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Antibacterial activity of the seeds, roots and shoots of Lotus populations

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ABSTRACT

In this study the antibacterial activity of the ethanol, ethyl acetate and chloroform extracts from the seeds, roots and shoots of *Lotus aegaeus*, *Lotus angustissimus*, *Lotus corniculatus*, *Lotus gebelia*, *Lotus palustris* populations grown naturally in Turkey were investigated by using the disc diffusion and agar dilution method, against main plant pathogenic bacteria (*Clavibacter michiganensis*, *Agrobacterium tumefaciens*, *Erwinia caratovora*, *Pseudomonas phaseolicola*). According to results, the shoot ethyl acetate extract of *L. aegaeus* and shoot extracts of *L. corniculatus* against *C. michiganensis* and shoot extracts of all solvents of *L. angustissimus* against *P. phaseolicola* showed high antibacterial activity. This is the first report of antibacterial activity of the Lotus species against plant pathogens. In the study these stated effective extracts showed higher antibacterial effects by comparison with used chemical preservatives against sensitive bacteria. This study offers that active compounds present in Lotus species could play a big role in naturally plant preservation against plant diseases.

Key words: Antimicrobial, Forage crop, Legume crop, Natural preservative.

INTRODUCTION

Plant pathogens are considered economically important agricultural microorganisms in the world. They cause the destruction to a large number of crops during the season. So, mostly the chemical pesticides provide the controlling of the plant pathogens (Badawy and Rabea, 2011).

Even though pharmacological industries have provided a number of new drugs and herbicides in the last decades, resistance to these has increased by microorganisms. Chemical preservatives have been used in agriculture for years. However, an increasing perception by consumers that synthetic compounds may lead to health problems has led to a reduced acceptance for their use in crops. This situation improves the research on natural antimicrobial agents from plants (Mulaudzi *et al*., 2011). These plants having compounds with potentially significant therapeutic application against plant pathogens including bacteria, fungi, and virus, are known by their activity substances such as phenolics, alkaloids and terpenoids (Hemphill and Cobiac, 2006).

In direction of these strategies, some convincing results have been reported using natural products such as chitosan which is known safety alternative to hazardous pesticides with negligible risk to human health and the environment (Muzzarelli, 1983, Badawy, 2009, Badawy and Rabea, 2011).

The activity of an antimicrobial compound depends on the type, genus, species and strain of the target microorganism, besides the environmental factors such as pH, water activity, temperature, atmospheric composition and initial microbial load of the substrate (Negi, 2012). In studies, these should be taken into account.

Legumes are among the main protein sources for human beings and animals (Patil *et al*., 2015; Armand *et al.*, 2016) Lotus is a perennial legume crop and grown in nature. It is an important forage for ruminant nutrition with a wide ecological and latitudinal range and is particularly well suited to relatively infertile soils (Frame, 2005).

Lotus plants can accumulate condensed tannins, and this has been reported to have beneficial effects on wool growth in weaned lambs (Merkouropoulos *et al*., 2017), in essential amino acid absorption (Dalmarco *et al*., 2010), on the ovulation rate (Rochon *et al*., 2004), milk yield and protein and lactose production (Wang *et al*., 1996).

There are many reports showing many constituents including flavonoids, anthocyanins, sterols, tannins and alkaloids *in Lotus corniculatus* (Robbins *et al*., 2003; Reynaud and Lussignol, 2005).

Studies show that *Lotus corniculatus* has antibacterial activity against some human pathogen microorganisms (Dalmarco *et al.*, 2010; Girardi *et al*., 2014). Nevertheless, there is no report concerning the investigation of antibacterial activity of different Lotus species against plant pathogens.

The main objective of this study is to determine the *in vitro* antibacterial activity of different parts of Lotus populations.

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MATERIALS AND METHODS

Plant materials: Lotus populations were collected in 2016 and 2017 from nature flora inTurkey during flowering period. The second year populations were collected from same areas from where it was educated in the previous year. The identification of these specimens was carried out using the Flora of Turkey (Davis, 1966-1988). The information of the populations is given in Table 1.

