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ORIGINAL RESEARCH PAPER

Investigations on Green Preparation of Heavy Metal Saponin Complexes

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ABSTRACT
Green preparation of heavy metal saponin complexes has been successfully optimized by direct combination between crude extract of Olea Europaea and Citrus Aurantium with divalent heavy metals, Pb^{2+} and Cd^{2+}. The main operating factors affecting preparation process were investigated and evaluated in terms of setting time, heavy metal ion concentration, crude extract concentration, and pH value of the medium. Saponin complexes had been prepared using the optimum concentrations of heavy metal ions (120 ppm) and optimum concentration of crude extract (600 ppm) in the slightly alkaline medium. The presence of saponin in plants was confirmed by chemical tests and UV/Vis analysis. Amount of prepared saponin complexes has the order: (Pb/Olive) > (Cd/Olive) > (Pb/Citrus) > (Cd/Citrus). In this process, saponins was isolated and heavy metals were eliminated by a simple, faster and without a huge amount of solvents. The process itself seems to be green isolation of saponins from plants, green removal of heavy metal from aqueous waste streams or green preparation of heavy metal saponin complexes. The process exhibits several advantages and hence benefits, among of them are shorter setting time, higher volume reduction factor and no chemical or solvents used. Direct combination between heavy metals solution and plant extract solution to prepare saponin complex could be considered three in one process. During preparation of the complex, saponin isolated or extracted by heavy metals and the heavy metal eliminated or removed by saponin solution.

Keywords: *Citrus Aurantium, Complex, Saponin, Heavy Metals, Olea Europaea*

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INTRODUCTION

Saponins are naturally occurring glycosides of steroids, alkaloids and triterpenoids. They are found in more than 500 kind of plant kingdom. Different families exhibit strong positive test of saponin such as: Liliaceae, Dioscoreaceae, Solanaceae, Sapindaceae and Agavaceae, so it may be considered as a good source of natural saponin. An aqueous solution of saponin produces foam with shacking but an alcoholic solution inhibits it's foam property. Saponins in pure solid state are an amorphous, and having high molecular weight. They can be usually extracted using two techniques: (1) liquid – liquid extraction/partitioning or (2) column chromatography. In the first technique, n-butanol is a good solvent for saponin extraction from aqueous solutions. In the second technique, silica gel (SiO_2) or alumina (Al_2O_3) are the adsorbent phase and a mixture of chloroform

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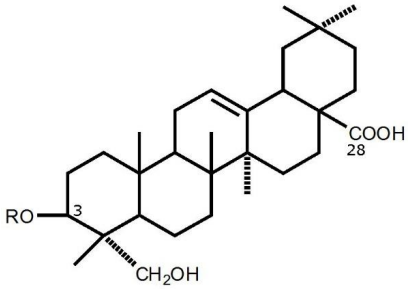
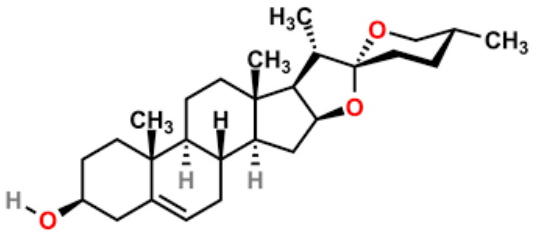
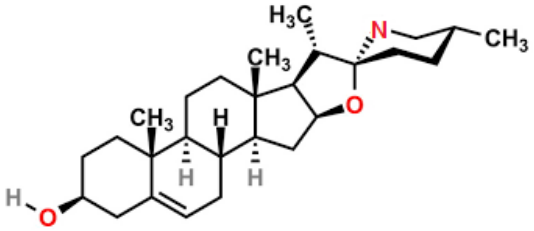


Triterpene glycosides (30C)



Steroid glycosides (27C)



Alkaloid glycosides

Fig. 1. Classifications of sapogenin (27C).

and methanol as mobile phase for separation of saponin. Recently, high performance liquid chromatography (HPLC), gas chromatography (GC), sephadex chromatography, paper chromatography and thin layer chromatography (TLC), can be used for isolation of saponin. Complete chemical structure of saponin were elucidated by: ultraviolet (UV), infrared (IR), proton/carbon nuclear magnetic resonance (^{1}H NMR - ^{13}C NMR), and Mass spectroscopy (MS) [1]. Saponins possess several biological activities such as antioxidant, immunostimulant, antihepatotoxic, antibacterial, anticarcinogenic, antihypoglycemic, and antimolluscicidal [2-4]. Saponin extracts have been used to improve the feed efficiency for cattle and lambs [5].

