The Journal of Animal & Plant Sciences, 29(2): 2019, Page: 453-460
ISSN: 1018-7081

SILVER NANOPARTICLES: ECOFRIENDLY SURFACE STERILIZATION OF PLANT SEEDS IN DIFFERENT SHAPES AND SIZES

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ABSTRACT

In this study, silver nanoparticles obtained via green synthesis were utilized for the surface sterilization of seeds in different shapes and sizes. Water extracts of dried *Alkanna tinctoria* rhizomes and *Pinus nigra* leaves were utilized in the bioreduction of silver ions. *Centaurea cyanus* L., *Digitalis purpurea* L., *Calendula officinalis* L., *Lavandula officinalis* L. and *Melissa officinalis* L. seeds were exposed to silver nanoparticle colloidal solutions (Alkanna-NP and Pinus-NP) for 1-20 mins for surface sterilization. Germination and sterilization percentages of the seeds were recorded during four weeks. Pinus-NP was found more effective on surface sterilization (85.11%) than Alkanna-NP (74.77%). The germination percentages of the different genus seeds were recorded according to the applied silver nanoparticle sterilization (Alkanna- NP and Pinus-NP), respectively: *C. cyanus* 40.56% and 57.78%; *D. purpurea* 89.44% and 52.22%; *C. officinalis* 18.87% and 13.89%; *L. officinalis* 15.56% and 17.22%. No germination was observed at *M. officinalis* seeds. There was no negative effect was detected during culture period on both seeds and sterile plantlets. Silver nanoparticles were also found more effective on rough-coated seed surface sterilization with regard to NaOCl. It was proved that both Alkanna-NP and Pinus-NP can be used as sterilization agents in plant tissue cultures.

Keywords: surface sterilization, plant seed, silver nanoparticle, *Alkanna tinctoria*, *Pinus nigra.*

INTRODUCTION

Nanotechnology is the science of nano-objects having dimensions roughly within the 1–100 nm range (Liu *et al*, 2012). "Nanobiotechnology" term the combination of nanotechnology and biotechnology terms and is the intersection of nanotechnology, biomedical sciences and molecular biology. In both nanotechnology and nanobiotechnology, the main area of interest is nanoparticles. Silver nanoparticles are the most commonly used ones amongst the other metal nanoparticles, such as gold, zinc, etc. They have strong antiseptic, antibacterial, antifungal and antiviral effects depending on their sizes and shapes. Moreover, they are eco-friendly elements which have relatively low toxicity towards to environment and human (Pal *et al*., 2007; Yah and Simate, 2015; Rudramurthy *et al*., 2016).

Plants are the main source of many natural products. They are used in many areas, such as pharmaceutical, cosmetical, agrochemical, and biomedical applications. Nowadays, plant extracts are being used as bioreductors in nanobiotechnological studies in order to synthesize silver nanoparticles from silver ions and this method is called as "green synthesis" or "green chemistry procedure" of silver nanoparticles (Jha and Prasad, 2010; Kaviya *et al*., 2011). Silver nanoparticles have also been used in plant biotechnology as elicitors and surface sterilization agents for plant explants (Bhat and Bhat, 2016; Fazal *et al*., 2016; Ghanati and Bakhtiarian, 2016; Nartop, 2017; Nartop 2018a).

Surface sterilization is the first and the most important step of plant biotechnology. Mostly utilized surface sterilization agents are sodium chlorite, ethanol, calcium hypochlorite, mercuric chlorite, surfactants and antibiotics. They are toxic to all living organisms and not eco-friendly. Nevertheless, they have also very toxic effects on plant cells and tissues. They cause necrosis, browning and growth inhibition on plant cells and tissues in culture conditions (Çölgeçen *et al*., 2011; Mbah and Wakil, 2012). Therefore, well-chosen surface sterilization agent is the key to successful procedures for plant biotechnology.

In this study, silver nanoparticles synthesized via green synthesis with water extracts of *Alkanna* rhizomes and *Pinus* leaves were used as surface sterilization agent on medicinal plants' seeds which have different shapes and sizes. Germination and surface sterilization percentages of the seeds were recorded in order to determine the effects of silver nanoparticles at different sterilization duration periods (1 min, 5 mins, 10 mins and 20 mins).

