Implementation of biotechnology for production of hypericin as antibladder cancer photosensitizer compound from egyptian hypericum sinaicum

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Photodynamic therapy (PDT) has been categorized as a new therapeutic approach for cancer. PDT includes the combination of a photosensitizer compound, which is taken up and retained by tumor cells, and visible light of an appropriate wavelength matching the absorption spectrum of the compound. Hypericin is a plant-derived compound and is a powerful natural photosensitizer. The focus of our interest was to promote the production of hypericin from Egyptian Hypericum sinaicum culture in vitro by using biotechnological applications, employ hypericin-PDT for the treatment of bladder cancer in vivo and to suggest the possible mechanisms underlying this action. Eighty adult albino rats were assigned into eight groups. The 1st group served as negative control. The 2nd group received N-butyl-N-(4-hydroxybutyle)-nitrosamine (BBN); 0.1 g in 100 ml of drinking water for a period of 8 weeks for induction of bladder cancer and served as positive control. The 3rd, 4th and 5th groups received intraperitoneally (i.p) 5, 7.5 and 10 mg/kg hypericin-PDT respectively. The 6th, 7th and 8th were bladder cancer bearing groups treated i.p with 5, 7.5 and 10 mg/kg hypericin-PDT respectively. Histopathological investigation of bladder specimens in all groups was carried out. Treatment with hypericin-PDT could restore the structural organization of bladder and maintain the architectural integrity of bladder cells. The outcomes of this investigation offer a convincing evidence for the importance of biotechnological approaches in improving the production of natural compounds. Also, this study provides spotlight on hypericin-PDT as an appropriate therapeutic modality for bladder cancer. The mechanisms behind this effect seem to depend on promoting apoptotic pathway, targeting tumor vasculature and activating immune response.

Keywords: Hypericum sinaicum, Hypercin, Photodynamic therapy, Bladder cancer, Rat model.

INTRODUCTION

Plant-based medications have been found to be the subject of a multitude of studies. During the last decade, there have been many studies directed towards the recognition of botanical compounds for prevention and intervention of various human diseases (Busby and Kamat, 2006). Additionally, with the identification of new therapeutic candidates, plants are also poised to continue with a critical role in the modern medicine era (Suman et al., 2013). Hypericum spp. are commonly recommended by traditional medicine practitioners as a promising medicinal remedy, but the study of the pharmacological
activity of their active compounds still continues. *Hypericum sinaicum* L. is one of the endemic species in Sant Katherin Protectorate, South Sinai, Egypt (Boulos, 2002).

Hypericin is a naturally occurring chromophore found in many widely spread plants of the *Hypericum* gender. Hypericin and its analogues have been found in special morphological structures, so called dark nodules, present in the aerial parts of hypericin-producing *Hypericum* spp. It is a promising natural photosensitizer in the PDT of different oncological ailments (Jendzelovska et al., 2016). The accumulation of hypericin has been shown to be significantly higher in neoplastic tissue than in normal tissue showing high tumoricidal efficacy (Chen et al., 2002). Regarding its redistribution in the subcellular organelles, hypericin has been shown to be accumulated in the membranes of the endoplasmic reticulum, the Golgi apparatus, lysosomes and mitochondria (Ali and Olivo, 2002). The photosensitizing effects of hypericin are generally described as oxygen-dependent to generate ROS in the presence of light (at wavelengths around 600 nm) resulting in cell death and tissue destruction. This concept is strongly supported by the observations that hypericin in a hypoxic environment does not exhibit photocytotoxicity (Agostinis et al., 2002).

As a consequence of the high bioactivity of hypericin, its content has been assessed in *Hypericum* cultures subjected to different biotechnological implementations that focused on its preservation (Brunáková et al., 2015). The best treatments for callus induction, in vitro regeneration culture of *Hypericum sinaicum* and the extraction of hypericin are mentioned in our previous study (Khlifa et al., 2016a).

Bladder cancer is one of the most common sites of cancer in the urinary tract and it compresses the second type of cancer after prostate cancer among urinary tract tumors. Bladder cancer is the fourth most frequent cancers in men after prostate, pulmonary and colonic cancers (Oliveria et al., 2008). Urothelial cell carcinoma (UCC) represents more than 90-95 % of all bladder tumors followed by squamous cell carcinoma (SCC) which accounts 3-5% of bladder tumors in Western countries. Off note, SCC is the most prevalent form of cancers in men overall in countries where the *Schistosoma haematobium* parasite is endemic. Adenocarcinoma represents the third most common type of bladder cancer accounting 0.5-2% of bladder tumors (Tyczynski and Parkin, 2003).

