RESULTS AND DISCUSSION

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Saponins are natural plant materials having broad biological and pharmacological activities (Paris, 1963). Saponins were reported to be potent molluscicides and nematicides against snails and nematodes (Mahran *et al.*, 1974) and (Rohde and Jenkins 1958). There is no evidence to suggest that consumption by humans is harmful. Ahemolytic saponins are highly toxic to mammals when administered intravenously but oral toxicity is very much lower due to failure to cross the gut and enter the blood stream.

The chemical molluscicides and nematicides are highly toxic, persistent and broad spectrum compounds. Therefor the attention was drawn to use safe and biodegradable compounds of plant origin. Plant saponin is one of the most promising agent which used as molluscicides and nematicides. Many factors make it difficult or expensive to obtain natural saponins in commercially reasonable quantities. The long maturation period of the plant is one of these factors. These difficulties leaded to involve tissue culture techniques as well as genetic transformation techniques either to produce saponins on commercial scale or to serve theoretical researches on saponin biosynthetic processes.

The development of both *Azotobacter sp.* and *E.coli* capable of producing saponin in economical value by genetic manipulation is of

great importance. *Azotobacter* with saponin producing ability could be used as biocontrol agent for snails [the intermediate hosts of schistosoma that cause one of the most dangerous disease in Egypt (Schistosomiasis)]. *Azotobacter* are also free living, nitrogen fixing microorgansisms. They are found in soils . The natural occurrence of both nitrogen fixation and saponin producing ability in a single organism is very effective to control plant parasitic nematodes.

E. coli is a promising industrial strain due to its high growth rate in simple medium and its ability to express the eukaryotic gene (s) which means that *E.coli* might has some kind of trans- splicing (Andrey *et al.*, 1992). On the other hand, *F.coli* cannot be used as biocontrol agent in the field for many reasons: (1) There is potential risks associated with the release of genetically engineered *E.coli* into natural environments (Curtiss, 1976; Sharples, 1983, and Rissler, 1984), including the possible transfer of the novel genetic information to indigenous soil microbes. (2) *E. coil* preparations that remain viable under field conditions is not easily applied. So *E.coli* strain capable of producing saponin is used for industrial production of saponins.

Isolation and properties of saponins from *Saponaria officinalis, Asparagus officinalis,* and *Poinciana regia*

Saponins are found in about 70 plant families (Paris, 1963). Methods for qualitative and quantitative estimation of saponin depend on its biological properties such as hemolytic, molluscicidal and nematicidial effects. A colour reaction was also developed to determine saponin types (Stahl 1969).

In this study, three plants belongin to families previousely known to encompass saponin producing plants were chosen. They were *Saponaria officinalis* (family rosaceae), *Asparagus officinalis* (famili liliaceae) and *Poinciana regia* (family leguminosae). Saponin concentration produced by three plants was estimated by hemolysis (Monkiedje et al., 1990). Saponin types produced were determined with a procedure similar to Stahl (1969). Saponin from natural producing plant was biologically assayed by studying their mortalizing action on snails and nematodes.

The information from this experiment will be useful in evaluating the potential for screening the most effecient plant strain to be used as a source of donor DNA. Also a comparative studies between hemolytic activity and biological activities (molluscicidal and nematicidal activities) of plant saponin could be done.

Table (1) gaves three different plants used, their saponin type and saponin conc. in mg saponin mg plant, it also gives the LC_{50} concentrations of plants to both snails and nematodes. Table (1) showed that the different types of saponin produced by the 3 plants: triterpenoid saponin was produced by both *saponaria* and *poinciana* while steroid saponin was produced by *Asparagus*. It was observed that saponin conc. required for nematodes mortality was higher than conc. required for snail mortality. LC_{50} concentrations of the 3 plants used (*Saponaria*, *Asparagus* and *Poinciana*) were (35, 40 and 140) mg/ml respectively to snails (*Biomphalaria alexandrina* and bleeding of snails was noticed and (100, 100 and 280) mg/ml respectively to nematodes (*Meloidagyne incogenita*). This indicate that *saponaria* and *Asparagus* plants have the best potent molluscicidal and nematicidal properties.

Also it was found that saponin concentration (measured by hemolysis) according to Monkiedje (1990) of a queous extract of *Saponaria officinalis* (0.015 mg / mg of plant) is more than saponin concentration of *Asparagus officinalis* (0.012mg/ gm) and *Poinciana regia* (0.054 mg/mg of plant). i.e. *Saponaria* produces much higher saponin than the other two plants. So, *Saponaria* was the best potent plant for saponin production. On the other hand, triterpenoid saponins from *Poinciana regia* and *Saponaria officinalis* are powerful haemolysers than steroid saponin from *Asparagus officinalis*.

