World Applied Sciences Journal 23 (4): 500-506, 2013
ISSN 1818-4952
© IDOSI Publications, 2013
DOI: 10.5829/idosi.wasj.2013.23.04.368

In vitro Antimicrobial, Insecticidal, Antitumor Activities and Their Phytochemical Estimation of Methanolic Extract and its Fractions of *Medicago lupulina* Leaves

Nizam Baloch, Sajid Nabi
and Yasser M.S.A. Al-Kahraman
Institute of Biochemistry, University of Balochistan, Quetta, Pakistan

Submitted: Feb 2, 2013; **Accepted:** Mar 8, 2013; **Published:** Jun 28, 2013

Abstract: The aim of the present investigation deals with biological evaluation of *Medicago lupulina* leaves. For this purpose, different biological assays of methanolic extract (Crude) and its fractions that are chloroform fraction, *n*-hexane fraction, Ethyl acetate fraction, *n*-butanol fraction and aqueous fraction were carried out. The results from the agar diffusion method indicated that Crude showed maximum antibacterial activity against *Staphylococcus aureus* with the inhibition zone (29.02±0.18mm), whereas chloroform fraction also showed strong activity against *Staphylococcus aureus* (26.02±0.04mm). On the other hand, Crude showed maximum activity against *Candida albicans* and *Candida glaberata* with % inhibition of (66.02%±0.2) and (72.16%±0.09) respectively and Chloroform fraction showed good activity against *Candida glaberata* with % inhibition of (62.03%±0.09). Furthermore, Crude showed maximum insecticidal % mortality against *Tribolium castaneum* with (86%) mortality whereas (75%) mortality against *Rhyzopertha dominica* and Chloroform fraction showed maximum activity against *Tribolium castaneum* with (70%) mortality. Moreover, Crude showed tremendous antitumor activity with 89.40% inhibition and Chloroform fraction showed moderate level of tumor inhibition with 62.16% inhibition. Furthermore, the phytochemical estimation of Crude and its fractions showed the presence of Alkaloids, Flavonoids, Phenols, Tannins and Diterpenes.

Key words: Antimicrobial · Insecticidal · Antitumor · Phytochemical Estimation · *Medicago lupulina* Leaves

INTRODUCTION

Plant-derived drugs remain an important resource, especially in developing countries, to combat severe diseases. Approximately 60-80% of the world's population still relies on traditional medicines for the treatment of common illnesses [1, 2]. Plants contain several compounds that have potent biological activity [3]. For example, phenolic compounds and essential oils play a vital role as powerful natural biological agents [4-7]. Antibiotic use has led to the emergence of infectious bacteria that are resistant to one or more antibiotics [8]. This situation has resulted in the failure of the treatment of many microbial diseases. A number of previous investigations has indicated that many medicinal plant extracts constitute a class of potent natural antimicrobial agents [9, 10]. Therefore, many researchers have focused on the investigation of aromatic and medicinal plants as a source of new antimicrobial substances [11, 12].

Medicago lupulina can be seen through the old world: all of Europe, a great part of Asia, including China, Korea and Taiwan, as well as the Indian sub-continent, North Africa, the islands of the Atlantic (the Canaries, Madeira) and throughout the United States, including Hawaii. It grows in grassy places and roadsides, often occurring as a garden weed on acidic and calcareous soils.

Aqueous extracts of the plant have antibacterial properties against micro-organisms. The plant is lenitive. This plant has agents that are capable of easing pain or discomfort. Legume isoflavones seem to be estrogenic and are believed by some NCI scientists to prevent cancer.

Corresponding Author: Yasser M.S.A. Al-Kahraman, Institute of Biochemistry, University of Balochistan, Quetta, Pakistan
Tel: 00923318051961 Fax: 0092819211277.

Medicago lupulina is sometimes used as a fodder plant. While being of good value, it isn't a very productive fodder. It is sometimes used in the composition of artificial meadows, especially when implanted in dry lands. It is a common sight in natural pastures. It is also one of the flowers that can be used to create honey [13].

