Original Article

Screening of Indian medicinal plants for cytotoxic activity by Brine Shrimp Lethality (BSL) assay and evaluation of their total phenolic content

Abstract

Objective: Plant-derived cytotoxic constituents and polyphenolic compounds have played an important role in the development of clinically useful anticancer agents. In this context, we have selected six Indian medicinal plants based on the literature claims and an attempt was made to evaluate the cytotoxic potential and total phenolic content (TPC) of their methanol extracts and fractions. **Materials and Methods:** Six plants have been selected for the study, namely, *Artemisia absinthium* Linn. (Asteraceae), *Oroxylum indicum* (Linn.) Vent. (Bignoniaceae), *Heliotropium indicum* Linn. (Boraginaceae), *Amorphophallus sylvaticus* (Roxb.) Kunth. (Araceae), *Mimosa pudica* Linn. (Mimosaceae), and *Premna serratifolia* Linn. (Verbenaceae). Authenticated plant materials were subjected to extraction with methanol by cold maceration and hot percolation methods. The extracts were fractionated into four fractions (F1, F2, F3, and F4). Preliminary phytochemical investigation was carried out for all extracts and fractions. All extracts and their fractions were subjected to cytotoxicity screening by brine shrimp lethality (BSL) bioassay. The plants with significant cytotoxicity were evaluated for TPC by using Folin-Ciocalteu reagent. **Results:** F1, F2, and F3 fractions of *A. absinthium* and *P. serratifolia* and F1 fraction of *M. pudica* have shown significant cytotoxicity (lethal concentration (LC_{50}) < 100 ppm) compared with other fractions. F1, F2, and F3 fractions of *A. absinthium* show the LC_{50} values 32.52, 14.27, and 24.02, respectively; F1, F2, and F3 of *P. serratifolia* show LC_{50} values 7.61, 4.01, and 10.91 and same for F1 fraction of *M. pudica* was 34.82 μg/ml, respectively. TPC was found to be significantly higher (39.11 mg gallic acid equivalent (GAE)/g) in *P. serratifolia* compared with other two plants. **Conclusion:** The cytotoxicity screening system confirmed the proposed anticancer plants used by traditional healers and literature claims.

Key words:
Artemisia absinthium, amorphophallus sylvaticus, heliotropium indicum, mimosa pudica, oroxylum indicum, premna serratifolia

Introduction

Various medicinal plants are being used in cancer therapy due to their cytotoxic property.[1] Plant-derived cytotoxic constituents have played an important role in the development of clinically useful anticancer agents.[2] Cytotoxic constituents such as flavonoids, terpenes, and caffeic acid of propolis — a honeybee product — induces anticancer effects,[3] and various cytotoxic constituents have been isolated from plants and are being developed as anticancer agents. Plant-derived polyphenols have been shown to have cancer-preventing activities in laboratory studies.[4] Polyphenols such as flavonoids are being used to prevent various types of cancers.[5] Epigallocatechin gallate (EGCG), a green tea constituent and other polyphenols with 1,2-benzenediol moieties, effectively prevented tumors.[6] Considering these all facts, natural products in the treatment of cancer as complementary and alternative

Mahesh Biradi, Kirankumar Hullatti

Department of Pharmacognosy, KLE University's College of Pharmacy, Nehru Nagar, Belgaum, Karanakata, India

Address for correspondence:
Dr. Kirankumar Hullatti,
Department of Pharmacognosy, KLE University's College of Pharmacy,
Nehru Nagar, Belgaum - 590 010, Karnataka, India.
E-mail: drhullatti@klepharm.edu

therapy are accepted increasingly with growing scientific evidences of biomedical research and clinical trials.[7]

Scanty research has been carried out on extracts of the selected medicinal plants for their anticancer potential and only extraction level study has been carried out. Hence, some Indian medicinal plants have been selected based on the Ayurvedic text and folklore claims with literature survey and tested for their cytotoxicity by BSL bioassay and total phenolic content (TPC) by Folin — Ciocalteu method using ultraviolet–visible (UV-VIS) spectroscopy. With this regard, in the present study an attempt was made to evaluate the cytotoxic potential and total polyphenols of six medicinal plants, which in turn leads to identify the anticancer plants and isolation of the cytotoxic constituents from the same. These cytotoxic constituents could be developed as anticancer biomolecules for the treatment of cancer.

