

Preliminary Evaluation of *In Vitro* Activity of *Camelia sinensis*, *Hypericum perforatum* and *Citrus X paradisi* extracts

Ioana Cuc-Hepcal¹, Claudiu Morgovan¹, Florina-Maria Andrica^{2*},
Ioana Zinuca Pavel², Andrei Motoc²

¹"Vasile Goldis" Western University of Arad, 310086, Arad, Romania

²"Victor Babes" University of Medicine and Pharmacy Timisoara, 300041, Timisoara, Romania

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Abstract

Citrus X paradisi (CP), *Camelia sinensis* (Green tea – GT) and *Hypericum perforatum* (HP) are natural products with antioxidant properties commonly used in traditional medicine as alternative treatments for various chronic disorders including cardio-vascular diseases, diabetes and cancer. The aim of this study was to evaluate: a) the antioxidant properties of CP, GT (ethanolic extract and infusion) and HP extract using DPPH method and b) the anti-migratory effects of CP, GT and HP extracts on B164A5 murine melanoma cells using scratch assay technique. Our results indicated that the CP, the GT and the HP extracts had shown potent antioxidant effects and a slight anti-migratory effect on B164A5 murine melanoma cells.

Keywords: *Citrus X paradisi*, *Camelia sinensis*, *Hypericum perforatum*, antioxidant properties

1. Introduction

A great amount of interest was invested in the study of functional foods with antioxidant properties regarding their underlying mechanism that suppresses oxidative stress in various degenerative diseases [1]. A challenge in this regard is represented by the incompletely elucidated mode of action of natural products on biological systems [2].

Nowadays, an increasing number of studies have been developed to analyze the pharmacological properties of various plants including *Citrus X paradisi* (CP), *Camelia sinensis* and *Hypericum perforatum* (HP), plants that are well known for their antioxidant activity.

Grapefruit (CP) is rich in flavonoids, including naringenin and hesperetin, which are responsible for several biological effects, including: anti-inflammatory, antioxidant, anti-atherogenic, antidiabetic and antidyslipidemic effects [3,4]. A previous study has shown that *in vivo*

administration of naringenin was associated with a low toxicity [5]. The limonoids extracted from CP have exhibited apoptotic effects on colon cancer (SW480) cells [6]. Another bioactive substance from CP with antiproliferative properties is bergamottin, which induced apoptosis in multiple myeloma (MM) cells [7]. Psoralen, a natural furanocoumarin from *Citrus sp.* has proved a stimulatory effect on melanoma cells growth [8].

Green tea (GT) is one of the most consumed beverages all over the world, being extracted from *Camelia sinensis L.* Depending on their degree of fermentation, there were described three types of tea obtained from *Camelia sinensis*: GT (non-fermentated), oolong tea (semi-fermentated) and black tea (fully-fermentated). According to the previous data, GT has demonstrated a stronger antioxidant activity than black tea due to their high level of epigallocatechin gallate [9]. Epigallocatechin gallate, (+)-gallocatechin and (-)-epigallocatechin (EGC) proved to be the most active anticancer compound from GT on MCF-7 breast

cancer, HT-29 colon cancer and UACC-375 melanoma cells. Catechins are the most active polyphenols of GT, responsible for GT antioxidant effect. A cup of GT contains approximately 30-40% catechins [10]. A recent *in vitro* study showed that GT polyphenols have an inhibitory effect on A375 and Hs294t melanoma cell lines by decreasing cyclins levels and cyclin dependent kinase [11].

HP known as St John's Wort is generally found in temperate regions and it was described to possess a high content in flavonoids, phloroglucinols and naphthodiathrones [12]. Among them, hypericin and hyperforin, are the most biologically active compounds responsible for the antiproliferative effect of HP [13]. Kleemann *et al.* showed that hypericin used as photosensitizer in photodynamic therapy proved an antiproliferative effect on melanoma cells through inducing necrosis, apoptosis and autophagy related cell death [14].

The anti-inflammatory, antidepressant, antiviral and antibacterial properties of HP are well known in the folk medicine and this plant was widely used as traditional remedy [13, 15,16].

The aim of this study was to evaluate the antioxidant and antimigratory effects of CP juice, GT ethanolic extract, GT infusion and HP ethanolic extract.