In continuation of our previous work [14-16] the aim of this study was to screen for medicinal plant extracts of this province that could be useful for the development of new tools for the control of infectious diseases. While pursuing this goal, we initiated a systematic evaluation of extracts and fractions from the "*Medicago lupulina*" plant species in bioassays such as (a) Antimicrobial activity (b) Insecticidal activity (c) Antitumor activity and (d) their Phytochemical Estimation.

MATERIALS AND METHODS

Plant Material: The leaves of *Medicago lupulina* were collected from Soorab, Balochistan province, Pakistan.

Extraction and Fractionation: Fresh leaves were washed, sliced and dried under shade for 15 days. The leaves' extract was prepared in analytical grade methanol (3 kg in 8L) for 72 hours. Then the methanol was removed and residue was immersed in methanol for a further seven days. Thereafter, the methanol was decanted and filtered with Whatman filter paper. The filtrate was subsequently concentrated under reduced pressure at 45° C in rotatory evaporator (Stuart RE 300) and dried to constant weight (460 g) in vacuum oven (LINN high therm) at 45° C. This was crude methanolic leaves' extract (CME). The CME was than further fractionalized, where 250g of CME was suspended in 250ml of distilled water. This aqueous suspension was further subjected to solvent-solvent extraction for four fractions, namely, *n*-hexane fraction (NHF), chloroform fraction (CHF), acetone fraction (ACE) and aqueous fraction (AQF).

Biological Activities: Following biological activities were performed on the extract and its fractions.

Preparation of the Tested Organisms

Preparation of Standard Bacterial Suspensions: The average number of viable, *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus* organisms per ml of the stock suspensions was determined by means of the surface viable counting technique [17]. About (108- 109) colony forming units per ml was used. Each time, a fresh stock suspension was prepared; the experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

Preparation of Standard Fungal Suspensions: The fungal cultures (*Microsporum canis, Candida albicans, Aspergillus flavus and Candida glaberata)* were maintained on Saboraud Dextrose Agar, incubated at 25° C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in (100 ml) of sterile normal saline and the suspension was maintained for further use.

Antimicrobial Activity

Testing for Antibacterial Activity: The cup-plate agar diffusion method was used [18] to assess the antibacterial activity of the prepared extracts. 0.6 ml of standardized bacterial stock suspensions of 108 -109 colony forming units per ml was thoroughly mixed with 60 ml of sterile nutrient agar. 20 ml of the inoculated nutrient agar were distributed into sterile Petri dishes. The agar was left to set and in each of these plates, 4 cups, 10mm in diameter, were cut using a sterile cork borer No. 4 and the agar discs were removed. Alternate cups were filled with 0.1ml of each extracts using micropipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37° C for 18 hours. Two replicates were carried out for each extract against each of the test organism. Simultaneously, addition of the respective solvents instead of extracts was carried out as controls. After incubation, the diameters of the growth inhibition zones were measured, averaged and the mean values were tabulated (Table 1).

Testing for Anti-fungal Activity: The same method as for bacteria was followed. Instead of nutrient agar media, yeast and mould extract agar was used. The inoculated medium was incubated at 25°C for two days for *Microsporum canis* and *Candida albicans* and three days for *Candida glaberata* and *Aspergillus flavus.*

Insecticidal Activity: Crude extract and all fractions were evaluated against different insects viz. *Tribolium castaneum, Sitophilus oryzea, Callosbruchus analis and Rhyzopertha dominica*. The test sample was prepared by

dissolving 200 mg of crude fractions in 3 ml acetone and loaded in a Petri dishes covered with the filter papers. After 24 hours, 10 test insects were placed in each plate and incubated at 27°C for 24 hours with 50% relative humidity in growth chamber. The results were analyzed as percentage mortality, calculated with reference to the positive and negative controls. Permethrin was used as a standard drug, while Permethrin, acetone and test insects were used as positive and negative controls [19-23].