Materials and Methods

Chemicals and reagents

Methanol, ethanol, petroleum ether, n-hexane, chloroform, dichloromethane, sulfuric acid, ammonia, citric acid, Folin — Ciocalteu reagent, sodium carbonate, distilled water were procured from KLEU's College of Pharmacy, Belgaum.

Selection of plant materials

Based on the Ayurvedic literature and information from the traditional healers, the following six plants have been selected for this study: *Artemisia absinthium* Linn. (Asteraceae), *Oroxylum indicum* (Linn.) Vent. (Bignoniaceae), *Heliotropium indicum* Linn. (Boraginaceae), *Amorphophallus sylvaticus* (Roxb.) Kunth. (Araceae), *Mimosa pudica* Linn. (Mimosaceae), and *Premna serratifolia* Linn. (Verbenaceae).[8-13] Scanty research has been carried out on various extracts of these selected medicinal plants originated in India for their anticancer potential. Thus, these plants have been chosen for the study.

Collection and authentication

Plant materials have been collected from different places of Karnataka state, India. Whole plant of *A. absinthium* and leaves of *O. indicum* were collected from Horticulture Department, Agricultural Sciences, Bagalkot during December 2010. Aerial parts of *H. indicum* were collected from the local region of Jamboti and tubers of *A. sylvaticus* were collected from Amrut Kesari, Bangalore during December 2010. Leaves of *P. serratifolia* and whole plant of *M. pudica* were collected from Indian Council for Medical Research (ICMR) Campus, Belgaum during January 2011.

The plants *A. absinthium*, *P. serratifolia*, and *M. pudica* were authenticated by Dr. Harsha Hegde, Scientist 'B' ICMR, Belgaum, India. The voucher specimens of the plants (Accession numbers RMRC-937, RMRC-554, and RMRC-553, respectively) are deposited in ICMR Herbarium repository. And remaining plants *O. indicum*, *H. indicum*, and *A. sylvaticus* were authenticated (Accession Numbers of the plants are RLSIB/Bot/04-06) by Dr. Bendigeri PB, Professor and Head of Department (HOD), Raja Lakhamagouda Science (RLS) Institute, Belgaum, India.

Extraction

Extraction of all plant materials was done by cold maceration and hot percolation methods with methanol. The collected plant materials were processed by washing it thoroughly under running water and dried in shade/in tray driers with temperature not exceeding 45°C. The dried materials were powdered using grinder and stored in an air tight container at room temperature for the further use. Dried powdered plant material was subjected to extraction with methanol (drug to solvent ratio 1:4) by cold maceration for 24 h. Then extract was filtered off and the marc was subjected to hot percolation by Soxhlet using methanol (drug to solvent ratio 1:3) at 60°C for 6-8 h. Then extract was filtered in hot condition. Both the filtrates were pooled and subjected for concentration at 45°C under vacuum using Rotavapor (IKA RV-10 Digital). Concentrated extracts were kept in previously labeled closed container.

Fractionation

All plant extracts were fractionated into four fractions (F1, F2, F3, and F4) by adopting the method given by Cos *et al*., 2006[14] with slight modifications [Figure 1] to separate all phytoconstituents according to the nature of their solubility in various solvents.

Preliminary phytochemical investigation

All extracts and fractions were subjected for preliminary phytochemical investigation by performing various chemical tests to determine the secondary metabolites present in the extract/fraction obtained. All chemical tests were performed according to the procedure given in the Textbook of Pharmacognosy by Kokate *et al*., 2010.[15]

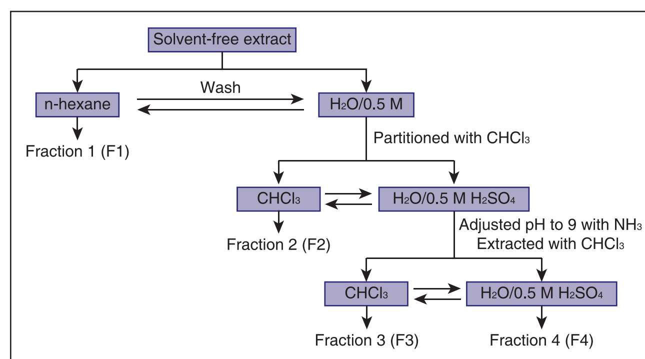

Figure 1: Flow chart for the preparation of various fractions from crude methanol extracts. Fractionate the extract into different class of phytocompounds according to the modified protocol. $CHCl_3$ – Chloroform, H_2O – Water, NH_3 – Ammonia, H_2SO_4 – Sulfuric acid

The results of the phytochemical investigation along with the yield of the plant extract and fractions are depicted in Table 1.