The goal of the current work was to utilize the best biotechnological procedure to induce the production of hypericin from Egyptian *Hypericum sinaicum*, explore the potentiality of hypericin-PDT against bladder cancer induced in rat model (in vivo) and to propose the possible mechanisms behind its anticancer effect.

**MATERIALS AND METHODS**

**Hypericum sinaicum seeds**

Seeds of *Hypericum sinaicum* (Fig. 1) were obtained from Sant Katherin Protectorate, South Sinai governorate, Egypt.

**Hypericin (HY) standard**

Hypericin standard authentic sample (Fig. 2) was secured from Sigma –Aldrich Company (USA) with molecular formula (C30H16O8) and molecular weight (504.45).

**N-Butyl-N-(4-hydroxybutyl) nitrosamine (BBN)**

N-Butyl-N-(4-hydroxybutyl) nitrosamine (BBN) is a chemical carcinogen compound, acquired from Sigma-Aldrich Company (USA) and it is used to induce urinary bladder cancer in rat model (Kawahara et al., 1987). It is a registered trademark of Sigma-Aldrich Company LLC as shown in Fig (3).

**Establishment of sterilized *Hypericum sinaicum* L. seedlings, shoots induction and multiplication**

All cultures were incubated under 26 ± 1°C in a growth room with a daily16-hr photoperiod under standard cool white fluorescent lamps at light intensity 20-25µmol m⁻²s⁻¹ in light, and darkness conditions. The experiment consisted of ten jars per treatment (Khlifa et al., 2016b).

**Hypericin extraction procedure**

Regenerated shoots from the best treatments were air dried on a filter paper to wipe away the excess water. Then the samples were dried in oven for 3 days at 50° C and grounded with a coffee grinder. Grounded samples were extracted following the method described by Taha (1999).

**Hypericin estimation (µg/g DW)**

Chemical qualitative analysis of hypericin was done using different chromatographic methods: (I) Thin layer chromatography (TLC) described by Stahl (1988) as a preliminary study for naphthodianthrones qualitative determination was carried out. Twenty microliters of regenerated...
crude extract and HY standard solutions were loaded onto a normal phase TLC plate using microcaps and allowed to evaporate. The plate loaded with the samples was carefully placed into the tank contains mobile phase (30 ml of ethyl acetate: 1.5 ml formic acid: 1.5 ml acetic acid: 7 ml distal water). Then, the TLC plate was taken out when the mobile phase was about 1 cm away from the top of the plate. Then, plate was allowed to dry in a fume hood for 15 min. The developed plate was visualized for hypericin by UV documentation apparatus at wave length 590 nm. The compounds present on the plate, the distance between the spots and the end of the mobile phase, and the Rf value of the hypericin compound was calculated and recorded.

(II) High performance liquid chromatography (HPLC) was used as quantitative determination method of naphthodianthrones. One hundred microliters of the sample was placed into a plastic auto-sampler vial, further, capped with aluminum vial sealed for HPLC analysis. The HPLC system was composed of a binary pump (Model 1525, Waters Corporation, Milford, MA, USA), a fluorimetric detector (Model 474, Waters; λex=236 nm/λem=592 nm), an autosampler (Model 717plus, Waters) and a personal computer with Breeze data acquisition and integration software (Waters). Chromatographic separation was performed at ambient temperature on a C8 column (Symmetry 4.6 x 150 mm, 5 µm, Waters) fitted with a C8 guard column (Symmetry 3.9 x 20 mm, 5 µm, Waters) using gradient eluents. Eluent A consisted of 60 mM phosphate buffer pH 6.8 and eluent B consisted of 80%:20% (v/v) acetonitrile: H2O. The mobile phase started with eluent A: eluent B at 80: 20, the gradient program of mobile phase (concentrations and run time) are shown in Table (1).

The total run time was 100 min., the flow rate of mobile phase was 1 ml/ min. and the injection volume was 20μl.