MATERIALS AND METHODS

Materials: Commercial seeds of medicinal plants, which are also grown as garden plants such as *Centaurea cyanus* L. (syn. *Cyanus segetum* L.), *Digitalis purpurea* L. (syn. *Digitalis alba* Schrank), *Calendula officinalis L.*

(syn. *Calendula prolifera hort. ex* Steud.), *Lavandula officinalis* L. (syn. *Lavandula angustifolia* Mill.) and *Melissa officinalis* L. (syn. *Thymus melissa* E.H.L.Krause) were used for the surface sterilization studies. Dried *Alkanna tinctoria* rhizomes were obtained from a local market. *Pinus nigra* leaves were collected from the trees of Namık Kemal University, Faculty of Engineering (Çorlu, Tekirdağ, Turkey) in May 2017. Water extracts of *A. tinctoria* rhizomes and *P. nigra* leaves were used as bioreductors for silver nanoparticle formation.

Methods: Dried *A. tinctoria* rhizomes and *P. nigra* leaves were surface-cleaned with running tap water and rinsed in distilled water twice. After they were dried, they were ground with a mortar. Five grams of each were mixed with 100 ml distilled water and kept in water bath at 80°C for one hour. The mixtures were filtered through filter paper and kept in glass bottles for one day at room temperature (22±2°C).

Five milliliters of *A. tinctoria* rhizomes *and P. nigra l*eaves water extracts were mixed with 95 ml of 1 mM silver nitrate solution and incubated at room temperature (22±2°C) for two hours. At the end of bioreduction,these colloidal solutions (Alkanna-NP and Pinus-NP) were used for surface sterilization of seeds.

The silver nanoparticle formations in Alkanna-NP and Pinus-NP were confirmed by color and pH changes. The UV-vis spectra of Alkanna-NP and Pinus-NP were also monitored at 320-500 nm by using UV-visible spectrophotometer (Shimadzu UV-1201V). Distilled water was used as blank to adjust the baseline.

Alkanna-NP and Pinus-NP were used for the surface sterilization of *C. cyanus*, *D. purpurea*, *C. officinalis*, *L. officinalis* and *M. officinalis.* The seeds were washed with excess tap water and dried on filter paper. After that, the seeds were exposed to Alkanna-NP and Pinus-NP for 1, 5, 10 and 20 mins. The seeds were not rinsed with sterile distilled water after Alkanna-NP and Pinus-NP sterilization and placed directly in semi-solid WPM (Lloyd and McCown, 1980) medium supplemented with 3% sucrose and 0.7% agar (pH=5.8). In control groups, instead of Alkanna-NP and Pinus-NP, the seeds were exposed to 1% NaOCl for 20 mins for surface sterilization. They were rinsed in sterile distilled water three times before culture. Each experiment was conducted with 15 seeds with three replicates and they were incubated at 22±2°C with 16 h light/8 h dark photoperiods. At the fourth week, sterile and germinated seed percentages were calculated by observations of seeds in culture.

Germination and surface sterilization experiments were repeated three times. Experiments were implemented in a factorial randomized plots design with three factors; seed type:*C. cyanus*, *D. purpurea*, *C. officinalis*, *L. officinalis* and *M. officinalis;* nanoparticle type: Alkanna-NP, Pinus-NP; sterilization duration: 1, 5, 10, 20 mins. Data were analyzed with one-way ANOVA test and TUKEY tests were also performed ($p \leq 0.05$).

