Biotechnological and phytochemical studies on *Sabal yapa* Becc. growing in Egypt

Nagwa M. Ammar [(1)], Hussein S. Taha [(2)], Mohammed S. Hefnawy [(3)] and Ahmed H. Afifi [(1)]
1-Pharmacognosy Department, National Research Center, El Tahreer Street, Dokki, Cairo, Egypt.
2-Plant Biotechnology Department, National Research Center, El Tahreer Street, Dokki, Cairo, Egypt.
3- Pharmacognosy Department, Faculty of Pharmacy, Cairo University.
hussein.taha2@yahoo.com

Abstract: This study aim to production of the biologically active lipoidal fraction from *Sabal yapa* Becc., Family Palmae, using tissue culture techniques, which previously proved its remarkable and significant antiandrogenic activity. Immature embryos of *S .yapa* unripe fruits were cultured on fortified MS media with different type of growth regulators, and incubated under light or dark conditions. Calli growth and lipoidal content were evaluated. MS medium augmented with 10 mg/l 2,4-D and 3mg/l of 2iP and BAp showed the best modified medium for calli production under light or dark conditions. The highest mass of calli production was recorded after the 5th week of cultivation. However, the significant growth rate (mg/day) was recorded during the 3rd week of cultivation. Moreover, fractionation of lipoidal calli, was analyzed by GLC and compared with that of the *in vivo* fruit. The obtained results revealed that the lipoidal content was 0.85% in *in vivo* fruit while it recorded 0.64 and 0.68 (%) with calli resulted under light or dark conditions, respectively. The maximum percentage of unsaponifiable fraction (59.6 %) was recorded in fruit, however, it recorded 20.83 and 17.53 (%) with calli produced under light or dark conditions, respectively. Although, the highest percentage of the total fatty acids fraction 70.8 and 74.1 (%) were recorded with calli governed under light or dark conditions, respectively, it was 32.3 (%) in *in vivo* fruit. Further, we recommend the exploitation of plant tissue culture techniques for the preparation and production of the biologically active non-polar fraction of *S. yapa* fruit at semi-industrial scale using bioreactors.
[Nagwa M. Ammar, Hussein S. Taha, Mohammed S. Hefnawy and Ahmed H. Afifi. **Biotechnological and phytochemical studies on *Sabal yapa* Becc. growing in Egypt**. Journal of American Science 2011;7(2):14-20]. (ISSN: 1545-1003). http://www.americanscience.org.

Keywords: Sabal yapa, Palmae , non-polar extract, callus, lipoidal fraction.

1. Introduction

The difficulties of obtaining the source plants led to an increasing interest in developing alternative for the production of plant secondary metabolites. There has been a considerable interest in plant cell cultures as a potential alternative to traditional agriculture for the industrial production of secondary products (Dicosmo and Misawa, 1995). Moreover, plant tissue cultures have long been regarded as a source of commercially important steroids, alkaloids, and terpenes for pharmaceutical industry (Taha 1999; Zaho and Verpoorte 2007; Zarate and Verpoorte 2007).

The genus sabal was reported to be rich in saturated and unsaturated fatty acids, mainly lauric, myristic, palmitic; oleic acids and contain fatty alcohols and sterols (Cristoni *et al.* 1997). The lipidosterolic extract from some species of sabal was reported to possess as anti-androgenic activity through the inhibition of 5α-reductase isoenzymes (Iehle *et al*. 1995; Weisser *et al*. 1997; Bayne *et al*. 2000). Furthermore this extract proved to be effective in treatment of problems of micturation caused by Benign Prostate Hyperplasia (BPH), which is a condition experienced by a majority of aging men (Bach and Ebeling 1996; Gerber *et al.* 1998; Boyle *et al.* 2000).

2. Materials and Methods

Plant material:

Unripe fruits of *S. yapa* were secured from the available tree in the Orman garden, Giza, Egypt.