The aglycone or non-saccharide portion of the saponin molecule is called the sapogenin. Depending on the type of sapogenin present, the saponins can be divided into three major classes: triterpens glycosides, steroid glycosides, and alkaloid glycosides as shown in Fig. 1 [2]. Triterpens glycosides are normally hydroxylated at C-3 and carboxylated at C-28. Saponins have surfactant properties, so that they can be used as important agents for heavy metal removal for aqueous wastes and remediation of contaminated sites. Since saponins have been served as a natural chelating agent to eliminate problems due to several heavy metals such as: Cr, Cd, Cu, Pb, Zn, and As from soil and waste water [6-9].

Extraction of saponin has been performed using water or polar organic solvents like methanol, ethanol and n-buthanol by immersion of the leaves of the plant for 18 to 72 h, even for 15 days, and by elimination of lipids and fats/oils by extraction with non-polar solvents like hexane or petroleum ether [10]. Some authors have hydrolyzed saponin extracts by acidic medium for characterizing them like sapogenins and thus avoid a probably interference of other compounds. Saponin removes heavy metals effectively from soil by desorption, ion exchange or complex formation mechanism. It was also demonstrated that heavy metals were retained due to some form of complexation with carboxyl group in saponin [11]. Stronger bond between the metals and saponin as a complex must be responsible for metal removal from soil and aqueous solutions [12, 13].

Conventional methods of extraction and purification of saponins have some disadvantages. They require mainly longer extraction time, large amounts of solvent, and may have lower efficiency. Compared to traditional methods, isolation of saponins by heavy metals have many advantages, such as shorter extraction time, no solvent required, and higher extraction rate.

The objective of the present work is using the direct combination technique simultaneously: (1) to investigate the green method for the preparation of heavy metal saponin complexes, (2) to isolate saponines from *Olea Europaea* (Olive) and *Citrus Aurantium* and (3) to remove and/or eliminate heavy metal using plant extract. Setting time, the effect of heavy metal concentration, effect of crude extract concentration and pH were studied to optimize the green preparation method.

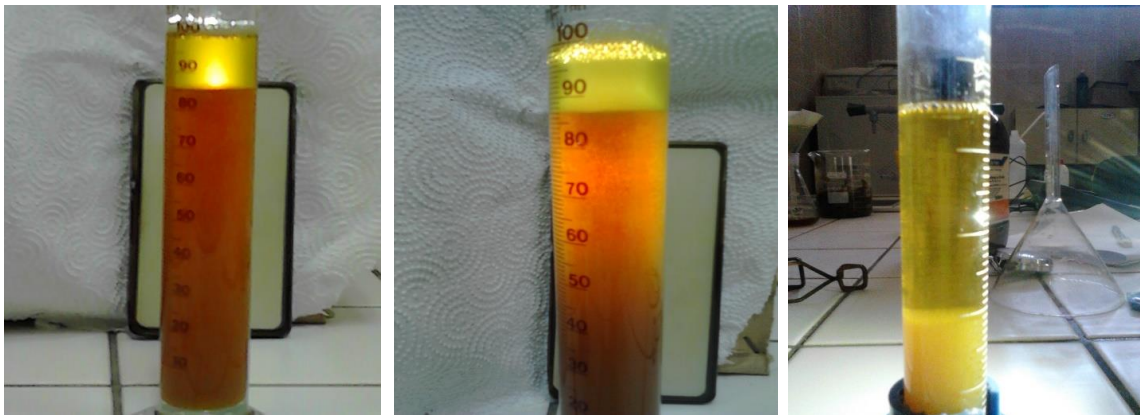


Fig. 2. Settling down of the prepared heavy metal saponin complex with time.