The Percentage Mortality Was Calculated by the Formula:

$$\text{Growth regulation (\%)} = \frac{\text{Number of insects alive in test}}{\text{Number of insects alive in control}} \times 100$$

Crown Gall Tumor Inhibition (Potato Disc) Assay: Antitumor potato disc assay was performed for *Medicago lupulina* leaves by using *Agrobacterium tumefaciens* (At- 10). *Medicago lupulina* leaves' extract and its fractions were tested for *in vitro* antitumor activities. Reported as [24].

Preparation of Potato Discs: Fresh, red and disease free potato tubers were surface sterilized by soaking in 0.1% HgCl solution in water for 1 minute. A core cylinder of tissue was removed from tuber by means of sterilized cork borer. 2 cm end of each tissue cylinder was discarded and remainder was cut into discs of uniform thickness by a special aseptic cutter.

Preparation of Agar Plates and Treatment: These potato discs were then transferred to petri plates each containing 25 ml of 1.5 % agar (1.5 g agar/100 ml distilled water). Five potato discs were placed on each plate and three plates were used for each test sample along with same number of plates for vehicle control (DMSO) and reference drug (Vincristine). As a stock solution, 10 mg of each compound was dissolved in 1 ml of DMSO in separate test tubes. Then 0.5 ml of stock (10 mg/ml) of the test sample was added to 2 ml of a broth culture of *Agrobacterium tumefaciens* (At-10, a 48 hours culture containing 5×109 cells/ml) and 2.5 ml of autoclaved distilled water to make 1000ìg/ml final concentration. One drop (10ìl) was drawn from these test tubes using a sterile pipette and it was used to inoculate each potato disc, spreading it over the disc surface. The process starting from the cutting of the potatoes to the inoculation was completed in 30 minutes in order to avoid contamination. The lids of the petri plates were taped down with parafilm to minimize moisture loss.

Incubation and Analysis: The petri plates were incubated at 28° C for 21 days and the number of tumors was counted with the aid of dissecting microscope after staining with Lugol's solution (5 % I2, 10 % KI in distilled water). The numbers of tumors in vehicle control (DMSO) were used as a reference for activity. The results were derived from the number of tumors on test discs versus those on the vehicle control disc. Percentage tumor inhibition was calculated by using formula as shown below. Twenty percent or more inhibition was considered as significant activity.

$$\text{\% tumor inhibition} = \frac{\text{(1-Number of tumors in sample)}}{\text{(Number of tumors in control)}} \times 100$$

Phytochemical Study: Phytochemical Screening: Phytochemical screening for major bioactive constituents like Alkaloids, Phenolics, Flavonoids and Tannins were determined by using standard phytochemical methods [25, 26].

Determination of Total Phenolics: Total phenolic content of methanolic extract of *Medicago lupulina leaves* was determined by FolinCiocalteu method phenolic content was expressed as Gallic acid equivalents (GAE mg/g dry weight of extract) and the values were presented as mean ±SD of triplicate analysis with slight modifications [27]. 200ìl of sample (1mg/ml) was added to 100ìl diluted (1:10) Folin Ciocalteu reagent and equilibrated for few minutes. Then 800ìl of 2.5 % aqueous Na_2CO_3 was added and mixture was allowed to stand for 60 minutes at room temperature with intermittent shaking. The absorbance of the blue color solution was measured at 765 nm on UV visible spectrophotometer (Shimadzu UVPC-1700 (Japan)). Gallic acid (50 mg %) was used as standard. The absorbance of solution was compared with Gallic acid calibration curve.

Determination of Total Flavonoids: Total flavonoid content was determined by aluminum chloride colorimetric method [28]. This method is based on the formation of a complex flavonoid-aluminium, having the absorbance maximum at 430 nm. 0.5 ml of plant extract (1mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml

of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 15 minutes, the absorbance of the reaction mixture was measured at 430 nm on UV-visible spectrophotometer (Shimadzu UVPC-1700 (Japan)). Total flavonoid contents of berry sample were expressed as rutin equivalents (RE mg /g dry weight of extract) through the calibration curve with rutin as standard.