2. Materials and Methods

Materials

Reagents

Reagents used in the experiments were of analytical grade, ethanol 96% (v/v) and the bi-distilled water were purchased from Chemical Company SA, Iasi, Romania and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Batch No: # STBF5255V) from Sigma Aldrich, Germany. The ascorbic acid was purchased from Lach-Ner Company. The specific media and supplements for cell culture, including: Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), saline phosphate-buffered (PBS), penicillin/streptomycin and trypsin-EDTA solution were purchased from Sigma Aldrich (Munich, Germany).

The GT was produced by the National Health Products CO., LTD. and purchased from Naturalia Dict SRL, China; the CP juice was obtained by

direct spoliation of fresh CP fruits. HP plants were harvested from the spontaneous flora, being collected between May-August.

Cells

B164A5 murine melanoma cells were purchased from European Collection of Authenticated Cell Cultures (ECACC) as frozen vial.

Methods

Preparation of extracts

The GT extract was prepared as follows: 1 g of GT leaves was mixed with 50 mL ethanol 50% and continuously stirred for 90 minutes at 25°C and 250 rpm using an orbital shaker. The GT aqueous infusion: 5 g of GT leaves were mixed with 250 mL of hot distilled water and then the mixture was allowed to stand for 5 minutes. After the preparation, both the extract and the infusion were filtered, in the case of ethanolic extract the solvent was removed using a rotary evaporator and the resulted solution was analyzed. The CP extract was obtained directly from the fruit by spoliation and the juice has also been filtered. The HP extract was prepared using fresh plants which were dried, macerated for ten days using ethanol 80% at 25°C temperature. Then the final solution was filtered. The solvent has been evaporated using a rotary evaporator and the resulted aqueous extract was used for DPPH and scratch assays evaluations.

DPPH Radical Scavenging Assay

The antioxidant activity of the extracts was evaluated by DPPH radical scavenging assay which was originally described by Blois [17]. It was used the following protocol: 1 mmol·L⁻¹ ethanolic solution of DPPH was prepared and stored at 4°C in the dark place. As control, it was prepared a solution of ascorbic acid 0.167 mmol·L⁻¹ in ethanol 96% (v/v).

From each sample (GT– extract and infusion, CP juice and HP extract) it was prepared three dilutions as follow: 1:1; 1:10; 1:20.

0.5 mL of the samples solution (including the ascorbic acid solution) was mixed with 0.5 mL DPPH and 2 mL ethanol 96%; the mixture was analyzed using an Uvi Line 9400 Spectrophotometer from SI Analytics at 516 nm for 20 minutes (1200 seconds).

Antioxidant activity was calculated using the following formula:

$$AAO \% = 100 - \frac{A_{516(t)}}{A_{516(t=0)}} \cdot 100$$

Where: AAO – antioxidant activity;

$A_{516(t)}$ – absorbance measured at 516 nm at a specific time;

$A_{516(t=0)}$ – absorbance measured initially at 516 nm (without sample).

Scratch assay technique

B164A5 cells were cultured in DMEM supplemented with 1% penicillin/streptomycin 10000 UI/mL and 10% FCS.

A number of 2×10^5 cells/well were seeded in 12-wells culture plates. When the cells acquired the appropriate confluence, scratches were made using a pipette tip (10 μ l). The media was removed and the cells were washed with PBS and stimulated with CP, GT and HP extracts using the following dilution (1:10). The samples were incubated for 24 h and there were taken pictures at different time points (0, 3 and 24h) using Optika Microscope Optikam Pro Cool 5.

Statistical analysis

The data were statistically analyzed using OriginLab - graphing and data analysis software.

3. Results and discussion

Antioxidant activity

In figure 1 is presented the AAO of all three dilutions of CP juice. As we can see, the samples have shown AAO comparatively with that of ascorbic acid, which was used as positive control.

The aqueous sample diluted 1:1 had the highest AAO (93%) comparatively with AAO of ascorbic acid (96%). At higher dilutions, the AAO of the samples decreased, which was in perfect correlation with the literature [18], which says that the AAO increases with the increasing of the concentration.

In the figure 2 is presented the AAO of all three dilutions of the GT extract. In the case of the GT extract, the AAO increased with the decreasing of

the concentration, because the dilution 1:20 of the GT extract presented the highest AAO (90%) than the sample with dilution 1:1 (88%) and the sample with dilution 1:10 (89%). Comparatively with the AAO of the ascorbic acid (96%), all three samples had a higher AAO (around 90%) after 1200 seconds.

This could be explained by the fact that the GT extract had a higher concentration of polyphenols, catechins, and flavonoids as compared to the samples prepared by dilution.