1- Establishment of calli cultures from immature embryos of *S. yapa*:

The immature embryos were excised from sterilized unripe fruits and aseptically cultured on 150 ml glass jars containing 25 ml modified MS medium (Murashige and Skoog, 1962).
The following MS media were used as follows:

- MS_1) MS-basal medium free growth regulators
- MS_2) MS + 10 mg/l 2,4-D
- MS_3) MS + 10 mg/l 2,4-D +3 mg/l 2iP
- MS_4) MS + 10 mg/l 2,4-D +3 mg/l BAp
- MS_5) MS + 10 mg/l 2,4-D +3 mg/l 2iP+3mg/l BAp

Activated charcoal was added to all culture media at 3gm/l as antioxidant substance. One immature embryo was cultured /jar, and cultures of all treatments were divided into two groups, the first group was maintained under light condition (16h/day

photoperiod at intensity of 3000 lux from white cool light of fluorescent lamps) while the second group was maintained under dark condition, these jars were incubated in growth chamber at 26±1°C for 4 weeks.

The following data were recorded after four weeks of cultivation:

1- Percentage of survival.
2- Percentage of calli formation.
3- Fresh weight (mg/embryo).
4- Dry weight (mg/embryo).
5- Dry matter content (%).

2- Mass calli production:

An equal weight (250 mg/jar) of calli cultures derived from immature embryos were re-cultured onto the same MS medium contained the optimum concentration of growth regulators (MS_5). The comparative study was done under light or dark conditions and incubated at 26 ± 1° C. The following parameters were recorded during five weeks of cultivation:

1- Fresh weight (mg/jar).
2- Dry weight (mg/jar).
3- Dry matter content (%).
4- Determination of growth dynamics for embryo calli cultures during five weeks of cultivation under light or dark conditions:

The following parameters of growth dynamics were recorded during the five weeks of cultivation:

Growth value (G_V) of immature embryo calli cultures was calculated during the experiment time according to Szoke *et al*.(1979).

Growth rate (Gr) of calli cultures (mg/day) was determined weekly as described by Dung *et al.* (1981).

All comparative studies were applied under light or dark conditions.

The obtained results were statistically analyzed according to Snedcor and Cochran (1972).

3- Chemical analysis:

The obtained calli under light and dark conditions were collected individually, lyophilized, pulverized and extracted with petroleum ether, the obtained extract was subjected to saponification as previously described, and both the unsaponifiable matter and the fatty acid methyl esters were analyzed using GLC.

3. Results

1- Calli initiation and mass production:

There no callus and survival embryos were formed on free growth regulators of MS medium (MS_1). However fortified MS medium with 2,4-D in concentration of 10 mg/l showed highly significant embryos survival and calli formation under light (13.2, 9.4 %) and dark (20.8, 12.9 %) conditions, respectively. These previous percentages were enhanced by the addition of either 2iP or BAp in concentration of 3 mg/l which gave 31.2, 21.6 (%) or 31.4, 25.2 (%) under light condition, respectively and 43.2, 31.9(%) or 50.6, 46.3 (%) under dark condition, respectively. Moreover, augmented of MS medium with a combination of 10 mg/l 2,4 D and 3mg/l each of 2iP and Bap gave highest value of embryos survival which gave 51.8 % under light and 64.8 % under dark conditions. In addition, the percentage of calli formation 42.4 and 71.5 was recorded under light and under dark condition, respectively. Furthermore, the previous combination of growth regulators showed the best results of mass calli production in terms of calli fresh and dry weights (mg/embryo) and the percentage of calli dry matter content recorded 79.8, 0.65, 0.809 (%) and 91.7, 0.82, 0.895 (%) in case of light and dark incubation condition, respectively. In a view glance for the previous data, it can be concluded that, culturing of immature embryos of *Sabal yapa* on MS medium fortified with a 10 mg/l 2,4 D and 3 mg/l each of 2iP and BAp and the incubation under dark condition gave the best results of calli production as compared with incubation under light condition, at 26±1°C (Table 1).

2- Callus growth dynamics:

Calli derived from immature embryos were gradually increased to the 5th week of cultivation. The highest significant and promising growth values of immature embryos calli production 0.14 and 7.21 were recorded after the 5th week of cultivation under dark and light condition, respectively (Fig. 3). However, the highest value of growth rate was recorded during the 3rd week of cultivation. They were 102 and 177.7 (mg/day) under light and dark conditions, respectively (Fig. 4).