Alkaloid Estimation: 2.5g of the plant powder was extracted using 100ml of 20% acetic acid in ethanol. The solution was covered for almost 4 hours. Filtrate was concentrated to 25ml. Concentrated ammonium chloride was added stepwise for precipitation. The whole solution was kept as such so that precipitate should settle down. Collected precipitate was washed with dilute ammonium hydroxide and finally filtered. Filtrate was discarded and pellet obtained was dried and weighed [29, 30].

Tannins Estimation: The tannin content in samples was estimated by the method of Price and Butler [31]. Different aliquots of sample were taken and final volume to 3 ml was adjusted by distilled water. The samples after vortexing were mixed with 1ml of 0.016M $K_3Fe(CN)_6$, followed by 1 ml of 0.02M $FeCl_3$ in 0.10 M HCl. Vortexing was repeated and the tubes were kept as such for 15 min. 5 ml of stabilizer (3:1:1 ratio of water, H_2PO_4 and 1% gum Arabic) was added followed by revortexing. Absorbance was measured at 700 nm against blank. Standard curve was plotted using various concentrations of 0.001M Gallic acid.

RESULTS AND DISCUSSIONS

Antibacterial Activity: The antibacterial activity of the methanolic extract and different fractions from leaves of *Medicago lupulina* possess good antibacterial activity against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi and Staphylococcus aureus*. Table 1 shows the zone of inhibition against different species of gram positive and gram negative bacteria. The results from the agar diffusion method indicated that 100% methanolic extract showed good activity against *Staphylococcus aureus*, with the inhibition zone (29.02±0.18 mm). Moderate activity of Crude was exhibited against both *Pseudomonas aeruginosa* and *Salmonella typhi* with the inhibition zone (18.06±0.10) and (17.09±0.02) respectively. Least activity was exhibited against *Escherichia coli* with the smallest inhibition zone (14.02±0.08mm). Chloroform fraction showed strong activity against *Staphylococcus aureus* with (26.02±0.04mm) zone inhibition and moderate activity against both *Bacillus subtilis* and *Pseudomonas aeruginosa* with the inhibition zone (18.42±0.11) and (16.06±0.08) respectively. *n*-hexane fraction showed good activity against *Staphylococcus aureus* with (19.49±0.10mm) zone inhibition. Least activity was exhibited against *Pseudomonas aeruginosa* with inhibition zone (10.02±0.11mm). *Et*-acetate fraction showed moderate activity against *Staphylococcus aureus* with the inhibition zone (15.06±0.01mm). *n*-butanol and Aqueous fraction showed low activity against *Staphylococcus aureus* with the inhibition zones (13.04±0.01mm) and (11.52±0.02) respectively, while were resistant to the rest of species of bacteria. The antimicrobial activity of the tested extract and fractions is comparable with the standard drugs, Impenum.

Antifungal Activity: The antifungal activity of the methanolic extract and different fractions from leaves of *Medicago lupulina* possess good antifungal activity against *Microsporum canis, Candida albicans, Aspergillus flavus* and *Candida glaberata*. Table 2. Shows % inhibition against different species of fungi compared to the standard drug (Miconazole and Amphotericin B). The result indicated that Crude showed maximum activity against *Candida glaberata* and *Candida albicans* with % inhibition of (72.16%±0.09) and (66.02 %±0.15) respectively and showed least % inhibition against *Aspergillus flavus* with (12.03%±0.06.) inhibition. Chloroform fraction showed good activity against *Candida glaberata* and *Candida albicans* with % inhibition of (62.03%±0.09) and (58.38%±0.10) respectively and showed least % inhibition against *Aspergillus flavus* with (09.22%±0.09) inhibition. *n*-hexane fraction showed moderate % inhibition against *Candida glaberata* with (52.13%±0.06) inhibition. *Et*-acetate and *n*-butanol fractions showed moderate and low % inhibition whereas all fungal species showed complete resistance towards aqueous fraction.

