7-glucoside) from the flowers (Glennie and Jain 1980) and triterpenoids, were isolated from the roots and aerial parts (Joshi *et al*. 1983). Some work concerning their toxicity due to galegine (Kingsbury 1964, Oelrichs *et al*. 1981, Eichholzer *et al*. 1982, Petricic and Kalodera 1982, Keeler *et al*. 1986, Lopez *et al*. 1996), was also carried out. Allelopathic effects have also been noted and attributed to its dominant coverage and success in inhibiting native plant growth (Goel 1987, Inderjit *et al*. 1999). In continuation to our earlier studies (Jain *et al*. 2007a, b, 2008), we now report the biosynthetic and biological potentialities of *V. encelioides* cell cultures.

MATERIALS AND METHODS

Plant materials: Seeds of *V. encelioides* were collected from the fields of University in the month of August, 2006. The Botanical identity was confirmed by the Herbarium Section, Botany Department, University of Rajasthan, Jaipur, India. A voucher specimen was deposited at the Herbarium, University Department of Botany, Jaipur, India and in our laboratory for further reference (RUBL 12977).

Mature scarified seeds were surface-sterilized by dipping in 70% ethanol for 30 seconds, followed by treatment with 0.1% $HgCl_2$ for 2 min and subsequent washings (4 or 5 times) with sterile distilled water. Seeds were then transferred on the MS solid medium (Murashige and Skoog 1962) with 3% sucrose. The medium was gelled with 0.8% agar (HiMedia) and its pH was adjusted to 5.8 before autoclaving. The culture flasks were autoclaved at 15 lbs/psi pressure for 20 min. The seeds were incubated for germination at 25º±2ÚC under fluorescent light of 300-400 Lux intensity for 16 hr photoperiod, diurnally.

Establishment of cell cultures: Hypocotyl explants (~1 cm in size) were sectioned from 12-15 d old seedlings (about 5.0 cm long) and cultured on MS medium without or with IAA (0.00-1.00 mg/l), NAA (10-20 mg/l), Kn (0.4-1.0 mg/l) and BAP (0.5-5.0 mg/l), either individually or in different combinations, resulting in callus formation. Growth indices (GI) were calculated (final dry weight of callus – initial dry weight of callus / initial dry weight of callus) at varying growth intervals (2, 4, 6, 8 and 10 weeks) in five replicates and the mean values were computed. The moisture content of callus was also calculated (%). Callus was later powdered and used for phytochemical and biological screening.

Preparation of the extracts: The callus harvested at the transfer age of 8 weeks was kept at 100º C for 5 minutes so as to inactivate any enzymatic activity , and later at 60º C, till a constant weight was achieved. 10 g seeds and seed-callus were separately powdered and extracted in a Soxhelt apparatus with 100 ml of ethanol (60ºC for 12 hr). The samples were then filtered through Whatman No. 1 paper in a Buchner funnel, the filtrate was freeze dried and weighed.

Phytochemical studies: Thin layer chromatography (TLC) profile of *in vivo* and *in vitro* extracts was carried out on silica gel G plates (0.4-0.5 mm) along with the reference markers (Harborne 1973) using heptane-benzene-alcohol (100:100:1) as mobile phase. Separated constituents were visualized by spraying with 10% alcoholic H_2SO_4. A portion of the plate containing the seed-callus extract and the reference markers were visualized under UV light (254 nm) and exposed to iodine vapors for ~10 min. Several spots coinciding with reference compounds were marked, scrapped off from the parallel lanes of unsprayed plates, eluted with methanol, filtered, evaporated to dryness, reconstituted and crystallized in methanol. The melting points of the isolated compounds were determined in capillary tubes (Toshniwal Melting Point Apparatus). Each compound was subjected to IR (Perkin Elmer 337, Grating Infra Red Spectrophotometer) and NMR analyses (model Brukur-DPX- 300 MHz, using $CDCl_3$ and DMSO- d6 as an internal reference) and identified (Dev *et al*. 1989, Yamaguchi 1970).