RESULTS AND DISCUSSION

When the water extracts of plants were mixed with silver nitrate solution, their color started to change from lighter color to its darker tones (Figure 1). In my previous study, water extracts of twelve plants were used as bioreductor in silver nanoparticle biosynthesis. Amongst these extracts, *Alkanna* rhizome and *Pinus* leaf extracts were found to form the lightest-colored colloidal silver nanoparticles (Alkanna-NP and Pinus-NP), whereas clove (*Syzygium aromaticum*) flower extract formed the darkest and the thickest suspension (Syzygium-NP) (*unpublished data*). Following this, another study was established; Alkanna-NP and Syzygium-NP were utilized for surface sterilization of *Lamiaceae* seeds in order to see the efficacy of light (Alkanna-NP) and dark-thick (Syzygium-NP) colloidal solutions on surface sterilization of seeds. The highest sterilization percentages (100%) were obtained from Alkanna-NP. This result was elucidated with SEM analysis; Alkanna-NP was found more spherical and smaller than Syzygium-NP (Nartop, 2018b). It has known that antibacterial effects of silver nanoparticles were strongly affected by sizes and shapes of plant seeds. Smaller and spherical nanoparticles enhance the extent of bacterial elimination and are more effective than bigger ones, due to their larger contact surface (Pal *et al*., 2007). Moreover, in this study, silver nanoparticles were found to engage with each other, narrow their surface area as their colloidal solutions get older and precipitation can even be observed at the bottom of a glass bottle. In rosemary stem cultures, when colloidal solutions of silver nanoparticles filtered through 0.22 µm sterile filter, surface sterilization percentages of explants were enhanced up to 96.67%, which also confirms the stronger antibacterial effect of smaller (unengaged) nanoparticles (Nartop, 2018a).

In this study, the color of Alkanna-NP changed from lighter yellow to a darker tone. In Pinus-NP, the color change was more distinct; it changed from very light pink colour to a darker tone. These color changes were the sign of bioreduction in the mixtures. The coloration was caused by the excitation of surface plasmon resonance of conducting electrons in the silver nanoparticles.Silver ions are converted to elemental silver when they come across bioreductors. Plant secondary metabolites, especially phenolic compounds play bioreductor role in extracts of plants. As bioreduction finished, color changes of the mixture stop (Sinha and Paul, 2014; Kumar *et al*., 2017; Nartop, 2017; Nartop 2018a). The colour changes of Alkanna-NP and Pinus-NP became stable after two hours, which means the

bioreduction was completed. Therefore, Alkanna-NP and Pinus-NP mixtures were not used immediately for surface sterilization and were waited for two hours in room conditions (22±2°C) prior to use.

Another sign of bioreduction is pH change of the mixtures (Maria *et al*., 2015). In this study, pH alterations ofthe mixtures were recorded prior to surface sterilization. pH decreased in both mixtures after two hours; from 6.33 to 6.12 in Alkanna-NP and from 4.97 to 3.83 in Pinus-NP.

Silver nanoparticle biosynthesis was also confirmed by UV-Vis spectrophotometry. The optical properties of silver nanoparticles are commonly calculated by UV-Vis absorption spectroscopy.Because of surface plasmon resonance, a strong absorption of electromagnetic waves is exhibited by metal nanoparticles in the visible range (Amin *et al*., 2012). Alkanna-NP and Pinus-NP showed characteristic bands in UV-Visible region; 430 nm and 425 nm, respectively (Figure 2). Similar results were reported in green synthesis of silver nanoparticle studies (Amin *et al*., 2012; Sinha and Paul, 2014; Kumar *et al*., 2017; Nartop, 2017; Nartop 2018a).

For surface sterilization with silver nanoparticles,the seeds of *C. cyanus*, *D. purpurea*, *C. officinalis*, *L. officinalis* and *M. officinalis* were selected because of their different shapes and sizes. (Figure 3). In general, surface sterilization of rough-coated seeds is harder than smooth ones. Since it is not always possible to reach all recess ledge area on rough-coated seeds, surface sterilization percentages decrease. Moreover, surface sterilization of small seeds is always easier and practical than the bigger ones. Therefore, the seeds in different sizes with rough or smooth coats were chosen for surface sterilization. The seed-coats of *C. cyanus* is not rough, but have a tuft-like accumulation of hairs at the top (Figure 3a). *D. purpurea* seeds are not rough and they are very small (approximately 1 mm length) (Figure 3b). The seeds of *C. officinalis* is rough and the biggest ones amongst the seeds used in this study (Figure 3c). *L. officinalis* and *M. officinalis* seeds have smooth coats and are not bigger than 2 mm (Figure 3d and 3e).