Insecticidal Activity: Methanolic extract and its fractions from leaves of *Medicago lupulina* were evaluated for its insecticidal activity against *Tribolium castaneum, Sitophilus oryzea, Rhyzopertha dominica* and *Callosbruchus analis.* (Table 3) shows the % mortality of

Table 1: Antibacterial Activity of leaves of *Medicago lupulina*

Bacterial species	Zone of Inhibition of Std. drug* (mm)	Zone of inhibition (mm) Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Bacillus subtilis	36.06±0.03	21.13±0.01	13.18±0.04	18.42±0.11	14.08±0.03	-	-
Escherichia coli	35.11±0.02	14.02±0.08	-	12.49±0.03	-	-	-
Pseudomonas aeruginosa	32.01±0.09	18.06±0.10	10.02±0.11	16.06±0.08	10.39±0.04	-	-
Salmonella typhi	40.12±0.01	17.09±0.02	-	-	-	-	-
Staphylococcus aureus	43.22±0.08	29.02±0.18	19.49±0.10	26.02±0.04	15.06±0.01	13.04±0.18	11.52±0.02

*Impenum(10ìg disc)

Table 2: Antifungal activity of leaves of *Medicago lupulina*

Fungal species	% Inhibition of Std. drug*	% inhibition Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Microsporum canis	98.04%±0.02 Miconazole	52.11%±0.3	36.46%±0.10	46.02%±0.06	30.06%±0.02	-	-
Candida albicans	110.08%±0.02 Miconazole	66.02%±0.15	45.02%±0.08	58.38%±0.10	40.10%±0.09	38.02%±0.18	35.49%±0.10
Candida glaberata	110.25%±0.06 Miconazole	72.16%±0.09	52.13%±0.06	62.03%±0.09	48.12%±0.06	35.62%±0.10	30.53%±0.04
Aspergillus flavus	20.12%±0.06 Amphotericin B	12.03%±0.06	-	09.22%±0.09	-	-	-

Percent inhibition activity, 0-39= Low (non-significant); 40-59= moderate; 60-69= Good; above 70= Significant

Table 3: Antitumor activity of CME and its Fractions of *Medicago lupulina* leaves.

Extract/ Fractions	Average number of tumors [a]± SE	% inhibition of Tumors [b, c]
Crude	1.8±0.12	89.40
n-hexane	9.2±0.06	16.22
Chloroform	2.2±0.20	62.16
Et-acetate	-	-
n-butanol	6.2±0.55	26.32
Aqueous	-	-
Vincristine Std. drug	0.0±0.0	100
Vehicle Control	8.4±0.92	-

[a])Potato disc antitumor assay, Concentration:1000μg/ml in DMSO. [b]) More than 20% tumor inhibition is significant. [c]) Data represents mean value of 15 replicates.

Table 4: Quantitative estimation of phytoconstituents present in methanol extract and its fractions of *Medicago lupulina* leaves.

Phytoconstituents	Quantity (mg/g plant extract and it is fractions)
Alkaloids	18.02±0.03
Phenolics	20.20±0.08
Flavonoids	22.04±0.02
Tannins	16.28±0.06
Diterpenes	17.52±0.01

Table 5:

Funga l species	% Inhibition of Std. drug*	% inhibition Crude	*n*-hexane	Chloroform	Et-acetate	*n*-butanol	Aqueous
Tribolium castaneum	100	86	30	70	30	40	-
Sitophilus oryzea	100	60	35	60	-	-	-
Rhyzopertha dominica	100	75	30	60	40	50	-
Callosobrushus analis	100	50	-	50	-	-	-

different species of insects as compared to standard drug (Permethrin). Crude showed maximum insecticidal % mortality against *Tribolium castaneum* with (86%) mortality whereas (75%) mortality against *Rhyzopertha dominica.* Least was against *Callosbruchus analis* with 50% mortality. Chloroform fraction also showed good activity against *Tribolium castaneum* with (70%) mortality and (60%) against both *Sitophilus oryzea and Rhyzopertha dominica*. *n*-hexane, Et-acetate and *n*-butanol fractions showed low insecticidal activities with % mortality less than and equal to 50%. Aqueous fraction did not show any insecticidal activity.