Antimicrobial efficacy: Pure cultures of test bacteria, *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 11298), *Pseudomonas aeruginosa* (ATCC 25668) and *Staphylococcus aureus* (ATCC 9144) were obtained from the IMTECH,

Chandigarh, India. These cultures were grown and maintained on Nutrient Broth medium (NB) at 27°C for 48 hr. Similarly, test fungi, *Aspergillus niger* (ATCC 322), *Candida albicans* (ATCC 4718), *Penicillium crysogenum* (ATCC 5476), and *Tricophyton rubrum* (ATCC 2327), obtained from IARI, New Delhi, India, were cultured on Potato dextrose agar medium (PDA) at 37°C for 48 hr. Antimicrobial activity of the alcoholic extracts of seed and seed-callus were performed by disc diffusion method using NB and PDA medium (Gould and Bowie 1952). Whatman No. 1 filter paper discs were enriched with 4 mg of each extract and used. The density of microorganisms was adjusted as per McFarland 0.5 standard. 100 µl suspension of each microorganism was inoculated in the petri plates containing NB or PDA medium. The discs of test extracts were used and the plates incubated (SEW, N. Delhi, India) at 37°C for 24 hr. The diameter of the inhibition zone (mm) was measured (Inhibition zone recorder, HiMedia, India), and in each case, the activity index (AI) was also calculated. Three replicates were used and the average value was recorded. Parallel negative control and streptomycin (10 mcg/disc) and ketonocozole (10 mcg/ disc) were used as positive controls.

Antioxidant potentialities: 2, 2- Diphenyl-1-picrylhydrazyl (DPPH), and quercetin were obtained from HiMedia, India. The method used by Fogliano *et al.* (1999) was adopted with suitable modifications to our experimental conditions. For qualitative assay, extracts, and quercetin as standard (20 mg), were dissolved in 1 ml methanol, out of which 1 µl was applied on TLC plates (Silica gel 60 $F_{254;}$ 20x20 cm). These plates were sprayed with DPPH (0.002 g/10 ml) and exposed to daylight until discoloring of the background (6 hr). The resulting yellow colour on the plates was determined as active antioxidant constituents. This method was also used for positive and negative control. For quantitative assay, each of the extract (0.0008 g) was dissolved separately in 10 ml of methanol and various concentrations (80, 60, 40, 20 and 10 µg) were prepared. Each of the 2.5 ml test extract was mixed with DPPH (0.002 g/ 10 ml) and allowed 30 min for the reaction occur. The absorbance of the colour developed was measured at 517 nm by UV spectrophotometer (Varian type Cary PCB 150 Water Peltier System with Standard Cuvettes). The negative control and standard quercetin as positive control were also subjected to the same procedure. Three replicates were used and the average absorption was noted for each concentration. Data were processed using EXCEL and the concentrations that cause 50% reduction in absorbance (RC_{50}) were calculated. Percent inhibition of DPPH was calculated by following equation (Lee *et al*. 1998):

$$\% \text{ Inhibition} = 1- (A_1/A_2) \times 100$$

where, A_1 is the absorbance of the test sample and A_2 as the absorbance of control reaction.

RESULTS AND DISCUSSION

Callogenesis: Induction of callus from the hypocotyl explants of *in vitro* germinated scarified seeds was observed after 5 days of inoculation (Fig. 1A). Out of the variable hormonal concentrations used, better results in term of callus formation, were obtained on MS medium supplemented with NAA+BAP (1+3 mg/l), followed by 1+2 mg/l concentration of hormones (Table 1). The callus was initially white, friable and profuse in nature, which on subsequent subculturings turned whitish-green (Fig 1B). The growth indices exhibited a