Brine shrimp lethality (BSL) bioassay

Cytotoxicity of the all plant extracts and fractions were determined by BSL bioassay, a method developed by McLaughlin and Rogers, 1998.[16] The brine shrimp (*Artemia salina* Lich.) eggs were procured from Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal, India. Previously filtered sea water (3 l) was added into the hatching chamber; sprinkle shrimp eggs (50 mg) to one side of the divided tank (larger darkened compartment) after washing with sodium hypochlorite (bleaching solution) followed by water. Allow 2 days (48 h) for the shrimp to hatch and mature as nauplii (hatched shrimp). Another smaller side of the compartment was illuminated with a lamp (40 W bulb). As the nauplii are phototropic in nature, they will move toward smaller illuminated compartment through the holes made on compartment divider.

Samples and standard (potassium dichromate) were prepared in vials for testing to get final concentration of 10, 100, and 1000 μg/ml; all the samples and standard were prepared in triplicate.

Nauplii were drawn from the hatching chamber using bulb pipette against light background and exactly 10 shrimps were transferred to each test tubes. Then drug samples were added to each test tube that was previously marked in triplicate for each extract/fraction. The sea water was added to each test tube to make the volume up to 5 ml. A drop of dry yeast suspension (3 mg in 5 ml sea water) was added to each test tube as a food for shrimps. The test tubes were maintained under illumination. After 24 h, number of survivors were counted and recorded and the lethal concentration (LC_{50}) values were calculated by means of Statistical Package for the Social Sciences (SPSS)-20 software. The fractions with LC_{50} values < 100 ppm were selected for further studies.[17,18]

Total phenolic content

TPC of methanolic extract and aqueous fraction of above active plants were determined by colorimetric method using

Table 1: Phytochemical screening of methanol extracts and fractions of the selected plants

Plant	Extract/fractions	Percent yield	Presence of secondary metabolites
A. absinthium	AA/ME	10.66	Steroids, triterpenoids, alkaloids, flavanoids, tannins
	AA/F1	3.92	Steroids, triterpenoids *(sesquiterpene lactones — absinthin, anabsinthin, artabasin)
	AA/F2	1.98	Triterpenoids *(absinthin, anabsinthin, artabasin)
	AA/F3	4.04	Alkaloids
	AA/F4	83.0	Flavanoids (artemetin (II), rutin), tannins
P. serratifolia	PS/ME	21.93	Sterols, triterpenoids, alkaloids, glycosides, flavanoids, tannins
	PS/F1	18.07	Sterols (betasitosterol), triterpenoids
	PS/F2	13.82	Triterpenoids
	PS/F3	11.75	Alkaloids (isoxazole-premnazole)
	PS/F4	53.10	Glycosides, flavanoids (scutellareine, pectolinarin), tannins
M. pudica	MP/ME	14.84	Steroids, triterpenoids, alkaloids, glycosides, flavanoids
	MP/F1	11.27	Steroids, triterpenoids
	MP/F2	11.80	Triterpenoids
	MP/F3	10.33	Alkaloids
	MP/F4	61.74	Glycosides, flavanoids (C-glycosylflavones)
O. indicum	OI/ME	16.80	Steroids, triterpenoids, alkaloids, glycosides, flavanoids, tannins
	OI/F1	3.12	Steroids, triterpenoids
	OI/F2	3.68	Triterpenoids
	OI/F3	2.62	Alkaloids
	OI/F4	85.37	Flavanoids (baicalein, scutellarein, aloe-emodin, chrysin, and oroxylin A), tannins
H. indicum	HI/ME	14.66	Steroids, triterpenoids, alkaloids, saponins, tannins
	HI/F1	7.24	Steroids, triterpenoids
	HI/F2	4.28	Triterpenoids
	HI/F3	3.13	Alkaloids (indicine N-oxide, echinatine, supinine)*
	HI/F4	81.88	Saponins, tannins
A. sylvaticus	AS/ME	2.66	Steroids, triterpenoids, alkaloids, flavanoids, saponins
	AS/F1	27.53	Steroids, triterpenoids
	AS/F2	4.23	Triterpenoids
	AS/F3	3.39	Alkaloids
	AS/F4	62.08	Flavanoids, saponins