Antitumor Activity: The antitumor activities of *Medicago lupulina* leaves extract and its fractions showed good and moderate levels of tumor inhibition. Crude of *Medicago lupulina* leaves showed 89.40% inhibition which is significant level of tumor inhibition that is comparable to Standard drug Vincristine (100 % tumor inhibition) and Chloroform fraction showed moderate level of tumor inhibition with 62.16% inhibition.

Preliminary Phytochemical Screening: Phytochemical analysis showed the presence of Alkaloids, Flavonoids, Tannins, Phenols and Diterpenes, whereas terpenoids and cardiac glycoside were completely absent (Table 4).

Conflict of Interest Statement: We declare that we have no conflict of interest.

ACKNOWLEDGEMENT

The Authors are thankful to the University of Balochistan, Quetta, Pakistan for providing the facilities and also Mir Shah Ali Bugti for providing the plant.

REFERENCES

1. WHO, 2002. Traditional Medicine Strategy 2002-2005. Geneva.
2. Zhang, X., 2004. Traditional medicine: its importance and protection. In: Twarog S, Kapoor P (eds). Protecting and promoting traditional knowledge: systems, national experiences and international dimensions. Part I. The role of traditional knowledge in healthcare and agriculture. New York: USA, pp: 3-6.
3. Mokbel, M.S. and F. Hashinaga, 2006. Evaluation of the antioxidant activity of extracts from buntan (*Citrus grandis* Osbeck) fruit tissues. Food Chem., 94: 529-534.
4. Cutter, C.N., 2000. Antimicrobial effect of herb extracts against *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella typhimurium* associated with beef. Journal of Food Protect., 63: 601-607.
5. Hao, Y.Y., R.E. Brackett and M.P. Doyle, 1998. Efficacy of plant extracts in inhibiting *Aeromonas hydrophilia* and *Listeria monocytogenes* in refrigerated cooked poultry. Food Microbiol., 15: 367-378.
6. Lis-Balchin, M. and S.G. Deans, 1997. Bioactivity of selected plant essential oils against Listeria monocytogenes. J. Appl. Microbiol., 82: 759-762.
7. Puupponen-Pimia, R., L. Nohynek, C. Meier, M. Kahkonen, M. Heinonen, A. Hopia and K.M. Oksman-Caldentey, 2001. Antimicrobial properties of phenolic compounds from berries. J. Appl. Microbiol., 90: 494-507.
8. Falagas, M.E. and I.A. Bliziotis, 2007. Pandrug-resistant Gram negative bacteria: The dawn of the post-antibiotic area? Int. J. Antimicrob. Ag., 29: 630-636.
9. Rayne, S. and G. Mazza, 2007. Biological activities of extracts from sumac (*Rhus* spp.): a review. Plant Foods Hum. Nutr., 62: 165-175.
10. Suffredini, I.B., H.S. Sader, A.G. Gonçalves, A.O. Reis, A.C. Gales, A.D. Varella and R.N. Younes, 2004. Screening of antibacterial extracts from plants native to the Brazilian Amazon rain forest and Atlantic forest. Braz. J. Med. Biol. Res., 37: 379-384.
11. Recio, M.C. and J.L. Rios, 1989. A review of some antimicrobial compounds isolated from medicinal plants reported in the literature. Phytother Res., 3: 117-125.
12. Silver, L.L. and K.A. Bostian, 1993. Discovery and development of new antibiotics: the problem of antibiotic resistance. Antimicrob. Agents Ch., 37: 377-383.
13. Duke, J.A. and E.S. Ayensu, 1985. Medicinal Plants of China Reference Publications, Inc. Details of over 1,200 medicinal plants of China and brief details of their uses. Often includes an analysis, or at least a list of constituents. Heavy going if you are not into the subject.
14. Sajid Nabi. Nisar Ahmed. Muhammad Javed Khan. Zahoor Bazai. Masoom Yasinzai. Yasser, MSA Al-Kahraman, 2012. *In Vitro* Antileishmanial, Antitumor Activities and Phytochemical Studies of Methanolic Extract and its Fractions of *Juniperus Excelsa* Berries. World Applied Sciences Journal, 19(10): 1495-1500.