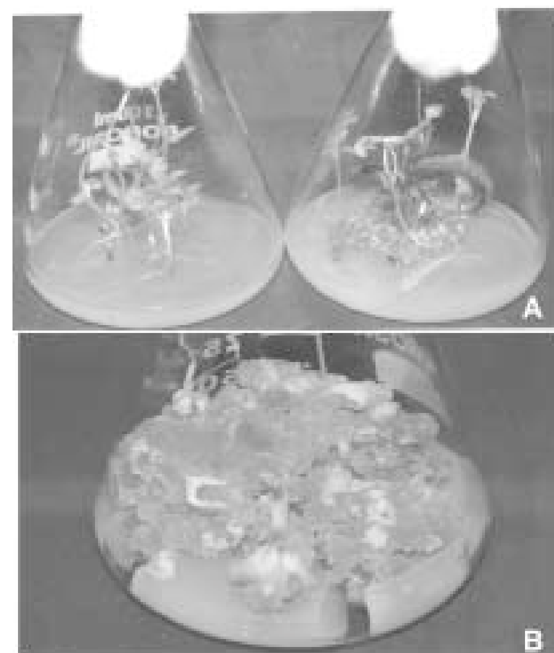

Fig. 1. (A, B). Callus induction from *V. encelioides*
(A) Germinated seeds on MS medium, (B) 8 weeks old callus.

Table 1. Callus induction from scarified seeds *V. encelioides.*

Hormones used	Concentration (mg/l)	Explant response (%)	Callus growth	Colour	Texture
NAA+BAP	0.50+1.00	33	++	BG	FR
	0.50+2.00	31	+	"	"
	0.50+3.00	28	+	BN	"
	1.00+1.00	52	++	"	"
	1.00+2.00	71	+++	BG	"
	1.00+3.00	82	++++	BG	"
NAA+Kn	5.00+0.20	40	+	GN	"
	10.00+0.40	59	++	BN	"
	15.00+0.60	33	+	"	"

+, Low; ++, Moderate; +++, High; ++++, Intense. BG, Brownish green; BN, Brown; GN, Green; FR, Friable.

gradual increase from 2 weeks onwards, following a sigmoid pattern of growth reaching maxima in 8 week-old cultures. The moisture content was highest (95.76%) in 10 week-old cultures (Fig 2).

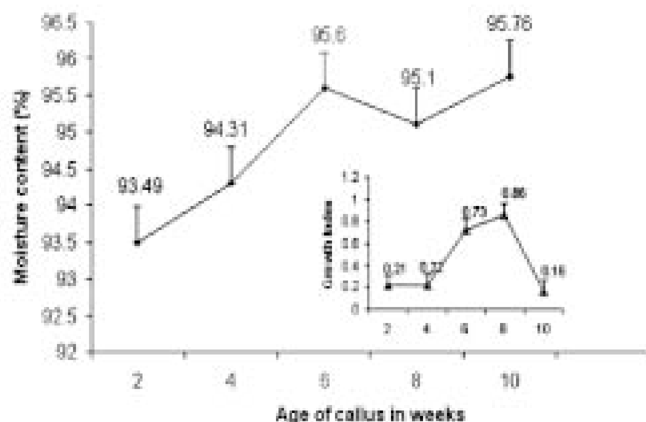

Fig. 2. Moisture content and growth index of seed-callus of *V. encelioides.*

Phytochemical studies: The total yield of crude extracts was found to be variable (seeds 18.60% > seed-callus 1.33%). From the cell cultures of this plant, several triterpenoidal compounds viz. friedelin, epifriedelinol, lupeol, α- and β-amyrins, stigmasterol, betulin and β-sitosterol, were isolated and identified on the basis of their chromatographic behaviour, melting points and spectral analysis (Table 2, Fig 3).

Antimicrobial activity: Pure methanol (control) was inactive on the all tested microorganisms and activity was varied related to test organisms. While comparing the data of antimicrobial screening, it is evident that seed-callus extract showed appreciable antibacterial activity (IZ 10 mm) against *B. subtilis* and notable antifungal activity against *C. albicans* and *T. rubrum* (IZ 15 mm, Table 3, Fig 4).