C. cyanus seeds germination started at the second day of culture. *D. purpurea* and *C. officinalis* seeds started to germinate at the third day of culture. During the first two weeks of culture, no germination was detected in *L. officinalis* seeds. They were started to germinate at the 16^{th}-20^{th} days of culture. In these four plant genus, a rapid growth was observed after the germination. Two week-old plantlets obtained from the germinated seeds were shown in Figure 4. No adverse effect was observed on sterile plantlets when they compared to the control groups. They showed an accordance with control group plantlets: they all started to germinate within the same days and they came to approximately the same height as the controls. This result shows that there is no need to remove silver nanoparticles from the seeds prior to culture, which also makes this application more practical; no need to provide sterile distilled water and more sterile erlenmeyers or flasks while working in sterile conditions. In *M. officinalis* seeds, no germination was observed during two months of culture. Winiarczyk *et al*. (2016) mentioned a low rate of germination of *M. officinalis* seeds. Some chemical treatments have been used to overcome dormancy problems of seeds, such as H_2SO_4 solution application (Ramak *et al*., 2011). In this study, *M. officinalis* seeds were treated with silver nanoparticles, but silver nanoparticles did not work as chemical stratification agent. Therefore, *M. officinalis* seeds were only evaluated for their surface sterilization percentages.

Germination and sterilization percentages of *C. cyanus*, *D. purpurea*, *C. officinalis*, *L. officinalis* and *M. officinalis* were given in Table 1. According to ANOVA analysis, germination percentages highly affected by the three factors; nanoparticle type, seed type, nanoparticle type*seed type interaction, nanoparticle type*seed type*sterilization duration interaction were found statistically significant at $p \leq 0.01$ ($F = 4.44$; $MSE = 87.8$). Alkanna-NP (32.89%) had a higher effect on germination than Pinus-NP (28.22%), whereas this percentage was found 23.11% in control groups. Germination percentage of *D. purpurea* seeds was found 70.83% and this was the highest percentage of germination amongst the seeds tested, whereas the control group had the 68.89% germination percentage. Besides, according to nanoparticle type*seed type*sterilization duration interaction, the highest germination percentage (93,33%) was also obtained from *D. purpurea* seeds which were exposed to Alkanna-NP for 1 min. This result was 35.48% higher than its control group and sterilization percentages were 100% in both groups. In *C. cyanus* seeds, germination percentages were also ascended while using nanoparticle sterilization; in control groups, germination percentages were 22.22%, whereas 40,55% and 57,78% were obtained from Alkanna-NP and Pinus-NP sterilizations, respectively. These result showed that silver nanoparticles have better effects than NaOCl for germination of hairy seeds. *L. officinalis* seeds were not germinated in control group, however, 15,55% and 17,22% germination percentages were obtained in Alkanna-NP and Pinus-NP sterilizations, respectively. Sterilization duration had no statistically significant effect on germination percentages. However, the highest germination percentage (33.56%) was observed in 10 min sterilization. In 5 min, 20 min and 1 min, germination percentages were 30.66%, 29.33% and 28.67%, respectively. These findings were higher than 23.11%, which was obtained from the control groups. This result showed that there was no adverse effect of silver nanoparticles on seed germination, moreover, this procedure enhanced seed germination up to 45.22%.

In surface sterilization, nanoparticle type, seed type, sterilization duration, nanoparticle type*seed type interaction, nanoparticle type*sterilization duration interaction, seed type*sterilization duration interaction were found statistically significant at $p \leq 0.01$ ($F=1.24$; MSE: 155.2). Pinus-NP (85.11%) had a higher effect on surface sterilization than Alkanna-NP (74.77%), whereas this percentage was found 84,4% in control groups. This result shows that in surface sterilization, Pinus-NP was as effective as 20% NaOCl for 20 mins. In seed type, *D. purpurea*, *L. officinalis* and *M. officinalis* were found in the same group, statistically (100%, 95.83% and 94.45%, respectively). Because of the smooth-coats of these seeds, this result is predictable. On the other hand, the results were interesting in *C. cyanus* and *C. officinalis*. In *C. cyanus*, the average sterilization percentage was found 57.78% which was very low than the control group (100%). However, sterilization percentage was rising gradually with longer sterilization durations with both Alkanna-NP and Pinus-NP. In order to get better results for surface sterilization of hairy seeds like *C. cyanus*, sterilization duration may be prolonged. In silver nanoparticle surface sterilization, the average germination percentage (49,17%) was approximately two times higher than the control group (22,22%) in *C. cyanus*, which means there is no negative effect of silver nanoparticles while long exposures. In *C. officinalis*, it was very obvious that silver nanoparticle sterilization was far better than NaOCl sterilization. Sterilization percentages were enhanced 3.2 and 3.4 times than the control group with Alkanna-NP and Pinus-NP sterilizations, respectively. These results showed that using silver nanoparticles for surface sterilization of rough-coated seeds may be more beneficial than NaOCl. Effects of sterilization durations were gradually increased as the duration prolonged; 90.67% in 20 mins, 84% in 10 mins, 81.11% in 5 mins and 64% in 1 min.