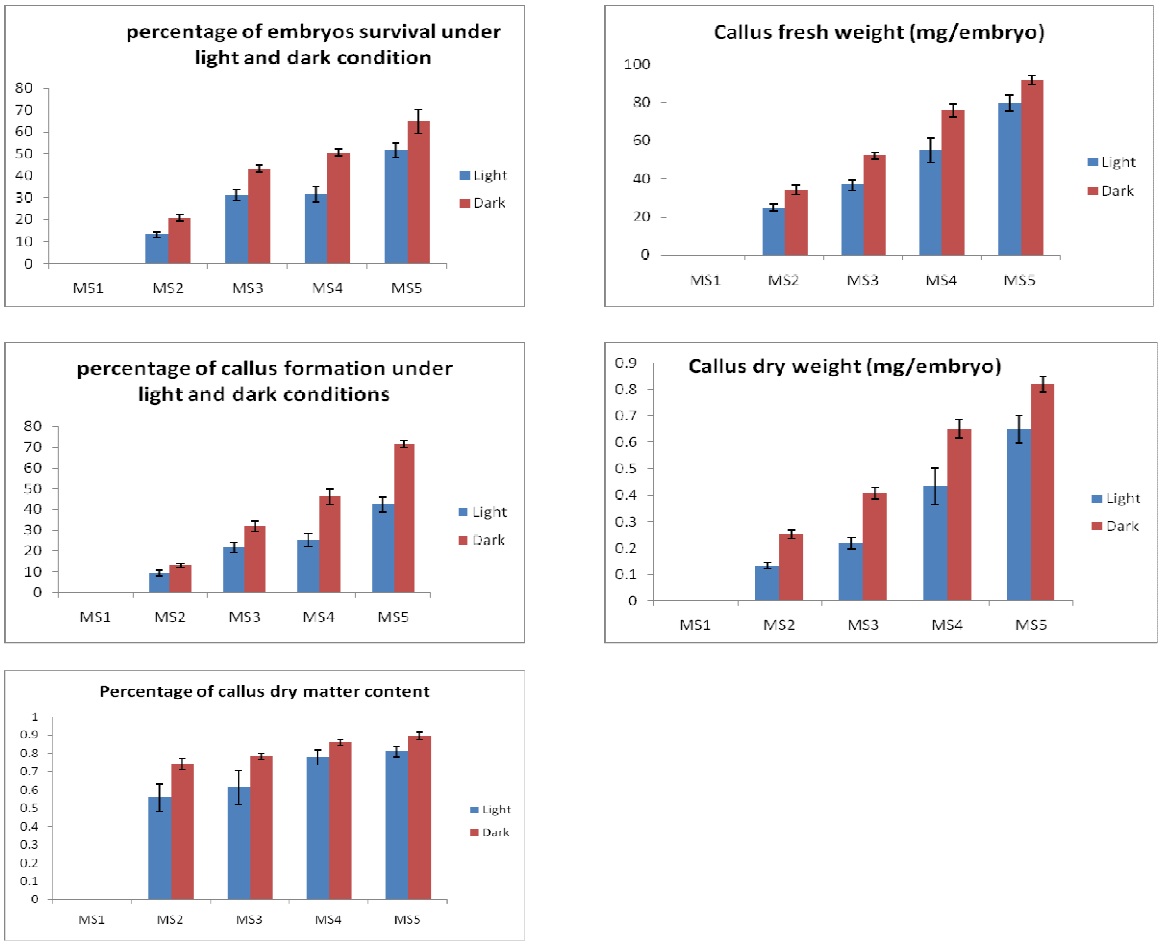

Fig (1). Effect of fortified MS-medium with different growth regulators and incubation under light and dark incubation conditions on establishment of calli culture from immature embryos of *Sabal yapa*

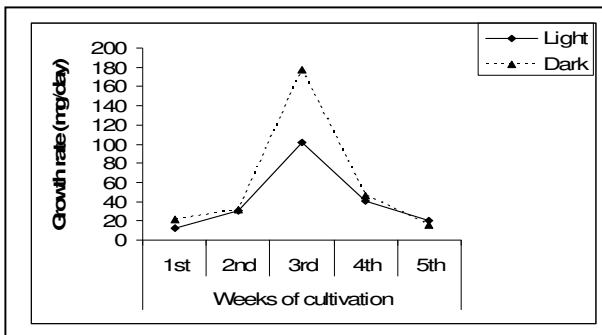

Fig (3). Growth rate G_r (mg/day) of calli cultures derived from immature embryos of *S. yapa* cultured on MS-medium supplemented with 10mg/l 2,4-D, 3mg/l 2iP and 3mg/l BAp for five weeks and incubated under light

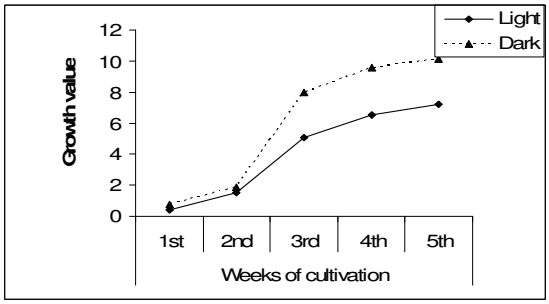

Fig (4). Growth values (G_V) of calli cultures derived from immature embryos of *S. yapa* cultured on MS-medium supplemented with 10mg/l 2,4 D, 3mg/l 2iP and 3mg/l BAp for five weeks and incubated under light or dark conditions.