Table 2. Chromatographic and chemical characterstics of isolated compounds from seed-callus of *V. encelioides.*

Isolated compounds	R_F (×100)	Colour after spray	m.p. (°C)	IR (ν_{max}) cm-1(KBr)
Friedelin	81	Pink	198-200	1720, 1380, 1365, 1255, 1230, 1200, 920
Epifriedelinol	48	Brown	279-281	3410, 1470, 1360, 1260, 1100, 990, 970
Lupeol	36	Violet	212-213	3450, 1650, 1140
α- Amyrin	26	Pink	183-184	3350, 1640, 1480, 1360, 1130, 1050, 930
β- Amyrin	20	Pink	197-198	3350, 1650, 1190, 1140, 1100, 1050
Stigmasterol	15	Pink	166-167	3400, 1640, 1470, 1300, 1110, 820
Betulin	13	Pink	245-246	3490, 1655, 1060, 1010, 880
β- Sitosterol	06	Blue	136-137	1730, 1640, 1240, 735, 725

Solvent system: Heptane – Benzene-Alcohol (100:100:1); Spraying reagent: 10% H_2SO_4.

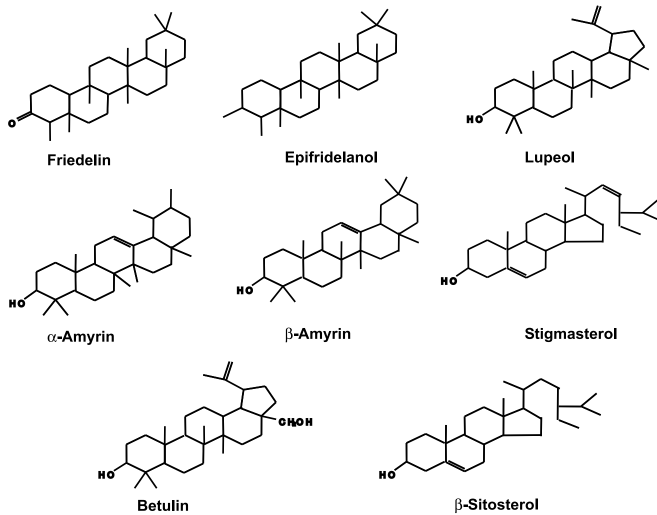

Fig. 3. Isolated compounds from *V. encelioides* seed-callus.

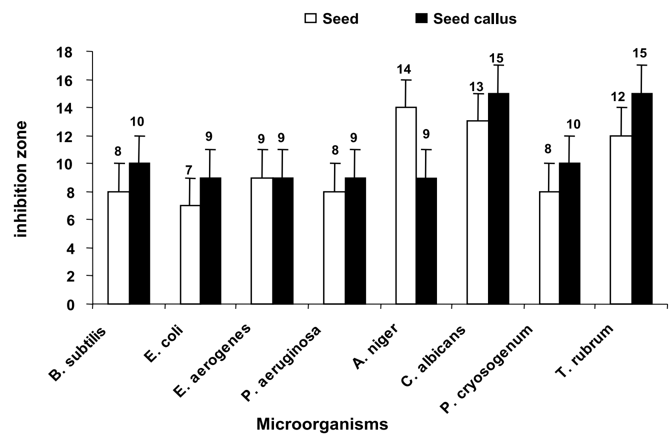

Fig. 4. Antimicrobial activity of seed and seed-callus of *V. encelioides*

Table 3. Antimicrobial activity of *V. encelioides* seed and seed-callus.

Microorganisms		Seed[a]	Seed-callus[a]
Bacteria			
Bacillus subtillis	IZ[b]	8	10
	AI[c]	0.26	0.33
Enterobacter aerogenes	IZ	9	9
	AI	0.45	0.45
Escherichia coli	IZ	7	9
	AI	0.31	0.40
Pseudomonas aeruginosa	IZ	8	9
	AI	0.26	0.30
Fungi			
Aspergillus niger	IZ	14	9
	AI	0.77	0.50
Candida albicans	IZ	13	15
	AI	0.65	0.75
Penicillium crysogenum	IZ	8	10
	AI	0.36	0.45
Tricophyton rubrum	IZ	12	15
	AI	0.66	0.83

[a]Test samples 4 mg/disc. Standard test drugs: streptomycin for bacteria, ketonocozole for fungi (10 mcg /disc). [b]IZ= Inhibition zone (in mm) including the diameter of disc (6 mm). [c]AI= Activity index= inhibition area of the sample/inhibition area of the standard.