It's known that plant extracts have antimicrobial effects (Cowan, 1999). In order to test the antibacterial activity and surface sterilization performance of *A. tinctoria* rhizomes and *P. nigra leaves* water extracts, they were applied to the seeds for 20 mins. However, all the seeds were contaminated at the 4th day of culture, which means the water extracts have no sufficient antibacterial activity alone for surface sterilization of these seeds.

Because of their antibacterial effects, silver nanoparticles can be used as surface sterilization agents in plant biotechnology. No adverse effect was observed regarding silver nanoparticle utilization for surface sterilization in both smooth-coated and rough-coated seeds. The rough-coated seeds of *C. officinalis* had a higher germination and sterilization percentages when they were treated with silver nanoparticles. The seeds germinated at high percentages (up to 93.33%) and formed healthy sterile plantlets. Data obtained from this study bring a new perspective to surface sterilization problem faced in plant cell and tissue cultures and show that nanotechnology can be used in plant biotechnology as well. This method may also be helpful than currently used methods, especially for delicate plant materials which are easily damaged by commonly used surface sterilizing agents. These studies about silver nanoparticles associate plant biotechnology and nanotechnology for better, more practical and beneficial applications.

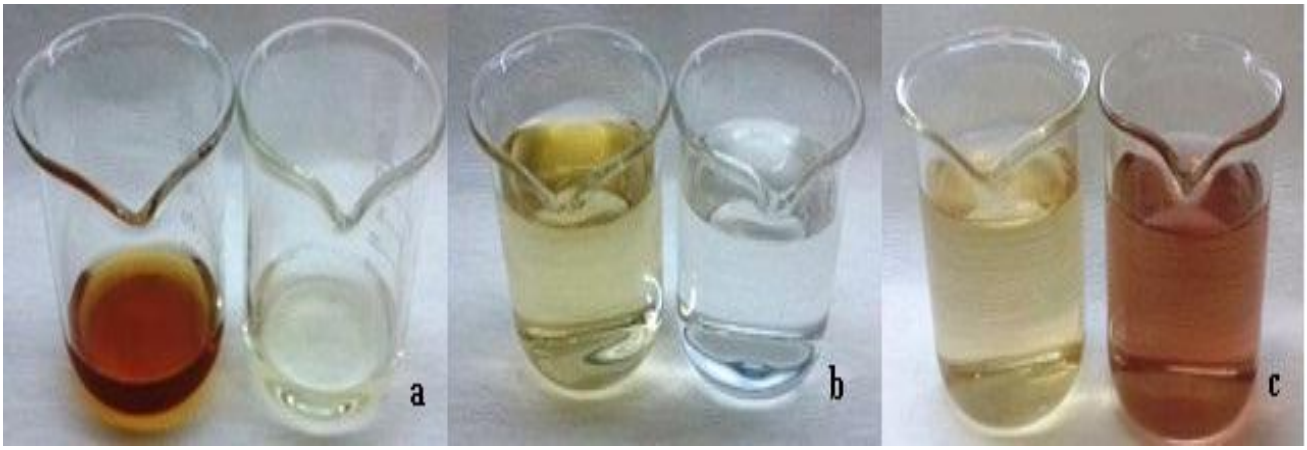
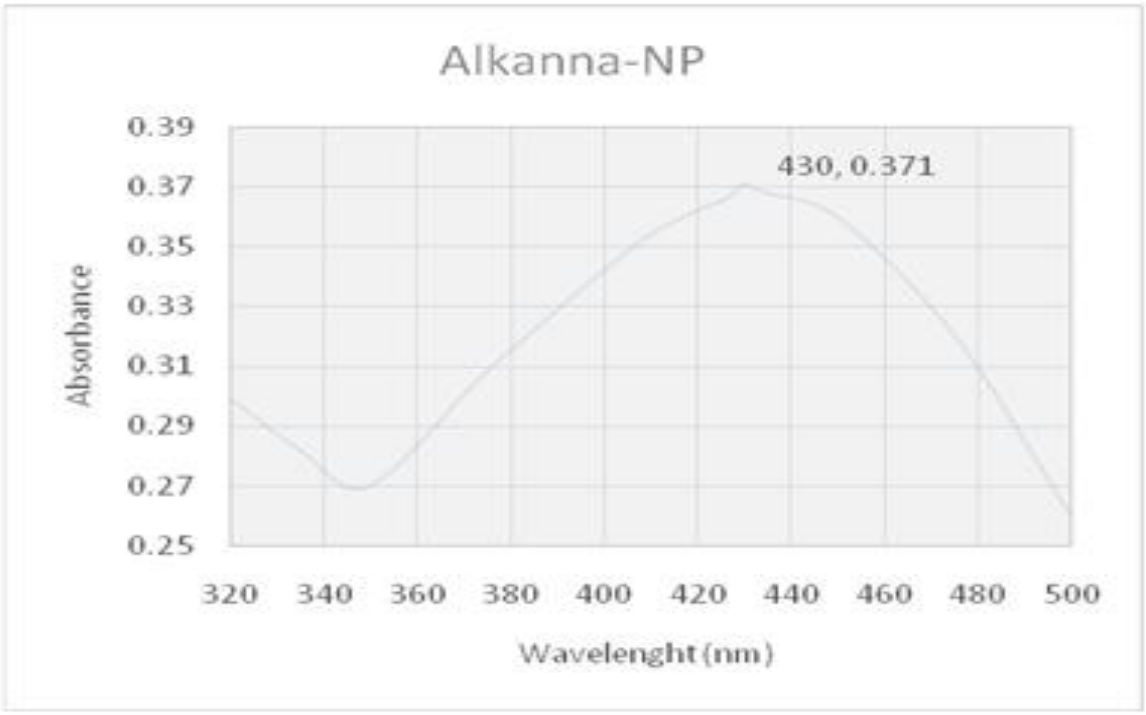


Figure 1. Plant extracts and silver nanoparticle formation; (a) *A. tinctoria* (left) and *P. nigra* (right) water extracts; Mixtures of silver nitrate solution and plant extracts (b) before silver nanoparticle formation and (c) after 2 h.