Preparation of extracts: Fresh shoots, roots and seeds were dried. The dried samples were obtained by maceration of 2.5 g dry powder in 25 mL of chloroform, ethyl acetate and ethanol for 24 h. Extracts were filtered through a Whatman filter paper and evaporated under reduced pressure at 35°C using rotary vacuum evaporator (Boulaaba *et al*., 2015).

Test strains and culture media: *Pseudomonas phaseolicola, Clavibacter michiganensis, Erwinia caratovora* and *Agrobacterium tumefaciens* were selected as a plant pathogen materials. Because they are the most serious pathogens of a wide range of crops in Turkey and also worldwide. The losses due to development of these pathogens were very high. In the study, bacteria strains were obtained from ATCC (American Type Culture Collection, Rockville, MD). The antibacterial activity of the Lotus species dissolved in different solvents were assayed against *Pseudomonas phaseolicola* (11355), *Clavibacter michiganensis* (4450), *Erwinia caratovora* (15390) and *Agrobacterium tumefaciens* (33970) as plant pathogens. Bacterial suspension concentrations were set at 10^8 cells/mL.

Antibacterial assay: The antibacterial activity of the samples were screened by the agar disc diffusion assay (Hajlaoui *et al.*, 2008). Bacterial strains were first grown on Mueller Hinton medium at 37°C for 24 h (Haritha and Maheswari, 2007) prior to seeding on to the nutrient agar. Overnight cultures were diluted with broth and the final bacterial cell concentrations were adjusted to 108 cells/mL by measuring spectrophotometrically at A_{600} nm, respectively. One hundred microliters of each suspension with 108 bacteria/mL was added to agar-plated petri dishes. Sterile paper discs (6 mm diameter) were placed on agar to include a 15 µL sample. These plates, after standing at 4°C for 2 h, were incubated at 37°C for 24 h for bacteria.

Cefotaxime, Rifampicin and Agrigent Plus were assessed as positive control reference antibiotics (Villa-Ruano *et al.*, 2015). The diameters of the inhibition zones were measured in millimetres.

MIC assay: Minimal inhibition concentration values were determined using the agar dilution method defined by Vander Berghe and Vietinck (1991) with minor modifications. Samples were dissolved in chloroform, ethyl acetate and ethanol with physiological tris buffer 1:4 and mixed with an equal amount of 3% agar solution (Mueller Hinton agar). Samples were tested at concentrations of 0.313, 0.625, 1.250, 2.500, 5.000, 10.000 mg/mL. Four hundred milliliters from each solution were added to the tissue culture plate wells. Once solidified, each well was inoculated with 10 µL of freshly prepared suspension of 10^8 bacteria per 1 mL and incubated. Bacterial growths were assessed after incubation using a stereomicroscope.

Statistical analysis: The experiment were designed in randomized plots with three replications. The assumptions of data normality and homogeneity of variance, which are prerequisite for ANOVA, were tested with the Kolmogorov-Smirnov test and the Bartlett's test, respectively. The data of microorganisms obtained from different species, part and solvent were analysed by the three-way ANOVA with main effect and interactions effect, after means compared with LSD test. The results of the Tukey's test were presented in the form of letters displayed in the form of letters and numbers. The means were displayed as mean±standard error of the mean. The alpha level was set at 5%. All calculations were performed with Minitab 17 statistical software.

RESULTS AND DISCUSSION

Antibacterial activity of Lotus has been reviewed during last years in multiple studies (Dalmarco *et al.*, 2010; Girardi *et al*., 2014) especially in *Lotus corniculatus*. In this study, we attempted to assess the value of different parts of five Lotus species using different solvents as an antibacterial therapeutic agent.