3- Investigation of calli lipoidal content :

Data presentes in Table (2) show the quantitative and qualitative determinations of lipoidal mater of *S.yapa* derived from either *in vivo* whole fruits or *in vitro* calli. Data revealed that the yield of the lipoidal matter of *in vivo* whole fruits (0.85%) was slightly higher than that of calli produced under either light or dark conditions (0.64 and 0.68 %), respectively. Moreover, the percentage of unsaponifiable fraction was higher in *in vivo* whole fruits (59.6%) than in the calli produced under light or dark conditions (20.83 and 17.53%), respectively. While, the percentage of fatty acids fraction was much higher in calli produced under light or dark conditions (70.8 and 74.1%), respectively than in *in vivo* whole fruits (32.3%).

In addition, as shown in Table (3) the percentage of identified hydrocarbons in USM of *in vivo* whole fruits of *S.yapa* 94.98 (%) it was 81.6 and 95.4 (%) in calli derived under light or dark conditions, respectively. Sitosterol, campesterol and stigmasterol were identified; however, heneicosane (C21) was the main identified hydrocarbon in the USM of *in vivo* whole fruits. In contrast, the sterols cholesterol, β-cholesterol was absent in the USM of the calli derived under dark condition while stigmasterol was the only sterol which identified in the USM of the calli derived under light condition.

The percentage of the identified unsaturated fatty acids in *in vivo* whole fruits was 36.87, and it was 22.6 and 39.8 (%) in calli incubated under light or dark conditions, respectively. Oleic acid represented the main unsaturated fatty acid in *in vivo* whole fruits (33.8%), and in calli derived from light or dark conditions (13.27 and 48.19%), respectively. While, the percentage of the identified saturated fatty acids in *in vivo* whole fruits recorded 58.54 %; however, it was 41.47 and 27.5 (%) in calli incubated under light or dark conditions, respectively. Lauric acid represented the main saturated fatty acid in the whole fruits (31.1%) while, arachidic acid was the main saturated fatty acid in calli incubated under light condition. However, palmitic acid was the main saturated fatty acid in calli incubated under dark condition.

Table (2). GLC analysis of FAME of *S.yapa in vivo* whole fruits and *in vitro* derived calli under light or dark conditions.

Peak number	Retention time	Area percentage (%)			Comparable with
		In-vivo (Whole fruits)	In vitro (Calli)		
			Dark	Light	
1	7.007	0.25	-	-	Caprylic acid
2	9.137	0.603	-	-	Capric acid
3	11.878	29.2	2.272	6.975	Lauric acid
4	15.293	15.01	3.55	6.975	Myristic acid
5	15.986	-	0.1562	7.998	Myrisoleic acid
6	19.110	9.19	24.495	5.0375	Palmitic acid
7	20.04	0.32	1.1502	7.2695	Palmitoleic acid
8	21.207	0.07	0.2982	6.5875	Stearic acid
9	23.284	33.8	48.1948	13.268	Oleic acid
10	24.090	7.05	7.2136	7.812	Linoleic acid
11	24.470	-	0.2982	6.3085	Linolenic acid
12	26.324	0.67	3.0814	15.562	Arachidic acid
13	30.06	0.53	1.9596	2.5265	
14	34.06	0.27	0.3124	4.3245	Lignoceric acid

Table (3). GLC analysis of USM of *S.yapa in vivo* whole fruits and *in vitro* derived calli under light or dark conditions.