Antioxidant activity: The results of antioxidant activity are given in Table 4. Highest antioxidant activities as % of DPPH inhibition, were 72.70 % in seed followed by and 65.20 % in seed-callus extract at 80 µg/ml concentrations. The activity increased in both the extracts with an increasing amount of extract which was found to be concentration-dependent (Fig 5). Callus extract had slightly weaker radical inhibitor than the extract of the seed. However, due to weaker antioxidant activity RC_{50} value could not be recorded in both the extracts.

Table 4. Percent inhibition of DPPH by *V. encelioides* seed and seed-callus extracts

Plant parts	% Inhibition of DPPH				
	10 µg/ml	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml
Seed	60.20	61.10	61.80	65.00	72.70
Seed-callus	55.80	59.00	59.10	62.10	65.20
Quercetin	62.42	80.58	93.38	93.82	94.71

% inhibition = 1- (Absorbance of sample/Absorbance of control) x100.

Earlier studies have established that there was a direct relationship between the antioxidant activity and total phenolic content in selected herbs, vegetables and fruits. Phenolic compounds are known to contribute maximally for antioxidant activity (Zheng and Wang 2001, Sun *et al*. 2002). The present results, however, indicate that both *in vivo* and *in vitro* extracts of *V. encelioides* are rich in triterpenoidal compounds. Perhaps due to the presence of these compounds, low antioxidant activity was found as compared to appreciable antimicrobial

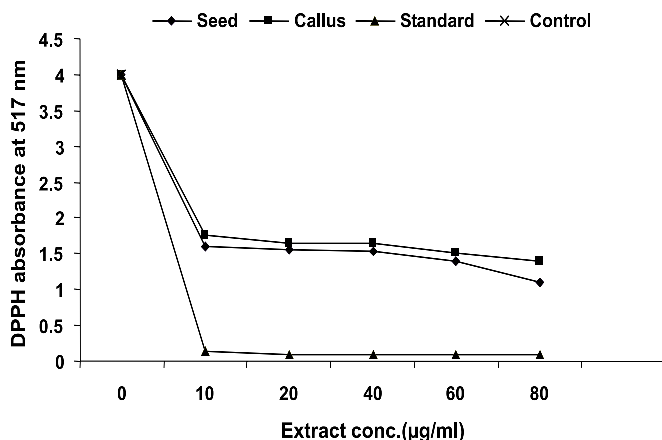

Fig. 5. Antioxidative assay of seed and seed-callus of *V. encelioides*

activity in this plant. Earlier studies have also proved the bioactive potentials of these isolated compounds (Jain *et al*. 2001).

In conclusion, a simple, reproducible and efficient protocol has been developed for the formation of callus and for its mass production. Also, similar biosynthetic potential to synthesize several triterpenoids *in vivo* and *in vitro* in *V. encelioides*, is attributed to chemical defense against herbivory or a variety of ecological functions.

ACKNOWLEDGEMENT

The authors are thankful to the University Grants Commission, New Delhi, India, for the financial support.

REFERENCES

Dev, S., Gupta, A.S. and Patwardhan, S.A. (1989). Handbook of Triterpenoids. Triterpenoids. Vol. II: Pentacyclic and Hexacyclic Triterpenoids. CRC Press, Inc., Florida.

Eichholzer, J.V., Lewis, I.A.S., MacLeod, J.K., Oelrichs, P.B. and Vallely, P.J. (1982). Galegine and a new dihydroxyalkylacetamide from *Verbesina encelioides*. *Phytochem.* **21:** 97-99.

Fogliano, V., Verde, V., Randazzo, G. and Retieni, X. (1999). A method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. *J. Agric. Food Chem.* **47:** 1035-1040.

Glennie, C.W. and Jain, S.C. (1980). Flavonol 3,7- diglycosides of *Verbesina encelioides. Phytochem.* **19:** 157-158.

Goel, U. (1987). Allelopathic effects of *Verbesina encelioides* Cav. *Annals Arid Zone* **26:** 287-291.

Gould, J.C. and Bowie, J.H. (1952). The determination of bacterial sensitivity of antibiotics. *Edin. Med. J.* **59:** 178.