15. Muhammad Javed Khan. Nizam U. Baloch. Sajid Nabi. Nisar Ahmed. Zahoor Bazai. Masoom Yasinzai. Yasser M.S.A. Al-Kahraman, 2012. Antileishmanial, Cytotoxic, Antioxidant Activities and Phytochemical Analysis of Rhazya Stricta Decne Leaves Extracts And Its Fractions. Asian Journal of Plant Science and Research, 2(5): 593-598.
16. Abdul Aziz Khan. Nizam U. Baloch. Sajid Nabi. Muhammad Javed Khan. M. Sharif Jamali. Yasser M.S.A. Al-Karhaman, 2012. In Vitro Antimicrobial and Insecticidal Activity Activity of Methanolic Extract and Its Fractions of *Berberis Baluchistanica* Roots. World Journal of Pharmaceutical Research, 2(1): 219-226.
17. Miles, A.A. and S.S. Misra, 1938. Estimation of bacterial power of blood. J. Hyg., 38: 7.
18. Kavanagh, F., 1972. Analytical Microbiology. F. Kavanagh (ED), VOLII, Academic press, New York and London, pp: 11.
19. Abbott. W.S., 1925. A method of computing effectiveness of insecticides. J. Econ. Ent., 18(2): 265-67.
20. Atta-ur-Rahman Choudhary, M.I. and J.T, William, 2001. Bioassay techniques for drug development. Harwood academic Publisher, pp: 67-68.
21. Collins. P.J., 1998. Resistance to grain protectants and fumigants in insect pests of stored products in Australia. In: (Ed.): H.J. Banks, E.J. Wright and K.A. Dameevski. CSIRO Stored Grain Research Laboratory, Presented in Australian Post Harvest Technical Conference, pp: 55- 57.
22. Rashid, R., M. Farah and M.N. Mirza, 2009. Biological screening of Salvia cabulica. Pak. J. Bot., 41(3): 1453-1462.
23. Tabassum, R., S.N.H.M. Naqvi, A. Azmi, S.M. Nurulain and M.F. Khan, 1997. Residual effect of a neem fraction, nimolicine and an insect growth regulator, dimilin, against stored grain pest Callosobruchus analis. Proc. Pakistan Congr. Zool., 17: 165-170.
24. Ahmad, M.S., M. Hussain, M. Hanif, S. Ali, M. Qayyum and B. Mirza, 2008. Di and Tri organotin [IV] Esters of 3,4-Methylenedioxyphenylpropenoic Acid: Synthesis, Spectroscopic Characterization and Biological Screening for Antimicrobial, Cytotoxic and Antitumor Activities. Chem. Bio. Drug. Design. 71: 568-576.
25. Jennifer, M.A., 2001. J. Antimic. Chemotherapy, 48: 5-16.
26. Harborne, I.B., 1973. Phytochemical methods. A guide to modern techniques of plant analysis. 2nd Ed. Chapman and Hall, New York press, pp: 88-185.
27. Trease, E.G. and W.C. Evan, 2007. Text book of pharmacognosy. 12th Ed. Oxford, Alden Press, pp: 343-383.
28. Chang, C., M. Yang and H. Wen, 2002. Chem J. Food Drug Anal., 10: 178-182.
29. Edeoga, H.O., D.E. Okwu and B.O. Mbaebie, 2005. Afr J. Biotechnol. 4: 685-688.
30. Okwu, D.E. and C. Josiah, 2006. African J. Biotechnology, 5(4): 357-361.
31. Price, M.L. and L.G. Butler, 1977. J. Agric. Food. Chem., 25: 1268-1273.