Peak number	Retention time	Area percentage (%)			Comparable with
		In vivo (whole fruit)	In vitro (calli)		
			Light	Dark	
1	2.335	0.21	-	-	-
2	4.434	0.26	-	0.163809	n-decane
3	5.704	0.17	-	0.083105	-
4	7.206	0.45	-	0.055495	n-dodecane
6	9.116	3.5	0.751	0.073955	tetradecane
7	11.197	20.7	0.168	0.251886	Hexadecane
8	13.380	12.3	1.376	2.493242	Heptadecane
9	14.409	2.5	1.433	0.129232	Octadecane
10	15.567	8.1	32.7	19.35281	Nonadecane
11	16.517	9.9	1.502	1.782878	Eicosane
12	17.880	31.9	32.76	59.66856	Heneicosane
13	18.001	-	6.468	-	-
14	18.310	-	7.765	-	-
16	19.216	1.8	1.436	1.631449	Tetracosane
17	20.960	0.6	2.352	1.438461	Pentacosane
18	21.175	-	2.917	1.305722	-
19	21.293	0.36	-	-	-
20	22.071	1.1	4.451	2.813	-Hexacosane
21	24.410	0.7	1.608	0.686839	Heptacosane
22	25.844	0.37	0.219	1.006208	Octacosane
23	27.913	0.8	0.859	2.58838	Nonacosane
27	29.416	0.18	-	-	Cholesterol
28	30.161	0.29	-	0.755591	Campesterol
29	31.031	0.31	0.753	1.483642	stigmasterol
30	33.380	0.27	-	0.998	ß-sitosterol

4. Discussion:

Callus is basically a more or less non organized tumor tissue which usually arises on wounds of differentiated tissues and organs. The initiation of calli formation is referred to as calli induction. Monocotyledons are less likely to form callus tissue than dicotyledons and auxins are often needed as a hormonal stimulant for callus induction (Tisserat 1979; Ibrahim 1999). An exogenous supply of regulators is often recommended to initiate callus formation on an explants, the exogenous regulator requirements regarding the type of regulator, its concentration and auxin/cytokinin ratio depends strongly on the type and endogenous hormones content (Pierik 1987).

The obtained results showed that, supplementation of MS-medium with different combinations of growth regulators had a promising effect on callus production from immature embryos of *S. yapa* and the best results were achieved when MS medium was augmented with 10 mg/l 2,4-D + 3 mg/l 2iP + 3 mg/l Bap, so it can be concluded that supplementation of culture medium with auxins and cytokines achieved of mass callus production.

The obtained results seem to be in accordance with the published data by Shatnawi *et al*.,(1997) how reported that MS medium supplemented with 0.1 mg/l NAA and 5 mg/l 2iP showed the best results of calli production from auxiliary buds of *Phoenix dactylifera* and it was superior of MS medium supplemented with 2,4-D alone at different concentrations. In addition, Sharma *et al.,*(1984) reported that best results of callus formation from shoot tips of date palm were obtained when MS medium supplemented with 100 mg/l 2,4-D and 5mg/l BA was used. Further, Al-Ghamdi (1993) mentioned that good calli initiation and growth was obtained on MS medium supplemented

with 100 mg/l 2,4-D and 3 mg/l 2iP. However, Saker *et al*., (1998) initiated and proliferated calli from immature embryos of *Phoenix dactylifera* on MS medium supplemented with 10 mg/l 2,4-D. In addition , Zaid (1987) found that MS medium fortified with 100 mg/l 2,4-D and 3 mg/l 2iP promoted calli initiation from embryos of date palm during 4-8 weeks.

Regarding the effect of culture conditions on callus formation, the incubation under dark condition showed the best results compared the incubation under light condition. In contrast, the growth value and growth rates were higher than in case of incubation under light conditions. These results are in harmony with data have been published by Tisserat (1981), Zaid (1987), Bawa (1993) and Veramendi and Navarro (1996).

Analysis of the non-polar extract of *in vitro* obtained calli revealed that the yield of the lipoidal matter of calli incubated under light and dark conditions was 0.64 and 0.69 (%), respectively, which was significant compared to that of whole fruits (0.85%). While, the total percentage of the fatty acids in the lipoidal matter of calli incubated under light (70.8 %) or dark (74.4 %) conditions, it was 32.2 (%) in whole fruits. Moreover, oleic acid (which may be responsible for the antiandrogenic activity) was present in the lipoidal matter of the obtained calli in a reasonable concentration. It present in higher concentration in the fatty acid mixture of calli incubated under dark condition compared with incubated calli under light condition. Further, it can be conclude that, the exploitation of tissue culture techniques for the preparation of the biologically active non-polar extract of *S. yapa* fruits proved to be a successful alternative for the extraction of the whole fruit with the advantages of overcoming the difficulties of obtaining the source plants.