The values given here were the means of the two study years, as no significant differences observed among antibacterial activities of Lotus populations grown in 2016 and 2017 years. The extracts from Lotus populations exhibited different degree of growth inhibition against tested bacterial strains (Fig 1-4). The minimal inhibition concentration values are shown in Table 2. In the study, chloroform, ethyl acetate and ethanol solvents without extracts did not show any inhibitory effect as a control.

The results against *C. michiganensis* showed difference statistically (p=0.007**). As depicted in Fig 1, all the seed and shoot extracts showed antibacterial activity against *C. michiganensis*. These extracts could play a big

Table 1: Information about collected populations.

Species	Province	District	Altitude (m)
L. aegaeus	Manisa	Turgutlu	784
L. angustissimus	Sinop	Gerze	511
L. corniculatus	Rize	Çamlıhemşin	639
L. gebelia	Kahramanmara's	Göksun	1220
L. palustris	Antalya	Serik	923

Table 2: Results of MIC values (mg/mL) of the extracts.

Species	Parts	Solvents	*C.m.*	*A.t.*	*E.c.*	*P.p.*
L. aegaeus	Root	Chloroform	10.00	NT	NT	NT
		Ethanol	5.00	NT	NT	NT
		Ethyl acetate	5.00	NT	NT	NT
	Seed	Chloroform	2.50	NT	NT	10.00
		Ethanol	2.50	NT	NT	10.00
		Ethyl acetate	2.50	NT	NT	10.00
	Shoot	Chloroform	1.25	NT	NT	10.00
		Ethanol	1.25	NT	NT	5.00
		Ethyl acetate	0.31	10.00	NT	5.00
L. angustissimus	Root	Chloroform	NT	NT	NT	NT
		Ethanol	NT	NT	NT	NT
		Ethyl acetate	NT	NT	NT	NT
	Seed	Chloroform	5.00	NT	NT	10.00
		Ethanol	5.00	NT	NT	2.50
		Ethyl acetate	2.50	NT	NT	5.00
	Shoot	Chloroform	2.50	NT	NT	2.50
		Ethanol	1.25	NT	NT	5.00
		Ethyl acetate	1.25	10.00	NT	0.63
L. corniculatus	Root	Chloroform	NT	NT	NT	NT
		Ethanol	NT	NT	NT	NT
		Ethyl acetate	NT	NT	NT	NT
	Seed	Chloroform	5.00	NT	NT	10.00
		Ethanol	5.00	NT	NT	10.00
		Ethyl acetate	2.50	NT	NT	5.00
	Shoot	Chloroform	0.63	NT	NT	5.00
		Ethanol	1.25	NT	NT	5.00
		Ethyl acetate	0.63	NT	NT	1.25
L. gebelia	Root	Chloroform	NT	NT	NT	NT
		Ethanol	NT	NT	NT	NT
		Ethyl acetate	NT	NT	NT	NT
	Seed	Chloroform	5.00	NT	NT	5.00
		Ethanol	10.00	NT	NT	5.00
		Ethyl acetate	10.00	NT	NT	5.00
	Shoot	Chloroform	2.50	NT	NT	5.00
		Ethanol	10.00	NT	NT	5.00
		Ethyl acetate	1.25	NT	NT	5.00
L. palustris	Root	Chloroform	NT	NT	NT	NT
		Ethanol	NT	NT	NT	NT
		Ethyl acetate	NT	NT	NT	10.00
	Seed	Chloroform	10.00	NT	NT	10.00
		Ethanol	10.00	NT	NT	10.00
		Ethyl acetate	10.00	NT	NT	10.00
	Shoot	Chloroform	2.50	NT	NT	5.00
		Ethanol	5.00	NT	NT	5.00
		Ethyl acetate	2.50	NT	NT	2.50