EXPERIMENTAL

Materials, Chemicals and Instruments

Leaves of *Olea Europaea (Oleaceae)*, *Citrus Aurantium (Rutaceae)* and *Ficus Carica (Moraceae)* were collected during spring 2015 from Tripoli region, Libya. The leaves of the three plants were cleaned with tap water followed by deionized water to remove dust and dried in the shade at about 30°C and then cut it off to small pieces before extraction. All reagents used in this study were analytical graded and used without further purification. De-ionized water was used in all experimental investigations. Silver (I) acetate CH_3COOAg, Manganese (II) chloride tetrahydrate $MnCl_2.4H_2O$, and Mercury (II) chromate $HgCrO_4$ are from MIDLEKS EST, GENEVA. Cadmium (II) nitrate tetrahydrate $Cd(NO_3)_2.4H_2O$ and zinc (II) sulfate hyptahydrate $ZnSO_4.7H_2O$ are from LANCASTAR, ENGLAND. Lead (II) acetate $Pb(CH_3COO)_2$ is from SURE CHEM PRODUCTS LTD, ENGLAND. Nickel (II) chloride hexahydrate $NiCl_2.6H_2O$ and cobalt (II) chloride hexahydrate $CoCl_2.6H_2O$ are from BDH. Petroleum ether, n-hexane and absolute ethyl alcohol are from MERCK. UV/visible (UV/Vis.) spectrum of different prepared heavy metal saponin complexes was carried out using Shimadzu UV–Visible spectrophotometer model160A, JAPAN.

Preparation of Crude Extracts and Heavy Metal Saponin Complex

At first, fat and lipids have been removed from plants using petroleum ether (60-80°C) followed by n-hexane. Aqueous and ethanolic crude extracts were individually prepared from about 500 g of each plant by digestion of the plant materials with a desirable amount (~ 600 mL) of the solvent in 2 L glass beakers at 55°C in a thermostatic water bath for 6h. The extract solution, was cooled and stored overnight at ambient temperature, then separated via decantation and evaporated to almost dryness. The crude extract residue was weighed and tested for the presence of saponin [14]. The heavy metal saponin complex was prepared by direct combination between the heavy metal aqueous solution and the crude extract solution of *Olea Europaea* and *Citrus Aurantium* with appropriate concentration in a 100 ml graduated glass cylinder. Some complexes were prepared in a very low acidic medium, and the others were prepared in neutral or slightly alkaline medium. Once the flocks of the complex formed, it starts to settle down in the bottom of the cylinder. The volume and dry weight of the prepared complexes depends on setting time, the type of plant, the type and concentration of heavy metal, and pH value of the solution.

Investigations

Different investigations were carried out during the preparation of heavy metal saponin complexes to optimize the preparation process. In brief, setting time was investigated by measuring the volume (mL) of the precipitate with time (min.) that required for the flocks of the complex to separate and precipitate completely from the solution. The influence of heavy metal concentration (ppm) on the complex formation was studied by using different concentrations of heavy metal and the weight (mg) of dry complex was measured as a function of concentration. Also, the effect of concentration (ppm) of ethanolic extract and pH value on the preparation of heavy metal saponin complex was investigated in terms of the weight and the volume of the prepared complex respectively. Fig. 2 shows

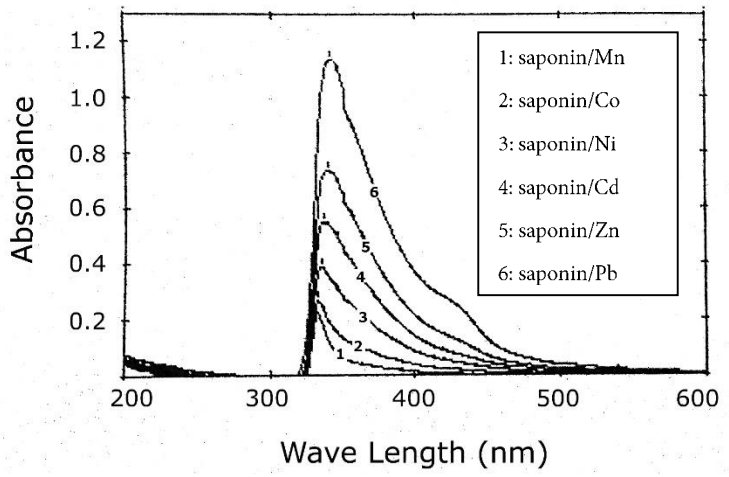


Fig. 3. UV/Vis. spectrum of different heavy metal saponin complexes.

the heavy metal saponin complex during settling down to the bottom of the cylinder.