The linearity of a method was tested for the concentration of 50 µg/ ml of standard hypericin using the equation of a straight line y= ax+b, the regression coefficient r= 0.9995 (Fig.4).

Figure. (1): Seeds of *H. sinaicum* and germinated plant in Sant Katherin Protectrate South Sinai, Egypt

Figure. (2): Chemical structure of hypericin

Figure. (3): Chemical structure of N-pseudo-N-(4-hydroxybutyl) nitrosamine (BN).
Bioassay

The biological experiment was conducted on male *Wistar* albino rats weighing 180 - 200 g obtained from the Animal House Colony of the National Research Centre, Giza, Egypt. The animals were housed in plastic cages with hard wood chips at room temperature of 22± 1 °C, humidity of 45% to 55%, and 12 hours of light/dark cycle. The animals had access to commercial standard rat food pellets and water *ad libitum*. The biological design of this study was approved by the Ethical Committee for Medical Research of the National Research Centre (Approval Number 11/080). For establishing bladder cancer, 0.1 g of BBN was dissolved in 100 ml of drinking water of rats for a period of 8 weeks as described by Kawahara et al., (1987). After this time, most of the animals had developed multiple neoplasia spread all over the bladder wall.

The protocol of photodynamic therapy (PDT) in this study was designed as follows: The hypericin extracted from crude extract of *H. sinaicum* was dissolved in water and injected intraperitoneally (i.p) at doses of 5, 7.5, 10 mg/Kg b.wt. The animals were kept under conditions of subdued lighting until commencement of PDT. A filtered halogen light source (Zeiss KL 1500) was filtered with a customizes 560- to 640 nm band pass filter. After the drug- light interval of 2 h, the animals were anethetized with 1:1 cocktail of ketamine hydrochloride and valium. Then after the bladders were exposed to light at a dose of 30 J/cm² x delivered at a fluence rate of 25 mW/cm² (Thong et al., 2006).

Animals and experimental groups

Eighty adult male rats of Wistar strain were randomized into eight groups (n= 10). The 1st group received the vehicle (tap water) and served as negative control group. While, the 2nd group received 0.1 gm of BBN dissolved in 100 ml of drinking water for a period of 8 weeks for induction of bladder cancer and left untreated (positive control group). The 3rd, 4th and 5th groups received i.p doses of hypericin-PDT in doses of 5 mg/kg (Bhuvaneswari et al., 2008), 7.5 and 10 mg/kg (Sanovic et al., 2011) respectively five times/week for a period of 6 weeks. The 6th, 7th and 8th groups received BBN in drinking water for induction of bladder cancer and then treated i.p with hypericin-PDT in dose of 5, 7.5 and 10 mg/kg, respectively five times/week for a period of 6 weeks.

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Table (1) Gradient program of mobile phase

<table>
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<tr>
<th>Time [min]</th>
<th>Eluent A (%)</th>
<th>Eluent B (%) *</th>
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<tr>
<td>0</td>
<td>80</td>
<td>20</td>
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<tr>
<td>18</td>
<td>0</td>
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<td>30</td>
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+1 additional minute of column flushing with A: B 80%: 20% before injection Where: *Eluent A was 60 mM phosphate buffer pH 6.8 **Eluent B was 80% acetonitrile: 20% H₂O.

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Figure (4): Hypericin calibration curve

The calibration curve for hypericin was obtained using a standard curve approach. The equation for the calibration curve is given by:

\[ y = 7020.1x + 15.467 \]

The coefficient of determination, \( R^2 \), is 0.9995.
Histopathological examination of bladder

After the experiment was over, autopsy samples were taken from the urinary bladder of sacrificed rats in the different groups and fixed in 10% formol saline solution for twenty four hours. Washing was done using tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin wax at 56 degree in hot air oven for twenty four hours. Paraffin wax tissue blocks were prepared for sectioning at 4 microns thickness by rotary microtome. The obtained tissue sections were collected on glass slide, deparaffinized, and stained by hematoxylin and eosin stain (H&E stain) for routine examination through the light electric microscope (Banchroft et al., 1996).