*The constituents that are responsible for anticancer (cytotoxic) activity. The percent yield of the extract was calculated based on the weight of air-dried plant material and percent yield of the fractions was calculated based on dry weight of the respective extracts; AA – *A. absinthium*, ME – Methanolic extract, F – Fraction; PS – *P. serratifolia*; MP – *M. pudica*; OI – *O. indicum*; HI – *H. indicum*; AS – *A. sylvaticus*

Folin — Ciocalteu assay according to Rebiai *et al*., 2011 with minor changes. In brief, 0.1 ml of Folin — Ciocalteu reagent was mixed with sample solutions (0.05-0.25 ml corresponds 50-250 μg) and incubated for 3 min at room temperature. Then, 2 ml of 20% sodium carbonate (w/v) solution was added and volume was adjusted to 10 ml with distilled water and heated in water bath for 1 min. The mixture was allowed to stand in the dark for 30 min before measuring the absorbance at 685 nm using UV-VIS spectrophotometer (SICAN 2301) against blank containing distilled water omitting sample extract.

TPC values were determined from a calibration curve prepared with a series of gallic acid standards (5-25 μg/ml; y = 0.006 ×; R^2 = 0.9984) that is depicted in Figure 2. Average mean of five serial sample dilutions have been taken for interpreting the result.[19,20]

Statistical analysis

Each experiment was performed in triplicate. Statistical calculations were done by using SPSS 20.0 software (IBM SPSS Statistics). The percentage lethality was calculated from the mean survival larvae of extracts treated tubes and control. The LC_{50} values were obtained by best-fit line method.

Results

Extraction and fractionation

The percent yield of the extract was calculated based on the weight of air-dried plant material, and percent yield of the fraction was calculated based on dry weight of the respective extracts. Percent yield of the same are depicted in Table 1.

Preliminary phytochemical investigation

Phytochemical study reveals the presence of alkaloids, flavanoids, steroids, triterpenoids, and tannins in common in methanol extract of all plants, whereas flavonoids were absent in *H. indicum* and glycosides were present in *O. indicum*, *P. serratifolia*, and *M. pudica*. Results of the same are depicted in Table 1.

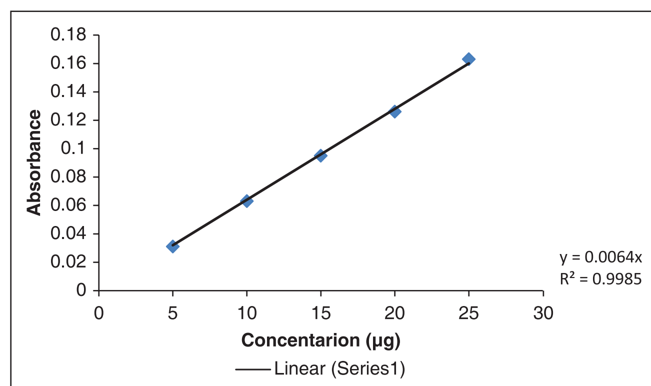

Figure 2: Standard calibration curve of gallic acid with linear regression. TPC values were determined from a calibration curve prepared with a series of gallic acid standards (5-25 μg/ml; y – 0.006 ×; R^2 – 0.9984). Average mean of five serial sample dilutions have been taken for interpreting the result. TPC – Total phenolic content

Brine shrimp lethality (BSL) bioassay

Cytotoxicity screening of all extracts and their fractions were done by BSL bioassay. Fractions (F1, F2, and F3) of *A. absinthium* and *P. serratifolia* and F1 fraction of *M. pudica* have shown significant cytotoxic activity (LC_{50} < 100 ppm) compared with other fractions. The extract/fractions were almost 100% lethal to the brine shrimp at the concentration of 1000 μg/ml. Active fractions with LC_{50} < 100 ppm were considered significant. Results are expressed as the mean ± standard error of the mean (S.E.M.) of three independent experiments. Cytotoxicity (mean % death after 24 h with LC_{50} values) of various extracts and fractions was compared with those of the control and is shown in Table 2.

Total phenolic content

The plants that show significant cytotoxic property were evaluated for TPC by using Folin — Ciocalteu reagent, and TPC was found to be significantly higher in *P. serratifolia* compared with the other two plants. Results are expressed as microgram of gallic acid equivalents (GAE) per gram fresh weight (mg GAE/g FW) with S.E.M., which are shown in Table 3.