NT: Not Tested, *C.m*: *Clavibacter michiganensis*, *A.t*: *Agrobacterium tumefaciens*, *E.c*: *Erwinia caratovora*, P.p: *Pseudomonas phaseolicola*

role to prevent the disease of many crops like corn, potatoes and alfalfa infected by *C. michiganensis*. This is the first report for Lotus extracts against *C. michiganensis*. In the study, the shoot ethyl acetate extract of *L. aegaeus* and shoot extracts of *L. corniculatus* showed the highest antibacterial activity. Here, the more impressive result is these values (26.00 and 24.17 mm respectively) are higher than Rifampicin (22.33 mm) as a positive control. This result is very important because the use of herbicide and drugs for *C. michiganensis* may lead to health and environment problems. According to results, ethyl acetate solvents showed higher values in *L. aegaeus* and *L. angustissimus*. On the other hand, chloroform extracts showed higher values in *L. gebelia* and *L. palustris*. In the study root extracts did not show any activity except the ethyl acetate and ethanolic root extracts of *L. aegaeus*.

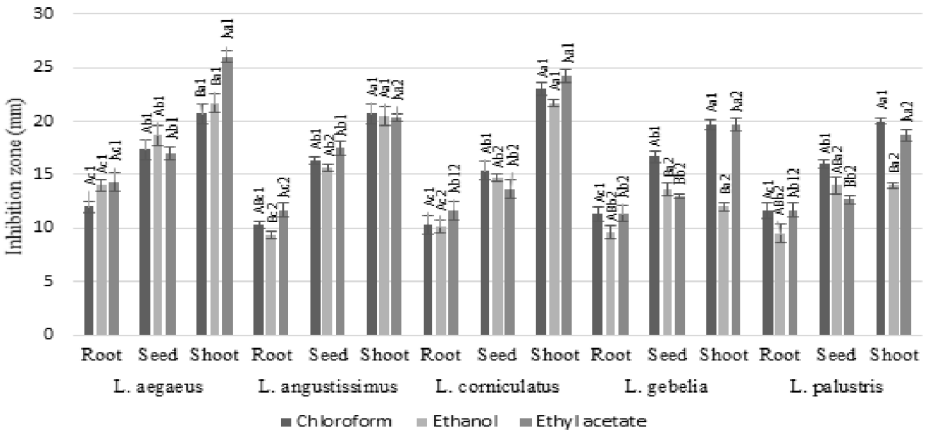

Fig 1: Inhibition zones (mm) of the extracts against *Clavibacter michiganensis.*
solvent means that do not share a common upper case letter are significantly different (Tukey, p<0.05)
in the same species, part means that do not share a common lower case letter are significantly different (Tukey, p<0.05)
in the same part, species means that do not share a common number are significantly different (Tukey, p<0.05)
p-value: 0.007

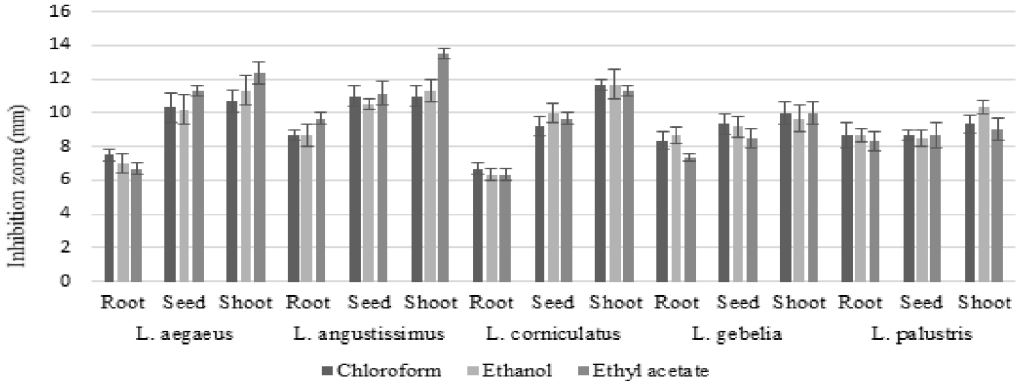

Fig 2: Inhibition zones (mm) of the extracts against *Agrobacterium tumefaciens.*

The results against *A. tumefaciens* did not show difference statistically. As shown in Fig 2, no extract showed antibacterial activity against *A. tumefaciens* except the ethyl acetate shoot extracts of *L. aegeus* and *L. angustissimus*, however there is no statistical difference. It showed the value of 12.34, 13.50 mm respectively against *A. tumefaciens.* Although the value was lower than Cefotaxime (21.66 mm) as a positive control, this may be a meaningful result for future.