Table (1): Detection of saponin in three different plant sources *Saponaria officinalis, Asparagus officinalis* and *Poinciana regia.*

Plant source	**Saponin type**	**Saponin conc. mg/mg plant**	**Snail LC_{50}**		**Nematode LC_{50}**	
			plant*	**Saponin****	**Plant***	**Saponin****
Saponaria officinalis	Triterpenoid	0.0150	35	0.52	100	1.5
Asparagus officinalis	Steroid	0.0120	40	0.48	100	1.2
Poinciana regia	triterpeniod	0.0054	140	0.756	280	1.512

* mg of plant / ml water.

** expected saponin conc. by mg saponin / mgrams of plant used.

According to (Kamel, 1995) triterpenoid saponins being known to be powerful haemolysers than steroid saponin.

It was expected that LC_{50} concentration of saponin determined by hemolysis of aqueous extracts of *Saponaria*, *Asparagus* and *Poinciana* in mg/ml were 0.52, 0.48 and 0.756 respectively to snails and 1.5, 1.2 and 1.512 mg/ml respectively to nematodes. Contrarily of these it was found that LC_{50} concentration of saponin determined by standared curve of white saponin (Merck constructed for mortalizing action) were 0.015 and 80 mg/ml to snails and nematodes respectively. These results could be explained by saponins exert different biological activities which may not be correlated because each activity has its own responsible functional structure, within the molecule the bisdesmosidic saponin for example show some hemolytic activity while no molluscicidal activity was shown (Kamel 1995). Masauki and Tanaka (1990) found that free carboxylic acid of α hederin (saponin from *Hedera rhombea*) was important for its hemolytic activity, whereas the terminal rhamnose is more important for anti fungal activity.

It was noticed by El- Gengaihi *et al.*, (1988) that *Sapnaria* showed LC_{50} above 1000 ug/ml for snails and bleeding of snails was observed. Sati. (1984) investigated *Asparagus Plumosus* and *Curillus glycosides* with molluscicidal activity and found that an oligospirostanoside was the most active of all the spirostanol glycosides tested and in highly diluted solution (1.5 ug/ml) killed the snails (*Biomphalaria glabrata*) in 18 hr. Another root saponin from

Asparagus curillus was lethal at 5 ug/ml concentration in 24 hr. Monkiedje (1990) observed that LC_{50} and LC_{90} conc. of endod saponin (*Phytolacca Dodecondra*) to the snails [*Biomphalaria* glabrata (albino)] were 2.57 and 2.92 mg/L respectively.

Estimation of DNA purity

DNA prepared from plants (*Saponaria, Asparagus* and *Poinciana*) were used as transforming agents of saponin production gene (s). It was necessary to investigate the purity of these preparations by observing the ratio of O.D. at 260 nm (λ max of nucleic acid) to O.D. at 280 nm (λ max of protein). This study is concerned with the quantitative and qualitative evaluation of DNA isolated from different plant sources by different methods. The qualitative study is needed to evaluate the transforming ability of DNA used.

Data represented in table (2) gave the purity of DNA extracted from different plant sources by different methods. It was observed that *saponaria* and *poinciana* DNA gave a ratio between nucleic acid and protein of $\simeq 1.5$ regardless the plant species or the method of isolation. This indicated that the sample had 50% protein and 50% nucleic acid (Maniatis et al., 1982) while with *Asparagus* DNA the ratio was largely lower than 1.5 indicating that the sample was highly rich in protein. Doyle and Doyle (1987) suggested that it must be made a further purification extraction in treating sample rich in protein and starch, but the low absorption of nucleic acid gave an early indication

Table (2): Estimation of DNA purity.

Sources of DNA	Method of extraction	O.D (260nm)	O.D (280nm)	O.D260* / O.D 280
Saponaria officinalis roots	Bendich & Bolton (1967)	1.800	1.100	1.64
Saponaria officinalis roots	Doyle & Doyle (1987)	1.81	1.15	1.57
Poinciana regia seeds	Bonner (1965)	2.68	1.64	1.63
Poinciana regia seeds	Bendich & Bolton (1967)	1.83	1.2	1.50
Asparagus officinalis roots	Doyle &Doyle (1987)	0.200	0.400	0.50

* 50% protein /50% DNA mixtures have O.D. 260/O.D280 ratios of 1.5

of the low content, and low transforming ability of *Asporagus* DNA.Spizizen (1958) found that protein removal increased the transforming ability 100-1000 fold, suggesting that protein removal may produce smaller molecular species readinly able to penetrate the recipient cells. On the other hand, he also suggested that a highly purification of DNA from protein would presumably make the DNA more susceptible to recipient DNase.

In this study, we didn't exclude the RNA fraction. Spizizen (1958) found that the presence of RNA component appeared to have potentiating activity and its complete removal lowered the biolgical activity of DNA about ten fold. The partialy restored this loss in activity by the addition of RNA fraction.

Transfer of gene (s) required for saponin production from *Saponaria officinalis, Asparagus officinalis* and *Poinciana regia to Azotobacter Sp.*

It was decided to determin whether gene (s) required for saponin production from saponin - producing plants could be transferred to *Azotobacter sp.*; *A. vinelandii* and *A. chroococcum.* The expression of saponin producing ability was screened using hemolysis technique (i.e. the transformants exhibited hemolysis zones on blood agar plates).