RESULTS AND DISCUSSIONS

Saponin and Heavy Metal Saponin Complexes

The aqueous and/or ethanolic extracts of three plants, *Olea Europaea*, *Citrus Aurantium* and *Ficus Carica* has been investigated for the test of saponin. According to Frothing test [15], *Olea Europaea* and *Citrus Aurantium* gave a positive response to the stable foam test, forming an emulsion with an ethanolic solution while *Ficus Carica* gave a negative test of saponin. Salkowski test developed a deep red solution with triterpenoid saponins in *Olea Europaea* and *Citrus Aurantium* while Liebermann-Burchard test show a negative test of steroides. Accordingly, *Ficus Carica* was excluded for more investigations. Ultraviolet/visible (UV/Vis.) analysis of different heavy metal saponin complexes prepared by direct combination between the aqueous extract of *Olea Europaea* and divalent metal ions (Pb^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}) were carried out. UV/Vis. Spectrum was shown in Fig. 3, from which we notice that all saponin complexes exhibit major absorption peaks in the narrow wavelength range of ~ 320 to 400 nm with absorbance value in the range of 0.2 to 1.2. This result can be attributed to the fact that the spectra of different saponins are typical, but different from those of other metabolites like flavonoids, which are a kind of phenols frequently extracted along with the saponins during the extraction procedures [16]. Most of saponin itself has a normal absorption peak in the range of ~ 250 to 350 nm, and hence the peaks of the prepared complexes were shifted towards longer wavelength (red shift), and this result may be due to a complex formation between saponin and heavy metals. The above mentioned results prove that saponin can easily separate/isolated by heavy metals through direct combination method. Fortunately, from an environmental point of view, heavy metal can easily eliminate from its aqueous effluents by a natural saponin and, may be led to a more developed treatment method for so many hazardous waste streams. On the other side, from the chemical point of view, a new class of organo-metallic complexes have been prepared successfully by the direct combination/chelation between heavy metal and natural saponin. Also the absorbance of the prepared complexes were in the following sequence:

Saponin/Pb > Saponin/Zn > Saponin/Cd > Saponin/Ni > Saponin/Co > Saponin/Mn

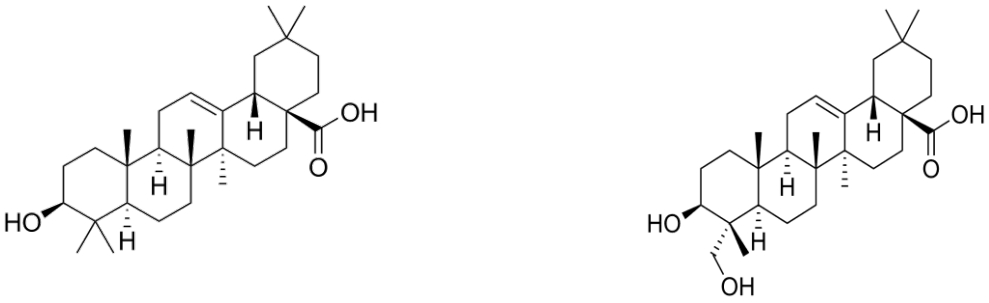
The differences in absorbance may reflect the differences in the degree of coloration of the prepared complexes.