RESULTS

Direct regeneration and multiplication of *H. sinaicum*

The best direct regeneration and multiplication of *Hypericum sinaicum* was produced from nodal segment cultured on Murashige and Skooge (MS) medium in the presence of naphthyl acetic acid (NAA) 0.1mg/l combined with 1mg/l benzyl adenine (BA) under light condition for 60 days as shown in Fig. (5). The results of this experiment were completely described in our previous article (Khlifa et al., 2016b).

Chemical analysis

Hypericin contents (µg/g dry weight)

Fig. (6) represents total hypericin content extracted from *in vitro* regenerants produced from nodal segment and authentic hypericin. The content of hypericin is expressed as µg/ g dry weight and the hypericin content in direct shootlets regeneration from nodal segment recorded 31.59µg/g dry weight.

Anticancer effect of hypericin-PDT

The obtained histopathological findings of bladder tissue section of rat from the 1<sup>st</sup> group which was kept as negative control group revealed no histopathological alteration and the normal histological structure of the pseudostratified mucosal epithelium and the underlying lamina propria and muscularis were observed (Fig. 7). However, most of the mucosal lining epithelium cells of the bladder tissue section of rat from the 2<sup>nd</sup> group afflicted with bladder cancer (positive control group) showed hyperplasia and dysplasia with atypia, hyperstratification and thickening (Fig. 8).

Microscopic investigation of bladder tissue sections of normal rats from the 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> groups that received different doses of hypericin-PDT (5, 7.5 and 10 mg/kg) respectively showed normal histological structure of the lining transitional mucosal epithelium with underlying lamina propria and muscular as recorded in Figs. (9, 10 and 11), respectively. Meanwhile, histological examination of bladder tissue section of the rat from the 6<sup>th</sup> group that was treated with hypericin-PDT (5 mg / kg) after induction of bladder cancer showed mild hyperplasia and stratification of the mucosa (Fig. 12). Optical micrograph of bladder tissue section of the rat from the 7<sup>th</sup> group that was treated with hypericin-PDT (7.5 mg/ kg) after induction of bladder cancer showed normal mucosal lining epithelium with congestion of blood vessels in the lamina propria (Fig. 13). Likewise, optical micrograph of bladder tissue section of the rat from the 8<sup>th</sup> group that was treated with hypericin-PDT (10 mg/kg) showed normal histological architecture of the lining mucosal epithelium and the underlying lamina propria with a slight congestion of blood vessels in the lamina propria (Fig. 14)

![Figure. (5): Effect of MS medium supplemented with 0.1 mg/l NAA and 1mg/l BA on direct shootlets regeneration of nodal segment and multiplication at light.](image_url)
Figure. (6): HPLC of standard hypericin(A) and hypericin extracted from direct shootlet regeneration of nodal segment (B).

Figure. (7): Optical micrograph of a cross-sectioned bladder tissue of rat in the negative control group showing normal histological structure of the mucosal lining epithelium (mu) with underlying lamina propria (p) and muscular layer (ml).

Figure. (8): Optical micrograph of a cross-sectioned bladder tissue of rat bearing bladder cancer (positive control group) showing hyperplasia and dysplasia of the mucosal lining epithelium (mu) with multiple stratifications and thickening.

Figure. (9): Optical micrograph of a cross-sectioned bladder tissue of normal rat received 5mg/kg hypericin-PDT for 6 weeks showing normal histological structure of the lining transitional mucosal epithelium (mu) with underlying lamina propria and muscolous (ml).
Figure. (10): Optical micrograph of a cross-sectioned bladder tissue of normal rat received 7.5mg/kg hypericin-PDT for 6 weeks showing normal histological structure of the lining transitional mucosal epithelium (mu) with underlying lamina propria and muscolous (ml).

Figure. (11): Optical micrograph of a cross-sectioned bladder tissue of normal rat received 10 mg/kg hypericin-PDT for 6 weeks showing normal histological structure of the lining transitional mucosal epithelium (mu) with underlying lamina propria and muscolous (ml).

Figure. (12): Optical micrograph of a cross-sectioned bladder tissue of rat bearing bladder cancer and treated with 5 mg/kg hypericin-PDT for 6 weeks showing mild hyperplasia and stratification of the mucosa (mu).