In figure 3 is presented the AAO of all three dilutions of the aqueous GT infusion.

In this case, the results are different, because the AAO was no longer in correlation with the concentration. Thereby, the sample diluted 1:20 from the aqueous GT infusion, presented a higher AAO (44%) towards the sample diluted 1:10 (34%). Both samples (dilution 1:10 and 1:20) had AAO much lower at the initial moment (t=0 seconds) then the AAO at the final moment (t=1200 seconds).

In figure 4 is presented the AAO of all three dilutions of the aqueous HP extract.

In the case of HP aqueous extract, we could conclude that also for this extract the AAO was not concentration dependent – the AAO increased with the decreasing of concentration. We could see from the chart that the sample diluted 1:20 not touch the equilibrium in 1200 seconds, this sample still reacted with the standard antioxidant solution (DPPH) after 20 minutes. If the sample will be analyze longer, it could be observed that the AAO at the final moment will be higher than the sample diluted 1:10.

Figure 5 presents the highest AAO of all four samples which were analyzed (CP juice, GT extract, GT infusion and HP) comparatively with the AAO of the control (ascorbic acid). All samples analyzed had a significant AAO, and the nearest value as compared to ascorbic acid was recorded for the sample diluted 1:1 from the CP juice.

Scratch assay results

The anti-migratory effects of the tested extracts were evaluated using the scratch assay technique, a wound healing-type method. In order to verify the effects of the extracts after stimulation, the pictures were taken at 0, 3 and 24h post-stimulation. The CP extract stimulation induced a slight anti-migratory effect on

B164A5 murine melanoma cells as compared to control cells (figure 6).

In the case of HP extract it was observed a similar effect as the one described for the CP extract (figure 7). The GT extracts stimulation of B164A5 induced significant changes of the cells shape (normally these cells have a fibroblastic-like shape, but as it can be seen in figure 8 the cells became round a shape that is specific for apoptotic cells), changes that were observed at an early time point – 3h. The signs observed after GT extract stimulation led us to the conclusion that the concentration used in the study induced cells apoptosis and in order to evaluate the anti-migratory effects will be recommended lower concentrations (figure 8).

The migratory capacity of cancer cells play a major role in the process of cancerogenesis and metastasis

and finding compounds that might suppress this effect represent an important step in elucidating new mechanisms involved in the development of cancer.

Apigenin from CP which is a polyphenolic flavone proved to have an apoptotic effect in HER2 breast cancer cells [19].

Beneficial effects of GT have been related to their high content of polyphenols, especially flavonoids, as epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin [20].

Previous *in vitro* studies have shown that HP had an inhibitory effect on UCT Mel-1 and A375 melanoma cells but, a low antiproliferative effect on MCF-7 breast cancer cells [21].

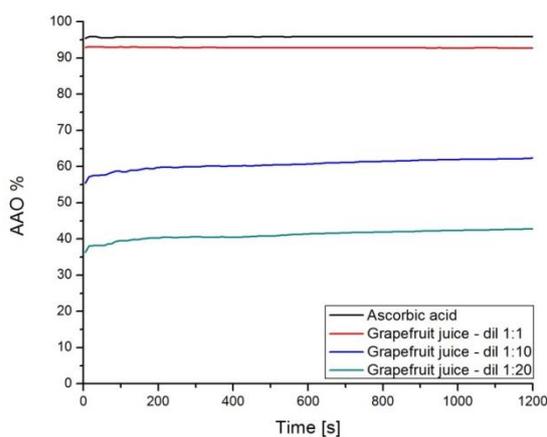


Figure 1. The AAO of all three aqueous dilutions of CP juice in time of ascorbic acid, used as positive control.

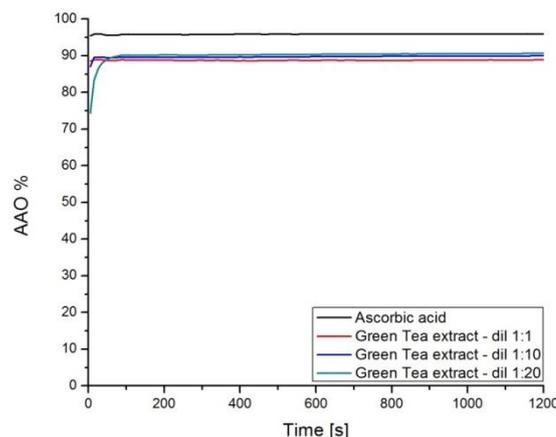


Figure 2. AAO of all three dilutions of the ethanolic GT extract in time.