References

1. Al-Ghamdi, A.S. (1993). True-to-type date palm (*Phoenix dactylifera* L.) produce through tissue culture tecnique. The Third Symposium on Date Palm in Saudi Arabia, 1993.pp. 57
2. Bach, D. and Ebeling, L. (1996). Long term drug treatment of BPH. Phytomedicine 3(2), 105- 111
3. Bawa ,M.O. (1993). The first tissue culture work on date palm. The Third Symposium on Date Palm in Saudi Arabia, 1993, 68 pp
4. Boyle, P., Robertson, C., Lowe, F. and Roehrborn, C. (2000). Meta-analysis of clinical trials of Permixon in the treatment symptomatic benign prostatic hyperplasia. Urology, 55, 533-539
5. Dicosmo, F. and Misawa, M. (1995). Plant cell and tissue culture alternative for metabolite production. Biotechnology Advances, 13 (3), 425-453
6. Dung, N-N., Szoke, E. and Verzar-Petri, G. (1981). The growth dynamics of callus tissues of root and leaf origin in *Datura inoxia*. Acta. Bot., Acad. Sci. Hung 27, 325-333
7. Gerber, G.S., Zagaja, G.P., Bales, G.T., Chodak, G.W and Contreras, B.A. (1998). Saw palmetto *Serenoa repens* in men with lower urinary tract symptoms: effect on urodynamic parameters and voiding symptoms. Urology 51, 1003- 1007
8. Ibrahim, A.I. (1999). Somaclonal variation during micro- propagation of date palm *via* embryogenesis. The First International Conference on Plant Tissue Culture and Its Applications, Cairo, Egypt, p 189
9. Pierik, R.L.M. (1987). In vitro Culture of Higher Plants, Martinus Nizhoff Publishes, Boston
10. Saker, M.M., Moursy, H.A. and Bekheet, S.A. (1998). In vitro propagation of Egyptian date palm: morphogenic responses of immature embryos. Bulletin of Faculty of Agriculture, University of Cairo 49(2), 203-214
11. Sharma, D.R., Dawrara, S. and Chowdruy, J.B. (1984). Somatic Embryogenesis and plant regeneration in date palm *Phoenix dactylifera* L. cv. khadrawi through tissue culture. Indian Journal of Experimental Biology 22, 596-598
12. Shatnawi, A., Hussain, A. and Quraishi, A.(1997). In vitro response of date palm cv. dhaki to plant growth regulators. Sarhad J. Agriculture 13(1), 45-53
13. Snedecor, G.W. and Cochran, W.G. (1972). Statistical Methods (7th Edn), Iowa State Univ. Press, 504pp
14. Szoke, E., Kuzovkina, I.N., Verzar-Petri, G. and Smirnov, M.A. (1979). The effect of the growth regulators on biomass formation in callus cultures of *Matricaria chamomilla* L. Herba Hungarica 18, 41-57
15. Taha, H.S. (1999). In vitro studies on *Hypericum perforatum* L. Ph.D. Thesis, Faculty of Agriculture, Cairo University.
16. Tisserat, B. (1979). Propagation of date palm *Phoenix dactylifera* L. *in vitro*. J. Exp. Bot 30, 1275-128
17. Tisserat, B .(1981). Production of free living palms through tissue culture. Date Palm J. 1, 43-54
18. Veramendi, J. and Navarro, L. (1996). Influence of physical conditions of nutrient medium and sucrose on somatic embryogenesis of date palm. Plant Cell, Tissue and Organ Culture 45 (2), 159-164

19. Weisser, H., Behnke, B, Helpap, B., Bach, D. and Krieg, M. (1997). Enzyme activities in tissue of human benign prostatic hyperplasia after three months treatment with the *Sabal serrulata* extract IDS89 (Strogen) or placebo. Eur. Urol. 31(1), 97- 101
20. Zaid, A. (1987). Morphogenic variation in Palm Embryos Cultured In Vitro. Date palm J. 5(1), 36-47
21. Zarate, R. and Verpoorte, R.(2007). Strategies for the genetic modification of the medicinal plant *Catharanthus roseus* (L.) G. Don. Phytochem Rev 6: 475–491
22. Zhao, J. and Verpoorte, R.(2007). Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. Phytochem Rev 6: 435–457

12/2/2010