As seen in Fig 3 all the extracts did not show any antibacterial activity against *E. caratovora*. Rifampicin as a positive control showed antibacterial activity with the value of 15.33 mm against *E. caratovora* in the study.

As seen in Fig 4, many of the extracts showed antibacterial activity against *P. phaseolicola*. In addition to this, the interaction was determined between species and parts (p=0.017*). In the study the shoot extracts of *L. angustissimus* were the most tolerant extracts (19.44 mm, as a mean of solvents). On the other hand, all the seed and shoot extracts showed higher antibacterial activity than Agrigent Plus as a positive control (11.66 mm). In the study the ethyl acetate extracts showed higher antibacterial activity than the other extracts statistically (p=0.000***). The study showed that, in all species the activity situation is like shoot>seed>root against *P. phaseolicola.* This result was very important in terms of the prevention of herbicides and drugs against *P. phaseolicola*.

In Table 2, the extracts having antibacterial activity were tested in terms of minimal inhibition concentration. As seen, many of the extracts inhibited the bacterial growth even at low doses. The most effective result was determined in ethyl acetate shoot extract of *L. aegaeus* against *C. michiganensis* in parallel with the disc diffusion results with the value of 0.31 mg/mL. And then followed by the chloroform and ethyl acetate shoot extracts of *L. corniculatus* against *C. michiganensis* and ethyl acetate shoot extract of *L. angustissimus* against *P. phaseolicola* with the value of 0.63 mg/mL. These results are very important, because it gives information about the usage doses of the extracts having antibacterial activity.