Discussion

Three of six medicinal plants were found to have cytotoxic property. The results revealed that the active principles with cytotoxic property were mainly distributed in n-hexane and chloroform fractions of *A. absinthium* and *P. serratifolia*, wherein *M. pudica* the hexane portion was the most active fraction. The cytotoxic plants were evaluated for TPC and it was found to be significantly higher in *P. serratifolia* compared with other plants.

Conclusion

The cytotoxicity screening system confirmed the proposed anticancer plants used by traditional healers and literature claims. This screening method could apply to plant extracts to facilitate the isolation of biologically active compounds. The active fractions of these plant extracts could be taken up for the isolation of certain probable molecules with cytotoxic property that would help us in finding new anticancer molecules.

Acknowledgments

Authors are thankful to the Principal, KLE University's College of Pharmacy, Belgaum for providing facilities to carry out this research work.

Table 2: Cytotoxicity effect of the plants extracts and fractions on BSL assay

Plant	Extract/fraction	Mean percent death after 24 h (concentration in μg/ml)			LC_{50} (ppm)
		10	100	1000	
Control	Normal	–	–	–	0
Potassium dichromate	Std	40.0±0.00	100	100	11.49
A. absinthium	AA/ME	0	20.0±0.00	95.0±0.33	160.06
	AA/F1	20.0±0.00	83.33±0.33	100	32.52*
	AA/F2	40.0±0.58	100	100	14.27*
	AA/F3	33.33±0.33	86.66±0.33	100	24.02*
	AA/F4	6.67±0.33	26.66±0.33	93.33±0.33	147.84
P. serratifolia	PS/ME	6.67±0.33	33.33±0.33	100	125.52
	PS/F1	60.0±0.00	83.33±0.33	100	7.67*
	PS/F2	73.33±0.33	100	100	4.01*
	PS/F3	53.33±0.33	90.0±0.00	100	10.91*
	PS/F4	6.67±0.33	30.0±0.00	100	115.83
M. pudica	MP/ME	3.33±0.33	20.0±0.00	80.0±0.00	215.47
	MP/F1	20.0±0.00	100	100	34.82*
	MP/F2	6.67±0.33	30.0±0.00	73.33±0.33	214.54
	MP/F3	10.0±0.00	33.33±0.33	90.0±0.00	132.75
	MP/F4	10.0±0.00	20.0±0.00	35.0±0.33	>1000
O. indicum	OI/ME	46.66.0±0.58	33.33±0.33	96.66±0.33	40.64
	OI/F1	26.67±0.33	30.0±0.00	83.33±0.33	129.86
	OI/F2	16.66±0.88	30.0±0.00	83.40±0.66	159.13
	OI/F3	3.40±0.33	6.70±0.00	23.40±0.33	>1000
	OI/F4	3.33±0.66	10.0±0.00	23.40±0.33	>1000
H. indicum	HI/ME	16.66±0.88	36.66±0.33	70.0±0.00	226.39
	HI/F1	20.0±0.00	30.0±0.00	80.0±0.00	187.89
	HI/F2	23.33±0.33	33.33±0.33	80.0±0.00	144.93
	HI/F3	6.67±0.33	20.0±0.00	100	152.38
	HI/F4	10.0±0.00	20.0±0.00	50.0±0.00	>1000
A. sylvaticus	AS/ME	40.0±0.00	70.0±0.00	84.0±0.33	21.25
	AS/F1	23.33±0.33	33.33±0.33	83.33±0.33	128.85
	AS/F2	13.33±0.33	30.0±0.00	86.66±0.33	158.34
	AS/F3	13.33±0.33	33.33±0.33	90.0±0.00	132.76
	AS/F4	20.0±0.00	30.0±0.00	90.0±0.00	122.64

Active fractions (LC_{50} < 100 ppm) are highlighted with asterisk. Cytotoxicity effects (mean percent death after 24 h with LC_{50} values) of the plants are expressed in terms of mean ± S.E.M. of three independent experiments. BSL – Brine shrimp larvae, LC – Lethal concentration, ppm – Parts per million, Std – Standard, AA – *A. absinthium*; ME – Methanolic extract; F – Fraction; PS – *P. serratifolia*, MP – *M. pudica*, OI – *O. indicum*, HI – *H. indicum*, AS – *A. sylvaticus*, S.E.M. – Standard error of the mean

Table 3: TPC of cytotoxic plants

Plant	TPC (mg GAE/g FW) Mean ± S.E.M.	
	ME	Aqueous extract (F4)
A. absinthium	10.47±0.46	1.30±0.07
P. serratifolia	39.11±2.93	8.43±0.29
M. pudica	12.48±0.37	0.40±0.13