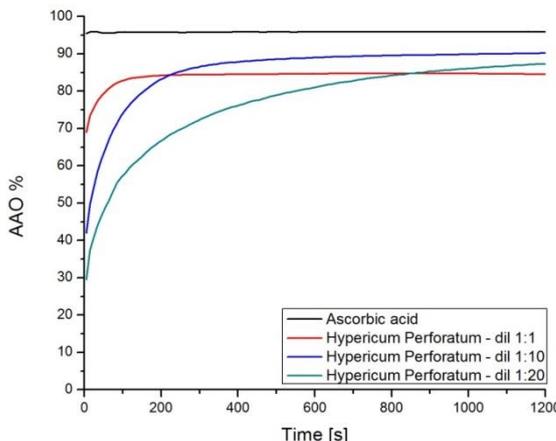
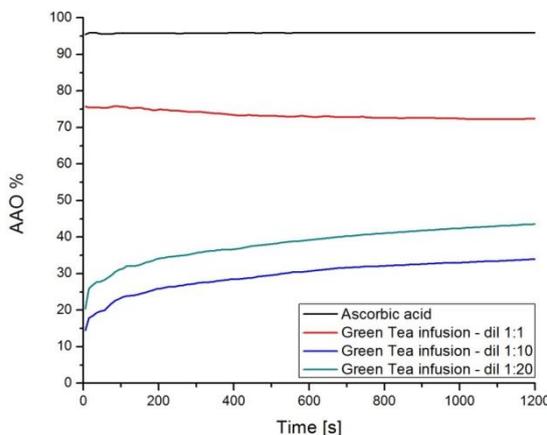


Figure 3. AAO of all three dilutions of the aqueous GT infusion in time

Figure 4. AAO of all three dilutions of the aqueous HP extract in time.

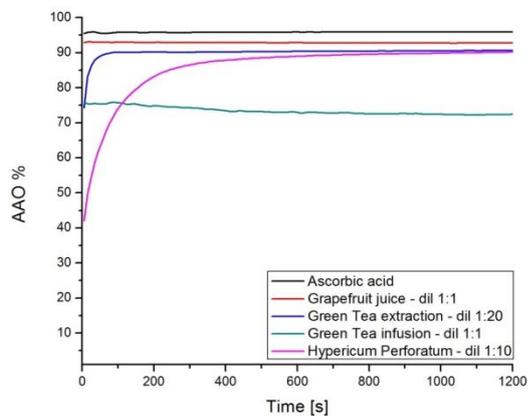


Figure 5. AAO of the samples analyzed in time.

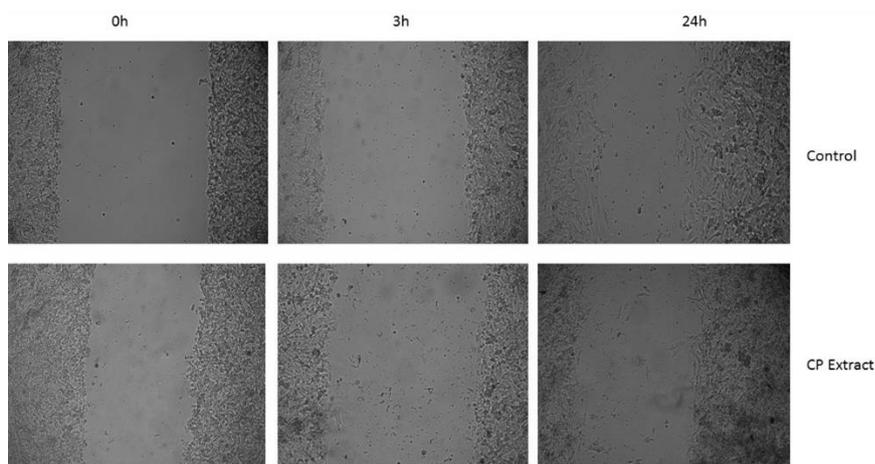


Figure 6. CP effect on B164A5 murine melanoma cells (control 0h; control 3h; control 24; CP at 0h; CP at 3h; and CP at 24h)