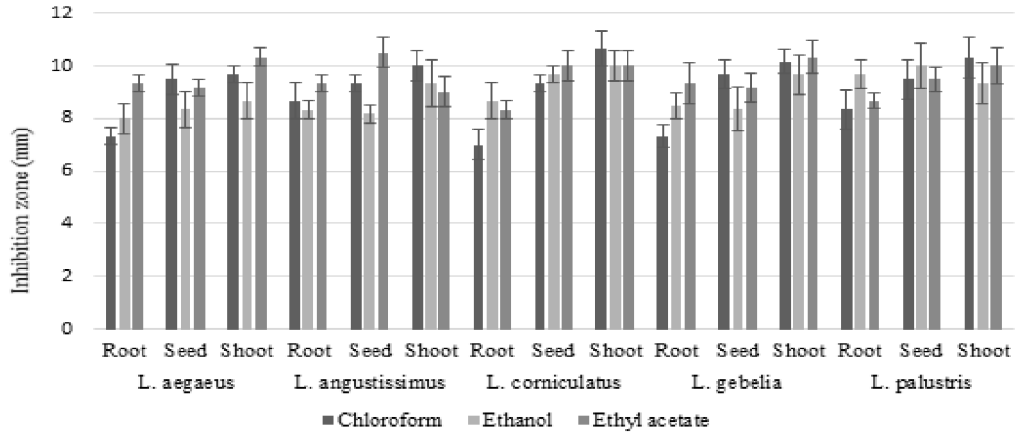

Fig 3: Inhibition zones (mm) of the extracts against *Erwinia caratovora.*

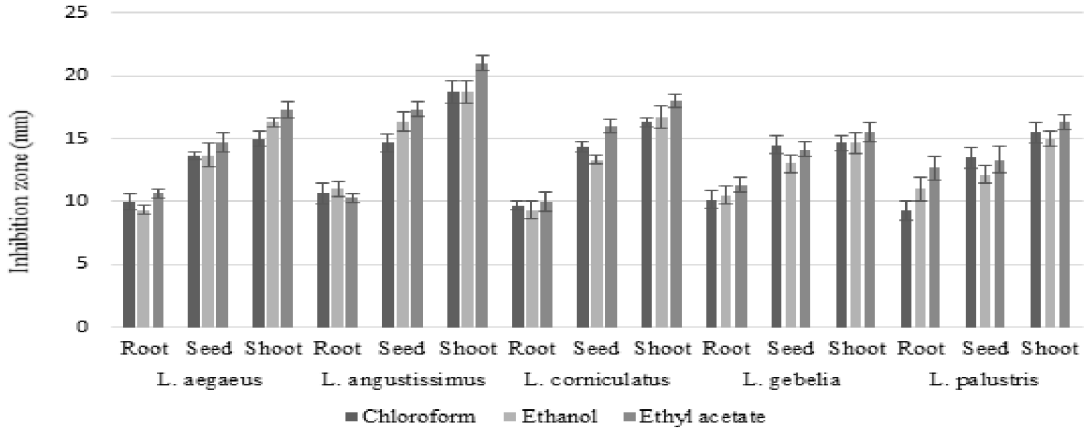

Fig 4: Inhibition zones (mm) of the extracts against *Pseudomonas phaseolicola.*

Today, reports demonstrate that many plants have antimicrobial activity against pathogens. However not many reports determined the antimicrobial activity against plant pathogens for plant protection.

Although studies have been conducted about antibacterial activity in *Lotus corniculatus*, no study was conducted to determine the effect of different Lotus species against plant pathogens, so this study will be beneficial in observing the antibacterial activity of Lotus plants with several factors.

CONCLUSION

This two years replicate study emphasizes antibacterial properties of the parts of Lotus species with different solvents. The results of present study clearly indicate that the antibacterial activity vary with the species, parts and solvents in Lotus.

In the study, the value of the results were higher than chemical preservatives in the shoot ethyl acetate extract of *L. aegaeus* and shoot extracts of *L. corniculatus* against *C. michiganensis* and shoot extracts of all solvents of *L. angustissimus* against *P. phaseolicola*. This is the first report having antibacterial activity against plant pathogens in Lotus species. In the study, it is suggested that active compounds present in Lotus species could play a big role in plant protection.

REFERENCES

Armand, N., Amiri, H., and Ismaili, A. (2016). The effects of spraying methanol solution and drought stress on level of antioxidant enzyme activity, growth, biomass and yield of beans (*Phaseolus vulgaris* L. cv. COS16). *Legume Research: An International Journal*, **39**(3): 385-395.

Badawy, M.E. (2010). Structure and antimicrobial activity relationship of quaternary N alkyl chitosan derivatives against some plant pathogens. *Journal of Applied Polymer Science*, **117**(2): 960-969.

Badawy, M.E. and Rabea, E.I. (2011). A biopolymer chitosan and its derivatives as promising antimicrobial agents against plant pathogens and their applications in crop protection. *International Journal of Carbohydrate Chemistry*. 1-29.

Boulaaba, M., Snoussi, M., Saada, M., Mkadmini, K., Smaoui, A., Abdelly, C., and Ksouri, R. (2015). Antimicrobial activities and phytochemical analysis of *Tamarix gallica* extracts. *Industrial Crops and Products*, **76**: 1114-1122.

Dalmarco, J.B., Dalmarco, E.M., Koelzer, J., Pizzolatti, M.G., and Fröde, T.S. (2010). Isolation and identification of bioactive compounds responsible for the anti-bacterial efficacy of *Lotus corniculatus* var. São Gabriel. *International Journal of Green Pharmacy*, **4**(2): 108-114.

Davis, P.H. (1966-1988). Flora of Turkey and the East Aegean Islands, Vols. 1-10. Edinburgh University Press, Edinburgh.

Frame, J. (2005). Forage legumes for temperate grasslands. Rome: FAO, Enfield, NH: Science Publishers Inc. pp 84-90.