Heavy metal saponin complexes were prepared using the direct combination method between aqueous extract of *Olea Europaea* and *Citrus Aurantium* with different heavy metal ions. Experimental set up and the obtained results were summarized in table 1 which show that the medium of the crude extract solution is weak acidic (pH = 5.16 and 6.35) that may be due to the founding of triterpenoid saponin as organic acid containing carboxyl group (-COOH) at carbon number 28. The above mentioned fact support the presence of triterpenoid saponin such as Oleanolic acid and Hederagenin as shown in Fig. 4 [17]. This

Table 1. Experimental results for preparation of various heavy metal saponin complexes from aqueous extracts.

Plant	Test of saponin	pH of crude extract	Heavy metal ion	pH of complex formation	Final setting Time (min.)	Volume of wet complex (mL)
Olea Europaea (Oleaceae)	++ ve	6.35	Pb^{2+}	10.5	80	9
			Cd^{2+}	10.5	89	8
			Ni^{2+}	10.5	130	23
			Hg^{2+}	6.35-10.5	Nil	Nil
			Ag^{+}	6.35-10.5	Nil	Nil
			Mn^{2+}	6.35	110	7
			Co^{2+}	6.35	112	7
			Zn^{2+}	6.36	120	8
Citrus Aurantium (Rutaceae)	+ ve	5.16	Pb^{2+}	10.5	90	8
			Cd^{2+}	10.5	120	8
			Ni^{2+}	10.5	135	19
			Hg^{2+}	5.16-10.5	Nil	Nil
			Ag^{+}	5.16-10.5	Nil	Nil
			Mn	5.16	115	7
			Co^{2+}	5.16	118	7
			Zn^{2+}	5.16	120	7

Concentration of heavy metal ion = 150 ppm
Total volume of reaction mixture = 50 mL
Temperature ≈ 21±2



Oleanolic acid Hederagenin

Fig. 4. Oleanolic acid and Hederagenin as an aglycone of triterpenoid saponins.

Fig. represents the organic part of the heavy metal saponin complex. Six heavy elements only (Pb, Cd, Mn, Co, Ni, and Zn) succeeded for the preparation of saponin complexes while, Hg and Ag were failed along a wide/broad range of pH values (5.16-10.5) for both plants. Succeeded heavy metals would divide into two categories: the first group which precipitated with saponin in the slightly alkaline medium. This group involves Pb, Cd, and Ni. The second group which is precipitated at pH values of the crude extract of the plants (weak acidic medium), and this group involves Mn, Co, and Zn. The final volume of wet complexes after adequate setting times varied from the minimum volume ≈ 7 mL (for Mn & Co) to the maximum volume ≈ 23 mL (for Ni). The final setting time varied from 80 to 135 min. In general, setting time and the volume of the prepared wet complexes are closely related to each other for both *Olea Europaea* and *Citrus Aurantium*. Nickel has the highest values of the volume and setting time. This result reflects that the density of nickel – saponin complex is different completely from that, any other metal – saponin complexes and it may be explained by precipitation of other organic compounds rather than saponin with nickel. For some unknown circumstances, saponin itself may be not precipitated with nickel. From table 1, lead (Pb) and cadmium (Cd) were chosen for more investigations while silver (Ag), mercury (Hg) and nickel (Ni) were excluded due to its low reactivity towards saponins. Manganese (Mn), cobalt (Co) and zinc (Zn) also excluded due to its low availability and durability in different industrial waste streams.

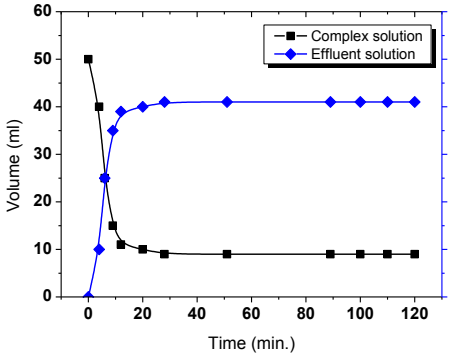


Fig. 5. Setting time of Olive saponin complex with Pb^{2+}.