Azotabacter has some intrinsic biological properties that it is found in fresh water like; ponds, lakes and marsh water (Becking 1981) in which snails are found . The development of *Azotobacter* organisms by genetic manipulation that is capable of both living in fresh water and producing saponin is very effective to control snails which are the intermediate hosts of schistosoma (Which cause schistosomiasis).

Azotobacter are free living , nitrogen fixing micro organism. *Azotobacter* with nematicidal activity can control the root- knot nematode *Meloidogyne incognita*. The development of such strains by genetic manipulation are effective in increasing crop yields together with their benefit in fixing nitrogen.

The inhibitory effect of saponin on *Azotobacter* growth

Azotobacter cells were tested for their abilities to grow on *Azotobacter* growth medium (AG) contains 30 µg/ml saponin. This experiment had been done to determine the inhibitory effects of saponin on *Azotobacter* growth which aid in studying, characterizing and analysis of other genes involved in saponin production ability. It was observed that *Azotobacter vinelandii* parent strain did not grow on AG medium contains 30µg/ml saponin while *Azotobacter chroococcum* grew well . On the other hand when *A. vinelandii* transfomed with

DNA of saponin producing plants and retested for growth in the presence of saponin, it was found that all *Azotobacter* transformants were able to grow in the presence of saponin (30 μg/ml).

This finding may be explained by the presence of gene (s) for saponin resistance element (s) in *A. chrooococcum*. So it grew normally in the presence of saponin, but *A. vinelandii* showed no gorwth in the presence of saponin due to the absence of saponin resistance element (s). Elements of saponin resistance were previously recognized in a plant pathogenic fungi by Bowyer *et al.*, (1995) as saponin detoxifying enzyme. Heftmann (1965) found several species of fungi produce saponases (saponin hydrolyzing enzymes) adaptively in presence of saponin. Leaves of Agave and Yucca species which contain saponin also contain saponases (Heftmann, 1965). All these observations make reasonable to suggest that plant DNA preparation would have saponin- resistance element(s), and *A. vinelandii* aquire these elements from plant DNA by transformation.

Azotobacter vinlandii transformation

(Saponin - resistant transformants)

When *Azotobacter vinelandii* was subjected to DNA preparations from saponin producing plants (listed in table 2), it exhibited no response to transfer ability of saponin production. This

was detected by the absence of hemolytic zones on blood agar plates. The inablility to produce *Azotobacter vinelamdii* transformants capable of producing saponin indicated that saponin producing ability may require many different gene (s) such as (saponin- resistant gene(s) which are difficult ot be introduced into *Azotobacter vinelandii* at the same time with saponin production gene (s). It also might be due to the presence of saponin- resistant gene (s) far apart from saponin production gene (s) in plant DNA preparations. it was found that *Azotobcater vinelandii* acquire saponin- resistant element (s) with higher frequency. Transformants were selected for resistantce, using 30μg/ml AG medium supplemented with saponin. This resistant element(s) may be found as (saponases) saponin hydrolyzing enzymes, which transferred from plants to *A. vinelandii*. The final conclusion from these results was that it may be necessary to devise a protocol to insure that saponin producing gene (s) will be inserted into *Azotobacter vinelandii* simultineously with saponin hydrolyzing gene (s).

Azotobacter chroococcum transformation:

In this experiment a protocal that yield a high frequency of transformation with various donor DNA (listed in table 2) was developed. The procedure reported here was a modification of Glick *et al.*, (1985). Transformants were screened for saponin production ability on blood agar plates. The transformant exhibit hemolysis zone, as

shown in Fig. (2,3) were selected and isolated for further studies. Transformation frequency was calculated as the number of transformants per the total number of viable cells/ μg DNA. Donor DNA wer extracted from *Saponaria*, *Asparagus* and *Poinciana* by different methods listed in table (2) to evaluate the transforming ability of donor DNA isolated by different methods. *A. chroococcum* was used as recipient. Table (3) gave the DNA sources, the method of DNA extraction, the number of transformants with saponin producing ability and transformation frequencies. It was found that transformation frquency varied according to the transforming DNA type. The highest frequency was obtained with *Saponaria* DNA as shown in table (3), Fig.(4) indicating that this plant was the best donor for saponin production gene (s).

Poinciana DNA had lower transforming activity than *Saponaria* DNA. No transformants were obtained with Poinciana DNA isolated by the method of Bendich and Bolton (1967). This result was logically expected from table (1) which showed that *Poinciana* had the lowest saponin producing ability or it may be due to their low efficient of saponin producing system.

Although *Asparagus* was rich in saponin as shown in table (1) its DNA was inactive as transfoming agent. This may be due to, the high content of protein reduced the transforming ability of *Asparagus*