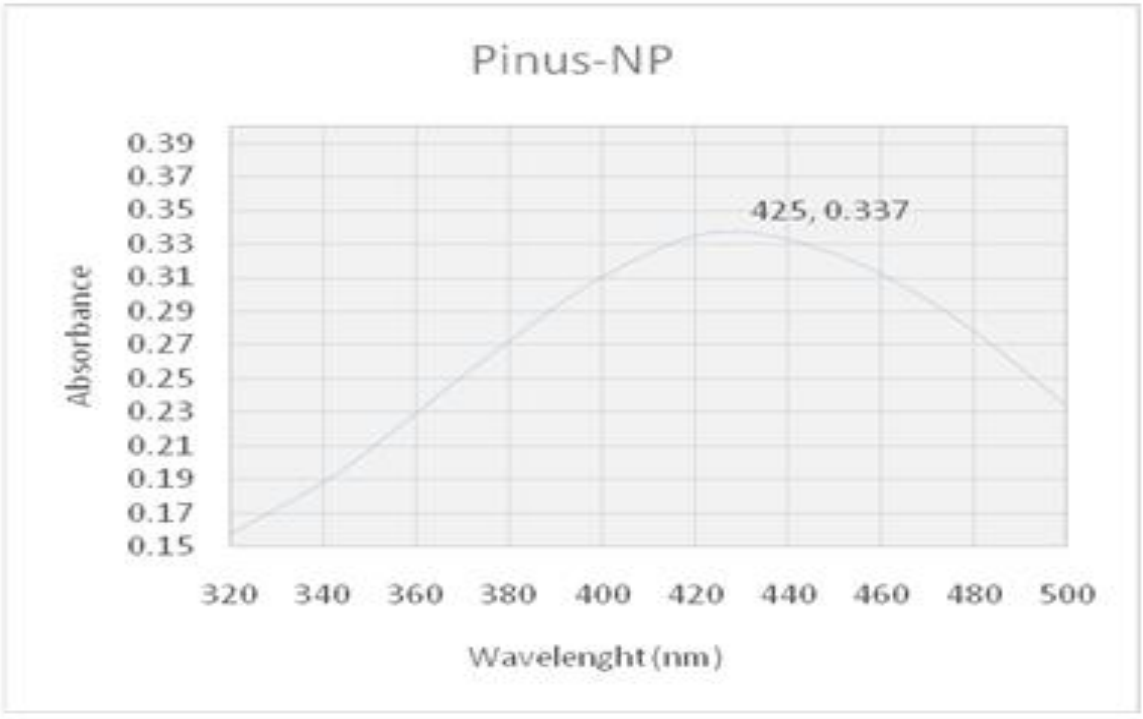


Figure 2. UV-Vis chromatograms of Alkanna-NP and Pinus-NP

Figure 3. Seeds of (a) *Centaurea cyanus*, (b) *Digitalis purpurea*, (c) *Calendula officinalis*, (d) *Lavandula officinalis* and (e) *Melissa officinalis.*

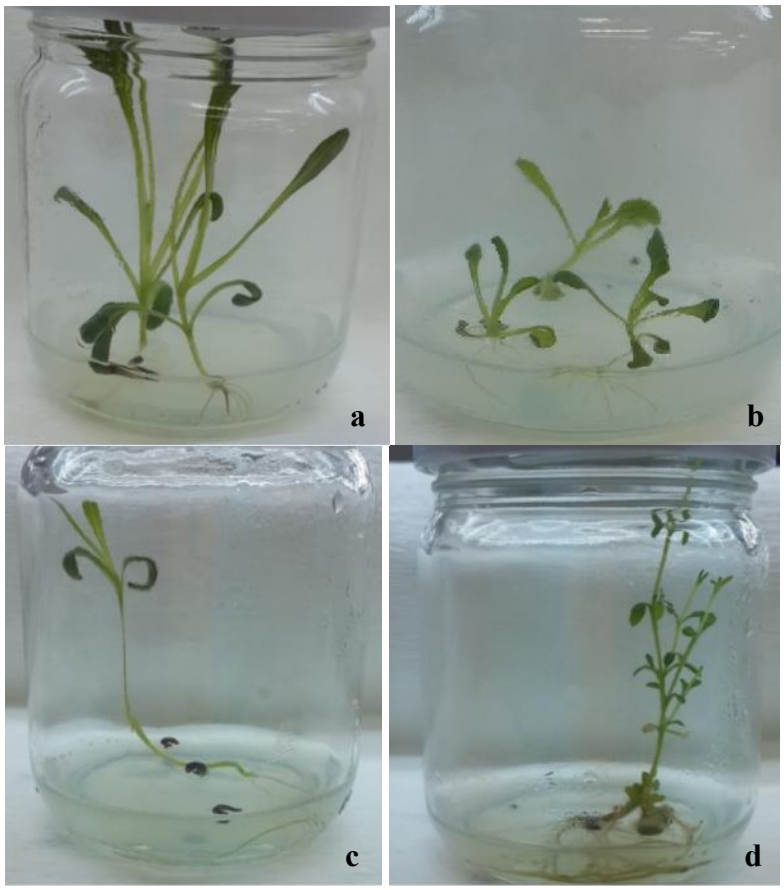


Figure 4. Germinated seeds of (a) *Centaurea cyanus*, (b) *Digitalis purpurea*, (c) *Calendula officinalis* and (d) *Lavandula officinalis*.