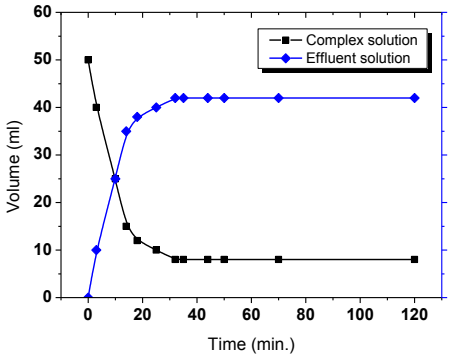


Fig. 6. Setting time of Citrus Aurantium saponin complex with Pb^{2+}.

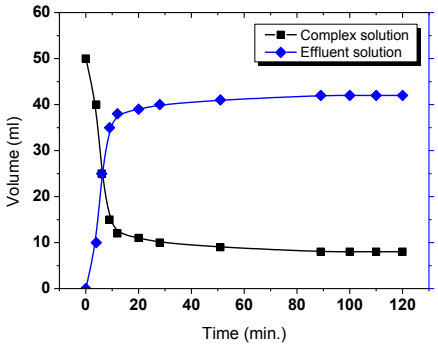


Fig. 7. Setting time of Olive saponin complex with Cd^{2+}.
Volume of reaction mixture = 50 ml
Temperature ≈ 21±2

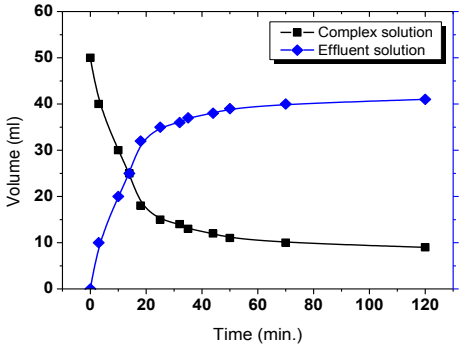


Fig. 8. Setting time of Citrus Aurantium saponin complex with Cd^{2+}.
Metal ion concentration = 100 ppm
pH ≈ 10.5±0.05

Setting Time

The volume of the prepared wet complexes particulates and the volume of supernatant solution were followed along different time intervals and the obtained results was presented in Figs. 5-8 for four types of complexes. As shown in these Figs., the same trends were observed for Pb^{2+}/Cd^{2+} with *Olea Europaea* and *Citrus Aurantium*. Within the first 20 min., the volume of the complex particulates was decreased to the minimal value, while the volume of effluent solution was increased to the maximal value. A steady state and the final setting time were reached after about ~ 30 min. for both Pb^{2+} and Cd^{2+} with the two plants. The final volume of the prepared complexes is equal to 8 mL. In hazardous waste treatment technology, the volume reduction factor (VRF) is defined as [18]:

$$VRF = \frac{Volume\ of\ intial\ waste\ form}{Volume\ of\ the\ final\ waste\ form}$$

Accordingly, VRF for the removal of the heavy metal process was calculated and was found to be equal to 6.25 which is considered a relatively high volume reduction value. The more efficient decontamination process is associated with the higher VRF and vice versa.

Concentrations of Heavy Metal and Crude Extract

Amount of dry complexes (mg) as a function of the concentration (ppm) of Pb^{2+} and Cd^{2+} was investigated for *Olea Europaea (Olive)* and *Citrus Aurantium* as shown in Fig. 9 and 10. From these Figs., it was noticed that the amount of complexes is directly proportion to the concentration of heavy metal along the first stage of the curve. The optimum concentration of heavy metal which give the maximum amount of saponin complexes is 120 ppm for both Pb^{2+} and Cd^{2+}. After the maximum value, any more concentration of heavy metals is accompanied by decreasing in the amount of the prepared complexes. At 120 ppm of heavy metals, maximum and minimum amount of the prepared complexes is equal to 3300 and 2000 mg for Pb/Olive and Cd/Citrus respectively. The obtained amount of the prepared complexes has the following order:

(Pb/Olive) > (Cd/Olive) > (Pb/Citrus) > (Cd/Citrus)