Harborne, J.B. (1973). Phytochemical Methods. Chapman and Hall, London.

Inderjit, Asakawa, C. and Dakshini, K.M. (1999). Allelopathic potential of *Verbesina encelioides* root leachate in soil. *Can. J. Bot.* **77:** 1419-1424.

Jain, S.C., Purohit, M. (1985). Establishment of callus culture of three medicinally important plants and investigation of their metabolites. I. *Herba Pol.* **31:** 35-37.

Jain, S.C. and Purohit, M. (1989). Studies on the callus cultures of selected Indian Medicinal Plants: II. Quantification of different metabolites. *Herba Pol.* **35:** 35-38.

Jain, S.C., Purohit. M. and Sharma, R. (1988). Pharmacological evaluation of *Verbesina encelioides. Phytother. Res.* **2:** 146-148.

Jain, S.C., Jain, R., Singh, R. and Menghani, E. (2007a). Studies on *Verbesina encelioides*: Chemistry and bioactivity. Proc. of NSPB, pp. 439-445. CIMAP, Lucknow.

Jain, S.C., Jain, R., Singh, R. and Menghani, E. (2007b). Galegine- Antimicrobial principle from *Verbesina encelioides. Indian Drugs* **44:** 5-7.

Jain, S.C., Singh, R. and Jain, R. (2008). Antimicrobial and antioxidant potentials of *Verbesina encelioides* (Cav.) Benth. & Hook. Fil. ex Gray. *Res. J. Med. Plants* **2:** 61-65.

Jain, S.C., Singh, B. and Jain, R. (2001). Antimicrobial activity of triterpenopids from *Heliotropium ellipticum* . *Fitoterapia* **72:** 666-668.

Joshi, K.C., Singh, P. and Singhi, C.L. (1983). Chemical constituents of *Verbesina encelioides* and *Holmkioldia sanguinea. J. Chem. Soc*. **60:** 905-906.

Keeler, R.F., Johnson, A.E. and Stuart, L.S. (1986). Toxicosis from and possible adaption to *Galega officinalis* in

sheep and the relationship to *Verbesina encelioides* toxicosis. *Vet. Hum. Tox.* **28:** 309-315.

Kingsbury, J.M. (1964). Poisonous Plants of the United States and Canada. Prentice - Hall, Inc., New Jersey.

Lee, S.K., Mbwambo, Z.H., Chung, H.S., Luyengi, L., Games, E.J.C. and Mehta, R.G. (1998). Evaluation of the antioxidant potential of natural products. *Combinatorial Chemistry & High Throughput Screening.* **1:** 35-46.

Lopez, T.A., Campero, C.M., Chayer, R., Cosentino, B. and Caracino, M. (1996). Experimental toxicity of *Verbesina encelioides* in sheep and isolation of galegine. *Vet. Hum. Tox.* **38:** 417-419.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15:** 473-497.

Oelrichs, P.B., Vallely, P.J., MacLeod, J.K. and Lewis, I.A.S. (1981). Isolation of galegine from *Verbesina encelioides*. *J. Nat. Prod.* **44:** 754-755.

Petricic, J. and Kalodera, Z. (1982). Galegine in the goat's rue herb: Its toxicity, antidiabetic activity and content determination. *Acta. Pharm. Jugosl.* **32:** 219-223.

Sun, J., Chu, Y.F., Wu, X.Z. and Liu, R.H. (2002). Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.* **50:** 7449-7454.

Tiwari, H., Rao, P. and Sambasiva, V. (1978). Constituents of *Verbesina encelioides*: Isolation of triterpenoids from a *Verbesina species. Indian J. Chem.* **16:** 1133.

Toribio, M.S., Oriani, D.S., Fernandez, J.G. and Skliar, M.I. (2005). Antimicrobial activity of *Verbesina encelioides. In Vet.* **7:** 41- 45.

Yamaguchi, K. (1970). Spectral Data of Natural Products. Vol. I. American Elsevier, New York.

Zheng, W. and Wang, S.Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *J. Agric. Food Chem.* **49:** 5165-5170.