Girardi, F.A., Tonial, F., Chini, S.O., Sobottka, A.M., Scheffer-Basso, S.M., and Bertol, C.D. (2014). Phytochemical profile and antimicrobial properties of Lotus spp.(Fabaceae). *Anais da Academia Brasileira de Ciências*, **86**(3), 1295-1302.

Hajlaoui, H., Snoussi, M., Ben Jannet, H., Mighri, Z., and Bakhrouf, A. (2008). Comparison of chemical composition and antimicrobial activities of *Mentha longifolia* L. ssp. longifolia essential oil from two Tunisian localities (Gabes and Sidi Bouzid). *Annals of Microbiology*, **58**: 513-520.

Haritha, P. and Maheswari, K.U. (2007). Effect of processing on the antinutritional and antimicrobial activity of sicklesenna seeds (*Cassia tora* L.). *Legume Research-An International Journal*, **30**(2): 108-112.

Hemphill, L. and Cobiac, L. (2006). Health benefits of herbs and spices: the past, the present, the future. *The Medical Journal of Australia*, **185**: 5-6.

Merkouropoulos, G., Hilioti, Z., Abraham, E.M., Lazaridou, M. (2017). Evaluation of Lotus corniculatus L. accessions from different locations at different altitudes reveals phenotypic and genetic diversity. *Grass and Forage Science*, **72**(4): 851-856.

Mulaudzi, R.B., Ndhlala, A.R., Kulkarni, M.G., Finnie, J.F., and Van Staden, J. (2011). Antimicrobial properties and phenolic contents of medicinal plants used by the Venda people for conditions related to venereal diseases. *Journal of Ethnopharmacology*, **135**: 330-337.

Muzzarelli, R.A. (1983). Chitin and its derivatives: new trends of applied research. *Carbohydrate Polymers*, **3**(1): 53-75.

Negi, P.S. (2012). Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. *International Journal of Food Microbiology*, **156**(1): 7-17.

Patil, S.L., Mishra, P.K., Loganandhan, N., Math, S.K.N., Manikatti, S.M., Seshadri, B.N. (2015). Suitable chickpea cultivars for rainfed situations in black soils of South India. *Legume Research-An International Journal*, **38**(2): 229-234.

Reynaud, J. and Lussignol, M. (2005). The flavonoids of *Lotus corniculatus*. *Lotus Newsletter*, **35**(1): 75-82.

Robbins, M.P., Paolocci, F., Hughes, J.W., Turchetti, V., Allison, G., Arcioni, S., Morris, P., Damiani, F. (2003). Sn, a maize bHLH gene, modulates anthocyanin and condensed tannin pathways in *Lotus corniculatus*. *Journal of Experimental Botany*, **54**(381): 239-248.

Rochon, J.J., Doyle, C.J., Greef, J.M., Hopkins, A., Molle, G., Sitzia, M., Scholefield, D., Smith, C.J. (2004). Grazing legumes in Europe: a review of their status, management, benefits, research needs and future prospects. *Grass and Forage Science*, **59**(3): 197-214.

Vander Berghe, D.A. and Vietinck, A.J. (1991). Screening methods for antibacterial and antiviral agents from higher plants. In Methods in Plant Biochemistry (P.M. Dey and J.B. Harborne, eds.) Academic Press, London, 47-67.

Villa-Ruano, N., Pacheco-Hernández, Y., Cruz-Durán, R., and Lozoya-Gloria, E. (2015). Volatiles and seasonal variation of the essential oil composition from the leaves of *Clinopodium macrostemum* var. *laevigatum* and its biological activities. *Industrial Crops and Products*, **77**: 741-747.

Wang, Y., Douglas, G.B., Waghorn, G.C., Barry, T.N., Foote, A.G. (1996). Effect of condensed tannins in *Lotus corniculatus* upon lactation performance in ewes. *The Journal of Agricultural Science*, **126**(3): 353-362.