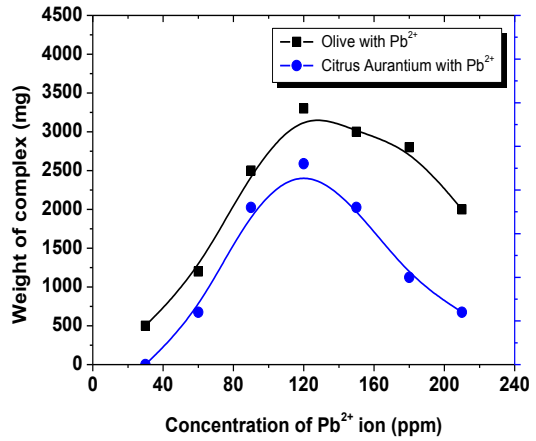


Fig. 9. Effect of Pb^{2+} ion concentration.
Aqueous Crude extract concentrations = 5000 ppm
pH ≈ 10±0.05

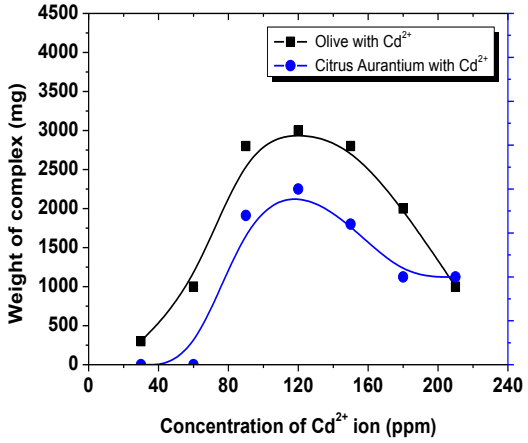
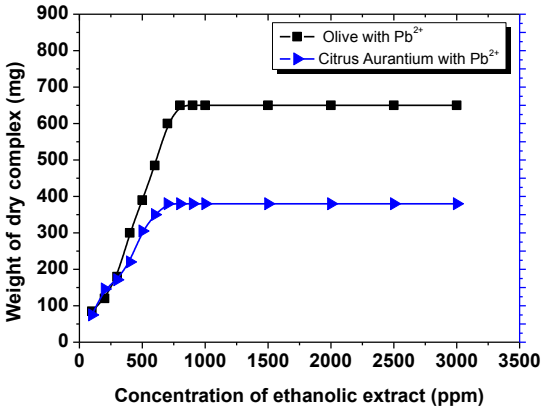
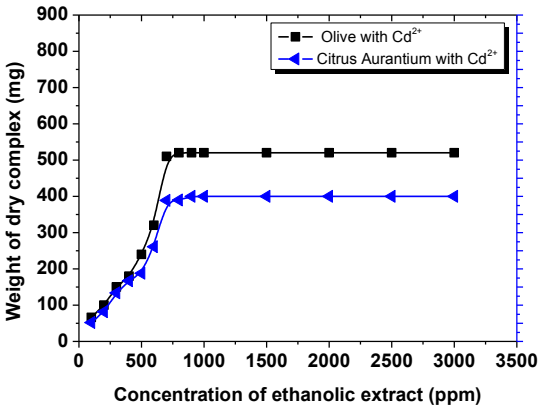


Fig. 10. Effect of Cd^{2+} ion concentration.
Volume of reaction mixture = 100 ml
Temperature ≈ 21±2



Heavy metal concentrations = 120 ppm
pH ≈ 10±0.05



Heavy metal concentrations = 120 ppm
pH ≈ 10±0.05

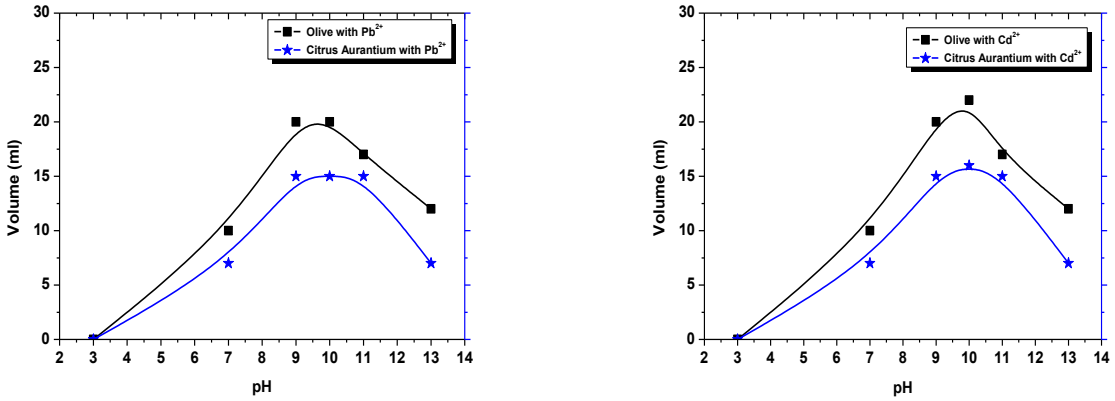
Fig. 11. Effect of crude extract concentration.