The values represent the mean ± S.E.M. of five independent experiments and expressed in terms of milligram of gallic acid equivalents/gram fresh weight (mg GAE/g FW); TPC – Total phenolic content; GAE – Gallic acid equivalent; FW – Fresh weight; ME – Methanolic extract; F – Fraction, S.E.M. – Standard error of the mean

References

1. Balachandran P, Govindarajan R. Cancer — An ayurvedic perspective. Pharmacol Res 2005;51:19-30.
2. Cragg GM, Newman DJ. Plant as a source of anti-cancer agents. J Ethnopharmacol 2005;100:72-9.
3. Watanabe MA, Amarante MK, Conti BJ, Sforcin JM. Cytotoxic constituents of propolis inducing anticancer effects: A review. J Pharm Pharmacol 2011;63:1378-86.
4. Lambert JD, Hong J, Yang GY, Liao J, Yang CS. Inhibition of carcinogenesis by polyphenols: Evidence from laboratory investigations. Am J Clin Nutr 2005;81:284-91S.
5. Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: Promising anticancer agents. Med Res Rev 2003;23:519-34.
6. Golden EB, Lam PY, Kardosh A, Gaffney KJ, Cadenas E, Louie SG, *et al*. Green tea polyphenols block the anticancer effects of bortezomib and other boronic acid-based proteasome inhibitors. Blood 2009;113:5927-37.
7. Feng Y, Wang N, Zhu M, Feng Y, Li H, Tsao S. Recent progress on anticancer candidates in patents of herbal medicinal products. Recent Pat Food Nutr Agric 2011;3:30-48.
8. Anonymous. The ayurvedic pharmacopoeia of India. 1st ed. Vol. I. New Delhi: Ministry of Health and Family Welfare, Department of AYUSH; 2008. p. 1-157.
9. Anonymous. The Ayurvedic Pharmacopoeia of India. 1st ed. Vol. I New Delhi: Ministry of Health and Family Welfare, Department of AYUSH; 2008. p. 105.
10. Anonymous. Agro-techniques of selected medicinal plants. New Delhi: Ministry of Health and Family Welfare, National Medicinal Plants Board, Department of AYUSH 2008;2:151-4.

11. Khare CP. Indian medicinal plants an illustrated dictionary. Berlin/Heidelberg: Springer-Verlag; 2007.
12. Harminder, Singh V, Chaudhary AK. A Review on the Taxonomy, Ethnobotany, Chemistry and Pharmacology of Oroxylum indicum Vent. Indian J Pharm Sci 2011;73:483-90.
13. Schmelzer GH, Gurib-Fakim A. Heliotropium indicum L. Prota 11 (1): Medicinal plants [online]. Netherlands 2006. Available from: Database. prota.org/PROTAhtml/Heliotropium%20indicum_En.htm [Last accessed on 2012 Jun 28].
14. Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. J Ethnopharmacol 2006;106:290-302.
15. Kokate CK, Purohit AP, Gokhle SB. Text Book of Pharmacognosy. 45th ed. Pune: Nirali Prakashan; 2010. p. A1-6.
16. McLaughlin JL, Rogers LL. Use of biological assays to use botanicals. Drug Info J 1998;32:513-4.
17. Rahman A, Choudary MI, Thomsen WJ. Bioassay techniques for drug development, Singapore: Taylor and Francis e-Library. Harwood Academic Publishers; 2005. p. 8-10.
18. Gibson KA, Reese RN, Halaweish FT, Ren Y. Isolation and characterization of a bactericidal withanolide from Physalis virginiana. Pharmacogn Mag 2012;8:22-8.
19. Rebiai A, Lanez T, Belfar ML. *In vitro* evaluation of antioxidant capacity of algerian propolis by spectrophotometrical and electrochemical Assays. Int J Pharmacol 2011;7:113-8.
20. Scalbert A, Monties B, Janin G. Tannins in wood: Comparison of different estimation methods. J Agri Food Chem 1989;37:1324-9.

How to cite this article: Biradi M, Hullatti K. Screening of Indian medicinal plants for cytotoxic activity by Brine Shrimp Lethality (BSL) assay and evaluation of their total phenolic content. Drug Dev Ther 2014;5:139-44.

Source of Support: Nil. **Conflict of Interest:** None declared