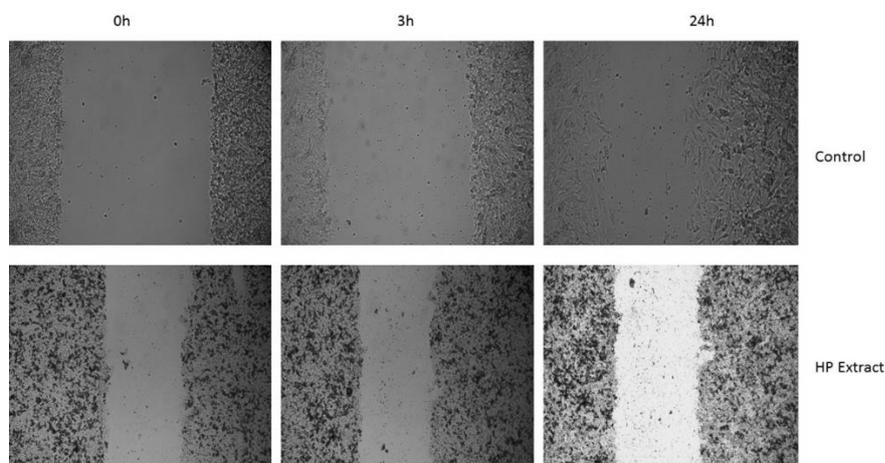


Figure 7. HP effect on B164A5 murine melanoma cells (control 0h; control 3h; control 24; HP at 0h; HP at 3h; and HP at 24h)

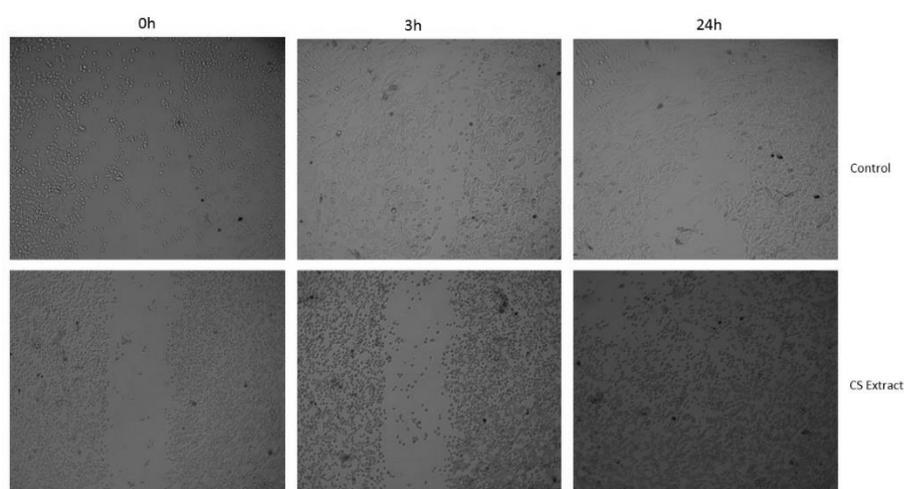


Figure 8. GT effect on B164A5 murine melanoma cells (control 0h; control 3h; C: control 24; GT at 0h; GT at 3h; and GT at 24h)

Conclusions

This study was developed in order to verify the possible anti-migratory effects of the CP, GT and HP extracts on B164A5 murine melanoma cells. Our results indicated a slight anti-migratory effect at the concentration tested for CP and HP extracts, whereas the same concentration of GT extract induced a noxious effect (apoptosis) on these cells. All evaluated vegetal sources had revealed potent antioxidant properties according to the DPPH method. Further studies are required in order to establish the exact mechanism of action of these extracts and a possible link between the anti-migratory effect and the antioxidant activity exhibited by the compounds.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