Figure. (13): Optical micrograph of a cross-sectioned bladder tissue of rat bearing bladder cancer and treated with 7.5 mg/kg hypericin-PDT for 6 weeks showing normal mucosal lining epithelium (mu) with congestion in blood vessels (v) in lamina propria.
Hypericin antagonizes urinary bladder cancer in vivo

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Figure. (14): Optical micrograph of a cross-sectioned bladder tissue of rat bearing bladder cancer and treated with 10 mg/kg hypericin-PDT for 6 weeks showing normal histological structure of the lining mucosal epithelium and the underlying lamina propria with a slight congestion of blood vessels in the lamina propria (v).

So, the doses of hypericin-PDT (7.5 and 10 mg/kg) for a period of 6 weeks are recommended as they showed the best results for recovery from bladder cancer.

DISCUSSION

Hypericin was obtained from different in vitro cultures of *H. sinaicum* as reported in our previous articles (Khlifa et al., 2016a,b). TLC-densitometry is cost-effective due to the short time of analysis and the low consumption of solvent; up to eight samples can be chromatographed simultaneously on a single plate in comparison with HPLC method which is simple, rapid, reliable and reproducible but costly. In agreement of our results Mulinacci et al., (2000) mentioned that hypericin and pseudohypericin are easily identified at 590 nm. Moreover, Camas et al., (2012) investigated the presence of the naphthodianthrones hypericin by HPLC technique. Hypericin as natural fluorescent photosensitizer (PS) possesses several properties that make it suitable for PDT and photodynamic diagnosis (PDD) for detection and treatment of tumors (Jendzelovska et al., 2016).

PDT constitutes a non-invasive therapeutic modality with a good benefit in the treatment of different type of cancer (Agostinis et al., 2011). PDT includes the administration of a non-toxic photosensitizer that accumulates preferentially in the target tissue, followed by its local illumination with harmless visible light of a proper wavelength, to stimulate and excite the photosensitizer. These photoreactions result in oxygen dependent production of cytotoxic ROS, leading to cell death and tissue damage. Meanwhile, PDT is a multifactorial process and the level of cellular photo damage is based on several factors, involving the permeability of the cell, the localization of the photosensitizer in the subcellular organelles, the molecular oxygen quantity, the dose of light, the types of ROS and the type of cancer cells (Jendzelovska et al., 2016).

To better understanding of hypericin-PDT-induced response in the treatment of bladder cancer and to implicating hypericin-PDT as a promising anticancer therapeutic approach, the condition should be optimized. These conditions include suitable hypericin dosage, light dosimetry, fluence rate and time intervals. Herein, we used three doses of hypericin (5, 7.5, 10 mg/kg b.wt) based on previous most published studies (Bhuvaneswari et al., 2008, Vandenbogaerde et al., 1996; Zupko et al., 2001, Cavarga et al., 2001; Sanovic et al., 2011, Noell et al., 2011 and Blank et al., 2004). Also, the light dosimetry and fluence rate were selected according to the study of Thong et al., (2006).

The optimal drug-light interval for treatment of bladder cancer was adjusted depending on the study Kubin et al., (2008). Selective hypericin uptake in rat bladder tumors has been reported by Kamuhabwa et al., (2002). These investigators mentioned the selectivity of hypericin uptake in bladder tumors and subsequently, hypericin-PDT-mediated tumor destruction was observed without damage of normal tissue (Kamuhabwa et al., 2003). In the study of Kamuhabwa et al., (2002), the experimental model of female Fisher rats with an orthotopic superficial transitional cell carcinoma (TCC) was used and hypericin was injected directly into the bladder via the catheter. It has been observed that hypericin accumulates selectively in the bladder urothelial tumors and the normal urothelium in a ratio of 12:1, and no hypericin was found in normal bladder submucosa and muscle layers. In addition, hypericin was not detected in plasma; so, systemic adverse events should not appear. Moreover, Vandepitte et al., (2010) observed that Polyvinylpyrrolidone hypericin (PVP–hypericin) is uniformly
distributed of instilled in all cell layers of the malignant urothelium, whereas its uptake by the epithelium of normal bladder is very restricted.