The above mentioned result is in agreement with the test of saponin in table 1 and may be attributed to the fact that Olive leaves is more rich in saponin than Citrus leaves [19]. Also, lead and cadmium ions with low concentration (below 120 ppm) can successfully remove from its aqueous solutions by natural saponin via direct precipitation/flocculation method.

A variation of the amount of the prepared complexes with a long range of ethanolic crude extract concentrations using different systems has been investigated as shown in Fig. 11. It was found that for all investigated systems, the amount of the prepared complex was developed as the concentration of crude extract increased till reached to about ~ 600 ppm. After this concentration, the amount of the prepared complexes does not change with the concentration of the extract. The maximum amount of heavy metal saponin complexes could be obtained within the concentration range 600-1000 ppm of ethanolic extract. Again the obtained amount of prepared complexes has the following sequence:

(Pb/Olive) > (Cd/Olive) > (Pb/Citrus) > (Cd/Citrus)

Relatively high concentration of crude extract that required for the elimination of heavy metals may be due to the presence of so many numerous high molecular weight compounds in *Olea Europaea (Olive)* and *Citrus Aurantium* as a natural product rather than saponin such as falvonoids, rotenoids, terpenoids, tannins, chalcons, and alkaloids [20].



Concentration of ethanolic extract = 5000 ppm
Volume of reaction mixture = 100 ml

Concentration of heavy metal = 120 ppm
Temperature ≈ 21±2

Fig. 12. Influence of pH value on the volume of saponin complex.

Influence of pH

Influence of pH value on the volume of saponin complexes was studied with different systems and the obtained results was drawn as in Fig. 12 which show that highly acidic and/or highly basic medium is not the favorite medium for the preparation of heavy metal saponin complexes. These results reflect the over sensitivity of the natural products towards severing conditions. Chemical destruction of the natural products like saponin may be the predominant and probable actions in the presence of hydrogen ions [H^+] or hydroxide anions [OH^-] in very high concentrations. In general, the volume of saponin complexes prepared in strong acidic or alkaline medium is less than that prepared in the slightly alkaline. The maximum yield of the prepared saponin complexes appears between pH ≈ 9-11. The general behavior in Fig. 12 seems not affected by the type of plant or/and the type of heavy metals only, but affected also by the pH value of the medium. The maximum volume is in the range 15-20 mL and was obtained at pH = 10.50±0.05.

CONCLUSIONS

Heavy metal saponin complexes have been prepared successfully by direct combination between heavy metal aqueous solutions and the crude extract aqueous solutions of *Olea Europaea (Olive)* and *Citrus Aurantium* in the slightly alkaline medium. Setting time, heavy metal concentrations, crude extract concentrations, and pH are the main operating factors affecting the preparation process. The presence of saponin in the plants was confirmed by chemical tests and UV/Vis analysis. For all investigated cases, final setting time does not exceed 30 min under the effect of gravity. The optimum concentration of heavy metal and crude extract is equal to 120 and (600-1000) ppm respectively. In general, the amount of heavy metal saponin complexes was in the order:

(Pb/Olive) > (Cd/Olive) > (Pb/Citrus) > (Cd/Citrus).

In this process, (1) saponins were isolated faster than traditional chromatography techniques (2) heavy metals have been removed by saponin (3) a chemical complex have been prepared by direct combination between heavy metal and saponin So, the process may consider to be a simple and green way for the preparation of heavy metal saponin complexes.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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