References

1. Waly MI, Ali A, Guizani N, Al-Rawahi AS, Farooq SA, Rahman MS. Pomegranate (*Punicagranatum*) peel extract efficacy as a dietary antioxidant against azoxymethane-induced colon cancer in rat. *Asian Pac J Cancer Prev.* **2012**, *13*(8), 4051-5.
2. Faria A, Monteiro R, Mateus N, Azevedo I, Calhau C. Effect of pomegranate (*Punicagranatum*) juice intake on hepatic oxidative stress. *Eur J Nutr.*, **2007**, *46*(5), 271-8.
3. Habauzit V, Verny MA, Milenkovic D, Barber-Chamoux N, Mazur A, Dubray C, et al. Flavanones protect from arterial stiffness in postmenopausal women consuming grapefruit juice for 6 mo: a randomized, controlled, crossover trial. *Am J Clin Nutr.*, **2015**, *102*(1), 66-74.
4. Murunga AN, Miruka DO, Driver C, Nkomo FS, Cobongela SZ, Owira PM. Grapefruit Derived Flavonoid Naringin Improves Ketoacidosis and Lipid Peroxidation in Type 1 Diabetes Rat Model. *PLoS One.* **2016**, *11*(4), e0153241.
5. Sirovina D, Orsolcic N, Gregorovic G, Koncic MZ. Naringenin ameliorates pathological changes in liver and kidney of diabetic mice: a preliminary study. *Arh Hig Rada Toksikol.*, **2016**, *67*(1), 19-24.
6. Chidambara Murthy KN, Jayaprakasha GK, Patil BS. Obacunone and obacunone glucoside inhibit human colon cancer (SW480) cells by the induction of apoptosis. *Food Chem Toxicol.* 2011;*49*(7):1616-25.
7. Kim SM, Lee JH, Sethi G, Kim C, Baek SH, Nam D, et al. Bergamottin, a natural furanocoumarin obtained from grapefruit juice induces chemosensitization and apoptosis through the inhibition of STAT3 signaling pathway in tumor cells. *Cancer Lett.* 2014;*354*(1):153-63.
8. Wu S, Han J, Feskanich D, Cho E, Stampfer MJ, Willett WC, et al. Citrus consumption and risk of cutaneous malignant melanoma. *Journal of Clinical Oncology.* 2015;*JCO.* 2014;*57.* 4111.
9. Ferrara L, Montesano D, Senatore A. The distribution of minerals and flavonoids in the tea plant (*Camellia sinensis*). *Farmaco.* **2001**, *56*(5-7), 397-401.
10. Kanwar J, Taskeen M, Mohammad I, Huo C, Chan TH, Dou QP. *Recent advances on tea polyphenols.* Front Biosci (Elite Ed). **2012**, *4*, 111-31.

11. Prasad R, Katiyar SK. Polyphenols from green tea inhibit the growth of melanoma cells through inhibition of class I histone deacetylases and induction of DNA damage. *Genes & cancer*. 2015;6(1-2):49.
12. Priyadarshi S, Valentine B, Han C, Fedorova OV, Bagrov AY, Liu J, et al. Effect of green tea extract on cardiac hypertrophy following 5/6 nephrectomy in the rat. *Kidney international*.,2003, 63(5), 1785-90.
13. Mirmalek SA, Azizi MA, Jangholi E, Yadollah-Damavandi S, Javidi MA, Parsa Y, et al. Cytotoxic and apoptogenic effect of hypericin, the bioactive component of *Hypericum perforatum* on the MCF-7 human breast cancer cell line. *Cancer Cell Int*. 2015, 16, 3.
14. Kleemann B, Loos B, Scriba TJ, Lang D, Davids LM. St John's Wort (*Hypericum perforatum* L.) photomedicine: Hypericin-photodynamic therapy induces metastatic melanoma cell death. *PloS one*. 2014;9(7):e103762.
15. He M, Wang Y, Hua W, Zhang Y, Wang Z. De novo sequencing of *Hypericum perforatum* transcriptome to identify potential genes involved in the biosynthesis of active metabolites. *PLoS One*.,2012, 7(7), e42081.
16. Franco P, Potenza I, Moretto F, Segantin M, Grosso M, Lombardo A, et al. *Hypericum perforatum* and neem oil for the management of acute skin toxicity in head and neck cancer patients undergoing radiation or chemo-radiation: a single-arm prospective observational study. *RadiatOncol.*, 2014, 9, 297.
17. Zhang JJ, Yang XW, Ma JZ, Liu X, Yang LX, Yang SC, et al. Hypercohesones D-G, New Polycyclic Polyprenylated Acylphloroglucinol Type Natural Products from *Hypericum cohaerens*. *Nat Prod Bioprospect.*,2014, 4(2), 73-9.
18. Pyrzynska K, Pękal A. Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples. *Analytical Methods*.2013, 5(17), 4288-95.
19. Harrison ME, Power Coombs MR, Delaney LM, Hoskin DW. Exposure of breast cancer cells to a subcytotoxic dose of apigenin causes growth inhibition, oxidative stress, and hypophosphorylation of Akt. *Exp Mol Pathol.*,2014, 97(2), 211-7.
20. Cabrera C, Artacho R, Gimenez R. Beneficial effects of green tea--a review. *Journal of the American College of Nutrition*.2006, 25(2),79-99.
21. Chacko SM, Thambi PT, Kuttan R, Nishigaki I. Beneficial effects of green tea: a literature review. *Chinese medicine*. 2010,5, 13.