Table 1. Germination and sterilization percentages of Alkanna-NP and Pinus-NP surface sterilizations.

Plant Seed	Sterilization Duration	Sterilization Percentages (%)*			Germination percentages (%)**		
		Alkanna-NP	Pinus-NP	Avr. (%)	Alkanna-NP	Pinus-NP	Avr. (%)
Centaurea cyanus	*Control*	*100,00±0,00*	*100,00±0,00*	*100*	*22,22±5,89*	*22,22±5,89*	*22,22*
	1 min	0,00±0,00 e	55,56±8,90 bcd	57,78	15,56±2,22 hij	71,11±5,89 abcd	49,17
	5 min	53,33±10,20 bcd	68,89±17,38 abc		44,44±2,22 degfh	57,78±5,89 cde	
	10 min	55,56±2,22 bcd	64,44±4,45 abc		57,78±8,90 cde	55,56±5,89 de	
	20 min	75,56±12,39 ab	88,89±8,02 ab		44,44±2,22 defgh	46,67±15,41 defg	
	Mean %	46,11	69,44		40,55	57,78	
Digitalis purpurea	*Control*	*100,00±0,00*	*100,00±0,00*	*100*	*68,89±4,45*	*68,89±4,45*	*68,89*
	1 min	100,00±0,00 a	100,00±0,00 a	100	**93,33±3,85 a**	51,11±2,22 def	70,83
	5 min	100,00±0,00 a	100,00±0,00 a		88,89±5,89 ab	60,00±6,67 bcde	
	10 min	100,00±0,00 a	100,00±0,00 a		86,67±3,85 abc	51,11±8,02 def	
	20 min	100,00±0,00 a	100,00±0,00 a		88,89±5,89 ab	46,67±3,85 defg	
	Mean %	100	100		89,44	52,22	
Calendula officinalis	*Control*	*22,22±5,89*	*22,22±5,89*	*22,22*	*24,44±4,45*	*24,44±4,45*	*24,44*
	1 min	17,78±2,22 de	33,33±0,00 cde	51,67	15,56±2,22 hij	6,67±3,85 ij	16,39
	5 min	33,33±10,20 cde	55,56±4,45 bcd		17,78±2,22 ghij	11,11±4,45 ij	
	10 min	71,11±4,45 abc	60,00±13,89 abc		35,56±4,45 efghi	13,33±0,00 ij	
	20 min	66,67±6,67 abc	75,56±18,21 ab		6,67±3,85 ij	24,44±2,22 fghij	
	Mean %	47,22	56,11		18,89	13,89	
Lavandula officinalis	*Control*	*100,00±0,00 a*	*100,00±0,00*	*100*	*0,00±0,00*	*0,00±0,00*	*0*
	1 min	66,67±11,56 abc	100,00±0,00 a	100	20,00±11,56 ghij	13,33±0,00 ij	16,39
	5 min	100,00±0,00 a	100,00±0,00 a		17,78±5,89 ghij	8,89±4,45 ij	
	10 min	100,00±0,00 a	100,00±0,00 a		13,33±3,85 ij	22,22±5,89 fghij	
	20 min	100,00±0,00 a	100,00±0,00 a		11,11±11,10 ij	24,44±8,02 fghij	
	Mean %	91,67	100		15,55	17,22	
Melissa officinalis	*Control*	*100,00±0,00*	*100,00±0,00*	*100*	*0,00±0,00*	*0,00±0,00*	*0*
	1 min	66,67±19,27 abc	100,00±0,00 a	100	0,00±0,00 j	0,00±0,00 j	0
	5 min	100,00±0,00 a	100,00±0,00 a		0,00±0,00 j	0,00±0,00 j	
	10 min	88,89±11,12 ab	100,00±0,00 a		0,00±0,00 j	0,00±0,00 j	
	20 min	100,00±0,00 a	100,00±0,00 a		0,00±0,00 j	0,00±0,00 j	
	Mean %	88,89	100		0	0	

* F = 1,24; MSE = 155,2; p≤0,05
** F = 4,44; MSE = 87,8; p≤0,05

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