The obtained histological findings showed great recovery from bladder tumor when using high doses of hypericin-PDT (7.5 and 10 mg/kg) for 6 weeks where the congestion in the blood vessels in the lamina propria was only noticed. This finding is in keeping with the photophysical processes that are taken place during PDT. The photophysical processes are well explained by Castano et al., (2005) who reported, that the PS absorbs light and boosts from the resting state (low energy at which the electrons occupy opposite spins) to the first excited singlet state. The excited singlet state of the PS is then submitted to the intersystem crossing whereby the spin of the excited electron is inverted to form the excited triplet-state (electron spins parallel). The PS excited triplet is further subjected to two types of reactions; type 1 reaction, during which it is combined directly with the substrate like protein or lipid molecules of the cell membrane, and type 2 reaction in which the triplet PS could transfer its energy directly to molecular oxygen, to generate excited state singlet oxygen. Both of type 1 and type 2 reactions could produce additional reactions resulting in the generation of toxic products (ROS) that cause toxicity to various cell structures and macromolecules (DNA, lipids, and enzymes). It is worth noting that only molecules and structures that nearby the areas of the yielded ROS (areas of PS accumulation) are directly affected by PDT. In agreement with our results Kamuhabwa et al., (2002, 2003) reported that, hypericin can be used not only for diagnosis but also for photodynamic therapy (PDT) of superficial bladder tumors. This suggestion is further supported by Wang et al., (2010).

Three separate mechanisms (but probably interrelated) are implicated in the noticeable regression of tumors during PDT:(i) The ROS could kill tumor cells directly by apoptosis. The light-dependent suppressing impact of hypericin against different enzymes engaged in governing of cell survival and proliferation (Ser/Thr kinases, tyrosine kinases, etc.) has been reported (Kubin et al., 2005). These actions could also participate in the cytotoxic and antiproliferative insults of hypericin-PDT; (ii) Damaging of the tumor-associated vasculature leads to the deprivation of tissue from nutrients and oxygen with consequent infarction of the tumor. Hypericin-PDT modality appeared to completely eliminate tumors by preferentially targeting their vasculature (Sanovic et al., 2011). Photodynamic therapy with hypericin has been found to evoke an acute and potent vascular impact that is accountable for tumor damage. Researches have also demonstrated that the damage of tumor vasculature occurs as a result of deprivation of oxygen and nutrient (Chen et al., 2002). The exhaustion of oxygen during PDT and the destruction of tumor microvasculature provides oxygen shortage within the treated tumor (Dougherty et al., 1998) and (iii) The stimulation of immune responses against tumor cells (Castano et al., 2005, Sanovic et al., 2011). Hypericin-PDT-induced activation of anticancer immunity was also reported in the studies utilizing various in vivo experimental models (Garg et al., 2012a, 2015b, 2016, Zheng et al., 2016). The data of these independent experimental groups propose the high potential of hypericin -PDT in the production of anticancer vaccines.

The antitumor activity of hypericin is well known to be oxygen dependent suggesting that the three mechanisms of photodynamic action may be implicated in the biological photo activity of hypericin. In 2010, the induction of multiple and interrelating signaling pathways has been proposed during PDT with hypericin. These are explained by Karioti and Bilia (2010) as shown in Fig. (15): (1) High doses of light lead to necrosis. (2) Medium doses of light stimulate different apoptotic cascades: (2a) aggravation of caspase 8 and final triggering of the caspases 3, 6, 7 (2b) Releasing of mitochondrial cytochrome c, in association of releasing mitochondrial Ca2+ lead to stimulation of the caspases 3, 6, 7 via stimulation of caspase 9. Bax/Bidn proapoptotic proteins increase the release of cytochrome c, while the antiapoptotic Bcl-2 suppresses the cytochrome c reflux (2c) Releasing of Ca2+ from the endoplasmic reticulum stimulates the release of cytochrome c from mitochondria. (3) The reduction of the ERKs activates cytostatic responses. (4) Low doses of light favor the MAPKs pathways resulting in cell survival; stimulation of MAPKs JNK1 and p38α cascade leads to the survival of the cell and angiogenesis.

**CONCLUSION**
Finally it could be concluded that, biotechnological modalities have a fundamental role in producing and improving natural products such as hypericin. Concerning the in vivo study it is suggested that, the dose range of hypericin-PDT (7.5 -10 mg/kg daily) for 6 weeks is recommended for the regression of early stage of bladder cancer via the activation of apoptotic signaling axis damaging of
the tumor vasculature and induction of anticancer immunity.

**CONFLICT OF INTEREST**
There is no conflict of interest here.

**REFERENCES**


